Original Article

Effects of the Consumption of Fish Meals on the Carotid Intima-Media Thickness in Patients with Hypertension: A Prospective Study

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***Aim*: The composition of dietary fat affects various modifiable cardiovascular risk factors and cardiovascular outcomes in the general population. We investigated the effects of the regular consumption of fish meals on the fatty acid composition of red blood cell (RBC) membranes and the relationship of this parameter with the carotid intima-media thickness (IMT), an early marker of atherosclerosis.**

***Methods*: In 56 hypertensive patients, we measured the carotid IMT using ultrasound imaging and the RBC membrane fatty acid composition using gas-chromatography and calculated the polyunsaturated to saturated fatty acid (PUFA/SFA) ratio. The patients received intensive nutritional counseling and three weekly meals of fish containing elevated amounts of PUFA, in order to increase the membrane PUFA content. The RBC membrane fatty acid composition and IMT were reassessed after one year.**

***Results*: At baseline, the membrane PUFA/SFA ratio was inversely related to the carotid IMT, and the relationship was independent of all major cardiovascular risk factors. At follow-up, the PUFA/SFA ratio increased in the RBC membranes of 25 (45%) of 56 patients. The regular consumption of fish meals resulted in a decreased carotid IMT only in the patients with an increased membrane PUFA/SFA ratio. Changes in the PUFA/SFA ratio induced by the dietary intervention were inversely related to the changes in the IMT, independent of variations in body mass, blood pressure and plasma lipids.**

***Conclusions*: In hypertensive patients, a low RBC membrane PUFA/SFA ratio is associated with more prominent vascular damage, and the regular consumption of fish reduces the carotid IMT in patients in whom dietary intervention affects the membrane fatty acid composition.**

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Key words: Red blood cell, Polyunsaturated fatty acids, Carotid intima-media thickness, Nutritional counseling, Atherosclerosis

Introduction

It is generally accepted that an elevated level of dietary fat consumption is associated with an increased cardiovascular risk, primarily due to the association with high plasma lipid levels. Substantial evidence indicates that the type of dietary fat accounts for the risk of cardiovascular disease more so than the total amount of daily fat intake[1)]. For instance, the "Mediterranean diet" is rich in unsaturated fats and has proven to be more beneficial than a generic low-fat diet with respect to modifiable cardiovascular risk factors[2)]. In addition, the dietary substitution of saturated with unsaturated fats decreases the risk of coronary events and death[3)]. Diets rich in saturated fats increase the total and low-density lipoprotein (LDL) cholesterol levels in the plasma[4)], thereby increasing the risk of coronary heart disease[5)]. Conversely, mono and polyunsaturated fats reduce the total and LDL cholesterol levels[6)], and their dietary supplementation

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has been reported to improve cardiovascular outcomes in the general population[3]. Because fatty acids are normal constituents of cell membranes, measurements of the ratio of polyunsaturated to saturated fatty acid components (PUFA/SFA) in the red blood cell (RBC) membrane reflect the fatty acid composition of the diet[7] and have been used as a biomarker of fatty acid intake in epidemiological studies[8].

The development of subclinical vascular changes is critical in the disease continuum linking risk factors, such as hypertension, to cardiovascular events and death. There is evidence that interventions to correct these changes decrease cardiovascular risks[9, 10]. In hypertensive patients, reducing the dietary fat content decreases the cardiovascular risk[11], and previous evidence indicates that dietary supplementation with PUFA of the n-3 family (n-3 PUFA) reduces arterial blood pressure[12]. Moreover, the incidence of hypertension has been demonstrated to be increased in women with a low PUFA/SFA ratio in the RBC membrane[13]. In the present study, we prospectively investigated the effects of dietary interventions with supplementation of fish meals containing elevated amounts of PUFA on the fatty acid composition of the RBC membrane in hypertensive patients and evaluated the relationship of this parameter with changes in the carotid intima-media thickness (IMT).

Patients and Methods

Study Patients

Following public advertisement of the present study characteristics in the local media, 67 consecutive patients with treated mild-to-moderate arterial hypertension were screened at our Hypertension Clinic, 56 of whom who fulfilled the inclusion criteria were enrolled in the follow-up study. The diagnosis of hypertension was made according to current guidelines[14]. We excluded patients younger than 18 and older than 70 years of age, current and former smokers, heavy alcohol drinkers (more than 30 g/day), vegetarians and patients with a body mass index (BMI) of more than 35 kg/m^2, uncontrolled blood pressure, secondary forms of hypertension as determined based on exhaustive diagnostic testing[15] and/or a history of clinically relevant cardiovascular complications, diabetes or advanced renal failure (a glomerular filtration rate of less than 30 mL/min/1.73 m^2 according to the MDRD formula). The exclusion criteria were defined to avoid possible interference with the carotid IMT. The patients were representative of the hypertensive population of northeast Italy and, at inclusion, were taking antihypertensive agents (52% angiotensin receptor blockers, 34% diuretics, 34% beta blockers, 18% calcium channel blockers, 9% ACE inhibitors and 12% alpha blockers) with an office blood pressure level persistently below 140/90 mm Hg. The baseline examination included an assessment of demographic, anthropometric and biochemical variables, 24-hour ambulatory blood pressure monitoring (ABPM), carotid IMT and the content of different types of fatty acids in the membrane of RBCs. The clinical and biochemical variables were reassessed at three, six months and one year, during which time the antihypertensive treatment was not modified. The assessment of the ABPM variables, carotid IMT and RBC membrane fatty acid composition was repeated after one year. The cardiovascular risk was estimated at the beginning and end of the study according to the Framingham score[16].

Prior to the start of the study, each patient individually participated in a program of nutritional counseling following current guidelines[17]. A physician and dietician instructed the patients to perform regular physical activity (brisk walking for 30-45 minutes at least three times a week), reduce salt and alcohol intake, limit the intake of meat, cheese (no more than three times per week) and eggs (no more than once per week), consume daily fruits and vegetables and use olive or sunflower oil instead of butter or margarine. In addition, the patients were required to consume at home a fish meal (Brown trout, *Salmo trutta fario*) provided by an independent local producer (Friultrota®, San Daniele del Friuli, Italy) at least three times per week. The farmed fish was raised in special conditions in order to enrich its content of n-3 PUFA so that one serving (100 g) of fish contained 4.0 g PUFA (2.4 g as n-3 PUFA), 3.6 g monounsaturated fatty acids (MUFA) and 3.1 g SFA (for details on the nutritional data for the fish see *Appendix A* in the online supplemental material). The nutritional counseling program was repeated individually at three and six months of follow-up. A food frequency questionnaire and physical activity survey were evaluated at the beginning and end of the study, the results of which are reported in *Appendix B* of the online supplemental material. The study protocol was approved by the local Institutional Review Board, and each participant provided their written informed consent.

Laboratory Methods

At baseline and at three and six months and one year of follow-up, venous blood samples were collected from the antecubital vein in the morning after an overnight fast for measurement of general biochemistry parameters, including the plasma lipid frac-

Table 1. Clinical and laboratory characteristics of the study patients at baseline

Variable	All patients	Quartiles of PUFA/SFA ratio in RBC membranes				*p* for trend
		I	II	III	IV	
Patients (n)	56	14	14	14	14	–
Age (years)	64 ± 7	64 ± 10	64 ± 6	62 ± 9	65 ± 4	0.872
Males [n (%)]	27 (48)	8 (57)	8 (57)	8 (57)	3 (21)	0.147
CV risk score (%)	25 ± 12	29 ± 15	27 ± 10	25 ± 14	19 ± 8	0.009
BMI (Kg/m^2)	28.7 ± 4.8	29.0 ± 4.2	30.3 ± 6.6	27.4 ± 2.3	28.0 ± 5.1	0.204
24-h SBP (mm Hg)	133 ± 16	135 ± 16	139 ± 21	132 ± 15	125 ± 10	0.059
24-h DBP (mm Hg)	78 ± 9	77 ± 8	81 ± 13	79 ± 6	76 ± 7	0.711
Total cholesterol (mmol/L)	5.67 ± 1.02	5.28 ± 1.22	5.67 ± 1.09	5.83 ± 0.70	5.88 ± 0.98	0.084
HDL cholesterol (mmol/L)	1.30 ± 0.33	1.14 ± 0.26	1.16 ± 0.23	1.29 ± 0.23	1.61 ± 0.39	< 0.001
LDL cholesterol (mmol/L)	3.70 ± 0.85	3.52 ± 0.96	3.73 ± 1.14	3.78 ± 0.54	3.75 ± 0.70	0.232
Triglycerides (mmol/L)	1.45 ± 0.77	1.38 ± 0.97	1.66 ± 0.69	1.62 ± 0.85	1.14 ± 0.41	0.536
Plasma glucose (mmol/L)	5.60 ± 1.17	6.22 ± 1.55	5.44 ± 0.94	5.60 ± 1.00	5.16 ± 0.89	0.019
eGFR (mL/min/1.73 m^2)	75 ± 14	73 ± 18	72 ± 13	74 ± 11	80 ± 12	0.162
UAC (mg/mmol)	0.44 [0.24-0.84]	0.43 [0.24-0.68]	0.44 [0.19-0.60]	0.43 [0.26-0.72]	0.76 [0.23-1.17]	0.190
α1-blockers [n (%)]	7 (13)	3 (21)	3 (21)	0 (0)	1 (7)	0.221
β-blockers [n (%)]	19 (34)	7 (50)	3 (21)	6 (43)	3 (21)	0.255
Diuretics [n(%)]	19 (34)	7 (50)	6 (43)	2 (14)	4 (29)	0.195
CCBs [n (%)]	10 (18)	5 (36)	4 (29)	0 (0)	1 (7)	0.080
ACE-i [n (%)]	5 (9)	3 (21)	1 (14)	1 (14)	0 (0)	0.243
ARBs [n (%)]	29 (52)	7 (50)	9 (64)	7 (50)	6 (43)	0.715
Statins [n (%)]	10 (18)	3 (21)	2 (14)	1 (7)	4 (29)	0.487
Antiplatelet [n (%)]	5 (9)	1 (7)	2 (14)	1 (7)	1 (7)	0.883
SFA (% weight)	47 ± 6	55 ± 3	49 ± 2	43 ± 2	40 ± 2	< 0.001
PUFA (% weight)	33 ± 5	26 ± 3	31 ± 2	35 ± 2	39 ± 3	< 0.001
MUFA (% weight)	0.72 ± 0.19	19 ± 3	20 ± 2	21 ± 4	21 ± 3	0.151
PUFA/SFA ratio	20 ± 3	0.48 ± 0.07	0.63 ± 0.06	0.81 ± 0.03	0.96 ± 0.08	< 0.001
n-3 PUFA (% weight)	5.9 ± 1.8	3.9 ± 1.2	5.5 ± 1.2	6.4 ± 1.4	7.8 ± 1.1	< 0.001
n-6 PUFA (% weight)	27 ± 4	22 ± 2	25 ± 1	29 ± 2	31 ± 2	< 0.001
n-6/n-3 PUFA	4.9 ± 1.4	5.9 ± 1.6	4.8 ± 1.1	4.7 ± 1.3	4.1 ± 0.6	0.001
Carotid IMT (mm)	0.96 ± 0.22	1.04 ± 0.27	1.01 ± 0.17	0.94 ± 0.25	0.84 ± 0.13	0.006

The data are expressed as the mean ± SD and were analyzed using a non-parametric test for trends across ordered quartiles, with the exception of gender and drug therapy, which are expressed as absolute numbers (percentages) and were analyzed according to Pearson's χ^2 test. The estimated glomerular filtration rate (eGFR) was calculated using the MDRD formula. PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; RBC, red blood cell; CV, cardiovascular risk score according to the Framingham Heart Study; 24h SBP, mean 24-hour systolic blood pressure; 24h DBP, mean 24-hour diastolic blood pressure; UAC, urinary albumin to creatinine ratio expressed as the median [interquartile range]; CCB, calcium channel blockers; ACE-i, angiotensin-converting enzyme inhibitors; ARBs, angiotensin receptor blockers; MUFA, monounsaturated fatty acids; n-3, polyunsaturated fatty acids of the n-3 family; n-6 polyunsaturated fatty acids of the n-6 family; IMT, intima-media thickness.

tions[18]. The fatty acid composition of the RBC membrane was assessed using gas-chromatography with a GC analyzer (GC 3300, Varian Inc., Milan, Italy) equipped with a high polar capillary column (CP-Wax 58 FFAP CB, Varian Inc., Milan, Italy). The amount of fatty acids is expressed as the % weight of the total fatty acid content of the RBC membrane.

ABPM was performed using a validated device (TM-2430, A&D Company Limited, Tokyo, Japan), and the data were analyzed with a property software program (SIGMA 2000, I-TECH, Milan, Italy), as previously reported[19]. The carotid arteries were evaluated by the same trained operator using B-mode ultrasonography (Aplio CV, Toshiba, Japan), as previously described[20]. For the analysis of the carotid IMT, we used the measurement obtained for the left common carotid artery, unless otherwise specified. A detailed description of the methods is presented in *Appendix C* of the online supplemental material.

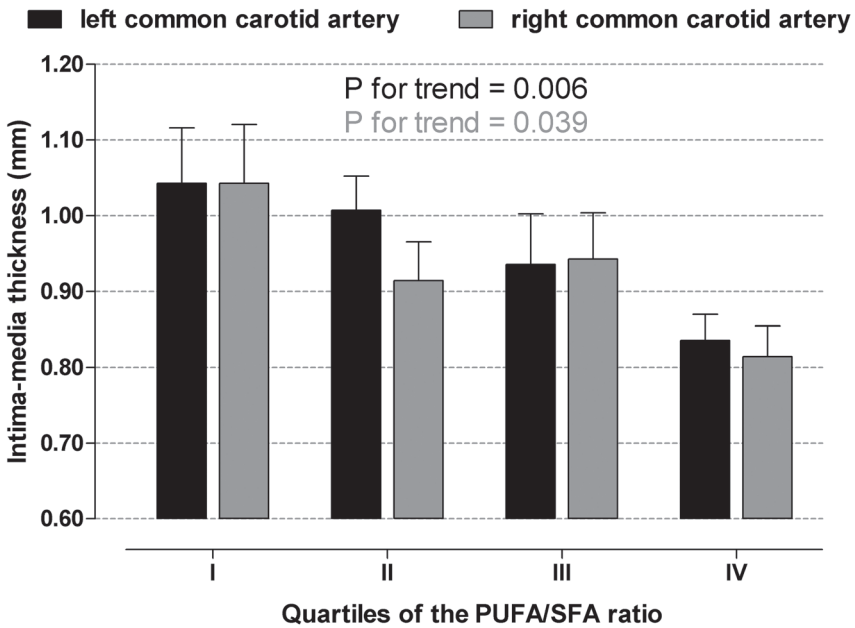

Fig. 1. Bar graph showing the left and right common carotid intima-media thickness across the quartiles of the red blood cell membrane polyunsaturated to saturated fatty acids (PUFA/SFA) ratio in the patients with hypertension. The analysis was performed using the non-parametric test for trends across ordered groups, and the bars represent the mean ± SEM.

Statistical Analysis

The data are presented as the mean ± SD or the median [interquartile range] for non-normally distributed variables. The normality of the distribution was assessed according to the Shapiro-Wilk test. For statistical purposes, the baseline data were divided into quartiles of the PUFA/SFA ratio in the RBC membrane. Comparisons between two means were made using Student's *t*-test and between quartiles using a non-parametric test for trends across ordered groups according to the method of Cuzick[21]. A frequency analysis was performed using the Pearson χ^2 test, and an analysis of longitudinal data and confounders was carried out according to two-way ANCOVA for repeated measurements. A post-estimation pairwise comparison was made on margins of the linear predictions calculated after ANCOVA with the Bonferroni correction to account for multiple comparisons. Prediction results are reported as the mean difference with 95% CI. The correlations between continuous variables were assessed according to the Pearson's R coefficient. The multivariate analysis was performed using a linear regression model, and the standardized coefficient (β) was reported for independent variables. Baseline predictors of a reduction in the carotid IMT were evaluated according to a logistic regression analysis, and the odds ratio (OR) and 95% CI were reported. We considered a probability value of less than 5% to reject the null hypothesis and accept a difference as significant. The statistical analysis was performed with the STATA® Ver. 12.1 software program (StataCorp LP, College Station, TX, USA).

Results

Cross-Sectional Study

The patients were divided into quartiles of the PUFA/SFA ratio in the RBC membrane. The baseline clinical and biochemical characteristics of the groups are summarized in **Table 1**. The carotid IMT (**Fig. 1**), cardiovascular risk score and plasma glucose level progressively decreased, while the HDL cholesterol level increased, across the RBC membrane PUFA/SFA ratio quartiles. No differences in age, gender, BMI, blood pressure, other plasma lipids, renal function parameters or drug therapy were observed across the PUFA/SFA quartiles.

The total and HDL cholesterol levels were found to be directly, while the plasma glucose level was found to be inversely, related to the total n-6 PUFA concentration ($r=0.285$, $p=0.033$; $r=0.585$, $p<0.001$; and $r=-0.380$, $p=0.004$, respectively), total PUFA con-

Table 2. Change in the variables according to the change in the polyunsaturated to saturated fatty acid ratio (Δ PUFA/SFA ratio) at the end of the follow-up period

Variable	All patients (n=56)		Δ− PUFA/SFA ratio (n=31)		Δ+ PUFA/SFA ratio (n=25)	
	Baseline	Follow-up	Baseline	Follow-up	Baseline	Follow-up
Age (years)	64±7	−	63±8	−	65±7	−
Male [n (%)]	27 (48)	−	10 (32)	−	17 (68)	−
CV risk score (%)	25±12	23±11*	21±11	19±9	29±12	27±11
BMI (Kg/m^2)	28.7±4.8	28.8±5.1	28.6±5.5	28.4±5.6	28.8±3.8	29.3±4.6
24-h SBP (mm Hg)	133±16	131±12	131±17	131±12	136±14	134±13
24-h DBP (mm Hg)	78±9	77±8	79±9	78±7	79±8	77±7
Total cholesterol (mmol/L)	5.67±1.02	5.99±1.25†	5.90±0.85	6.24±1.16*	5.39±1.14	5.70±1.32*
HDL cholesterol (mmol/L)	1.30±0.33	1.44±0.45‡	1.42±0.34	1.58±0.52†	1.14±0.23	1.27±0.26*
LDL cholesterol (mmol/L)	3.70±0.85	3.89±1.11	3.88±0.67	4.09±1.11	3.47±0.98	3.63±1.09
Triglycerides (mmol/L)	1.45±0.77	1.44±1.06	1.29±0.56	1.21±1.12	1.65±0.94	1.73±0.93
Plasma glucose (mmol/L)	5.60±1.17	5.40±0.90	5.38±1.00	5.27±0.89	5.83±1.33	5.49±0.89
eGFR (mL/min/1.73 m^2)	75±14	84±19‡	76±11	87±18‡	73±16	80±20*
α1-blockers [n (%)]	7 (13)	−	2 (6)	−	5 (20)	−
β-blockers [n (%)]	19 (34)	−	9 (29)	−	10 (40)	−
Diuretics [n(%)]	19 (34)	−	8 (26)	−	11 (44)	−
CCBs [n (%)]	10 (18)	−	2 (6)	−	8 (32)	−
ACE-i [n (%)]	5 (9)	−	1 (3)	−	4 (16)	−
ARBs [n (%)]	29 (52)	−	16 (52)	−	13 (52)	−
Statins [n (%)]	10 (18)	−	7 (23)	−	3 (12)	−
Antiplatelet [n (%)]	5 (9)	−	3 (10)	−	2 (8)	−
SFA (% weight)	47±6	47±5	43±4	50±3‡	52±4	44±4‡
PUFA (% weight)	33±5	32±5	36±4	29±4‡	28±4	35±5‡
PUFA/SFA ratio	0.72±0.19	0.69±0.19	0.85±0.13	0.58±0.11‡	0.55±0.11	0.82±0.18‡
MUFA (% weight)	20±3	21±2	21±3	22±2	20±3	21±2
n-3 PUFA (% weight)	5.9±1.8	5.8±2.3	6.8±1.6	4.9±1.9‡	4.8±1.4	6.9±2.2†
n-6 PUFA (% weight)	27±4	26±4	29±3	24±4‡	24±3	28±4‡
n-6/n-3 PUFA	4.9±1.4	5.4±3.1	4.6±1.4	5.0±1.4	5.1±1.2	4.2±1.0*
Carotid IMT (mm)	0.96±0.22	0.86±0.20†	0.90±0.20	0.90±0.18	1.01±0.21	0.81±0.19‡

The data are expressed as the mean±SD, unless otherwise indicated. The analysis was performed using two-way ANOVA for repeated measurements with the Bonferroni correction to account for multiple comparisons. The estimated glomerular filtration rate (eGFR) was calculated using the MDRD formula. CV, cardiovascular risk score, according to the Framingham Heart Study; 24h SBP, mean 24-hour systolic blood pressure; 24h DBP, mean 24-hour diastolic blood pressure; CCB, calcium channel blockers; ACE-i, angiotensin-converting enzyme inhibitors; ARBs, angiotensin receptor blockers; SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; n-3, polyunsaturated fatty acids of the n-3 family; n-6, polyunsaturated fatty acids of the n-6 family; IMT, intima-media thickness.
*$p<0.05$, †$p<0.01$, ‡$p<0.001$, with respect to the corresponding baseline value

centration (r=0.272, p=0.043; r=0.558, $p<0.001$; and r=−0.370, p=0.005, respectively) and PUFA/SFA ratio (r=0.265, p=0.048; r=0.580, $p<0.001$; and r=−0.298, p=0.026, respectively). The HDL cholesterol level was also related directly with the total n-3 PUFA concentration (r=0.359, p=0.007) and inversely with the SFA concentration (r=−0.492, $p<$ 0.001). Meanwhile, the carotid IMT was found to be inversely related with the total n-6 PUFA concentration (r=−0.385, p=0.003), total PUFA (r=−0.357, p=0.007) and PUFA/SFA ratio (r=−0.325, p=0.015), a relationship that, according to the multivariate linear regression analysis, was independent of age, gender, BMI, 24-hour ambulatory systolic blood pressure, total cholesterol, HDL cholesterol and fasting plasma glucose (the details for the univariate and multivariate analyses are shown in **Tables 1** and **2** of *Appendix D* of the online supplemental material). In addition, the relationships between the HDL cholesterol level and the n-3 PUFA (β=0.338; p=0.011), n-6 PUFA (β=0.506; $p<0.001$) and total PUFA concentrations (β=0.490; $p<0.001$), PUFA/SFA ratio (β=0.499; $p<$

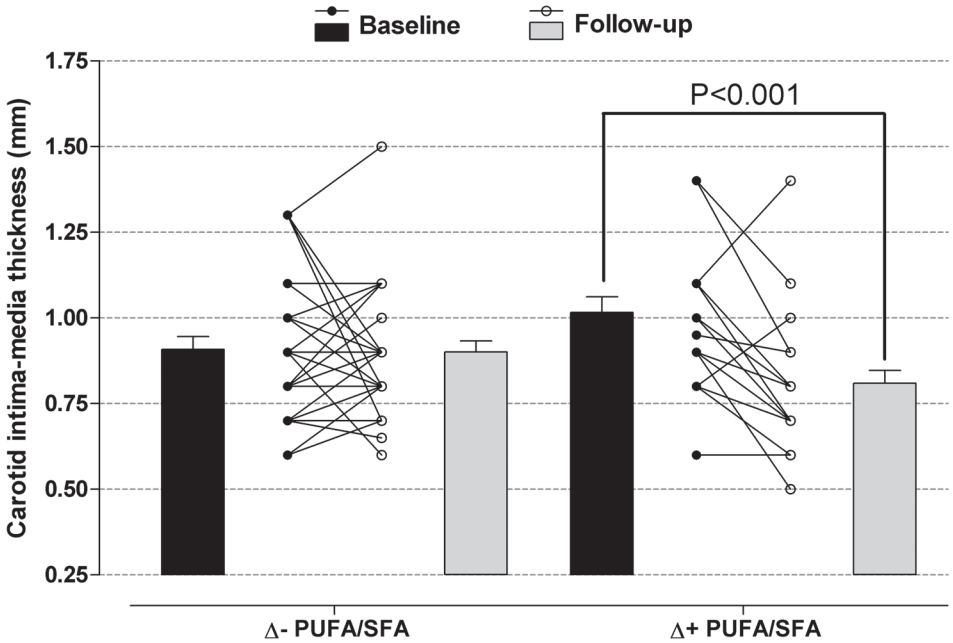

Fig. 2. Changes in the carotid intima-media thickness in the patients with hypertension who did (Δ+ PUFA/SFA) or did not (Δ− PUFA/SFA) exhibit an increased red blood cell membrane polyunsaturated to saturated fatty acids ratio after receiving nutritional counseling with supplementation of three weekly meals of fish. The measurements were obtained at baseline and after one year of follow-up. The comparisons were made using two-way ANOVA for repeated measurements with the Bonferroni correction to account for multiple comparisons. The bars represent the mean ± SEM.

0.001) and SFA concentration ($\beta = -0.409$; $p = 0.001$) were independent of the other cardiovascular risk factors.

Longitudinal Study

The patient characteristics and fatty acid composition of the RBC membrane at baseline and after one year of follow-up are shown in **Table 2** (the analytical data for each fatty acid are reported in *Appendix E* of the online supplemental material). The carotid IMT and cardiovascular risk score decreased in the hypertensive patients after the dietary intervention, whereas the total and HDL cholesterol levels increased. The hypertensive patients were divided in two groups: those in whom the PUFA/SFA ratio in the RBC membrane increased with dietary fat modification (Δ+ PUFA/SFA; $n = 25$) and those in whom the PUFA/SFA ratio either did not change or decreased (Δ− PUFA/SFA; $n = 31$). In the former group, the decrease in the total SFA concentration and increase in both the n-6 and n-3 PUFA concentrations explained the change in the PUFA/SFA ratio. The n-6/n-3 PUFA ratio decreased significantly in Δ+ PUFA/SFA patients, indicating a relatively greater enrichment in the n-3 type content that remained unchanged in the Δ− PUFA/SFA patients.

Following nutritional counseling, the total and HDL cholesterol levels increased and the renal function improved significantly, independent of changes in the RBC membrane fatty acid composition. Conversely, a significant decrease in the carotid IMT was observed only in the patients with an increased PUFA/SFA ratio in the RBC membrane after the dietary intervention (**Fig. 2**). Considering all patients, the changes in carotid IMT were inversely related to the changes in the PUFA/SFA ratio (**Fig. 3**); this relationship was independent ($\beta = -0.328$; $p = 0.009$) of the changes in blood pressure, BMI, total and HDL cholesterol, triglycerides, glucose and renal function parameters (mean carotid IMT reduction adjusted for confounders −0.21 mm; 95% CI from −0.34 to −0.08 mm; $p < 0.001$).

After the dietary intervention, the PUFA/SFA ratio in the RBC membrane decreased in the patients with an increased carotid IMT (from 0.82 ± 0.17 to 0.59 ± 0.13; $p < 0.001$), whereas the same ratio increased in the patients with a reduced IMT (from 0.65 ± 0.18 to 0.76 ± 0.20; $p = 0.022$). The proportion of patients with an increased RBC membrane PUFA/SFA ratio

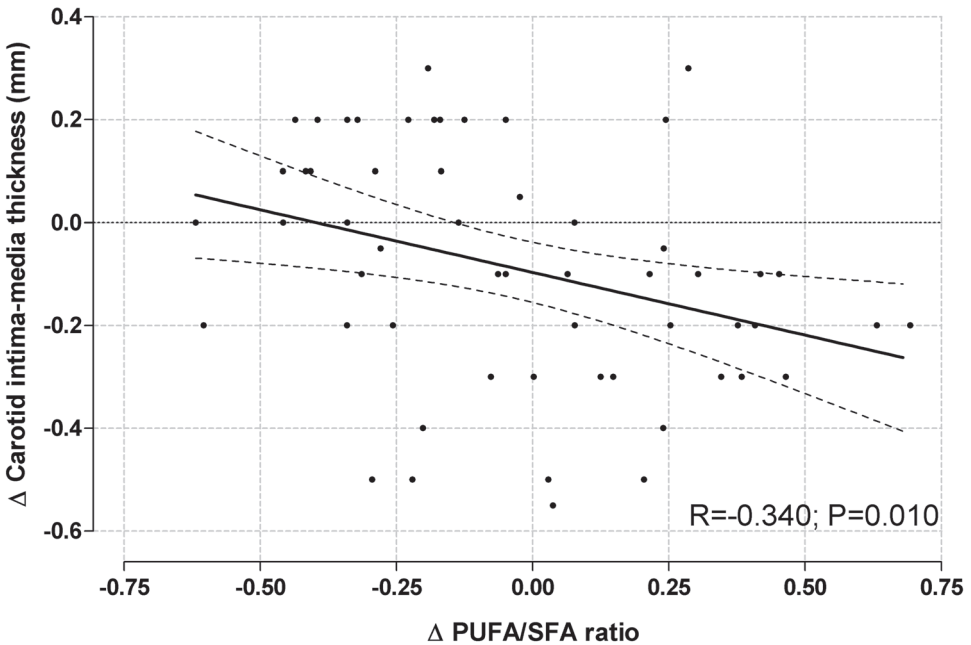

Fig. 3. Scatterplot and linear regression analysis with 95% CI of the changes (Δ) in the carotid intima-media thickness and changes (Δ) in the polyunsaturated to saturated fatty acids ratio (PUFA/SFA) in the red blood cell membrane after one year of dietary intervention with nutritional counseling and fish meal supplementation.

(**Fig. 4A**) or reduced carotid IMT (**Fig. 4B**) after the dietary intervention decreased progressively in association with an increasing PUFA/SFA ratio at baseline. In a logistic regression analysis including age, gender and baseline BMI, 24-hour systolic blood pressure, total and HDL cholesterol, triglycerides, glucose, eGFR and drug therapy, the baseline RBC membrane PUFA/SFA ratio quartile was found to be an independent predictor of a dietary-induced increase in the PUFA/SFA ratio in the RBC membrane and a decrease in the carotid IMT (**Table 3**).

Discussion

The findings of this study demonstrate that the fatty acid composition of RBC membranes, as assessed according to the PUFA/SFA ratio, is inversely and independently related to the carotid IMT in hypertensive patients without evidence of clinically relevant cardiovascular complications. Nutritional counseling with regular consumption of three weekly meals of fish containing elevated amounts of PUFA for one year significantly decreased the carotid IMT only in those patients in whom the PUFA/SFA ratio in the RBC membrane increased, indicating that the composition of dietary fat affects early markers of hypertensive vascular damage.

Clinical studies of dietary interventions are often biased due to inadequate compliance of the involved subjects to the dietary prescriptions. With regard to dietary fat composition, it has been shown that this parameter can be reliably assessed based on measurements of the concentrations of fatty acids in the plasma or membranes of circulating cells[7, 8]. Measuring the fatty acid composition in the RBC membrane has some advantages over evaluating the plasma levels, as, due to the long lifespan of these cells, this parameter reflects the long-term exposure of the cells to circulating lipids[22]. Moreover, the fatty acid composition of the RBC membranes reflects the fatty acid composition in other cell types, including cardiomyocytes[23], and has been used in past interventional studies to demonstrate the protective effects of PUFA on the cardiovascular system[24]. In fact, modification of the fatty acid composition of cell membranes is critical for the effects of PUFA on the cardiovascular system, inasmuch as an increased PUFA level results in a reduction in vascular proinflammatory and prothrombotic responses[25]. In this study, we chose to measure the fatty acid composition of RBC membranes in order to verify compliance with the dietary prescription of our hypertensive patients because this issue is critical in all studies involving long-lasting dietary manipulations. After one year, we observed an increase

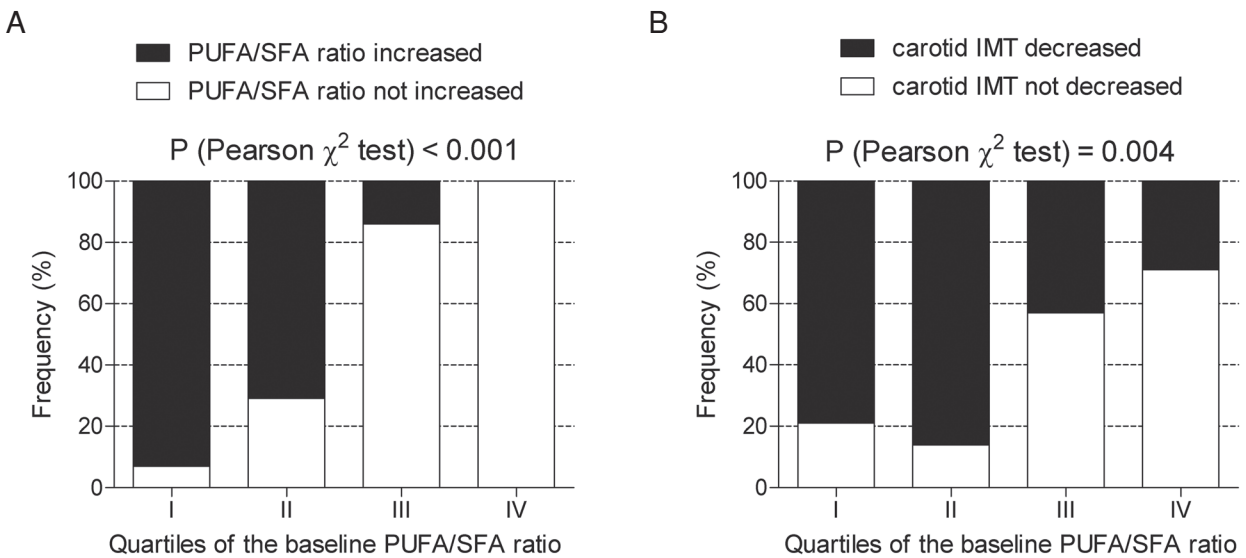

Fig. 4. Bar graph showing the percentage of patients with an increased (A) polyunsaturated to saturated fatty acid (PUFA/SFA) ratio in the red blood cell membrane and a decreased (B) carotid intima-media thickness (IMT) after dietary intervention according to the quartiles of the baseline PUFA/SFA ratio. The analysis was performed according to the Pearson χ^2 test.

in the PUFA/SFA ratio in only 45% of our uncomplicated hypertensive subjects, and only in these patients did we find a significant decrease in the carotid IMT. Various reasons may explain the low rate of success of nutritional counseling in increasing the RBC membrane PUFA/SFA ratio, including differences in the baseline dietary habits of the patients in terms of the consumption of fish or other foods rich in PUFAs, leading to saturation of some fatty acids in cell membranes[26]. This hypothesis is suggested by the observation that the baseline RBC membrane PUFA/SFA ratio was found to be the strongest predictor of both an increase in the PUFA/SFA ratio and a decrease in the IMT obtained with fish meals. However, the food frequency questionnaires collected at the beginning of the study did not show any substantial differences between the "responders" and "non-responders" to the dietary intervention. Another possibility is the low compliance of some patients to the dietary prescription during the study or interference of other variables that may affect lipid metabolism. Although the food frequency questionnaires and physical activity surveys collected during the study did not show any substantial differences between the "responders" and "non-responders," this possibility cannot be excluded.

In this study, we demonstrated a beneficial effect of enriching cell membranes with both n-3 and n-6 PUFA on carotid IMT reduction, although the changes in the n-6/n-3 PUFA ratio observed in the patients with a decreased IMT following regular consumption of fish meals indicates the major relevance of the n-3 type. While the protective effects of n-3 PUFA on the cardiovascular system are well established[25], the effects of n-6 PUFA are under debate. Because arachidonic acid, the metabolic precursor of a variety of proinflammatory molecules, is an n-6 PUFA, these fatty acids have traditionally been considered to be potentially detrimental to the cardiovascular system. However, experimental evidence indicates that n-6 PUFA have anti-inflammatory, lipid-lowering and insulin-sensitizing actions[27], and clinical studies have demonstrated that dietary replacement of SFA or refined carbohydrates with n-6 PUFA can reduce cardiovascular risks[27, 28]. Based on this evidence, the American Heart Association suggests a daily n-6 PUFA intake of at least 5% of total dietary calories[27].

The carotid IMT is an early marker of atherosclerotic damage that precedes plaque formation and is a good predictor of coronary and cerebrovascular events in hypertensive patients[9]. In agreement with population studies that have measured the PUFA/SFA ratio in the plasma[10], the PUFA/SFA ratio in the RBC membrane was inversely related to the carotid IMT in our hypertensive patients. In addition, and most importantly, we found a significant decrease in the carotid IMT only in the patients in whom the RBC membrane PUFA/SFA ratio increased following regular consumption of fish meals. In these patients, the decrease in the carotid IMT was independent of other confounders, including major cardiovascular risk

Table 3. Multivariate logistic regression analysis of predictors of an increase in the PUFA/SFA ratio (A) and a decrease in the carotid IMT (B) after the dietary intervention

(A) PUFA/SFA ratio increase in the red blood cell membrane (yes=1/no=0)

Baseline predictor	Odds Ratio	95% Confidence Interval	*p*
Age (years)	1.102	0.931-1.305	0.260
Male sex (yes=1/no=0)	63.407	0.467-8604	0.098
BMI (Kg/m^2)	0.877	0.528-1.457	0.613
24-h SBP (mm Hg)	0.950	0.863-1.045	0.292
Total cholesterol (mmol/L)	0.682	0.148-3.135	0.623
HDL cholesterol (mmol/L)	101.19	0.003-3446314	0.386
Triglycerides (mmol/L)	4.230	0.472-37.88	0.197
Plasma glucose (mmol/L)	0.781	0.204-2.989	0.719
eGFR (mL/min/1.73 m^2)	0.995	0.850-1.165	0.952
PUFA/SFA ratio (quartile)	0.007	0.0002-0.285	0.009
Statins use (yes=1/no=0)	0.090	0.0003-28.97	0.414

(B) Carotid IMT reduction (yes=1/no=0)

Baseline predictor	Odds Ratio	95% Confidence Interval	*p*
Age (years)	1.672	1.128-2.480	0.011
Sex male (yes=1/no=0)	4.432	0.090-218.03	0.454
BMI (Kg/m^2)	0.920	0.662-1.277	0.618
24-h SBP (mm Hg)	1.000	0.904-1.107	0.997
Total cholesterol (mmol/L)	4.700	0.678-32.57	0.117
HDL cholesterol (mmol/L)	0.384	0.0001-1326	0.818
Triglycerides (mmol/L)	0.301	0.022-4.204	0.373
Plasma glucose (mmol/L)	0.055	0.005-0.657	0.022
eGFR (mL/min/1.73 m^2)	1.037	0.892-1.206	0.632
PUFA/SFA ratio (quartile)	0.003	0.00002-0.466	0.024
β-blockers use (yes=1/no=0)	0.124	0.003-5.795	0.287
Diuretics use (yes=1/no=0)	0.0003	4.10e^{-08}-1.941	0.070
CCBs use (yes=1/no=0)	0.020	5.86e^{-06}-71.24	0.350
ACE-i/ARBs use (yes=1/no=0)	0.764	0.039-14.62	0.858
Statins use (yes=1/no=0)	0.619	0.010-38-72	0.820
Antiplatelet drug use (yes=1/no=0)	18.098	0.003-108054	0.514

The PUFA/SFA ratios in the red blood cell membrane and carotid IMT were included as the dependent variable. The estimated glomerular filtration rate (eGFR) was calculated according to the MDRD formula. PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; IMT, intima-media thickness; 24-h SBP, average 24-hour systolic blood pressure; CCB, calcium channel blockers; ACE-i, angiotensin-converting enzyme inhibitors; ARBs, angiotensin receptor blockers.

factors and the use of antihypertensive agents and/or statins, suggesting that an effective increase in PUFA consumption is beneficial with respect to early markers of atherosclerotic vascular damage. These findings are consistent with those of past studies demonstrating that dietary interventions with a reduced intake of saturated fat and increased intake of unsaturated fat decrease the carotid IMT in patients with high cardiovascular risks[29] and postmenopausal women[30], although the decrease in IMT appears to be mediated by a weight loss-related decline in blood pressure[31]. In the PREDIMED study[29], Murie-Fernandez *et al.* reported the beneficial effects of a Mediterranean diet on the carotid IMT only in those patients with higher baseline IMT values. Consistent with this observation, in our patients, the baseline carotid IMT value was found to be associated with a greater decrease in the IMT after the dietary intervention. The present prospective study is the first to suggest that dietary counseling with fish meal supplementation may reverse the IMT values in hypertensive patients without affecting either the BMI or blood pressure levels.

However, this study has limitations that should be addressed. First, this is an observational study that lacked a predefined control group treated without dietary intervention. Because the aim was to investigate the effects of changes in the membrane fatty acid composition induced by the regular consumption of fish meals on the carotid IMT, the patients in whom the membrane PUFA/SFA ratio did not increase were treated as a post-hoc control group. Second, the size of the patient sample may have limited the power of the study to detect additional relationships between RBC membrane fatty acids and plasma lipid variables. Third, the use of antihypertensive agents may have affected the carotid IMT changes observed in association with fish meal consumption, although all drugs were maintained unchanged across the study period, and no differences were found between the patients treated with different agents. Fourth, in this study we did not measure specific markers of inflammation, such as high-sensitivity C-reactive protein, that may be involved in the progression of vascular damage in patients with cardiovascular conditions.

In conclusion, this study demonstrated that the fatty acid content of the RBC membrane is independently related to the carotid IMT and HDL cholesterol level in patients with well-controlled hypertension and no cardiovascular complications. Nutritional counseling with regular consumption of fish meals was found to decrease the carotid IMT only in those patients who exhibited significant changes in membrane fatty acid composition with an increase in the n-3 and n-6 PUFA content. These findings provide evidence that hypertensive vascular damage may benefit from changes in dietary fatty acid composition, even at the earliest stages of disease, supporting the hypothesis that the consumption of PUFA-enriched foods may improve cardiovascular outcomes. Nevertheless, this hypothesis must be tested in larger interventional studies with a longer follow-up period and appropriate control of compliance with the dietary prescriptions.

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Conflicts of Interest

All authors disclose no financial relationships (within the past 24 months) with biotechnology manufacturers, pharmaceutical companies or other commercial entities with an interest in the subject matter or materials discussed in this manuscript. The local fish producer was not involved in the phases of study proposal, design, implementation, analysis or presentation. Furthermore, the authors did not receive any financial incentives in relation to the producer.

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Appendix A

Nutritional table of one serving (100 g) of fish meal (trout)

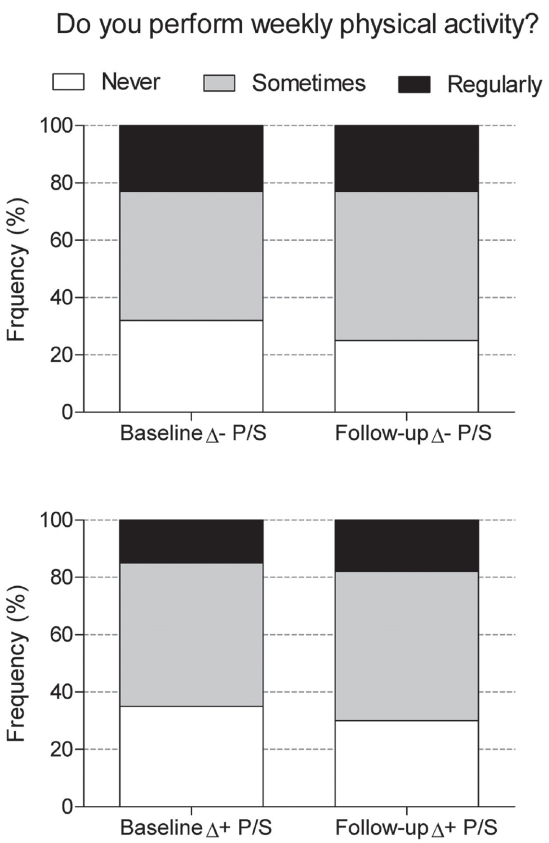
Total energy (Kcal)	195
Protein (g)	24.6
Carbohydrates (g)	<0.7
Fat (g)	10.7
➢ Saturated (g)	3.1
➢ Monounsaturated (g)	3.6
➢ Polyunsaturated (g)	4.0
➢ *n*-3 PUFA (g)	2.4
Cholesterol (mg)	60
Sodium (g)	0.87
Fiber (g)	<0.5

Appendix B

Table 1. Food Frequency Questionnaire results in portions or drinks per month

Type of food	All patients		Δ− PUFA/SFA (n=31)		Δ+ PUFA/SFA (n=25)	
	Baseline	Follow-up	Baseline	Follow-up	Baseline	Follow-up
Pasta	15 [12-18]	16 [12-20]	15 [10-16]	16 [12-20]	16 [12-18]	16 [12-20]
Bread	48 [30-60]	60 [30-60]	45 [28-60]	60 [12-60]	48 [30-60]	60 [32-60]
Rice	6 [4-10]	8 [4-9]	8 [4-10]	8 [4-12]	4 [4-6]	5 [4-8]
Milk	23 [0-30]	30 [0-30]	22 [0-30]	19 [0-30]	24 [12-30]	30 [23-30]
Cheese	12 [8-17]	9 [4-16]	12 [8-16]	8 [2-28]	12 [8-17]	11 [8-12]*
Meat	12 [8-12]	12 [8-12]	12 [8-16]	12 [8-12]	12 [12-12]	12 [8-12]
Cold cut	7 [4-8]	8 [3-10]	7 [4-8]	6 [2-9]	8 [4-8]	9 [4-12]
Eggs	4 [3-8]	4 [3-5]	7 [4-8]	4 [1-8]†	4 [3-4]	4 [4-5]
Fish	11 [8-12]	12 [12-13]‡	10 [8-12]	12 [12-12]*	12 [8-12]	12 [12-13]‡
Vegetables	61 [54-65]	63 [57-68]	60 [40-64]	63 [60-64]	62 [60-65]	62 [52-68]
Fruits	60 [30-61]	59 [30-60]	60 [30-61]	56 [30-60]	60 [59-60]	60 [40-90]
Confectionery	12 [3-15]	6 [2-12]†	12 [2-15]	4 [0-14]*	12 [4-16]	7 [2-8]†
Sugar drink	0 [0-3]	0 [0-4]	0 [0-7]	0 [0-6]	0 [0-7]	1 [0-4]
Coffee	62 [30-90]	60 [38-90]	64 [30-90]	70 [60-90]	60 [30-90]	60 [30-90]
Alcohol	20 [3-60]	29 [0-60]	12 [0-24]	16 [0-60]	39 [9-60]	29 [8-62]

The patients were subdivided according to the presence (Δ+)/absence(Δ−) of an increased polyunsaturated to saturated fatty acids ratio (Δ PUFA/SFA ratio) in the red blood cell membrane at the end of the follow-up period. The data are expressed as the median [interquartile range]. The statistical analysis was performed using the non-parametric Wilcoxon signed-rank test.
*$p<0.05$, †$p<0.01$, ‡$p<0.001$ vs. baseline.



Appendix C

Measurement of Fatty Acids in the Red Blood Cell Membrane

Seven mL of whole blood was collected in EDTA-K_3 and immediately centrifuged at 4,000 rpm for five minutes at 4℃. The supernatant and buffy coat were discarded, and the red blood cells were washed three times with a 7.4 pH phosphate-buffered saline (PBS) solution with the following composition: 137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na_2HPO_4, 2.0 mmol/L KH_2PO_4. Lipid extraction from the red blood cell membranes was then performed with a chloroform/methanol mixture in accordance with Folch's method[1]. Twenty mL of chloroform/methanol (2/1) was added to one mL of washed erythrocytes, and the mixture was agitated in an orbital shaker for 20 minutes at room temperature. The liquid phase was recovered via paper filtration and washed with 4 mL of a 0.9% NaCl solution. Following vortexing and centrifugation, the lower chloroform phase with the lipid extract was collected, and the solvent was evaporated at 40℃ under a nitrogen stream. The extract was dissolved in 1 mL of toluene with 50 ppm of the antioxidant butylated hydroxytoluene (BHT) and stored at −80℃ until the GC analysis.

The analysis of the fatty acid composition of the red blood cell membranes was performed after acid-catalyzed transmethylation of the lipid extract in order to obtain fatty acid methyl-esters (FAMEs) suitable for the GC analysis[2]. Two mL of 1% sulphuric acid in methanol was added to the 1 mL lipid extract solution in a stoppered tube left at 50℃ overnight. The mixture was then washed with 5 mL of 5% NaCl solution, and the FAMEs were extracted with n-hexane. Finally, the solvent hexane was removed via evaporation at 40℃ in a stream of nitrogen, and the extracted FAMEs were reconstituted in 100 μL of n-heptane for the GC analysis.

The GC analysis was performed using a 2-μL sample of n-heptane reconstituted FAMEs with a high polar capillary column (0.53-mm internal diameter and 1-μm film thickness) in which the phase was a nitroterephthalic acid-modified, chemically bonded, polyethylene glycol polymer (CP-Wax 58 FFAP CB, Varian Inc., Milan, Italy). The GC analyzer (GC 3300, Varian Inc., Milan, Italy) was provided with an on-column injector and a flame ionized detector (FID) heated at 220 and 280℃, respectively. The column heating program was as follows: 170℃ for one minute, from 170 to 200℃ at 10℃/min. and from 200 to 250℃ at 2℃/min. up to the end of the run. The carrier gas was helium, and the run time for each sample was approximately 30 minutes. We used nonadecanoic acid (C19:0) as the internal standard added to the lipid extract prior to transmethylation. Resolved peaks were identified using pure reference compounds in a standard mix or natural extract (cod liver oil), and peak's area was measured with an electronic integrator. The fatty acid composition of the red blood cell membrane was expressed as the weight percent (peak area percentage of the total fatty acid peak area). A single fatty acid was reported in the table as the number of carbon molecules separated by the number of double bonds present in the molecule, with the family type specified when appropriate. The PUFA/SFA ratio was then calculated. All chemicals were purchased from Sigma-Aldrich (Milan, Italy), and all solvents were of HPLC grade.

Ambulatory Blood Pressure Monitoring

The TM-2430 device (A&D Company Limited, Tokyo, Japan) has been previously validated for ambulatory blood pressure monitoring according to the 1990 and 1993 British Hypertension Society protocols[3]. The device was programmed to record blood pressure every 15 minutes during the day (8.00 am to 10.00 pm) and every 30 minutes during the night (10.00 pm to 8.00 am). Only recordings with more than 80% of valid readings were considered acceptable, and non-valid recordings were repeated the following day. The mean systolic and diastolic blood pressure value during the 24-hour monitoring period was used for the statistical analysis.

Carotid Artery Intima-Media Thickness (IMT)

The right and left carotid arteries were examined with a duplex scanner using a 7-MHz linear array transducer. The carotid IMT and longitudinal diameter were measured with an electronic caliper on the far wall of the distal segment of the common carotid arteries frozen in end-diastole, 1.0 cm proximal to the beginning of the carotid bulb. The IMT was defined as the distance between the leading edge of the lumen-intima interface and the leading edge of the media-adventitia interface. The carotid longitudinal diameter was defined as the distance between the far and near leading edge of the media-adventitia echo. The mean value of three carotid IMT and longitudinal diameter measurements obtained in wall segments free from plaque was calculated.

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Appendix D

Table 1. Univariate correlation analysis of the cross-sectional data

Fatty Acids	IMT		Age		BMI		24h SBP		Total Cholesterol		HDL Cholesterol		Plasma Glucose	
	R	P	R	P	R	P	R	P	R	P	R	P	R	P
SFA	0.249	0.064	0.058	0.669	0.186	0.170	0.173	0.202	−0.187	0.167	−0.492	<0.001	0.217	0.108
PUFA	−0.357	0.007	−0.026	0.869	−0.125	0.360	−0.247	0.066	0.272	0.043	0.558	<0.001	−0.370	0.005
PUFA/SFA ratio	−0.325	0.015	−0.042	0.761	−0.155	0.255	−0.224	0.098	0.265	0.048	0.580	<0.001	−0.298	0.026
MUFA	0.163	0.230	0.170	0.210	−0.086	0.528	0.019	0.888	−0.163	0.229	−0.013	0.922	0.030	0.828
n-3 PUFA	−0.206	0.128	0.150	0.270	−0.207	0.128	−0.255	0.058	0.175	0.197	0.359	0.007	−0.254	0.059
n-6 PUFA	−0.385	0.003	−0.098	0.471	−0.073	0.591	−0.216	0.110	0.285	0.033	0.585	<0.001	−0.380	0.004

A correlation analysis of the groups of fatty acids, cardiovascular risk factors and carotid intima-media thickness (IMT) values at baseline. The table shows Pearson's "R" coefficient and the probability (P). 24h SBP: mean 24-hour systolic blood pressure; SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; n-3, polyunsaturated fatty acids of the n-3 family; n-6, polyunsaturated fatty acids of the n-6 family.

Table 2. Multivariate analysis of the cross-sectional data with the carotid intima-media thickness (IMT) as the dependent variable

Fatty Acids		Male gender	Age	BMI	24h SBP	Total Cholesterol	HDL Cholesterol	Plasma Glucose
	β; P	β; P	β; P	β; P	β; P	β; P	β; P	β; P
SFA	0.248; 0.086	−0.087; 0.553	0.425; 0.001	0.252; 0.053	0.132; 0.322	0.237; 0.089	−0.076; 0.632	0.102; 0.439
PUFA	−0.484; 0.002	−0.097; 0.476	0.459; <0.001	−0.240; 0.046	0.088; 0.480	0.260; 0.046	0.052; 0.733	0.003; 0.981
P/S	−0.418; 0.007	−0.083; 0.555	0.445; <0.001	−0.254; 0.041	0.095; 0.456	0.256; 0.056	0.034; 0.829	0.053; 0.680
n-3	−0.319; 0.026	−0.049; 0.740	0.497; <0.001	−0.265; 0.039	0.083; 0.534	0.276; 0.047	−0.080; 0.586	0.078; 0.547
n-6	−0.489; 0.002	−0.137; 0.318	0.419; 0.001	−0.217; 0.071	0.120; 0.328	0.241; 0.064	0.057; 0.710	−0.004; 0.974

A multivariate linear regression analysis of the dependent variable IMT and independent variables fatty acids and cardiovascular risk factors. 24h SBP: mean 24-hour systolic blood pressure; P/S, polyunsaturated to saturated fatty acids ratio; n-3, polyunsaturated fatty acids of the n-3 family; n-6 polyunsaturated fatty acids of the n-6 family; β, standardized coefficient.

Appendix E

Table 1. Changes in the fatty acid composition of red blood cell membrane after the dietary intervention

Fatty acids (% weight)	Δ− PUFA/SFA (n=31)		Δ+ PUFA/SFA (n=25)	
	Baseline	Follow-up	Baseline	Follow-up
14:0	0.3 ± 0.2	0.3 ± 0.1	0.4 ± 0.2	0.3 ± 0.1
16:0	25.0 ± 3.1	27.6 ± 2.5*	30.0 ± 4.2	25.0 ± 2.5‡
18:0	15.9 ± 1.2	17.5 ± 1.6†	17.6 ± 1.3	15.0 ± 1.8‡
20:0	0.3 ± 0.3	0.4 ± 0.3	0.5 ± 0.5	0.5 ± 0.3
22:0	0.5 ± 0.3	1.5 ± 0.4‡	0.7 ± 0.4	0.9 ± 0.4
24:0	1.3 ± 1.0	3.1 ± 1.1‡	1.6 ± 0.9	2.4 ± 1.0
16:1	0.4 ± 0.3	0.4 ± 0.2	0.5 ± 0.4	0.4 ± 0.2
18:1 n-9	16.4 ± 1.9	15.4 ± 2.2	15.6 ± 2.1	15.0 ± 1.6
18:1 n-7	1.8 ± 0.6	2.1 ± 0.6	1.6 ± 0.6	2.2 ± 0.5†
20:1	0.4 ± 0.3	0.2 ± 0.2	0.7 ± 0.9	0.4 ± 0.3
24:1	2.0 ± 3.4	3.7 ± 1.1*	1.4 ± 2.0	2.9 ± 1.0
18:2 n-6	10.8 ± 1.6	8.5 ± 1.5‡	9.6 ± 1.5	10.3 ± 2.0
20:3 n-6	1.7 ± 0.4	1.9 ± 1.2	1.5 ± 0.4	2.3 ± 2.2
20:4 n-6	13.9 ± 2.3	11.7 ± 3.2†	10.7 ± 2.9	13.2 ± 2.3‡
22:4 n-6	2.5 ± 0.7	1.8 ± 0.6‡	1.7 ± 0.8	2.1 ± 0.5*
22:5 n-6	0.4 ± 0.5	0.4 ± 0.5	1.0 ± 0.9	0.3 ± 0.3‡
18:3 n-3	0.4 ± 0.5	0.6 ± 1.0	0.7 ± 0.5	1.1 ± 1.8
20:5 n-3	0.8 ± 0.3	0.6 ± 0.4	0.6 ± 0.3	0.8 ± 0.3
22:5 n-3	1.5 ± 0.5	0.9 ± 0.4‡	1.0 ± 0.5	1.4 ± 0.4†
22:6 n-3	4.1 ± 1.2	2.7 ± 1.6†	2.7 ± 1.4	3.6 ± 1.0*

The patients were subdivided according to the presence (Δ+)/absence(Δ−) of an increased polyunsaturated to saturated fatty acids ratio (Δ PUFA/SFA ratio) at the end of the follow-up period. The data are expressed as the mean ± SD. The analysis was performed using two-way ANOVA for repeated measurements with the Bonferroni correction to account for multiple comparisons.
*$p < 0.05$, †$p < 0.01$, ‡$p < 0.001$ vs. baseline