MEDITERRANEAN-STYLE DIET EFFECT ON THE STRUCTURAL PROPERTIES OF ERYTHROCYTE CELL MEMBRANE OF HYPERTENSIVE PATIENTS: THE PREDIMED STUDY

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ABSTRACT
A currently ongoing randomized trial has revealed that the Mediterranean diet, rich in virgin olive oil or nuts, reduces systolic blood pressure in high-risk cardiovascular patients. Here, we present a structural sub-study to assess the effect of a Mediterranean-style diet supplemented with nuts or virgin olive oil on erythrocyte membrane properties in 36 hypertensive participants after one year intervention. Erythrocyte membrane lipid composition, structural properties of reconstituted erythrocyte membranes and serum concentrations of inflammatory markers are reported. After the intervention, the membrane cholesterol content decreased, while that of phospholipids increased in all dietary groups; the diminishing cholesterol/phospholipid ratio could be associated with an increase in the membrane fluidity. Moreover, reconstituted membranes from the nuts and virgin olive oil groups showed a higher propensity to form a non-lamellar H_{II} structure, that was related to an increase in phosphatidylethanolamine lipid class. These data suggest that the Mediterranean-style diet affect the lipid metabolism that is altered in hypertensive patients, influencing the structural membrane properties. The erythrocyte membrane modulation described provides insight in the structural bases underlying the beneficial effect of a Mediterranean-style diet in hypertensive subjects.

Keywords: Mediterranean diet, lipids, membrane structure, cardiovascular disease, hypertension
INTRODUCTION

Cardiovascular disease constitutes the main cause of death in industrialized countries (1), and hypertension is one of the main modifiable cardiovascular risk factors, especially in the elderly (2). Health diet and lifestyle constitutes the first step in the guidelines for management of hypertension (3). In this context, the type and amount of dietary lipids influences the lipid composition of cell membranes (4-5), and modulates the interactions with proteins involved in the regulation of blood pressure (6). Thus, the changes in membrane properties induced by dietary lipids may have important consequences on the blood pressure regulation.

The Mediterranean-style diet (MD) is characterized by a high consumption of virgin olive oil (VOO) and nuts, which are rich natural sources of oleic (18:1, n-9) and α-linolenic (18:3, n-3) acids, respectively. The PREDIMED (PREvencion con DIeta MEDiterranea) study is a large-scale randomized trial aimed to assess the effects of a MD enriched with VOO or nuts on primary prevention of cardiovascular disease in patients at high-risk for coronary heart disease (CHD). The results of the 3-month intervention on the first 772 patients entering the study showed that, compared with a low-fat diet, the MD rich in VOO or nuts reduced systolic blood pressure, serum total cholesterol and triglycerides (TG) concentrations, and increased serum high-density lipoprotein (HDL)-cholesterol concentration (7). Although there is evidence indicating that dietary lipids can have a positive effect on cardiovascular risk factors, the mechanisms and effects on the molecular and structural bases underlying the physiological process are largely unknown.

Several studies support the involvement of plasma membrane properties in the
modulation of membrane protein activities and cell physiology. The structural properties and function of cell membranes appear to be modified in hypertensive humans and animal models of hypertension (8-10). Changes in membrane lipid composition of hypertensive subjects have been associated with alterations in the transmembrane fluxes of Na\(^+\) and K\(^+\), including Na\(^+\)-Li\(^+\) countertransport, which is a marker of essential hypertension (10,11) and in cell signaling proteins that participate in the control of blood pressure (12). On the other hand, it has been reported that dietary lipids has an effect on membrane lipid composition and cell signaling proteins (4-5; 12). Considering that changes in the dietary lipid composition yield to variations in the biophysical properties of the plasma membrane, it is likely that cellular functional changes could result from alterations in the structure of the lipid membrane properties influenced by the diet. Thus, the changes in membrane properties induced by dietary lipids may have important consequences on blood pressure regulation.

Dietary habits could play a role as an environmental factor altering some targeted molecular functions in the cell and through them influencing cardiovascular risk factors. In fact, the MD has been associated with changes in membrane structure and function. Consumption of olive oil-rich diets increases the concentration of oleic acid in plasma membrane lipids of different rat and human cells, with beneficial consequences on membrane functionality (13-16). In contrast, very little is currently known regarding the effects of nuts, another key ingredient of the MD, on membrane lipid composition and structure.

The present study was undertaken to examine the structural bases underlying the effect of the MD on the cardiovascular system, in parallel with the PREDIMED study in progress...
With this aim, we conducted a structural sub-study to assess the effect of the MD supplemented with nuts or VOO on the erythrocyte membrane properties in a group of participants recruited from the parent study after one year intervention. This is the first time that membrane structural analyses are included in an intervention study with a MD.
MATERIALS AND METHODS

Materials

N-(2-Hydroxy ethyl) piperazine-N’-(2-ethanesulfonic acid) sodium salt (Hepes) were obtained from Sigma Chem. Co. (Madrid, Spain). Lipid standards, cholesterol, 1,2-dipalmitoyl-sn-glycero-3-phosphatidylethanolamine, 1,2-Diacyl-sn-glycero-3-phosphocholine, 1,2-Diacyl-sn-glycero-3-phospho-L-serine, N-Acyl-4-sphingenyl-1-O-phosphorylcholine and lisophosphatidylcholine (LPC) were purchased from Sigma-Aldrich (Madrid, Spain). Solvents used for lipid extraction and HPLC-grade solvents were from Romil (Cambridge, UK). The HPLC column was purchased from Merck (Darmstadt, Germany).

Methods

Study design

The PREDIMED study is a large, parallel-group, multicenter, randomized, controlled, 5-year trial (7), whose aim is to assess the effects of the MD on the primary prevention of cardiovascular disease (http://www.predimed.org). Near 7500 high-risk participants have been divided into three intervention groups and each group receives a specific diet: one third a MD enriched with VOO, another third a MD enriched with mixed nuts and the remaining third a low-fat diet. The present study reports the first year effects of these dietary interventions on the structural membrane properties of the erythrocyte plasma membranes from 36 hypertensive subjects participating in the PREDIMED study.
Subjects

The first 36 hypertensive participants entering in the PREDIMED study from two nodes (Sevilla and Malaga, Spain) were divided into three groups and assigned to the following interventions: a MD enriched with VOO (MD+VOO group), a MD enriched with nuts (MD+nuts group) or to a low-fat diet (LF group). Each group consisted of 12 subjects to ensure adequate sample size in order to conduct the X-ray diffraction study, as well as to obtain sufficient statistical significance. Blood pressure was measured and blood samples were collected from all subjects before the dietary intervention (baseline) and after 1 year intervention with the corresponding diet as described in Estruch et al. (7). All the protocols used in this study followed the principles of the Declaration of Helsinki and were approved by the Institutional Committee of Human Research (Hospital Universitario Virgen del Rocio, Sevilla, Spain). All procedures followed were in accordance with institutional guidelines and the subjects gave their informed consent to participate in the study.

Dietary Intervention

Participants in the PREDIMED study were given written recommendation for a traditional MD and 3-month allotments of free VOO (1 L/week) or mixed nuts (30 g/day, as 15 g walnuts, 7.5 g hazelnuts and 7.5 g almonds). A 137-item food validated frequency questionnaire and a 14-item questionnaire, an extension of a questionnaire designed to assess the degree of adherence to the traditional MD was used (7). Please see http://hyper.ahajournals.org for detailed information.
**Serum inflammatory markers**

Fasting blood samples were obtained at baseline and after subjects had received the dietary intervention for one year, kept at 4°C for during transportation from the hospital to the laboratory (<1h) and then stored at -80°C until required for biochemical analyses. Measurements of high-sensitive C-reactive protein (CRP), interleukin-6 (IL-6), E-selectine and P-selectine were taken. Serum concentrations of high-sensitivity CRP were analyzed by particle-enhanced immunonephelometry. Serum IL-6, E-selectine and P-selectine were measured in duplicate using standard enzyme-linked immunoadsorbent assay.

**Erythrocyte model membrane preparation**

Erythrocyte membranes were isolated as described previously (16). Briefly, blood samples were collected in heparinised tubes and centrifuged at 1750 g and 4°C for 10 min. The erythrocyte pellets were washed twice with 110 mM MgCl₂. Erythrocyte membranes from the 12 participants of each group were mixed and used to reconstitute model membranes with a lipid composition representative of the three, MD+VOO, MD+nuts and LF, group patients. Total lipids of each erythrocyte membrane group were extracted with chloroform/methanol (2:1, v:v) as described previously (17). Multilamellar lipid vesicles (MLV) 15% (w/w) with total lipid extracts were prepared in 10 mM Hepes, 100 mM NaCl, 1 mM EDTA, pH 7.4 (Hepes buffer) (12). Lipid mixtures were hydrated, thoroughly homogenized with a pestle-type minihomogenizer (Sigma) and vortexed until a homogeneous mixture was obtained. Then, the suspensions were submitted to five temperature cycles (heated up to 70°C and cooled down to 4°C). Samples for X-ray scattering experiments were
stored at -80°C under argon and allowed to equilibrate at 4°C during 48 hours before measurements were taken.

**Lipid composition analyses**

Lipid and phospholipid classes were separated by HPLC in a single chromatogram following a modification of the method by Perona et al. (18). Briefly, triplicates of the lipid extracts were dissolved in chloroform:methanol (2:1, v:v), passed through 0.2-µm filters and subsequently analyzed by liquid chromatography (2695 Alliance, Waters Co., Milford, USA), using a Lichrosphere column (250 x 4.6 mm, 5-µm particle size) and an evaporative light-scattering detector (ELSD, Waters 2420, Waters Co., Milford, USA). A ternary gradient of hexane, 2-propanol and methanol was applied with a flow rate of 0.8 ml/min. Commercial purchased lipid standards were used to identify and quantify the lipid classes. The amounts of cholesterol and phospholipids were quantified using calibration curves from lipid standards. The quantification was based on regression analyses of curves with correlation coefficients higher than 0.999.

Fatty acid methyl esters were analyzed by gas chromatography using a Hewlett-Packard 5890 series II gas chromatograph equipped with a flame ionization detector (Hewlett-Packard Co, Avondale, USA) and a Supelcowax 10 capillary silica column (60 m and 0.25 mm internal diameter: Sulpelco Co, Bellefonte, USA). Fatty acid methyl esters were identified by comparison of their retention time against those of standards and quantified by internal standardization (tricosanoic methyl ester, 23:0), using peak area integration (19).
X-ray diffraction studies

Small and Wide-Angle (SAXS and WAXS) Synchrotron radiation X-ray scattering analysis were conducted using standard procedures on the Soft Condensed Matter beamline A2 of Hasylab at the Deutsches Elektronen Synchrotron (DESY). The data collection conditions were as described previously (12). Samples were heated from 10 to 70 °C. To work in quasiequilibrium conditions, the systems were allowed to equilibrate for 15 min at each temperature before measurements. Then, they were kept at the highest temperature for 15 min and finally cooled down to the lowest temperature at the same scan rate. Positions of the observed peaks were converted into distances, $d$, after calibration with the standards rat tendon tail and poly-(ethylene therephtalate) for the SAXS and WAXS regions, respectively. Interplanar distances, $d_{hkl}$, were calculated according to the equation:

$$s = 1/d_{hkl} = (2\sin \theta )/\lambda$$

(Eq. 1)

where $s$ is the scattering vector, $2\theta$ is the scattering angle, $\lambda$ (0.150 nm) is the X-ray wavelength and $hkl$ are the Miller indexes of the scattering planes.

Statistical analysis

Data are reported as the mean value ± standard deviation (SD) unless otherwise stated. Variables were examined for normality and skewness (Kolmogorov and Levene tests). We transformed values with a skewed distribution (CRP, interleukin-6, E-selectine and P-selectine) to their natural logarithm for analyses. Differences within and between groups were analyzed using the one-factor ANOVA analysis and the paired t-test, when indicated. Values were con-
sidered significantly different when P value was < 0.05. Analyses were performed using SPSS software version 14.0 (SPSS Inc., Chicago, IL, USA).
RESULTS

Systolic and Diastolic Pressure

Blood pressure was measured at baseline and after one year of intervention in all groups. There was a significant reduction of systolic blood pressure in both groups consuming the MD enriched with nuts or VOO (P < 0.05). In contrast, no changes were found in those following the low-fat diet during the same period time (Figure 1). No changes were observed in weight, body mass index (BMI) and energy consumption in any of the experimental groups after one-year intervention period (data not shown).

Inflammatory markers

Figure 2 shows the differences in serum concentration of CRP, IL-6, E-selectine and P-selectine at baseline and after one year of intervention in the three groups. Only the reduction observed after consumption of the MD enriched with VOO in plasma concentration of CRP (p<0.01), IL-6 (p<0.001), E-selectin (p<0.01) and P-selectin (p<0.05) achieved statistical significance.

Erythrocyte membrane lipid composition

The effect on the lipid and fatty acid composition of erythrocyte membranes from hypertensive patients of a MD enriched in VOO or nuts, or a low-fat diet was evaluated. The lipid composition of erythrocyte membranes was modified after 1-year dietary intervention in MD+nuts and MD+VOO groups (Table 1). TG concentrations were strikingly reduced in
both groups and cholesteryl esters only in the MD+nuts group. Conversely, the phospholipid concentration was increased, but the difference was only significant after following the MD+nuts diet or the low-fat diet. Significant differences were found in phospholipid classes in all groups studied (Table 2). Phosphatidylethanolamine (PE) concentration was increased in those consuming the MD enriched with nuts or VOO but was decreased in erythrocyte membranes of volunteers consuming the low-fat diet. PC was reduced in all groups after the dietary intervention but only significantly in the MD+nuts and LF groups. Sphyngomieline (SM) was only reduced in patients on the MD+VOO. Changes in the content of LPC were highly dependent on the diet; unchanged in the MD+nuts, reduced in the MD+VOO and greatly increased in the low-fat diet.

In the fatty acid analysis (Table 3), the concentration of stearic acid increased in the MD+nuts and MD+VOO groups and also palmitic acid in the MD+nuts group, compared with the low-fat diet group. The concentration of oleic acid remained unchanged in all groups, but that of palmitoleic acid (16:1, n-7) was higher in the MD+nuts and MD+VOO groups and that of vaccenic acid (18:1, n-7) in the MD+VOO group. Polyunsaturated fatty acids were only modified by the MD+nuts diet, by increasing the content of α-linolenic acid (18:3, n-3) and reducing that of linoleic and eicosadienoic (20:2, n-6) acids. In turn, no significant changes were observed in the FA composition of the membranes corresponding to the low-fat diet.
Structural influence on the supramolecular organization of erythrocyte model membranes

For each group of patients (MD+VOO, MD+nuts and LF), MLV were prepared with the total lipid extract from the mixture of the erythrocyte membranes from the 12 participants in the basal experiment and after 1-year dietary intervention. The reconstituted model membranes, with a lipid composition representative of the erythrocyte membrane of the participants assigned to each group, were analyzed by X-ray diffraction (Figure 3 and Table 4). Membranes displayed clear X-ray diffraction patterns composed by a lamellar L\textsubscript{\alpha} phase, transforming into a nonlamellar H\textsubscript{II} structure upon heating. The thermotropic behaviour depended on the membrane group. Control model membrane samples obtained from the basal experiment (VLF, VOO, and VDF) showed a lamellar L\textsubscript{\alpha} phase with a repeat distance (d) in the range 7.2 - 7.8 nm at 37 ºC, that developed alone or in coexistence with a H\textsubscript{II} phase with a d value ~ 10.0 nm at 50 ºC. Since the beginning, the H\textsubscript{II} phase coexisted with the L\textsubscript{\alpha} phase in VLF membranes and it appeared at ~ 25 or 50 ºC in VDF or VOO membranes. All samples exhibited reversible thermotropic behavior on cooling. We studied the effect of the specific diet by analyzing the model membranes samples (VLF1, VOO1 and VDF1) obtained at 1 year term and comparing the respective structural properties in each group. In MD+nuts group, VDF1 membranes showed a decrease in the structural parameters of the L\textsubscript{\alpha} phase (d = 7.7 and 7.1 nm at 37 ºC for VDF and VDF1, respectively) and an increase in the compressibility factor. Since 20-25 ºC and up to 70 ºC, the L\textsubscript{\alpha} phase developed in coexistence with a H\textsubscript{II} phase (d = 10.7 and 10.4 nm at 50 ºC for VDF and VDF1, respectively) for both groups. However, it is worth noticing that VDF membranes displayed a more
structured HII phase. In MD+VOO group, VOO and VOO1 membranes showed subtle differences in their thermotropic behavior. The Lα phase developed up to 42-50 °C as a sole phase (d ~ 7.8 nm) with no compressibility factor, and then went into a HII phase with a different rood diameter (d ~ 9.9 and 9.4 nm for VOO and VOO1 at 50 °C). Indeed, VOO1 presented a broad diffraction peak at 3.5 nm that developed up to 30 °C and was identified as a cholesterol rich domain organized in the bilayer in coexistence with the Lα phase. In the LF group, VLF and VLF1 (data not shown) membranes did not exhibit significant differences in their thermotropic behavior and the phases shown coexisted during the temperature range studied with similar structural parameters (Lα phase with d = 7.2nm at 37 °C and HII phase with d = 9.9 nm at 50°C).
DISCUSSION

The PREDIMED study is a large-scale randomized trial designed to assess the effects of the MD enriched in VOO or nuts on cardiovascular outcomes in high-risk CHD patients. The results of this study in the first 772 patients after 3-month intervention showed that, compared with a low-fat diet, the MD rich in VOO or nuts reduced systolic blood pressure, serum total cholesterol and TG concentrations, and increased serum HDL-cholesterol concentration (7). In the current study, we have explored molecular and structural bases underlying the effect of a MD supplemented with nuts or VOO and compared it to that of a low-fat diet.

The data presented clearly establish in vivo that dietary lipid management can modulate the structural properties of erythrocyte membranes in addition to a decrease in blood pressure. Membrane lipid composition and structural properties of reconstituted erythrocyte membranes were altered after one-year intervention by supplementing a MD diet with nuts or VOO as natural rich sources of α-linolenic or oleic acid, respectively. The main changes in lipid composition were observed in cholesterol and phospholipid concentrations. Cholesterol content decreased while that of phospholipids increased in all groups studied, although the difference was not significant in the MD+VOO group. Previous studies have shown that the hydrophobic core of erythrocyte membranes is less fluid in hypertensive rats and have a high index of cholesterol/phospholipid ratio (20). In addition, it has been reported that the increased cholesterol/phospholipid ratio in erythrocyte membranes of hypertensive patients, is associated with alterations of the Na$^+$-Li$^+$ countertransport activity (10,11), which can be normalized by short-term VOO intake (21). In the present study, the changes in the lipid composition that result in a reduction of the cholesterol/phospholipid ratio, could be
associated to an increase in the membrane fluidity. Although fatty acid composition also contributes to the modulation of membrane fluidity (22) only minor changes were found in the PL fatty acid profile of the three groups of patients analyzed.

Despite the similar increase found in phospholipids in the three groups studied, not all lipid classes were modified to the same extent. For instance, PE was reduced after following the low-fat diet but increased after both MD diets supplemented with nuts or VOO. Low membrane PE concentrations have been reported in spontaneously-hypertensive rats (SHR) compared with normotensive animals (22,23). In this latter strain, VOO consumption led to increased PE content in membranes compared with high-oleic sunflower oil (HOSO) (9). It is worth noticing that in contrast with VOO, HOSO was unable to exhibit beneficial effects on blood pressure in hypertensive subjects (16). Another interesting observation was that the low-fat and MD+VOO diets differed in their effects on SM and LPC concentrations in erythrocytes membranes. These two lipid classes were reduced in the group receiving VOO and increased in the group assigned to the low-fat diet. Previous studies suggest that SM content is also increased in SHR (24). Actually, a hallmark of sphingolipids is that they bring local order to fluid membranes (25), by forming structural microdomains (e.g. “lipid rafts”) enriched in cholesterol and SM with a high structural order. It is now becoming clear that these lipid microdomains play a role in the cell signaling (26). Some proteins (e.g. G proteins), that participate in cell signal transduction and are involved in the physiological process of the control of blood pressure, have been associated with lipid rafts (27). Interestingly, a growing body of data indicates that multiple signal transduction events in the heart occur via plasma membrane receptors located in signaling microdomains (28).
Changes in the lipid composition due to the diet style were associated with subtle differences in the structural properties of the reconstituted membranes from erythrocytes. Reconstituted membranes from MD+nuts and MD+VOO groups after 1 year intervention showed a higher propensity to form non-lamellar H_{II} structures that correlated with an increase in PE lipid class observed in their respective lipid composition. In model membranes, an experimental correlation between a H_{II}-phase propensity and an increase in G protein localization or PKC activity has been shown (29-30), demonstrating the influence of the membrane structure on cell signaling proteins that participate in the control of blood pressure. Thus, membrane structural changes induced by the MD diet style may have a cellular functional implication.

On the other hand, when serum inflammatory markers were analyzed, a reduction in CRP, IL-6, E-selectin and P-selectin concentration was observed after both MD interventions, although only the differences observed in the MD + VOO group achieved statistical significance. Leukocytes and thrombocytes have been causally related with atherogenesis and vascular thrombosis occlusion. However, more recently, an increased appreciation has been noticed for erythrocyte as a cell involved in atherosclerotic plaque destabilization (31). Recently, Tziakas et al. (32) have shown that interleukin-8 is increased in the membrane of circulating erythrocyte in patients with acute coronary syndrome. The results of our study also show a possible link between changes in erythrocyte membrane properties and serum inflammatory markers after a MD intervention, especially when this diet is supplemented with VOO.
PERSPECTIVES

Cardiovascular disease has a multifactorial etiology. Genetic and environmental factors apparently form the basis for structural membrane properties and function. Considering the *in vivo* approach of this study, the dietary fat management constitutes an external factor able to reduce the blood pressure and serum inflammatory markers and modulate the structural erythrocyte membrane properties. Adjustment in the lipid composition and structural properties of erythrocyte membranes due to MD diets supplemented with nuts or VOO are most probably related with changes in the physicochemical properties of the lipid microenvironment of membrane proteins. The complexity of biological membranes makes difficult to assign specific changes in membrane structure to membrane-dependent functions (e.g. the function of membrane proteins that participate in cell signaling). The alterations in the structural blood cells properties reported could reflect changes in other cell types related to the control of blood pressure and could account for the statistically significant reductions in blood pressure observed in those groups of participants in the PREDIMED study.
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CONFLICTS OF INTERESTS

None.
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22. Vazquez CM, Zanetti R, Ruiz-Gutierrez V. Lipid composition and fluidity in the


FIGURE LEGENDS

FIGURE 1. Systolic (A) and diastolic (B) pressures of hypertensive subjects assigned to a Mediterranean diet enriched with nuts or virgin olive oil (VOO), or to a low-fat diet at baseline (black bars) and after one-year intervention (grey bars). *, p<0.05 compared to baseline.

FIGURE 2. Serum concentrations of C-reactive protein (A), interleukin-6 (IL-6, B), E-selectin (C) and P-selectine (D) of hypertensive subjects assigned to a Mediterranean diet enriched with nuts or virgin olive oil (VOO), or to a low-fat diet at baseline (black bars) and after one-year intervention (grey bars). *, p<0.05; **, p<0.01; ***, p<0.001, compared to baseline.

FIGURE 3. Left panel: X-ray diffraction sequence of the scattering patterns of model erythrocyte membranes reconstituted with the total lipid extract from the mixture of the erythrocyte membranes from the 12 hypertensive patients assigned to a low-fat diet (LF diet) or to a Mediterranean diet enriched with nuts (MD+nuts, MD+nuts intervention) or virgin olive oil (MD+VOO, MD+VOO intervention) at baseline (LF diet, MD+nuts, MD+VOO) and after one-year intervention (MD+nuts intervention, MD+VOO intervention). The sequence of patterns was acquired in quasi-equilibrium conditions after equilibrating the sample during 15 min at each temperature. Successive diffraction patterns were collected for 20 s. The L\(_\alpha\) phase was identified by the two order reflection peaks on the SAXS and the absence of peaks on the WAXS region. The H\(_\beta\) phase was identified by three-four higher-order reflection peaks indicated. Only the heating sequence is shown from 10 °C to 70 °C. Right panel: Dependence of the repeat distance with temperature for the
reconstituted membranes. Phases represented are L\(\alpha\) (-○-) and H\(\beta\) (-■-). The broad diffraction peak at 3.5 nm (-▲-) was identified as a cholesterol rich domain organized in the bilayer. LF diet at one year intervention (data not shown) was similar to LF diet at baseline.
TABLE 1. Lipid composition (mg/100 mg) of erythrocyte cell membranes from hypertensive patients assigned to a Mediterranean diet enriched with nuts (MD+nuts) or virgin olive oil (MD+VOO) or to a low-fat (LF) diet at baseline and after one-year intervention.

<table>
<thead>
<tr>
<th>Lipid class</th>
<th>Low-fat Baseline</th>
<th>Intervention</th>
<th>MD+Nuts Baseline</th>
<th>Intervention</th>
<th>MD+VOO Baseline</th>
<th>Intervention</th>
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<tr>
<td>CE</td>
<td>1.33±0.80</td>
<td>1.18±0.36</td>
<td>3.50±0.73</td>
<td>0.55±0.57†</td>
<td>4.54±1.15</td>
<td>3.70±0.51</td>
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<tr>
<td>TG</td>
<td>0.77±0.52</td>
<td>0.60±0.29</td>
<td>1.12±0.05</td>
<td>0.25±0.12†</td>
<td>1.88±0.50</td>
<td>0.97±0.21†</td>
</tr>
<tr>
<td>C</td>
<td>20.22±2.40</td>
<td>16.39±1.10*</td>
<td>18.99±0.10</td>
<td>17.48±0.09*</td>
<td>35.20±5.38</td>
<td>33.13±4.92</td>
</tr>
<tr>
<td>PL</td>
<td>77.68±0.71</td>
<td>81.82±0.57*</td>
<td>76.39±1.88</td>
<td>81.73±0.59†</td>
<td>58.38±6.93</td>
<td>62.20±5.17</td>
</tr>
</tbody>
</table>

Data are mean values ± SD from three independent analyses, corresponding to a subgroup of subjects (n = 12). Abbreviations: CE, cholesterol esters; TG, triglycerides; C, cholesterol; and PL, phospholipids. *, p<0.01; †, p<0.001 compared to the baseline.
TABLE 2. Phospholipid composition (mg/100 mg) of erythrocyte cell membranes from hypertensive patients assigned to a Mediterranean diet enriched with nuts (MD+nuts) or virgin olive oil (MD+VOO) or to a low-fat (LF) diet at baseline and after one-year intervention.

<table>
<thead>
<tr>
<th></th>
<th>Low-fat</th>
<th>MD+Nuts</th>
<th>MD+VOO</th>
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<tbody>
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<td></td>
<td>Baseline</td>
<td>Intervention</td>
<td>Baseline</td>
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<tr>
<td><strong>PE</strong></td>
<td>25.10±0.81</td>
<td>19.84±1.88‡</td>
<td>21.55±1.95</td>
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<tr>
<td><strong>PS</strong></td>
<td>2.44±1.86</td>
<td>3.19±0.67</td>
<td>1.83±0.93</td>
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<tr>
<td><strong>PC</strong></td>
<td>44.73±1.73</td>
<td>41.13±1.25‡</td>
<td>46.53±1.73</td>
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<tr>
<td><strong>SM</strong></td>
<td>24.68±1.73</td>
<td>27.25±2.46</td>
<td>28.43±1.77</td>
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<tr>
<td><strong>LPC</strong></td>
<td>3.06±2.25</td>
<td>8.60±3.78*</td>
<td>1.66±0.23</td>
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</tbody>
</table>

Data are mean values ± SD from three independent analyses, corresponding to a subgroup of subjects (n = 12). Abbreviations: PL, phosphoilipid; PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; SM, sphingomyeline; LPC, lisophosphatidylcholine. *, p<0.05; †, p<0.01; ‡, p<0.001 compared to the baseline.
### TABLE 3. Fatty acid composition (mg/100 mg) of erythrocyte cell membranes from hypertensive patients assigned to a Mediterranean diet enriched with nuts (MD+nuts) or virgin olive oil (MD+VOO) or to a low-fat (LF) diet at baseline and after one-year intervention.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Low-fat Baseline</th>
<th>Intervention</th>
<th>MD+Nuts Baseline</th>
<th>Intervention</th>
<th>MD+VOO Baseline</th>
<th>Intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>20.60±0.74</td>
<td>21.12±1.05</td>
<td>20.18±1.37</td>
<td>23.15±1.64</td>
<td>21.49±1.20</td>
<td>22.12±1.60</td>
</tr>
<tr>
<td>16:1 n-7</td>
<td>1.22±0.53</td>
<td>1.29±0.20</td>
<td>1.24±0.39</td>
<td>0.79±0.16</td>
<td>4.07±1.47</td>
<td>1.22±0.45‡</td>
</tr>
<tr>
<td>18:0</td>
<td>12.02±1.33</td>
<td>11.04±1.77</td>
<td>13.13±3.54</td>
<td>18.10±4.50</td>
<td>12.42±1.06</td>
<td>15.30±2.25*</td>
</tr>
<tr>
<td>18:1 n-9</td>
<td>21.82±3.36</td>
<td>23.50±5.29</td>
<td>21.88±2.05</td>
<td>20.59±2.34</td>
<td>21.28±2.91</td>
<td>21.68±1.84</td>
</tr>
<tr>
<td>18:1 n-7</td>
<td>1.51±0.06</td>
<td>1.67±0.18</td>
<td>1.50±0.22</td>
<td>1.35±0.17</td>
<td>1.66±0.18</td>
<td>1.37±0.14†</td>
</tr>
<tr>
<td>18:2 n-6</td>
<td>19.37±2.13</td>
<td>17.89±4.23</td>
<td>21.10±3.3</td>
<td>14.91±2.44†</td>
<td>16.15±0.94</td>
<td>15.63±2.35</td>
</tr>
<tr>
<td>18:3 n-3</td>
<td>0.77±0.07</td>
<td>0.71±0.10</td>
<td>0.72±0.23</td>
<td>1.32±0.33†</td>
<td>0.68±0.38</td>
<td>0.72±0.27</td>
</tr>
<tr>
<td>20:2 n-6</td>
<td>2.06±0.58</td>
<td>1.56±0.44</td>
<td>1.91±0.46</td>
<td>1.50±0.16*</td>
<td>1.62±0.25</td>
<td>1.41±0.27</td>
</tr>
<tr>
<td>20:4 n-6</td>
<td>12.13±1.01</td>
<td>11.77±1.21</td>
<td>11.50±2.47</td>
<td>11.12±2.39</td>
<td>11.94±1.33</td>
<td>11.40±1.94</td>
</tr>
<tr>
<td>20:5 n-3</td>
<td>0.77±0.23</td>
<td>1.17±0.48</td>
<td>0.93±0.28</td>
<td>1.06±0.32</td>
<td>0.69±0.21</td>
<td>1.28±0.72</td>
</tr>
<tr>
<td>22:4 n-6</td>
<td>1.32±0.56</td>
<td>1.60±0.22</td>
<td>ND</td>
<td>ND</td>
<td>2.09±0.56</td>
<td>1.61±0.65</td>
</tr>
<tr>
<td>22:5 n-3</td>
<td>1.63±0.83</td>
<td>1.33±0.03</td>
<td>1.26±0.43</td>
<td>1.50±0.45</td>
<td>1.51±0.35</td>
<td>1.32±0.31</td>
</tr>
<tr>
<td>22:6 n-3</td>
<td>4.77±1.48</td>
<td>5.35±0.33</td>
<td>4.64±0.81</td>
<td>4.61±1.17</td>
<td>4.40±0.91</td>
<td>4.93±0.68</td>
</tr>
</tbody>
</table>

Data are mean values ± SD from three independent analyses, corresponding to a subgroup of subjects (n = 12). *, p<0.05; †, p<0.01; ‡, p<0.001 compared to the baseline. ND: not detected.
TABLE 4. Structural properties of reconstituted erythrocyte membrane from hypertensive patients assigned to a Mediterranean diet enriched with nuts (VDF, VDF1) or virgin olive oil (VOO, VOO1) or to a low-fat diet (VLF, VLF1) at baseline (VDF, VOO, VLF) and after one-year intervention (VDF1, VOO1).

<table>
<thead>
<tr>
<th>Sample</th>
<th>*Δ T_{Lα} (°C)</th>
<th>†d_{Lα} (nm)</th>
<th>†d_{HII} (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLF</td>
<td>10 – 10 (70)</td>
<td>7.25</td>
<td>9.94</td>
</tr>
<tr>
<td>VDF</td>
<td>10 – 25 (70)</td>
<td>7.62</td>
<td>10.73</td>
</tr>
<tr>
<td>VDF1</td>
<td>10 – 20 (70)</td>
<td>7.03</td>
<td>10.41</td>
</tr>
<tr>
<td>VOO</td>
<td>10 – 50 (60)</td>
<td>7.88</td>
<td>9.97</td>
</tr>
<tr>
<td>VOO1</td>
<td>10 – 42 (50)</td>
<td>7.77</td>
<td>9.42</td>
</tr>
</tbody>
</table>

* Temperature range where the L_{α} phase was observed is shown in Δ T_{Lα}. The parenthesis on the right indicates the temperature limit of the L_{α} phase in the L_{α} + H_{II} temperature range coexistence. † Repeat distance, d_{Lα} at 40°C and d_{HII} at 50°C. VLF1 data (not shown) were similar to VLF.
FIGURE 2

A

C-reactive protein (mg/L)

Lowfat MD+nuts MD+VOO

B

IL-6 (ng/mL)

Lowfat MD+nuts MD+VOO

C

E-selectin (ng/mL)

Lowfat MD+nuts MD+VOO

D

P-selectin (ng/mL)

Lowfat MD+nuts MD+VOO

***

*
FIGURE 3

(A) LF diet

(B) MD+nuts

(C) MD+nuts intervention

(D) MD+VOO

(E) MD+VOO Intervention

(F) $H_\parallel$

(G) $T_{max}$

(H) M

(I)

(J)

Scattering Intensity (a.u.) vs. $s$ (nm$^{-1}$)

Repeat Distance (nm) vs. Temperature ($^\circ$C)

FIGURE 3