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Results of life span carcinogenicity bioassay on Sprague-Dawley rats exposed to aspartame since foetal life

Risultati dell'esperimento di cancerogenesi a lungo termine su ratti Sprague-Dawley esposti ad aspartame a partire dalla vita fetale

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Summary

The artificial sweetener aspartame (APM), over two hundred times sweeter than sucrose, is an additive contained in over 6,000 products, including almost 500 drugs. As part of the Ramazzini Institute's mega-experiment project to test the carcinogenic potential of numerous products commonly used in the diet of millions of people, the Authors report the full results of the lifespan carcinogenicity bioassay on APM administered in animal feed from foetal life until natural death. A total of 470 Sprague-Dawley (S-D) rats were divided by number and sex into three different groups of 70-95 animals. APM was administered in their feed at concentrations of 2,000, 400 or 0 ppm to simulate a daily APM intake of 100, 20 or 0 mg/kg b.w., respectively. The results have shown: 1) a significantly increased dose-related incidence ($p \leq 0.05$) of females bearing benign tumours, particularly in the group of 2,000 ppm ($p \leq 0.05$); a significantly increased dose-related incidence ($p \leq 0.01$) of males bearing malignant tumours, in particular in the 2,000 ppm group ($p \leq 0.01$); 2) a signifi-

Riassunto

Il dolcificante artificiale aspartame (APM), oltre 200 volte più dolce dello zucchero, è un additivo contenuto in oltre 6.000 prodotti, inclusi circa 500 medicinali. Nell'ambito dei progetti sui mega esperimenti dell'Istituto Ramazzini, volti a testare il potenziale cancerogeno di numerosi prodotti comunemente usati nella dieta da milioni di persone, nel presente lavoro vengono riportati i risultati completi del saggio di cancerogenicità a lungo termine sull'APM somministrato nel cibo dalla vita fetale fino a morte spontanea. Un totale di 470 ratti Sprague-Dawley (S-D) sono stati equamente divisi per numero e sesso in 3 differenti gruppi di 70-95 animali. L'APM è stato somministrato nel cibo alle concentrazioni di 2.000, 400 o 0 ppm per simulare rispettivamente un'assunzione giornaliera di APM di 100, 20 o 0 mg/kg p.c. I risultati hanno mostrato: 1) un significativo aumento dose-correlato dell'incidenza ($p \leq 0,05$) di femmine portatrici di tumori benigni, particolarmente per quanto riguarda il gruppo 2.000 ppm ($p \leq 0,05$); un aumento significativo dose-correlato ($p \leq 0,01$) dell'incidenza di maschi portatori di tumori maligni,

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cantly increased dose-related incidence (p≤0.05) of mammary adenocarcinomas in females, particularly in the 2,000 ppm group (p≤0.05); 3) a significantly increased incidence (p≤0.05) of lymphomas/leukaemias in males treated at 2,000 ppm; and 4) a significantly increased dose-related incidence (p≤0.01) of lymphomas/leukaemias in females, particularly in the 2,000 ppm group (p≤0.01). Our experimental conditions confirm the carcinogenic potential of APM in rats. The data also show that when the lifespan treatment starts from foetal life the carcinogenic effects are heightened. Eur. J. Oncol., 16 (2), 81-97, 2011

Key words: **aspartame, artificial sweetener, Sprague-Dawley rats, carcinogenicity, prenatal exposure, life span observation**

in particolare nel gruppo 2.000 ppm (p≤0,01); 2) un significativo aumento dose-correlato dell'incidenza (p≤0,05) di carcinomi mammari nelle femmine, in particolare nel gruppo 2.000 ppm (p≤0,05); 3) un significativo aumento dell'incidenza (p≤0,05) di linfomi/leucemie nei maschi trattati con 2.000 ppm; e 4) un significativo aumento dell'incidenza dose-correlato (p≤0,01) di linfomi/leucemie nelle femmine, particolarmente nel gruppo 2.000 ppm (p≤0,01). Nelle nostre condizioni sperimentali è stato confermato l'effetto cancerogeno dell'APM nei ratti. Inoltre i dati mostrano che quando il trattamento per tutta la vita inizia dalla vita fetale, gli effetti cancerogeni risultano amplificati. Eur. J. Oncol., 16 (2), 81-97, 2011

Parole chiave: **aspartame, dolcificante artificiale, ratti Sprague-Dawley, cancerogenicità, esposizione prenatale, osservazione per tutta la vita**

Introduction

Consumerism and social changes have profoundly altered dietary habits in industrialized countries. Unhealthy so-called "fast food", "junk food", together with "pre-packed food", now mainly replace traditional cooking. As a consequence the new diet may contain various types of chemical agents, namely additives, flavouring, colouring and preservatives.

The paucity of scientific data on the potential long-term carcinogenic risks for human beings using such types of food led the Ramazzini Institute (RI) as of 1985 to develop an integrated set of mega-experiments to test the carcinogenic potential of many common products: beverages like ethyl alcohol (1) and Coca Cola (2); integrators like Vitamin A (3) and Vitamin C and E; contaminants like Mancozeb (4), a well-known fungicide; additives like aspartame and sucralose; and preservatives like formaldehyde (5). Particular interest has been aroused in the scientific community, consumers, the food industry and health-care organizations by recent reports from the RI on the carcinogenic potential of aspartame (APM) which, after saccharine, is the most widely used artificial sweetening agent in the world with over 17,000 tons produced every year (6). Some of APM's trade names are NutraSweet, Equal, or E951 (generally in ingredient lists).

APM was discovered accidentally in 1965 when a researcher at the G.D. Searle Company was testing an anti-ulcer drug (7). Currently, as a food additive, APM is present in more than 6,000 products (e.g. soft drinks, light nectars, chewing gum, candy, yogurt) including over 500 pharmaceuticals (e.g. vitamins, sugar-free cough drops, antibiotics and paediatric drugs).

Among the hundreds of millions of people around the world using APM, some of the most frequent consumers include infants, children and women of childbearing age whose daily consumption has been estimated to be 2.5-5 mg/kg b.w. (8). The current accepted daily intake (ADI) of APM in the USA and European Community is 50 mg/kg b.w. and 40 mg/kg b.w., respectively.

When metabolized, APM is transformed into its three constituents: phenylalanine (Phe), aspartic acid (Asp) and methanol (MetOH).

Apart from being used directly for protein synthesis, amino acid Phe is transformed into alanine plus oxalacetate (9) while Asp is transformed into

tyrosine and to a lesser extent, into phenylethylamine and phenylpyruvate (10). Phe plays an important rôle in neurotransmitter regulation, whereas Asp is also thought to play a rôle as an excitatory neurotransmitter in the central nervous system.

MetOH is metabolized to formaldehyde and thereafter to formic acid and finally to CO_2 and water, via the formation of 10-formyl tetrahydrofolate (11, 12).

MetOH is not subject to metabolism within the enterocyte but rapidly enters the portal circulation and is oxidized in the liver to formaldehyde, a highly reactive chemical which binds strongly to proteins (13) and nucleic acids (14) forming formaldehyde adducts. In a study in which APM, 14C-labelled in methanol carbon, was given orally to adult male Wistar rats for 10 days, it was shown that the carbon adducts of protein and DNA could only have been generated from formaldehyde derived from APM MetOH. Moreover, it was suggested that the amount of formaldehyde adducts may be cumulative (15). Several reviews have concluded that APM is metabolized in all species in the same way (16).

No *in vitro* or *in vivo* data have shown that APM is genotoxic (17-20).

Epidemiological studies performed among users of artificial sweeteners, including APM, have not shown any increased carcinogenic risks when compared to unexposed groups of people (21, 22), except in one old study which showed an association between increased risk of brain cancer and use of APM (23).

Before the RI carcinogenicity bioassays in rodents, three long-term carcinogenicity studies were performed in the 1970s-1980s, two on rats and one on mice, by the industry producing APM. The results of these studies did not show any carcinogenic effects after exposure from foetal/juvenile age (4 weeks) until 104 weeks of age, at which stage the animals were sacrificed (24). Going on these data, the Food and Drug Administration (FDA) first approved the use of APM in solid food in 1981 (24, 25), then the authorization was extended to soft drinks in 1983 (26), and was finally approved as a general sweetener in 1996 (27). The European Community (EC) approved the general use of APM in 1994 (28), which was then confirmed by the European Food Safety Agency (EFSA) in 2006 (29).

Other experiments were published in 1981 by a Japanese group and, more recently, the US National Toxicology Program (NTP) tested the potential carcinogenic effects of APM in transgenic mice models (19, 30, 31).

In our opinion, the safety of APM with regard to its potential long-term toxic and carcinogenic effects has not been demonstrated by the aforementioned studies because of the small number of animals used per sex/per group and the duration of the experiments (never exceeding 110 weeks of age, corresponding to 2/3 of the life span of these animals).

For these reasons we planned a project encompassing several experiments on rats and mice in which APM was administered in the feed at various doses to large groups of rats or mice divided by sex, starting the treatment at various ages and lasting for various periods. All the animals were then kept under observation until spontaneous death so as to allow APM to express its full carcinogenic potential.

In the first experiment, including 1,800 Sprague-Dawley rats (100-150 per sex/per group), we demonstrated that APM, administered from 8 weeks of age for the life span to Sprague-Dawley rats, induced a significant increased incidence of lymphomas/leukaemias and of neoplastic lesions of the renal pelvis and ureter in females, and a significant increased incidence of malignant schwannomas of the peripheral nerves in males (6, 32, 33).

In a second experiment, conducted on 470 Sprague-Dawley rats the treatment started during prenatal life and lasted until spontaneous death. Partial results from this experiment, published in 2007 (34), showed a significant dose-related increase in mammary adenocarcinomas and lymphomas/leukaemias among females, and a significant increase in the incidence of malignant tumours in males. An increased incidence of mammary adenocarcinomas was also observed among males treated at the highest dose.

In a third experiment, conducted on 852 male and female Swiss mice, we demonstrated that APM, administered in feed for the whole life span starting from prenatal life, caused in males a significant increased incidence of hepatocellular carcinomas and alveolar/bronchiolar carcinomas (35).

The present paper reports the incidence of each histotype of benign and malignant tumours from the

experiment in which APM was administered to Sprague-Dawley rats for the life span from prenatal life. The results will be discussed also in reference to the comments of several Authors on our work.

Materials and methods

The APM used was produced by Ajinomoto and supplied by Giusto Faravelli S.p.A. Milan, Italy. Its purity, determined by infrared absorption spectrophotometer assay, was >98.7%: diketopiperazine was <0.3% and L-phenylalanine was <0.5%. APM was added to the standard pelleted diet at concentrations of 2,000, 400 or 0 ppm in order to simulate an assumed daily APM intake of 100, 20 or 0 mg/kg b.w. The daily APM assumption in mg/kg b.w. was calculated considering an average male-female body weight of 400 g and a daily feed consumption of 29 g/day throughout the experiment. The concentrations and the homogeneity of APM in the feed were measured from samples collected at three steps of pellet production (start, middle and end). The stability of APM in the feed was analyzed before starting the study and periodically confirmed throughout the bioassay. The feed was supplied *ad libitum*.

Male and female Sprague-Dawley rats from the colony of the Cesare Maltoni Cancer Research Center (CMCRC)/Ramazzini Institute (RI) were used. Having used this colony of rats for nearly 30 years, the CMCRC laboratory possesses a large amount of historical data on tumour incidence among untreated rats, adding to the information on the basic tumorigram of this strain of rats.

The rats in this experiment were born from strictly outbred matching. Since female breeders were being treated, the animals in the experimental groups were predetermined.

At 4-5 weeks of age all the males and females of each litter were identified by ear punch, separated by sex and assigned to the respective dose groups of 70-95 males and females, depending on the APM concentration being administered to the breeder. The rats were then housed in groups of 5, in makrolon cages with stainless-steel wire tops and a shallow layer of white wood-shavings as bedding, and kept in a room used only for this experiment, at a controlled temperature of 23±2°C and relative humidity of 50-60%.

Treatment began during foetal life, administering APM in the feed to female breeders from the 12th day of pregnancy, when organogenesis is completed and before the time when many tissues and organs are refractory to the effects of carcinogenic agents (36). The breeders were sacrificed after weaning and treatment of the offspring lasted until their natural death. Control animals received the same feed without APM.

All animals were observed until natural death. The experiment was conducted according to Italian law regulating the use and humanitarian treatment of animals for scientific purposes (37), and following the principles of Good Laboratory Practices.

Mean daily drinking water and feed consumption were measured per cage, and body weight was measured individually, beginning at 6 weeks of age and continuing once a week for the first 13 weeks, then every two weeks until 110 weeks of age. Measurement of body weight continued every 4 weeks until the end of the experiment. In order to detect and register all gross lesions, the animals were clinically examined every 2 weeks in the course of the experiment. Moreover, a patrol was performed three times daily from Monday to Friday and twice on Saturdays/Sundays and holidays to check the status and behavior of the animals and to limit *post mortem* modifications. In this way, deceased animals, once registered, were kept refrigerated up to a maximum of 16-19 hours at 4°C before necropsy.

Necropsy

The biophase, i.e. the in-life experimental phase, ended at 147 weeks, with the death of the last animal at the age of 144 weeks. Upon death, all animals underwent complete necropsy. Histopathology was routinely performed on the following organs and tissues of each animal from each group: skin and subcutaneous tissue, mammary gland, the brain (3 sagittal sections), pituitary gland, Zymbal glands, salivary glands, Harderian glands, cranium (5 sections, with oral and nasal cavities and external and internal ear ducts), tongue, thyroid, parathyroid, pharynx, larynx, thymus and mediastinal lymph

nodes, trachea, lung and mainstream bronchi, heart, diaphragm, liver, spleen, pancreas, kidneys, adrenal glands, oesophagus, stomach (fore and glandular), intestine (4 levels), urinary bladder, prostate, vagina, gonads, interscapular brown fat pad, subcutaneous and mesenteric lymph nodes, and other organs or tissues with pathological lesions. All organs and tissues were preserved in 70% ethyl alcohol, except for bones or calcified lumps which were fixed in 10% formalin and then decalcified with 10% formaldehyde and 20% formic acid in water solution.

Histopathology

Organ and tissue specimens were trimmed, following the CMCRC/RI Laboratory Standard Operating Procedures: in particular pathological specimens were trimmed to allow for the largest possible surface, including some normal adjacent tissue.

Trimmed specimens were processed as paraffin blocks, and 3-5 µm sections of every sample were obtained for routine staining with hematoxylin and eosin (HE). All slides were examined under light microscope by the same group of pathologists, following the same criteria of histopathological evaluation and classification. A senior pathologist reviewed all tumours and all other lesions of oncological interest.

Statistics

Statistical evaluations of the incidence and dose-response relationship of neoplastic lesions were performed using the Cox regression model and/or a logistic regression model with a time covariate (38). The resultant p-values are reported in the tables.

Results

The study proceeded smoothly without any unexpected contretemps. The feed consumption was not modified by APM addition in either male (M) or female (F) rats (fig. 1 and 2). There were no sizable changes in the body weight apart from a slight increase among treated females probably due to the higher onset of mammary tumours (fig. 3). Concerning survival (fig. 4 and 5), a slight decrease was observed in male and female treated rats compared to the controls. Oncological results are reported in Tables 1-7. Multiple tumours of different type and site, of different type within the same site, of the same type in bilateral organs, of the same type in the skin, in subcutaneous tissue, in mammary glands, or at distant sites of diffuse tissue (e.g. bones and skeletal muscle) were counted as single/independent tumours. Multiple tumours of the same

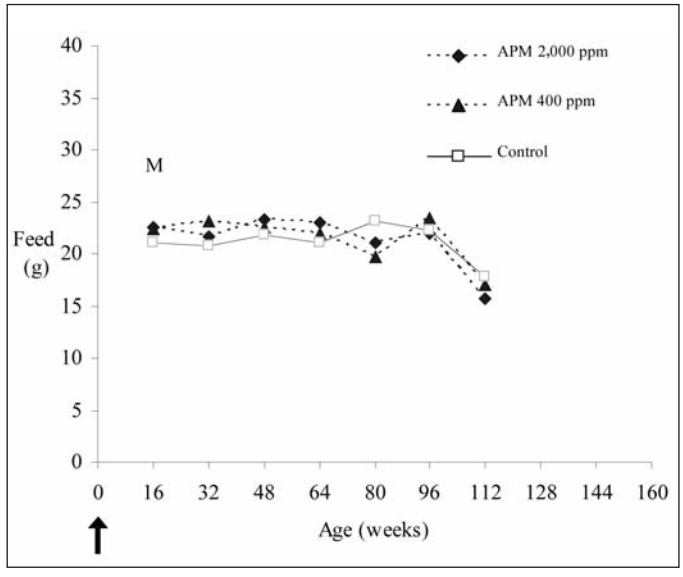

Fig. 1. Effects of APM exposure on feed consumption in S-D rats: mean daily feed consumption in males (M)

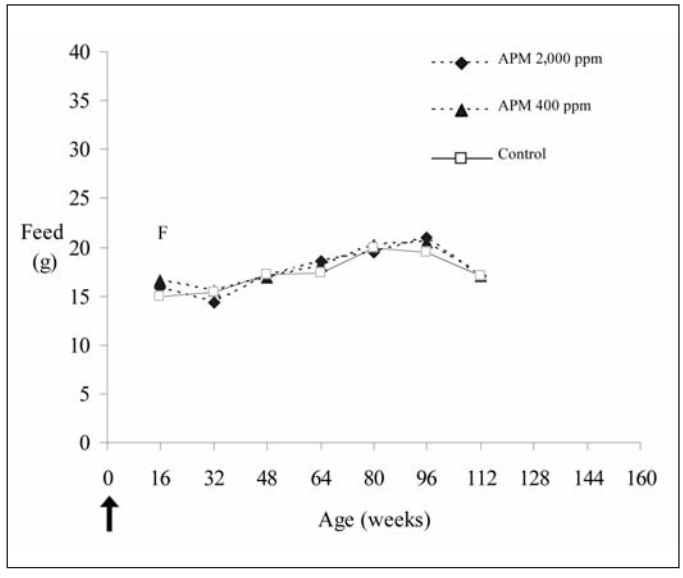

Fig. 2. Effects of APM exposure on feed consumption in S-D rats: mean daily feed consumption in females (F)

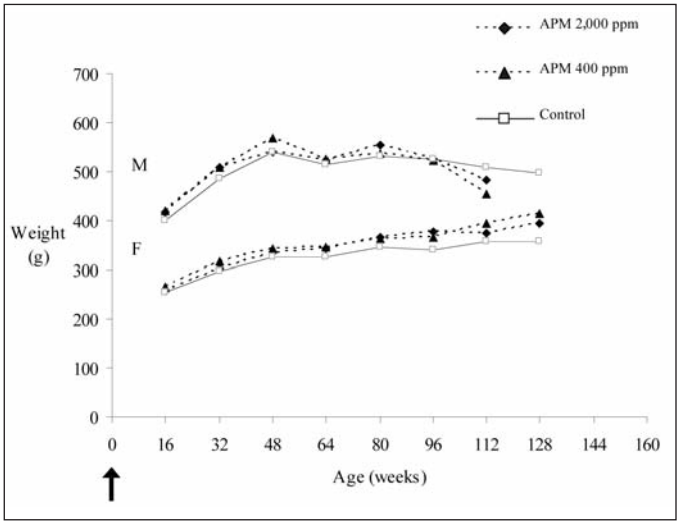

Fig. 3. Effects of APM exposure on body weight in S-D rats: mean body weights (males=M; females=F)

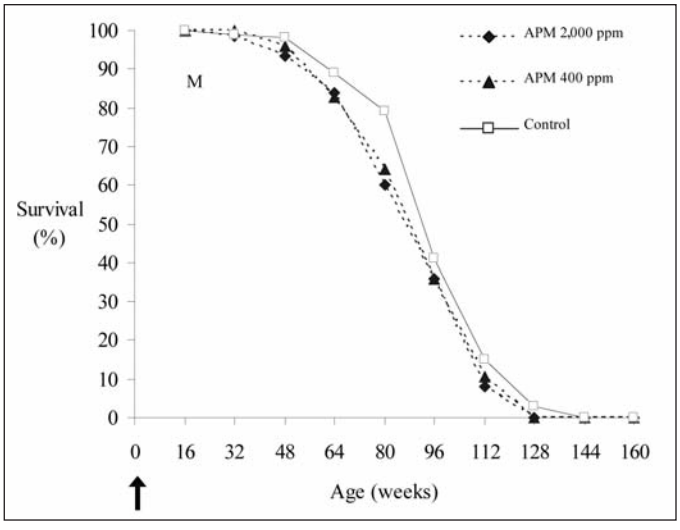

Fig. 4. Effects of APM exposure on survival in S-D rats: survival in males (M)

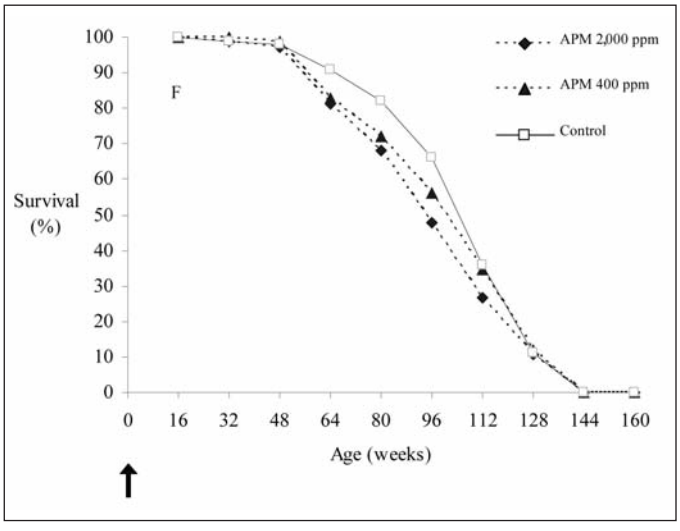

Fig. 5. Effects of APM exposure on survival in S-D rats: survival in females (F)

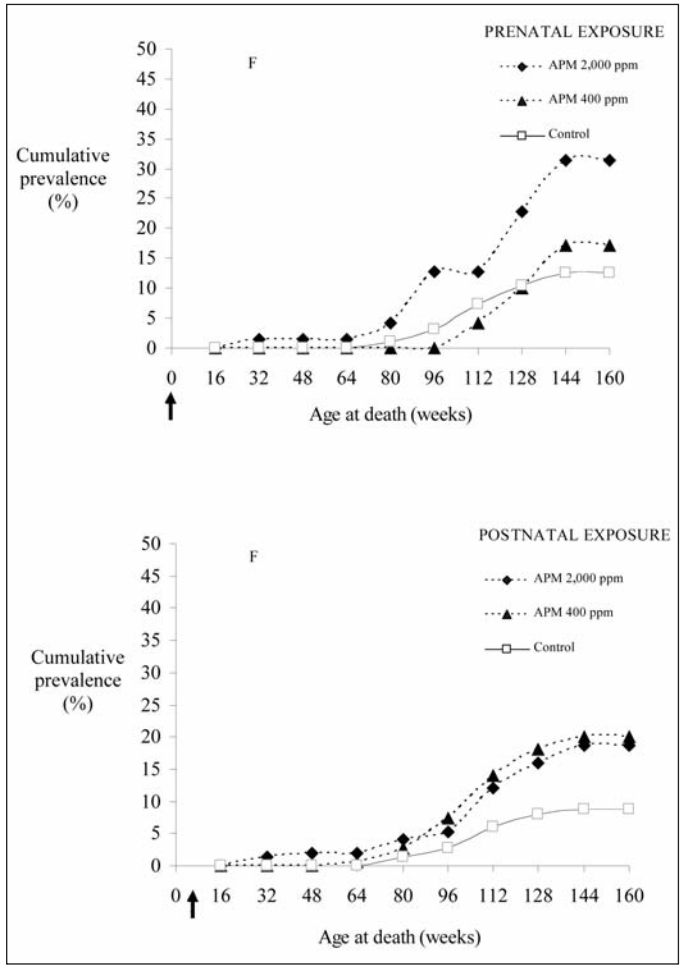

Fig. 6. Cumulative prevalence of death by age in female (F) S-D rats bearing hemolymphoreticular neoplasias. Arrows indicate the start of the experiment

type in the same tissue and organ, apart from those listed above, were counted only once.

The occurrence of total benign and malignant tumours in males and females is shown in Table 1. The noteworthy differences among the groups are listed below.

Benign and malignant tumours

The overall incidence of animals bearing benign tumours is reported in Table 2. A significant dose-related increase ($p \leq 0.05$) was observed in females. The incidence was significantly increased in females treated with 2,000 ppm ($p \leq 0.05$). A significant increase ($p \leq 0.05$) in the incidence of uterus polyps and osteomas of the head was observed in the 400 ppm female treated group as compared to controls (Table 1).

Table 1 - Long-term carcinogenicity bioassay on ASPARTAME, administered with the feed, supplied *ad libitum*, to Sprague-Dawley rats since foetal life: number and percentage of male and female Sprague-Dawley rats bearing various type of benign and malignant tumours[a]

Site	Histotype	Groups											
		I: 2,000 ppm				II: 400 ppm				III: 0 ppm (control)			
		Male		Female		Male		Female		Male		Female	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Skin													
	Dermatofibroma	0	-	0	-	1	1.4	0	-	1	1.1	0	-
Subcutaneous tissue													
	Fibroma and fibroadenoma	1	1.4	1	1.4	1	1.4	0	-	2	2.1	0	-
	Lipoma and fibrolipoma	0	-	0	-	3	4.3	1	1.4	3	3.2	0	-
	Fibroangioma	0	-	0	-	0	-	0	-	0	-	1	1.1
	Fibrosarcoma	2	2.9	0	-	0	-	0	-	1	1.1	0	-
	Fibroliposarcoma and liposarcoma	0	-	0	-	0	-	0	-	1	1.1	0	-
	Haemangiosarcoma	1	1.4	0	-	0	-	0	-	0	-	0	-
Mammary glands													
	Adenoma, fibroma and fibroadenoma	0	-	34(54)	48.6	3	4.3	38(52)	54.3	2	2.1	45(65)	47.4
	Lipoma and fibrolipoma	0	-	0	-	0	-	0	-	1	1.1	0	-
	Adenocarcinoma	2	2.9	11(15)	15.7*	0	-	5(6)	7.1	0	-	5(6)	5.3♦
	Fibrosarcoma	0	-	0	-	0	-	0	-	0	-	1	1.1
	Liposarcoma	0	-	1	1.4	0	-	2	2.9	0	-	0	-
Zymbal glands													
	Squamous cell carcinoma	1	1.4	0	-	0	-	1	1.4	1	1.1	0	-
Ear ducts													
	Papilloachantoma	0	-	1	1.4	0	-	1	1.4	0	-	0	-
	Squamous cell carcinoma	4	5.7	4(5)	5.7	1	1.4	7	10.0	1	1.1	8	8.4
Nasal cavities													
	Respiratory adenoma	0	-	0	-	0	-	0	-	1	1.1	0	-
	Olfactory adenoma	0	-	1	1.4	1	1.4	1	1.4	0	-	1	1.1
Oral cavity, lips and tongue													
	Acanthoma	0	-	1	1.4	1	1.4	0	-	0	-	3	3.2
	Squamous cell carcinoma	1	1.4	3	4.3	0	-	1	1.4	0	-	2	2.1
Salivary gland													
	Adenocarcinoma	1	1.4	0	-	1	1.4	1	1.4	0	-	0	-
	Squamous cell carcinoma	0	-	0	-	0	-	0	-	1	1.1	0	-

(continued)

Table 1 ***(continued)*** **-** Long-term carcinogenicity bioassay on ASPARTAME, administered with the feed, supplied *ad libitum*, to Sprague-Dawley rats since foetal life: number and percentage of male and female Sprague-Dawley rats bearing various type of benign and malignant tumours[a]

Site	Histotype	Groups											
		I: 2,000 ppm				II: 400 ppm				III: 0 ppm (control)			
		Male		Female		Male		Female		Male		Female	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Lung													
	Alveolar/Bronchiolar adenoma	0	-	0	-	2	2.9	1	1.4	0	-	1	1.1
	Fibroma	1	1.4	0	-	0	-	1	1.4	1	1.1	0	-
	Haemangioma and fibroangioma	0	-	0	-	0	-	0	-	0	-	1	1.1
Stomach													
- Forestomach	Acanthoma	1	1.4	1	1.4	3	4.3	2	2.9	4	4.2	3	3.2
- Glandular	Adenocarcinoma	0	-	0	-	0	-	1	1.4	0	-	0	-
stomach	Leiomyosarcoma	0	-	0	-	0	-	0	-	0	-	1	1.1
Intestine													
	Adenomatous polyp	0	-	1	1.4	0	-	0	-	0	-	0	-
	Leiomyoma	0	-	0	-	0	-	0	-	0	-	1	1.1
Liver													
	Hepatocellular adenoma	0	-	1	1.4	0	-	0	-	0	-	0	-
	Cholangioma	1	1.4	1	1.4	1	1.4	2	2.9	0	-	0	-
	Hepatocellular carcinoma	0	-	0	-	1	1.4	0	-	1	1.1	0	-
Pancreas													
	Exocrine adenoma	0	-	1	1.4	0	-	0	-	0	-	0	-
	Islet cell adenoma	5	7.1	2	2.9	4	3.0	2	2.9	9	9.5	0	-
	Islet cell carcinoma	0	-	0	-	0	-	0	-	1	1.1	0	-
Soft tissues													
	Lipoma	0	-	0	-	0	-	0	-	0	-	1	1.1
Peritoneum													
	Mesothelioma	0	-	0	-	0	-	0	-	1	1.1	1	1.1
	Liposarcoma	0	-	0	-	0	-	0	-	1	1.1	1	1.1
Kidneys													
	Renal tubular adenoma	0	-	1	1.4	0	-	0	-	0	-	0	-
	Nephroblastoma	0	-	0	-	0	-	0	-	1	1.1	0	-

(continued)

Table 1 ***(continued)*** **-** Long-term carcinogenicity bioassay on ASPARTAME, administered with the feed, supplied *ad libitum*, to Sprague-Dawley rats since foetal life: number and percentage of male and female Sprague-Dawley rats bearing various type of benign and malignant tumours[a]

Site	Histotype	Groups											
		I: 2,000 ppm				II: 400 ppm				III: 0 ppm (control)			
		Male		Female		Male		Female		Male		Female	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Pelvis													
	Papilloma	1	1.4	0	-	2	2.9	6(7)	8.6	0	-	0	-
Testes													
	Interstitial cell adenoma	2	2.9			0	-			3	3.2		
Ovaries													
	Cystoadenoma			4	5.7			1	1.4			3	3.2
	Granulosa and theca cell tumour			0	-			1	1.4			1	1.1
	Sertoli cell tumor			0	-			0	-			1	1.1
	Fibroma			0	-			2	2.9			1	1.1
	Haemangioma and fibroangioma			0	-			0	-			1	1.1
	Adenocarcinoma			2(3)	2.9			0	-			0	-
Uterus													
	Polyp			23	32.9			27	38.6■			24	25.3
	Haemangioma and fibroangioma			0	-			1	1.4			1	1.1
	Leiomyoma			0	-			0	-			1	1.1
	Adenocarcinoma			0	-			1	1.4			2	2.1
	Squamous cell carcinoma			0	-			1	1.4			0	-
	Leiomyosarcoma			0	-			0	-			3	3.2
	Malignant Schwannoma			2	2.9			0	-			3	3.2
Vagina													
	Polyp			0	-			0	-			2	2.1
Pituitary gland													
	Adenoma	30	42.9	13	18.6	28	40.0	13	18.6	44	46.3	25	26.3
	Adenocarcinoma	0	-	0	-	0	-	0	-	0	-	2	2.1
Thyroid gland													
	Follicular adenoma	1	1.4	1	1.4	0	-	0	-	1	1.1	0	-
	C-cell adenoma	2	2.9	1	1.4	3	4.3	8	11.4	1	1.1	7	7.4
	C-cell carcinoma	0	-	1(2)	1.4	0	-	0	-	2(3)	2.1	1	1.1

(continued)

Table 1 *(continued)* - Long-term carcinogenicity bioassay on ASPARTAME, administered with the feed, supplied *ad libitum*, to Sprague-Dawley rats since foetal life: number and percentage of male and female Sprague-Dawley rats bearing various type of benign and malignant tumours[a]

Site	Histotype	Groups											
		I: 2,000 ppm				II: 400 ppm				III: 0 ppm (control)			
		Male		Female		Male		Female		Male		Female	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Parathyroid glands													
	Adenoma	2	2.9	0	-	0	-	0	-	1	1.1	1	1.1
Adrenal glands													
	Cortical adenoma	0	-	5	7.1	1	1.4	3	4.3	2	2.1	7	7.4
	Pheochromocytoma	11(12)	15.7	17(22)	24.3	7(9)	10.0	22(26)	31.4	17(19)	17.9	23(26)	24.2
	Cortical adenocarcinoma	0	-	1	1.4	0	-	2(3)	2.9	0	-	1	1.1
	Pheochromoblastoma	0	-	1	1.4	0	-	3	4.3	2	2.1	0	-
Central nervous system													
- Brain	Granular cell tumour (benign)	0	-	1	1.4	0	-	0	-	0	-	1	1.1
	Oligodendroglioma	0	-	1	1.4	0	-	0	-	0	-	0	-
- Meninges	Benign meningioma	1	1.4	0	-	0	-	3	4.3	1	1.1	1	1.1
Pheripheral nervous system													
- Cranial nerves	Malignant Schwannoma	1	1.4	0	-	1	1.4	0	-	1	1.1	0	-
- Other peripheral nerves	Benign Schwannoma	0	-	0	-	0	-	0	-	0	-	1	1.1
Bones													
- Head	Condroma	0	-	1	1.4	0	-	0	-	0	-	0	-
	Osteoma	0	-	4	5.7	1	1.4	6	8.6■	0	-	1	1.1
	Osteosarcoma	4	5.7	3	4.3	3	4.3	4	5.7	1	1.1	3	3.2
- Other	Osteosarcoma	0	-	1	1.4	0	-	1	1.4	0	-	1	1.1
Heart													
	Mixoma	0	-	0	-	0	-	0	-	1	1.1	1	1.1
Thymus													
	Benign thymoma	0	-	4	5.7	0	-	5	7.1	0	-	6	6.3
	Malignant thymoma	1	1.4	0	-	0	-	0	-	0	-	0	-

(continued)

Table 1 ***(continued)*** **-** Long-term carcinogenicity bioassay on ASPARTAME, administered with the feed, supplied *ad libitum*, to Sprague-Dawley rats since foetal life: number and percentage of male and female Sprague-Dawley rats bearing various type of benign and malignant tumours[a]

Site	Histotype	Groups											
		I: 2,000 ppm				II: 400 ppm				III: 0 ppm (control)			
		Male		Female		Male		Female		Male		Female	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Spleen													
	Fibroma	0	-	0	-	0	-	0	-	1	1.1	0	-
	Haemangioma and fibroangioma	0	-	1	1.4	1	1.4	0	-	0	-	0	-
Haemolymphoreticular tissues													
	Lymphomas and leukaemias	12	17.1*	22	31.4**	11	15.7	12	17.1	9	9.5	12	12.6♦♦
Unknown origin													
	Osteosarcoma	1	1.4	0	-	1	1.4	0	-	0	-	0	-

[a] Between brackets the number of tumours (one animal can bear more than one tumour)

* 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 ($p \leq 0.05$) associated with the Cox Regression Model for the analysis of the trend

▪ Statistically significant ($p \leq 0.05$) using logistic regression with a time covariate

♦♦ Near the control incidence are the p-values ($p \leq 0.01$) associated with the Cox Regression Model for the analysis of the trend

Table 2 - Long-term carcinogenicity bioassay on ASPARTAME, administered with the feed, supplied *ad libitum*, to male (M) and female (F) Sprague-Dawley rats since foetal life

BENIGN TUMOURS

Group No.	Concentration (ppm)	Animals		Benign tumours [a]			
				Tumour-bearing animals		Total Tumours	
		Sex	No.	No.	%	No.	Per 100 animals
I	2,000	M	70	44	62.9	60	85.7
		F	70	58	82.9*	146	208.6
		M+F	140	102	72.9	206	147.1
II	400	M	70	43	61.4	65	92.9
		F	70	54	77.1	166	237.1
		M+F	140	97	69.3	231	165.0
III	0	M	95	66	69.5	98	103.2
	(control)	F	95	75	78.9♦	189	198.9
		M+F	190	141	74.2	287	151.1

[a] Benign tumour rates are based on the number of animals examined at necropsy

* Statistically significant ($p \leq 0.05$) using logistic regression with a time covariate

♦ 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 3 - Long-term carcinogenicity bioassay on ASPARTAME, administered with the feed, supplied *ad libitum*, to male (M) and female (F) Sprague-Dawley rats since foetal life

MALIGNANT TUMOURS

Group No.	Concentration (ppm)	Animals		Malignant tumours [a]			
				Tumour-bearing animals		Total Tumours	
		Sex	No.	No.	%	No.	Per 100 animals
I	2,000	M	70	28	40.0**	31	44.3
		F	70	37	52.9	60	85.7
		M+F	140	65	46.4	91	65.0
II	400	M	70	18	25.7	19	27.1
		F	70	31	44.3	44	62.9
		M+F	140	49	35.0	63	45.0
III	0	M	95	23	24.2♦♦	26	27.4
	(control)	F	95	42	44.2	48	50.5
		M+F	190	65	34.2	74	38.9

[a] Malignant tumour rates are based on the number of animals examined at necropsy

** 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

The overall incidence of animals bearing malignant tumours is reported in Table 3. A significant dose-related increase ($p \leq 0.01$) in the incidence occurred in males, where the difference proved significant ($p \leq 0.01$) at 2,000 ppm compared to the control group.

Table 4 - Long-term carcinogenicity bioassay on ASPARTAME, administered with the feed, supplied *ad libitum*, to male (M) and female (F) Sprague-Dawley rats since foetal life

MAMMARY ADENOCARCINOMAS

Group No.	Concentration (ppm)	Animals		Adenocarcinomas [a]			
				Tumour-bearing animals[b]		Total Tumours	
		Sex	No.	No.	%	No.[c]	Per 100 animals
I	2,000	M	70	2	2.9	2	2.9
		F	70	11	15.7*	15	21.4
		M+F	140	13	9.3	17	12.1
II	400	M	70	0	-	0	-
		F	70	5	7.1	6	8.6
		M+F	140	5	3.6	6	4.3
III	0	M	95	0	-	0	-
	(control)	F	95	5	5.3♦	6	6.3
		M+F	190	5	2.6	6	3.2

[a] Mammary adenocarcinoma rates are based on the number of animals examined at necropsy

[b] In the past 20 years the overall incidence of mammary adenocarcinomas in the females of our colony was 9.0% (range, 4.0- 14.2%) among 2,424 females

[c] Number of tumours: an animal can bear multiple tumours

* 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

Mammary adenocarcinomas

Incidences of mammary adenocarcinomas in males and females are reported in Table 4. A significantly increased dose-related incidence of animals bearing carcinomas occurred in females ($p \leq 0.05$), in particular among females treated at the higher dose ($p \leq 0.05$). The increased incidence (2.9%), albeit not significant, observed in males treated at the higher dose compared to 0% in controls, cannot be disregarded, since mammary adenocarcinomas are extremely rare tumours among the 2,415 male historical control Sprague-Dawley of our colony (overall 0.5%; range 0-1.3).

Morphologically, adenocarcinomas of the mammary gland presented a broad range of histological patterns, i.e. the neoplastic tissue was arranged in papillary, ductular, or alveolar architectures, or combined. Lung metastases were observed in one female from the higher treated group.

Lymphomas/leukaemias

The occurrence of lymphomas/leukaemias is reported in Table 5. A significant increased incidence ($p \leq 0.05$) was observed among males treated at 2,000 ppm; a non-significant increased incidence (15.7% *versus* 9.5% in controls) was also observed at 400 ppm. A significant increased dose-related incidence ($p \leq 0.01$) was observed among females. When compared to the control group, the incidence of lymphomas and leukaemias in females was significantly increased ($p \leq 0.01$) at 2,000 ppm; a non-significant increased incidence was also found at 400 ppm (17.1% *versus* 12.6% in controls).

Various different morphological aspects of lymphomas/leukaemias were observed. Lymphocytic lymphomas were mainly localized in the thymus and lymphnodes, showing a predominance of small-medium-sized cells with hyperchromatic irregular nuclei. Lymphoepithelioid cells (Lennert) of the Kiel classification (39) were present. Lymphoblastic lymphomas/leukaemias were composed of undifferentiated lymphoid cells. Lympho-immunoblastic lymphomas were more frequently observed in males, involving mainly lung and mediastinal/peripheral nodes, and showed various different stages of lymphoplasmacytic differentiation.

Histiocytic sarcomas often involved visceral organs; spindle-shaped and histiocytic (epithelioid)

Table 5 - Long-term carcinogenicity bioassay on aspartame, administered with the feed, supplied *ad libitum*, to male (M) and female (F) Sprague-Dawley rats since foetal life

LYMPHOMAS/LEUKAEMIAS

Group No.	Concentration (ppm)	Animals		Lymphomas/ leukaemias [a, b]	
		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	M	95	9	9.5
	(control)	F	95	12	12.6♦♦
		M+F	190	21	11.1

[a] Lymphomas/leukaemias rates are based on the number of animals examined at necropsy

[b] In the past 20 years the overall incidence of lymphomas/leukaemias among 2,415 males and 2,424 females historical controls was 20.5% (range, 8.0-30.9%) and 12.8% (range, 4.0-25.0%) respectively

* 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 the analysis of the trend

proliferating cells had a monomorphic or polymorphic appearance. Multinucleate giant cells were also present.

Leukaemias of myelo-monocytic origin were also observed. Myeloid leukaemia presented myeloblasts varying in size, at different stages of differentiation; a large number of promyelocytes were often present. Monoblasts and promonocytes were a feature of monocytic leukaemia. In both these leukaemia histotypes, the liver and spleen were the organs most involved, and many neoplastic cells were found in the blood vessels.

Discussion

In our laboratory we showed for the first time that APM, administered in the feed from 8 weeks of age until death, causes a significant increased incidence of malignant tumours, in particular lymphomas/leukaemias in females (6, 32, 33).

The present study in which the APM treatment (2,000, 400, and 0 ppm in feed) started from foetal life and lasted until spontaneous death confirmed the carcinogenic potential of APM. We found in males: 1) a significantly increased dose-related incidence of animals bearing malignant tumours ($p \leq 0.01$), in particular in the group treated at 2,000 ppm ($p \leq 0.01$); 2) a significant increased incidence ($p \leq 0.05$) of lymphomas/leukaemias among the animals treated at 2,000 ppm; and 3) an increased incidence of mammary adenocarcinomas at the highest dose in males, although not significant, cannot be disregarded in view of the rarity of this type of tumour in this sex.

In females we observed: 1) a significant increased dose-related incidence of lymphomas/leukaemias ($p \leq 0.01$), in particular in the 2,000 ppm group ($p \leq 0.01$); and 2) a significant increased dose-related incidence of mammary adenocarcinomas ($p \leq 0.05$), in particular at 2,000 ppm group ($p \leq 0.05$).

In comparing the same life span exposure (2,000 and 400 ppm) starting from foetal life or from postnatal life, we have shown: 1) a significant increased incidence of mammary adenocarcinomas (Table 6) and 2) a clear increase in the incidence of lymphomas/leukaemias in females (Table 7).

Moreover, when comparing the cumulative prevalence by age of death of animals with haemolym-

Table 6 - Comparison of the incidence of mammary adenocarcinomas in female Sprague-Dawley rats between prenatal and postnatal exposure to APM

APM dose, ppm (mg/kg b.w.)	Percent of female animals bearing mammary adenocarcinomas	
	Prenatal exposure (No. of animals at start)	Postnatal exposure (No. of animals at start)
2,000 (100)	15.7 (70)*	8.0 (150)
400 (20)	7.1 (70)	10.7 (150)
0 (0)	5.3 (95)♦	5.3 (150)

* 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 7 - Comparison of the incidence of lymphomas/leukemias in female Sprague-Dawley rats between prenatal and postnatal exposure to APM

APM dose, ppm (mg/kg b.w.)	Percent of female animals bearing lymphomas/leukemias	
	Prenatal exposure (No. of animals at start)	Postnatal exposure (No. of animals at start)
2,000 (100)	31.4 (70)**	18.7 (150)
400 (20)	17.1 (70)	20.0 (150)
0 (0)	12.6 (95)♦♦	8.7 (150)

** 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

phoreticular neoplasias, it is evident that prenatal exposure accelerates the onset of these lesions in females (fig. 6).

The results of our studies on rats have received severe criticism from international agencies, as well from some Authors, which personally we do not accept. The first reaction came from the European Food Safety Authority (EFSA) (29) which, referring to the significantly increased incidence of lymphomas/leukaemias, stated that "the increased incidence of lymphomas/leukaemias reported in treated rats was unrelated to APM… the most plausible explanation is that they developed in a colony suffering from chronic respiratory disease". The FDA agreed with the opinion expressed by the EFSA (40).

The same criticism was reported by the EFSA (41) as to the second study on rats exposed from prenatal life until spontaneous death.

More recently, Schoeb *et al*. (42) stated: a) "the most frequently reported hematopoietic neoplasm was lympho-immunoblastic lymphoma, the most affected organ was the lung and, in almost half of the rats with this diagnosis, the lung was the only affected organ"; b) "because lymphocyte and plasma cell accumulation in the lung is characteristic of *Mycoplasma pulmonis* disease, and because *Mycoplasma pulmonis* disease can be exacerbated by experimental manipulations, including chemical treatment, we suggest that a plausible alternative explanation for the reported results of these bioassays is that the study was confounded by *Mycoplasma pulmonis* disease and that lesions of the disease were interpreted as lymphoma"; and they concluded c) "The weight of available evidence favors the hypothesis that lesions of *Mycoplasma pulmonis* disease were interpreted as lymphomas in APM bioassay". There were other criticisms reported in confidential literature to which we do not reply.

Our answers are as follows. Regarding infections, we have already said that creatures that die naturally are subject to infectious pathologies, whether they be rodents or humans (32). Regarding the lymphoimmunoblastic lymphomas of the lung, we reported that the diffusion of neoplastic tissue sometimes involved the lung and concurrently various other organs (liver, spleen, mediastinal, and peripheral lymphnodes) confirming that the hematopoietic neoplasms are systemic and not secondary to local inflammation of the lung (34).

Concerning the hypothesis that the exacerbation of *Mycoplasma pulmonis* disease by experimental manipulation, including chemical treatment, may be responsible for dose-related pulmonary lesions, we answer that, out of 49 agents reported by our Institute to be carcinogenic in our experimental conditions (43), only 8 (3 in both males and females and 5 only in females) induced hematopoietic neoplasms and vinyl chloride and benzene, very well known toxic agents, were not among them. Moreover, it cannot be disregarded that among these 8 chemicals, formaldehyde per se or as a metabolite of MetOH, Methyl tert-Butyl Ether (MTBE) and APM, was involved. This detail should be taken into due consideration and properly investigated.

Finally we wish to point out that, after our first communication on the carcinogenicity of APM (6, 32) up to now no action has been taken to review the current regulations governing the use of APM. At the same time no research has been conducted or planned by the agencies or the industry which criticized our studies, in spite of several calls from the scientific community (44-48) to take proper action to prevent the exposure of people, in particular children and women of childbearing age, to an agent which has proved carcinogenic in rodents and is potentially so to humans.

Conclusions

In our experimental conditions APM, administered in feed to rats and mice, has proved to induce an increased incidence of various types of malignant tumours in two animal species, and in both sexes. The current regulations, which are still based on inadequate studies conducted in the early Seventies, urgently need to be re-evaluated therefore.

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