Associations between erythrocyte fatty acids and Mediterranean diet in Greek volunteers

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Abstract

Aim: The aim of the present study was to investigate the relation of erythrocyte fatty acids with the Mediterranean diet and traditional cardiovascular risk factors.

Patients and methods: Healthy volunteers were recruited (n=106) and erythrocyte fatty acid composition was determined by gas-liquid chromatography. Dietary variables were collected (FFQ and 24h recalls) and DXA body composition analysis was performed.

Results: In multiple linear regression models eicosapentaenoic (EPA), docosahexaenoic (DHA) and the Omega-3 index (% EPA+DHA), were positively related with age and the MedDietScore independent of sex, abdominal adiposity and energy/BMR. Moreover, EPA and DHA were positively related to fish consumption (r=0.438, P<0.001 and r=0.518, P<0.001, correspondingly) and the intake of representative food groups of the Mediterranean diet (i.e. legumes, fruits and vegetables). Oleic acid was positively related to HDL-cholesterol (r=0.309, P=0.002), LDL-cholesterol (r=0.243, P=0.01) and triacylglycerols (r=0.243, P=0.01). Saturated fatty acids were positively associated with insulin (r=0.196, P=0.05).

Conclusion: The results of our study generate hypothesis for candidate fatty acids to serve as targets in the diet-disease interplay.

Key words: Diet; Mediterranean diet; fatty acids; erythrocytes; omega-3 fatty acids

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1. Introduction

A cornerstone of lifestyle modifications for cardiovascular risk reduction is the adoption of a healthy dietary pattern, such as the Mediterranean Diet [1]. In this context, diet's macronutrient content and the type of ingested fat, in particular, is important as it affects plasma lipoproteins and cardiovascular risk [1]. However, inherent errors in the estimation of fat intake through traditional dietary tools based on self-report, underline the necessity of nutritional biomarkers [2].

Erythrocyte fatty acids constitute a good example of nutritional biomarker, as they reflect both endogenous metabolism and dietary fatty acid intake for a time interval of about 120 d, which corresponds to their life time [2]. They are considered a longer term biomarker than plasma fatty acids, for which perturbations are evident upon days or weeks [2]. Moreover, erythrocytes are incapable to de novo synthesize phospholipids, elongate or desaturate fatty acids [3]. This means that fatty acids are incorporated in erythrocytes by direct exchange of phospholipids from plasma lipoproteins [4] or are formed by acylation of lysophospholipids in the membrane or plasma [5].

Erythrocytes contain all major classes of fatty acids, i.e. saturated (SFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA). Much attention has been focused on the erythrocyte content of omega-3 fatty acids, eicosapentaenoic and docosahexaenoic acids (EPA and DHA, correspondingly) or their sum (% EPA+DHA), also known as the Omega-3 Index, which is characterized as an emerging cardiovascular risk factor [6].

Several studies have been conducted so far to investigate the factors that influence the different types of erythrocyte fatty acids, such as age, sex, adiposity, dietary intake, smoking habits and physical activity [2, 7]. Only one study has shown an association of circulating fatty acids with the Mediterranean diet, but the measurements were in plasma [8].

Given that the assessment of erythrocyte fatty acid determinants is crucial for the examination of diet-disease risk relationship, the present study aimed at testing the direction and magnitude of correlations between erythrocyte fatty acids, the adherence to the Mediterranean diet and traditional risk factors for cardiovascular disease. In order to test this hypothesis Healthy volunteers were recruited (n=106) and erythrocyte fatty acid composition was determined by gas-liquid chromatography. Dietary information was collected with both a food frequency questionnaire and dietary recalls and DXA body composition analysis were performed. In multiple linear regression models eicosapentaenoic (EPA), docosahexaenoic (DHA) and the Omega-3 index (% EPA+DHA), were positively related with age and the MedDietScore independent of sex, abdominal adiposity and energy/BMR. The observed positive relation of omega-3 fatty acids with the Mediterranean diet sheds light to the mechanisms of its beneficial cardioprotective effects.

2. Patients and Methods

2.1 Study population

One hundred and six subjects (48 men, 44±13 years) were studied. By the design of the study men were age- and BMI- matched to women. The exclusion criteria and the assessment of smoking habits, physical activity and anthropometric variables are described elsewhere [9]. It is noted that the participants did not use any food supplements, fish oils or drugs. The protocol was approved by the Bioethics Committee of Harokopio University and was in accordance with the Declaration of Helsinki (1989) of the World Medical Association. All participants gave their written informed consent.

2.2 Biochemical measurements

Serum glucose, triacylglycerols, total cholesterol and HDL cholesterol were determined enzymatically in fasting samples (ACE analyzer, Schiapparelli Biosystems, Inc, New Jersey, USA) using reagents from Alfa Wassermann (Woerden, The Netherlands). LDL cholesterol was calculated with the Friedewald Formula. Moreover, serum insulin levels were determined with a commercially available ELISA kit (Invitrogen).

2.3 Erythrocytes fatty acids determination

The exact procedure of erythrocytes isolation and extraction and methylation of fatty acids is described below. After an overnight fasting period antecubital venous blood was collected into 6 ml EDTA-containing vacutainer tubes. Plasma and RBC were separated by centrifugation (1500×g, 10 min). RBCs were washed 3 times with saline solution (0.9% NaCl), BHT was added (final concentration 0.11 mg/mL) and samples were then stored at -80oC. For the extraction of lipids 300 μL RBCs were haemolysed with equal volume of distilled water and suspended in 3.1 mL isopropanol. After vigorous shaking and incubation for 1 hour, 2 mL of chloroform were added. Subsequently vigorous shaking and incubation for 1 hour were performed and the samples were centrifuged at 500×g for 30min. The supernatant was transferred in a Teflon lined screw-capped tube and kept in -20oC until methylation [10].

For the preparation of fatty acids methyl esters (FAME), the solvent was evaporated under a stream of nitrogen, and 500 μL of acetyl chloride was slowly added under continuous stirring. Tubes were tightly closed with Teflon-lined caps and the RBC extracts were subjected to methanolysis at 90°C for 1.5 hour. The obtained FAME were extracted with hexane containing internal standard (methyl nonanoate, at a final concentration of 0.24 mg/mL) and BHT (final concentration of 0.11 mg/mL).

The fatty acids profile of erythrocyte lipids was determined by gas chromatography of fatty acids methyl esters (FAME) by means of an Agilent HP-6890 (Avondale, PA, USA) gas chromatograph equipped with flame ionization detector, split-splitless injector, and an HP 6890 autosampler,by injecting an aliquot (1μL) of erythrocyte FAME into the gas chromatograph at a split ratio 1:10. Separation of FAME was achieved on a SGE (Melbourne, Australia), BPX70 capillary column (60 m long, 0.25 mm internal diameter), coated with a 0.25-μm thick film of cyanopropyl silicone. Helium was used as the carrier gas at a flow rate of 0.8 mL/min, injector and detector were held at 230 and 290° C, respectively. The oven temperature program was: 130-220 °C at2 °C/min, held for 7 min and finally 220–250 °C at 20° C/ min where it was held for 6.5 min. Peaks identification was accomplished by means of a standard mixture of 37 FAME (Sigma L9405, St Louis, MO, USA). The FAME standard was injected periodically to record slight changes in retention times, while it additionally served for the calculation of response factors, which were applied to the areas derived from the chromatographic traces. Of 46 fatty acids identified, the 34 that comprised >0.01% of total fatty acids are reported [11]. In the current work trans isomers are not included. The omega-3 index was calculated as the sum of erythrocyte EPA and DHA [6].

2.4 Anthropometry and body composition

Weight, height as well as waist and hip circumferences were measured as previously described [9]. Body composition was assessed by DXA (Lunar, Corporation, Brussels, Belgium). Besides the standard body composition analysis, for each subject a "region of interest" (ROI) representing abdominal fat was manually defined as a quadrilateral box around the L1-L4 area [9].

2.5 Dietary assessment

Two non-consecutive multiple-pass 24h-recalls were collected. Energy and macronutrient intake were assessed with the Nutritionist Pro™ software (Axxya Systems, Stafford, TX) expanded with analyses of local food items [12]. Moreover, a semi-quantitative FFQ (150 items), developed in our Institution for the study purposes, was administered. The adoption of the Mediterranean diet was assessed a priori with the use of the MedDietScore [13], which includes the following food categories: fruit, vegetables, potatoes, non refined cereals, fish, legumes, olive oil, alcohol, as well as red meat, poultry and full-fat dairy products (reverse scale). The score range is 0-55, with higher values indicating greater adherence to the Mediterranean diet.

2.6 Statistical analysis

Normality was tested with the Kolmogorov-Smirnoff criterion. Normally distributed continuous variables

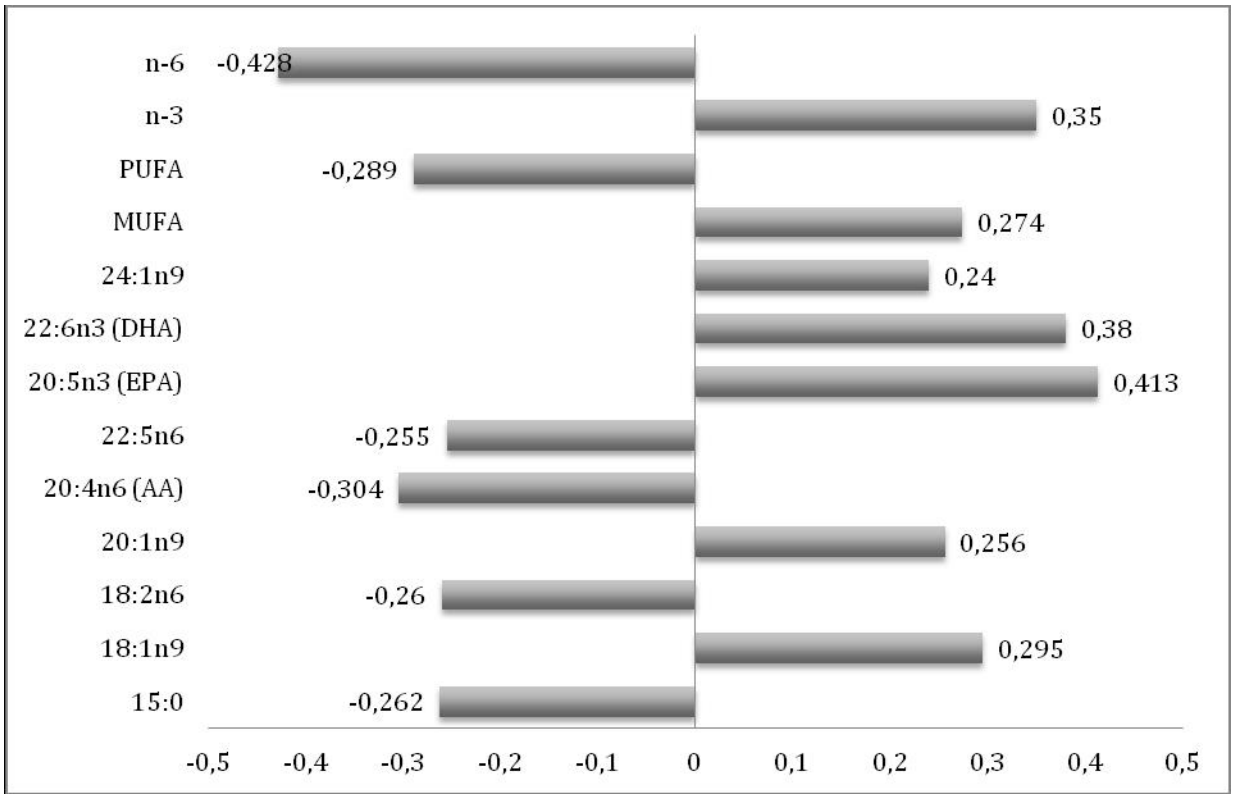

Figure 1: *Spearman or Pearson correlation coefficients of selected erythrocyte fatty acids with age. Pearson correlation coefficients are shown for 20:4n6 (AA), 20:5n3 (EPA), 22:6n3 (DHA), 24:1n9, MUFA, PUFA, n-3 and n-6. Spearman correlation coefficients are shown for 18:1n9 and18:2n6. All the correlations shown are significant (P<0.05). The rest fatty acids were not correlated with age.*

are presented as mean values± standard deviation, while skewed variables as median and 25th -75th quartiles. Categorical variables are presented as relative frequencies (%). T-test or Mann-Whitney test was applied for comparisons of parametric/log-transformed or non-parametric variables, respectively. In case that raw data included a high percentage of zero values (like full-fat dairy, low-fat dairy, whole-wheat products, coffee, alcohol and sweets), the Mann-Whitney non-parametric ranked test was used for comparisons. Pearson and Spearman partial correlation coefficients were evaluated. Adjustments were made for age, sex, BMI and EI/BMR. Results from multiple linear regression models are presented as b-coefficients and standard error. All reported P-values were two-sided (significance level 5%). SPSS 18 software was used for statistical analysis (SPSS Inc., Chicago, Il, USA).

3. Results

The main descriptive characteristics of the participants are shown on **Supplementary 1_Table_descriptives**. There was no significant difference between men and women regarding age, BMI and smoking habits. Differences in body composition as well as lipid and glycemic parameters were evident, with men having a worse metabolic profile than that of women.

3.1 Erythrocytes fatty acid composition and their relation to basic characteristics

Table 1 presents the erythrocytes fatty acid composition. As it is shown, the most abundant fatty acids in erythrocytes were SFA (36.9 %), followed by PUFA (35 %) and MUFA (17.8 %). For simplicity reasons the results from this point forward are shown for fatty acids found at concentrations great-

Table 1. Fatty acid composition in erythrocytes (%)

% of total fatty acids	Mean (Standard deviation) or Median (25th -75th quartiles)			
	Total	Men	Women	P-value
14:0	0.23 (0.09)	0.21 (0.08)	0.24 (0.09)	0.08
15:0	0.19 (0.1-1.7)	0.60 (0.10-1.73)	0.16 (0.11-1.66)	0.9
16:0	17.4 (16.8-18.0)	17.5 (17.0- 17.9)	17.4 (16.3- 18.0)	0.5
16:1n9	0.00 (0.00-1.0)	0.00 (0.00-0.1)	0.00 (0.00-0.1)	0.9
16:1n7	0.25 (0.19- 0.31)	0.23 (0.19- 0.31)	0.27 (0.22-0.31)	0.3
17:0 [a]	0.29 (0.06)	0.28 (0.03)	0.29 (0.08)	**0.005**
17:1	0.16 (0.00- 0.84)	0.08 (0.00-0.75)	0.25 (0.00-0.08)	0.7
18:0	14.4 (13.9- 14.8)	14.6 (14.1- 14.9)	14.2 (13.8-14.7)	**0.05**
18:1n9 (oleic acid)	12.7 (11.9-13.5)	13.1 (12.2- 13.9)	12.4 (11.6- 13.0)	0.01
18:1n7	0.7 (0.00-0.89)	0.70 (0.00-0.92)	0.69 (0.00- 0.88)	0.8
18:2n6 (linoleic acid)	8.6 (7.9- 9.4)	8.5 (7.9-9.2)	8.8 (7.9-9.7)	0.7
20:0	0.34 (0.07)	0.32 (0.07)	0.36 (0.06)	**0.01**
20:1n9	0.26 (0.19- 0.32)	0.27 (0.21-0.33)	0.26 (0.18-0.31)	0.3
20:3n9	0.00 (0.00- 0.13)	0.00 (0.00-0.13)	0.00 (0.00-0.13)	0.8
20:3n6	1.36 (0.33)	1.41 (0.38)	1.32 (0.28)	0.1
20:4n6 (arachidonic acid)	12.1 (1.8)	11.8 (1.7)	12.3 (1.8)	0.1
22:0	0.14 (0.00- 0.44)	0.11 (0.00-0.24)	0.15 (0.00-0.56)	0.1
22:1n9	0.00 (0.00- 0.09)	0.00 (0.00-0.07)	0.00 (0.00-0.10)	0.6
22:5n6	0.43 (0.17)	0.43 (0.15)	0.44 (0.18)	0.7
24:1n9	3.01 (0.9)	3.03 (0.9)	3.00 (0.9)	0.8
20:5n3 (EPA) [a]	0.50 (0.28)	0.51 (0.28)	0.49 (0.28)	0.6
22:6n3 (DHA)	5.07 (1.3)	5.13 (1.38)	5.02 (1.24)	0.6
22:4n6	2.5 (2.1- 3.1)	2.5 (2.2- 3.1)	2.5 (2.1- 3.1)	0.6
22:5n6	0.44 (0.35- 0.52)	0.45 (0.36- 0.50)	0.43 (0.34-0.52)	0.9
22:5 n3 (DPA[b])	2.1 (1.8-2.2)	2.1 (1.9- 2.3)	2.1 (1.8-2.2)	0.1
22:6 n6	0.8 (0.55-1.9)	0.8 (0.5-1.9)	0.8 (0.6-1.9)	0.8
24:0	2.7 (0.7)	2.7 (0.6)	2.7 (0.8)	0.7
24:1n9 (nervonic acid)	3.0 (0.9)	3.0 (0.9)	3.0 (0.9)	0.9
SFA	36.9 (34.9-38.0)	37.2 (35.4-38.0)	36.7 (34.4-38.1)	0.5
MUFA	17.89 (3.2)	18.3 (3.6)	17.4 (2.8)	0.1
PUFA	35.2 (2.4)	35.1 (2.2.)	35.2 (2.6)	0.7
n-6	27.3 (3.1)	27.1 (2.8)	27.4 (3.3)	0.6
n-3	7.7 (1.7)	7.8 (1.8)	7.6 (1.7)	0.5
Omega-3 index	5.5 (1.5)			

Data are presented as mean (standard deviation) for normally distributed variables. Otherwise data are presented as median (lower- upper quartile) (25th- 75th). Student t-test or Mann-Whitney test was used to compare means.
[a]Values were log- transformed prior to statistical comparisons. [b]DPA: Docosapentaenoic acid

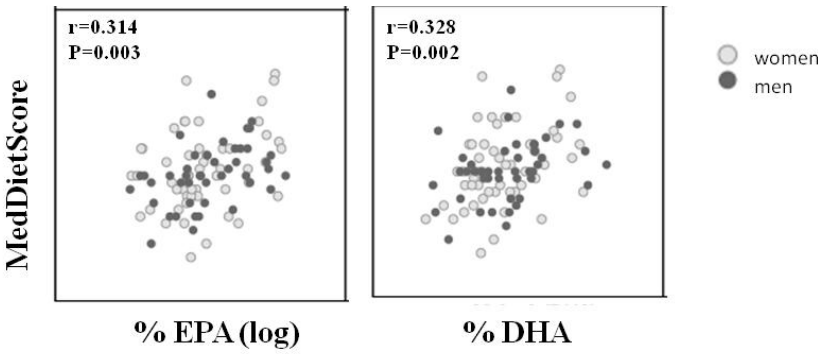

Figure 2: *Relation of EPA and DHA with the MedDietScore. Pearson partial correlation coefficients adjusted for age, sex, BMI and energy/BMR.*

er than 2% (with the exception of EPA due to its clinical importance).

Several differences were observed across sex and age. Men had higher 18:0 and 18:1ω9 and lower 20:0 than women. As far as age is concerned, omega-6 fatty acids were negatively associated with age while several MUFA and omega-3 fatty acids were positively associated with age (all Ps<0.05) (**Figure 1**). It is noted that the observed correlations remained significant after adjustment for lipid levels, with the exception of 18:1ω9. The positive association of MUFA with age was attenuated after adjustment for glucose levels. No significant association was found between smoking and the investigated fatty acids.

Given the strong differentiation of men and women in body composition, sex specific analysis was performed. Pearson or Spearman partial correlation coefficients are presented below after adjustments for age. In women MUFA were negatively associated with arms lean tissue (r= -0.286, P=0.04). As far as men are concerned, EPA was negatively associated with waist circumference (r=- 0.459, P=0.03), hip circumference (r=- 0.338, P=0.03), BMI (r=- 0.468, P=0.02), abdominal fat (as calculated from a special ROI) (r=- 0.289, P=0.06), arms fat (r=-0.324, P=0.01) and legs fat (r=-0.397, P=0.01). Moreover, erythrocyte omega-3 fatty acids were negatively associated with waist circumference and BMI (r=-0.387, P=0.01; r=- 0.397, P=0.01) in men. The physical activity in terms of MET-min/week was borderline correlated with erythrocyte 18:1ω9 (r= 0.187, P=0.07) after adjustment for age, sex and BMI.

Regarding the relations of erythrocyte fatty acids to glycemic and lipemic profile, glucose was negatively associated with 18:0 (r= -0.227, P=0.02), 20:0 (r=-0.216, P=0.030 and 24:1n9 (r=-0.204, P=0.05) while insulin was positively associated with SFA (r=0.196, P=0.05). Erythrocyte 18:1n9 was positively associated with lipids (correlation coefficients for total-, LDL-cholesterol and triacylglycerols were r=0.309, P=0.002; r=0.243, P=0.01 and r=0.196, P=0.05, correspondingly). All correlations were adjusted for age, sex and BMI.

3.2 Dietary intake and relation of erythrocyte fatty acids to nutrient and food group intake

The macronutrient intake of the participants is described elsewhere [12]. Briefly, the mean energy intake of the participants was 1803 Kcal with 42% of the energy deriving from fat (20% MUFA, 12.5% SFA and 5.4% PUFA), 15% from protein and 43% from carbohydrate. The food group intake of the participants is presented in Supplementary2_Table_ Food group intake of participants. The mean value of the MedDietScore was 32.5± 5.2. No difference was documented between men and women with the exception of energy intake and alcohol. Erythrocyte EPA was strongly correlated with total fat intake (r=-0.288, P=0.01), MUFA (r=0.245, P=0.03), PUFA (r=0.238, P=0.04),

Table 2. Partial correlation coefficients between individual fatty acids, fatty acid classes and food groups.

Selected fatty acids* (% of total)	Fruits	Vegetables	Legumes	Fish	Red meat	Refined products	Nuts	Sweets	Herbal decoctions	Coffee
					Servings/month					
16:0	ns	ns	r=0.216 P=0.04	ns	ns	ns	r=-0.291 P=0.006	ns	ns	ns
20:4 n6	ns	ns	ns	ns	ns	ns	ns	ns	0.317 P=0.002	ns
20:5 n3 (EPA)	r=0.316 P=0.003	r=0.221 P=0.03	r=0.225 P=0.03	r=0.438 P<0.001	Ns	ns	ns	ns	ns	r=-0.219 P=0.03
22:6 n3 (DHA)	r=0.223 P=0.03	r=0.230 P=0.03	r=0.240 P=0.02	r=0.518 P<0.001	ns	ns	ns	ns	ns	ns
24:1 n9 (Nervonic acid)	ns	ns	ns	ns	ns	ns	r=-0.198 P=0.06	r=-0.031 P=0.003	r=-0.258 P=0.01	ns
SFA	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
MUFA	ns	ns	ns	ns	r=-0.243 P=0.02	ns	ns	ns	r=-0.278 0.008	ns
PUFA	ns	ns	ns	r=0.182 P=0.08	ns	r=-0.198 P=0.06	ns	ns	r=0.401 P<0.001	ns
n-3	r=0.267 P=0.01	r=0.257 P=0.01	r=0.274 P=0.009	r=0.482 P<0.001	ns	ns	ns	ns	r=0.273 P=0.010	r=-0.200 P=0.06
n-6	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Spearman partial correlation coefficients adjusted for age, sex, BMI and energy/BMR.
ns: non significant
**The table includes only the fatty acids, for which at least one significant correlation with food intake was documented.*

18:1ω9 (r=0.215, P=0.06) after adjustment for age, sex, BMI and energy/BMR. Similarly, erythrocyte omega-3 fatty acids were correlated with PUFA intake (r=0.215, P=0.06). 18:1n9 was negatively associated with caffeine intake (r=- 0.392, P<0.001). Moreover, 16:0 was positively associated with protein intake (r=0.254, P=0.05). All reported correlations have been adjusted for age, sex, BMI and energy/BMR.

Table 2 presents the Spearman partial correlation coefficients of erythrocyte fatty acids with several food groups intake, as they derived from the FFQ analysis, after adjustment for age, sex, BMI and energy/BMR. The correlation coefficients ranged from 0.2 to 0.4. Indeed, as it is shown, EPA and DHA had strong correlations with fruit, vegetable, legumes and fish intake. Additionally, EPA was negatively correlated with coffee intake. In total, MUFA were negatively correlated with meat and herbal decoctions intake, whereas PUFA were positively correlated with fish, herbal decoctions intake and negatively correlated with refined products. Moreover, food groups, which represent major dietary fat sources, such as cheese, other dairy products, nuts, eggs and sweets showed either none or few correlations with erythrocyte fatty acids. On **Figure 2** the relation of EPA and DHA with MedDietScore is depicted, which remained significant after adjustments for age, sex, BMI and energy/BMR and further adjustment for LDL-cholesterol and glucose concentration.

The results of multiple linear regression analysis are shown on **Table 3**. In multi-adjusted mod-

Table 3. **Multiple linear regression models with EPA and DHA as dependent variables.**

	Erythrocyte EPAa (% of total)			Erythrocyte DHA (% of total)		
	B	**SE**	**P**	**B**	**SE**	**P**
Age (years)	0.006	0.002	**0.001**	0.028	0.010	**0.007**
Sex (Male)	0.05	0.044	0.2	0.283	0.257	0.2
Abdominal obesity (ROI fat, kg)	0.0001	0.03	0.9	0.011	0.015	0.4
MedDietScore	0.014	0.004	**0.03**	0.084	0.025	**0.001**
Energy/BMR	-0.052	0.045	0.2	-0.246	0.261	0.349

The R-square for the models presented was 0.28 and 0.26 for EPA and DHA, correspondingly.
[a]EPA was logarithmised prior to statistical analysis.

els for several co-variants, age and Mediterranean diet adherence remained significant predictors of the erythrocyte EPA and DHA. It is noted that the role of abdominal adiposity was no longer significant. The results were similar for the omega-3 index as well.

4. Discussion

The present study investigates the relation of erythrocyte fatty acids with the Mediterranean diet and traditional cardiovascular risk factors, i.e. age, sex, adiposity, diet, physical activity, lipemic and glycemic biomarkers in apparently healthy Greek subjects. Omega-3 fatty acids were positively associated with the MedDietScore and antioxidant-rich foods and several other associations were documented: omega-3 fatty acids were positively and omega-6 fatty acids negatively related with age, omega-3 fatty acids were negatively associated with abdominal adiposity in men while several associations were documented for particular erythrocyte fatty acids with markers of glycemia/ lipemia.

To the best of our knowledge, this is the first study to report erythrocyte fatty acids composition in healthy Greek subjects. Indeed, only few studies have measured erythrocyte fatty acids in high-risk individuals [10, 14] while for apparently healthy Greek subjects only plasma fatty acid status has been described [8]. In the present study the most abundant fatty acids in erythrocytes were SFA, followed by PUFA, and MUFA. The fatty acids with the highest percentage concentration were 16:0, 18:0, 18:1n9, 20:4n6, 18:2n6 and long chain PUFA, which is in line with the relevant literature [2]. The omega-3 index was 5.5, which is slightly higher than that reported for US residents [7] but lower than healthy samples from Germany, Spain and Korea [6]. The concentration of omega-6 in our study was lower than that reported in other studies for healthy/control subjects [15, 16]. The concentration of omega-3 was lower [16], comparable [15] or higher than other reports [7]. DHA concentration was similar to that reported for Italian subjects [16] or higher than that previously reported [7, 15].

The positive relation of erythrocyte EPA and omega-3 fatty acids with the dietary intake of PUFA reflects their value as dietary biomarkers [2]. The fact that EPA was also positively correlated with dietary MUFA and oleic acid intake may reflect a higher adherence to the Mediterranean diet. This hypothesis is also supported by the positive correlation of EPA (and DHA) with the MedDietScore and related food groups (i.e. fish, fruit, vegetables and legumes). The correlations of EPA and DHA food groups of plant origin (fruits, vegetables and legumes) may be explained by the associations of these food groups

with fish intake (r=0.233; P=0.02, r=0.381; P<0.001 and r=0.381, P<0.001, respectively) and the adoption of the Mediterranean diet as an entity. To the best of our knowledge, this is the first study to document a positive relation of erythrocyte EPA, DHA and omega-3 index with the proximity to the Mediterranean diet, which is independent of age, sex and adiposity, as well as other markers, such as glucose and triacylglycerol levels (data not shown). The ATTICA study has also revealed a positive correlation of omega-3 fatty acids with the adherence to the Mediterranean Diet, but the measurements were in plasma [8].

As far as the inverse relations of caffeine intake with oleic acid and coffee consumption with EPA are concerned, they deserve further investigation. Caffeine is known to stimulate lipolysis [17] but its relation with individual fatty acids is not clear. Moreover, is should be mentioned that erythrocyte oleic, can be also endogenously synthesized and thus is not considered a very good marker of MUFA dietary intake [2]. The positive correlation of PUFA and 20:4n6 with herbal decoctions needs further explanation. Antioxidants such as alkyl gallate inhibit desaturases resulting in reduced biosynthesis of 20:4n6 [18]. In this context, the observed associations may be better explained by dietary preferences rather than herbals antioxidant content. Indeed, in our study a pattern rich in herbal decoctions, nuts and legumes was identified [12].

The positive association of EPA and DHA with age is in accordance with previous studies in various populations [7, 19]. It has been proposed that the relation of age to various fatty acids can be explained by differences in dietary intake [2]. However, the associations of fatty acids with age are independent of diet, in most studies [19] and in the present study as well (the associations were independent of fish intake and the MedDietScore, data not shown). This suggests that other factors may influence this relation. For example, the conversion of a-linolenic to EPA is increased with age (increased endogenous production) [20] and older men incorporate EPA into plasma phospholipids more efficiently than younger men [21]. The present study reported an inverse relation of 20:4n6 with age, while other studies have mostly shown that it remains stable across lifespan [7, 22]. As far as the positive relation of age with MUFA is concerned, it has been also documented in other studies [7].

In the present study women had lower 18:1n9, which may occur as a result of sex differences in dietary intake and metabolism. Indeed, women had lower absolute intakes of total fat, MUFA and SFA although the intakes as % of energy were not different between genders. In this context the intakes of 18:1n9 and 18:0 (which can be converted to 18:1n9) may be lower in women resulting in lower erythrocyte concentrations of these fatty acids, as demonstrated by our results. In parallel, women had higher 20:0. Although this may mean that elongation route of 18:0 is preferable to that of its desaturation by delta-9 desaturase in women no difference in the calculated enzymatic activity was found (ratio of erythrocyte 16:1 n-7/16:0, data not shown). In this context, our results are in line with the role of the saturated fatty acid 18:0 and delta-9 desaturase in obesity and insulin resistance [23], since women had a better cardiometabolic profile and less abdominal adiposity than men. In addition, it is reported that exercise may increase 18:1n7, 18:1n9, 22:6n3, total MUFA and PUFA in skeletal muscle [24], which is in line with our observations in erythrocytes (increases in 18:1n9).

An inverse relation of omega-3 fatty acids, and especially EPA, with markers of adiposity was documented, which is in line with the literature [25, 26]. However, in the present study, the association of omega-3 fatty acids with adiposity was no longer significant in multi-adjusted linear regression models.

The positive relation of SFA with insulin may be viewed in the context of their association with metabolic syndrome [27]. However, the inverse relation of 18:0 and 20:0 with glucose levels deserves further investigation. A special mention has to be made for nervonic acid, a brain phospholipids component [28]. In our study it was negatively associated with glucose levels, which agrees with its protective effects regarding insulin resistance and other obesi-

ty-related components reported by others [29]. The positive association of erythrocyte oleic acid with lipid parameters may be explained by the fact that dietary stearic acid is converted to oleic acid [2].

The strengths of the study include erythrocytes fatty acids measurement, which is an objective biomarker reflecting diet for the last 120 days [2]. The limitations of the present study include its cross-sectional nature, which cannot confirm any cause-effect relationship. Another issue is the assessment of dietary intake through classic nutritional tools, i.e. FFQ and 24h recalls. The FFQ was administered once and subjects may have difficulties in the estimation of their yearly intake. In order to control for the potential deriving errors of over- or under- estimation correlations were adjusted for energy/BMR. Moreover, multiple dietary recalls were collected for the assessment of macronutrients intake.

In conclusion, this is the first study to report the values of erythrocyte fatty acids in Greek healthy volunteers and their correlations with diet and traditional risk factors for cardiovascular disease. The positive relation of omega-3 fatty acids the Mediterranean diet may indicate another mechanism by which Mediterranean diet exerts its beneficial cardioprotective effects. Moreover, neglected fatty acids such as nervonic acid may have positive metabolic effects. The results of our study generate hypothesis for candidate fatty acids to serve as targets in the diet- disease interplay.

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Conflict of interest

There is no conflict of interest.

Role of funding source

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Abbreviations

EPA: eicosapentaenoic; DHA: docosahexaenoic acids; MET: metabolic equivalents; FFQ: food frequency questionnaire; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; SFA: saturated fatty acids; FAME: Fatty acids methyl esters.

Supplementary 1_Table_descriptives: Clinical and biochemical characteristics of the participants.

	Total (n=106)	Men (n=48)	Women (n=58)	P
Age (years)	44± 13	44± 13	44± 14	0.8
Current smokers (%)	30.5	34.7	27.6	0.6
MET (min per week)	968±982	1043±1046	951±954	0.6
BMI (kg/m²)	27± 5	28± 4	26± 6	0.2
Waist circumference (cm)	85.2± 14.8	92.7± 9.2	78.7± 15.8	**<0.001**
Hip circumference (cm)	104.6± 12.1	104.7± 7.0	104.5± 15.2	0.9
Waist-to-hip ratio	0.81± 0.12	0.88± 0.06	0.75± 0.12	**<0.001**
% Total body fat	32.6±9.1	26.7±5.2	37.2±8.4	**<0.05**
DXA ROI fat (kg)	2.8±1.2	3.0±0.9	2.7±1.5	0.1
Legs fat (kg)	7.0 (5.7-9.7)	6.1 (5.0-8.2)	8.5 (6.4-11.1)	**<0.05**
Arms lean tissue (kg)	5.1± 1.6	3.81±0.60	6.67±1.18	**<0.05**
Legs lean tissue (kg)[a]	15.3±3.9	12.4±1.9	18.7±2.8	**<0.05**
Total-cholesterol (mmol/L)	5.5± 1.0	5.6±0.9	5.4±1.1	0.3
LDL-cholesterol (mmol/L)	3.8 ±0.9	3.9±0.7	3.7±0.9	0.3
HDL-cholesterol (mmol/L)	1.2±0.2	1.0±0.2	1.3±0.3	**<0.001**
Triacylglycerols (mmol/L)[a]	0.9 (0.7-1.4)	1.3 (0.9-1.7)	0.7 (0.6-1.0)	**<0.001**
Glucose (mmol/L)	5.1±0.6	5.2± 0.4	5.0± 0.7	0.01
Insulin (U/mL)[a]	14.2 (12.6-15.9)	14.5 (12.9-16.6)	13.9 (12.4- 15.8)	0.3
HOMA[a]	3.1 (2.8-3.8)	3.4 (3.0-3.9)	3.1 (2.7-3.4)	0.07

Data are presented as mean ± standard deviation for normally distributed variables. Otherwise data are presented as median (lower- upper quartile) (25th- 75th). Student t-test was used to compare means. HOMA: Homeostasis model assessment. aVariables were logarithmised prior to statistical comparisons.

Supplementary 2_Table: Food group intake of participants

Servings/ month	Total	Men	Women	P
Full fat dairy (milk and yogurt)* (250 ml milk, 200 g yogurt)	0 (0-14)	0 (0- 6)	1 (0- 24)	0.4
Low fat dairy (milk and yogurt)* (250 ml milk, 200 g yogurt)	14 (0-58)	10 (0- 62)	20 (0- 60)	0.6
Cheese (60 g)	24.4 ± 18.4	25.8± 20.5	23.2± 16.5	0.5
Fruits (1 item)	60.7± 40.2	56.9± 44.7	63.7± 36.1	0.4
Vegetables (1 cup raw, ½ cup boiled)	22± 7.6	21.2± 7.2	22.8± 8	0.1
Legumes (1 cup)	8.2± 5.1	8.0± 3.8	8.3± 6.1	0.8
Fish (125 g)[a]	10 (6- 16.4)	10 (6.4- 16.0)	10 (4-18)	0.9
Red meat (125 g)	19.2± 12.5	22.6± 14.7	16.3± 9.6	0.1
Poultry (125 g)**	12 (4- 12.5)	12 (4.2- 12.5)	12 (4- 12.5)	0.7
Whole-wheat products* (1 slice of bread, 2 melba toasts)	27 (6-60)	28 (7- 70)	22 (5- 48)	0.2
Refined cereals (1 slice of bread,1/2 cup pasta)**	31 (19- 44)	30 (16- 42)	33 (20- 51)	0.08
Eggs (1 item)*	2 (2-6)	4 (2-6)	2 (2-6)	0.9
Nuts (40 g)*	4 (2-8)	4 (2-8)	2 (2-6)	0.1
Sweets* (1 piece of cake, 3 biscuits)	12.6 (5- 18)	12 (5.4-17)	12.8 (5-21)	0.6
Herbal decoctions* (1 cup of 240 mL)	2 (0-14)	2 (2- 12)	2 (0- 15)	0.9
Coffee (1 cup 240 mL)*	42 (14- 58)	42 (15- 56)	43 (7- 59)	0.5
Alcoholic beverages (total) (125 mL wine, 330 mL beer, 40 mL whiskey)*	12 (0- 28)	28 (9- 42)	0 (0- 12)	**<0.001**

Data are presented as mean ± standard deviation for normally distributed variables. Otherwise data are presented as median (lower- upper quartile) (25th- 75th). Student t-test or Mann-Whitney test was used to compare means.

[a]Values were log- transformed prior to statistical comparisons.

**Due to the high percentage of zero values, variables were not log-transformed and instead non-parametric tests were used for comparisons. Abstainers from food groups: full-fat dairy, 45.5 %; low-fat dairy, 33 %; whole-wheat products, 10 %; eggs, 23%; nuts, 16%; herbal drinks, 23%; coffee,13%; alcoholic beverages, 36%.*

***Despite transformations the variables remained non-normally distributed. For this reason non-parametric tests were used for comparisons.*

Περίληψη

Συσχετίσεις μεταξύ των λιπαρών οξέων των ερυθροκυττάρων και της Μεσογειακής Διατροφής σε Ελληνικό πληθυσμό.

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Σκοπός: Σκοπός της παρούσας μελέτης ήταν η διερεύνηση της σχέσης των λιπαρών οξέων ερυθροκυττάρων με τη μεσογειακή διατροφή και τους κλασσικούς καρδιαγγειακούς παράγοντες κινδύνου.

Ασθενείς και μέθοδοι: Στρατολογήθηκαν υγιείς εθελοντές (n= 106) και ο προσδιορισμός των λιπαρών οξέων ερυθροκυττάρων έγινε με αέρια-υγρή χρωματογραφία. Συλλέχθηκαν διατροφικά δεδομένα (FFQ και ανακλήσεις 24ώρου) και διεξήχθη ανάλυση σύστασης σώματος DXA.

Αποτελέσματα: Στα μοντέλα πολλαπλής γραμμικής παλινδρόμησης, το εικοσιπενταενοϊκό (EPA), το εικοσιδυοεξαενοϊκό (DHA) και ο δείκτης Omega-3 Index (% EPA + DHA) συσχετίστηκαν θετικά με την ηλικία και το MedDietScore ανεξάρτητα από το φύλο, την κοιλιακή παχυσαρκία και το λόγο ενεργειακή πρόσληψη/BMR. Επιπλέον, το EPA και το DHA συσχετίστηκαν θετικά με την κατανάλωση ψαριών (r = 0,438, P <0,001 και r = 0,518, P <0,001 αντίστοιχα) και την πρόσληψη αντιπροσωπευτικών ομάδων τροφίμων της μεσογειακής διατροφής (όσπρια, φρούτα και λαχανικά). Το ελαϊκό οξύ συσχετίστηκε θετικά με την HDL-χοληστερόλη (r = 0,309, P = 0,002), την LDL-χοληστερόλη (r = 0,243, P = 0,01) και τις τριακυλγλυκερόλες (r = 0,243, P = 0,01). Τα κορεσμένα λιπαρά οξέα συσχετίστηκαν θετικά με την ινσουλίνη (r = 0,196, P = 0,05).

Συμπέρασμα: Η θετική σχέση των ωμέγα-3 λιπαρών οξέων με τη μεσογειακή δίαιτα φωτίζει τους μηχανισμούς με τους οποίους το διατροφικό αυτό πρότυπο ασκεί την καρδιοπροστατευτική του δράση.

Λέξεις ευρετηρίου: Μεσογειακή Διατροφή; λιπαρά οξέα; ερυθροκύτταρα. ωμέγα-3 λιπαρά οξέα

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