RESEARCH ARTICLE

Arachidonic acid/docosahexaenoic acid-supplemented diet in early life reduces body weight gain, plasma lipids, and adiposity in later life in ApoE*3Leiden mice

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Scope: This study addresses whether early life arachidonic acid (ARA)/docosahexaenoic acid (DHA) supplementation or eicosapentaenoic acid (EPA)/DHA (Omacor) supplementation affects body weight gain, lipid metabolism, and adipose tissue quantity and quality in later life in ApoE*3Leiden-transgenic mice, a humanized model for hyperlipidemia and mild obesity.

Methods and results: Four-week-old male ApoE*3Leiden mice were fed chow diet with or without a mixture of ARA (0.129 wt%) and DHA (0.088 wt%) or Omacor (0.30 wt% EPA, 0.25 wt% DHA). At age 12 weeks, mice were fed high-fat/high-carbohydrate (HFHC) diet without above supplements until age 20 weeks. Control mice received chow diet throughout the study. Mice receiving ARA/DHA gained less body weight compared to control and this effect was sustained when fed HFHC. Omacor had no significant effect on body weight gain. Plasma cholesterol and triglycerides were significantly lowered by both supplementations. At 20 weeks, epididymal fat mass was less in ARA/DHA-supplemented mice, while Omacor had no significant effect on fat mass. Both ARA/DHA and Omacor reduced inguinal adipocyte cell size; only ARA/DHA significantly reduced epididymal macrophage infiltration.

Conclusion: This study shows that early life ARA/DHA, but not Omacor supplementation improves body weight gain later in life. ARA/DHA and to a lesser extent Omacor both improved adipose tissue quality.

Keywords:
Adiposity / ApoE*3Leiden mice / Early life / Obesity / PUFA

1 Introduction

Obesity is an important risk factor for metabolic diseases such as type 2 diabetes and has become a major health problem over the last decades. The prevalence of obesity has reached epidemic proportions and the escalating number of children and adolescents with severe overweight worldwide is of concern.

Excessive energy intake, lack of physical activity, and (epi)genetic susceptibility are underlying causes of obesity. In addition, poor food quality (e.g. energy dense foods such as those rich in unsaturated fat and sugars) may play an important role in the development of obesity [1]. Further, the shift towards increased dietary n-6 and decreased *n*-3 polyunsaturated fatty acids (PUFAs) is thought to be associated with the observed increase in obesity [2, 3]. Indeed, an increased dietary intake of *n*-6 PUFAs, especially in periods of growth and development, has been reported to coincide with an increased fat mass gain [4, 5]. In contrast, dietary intake of *n*-3 PUFAs reduces body weight gain and adiposity [6]. Besides, *n*-3 PUFAs are associated with several beneficial effects on

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Abbreviations: ARA, arachidonic acid; **DHA**, docosahexaenoic acid; **EPA**, eicosapentaenoic acid; **LC-PUFA**, long-chain polyunsaturated fatty acid

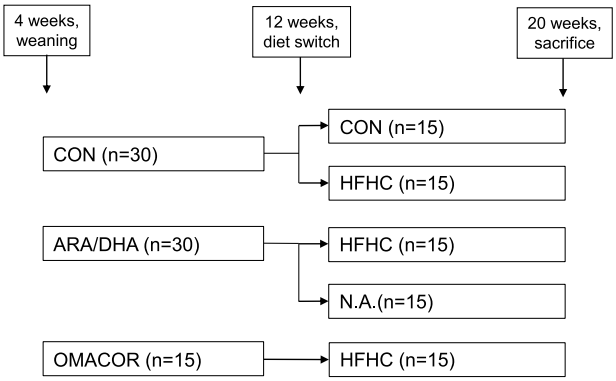

Figure 1. Schematic overview of experimental design. CON, Chow diet; HFHC, high-fat high-carbohydrate diet, ARA, arachidonic acid supplementation; DHA: docosahexaenoic acid supplementation. Omacor contains eicosapentaenoic acid (EPA) and DHA. NA, not available.

health such as improvement of insulin sensitivity, reduced accumulation of liver and muscle fat [7], increased glucose utilization in skeletal muscle [8], and enhanced mitochondrial and peroxisomal beta-oxidation [9].

Accumulating evidence suggests that the susceptibility for metabolic diseases may be influenced early in life [10, 11]. It has been suggested that the type, quantity, and ratio of *n*-3 and *n*-6 PUFAs that are consumed during childhood may determine the adult metabolic profile and risk factors of disease [12]. Arachidonic acid (ARA) and docosahexaenoic acid (DHA) are *n*-6 and *n*-3 long chain (LC)-PUFAs, respectively, proven to support brain and visual development in infants [13]. Their dietary supplementation early in life is therefore considered to be health beneficial. When provided during perinatal stage, LC-PUFAs exhibited promising potency in the prevention and treatment of obesity. Donahue et al. demonstrated an enhanced maternal–fetal *n*-3 LC-PUFA status to be associated with lower childhood adiposity [14], however, data on the effects of early life dietary LC-PUFAs intervention on obesity are lacking.

The present study addresses two important outstanding questions. Firstly, can dietary supplementation with a well-defined *n*-6/*n*-3 LC-PUFA mixture of ARA/DHA as present in breast milk or a clinically applied *n*-3 LC-PUFA mixture EPA/DHA (OMACOR®) during early life reduce body weight gain? Secondly, do these PUFA supplementations in early life beneficially affect body weight gain and metabolism in later life under adipogenic conditions?

To this end, male ApoE3Leiden-transgenic mice, a humanized animal model for hyperlipidemia and mild obesity [15, 16] were fed a chow diet rich in ARA/DHA or OMACOR for 8 weeks in early life (from weaning at 4 weeks to 12 weeks of age), after which the mice were exposed for 8 weeks to a mildly obesogenic high-fat/high-carbohydrate (HFHC) diet. Parameters that were evaluated include body weight, adiposity quantity and quality, as well as metabolic parameters.

2 Materials and methods

2.1 Ethics statement

The animal experiments were approved by an independent institutional ethical committee on animal care and experimentation (Zeist, The Netherlands), approval number DEC2844.

2.2 Animals and diets

Four-week old male heterozygous ApoE*3Leiden-transgenic mice originated from the SPF breeding stock at TNO-Metabolic Health Research, Leiden, The Netherlands. Mice were housed in Macrolon cages (3–5 mice per cage) in clean conventional animal rooms (relative humidity 50–60%, temperature ~21°C, light cycle 7 a.m.–7 p.m.) and had access to acidified tap water and diet ad libitum.

A blood sample for baseline measurements was taken by tail incision after 4 h of fasting and weaning was commenced. Mice were divided into three groups matched for body weight (Fig. 1). A first group ($n = 30$; control group; "CON" group) received chow diet (Sniff R/M diet V1530, Uden, The Netherlands; Supporting Information Table S1). A second group ($n = 30$) was fed chow diet supplemented with 0.129% (w/w) ARA (Martek, Columbia, MD) and 0.088% (w/w) DHA (Martek; "ARA/DHA" group). A third group ($n = 15$) also received chow diet but supplemented with OMACOR® (Pronova, Biocare, Lysaker, Norway; 0.66% w/w), which equals 0.30% (w/w) EPA and 0.25% (w/w) DHA ("OMACOR" group). This period of week 4 to week 12 was defined as "early life".

At age 12 weeks, 8 weeks after starting the experimental diets, the CON group was split into two: one group ($n = 15$) continued on chow diet ("CON→CON"), the other group ($n = 15$) was switched to a high-fat high-carbohydrate diet (HFHC; Arie Blok Diets, Woerden, the Netherlands) containing 23.6% (w/w) beef tallow, 39.7% (w/w) dextrose, 18.9% (w/w) casein, 4.4% (w/w) linoleic acid, and 0.44% (w/w) alpha-linolenic acid, based on the diets used by Madsen et al. [2] (Supporting Information Table S1). This group is designated as "CON→HFHC". The ARA/DHA group was split into two groups as well: one group ($n = 15$) was switched to HFHC ("ARA/DHA→HFHC") and one group ($n = 15$) was switched to a diet containing AHA/DHA. Because no additional effects of continuation of ARA/DHA on body weight were observed, this group was not further addressed. The OMACOR group was switched to HFHC diet ("OMACOR→HFHC"). The period from week 12 to week 20 was defined as "later life".

Body weight (individually) and food intake (at cage level) were monitored over time and blood samples were taken by tail incision after 4 h of fasting (9 a.m.–1 p.m.) at age 4, 8, 12, 16, and 20 weeks. At age 20 weeks, the mice were sacrificed by CO_2 and organs were collected and weighed. Heart, liver, and adipose tissues (epididymal, omental, and inguinal fat) were snap frozen in liquid N_2 and stored at −80°C until use.

2.3 Plasma analyses

Total plasma cholesterol and triglyceride levels were measured enzymatically using kits number 11489437 and 11488872 (Roche Diagnostics, Almere, The Netherlands), respectively [17]. For size fractionation of lipoproteins, 50 μL of pooled plasma per group was applied to a 25-mL Superose 6B column (Pharmacia AB, Uppsala, Sweden) connected to an ÄKTA-fast protein liquid chromatography (FPLC) system (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and eluted at a constant rate of 50 μL/min with PBS (pH 7.4). The effluent was collected in 50 μL fractions (24 fractions in total). Cholesterol concentrations in the fractions were measured enzymatically as described above [18]. Plasma glucose was measured using the glucose hexokinase method (Instruchemie, Delfzijl, The Netherlands). Plasma levels of leptin, E-selectin, total insulin (all R&D Systems), and serum amyloid A (SAA; Biosource) were determined by ELISA as reported [16].

2.4 Adipose tissue analysis

Adipose tissues were fixed in formalin, embedded in paraffin, and sliced (5 μm sections). The slices were stained with hematoxylin, and adipocyte sizes were analyzed as described previously [19]. Briefly, computer-assisted morphometric analysis was carried out using an Olympus BX51 microscope, applying CELL^D software (Olympus, Zoeterwoude, The Netherlands). A custom-made module within the CELL^D software was developed for the analysis of adipocyte size; only adipocytes fully within the photographed frame were taken into account (Supporting Information Fig. S1). A total area of approximately 1 mm^2 adipose tissue was analyzed for each type of adipose tissue of each mouse and the total area covered was normalized to 1 mm^2 to allow comparison between groups.

In addition, the slides were stained immunohistochemically for the presence of macrophages and crown-like structures [20] with MAC-3 antibody (BD-Bioscience Pharmingen). MAC-3 positive cells were counted per 1 mm^2.

2.5 Statistical analysis

Changes over time were evaluated with repeated measures ANOVA with factors treatment and time followed by least significant difference (LSD) post-hoc analysis. Differences between groups at a specific time point were analyzed with one-way ANOVA followed by Dunnett's multiple comparison post-hoc analysis relative to CON at week 12 of age and CON→HFHC at week 20 of age. In some cases, where indicated, Student's *t*-test was applied. $p < 0.05$ was considered significant. Results are shown as mean ± SEM.

3 Results

3.1 Effects of ARA/DHA and OMACOR on body weight

Seventy-five male ApoE*3Leiden mice, four weeks of age, were divided into three groups matched for body weight, and fed a chow diet (CON group, $n = 30$) or chow diet supplemented with ARA/DHA ($n = 30$) or OMACOR ($n = 15$) for 8 weeks. During early life feeding from 4 weeks of age to 12 weeks of age, average body weight of CON group increased from 12.9 ± 0.2 g at the start of the experimental period to 26.0 ± 0.3 g (Fig. 2A). Compared to CON group at 12 weeks of age, average body weight of the ARA/DHA group was significantly lower (24.7 ± 0.3 g), while that of the OMACOR group (25.3 ± 0.3 g) was not significantly different from CON. No differences in food intake between groups was observed (Table 1).

Subsequently, at 12 weeks of age, all groups (except a chow control, CON→CON) were switched to HFHC diet for 8 weeks. The average weight gain in the CON→HFHC group was 2.5 ± 0.3 g and significantly greater compared to CON→CON (1.7 ± 0.3 g; $p < 0.05$). The final body weight was 28.4 ± 0.6 g in the CON→HFHC group and 27.8 ± 0.6 g in the CON→CON groups, respectively (Fig. 2B). Notably, the average body weight of the ARA/DHA→HFHC mice remained significantly lower (26.4 ± 0.5 g, $p < 0.05$) than that of the CON→HFHC group, demonstrating that the weight gain tended to be less (1.6 ± 0.3 g). By contrast, OMACOR→HFHC mice gained more weight during HFHC feeding in later life (2.6 ± 0.3 g) and their body weight at age 20 weeks was comparable to CON→HFHC mice (27.5 ± 0.5 g). No differences in food intake between HFHC fed groups were observed (Table 2).

3.2 Effect of ARA/DHA on cardiometabolic risk factors

Total plasma cholesterol at early life, in 12-week old mice, was significantly lower in the ARA/DHA group (2.0 ± 0.3 mM, $p < 0.001$) and in the OMACOR group (1.5 ± 0.1 mM, $p < 0.001$) as compared to the CON group (2.8 ± 0.1 mM) (Fig. 3A). Lipoprotein profile distribution analysis (Fig. 3E), shows that ARA/DHA mainly lowered cholesterol in the (V)LDL-sized particles, whereas OMACOR mainly lowered cholesterol in LDL and HDL-sized particles.

At later life, 8 weeks of HFHC feeding increased plasma cholesterol to 5.3 ± 0.2 mM in the CON→HFHC group. In the ARA/DHA→HFHC group, plasma cholesterol tended to be lower at the end of the 8 weeks HFHC feeding period (4.8 ± 0.2 mM, $p = 0.06$) (Fig. 3B). This was reflected in cholesterol reduction in LDL-sized particles (Fig. 3F). OMACOR →HFHC had no sustaining effect on plasma

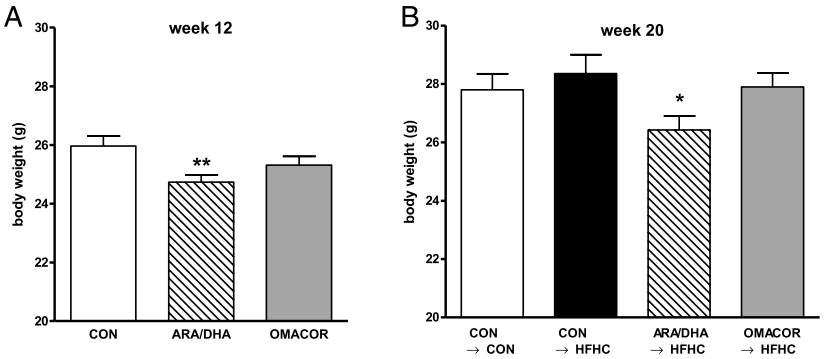

Figure 2. Body weight at week 12 of age (A) and at week 20 of age (B). Data are shown as means ± SEM. $^{**}p < 0.01$ compared to chow, $^{*}p < 0.05$ compared to CON→HFHC.

Table 1. Average food intake in early life, plasma glucose, insulin, E-selectin and Serum Amyloid A at week 12 (end of early life period)

	CON	ARA/DHA	OMACOR
Food intake (g/day)	5.3 ± 0.2	5.3 ± 0.2	4.9 ± 0.2
Glucose (mM)	10.5 ± 0.2	10.0 ± 0.2	10.4 ± 0.3
Insulin (ng/mL)	0.48 ± 0.06	0.24 ± 0.05**	0.28 ± 0.08
E-selectin (ng/mL)	83.5 ± 2.6	87.6 ± 5.2	77.9 ± 3.4
Serum amyloid A	6.6 ± 1.3	6.2 ± 1.6	4.9 ± 0.7

$^{**}p < 0.01$ compared to CON.

cholesterol, and levels (5.1 ± 0.1 mM) were comparable to CON→HFHC.

Similar to plasma cholesterol, plasma triglycerides were markedly reduced by ARA/DHA (1.43 ± 0.05 mM, $p < 0.001$) and OMACOR (1.38 ± 0.06 mM, $p < 0.001$) in early life relative to CON (1.98 ± 0.11 mM). Eight weeks of subsequent HFHC feeding decreased plasma triglycerides in the CON-HFHC group (0.71 ± 0.04 mM), similarly as observed previously with high-fat diets [16]. Comparably, lower triglyceride levels were found in the ARA/DHA→HFHC group (0.65 ± 0.04 mM), while OMACOR→HFHC mice displayed even slightly lower plasma triglycerides levels (0.60 ± 0.03 mM; $p < 0.05$ with Student's *t*-test).

Plasma glucose levels were comparable between groups at all time points (Tables 1 and 2), although plasma glucose tended to be lower in the ARA/DHA→HFHC group (8.7 ± 0.3 mM) compared to CON→HFHC (9.5 ± 0.3 mM; $p = 0.06$). Fasting insulin levels were normal and low in CON group (0.48 ± 0.06 ng/mL), and were significantly reduced with ARA/DHA supplementation (0.24 ± 0.05 ng/mL; $p < 0.01$) and tended to be reduced with OMACOR supplementation (0.28 ± 0.08 ng/mL) (Table 1) in early life. Fasting insulin levels in later life were comparable between groups (Table 2). Vascular inflammation was assessed by analyzing circulating E-selectin levels. No significant differences were observed after treatment with ARA/DHA or OMACOR in early life (Table 1). Subsequent HFHC feeding resulted in a significant increase in E-selectin in the CON→HFHC group (100.0 ± 3.8 ng/mL) compared to CON→CON (79.9 ± 2.7; $p < 0.01$). E-selectin in the ARA/DHA→HFHC group (106.7 ± 6.6 ng/mL) was comparable to CON→HFHC, while E-selectin in the OMACOR→HFHC group was significantly lower (88.7 ± 2.1 ng/mL; $p < 0.05$ with Student's *t*-test) (Table 2). Plasma Serum Amyloid A (SAA) concentrations, a marker for systemic inflammation, were comparable between groups at all time points (Tables 1 and 2).

In early life, plasma leptin levels were very low or below the detection limit in most mice (Fig. 4A). No effects were observed with either ARA/DHA or OMACOR treatment. When mice were switched to HFHC, leptin levels markedly increased to 3.0 ± 0.6 ng/mL in the CON→HFHC group, while leptin levels of CON→CON mice remained low (0.8 ± 0.3 ng/mL). Leptin levels of ARA/DHA→HFHC mice (0.8 ± 0.3 ng/mL), remained comparably low as chow reference

Table 2. Average food intake in later life, plasma glucose, insulin, E-selectin and Serum Amyloid A at week 20 (end of later life period)

	CON→CON	CON→HFHC	ARA/DHA→HFHC	OMACOR→HFHC
Food intake (g/day)	4.2 ± 0.1	2.5 ± 0.1	2.4 ± 0.1	2.6 ± 0.1
Glucose (mM)	10.8 ± 0.3*	9.5 ± 0.3	8.7 ± 0.3	9.5 ± 0.3
Insulin (ng/mL)	0.68 ± 0.11	0.63 ± 0.16	0.67 ± 0.18	0.85 ± 0.13
E-selectin (ng/mL)	79.9 ± 2.7**	100.0 ± 3.8	106.7 ± 6.6	88.6 ± 2.1
Serum amyloid A	7.2 ± 1.8	6.1 ± 0.7	5.5 ± 1.2	8.1 ± 1.0

$^{**}p < 0.01$ compared to CON→HFHC; $^{*}p < 0.05$ compared to CON→HFHC.

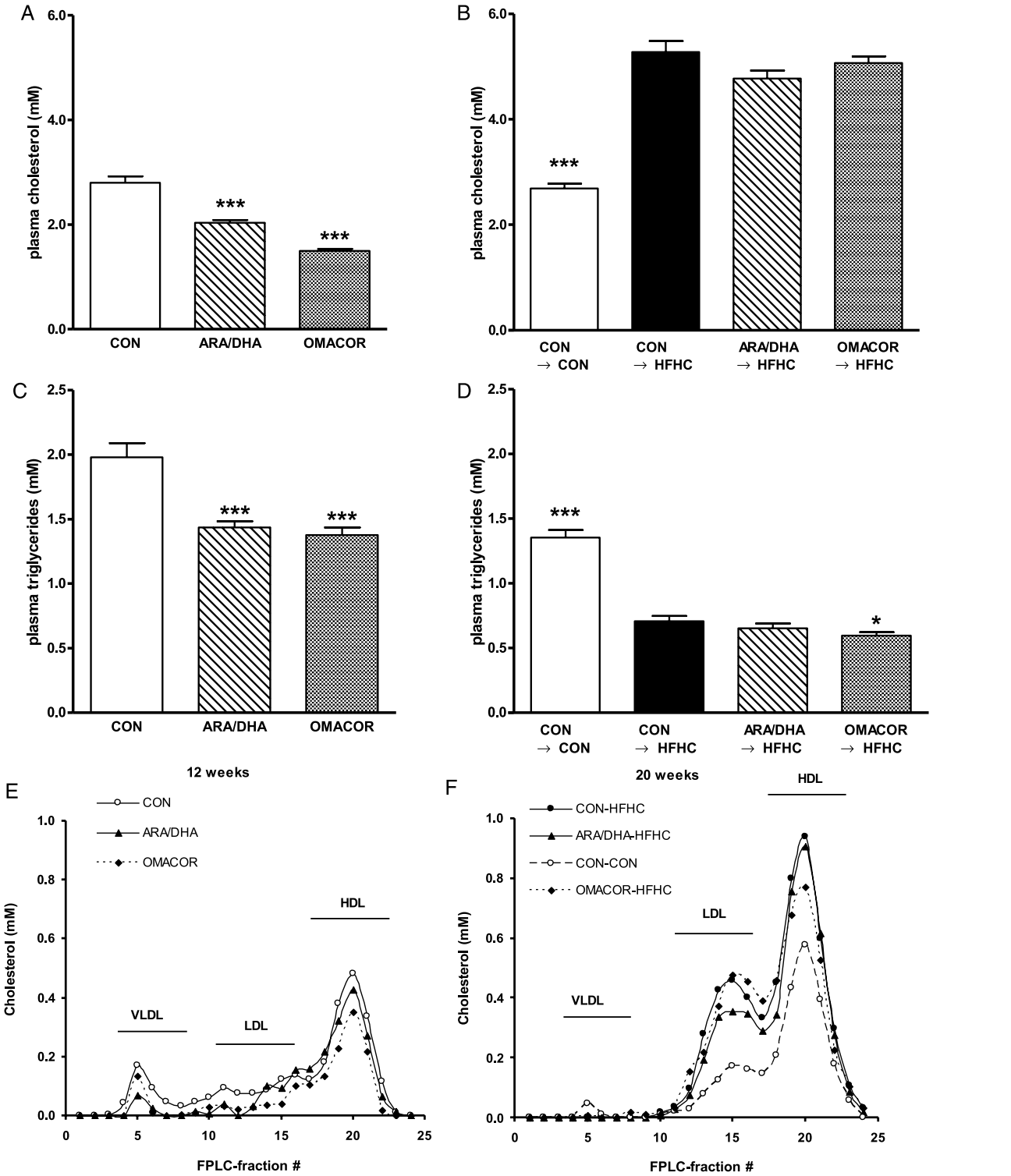

Figure 3. Plasma cholesterol at the end of early life at week 12 (A) and in later life at week 20 (B). Plasma triglyceride levels at the end of early life at week 12 (C) and in later life at week 20 (D). ÅKTA-profile in pooled plasma for cholesterol at week 12 (E) and week 20 (F). Data are shown as means ± SEM. ***$p < 0.001$ compared to CON, *$p < 0.05$ compared to CON→HFHC.

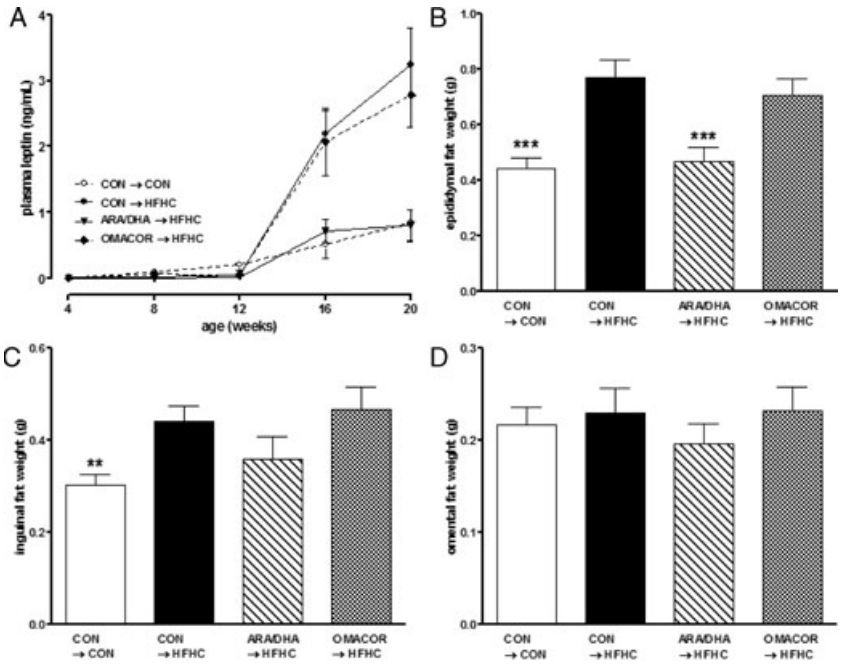

Figure 4. Plasma leptin over time (A) and weights of adipose tissue pad; epididymal adipose tissue (B), inguinal adipose tissue (C), and omental adipose tissue (D). ***$p < 0.001$, **$p < 0.01$ compared to CON→HFHC.

mice whereas OMACOR→HFHC mice had elevated leptin levels (2.8 ± 0.5 ng/mL) similar to CON→HFHC controls.

3.3 Effect of ARA/DHA and OMACOR on adipose tissue cell size and macrophage content

At age 20 weeks, mice were sacrificed and adipose tissue was collected from various depots. Epididymal fat mass was significantly increased in the CON→HFHC group (722 ± 63 mg) compared to CON→CON (445 ± 39 mg). Remarkably, epididymal fat mass of ARA/DHA→HFHC group (471 ± 49 mg) was comparable to CON→CON group, while OMACOR→HFHC (707 ± 61 mg) did not reduce the increase in fat mass induced by HFHC feeding (Fig. 4B). Inguinal fat mass was also significantly increased in the CON→HFHC group (410 ± 34 mg) compared to CON→CON group (302 ± 24 mg). Inguinal fat of ARA/DHA→HFHC (360 ± 48 mg) and OMACOR→HFHC (465 ± 51 mg) was not significantly different from CON→HFHC (Fig. 4C). Omental fat mass did not significantly differ among the groups (Fig. 4D).

We subsequently analyzed adipocyte size of each fat depot (Fig. 5). Adipocytes of all three adipose depots were larger in the CON→HFHC group compared to CON→CON. ARA/DHA→HFHC resulted in significantly smaller adipocytes than in CON→HFHC, specifically for the inguinal depot (Fig. 5B). Similarly, OMACOR →HFHC mice had also smaller inguinal adipocytes, without significant effects on epididymal and omental adipocyte size.

Immunohistochemical analysis of macrophages in adipose tissue showed that ARA/DHA→HFHC significantly reduced the content of resident MAC-3-positive cells in the epididymal adipose depot relative to the CON→HFHC group (9.3 ± 0.7 cells versus 15.9 ± 2.1 cells, $p < 0.05$) (Fig. 5D). OMACOR→HFHC mice also had less epididymal macrophages (10.9 ± 0.7), although this was not significant. There were no significant differences in macrophage content for the other depots. Notably, no crown-like structures were observed in any depot or group.

4 Discussion

Nutritional programming in early postnatal life may influence the development of obesity and metabolic diseases in later life. Recent scientific data have generated considerable interest in the specific impact of early nutritional factors [21], such as LC-PUFAs. This study addressed whether early ARA/DHA (a mixture of *n*-6 and *n*-3 LC-PUFAs) supplementation has beneficial effects on body weight development and lipid metabolism in later life. This was tested in ApoE*3Leiden-transgenic mice, a humanized animal model for hyperlipidemia with mild obesity. The results show that early ARA/DHA dietary supplementation reduces plasma levels of metabolic parameters such as cholesterol and later in life diminishes obesity and improves adipose tissue quantity and quality. EPA/DHA (*n*-3 LC-PUFAs only) had clear effects on lipid lowering in early life, while no effects on body weight and adipose tissue mass were observed in later life. Together, this suggests that a qualitative difference in LC-PUFA composition may impact outcomes.

In line with our finding that early ARA/DHA supplementation reduced body weight gain, other studies also reported on reduced body weight gain by *n*-3 LC-PUFAs only. Notably, these studies started treatment either during pregnancy [22]

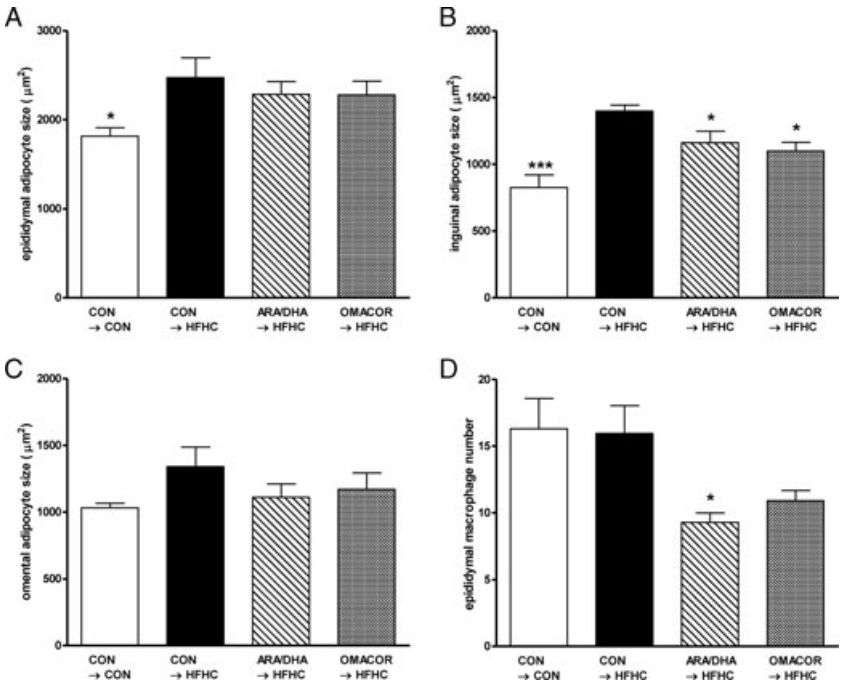

Figure 5. Adipocyte size and macrophage infiltration measurements. Mean adipocyte size is shown for epididymal (A), inguinal (B), and omental (C) adipose tissue ($n = 5$ per group). Macrophage infiltration in epididymal adipose tissue is illustrated in D. ***$p < 0.001$, *$p < 0.05$ compared to CON→HFHC.

or later in life [6, 23, 24]. We did not find a significant effect of postnatal nutrition with EPA/DHA on body weight in later life when feeding a Western style diet. Oosting et al. also did not find a significant effect of postnatal nutrition with *n*-3 LC-PUFAs on body weight at adulthood when feeding mice a Western style diet [25]. It should be noted that Oosting used C57BL/6 mice while we used ApoE*3Leiden mice, a model with more human-like plasma cholesterol levels and lipid metabolism and a mild increase in body weight.

The effects of ARA/DHA supplementation in early life on body weight gain were accompanied by reduced adiposity, more specifically reduced epididymal adipose tissue weight and smaller inguinal adipocyte cell size. Parallel to the effects on adipose tissue, ARA/DHA supplementation substantially reduced plasma leptin to healthy control levels in comparison to mice on unsupplemented diet. EPA/DHA in early life had no significant effect on body weight and adipose tissue weight, while inguinal adipocyte cells were smaller, similar as ARA/DHA supplementation. Despite the lack of effect on body weight by postnatal *n*-3 LC-PUFA, Oosting et al. also reported reduced body adiposity, smaller epididymal adipocytes, and reduced leptin levels at adulthood after Western style diet [25]. In two other studies, *n*-3 LC-PUFA (EPA/DHA) in adult life prevented fat accumulation with preferential reduction in abdominal fat depots in mice [6, 26]. Interestingly, in human subjects with diabetes, *n*-3 LC-PUFAs decreased the size of fat cells [27]. The reduced adipocyte size by ARA/DHA and EPA/DHA may be the result of inhibition of adipocyte differentiation, as *n*-3 PUFAs have been reported to inhibit adipocyte differentiation in vitro [28].

Not only adipose quantity, but also quality was affected by early life ARA/DHA supplementation. More specifically, ARA/DHA improved adipose tissue quality by reduced numbers of macrophages in epididymal adipose tissue. A similar trend was observed with EPA/DHA. The level of adipose tissue inflammation was still rather low, and no crown-like structures were observed. It has been suggested that *n*-3 LC-PUFAs have anti-inflammatory effects in adipose tissue [29]. Indeed, high-fat diet-induced adipose inflammation in *db/db* mice is prevented by *n*-3 LC-PUFA [30]. The beneficial effect of DHA on adipose quality was further substantiated by recent finding by Titos et al. These authors showed a switch to a less pro-inflammatory macrophage phenotype (M1 to M2) by DHA [31].

Plasma cholesterol levels, risk factor for cardiovascular disease [32], were lowered by both ARA/DHA and EPA/DHA supplementation in early life and the effects were sustained in later life during HFHC feeding. The cardioprotective effects of *n*-3 LC-PUFAs are well recognized [33]. A possibly protective effect of ARA/DHA or EPA/DHA in early life on cardiovascular disease should be further explored.

This study evaluated the effects of ARA/DHA, an *n*-6/*n*-3 PUFAs mixture, and the effects of EPA/DHA, a mixture of two *n*-3 PUFAs on body weight and lipid metabolism. The effects on plasma lipids of the two supplementations in early life were comparable. In later life, the most remarkable difference was on body weight and adipose tissue mass. Mice supplemented with ARA/DHA in early life showed lower adipose tissue mass while EPA/DHA supplementation in early life had no effect on adipose tissue mass in later life. Qualitative and quantitative differences in LC-PUFA composition between the two groups may explain the observed differences.

The specific mechanisms underlying the observed effects remain to be determined. It has been reported that

intake of LC-PUFAs during pregnancy and early in life have beneficial effects on body weight, adipose tissue, and plasma levels of the adipokines, adiponectin, and leptin [34, 35]. Lefils et al. showed that dietary DHA decreased leptin and increased adiponectin and that these effects were maintained even when the animals had been switched to DHA-free diet [36]. We also observed that leptin levels of ARA/DHA→HFHC mice remained low after switching to ARA/DHA-free HFHC diet. Obesity is strongly linked to inflammation [37]. Metabolic products derived from *n*-3 LC-PUFAs, such as 17S-hydroxy-DHA, resolvins, and protectins, may play a role in the long-term resolution of inflammation [38]. Furthermore, the G protein–coupled receptor 120, an *n*-3 LC-PUFA receptor or sensor, has been shown to mediate repression of macrophage-induced tissue inflammation [39]. To what extent the findings of the current study are mediated through the effects on adipokines and chronic low-grade inflammation is the topic of further research.

In addition to the beneficial effects on adipokine expression and inflammation, ARA, EPA, and DHA have been reported to play important roles in brain development, neurogenesis, and the expression and function of various neurotransmitters and their receptors in the brain which could be relevant in decreasing obesity [40]. LC-PUFAs reportedly may act on the mesocorticolimbic and the endocannabinoid pathways and decrease the reward associated with food, thereby reducing appetite, food intake, and ultimately reducing overweight and obesity [41]. However, the latter neural effects of ARA/DHA may not play a role in our study since no effect on food intake was observed. Finally, *n*-3/*n*-6 LC-PUFAs have been found to exert beneficial effects on thermoregulation in the brain [42], which may stimulate energy expenditure and thereby reduce body weight. Future studies will help to clarify whether increased energy expenditure may contribute to the diminished body weight gains observed in the present study.

Taken together, these data show that ARA/DHA supplementation in early life may have sustained effects on lower body weight gain and adipose quantity and quality when exposed to a mild obesogenic in later life. The beneficial effects may depend on the specific combination of *n*-6 LC-PUFA ARA and *n*-3 LC-PUFA DHA.

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5 References

[1] Symonds, M. E., Sebert, S., Budge, H., The obesity epidemic: from the environment to epigenetics - not simply a response to dietary manipulation in a thermoneutral environment. *Front. Genet.* 2011, *2*, 24.

[2] Madsen, L., Pedersen, L. M., Liaset, B., Ma, T. et al., cAMP-dependent signaling regulates the adipogenic effect of n-6 polyunsaturated fatty acids. *J. Biol. Chem.* 2008, *283*, 7196–7205.

[3] Ailhaud, G., Guesnet, P., Cunnane, S. C., An emerging risk factor for obesity: does disequilibrium of polyunsaturated fatty acid metabolism contribute to excessive adipose tissue development?. *Brit. J. Nutr.* 2008, *100*, 461–470.

[4] Ailhaud, G., Massiera, F., Weill, P., Legrand, P. et al., Temporal changes in dietary fats: role of n-6 polyunsaturated fatty acids in excessive adipose tissue development and relationship to obesity. *Prog. Lipid Res.* 2006, *45*, 203–236.

[5] Massiera, F., Saint-Marc, P., Seydoux, J., Murata, T. et al., Arachidonic acid and prostacyclin signaling promote adipose tissue development: a human health concern?. *J. Lipid Res.* 2003, *44*, 271–279.

[6] Flachs, P., Rossmeisl, M., Bryhn, M., Kopecky, J. Cellular and molecular effects of n-3 polyunsaturated fatty acids on adipose tissue biology and metabolism. *Clin. Sci. (Lond.)* 2009, *116*, 1–16.

[7] Simoncikova, P., Wein, S., Gasperikova, D., Ukropec, J. et al., Comparison of the extrapancreatic action of gamma-linolenic acid and n-3 PUFAs in the high fat diet-induced insulin resistance. *Endocr. Regul.* 2002, *36*, 143–149.

[8] Storlien, L. H., Jenkins, A. B., Chisholm, D. J., Pascoe, W. S. et al., Influence of dietary fat composition on development of insulin resistance in rats. Relationship to muscle triglyceride and omega-3 fatty acids in muscle phospholipid. *Diabetes* 1991, *40*, 280–289.

[9] Halvorsen, B., Rustan, A. C., Madsen, L., Reseland, J. et al., Effects of long-chain monounsaturated and n-3 fatty acids on fatty acid oxidation and lipid composition in rats. *Ann. Nutr. Metab.* 2001, *45*, 30–37.

[10] Fernandez-Twinn, D. S., Ozanne, S. E., Early life nutrition and metabolic programming. *Ann. N. Y. Acad. Sci.* 2010, *1212*, 78–96.

[11] Symonds, M. E., Gardner, D. S. Experimental evidence for early nutritional programming of later health in animals. *Curr. Opin. Clin. Nutr. Metab. Care* 2006, *9*, 278–283.

[12] Weisinger, H. S., Armitage, J. A., Sinclair, A. J., Vingrys, A. J. et al., Perinatal omega-3 fatty acid deficiency affects blood pressure later in life. *Nat. Med.* 2001, *7*, 258–259.

[13] Helland, I. B., Smith, L., Saarem, K., Saugstad, O. D. et al., Maternal supplementation with very-long-chain n-3 fatty acids during pregnancy and lactation augments children's IQ at 4 years of age. *Pediatrics* 2003, *111*, e39-e44.

[14] Donahue, S. M., Rifas-Shiman, S. L., Gold, D. R., Jouni, Z. E. et al., Prenatal fatty acid status and child adiposity at age 3 y: results from a US pregnancy cohort. *Am. J. Clin. Nutr.* 2011, *93*, 780–788.

[15] Zadelaar, S., Kleemann, R., Verschuren, L., de Vries-Van der Weij, J. et al., Mouse models for atherosclerosis and pharmaceutical modifiers. *Arterioscler. Thromb. Vasc. Biol.* 2007, *27*, 1706–1721.

[16] Kleemann, R., van Erk, M., Verschuren, L., van den Hoek, A. M. et al., Time-resolved and tissue-specific systems analysis of the pathogenesis of insulin resistance. *PLoS One.* 2010, *5*, e8817.

[17] Wielinga, P. Y., Yakala, G. K., Heeringa, P., Kleemann, R. et al., Beneficial effects of alternate dietary regimen on liver inflammation, atherosclerosis and renal activation. *PLoS One.* 2011, *6*, e18432.

[18] Verschuren, L., Kleemann, R., Offerman, E. H., Szalai, A. J. et al., Effect of low dose atorvastatin versus diet-induced cholesterol lowering on atherosclerotic lesion progression and inflammation in apolipoprotein E*3-Leiden transgenic mice. *Arterioscler. Thromb. Vasc. Biol.* 2005, *25*, 161–167.

[19] Verschuren, L., Kooistra, T., Bernhagen, J., Voshol, P. J. et al., MIF deficiency reduces chronic inflammation in white adipose tissue and impairs the development of insulin resistance, glucose intolerance, and associated atherosclerotic disease. *Circ. Res.* 2009, *105*, 99–107.

[20] Cinti, S., Mitchell, G., Barbatelli, G., Murano, I. et al., Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *J. Lipid Res.* 2005, *46*, 2347–2355.

[21] Miralles, O., Sanchez, J., Palou, A., Pico, C., A physiological role of breast milk leptin in body weight control in developing infants. *Obesity. (Silver. Spring).* 2006, *14*, 1371–1377.

[22] Korotkova, M., Gabrielsson, B. G., Holmang, A., Larsson, B. M. et al., Gender-related long-term effects in adult rats by perinatal dietary ratio of n-6/n-3 fatty acids. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2005, *288*, R575–R579.

[23] Kasbi, C. F., Andre, A., Prieur, X., Loirand, G. et al., n-3 PUFA prevent metabolic disturbances associated with obesity and improve endothelial function in golden Syrian hamsters fed with a high-fat diet. *Brit. J. Nutr.* 2012, *107,* 1305–1315.

[24] Sato, A., Kawano, H., Notsu, T., Ohta, M. et al., Antiobesity effect of eicosapentaenoic acid in high-fat/high-sucrose diet-induced obesity: importance of hepatic lipogenesis. *Diabetes* 2010, *59*, 2495–2504.

[25] Oosting, A., Kegler, D., Boehm, G., Jansen, H. T. et al., N-3 long-chain polyunsaturated fatty acids prevent excessive fat deposition in adulthood in a mouse model of postnatal nutritional programming. *Pediatr. Res.* 2010, *68*, 494–499.

[26] Ruzickova, J., Rossmeisl, M., Prazak, T., Flachs, P. et al., Omega-3 PUFA of marine origin limit diet-induced obesity in mice by reducing cellularity of adipose tissue. *Lipids* 2004, *39*, 1177–1185.

[27] Skurnick-Minot, G., Laromiguiere, M., Oppert, J., Quignard-Boulange, A. M. et. al., Whole-body fat mass and insulin sensitivity in type 2 diabetes women: effect of n-3 polyunsaturated fatty acids. *Diabetes* 2004, *53*, A44.

[28] Kim, H. K., Ia-Fera, M., Lin, J., Baile, C. A., Docosahexaenoic acid inhibits adipocyte differentiation and induces apoptosis in 3T3-L1 preadipocytes. *J. Nutr.* 2006, *136*, 2965–2969.

[29] Oliver, E., McGillicuddy, F., Phillips, C., Toomey, S. et al., The role of inflammation and macrophage accumulation in the development of obesity-induced type 2 diabetes mellitus and the possible therapeutic effects of long-chain n-3 PUFA. *Proc. Nutr. Soc.* 2010, *69*, 232–243.

[30] Todoric, J., Loffler, M., Huber, J., Bilban, M. et al., Adipose tissue inflammation induced by high-fat diet in obese diabetic mice is prevented by n-3 polyunsaturated fatty acids. *Diabetologia.* 2006, *49*, 2109–2119.

[31] Titos, E., Rius, B., Gonzalez-Periz, A., Lopez-Vicario, C. et al., Resolvin D1 and its precursor docosahexaenoic acid promote resolution of adipose tissue inflammation by eliciting macrophage polarization toward an M2-like phenotype. *J. Immunol.* 2011, *187*, 5408–5418.

[32] Blum, C. B., Levy, R. I., Role of dietary intervention in the primary prevention of coronary heart disease. Individuals with high-normal or elevated serum cholesterol levels should be placed on cholesterol-lowering diets. *Cardiology* 1987, *74*, 2–21.

[33] Cottin, S. C., Sanders, T. A., Hall, W. L., The differential effects of EPA and DHA on cardiovascular risk factors. *Proc. Nutr. Soc.* 2011, *70*, 215–231.

[34] Korotkova, M., Gabrielsson, B. G., Holmang, A., Larsson, B. M. et al., Gender-related long-term effects in adult rats by perinatal dietary ratio of n-6/n-3 fatty acids. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2005, *288*, R575– R579.

[35] Korotkova, M., Gabrielsson, B., Lonn, M., Hanson, L. A. et al., Leptin levels in rat offspring are modified by the ratio of linoleic to alpha-linolenic acid in the maternal diet. *J. Lipid Res.* 2002, *43*, 1743–1749.

[36] Lefils, J., Geloen, A., Vidal, H., Lagarde, M. et al., Dietary DHA: time course of tissue uptake and effects on cytokine secretion in mice. *Brit. J. Nutr.* 2010, *104*, 1304–1312.

[37] Gregor, M. F., Hotamisligil, G. S., Inflammatory mechanisms in obesity. *Annu. Rev. Immunol.* 2011, *29*, 415–445.

[38] Gonzalez-Periz, A., Horrillo, R., Ferre, N., Gronert, K. et al., Obesity-induced insulin resistance and hepatic steatosis are alleviated by omega-3 fatty acids: a role for resolvins and protectins. *FASEB J.* 2009, *23*, 1946–1957.

[39] Oh, D. Y., Talukdar, S., Bae, E. J., Imamura, T. et al., GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. *Cell* 2010, *142*, 687–698.

[40] Das, U. N., Is obesity an inflammatory condition? *Nutrition* 2001, *17*, 953–966.

[41] Golub, N., Geba, D., Mousa, S. A., Williams, G. et al., Greasing the wheels of managing overweight and obesity with omega-3 fatty acids. *Med. Hypotheses* 2011, *77*, 1114–1120.

[42] Yehuda, S., Carasso, R. L., Modulation of learning, pain thresholds, and thermoregulation in the rat by preparations of free purified alpha-linolenic and linoleic acids: determination of the optimal omega 3-to-omega 6 ratio. *Proc. Natl. Acad. Sci. USA* 1993, *90*, 10345–10349.

