$CD142^+/CD61^+$, $CD146^+$ and $CD45^+$ microparticles predict cardiovascular events in high risk patients following a Mediterranean diet supplemented with nuts.

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Short running title: $CD142^+/CD61^+$, $CD146^+$ and $CD45^+$ cMPs predict CVE

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Abstract

Circulating microparticles (cMPs) are small phospholipid-rich microvesicles shed by activated cells that play a pivotal role in cell signalling related to the pathogenesis of atherothrombosis. We aimed to investigate the prognostic value of cMPs released from different vascular cells for cardiovascular event (CVE) presentation in asymptomatic patients at high cardiovascular risk factors under nutritional and pharmacologic treatment. This is a nested case-control study of 50 patients from the 5-year follow-up prospective PREDIMED trial enrolled in the nuts arm of the Mediterranean diet (MedDiet-nuts). We randomly selected 25 patients who had suffered a CVE during follow-up and pair-matched them for sex, age, and classical CV risk factors to 25 patients who remained asymptomatic (no-CVE). Total Annexin V-$(AV)^+$ cMPs and cMPs from cells of the vascular compartment were quantified by flow cytometry at baseline and after one year follow-up. MedDiet-nuts and pharmacological treatment neither modified levels nor source of MP shedding in CVE patients. However, no-CVE patients showed 40-86% decreased total AV^+, PAC-$1^+/AV^+$, $CD61^+/AV^+$, $CD142^+/CD61^+/AV^+$, $CD62P^+/AV^+$, $CD146^+/AV^+$, $CD63^+/AV^+$ and $CD11a^+/AV^+$ cMPs at one year follow-up ($P \leq 0.046$, all). $CD142^+/CD61^+/AV^+$, $CD146^+/AV^+$ and $CD45^+/AV^+$ cMPs were decreased in no-CVE patients compared to CVE patients. A ROC-curve clustered model for $CD142^+/CD61^+/AV^+$, $CD45^+/AV^+$ and $CD146^+/AV^+$ cMPs predicted a future CVE [$P<0.0001$, AUC=0.805 (0.672 to 0.938)]. In patients at high CV risk profile treated with a controlled MedDiet supplemented with nuts and receiving up-to-date CV drug treatment, reduced cMPs derived from activated platelets, leukocytes and endothelial cells are predictive of protection against CVE within the next 4 years.

Keywords: circulating microparticles, platelets, tissue factor, cardiovascular event, leukocytes.

Introduction

Circulating microparticles (cMPs) are a subtype of phospholipid-rich vesicles of 0.1–1.0 μm in size shed from the plasma membrane of cells when activated or when undergoing apoptosis or necrosis. MPs have been shown to reflect cellular activation and/or tissue damage occurring in vivo (1). cMPs contribute to both vascular disease initiation and progression and ensuing clinical outcomes. Indeed, cMPs have recently emerged as mediators of cell-to-cell communication acting as biological messengers with key roles in various pathophysiological states. Elevated platelet MP levels have been documented after an ischemic stroke (2), in patients with severe hypertension (3) and in those at increased risk of coronary heart disease (4), and have also been found to enhance thrombosis in atherosclerotic plaques (5). Endothelial MPs affect vascular tone, permeability and haemostasis, and high endothelial cMPs correlate with loss of flow-mediated dilation, arterial stiffness (6), and severe hypertension (3). Moreover, circulating lymphocyte-derived MPs appear to be markers of lipid-rich atherosclerotic plaques in patients with familial hypercholesterolemia patients (7). Besides, the surface of MPs contains phosphatidylserine (PS), a negatively charged phospholipid that plays a crucial role in the assembly of the activation complexes of the plasma coagulation system and thus displays pro-coagulant activity (8). Therefore, cMPs have emerged as new biomarkers of cardiovascular disease (CVD) and strategies to decrease cMPs may reduce CVD risk.

Dietary and lifestyle interventions are the first line in the prevention and treatment of CVD. Indeed, the Mediterranean Diet (MedDiet) has been shown to decrease the incidence of major CVD by 30% (9). Several mechanisms have been proposed to explain this protective effect, such as decreasing pro-atherogenic gene expression (10), cell adhesion molecules and inflammatory biomarkers (11), besides the potency of classical CV risk factors (12). Nevertheless, CVD is still the primary cause of death worldwide (13), principally caused by atherosclerosis. In the atherosclerotic process, stress activated cells of the vascular compartment release signals such as cytokines and MPs that affect the arterial circulation, plaque progression and vulnerability, finally leading to cardiovascular events (CVE).

Therefore, we aimed to investigate in asymptomatic patients with high cardiovascular risk factors under nutritional and pharmacologic treatment whether the activation of the cells of the vascular compartment are predictive of CVE presentation.

Materials and Methods

Study design

The PREDIMED interventional trial (www.predimed.es; ISRCTN35739639) is a long-term follow-up randomized, controlled, multicenter, and parallel-group study designed to evaluate the efficacy of the MedDiet on the primary prevention of CVD. The detailed study design is extensively described elsewhere (9, 14). Briefly, patients were randomly assigned to one of the three dietary interventions: MedDiet supplemented with extra virgin olive oil, MedDiet supplemented with nuts (15 g of walnuts, 7.5 g of hazelnuts, and 7.5 g of almonds, MedDiet-nuts) and a low fat diet following the American Heart Association (AHA) guidelines, with no caloric restriction in any of the intervention groups, nor physical activity advice.

The inclusion criteria were as follows: men from 55 to 80 years of age or women from 60 to 80 years of age; no CVD at enrolment; type 2 diabetes mellitus or at least three of the following cardiovascular risk factors: smoking, hypertension, elevated low-density lipoprotein cholesterol levels, low high-density lipoprotein cholesterol levels, overweight or obesity, or a family history of premature coronary heart disease. Exclusion criteria were: documented history of CVD, immunodeficiency, illegal drug use or alcoholism and cancer or any other severe chronic illness.

Patients

Fifty patients from the MedDiet-nuts arm of the PREDIMED trial were included in the present nested case-control study. Twenty-five patients with a documented CVE during follow-up (from now, CVE patients) were randomly selected according to a computer-generated table. CVE was composed by acute myocardial infarction (AMI), stroke or cardiovascular death (CV death). Control patients (25 high CV risk patients without documented CVE at the end of the study, called from now no-CVE) were pair-matched for sex, age, body mass index (BMI), diabetes, hypertension and hyperlipidemia.
As a measure of compliance, at baseline and after 12 months of follow-up, the participants filled out a validated food frequency questionnaire and a 14-item questionnaire assessing adherence to the MedDiet (15). The Minnesota leisure-time physical activity questionnaire was administered to record physical activity. The dietary information was converted into nutrient data by using the Food Processor Nutrition and Fitness Software (Esha Research). Medication use, anthropometric parameters, blood pressure and other main cardiovascular risk factors were also recorded. Framingham Risk Score (FRS) for coronary heart disease (% of risk at 10 years) was calculated using the high-risk Framingham Heart Study equations of the National Cholesterol Education Program (16).

Blood sampling

Two sample measurements were considered: at baseline and after one year of dietary intervention. Fasting venous blood was withdrawn into 3.8% trisodium citrate-anticoagulant tubes. Blood cells were removed by centrifugation (1500×g, 15 min) at room temperature (RT). Plasma was carefully aspirated, and 500 µL aliquots were immediately frozen and stored at -80ºC until processing for isolation and quantification of cMPs.
Additionally, biochemical and haematological parameters were quantified by standardized methods.

Circulating microparticles isolation and quantification

The cMP fraction was isolated from plasma by a two-step high-speed centrifugation. Briefly, 500 µL of frozen plasma aliquots were thawed on melting ice for 1 hour and centrifuged again at 1,500×g, 10 min, RT to guarantee the complete cell removal. Then, 250 µL of plasma was transferred to another vial and centrifuged at 20,000×g for 30 min at RT to pellet cMPs. The supernatants were discarded and the cMP-enriched pellet was washed once with citrate-phosphate buffered saline (PBS) solution (citrate-PBS; 1.4 mmol/L phosphate, 154 mmol/L NaCl, 10.9 mM trisodium citrate, pH 7.4) before a second equal centrifugation step was made. Finally, the remaining cMP pellets were resuspended in 100 µL citrate-PBS.

Triple-label flow cytometric analysis was performed as described before (17, 18). Briefly, 5 µL of washed cMP suspensions were diluted in 30 µL PBS buffer containing 2.5 mM $CaCl_2$ (Annexin Binding Buffer, ABB). Thereafter, combinations of 5 µL of CF405M-conjugated Annexin V (AV, BD-horizon) with two specific monoclonal antibodies (mAb, 5 µL each, **Table 1** and **Supplemental Table 1**) labelled with fluorescein isothiocyanate (FITC) and phycoerythrin (PE), or the isotype-matched control antibodies were added in a final volume of 50 µL ABB (working dilution 1:10 for each antibody). Samples were incubated 20 min at RT in the dark and diluted with ABB before being immediately analyzed on a FACSCantoII™ flow cytometer. Acquisition was performed at 1 minute per sample. Forward scatter (FSC), side scatter (SSC) and fluorescence data were obtained with the settings in the logarithmic scale. cMPs were identified and quantified based on their FSC/SSC characteristics according to their size, binding to AV and reactivity to cell-specific mAb.
Gate limits were established following the criteria previously described (18), as depicted in **Figure 1**. The lower detection limit was placed as a threshold above the electronic background noise of the flow cytometer and the upper threshold for FSC to 1 µm was set with the Megamix-Plus FSC beads (BioCytex, Marseille, France). To identify positive stained events, thresholds were also set based on samples incubated with the same final concentration of isotype-matched control antibodies after titration experiments. Before all analyses, fluorescence minus one experiments were performed to compensate the spectral overlap between FITC and PE. AV binding level was corrected for autofluorescence using fluorescence signals obtained with microparticles in a calcium-free PBS.
Data were analyzed with the FACSDiva™ software (version 6.1.3, Becton Dickinson). The cMP concentration (number of cMPs per µL of plasma) was determined according to Nieuwland's formula (8), based on sample's volume, flow cytometer's flow rate (FR) and the number of fluorescence-positive events (N), as follows: $cMPs/\mu L = N \times (V_f/V_a) \times (_{Vt/}FR) \times (1/V_i)$ [where V_f(µL) = final volume of washed cMP suspension, V_a(µL) = volume of washed cMP suspension used for each labeling analysis, V_t(µL) = total volume of cMP suspension before fluorescence-activated cell sorting analysis, FR(µL/min) = flow rate of the cytometer at low mode (the average volume of microparticle suspension analyzed in one minute), 1 is the µL unit of volume, and V_i(µL) = original volume of plasma used for microparticle isolation]. FR was measured before each experiment. To reduce background noise buffers were prepared on the same day and filtered through 0.2 µm pore size filters under vacuum.

Statistical analysis
Sample size was determined with the ENE 3.0 statistical program (GlaxoSmithKline, Brentford, United Kingdom) assuming a loss of 0% participants because samples were already frozen. To detect mean differences in the number of $CD61^+/AV^+$ cMP of 160 units with a conservative SD of 150, 14 patients would be needed to complete the study (α risk=0.05, power=0.9). However, to obtain greater statistical power, the sample size was nearly doubled. The number of $CD61^+/AV^+$ cMP was used to determine the sample size but all cMPs were considered primary outcomes.
Statistical analyses were performed using the SPSS Statistical Analysis System (version 22.0). Descriptive statistics [mean ± sd or n (%)] were used to describe the baseline characteristics of the patients and the outcome variables. To compare baseline characteristics between no- and CVE patients, a Chi-square analysis of

frequencies of qualitative variables (risk factors and medications) and Mann-Whitney U test for quantitative variables were performed.
To analyze changes in cMPs, all variables with a skewed distribution were transformed to their natural logarithms for parametric analyses and are shown as antilogarithmic values to facilitate the interpretation of the results. Changes after one year intervention for no- and CVE patients separately were analyzed with a *t*-test for related samples. A *t*-test for unpaired samples was used to compare the differences of changes in outcome variables after one year intervention between no- and CVE patients.
ROC curve analyses were performed to identify the threshold concentration of cMPs able to discriminate between patients and controls, and the corresponding area under the curve (AUC) with its 95% confidence interval (CI) was calculated. A cut-off level of cMPs was determined with the shortest distance from upper left corner of the ROC curve, minimizing $[(1\text{-sensitivity})^2 + (1\text{-specificity})^2]$. Multivariable models for the prediction of a CVE were performed with a logistic regression model with cMP levels from different cell origins by creating predicted probabilities, which then were transferred to the ROC curve algorithm to estimate the likelihood of a future CVE by calculating the corresponding AUC along with their 95 % CI. P was considered significant when <0.05.

Results

Baseline characteristics

Table 2 shows the baseline characteristics of the 50 patients included in the study. Per protocol, there were no significant differences in sex, age, body weight, BMI, blood pressure, presence of CV risk factors or cardiovascular drugs between patients who suffered a CVD event or remained asymptomatic. CVE patients suffered a CVE during a mean follow-up time of 3.8±1.5 years, while no-CVE patients were free of documented CVE throughout the study (mean follow-up of 4.9±1.7 years). Overall, 11 patients suffered an AMI, 10 suffered a stroke and 4 died from CVD (2 died from a stroke, 1 died from a coronary artery disease and 1 died from congestive heart failure). As presented in **Table 1**, baseline levels of cMPs showed no statistical differences between no- and CVE patients.

Compliance with the intervention

According to the 14-item questionnaire, adherence to MedDiet increased by two points after one year of MedDiet-nuts intervention ($P<0.0001$, *t* test). No differences were observed in the changes of the adherence to the MedDiet after one year between no- and CVE patients (data not shown). As shown in **Table 3**, total energy, protein, and saturated fatty acids intakes remained constant after one year intervention. However, consumption of total fat, monounsaturated fatty acids, polyunsaturated fatty acids, α-linolenic acid and marine (n-3) fatty acids increased, and consumption of carbohydrates decreased after one year intervention, indicating good compliance with the dietary instructions.
Moreover, no differences were observed in the changes of energy and nutrient intakes after one year between no- and CVE patients (Supplemental Table 2).
In addition, as shown in **Table 4**, no significant changes in anthropometric, biochemical or haematological parameters were observed after one year intervention. Again, no differences were observed in the changes of these parameters between no- and CVE patients after one year follow-up (Supplemental Table 3).

Changes in circulating microparticles after one year MedDiet-Nuts intervention
MedDiet-nuts intervention did not influence MP shedding in CVE patients. However, in no-CVE patients one year of MedDiet-nuts intervention decreased by ~49% AV^+ (*P*=0.014), ~69% PAC-$1^+/AV^+$ (*P*=0.016), ~40% $CD61^+/AV^+$ (*P*=0.011), ~49% $CD142^+/CD61^+/AV^+$ (*P*=0.046), ~40% $CD62P^+/AV^+$ (*P*=0.038), ~86% $CD146^+/AV^+$ (*P*=0.034), ~59% $CD63^+/AV^+$ (*P*=0.040) and ~64% $CD11a^+/AV^+$ cMPs (*P*=0.040) **(Figure 2** and **Table 1).**
The comparison of the changes in cMPs after one year follow-up between no-CVE and CVE patients is shown in **Figure 3**. Platelet-derived MPs carrying TF ($CD142^+/CD61^+/AV^+$), cMPs from endothelial cells ($CD146^+/AV^+$) and leukocyte-derived cMPs ($CD45^+/AV^+$) were decreased in no-CVE patients compared to CVE patients, who showed increased shedding after one year MedDiet-nuts intervention (*P*=0.047, 0.045 and 0.020, respectively).

cMPs as a prognostic marker for CVE presentation
In order to evaluate the predictive power of cMPs for CVE presentation, ROC curve analyses were performed for $CD142^+/CD61^+/AV^+$, $CD146^+/AV^+$ and $CD45^+/AV^+$ cMP levels after one-year follow-up. $CD45^+/AV^+$ cMPs at 112.03 cMPs/µl of PFP (*P*=0.035) properly discriminated between no- and CVE patients with a 64% sensitivity and 60.9% specificity [area under de curve (AUC)=0.669 (95% CI 0.512 to 0.825)] (**Figure 4A**) followed by $CD142^+/CD61^+/AV^+$ cMPs at a cut-off point of 102.6 cMPs/µl of PFP, *P*=0.045, 69.6% sensitivity and 56% specificity, AUC=0.669 (0.512 to 0.825) (**Figure 4B**). $CD146^+/AV^+$ cMPs analyzed independently were not predictive of a future CVE [*P*= 0.068, AUC= 0.655 (0.499 to 0.812)].
When both $CD45^+/AV^+$ and $CD142^+/CD61^+/AV^+$ MPs were considered together, the predictive power of a future CVE increased [*P*= 0.019, AUC= 0.698 (0.548 to 0.848)], as depicted in **Figure 4C**. The best AUC was reached when the three types of cMPs were included in the multivariable model [*P*< 0.0001, AUC= 0.805 (0.672 to 0.938)] (**Figure 4D**). Therefore, $CD142^+/CD61^+/AV^+$, $CD45^+/AV^+$ and $CD146^+/AV^+$ cMPs considered together best predicted a future CVE. In these patients the FRS was not predictive of a future CVE [*P*= 0.648, AUC= 0.538 (0.372, 0.703)] because both cases and controls had similar cardiovascular risk burden. The incorporation of $CD142^+/CD61^+/AV^+$, $CD45^+/AV^+$ and $CD146^+/AV^+$ cMPs to the FRS increased the predictive power significantly [*P*< 0.0001, AUC= 0.806 (0.672 to 0.939), Supplemental Figure 1]. Therefore, cMPs show higher predictive degree for CVD presentation than the commonly used risk score.

Discussion

As shown by the area under the ROC curve, the main finding of this nested case-control study is that cMPs from platelets and leukocytes predict CVE presentation in PREDIMED participants undergoing a MedDiet-nuts intervention. The participation of endothelial cell passivation is less prominent, as shown by the low number of $CD146^+/AV^+$ cMPs compared to cMPs derived from other parental cells. Therefore, MP shedding from cells of the vascular compartment relate to CVD progression. In patients who remained asymptomatic after 5 years of follow-up, the MedDiet-nuts was associated with a novel CV protective effect, by decreasing MP shedding from

platelets, endothelial cells and leukocytes. MP shed from cells of the vascular compartment have procoagulant activity, accelerating the onset and progression of atherosclerotic lesion. Therefore, our results suggest that MP shedding is one of the pathways, among others, which partially explains the presentation of a CVE in patients receiving conventional cardiovascular drug therapy and dietary intervention. The MedDiet-nuts intervention did not alter glycaemia, cholesterol or blood pressure in this subset of the PREDIMED cohort. Interestingly, cMPs seem to provide information on subtle blood compartment cell changes, therefore becoming more sensitive markers to reflect changes in CV risk than the conventional used scores such as the FRS, which was unchanged after one year in neither CVE patients nor no-CVE patients. Hence, the combined analysis of these MPs improved the prediction of CVE presentation of current predictive models used in the clinical setting.

To the best of our knowledge, this is the first time that long-term MedDiet-nuts effects on MP shedding of the main cells of the vascular compartment have been analyzed considering separately patients with and without a future CVE. In a similar cohort at high CV risk, we have recently observed that a MedDiet supplemented with extra virgin olive oil decreased PAC-1^+/AV^+, $CD3^+$/$CD45^+$/AV^+ and Smooth Muscle Actin-α^+/AV^+ MP shedding by 38-63% in no-CVE patients while CVE patients showed increased $CD3^+$/$CD45^+$/AV^+ and $CD14^+$/AV^+ cMPs by 116% and 88% respectively (17), indicating that the whole dietary pattern of MedDiet influences MP shedding.

There is little information on the relationship between diet and MP shedding, and the few studies performed evaluated short-term outcomes. An oat-enriched diet during 8 weeks reduced fibrinogen- and tissue factor platelet-derived cMPs and monocyte-derived $CD11b^+$ cMPs in type 2 diabetic patients, whereas a standard dietary advice (consisting in decreasing fat and sugar intake) reduced fibrinogen and platelet MPs, compared to their habitual intake (19). Fish-oil supplementation has been shown to decrease the number of endothelial-derived cMPs but not platelet-derived MPs in moderate CV risk patients compared to a placebo of corn oil during 8 weeks, independently of their eNOS genotype (20). On the other hand, Del Turco et al (21) observed a decrease in platelet and monocyte but not endothelial-derived or tissue factor positive cMPs after 12 weeks of n-3 fatty acid supplementation in AMI survivors. In our study patients, marine n-3 fatty acids intake remained constant throughout the study and no correlations existed between changes of nutrients intake and changes in cMPs (data not shown), both in no-CVE and CVE patients. Therefore, changes in MP shedding may be attributable to the whole dietary pattern rather than to a single nutrient.

A previous study evaluated the short-term effects of a MedDiet intervention on endothelial-derived MP shedding (22). In that study, 20 elderly subjects at low-to-moderate CV risk, received for 4 weeks a MedDiet enriched with virgin olive oil, a diet rich in saturated fatty acids, or a low-fat, high-carbohydrate diet enriched in n-3 polyunsaturated fatty acids in a crossover design. Marin *et al*. observed that endothelial-derived ($CD31^+$) and activated endothelial-derived cMPs ($CD144^+$/$CD62E^+$) decreased after the MedDiet in comparison to the diet enriched in n-3 fatty acids and this diet was also associated with a reduction of these cMPs compared to the saturated fat-rich diet (22). Accordingly, in our moderate-to-high risk population subset, we observed a significant decrease in endothelial-derived MPs after MedDiet-nuts in no-CVE patients after one year intervention compared to baseline. We did not observe the same effect, however, after a MedDiet supplemented with extra virgin olive for 1 year

(17). The differences in observed effects may be partially attributed to the differences in the dietary patterns in these studies.

We observed decreased platelet-derived cMPs (PAC-1^+, $CD61^+/AV^+$, $CD142^+/CD61^+/AV^+$, $CD62P^+/AV^+$ and $CD63^+$) in no-CVE patients after one year follow-up. cMPs from platelets have been shown to enhance platelet deposition and thrombus formation in atherosclerotic plaques (5). Therefore, decreasing the levels of platelet-derived MPs may delay the progression of atherosclerotic lesions.

Remarkably, no-CVE patients showed a 50% reduction of AV and platelet-originated cMPs carrying TF and a 64% reduction of CD11a- loaded MP shedding. AV binds phosphatidylserine, which plays a crucial role in the assembly of the activation complexes of the plasma coagulation cascade and thus exhibitis pro-coagulant activity (8). In addition, TF is one of the main pro-coagulant factors, but recent findings indicate that beyond thrombosis TF is involved in other processes such as transcellular signaling or angiogenesis (23). CD11a is expressed in leukocytes and plays a central role in leukocyte intercellular adhesion (24) thus contributing to the progression of atherosclerotic lesion. Therefore, a reduction in the concentration of AV-, TF- and CD11a-positive cMPs suggests a reduced endothelial damage and systemic inflammation after one year MedDiet-nuts intervention in no-CVE patients. In addition, a reduction of cMPs bearing PAC-1 and CD62P was also observed in no-CVE patients after one year of dietary intervention. Nomura *et al*. (25) observed elevated $CD62P^+$ (P-selectin) and PAC-1^+ ($\alpha_{IIb}\beta_3$-integrin) platelet-derived cMPs in patients with arteriosclerosis obliterans compared to matched controls, and higher platelet expression of CD62P and PAC-1 was also associated with carotid atherosclerosis in convalescent stroke patients (26) independently of the type of stroke. Circulating $CD62P^+$ MPs have been found to be increased in hypertensive patients compared to controls (3, 27). Matsumoto *et al*. (28) found $CD62P^+$ and PAC-1^+ cMPs elevated in patients with AMI compared to patients with stable angina, suggesting that these molecules are involved in the complications of coronary artery disease. As reviewed in (29), selectins such as CD62P may contribute to the development of several CVD like atherosclerosis, arterial and deep vein thrombosis and ischemia-reperfusion injury, among others.

This study is not exempt of limitations. Although flow cytometry is the most widely used method for characterization and quantification of cMPs, its limit of detection (according to beads signal) is comprised between 0.1 and 0.3 µm diameter particle size. Therefore, information on the smaller microparticles is missing. In addition, we have used a dim fluorochrome (FITC) which has a spectral overlap into PE. Nevertheless, we performed fluorescence minus one experiments before starting the study to compensate this overlapping. Despite these technical limitations, flow cytometry is used in the clinical setting and therefore, a reliable tool to improve the assessment of individual CV risk.

Blood pressure was quite elevated in these patients despite the antihypertensive therapy, probably because it was measured before the administration of the hypertensive medication (or any other medication) on the morning of blood sampling. Nevertheless, no statistical differences were observed between no- and CVE patients at baseline or after one year follow-up. Finally, more no-CVE patients than CVE patients were treated with statins. This could potentially bias the results because statins are known to decrease MP shedding (30). Nevertheless, no differences in cMPs according to statin therapy were observed either in no-CVE or in CVE patients at baseline (not shown) and after one year follow-up the statin-treated patients were still

on statin therapy. Therefore changes in MP shedding at one year may not be attributed to statins.
In summary, the effects of a MedDiet-nuts on MP shedding have been analyzed separately in no- and CVE patients in relation to CVD presentation. Importantly the MedDiet-nuts intervention has been shown to be protective against CVD presentation over a conventional AHA-low fat diet in the PREDIMED study (9). However, the sensitive analysis of MP shedding can identify the individuals who remained protected under this diet from those who developed a CVE.
The underlying mechanisms by which CVE patients do not respond to the protective effects of the diet in the same manner as no-CVE individuals remains to be elucidated.
In conclusion, emerging cellular markers of vascular damage and integrity appear to be sensitive to identify patients at higher risk to develop a CVE. Overall, cMPs from platelets, endothelial cells and leukocytes are potential prognostic biomarkers of a future CVE in high CV risk patients following a Mediterranean diet rich in nuts.

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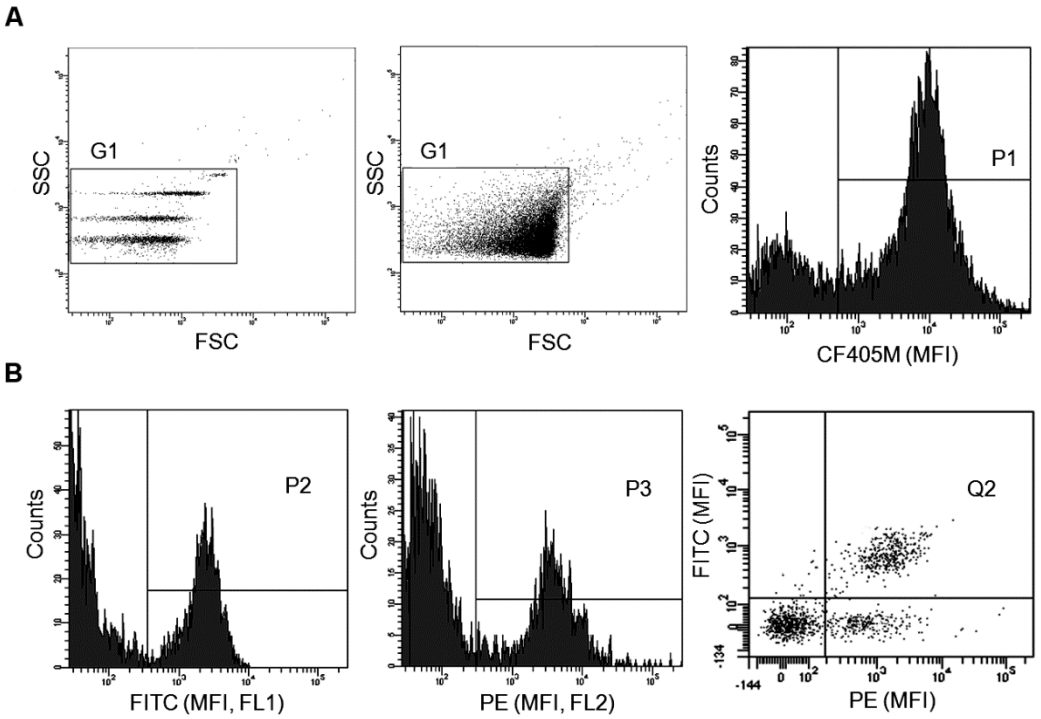
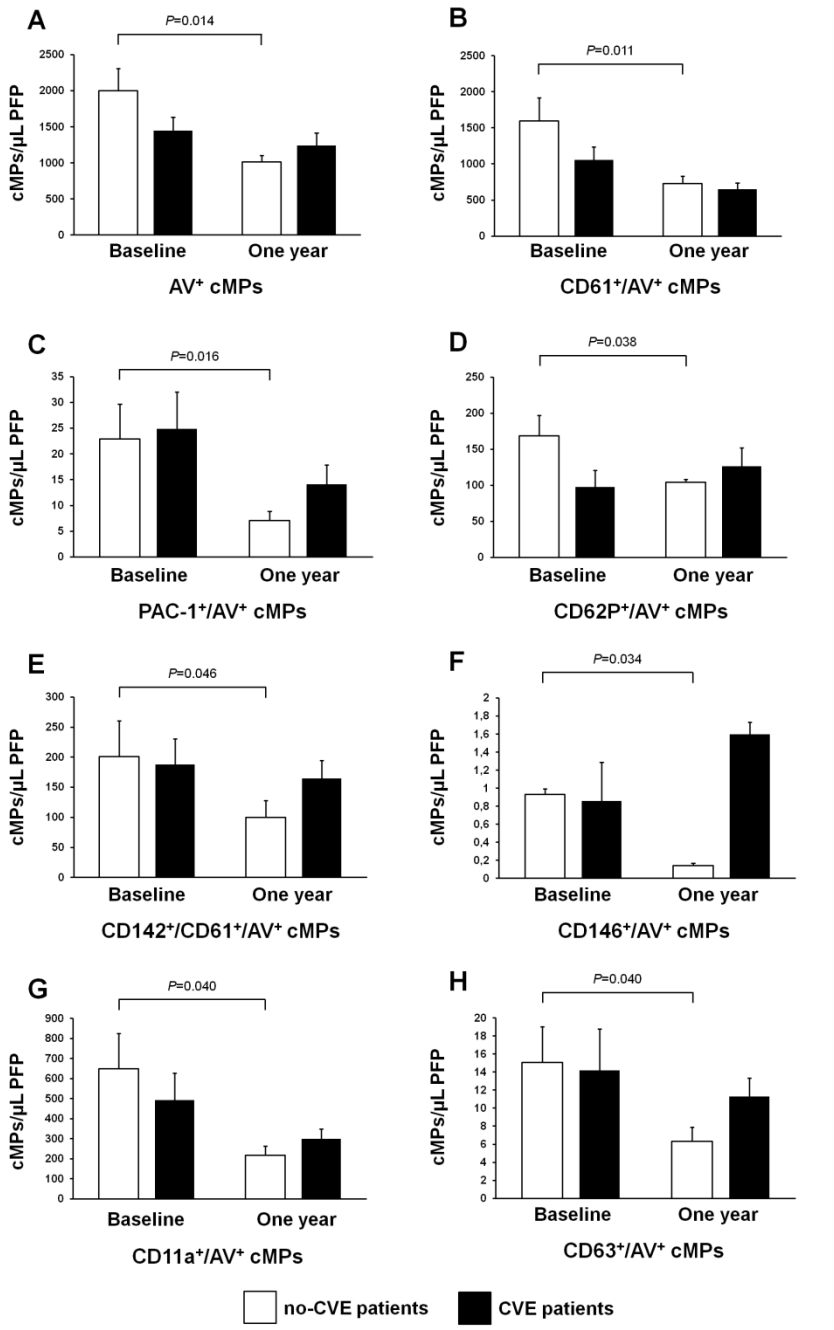
Legends to Figures

Figure 1. Gating and staining strategy for the detection of circulating microparticles in the FACS analysis.



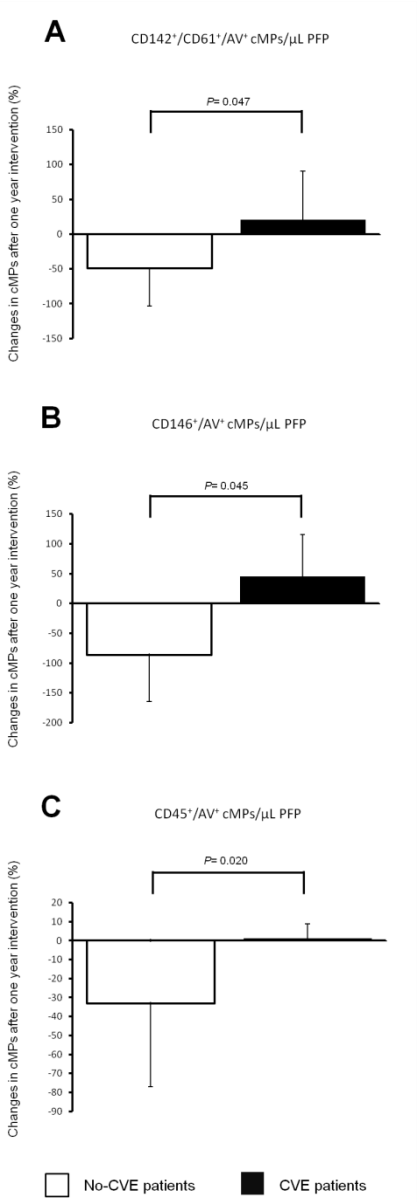
A) Gate limits (G1) in the FSC/SSC plot for microparticle quantification (defined as >0.1µm to <1µm) were established before analyses using the Megamix-Plus FSC beads. B) Gate limits were set according to beads signal and according to cMPs size and granularity (G1). C) Total cMPs Annexin V-CF405M^{+} cMPs (P1) were selected from G1. D) cMPs binding FITC-labeled antibodies were selected from P1 and quantified (P2). E) cMPs binding PE- labeled antibodies were also selected from P1 and quantified (P3). F) Double staining with FITC- and PE-labeled antibodies from P1 (Annexin V^{+} cMPs) was quantified from Q2 region. Used antibodies labeled with FITC or PE are listed in Supplemental Table 1. Before all analyses, fluorescence minus one experiments were performed to compensate the spectral overlap between FITC and PE. Compensations used were as follows: FL2- 27.2% for FL1 and FL1- 2.1% for FL2. CF405M is a blue fluorescent dye quantified in the Pacific blue channel. cMPs indicate circulating microparticles; FITC, fluorescein isothiocyanate (quantified in the FL1 channel); MFI, mean fluorescence intensity and PE, phycoerythrin (quantified in the FL2 channel).

Figure 2. Circulating microparticles at baseline and after one year of intervention in patients who will suffer a cardiovascular event and in patients who will not.



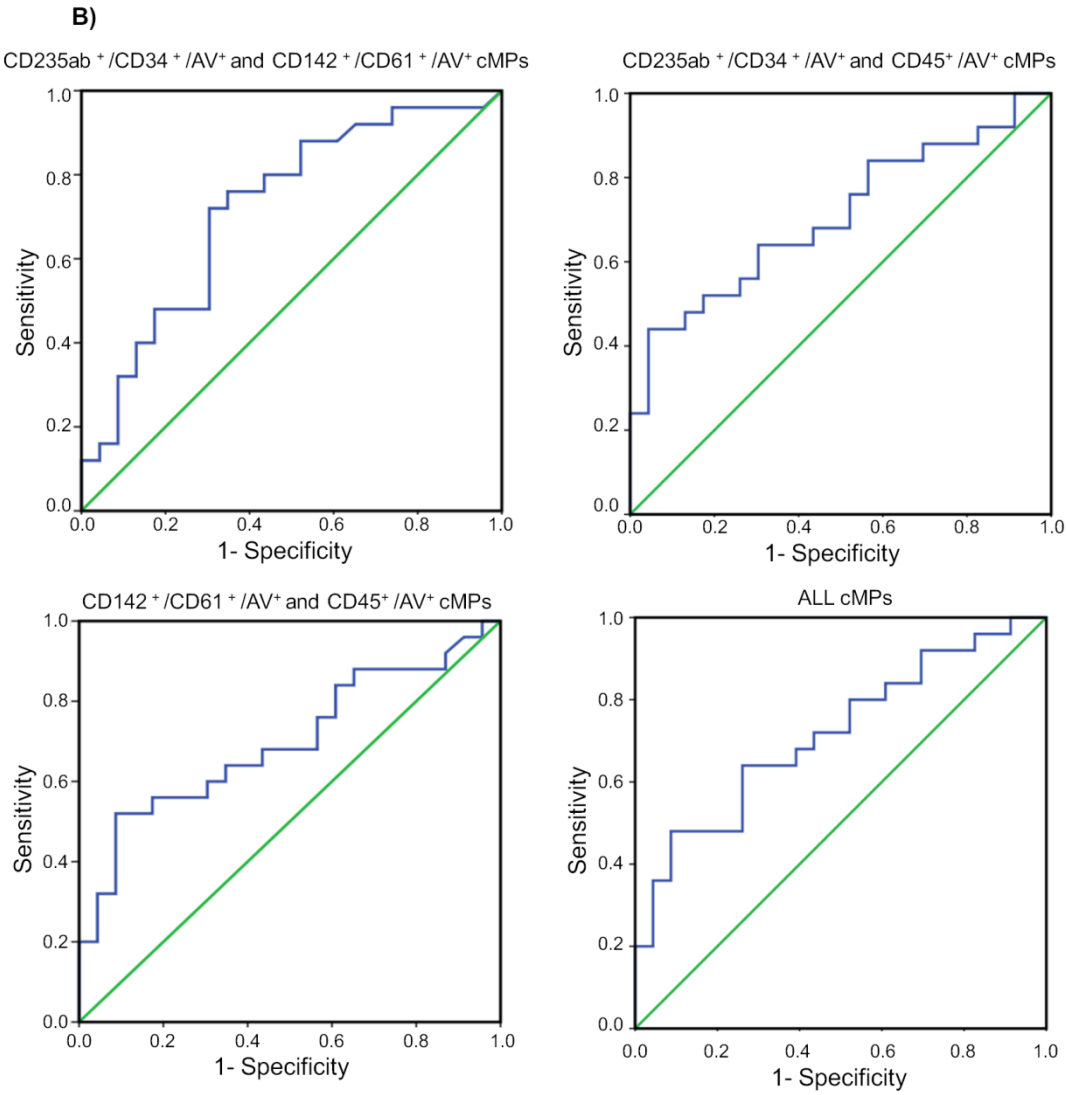
P value for the differences between baseline and after one year of intervention for no-CVE patients (*t*-test for related samples). cMPs denote circulating microparticles; no-CVE, patients without cardiovascular disease; CVE, patients who will suffer a cardiovascular event; AV, Annexin V; and PFP, platelet-free plasma. Used markers were: A) Annexin V for total cMPs; B) CD61 for platelet-derived cMPs; C) PAC-1 ($\alpha_{IIb}\beta_3$-integrin) and D) CD62P (P-Selectin) for activated platelets-derived cMPs; E) CD142/CD61, for platelet-derived cMPs carrying tissue factor; F) CD146 for endothelial-derived cMPs; and G) CD11a and H) CD63 for were markers of cell activation.

Figure 3. Changes in circulating microparticles after one year intervention in patients who will suffer a cardiovascular event and in patients who will not.



P value from the comparison of the differences in cMPs after one year intervention between no- and CVE patients (*t*-test for unrelated samples). A) Changes in $CD142^+$/ $CD61^+/AV^+$ cMPs after one year of intervention; B) Changes in $CD146^+/AV^+$ cMPs after one year of intervention and C) Changes in $CD45^+/AV^+$ cMPs after one year of intervention. cMPs denotes circulating microparticles; CVE, patients who will suffer a cardiovascular event; no-CVE, patients without cardiovascular disease; AV, Annexin V; PFP, platelet-free plasma; Used markers were CD61 for platelets, CD142 for tissue factor, CD146 for endothelial cells and CD45 for leukocytes.

Figure 4. ROC curve analyses to identify the threshold level of cMPs capable of predicting a future CVE.



A) ROC curve analysis to determine the threshold of leukocyte-derived ($CD45^+/AV^+$) cMPs that predicts a future CVE. $CD45^+/AV^+$ cMPs at a cut-off point of 112.03 cMPs/µl of PFP (*P*=0.035) properly discriminated between no- and CVE patients with a 64% sensitivity and 60.9% specificity [area under de curve (AUC)=0.649 (95% CI 0.487 to 0.812)]; B) ROC curve analysis to determine the threshold of platelet-derived ($CD142^+/CD61^+/AV^+$) cMPs that predicts a future CVE. $CD142^+/CD61^+/AV^+$ cMPs at a cut-off point of 102.6 cMPs/µl of PFP, *P*=0.045, 69.6% sensitivity and 56% specificity, AUC=0.669 (0.512 to 0.825) also discriminate between no- and CVE patients; C) ROC curve analysis considering together $CD45^+/AV^+$ and $CD142^+/CD61^+/AV^+$ cMPs [*P*= 0.019, AUC= 0.698 (0.548 to 0.848)] to increase the predictive power, and D) ROC curve analysis considering together $CD45^+/AV^+$, $CD142^+/CD61^+/AV^+$ and $CD146^+/AV^+$ cMPs [*P*= 0.001, AUC= 0.798 (0.660 to 0.936)] which best predicted the presentation of a cardiovascular event. cMPs denotes circulating microparticles; CVE, patients who will suffer a cardiovascular event; no-CVE, patients without cardiovascular disease; AV, Annexin V; PFP, platelet-free plasma and CD142, tissue factor. CD146 is a marker of endothelial-derived cMPs.

Table 1. Circulating microparticles at baseline and after one year dietary intervention in the 50 patients studied.

	no-CVE (n=25)			CVE (n=25)		
cMPs AV$^+$/µL PFP	Baseline	One year	P^1	Baseline	One year	P^2
Total	**1999.88 ± 1540.05**	**1016.37 ± 444.27**	**0.014**	1447.86 ± 941.49	1244.19 ± 876.72	0.502
Platelet-derived MPs						
PAC-1$^+$	**22.92 ± 33.95**	**7.06 ± 8.88**	**0.016**	24.88 ± 35.75	14.12 ± 18.86	0.270
CD62P$^+$	**168.92 ± 197.67**	**104.32 ± 104.83**	**0.038**	97.48 ± 80.66	126.5 ± 139.94	0.581
PAC-1$^+$/CD62P$^+$	0.68 ± 1.34	0.45 ± 1.8	0.633	0.51 ± 2.36	0.18 ± 0.88	0.329
CD61$^+$	**1278.58 ± 1204.92**	**727.25 ± 534.24**	**0.011**	944.98 ± 708.08	652.16 ± 415.39	0.100
CD142$^+$/CD61$^+$	**201.15 ± 298.51**	**99.77 ± 140.52**	**0.046**	188.03 ± 211.7	164.91 ± 146.87	0.128
Endothelial-derived MPs						
CD146$^+$	**0.94 ± 1.55**	**0.14 ± 0.67**	**0.034**	0.87 ± 2.05	1.64 ± 3.32	0.225
CD62E$^+$	142.16 ± 93.96	112.23 ± 72.25	0.220	136.45 ± 122.21	146.29 ± 108.45	0.631
CD146$^+$/CD62E$^+$	0.28 ± 0.99	0 ± 0	0.186	0.28 ± 1.35	0.61 ± 3.03	0.328
Hematopoietic-derived MPs						
CD34$^+$	178.83 ± 129.7	117.54 ± 45.25	0.058	197.65 ± 133.23	156.07 ± 78.9	0.235
Erythrocyte-derived MPs						
CD235ab$^+$	510.38 ± 526.1	326.7 ± 324.1	0.068	347.81 ± 270.54	314.95 ± 182.16	0.390
Leukocyte-derived MPs						
CD45$^+$	119.96 ± 97.99	101.32 ± 47.58	0.523	137.96 ± 72.85	148.49 ± 81.98	0.980
CD3$^+$/CD45$^+$	0.56 ± 1.87	1.62 ± 4.9	0.303	2.57 ± 9.99	1.46 ± 5.08	0.813
CD14$^+$	11.72 ± 13.88	13.66 ± 13.76	0.760	14.77 ± 13.88	8.94 ± 6.83	0.080
CD3$^-$/CD14$^-$ /CD45$^+$	97.92 ± 88.33	78.27 ± 46.26	0.413	105.27 ± 71.55	114.06 ± 87.69	0.786
CD14$^+$/CD11a$^+$	0.94 ± 1.79	0.97 ± 4.35	0.911	1.27 ± 3.59	1.74 ± 4.41	0.181
Activated cells						
CD142$^+$	439.9 ± 584.05	205.51 ± 235.07	0.076	439 ± 555.49	294.22 ± 216.72	0.129
CD11a$^+$	**648.11 ± 887.54**	**218.48 ± 230.35**	**0.040**	492.36 ± 673.65	298.61 ± 255.43	0.124
CD63$^+$	**15.07 ± 19.72**	**6.34 ± 7.73**	**0.040**	14.18 ± 23.11	11.29 ± 10.16	0.467
CD62L$^+$	96.01 ± 109.31	58.55 ± 46.53	0.053	66.57 ± 49.67	74.61 ± 41.13	0.639
CD63+/CD62L$^+$	0.39 ± 1.44	1.44 ± 6.61	0.380	0.85 ± 4.06	1.19 ± 5.07	0.179

Circulating microparticles were measured by flow cytometry. Results are expressed as mean ± sd. P^1: *P* value for the differences after one year of intervention for no-CVE patients (t test for related samples). P^2: *P* value for the differences after one year of intervention for CVE patients (t test for related samples). cMP denotes circulating microparticles; no-CVE, patients without cardiovascular disease; CVE, patients who will

suffer a cardiovascular event; AV, Annexin V; and PFP, platelet free plasma. Used markers were CD61 for platelet, CD146 for endothelial cells, CD45 for total leukocytes, and CD3 for lymphocyte and CD14 for monocyte origins accounting for agranulocytes. Other leukocytes were inferred subtracting agranulocytes subpopulation from leukocytes fraction and defined as $CD45^{+}/CD3^{-}/CD14^{-}$. The other CDs were used as biomarkers of cell activation (see Supplemental Table 1).

Table 2. Baseline characteristics of the 50 subjects included in the study.

	No-CVE	CVE	*P*
n	25	25	
Males [n (%)]	13 (52)	13 (52)	1.000
Age	68 ± 5	69 ± 6	0.437
Body Weight (Kg)	74.5 ± 10.6	75.5 ± 9.0	0.479
BMI (Kg/m^2)	29.3 ± 3.7	29.7 ± 4.4	0.607
SBP (mmHg)	152 ± 25	156 ± 20	0.366
DBP (mmHg)	82 ± 11	84 ± 8	0.690
Glucose (mg/dL)	125 ± 39	132 ± 63	0.627
Triglycerides (mg/dL)	133 ± 75	149 ± 88	0.491
Total cholesterol (mg/dL)	198 ± 29	210 ± 36	0.120
LDL cholesterol (mg/dL)	128 ± 27	132 ± 36	0.206
HDL cholesterol (mg/dL)	52.3 ± 11.1	55.3 ± 14.5	0.428
Leukocytes (x10^9/L)	6.5 ± 1.3	5.8 ± 1.6	0.123
Neutrophils (%)	54.4 ± 23.6	56.2 ± 20.8	0.284
Lymphocytes (%)	33.6 ± 6.8	31.4 ± 8.1	0.352
Monocytes (%)	6.5 ± 2.1	9.6 ± 14.3	0.339
Eosinophils (%)	2.7 ± 1.1	2.7 ± 1.3	1.000
Basophils (%)	0.7 ± 0.3	0.5 ± 0.4	0.071
Family history of CVD [n (%)]	8 (32)	7 (28)	0.297
Current smokers [n (%)]	6 (24)	7 (28)	1.000
Diabetes [n (%)]	13 (52)	12 (48)	0.779
Dyslipidemia [n (%)]	16 (64)	16 (64)	1.000
Hypertension [n (%)]	18 (72)	19 (76)	0.750
Medication [n (%)]			
Statins	12 (48)	7 (28)	0.149
Insulin	0 (0)	2 (8)	0.153
Hypoglycemiants	7 (28)	9 (36)	0.548
Antiplatelet agents	6 (24)	6 (24)	1.000
Angiotensin-converting-enzyme inhibitor	8 (32)	6 (24)	0.533
Diuretics	4 (16)	8 (32)	0.190
Calcium channel blockers	3 (12)	3 (12)	1.000
B- Blockers	2 (8)	1 (4)	0.556
A- Blockers	1 (4)	0 (0)	0.317
Angiotensin II receptor antagonists	4 (16)	6 (24)	0.484

Results are expressed as mean ± sd or n (%) when indicated. *P* value from the Chi-square analysis for frequencies of qualitative variables (risk factors and medications) and from Mann-Whitney for quantitative variables. No-CVE, patients without cardiovascular disease; CVE, patients who will suffer a cardiovascular event; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; and CVD, cardiovascular disease.

Table 3. Differences in energy and nutrient intakes after 1 year intervention of the subjects included in the study.

	Baseline	One year	*P*
Energy (kcal/d)	2461.86 ± 686.82	2484.28 ± 522.40	0.814
Proteins (g/day)	99.50 ± 26.94	100.53 ± 22.54	0.768
Carbohydrates (g/day)	276.04 ± 100.56	243.76 ± 64.05	0.036
Total fat (g/day)	102.33 ± 32.94	117.80 ± 30.42	0.002
SFA (g/day)	27.61 ± 10.43	25.91 ± 9.14	0.185
MUFA (g/day)	49.57 ± 16.63	57.51 ± 15.33	0.003
PUFA (g/day)	15.96 ± 6.08	23.84 ± 7.26	<0.0001
α-linolenic acid (g/day)	1.53 ± 0.81	2.99 ± 1.17	<0.0001
Marine (n-3) fatty acids (g/day)	0.75 ± 0.43	0.89 ± 0.46	0.029

Results are expressed as mean ± sd. *P* value for the comparison between baseline and after one year intervention for the 50 subjects included in the study (*t*-test for related samples). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids and PUFA, polyunsaturated fatty acids.

Table 4. Differences in anthropometric and biochemical and haematological parameters after 1 year intervention of the subjects included in the study.

	Baseline	One year	*P*
Body Weight (Kg)	75.00 ± 11.74	75.87 ± 12.28	0.075
BMI (Kg/m^2)	29.49 ± 4.53	29.83 ± 4.72	0.072
SBP (mmHg)	153.46 ± 22.99	153.30 ± 26.61	0.972
DBP (mmHg)	82.62 ± 9.85	82.08 ± 11.29	0.768
Heart rate (beats/min)	70.35 ± 11.67	69.51 ± 13.08	0.400
Glucose (mg/dL)	128.59 ± 51.92	123.85 ± 40.47	0.284
Triglycerides (mg/dL)	141.60 ± 83.54	137.54 ± 73.40	0.603
Total cholesterol (mg/dL)	209.14 ± 34.63	205.16 ± 35.22	0.232
LDL cholesterol (mg/dL)	128.65 ± 33.05	127.76 ± 29.19	0.781
HDL cholesterol (mg/dL)	54.24 ± 12.90	52.44 ± 12.37	0.057
Leukocytes (x10^9/L)	6.22 ± 1.63	6.32 ± 1.68	0.543
Neutrophils (%)	55.76 ± 11.87	57.43 ± 8.23	0.355
Lymphocytes (%)	32.14 ± 1.20	31.46 ± 1.26	0.400
Monocytes (%)	8.23 ± 1.69	6.65 ± 0.33	0.336
Eosinophils (%)	2.75 ± 0.18	2.90 ± 0.23	0.514
Basophils (%)	0.57 ± 0.05	0.61 ± 0.05	0.570

Results are expressed as mean ± sd. *P* value for the comparison between baseline and after one year intervention for the 50 subjects included in the study (*t*-test for related samples). BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high density lipoprotein cholesterol; and LDL, low density lipoprotein cholesterol.