Potential Carcinogenic Risks of Aspartame

Morando Soffritti, Angela Guaragna, and Marco Manservigi

Abstract

Aspartame is an intense artificial sweetener with a sweet test approximately 200 times that of sucrose and used as additive in more than 6,000 products.

Aspartame was invented by GD-Searle in 1965 and submitted for pre-marketing safety evaluation in early 1980s. The studies conducted by GD-Searle to evaluate the potential carcinogenic risks of aspartame did not show any effect. Because of the great commercial diffusion of aspartame, in 1997 the Ramazzini Institute started a large experiments project on rodents to test the carcinogenic effects of aspartame in our experimental model with more sensitive characteristics, namely large number of rats and mice, observation until natural death. Overall the project included the study of 2,270 rats and 852 mice starting the treatment from prenatal life or in mature age and lasting all life.

These studies have shown that aspartame is a carcinogenic agent inducing a significant dose-related increased incidence of several types of malignant tumors and, among them, haematological neoplasias. Later this effect was confirmed by an epidemiological study conducted by a group of the Harvard University.

Keywords

Aspartame • Food additive • Carcinogenic bioassay • Rat • Mice • Artificial sweetener • Carcinogenic effects • EFSA • FDA • GD-SEARLE

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Abbreviations

ADI	Admitted daily dose
APM	Aspartame
CI	Confidence interval
DKP	5-benzyl-3, 6-dioxo-2 piperazine acetic acid
EFSA	European Food Safety Authority
FDA	Food Drug Administration
GR	Glutathione reductase
GSH	Glutathione
IARC	International Agency for Research on Cancer
MPL	Maximum permitted level
RR	Relative risk
US-NCI	United States – National Cancer Institute
US-NTP	United States – National Toxicology Program
WHO	World Health Organization

1 Introduction

The use of artificial sweeteners as substitutes for sucrose began during World Wars I and II when the use of saccharin became prevalent due to its low cost and the wartime shortage of table sugar. Since the 1970s, the growing obesity problem in industrialized countries, due in part to fast food and soft drink consumption, has led to an increased demand for reduced-calorie foodstuffs. Given the lucrative market for these so-called "diet" or "light" products, additional intense artificial sweeteners have emerged, including aspartame, cyclamate, acesulfame-K, sucralose, and neotame. These substances are used in various food/beverages/drugs labeled as diet/sugar-free, including candy, chewing gum, ice cream, beverages, yogurt, and baked goods. They are also present in almost 500 drugs (including pediatric drugs) as cough drops and syrups, vitamins, antibiotics, etc. [1, 2].

With the expansion of the artificial sweetener market, concern has arisen among consumers regardings the safety of these sweeteners and their possible long-term health effects, in particular the potential carcinogenic risks.

Given the fact that wide consumption of artificial sweeteners emerged in the 1980s and 1990s, epidemiological studies are by definition limited, the exposure to such compounds being widely diffused among the population.

Moreover, most long-term carcinogenicity bioassays on artificial sweeteners performed on rodents in the past have not been adequately designed to assess the carcinogenic risk. The sensitivity of these studies in detecting risk has been greatly limited by the following factors: (1) the number of animals per sex per group was usually 50 or less; (2) experiments were usually truncated at 104 weeks (or earlier) from the start of the experiment, thus not allowing the tested compound to express its carcinogenic potential; (3) conduct of the experiments was often inadequate with incomplete or nonsystematic histopathological analysis for all organs and tissues.

The purpose of this review is to summarize the existing literature regarding general data on the technical characteristics and the biological and toxicological effects of aspartame (APM). Unpublished studies dealing with these issues were performed by GD-Searle in the 1970s. These studies were provided for EFSA in 2011 after a public call was launched. The results of the GD-Searle studies are derived from the documentation posted online by EFSA and made available to the scientific community and interested stakeholders [3]. The references of the GD-Searle studies are here identified with the letter E plus the code number and the year (i.e., E18, 1972). In this review, particular emphasis will be deserved on what we know now about the carcinogenic potential of APM. Indeed, this is a major public health concern, if we consider that children and women in childbearing age are among the major consumers, and this prolonged experience may represent a potential risk not only for children but also in adult age.

2 Technical Characteristics, Production, Uses, and Stability of APM in Food and Beverages

Aspartame (APM) is a dipeptide of L-phenylalanine methyl ester and L-aspartic acid bearing an amino group at the α-position from the carbon of the peptide bond (Fig. 1). APM was first synthesized in 1965 by the US GD-Searle and first sold under the name "Nutrasweet." APM has a molecular formula of C_{14} H_{18} N_2 O_5 with a molecular weight of 294.31 g/mol and the CAS Registry Number is 22839-47-0. APM is a white odorless, crystalline powder with a sweet taste approximately 200 times that of sucrose. At room temperature and at pH7, the solubility in water is 10 g/l and it is insoluble in oil [4]. The major degradation products of APM are 5-benzyl-3,6-dioxo-2-piperazine acetic acid (DKP), methanol, L-phenylalanine, and L-aspartic acid. Aspartame is used in >6,000 products, and the maximum permitted levels (MPL) of APM in food and beverage products range from 25 to 6,000 mg/kg, except for tabletop sweeteners for which there is *quantum satis* authorization [5]. The highest MPL of APM is for chewing gum (MPL 5,500 mg/kg) and breath refreshing microsweets (6,000 mg/kg); coca, chocolate, and dietary food products have a range of 1,000–2,000 mg/L (Commission Regulation-EU-No 1129/2011). Moreover, it has been reported that in the USA, the annual use of APM in food products was

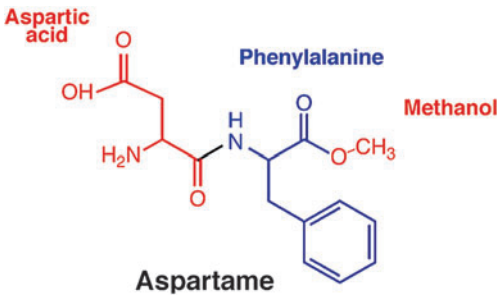


Fig. 1 Molecular structure of aspartame and its three components

Table 1 Estimated exposure (mg/kg b.w./day) to Aspartame (*APM*) based on its use as a food additive using the maximum permitted levels (*MPLs*), in five population groups[a]

Estimated exposure (mg/kg b.w./day)	Toddlers (12–35 months)	Children (3–9 years)	Adolescents (10–17 years)	Adults (18–64 years)	The elderly (>65 years)
> Mean level	3.2–16.3	2.3–12.8	0.8–4.0	0.8–8.6	0.5–4.4
> High level	11.8–36.9	7.1–32.9	2.3–13.3	2.5–27.5	1.5–23.5

[a]From EFSA, 2013

estimated at 5,000–5,500 tons and that most (~ 85%) was used for diet soda [6]. Overall, hundreds of millions of people in the world consume APM on a daily basis. An estimated exposure to APM based on its use as a food additive in the population stratified in five groups by age is reported in Table 1 [5].

The US Food and Drug Administration (FDA) approved the use of APM in a limited number of dry foods in 1981 [7], in soft drinks in 1983 [8], and in all foods in 1996 [9]. In 1994, the use of APM was approved by Europe [10]. Currently, the daily admitted dose (ADI) in the USA and in Europe is 50 mg/kg b.w. and 40 mg/kg b.w., respectively. In 2013, EFSA carried out a reevaluation of APM concluding that APM is safe for human consumption [5].

The stability of APM in solid or solution state is affected mainly by temperature, moisture, pH, and storage time. The major degradation products of APM are L-phenylalanine, aspartic acid, methanol, and 5-benzyl-3,6-dioxo-2-piperazine acetic acid (DKP).

Solid-state stability studies showed that at temperatures higher than 80 °C, APM may release methanol to form DKP [11]. Under dry conditions, conversion of APM into DKP is slow (5% per 100 h at 105 °C), while at higher temperatures, the rate of conversion to DKP increases (around 50% per 80 h at 120 °C and 100% per 30 h at 150 °C) [12].

The degradation products of APM in solution have been tested at different pH levels. The main degradation product found at pH 2–6 was L-phenylalanine methyl ester; at pH 7–10, the chief degradation product was DKP and at pH 12, the L-aspartyl-phenylalanine [13].

The content of APM and its degradation products have been tested in several products. The degradation of APM was high in fruit cream (40%), milk chocolate (26%), and fruit yogurt; 12–21% decomposition of APM in cola drink and fruit cream was in the form of DKP [14]. In soft drinks, the degradation of APM into DKP at a temperature of 25 °C for 2 months has been found four times higher than at 4–5 °C [15].

3 Absorption, Distribution, and Metabolism of APM

The metabolism of APM has been investigated in mouse, rat, rabbit, dog, and monkey, using different radiolabeled forms of ^{14}C-APM located either on the phenylalanine or aspartic acid moiety or on the methyl group.

In mice, [14 C(U)-phe]-APM was administered by gavage at the dose of 20 mg/kg b.w. The radioactivity found in plasma, urine, feces, and expired air clearly demonstrated that APM was hydrolyzed in the gut before the absorption of its phenylalanine moiety occurred (E 18, 1972)[1]. Studies conducted on rats, primates, and man receiving APM by oral ingestion have shown that APM is hydrolyzed in the lumen of the gastrointestinal tractor within the mucosal cells lining the GI tract, its half-life in the GI tract being in the order of a few minutes [16]. This short time was mediated in the GI tract by enzymes of the intestine, including esterases and peptidases [17, 18]. The metabolites are methanol, aspartic acid, and phenylalanine. APM releases a maximum of 10% methanol by weight [19]. The metabolic process is very efficient and the amount of APM that enters the blood stream is undetectable [17, 20]. Methanol is oxidized in the liver to formaldehyde and then to formic acid. The enzymes involved depend on the species: in rats the metabolism of methanol to formaldehyde is mediated through a catalase-peroxidase system; in primates and humans, an alcohol dehydrogenase is responsible [19].

In humans, APM is hydrolyzed in the intestine as in animals. Qualitatively and quantitatively, the metabolites are also the same. Doses of APM higher than 100–200 mg/kg b.w. peak blood levels were around 13–26 mg methanol/l blood and persisted for several hours in the blood. After 24 h, the methanol was under the limit of detection [21].

Stegink [22] found that, following a single oral administration of APM at various dose levels ranging from 34 to 200 mg/kg b.w., a significant dose-related increase in plasma phenylalanine was detected between 30 min and 2 h following dining; concerning plasma aspartate, no significant dose-related increase was observed after dosing. The author concluded that a rapid metabolization of aspartic acid occurred at all doses. In another study conducted to evaluate the effects of APM on its metabolism after repeated ingestions, no accumulation of plasma phenylalanine or aspartate or methanol concentrations was observed [23]. Other studies were conducted to test the effects of oral administration of APM (at doses ranging from 600 to 8,100 mg per person per day for 27 weeks) on plasma levels of phenylalanine and aspartic acid, in obese individuals, diabetics, and children (E 23, 1972; E 24, 1972; E 60, 1973; E 61, 1972; E 64, 1972). No significant differences were observed in plasma amino acid concentrations between treated subjects and controls.

In conclusion, pharmacokinetic studies on APM showed that unchanged APM was not detectable in plasma. After ingestion, APM is hydrolyzed in the intestinal tract to methanol, phenylalanine and aspartic acid, and metabolites which are then absorbed and metabolized.

[1]Code of the reference as reported on the documentation posted on EFSA website [3].

4 Acute, Subacute Toxicity, and Genotoxicity

The acute effects of APM were tested in mice, rats, and rabbits (E 46, 1973). Male mice were treated by gavage at 1,000 or 5,000 mg/kg b.w. or by i.p. injection at 200 or 450 mg/kg b.w.; rats were treated by gavage at 2,033 or 5,000 mg/kg b.w. or by i. p. injection at 2,033 mg/kg b.w.; rabbits were treated by gavage in the dose range of 2,000–5,000 mg/kg b.w. The animals were kept under clinical observation for 7 days, and no remarkable changes of the behavior and motility were registered. No mortality was observed. The LD_{50} dose level was estimated by the authors higher than the ones treated in the various species.

Subacute tests were conducted on mice, rats, and beagle dogs. Mice of 8 weeks of age were treated with APM in feed at the dose levels of 0, 3,000, 5,000, and 13,000 mg/kg b.w. for 4 weeks. No significant difference in body weight and no clinical changes or mortality was observed (E 2, 1972). Groups of five male and five female 7 weeks old CD rats were treated with APM at the dose of 0, 2,000, 4,000, and 10,000 ppm in feed for 8 weeks. Decreased feed consumption at the highest dose was observed in females, without consequence for body weight and without mortality (E 3, 1972). Groups of ten male and ten female CD rats were treated with APM in feed at the dose of 0, 5, and 125 mg/kg b.w. for 8 weeks. No changes in hematological and clinical chemistry tests in urine analysis were observed, as well as no clinical alterations or mortality. Among males of the highest dose level, an increased liver to body weight ratio compared to controls was observed. Bile duct hyperplasia and pericholangitis were present in all treated and untreated rats (E 20, 1969). Three groups of male Wister rats were daily exposed for 6 months to 500 or 1,000 mg/kg b.w. of APM dissolved in water and administered by gavage. Rats that received 1,000 mg/kg b.w. showed a significant serum increase in activities of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and gammaglutamyltransferase. The concentration of reduced glutathione (GSH) and the activities of glutathione peroxidase and glutathione reductase (GR) were significantly decreased in the liver of rats exposed to 1,000 mg/kg b.w./daily. The levels of GSH were significantly decreased in both treated groups and controls. Concerning the brain, it was reported that the concentration of GSH and GR activity were significantly reduced in rats exposed to 1,000 mg/kg b.w./day of APM. GSH resulted significantly decreased also at the level of exposure to 500 mg/kg b.w./day. On the basis of these data, the authors concluded that long-term consumption of APM may induce liver toxicity and a dose-related correlation between toxic effects of APM and alteration in the glutathione-dependent system of the brain [24, 25].

The genotoxicity of APM was evaluated in the in vitro and in vivo studies.

Various strains of *Salmonella* Typhimurium (TA 1535; TA 1537; TA 1538; TA 98 and TA 100) were tested in the absence and presence of a rat liver metabolic activation system at dose levels from 10 to 5,000 mg/plate (E 97, 1978; E 101, 1978). Aspartame was not mutagenic in that tests. Other studies conducted on *Salmonella* Typhimurium resulted negative for genotoxicity [26–28]. A study to evaluate DNA damaging activity in the in vitro primary rat hepatocyte/DNA repair assay resulted negative [29].

In an in vivo bone marrow micronucleus test conducted on male Fischer 344 rats a rally exposed to 0, 500, 1,000, or 2,000 mg/kg b.w. for 3 days, no increase in the number of micronucleated erythrocytes was observed [26]. In other peripheral blood micronucleus tests, conducted in male and female transgenic mice (Tg.AC hemizygous, p53 haploinsufficient, or Cdkn2a deficient), after 9 months of exposure to APM at doses from 3.1 mg to 50 mg/kg diet, no clastogenic activity was observed in male and female Tg.AC hemizygous and Cdkn2a-deficient mice and in male p53 haploinsufficient mice. In female p53 haploinsufficient mice, the results of the test were considered positive [26].

Four male mice were treated with a single dose of 2,000 mg/kg b.w. APM and analyzed the DNA migration in the cells of the stomach, colon, liver, kidney, bladder, lung, brain, and bone marrow. No significant increase in the DNA migration was observed [1].

To assess the genotoxicity of APM, five groups of mice were treated with APM administered by gavage at the dose levels of 0, 250, 455, 500, and 1,000 mg/kg b.w. The treatment induced (1) micronuclei in bone marrow erythrocytes, (2) micronuclei in peripheral blood, and (3) chromosome aberrations in bone marrow erythrocytes [30].

5 The Historical Available Data on the Chronic Toxicity and Carcinogenicity of Aspartame

Food additives and any other ingredient used for human consumption must be submitted before marketing to several laboratory tests for regulatory safety evaluation. Guidelines to performing these tests, including chronic toxicity and carcinogenicity bioassays, were published in the mid-1960s by the US National Cancer Institute. The main recommendations concerned (1) the use of two species of animals, namely, rats and mice; (2) using at least 50 males and 50 females for each dose level, without restrictions on increasing the number in order to improve the statistical power of the studies; (3) duration of the treatment and observation for at least 104 weeks; and (4) complete necropsy of all animals in the experiment, a complete histopathological evaluation of control and high-dose-treated animals and a limited evaluation of the animals of other groups. Updating of the guidelines has been performed by the Environmental Protection Agency [31], by the FDA [32] and the Organization for Economic Cooperation and Development [33]. Meanwhile, bioassays have been conducted by academic and independent institutions not for regulatory purposes but to evaluate if, following a different protocol design and conduct, the sensitivity and specificity of the studies might be improved [34–36].

In the early 1970s, GD-Searle submitted three 2-year carcinogenicity bioassays, codified as E-33/34, E-70 on rats, and E-75 on mice, to the FDA with the intent to gain market approval for APM. These studies are summarized in Table 2.

In the first experiment (E-33/34, 1973), four groups of 40 male and 40 female Charles River Sprague-Dawley rats were treated at the doses of 1, 2, 4, 6, and 8% of APM in the feed starting from mature age and lasting to 104 weeks. One group of 60

Table 2 Long-term carcinogenicity bioassays on ASPARTAME administered orally with feed to male (M) and female (F) mice or rats: summary of experimental design and results of major studies

Authors	Animals				Treatment		Histopath. evaluation	Endpoints	Biophase results of the studies			Major carcinogenic results and statistical significance (pvalue) when available
	Species/strain	Group	No. M/F	Age at start	Dose (mg/kg/bw) in feed day	Duration (wks/LS[a])	(Complete/Limited)[b]		Mean feed consumption differences (%) M/F	Mean body weights differences (%) M/F	Survival (%) at the end of the study M/F	
GD Searle & Co., 1973 (E-33/34)	Rat Charles River (CD) Sprague-Dawley	I	60/60	NA[c]	0	104	Complete	Tumor incidence	–	–	38.4/46.7	*Animals bearing malignant tumors (%)*:
		II	40/40		1,000		Limited		−1.4/+1.0	0/+4.5	45.0/57.5	M: 16.7 (I, control); 17.5 (II); 7.5 (III); 12.5 (IV);7.5 (V)
		III	40/40		2,000		Limited		+2.6/+3.0	1.8/−3.2	52.5/50.0	F: 23.3 (I, control); 20.0 (II); 35.0 (III); 22.5 (IV); 25.0 (V)
		IV	40/40		4,000		Complete		−3.1/−8.2*	− 0.1/−3.5	57.5/35.0	*Animals bearing mammary cancers (%)*:
		V	40/40		6,000–8,000		Complete		−5.7*/−14.6* *(pvalueNA)	−12.3*/−15.1* *(pvalueNA)	52.5/25.0* *($p<0.05$)	F: 11.7 (I, control); 5.0 (II); 15.0 (III); 17.5 (IV); 17.5 (V)
												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 & Co., 1974 (E-70)	Rat Charles River (CD) Sprague-Dawley	I	60/60	Prenatal	0	104	Complete	Tumor incidence	–	–	41.7/48.4	*Animals bearing malignant tumors (%)*:
		II	40/40		2,000		Complete		+1.3/−1.7*	+ 2.2/−0.6	50.0/45.0	M:18.3 (I, control); 10.0 (II); 20.0 (III)
		III	40/40		4,000		Complete		−8.7*/−3.5* *(pvalueNA)	−2.4/−5.5* *(pvalueNA)	57.5/52.5	F: 18.3 (I, control); 20.0 (II); 27.5 (III) Final sacrifice probably at 106 weeks of age
GD Searle & Co., 1974 (E-75)	Mice CD1 Swiss	I	72/72	4 weeks	0	104	Complete	Tumor incidence	–	–	32.5/41.7	*Animals bearing malignant tumors (%)*:
		II	36/36		1,000		Limited		−3.5*/−2.6	−0.5*/+0.8	27.8/38.9	M: 16.9 (I, control); 9.4 (II); 8.6 (III); 22.2 (IV)
		III	37/35		2,000		Limited		−4.7*/−8.3	+ 0.7*/+ 0.5	25.8/41.7	F: 16.7 (I, control); 12.9(II); 12.9(III); 16.1(IV)
		IV	37/35		4,000		Complete		−7.5*/−9.0*	−0.2*/+ 1.4 *(pvalue NA)	25.0/41.7	Total number of mice available for histopathological evaluation: M: 65 (I, control); 32 (II); 35 (III); 27 (IV) F: 66 (I, control); 31 (II); 31 (III); 31 (IV) Final sacrifice at 108 weeks of age

(*continued*)

Table 2 (continued)

Authors	Animals				Treatment		Histopath. evaluation	Endpoints	Biophase results of the studies			Major carcinogenic results and statistical significance (pvalue) when available
	Species/strain	Group	No. M/F	Age at start	Dose (mg/kg/bw) in feed day	Duration (wks/LS[a])	(Complete/Limited)[b]		Mean feed consumption differences (%) M/F	Mean body weights differences (%) M/F	Survival (%) at the end of the study M/F	
Ishii [37]; Iwata [38]	Rat, Wistar	I	86/86	6 weeks	0	104	1. 10M+10F × group killed at 26 weeks; 2. 16M+16F × group killed at 52 weeks; 3. 60M+60F × group followed until 104 weeks	Brain tumors (6 section each brain)	Dose dependent decrease at 2 and 4 g/kg b.w. and at 4 g/kg b.w. APM+DKP in males and in all treated females (numerical data NA)	A dose-dependent decrease at 2 and 4 g/kg b. w. APM and at 4 g/kg b. w. APM*DKP in males and in all treated females (Numerical data NA)	43.3/81.7	No brain tumors were observed in animals killed at 26 and 52 weeks – Brain tumors in rats observed until 104 weeks M: 0 (I, control); 1.7 (II); 0 (III); 1.7 (IV); 0 (V) F: 1.7 (I, control); 0 (II); 3.3 (III); 0 (IV); 1.7 (V)
		II	86/86		1,000						26.7/66.7	
		III	86/86		2,000						46.7/85.0	
		IV	86/86		4,000						41.7/71.7	
		V	86/86		4,000						48.3/68.3	Final sacrifice at 110 weeks of age
					ASP+DKP[d] (3:1)							
National Toxicology Program [26]	Mice Tg-AC hemizygous[e]	I	15/15	6 weeks	0	40	Complete	Development of 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 related to exposure to APM
		II	15/15		520		Limited				–	
		III	15/15		1,040		Limited				–	Final sacrifice at 46 weeks of age
		IV	15/15		2,110		Limited				–	
		V	15/15		4,190		Limited				–	
		VI	15/15		7,920		Complete				–	

National Toxicology Program [26]	Mice p53 haploinsufficient[f]	I	15/15	7 weeks	0	40	Complete	Development of 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		520		Limited				–	
		III	15/15		1,040		Limited				–	Final sacrifice at 46 weeks of age
		IV	15/15		2,110		Limited				–	
		V	15/15		4,190		Limited				–	
		VI	15/15		7,920		Complete				–	
National Toxicology Program [26]	Mice Cdkn2a deficient[g]	I	15/15	7–9 weeks	0	40	Complete	Development of 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		520		Limited				–	
		III	15/15		1,040		Limited				–	Final sacrifice at 46 weeks of age
		IV	15/15		2,110		Limited				–	
		V	15/15		4,190		Limited				–	
		VI	15/15		7,920		Complete				–	

[a]*LS* Life Span
[b]Histopathological evaluation: *C* complete; *L* limited
[c]*NA* not available
[d]*DKP* diketopiperazine
[e]Model susceptible for development of high incidence of skin papillomas in response to topical application of TPA (12-O-tetradecanoyl-phorbol-13-acetate)
[f]Model susceptible for development of high incidence of lymphomas or sarcomas
[g]Model susceptible for development of high incidence of brain tumors, lymphomas and fibrosarcomas

males and 60 females served as control. At the highest dose level, both males and females ate significantly less as well as the body weight was significantly decreased. The survival in males was higher at the highest dose than among controls (52.5% vs. 38.4%), the reverse with females treated at the highest dose versus controls (25% vs. 46.7%). Incidence of malignant tumors in males was higher in controls than in the animals treated at the highest dose (16.7% vs. 7.5%). No differences in females were observed.

In a second experiment (E-70, 1974), two groups of 40 male and 40 female Charles River Sprague-Dawley rats were exposed to APM in the feed at the dose levels of 2 or 4% from prenatal life and lasting to 104 weeks. Both males and females exposed at the higher doses ate significantly less than controls. The body weight was significantly lower in females exposed at the highest dose compared to controls. No difference was observed in survival among the groups of both genders. No difference was observed in survival among the groups. No significant difference in the incidences of malignant tumors was observed in treated males and females compared to controls.

A third experiment was performed on four groups of male and female Swiss CD1 mice treated with APM in feed at the dose of 0, 1, 2, and 4% for 104 weeks starting at 4 weeks of age. Compared to controls, it showed (1) a significant decreased feed consumption among males of all treated groups, (2) no substantial difference in mean body weight among the groups, and (3) no significant difference in the incidence of malignant tumors observed between treated and control rats.

During the period 1981–2005, other chronic toxicity/carcinogenicity bioassays were conducted. These studies are summarized on Table 2.

The first experiment [37] was conducted on five groups of 86 male and 86 female Wistar rats treated with APM in feed at the daily dose levels of 0, 1,000, 2,000, 4,000, and 4,000 mg/kg b.w. (the last as a mixture of APM plus DKP, 3:1) for 104 weeks starting from 6 weeks of age and then sacrificed. Two interim sacrifices of 10 M and 10 F and 16 M and 16 F were performed after 26 and 52 weeks of treatment, respectively. A dose-dependent decrease in feed consumption was observed in males treated at 2,000 and 4,000 mg/kg b.w. daily dose levels of APM and in all treated females as well as concerns mean body weights. No significant differences of brain tumor incidence were observed among the groups. In 2006, a reevaluation of all neoplastic and nonneoplastic lesions of the Ishii study was performed. No differences were observed in the neoplastic incidences among males and females treated with APM versus controls [38]. In 2005, the US NTP published the results of three studies conducted on genetically modified mice models [26]. According to the NTP report, there were no neoplasms or nonneoplastic lesions related to APM exposure (see Table 2).

Overall, these long-term studies did not show that exposure to APM in feed could produce carcinogenic effects in rats and mice.

6 The Carcinogenicity Bioassays on APM Conducted by Ramazzini Institute on Rats and Mice

In the early 1997, the Ramazzini Institute started a large project of life-span carcinogenicity bioassays on rats and mice to test the carcinogenic effects of exposure to APM administered with feed. The reasons for performing these studies were to test the potential of APM for carcinogenicity using an experimental model with more sensitive characteristics, namely, a large number of animals per sex per group, exposure, and observation until their natural death. This model used at the Cesare Maltoni Cancer Research Center of the Ramazzini Institute in the past 40 years allowed us to show for the first time that several agents widely used in the working place and diffused in the general environment are carcinogenic, such as vinyl chloride, benzene, formaldehyde, xylene, trichlorethylene, and others [34, 35].

The Ramazzini Institute project on APM included three life-span bioassays on APM already published, the first on rats exposed from 8 weeks of age until death [39, 40], the second on rats exposed from prenatal life until death [41], and the third on mice exposed prenatally until death [42]. The studies were conducted according to the procedures usually followed for the studies performed in Good Laboratory Practices, which means periodical measuring of feed consumption and body weight, daily clinical control of health and behavior, full necropsy of each animal after death, full histopathological evaluation of all organs and tissues of all animals in each group, and statistical elaboration of the results.

6.1 Carcinogenicity Bioassay on APM Administered in Feed to Sprague-Dawley Rats from 8 Weeks of age until Natural Death (Exp. BT6008)

Six groups of 200–300 male and female Sprague-Dawley rats were exposed to APM administered in feed at the concentration of 100,000, 50,000, 10,000, 2,000, 400, and 80 ppm from 8 weeks of age until natural death; 300 males and females served as controls [40]. Overall, the study encompassed 1,800 rats and the biophase proceeded smoothly and well. A slight decrease in feed consumption was observed at the highest dose in both males and females without affecting the body weight. No difference in survival was observed among males; a slight decrease in females was observed in controls versus treated rats from 104 weeks of age. The results of the carcinogenic effects are reported in Tables 3, 4, and 5.

The data showed (1) a significant dose-related increased incidence of total malignant tumors in males and females (Table 3); (2) a significant dose-related increased incidence of transitional cell carcinomas of the renal pelvis in females, particularly at the highest dose (Table 4). Some of the lesions appeared highly invasive, extending from the pelvis surface right into the renal parenchyma, showing anaplastic cellular morphology, a lobular growth pattern originating from a broad base of papillary growth with some areas of mineralization (Fig. 2a, b); (3) a significant dose-related increased incidence of malignant schwannomas of the

Table 3 Long-term carcinogenicity bioassay on aspartame, administered with the feed supplied ad libitum, from 8 weeks of age until natural death, to male (M) and female (F) Sprague-Dawley rats (Experiment BT6008)

Results: benign and malignant tumors											
Group No.	Concentration (ppm)	Animals		Benign tumors				Malignant tumors			
				Tumor-bearing animals[a,b]		Tumors[c]		Tumor-bearing animals[a,b]		Tumors[c]	
		Sex	No.	No.	%	No.	Per 100 animals	No.	%	No.	Per 100 animals
I	100,000	M	100	66	66.0	92	92.0	43	43.0	55	55.0
		F	100	88	88.0	245	245.0	51	51.0	64	64.0
		M+F	200	154	77.0	337	168.5	94	47.0	119	59.5
II	50,000	M	100	71	71.0	120	120.0	38	38.0	45	45.0
		F	100	87	87.0	231	231.0	58	58.0[##]	84	84.0
		M+F	200	158	79.0	351	175.5	96	48.0	129	64.5
III	10,000	M	100	77	77.0[**##]	127	127.0	34	34.0	42	42.0
		F	100	85	85.0	221	221.0	40	40.0	62	62.0
		M+F	200	162	81.0	348	174.0	74	37.0	104	52.0
IV	2,000	M	150	87	58.0	131	87.3	60	40.0	69	46.0
		F	150	121	80.7	265	176.7	67	44.7	86	57.3
		M+F	300	208	69.3	396	132.0	127	42.3	155	51.7
V	400	M	150	99	66.0	148	98.7	48	32.0	52	34.7
		F	150	120	80.0	278	185.3	70	46.7	95	63.3
		M+F	300	219	73.0	426	142.0	118	39.3	147	49.0

VI	80	M	150	82	54.7	120	80.0	44	29.3	49	32.7
		F	150	112	74.7	256	170.7	64	42.7	85	56.7
		M+F	300	194	64.7	376	125.3	108	36.0	134	44.7
VII	0 (control)	M	150	93	62.0	124	82.7	53	35.3*	59	39.3
		F	150	123	82.0*	310	206.7	55	36.7**	69	46.0
		M+F	300	216	72.0	434	144.7	108	36.0	128	42.7

[a]Near the dosed group incidence are the p-values corresponding to pairwise comparisons between the controls and that dosed group
[b]Near the control incidence are the p-values associated with the trend test
[c]Multiple tumors of different type and site, or of different type in the same site, or of the same type in bilateral organs, in the skin and in the subcutaneous tissues (i.e., bones, skeletal muscles, etc.), were plotted as single/independent tumors. Multiple tumors of the same type in the same tissue and organ, a part those above mentioned, were plotted only once
*Statistically significant ($p \leq 0.05$) using Cochran-Armitage test
**Statistically significant ($p \leq 0.01$) using Cochran-Armitage test
##Statistically significant ($p \leq 0.01$) using poly-k test (k=3)

Table 4 Long-term carcinogenicity bioassay on ASPARTAME, administered with the feed supplied ad libitum, from 8 weeks of age until natural death, to male (M) and female (F) Sprague-Dawley rats (Experiment BT6008)

Results: lesions of oncological interest of the TCE[a] of the kidney pelvis and ureter								
Group No.	Concentration (ppm)	Animals		No. of organs examined	Animals bearing tumors of the pelvis and ureter[b,c]			
					PAA[a]		CA[a,d]	
		Sex	No.		No.	%	No.	%
I	100,000	M	100	100	0	–	1	1.0
		F	100	100	3	3.0	4	**4.0**[#]
		M+F	200	200	3	1.5	5	2.5
II	50,000	M	100	100	0	–	1	1.0
		F	100	99	1	1.0	3	**3.0**
		M+F	200	199	1	0.5	4	2.0
III	10,000	M	100	100	0	–	1	1.0
		F	100	100	1	1.0	3(4)	**3.0**
		M+F	200	200	1	0.5	4	2.0
IV	2,000	M	150	150	0	–	1	0.7
		F	150	150	1	0.7	3(4)	**2.0**
		M+F	300	300	1	0.3	4	1.3
V	400	M	150	149	1	0.7	0	–
		F	150	150	1	0.7	3	**2.0**
		M+F	300	299	2	0.7	3	1.0
VI	80	M	150	149	0	–	0	–
		F	150	150	1	0.7	1	**0.7**
		M+F	300	299	1	0.3	1	0.3
VII	0 (control)	M	150	150	0	–	0	–
		F	150	150	0	–	0	–[*#]
		M+F	300	300	0	–	0	–

[a]*TCE* transitional cell epithelium, *PAA* papilloma with atypia, *CA* carcinoma
[b]Near the dosed group incidence are the p-values corresponding to pairwise comparisons between the controls and that dosed group
[c]Near the control incidence are the p-values associated with the trend test
[d]Between brackets the number of tumors (one animal can bear bilateral tumors)
[*]Statistically significant ($p \leq 0.05$) using Chocran-Armitage test
[#]Statistically significant ($p \leq 0.05$) using poly-k test (k = 3)

peripheral nervous system in males (Table 5). The tumors originated mainly from Schwann cells of cranial nerves and, as shown in Fig. 3a–e, were composed of cystic cavities containing proteinaceous fluid and blood cells (a feature called Antoni type B pattern); the neoplastic cells were arranged in fascicles with dense cellularity and nuclear palisading (called Antoni type A); moreover, the neoplastic cells proved positive for S-100 proteins as evidenced by the dark brown staining and by Verocay bodies (Fig. 3e); (4) a significant dose-related increased incidence of lymphomas/

Table 5 Long-term carcinogenicity bioassay on aspartame, administered with the feed supplied ad libitum, from 8 weeks of age until natural death, to male (M) and female (F) Sprague-Dawley rats (Experiment BT6008)

Results: malignant schwannomas of the peripheral nervous system and hematological neoplasias (HN)[a]											
Group No.	Concentration (ppm)	Animals		Tumor-bearing animals							
				Peripheral nerves						Animals bearing HN[a, b]	
				Cranial		Others		Total[b]			
		Sex	No.	No.	%	No.	%	No.	%	No.	%
I	100,000	M	100	3	3.0	1	1.0	4	4.0	29	29.0
		F	100	1	1.0	1	1.0	2	2.0	25	25.0$^{\#\#}$
		M+F	200	4	2.0	2	1.0	6	3.0	54	27.0
II	50,000	M	100	3	3.0	0	–	3	3.0	20	20.0
		F	100	1	1.0	0	–	1	1.0	25	25.0$^{\#\#}$
		M+F	200	4	2.0	0	–	4	2.0	45	22.5
III	10,000	M	100	2	2.0	0	–	2	2.0	15	15.0
		F	100	1	1.0	0	–	1	1.0	19	19.0$^{\#}$
		M+F	200	3	1.5	0	–	3	1.5	34	17.0
IV	2,000	M	150	2	1.3	0	–	2	1.3	33	22.0
		F	150	1	0.7	2	1.3	3	2.0	28	18.7$^{\#}$
		M+F	300	3	1.0	2	0.7	5	1.7	61	20.3
V	400	M	150	1	0.7	2	1.3	3	2.0	25	16.7
		F	150	0	–	0	–	0	–	30	20.0$^{\#\#}$
		M+F	300	1	0.3	2	0.7	3	1.0	55	18.3
VI	80	M	150	1	0.7	0	–	1	0.7	23	15.3
		F	150	1	0.7	1	0.7	2	1.3	22	14.7
		M+F	300	2	0.7	1	0.3	3	1.0	45	15.0
VII	0 (control)	M	150	1	0.7	0	–	1	0.7$^{*\#}$	31	20.7$^{*\#}$
		F	150	0	–	0	–	0	–	13	8.7$^{**\#}$
		M+F	300	1	0.3	0	–	1	0.3	44	14.7

[a] *HN* hematological neoplasias
[b] Near the control incidence are the p-values associated with the trend test
* Statistically significant ($p \leq 0.05$) using Cochran-Armitage test
** Statistically significant ($p \leq 0.01$) using Cochran-Armitage test
$^{\#}$ Statistically significant ($p \leq 0.05$) using poly-k test (k = 3)
$^{\#\#}$ Statistically significant ($p \leq 0.01$) using poly-k test (k = 3)

leukemias in females, particularly in the range of APM exposure from 100,000 to 400 ppm (Table 5). The characteristics of the hematopoietic neoplasms were defined by morphology as lymphocytic lymphoma, lymphoimmunoblastic lymphoma, histiocytic sarcoma, and myeloid and monocytic leukemia. In some cases, immunohistochemical stainings were used to confirm the kind of neoplastic lesion.

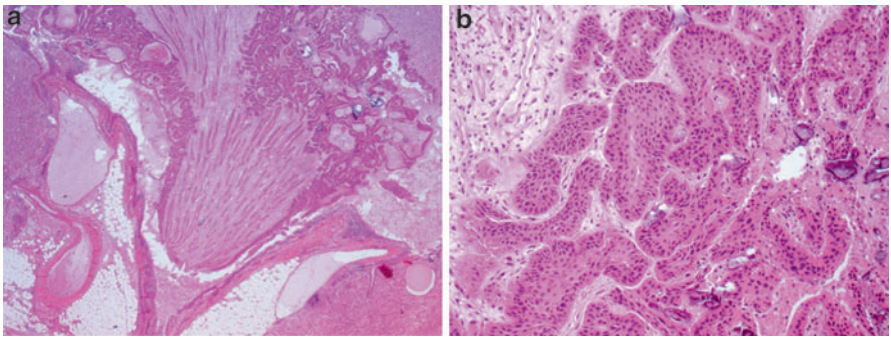


Fig. 2 Transitional cell carcinoma of the renal pelvis. (**a**) Highly invasive carcinoma extending from the pelvis surface to the renal parenchyma (HE 25X). (**b**) Higher magnification (HE 200X)

The hematopoietic neoplasms involved the thymus and mediastinal lymph nodes (Fig. 4a, b), the spleen (Fig. 5a, b), the lung (Fig. 6a–f), the liver with invasion of blood vessels as in the case of myeloid leukemias (Fig. 7a, b), multiple organs as in the case of histiocytic sarcoma (Fig. 8a–c), or multiple organs with invasion of the blood vessels as in the case of monocytic leukemias (Fig. 9a, b).

This experiment showed for the first time that APM administered in feed to rats induces a significant dose-related increased incidence of several malignant tumors, even at a dose of 400 ppm (simulating an APM daily assumption of 20 mg/kg b.w. in rat).

6.2 Carcinogenicity Bioassay on APM Administered in Feed to Sprague-Dawley Rats from Prenatal Life until Natural Death (Exp. BT6009)

Two groups of 140 male and female Sprague-Dawley rats were exposed to APM administered in the feed at the concentration of 2,000 and 400 ppm from prenatal life (11^{th} day of gestation) until natural death; one group of 190 males and females served as controls [41]. Overall, the study encompassed 470 rats. No substantial difference in feed consumption was observed in males and females of treated rats compared to controls or again in mean body weights and survival [41].The results of the carcinogenic effects of APM are reported in Tables 6, 7, and 8.

The data showed (1) a significant increased incidence (dose-related) of malignant tumors in males treated at the highest dose (Table 6), (2) a significant increased incidence (dose-related) of mammary adenocarcinomas (Fig.10a, b) in females treated at the highest dose (Table 7), and (3) a significant increased incidence

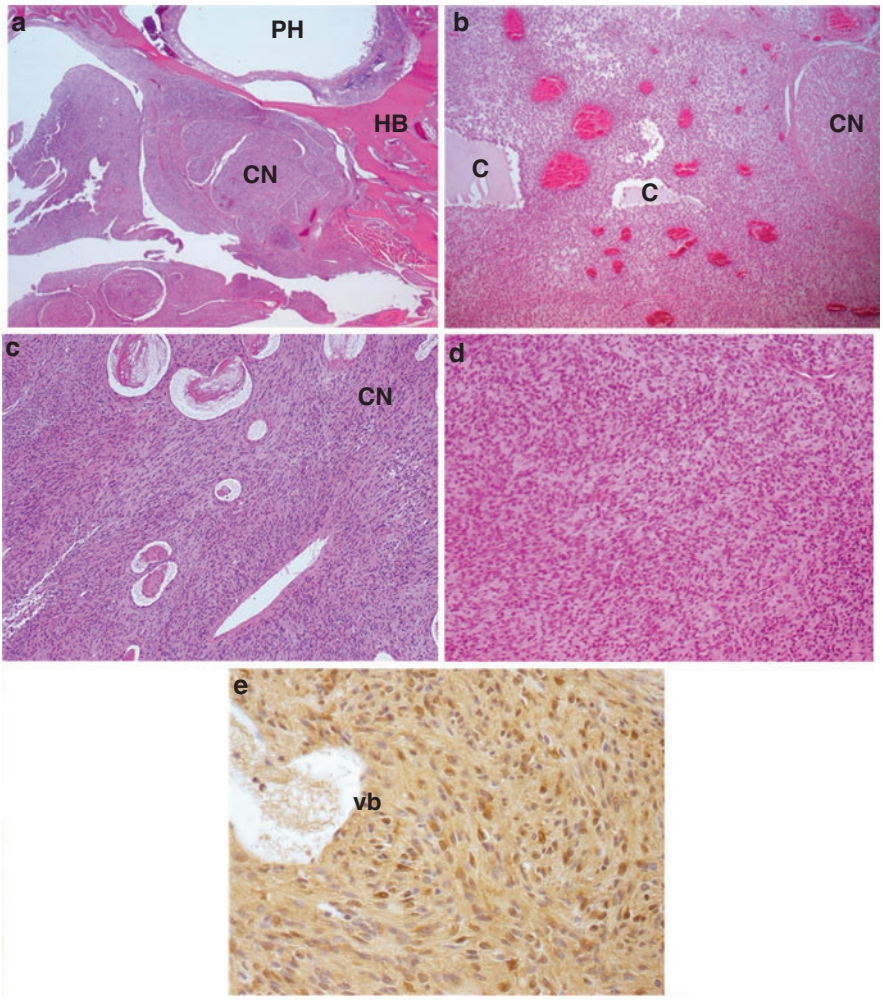


Fig. 3 Malignant schwannoma of cranial nerves. (**a**) The tumor originates from Schwann cells of the cranial nerves (*CN*) observed in this case along the sides of the pharynx (*PH*) in the head section (*HB*, head bone). (**b**) The tumor is composed of cystic cavities (*C*) (this feature is known as the Antoni type B pattern). (**c**) The neoplastic cells are arranged in fascicles with dense cellularity. (**d**) Nuclear palisading may be observed (this pattern is known as Antoni type A). (**e**) Positivity for S-100 protein evidenced by the dark brown staining of the neoplastic cells; Verocay bodies (*Vb*) are also present (**a**: HE 25X; **b**: HE 100X; **c**: HE 100X; **d**: HE 100X; **e**: 200X)

(dose-related) of hematological neoplasias both in males and females treated at the highest dose (Table 8).

This study confirmed the carcinogenic effects of APM in rats and moreover clearly demonstrated an increased carcinogenic effect when life-span exposure to APM started from prenatal life.

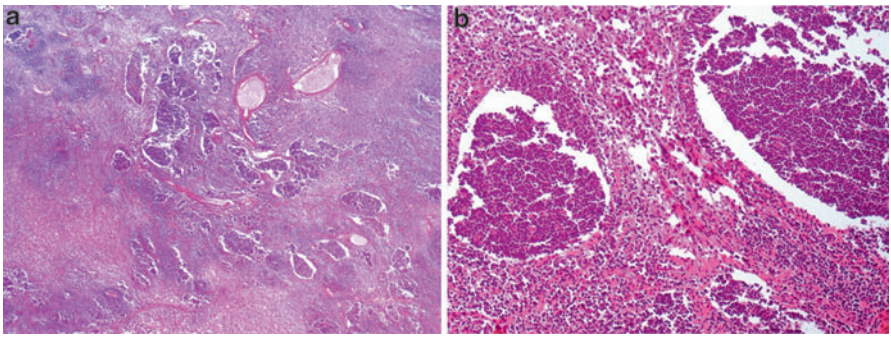


Fig. 4 Lymphocytic lymphoma in the thymus. (**a**) The architecture of the thymus is not maintained (HE 25X). (**b**) The cells are small to medium size resembling normal circulating lymphocytes that are arranged in high-density cell packages of probably monoclonal origin (HE 200X)

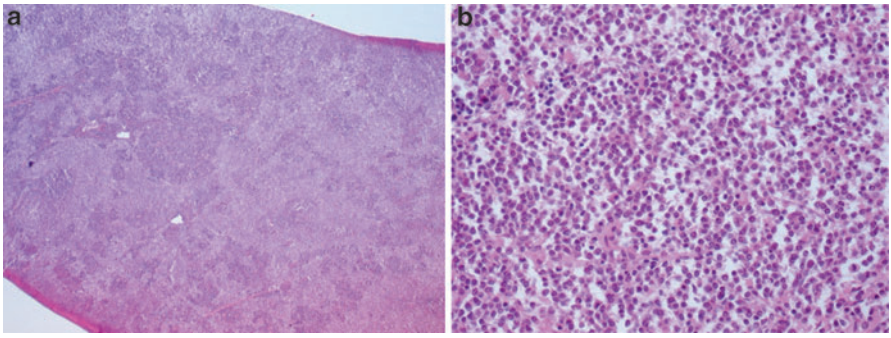


Fig. 5 Lymphoblastic lymphoma in the spleen. (**a**) The architecture of the spleen is not maintained (HE 25X). (**b**) The cells are non-cohesive, medium-sized to large lymphoblasts. The nuclear to cytoplasmic ratio is high and the cytoplasm is moderate in amount and often basophilic (HE 200X)

6.3 Carcinogenicity Bioassay on APM Administered in Feed to Swiss Mice from Prenatal Life until Natural Death (Exp. BT6010)

Four groups of 128–225 male and female Swiss mice were treated with APM in feed at the concentrations of 32,000, 16,000, 8,000, 2,000, or 0 ppm from prenatal life until natural death [42]. The biophase proceeded smoothly and without any problem. During the study, no substantial differences among the male and female treated groups compared to controls were observed in feed consumption and mean body weights. Compared to the control group, slight decreased survival was

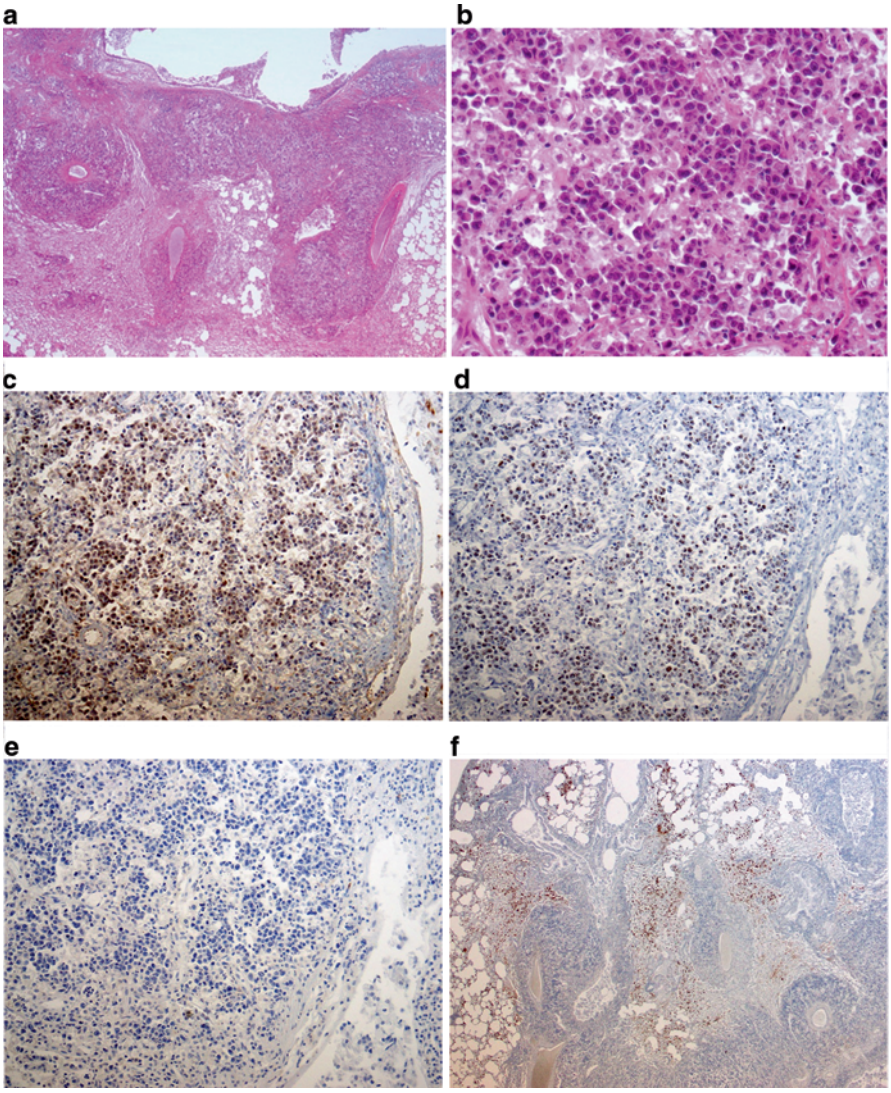


Fig. 6 Lymphoimmunoblastic lymphoma in the lung. (**a**) The pattern of organ involvement is aggressive with diffuse infiltration along the vascular tree in the lung (HE 25X). (**b**) The cells are large, non-cohesive, and monotypic; sometimes plasma cells may be present (HE 400X). (**c**) Pax5-positive staining in the zone occupied by the tumor shows the origin to be B-lymphocytes (200X). (**d**) Ki67-positive staining indicates a high index of cellular proliferation in the same area (200X). (**e**) CD68-negative staining in the zone occupied by the tumor (25X). (**f**) CD68-positive staining in the areas surrounding the lymphoma indicates that the macrophages are external to the tumor, and the lymphoma is not confused or mixed with inflammatory reaction (200X)

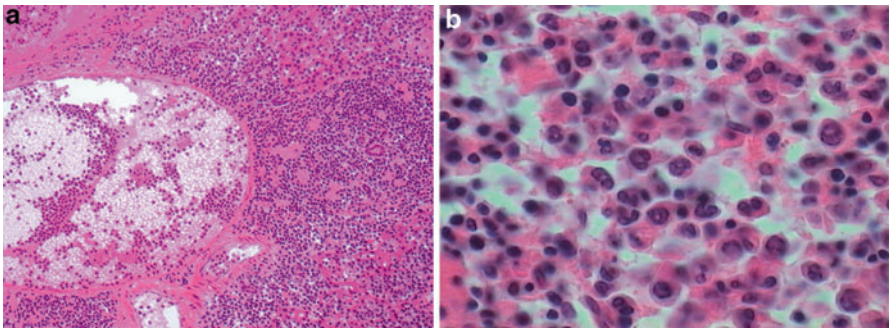


Fig. 7 Granulocytic or myeloid leukemia in the liver. (**a**) Basophilic cells are invading the blood vessels (HE 400X). (**b**) The cells are large and resemble developing or immature granulocytes. The cells may show some ring or lobed forms and others may be blastic in appearance (HE 1000X oil)

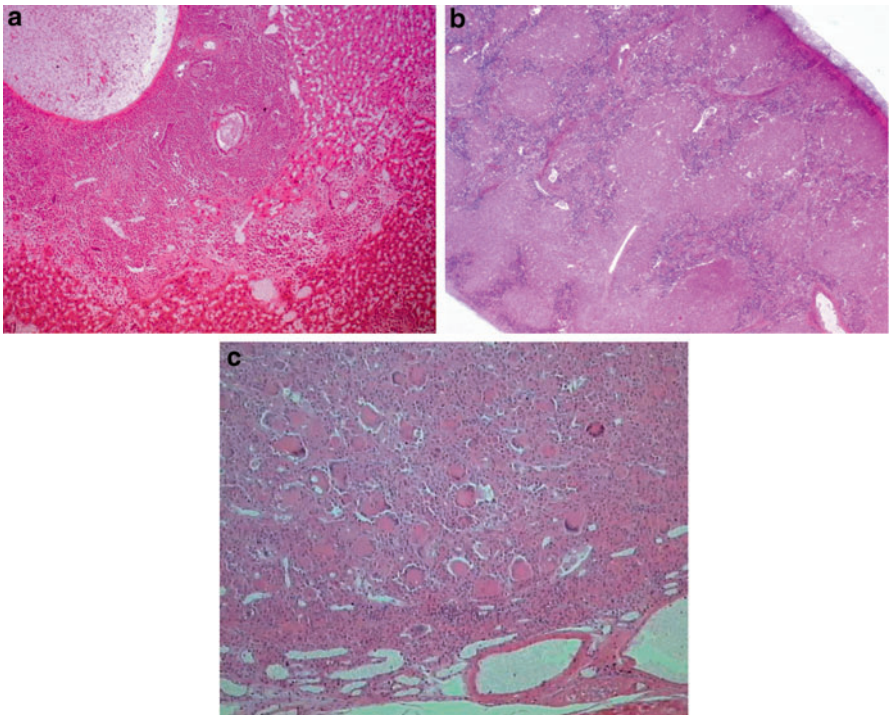


Fig. 8 Histiocytic sarcoma. The neoplasm involves the liver (**a**), spleen (**b**), and thymus (**c**). (**a**) In the liver, the tumor is characterized by a uniform monomorphic population of rounded cells with foamy eosinophilic cytoplasm (HE 100X). (**b**) In the spleen, the neoplastic cells invade the white pulp giving a pink color to the organ (HE 25X). (**c**) In the thymus, many multinucleated giant cells are scattered throughout the tumor (HE 200X)

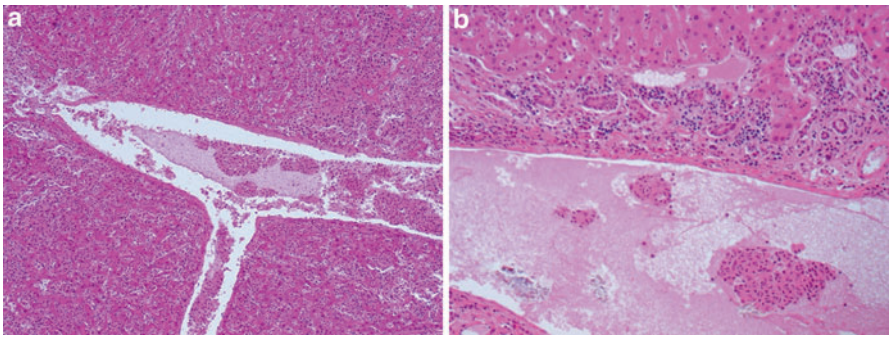


Fig. 9 Monocytic leukemia in the liver. The neoplastic cells appear immature and invade the blood vessels (HE 25X). (**b**) Higher magnification (HE 400X)

observed in treated males and females from 104 weeks of age until the end of the study [42].

The carcinogenic effects of APM in treated mice compared to controls are reported in Tables 9, 10, and 11. The data show (1) no significant difference among the treated groups versus control group in the incidences of animals bearing benign and malignant tumors (Table 9); (2) a significant increased incidence (dose-related) of alveolar carcinomas of the lung in males treated with APM at 32,000 and 16,000 ppm and a significant dose-related difference in the incidence of alveolar/bronchial adenoma in treated groups versus control group (Table 10). Microscopically, alveolar/bronchial adenomas presented different patterns of growth with a sharp demarcation from the adjacent parenchyma. The most common feature was papillary type with deeply basophilic cells (Fig. 11a, b). Alveolar/bronchial carcinomas presented an irregular nodular growth that tended to occupy the entire lobe invading the surrounding parenchyma. The most frequent feature was the papillary growth pattern (Fig. 12a, b); (3) a significant increased (dose-related) incidence of hepatocarcinoma of the liver in males treated at 32,000 and 16,000 ppm of APM compared to controls, as well as a dose-related (not significant) increased incidence of hemangiosarcomas (Table 11). The liver tumors were usually grossly described as nodules, varying in size and color. Microscopically, they were distinguished as adenomas and hepatocarcinomas. Hepatocellular adenoma appeared as a very-well-demarcated nodular lesion causing distinct compression of the adjacent parenchyma. It appeared solid and moderately trabecular and the cells occurred in irregular plates two to three layers thick (Fig. 13a, b). The hepatocellular carcinoma was not well demarcated from the surrounding tissue and was characterized by an abnormal growth pattern with cellular atypia. The trabecular pattern was the most frequent type (Fig. 14a, b).

Table 6 Long-term carcinogenicity bioassay on ASPARTAME, administered with the feed, supplied ad libitum, from prenatal life until natural death, to male (M) and female (F) Sprague-Dawley rats (Experiment BT6009)

Results: benign and malignant tumors											
Group No.	Concentration (ppm)	Animals		Benign tumors				Malignant tumors			
				Tumor-bearing animals		Tumors		Tumor-bearing animals		Tumors	
		Sex	No.	No.	%	No.	Per 100 animals	No.	%	No.	Per 100 animals
I	2,000	M	70	44	62.9	60	85.7	28	40.0**	31	44.3
		F	70	58	82.9°	146	208.6	37	52.9	60	85.7
		M+F	140	102	72.9	206	147.1	65	46.4	91	65.0
II	400	M	70	43	61.4	65	92.9	18	25.7	19	27.1
		F	70	54	77.1	166	237.1	31	44.3	44	62.9
		M+F	140	97	69.3	231	165.0	49	35.0	63	45.0
III	0 (control)	M	95	66	69.5	98	103.2	23	24.2***	26	27.4
		F	95	75	78.9****	190	200.0	42	44.2	48	50.5
		M+F	190	141	74.2	288	151.6	65	34.2	74	38.9

°Statistically significant ($P \leq 0.05$) using logistic regression with a time covariate

**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 the analysis of the trend

****Near the control incidence are the p-values ($P \leq 0.05$) associated with the logistic regression with a time covariate for the analysis of the trend

Table 7 Long-term carcinogenicity bioassay on ASPARTAME, administered with the feed, supplied ad libitum, from prenatal life until natural death, to male (M) and female (F) Sprague-Dawley rats (Experiment BT6009)

Results: mammary benign and malignant tumors											
Group No.	Concentration (ppm)	Animals		Mammary benign tumors				Carcinomas			
				Tumor-bearing animals		Tumors		Tumor-bearing animals		Tumors	
		Sex	No.	No.	%	No.	Per 100 animals	No.	%	No.	Per 100 animals
I	2,000	M	70	0	–	0	–	2	2.9	2	2.9
		F	70	34	48.6	54	77.1	11	15.7*	15	21.4
		M+F	140	34	24.3	54	38.6	13	9.3	17	12.1
II	400	M	70	3	4.3	3	4.3	0	–	0	–
		F	70	38	54.3	52	74.3	5	7.1	6	8.6
		M+F	140	41	29.3	55	39.3	5	3.6	6	4.3
III	0 (control)	M	95	2	2.1	3	3.2	0	–	0	–
		F	95	45	47.4	65	68.4	5	5.3**	6	6.3
		M+F	190	47	24.7	68	35.8	5	2.6	6	3.2

*Statistically significant ($P \leq 0.05$) using Cox Regression Model

**Near the control incidence are the p-values ($P \leq 0.05$) associated with the Cox Regression Model for the analysis of the trend

Table 8 Long-term carcinogenicity bioassay on ASPARTAME, administered with the feed, supplied ad libitum, from prenatal life until natural death, to male (M) and female (F) Sprague-Dawley rats (Experiment BT6009)

Results: haematological neoplasias					
Group No.	Concentration (ppm)	Animals		Animals with Haematological neoplasias	
		Sex	No.	No.	%
I	2,000	M	70	12	17.1*
		F	70	22	31.4**
		M+F	140	34	24.3
II	400	M	70	11	15.7
		F	70	12	17.1
		M+F	140	23	16.4
III	0 (control)	M	95	9	9.5
		F	95	12	12.6***
		M+F	190	21	11.1

*Statistically significant ($P \leq 0.05$) using Cox Regression Model
**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

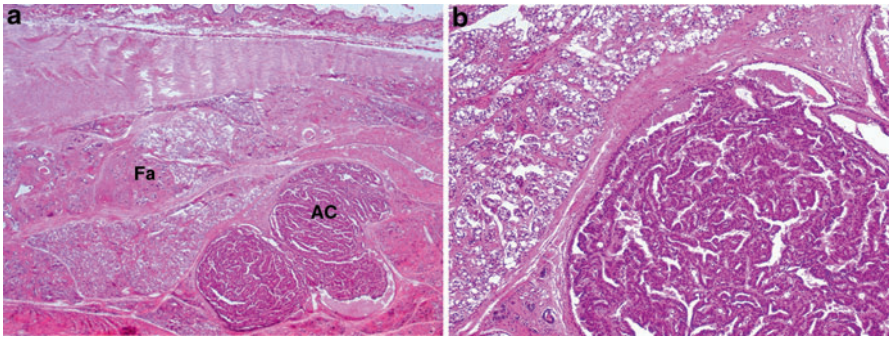


Fig. 10 Mammary gland adenocarcinoma. (**a**) Adenocarcinoma (*AC*) in fibroadenoma (*Fa*) diagnosed in a mammary lump from a female rat (HE 25X). (**b**) Note the papillary and ductular growth with anaplastic glandular features (HE 100X)

7 Epidemiological Studies on Cancer Risks and Exposure to Aspartame

Not until the early 2000s, there were two epidemiological case-control studies conducted to evaluate the carcinogenic risks among people who consumed products containing APM. The first study [43] showed an increased (nonsignificant) risk of brain tumors correlating with consumption of drinks containing APM. A second study [44] showed an increased trend of medulloblastoma in children born by

Table 9 Long-term carcinogenicity bioassay on ASPARTAME, administered with the feed, supplied ad libitum, from prenatl life until natural death to male (M) and female (F) Swiss mice (Experiment BT6010)

Results: benign and malignant tumors											
Group No.	Concentration (ppm)	Animals		Benign tumors				Malignant tumors			
				Tumor-bearing animals		Tumors		Tumor-bearing animals		Tumors	
		Sex	No.	No.	%	No.	Per 100 animals	No.	%	No.	Per 100 animals
I	32,000	M	83	22	26.5	29	34.9	57	68.7	87	104.8
		F	62	32	51.6	64	103.2	40	64.5	55	88.7
		M+F	145	54	37.2	93	64.1	97	66.9	142	97.9
II	16,000	M	64	26	40.6	33	51.6	39	60.9	63	98.4
		F	64	30	46.9	51	79.7	44	68.8	61	95.3
		M+F	128	56	43.8	84	65.6	83	64.8	124	96.9
III	8,000	M	62	20	32.3	26	41.9	45	72.6	66	106.5
		F	73	30	41.1	49	67.1	47	64.4	61	83.6
		M+F	135	50	37.0	75	55.6	92	68.1	127	94.1
IV	2,000	M	103	35	34.0	49	47.6	58	56.3	76	73.8
		F	122	53	43.4	81	66.4	90	73.8	117	95.9
		M+F	225	88	39.1	130	57.8	148	65.8	193	85.8
V	0 (control)	M	117	42	35.9	53	45.3	66	56.4	94	80.3
		F	102	39	38.2	56	54.9	69	67.6	92	90.2
		M+F	219	81	37.0	109	49.8	135	61.6	186	84.9

Table 10 Long-term carcinogenicity bioassay on ASPARTAME, administered with the feed, supplied ad libitum, from prenatal life until natural death to male (M) and female (F) Swiss mice (Experiment BT6010)

Results: tumors of the lung									
Group No.	Concentration (ppm)	Animals		Tumor-bearing animals[a]					
				Alveolar/ bronchiolar Adenomas		Alveolar/ bronchiolar Adenocarcinomas		Total	
		Sex	No.	No.	%	No.	%	No.	%
I	32,000	M	83	6	7.2	11	13.3**	17	20.5
		F	62	3	4.8	2	3.2	5	8.1
		M+F	145	9	6.2	13	9.0	22	15.2
II	16,000	M	64	7(1)	10.9	8(3)	12.5*	15(4)	23.4
		F	64	2	3.1	7	10.9	9	14.1
		M+F	128	9	7.0	15	11.7	24	18.8
III	8,000	M	62	7(1)	11.3	7	11.3	14(1)	22.6
		F	73	3	4.1	6	8.2	9	12.3
		M+F	135	10	7.4	13	9.6	23	17.0
IV	2,000	M	103	9(2)	8.7	6	5.8	15(2)	14.6
		F	122	9	7.4	10	8.2	19	15.6
		M+F	225	18	8.0	16	7.1	34	15.1
V	0 (control)	M	117	8(1)	6.8[b]	7	6.0*	15(1)	12.8***
		F	102	4	3.9	7	6.9	11	10.8
		M+F	219	12	5.5	14	6.4	26	11.9

[a]Between brackets are reported the number of animals bearing multiple tumors
[b]Near the control incidence are the p-values associated with the trend test
*Statistically significant ($P \leq 0.05$), using Cox proportional hazard model
**Statistically significant ($P \leq 0.01$), using Cox proportional hazard model
***Statistically significant ($P \leq 0.05$) using logistic analysis

mothers who frequently consumed diet soda during the preconception period and during pregnancy.

After the publication of the results of the Ramazzini Institute bioassays, some groups were motivated to perform epidemiological studies to evaluate the potential carcinogenic risks among consumers of products containing APM, particularly in diet beverages. Of these, two prospective studies, one conducted at the US National Cancer Institute (NCI) and the second at the University of Harvard, dealt with hematopoietic cancers.

The NCI study was conducted on 473,984 males and females aged 50–71 who were surveyed in 1995 and followed up until 2000 for signs of gliomas (315 cases) and hematopoietic tumors (1,885 cases). The authors reported that for a daily intake of APM > 900 mg/day, no significant increased risk of hematopoietic neoplasms (RR 0.98, 95% CI 0.76–1.27) or gliomas (RR 0.73, 95% CI 0.46–1.19) was observed [45]. However, it must be noted that the limited duration of exposure,

Table 11 Long-term carcinogenicity bioassay on ASPARTAME, administered with the feed, supplied ad libitum, from prenatal life until natural death to male (M) and female (F) Swiss mice (Experiment BT6010)

Results: preneoplastic and neoplastic lesions of the liver													
Group No.	Dose (ppm)	Animals		Bearing animals									
		Sex	No.	Foci		Hepatocellular adenoma		Hepatocellular carcinoma		Hemangioma		Hemangiosarcoma	
				No.	%	No.	%	No.	%	No.	%	No.	%
I	32,000	M	83	2	2.4	2	2.4	15	18.1**	0	–	8	9.6
		F	62	0	–	0	–	0	–	0	–	1	1.6
		M+F	145	2	1.4	2	1.4	15	10.3	0	–	9	6.2
II	16,000	M	64	2	3.1	6	9.4	10	15.6*	1	1.6	5	7.8
		F	64	1	1.6	0	–	2	3.1	2	3.1	3	4.7
		M+F	128	3	2.3	6	4.7	12	9.4	3	2.3	8	6.3
III	8,000	M	62	2	3.2	4	6.5	9	14.5	1	1.6	5	8.1
		F	73	1	1.4	2	2.7	0	–	0	–	0	–
		M+F	135	3	2.2	6	4.4	9	6.7	1	0.7	5	3.7
IV	2,000	M	103	2	1.9	10	9.7	12	11.7	2	1.9	5	4.9
		F	122	1	0.8	6	4.9	2	1.6	3	2.5	3	2.5
		M+F	225	3	1.3	16	7.1	14	6.2	5	2.2	8	3.6
V	0 (control)	M	117	5	4.3	9	7.7	6	5.1***	2	1.7	5	4.3
		F	102	2	2.0	1	1.0	0	–	0	–	4	3.9
		M+F	219	7	3.2	10	4.6	6	2.7	2	0.9	9	4.1

*Statistically significant ($P \leq 0.05$), using Cox proportional hazard model
**Statistically significant ($P \leq 0.01$), using Cox proportional hazard model
***Near the control incidence is the p-values ($P \leq 0.01$) associated with trend test

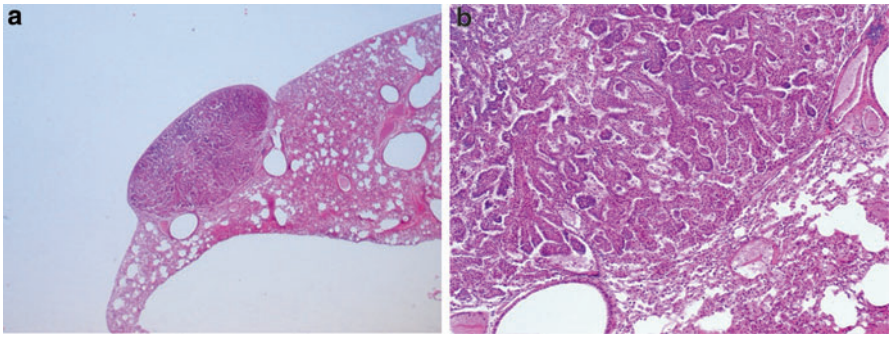


Fig. 11 Bronchiolo-alveolar adenoma in mice. (**a**) Papillary structure (HE 25X). (**b**) Higher magnification (HE 200X)

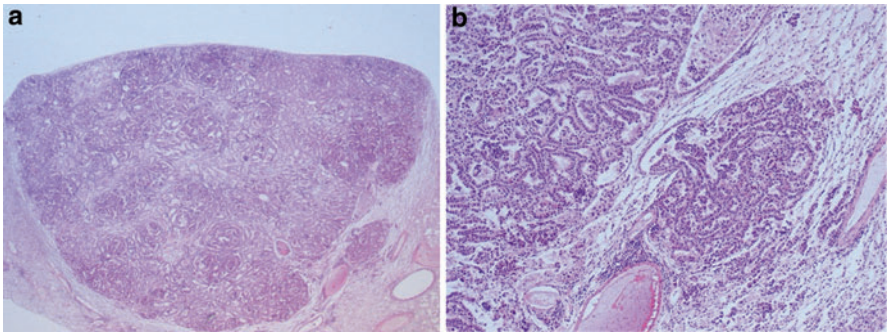


Fig. 12 Bronchiolo-alveolar carcinoma in mice. (**a**) Carcinoma with irregular growth invading large parts of the lobe (HE 25X). (**b**) The most frequent feature is the papillary growth pattern (HE 200X)

the limited follow-up, and the low exposure levels greatly reduce the power to detect an effect.

The Harvard Study [6] conducted a prospective study on diet soda containing APM in relation to cancers in two cohorts: (1) the Nurses' Health Study, which started in 1976, and includes 121,701 female registered nurses; and (2) the Health Professional Follow-up Study which began in 1986 and includes 51,529 male health professionals. The study design covered diet soda consumption from 1984 for women and for both genders from 1986. The diet was reassessed every 4 years until 2006. The final study population included 77,218 women and 47,810 men. The authors concluded that in men a statistically significant increase was observed in the risk of non-Hodgkin lymphoma in individuals who consumed >1 serving of diet soda/day. Moreover, in men, the risk of multiple myeloma increased linearly with increased consumption, and a statistically significant increase was observed in

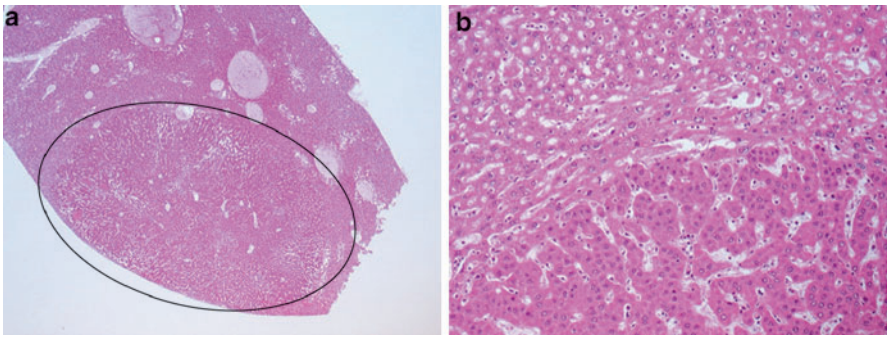


Fig. 13 Hepatocellular adenoma. (**a**) Hepatocellular adenoma (HE 25X). (**b**) Higher magnification (HE 200X)

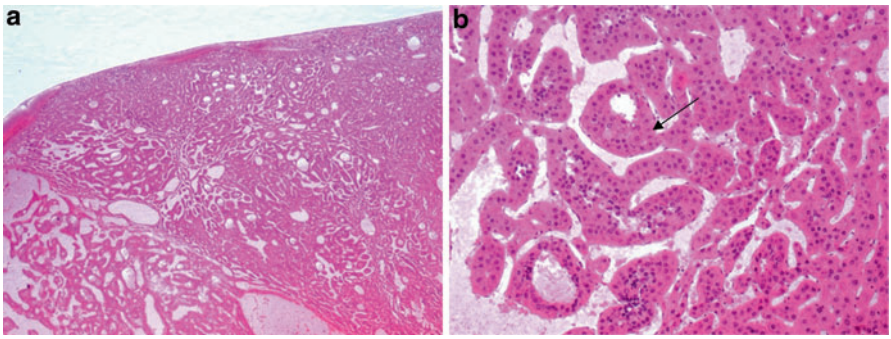


Fig. 14 Hepatocellular carcinoma. (**a**) Hepatocellular carcinoma (HE 25X). (**b**) Higher magnification (HE 200X)

individuals who consumed >1 diet soda/day. The authors concluded that their data provide some support for the evidence that APM induces hematopoietic neoplasias in animals chronically exposed to APM. However, the authors claimed that because this was the first demonstration of the carcinogenic effects of APM in humans, and because in the experimental studies [40, 41] the hematopoietic neoplasias were shown in females and not in males, the results need confirmation by other cohort studies in order to rule out chance as a possible explanation.

8 Conclusions

Aspartame is an artificial sweetener discovered by GD-Searle in 1965. The tests to evaluate its safety before commercialization were performed by the producer in the 1970s. Queries regarding the conduct of these studies were raised in the 1980s and

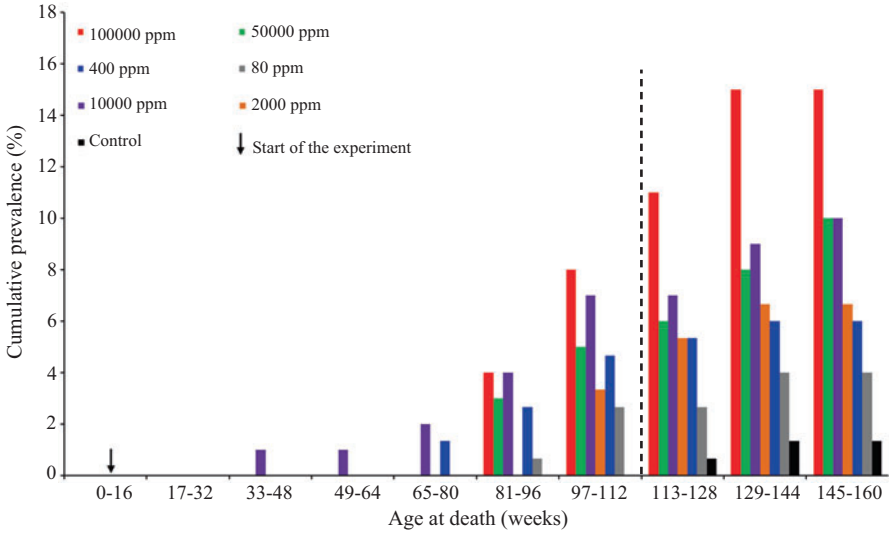


Fig. 15 Cumulative prevalence of female animals with carcinoma and atypical lesions of the pelvis and ureter, by age at death

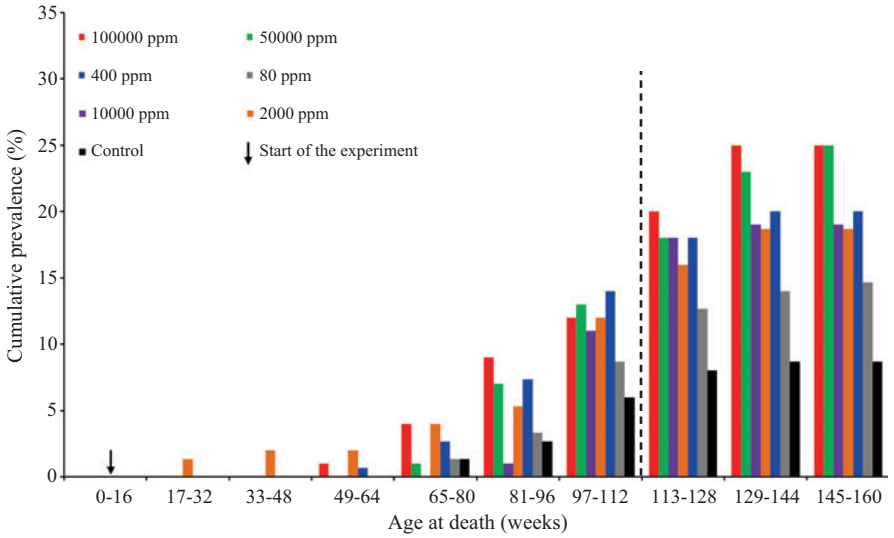
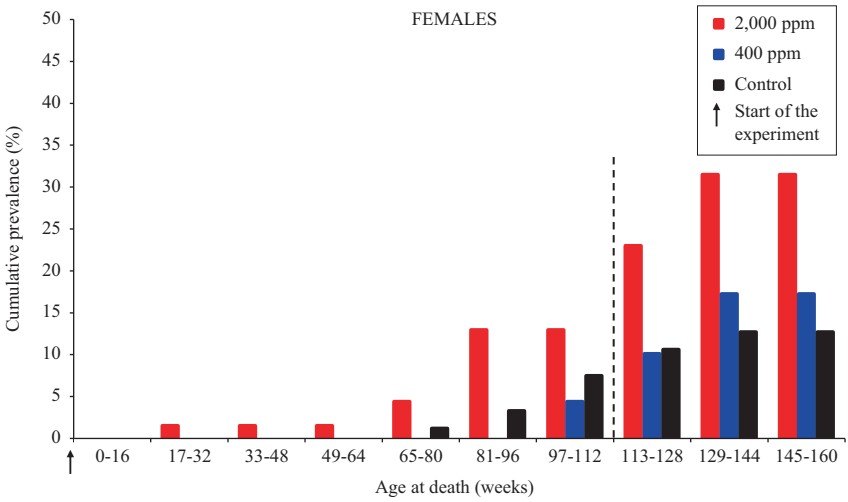


Fig. 16 Cumulative prevalence of female animals with hemolymphoreticular neoplasias, by age at death

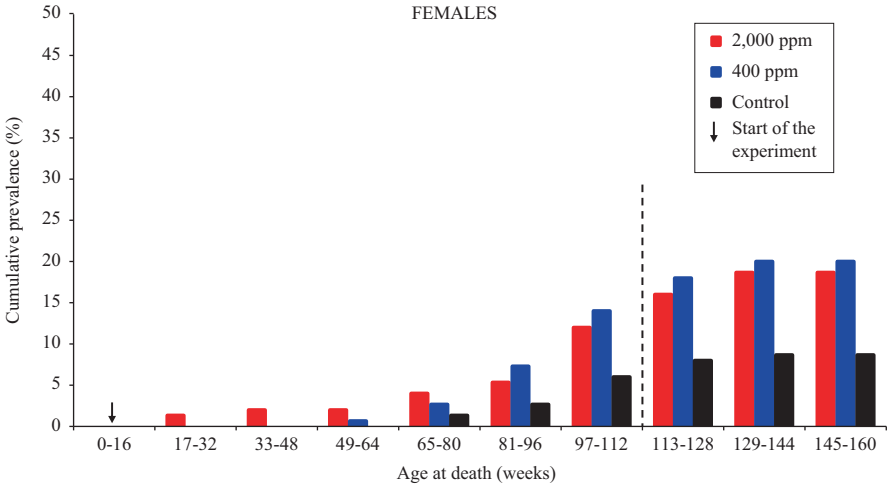
are still present today. Because of the large-scale use of APM in more than 6,000 products and because pregnant women and children are the main consumers, it became urgent we perform new studies to evaluate the potential carcinogenic effects of APM using a more sensitive experimental model. For this reason, at the end of the 1990s, the Ramazzini Institute started a large project of carcinogenicity bioassays on rats and mice using the experimental methods employed from more than 40 years [34, 35].

The results of these studies showed that APM may induce a significant dose-related increased incidence of multiple malignant tumors in two species, rats and mice, and in both genders. The consistency and robustness of the evidence as to the carcinogenic potential of APM are supported by the following considerations due to the distinctive characteristics of the method followed by the Ramazzini Institute in conducting the carcinogenicity bioassays: (1) life-span observation of the animals enabled us to detect the tumor trend in the last part of the life-span which is the time that brings out the difference between treated and untreated experimental animals. As shown by the cumulative prevalence of carcinomas and atypical lesions of the pelvis and ureter, represented in Fig. 15, if the experiment had been truncated at 110 weeks of age, probably the dose-related significance of the increased incidences of these lesions would not have been so clear. The same pattern holds true for what concerns hematological neoplasias in females. The cumulative prevalence of females bearing hematological neoplasias shows the difference between treated and untreated animals after 112 weeks of age (Fig. 16); (2) exposure starting from prenatal life increases the carcinogenic effects of APM. Indeed as shown in Fig. 17, the cumulative prevalence of hematological neoplasias in females exposed from prenatal life is higher than the cumulative prevalence in females exposed from mature age; (3) if the animals are allowed to survive until natural death, it is possible to observe the diffusion of hematological neoplasias into multiple organs and tissues. In the study where exposure to 2,000 ppm APM started from prenatal life, out of 22 females bearing hematological neoplasias, 14 involved the lung and other organs, while eight involved other organs though not the lung. Concerning lymphoimmunoblastic lymphoma in lung, the histopathological diagnosis of this type of lesions was confirmed by immunohistochemical stainings (Fig. 6c-f); (4) moreover, the prospective epidemiological study performed by the Harvard group supported the findings of the Ramazzini Institute in showing a positive association between APM exposure and hematological neoplasias in humans.

However, it cannot be forgotten that the evidence of the carcinogenic effects of APM documented by the experimental studies of the Ramazzini Institute and by the epidemiological results from the group at Harvard generated intense and coordinated criticism by spokespersons for the chemical industry in Europe, Japan, and the USA, as well as from EFSA and FDA, which led to a prompt reply by the Ramazzini Institute in a commentary published in 2014 [46].



Life span feed carcinogenicity s tu d y of **ASPARTAME (BT 6009)**: cumulative prevalence of animals with **hemolymphoreticular neoplasias**, by age at death



Life span feed carcinogenicity study of **ASPARTAME (BT 6008)**: cumulative prevalence of animals with **hemolymphoreticular neoplasias**, by age at death

Fig. 17 Comparison of cumulative prevalence of female animals with hemolymphoreticular neoplasias, by age at death, in experiments starting at 8 weeks of age or during fetal life

Finally, if we consider that in our experimental conditions, the carcinogenic effects of APM are evident in female rats even at 400 ppm in feed, equivalent at a daily dose of 20 mg/kg b.w., much less than the current ADI for humans in Europe (40 mg/kg b.w.), we must conclude that it is urgent that agencies, such as the WHO, EFSA, FDA, international scientific institutions like IARC, and national public health institutions, take a stand and re-examine their evaluation on APM. Moreover,

we recommend pregnant women and children to abstain from consuming products containing APM.

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