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ORIGINAL ARTICLE

Effect of DHA supplementation in a very low-calorie ketogenic diet in the treatment of obesity: a randomized clinical trial

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Abstract A VLCK diet supplemented with DHA, commercially available, was tested against an isocaloric VLCK diet without DHA. The main purpose of this study was to compare the effect of DHA supplementation in classic cardiovascular risk factors, adipokine levels, and inflammation-resolving eicosanoids. A total of obese patients were randomized into two groups: a group supplemented with DHA ($n = 14$) (PnK-DHA group) versus a group with an isocaloric diet free of supplementation ($n = 15$) (control group). The follow-up period was 6 months. The average weight loss after 6 months of treatment was 20.36 ± 5.02 kg in control group and 19.74 ± 5.10 kg in PnK-DHA group, without statistical differences between both groups. The VLCK diets induced a significant change in some of the biological parameters, such as insulin, HOMA-IR, triglycerides, LDL cholesterol, C-reactive protein, resistin, TNF alpha, and leptin. Following DHA supplementation, the DHA-derived oxylipins were significantly increased in the intervention group. The ratio of proresolution/proinflammatory lipid markers was increased in plasma of the intervention group over the entire study. Similarly, the mean ratios of AA/EPA and AA/DHA in erythrocyte membranes were dramatically reduced in the PnK-DHA group and the anti-inflammatory fatty acid index (AIFAI) was consistently increased after the DHA treatment ($p < 0.05$). The present study demonstrated that a very low-calorie ketogenic diet supplemented with DHA was significantly superior in the anti-inflammatory effect, without statistical differences in weight loss and metabolic improvement.

Keywords DHA · Ketosis · Protein diet · Weight loss · Pronokal method · PnK method

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Introduction

The prevalence of overweight is increasing globally and has become a serious public health problem [1]. Overweight increases the risk of chronic metabolic diseases such as type 2 diabetes and cardiovascular disease [2], and a low-grade inflammatory status in obese persons has been proposed as one of the mediating processes in metabolic disease development, such as cardiovascular diseases and diabetes [3], too. Thus, prevention and treatment of obesity is important to reduce the burden of the disease.

The promotion of lifestyle changes has a solid rationale, but this policy is doomed to failure frequently [4] and bariatric surgery has been shown to be unequivocally effective in the long-term perspective and able to reduce cardiovascular morbidity and mortality [5], but such techniques are unsuitable as population-wide treatments [6]. Hence, effective medical treatments are needed. However,

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most drugs that have been used to treat obesity have been withdrawn from the market due to incorrect use or side effects [7–9].

The leading non-pharmacological approach is the use of diets, particularly low-calorie and very low-calorie diets. In the last few years, the low-carbohydrate diet has gained progressive recognition over other dietary treatments [10], and the low-carbohydrate ketogenic diet in particular has been closely examined [11]. In particular, Pronokal® Method, a very low-calorie ketogenic diet (VLCK diet) was significantly more effective than a standard low-calorie diet [12].

On the other hand, dietary factors substantially modulate obesity-related inflammation [13]. An increased ratio of dietary n6:n3 PUFAs has been linked to the risk of chronic inflammatory diseases [14]. Recent studies have identified docosahexaenoic acid (DHA) as a precursor of a new type of potent anti-inflammatory and inflammation-resolving eicosanoids, known as resolvins and protectins, which actively help terminate an inflammatory response [15].

In the present work, a very low-calorie ketogenic diet supplemented with DHA, commercially available, was tested against the isocaloric ketogenic diet without DHA. The main purpose of this study was to compare the effect of DHA supplementation in usual cardiovascular risk factors, serum adipokine levels, and inflammation-resolving eicosanoids.

Materials and methods

This study was a nutritional intervention, simple-blinded, randomized, controlled, prospective clinical trial during 6 months, and performed in a single center https://clinicaltrials.gov/show/NCT01865448. The patients attending the Endocrinology and Nutrition Department at the Hospital Clinico Universitario of Valladolid to receive treatment for obesity were consecutively enrolled in this study. Apart from obesity and prediabetes, participants were generally healthy individuals. The inclusion criteria were age 18–65 years, body mass index (BMI) between 30 and 35, stable body weight in the previous 3 months, and desire to lose weight. None of the participants had serious medical condition.

The exclusion criteria were as follows: type 1 diabetes mellitus and type 2 diabetes mellitus, obesity induced by other endocrine disorders or by drugs, use of any weight loss diet or pills in the previous 6 months, metabolic disorders, taking nonsteroidal anti-inflammatory drugs, glitazones, pregnancy, history of cancer, immunological pathologies, potentially modifiable markers of inflammation, abuse of narcotics or alcohol, severe hepatic insufficiency, any type of renal insufficiency or gout episodes, kidney litiasis, uncontrolled hypertension, and hydroelectrolytic alterations. Females of child-bearing potential, pregnant, breast-feeding, intending to become pregnant, or not using adequate contraceptive methods were excluded. All participants provided written informed consent and the Institutional Review Board (Comité Ético de Investigación Clínica, Hospital Clínico Universitario, Valladolid) approved the study (C.I: 40/13, PNK-DHA2013-01). Participants received no monetary incentive. A total of 34 patients were enrolled (Fig. 1) and 29 subjects finished the study (Table 1) (clinicaltrial.gov: NCT01856686).

Interventions

Using a controlled, open design clinical trial, the patients were randomized and allocated to receive either a very low-calorie ketogenic diet (VLCK) (table of random numbers)—one with DHA (PnK-DHA group) or an isocaloric VLCK diet without DHA (control group). This diet in each group was followed by a no-ketogenic low-calorie diet with reintroduction of natural foods, with the same calorie intake for both. The intervention included an evaluation by the specialist physician conducting the study, an assessment by an expert dietician, group meetings, and exercise recommendations for both groups. The

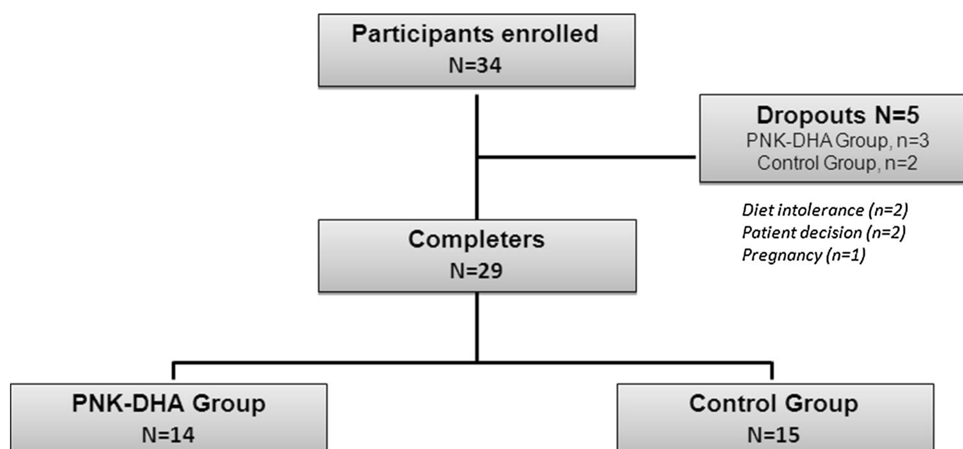

Fig. 1 Consort flow diagram

Table 1 Characteristics of the sample at baseline

	Control group ($n = 15$)	PnK-DHA group ($n = 14$)	*p*
Age (years),	44.3 ± 11.7	47.4 ± 9.1	n.s.
Sex *n*(%)			
Male	7 (46.7 %)	5 (35.7 %)	
Female	8 (53.3 %)	9 (64.3 %)	
Weight (kg)	92.2 ± 13.1	92.05 ± 8.7	n.s.
BMI (kg/m^2)	32.95 ± 1.9	33.4 ± 1.4	n.s.
Waist circumference (cm)	109.4 ± 7.8	109.1 ± 7.95	n.s.

Results are expressed as mean ± SD, except sex. n.s. means not significant (independent samples *t* test)

group meetings and evaluations took place in a hospital setting at 0, 30, 60, and 180 days from the start of the study. In these meetings, the patients received diet instructions, individual supportive counsel, and encouragement to exercise on a regular basis using a formal exercise program. In addition, a program of phone call reinforcement was available, and a phone number to address any doubts was provided to all participants.

PnK-DHA group

This group followed a ketogenic diet according to a commercial weight loss program (PnK® Method) [16].

The intervention stage consists of a ketogenic diet, very low in calories (600–800 kcal/day), low in carbohydrates (<50 g daily from vegetables), and low in lipids (only 10 g of olive oil per day). The amount of high-biological-value proteins ranged between 0.8 and 1.2 g per each kg of ideal body weight, to ensure meeting the minimal body requirements and to prevent the loss of lean mass. This method produces three ketogenic steps based on a high-biological-value protein preparation diet and natural foods. Each protein preparation contained 15 g protein, 4 g carbohydrates, and 3 g fat, and provided 90–100 kcal. In step 1, the patients eat high-biological-value protein preparations five times a day, and vegetables with low glycemic index. In step 2, one of the protein servings is substituted by a natural protein (e.g., meat and fish) either at lunch or at dinner. In step 3, a second serving of the natural protein substituted the second serving of biological protein preparation. Throughout these ketogenic steps, supplements of vitamins and minerals, such as K, Na, Mg, Ca, and omega-3 and omega-6 fatty acids, were provided in accordance with the international recommendations [17]. In this method, the intervention stage is maintained until the patient loses most of weight loss target (ideally 80 %) [16], although in this study the ketogenic step was maintained for 2 months and then the "key to success" stage started with a no-ketogenic low-calorie diet. At this point, the patients underwent a progressive incorporation of different food groups to guarantee the long-term maintenance of the weight loss. This period continued until completing 6 months of the study.

In this previous scheme, the patients of intervention group received DHA (Polaris®, Pleuven, France) oral supplementation of DHA 500 mg/day during the first 60 days and 250 mg/day till 180 days and the patients of control group received a placebo pill with the same scheme.

Primary outcome measures

Proinflammatory and pro-resolving PUFA metabolite mediators (lipid mediator profile)

The main outcome was modified inflammation-resolving eicosanoid levels. Lipidomic analysis of PUFA metabolite mediators (AA-derived oxylipins: 15-HETE, 12 -HETE, 8-HETE, 5-HETE, TXB2, PGE2, LTB4; and DHA-derived oxylipins: 17-HDOHE, 14-HDOHE, 7-HDOHE, 4-HDOHE, RVD2, PD1, 7SMAR1, 7RMAR1) were measured on plasma samples. The extraction protocol and LC/MS/MS analysis were performed by AMBIOTIS SAS (France) as described in Le Faouder et al. [18] adapted from AMBIOTIS Standard Operating Procedure.

To investigate changes in the lipid mediator (LM) profile in human peripheral blood, we carried out targeted LM lipidomics using an LC–MS/MS system. Using this approach, we profiled human plasma. In these samples, we identified LM from each of the DHA and AA bioactive metabolomes. We measured DHA-derived lipoxygenase (LOX) products, 17-HDOHE, and 14-HDOHE, the monohydroxy markers for RvD2, PD1, and MaR1 biosynthesis pathways, respectively, as well as 7-HDOHE and 4-HDOHE. In addition to special pro-resolving mediators (SPMs), we also identified significant levels of proinflammatory lipid mediators of cyclooxygenase (COX) (PGE2 and TXB2) and LOX (LTB4, 5-HETE, 8-HETE, 12-HETE, and 15-HETE) products biosynthesized from endogenous sources of AA. Considerable acute inflammatory alterations occur in obese patients and since the induction of SPM biosynthesis is critical for subsequent resolution, we examined if altered lipid mediator levels could be found after a control diet for 2 and 6 months.

Secondary outcome measures

Anthropometric parameters

The body weight, waist circumference (WC), and body mass index (BMI) were the secondary outcome measures. At each visit, patients were weighted on the same calibrated scale (Seca 200 scale, Medical Resources, EPI Inc OH, USA). BMI was calculated as body weight in Kg, divided by the square of the body height in meters. WC was measured with a standard flexible non-elastic metric tape over the midpoint between the last rib and the iliac crest, with the patient standing and exhaling.

Biochemical parameters

Plasma glucose levels were determined using an automated glucose oxidase method (Glucose Analyzer 2, Beckman Instruments, Fullerton, California). Insulin was measured by RIA (RIA Diagnostic Corporation, Los Angeles, CA) with a sensitivity of 0.5mUI/L (normal range 0.5–30 mUI/L) [19] and the homeostasis model assessment for insulin resistance (HOMA-IR) was performed using these values [20]. C-reactive protein (CRP) was measured by immunoturbidimetry (Roche Diagnostics GmbH, Mannheim, Germany), with a normal range of (0–7 mg/dl) and an analytical sensitivity of 0.5 mg/dl. Serum total cholesterol and triglyceride concentrations were determined by enzymatic colorimetric assay (Technicon Instruments, Ltd., New York, N.Y., USA), while HDL cholesterol was determined enzymatically in the supernatant after precipitation of other lipoproteins with dextran sulfate-magnesium. LDL cholesterol was calculated using Friedewald formula.

Resistin was measured by ELISA (Biovendor Laboratory, Inc., Brno, Czech Republic) with a sensitivity of 0.2 ng/ml with a normal range of 4–12 ng/ml [21]. Leptin was measured by ELISA (Diagnostic Systems Laboratories, Inc., Texas, USA) with a sensitivity of 0.05 ng/ml and a normal range of 10–100 ng/ml [22]. Adiponectin was measured by ELISA (R&D systems, Inc., Mineapolis, USA) with a sensitivity of 0.246 ng/ml and a normal range of 8.65–21.43 ng/ml [23]. Interleukin 6 and TNF alpha were measured by ELISA (R&D systems, Inc., Mineapolis, USA) with a sensitivity of 0.7 and 0.5 pg/ml, respectively. Normal values of IL6 and TNF alpha were 1.12–12.5 pg/ml and 0.5–15.6 pg/ml, respectively.

Red blood cell (RBC) membrane fatty acid analysis

The composition of fatty acids was determined using the method by Lepage and Roy [24]. Venous blood samples were taken in K2-EDTA-containing tubes and the erythrocytes were separated from the plasma by centrifugation (3000 rpm, $1500\times g$, for 10 min) and washed with an equal volume of saline. These erythrocytes resuspended with saline were stored in fresh 0.01 % BHT-treated Eppendorf vials at −80 °C. Afterwards, aliquots of 200 µL of erythrocyte suspensions were converted to fatty acid methyl esters by reaction with acetyl chloride for 60 min at 100 °C. Gas chromatography analysis was performed on a Shimadzu GCMS-QP2010 Plus gas chromatograph/mass spectrometer (Shimadzu, Kyoto, Japan). Fatty acid methyl ester peaks were identified through mass spectrometry and by comparing the elution pattern and relative retention times of FAME with reference FAME mixture (GLC-744 Nu-Chek Prep. Inc., Elysian MN, USA). The results were expressed in relative amounts (molar percentage of total fatty acids). The omega-3 index [25] was calculated as erythrocyte (EPA + DHA)/(total fatty acids) × 100 %. The anti-inflammatory fatty acid index (AIFAI) [26] was calculated as the ratio of the total mol % of 20:5n-3, 22:6n-3, and 20:3n-6 divided by mol % of 20:4n-6. The anti-inflammatory AA/EPA and AA/DHA indexes were calculated as the ratio (mol %) of 20:5n-3 and 22:6n-3 to 20:4n-6, respectively.

Adverse effects, dropouts, and satisfaction with the treatments

During all visits, the patients completed a questionnaire of side effects and dropouts, and the reasons provided were recorded.

Statistical analysis

This study was a nutritional intervention, open, randomized, controlled, prospective clinical trial for 1 year, and performed in a single center. A total of 29 obese subjects were studied, 15 patients were in the PnK-DHA group and 14 patients were in the control group, because the groups should have at least 13 patients on each arm to detect differences in the inflammation-resolving eicosanoids over 10 %, with a potency of 90 %. The significance level was established at $p < 0.05$.

The data are presented as mean ± SD. The differences between the two groups were determined comparing the data in each visit using Fisher's exact test for categorical variables and Student's *t* test. Additionally, the values between visits are compared to analyze the evolution for each treatment group separately by paired *t* test and for comparison between two groups by analysis of covariance (ANCOVA) and unpaired *t* test. Variables that were not normally distributed were analyzed by non-parametric tests (Mann–Whitney *U* test or Kruskal–Wallis test). For completer-only analysis, there was no formal imputation,

therefore all the estimates were obtained using all the available data (available data only-ADO), with the exception of proinflammatory and pro-resolving PUFA metabolite mediators, on which the value was zero when the result of different tests was undetectable (imputed values). Analyses were performed using the SAS software version 9.1.3 (SAS Institute Inc). The level of significance was set to 5 %.

Funding source

The funding for the study as well as the high-biological-value protein preparations were provided by PNKDIET, SLU. (Barcelona, Spain) free of charge to the patients. The funding source had no involvement in the study design, recruitment of patients, study interventions, data collection, or interpretation of the results.

Results

Of the 34 participants that were initially enrolled in the study, a total of 15 in the PnK-DHA group and 14 in the control group completed the study and 5 patients dropped out of the study within 6 months: 3 patients in PnK-DHA group (1 vomiting, 1 dropout unrelated to study, and 1 pregnancy) and 2 patients in control group (1 vomiting and 1 dropout unrelated to study) (Fig. 1). The 29 patients who completed the study (completers) exhibited the following characteristics at enrollment: mean age 45.83 ± 10.46 years (range 24–65), BMI 33.16 ± 1.66 kg/m^2(range 30.5–47.3), and waist circumference 109.29 ± 7.73 cm (range 90.0–151.0). In addition, 58.6 % were women and all the patients were obese (Table 1).

Changes in the lipid mediator profile

The 12-LOX pathway metabolites like 12-HETE and 14-HDOHE were mainly found in higher levels, followed by the 5-LOX metabolites (5-HETE, 7-HDOHE, and 4-HDOHE) and the 15-LOX metabolites (15-HETE and 17-HDOHE) (Table 2). In the COX pathway group, the concentration of the metabolite TBX2 was significantly higher than those of PGE2 and 5-LOX metabolite LTB4. The proresolution lipid mediators (SPMs) such as RvD2, MaR1, and PD1 either were detected at very low concentrations or were below the detection limit.

After 2 months of treatment, the LOX pathway metabolites and the COX pathway metabolites PGE2 and TBX2 were all present at higher concentrations, whereas 14-HDOHE and SPM were decreased (Table 2). After 4 additional months of treatment, differences with basal levels were less significant, while no changes in PGE2, 17-HDOHE, RvD2, and PD1 were observed. In addition MAR1 became detectable. We used the proresolution index (ratio of summation of pro-resolving mediators to summation of inflammatory mediators) [27] defined previously as a parameter that reflects the relatively abundant metabolites derived from DHA compared with those derived from AA. At baseline, the participants of the control group showed a proresolution index of 0.72 ± 0.23, i.e., the levels of AA-derived metabolites exceeded those derived from DHA. This ratio was decreased to 0.55 ± 0.20 after 2 months and returned nearly to baseline after 6 months of control treatment (Table 2). Instead, in the PnK-DHA group the ratio of proresolution to proinflammatory lipid markers decreased from 0.78 ± 0.35 only to 0.72 ± 0.19 and was increased to 0.84 ± 0.36 at 180 days, remaining higher over the entire study compared to the control treatment (Table 2)

Following DHA supplementation in the PnK-DHA group, almost all of the quantified DHA-derived oxylipins were significantly high compared to both baseline and control treatment plasma levels except for 14-HDOHE and related SPM that showed similar levels, over the entire study. In addition to the DHA products, metabolites originating from AA pathways were significantly decreased compared to concentrations following the control treatment (Table 2)

Anthropometric, biochemical, and metabolic parameters

Anthropometric parameters are shown in Table 3. Body weight, body mass index, and waist circumference improved in both groups without any statistical differences. The average weight loss after 6 months of treatment was 20.36 ± 5.2 kg in the control group and 19.74 ± 5.1 kg in the PnK-DHA group (both with $p < 0.0001$), without statistical differences between both groups (Table 3).

Biochemical and metabolic parameters measured during the treatment and follow-up periods are depicted in (Table 4). As shown, both very low-calorie-ketogenic diets (PnK-DHA group and control group) induced a significant change in some of the biological parameters that were clinically meaningful, such as insulin, HOMA-IR, tryglycerides, total cholesterol, LDL cholesterol, and C-reactive protein. Moreover, TNF alpha, resistin and leptin also showed significant changes while IL6 and adiponectin concentrations remained unchanged in PnK-DHA group and IL-6 increased in control group (Table 4).

RBC membrane fatty acids

RBCs showed distinct fatty acid (FA) profiles before and after control treatment. Treatment did not affect saturated (SFA), monounsaturated (MUFA), and polyunsaturated fatty acid (PUFA) global composition. The relative

Table 2 Concentration of lipid mediators in serum samples

	Control group			PnK-DHA group		
	Baseline	2 months	6 months	Baseline	60 days	180 days
AA-derived oxylipins						
15-HETE	51.14 ± 84.24	101.37 ± 136.54 ¥	65.65 ± 32.65	23.76 ± 38.35	49.51 ± 49.77	72.95 ± 51.50 ¥
12 -HETE	4985.94 ± 4301.86	6526.37 ± 3922.55 ¥	3652.28 ± 3488.85	5359.26 ± 3431.47	5148.86 ± 1817.86	3226.87 ± 1431.22 ¥†
8-HETE	43.74 ± 42.24	92.05 ± 69.45	70.10 ± 28.45 ¥	57.85 ± 51.38	88.25 ± 37.00 ¥	82.88 ± 36.02 ¥
5-HETE	424.39 ± 209.93	512.39 ± 362.39	313.75 ± 104.23	369.63 ± 106.82	399.09 ± 126.57	369.10 ± 149.68
TXB2	118.20 ± 133.33	177.96 ± 249.02	132.57 ± 76.49	131.26 ± 95.62	149.31 ± 123.06	148.01 ± 71.79
PGE2	2.82 ± 6.93	16.58 ± 36.39	28.73 ± 57.82¥	0.14 ± 0.34	5.58 ± 10.77	13.31 ± 15.46 ¥
LTB4	15.08 ± 29.88	43.41 ± 128.46¥	13.34 ± 18.30	*	1.49 ± 5.58	16.31 ± 18.99 ¥†
DHA-derived oxylipins						
17-HDOHE	96.56 ± 87.70	172.24 ± 123.32 ¥	177.95 ± 120.73 ¥	133.92 ± 80.78	213.17 ± 109.31 ¥	286.03 ± 214.13 ¥
14-HDOHE	3668.74 ± 3540.69	3320.96 ± 2520.22	2760.07 ± 3984.42	4071.54 ± 3150.66	3423.64 ± 1452.23	2575.02 ± 1833.92 ¥
7-HDOHE	128.55 ± 52.83	118.46 ± 42.42	149.72 ± 47.26 †	120.83 ± 49.09	140.10 ± 59.71	190.58 ± 56.36 ¥†
4-HDOHE	268.36 ± 148.25	298.81 ± 102.32	207.26 ± .94.35	288.93 ± 147.32	346.78 ± 130.19	306.24 ± 171.67
RVD2	64.89 ± 74.55	29.15 ± 37.23	31.08 ± 33.48	50.68 ± 91.41	23.16 ± 39.62	31.73 ± 44.41
PD1	6.27 ± 7.04	3.70 ± 4.99	4.51 ± 3.11	3.80 ± 5.27	6.06 ± 5.69	6.31 ± 3.03
7SMAR1	*	*	5.52 ± 8.81 ¥†	*	*	7.35 ± 8.52 ¥†
7RMAR1	*	*	6.01 ± 3.29 ¥†	*	*	6.05 ± 6.95 ¥†
Sum of proinflammatory mediators	5641.30 ± 4357.98	7470.14 ± 4105.76	4276.89 ± 3589.04	5941.89 ± 3563.60	5842.09 ± 1944.08	3931.00 ± 1654.83 ¥†
Sum of pro-resolving mediators	4233.38 ± 3652.42	3943.32 ± 2545.83	3342.11 ± 4132.21	4669.69 ± 3334.98	4152.90 ± 1545.52	3409.31 ± 2065.58
Proresolution index	0.72 ± 0.23	0.55 ± 0.20 ¥	0.71 ± 0.27	0.78 ± 0.35	0.72 ± 0.19 §	0.84 ± 0.36

The concentration of lipid mediators in serum of obese patients was measured by HPLC–MS/MS. Results in pg/ml are expressed as mean ± SD. ¥, †, and § denote significant difference ($p < 0.05$, Student's paired samples *t* test or independent samples *t* test) in intragroup analysis versus baseline (¥) or versus 2 months (†); and in intergroup analysis at the same time (§). Various values below the quantification limit set to 0.05 ng/ml. *Below limits of detection (detection limit was ∼2 pg in tissue matrix). Proresolution index is the ratio of sum of pro-resolving mediators to sum of proinflammatory mediators

Table 3 Changes in anthropometric variables (mean ± SD)

	Control group				PnK-DHA group			
	0 time	30 days	60 days	180 days	0 time	30 days	60 days	180 days
BMI (Kg/m2)	33 ± 1.9	29.7 ± 1.7*	27.4 ± 1.8*	25.6 ± 1.9*	33.4 ± 1.4	30.2 ± 1.7*	28.2 ± 1.4*	26.2 ± 1.6*
Weight (kg)	92.2 ± 13.1	82.8 ± 11.5*	76.6 ± 10.4*	71.8 ± 11.4*	92.1 ± 8.7	83.1 ± 7.2*	77.6 ± 6.9*	72.3 ± 7.1*
Fat mass (kg)	30.3 ± 6.1	25.0 ± 4.4*	19.8 ± 5.4*	16.8 ± 4.2*	34.4 ± 5.3	31.2 ± 4.8*	28.5 ± 6.1*	26.3 ± 5.3*
WC (cm)	109.2 ± 7.8	100.7 ± 7.3*	95.8 ± 7.2*	87.4 ± 7.4*	109.1 ± 8	101.4 ± 7.3*	96.1 ± 7.0*	89.1 ± 5.2*

WC Waist circumference

* $p < 0.05$, in each treatment group with basal values. No statistical differences between groups analysis of covariance (ANCOVA)

Table 4 Biochemical and metabolic parameters (mean ± SD)

	control group				PnK-DHA group			
	0 time	30 days	60 days	180 days	0 time	30 days	60 days	180 days
Glucose (mg/dl)	101.6 ± 11.3	78.0 ± 5.6*	79.6 ± 8.8*	88.9 ± 7.6*	105.0 ± 17.5	80.4 ± 5.9*	83.1 ± 7.2*	89.0 ± 7.7*
Total ch. (mg/dl)	212.4 ± 37.8	146.3 ± 24.1*	169.6 ± 26.6*	183.4 ± 31.2*	195.8 ± 41.9	140.2 ± 35.4*	159.9 ± 41.4*	177.1 ± 43.2*
LDL-ch. (mg/dl)	139.4 ± 33.0	88.4 ± 23.8*	106.0 ± 21.7*	119.2 ± 28.9*	117.6 ± 35.8	83.4 ± 34.2*	94.2 ± 25.0*	108.9 ± 35.5
HDL-ch. (mg/dl)	49.4 ± 12.2	42.0 ± 9.1*	47.7 ± 15.0	49.5 ± 9.8	49.4 ± 13.8	39.9 ± 11.8*	43.3 ± 14.0*	51.4 ± 15.3
TG (mg/dl)	135.0 ± 50.6	89.1 ± 23.9*	86.2 ± 21.1*	78.5 ± 27.7*	150.6 ± 71.2	96.6 ± 26.7*	85.4 ± 25.0*	83.9 ± 31.4*
Insulin (mUI/L)	12.3 ± 8.5	4.6 ± 1.9*	4.1 ± 2.4*	4.8 ± 2.5*	14.4 ± 5.5	5.1 ± 2.8*	5.1 ± 2.3*	5.3 ± 1.9*
HOMA-IR	3.1 ± 2.2	0.8 ± 0.3*	0.8 ± 0.5*	1.0 ± 0.6*	3.8 ± 1.9	1.0 ± 0.6*	1.1 ± 0.5*	1.2 ± 0.4*
CRP (mg/dl)	3.3 ± 1.4	2.6 ± 2.2*	2.3 ± 2.3*	1.9 ± 1.4*	3.8 ± 3.4	3.2 ± 3.6*	3.2 ± 2.9	2.1 ± 1.2*
Adiponectin (ng/ml)	8.6 ± 4.2	8.6 ± 4.1	11.3 ± 4.9	9.9 ± 4.8	7.9 ± 3.5	8.2 ± 2.8	8.6 ± 2.6	7.9 ± 3.4
Resistin (ng/ml)	5.3 ± 3.6	5.9 ± 4.5	4.7 ± 2.5	3.6 ± 2.4*	4.5 ± 2.2	5.4 ± 2.4	4.5 ± 1.9	3.4 ± 1.6 *
TNF alpha(ng/ml)	4.7 ± 2.3	4.8 ± 2.3	3.1 ± 2.1*	2.0 ± 0.4*	5.0 ± 2.5	4.3 ± 2.3	3.3 ± 1.8	2.0 ± 0.3*
IL6(ng/ml)	4.2 ± 2.1	4.9 ± 3.0	4.0 ± 2.1	6.3 ± 2.7*	4.1 ± 1.7	3.8 ± 1.6	4.3 ± 1.5	4.9 ± 2.0

Chol cholesterol, *TG* triglycerides, *CRP* C-reactive protein, *HOMA-IR* homeostasis model assessment

* $p < 0.05$, in each group with basal values. No statistical differences between groups by analysis of covariance (ANCOVA)

abundance of MUFAs, including palmitoleic acid (16:1n-7) and oleic acid (18:1n-9), was decreased in RBCs but nervonic acid (24:1n9) was increased. In general, n-3 PUFAs were incorporated at the expense of the n-6 PUFAs. Simultaneously with the increase in RBC AA (20:4n-6), EPA (C20:5n-3), DPA (C22:5n-3), and DHA (C22:6n-3) levels, the contents of linoleic acid (C18:2n-6), dihomo-γ-linolenic acid (C20:3n-6), and α-linolenic acid (C18:3n-3) were significantly decreased (Table 5).

DHA supplementation caused changes similar to those in the control treatment. As expected, supplementation resulted in a concomitant increase in EPA and DHA species in RBC indicating that all patients were compliant with the capsule intake. Simultaneously with the increase in RBC EPA and DHA levels, the contents of AA (20:4n-6) and oleic acid (C18:1n-9) showed the greatest decrease in relative abundance. On a concentration basis, a slight increase of PUFAs was detected in the total RBC and the overall percentage of MUFAs was lower after treatment.

Consequently, both treatments caused an increase of the omega-3 index in all subjects, being twice in the case of DHA supplementation. In the DHA treatment, the participants ingested two capsules (500 mg of DHA) daily for the first 60 days and one capsule daily in the subsequent 16 weeks. The highest increase of the omega-3 index was observed 60 days after starting DHA supplementation. At this time, the omega-3 index reached 6.81 ± 0.66 compared with 4.72 ± 1.07 at baseline. After decreasing DHA supplementation, the omega-3 index started to decline, but still remained significantly high for the following 180 days (6.60 ± 0.98) compared with the low baseline values. Similarly, the mean ratios of AA/EPA and AA/DHA were dramatically reduced in the PnK-DHA group at the end of the treatment period (−16.37 ± 21.35 and −0.81 ± 0.68 in the PnK-DHA group compared with −8.28 ± 12.12 and −0.23 ± 0.48 in the control group; $p < 0.05$ respect ratio AA/DHA). The AIFAI was consistently increased after the DHA treatment (12.72 ± 11.81 in the PnK-DHA group compared with 0.94 ± 8.04 in the control group; $p < 0.05$).

Table 5 Lipid profile of erythrocyte membrane (mean ± SD)

	Control group				PnK-DHA group			
	0 time	30 days	60 days	180 days	0 time	30 days	60 days	180 days
Myristic	1.33 ± 0.25	1.04 ± 0.27*	1.07 ± 0.29*	1.24 ± 0.34*	1.34 ± 0.18	1.12 ± 0.19*	1.15 ± 0.19*	1.28 ± 0.21*
Myristoleic	0.62 ± 0.27	0.63 ± 0.24*	0.67 ± 0.27*	0.69 ± 0.29*	0.65 ± 0.24	0.69 ± 0.22	0.72 ± 0.19*	0.74 ± 0.20*
Palmitic	17.78 ± 1.14	18.55 ± 0.66*	18.73 ± 0.75*	18.06 ± 0.62*	17.72 ± 0.90	18.48 ± 0.68*	18.51 ± 0.89*	18.04 ± 0.79*
Palmitoleic	0.75 ± 0.46	0.31 ± 0.15	0.25 ± 0.09	0.33 ± 0.14	0.76 ± 0.42	0.34 ± 0.12	0.25 ± 0.07	0.31 ± 0.16
Stearic	14.83 ± 1.17	15.03 ± 0.58	15.30 ± 0.57	15.33 ± 0.64	14.99 ± 0.76	15.02 ± 0.40	15.11 ± 0.31	15.41 ± 0.44
Oleic	13.95 ± 1.66	12.83 ± 0.52*	12.80 ± 0.59	12.79 ± 0.86	14.16 ± 2.19	12.67 ± 0.60*	12.38 ± 0.60	12.49 ± 0.84
Vaccenic	1.56 ± 0.21*	1.61 ± 0.28	1.70 ± 0.17	1.66 ± 0.32*	1.71 ± 0.13*	1.71 ± 0.13	1.71 ± 0.14	1.61 ± 0.12*
Elaidic	0.12 ± 0.04	0.10 ± 0.04*	0.10 ± 0.04*	0.12 ± 0.05*	0.12 ± 0.04	0.11 ± 0.04*	0.10 ± 0.05*	0.11 ± 0.04*
Linoleic	11.30 ± 1.48	8.72 ± 1.07	8.08 ± 0.86*	9.91 ± 0.95*	10.85 ± 1.61	8.65 ± 1.02	7.91 ± 1.09*	9.41 ± 1.10*
Linoelaidic	0.50 ± 1.74	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.05 ± 0.02	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.02
γ-Linolenic	0.11 ± 0.08	0.04 ± 0.02	0.02 ± 0.01	0.05 ± 0.03	0.12 ± 0.07	0.03 ± 0.02	0.02 ± 0.01	0.05 ± 0.03
α-Linolenic	0.12 ± 0.07	0.05 ± 0.01	0.04 ± 0.01	0.09 ± 0.03*	0.13 ± 0.09	0.05 ± 0.02	0.04 ± 0.01	0.07 ± 0.02
Stearidonic	0.01 ± 0.01	0.00 ± 0.00*	0.00 ± 0.01	0.01 ± 0.01*	0.00 ± 0.00	0.00 ± 0.00*	0.00 ± 0.00	0.00 ± 0.00*
Arachidonic	0.58 ± 0.08	0.57 ± 0.06*	0.56 ± 0.07*	0.58 ± 0.06*	0.57 ± 0.06	0.59 ± 0.07*	0.56 ± 0.05*	0.59 ± 0.04*
Gondoic	0.34 ± 0.06	0.38 ± 0.06*	0.43 ± 0.09*	0.38 ± 0.06*	0.35 ± 0.06	0.38 ± 0.06*	0.39 ± 0.07*¥	0.34 ± 0.05*¥
Eicosadienoic	0.40 ± 0.05	0.36 ± 0.05*	0.38 ± 0.07	0.39 ± 0.08*	0.40 ± 0.05	0.37 ± 0.05*	0.37 ± 0.06	0.39 ± 0.04*
Dihomo-γ-linolenic	2.16 ± 0.43	1.51 ± 0.37*	1.43 ± 0.35*	1.82 ± 0.39*	1.90 ± 0.29	1.39 ± 0.25*	1.29 ± 0.17*	1.76 ± 0.29*
AA	12.16 ± 1.32	14.02 ± 0.67	14.37 ± 0.58	12.97 ± 0.83	12.71 ± 1.55	13.86 ± 0.66	13.95 ± 0.69	13.03 ± 0.72
Eicosatrienoic	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
Eicosatetraenoic	0.01 ± 0.01	0.01 ± 0.00	0.00 ± 0.00	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.01
EPA	0.48 ± 0.21	0.57 ± 0.17*	0.71 ± 0.21*	0.79 ± 0.32	0.53 ± 0.27	0.58 ± 0.12*	0.80 ± 0.21*	0.96 ± 0.42
Behenic	1.99 ± 0.27	1.98 ± 0.21*	1.77 ± 0.28*	1.80 ± 0.28	2.02 ± 0.28	2.12 ± 0.19*¥	1.99 ± 0.21*¥	2.01 ± 0.29
Erucic	0.07 ± 0.02	0.10 ± 0.03*	0.11 ± 0.03*	0.09 ± 0.03*	0.08 ± 0.02	0.10 ± 0.03*	0.10 ± 0.03*¥	0.09 ± 0.02*
Docosadienoic	0.06 ± 0.03	0.06 ± 0.03*	0.08 ± 0.06*	0.06 ± 0.04	0.04 ± 0.03	0.07 ± 0.04*	0.08 ± 0.03*	0.06 ± 0.03
Adrenic	2.10 ± 0.78	2.33 ± 0.75*	2.21 ± 0.77*	1.98 ± 0.55*	2.25 ± 0.77	2.34 ± 0.79*	2.22 ± 0.63*	1.91 ± 0.60*
DTA	0.01 ± 0.01	0.02 ± 0.02	0.01 ± 0.02	0.02 ± 0.02	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.02	0.01 ± 0.01
Docosapentanoic	0.45 ± 0.12	0.44 ± 0.13	0.44 ± 0.10*	0.37 ± 0.10	0.40 ± 0.14	0.43 ± 0.09	0.39 ± 0.09*	0.33 ± 0.10
Docosatetranoic	0.01 ± 0.01	0.00 ± 0.01*	0.00 ± 0.01*	0.01 ± 0.01*	0.00 ± 0.01	0.00 ± 0.01*	0.00 ± 0.01*	0.00 ± 0.01*
DPA	1.11 ± 0.22	1.36 ± 0.42*	1.57 ± 0.32*	1.52 ± 0.31*	1.18 ± 0.30	1.34 ± 0.42*	1.43 ± 0.37*	1.40 ± 0.34*
DHA	4.20 ± 0.87	4.97 ± 0.78*	5.10 ± 0.85*	4.84 ± 0.92*	4.19 ± 0.89	5.27 ± 0.58*	6.01 ± 0.56*¥	5.64 ± 0.71*¥
Lignoceric	4.88 ± 0.56	5.30 ± 0.63	4.79 ± 0.54	4.83 ± 0.41	4.89 ± 0.65	5.30 ± 0.41	5.04 ± 0.54	4.83 ± 0.70
Nervonic	6.00 ± 0.95	7.06 ± 0.64	7.19 ± 0.55*	7.20 ± 0.62	5.82 ± 0.81	6.93 ± 0.49	7.40 ± 0.70*	7.05 ± 0.96
Saturated (SFA)	41.39 ± 1.96	42.48 ± 0.84	42.21 ± 1.03*	41.85 ± 1.04*	41.54 ± 1.16	42.62 ± 1.10	42.36 ± 0.89*	42.16 ± 0.97*
Unsaturated(UFA)	58.61 ± 1.96	57.52 ± 0.84	57.79 ± 1.03*	58.15 ± 1.04*	58.46 ± 1.16	57.38 ± 1.10	57.64 ± 0.89*	57.84 ± 0.97*

Table 5 continued

	Control group				PnK-DHA group			
	0 time	30 days	60 days	180 days	0 time	30 days	60 days	180 days
Monounsatur. (MUFA)	23.40 ± 1.30	23.02 ± 0.94*	23.26 ± 1.05	23.26 ± 1.40*	23.65 ± 2.15	22.93 ± 1.03*	23.04 ± 1.07	22.74 ± 1.43*
Polyunsatur. (PUFA)	35.21 ± 1.40	34.50 ± 0.62	34.52 ± 0.79*	34.89 ± 0.77	34.80 ± 1.34	34.45 ± 0.59	34.60 ± 0.49*	35.10 ± 0.78
Polyunsat. omega-6 (n-6)	29.25 ± 1.80	27.50 ± 1.12*	27.05 ± 0.80*	27.60 ± 0.99*	28.72 ± 1.79	27.17 ± 1.20*	26.27 ± 1.00*¥	26.99 ± 1.33*
Polyunsat.omega-3 (n-3)	5.96 ± 1.10	7.00 ± 1.11*	7.47 ± 1.17*	7.29 ± 1.26*	6.08 ± 1.15	7.28 ± 0.86*	8.33 ± 0.90*¥	8.11 ± 1.16*
Omega-6/omega-3	5.10 ± 1.17	4.05 ± 0.84*	3.72 ± 0.70*	3.91 ± 0.81*	4.90 ± 1.09	3.79 ± 0.58*	3.20 ± 0.44*¥	3.41 ± 0.61*
AA/EPA	28.55 ± 8.46	27.87 ± 14.39*	23.72 ± 14.64	20.27 ± 12.52	31.69 ± 20.45	24.73 ± 5.87*	18.57 ± 4.78	16.18 ± 6.96
AA/DHA	3.00 ± 0.65	2.89 ± 0.51*	2.90 ± 0.56*	2.77 ± 0.55*	3.14 ± 0.68	2.66 ± 0.30*¥	2.34 ± 0.23*¥	2.35 ± 0.36*¥
Omega 3 index (EPA ± DHA)	4.68 ± 0.97	5.55 ± 0.87*	5.81 ± 0.96*	5.63 ± 1.15*	4.72 ± 1.07	5.85 ± 0.63*	6.81 ± 0.66*¥	6.60 ± 0.98*¥
Anti-inflammatory fatty acid index (AIFAI)	56.72 ± 8.17	50.47 ± 6.23*	50.58 ± 6.94*	57.65 ± 9.54*	52.71 ± 8.69	52.31 ± 5.20*¥	58.21 ± 5.87*¥	64.53 ± 9.69*¥

* $p < 0.05$ in each group with basal values. (¥) $p < 0.05$ by comparison between groups. Fatty acids expressed as relative amounts (g of a specific fatty acid methyl ester per g of total fatty acid methyl ester)

Discussion

The main finding of this work is that a very low-calorie ketogenic diet supplemented with DHA improved inflammation-resolving eicosanoids compared with no supplemented isocaloric VLCK diet, and was effective in inducing loss of body weight.

In the present work, the very low-calorie ketogenic diets induced a rapid, intense, and persistent weight loss. The effect was rapid and maintained during 6 months. This result is remarkable considering that the ketogenic phase ended 45-60 days after starting the method, therefore the above results were obtained in the reeducation stage and out of ketosis. It has been previously found that a greater initial weight loss improves the long-term maintenance of the weight loss [28]. This hypothesis was confirmed in the present work as shown in a previous work of our group [12]. The very low-calorie ketogenic diet method in both branches was found to be superior than the weight loss that has been observed with most of the anti-obesity drugs that have been studied [29–34]. The dropout rate in this study was similar to that expected in any program with obese individuals, but the dropout rate due to side effects was negligible.

Our results are in agreement with a number of studies in which leptin has been consistently shown to decrease in response to weight loss [35–37]. Our results showed a decrease in TNF alpha concentrations after weight loss. Previous studies have demonstrated a decrease in interleukin levels with weight loss [38, 39], although others have failed to report a change [36]. We observed that IL-6 remained unchanged in PnK-DHA group and slightly increased in control group. Perhaps, our population has a low inflammatory background, as a group of obese patients without diabetes and cardiovascular disease.

During ketogenic diet, the adipose tissue produces considerable amounts of free fatty acids and fatty acid-derived bioactive lipid mediators with potent pro- and anti-inflammatory actions. Even though the RBC fatty acid profile showed favorable changes (increased omega-3 index and decreased AA/EPA and AA/DHA ratios), a significant decrease of the AIFAI in the ketogenic phase was observed which returned nearly to baseline after reeducation stage. As expected, after DHA supplementation, the AIFAI increased in both stages and the variation of omega-3 index and AA/EPA and AA/DHA ratios was higher than that with VLCK diet alone. It has been shown that DHA supplementation induces a time-dependent incorporation of omega-3 PUFAs into the cell membrane phospholipids [40, 41]. DHA reduced the content of AA in the biomembranes, possibly making AA less available for eicosanoid synthesis, which could modulate inflammation [42].

Furthermore, the provision of predominantly omega-3 or omega-6 lipids alters the downstream production of LMs.

An increased proportion of omega-3 PUFAs limits the omega-6 substrate for the production of AA and subsequent LM autacoids that amplify acute inflammation [43]. Moreover, the biosynthesis of the anti-inflammatory specialized pro-resolving mediators (SPMs) like resolvins and protectins is regulated by the availability of omega-3 PUFA EPA and DHA and the spatial and temporal control of specific lipoxygenase pathways [44, 45]. In the present study, we demonstrate that DHA supplementation induces functionally distinct LM profiles in plasma of patients under control diet. Raising the omega-3 index from 5.6 to 6.6 units is accompanied by a decrease in proinflammatory LMs and the maintenance of proresolution precursors, which serve as pathway markers of the active resolution of inflammation [44, 45]. Similar marked oxylipin changes were reported from studies on high-fat diet-induced obesity in response to a combined treatment using mild calorie restriction and dietary omega-3 PUFA [46].

Proresolution index defined as their respective relationships and summation index of bioactive mediators from AA and DHA provide information regarding the potential inflammatory and/or resolution status of a given target tissue [27]. Dietary DHA supplementation produces an increase of this ratio over the entire study. In contrast, in the control diet this index reached a valley value in the ketogenic phase, and returned nearly to baseline after reeducation phase. Even without dietary intervention, the individual differences in the plasma proresolution index correlated well with the AIFAI in RBCs. As AIFAI indexes increased, the synthesis of anti-inflammatory DHA-derived resolvins and protectins increased and AA-derived oxilipins decreased, which indicated that altered eicosanoid formation could be a crucial molecular mechanism underlying the effects of DHA on obesity-driven inflammation. This result suggests that DHA was efficiently metabolized even when AA was still present as the predominant long-chain PUFA. Also, this observation supports the hypothesis that DHA is able to exert anti-inflammatory effects in this model.

In summary, the present work demonstrated that a very low-calorie ketogenic diet supplemented with DHA was significantly superior in the anti-inflammatory effect without statistical differences in weight loss and metabolic improvement. Moreover, this study has the unique advantage of comparing the differential downstream SPM production under very low-calorie ketogenic diet, which to our knowledge has not been done previously. This kind of supplementation may optimize the positive effects of very low-calorie ketogenic diet on some cardiovascular risk markers and on obesity-related chronic low-grade inflammation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Glossary

12-HETE	15-Hydroxyicosatetraenoic acid
12-LOX	12-Lipoxygenase
14-HDOHE	14-Hydroxy docosahexaenoic acid
14-HDHA	14-Hydroxy docosahexaenoic acid
15-HETE	15-Hydroxyicosatetraenoic acid
15-LOX	12-Lipoxygenase
17-HDOHE	17-Hydroxy docosahexaenoic acid
17-HDHA	17-Hydroxy docosahexaenoic acid
4-HDOHE	4-Hydroxy docosahexaenoic acid
4-HDHA	4-Hydroxy docosahexaenoic acid
5-HETE	5-Hydroxyicosatetraenoic acid
5-LOX	5-Lipoxygenase
7-HDOHE	7-Hydroxy docosahexaenoic acid
7-HDHA	7-Hydroxy docosahexaenoic acid
7RMAR1	Nuclear matrix-associated protein RMAR1
7SMAR1	Nuclear matrix-associated protein SMAR1
8-HETE	8-Hydroxyicosatetraenoic acid
AA	Arachidonic acid
ADO	Available data only
AIFAI	Anti-inflammatory fatty acid index
ANCOVA	Analysis of covariance
BHT	Butylhydroxytoluene
BMI	Body mass index
CHOL	Cholesterol
COX	Cyclooxygenase
CRP	C-reactive protein
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl ester
GCMS	Gas chromatograph/mass spectrometer
HDL	High-density lipoprotein
HOMA-IR	Homeostatic model assessment of insulin resistance
HPLC	High-performance liquid chromatography
IL6	Interleukin 6
K2-EDTA	K2-Ethylenediaminetetraacetic acid
LC	Liquid chromatography
LDL	Low-density lipoprotein
LM	Lipid mediators
LOX	Lipoxygenase
LTB4	Leukotriene B4
MAR1	Nuclear matrix-associated protein MAR1
MS	Mass spectrometry

MUFA	Monounsaturated fatty acid
NS	Not significant
PD1	Programmed cell death protein 1
PGE2	Prostaglandin E2
PnK	Pronokal
PUFA	Polyunsaturated fatty acid
RBC	Red blood cell
RVD2	7(S), 16(R), 17(S)-resolvin D2
SD	Standard deviation
SFA	Saturated fatty acid
SPM	Proresolution lipid mediators
TNF	Tumor necrosis factor
TXB2	T-box transcription factor
VLKD	Very low-calorie ketogenic diet
WC	Waist circumference

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