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Breast Cancer see pp 11

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Breast Cancer see pp 11 Mammary Carcinin

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LIFESPAN EXPOSURE TO LOW DOSES OF ASPARTAME BEGINNING DURING PRENATAL LIFE INCREASES CANCER EFFECTS IN RATS

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Short running head: Carcinogenicity of Aspartame

Key words: artificial sweeteners, aspartame, carcinogenicity, lymphomas/leukemias, mammary cancers, prenatal exposure, Sprague-Dawley

Abbreviations:

ADI	acceptable daily intake
APM	aspartame
BW	body weight
CMCRC	Cesare Maltoni Cancer Research Center
EFSA	European Food Safety Authority
ERF	European Ramazzini Foundation
EU	European Union
FDA	Food and Drug Administration

Outline of section headers

Abstract

Introduction

Materials and Methods

Results

Discussion

Conclusions

References

Tables

Figure legends

Figures

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ABSTRACT

Background. In a previous study conducted at the Cesare Maltoni Cancer Research Center of the European Ramazzini Foundation (CMCRC/ERF), we demonstrated for the first time that aspartame (APM), administered with feed at various doses to 8 week-old Sprague Dawley rats for the lifespan, is a multipotent carcinogenic agent. Objective. The aim of this second study is to better quantify the carcinogenic risk of APM, beginning treatment during fetal life. Methods. The study was conducted on groups of 70-95 male and female Sprague Dawley rats, administered APM with feed at concentrations of 2000, 400, or 0 ppm from the 12th day of fetal life until natural death. Results. The results of the study show: a) a significant dose-related increase of malignant tumor-bearing animals in males ($p<0.01$), in particular in the group treated at 2000 ppm ($p<0.01$); b) a significant increase of the incidence in lymphomas/leukemias in males treated at 2000 ppm ($p<0.05$) and a significant dose-related increase of the incidence of lymphomas/leukemias in females ($p<0.01$), in particular in the group treated at 2000 ppm ($p<0.01$); c) a significant dose-related increase of the incidence of mammary cancer in females ($p<0.05$), in particular in the group treated at 2000 ppm ($p<0.05$). Conclusions. The results of this carcinogenicity bioassay not only confirm, but also reinforce the first experimental demonstration of APM's multipotential carcinogenicity at a dose level close to the acceptable daily intake (ADI) for humans. Furthermore, the study demonstrates that when lifespan exposure to APM begins during fetal life, its carcinogenic effects are increased.

INTRODUCTION

Aspartame (APM) is one of the most widely used artificial sweeteners in the world. First approved by the US Food and Drug Administration (FDA) for limited use in solid food in 1981, its authorization was extended to soft drinks in 1983 and then approved as a general sweetener in 1996 (FDA 1981; FDA 1983; FDA 1996). Likewise, the sweetener was approved for general use in the European Union (EU) in 1994 (EC Directive 1994). Today APM is present in > 6000 consumer packaged goods and in nearly 500 pharmaceutical products, including children's medicines (Aspartame Information Center 2005). In the United States, > 70% of aspartame sales are attributed to soft-drinks (American Dietetic Association 2004). The acceptable daily intake (ADI) of aspartame is currently 50 mg/kg body weight (bw) in the USA and 40 mg/kg bw in the EU for both children and adults. Daily assumption of artificial sweeteners by women of childbearing age and children has been estimated between 2.5 – 5.0 mg/kg bw (Butchko et al. 2002). In a study of Swedish diabetics, although the general APM intake was lower than the ADI, the worst-case calculation of intake in the children's group was reported to be 114% of ADI (Ilbäck et al 2003).

APM is metabolized in the gastric tract of rodents, non-human primates and humans to its three constituents: aspartic acid, phenylalanine and methanol. When absorbed, aspartic acid is transformed into alanine plus oxaloacetate (Stegink 1984); phenylalanine is transformed mainly into tyrosine and, to a lesser extent, phenylethylamine and phenylpyruvate (Harper 1984); and methanol is transformed into formaldehyde and then to formic acid (Opperman 1984).

In vitro and *in vivo* tests demonstrate that APM is not genotoxic. Likewise, long term carcinogenicity studies conducted by the manufacturers of aspartame using rats and mice in the '70s and '80s did not demonstrate any carcinogenic effects. We have reported a detailed review of the genotoxicity and carcinogenicity studies available to date on APM in previous publications. (Belpoggi et al. 2006; Soffritti et al. 2005; Soffritti et al. 2006). In our opinion, the small number of animals used per sex/per group and the termination of these experiments after 110 weeks of age rather than observing animals for the lifespan, represent limiting factors when evaluating the

carcinogenic risk or safety of artificial sweeteners such as aspartame. It was for this reason, together with the growing use of APM in industrialized countries, that we designed and performed a mega-experiment using 7 groups of Sprague-Dawley rats (100-150/sex/group), treated with APM in feed at various dose levels (including one very close to the ADI for humans), from 8 weeks of age until natural death. The study demonstrated for the first time that APM is a multipotential carcinogenic agent, capable of inducing, in our experimental conditions: a) a significant, dose-related increased incidence of malignant tumor-bearing animals in males ($p \leq 0.05$) and in females ($p \leq 0.01$), in particular in females treated at 50,000 ppm ($p \leq 0.01$); b) a significant dose-related increase in lymphomas/leukaemias in both males ($p \leq 0.05$) and females ($p \leq 0.01$), in particular in females treated at doses of 100,000 ($p \leq 0.01$), 50,000 ($p \leq 0.01$), 10,000 ($p \leq 0.05$), 2,000 ($p \leq 0.05$), or 400 ppm ($p \leq 0.01$); c) a significant, dose-related increased incidence ($p \leq 0.01$), of transitional cell carcinomas of the renal pelvis and ureter and their precursors (dysplasias) in females treated at 100,000 ($p \leq 0.01$), 50,000 ($p \leq 0.01$), 10,000 ($p \leq 0.01$), 2,000 ($p \leq 0.05$), or 400 ppm ($p \leq 0.05$); d) a significant, dose-related increased incidence of malignant schwannomas of peripheral nerves ($p \leq 0.05$) in males (Belpoggi et al. 2006; Soffritti et al. 2005; Soffritti et al. 2006).

Given the consolidated experience of the European Ramazzini Foundation (ERF) in the conduct of long term bioassays, and the large number of rodents used in the study, the results attracted the attention of the scientific community, consumer and industry associations, and the national and international agencies responsible for food safety, including the Italian Superior Council of Health, the European Food Safety Authority (EFSA), the US FDA, Health Canada, and others. Per their request, each of these agencies was provided with all available raw data related to the study.

To our knowledge, only EFSA has issued an official opinion on our study, releasing on May 5, 2006 a 40-page report in which they concluded that it is not necessary to revise their previous opinion on the absolute safety of APM (EFSA 2006).

Subsequent to our findings of hematopoietic cancers in rats, and in light of persistent concerns among the scientific community of an association between APM and brain cancers, Lim et al (2006)

published the results of a study which assessed the correlation between the consumption of aspartame-containing beverages and the incidence of these types of cancers. The findings were based on data derived from a prospective study conducted by the US National Institutes of Health and the American Association of Retired Persons, using a cohort of > 285,000 men and > 188,000 women between the ages of 50-71, who had satisfactorily responded to a self-administered food frequency questionnaire. Among the survey questions was the consumption of beverages (soda, fruit drinks, sweetened iced tea) potentially containing APM during the previous year. The questionnaires were mailed from 1995 to 1996 and the follow-up lasted until 2000. The conclusions of the study did not support the hypothesis that APM increases hematopoietic or brain cancer risks.

Recently a group of Italian authors (Gallus et al. 2007) published the results of an integrated network of case-control studies conducted in Italy between 1991-2004 on the potential correlation between artificial sweeteners (including APM) and cancer. The authors interviewed patients with histologically confirmed cancers of the oral cavity and pharynx (598), esophagus (304), colon (1225), rectum (728), larynx (460), breast (2569), ovary (1031), prostate (1294) and kidney (renal cell carcinoma 767). Controls were 7028 patients (3301 men and 3727 women) admitted to the same hospitals for acute, non-neoplastic disorders. Cases and controls were interviewed during their hospital stay, using a questionnaire on subjects' usual diet in the 2 years before diagnosis. The results reported a lack of association between artificial sweeteners and the risk of the aforementioned cancers.

As soon as we perceived the carcinogenic effects of APM during the elaboration of the data in our first mega-experiment, we planned an integrated program of long-term bioassays, beginning treatment from prenatal life, on a total of more than 4000 rats and mice in order to better quantify the carcinogenic risks of aspartame. In this report we present the results of a second study on APM in which male and female Sprague-Dawley rats were exposed to very low doses of APM in feed (100 or 20 mg/kg bw) from fetal life until natural death.

MATERIALS AND METHODS

The APM used was produced by Ajinomoto and supplied by Giusto Faravelli S.p.A. in Milan, Italy. Its purity, as determined by an infrared absorption spectrophotometer assay, was > 98.7%: diketopiperazine was < 0.3% and L-phenylalanine was < 0.5%. APM was added to the standard diet, used from more than 30 years at the Cesare Maltoni Cancer Research Center (CMCRC)/ERF, at concentrations of 2000; 400; or 0 ppm in order to simulate an assumed daily APM intake of 100; 20; or 0 mg/kg bw. The feed was supplied by the producer on monthly basis. The stability of the aspartame in feed was analyzed prior to the start of the study and periodically confirmed throughout the course of the biophase. The daily APM assumption in mg/kg bw was calculated considering the average body weight both for males and females as 400 g for the duration of the experiment and the daily consumption of feed as 20 g/day.

The feed was supplied *ad libitum* to groups of 70-95 male and female Sprague-Dawley rats from the colony of the CMCRC/ERF. The basic tumorigram of this strain of rats is well known and the susceptibility to cancer does not differ greatly from that of humans. Treatment began during fetal life, administering APM in feed to female breeders from the 12^{th} day of pregnancy, when organogenesis is completed and before which time many tissues and organs are refractory to the effects of carcinogenic agents (IARC 1973). The breeders were sacrificed after weaning and treatment of the offspring lasted until natural death. Control animals received the same feed without APM.

At 4-5 weeks of age, after weaning, the experimental animals were identified by ear punch, separated by sex and assigned to a respective dose group, depending on the APM concentration administered to the breeder. They were then housed, in groups of 5, in makrolon cages (41x25x15 cm), 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 temperature of 23 ± 2°C and relative humidity of 50-60%.

All animals were kept under observation until natural death. The experiment was conducted according to Italian law regulating the use and humane treatment of animals for scientific purposes (Decreto Legislativo N. 116 1992).

Mean daily drinking water and feed consumption were measured per cage, and body weight 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 2 weeks until the end of the experiment. In order to detect and register all gross lesions, the animals were clinically examined every 2 weeks for the duration of the experiment. In order to evaluate the status and behavior of the animals and to limit the *post mortem* modifications, a patrol was performed three times daily from Monday to Friday and twice on Saturdays, Sundays and holidays. Deceased animals were registered and kept refrigerated for a maximum of 16-19 hours at 4°C until necropsy.

The biophase 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 (five 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 mainstem bronchi, heart, diaphragm, liver, spleen, pancreas, kidneys, adrenal glands, esophagus, stomach (fore and glandular), intestine (four 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 which were fixed in 10% formalin and then decalcified with 10% formaldehyde and 20% formic acid in water solution. The normal specimens were trimmed, following the CMCRC/ERF Laboratory standard operating procedures. The pathological tissue was trimmed to

allow for the largest surface including normal adjacent tissue. Trimmed specimens were processed as paraffin blocks, and 3-5 μm sections of every specimen were obtained.

Sections were routinely stained with hematoxylin and eosin. All slides were examined microscopically by the same group of pathologists, following the same criteria of histopathological evaluation and classification. A senior pathologist reviewed all tumors and all other lesions of oncologic interest.

Statistical evaluations of the incidence and dose-response relationship of neoplastic lesions were performed using the Cox regression model (Cox 1972). The p-values are reported in the tables.

RESULTS

The experiment proceeded smoothly without unexpected occurrences. No relevant differences were observed in feed consumption between treated and untreated groups, in both males and females (Figure 1A, 1B). No differences were observed in water consumption in both males and females in the various groups. No difference in mean body weight was observed in the treated groups compared to the control (Figure 1C). A slight decrease, seemingly dose-related, in survival was observed between the treated groups and the control group in both males and females (Figure 1D, 1E).

Oncologic results are reported in Table 1 for males and Table 2 for females. Multiple tumors of different type and site, of different type in the same site, of the same type in bilateral organs, of the same type in the skin, in the subcutaneous tissue, in mammary glands, or at distant sites of diffuse tissue (i.e. bones and skeletal muscle) were plotted as single/independent tumors. Multiple tumors of the same type in the same tissue and organ, apart those listed above, were plotted only once.

Total malignant tumors. The incidence of malignant tumor-bearing animals occurred with a significant, dose-related increase in males ($p \leq 0.01$). A significant increase of the incidence of malignant tumors was observed in males treated at 2000 ppm ($p \leq 0.01$) compared to the control group (Table 1). Albeit not significant, a numeric increase of the incidence of animals bearing

malignant tumors was also observed among females exposed at 2000 ppm compared to the controls (Table 2). Tumor types which contributed most to this increased incidence are presented as follows:

Lymphomas/leukemias. The occurrence of lymphomas/leukemias in males and females is reported in Tables 1 and 2. The data show that APM causes a significant, dose-related increased incidence in females ($p \leq 0.01$). When compared to untreated control group, the increased incidence of lymphomas/leukemias in treated males and females was significant at 2000 ppm ($p \leq 0.05$ and $p \leq 0.01$ respectively). In males, the most frequent histotypes observed in the experiment were lymphoimmunoblastic lymphomas, mainly involving lung and mediastinal/peripheral nodes. In females, the most frequent histocytotypes were lymphocitic lymphomas and lymphoimmunoblastic lymphomas mainly involving the thymus, lung, spleen and peripheral nodes. The differential diagnoses were based on the morphological criteria regularly used in our laboratories, according to the guidelines of the International Classification of Rodent Tumors (IARC 1993). Lymphomas/leukemias (this term includes all types of hemolymphosarcomas and leukemias) are neoplasias arising from hemolymphoreticular tissues, and their aggregation is regularly used in experimental carcinogenesis. The reason, as has been widely noted, is that both solid and circulating phases are present in many lymphoid neoplasms, and distinction between them is artificial (Harris et al. 2001).

Mammary Carcinomas. The incidence of mammary gland carcinomas in males and females are reported in Tables 1 and 2. A dose-related increase in the incidence of carcinomas was observed in females ($p \leq 0.05$). The incidence of lesions in females exposed at 2000 ppm was significantly higher ($p \leq 0.05$) compared to the controls. Two carcinomas were also observed among males treated at 2000 ppm.

Historical controls. The overall incidence of lymphomas/leukemias among male and female Sprague-Dawley rats and mammary cancers in female Sprague-Dawley rats in our laboratory over the last 20 years is reported in the footnotes of the respective tables.

DISCUSSION

In our first mega-experiment, we demonstrated for the first time that APM is a multipotential carcinogenic agent inducing, among other cancers, a dose-related, significant increase in lymphomas/leukemias in females (Belpoggi et al. 2006; Soffritti et al. 2005; Soffritti et al. 2006).

In the present experiment, in which APM was administered in feed beginning during fetal life to Sprague-Dawley rats at doses of 2000 or 400 ppm (equivalent to an assumption of 100 and 20 mg/kg bw), we again confirmed that APM induces carcinogenic effects, namely: a) a significant dose-related increase of malignant tumor-bearing animals in males ($p<0.01$), in particular in the group treated at 2000 ppm ($p<0.01$); b) a significant increase of the incidence of lymphomas/leukemias in males treated at 2000 ppm ($p<0.05$) and a significant dose-related increase of the incidence of lymphomas/leukemias in females ($p<0.01$), in particular in the group treated at 2000 ppm ($p<0.01$); c) a significant dose-related increase of the incidence of mammary cancer in females ($p<0.05$), in particular in the group treated at 2000 ppm ($p<0.05$).

When comparing lifespan exposure beginning during post- and prenatal life, we have shown that prenatal exposure to APM clearly increases the incidence of lymphomas/leukemias in females (Table 3). Moreover, when comparing the cumulative prevalence by age of death of hemolymphoreticular neoplasias, it is clear that prenatal exposure to APM also accelerates the insurgence of these lesions in females (Figure 2A, 2B).

With regard to males, the incidence of lymphomas/leukemias in the concurrent control (9.5%) falls within the lower range of our historical controls (8.0-30.9%), and the incidence of lymphomas/leukemias in the group treated at the highest dose (17.1%) is close to the overall historical incidence (20.9%). Since the incidence of lymphomas/leukemias observed in males treated at 2000 ppm is close to double the concurrent control, we consider these effects to be related to APM exposure (Haseman et al. 1984; Haseman 1992; Haseman 1995).

The results of our second experiment further disprove the alternative hypothesis suggested by EFSA regarding the cause of lymphomas/leukemias in our colony, in which they consider the

incidence of lymphomas/leukemias observed in our first experiment "unrelated to APM given the high background incidence of chronic inflammatory changes in the lung" (EFSA 2006). First of all, as previously reported (Soffritti 2006), experimental animals which are allowed to die spontaneously are subject to infectious pathologies which are part of the natural dying process in both rodents and humans. Secondly, among the animals bearing lymphomas/leukemias, the diffusion of neoplastic tissue was observed not only in the lung, but also concurrently in various organs (liver, spleen, mediastinal and other lymph nodes). Finally, it should be noted that out of 49 agents reported to be carcinogenic in rats by the CMCRC/ERF, only 8 of these agents induced hemolymphoreticular malignancies. Of these, 3 were demonstrated in both males and females, namely formaldehyde (Soffritti et al 2002b), mancozeb (Belpoggi et al 2002a), di-isopropyl-ether (Belpoggi et al 2002b), and 5 in only females, namely toluene (Soffritti et al 2004), methyl alcohol (Soffritti et al 2002a), methyl tert-butyl ether (Belpoggi et al 1995), *tert*-amyl-methyl-ether (Belpoggi et al 2002b) and APM (Belpoggi et al. 2006; Soffritti et al. 2005; Soffritti et al. 2006).

The two aforementioned epidemiological studies published after our first mega-experiment merit general comment. Both studies consider the eating habits of a large population of males and females, age 50-70, in the 1990s. Given the timeframe of these surveys and the commercialization of aspartame in the 1980s, the subjects' potential use of the sweetener could not have exceeded 10-15 years. It is difficult to think that this limited adult period of exposure to APM could evidence or exclude a potential carcinogenic risk. The design of these studies underlines the importance of conducting an epidemiological study in which exposure to APM is monitored beginning from fetal life, particularly given the use of products containing APM by women of child-bearing age and children.

CONCLUSIONS

The results of our second long-term carcinogenicity bioassay on APM not only confirm, but also reinforce our first experimental demonstration of APM's multipotental carcinogenicity at a dose level close to the human ADI. Furthermore, the study demonstrates that when lifespan exposure to APM begins during fetal life, its carcinogenic effects are increased.

On the basis of the present findings, we believe that a review of the current regulations governing the use of aspartame cannot be delayed. This review is particularly urgent with regard to aspartame-containing beverages, heavily consumed by children.

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Table 1. Incidence of malignant tumors in male Sprague-Dawley rats in a prenatal lifespan carcinogenicity study of APM

Dose, ppm (mg/kg bw)	Animals at start	Malignant tumors[a]				Total animals bearing lymphomas/leukemias[b]		Total animals bearing mammary carcinomas	
		Tumor-bearing animals[c]		Total tumors					
		No.	%	No.	Per 100 animals	No.	%	No.	%
2,000 (100)	70	28	40.0**	31	44.3	12	17.1*	2	2.9
400 (20)	70	18	25.7	19	27.1	11	15.7	0	-
0 (0)	95	23	24.2**	26	27.4	9	9.5	0	-

[a] The tumor rates are based on the number of animals examined (necropsied)
[b] In male historical controls, out of 2265 rats, the overall incidence of lymphomas/leukemias is 20.6% (range 8.0-30.9%)
[c] p-value associated with the dose-response test is near the control incidence
** Significant ($p \leq 0.01$) using Cox regression model
* Significant ($p \leq 0.05$) using Cox regression model

Table 2. Incidence of malignant tumors in female Sprague-Dawley rats in a prenatal lifespan carcinogenicity study of APM

Dose, ppm (mg/kg bw)	Animals at start	Malignant tumors[a]				Total animals bearing lymphomas/leukemias[b,c]		Total animals bearing mammary carcinomas[c,d]	
		Tumor-bearing animals		Total tumors					
		No.	%	No.	Per 100 animals	No.	%	No.	%
2,000 (100)	70	37	52.9	60	85.7	22	31.4**	11(15)	15.7*
400 (20)	70	31	44.3	44	62.9	12	17.1	5(6)	7.1
0 (0)	95	42	44.2	48	50.5	12	12.6**	5(6)	5.3*

[a] The tumor rates are based on the number of animals examined (necropsied)

[b] In female historical controls, out of 2274 rats, the overall incidence of lymphomas/leukemias is 13.3% (range 4.0-25.0%), and of mammary cancers is 9.2% (range 4.0-14.2%)

[c] p-values associated with the dose-response test are near the control incidence

[d] Between brackets the number of tumors (one animal can bear multiple tumors)

** Significant ($p \leq 0.01$) using Cox regression model

* Significant ($p \leq 0.05$) using Cox regression model

Table 3. Comparison of the incidence of lymphomas/leukemias in female Sprague-Dawley rats beginning APM exposure from postnatal or prenatal life

Dose, ppm (mg/kg bw)	% of animals bearing lymphomas/leukemias	
	Postnatal exposure[a,b] (No. of animals at start)	Prenatal exposure[c] (No. of animals at start)
2,000 (100)	18.7 (150)	31.4 (70)
400 (20)	20.0 (150)	17.1 (70)
0 (0)	8.7 (150)	12.6 (95)

[a] Exposure began at 8 weeks of age and lasted until natural death (Experiment BT 6008)
[b] Data from Soffritti et al. (2006)
[c] Exposure began on the 12th day of fetal life and lasted until natural death (Experiment BT 6009)

FIGURE LEGENDS

Figure 1. *(A)* Mean daily feed consumption in males. *(B)* Mean daily feed consumption in females. *(C)* Mean body weights in males (M) and females (F). *(D)* Survival in males. *(E)* Survival in females. (─ ♦ ─ 2,000 ppm; ─ ▲ ─ 400 ppm; ─ □ ─ Control; ↑ start of the experiment)

Figure 2. Lifespan carcinogenicity study of aspartame administered with feed to Sprague-Dawley rats from fetal life until natural death: cumulative prevalence by age of death of female rats bearing hemolymphoreticular neoplasias when treatment began postnatally (A) or prenatally (B). (─ ♦ ─ 2,000 ppm; ─ ▲ ─ 400 ppm; ─ □ ─ Control; ↑ start of the experiment).

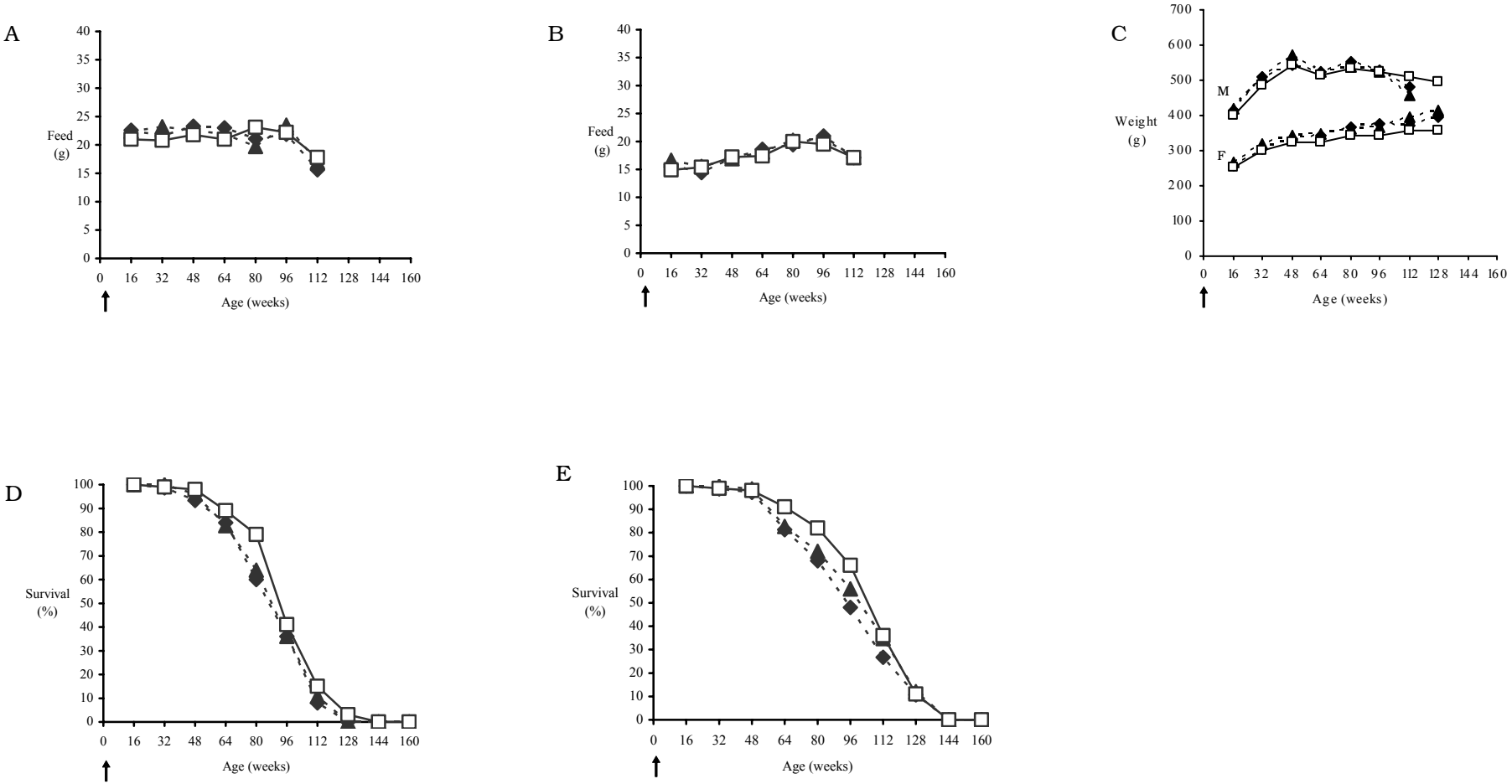
A
Feed (g)
Age (weeks)
B
Feed (g)
Age (weeks)
C
Weight (g)
M
F
Age (weeks)
D
Survival (%)
Age (weeks)
E
Survival (%)
Age (weeks)

Figure 1

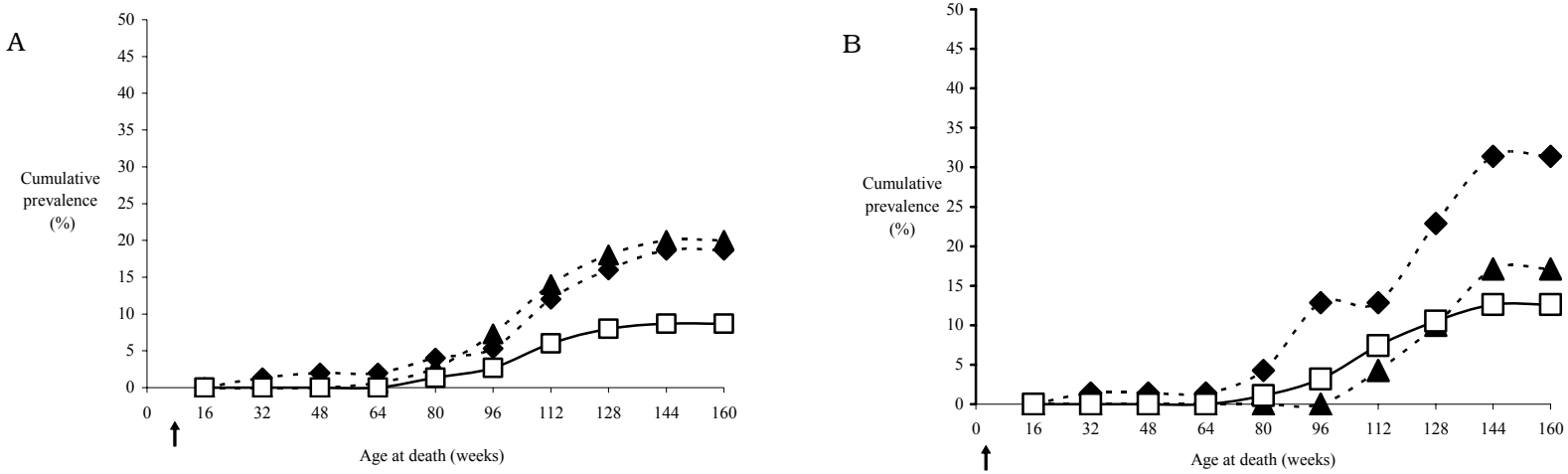
A
Cumulative prevalence (%)
50
45
40
35
30
25
20
15
10
5
0
0
16
32
48
64
80
96
112
128
144
160
Age at death (weeks)
B
Cumulative prevalence (%)
50
45
40
35
30
25
20
15
10
5
0
0
16
32
48
64
80
96
112
128
144
160
Age at death (weeks)

Figure 2