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Dietary polyunsaturated n-6 lipids effects on the growth and fatty acid composition of rat mammary tumors

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Abstract

The aim of this study was to analyze the effects of a polyunsaturated n-6 high-fat diet on rat DMBA-induced breast cancer at different stages of the carcinogenesis and to investigate if changes in the tumor fatty acid composition are one of the mechanisms by which dietary lipids could exert their effects. 14 fatty acids were evaluated in 6 lipid fractions. The results firstly showed that this high-fat diet stimulated the malignant mammary tumor growth, mainly all in the promotion group. The tumor lipid analysis indicated: 1) that each lipid fraction presented distinct major fatty acids (>5%) which were not the most abundant in the diet, except in the case of the triacylglicerides, suggesting the different resistance to dietary fatty acid modification of the tumor lipid fractions; 2) a higher arachidonic acid content in the fractions with less linoleic acid, above all in phospholipids, particularly in the phosphatidylethanolamine, indicating a different efficiency of conversion; 3) the three most abundant fatty acids in the dietary lipid (18:2n-6, 18:1n-9 and 16:0) were those which essentially displayed the differences between groups; thus, the high-fat diet changed the tumor lipid profile, increasing the 18:2n-6 relative content and decreasing that of the 18:1n-9; differences were significant in phosphatidylcholine, free fatty acids and triacylglycerides. Any change was obtained in the phosphatidylinositol. The greatest number of differences was found in the promotion group. Taken as a whole, our results suggest the different roles of lipid fractions in breast cancer cells and an association between cancer malignancy and the content of linoleic and oleic acids.

Keywords: Mammary carcinomas; DMBA-induced breast cancer; Fatty acids; Fatty acid composition; Polyunsaturated n-6 diet; PUFA

1. Introduction

Studies on the effects of dietary fat on mammary cancer carried out in several rodent experimental models have consistently showed that mice and rats develop mammary tumors more readily when they are fed high-fat as compared to low-fat diets (reviewed in [1]). There is also experimental evidence that dietary fat exerts its effect mostly during the promotion phase of breast cancer development [2]. However, the tumor-promoting capability depends on the type of fatty acids. Thus, diets containing high proportions of n-6 polyunsaturated fatty acids (PUFA) have greater enhancing effects than diets high in saturated fatty acids [2–5]. In contrast, fish oils that contain fatty acids derived from linolenic acid (18:3n-3) show an inhibiting effect on experimental mammary carcinogenesis [6,7]. Studies on the influence of diets rich in monoenoic fatty acids such as oleic acid (18:1n-9), contained in the olive oil, are inconclusive [8,9] but at this time there are several results which give them a protective antitumor effect [1,10,11]. More recently, it has been described that conjugated linoleic acid has also inhibitory effects on breast cancer [9].

These observations in animals have been correlated with human epidemiological data from different countries showing that breast cancer mortality increases with increasing

Abbreviations: C, control group; CE, cholesteryl esters; D, development group; DMBA, 7,12-dimethylbenz(α)anthracene; I, initiation group; FFA, free fatty acids; P, promotion group; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; TG, triacylglycerides.

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levels of dietary fat [3,12]. In the same way, the incidence of breast cancer in migrants approaches that of their country of adoption [13]. On the contrary, the results of case-control and prospective cohort studies have not provided strong evidence for an association between dietary fat and breast cancer [14,15] although there are a number of possible methodological reasons for this apparent discrepancy [16, 17]. Likewise, in the epidemiological studies within countries it has been observed that those who have dietary habits based on the so-called "Mediterranean diet", where olive oil is the most important source of fat, have intermediate rates of incidence and mortality from breast cancer [3,18]. However, the epidemiological studies of the intake of olive oil and breast cancer risk show discrepancy [11,19].

The mechanisms of action through which the dietary lipids participate in the growth of breast cancer are beginning to be known. Firstly, breast tumor cells require lipids as a metabolic fuel. In this respect it has been described that fatty acid synthase, a major enzyme of fatty acid biosynthesis, is produced in primary breast tumors and certain human and murine cancer cell lines. In human tumors its overexpression is associated with poor prognosis [20]. Furthermore, genes whose function is to activate final genes of fatty acid synthesis have been found both amplified and expressed in several breast cancer-derived cell lines [21]. Conversely, the pharmacological inhibition of fatty acid synthesis in breast cancer cells with fatty acid synthase overexpression caused reduced growth and programmed cell death, an effect that was reversed by addition of fatty acids to the growth media [22]. Added to this endogenous production, the lipids supplied in the diet can be used by the tumor cells in metabolic functions.

Secondly, abundant experimental data demonstrate that the dietary lipids can exert their effect on breast cancer through specific mechanisms. Those that stand out are: the influence on the stages of carcinogenesis [1]; the modification of the structure and the function of cell membranes [23,24], the enhanced tumor eicosanoid biosynthesis [25], the alteration of cell signal transduction pathways [26,27], the modulation of the specific gene expression [28,29], the increase of cell proliferation rates [30] and the immunosupressor effect [31,32]. These mechanisms would depend to a great extent on the type and quantity of lipids supplied in the diet.

The fact that all the cancerous cell types have high metabolic requirements to maintain their growth and that a great diversity of tumors can be inhibited through caloric restriction [3,18] suggests that the first of the described mechanisms is of unspecific type and related with the availability of energy. Interestingly, it has been described that caloric restriction has other kind of effects such as the increase of activities of antioxidant enzymes, the enhancement of DNA repair and the reduction of oncogene expression [33]. On the other hand, the fact that only certain types of tumors are stimulated by high-fat diets, principally breast, prostate and colon, [3,12,25] suggests that the second group of mechanisms, described as specific, have a more important role.

In the present work we have studied the effects of a polyunsaturated n-6 high-fat diet on rat experimental breast cancer at different stages of the carcinogenic process, and then we have focused the analysis of the mechanisms of such effects on the changes in the tumor fatty acid composition. This kind of approach should provide us information about the effects of the dietary lipids on the tumor cell structure and function and the signal transduction pathways. We have evaluated 14 fatty acids in 6 lipid fractions of breast tumors from rats fed a low (3%) and/or high (20%) fat corn oil diet.

2. Materials and methods

Diets. Two semi-synthetic diets were designed: a low-fat and a high-fat one, containing 18% or 23% casein, 67.9% or 45.9% dextrose, 3% or 20% corn oil, 5% cellulose, 5,9% salt-mixture and 0.24% vitamin mixture. As determined by gas chromatography, the fatty acid content in the corn oil used, expressed in weight percentage, was as follows: palmitic 11.1%, palmitoleic 0.2%, stearic 2.1%, oleic 28.2%, linoleic 57.1%, linolenic 0.9%, arachidic 0.3% and eicosenoic $<0.1\%$. Neither trans fatty acids nor phenolic antioxidants were present in the oil. Its concentration in α-tocopherol was 280 ppm. In order to maintain the normal lipid metabolism, diets were supplemented with methionine (0.51% in the low-fat diet and 0.66% in the high-fat diet), choline (1800 mg/Kg diet) and folic acid (5 mg/Kg diet). The definition, preparation and suitability of both experimental diets were previously described [34,35]. The diets were prepared weekly and stored in the dark at + 4°C.

Experimental model. Eighty, 22-days-old female Sprague-Dawley rats weighing in average 48.3 g (standard deviation = 3.5 g) were purchased from "Centre de Producció Animal" of the Universitat Autònoma de Barcelona - source ICO:OFA.SD (IOPSCaw)-. Animals were housed four to a cage in a room with controlled temperature, humidity and lighting. Animals were provided with fresh experimental diets and tap water *ad libitum.* Tumors were induced in all rats at 53 days of age by a single intra-gastric dose of 5 mg of 7,12-dimethylbenz(α)anthracene -DMBA- (Sigma-Aldrich, Madrid, Spain)/rat in 1 ml corn oil [36,37].

Experimental design. After weaning at 22 days of age, rats were randomly distributed into four experimental groups of 20 as follows: a control group (C) and an initiation group (I) in which the animals received only the low-fat diet or high-fat diet respectively throughout the experiment; a promotion group (P) where rats were fed the low-fat diet from weaning until carcinogenic induction and the high-fat diet from that time onwards; finally, a development group (D), whose animals received the low-fat diet until 157 days and

then the high-fat diet, once tumors had appeared. Animals in the group I were switched to the low-fat diet for two days before administration of DMBA and for one day after that in order to minimize the effects of dietary fat on the absorption of carcinogen. For the same reason, animals from the group P were fed the low fat diet the following day after DMBA was administered.

Once a week the rats were weighed and explored in order to discard any possible pathologies resulting from nutritional imbalances [38,39] and palpated for mammary tumors. When a tumor was first palpated, the date and the tumor location were recorded. Biological assays were finished 161 days after DMBA administration (average day of sacrifice) when the animals were on average 214 days old. Rats were killed by decapitation and a complete post-mortem examination was carried out. Mammary tumors and suspicious lesions were rapidly removed, measured, rinsed in normal saline, and divided for histopathological and lipid analyses. Samples for histological evaluation were fixed in 10% buffered formalin and later embedded in paraffin. The sections were stained with hematoxylin and eosin. The diagnosis of the mammary pathology was mainly based on the criteria of Young and Hallowes [40]. Samples for lipid analysis were flash frozen and stored in liquid nitrogen.

The care of the animals was in accord with institution guidelines. These regulations follow the current legislation applicable in our country to those animals used for experimentation and other scientific purposes. The organization and infrastructure of the animal facilities are set up in line with the Good Laboratory Practices.

Carcinogenesis parameters. Animals were monitored for mammary tumor appearance throughout the study and at the sacrifice. From this data, we calculated the following parameters: latency time, as the average time of tumor appearance; tumor incidence, as the percentage of animals bearing at least one palpable malignant mammary tumor; tumor multiplicity, as the cumulative total number of malignant mammary tumors per group; and tumor volume as the total volume of all malignant tumors in each experimental group. The functions that define each one of these carcinogenesis parameters were previously described [41].

Lipid Analysis. The fatty acid composition of mammary adenocarcinomas was studied. Previously we determined the frequency of appearance of all the fatty acids detected by gas chromatography in the tumors and we selected those that appeared regularly in at least one of the experimental group (data not shown). Following this criterion 14 fatty acids were chosen: 12:0, 14:0, 16:0, 16:1n-7, 18:0, 18:1n-9, 18:2n-6, 18:3n-3, 20:0, 20:1n-9, 20:2n-6, 20:3n-6, 20:4n-6 and 24:0. These were evaluated in 3 phospholipids (phosphatidylcholine -PC-, phosphatidylinositol -PI-, phosphatidylethanolamine -PE-), triacylglycerides -TG-, cholesteryl esters -CE- and non esterified fatty acids -FFA-.

Total lipids of mammary adenocarcinoma tissue samples were extracted with 2-propanol –Merck, Barcelona, Spain- (10:1 -ml:g-), centrifuged at 2000xg for 15 min at room temperature and the supernatant was dried under nitrogen and kept at -20°C. Aliquots of 500 μl of the extracts were subjected to thin layer chromatography on 0.25 mm silica gel plates (Merck 5721) that were developed with ethanol -Merck-/water/trichloroethylen -Merck- (54:10:36, by vol) for major phospholipids, and with 1,2 dichloroethane (Scharlau, Barcelona, Spain) for neutral lipids. The standards of oleic acid, cholesterol, triacylglycerides and cholesteryl esters (Matreya Inc, Pleassant Gap, PA) were used as markers for free fatty acids and neutral lipids and PC, PI, and PE (Matreya) were used as markers for phospholipid separation. The front of the first solvent was interrupted at 10.5 cm from the sample application area, and that of the second at 1 cm from the upper edge of the plate. Plates were stained with ethanol solution at 0.1% (wt/vol) of 2,7dichlorfluorescein (Merck) in order to visualize subsequently the various spots by means of UV light. The desired lipid spots (PC, PI, PE, FFA, TG, and CE) were scraped off the plates and the constituent fatty acids were transmethylated with 2 ml of 14% boron trifluoride-methanol (Supelco, Sigma-Aldrich, Madrid, Spain) at + 100°C for 1 h [42]. After the addition of 2 ml of supersaturated NaCl (Merck) and 4 ml of n-pentane (Fluka, Sigma-Aldrich, Madrid, Spain), the two phases were separated. The fatty acid methyl esters (organic phase) were evaporated at + 40°C under nitrogen and conserved at -20°C.

The evaporated matter was held in suspension in 500 μl of n-hexane (Merck) and aliquots (1–2 μl) were injected into the gas chromatograph (Hewlett Packard 5790, Hewlett Packard Española, S.A., Barcelona, Spain) with a flame ionization detector. The fatty acid methyl esters were analyzed on a 30m x 0.22mm capillary column with Carvowax 20M as a stationary phase (Supelcowax 10 - Supelco-). Helium was used as a carrier gas at a flow rate of 1.2 ml/min. The samples were injected in a split less mode. The column temperature was programmed as follows: +120°C (2min), +20°C/min until + 225°C, (60 min). The detector and injector temperatures were both + 250°C. The Hewlett Packard 2290A integrator was connected to the gas chromatograph. The peaks for fatty acid methyl esters were identified by comparison of their retention times to those of standard Supelco 7019, 7024 and 7033. The fatty acid contents of each lipid fraction were expressed as a percentage of the total fatty acids of that lipid fraction.

2.1. Statistical analysis

The analysis of the body weights all through the time was carried out using the methodology of Mixed-Effects Models. Average latency time of mammary malignant tumors was analyzed by the Mann-Whitney's U non-parametric test. The evolution of the other carcinogenesis parameters throughout the study was compared among the experimental groups using the Friedman's two factor non-

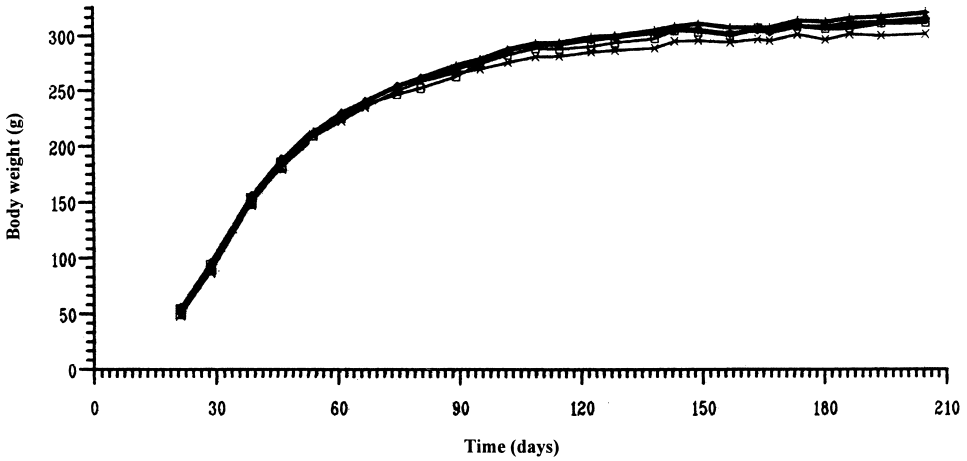

Fig. 1. Body weight evolution of the rats induced with the mammary carcinogen DMBA and fed low-fat and/or high-fat corn oil diets. See the text for the statistical significance. ×, Group C; +, group I; ◇, group P; □, group D.

parametric analysis of variance. The analysis of the qualitative tumor data at the end of the study (sacrifice) was carried out by χ^2 test and that of the quantitative data by Mann Whitney's U-test. Finally, the data of the tumor fatty acid composition were also analyzed using the Mann-Whitney's U test. The level of significance was established at $p < 0.05$.

3. Results

3.1. Animal weight gain

The body weights of the animals in each experimental group were taken throughout the study (Figure 1). The statistical analysis of the results showed that there were not differences between any of the high-fat diet groups and the control group. Moreover, the homogeneity of animal growth all through the experiment in each group was evaluated by means of the study of the coefficients of variance of body weight averages, as we previously described [35]. In all groups these coefficients were uniformly low, always lower than 10%.

3.2. Carcinogenesis parameters

The average time to first tumor development (average latency time-days) recorded in the different experimental groups was the following (mean±standard deviation): group C, 97.6 ± 25.9 (n = 18); group I, 90.6 ± 31.2 (n = 19); group P, 74.5 ± 27.4 (n = 17); group D, 97.8 ± 32.8 (n = 16). The medians were 96, 89, 64 and 96 respectively. Therefore, the latency time recorded for group P was the shortest ($P < 0.05$). The latency for groups I and D were not statistically different from that of control group.

The number of animals with palpable malignant mammary tumors (tumor incidence) was increasing throughout the study in all groups (Figure 2). The high fat diet groups I and P displayed the greatest values ($P < 0.01$), specially the group P. The evolution of this parameter in the group D was very similar than that of the control. At the sacrifice, the differences observed among groups were not statistically significant (Table 1, Figure 2).

The cumulative total number of malignant mammary tumors in each group (tumor multiplicity) also increased throughout the study (Figure 2). The tumor appearance in animals fed the low fat control diet –groups C and D- was slower in the first period of the experiment and its increase went faster from about the day 90. The multiplicity of adenocarcinomas was significantly higher ($P < 0.01$) in the group P than in the other groups, even at the sacrifice (Table 1). The differences in the group I were lower, they existed during less time and were not statistically significant. Differences did not exist between the groups D and C.

The behavior of the total tumor volume all through the experiment of each one of the experimental groups was very similar to that of the total number of tumors. Thus, the group P always showed a tumor volume significantly higher than the control, and in the group I the differences were lower and existed during less time although they were statistically significant too. The values of the group D were very similar to those of the control although the small differences were paradoxically statistically significant (Figure 2). At the sacrifice, this parameter could not be statistically analyzed because there was only one value per group.

3.3. Lipid analysis

We analyzed the fatty acid composition of six lipid fractions (3 phospholipids, free fatty acids, triacylglycerides and cholesteryl esters) in the mammary adenocarcinomas from rats fed a low-fat and/or high-fat diet. The relative

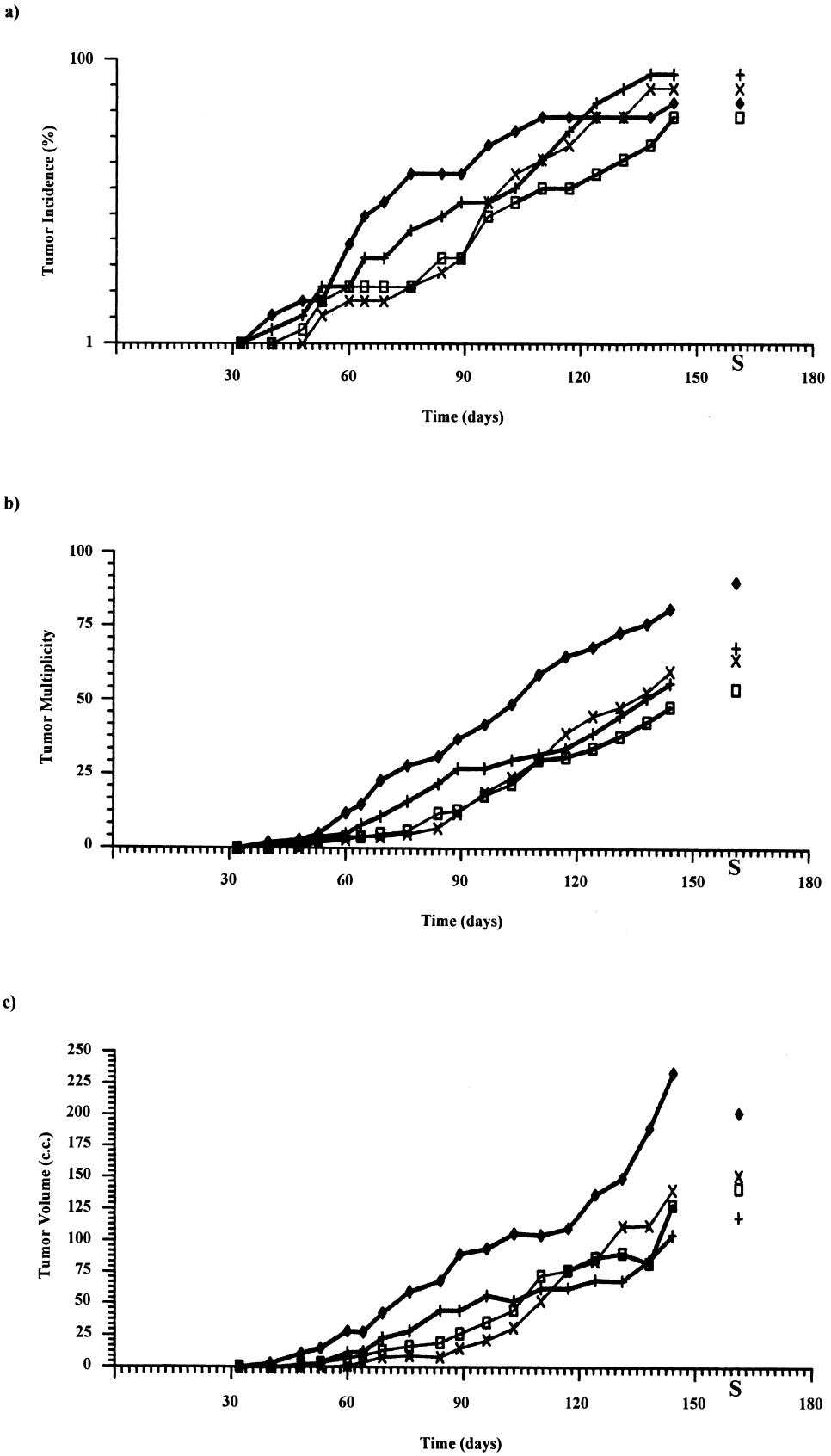

Fig. 2. Influence of dietary n-6 polyunsaturated lipids on tumor incidence (a), multiplicity (b) and volume (c) of the malignant mammary pathology in the course of the experiment. See the text for the statistical significance. ×, Group C; +, group I; ◇, group P; □, group D. S, sacrifice.

Table 1
Influence of dietary n-6 polyunsaturated lipids on tumor incidence, multiplicity and volume of the malignant mammary pathology at the end of the study[a]

Group	Tumor Incidence (%)	Tumor Multiplicity (total num. tumors)	Tumor Volume[b] (c.c.)	
C	90 (18/20)	64	m M ± SD n	0.24 1.68 ± 4.55 64
I	95 (19/20)	68	m M ± SD n	0.23 1.09 ± 2.47 66
P	85 (17/20)	90	m M ± SD n	0.45 1.47 ± 2.72 90
D	80 (16/20)	54	m M ± SD n	0.27 1.66 ± 4.05 54

[a] See the text for statistical significance at this point. [b] Tumor volume (c.c.) was measured using the formula $V = 4/3\pi$ (D1/2)(D2/2)(D3/2), where D1, D2 and D3 are the three diameters of the tumor. m: Median; M: mean; SD: standard deviation; n: sample size.

content of the 14 fatty acids studied, expressed as a percentage, for each lipid fraction in the various experimental groups is indicated in Tables 2 to 7. The content of linoleic, oleic and arachidonic acids has also been plotted in Figure 3.

The major fatty acids (those whose relative content was equal to or higher than 5%) were different in the several lipid fractions analyzed. The ordering of fatty acids within a specific lipid fraction according to their relative content (i.e. order of magnitude) and the comparisons held between experimental groups for each fatty acid were as follows:

Phospholipids. One unique feature in the 3 phospholipids studied is that although each one of them exhibited different major fatty acids, their order of magnitude was the same in the four experimental groups. In decreasing order, they were the following: in phosphatidylcholine, 1st) 16:0, 2nd) 18:1n-9, 3rd) 20:4n-6, 4th) 18:0 and 5th) 18:2n-6; in phosphatidylinositol, 1st) 18:0, 2nd) 18:1n-9, 3rd) 16:0 and 4th) 20:4n-6; and in phosphatidylethanolamine, 1st) 20:4n-6, 2nd) 18:0, 3rd) 18:1n-9 and 4th) 16:0 (Tables 2,3 and 4). When comparing among the experimental groups, the highest number of differences was found in the phosphatidylcholine. In this fraction, the 18:1n-9 showed a relative content significantly lower in the three groups fed the high-fat diet than in the control group. Conversely, the 18:2n-6 was significantly more abundant in the high-fat diet groups, being their values in direct relation to the administration time of this kind of diet. Significant differences were also observed in the C/P comparison for the minor fatty acids 14:0 and 16:1n-7. In both cases the average values of group P were lower than those of group C. Other differences obtained relate to 20:2n-6 in the C/D comparison and to 20:3n-6 in the C/I comparison (Table 2). With regards to the phosphatidylinositol, no significant differences between the groups were found (Table 3). Finally, in the phosphatidylethanolamine, differences were mainly found in group P compared with C (lower content of 18:1n-9 and higher content of 18:2n-6 and 24:0) except for the 16:0 whose content was greater in group D than in the control (Table 4).

Free fatty acids and neutral lipids. In contrast with what occurred with the phospholipids, the order of magnitude of the fatty acids in the free fatty acids fraction was variable depending on the experimental group. Only the two most abundant ones, 16:0 and 18:1n-9, maintained their order of magnitude in all groups. The other major fatty acids in this fraction were 18:0, 16:1n-7, 20:4n-6 and 18:2n-6 (Table 5). In the triacylglycerides, the two groups fed the high-fat diet for the longest period (I and P) showed a composition and a relative content of major fatty acids which reflected those of the type of the dietary fat (corn oil): 1st) 18:2n-6, 2nd) 18:1n-9, 3rd) 16:0 (Table 6). Finally, in the cholesteryl esters, the order of magnitude of the fatty acids was also different in relation to the experimental group. In general, the major fatty acids in this fraction were 16:0, 18:1n-9, 20:4n-6, 18:0, 18:2n-6 and 16:1n-7 (Table 7). The comparisons among the groups showed that in the free fatty acids fraction differences existed in 18:1n-9 and 18:2n-6. As to the former, its relative content was lower in groups I and P than in C. Instead, the 18:2n-6 was greater in the three groups fed the high-fat diet (Table 5). Among the six lipid fractions studied, that of the triacylglycerides was the one that displayed the greatest number of differences between the experimental groups. In this fraction, the relative content of the fatty acids 16:0, 16:1n-7, 18:1n-9 and 18:2n-6 was significantly different in tumors from the animals fed the high-fat diet and those of the control group, with the exception of 18:1n-9 in the C/D comparison. The content of the first three (16:0, 16:1n-7, 18:1n-9) was lower in the high-fat diet groups than in group C, and in the case of 18:2n-6 the opposite was true. Differences were also found between groups C and P for 12:0 and between groups C and I for 14:0 (Table 6). In the cholesteryl ester fraction, the only differences found were a greater content of 18:2n-6 in the groups P and D than in the control group, and a lower content of 20:4n-6 in the group I than in C (Table 7).

4. Discussion

The results of the analysis of the animal body weight evolution and the absence in the animals of clinical and anatomopathological signs caused by nutritional imbalance during the course of the experiment, demonstrated that our experimental semi-synthetic diets, even the high-fat diet which is imbalanced in its lipid content, can be consumed chronically without any adverse effect on the host. This is a key factor in order to be able to assure the specificity of the

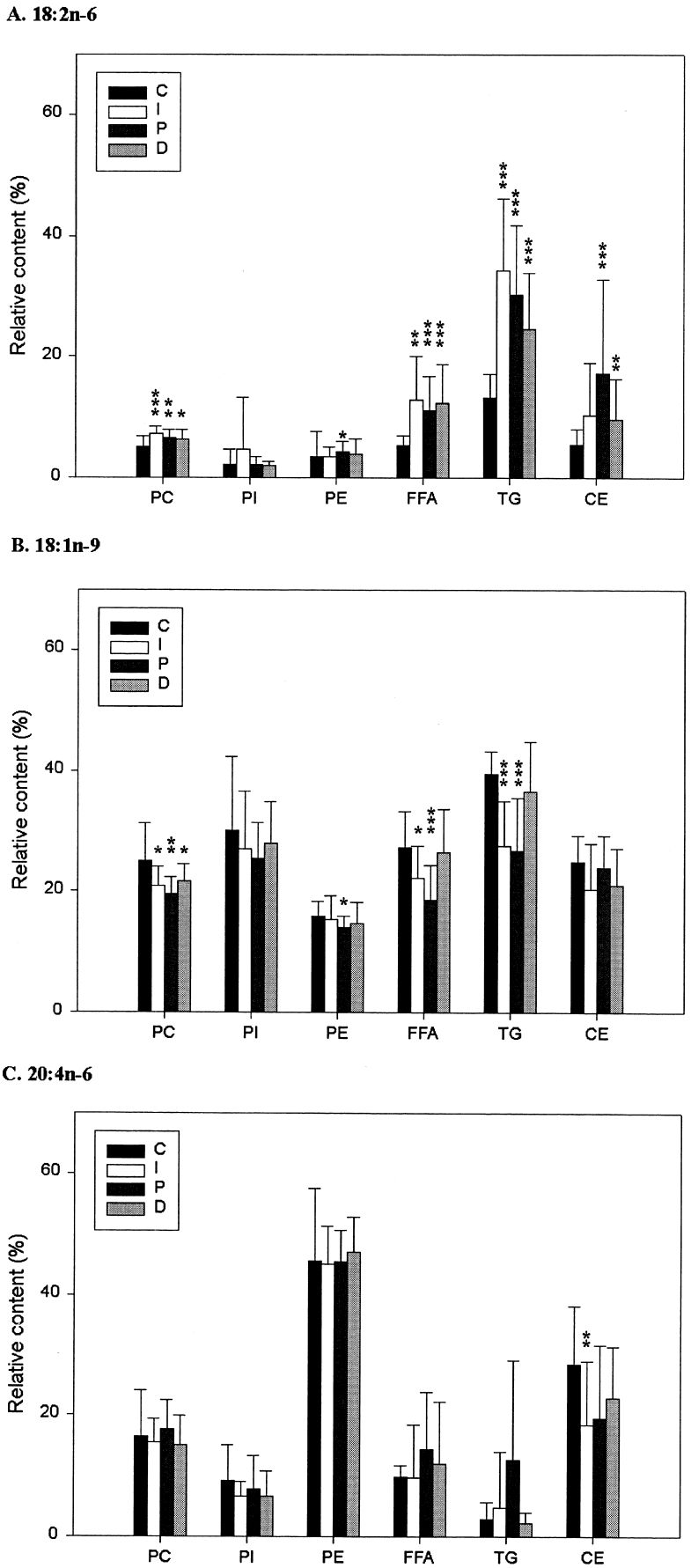

Fig. 3. Composition in linoleic acid (A), oleic acid (B) and arachidonic acid (C) of the different lipid fractions from malignant mammary tumors. Bars correspond to the control group C (black bar), initiation group I (white bar), promotion group P (dark gray bar) and development group D (light gray bar). The values are area percent (mean ± standard deviation). The statistically significant differences from the values of control group are indicated as follows: * $p < 0.05$, ** $p < 0.01$, ***$p \ll 0.01$.

Table 2
Fatty acid composition of phosphatidylcholine in malignant mammary tumors of rats fed experimental diets

Fatty acids	Group			
	C	I	P	D
12:0	0.09 ± 0.03 (6)	0.07 ± 0.04 (5)	0.12 ± 0.10 (8)	0.07 ± 0.05 (9)
14:0	1.48 ± 0.54 (16)	1.45 ± 0.67 (14)	1.07 ± 0.57 (15)*	1.32 ± 1.31 (17)
16:0	42.05 ± 5.04 (15)	42.93 ± 4.66 (14)	42.53 ± 5.72 (15)	40.08 ± 14.97 (17)
16:1n − 7	3.00 ± 0.89 (8)	2.41 ± 1.08 (10)	2.22 ± 0.31 (7)*	14.50 ± 22.00 (8)
18:0	8.76 ± 2.61 (16)	8.77 ± 1.26 (14)	9.57 ± 2.88 (15)	8.50 ± 1.73 (17)
18:1n − 9	25.03 ± 6.24 (16)	20.86 ± 3.18 (14)*	19.54 ± 2.75 (15)**	21.68 ± 2.77 (17)*
18:2n − 6	5.15 ± 1.71 (15)	7.27 ± 1.16 (14)***	6.63 ± 1.34 (15)**	6.28 ± 1.70 (16)*
18:3n − 3	— (1)	0.28 ± 0.26 (3)[a]	— (1)[a]	— (2)[a]
20:0	— (1)	0.34 ± 0.26 (3)[a]	0.24 ± 0.18 (3)[a]	0.59 ± 0.50 (7)[a]
20:1n − 9	3.50 ± 8.12 (9)	0.70 ± 0.44 (6)	2.04 ± 4.41 (8)	0.75 ± 0.36 (8)
20:2n − 6	0.41 ± 0.16 (8)	0.64 ± 0.33 (9)	0.54 ± 0.20 (8)	0.68 ± 0.23 (10)*
20:3n − 6	0.54 ± 0.12 (9)	0.75 ± 0.24 (10)*	0.68 ± 0.21 (9)	0.70 ± 0.24 (9)
20:4n − 6	16.40 ± 7.62 (16)	15.48 ± 3.88 (14)	17.67 ± 4.83 (15)	15.11 ± 4.82 (16)
24:0	— (1)	0.52 ± 0.28 (3)[a]	— (2)[a]	0.23 ± 0.13 (4)[a]

Values are area percent (mean ± standard deviation). Significantly different from the values of control group: * $p < 0.05$, ** $p < 0.01$, *** $p \ll 0.01$. —: fatty acid present on fewest occasions in the lipid fraction ($n \leq 3$). [a]: statistical comparison could not be carried out. The number of tumors analyzed was: 17 in control group (C), 15 in initiation group (I), 17 in promotion group (P) and 18 in development group (D). The sample size is indicated in parentheses.

results of the dietary lipid effects on experimental breast cancer.

The analysis of the carcinogenesis parameters showed that the high-fat n-6 polyunsaturated diet stimulated the malignant mammary tumor growth. The group P was the one that displayed the highest differences in all the carcinogenesis parameters studied followed by the group I. Thus, in the group P adenocarcinomas appeared earlier and the incidence of animals affected, the tumor yield and the tumor volume were also higher than in the other groups. On the other hand, the group D, which was fed the low-fat diet during a long time before receiving the high-fat diet, in general behaved like the control group although showed significant differences in the analysis of the total volume as a function of time. However, this fact could be explained by the limitations of the statistic test applied. The Friedman's test as a non-parametric analysis works with ranks and therefore it can detect statistical differences when the most points of a curve are above than those of the other although the quantitative differences are very small. This was the case when comparing the group D with the control.

All these results clearly suggest that the main effect of the high-fat corn oil diet on experimental mammary cancer was to stimulate the growth of the tumors during the promotion stage of the carcinogenesis. These results are in accordance with those obtained by other authors [1,2]. We

Table 3
Fatty acid composition of phosphatidylinositol in malignant mammary tumors of rats fed experimental diets

Fatty acids	Group			
	C	I	P	D
12:0	0.63 ± 0.86 (7)	0.69 ± 0.65 (5)	0.19 ± 0.17 (7)	0.27 ± 0.22 (4)[a]
14:0	3.01 ± 7.65 (11)	0.41 ± 0.23 (12)	0.40 ± 0.19 (12)	0.64 ± 0.48 (10)
16:0	14.29 ± 8.46 (15)	10.74 ± 4.37 (14)	10.86 ± 5.56 (14)	13.18 ± 8.72 (13)
16:1n − 7	3.00 ± 2.59 (8)	1.23 ± 0.48 (7)	3.40 ± 6.45 (7)	6.65 ± 6.16 (5)
18:0	44.51 ± 13.28 (15)	52.63 ± 7.86 (13)	51.62 ± 10.19 (14)	46.54 ± 15.61 (13)
18:1n − 9	30.01 ± 12.28 (14)	27.03 ± 9.50 (14)	25.42 ± 6.04 (14)	27.95 ± 6.93 (12)
18:2n − 6	2.24 ± 2.42 (6)	4.69 ± 8.55 (12)	2.17 ± 1.36 (12)	2.07 ± 0.62 (11)
18:3n − 3	2.05 ± 0.86 (3)	1.12 ± 0.88 (6)[a]	0.59 ± 0.41 (3)[a]	— (2)[a]
20:0	0.92 ± 0.48 (3)	0.93 ± 0.38 (6)[a]	0.50 ± 0.07 (6)[a]	0.64 ± 0.44 (5)[a]
20:1n − 9	— (2)	1.56 ± 1.49 (6)[a]	0.50 ± 0.06 (4)[a]	2.20 ± 3.35 (4)[a]
20:2n − 6	— (1)	2.29 ± 3.31 (6)[a]	— (2)[a]	1.81 ± 1.23 (4)[a]
20:3n − 6	— (2)	0.73 ± 0.10 (3)[a]	1.18 ± 0.88 (6)[a]	— (2)[a]
20:4n − 6	9.19 ± 5.86 (11)	6.70 ± 2.43 (10)	7.96 ± 5.37 (11)	6.73 ± 4.10 (12)
24:0	— (2)	1.52 ± 0.74 (6)[a]	2.70 ± 1.10 (4)[a]	3.15 ± 1.57 (4)[a]

Values are area percent (mean ± standard deviation). Significantly different from the values of control group: * $p < 0.05$, ** $p < 0.01$, *** $p \ll 0.01$. —: fatty acid present on fewest occasions in the lipid fraction ($n \leq 3$). [a]: statistical comparison could not be carried out. The number of tumors analyzed was: 17 in control group (C), 15 in initiation group (I), 17 in promotion group (P) and 18 in development group (D). The sample size is indicated in parentheses.

Table 4
Fatty acid composition of phosphatidylethanolamine in malignant mammary tumors of rats fed experimental diets

Fatty acids	Group			
	C	I	P	D
12:0	0.06 ± 0.03 (6)	0.05 ± 0.03 (6)	0.10 ± 0.05 (9)	0.18 ± 0.14 (9)
14:0	0.91 ± 2.31 (14)	0.33 ± 0.22 (13)	0.32 ± 0.22 (15)	0.27 ± 0.16 (14)
16:0	9.54 ± 2.84 (16)	10.07 ± 2.33 (14)	10.44 ± 2.17 (15)	12.12 ± 3.39 (17)*
16:1n − 7	1.40 ± 0.33 (12)	1.46 ± 1.15 (12)	2.33 ± 3.35 (10)	2.33 ± 3.29 (10)
18:0	20.37 ± 7.14 (16)	21.38 ± 2.81 (14)	18.80 ± 3.31 (15)	18.79 ± 5.31 (17)
18:1n − 9	15.89 ± 2.44 (16)	15.43 ± 3.82 (14)	14.12 ± 1.82 (15)*	14.75 ± 3.44 (17)
18:2n − 6	3.46 ± 4.17 (15)	3.53 ± 1.64 (14)	4.37 ± 1.74 (15)*	3.85 ± 2.57 (16)
18:3n − 3	— (1)	0.29 ± 0.14 (5)[a]	0.36 ± 0.43 (3)[a]	— (2)[a]
20:0	— (2)	0.25 ± 0.24 (5)[a]	0.18 ± 0.07 (3)[a]	0.22 ± 0.17 (4)[a]
20:1n − 9	4.87 ± 11.59 (7)	0.51 ± 0.33 (8)	0.30 ± 0.26 (4)[a]	0.49±0.79 (7)
20:2n − 6	0.58 ± 0.38 (9)	0.63 ± 0.30 (13)	4.39 ± 10.68 (8)	0.58 ± 0.32 (7)
20:3n − 6	0.61 ± 0.31 (7)	0.63 ± 0.25 (10)	0.50 ± 0.31 (6)	0.47 ± 0.28 (9)
20:4n − 6	45.72 ± 11.82 (16)	45.19 ± 6.17 (14)	45.51 ± 5.28 (15)	47.19 ± 5.66 (17)
24:0	1.65 ± 0.99 (5)	2.02 ± 1.55 (9)	3.14 ± 0.44 (10)**	1.99 ± 1.48 (8)

Values are area percent (mean ± standard deviation). Significantly different from the values of control group: * $p < 0.05$, ** $p < 0.01$, *** $p \ll 0.01$. —: fatty acid present on fewest occasions in the lipid fraction ($n \leq 3$). [a]: statistical comparison could not be carried out. The number of tumors analyzed was: 17 in control group (C), 15 in initiation group (1), 17 in promotion group (P) and 18 in development group (D). The sample size is indicated in parentheses.

have also shown, from the results obtained in the group D, that, in the experimental conditions of the present work, this kind of high-fat diet did not have any effect on the development of the mammary tumors once they had appeared. Furthermore, the fact that the differences between the groups P and C have not been found in the same way between the groups I and C, suggests that the high fat corn oil diet administered to the animals from weaning onwards (group I) could have changed the cell differentiation status of the mammary gland. The changes would lead the tissue far from the best conditions for the carcinogenic induction and therefore would make it more resistant to the carcinogen, decreasing the carcinogenesis yield. In this sense, it has been described an acceleration of the puberty in rats fed diets with a high lipid content [43]. New studies will be necessary in order to clarify this subject.

How dietary n-6 lipids enhance mammary tumor growth is not well understood. One of the proposed mechanisms is the modification of the structure and the function of cell and nuclear membranes [23,24]. In the present work we have focused on investigating the possibility that these dietary lipids influence the tumor development either directly or indirectly through modifications in the fatty acid composition of the tumor cell. The qualitative and/or quantitative

Table 5
Fatty acid composition of free fatty acids in malignant mammary tumors of rats fed experimental diets

Fatty acids	Group			
	C	I	P	D
12:0	0.96 ± 0.43 (11)	1.12 ± 1.56 (8)	0.75 ± 0.39 (14)	0.69 ± 0.39 (6)
14:0	2.22 ± 1.23 (16)	2.43 ± 1.69 (13)	1.84 ± 0.55 (14)	2.20 ± 1.13 (14)
16:0	34.02 ± 10.86 (16)	32.13 ± 6.00 (13)	37.78 ± 15.94 (15)	32.23 ± 6.96 (16)
16:1n − 7	12.93 ± 10.26 (10)	9.30 ± 12.88 (7)	12.59 ± 11.64 (3)[a]	7.69 ± 8.51 (7)
18:0	14.36 ± 3.24 (15)	15.94 ± 4.92 (12)	12.07 ± 3.48 (14)	12.92 ± 4.65 (15)
18:1n − 9	27.30 ± 5.99 (16)	22.22 ± 5.29 (13)*	18.56 ± 5.77 (15)***	26.45 ± 7.21 (16)
18:2n − 6	5.33 ± 1.65 (14)	12.84 ± 7.17 (12)**	11.06 ± 5.66 (15)***	12.27 ± 6.47 (16)***
18:3n − 3	— (2)	2.98 ± 1.13 (3)[a]	8.59 ± 8.87 (3)[a]	— (2)[a]
20:0	— (0)	— (1)[a]	— (0)[a]	— (0)[a]
20:1n − 9	3.18 ± 1.26 (3)	11.78 ± 5.36 (3)[a]	3.11 ± 2.02 (10)[a]	3.17 ± 2.49 (7)[a]
20:2n − 6	2.60 ± 2.37 (4)	5.39 ± 3.57 (3)[a]	— (0)[a]	— (1)[a]
20:3n − 6	— (0)	— (1)[a]	— (1)[a]	— (1)[a]
20:4n − 6	9.89 ± 1.84 (13)	9.80 ± 8.66 (8)	14.46 ± 9.30 (13)	12.01 ± 10.19 (12)
24:0	— (0)	— (0)[a]	— (0)[a]	— (0)[a]

Values are area percent (mean ± standard deviation). Significantly different from the values of control group: * $p < 0.05$, ** $p < 0.01$, *** $p \ll 0.01$. —: fatty acid present on fewest occasions in the lipid fraction ($n \leq 3$). [a]: statistical comparison could not be carried out. The number of tumors analyzed was: 17 in control group (C), 15 in initiation group (1), 17 in promotion group: (P) and 18 in development group (D). The sample size is indicated in parentheses.

Table 6
Fatty acid composition of triacylglycerides in malignant mammary tumors of rats fed experimental diets

Fatty acids	Group			
	C	I	P	D
12:0	0.14 ± 0.10 (15)	0.24 ± 0.21 (11)	0.53 ± 0.97 (15)**	0.27 ± 0.34 (13)
14:0	1.65 ± 0.57 (16)	1.17 ± 0.51 (13)*	1.68 ± 1.20 (15)	1.39 ± 0.72 (16)
16:0	27.56 ± 4.17 (16)	22.50 ± 13.89 (13)**	18.11 ± 7.87 (15)**	24.02 ± 8.67 (16)*
16:1n − 7	7.27 ± 2.38 (16)	3.08 ± 2.40 (12)***	4.97 ± 5.91 (10)**	4.24 ± 1.12 (12)**
18:0	5.00 ± 2.13 (16)	4.48 ± 2.30 (13)	7.40 ± 5.60 (14)	4.99 ± 1.73 (16)
18:1n − 9	39.51 ± 3.71 (16)	27.51 ± 7.46 (13)***	26.70 ± 8.86 (14)***	36.54 ± 8.34 (16)
18:2n − 6	13.32 ± 3.83 (16)	34.39 ± 11.87 (13)***	30.34 ± 11.47 (14)***	24.55 ± 9.35 (16)***
18:3n − 3	0.07 ± 0.06 (3)	0.71 ± 1.24 (6)[a]	4.16 ± 5.86 (4)[a]	0.19 ± 0.09 (3)[a]
20:0	0.31 ± 0.45 (6)	0.16 ± 0.07 (5)	0.66 ± 0.47 (6)	0.38 ± 0.45 (6)
20:1n − 9	3.13 ± 6.81 (11)	2.17 ± 4.10 (9)	2.99 ± 4.25 (12)	4.41 ± 9.92 (11)
20:2n − 6	0.59 ± 0.31 (8)	0.49 ± 0.22 (7)	0.60 ± 0.22 (9)	0.29 ± 0.12 (8)
20:3n − 6	0.37 ± 0.27 (7)	0.36 ± 0.16 (7)	0.49 ± 0.19 (7)	0.28 ± 0.12 (9)
20:4n − 6	3.00 ± 2.75 (15)	4.90 ± 9.17 (12)	12.68 ± 16.41 (14)	2.33 ± 1.78 (11)
24:0	— (1)	— (2)[a]	— (2)[a]	— (1)[a]

Values are area percent (mean ± standard deviation). Significantly different from the values of control group: * $p < 0.05$, ** $p < 0.01$, *** $p \ll 0.01$. —: fatty acid present on fewest occasions in the lipid fraction ($n \leq 3$). [a]: statistical comparison could not be carried out. The number of tumors analyzed was: 17 in control group (C), 15 in initiation group (1), 17 in promotion group: (P) and 18 in development group (D). The sample size is indicated in parentheses.

changes in the lipid profile of mammary tumors could affect both the cell membrane structure and function and cell energy store, what could lead the cell to a more malignant phenotype.

Firstly, when we analyze the presence of the 14 fatty acids in the six lipid fractions studied for each of the four experimental groups, we found that only three of them always were major (relative content ≥ 5%): palmitic acid (16:0), stearic acid (18:0) and oleic acid (18:1n-9). In close relation to them, we found arachidonic acid (20:4n-6) which was only minor (relative content <5%) in the triacylglycerides. Palmitoleic (16:1n-7) and linoleic (18:2n-6) acids would form an intermediate group characterized by being in low quantities in phospholipids. Finally, the remaining eight fatty acids were always minor.

One feature which differentiated phospholipids from free fatty acids and neutral lipids was that only in the former the type of major fatty acids and their order of magnitude were identical in the four experimental groups. The composition of the free fatty acids and the two neutral lipids were generally different depending on the experimental group. The results obtained from the two neutral lipids (triacylglicerides and cholesteryl esters) in the two groups fed the high-fat diet for the longest time (I and P) were similar for

Table 7
Fatty acid composition of cholesteryl esters in malignant mammary tumors of rats fed experimental diets

Fatty acids	Group			
	C	I	P	D
12:0	3.68 ± 3.89 (15)	2.44 ± 2.29 (13)	2.61 ± 3.63 (13)	2.66 ± 1.67 (13)
14:0	2.55 ± 1.33 (16)	2.42 ± 1.34 (14)	2.35 ± 1.11 (15)	2.49 ± 1.09 (15)
16:0	21.93 ± 10.06 (15)	24.71 ± 19.06 (14)	23.69 ± 7.99 (15)	23.54 ± 6.52 (16)
16:1n − 7	8.66 ± 6.94 (11)	7.28 ± 6.45 (12)	7.51 ± 8.69 (10)	7.98 ± 6.53 (9)
18:0	10.59 ± 2.77 (13)	10.11 ± 4.33 (14)	10.29 ± 5.31 (15)	10.69 ± 3.07 (15)
18:1n − 9	24.83 ± 4.44 (16)	20.36 ± 7.54 (14)	23.92 ± 5.39 (15)	20.95 ± 6.21 (16)
18:2n − 6	5.57 ± 2.42 (14)	10.37 ± 8.57 (12)	17.20 ± 15.66 (14)***	9.69 ± 6.59 (16)**
18:3n − 3	1.53 ± 1.32 (3)	— (2)[a]	3.51 ± 05.91 (5)[a]	— (2)[a]
20:0	— (1)	0.79 ± 0.26 (3)[a]	0.47 ± 0.25 (4)[a]	1.78 ± 0.86 (3)[a]
20:1n − 9	3.13 ± 2.87 (4)	9.95 ± 15.60 (7)[a]	0.89 ± 0.78 (4)[a]	2.81 ± 1.29 (8)[a]
20:2n − 6	4.13 ± 4.50 (3)	1.40 ± 0.39 (4)[a]	0.51 ± 0.33 (4)[a]	1.52 ± 1.28 (7)[a]
20:3n − 6	1.56 ± 0.61 (5)	2.63 ± 1.44 (4)[a]	1.19 ± 1.12 (5)	2.23 ± 1.02 (8)
20:4n − 6	28.43 ± 9.83 (15)	18.40 ± 10.66 (14)**	19.56 ± 12.10 (11)	22.88 ± 8.50 (16)
24:0	— (1)	— (1)[a]	— (1)[a]	— (2)[a]

Values are area percent (mean ± standard deviation). Significantly different from the values of control group: * $p < 0.05$, ** $p < 0.01$, *** $p \ll 0.01$. —: fatty acid present on fewest occasions in the lipid fraction ($n \leq 3$). [a]: statistical comparison could not be carried out. The number of tumors analyzed was: 17 in control group (C), 15 in initiation group (1), 17 in promotion group (P) and 18 in development group (D). The sample size is indicated in parentheses.

both, but different from those obtained from the other two groups (C and D) which were also similar to each other. Therefore, one important aspect to consider is the influence which the composition of the dietary lipid (corn oil) had on that of the tumor lipid fractions. In this respect it was seen that only in the triacylglicerides in groups I and P, the major fatty acids were the same ones and in the same order of abundance as the dietary lipid, i.e. the linoleic acid (18:2n-6) the most abundant, followed by the oleic acid (18:1n-9) and, in the third place, the palmitic acid (16:0). The composition of the rest of the lipid fractions were not as related to that of the diet. This situation was clear for the 18:2n-6, an essential fatty acid and so non-synthesizable by tumor cells. The same occurred with the 18:1n-9 and the 16:0 although in a less evident way as they were found in higher quantities than those of the 18:2n-6. Similar results for oleic acid have also been found by other authors in experimental tumors [44,45]. All these results would indicate the resistance to dietary fatty acid modification of the tumor lipid fractions. This behavior would be more noticeable in the phospholipids fractions and would not occur in the triacylglicerides. It must be pointed out that the mentioned resistance of these lipid fractions was maintained even when the levels of lipids in the diet were high.

Therefore, our results suggest that in breast cancer cells the metabolism of the fatty acids depend more on specific factors of tumor tissue than on the dietary lipid content. These results would agree with the fact that in breast cancer, particularly in humans, tumor cells express high levels of fatty acids synthesizing enzymes [20] and use endogenously synthesized fatty acids [22]. In marked contrast, lipogenic tissues and normal mammary cells preferentially utilize dietary lipids [22,46]. Likewise, they are consistent and extend those of Cohen et al [47] and Rees et al [48] carried out on other mammary carcinogenesis models using different rat strains and similar or distinct diets. Despite the experimental approach of these works being somewhat different from our own (in the former the authors studied the fatty acid content of total phospholipids and total neutral lipids, without determining the fractions which comprise them, and in the latter only the TG), the general conclusion was similar. Thus, the diet had less influence on the fatty acid composition of different phospholipids than in that of the triacylglicerides, where the differences would depend on the type and the quantity of lipid administered in the diet. The same results have been also found when studying the fatty acid composition of triacylglicerides from normal mammary gland [48].

Another fatty acid important to consider is the arachidonic acid (20:4n-6). Our results showed that this fatty acid was the most abundant in the PE, found in moderate quantities in PC and CE, low in PI and FFA, and very low in TG. By comparing the content of arachidonic and linoleic acids we observed a tendency to find more arachidonic acid in the fractions in which there was less linoleic acid (Figure 3). This situation was above all manifested in the phospholipids, particularly in the PE. Contrarily, in the TG in which the 18:2n-6 was high, the values for 20:4n-6 were low. Thus, it suggests that the efficiency of conversion of linoleic acid to arachidonic acid is different in the diverse lipid fractions, being more or less high in the phospholipids and low in the TG. In the N-ethyl-N-nitrosourea-induced breast cancer model, other authors have also described that in the PE fraction there is a evidence of increased conversion of linoleic acid to arachidonic acid in tumor tissue compared to normal tissue [49]. The lower quantities of 20:4n-6 found in our study in PI and PC, compared to those in PE, could also be on relation to the fact that this fatty acid in these fractions undergoes rapid use on being substratum from the synthesis of second messengers [50].

The different fatty acid composition described of the neutral lipids and phospholipids could probably be attributed to the different roles played by these lipid fractions in the economy of the normal and tumor cells: the former serving largely as energy store or with a structural role, and the latter for maintaining cell membrane structure and function (see below).

When we analyzed the results from a quantitative point of view, that is to say the differences that could exist between the average percentages exhibited by the different fatty acids in the various lipid fractions in the high-fat diet groups in relation to the control group, the results indicated that the most abundant fatty acids in the lipid fractions did not tend to be those that provided the statistically significant differences among groups. Thus, if we calculate the cumulative frequency with which each of the fatty acids studied displayed these differences, we may firstly observe that the three most abundant in the corn oil were, in the same order, those that showed the greatest number of differences (18:2n-6, 12; 18:1n-9, 8; 16:0 and 16:1n-7, 4; 14:0, 2; others -12:0, 20:2n-6, 20:3n-6, 20:4n-6, 24:0-, 1). Moreover, these fatty acids were normally found in low percentages in the lipid fractions with the exceptions previously mentioned for the 18:1n-9 and 16:0. Likewise, as we discussed above, in the triacylglycerides from the groups I and P the most abundant fatty acids were also those that displayed the statistical differences. Moreover, when we analyzed the total number of differences in the various lipid fractions, we found that the TG and PC displayed the highest number of them whereas the PI did not show none. Finally, on analyzing the differences by experimental groups, we found the greatest number of differences in the group P and the lowest number in the group D. These results are consistent with those obtained in the analysis of the carcinogenesis, where the group P displayed the highest yield and the group D the lowest.

We must also contemplate the nature of the differences obtained. In this respect, we found an opposite trend in the first two fatty acids with the greatest number of differences, i.e. 18:2n-6 and 18:1n-9. Thus, when differences existed, the 18:2n-6 content was always higher in the high-fat diet groups, basically P and I, than in control group, in all the lipid fractions and irrespective of its abundance in the frac-

tion or the experimental group compared. Conversely, the 18:1n-9 content was always lower in the groups fed the high-fat diet than in group C. Therefore, our results indicate that the high-fat corn oil diet change the tumor lipid profile: it increases the 18:2n-6 and decreases the 18:1n-9 content of the mammary tumors. This modification associated with a greater mammary carcinogenesis in the groups of the animals fed this high-fat diet, specially in the group P, and therefore it could be related to the mechanism of the stimulating action of this diet. Some experimental and epidemiological data support this hypothesis. In the first place, the animal studies clearly indicate that the 18:2n-6 is the fatty acid most directly implicated in the stimulation of experimental mammary tumors by high-fat diets [2–5]. Furthermore, the epidemiological data show that the breast cancer mortality rates in some Mediterranean countries, with a relatively high intake of oleic acid, mainly present in olive oil [51,52], are low in comparison to those of the rest of the developed world [3,18]. Moreover, some experimental studies, including one recently developed in our laboratory –data not shown-, have indicated olive oil to be protective relative to other sources of fats [1,10], probably related both with the capacity of the 18:1n-9 to interfere with the metabolism of the 18:2n-6 to 20:4n-6 [25] and its content in antioxidant compounds provided by tocopherols and phenolic compounds [11].

The above mentioned change in the tumor lipid profile because of the high-fat corn oil diet was mainly found, among the phospholipids, in the PC, and in the FFA and TG. In the PE it was only observed in the group P and, interestingly, it didn't exist in the PI, where any significant modification was found in the fatty acid composition because of the dietary lipid. The resistance to dietary fatty acid modification of PI has also been described for distinct types of lipids (maize, olive and fish oils) in both normal and tumor tissue of the mammary gland of a experimental model of breast cancer induced in the rat with N-ethyl-N-nitrosourea [49].

Some mechanisms by which this change in the fatty acid composition of mammary tumors could have accounted for the enhanced carcinogenesis may be postulated. Firstly, it has been described that the 18:2n-6, and its derivative arachidonic acid, once incorporated into the membrane phospholipids, can alter the physiochemical characteristics (micro viscosity or fluidity) of the membrane lipid matrix. These alterations in turn can influence the conformation, mobility and function of a wide variety of intrinsic and extrinsic membrane-bound proteins such as hormone receptors, ion gates and channels and bound-enzymes [24,27,53]. Likewise, increased unsaturation of the cellular membranes has been described to enhance their fluidity and has been associated with the processes of carcinogenesis [54–56]. The variations found in the present work in the unsaturated fatty acids content of the phospholipids, mainly in the PC, located fundamentally in the outer face of the membrane [57], suggest that changes in the fluidity of the membrane could have been produced in tumors because of the dietary lipids. Nevertheless, the final effect would depend on the relationship between the increase in fluidity produced by the rise in 18:2n-6 and its decrease due to the fall in 18:1n-9.

Secondly, the 18:2n-6, the 20:4n-6, the diacylglycerol and the eicosanoids synthesized from the arachidonic acid are second messengers in the signal transduction of hormones, growth factors and oncogenes and have been involved in the multiple steps of tumorogenesis and metastasis [26,27,58–61]. Interestingly, it has been described higher concentrations of arachidonic acid and prostaglandines in experimental breast tumors than in normal breast tissue [4,25,48], and the inhibitors of such prostaglandines reverse the tumor development [62].

The results obtained in the phospholipids and free fatty acids fractions would be in agreement with the mechanisms of action described of the unsaturated fatty acids on the cell membrane structure and function. Although these results were obtained at a specific point during the study, and therefore solely reflect the situation at that precise moment, when analyzed as a whole they suggest the existence of a dynamic process. In it, it is necessary to consider both the quantity and type of lipids supplied in the experimental diets, and the physiopathology of the tumor cell. In this respect, it must be firstly pointed out that in all the experimental groups the 18:1n-9 was one of the fatty acids present in relatively high quantities as much in the three phospholipids as in the free fatty acids studied. Conversely, the 18:2n-6 content was lower in all these fractions. The low values of 18:2n-6, even when in massive supply (as was the case of the high-fat diet), and the fact that it seems to be the fatty acid most directly implicated in experimental breast cancer raise the possibility that once incorporated into the tumor cell membrane it would undergo an important turnover, whether it was by conversion into 20:4n-6 or by use in the signal transduction pathways. Secondly, when comparing the values obtained for these two fatty acids between the normal diet (3% corn oil) group and the high-fat (20% corn oil) diet groups, we found a tendency in the latter to find a greater quantity of 18:2n-6 than in the control. This tendency could be solely the consequence of the saturation of the conversion mechanisms or the use of this fatty acid as we have previously postulated. However, it seems paradoxical that the quantity of 18:1n-9 was fewer in the phospholipids and the free fatty acids in the tumors from the high-fat diet groups than in those from the control group given that the corn oil contains a great quantity of this fatty acid. Despite this, our results are compatible with those of other studies, both epidemiological and in animal studies, as previously indicated. Furthermore, increases in 18:2n-6 and decreases in 18:1n-9 were also found by other authors in both phospholipid fractions of erythrocytes of women with breast disease and in the phosphatidylcholine fraction of breast tumors compared to normal breast tissue [63]. Although in none of these studies, including our own, has it been possible to formulate a mechanism of action which

would explain the differential behavior of both fatty acids in the mammary tumor cells, they suggest an association between greater malignancy of breast cancer with increases in linoleic acid and decreases in oleic acid in the tumors. The effect on the membrane fluidity and/or protein conformation, mobility and function would depend on both its relative variations as already indicated and the presence of other polyunsaturated fatty acids such as the 20:4n-6. Finally, we should consider that even though the tendency to present a greater quantity of 18:2n-6 and less 18:1n-9 in the high-fat diet groups have occurred in the three phospholipids studied, only in the phosphatildycholine has been clearly significant for both fatty acids. This result is interesting if we take into account the fact that the diacylglycerol resulting from phosphatidylcholine hydrolysis has been linked to sustained activation of protein kinase C, which induces long-term cellular responses such as tumor cell proliferation [58,59,64,65].

Analyzing the results obtained it seems that PE and PC have a distinct role in tumor cells. In the former the high 20:4n-6 content would be in accordance with a more structural role, increasing the unsaturation of the membrane. This situation seems to be characteristic of the mammary tumor cell as the 20:4n-6 is the most abundant fatty acid in the control group and even in those of the high-fat diet. In contrast, the composition in 18:2n-6 and 20:4n-6 of the PC suggests a more dynamic and more functional than structural role. It would be dynamic and functional due to the incorporation of these fatty acids into the membrane, the conversion and use in the intracellular signaling. But it would also be structural given that the high relative content of both of these fatty acids in this fraction would also contribute to the unsaturation of the membrane. Moreover the fact that this composition was modified by the high-fat diet suggests that all these processes would have enhanced in the tumors of animals fed this diet. The significant increase of 18:2n-6 in the free fatty acids of the I and P groups would also be compatible with this situation. All these mechanisms have been reported to be involved in mammary carcinogenesis.

In conclusion, the supply with large quantities of a n-6 polyunsaturated lipid (corn oil) into the diet of the animals generated changes in the fatty acid composition of the different lipid fractions of the mammary tumors induced in these animals. Some of these changes have also been found by other authors in other models and with other experimental designs. Likewise, they are compatible with those mechanisms related to carcinogenesis that have been described. Nevertheless, the results of this first phase of our study do not allow us to determine completely to what extent the differences obtained was due to the diet factor, or a some specific effect of the tissue that might occur in a different way between normal and tumor tissue. Future studies will be planned to test the effects of different levels of dietary intake of n-6 fatty acids on mammary gland fatty acid composition.

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References

[1] C.W. Welsch, Relationship between dietary fat and experimental mammary tumorigenesis: a review and critique, Cancer Res. 52 (1992) 2040s–2048s.
[2] E.L. Wynder, L.A. Cohen, J.E. Muscat, B. Winters, J.T. Dwyer, G. Blackburn, Breast cancer: weighing the evidence for a promoting role of dietary fat, J. Natl. Cancer Inst. 89 (1997) 766–775.
[3] K.K. Carrol, H.T. Khor, Dietary fat in relation to tumorigenesis, Progr. Biochem. Pharmacol. 10 (1975) 308–353.
[4] D.P. Rose, Effects of dietary fatty acids on breast and prostate cancers: evidence from in vitro experiments and animal studies, Am. J. Clin. Nutr. 66(suppl) (1997) 1513s–1522s.
[5] H. Bartsch, J. Nair, R. Owen Wyn, Dietary polyunsaturated fatty acids and cancers of the breast and colorectum: emerging evidence for their role as risk modifiers, Carcinogenesis 20 (1999) 2209–2218.
[6] L.M. Braden, K.K. Carrol, Dietary polyunsaturated fat in relation to mammary carcinogenesis in rats, Lipids 21 (1986) 285–288.
[7] W.T. Cave, Jr, Dietary ω–3 polyunsaturated fats and breast cancer, Nutrition 12(suppl) (1996) s39–s42.
[8] L.A. Cohen, D.O. Thompson, Y. Maeura, K. Choi, M.E. Blank, D.P. Rose, Dietary fat and mammary cancer. I. Promoting effects of different dietary fats on N-nitrosomethylurea-induced rat mammary tumorigenesis, J. Natl. Cancer Inst. 77 (1986) 33–42.
[9] C. Ip, Review of the effects of *trans* fatty acids, oleic acid, n-3 polyunsaturated fatty acids, and conjugated linoleic acid on mammary carcinogenesis in animals, Am. J. Clin. Nutr. 66(suppl) (1997) 1523s–1529s.
[10] I. Zusman, P. Gurevich, Z. Madar, A. Nyska, D. Korol, B. Timar, A. Zuckerman, Tumor-promoting and tumor-protective effects of high-fat diets on chemically induced mammary cancer in rats, Anticancer Res. 17 (1997) 349–356.
[11] M. Gerber, Olive oil, monounsaturated fatty acids and cancer, Cancer Lett. 114 (1997) 91–92.
[12] B. Armstrong, R. Doll, Environmental factors and cancer incidence and mortality in different countries with special reference to dietary practices, Int. J. Cancer 15 (1975) 617–631.
[13] K.K. Carroll, Dietary fat and breast cancer, Lipids 27 (1992) 793–797.
[14] D. Kritchevsky, Nutrition and breast cancer, Cancer 66 (1990) 1321–1325.
[15] A.S. Whittemore, B.E. Henderson, Dietary fat and breast cancer: Where Are We? J. Natl. Cancer Inst. 85 (1993) 762–763.
[16] J.L. Freudenheim, J.R. Marshall, The problem of profound mismeasurement and the power of epidemiological studies of diet and cancer, Nutr. Cancer 11 (1988) 243–250.
[17] R.L. Prentice, M. Pepe, S.G. Self, Dietary fat and breast cancer: a quantitative assessment of the epidemiological literature and a discussion of methodological issues, Cancer Res. 49 (1989) 3147–3156.
[18] K.K. Carroll, Experimental evidence of dietary factors and hormone-dependent cancers, Cancer Res. 35 (1975) 3374–3383.
[19] N.R. Simonsen, J. Fernandez-Crehuet, J.M. Martin-Moreno, J.J. Stain, J.K. Huttunen, B.C. Martin, M. Thamm, A.F.M. Kardinaal, P. van't Veer, F.J. Kok, L. Kohlmeier, Tissue stores of individual monounsaturated fatty acids and breast cancer: the EURAMIC Study, Am. J. Clin. Nutr. 68 (1998) 134–141.
[20] F.P. Kuhajda, Fatty-Acid Synthase and human cancer: new perspectives on its role in tumor biology, Nutrition 16 (2000) 202–208.
[21] J.T. Moncur, J.P. Park, V.A. Memoli, T.K. Mohandas, W.B. Kinlaw, The "Spot 14" gene resides on the telomeric end of the 11q13amplicon and is expressed in lipogenic breast cancers: impli-

cations for control of tumor metabolism, Proc. Natl. Acad. Sci. USA 95 (1998) 6989–6994.
[22] E.S. Pizer, Ch. Jackisch, F.D. Wood, G.R. Pasternack, E.N. Davidson, F.P. Kuhajda, Inhibition of fatty acid synthesis induces programmed cell death in human breast cancer cells, Cancer Res. 56 (1996) 2745–2747.
[23] W.T. Cave, Jr, J.J. Jurkowski, Dietary lipid effects on the growth, membrane composition, and prolactin-binding capacity of rat mammary tumors, J. Natl. Cancer Inst. 73 (1984) 185–191.
[24] M.T. Clandinin, S. Cheema, C.J. Field, M.L. Garg, J. Venkatraman, T.R. Clandinin, Dietary fat: exogenous determination of membrane structure and cell function, FASEB J. 5 (1991) 2761–2769.
[25] D.P. Rose, Dietary fatty acids and cancer, Am. J. Clin. Nutr. 66(suppl) (1997) 998s–1003s.
[26] C. Sumida, R. Graber, E. Nunez, Role of fatty acids in signal transduction: modulators and messengers, Prostag. Leukotr. Ess. 48 (1993) 117–122.
[27] A.H. Merrill Jr, J.J. Schroeder, Lipid modulation of cell function, Annu. Rev. Nutr. 13 (1993) 539–559.
[28] H.P. Glauert, Dietary fat, gene expression, and carcinogenesis. In *Nutrition and Gene Expression* (Berdanier, C.D. and Hargrove, J.L., eds.) pp. 247–268, CRC Press, Inc., USA, 1993.
[29] D.B. Jump, S.D. Clarke, Regulation of gene expression by dietary fat, Annu. Rev. Nutr. 19 (1999) 63–90.
[30] J.K. Tillotson, Z. Darzynkiewicz, L.A. Cohen, Z. Ronai, Effects of linoleic acid on mammary tumor cell proliferation are associated with changes in P53 protein expression, Int. J. Oncol. 3 (1993) 81–87.
[31] V. Utermohlen, M.A.M. Tucker, Possible effects of dietary n-6 series polyunsaturated fatty acids on the development of immune dysfunction and infection, Proc. Nutr. Soc. 45 (1986) 327–331.
[32] P.C. Calder, Dietary fatty acids and the immune system, Lipids 34 (suppl) (1999) S137–S140.
[33] D. Kritchevsky, Caloric restriction and experimental carcinogenesis, Toxicol. Sciences 52 (suppl) (1999) 13–16.
[34] E. Escrich, M. Solanas, R. Segura, Experimental diets for the study of lipid influence on induced mammary carcinoma in rats: I - Diet definition, In Vivo 8 (1994) 1099–1106.
[35] E. Escrich, M. Solanas, M.C. Ruiz de Villa, T. Ribalta, J. Muntané, R. Segura, Experimental diets for the study of lipid influence on induced mammary carcinoma in rats: II - Suitability of the diets, In Vivo 8 (1994) 1107–1112.
[36] C. Huggins, L.C. Grand, F.P. Brillantes, Mammary cancer induced by a single feeding of polynuclear hydrocarbons, and its suppression, Nature 189 (1961) 204–207.
[37] E. Escrich, Validity of the DMBA-induced mammary cancer model for the study of human breast cancer, Int. J. Biol. Markers 2 (1987) 197–206.
[38] Subcommittee on Laboratory Animal Nutrition, Committee on Animal Nutrition, Board on Agriculture and Renewable Resources, National Research Council, Nutrient requirements of laboratory rat. In *Nutrient Requirements of Domestic Animals* (Animals National Academy of Sciences, ed.), pp. 6–35, Washington, DC, 1978.
[39] A.E. Rogers, Nutrition. In *The Laboratory Rat* (Baker, H.J., Lindsey, J.R. and Weisbroth, S.H., eds) Vol 1, pp. 123–152, Academic Press, Inc., NewYork, 1979.
[40] S. Young, R.C. Hallowes, Tumours of the mammary gland, IARC Sci. Publ. 5 (1973) 31–73.
[41] E. Escrich, M. Solanas, C.H. Bailly, M.C. Ruiz de Villa, S. Saez, Effects of an androgenic derivative on the development of chemically-induced mammary carcinogenesis in the rat, Anticancer Res. 14 (1994) 539–544.
[42] W.R. Morrison, L.M. Smith, Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron trifluoride-methanol, J. Lipid Res. 5 (1964) 600–608.
[43] S. Innami, M.G. Yang, O. Mickelsen, The influence of high-fat diet on estrous cycles, sperm production and fertility of rats (37253), Proc. Soc. Exp. Biol. Med. 143 (1973) 63–68.
[44] S. Ruggieri, A. Fallani, Lipid composition of Morris hepatoma 5123c, and of livers and blood plasma from host and normal rats, Lipids 14 (1979) 781–788.
[45] K.H. Cheeseman, M. Collins, K. Proudfoot, T.F. Slater, G.W. Burton, A.C. Webb, K.U. Ingold, Studies on lipid peroxidation in normal and tumour tissues, Biochem. J. 235 (1986) 507–514.
[46] C.M. Williams, K. Maunder, The influence of dietary fatty acid composition on N-ethyl-N-nitrosourea-induced mammary tumour incidence in the rat and on the composition of inositol- and ethanolamine-phospholipids of normal and tumour mammary tissue, Brit. J. Nutr. 71 (1994) 543–552.
[47] G.R. Herzberg, The influence of dietary fatty acid composition on lipogenesis, Adv. Nutr. Res. 5 (1983) 221–253.
[48] L.A. Cohen, D.O. Thompson, K. Choi, R.A. Karmali, D.P. Rose, Dietary fat and mammary cancer. II. Modulation of serum and tumor lipid composition and tumor prostaglandins by different dietary fats: association with tumor incidence patterns, J. Natl. Cancer Inst. 77 (1986) 43–51.
[49] E.D. Rees, A.E. Shuck, H. Ackermann, Lipid composition of rat Mammary carcinomas, mammary glands, and related tissues: endocrine influences, J. Lipid Res. 7 (1966) 396–402.
[50] W.E.M. Lands, Biosynthesis of prostaglandins, Annu. Rev. Nutr. 11 (1991) 41–60.
[51] W.C. Willet, D.J. Hunter, M.J. Stampfer, G. Colditz, J.E. Manson, D. Spiegelman, B. Rosner, C.H. Hennekens, F.E. Speizer, Dietary fat and fiber in relation to risk of breast cancer, J. Am. Med. Assoc. 268 (1992) 2037–2044.
[52] D.J. Hunter, W.C. Willet, Diet, body build, and breast cancer, Annu. Rev. Nutr. 14 (1994) 393–418.
[53] Ch.D. Stubbs, A.D. Smith, The modification of mammalian membrane polyunsaturated fatty acid composition in relation to membrane fluidity and function, Biochim. Biophys. Acta 779 (1984) 89–137.
[54] G. Taraboletti, L. Perin, B. Bottazzi, A. Mantovani, R. Giavazzi, M. Salmona, Membrane fluidity affects tumor-cell motility, invasion and lung-colonizing potential, Int. J. Cancer 44 (1989) 707–713.
[55] A.B. Kier, Membrane properties of metastatic and non-metastatic cells cultured from C_3H mice injected with LM fibroblasts, Biochim. Biophys. Acta 1022 (1990) 365–372.
[56] A.B. Kier, M.T. Parker, F. Schroeder, Local and metastatic tumor growth and membrane properties of LM fibroblasts in athymic (nude) mice, Biochim. Biophys. Acta 938 (1988) 434–446.
[57] Ph.F. Devaux, A. Zachowski, Maintenance and consequences of membrane phospholipid asymmetry, Chem. Phys. Lipids 73 (1994) 107–120.
[58] E.A. Dennis, S.G. Rhee, M.M. Billah, Y.A. Hannun, Role of phospholipases in generating lipid second messengers in signal transduction, FASEB J. 5 (1991) 2068–2077.
[59] R. Graber, Ch. Sumida, E.A. Nunez, Fatty acids and cell signal transduction, J. Lipid Mediat. Cell 9 (1994) 91–116.
[60] K.V. Honn, D.G. Tang, X. Gao, I.A. Butovich, B. Liu, J. Timar, W. Hagmann, 12-Lipoxygenases and 12(S)-HETE: role in cancer metastasis, Cancer Metast. Rev. 13 (1994) 365–396.
[61] Y. Nishizuka, Studies and perspectives of protein kinase C, Science 233 (1986) 305–12.
[62] Y. Mizukami, A. Nonomura, M. Noguchi, T. Taniya, M. Thomas, S. Nakamura, I. Miyazaki, Effects of high and low dietary fat and indomethacin on tumour growth, hormone receptor status and growth factor expression in DMBA-induced rat breast cancer, Int. J. Tissue React. 14 (1992) 269–276.
[63] C.M. Williams, K. Maunder, Fatty acid compositions of inositol and choline phospholipids of breast tumors and normal breast tissue, Eur. J. Clin. Nutr. 47 (1993) 260–267.
[64] J.H. Exton, Signaling through phosphatidylcholine breakdown, J. Biol. Chem. 265 (1990) 1–4.
[65] M.M. Billah, Phospholipase D and cell signaling, Curr. Opin. Immunol. 5 (1993) 114–123.