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Dietary fish oil (MaxEPA) enhances pancreatic carcinogenesis in azaserine-treated rats

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Summary In the present study the putative chemopreventive effect of dietary fish oil (MaxEPA) on azaserine-induced pancreatic carcinogenesis in rats was investigated. Groups of rats were maintained on a semipurified low-fat (LF; 5 wt%) diet or on semipurified high-fat (HF; 25 wt%) diets containing 5 wt% linoleic acid (LA) and including 0.0, 1.2, 2.4, 4.7, 7.1 or 9.4 wt% MaxEPA. Animals fed a HF diet developed significantly higher mean numbers of atypical acinar cell nodules (AACNs), adenomas and carcinomas than animals fed a LF diet. Dietary MaxEPA caused a significant ($P<0.01$) dose-related increase in mean number of AACNs ($0.5<\varnothing<3.0$ mm). The mean number of adenomas and carcinomas remained similar among the groups. Cell proliferation was significantly lower in AACNs from animals fed HF containing 9.4% MaxEPA in comparison with HF without MaxEPA and with LF. LA levels had increased and arachidonic acid (AA) levels had decreased in blood plasma and pancreas with increasing dietary MaxEPA. Feeding MaxEPA resulted in significant decreases in 6-keto-prostaglandin (PG) $F_{1\alpha}$ ($P<0.05$) and $PGF_{2\alpha}$ ($P<0.01$) in non-tumorous pancreas, whereas PGE_2, $PGF_{2\alpha}$ and thromboxane B_2 (TXB_2) levels were significantly ($P<0.001$) higher in pancreatic tumour tissue than in non-tumorous pancreatic tissue. It is concluded that (i) dietary MaxEPA enhances dose-relatedly growth of putative preneoplastic AACNs in the pancreas of azaserine-treated rats; (ii) dietary MaxEPA inhibits the conversion of LA to AA, as well as the conversion of AA to TXB_2 or $PGF_{2\alpha}$ in non-tumorous pancreatic tissue; (iii) the high levels of PGE_2, $PGF_{2\alpha}$ and TXB_2 in pancreatic adenocarcinomas indicate a possible role for these eicosanoids in modulation of tumour growth.

Keywords: pancreatic carcinogenesis; rat; azaserine; fish oil; prostaglandins; cell proliferation

Polyunsaturated fatty acids (PUFAs) from the ω-3 family (abundant in fish oil) have been shown to inhibit tumour development in animal models for mammary (Jurkowski and Cave, 1985), colon (Reddy and Sugie, 1988) and pancreatic carcinogenesis. Information on effects of fish oil on pancreatic carcinogenesis is scarce and comes mainly from the studies of O'Connor *et al.* (1985), who observed that in azaserine-treated rats maintained on a 20% menhaden oil (MO) diet for 4 months, the number and size of pancreatic preneoplastic atypical acinar cell nodules (AACNs) were significantly reduced as compared with rats fed a 20% corn oil (CO) diet. In a subsequent 4 month study they found a decrease in the number of AACNs with increasing levels of MO in a 20% fat diet (O'Connor *et al.*, 1989). However, using the same model, we did not find any difference in AACN yield in rats fed 25% fat diets with a constant 5% linoleic acid (LA) level, either or not containing 9.4% fish oil (MaxEPA), for 6 months (Appel and Woutersen, 1994). Because of the latter unexpected and contrasting result and the lack of data on effects of fish oil on development of pancreatic tumours, we performed a 12 month study to investigate the effects of increasing levels of MaxEPA in a 25% fat/5% LA diet on pancreatic tumour development in azaserine-treated rats. Furthermore, the effects of dietary MaxEPA on cell proliferation in AACNs and normal acinar pancreatic tissue, as well as fatty acid profiles and prostaglandin levels in pancreatic tissue were examined.

Materials and methods

Animals and diets

Fifty-five 1 week pregnant female Wistar rats were obtained from Harlan-CPB, Austerlitz, The Netherlands. During pregnancy the rats were kept solitary, in stainless-steel cages fitted with wire-mesh floors and fronts and were fed a standard laboratory chow. Two weeks (± 1 day) after arrival the rats gave birth to a mean of eight pups. After 4 days the pups were sexed. All females, the surplus of male pups and the surplus of mothers were killed and a total of 210 male pups were divided among the remaining 26 mothers. One hundred and seventy-five pups were given an i.p. injection of 30 mg azaserine (Calbiochem-Behring, La Jolla, CA, USA) per kg body wt, which was dissolved freshly in 0.9% sodium chloride solution, at 14 and 21 days of age. Thirty-five control pups received injections with sodium chloride solution alone. Directly after the second injection the animals were weaned and randomly allocated to seven groups of 30 animals each (five control animals and 25 azaserine-treated animals). The animals were kept in stainless steel cages, with wire-mesh floors and fronts, five animals per cage and under standard laboratory conditions. One week after carcinogen treatment the rats were fed an AIN^{76}-based purified diet containing either 5 or 25 wt% fat. The control group received a 5 wt% lard (Best Food, The Netherlands; LF) diet, containing a marginal (0.61 wt%) but sufficient level of linoleic acid (LA; National Research Council Subcommittee, 1978). The experimental groups received a high-fat (25 wt%; HF) diet containing 5 wt% LA and including 0.0, 1.2, 2.4, 4.7, 7.1 or 9.4 wt% (0, 2.5, 5, 10, 15 and 20 en%) MaxEPA. The experimental design is summarised in Table I. The diets were compounded by mixing high-linoleic safflower oil (Unilever, Vlaardingen, The Netherlands) with high-oleic

Table I Experimental groups and carcinogen treatment

	Experimental groups						
	Low fat		*High fat*				
Treatment	*(5 wt %)*		*(25 wt %)*				
Wt% MaxEPA	0.0	0.0	1.2	2.4	4.7	7.1	9.4
Wt% linoleic acid	0.6	5.0	5.0	5.0	5.0	5.0	5.0
No. of rats							
Saline	5	5	5	5	5	5	5
Azaserine	25	25	25	25	25	25	25

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sunflower oil (Trisun, Contined, Bennekom, The Netherlands) and fish oil (MaxEPA; Seven Seas, Hull, UK). The safflower oil, the sunflower oil and the MaxEPA contained 0.55 g kg^{-1}, 0.44 g kg^{-1} and 1.80 g kg^{-1} vitamin E respectively. α-Tocopherol was added to all diets as extra antioxidant to a level of 0.450 g kg^{-1}. The composition of the AIN[76]-based diets and the fatty acid composition of the oils are summarised in Tables II and III. The diets were prepared monthly and stored at −20°C until use. The animals were fed daily to minimise oxidation of the polyunsaturated fatty acids. Peroxide values (as measured by means of the AOCS official method in terms of milliequivalents peroxide per kg) of the HF/9.4% MaxEPA-containing diet, stored at −20°C for 3 months, were below 1.0 and remained below 1.0 when exposed to air at room temperature for 24 h. Longer periods of exposure to air at room temperature caused a rapid increase in the peroxide value. The profiles of the dietary fatty acids of interest are depicted in Figure 1. Food consumption was measured daily during the first 3 months and on 7 consecutive days per month during the remainder of the study. The animals were weighed weekly during the first 3 months of the study and monthly thereafter.

Monitoring and autopsy

Three days before autopsy five saline-treated control rats and five azaserine-treated rats from the LF, HF/0.0% MaxEPA and the HF/9.4% MaxEPA groups had an Alzet osmotic pump (Alza, Palo Alto, USA, model 2001) implanted subcutaneously, containing 200 μl of a bromodeoxyuridine (BrdU) solution (Sigma, Brussels, Belgium; conc. 25 mg ml^{-1}). The release rate of this pump was 1 μl h^{-1}. Autopsy was performed 343, 344 or 345 days after the last injection of azaserine. The animals were anaesthetised with ether and exsanguinated by cannulating the abdominal aorta. Blood was collected in heparin-containing tubes, centrifuged at 1700 *g* for 20 min and stored at −80°C until analysis. The pancreas and liver were excised and weighed. About one-third of the pancreas of two animals per cage was snap frozen in liquid nitrogen and stored at −80°C until fatty acid or prostaglandin analyses. The remaining two-thirds of these pancreata plus all other pancreata and all livers were fixed in 10% neutral buffered formalin. Livers and pancreata of BrdU-treated animals were fixed for 24 h in formalin followed by 72 h in 70% ethanol. The organs were processed for microscopy by conventional methods, step-sectioned at 5 μm and collected on organosilane-coated slides. Parallel sections were stained with haematoxylin and eosin (H&E) or with a monoclonal antibody against BrdU (Organon Technics, the Netherlands) and examined by light microscopy. In the H&E-stained slides all pancreatic lesions were identified as acidophilic or basophilic atypical acinar cell foci (AACF), localised carcinoma (carcinoma *in situ*; CIS) or invasive carcinomas according to the criteria of Longnecker (1983) and Rao *et al.* (1982). Basophilic AACF were not scored because of insufficient yield. The area of the acidophilic AACF was determined by using an intraocular grid as described before (Woutersen *et al.*, 1986). The volumetric data were estimated using the method of Campbell *et al.* (1982). In slides stained for BrdU the labelling index (LI) was expressed as the percentage of brown-stained BrdU-positive nuclei of the total number of nuclei counted. To select a random sample of acinar cells, only nuclei that were located beneath the crossings of the horizontal and vertical lines in a 20 × 20 intraocular grid at high-power magnification (400 ×) were counted. In normal pancreatic tissue at least 1000 nuclei per pancreas were counted. A mean of 8.8 AACF per pancreas was evaluated (without discrimination between focus size) and a mean of 133 nuclei per AACF was counted.

Analytical procedures

Fatty acids Pancreatic microsomes were prepared by homogenising 100–200 mg of pancreatic tissue in 0.1 M Tris-KCl buffer, pH 7.4. Subsequently, the homogenate was centrifuged at 10 000 *g* for 30 min and the supernatant was centrifuged at 105 000 *g* for 60 min. The microsomal pellet was resuspended in 300 μl of buffer and stored at −30°C until

Table II Percentage fatty acid composition of the dietary lipids

Fatty acid	*Lard*	*SA*	*SO*	*MaxEPA*
C14:0	1.8	0.1	0.1	7.1
C16:0	25.6	7.0	3.8	17.5
C16:1	2.9	0.1	0.1	9.9
C18:0	14.2	2.6	4.0	4.2
C18:1	43.1	13.1	82.6	12.9
C18:2 (ω-6)	8.7	76.0	7.7	4.2
C18:3 (ω-3)	0.6	0.4	0.1	0.0
C20:0	0.2	0.3	0.3	2.5
C20:1	0.8	0.2	0.3	4.4
C20:4 (ω-6)	0.0	0.0	0.0	1.6
C20:5 (ω-3)	0.0	0.0	0.0	18.2
C22:0	0.1	0.2	0.9	0.0
C22:1	0.0	0.0	0.0	1.1
C22:4 (ω6)	0.0	0.0	0.0	1.2
C22:6 (ω3)	0.0	0.0	0.0	14.9
Total	98.0	99.9	100.0	99.7
ω-3/ω-6 (ratio)	0.07	0.01	0.01	4.7

SA, Safflower oil; SO, Sunflower oil (Trisun).

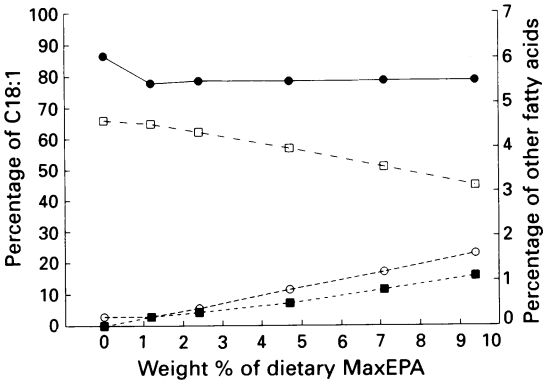

Figure 1 Percentage of selected dietary fatty acids. ●, Linoleic acid; □, oleic acid; ○, eicosapentaenoic acid; ■, docosahexaenoic acid.

Table III Weight percentage composition of the AIN[76]-based diets

Premix				*LF*	*HF/wt % MaxEPA*					
Diet components	LF	HF			0.0%	1.2%	2.4%	4.7%	7.1%	9.4%
Casein	20.00	25.00	Premix	95.00	75.00	75.00	75.00	75.00	75.00	75.00
DL-Methionine	0.30	0.37	Lard	4.74	–	–	–	–	–	–
Wheat starch	63.50	35.79	Safflower oil	0.26	4.63	4.67	4.69	4.80	4.91	5.04
Cellulose	5.00	6.18	Sunflower oil	–	20.37	19.15	17.95	15.49	13.04	10.53
Choline bitartrate	0.20	0.25	MaxEPA	–	–	1.18	2.36	4.71	7.05	9.43
AIN[76] minerals	3.50	4.32								
AIN[76] vitamins	1.00	1.24								
Calcium dihydrogen phosphate	1.50	1.85								
Total	95.00	75.00	Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00

fatty acid analysis. Total lipids were extracted from 50 µl aliquots of pancreatic microsomes or from 100 µl aliquots of blood plasma as described by Folch *et al.* (1957). Fatty acid composition was determined by gas-liquid chromatography. The samples were eluted on a capillary BD23 column (J&W Scientific) after saponification with sodium hydroxide in methanol and transmethylation of the fatty acids with boron-trifluoride in methanol.

Prostaglandins Pancreatic tissue (100–200 mg) was homogenised in 0.1 M phosphate-buffered saline (PBS; pH 7.4) containing 15% methanol. Before, during and after the homogenisation procedure the samples were kept on ice. The samples were applied to Sep-pak C_{18} columns (JT Baker, Phillipsburg, NJ, USA), and, after washing with 6 ml of 15% methanol/PBS and 6 ml of petroleum ether, eluted with 6 ml of methanol. After evaporation of the methanol under nitrogen, the samples were dissolved in 1.0 ml of potassium phosphate buffer (1.0 M; pH 7.4) and subsequently analysed by using enzyme immunoassay kits for PGE_2, $PFE_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and TXB_2 (Cascade Biochem Ltd, Reading, UK).

Statistics Food and energy intake and body and pancreatic weights were statistically evaluated by two-way analysis of variance followed by Dunnett's test, prostaglandin levels were evaluated by analysis of variance followed by Student's *t*-test, the number of pancreatic lesions was evaluated by two-sample *t*-test, or by one-way analysis of variance followed by linear trend tests with orthogonal contrasts. The number of tumour-bearing animals (incidence) was analysed by Pearson χ^2-test. Fatty acid compositions were evaluated by two-way analysis of variance using percentage of dietary MaxEPA and carcinogen-treatment as factors, and by one-way analysis of variance followed by linear trend tests with orthogonal contrasts.

Results

Food consumption and body and organ weights

Mean food consumption of rats maintained on an LF diet was significantly ($P<0.001$) higher than that in rats maintained on an HF diet. However, owing to a higher energy content of the HF diet, mean caloric intake was similar among LF and HF groups (Table IV).

Mean body weight gain over the study showed no significant differences among the groups (Figure 2). Mean terminal body weights of animals fed a HF diet were significantly higher relative to animals fed a LF diet ($P<0.05$; Table V). Azaserine treatment caused a consistent, significant increase in both absolute and relative pancreas weights in all groups ($P<0.001$) in comparison with saline-treated controls. Both absolute and relative liver weights were significantly higher in animals kept on a HF diet than in LF controls ($P<0.01$ and $P<0.05$ respectively).

Microscopy

Feeding a HF diet significantly enhanced tumour growth in comparison with the LF control diet, as reflected by a significantly higher number of tumour-bearing animals ($P<0.001$), number of AACNs ($P<0.01$) and total number of carcinomas ($P<0.05$). Including MaxEPA in the HF diet resulted in a significant dose-related linear increase in number of AACNs with both a diameter of 0.5–1.0 mm as well as a diameter of 1.0–3.0 mm ($P<0.01$). A similar effect was seen on the volume of pancreas occupied by AACNs ($P<0.01$). No such effect was seen on the number of adenomas and carcinomas or on the number of tumour-bearing animals (Table VI and Figure 3).

Cell proliferation

Labelling index in normal acinar cells was low (below 1%, Figure 4) and similar in all groups. The mean LI in AACNs

Table IV Food and energy consumption[a]

	Food	*Energy*
LF	15.4 ± 0.3***	238.7
HF/0.0% MaxEPA	12.6 ± 0.2	248.2
HF/1.2% MaxEPA	12.1 ± 0.2	238.4
HF/2.4% MaxEPA	12.1 ± 0.2	238.4
HF/4.7% MaxEPA	12.8 ± 0.3	252.2
HF/7.1% MaxEPA	12.9 ± 0.3	254.1
HF/9.4% MaxEPA	12.4 ± 0.2	244.3

[a]Food intake in g day^{-1} per animal; energy intake in kJ day^{-1}. Statistics: analysis of variance, ***$P<0.001$.

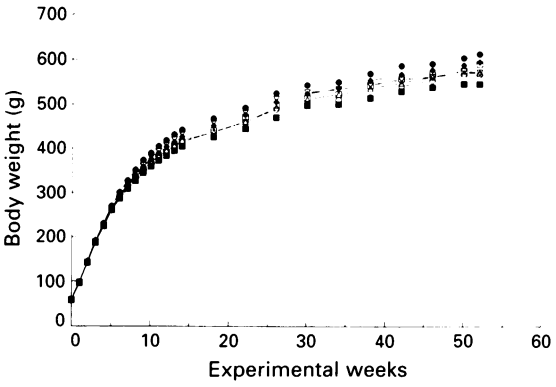

Figure 2 Body weight gain of azaserine-treated rats maintained on a low-fat diet or a high-fat diet containing increasing levels of MaxEPA for 12 months. ■, LF; ▽, HF/0.0% MaxEPA; ○, HF/1.2% MaxEPA; △, HF/2.4% MaxEPA; ◆, HF/4.7% MaxEPA; ●, HF/7.1% MaxEPA; □, HF/9.4% MaxEPA.

Table V Body and organ weight at autopsy[a]

Diet group		n	*Absolute weight (g)* *Body wt*[b]	*Pancreas wt*[c]	*Liver wt*[d]	*Relative weight (g kg^{-1})* *Pancreas wt*[c]	*Liver wt*[b]
LF	Sal	5	546 ± 22	1.07 ± 0.06	12.2 ± 1.0	1.97 ± 0.12	22.3 ± 1.2
	Aza	25	546 ± 10	1.47 ± 0.06	14.0 ± 0.4	2.69 ± 0.10	25.7 ± 0.5
HF/0.0% MaxEPA	Sal	4	588 ± 24	1.07 ± 0.08	15.1 ± 0.8	1.81 ± 0.06	25.6 ± 0.4
	Aza	22	571 ± 12	1.80 ± 0.14	14.6 ± 0.5	3.13 ± 0.19	25.4 ± 0.6
HF/1.2% MaxEPA	Sal	5	584 ± 36	0.93 ± 0.07	14.6 ± 0.9	1.58 ± 0.06	24.9 ± 0.3
	Aza	21	570 ± 13	1.57 ± 0.10	14.7 ± 0.5	2.76 ± 0.16	25.8 ± 0.6
HF/2.4% MaxEPA	Sal	5	566 ± 31	1.26 ± 0.07	14.6 ± 1.3	2.23 ± 0.11	25.6 ± 1.3
	Aza	23	591 ± 17	1.79 ± 0.18	15.7 ± 0.6	2.98 ± 0.21	26.5 ± 0.5
HF/4.7% MaxEPA	Sal	4	632 ± 41	1.32 ± 0.20	16.3 ± 1.2	2.17 ± 0.44	25.8 ± 0.6
	Aza	24	595 ± 16	1.96 ± 0.15	15.7 ± 0.6	3.28 ± 0.22	26.2 ± 0.5
HF/7.1% MaxEPA	Sal	5	637 ± 51	1.04 ± 0.09	16.5 ± 1.5	1.68 ± 0.22	25.9 ± 0.8
	Aza	23	613 ± 14	1.78 ± 0.11	16.4 ± 0.5	2.87 ± 0.15	26.7 ± 0.5
HF/9.4% MaxEPA	Sal	4	643 ± 53	1.04 ± 0.11	18.8 ± 1.9	1.65 ± 0.25	29.1 ± 0.8
	Aza	22	572 ± 17	1.95 ± 0.15	16.2 ± 0.7	3.37 ± 0.20	28.1 ± 0.8

[a]Values are means ± s.e.m.; [b]$P<0.05$ (LF *vs* HF); [c]$P<0.001$ (Sal *vs* Aza); [d]$P<0.01$ (LF *vs* HF).
LF, low fat; HF, high fat; Sal, saline-treated; Aza, Azaserine-treated.

from animals fed the LF, the HF and the HF diet including 9.4% MaxEPA was 28.4 ± 3.2, 24.8 ± 1.7 and 15.5 ± 1.2 respectively. Cell proliferation was significantly higher in AACNs in comparison with normal tissue ($P<0.001$). A high level of MaxEPA in the HF diet caused a significant ($P<0.05$) decrease of the LI in AACNs in comparison with AACNs from both the HF diet without MaxEPA and the LF diet.

Fatty acid analyses

Fatty acid profiles of blood plasma and pancreatic microsomes are presented in Tables VII and VIII. Statistics are only presented on oleic acid (OA; C18:1), linoleic acid (LA; C18:2), arachidonic acid (AA; C20:4), eicosapentaenoic acid (EPA; C20:5) and docosahexaenoic acid (DHA; C22:6).

In blood plasma the levels of AA (C20:4) and DHA (C22:6) were significantly higher in saline-treated *vs* azaserine-treated rats ($P<0.001$). In pancreatic microsomes the level of OA (C18:1) was significantly higher ($P<0.05$) and the levels of AA (C20:4), EPA (C20:5) and DHA (C22:6) were significantly lower ($P<0.001$, $P<0.05$ and $P<0.001$ respectively) in saline-treated rats in comparison with azaserine-treated rats.

In blood plasma and pancreas the levels of OA (C18:1) decreased and the levels of EPA (C20:5) and DHA (C22:6) increased, parallel to the dietary supply of these fatty acids. Levels of LA (C18:2) increased and AA (C20:4) decreased in both blood plasma and pancreas of saline- and azaserine-treated rats. Significant linear increases were observed in levels of LA (C18:2; $P<0.001$), EPA (C20:5; $P<0.001$) and DHA (C22:6; $P<0.001$) and significant linear decreases were observed in levels of OA (C18:1; $P<0.01$) and AA (C20:4; $P<0.001$; except for C20:4 in pancreas of saline-treated rats: $P=0.926$).

LA (C18:2) levels in microsomes from tumours were significantly lower in all diet groups measured ($P<0.05$, at least), whereas AA (C20:4) levels were significantly elevated in tumours from animals fed 2.4% and 4.7% MaxEPA in comparison with non-tumorous pancreas from azaserine-treated rats ($P<0.01$). No differences were found in EPA and DHA profiles, except for the DHA level in tumours from the group fed 2.4% MaxEPA, which was significantly higher in comparison with non-tumorous tissues ($P<0.001$). The profiles of LA (C18:2), AA (C20:4), EPA (C20:5) and DHA (C22:6) in pancreatic tumours are depicted in Figure 5.

Prostglandins

Pancreatic 6-keto-$PGF_{1\alpha}$- and $PGF_{2\alpha}$ levels were significantly lower in saline-treated rats maintained on the HF diet containing 4.7% and 9.4% MaxEPA, in comparison with saline-treated rats maintained on the HF diet without MaxEPA ($P<0.05$ and $P<0.01$, for the respective prostaglandins). In azaserine-treated rats $PGF_{2\alpha}$ levels were significantly lower ($P<0.05$) in pancreata from animals fed 9.4% MaxEPA in comparison with those from animals fed the HF diet without MaxEPA. Pancreatic $PGF_{2\alpha}$ and TXB_2 levels were inversely related to the percentage of dietary MaxEPA in both saline- and azaserine-treated rats. Parts of all grossly visible pancreatic rat tumours were analysed for prostaglandins. Microscopic examination of the grossly visible pancreatic lesions indicated that the number of adenocarcinomas in the group given high fat without MaxEPA was high enough ($n=3$) to justify statistical analysis. PGE_2, TXB_2, $PGF_{2\alpha}$, but not 6-keto-$PGF_{1\alpha}$ levels in pancreatic adenocarcinomas were significantly elevated ($P<0.001$) in comparison with non-tumorous pancreas from azaserine-treated rats (Figure 6).

Table VI Incidence and number of pancreatic (pre)neoplastic lesions in azaserine-treated rats maintained on a low-fat diet or a high-fat diet including 0.0, 1.2, 2.4, 4.7, 7.1 or 9.4 wt% MaxEPA for 12 months

Mean no. of (pre)neoplastic lesions[a]	*LF*	*HF/wt% MaxEPA*						P[b]
		0.0%	1.2%	2.4%	4.7%	7.1%	9.4%	
No. of rats	25	22	21	23	24	23	21	
Incidence (%)[c]	1 (4)[d]	9 (41)	9 (43)	10 (43)	14 (58)	13 (56)	14 (67)	
AACN ($0.0<ø<1.0$ mm)	0.76 ± 0.23[e]	11.00 ± 2.38	13.81 ± 3.00	18.54 ± 2.55	20.71 ± 3.12	17.75 ± 2.93	22.96 ± 3.92	0.008
AACN ($1.0<ø<3.0$ mm)	0.68 ± 0.48[f]	8.82 ± 2.78	9.46 ± 2.14	11.67 ± 2.21	17.71 ± 2.89	14.99 ± 3.08	18.35 ± 4.28	0.006
Adenoma ($ø>3.0$ mm)	0.16 ± 0.16	0.59 ± 0.46	0.55 ± 0.23	0.29 ± 0.11	0.79 ± 0.25	0.25 ± 0.11	0.74 ± 0.35	0.780
Carcinoma *in situ*	0.04 ± 0.04	0.36 ± 0.20	0.46 ± 0.22	0.50 ± 0.21	0.76 ± 0.21	0.92 ± 0.35	0.78 ± 0.19	0.068
Adenocarcinoma	0.00 ± 0.00	0.55 ± 0.17	0.23 ± 0.09	0.21 ± 0.09	0.68 ± 0.18	0.17 ± 0.10	0.26 ± 0.09	0.357
Total carcinoma	0.04 ± 0.04[f]	0.91 ± 0.33	0.68 ± 0.23	0.71 ± 0.23	1.44 ± 0.33	1.08 ± 0.36	1.04 ± 0.20	0.275
Vol% of pancreas[g]	2.76 ± 1.63	17.97 ± 4.41	22.63 ± 4.59	24.20 ± 3.59	35.66 ± 4.65	29.18 ± 5.05	36.25 ± 6.80	0.003

[a]Values are mean numbers of lesions per pancreas ± s.e.m. [b]*P* for linear trend tests among HF groups (orthogonal contrasts). [c]Incidence, the percentage of tumour-bearing rats. Incidence of tumours is significantly lower in the LF group in comparison with the HF groups ([d]$P<0.001$; Pearson χ^2-test). Number of lesions is significantly lower in the LF group in comparison with the HF groups ([e]$P<0.001$; [f]$P<0.05$; analysis of variance followed by Student's *t*-test). [g]Vol% of pancreas, the volume percentage of pancreas occupied by AACN.

Discussion

The results of the present study demonstrated a strong enhancing effect of HF (25 wt%) diet including 5 wt% LA on pancreatic carcinogenesis in azaserine-treated rats.

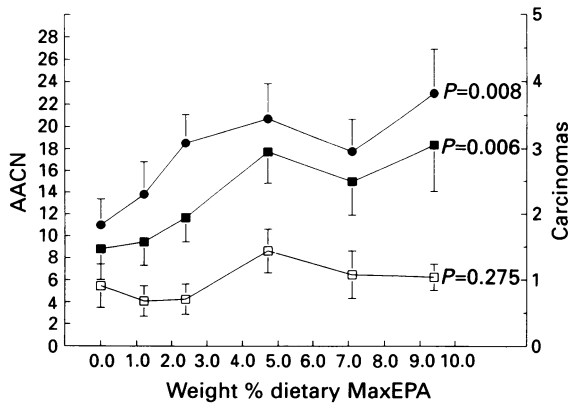

Figure 3 Mean number of (pre)neoplastic lesions (± s.e.m.) in the pancreas of azaserine-treated rats maintained on a high fat diet containing increasing levels of MaxEPA for 12 months. Statistics: analysis of variance followed by linear trend tests (orthogonal contrasts). —●—, AACN > 0.5 mm; —■— AACN 1–3 mm; —□—, carcinoma.

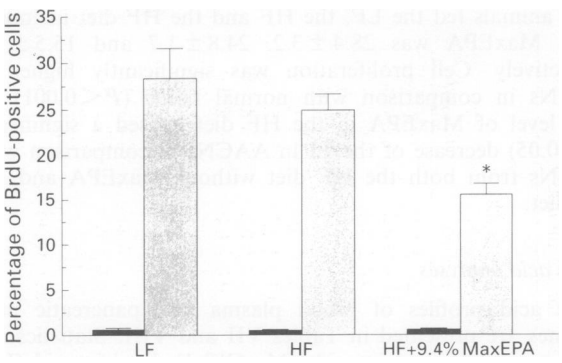

Figure 4 Labelling index of normal and preneoplastic pancreatic tissue (AACN) in rats maintained on a 5 wt% fat AIN[76]-based diet, a 25 wt% fat AIN[76]-based diet or a 25% fat AIN[76]-based diet containing 9.4 wt% MaxEPA for 12 months. Statistics: analysis of variance followed by Student's *t*-tests. $*P<0.05$. ■, Normal tissue; ■, AACN.

Moreover, including various amounts of fish oil (MaxEPA) in the HF diet resulted in a significant dose-related linear increase in the number of putative preneoplastic AACNs with a diameter between 0.5 and 3.0 mm. It appeared that long-chain PUFAs of the ω-3 series from fish oil in the diet are readily taken up by the rat, resulting in increased concentrations in both plasma and pancreas, concomitant with increasing concentrations in the feed. The presence of MaxEPA in the diet significantly decreased cell proliferation in AACNs and influenced the metabolism of linoleic acid, arachidonic acid and and hence prostaglandin synthesis substantially.

In a number of studies the chemopreventive effect of dietary fish oil has been reported in experimental mammary gland carcinogenesis (Jurkowski and Cave, 1985; Braden and Carroll, 1986; Ip *et al.*, 1986; Abou-El-Ela *et al.*, 1989), colon carcinogenesis (Minoura *et al.*, 1988; Reddy and Maruyama, 1986; Reddy and Sugie, 1988) and pancreatic carcinogenesis (O'Connor *et al.*, 1985; 1989). To investigate the effects of fish oil (containing high concentrations of ω-3 fatty acids), most investigators used mixtures of corn oil and fish oil or fish oil as such. Most of the studies that had the intention of varying ω-3 fatty acids in the diet, had an experimental design also resulting in a variation in dietary ω-6 fatty acid levels. Moreover, in those studies where fish oil was used as sole lipid source, the level of ω-6 fatty acid frequently was a deficient one.

O'Connor *et al.* (1985) reported that 20% MO caused a significant inhibition of the growth of azaserine-induced AACNs in rat pancreas as compared with a 20% CO diet. In a subsequent study (O'Connor *et al.*, 1989), they varied the ω-3/ω-6 ratio from 0.01 up to 7.0 by mixing CO with MO and observed a significant decrease in development of AACN with increasing ratio of ω-3/ω-6. They acknowledged that their dietary regimen also implied a variation of dietary LA from 0.6% up to 12.0%, which is within the range in which growth of AACN is significantly increased (4.4–8.5%; Roebuck *et al.*, 1985). In a previous study (Appel *et al.*, 1994), we varied the dietary LA levels from 2.0% to 15.0%, without influencing the chain length of the fatty acids, and observed an inverse rather than a positive dose–response relationship between LA and AACN development. In order to minimise the number of variables and their possible confounding effects, it seems of paramount importance to keep LA in the diet at a constant level when investigating the effects of other variables such as dietary fish oil. In the present study an increase of MaxEPA paralleled a decrease of oleic acid (OA). OA has been reported to enhance the growth of pancreatic AACNs (Khoo *et al.*, 1991). However, in this study pancreatic AACNs were absent in the group treated with azaserine alone and in the other groups the yield of AACNs was unusually low for the experimental protocol used (30 mg of azaserine per kg body wt at 14, 21 and 28 days of age, followed by a 6 month feeding period). In our studies dietary OA either correlated negatively (present results) or positively (Appel *et al.*, 1994) with the development of AACNs. Apart from the isolated study by Khoo *et al.*, OA has not been implicated as a promoter in carcinogenesis. Moreover, since OA cannot be metabolised to LA or other long-chain PUFAs and prostaglandins, the findings of Khoo *et al.* (1989) are hard to explain mechanistically. In our view, the influence of OA on pancreatic carcinogenesis seems to be of minor importance.

Apart from the importance of an adequate study design, the present results also emphasise that an enhancing effect on growth of early stages in pancreatic carcinogenesis (AACNs), does not necessarily lead to more carcinomas. Consequently, a long-term (12–15 months) study is needed to establish the modulating effects of dietary or other factors on the development of the ultimate pancreatic tumours.

The observation that after 12 but not after 6 months (Appel and Woutersen, 1994) an enhancing effect of fish oil on the growth of AACNs is seen, suggests that fish oil modulated pancreatic carcinogenesis in azaserine-treated rats only after a rather long period of feeding. Moreover, it cannot be excluded that prolonged feeding of diets high in MaxEPA for over 12 months will also finally modulate development of pancreatic carcinomas. The significantly higher LI of the acidophilic AACNs *vs* normal acinar tissue, as observed in the present study, is in agreement with the previously observed high growth potential of these putative preneoplastic lesions (Rao *et al.*, 1982). Although MaxEPA caused a dose-related increase in number of AACNs, the LI of AACNs in the pancreas of animals maintained on the HF diet including 9.4% MaxEPA was significantly lower than the LI of AACNs present in the pancreas of rats fed the HF diet without MaxEPA. This seemingly contradictory observation may be due to MaxEPA-induced differences in cell turnover, possibly caused by decreased apoptotic cell death. The relation between cell proliferation and programmed cell death is currently under investigation at our Institute.

The main compositional changes in fatty acids in blood plasma and pancreas as a result of increasing MaxEPA in the diet were a decrease in OA (C18:1) and increases in EPA (C20:5) and DHA (C22:6). An increasing level of dietary MaxEPA caused a shift in the ratio of EPA/DHA from equal or higher than 1 towards an EPA/DHA ratio of equal or less than 1, both in plasma and pancreas. An explanation for this interesting observation may be formation of DHA from EPA. This newly formed DHA accompanied by DHA from the diet accumulates, resulting in higher DHA levels than EPA levels in plasma and tissue (Mathias and Dupont, 1989). Furthermore, the presently observed fatty acid profiles

Table VII Fatty acid composition of plasma of untreated and azaserine-treated rats maintained on a high-fat diet containing increasing levels of MaxEPA for 12 months[a,b]

	Percentage of dietary MaxEPA											
	0.0%		*1.2%*		*2.4%*		*4.7%*		*7.1%*		*9.4%*	
Fatty acid	*Saline treated*	*Aza treated*	*Saline treated*	*Aza treated*	*Saline treated*	*Aza treated*	*Saline treated*	*Aza treated*	*Saline treated*	*Aza treated*	*Saline treated*	*Aza treated*
C14:0	0.1 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.5 ± 0.0	0.1 ± 0.0	0.5 ± 0.0	0.4 ± 0.1
C16:0	10.1 ± 0.1	13.8 ± 0.5	11.7 ± 0.2	13.5 ± 0.2	12.1 ± 0.2	16.1 ± 1.2	12.8 ± 0.4	15.3 ± 0.2	13.2 ± 0.1	17.0 ± 0.1	13.4 ± 0.2	17.8 ± 0.7
C16:1 *trans*	0.5 ± 0.0	0.9 ± 0.1	0.8 ± 0.0	0.9 ± 0.0	0.6 ± 0.0	0.9 ± 0.1	0.7 ± 0.1	0.8 ± 0.0	0.5 ± 0.0	0.7 ± 0.0	0.4 ± 0.0	0.6 ± 0.0
C16:1 *cis*	0.2 ± 0.0	0.3 ± 0.1	0.4 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.7 ± 0.1	0.6 ± 0.1	1.1 ± 0.1	0.6 ± 0.0	1.3 ± 0.3
C18:0	14.4 ± 0.7	14.5 ± 0.3	13.3 ± 0.3	13.2 ± 0.2	13.2 ± 0.4	16.2 ± 1.1	13.2 ± 0.5	14.3 ± 0.2	13.0 ± 0.5	12.3 ± 0.3	13.1 ± 0.3	12.5 ± 0.4
C18:1	27.4 ± 2.1	28.8 ± 1.9	27.8 ± 0.7	28.3 ± 1.0	26.7 ± 1.6	23.3 ± 1.0	24.4 ± 1.1	21.1 ± 0.4	18.9 ± 0.8	23.4 ± 0.9	16.7 ± 0.3	19.4 ± 0.7[c]
C18:2	13.8 ± 0.6	13.4 ± 0.6	14.0 ± 0.8	15.0 ± 0.2	15.2 ± 0.5	14.1 ± 0.8	15.5 ± 0.2	14.9 ± 0.1	16.8 ± 0.8	16.0 ± 0.3	17.3 ± 0.2	17.2 ± 0.1[c]
C18:3 (ω-6)	0.4 ± 0.0	0.5 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
C20:0	0.2 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
C20:1	0.4 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
C20:2	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
C20:3	0.4 ± 0.0	0.4 ± 0.0	0.6 ± 0.0	0.5 ± 0.0	0.7 ± 0.0	0.8 ± 0.0	1.0 ± 0.1	0.9 ± 0.0	1.0 ± 0.1	0.9 ± 0.0	1.1 ± 0.1	0.9 ± 0.0
C20:4	30.7 ± 2.0	26.3 ± 2.4	25.0 ± 1.0	23.3 ± 1.1	24.0 ± 1.1	22.2 ± 1.4	22.5 ± 0.8	24.3 ± 0.6	23.5 ± 1.4	18.8 ± 1.1	23.1 ± 0.6	17.6 ± 0.4[c]
C20:5	0.0 ± 0.0	0.0 ± 0.0	0.7 ± 0.1	0.8 ± 0.0	1.5 ± 0.0	1.3 ± 0.1	2.9 ± 0.1	2.6 ± 0.0	5.6 ± 0.4	4.0 ± 0.2	6.6 ± 0.7	6.2 ± 0.3[c]
C22:6	0.6 ± 0.0	0.0 ± 0.0	4.0 ± 0.2	3.1 ± 0.1	4.2 ± 0.2	3.4 ± 0.3	5.4 ± 0.2	4.0 ± 0.1	5.9 ± 0.3	4.4 ± 0.2	7.0 ± 0.1	5.2 ± 0.1[c]

[a]Values are mean percentages of total fatty acids ± s.e.m. ($n = 3$); Aza, azaserine. [b]Statistics: two-way analysis of variance or one-way analysis of variance followed by linear trend tests (orthogonal contrasts). [c]Statistically significant linear trend ($P < 0.001$, except for C18:1: $P < 0.01$).

Table VIII Fatty acid composition of pancreatic microsomes of untreated and azaserine-treated rats maintained on a high-fat diet containing increasing levels of MaxEPA for 12 months[a,b]

	Percentage of dietary MaxEPA											
	0.0%		*1.2%*		*2.4%*		*4.7%*		*7.1%*		*9.4%*	
Fatty acid	*Saline treated*	*Aza treated*	*Saline treated*	*Aza treated*	*Saline treated*	*Aza treated*	*Saline treated*	*Aza treated*	*Saline treated*	*Aza treated*	*Saline treated*	*Aza treated*
C14:0	0.4 ± 0.0	0.2 ± 0.0	0.5 ± 0.0	0.3 ± 0.0	0.6 ± 0.1	0.3 ± 0.1	0.9 ± 0.1	0.5 ± 0.0	1.2 ± 0.0	0.6 ± 0.0	1.2 ± 0.0	0.5 ± 0.0
C16:0	9.1 ± 0.7	13.5 ± 0.8	13.6 ± 1.0	12.4 ± 1.0	13.0 ± 0.6	14.5 ± 1.0	16.3 ± 1.0	13.0 ± 1.1	17.9 ± 1.4	15.5 ± 0.8	17.0 ± 0.7	15.5 ± 0.8
C16:1 *trans*	1.0 ± 0.1	0.9 ± 0.0	1.1 ± 0.1	0.7 ± 0.0	1.4 ± 0.2	0.8 ± 0.1	1.2 ± 0.1	0.7 ± 0.0	1.1 ± 0.1	0.7 ± 0.0	0.9 ± 0.0	0.6 ± 0.0
C16:1 *cis*	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.6 ± 0.1	0.5 ± 0.0	0.9 ± 0.1	0.6 ± 0.1	1.4 ± 0.2	1.0 ± 0.1	1.6 ± 0.1	1.0 ± 0.1
C18:0	5.6 ± 0.5	7.4 ± 0.6	7.5 ± 0.6	7.7 ± 0.4	7.0 ± 0.1	7.7 ± 0.3	7.9 ± 0.9	8.3 ± 1.1	8.6 ± 0.6	8.5 ± 0.3	7.3 ± 0.8	8.8 ± 0.7
C18:1	65.3 ± 1.0	51.3 ± 2.8	57.2 ± 1.9	52.4 ± 1.3	56.9 ± 1.6	48.4 ± 1.0	50.5 ± 5.6	45.6 ± 2.7	45.5 ± 2.8	46.7 ± 2.0	40.4 ± 3.2	35.6 ± 2.0[c]
C18:2	15.4 ± 0.2	13.6 ± 0.4	15.0 ± 0.3	14.7 ± 0.5	15.6 ± 0.1	15.2 ± 0.3	17.0 ± 0.6	17.8 ± 0.2	17.0 ± 0.2	17.9 ± 0.3	18.8 ± 0.3	20.7 ± 0.5[c]
C18:3 (ω-6)	1.0 ± 0.2	0.2 ± 0.0	1.6 ± 0.4	0.2 ± 0.0	1.6 ± 0.1	0.1 ± 0.0	1.1 ± 0.4	0.3 ± 0.1	1.5 ± 0.1	0.3 ± 0.1	1.0 ± 0.2	0.2 ± 0.0
C20:0	0.5 ± 0.1	0.2 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.8 ± 0.1	0.2 ± 0.0	0.6 ± 0.2	0.2 ± 0.0	0.7 ± 0.1	0.3 ± 0.0	0.4 ± 0.1	0.2 ± 0.0
C20:1	0.4 ± 0.0	0.7 ± 0.1	0.0 ± 0.0	0.5 ± 0.1	0.6 ± 0.0	0.6 ± 0.1	0.3 ± 0.1	0.4 ± 0.0	0.0 ± 0.0	0.7 ± 0.1	0.3 ± 0.0	0.6 ± 0.0
C20:2	0.0 ± 0.0	0.3 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
C20:3	0.0 ± 0.0	0.7 ± 0.3	0.0 ± 0.0	0.4 ± 0.2	0.0 ± 0.0	0.5 ± 0.1	0.3 ± 0.0	0.5 ± 0.0	0.0 ± 0.0	0.9 ± 0.2	0.0 ± 0.0	0.5 ± 0.1
C20:4	1.8 ± 0.5	8.6 ± 1.4	3.1 ± 0.5	5.8 ± 0.5	2.0 ± 0.3	6.1 ± 0.4	3.4 ± 0.8	4.2 ± 1.2	2.3 ± 0.4	4.0 ± 0.8	1.8 ± 0.3	4.1 ± 0.7[c]
C20:5	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.3 ± 0.0	0.4 ± 0.0	0.9 ± 0.1	1.3 ± 0.3	2.1 ± 0.7	1.4 ± 0.3	2.5 ± 0.3	1.7 ± 0.3	3.0 ± 0.6[c]
C22:6	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.2	1.1 ± 0.0	0.4 ± 0.1	1.2 ± 0.1	1.0 ± 0.2	1.7 ± 0.5	1.0 ± 0.2	1.9 ± 0.1	1.1 ± 0.1	2.3 ± 0.3[c]

[a]Values are mean percentages of total fatty acids ± s.e.m. Aza, azaserine ($n = 3$). [b]Statistics: two-way analysis of variance or one-way analysis of variance followed by linear trend tests (orthogonal contrasts). [c]Statistically significant linear trend ($P < 0.001$, except for C18:1: $P < 0.01$, and for C20:4 in saline-treated rats: $P = 0.926$).

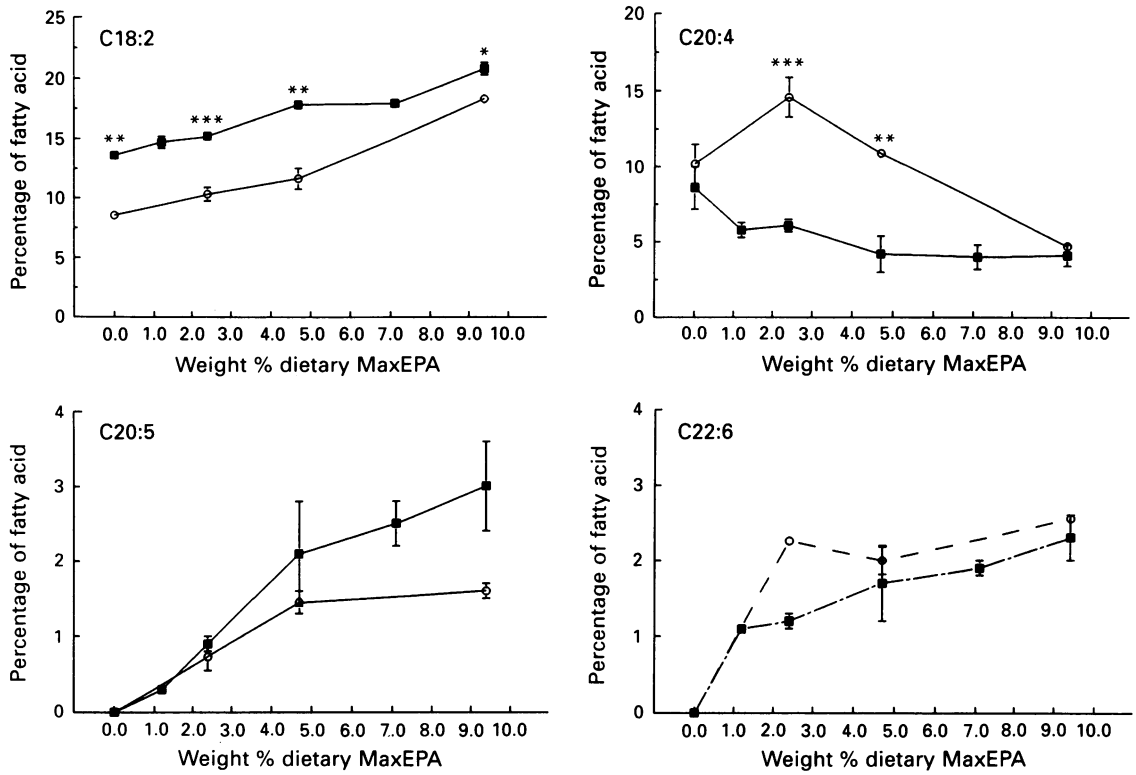

Figure 5 LA (C18:2), AA (C20:4), EPA (C20:5) and DHA (C22:6) levels in microsomes of pancreas tumours (○) and non-tumorous pancreas (■) of rats maintained on a high-fat diet containing increasing levels of MaxEPA.

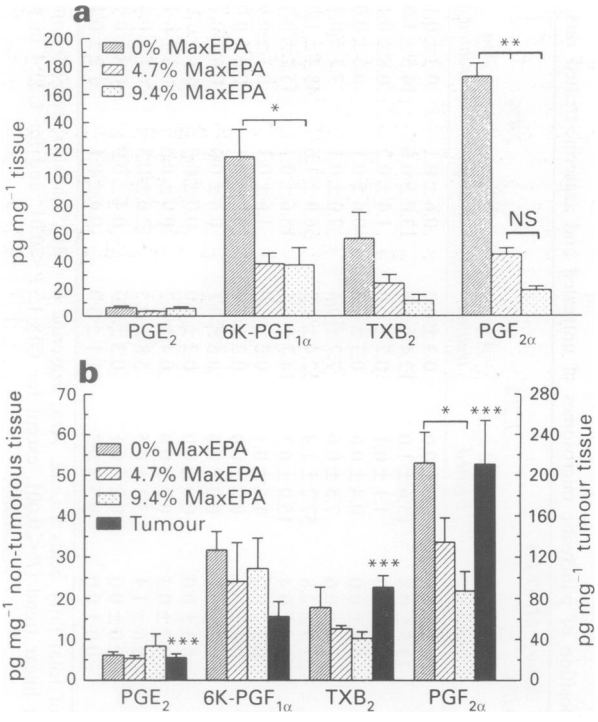

Figure 6 Prostaglandin levels in pancreas of (**a**) saline-treated rats and (**b**) azaserine-treated rats. Statistics: analysis of variance followed by Student's *t*-test. $*P<0.05$, $**P<0.01$, $***P<0.001$. NS, not significant.

show that feeding MaxEPA causes a decrease of AA (C20:4) levels in both blood plasma (saline- and azaserine-treated rats) and pancreatic tissue (azaserine-treated rats), which can be ascribed to either replacement of AA by EPA (Mathias and Dupont, 1989) or to an inhibition of the LA-converting enzyme δ^6-desaturase by EPA and/or DHA (Garg *et al.*, 1988). Evidence in favour of the latter process is given by the observed increase in plasma and pancreatic levels of LA in MaxEPA fed animals, although the amount of LA in the diet was kept constant.

The differences between fatty acid profiles in pancreatic tumours and non-tumorous tissue indicate an accelerated conversion of LA to AA in tumour tissue, which is reflected in consistently lower LA levels accompanied by higher AA levels, pointing to an altered fatty acid metabolism in neoplastic tissue.

LA may give rise to PGs of the 2-series via AA. It has been demonstrated that some of these PGs stimulate cell proliferation ($PGF_{1\alpha}$; Jimenez de Asua *et al.*, 1983) or have immunosuppressive properties (PGE_2; Plescia and Racis, 1988) and hence may either promote tumour growth or disturb inhibition of tumour development. ω-3 PUFAs may inhibit the formation of PGs derived from LA and AA, by either inhibiting the conversion of LA to AA or inhibiting the actual PG formation via cyclo-oxygenase. The dose-related decrease of TXB_2 and $PGF_{2\alpha}$ in non-tumorous pancreatic tissue of rats maintained on HF diet containing 0.0%, 4.7% or 9.4% MaxEPA, together with the increased LA levels in plasma and non-tumorous pancreas, demonstrate that ω-3 fatty acid influence LA/AA metabolism as expected. The decreased PG-levels in AACN-containing pancreatic tissue do not correlate with the increased mean number of AACNs when dietary MaxEPA is increased, suggesting no causal relationship between growth of preneoplastic acinar lesions and prostaglandins of the 2-series. PG levels are elevated, however, in pancreatic carcinomas indicating that they may play a role in the development of AACNs to ultimate pancreatic carcinomas.

From the present results it can be concluded that within the duration of this study (i) dietary MaxEPA has a dose-related enhancing effect on the development of AACNs but not on development of carcinomas in the pancreas of azaserine-treated rats and (ii) dietary MaxEPA inhibits the conversion of LA to AA, as well as the conversion of AA to TXB_2 or $PGF_{2\alpha}$ in non-tumorous pancreatic tissue and (iii) PGs may play a role in the growth/development of pancreatic adenocarcinomas, but not in the growth of AACNs.

Abbreviations
LA, linoleic acid; AA, arachidonic acid; OA, oleic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; AACN, atypical acinar cell nodule; AACF, atypical acinar cell focus; PG, prostaglandin; TXB_2, thromboxane B_2; PUFA, polyunsaturated fatty acid; CO, corn oil; MO, menhaden oil; LI, labelling index.

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