Conjugated linoleic acids in diet of female rats inhibit the breast cancer formation in their offspring

AGNIESZKA BIAŁEK – ANDRZEJ TOKARZ – PAWEŁ ZAGRODZKI

Summary

The aim of this study was to examine whether the enrichment of female rats diet with conjugated linoleic acids (CLA) influences breast cancer formation in their offspring, and to evaluate fatty acids distribution in tissues of progeny. The female offspring of animals with different diet modifications (oil or CLA) administered during pregnancy and breastfeeding was divided within groups into two subgroups: (1) – fed the same modified fodder as mothers, (2) – fed the standard fodder. Carcinogenic agent (7,12-dimethylbenz[*a*]anthracene) was administered to the offspring on 50th day of life. Mammary tumours occurred in all groups but the cancer morbidity decreased both among animals whose mothers were supplemented with CLA and among those animals whose diet was enriched in CLA also in adult life ($p = 0.0322$). Two CLA isomers present in dietary supplement in equal amounts were detected in serum and in tumour tissues, but the concentration of rumenic acid was higher. The mean concentration of CLA was higher in serum of animals without mammary tumours. Dietary supplementation also influenced concentrations of other fatty acids. Our results indicate that CLA decrease the risk of mammary carcinogenesis in rats. Their higher concentration in serum correlates with lower susceptibility to chemically induced tumorigenesis.

Keywords

conjugated linoleic acids; rumenic acid; breast cancer

Breast cancer is the most frequent type of cancer among women, both in developed and developing countries, and the third in global population [1]. Despite the fact that the etiology of most cases of this disease is unknown [2], among risk factors are numerous reproductive factors such as childbirth or breastfeeding, and nutritional factors [1, 3]. Quantity and quality of fat, especially the fatty acids profile in diet, are associated with many types of cancers. Results of many studies emphasize the great importance of conjugated linoleic acids in the modification of the cancerous process risk.

Conjugated linoleic acids (CLA) is a term that refers to the group of positional and geometric isomers of linoleic acid with conjugated double bonds in their chain. They are found in various types of food, milk, dairy products and meat from ruminants being the richest natural sources of CLA. *Cis*-9, *trans*-11 octadecadienoic acid (rumenic acid), is the predominant CLA isomer present in dietary products, constituting over 90% of all CLA isomers in these products [4]. The second important CLA isomer is *trans*-10, *cis*-12 octadecadienoic acid, which is found in dietary supplements with rumenic acid, usually in the ratio of one to one [5].

The interest in these fatty acids began in 1970s [6–8]. The results of scientific research show their health-promoting properties in different pathological states, e.g. obesity [5, 9], arteriosclerosis [10], cardiovascular diseases [11], inflammation [12, 13] and different types of cancer, in particular breast cancer [14–18]. Especially the antiobesity effect

Agnieszka Białek, Andrzej Tokarz, Department of Bromatology, Medical University of Warsaw, Banacha 1, 02-097 Warsaw, Poland.

Paweł Zagrodzki, Department of Food Chemistry and Nutrition, Jagiellonian University Medical College, Medyczna 9, 30-688 Cracow, Poland; H. Niewodniczański Institute of Nuclear Physics, Polish Academy of Sciences, Radzikowskiego 152, 31-342 Cracow, Poland.

Correspondence author:
Agnieszka Białek, e-mail: agnieszka.bialek@wum.edu.pl, tel.: +48 22 5720745, fax: +48 22 5720785

of CLA was examined and very well documented. *Trans*-10, *cis*-12 CLA influences the whole body weight, adipose tissue weight and internal organs weight, but its action depends on both the applied dose and the model organism.

The aim of this study was the assessment of the influence of diet supplementation of pregnant and breastfeeding female Sprague-Dawley rats with CLA on breast cancer formation in their offspring. The influence of this supplementation on fatty acids profile in the tissues of the progeny was also examined.

MATERIALS AND METHODS

Animals

This research and guiding principles in the care and use of laboratory animals were approved by Local Ethical Committee on Animal Experiments. Virgin female Sprague–Dawley rats (n = 8) were obtained from the Division of Experimental Animals, Department of General and Experimental Pathology (Medical University of Warsaw, Warsaw, Poland). They were housed in animal room at 21 °C, in a 12 h light : 12 h dark cycle. A standard diet composed of 22.0% protein, 4.0% fat, 30.0% starch, 5.0% fibre, 6.5% minerals (Labofeed H, Wytwórnia pasz "Morawski", Kcynia, Poland) was fed *ad libitum* during the entire experiment. After 1-week adaptation the animals were randomly divided into two groups of 4 rats each with different dietary supplementation regimes – with vegetable oil without CLA; substrate for the synthesis of CLA purchased from Pharma Nord (Vojens, Denmark); group K, or with CLA (Bio-C.L.A., Pharma Nord); group O. Both preparations were given intragastrically in the amount of 0.15 ml per day. The dose of CLA was established based on the value declared by the producer of Bio-C.L.A. (700 mg of CLA in one capsule) and constituted 1.0% of the diet. After 1 week, rats were mated with male Sprague-Dawley rats. Dietary supplementation of females lasted for the whole period of pregnancy and breastfeeding of their offspring. The progeny was separated from their mothers on the 30th day of age and, within the groups of supplementation, was divided into two subgroups of 8–10 rats each: first with the diet enriched with the dietary supplement that had been given to the mothers (vegetable oil – group K1, or Bio-C.L.A. – group O1) and the second subgroup that was fed the standard Labofeed H diet. Dietary supplementation of subgroups lasted for the following 21 weeks. Each offspring received intragastrically on 50th day of life a single dose of 80.00 mg·kg^{-1} body weight of carcinogenic agent – 7,12-dimethylbenz[*a*]anthracene (DMBA, approx. 95%; Sigma-Aldrich, Saint Louis, Missouri, USA) for the induction of mammary tumours. During the entire experiment, which was terminated at the end of 21st week, the rats were weighed weekly and palpated to detect the appearance of tumours. All animals were decapitated and exsanguinated and the mammary tumours were isolated. Tab. 1 shows the profiles of experimental groups.

Histopathological examination

There were no spontaneous tumours in maternal rats during the experiment. The effectiveness of mammary cancers induction in offspring was determined as the percentage of animals with tumours. Some of the tumours collected during necropsy (three in each group, selected randomly) were fixed in 10% formalin. Afterwards, they were identified as adenocarcinomas and papillary adenocarcinomas of mammary gland. Because of the procedure of histopathological examination, these tumours were not analysed regarding the fatty acids profile.

Preparation of experimental material.

Serum was obtained by centrifugation of blood for 10 min at 2000 ×g at 4 °C and stored at –20 °C until being analysed. Mammary tumours were collected during necropsy and were stored at –20 °C as well.

Tab. 1. Characteristics of experimental groups.

Group	Number	Supplementation of mother	Supplementation of children	Mammary tumour incidence	Total number of tumours	Age of tumour appearance	Total mass of tumours [g]	Mean number of tumours per individual
K1	10	+ oil	+ oil	80%	14	130 ± 20	78.55	1.4
O1	8	+ CLA	+ CLA	25%	4	105 ± 9	12.80	0.5
K2	9	+ oil	–	78%	11	120 ± 16	50.95	1.2
O2	9	+ CLA	–	33%	5	147 ± 15	9.57	0.6

Age of tumour appearance is expressed as day of life, values are mean ± standard deviation.

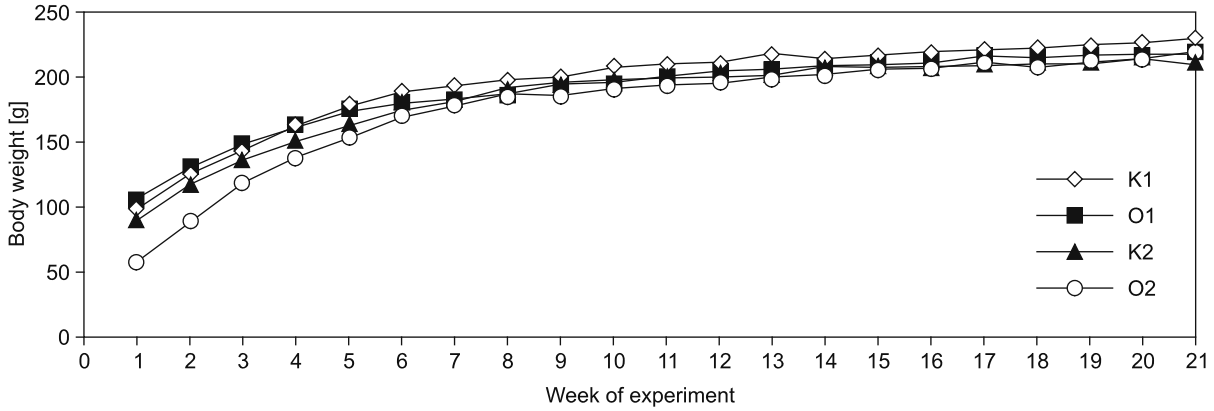

Fig. 1. Changes of average body weight during the experiment ($p > 0.05$).

Fatty acids analysis

Fatty acid analysis was made with gas chromatography (GC-17A gas chromatograph, Shimadzu, Kyoto, Japan) with a capillary column (BPX 70; 60 m × 0.25 mm i.d., film thickness 0.20 μm; SGE, Ringwood, Australia) and flame-ionization detection. The rat sera were thawed and samples of 100 μl were transesterified by the procedure of Bondia-Pons et al. with minor modifications [19], which were thoroughly described elsewhere [20].

Mammary tumours were thawed only once and three replicate samples of 0.20 g were taken for lipids extraction according to Folch et al. with a minor modification [21]. Purified organic extract was evaporated to dryness under a stream of nitrogen and weighed to estimate the content of lipids in tumour tissue. The residue was taken for the preparation of fatty acids methyl esters (FAME) according to the procedure previously described for serum.

Statistical analysis

All data are shown as mean values ± standard deviation. For variables with skew distribution, data were transformed to logarithms and re-transformed after calculations. Obtained results were evaluated with Statistica 9.0 (StatSoft, Tulsa, Oklahoma, USA) and GraphPad Prism 3.02 (GraphPad Software, La Jolla, California, USA). Due to the relatively small number of individuals in each group, the data were tested with Kruskal-Wallis test and verified with Dunn's multiple comparison test. *p*–value of 0.05 was considered significant. Cluster analysis was performed for medians of fatty acids concentration after standardization of the data.

RESULTS

The agent 7,12-dimethylbenz[*a*]anthracene administered intragastrically in a single dose of 80 mg·kg^{-1} body weight was effective in the induction of mammary tumours, as they appeared during the experiment in all investigated groups. They were identified as adenocarcinomas and papillary adenocarcinomas of mammary gland. The percentage of tumour-bearing animals in each group is shown in Tab. 1.

Tab. 2. Comparison of average masses of internal organs with statistical tests.

Organ	Group	Organ weight [g]	*p* value
Liver	K1	7.21 ± 1.24 [a]	0.008
	O1	6.65 ± 0.54	
	K2	5.98 ± 0.56 [a]	
	O2	6.15 ± 0.55	
Kidney	K1	1.90 ± 0.13 [b]	0.004
	O1	1.85 ± 0.13 [a]	
	K2	1.78 ± 0.16	
	O2	1.61 ± 0.13 [ab]	
Heart	K1	1.06 ± 0.14 [ab]	0.003
	O1	0.88 ± 0.10	
	K2	0.87 ± 0.15 [b]	
	O2	0.84 ± 0.06 [a]	
Spleen	K1	0.82 ± 0.14	0.030
	O1	0.58 ± 0.19	
	K2	0.71 ± 0.29 [a]	
	O2	0.49 ± 0.05 [a]	

Values of organ weights are given as mean ± standard deviation.
a – pairs of groups detected by Dunn test as significantly different, $p < 0.05$; b – pairs of groups detected by Dunn test as significantly different, $p < 0.01$.
p values were obtained by Kruskal-Wallis test for comparison of all groups.

The higher effectiveness of cancer induction was observed in groups K1 and K2 ($p = 0.0322$). In K1 group, 8 of 10 animals developed tumours: 2 animals had 3 tumours, 2 had 2 tumours, and 4 had 1 tumour. In K2 group, the number of tumour-bearing animals was 7 of 9. Among them, 1 had 4 tumours and 1 had 2 tumours, and the others had single tumours. CLA supplementation decreased the incidence of mammary cancer in investigated animals, both in O1 and in O2 groups. In O1 group, with permanent CLA supplementation, 2 of 8 animals developed tumours and in O2 group, only with maternal supplementation, 3 of 9 animals developed mammary cancer during the experiment. In O1 group, 1 rat had 3 tumours and 1 had 1 tumour, whereas in O2 group, 2 animals developed 2 tumours each and 1 had a single tumour. Despite the fact that in O1 group tumours appeared much earlier (on average at 15th week of life), their number per individual was the smallest. Moreover, cancer-preventive properties of CLA were observed in O2 group, where both the number and mass of tumours were similar to those in O1 group.

During the whole experiment the mean body weight of each group was checked weekly. There were no significant differences among them (Fig. 1).

Body mass growth was the most intensive for the first 5 weeks, when animals gained 90 g of weight each. For the next 16 weeks, their body mass increased on average by 45 g. Animals from O2 group, whose initial body mass was about 40 g less than others, gained similar final body weight, which indicates that they utilized the forage as effectively as other groups.

The comparison of average mass of organs (liver, heart, spleen, kidney) revealed that they were significantly influenced by the supplementation applied (Tab. 2).

The highest mass of organs was observed in animals from K1 group, whereas the mass of most

Tab. 3. Fatty acids profile in serum of investigated groups.

Fatty acid [%]	Group of animals				Kruskal-Wallis test *p* value
	K1	O1	K2	O2	
C12:0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.000
C14:0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.001
C15:0	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.1	0.4 ± 0.0	0.004
C16:0	17.0 ± 1.5	15.7 ± 0.8	18.1 ± 1.1	17.9 ± 1.5	0.006
C16:1	0.5 ± 0.1	0.5 ± 0.1	0.8 ± 0.2	0.7 ± 0.2	0.000
C17:0	0.6 ± 0.2	0.6 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	NS
C17:1	0.1 (0.0–0.1)*	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	NS
C18:0	16.2 ± 1.2	19.2 ± 1.6	15.7 ± 1.2	14.7 ± 1.9	0.000
C18:1 *n*-9 *trans*	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	–	NS
C18:1 *n*-9 *cis*	7.8 ± 1.1	5.9 ± 0.8	7.6 ± 2.1	7.5 ± 1.3	0.032
C18:2 *n*-6 *trans*	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	NS
C18:2 *n*-6 *cis*	18.6 ± 2.4	15.7 ± 1.7	18.9 ± 1.5	20.2 ± 2.5	0.002
C18:3 *n*-6	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	NS
C18:3 *n*-3	0.9 ± 0.1	0.7 ± 0.2	1.0 ± 0.3	1.2 ± 0.3	0.008
C20:0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	NS
cis-9, *trans*-11 CLA	0.0 (0.01–0.04)*	0.3 ± 0.1	0.0 (0.01–0.02)*	0.0 (0.01–0.03)*	0.000
trans-10, *cis*-12 CLA	0.0 (0.01–0.02)*	0.1 ± 0.0	0.0 (0.01–0.02)*	0.0 ± 0.0	0.000
C20:1	0.1 ± 0.0	0.3 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.000
C20:3 *n*-6	0.1 (0.0–0.2)*	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	NS
C20:4 *n*-6	24.7 ± 4.0	28.4 ± 1.5	23.7 ± 3.3	24.6 ± 2.5	0.012
C20:3 *n*-3	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	NS
C20:5 *n*-3	1.0 ± 0.2	0.9 ± 0.2	1.1 ± 0.30	0.9 ± 0.2	NS
C24:0	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	NS
C22:6 *n*-3	5.2 ± 1.9	5.7 ± 0.5	4.8 ± 1.0	4.4 ± 0.6	0.012

Data are expressed as percentage share of a single fatty acid in the pool of all fatty acids. Data are shown as mean values ± standard deviation.

(0.0 ± 0.0) – means that the percentage share of this fatty acid < 0.1%; (–) – means that this fatty acid was not present in this group; * – for variables with skew distribution, data were transformed to logarithms and re-transformed after calculations; data are shown as mean and confidence interval.

p value was lower than 0.05 for those fatty acids with significant differences among groups in Kruskal-Wallis test. NS – not significant differences among groups in Kruskal-Wallis test ($p > 0.05$).

organs from group O2 was the smallest (Tab. 2). Long-term supplementation with CLA had no effect on the weight of most examined organs, except for kidneys, the mass of which was significantly higher in O1 group in comparison with group O2 (Tab. 2).

FAME profiles of serum were determined using gas chromatography. In our experiment, 24 fatty acids were analysed in serum. The following fatty acids were found to be the main species in the serum of all investigated groups: C20:4 *n*-6 (arachidonic acid, AA), C18:2 *n*-6 *cis* (linoleic acid, LA), C16:0, C18:0 and C18:1 *n*-9 *cis* (oleic acid, OL) (Tab. 3). There were significant differences in the concentrations of the majority of fatty acids among the examined groups (Tab. 4). These differences were caused mainly by two-step supplementation of the diet with CLA. In group O1, higher concentration of stearic acid and lower concentration of miristic and palmitic acids in serum were found than in group K2 or O2. We also observed the lowest concentration of OL, LA and α–linolenic acid (ALA), and the highest concentration of C20:4 *n*-6 (AA) and C22:6 *n*-6 (docosahexaenoic acid, DHA) in group O1. The two-step supplementation of diet with vegetable oil decreased the mean concentration of C20:1 in serum. Unlimited consumption of standard fodder caused apparent similarity in most fatty acids concentration in serum of K2 and O2 groups. The highest C16:1 level was observed in the samples of serum obtained from rats in those groups, where the diet was not modified after maternal milk consumption. Also C12:0 in K2 group and C15:0 in O2 group were present in much higher amounts than in other groups.

Cluster analysis of FAME profiles revealed five clusters (Fig. 2). Cluster 1 included all animals from group O1 and one individual from group O2 (O2/1), with similar concentration of many fatty acids in serum, e.g. both CLA isomers, to O1 animals (89% specificity for the O1 group). Modification of the diet with continuous supplementa-

Tab. 4. Results of Dunn's multiple comparison test for fatty acids concentration in serum.

	Compared groups	Dunn's test *p* value
C12:0	K1 vs K2	$p < 0.05$
	K2 vs O2	$p < 0.001$
C14:0	O1 vs K2	$p < 0.001$
C15:0	K1 vs O2	$p < 0.05$
	O1 vs O2	$p < 0.01$
C16:0	O1 vs K2	$p < 0.01$
	O1 vs O2	$p < 0.01$
C16:1	K1 vs K2	$p < 0.05$
	O1 vs K2	$p < 0.001$
	O1 vs O2	$p < 0.05$
C18:0	O1 vs K2	$p < 0.01$
	O1 vs O2	$p < 0.001$
C18:1 *n*-9 *cis*	K1 vs O1	$p < 0.05$
C18:2 *n*-6 *cis*	O1 vs K2	$p < 0.05$
	O1 vs O2	$p < 0.001$
C18:3 *n*-3	O1 vs O2	$p < 0.01$
C20:1	K1 vs O1	$p < 0.001$
	K1 vs O2	$p < 0.05$
C20:4 *n*-6	O1 vs K2	$p < 0.05$
C22:6 *n*-3	O1 vs O2	$p < 0.01$
cis-9, *trans*-11 CLA	K1 vs O1	$p < 0.01$
	O1 vs K2	$p < 0.001$
	O1 vs O2	$p < 0.05$
trans-10, *cis*-12 CLA	K1 vs O1	$p < 0.001$
	O1 vs K2	$p < 0.01$
	O1 vs O2	$p < 0.05$

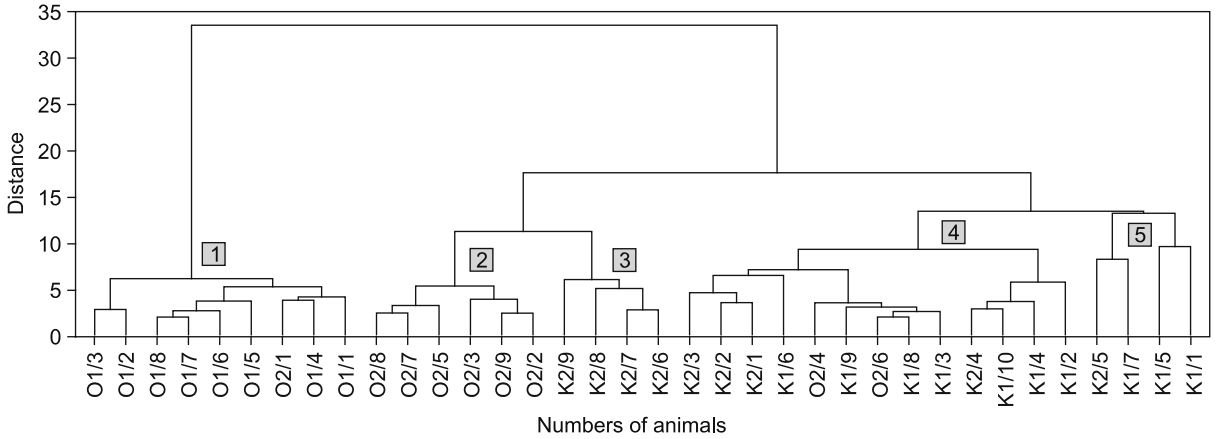

Fig. 2. Dendrogram of similarity in fatty acids profile in serum among the investigated individuals.

Method of grouping: Ward agglomeration procedure, function of the distance: Euclidean distance, Mojena's rate: d=9.26.

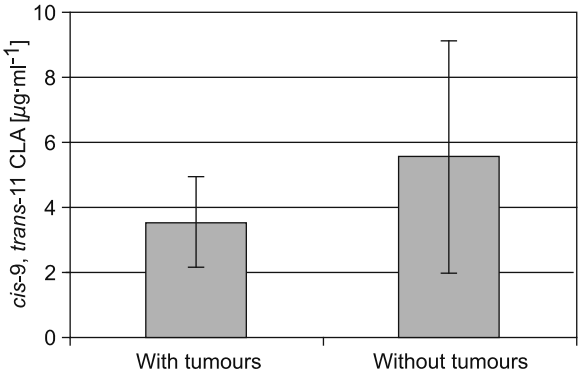

Fig. 3. Concentration of *cis*-9, *trans*-11 CLA in serum of O1 individuals with and without mammary tumours.

tion with CLA altered the profile of fatty acids in serum most significantly, therefore, cluster 1 was most distant from other clusters. The lack of dietary supplementation after breastfeeding in K2 and O2 groups led to similar profiles of fatty acids in sera. Because of that, clusters 2 and 3 were very similar. Clusters 4 and 5 were formed by animals from different experimental groups, which suffered from mammary tumours.

Two main CLA isomers identified as *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA, were detected in all samples of serum obtained from all investigated groups. Bio-C.L.A. used as the source of CLA for O (mothers) and O1 (progeny) consisted of several fatty acids, with prevailing share of two CLA isomers: *trans*-10, *cis*-12 CLA (33%) and *cis*-9, *trans*-11 CLA (31%) [20]. In O1 group, with continuous CLA intake, the percentage share of CLA isomers in total fatty acids amount was much higher than in other groups (Tab. 3). Rumenic acid was shown to be the most prominent CLA isomer in this group, while *trans*-10, *cis*-12 CLA constituted only (0.1 ± 0.0)% of fatty acids. Mean concentration of rumenic acid in serum of O1 rats was (5.03 ± 3.22) μg·ml^{-1} and its concentration differed among the examined individuals (from 2.53 μg·ml^{-1} to 11.19 μg·ml^{-1}). We also compared the rumenic acid concentration in serum of O1 group between tumour-bearing and non-tumour-bearing animals. Its concentration tended to be slightly higher in serum of O1 rats without noticeable tumours in comparison with O1 tumour-bearing animals ((5.53 ± 3.59) μg·ml^{-1} versus (3.53 ± 1.41) μg·ml^{-1}; Fig. 3).

The lipid contents in mammary tumours in both CLA supplemented groups (O1 and O2) were significantly higher than in tumours of K1 and K2 (p = 0.0486; Fig. 4). Over 30 fatty acids were identified and determined in the lipid fraction of breast tumours. The following fatty acids were the most common in the cancerous tissues obtained from suffering animals of all experimental groups (Tab. 5): C16:0, C18:1 *n*-9 *cis* (OL), C18:2 *n*-6 *cis* (LA), C20:4 *n*-6 (AA), and C18:0.

Because of the limited number of tumour-bearing animals, especially in O1 and O2 group, it was not possible to evaluate the obtained results with proper statistical tests, but some differences were apparent. LA and ALA concentration in the lipid fraction of mammary tumours from O1 group was the highest, whereas C20:4 *n*-6 (AA) was present in this group in the smallest concentration. Moreover, two-step supplementation of the diet with vegetable oil increased C20:5 *n*-3 (eicosapentaenoic acid, EPA) and C22:6 *n*-6 (DHA) concentration in cancerous tissues. C18:0 concentration was

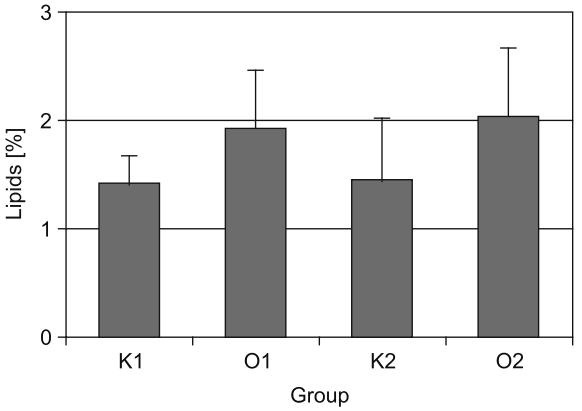

Fig. 4. Lipids content in mammary tumours of investigated groups (p = 0.0486).

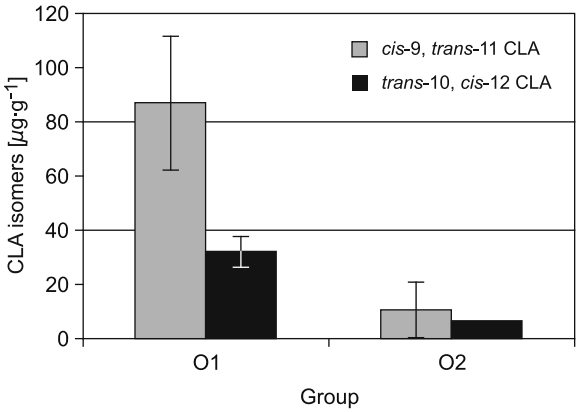

Fig. 5. Content of CLA isomers in mammary tumours of O1 and O2 groups.

Tab. 5. Fatty acids profile in mammary tumours of investigated groups.

Fatty acid [%]	Group of animals			
	K1	O1	K2	O2
C10:0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 (0.0–0.3)*
C12:0	0.3 ± 0.1	0.3 ± 0.0	0.4 ± 0.2	0.2 ± 0.0
C13:0	0.2 ± 0.1	0.1 ± 0.1	0.0 (0.0–0.1)*	0.2 ± 0.0
C14:0	1.0 ± 0.2	1.3 ± 0.1	1.2 ± 0.1	1.1 ± 0.1
C14:1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
C15:0	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0
C15:1	0.1 ± 0.1	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0
C16:0	22.4 ± 2.2	23.0 ± 1.0	22.6 ± 1.4	22.8 ± 0.8
C16:1	1.4 ± 1.3	1.6 ± 0.4	2.0 ± 0.4	3.1 ± 0.1
C17:0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 (0.0–0.4)*
C17:1	0.1 ± 0.0	0.1 ± 0.0	0.1 (0.0–0.4)*	0.0 ± 0.0
C18:0	11.6 ± 3.6	9.7 ± 3.0	10.6 ± 2.1	8.3 ± 2.7
C18:1 *n*-9 *cis*	17.6 ± 4.3	18.2 ± 0.8	18.0 ± 2.6	21.0 ± 4.4
C18:2 *n*-6 *trans*	0.1 (0.0–0.1)*	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
C18:2 *n*-6 *cis*	12.6 ± 5.4	20.8 ± 0.9	12.4 ± 4.3	15.2 ± 8.1
C18:3 *n*-6	0.0 ± 0.0	0.1 ± 0.0	0.1 (0.0–0.3)*	–
C18:3 *n*-3	0.8 ± 0.8	1.5 ± 0.4	1.0 ± 0.5	1.3 ± 0.9
C20:0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0
cis-9, *trans*-11 CLA	0.0 ± 0.0	0.7 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
trans-10, *cis*-12 CLA	0.0 ± 0.0	0.3 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
C20:1	0.1 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.1
C21:0	0.5 ± 0.2	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.1
C20:2	0.2 ± 0.1	0.1 ± 0.0	0.3 ± 0.2	0.3 ± 0.1
C20:4 *n*-6	13.7 ± 4.9	11.3 ± 1.2	13.0 ± 3.4	13.3 ± 8.2
C20:3 *n*-3	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0
C22:0	0.0 ± 0.0	0.0 ± 0.0	0.0 (0.0–0.1)*	0.1 ± 0.0
C20:5 *n*-3	0.2 ± 0.1	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0
C22:2	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
C23:0	0.2 ± 0.1	0.0 ± 0.0	0.2 ± 0.1	0.1 ± 0.1
C24:0	0.2 ± 0.1	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.1
C24:1	0.9 ± 0.3	0.7 ± 0.2	0.8 ± 0.3	0.5 ± 0.1
C22:6 *n*-3	2.2 ± 0.9	1.4 ± 0.1	1.8 ± 0.5	1.3 ± 0.5

Data are expressed as percentage share of single fatty acid in pool of all fatty acids. Data are shown as mean values ± standard deviation.
(0.0 ± 0.0) – means that the percentage share of this fatty acid was lower than 0.1%; (–) – means that this fatty acid was not present in this group; * – for variables with skew distribution, data were transformed to logarithms and re-transformed after calculations; data are shown as mean and confidence interval.

slightly higher in tumours from K1 and K2 groups. As far as C16:1 is concerned, we observed similar dependence as in serum. Its highest concentrations were observed in tumour-bearing individuals from K2 and O2 groups, whose diet was not modified in the adult life.

Although both CLA isomers were identified in the lipid fraction of tumours of all experimental groups (Tab. 5), their absolute amounts were measured only in O1 and O2 group. We also observed big variations among individuals in the concentration of CLA isomers, both in the lipid fraction and in the cancerous tissue, but some general tendencies could be determined. Comparison of their distribution in mammary tumours revealed the great similarity to their distribution in serum, because likewise in serum, *cis*-9, *trans*-11 CLA concentration tended to be much higher than *trans*-10, *cis*-12 CLA (Fig. 5).

DISCUSSION

In our experiment, cancer-chemopreventive properties of CLA were observed. Both the dose and the time of supplementation influenced the effectiveness of this action. Ip et al. got similar results as, in their experiment, 0.5%, 1.0% and 1.5% dose of CLA in diet reduced the number of mammary tumours in female rats [15]. Even smaller

doses of CLA in diet (0.05% to 0.5%) appeared to be effective, when given for 9 months [22]. Also vaccenic acid, which is endogenously converted into CLA, diminished the breast cancer risk in experimental animals [23, 24]. The effectiveness of anticancerogenic action in mammary glands of both main CLA isomers in 0.5% dose were comparable [25]. We observed smaller number of tumours among those animals, whose mothers were supplemented with CLA only during the pregnancy and breastfeeding. This action was almost as efficient as observed among those animals whose diet was modified with continuous CLA consumption. Our results are in line with those of Ip et al., who supplemented the diet of rats for 5 weeks before the carcinogenic agent administration and observed inhibition of mammary carcinogenesis [22]. Thompson et al. demonstrated also that supplementation of the diet with 1.0% of CLA for 1 month before DMBA administration was equally efficient as 6-months supplementation [26]. CLA, when given before the 50th day of life, that is during the maturation of mammary glands, induced changes in their structure, i.e. diminished the number and differentiation of terminal end buds [22]. It seems that the optimal dosage of conjugated dienes of linoleic acid to infants during pregnancy and breastfeeding is important for the protective effect of CLA.

The higher supply of conjugated linoleic acids in diet of pregnant and breastfeeding females induced their elevated levels in maternal milk and increased their consumption by the breastfed infants [27, 28]. Cao et al. observed that administration of CLA mix into mothers' diet in the amount of 1.0% and 2.0% increased their concentration in maternal milk by 4.2% and 8.6%, respectively [28]. Incorporation of natural CLA-rich foods (especially milk and dairy products) as well as other enriched dietary products into women's diet led to elevation of their concentration in human milk [29, 30].

We did not notice any significant differences in gained body mass among the examined groups. He et al. showed that supplementation of animal diet with synthetic isomers of CLA led to significantly lower body mass [31]. In contrast, Chin et al., who supplemented the diet of females with 0.25% and 0.5% CLA, observed in their offspring significantly higher birth weight. Moreover, continuation of the dietary supplementation with CLA led to higher body weight and stimulated the usage of food in comparison to control group [27]. Also Cao et al. indicated that progeny of female rats with 1.0% or 2.0% addition of CLA in diet gained weight more effectively [28]. Neither of these experiments lasted as long as ours, and the animals had not been treated with carcinogenic agent. Furthermore, the development of mammary tumours as a result of the DMBA administration, influences the total body mass indirectly, by adding the mass of the tumours. In accordance to our results, long-term application of CLA isomers mixture by Ip et al. in a dosage of 0.5–1.5% to rats with tumorigenesis stimulation with DMBA, did not influence significantly their total body mass [15].

In our experiment, we observed some significant differences in the mass of inner organs. However, the two-step supplementation of the diet with CLA did not cause too many of them because only kidneys' mass in O1 group (and in K1) was greater than in O2 group. Our results are similar to those of Ip et al. who did not note any differences in the mass of organs when the mixture of CLA isomers was applied [15]. This observation is also in agreement with that of He et al. [31] who did not observe any significant differences in the mass of organs after CLA supplementation. Also Turpeinen et al., who applied two dosages of *cis*-9, *trans*-11 CLA isomer, confirmed the lack of rumenic acid influence on the mass of inner organs [32]. Moreover Ip et al., who used in their experiment mice with cancer, observed that only *trans*-10, *cis*-12 CLA but not *cis*-9, *trans*-11 CLA caused a significant increase of mass of liver, heart and spleen [33].

Our results of cluster analysis indicate that breast cancer significantly influenced the fatty acids profile in serum. Moreover, this action differs from the influence of continuous CLA supplementation, because the distance among clusters 1 and 4 and 5 was the biggest. This effect was confirmed by other data. It was observed, that the profile of fatty acids in serum of patients suffering from different types of cancer differed from that in healthy people. There are many possible explanations of this fact but the most probable one is that these effects follow changes in the lipid metabolism, e.g. enhanced lipolysis or lipid peroxidation [34] or inhibited action of desaturases, mainly Δ6–desaturase, which is characteristic for cancer cells [35]. In plasma phospholipids from patients with bladder cancer, much lower levels of LA and its metabolites (except for C20:3 *n*-6 and C22:5 *n*-6) and ALA metabolites were detected, whereas levels of ALA did not differ [35]. The concentration of numerous fatty acids, e.g. *n*-3 and *n*-6 polyunsaturated fatty acids (PUFA), was much lower in phospholipids of patients with advanced cancer than in control group, but their neutrophils contained much more C20:4 *n*-6 (AA)

[34]. Results of many epidemiological studies suggest a correlation between elevated risk of cancerous process and concentration in serum of selected fatty acids, e.g. higher amounts of saturated fatty acids correlate with increased breast cancer risk in postmenopausal women, whereas for most of *n*-3 and *n*-6 PUFA, this dependence is opposite. TAKATA et al. found that higher concentration in serum of C22:0, C24:0 and *trans* C16:1 correlated with higher mammary cancer risk in smokers [36].

As far as fatty acids profile in serum is concerned, our observations were similar to those of other authors. BONDIA-PONS et al. found the same fatty acids to be the most prominent in the plasma of rats [19]. However, both the applied dosages and time of supplementation are of importance, and can modify fatty acids profile in serum as well as cause some difficulties in interpretation of data of other authors. CORL et al. who used four different levels of rumenic acid supply, observed significant differences only in C12:0, C14:1, *trans*-10 C18:1 and rumenic acid concentration [23]. Moreover, when TURPEINEN et al. administered to animals lower dosage of *cis*-9, *trans*-11 CLA, they observed a decrease in OL, LA, ALA and an increase in AA and DHA levels, whereas in group with twice as high dosage of rumenic acid, its influence on those fatty acids was opposite [32]. Consumption of dietary supplements rich in a mixture of two main CLA isomers significantly decreased concentrations in serum of saturated fatty acids and *n*-6 PUFA, especially AA, in comparison with their levels in serum of people who consumed CLA only in dietary products in standard contents [37]. SHAHIN et al. also found that the profile of fatty acids in different lipid fractions in serum depended on the consumption of butter and other fats [38].

Many authors claimed that determination of CLA concentration in plasma or serum reflects their dietary intake [19, 39]. SHAHIN et al. indicated that rumenic acid was preferentially incorporated into triacylglycerols in human plasma in comparison with cholesterol esters and phospholipids. Moreover, its amount in triacylglycerols increased mostly as the response to its higher supply in diet [38]. Also PETRIDOU et al. revealed preferential incorporation of CLA isomers into serum triacylglycerols and unequivocal impact of CLA supplementation on their concentration in serum [40]. Our results indicate that not only diet but also coexisting factors, such as pathological conditions or diseases e.g. cancers, also affect levels of CLA in serum. We detected much higher concentration of *cis*-9, *trans*-11 CLA in serum than the second CLA isomer present in dietary supplement. This observation is in agreement with our previous results [20]. BURDGE et al. who administered dietary supplement with *trans*-10, *cis*-12 CLA as the predominant isomer, detected much higher amounts of *cis*-9, *trans*-11 of CLA in plasma phosphatidylcholine [41]. Likewise, PETRIDOU et al. claimed that rumenic acid was the main CLA isomer of serum lipids [40]. Moreover, ZLATANOS et al. who gave to volunteers CLA supplement containing two major CLA isomers in equal proportions, detected in their serum only rumenic acid, whereas *trans*-10, *cis*-12 CLA was below the detection limit [37]. The total concentration of rumenic acid tended to be higher in plasma of non-tumour-bearing animals in comparison with tumour-bearing individuals. These results confirm our previous reports [20] and are in agreement with those of HOFFMAN et al., who detected lower concentration of CLA in mitochondrial fraction of cancerous tissue than in normal tissue [42].

Concerning fatty acids profile in mammary tumours, our data seemed to indicate some interesting relationships. Oleic acid was the predominant of all unsaturated fatty acids. JELIŃSKA et al. also determined its highest contents of all unsaturated fatty acids in phospholipids fraction of mammary tumours [43]. The two-step supplementation of diet with CLA isomers affected concentration of some fatty acids in cancerous tissue. BANNI et al. who applied 1.0% addition of CLA to diet, did not report changes in LA content in adipose tissue of mammary glands, but they observed a significant depletion of its metabolites, C18:3, C20:3 and C20:4 [39]. Moreover, EDER et al. observed much lower levels of both AA and sum of *n*-6 fatty acids in liver phospholipids after diet enrichment with CLA [44]. As observed by CAO et al., progeny of rat females supplemented with 1.0% of CLA was characterized by lower concentration of C20:4 in liver phospholipids, whereas in group of 2.0% CLA, the tendency was opposite. Concerning ALA levels, the observed trend was reverse, while LA concentrations increased in both groups [28]. As far as EPA and DHA concentration is concerned, we observed their minimal share in mammary tumours. JELIŃSKA et al. did not detect EPA in phospholipid fraction of mammary tumours, whereas DHA levels were much lower than in liver tissue [43]. Also SENKAL et al. observed a lower concentration of EPA than DHA in different cancers of gastrointestinal tract as well as in livers [45]. The study by HOFFMAN et al. revealed lower levels of DHA in cancerous than in normal tissues [42].

In this study, we detected the highest percentage of two major CLA isomers in mammary tumours in a group constantly supplemented with

CLA. The total content of conjugated linoleic acids in tumours from O1 group was much higher than in O2, however in O2 group, it was also possible to assess the concentration of these fatty acids. The higher supply of these compounds in the diet of mothers during pregnancy and breastfeeding caused their incorporation into the tissues of the offspring. These observations emphasize the importance of adequate mother's nutrition during pregnancy and breastfeeding. However, *trans*-10, *cis*-12 CLA content in mammary tumours, similar as in serum, was much lower than the content of rumenic acid. According to TSUZUKI, such diversity emerges from differences in the metabolism and not in the bioavailability. *Trans*-10, *cis*-12 octadecadienoic acid is known to activate β-oxidation and to facilitate its own metabolism [46].

CONCLUSIONS

Our findings confirm that conjugated linoleic acids can inhibit the development of chemically induced mammary tumours. Higher concentration of CLA in serum seems to be connected with a lower risk of mammary carcinogenesis. CLA content in diet influences also the profile of other fatty acids in serum and cancerous tissues, but coexisting conditions also play a role. The higher supply of CLA in the diet of mothers during pregnancy and breastfeeding causes the incorporation of CLA into tissues of children and can exert health-promoting effect in progeny's adult life.

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Ethical standards

This research and guiding principles in the care of laboratory animals were approved by Local Ethical Commission on Animal Experiments and they comply with the law of Poland.

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