$CD3^+/CD45^+$ and SMA-α^+ circulating microparticles are increased in individuals at high cardiovascular risk who will develop a major cardiovascular event.

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All authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

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Circulating microparticles (cMPs) are phospholipid-rich blebs of 0.1–1.0 μm in size shed from the plasma membrane of eukaryotic cells when injured, activated, or undergoing apoptosis. MPs have been shown to reflect cellular activation and/or tissue degeneration occurring in vivo [1]. Due to their molecular composition, cMPs are believed to contribute to vascular disease initiation and progression. Indeed, cMPs have recently emerged as mediators of cell-to-cell communication acting as biological messengers with key roles in various pathophysiological states [2]. Therefore, strategies to characterize MP shedding are being investigated in order to better characterize cardiovascular disease (CVD) risk.

Dietary and lifestyle interventions are the first line of treatment in the management of CVD risk, but information on the effects of dietary patterns on MP shedding is scanty. Moreover, whether cMPs relate to CVD in patients with high CVD risk pharmacologically treated as per guidelines and under a controlled dietary intervention remains unknown.

Therefore, the aim of this study was to evaluate whether MP shedding by cells of the vascular compartment in older subjects with moderate-to-high CVD risk (with at least 3 risk factors and no evidence of clinical CVD) under standard treatment would predict the presentation of future cardiovascular events (CVE).

The PREDIMED interventional trial (www.predimed.org; ISRCTN35739639) is a prospective 5-years follow-up randomized, controlled, multicenter, parallel-group feeding trial designed to evaluate the efficacy of two Mediterranean diets, one supplemented with nuts and the other one supplemented with extra virgin olive oil, on the primary prevention of cardiovascular disease. Both Mediterranean diets showed reduced major adverse cardiac events compared to participants in the control arm (advice on AHA-standard low fat diet) [3].

Fifty patients from the Mediterranean diet supplemented with extra virgin olive oil arm of the PREDIMED trial (see Supplemental Methods) were investigated in this nested case-control study (n=25 per group). Twenty-five patients that had a documented

cardiovascular event (CVE) during the follow-up trial (3.1±0.5 years after inclusion) were randomly selected according to a computer-generated table and 25 controls (within the same intervention arm and without documented CVE at the end of the study, called from now no-CVE) were matched for age, sex and CV risk factors to measure MPs. CVE was composed of acute myocardial infarction, stroke or CVD death. For MP determination, two sample measurements were considered: at baseline and after one year of follow-up. Detailed MP isolation and quantification is shown in Supplemental Methods. Briefly, the cMP fraction was isolated from plasma by a two-step high-speed centrifugation (20,000×g for 30 min at RT). Annexin V positive (AV^+) cMPs from platelets (CD61, PAC-1, CD62P), endothelial cells (CD146, CD62E), leukocytes (CD45), monocytes (CD14), lymphocytes (CD3), erythrocytes (CD235ab) and smooth muscle cells -SMC- (SMA-α) were characterized and quantified by triple-label flow cytometric analysis in a FACSCantoII™ flow cytometer as described before [4,5]. A *t*-test was performed to analyze: a) changes after one year for no- and CVE subjects separately; and b) differences of changes in outcome variables after one year between no- and CVE patients. To evaluate cMP levels as prognostic markers of CVD presentation, associated receiver-operating characteristic (ROC) curve analyses and the corresponding areas under the curve (AUC) with their 95% confidence intervals (CI) were calculated.

Baseline levels of cMPs (samples taken at entry with no control of dietary intervention and pharmacologically treated as per guidelines) were similar between no-CVE and CVE patients except for SMC-derived cMPs, which were significantly higher in no-CVE patients.

After one year of follow-up under controlled diet and drug treatment, $PAC\text{-}1^+/AV^+$, $CD3^+/CD45^+/AV^+$ and $SMA\text{-}\alpha^+/AV^+$ cMPs decreased in no-CVE patients (P=0.020, 0.041 and 0.026, respectively, *t*-test for related samples, Table 1). In CVE patients, $CD3^+/CD45^+/AV^+$ and $CD14^+/AV^+$ cMPs increased significantly (P=0.027 and 0.038, respectively). Therefore, after one year follow-up, no-CVE patients showed decreased

$CD3^+/CD45^+/AV^+$ and SMA-α^+/AV^+ MP levels compared to CVE patients, whose levels were increased (*P*=0.021 and 0.027, respectively, *t*-test for unpaired samples).

ROC analysis with the Framingham Risk Score (FRS) was not predictive of a future CVE (Figure 1, broken line) because both no-CVE and CVE patients presented similar cardiovascular risk burden. Therefore, we evaluated the predictive power of $CD3^+/CD45^+/AV^+$ (lymphocyte-derived) and SMA-α^+/AV^+ (SMC-derived) cMPs at one year of follow-up in patients with CVE. According to the ROC analysis depicted in Figure 1 (continuous line), when these MPs were added to FRS in the cluster model for CVE prediction, the AUC increased from 0.548±0.087 [95% CI: 0.377-0.719] (*P*=0.585) to 0.748±0.078 [95% CI: 0.596-0.900] (*P*=0.006), indicating that cMPs display a higher predictive value for CVE than the commonly used FRS.

In summary, individuals with cardiovascular risk factors without clinical atherosclerosis of one of the study arms of the PREDIMED trial following a Mediterranean Diet that had reduced major adverse cardiac events compared to participants in the control arm (advice on a standard low fat diet) [3], but that still suffered a CVE within the study follow-up period, showed increased MP shedding from lymphocytes and SMC after one year of intervention. Subject from this PREDIMED arm that did not have a future CVE show reduced MP shedding from these cells. Our results suggest that MP shedding relates to CVD progression. MP shed from cells of the vascular compartment have an active cross-talk function, accelerating the onset and progression of atherosclerotic lesion [6,7] and enhancing platelet deposition and thrombus formation on atherosclerotic plaques [8]. Increased MP shedding is mapping activated cells not protected by the conventional cardiovascular drug therapy (standard of care) and dietary intervention and such MPs may also be active participants in the progression of atherosclerosis leading to major CVE. To the best of our knowledge, this is the first time that the relationship between MP shedding from the main cells of the vascular compartment and CVD presentation has been analyzed in pharmacologically and nutritionally well-controlled patients.

Lymphocyte- and SMC-derived MPs add prognostic value to FRS as shown by the ROC curve analysis, potentially improving current predictive models used in the clinical setting. Therefore, leukocyte- and SMC-derived cMPs may be potential prognostic biomarkers of incident CVD, and combined analysis of FRS and these MP may improve the prediction of CVE presentation.

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Conflicts of interest

The authors report no relationships that could be construed as a conflict of interest.

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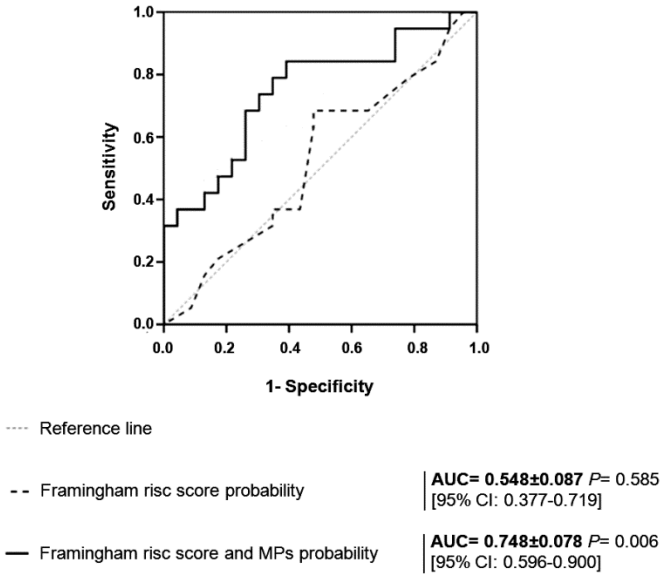
Table 1. Circulating microparticles at baseline and after one year intervention of the 50 subjects.

	no-CVE (n=25)			CVE (n=25)		
cMPs AV$^+$/µL PFP	Baseline	One year	P^1	Baseline	One year	P^2
Total	983.25 ± 527.55	1183.73 ± 646.41	0.224	1007.57 ± 530.41	1318.49 ± 1120.52	0.627
Platelet-derived MPs						
PAC-1$^+$	**13.18 ± 19.2**	**5.06 ± 9.65**	**0.020**	9.8 ± 16.93	15.77 ± 24.85	0.102
CD62P$^+$	66.43 ± 53.19	98.13 ± 79.31	0.189	100.67 ± 101.22	120.16 ± 124.34	0.478
PAC-1$^+$/CD62P$^+$	0.29 ± 1.38	0 ± 0	0.317	0 ± 0	0.34 ± 1.49	0.317
CD61$^+$	656.1 ± 470.15	773.45 ± 516.5	0.287	700.99 ± 600.8	972.61 ± 852.36	0.394
CD142$^+$/CD61$^+$	117.93 ± 128.4	154.55 ± 190.29	0.777	200.25 ± 229.07	232.65 ± 329.48	0.619
Endothelial-derived MPs						
CD146$^+$	0.34 ± 1.49	0.31 ± 1.42	1.000	0.29 ± 1.38	0.1 ± 0.46	0.317
CD62E$^+$	101.04 ± 52.34	105.93 ± 41.94	0.523	110.61 ± 79.32	141.56 ± 147.65	0.664
CD146$^+$/CD62E$^+$	0.27 ± 1.32	0.18 ± 0.88	0.317	0.27 ± 1.32	0.09 ± 0.45	0.317
Hematopoietic-derived MPs						
CD34$^+$	115.42 ± 47.77	123.85 ± 68.15	1.000	137.32 ± 102.61	175.17 ± 145.25	0.317
Leukocyte-derived MPs						
CD45$^+$	125.1 ± 81.74	136.12 ± 116.19	0.056	116.35 ± 82.06	127.48 ± 98.94	0.903
CD3$^+$/CD45$^+$	**0.21 ± 0.94**	**0.09 ± 0.45**	**0.041**	**0.1 ± 0.47**	**1.26 ± 2.09**	**0.027**
CD14$^+$	8.14 ± 8.42	7.47 ± 7.42	0.781	**5.8 ± 6.43**	**10.91 ± 13.85**	**0.038**
CD3$^-$/CD14$^-$/CD45$^+$	99.02 ± 76.79	122.09 ± 114.13	0.052	103.92 ± 83.3	99.22 ± 81.57	0.783
CD14$^+$/CD11a$^+$	0 ± 0	0 ± 0	1.000	0.29 ± 1.38	0.7 ± 1.54	0.144
Smooth muscle cell-derived MPs						
SMA-α$^+$	**8.16 ± 10.86**	**5.15 ± 7.63**	**0.007**	5.14 ± 5.58	6.22 ± 6.82	0.381
CD142$^+$/SMA-α$^+$	1.20 ± 1.72	0.94 ± 1.44	0.582	0.95 ± 1.19	0.60 ± 0.85	0.099
Activated cells						
CD142$^+$	251.49 ± 295.65	253.01 ± 283.73	0.775	308.8 ± 327.94	316.17 ± 376.83	0.884
CD11a$^+$	267.88 ± 314.57	281.33 ± 353.97	0.456	285.77 ± 309.56	333.39 ± 433.25	0.972
CD63$^+$	3.54 ± 5.52	7.5 ± 7.82	0.055	5.38 ± 6.25	4.6 ± 6.26	0.339
CD62L$^+$	49.17 ± 29.55	59.14 ± 46.18	0.429	59.73 ± 34.5	65.18 ± 71.57	0.689
CD63+/CD62L$^+$	1.08 ± 5.07	0.1 ± 0.47	0.317	0.49 ± 2.3	0.38 ± 1.8	0.317

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; PFP, platelet free plasma and MD-VOO, Mediterranean diet supplemented with extra virgin olive oil. Used markers were CD61 for platelets, CD146 for endothelial cells, CD45 for total leukocytes, CD3 for lymphocyte and CD14 for monocyte origins accounting for agranulocytes, and SMA-α for smooth muscle cells. Other leukocytes were inferred subtracting agranulocytes subpopulation from leukocytes fraction. The other CDs were used as biomarkers of cell activation (see Supplemental Table 1).

FIGURE CHAPTIONS

Figure 1. ROC curve analyses to identify cMPs capable of predicting a future CVE.



ROC curve analyses used to evaluate the combined effects of lymphocyte ($CD3^+/CD45^+/AV^+$)- and smooth muscle cell ($SMA\text{-}\alpha^+/AV^+$)-derived cMPs and the Framingham risk score (FRS) probabilities as predictors of a future cardiovascular event (CVE) with their corresponding areas under the curve (AUC) and their 95% confidence intervals (CI). cMPs indicates circulating microparticles.

Supplemental Material to Chiva-Blanch et al. "**CD3$^+$/CD45$^+$ and SMA-α$^+$ circulating microparticles are increased in individuals at high cardiovascular risk who will develop a major cardiovascular event.**"

Supplemental Methods

Study design

The PREDIMED interventional trial (www.predimed.org; ISRCTN35739639) is a 5-years follow-up randomized, controlled, multicenter, parallel-group feeding trial designed to evaluate the efficacy of a Mediterranean Diet (MedDiet) on the primary prevention of cardiovascular disease. The detailed study design is extensively described elsewhere [1,2]. The patients studied here belonged to the arm of MedDiet supplemented with extra virgin olive oil (MedDiet-EVOO).

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

Patients

We selected 50 patients from the MedDiet-EVOO arm of the PREDIMED trial. Twenty-five patients with a documented cardiovascular event (CVE) during the follow-up trial were randomly selected according to a computer-generated table. CVE was composed of acute myocardial infarction (AMI), stroke or cardiovascular disease (CVD) death. CVD death included coronary artery disease, AMI, stroke, or sudden CVD death. Control patients (25 high CV risk subjects within the same intervention arm and without documented CVE at the end of the study, called from now no-CVE) were matched for

sex, age, body weight, body mass index (BMI), systolic and diastolic blood pressure, total and HDL cholesterol, family history of premature CVD, smoking habit, diabetes, dyslipidemia, hypertension, statins and antiplatelet agents.

As a measure of compliance with the dietary intervention, at baseline and after one year of follow-up, patients filled out a 137-item validated food frequency questionnaire, and a validated 14-item questionnaire assessing adherence to the MD [3]. 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 cardiovascular risk factors were also recorded or measured. Additionally, a validated Spanish version of the Minnesota leisure-time physical activity questionnaire was administered to record physical activity [4]. Framingham Risk Score 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 [5].

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 ensure complete cell removal. Then, 250 µL of plasma were 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 mM phosphate, 154 mM NaCl, 10.9 mM trisodium citrate, pH 7.4) before a second equal centrifugation step. Finally, the remaining cMP pellets were resuspended in 100 µL citrate-PBS.

Triple-label flow cytometric analysis was performed as described before [6]. Five 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 V450-conjugated Annexin V (AV, BD-horizon) with two specific monoclonal antibodies (mAb, 5 µL each,

Supplemental Table 1) labeled with fluorescein isothiocyanate (FITC) or phycoerythrin (PE), or the isotype-matched control antibodies were added.

Supplemental Table 1. Cell surface molecules for circulating microparticle identification and characterization. Each tube contained 5 µL of the microparticle suspension, 30 µL of Annexin binding buffer (except for tube 1 that contained 30µL of citrate- phosphate buffered saline) and combinations of 5 µL of CF405M-conjugated Annexin V with two specific monoclonal antibodies FITC and PE labeled.

mAb	Alternative name	Expression
Annexin V	PS-binding protein	Widely expressed
IgG1γ	--	--
IgG1k	--	--
CD142	Tissue Factor	Widely expressed
CD61	β_3-integrin	Platelets
PAC-1	$\alpha_{IIb}\beta_3$-integrin	Activated Platelets
CD62P	P-Selectin	Activated Platelets
CD63	Lysosome-associated membrane protein-3	Activated Cells
CD146	Melanoma Cell Adhesion Molecule	Endothelial Cells
CD62E	E-Selectin	Endothelial Cells
CD34	Mucosialin	Progenitor and Stem Cells
CD3	T-cell co-receptor	T-Lymphocytes
CD45	Leukocyte Common Antigen	Leukocytes
CD11a	Lymphocyte function-associated antigen 1	Leukocytes
CD62L	L-Selectin	Leukocytes
CD14	LPS-receptor	Macrophages, monocytes
SMA-α	Smooth Muscle Actin α	Smooth muscle cells

mAb indicates monoclonal antibody; PS, phosphatidylserine; FITC, fluorescein isothiocyanate; PE, phycoerythrin; and LPS, lipopolysaccharide.

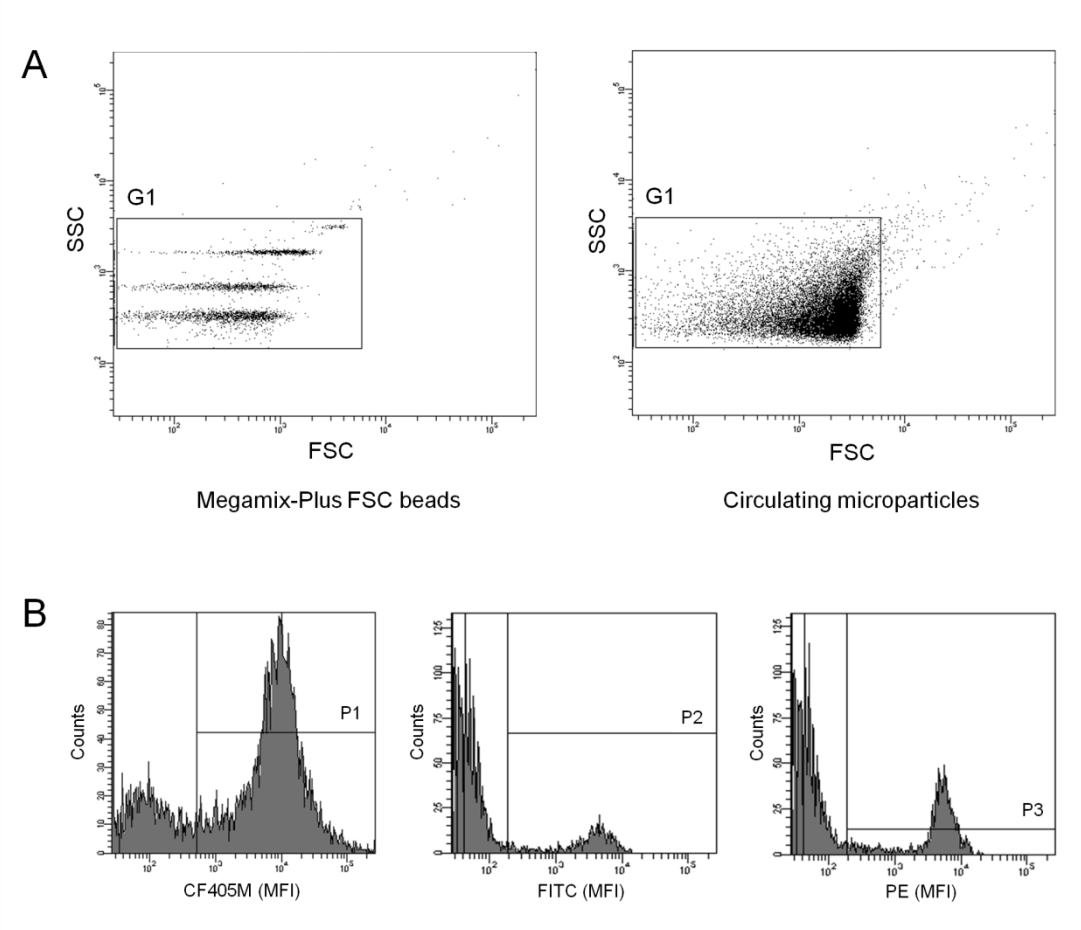
Samples were incubated 20 min at room temperature (RT) in the dark and diluted with ABB before being immediately analyzed on a FACSCantoIITM flow cytometer (except for MPs from smooth muscle cells -SMC-). For the detection and quantification of cMPs from SMC origin, 5 µL of the cMPs suspension were incubated 20 min at RT in the dark with 5 µL AV-V450 and 5 µL CD142-FITC (tissue factor, TF) in a final volume of 50 µL ABB. As previously described [7], cMPs were fixed with 450 µL of ABB/ 2% paraformaldehyde during 30 min and centrifuged at 20,000×g for 30 min to pellet cMPs. After eliminating the supernatant, cMPs were permeabilized with 20 µL of ABB/ 0.1% saponin for 20 min at RT in the dark. After permeabilizing, 5 µL of smooth muscle actin (SMA)-α-PE were added to the cMPs suspension and incubated for 20 min at RT in the dark and finally diluted with ABB prior to flow cytometry.

Acquisition was performed at 1 minute per sample. Flow rate was measured before each experiment [mean of 18.2 ± 0.4 µL/ min]. Forward scatter (FSC), side scatter (SSC) and fluorescence data were obtained with the settings in the logarithmic scale. The upper threshold for FSC and SSC to ≤1 µm was set with the Megamix-Plus FSC beads (BioCytex, Marseille, France, Supplemental Figure 1A). Megamix-Plus FSC beads for cytometer settings in microparticle analysis are a mix of beads of the following bead-equivalent diameters: 0.1 µm, 0.3 µm, 0.5 µm and 0.9 µm. According to beads signal, the lower detection limit was placed as a threshold above the electronic background noise of the flow cytometer for FSC and at the second logarithm for SSC. cMPs within the established gate limits (≥0.1 to ≤1 µm) were identified and quantified based on their binding to Annexin V and reactivity to cell-specific mAb (Supplemental Figure 1B).

To identify positive marked events, thresholds of fluorescence were also set based on samples incubated with the same final concentration of isotype-matched control antibodies after titration experiments. AV binding level was corrected for autofluorescence using fluorescence signals obtained with MPs in calcium-free buffer

(PBS). To reduce background noise, buffers were prepared on the same day and filtered through 0.2 µm pore size filters under vacuum.

Supplemental Figure 1. Gating and acquisition strategy for the detection of circulating microparticles.



A, Gating strategy to identify cMPs in the FACS analysis. Gate limits were established before analyses using the Megamix-Plus FSC beads (defined as >0.1µm to <1µm), and according to cMPs size and granularity (G1). **B**, Characterization of cMPs. Annexin V-CF405M^{+} cMPs (P1) were selected from G1. cMPs binding FITC^{+} (P2) and/or PE^{+} (P3) labeled antibodies (Supplemental Table 1) were selected from P1 and quantified. CF405M is a blue fluorescent dye. cMPs indicate circulating microparticles; FITC, fluorescein isothiocyanate; MFI, mean fluorescence intensity and PE, phycoerythrin.

Other leukocyte's percentage was inferred by subtracting agranulocytes (lymphocytes plus monocytes) from total leukocytes instead of labeling with specific mAb.

Data were analyzed with the FACSDivaTM software (version 6.1.3, Becton Dickinson). The cMP concentration (number of cMPs per µL of PFP) was determined according to Nieuwland's formula [8], based on sample's volume, flow cytometer's flow rate and the number of fluorescence-positive events (N), as follows: cMPs/µL = N x (Vf/Va) x (Vt/FR) x (1/Vi) [where Vf(µL) = final volume of washed MP suspension, Va(µL) = volume of washed MP suspension used for each labeling analysis, Vt(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 MP suspension analyzed in one minute), 1 is the µL unit of volume, and Vi(µL) = original volume of plasma used for MP isolation].

Statistical analyses

Sample size was determined with the ENE 3.0 statistical program (GlaxoSmithKline, Brentford, United Kingdom). To detect mean differences in the number of $CD61^+/AV^+$ cMP of 160 units with a conservative SD of 150, 14 subjects 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 software (v. 23.0). Descriptive statistics [mean ± sd or n (%)] were used to describe the baseline characteristics of the patients and 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 a 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 antilogarithm to facilitate interpretation of the results. A *t*-test was performed to analyze: a) changes

after one year for no- and CVE subjects separately; and b) differences of changes in outcome variables after one year between no- and CVE patients.

To evaluate cMP levels as prognostic markers of CVD presentation, associated receiver-operating characteristic (ROC) curve analyses and the corresponding areas under the curve (AUC) with their 95% confidence interval (CI) were calculated. Multivariable models for the prediction of a CVE were performed with a binary 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.

Supplemental Results

Baseline characteristics

Supplemental Table 2 shows the baseline characteristics of the 50 patients included in the study. There were no statistical differences in sex, age, body mass index, blood pressure, prevalence of family history of CVD, diabetes, dyslipidemia and hypertension, or tobacco or cardiovascular drugs use between no-CVE and CVE patients. CVE patients suffered a CVE within 2.1 ± 0.5 years after the second sampling (one year follow-up), while no-CVE patients did not present a documented CVE throughout the study follow-up period (mean of 5.0 ± 1.7 years of follow-up). Overall, 11 patients suffered an acute myocardial infarction (AMI), 11 suffered a stroke and 3 died because of CVD.

Baseline levels of cMPs (samples taken at entry with no control of dietary intervention and pharmacologically treated as per guidelines) were similar between no-CVE and CVE patients except for SMC-derived cMPs, which were significantly higher in no-CVE patients.

Supplemental Table 2. Baseline characteristics of the 50 patients included in the study.

	no-CVE	**CVE**	***P***
n	25	25	
Males [n (%)]	12 (48)	12 (48)	1.000
Age, years	69 ± 4	68 ± 6	0.521
Body Weight, Kg	76.9 ± 11.7	74.8 ± 11.0	0.613
Body mass index, Kg/m^2	29.7 ± 4.3	29.4 ± 3.0	0.959
Systolic blood pressure, mmHg	146 ± 14	160 ± 24	0.086
Diastolic blood pressure, mmHg	84 ± 10	84 ± 9	0.467
Total cholesterol, mg/dL	194 ± 27	214 ± 38	0.077
HDL cholesterol, mg/dL	53.3 ± 15.6	51.5 ± 13.8	0.670
Family history of CVD [n (%)]	5 (20)	8 (32)	0.332
Current smokers [n (%)]	8 (32)	8 (32)	1.000
Diabetes [n (%)]	19 (76)	15 (60)	0.209
Dyslipidemia [n (%)]	16 (64)	10 (40)	0.085
Hypertension [n (%)]	17 (68)	18 (72)	0.748
Framingham Risk Score, %*	12 ± 8	14 ± 8	0.293
Medication [n (%)]			
Angiotensin-converting-enzyme inhibitor	10 (40)	8 (32)	0.637
Diuretics	4 (16)	7 (28)	0.308
Antihypertensive agents	0 (0)	0 (0)	1.000
Statins	10 (40)	4 (16)	0.059
Insulin	3 (12)	2 (8)	0.640
Hypoglycemic drugs	13 (52)	8 (32)	0.150
Antiplatelet agents	6 (24)	8 (32)	0.630
Calcium channel blockers	3 (12)	3 (12)	1.000
beta- Blockers	1 (4)	2 (8)	0.555
alpha- Blockers	1 (4)	1 (4)	1.000
Angiotensin II receptor antagonists	4 (16)	1 (4)	0.161

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 for the comparison between no-CVE and CVE patients within each group of intervention. *Framingham Risk Score 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 [5]. no-CVE indicates patients without cardiovascular disease; CVE, patients who will suffer a cardiovascular event; HDL, high density lipoprotein; LDL, low density lipoprotein; CVD, cardiovascular disease.

Compliance with the intervention

According to the 14-item questionnaire, adherence to MedDiet increased by two points after one year of MedDiet-EVOO intervention ($P < 0.0001$, *t* test). As can be seen in Supplemental Table 3, total energy, protein, carbohydrates, total fat, saturated fatty acids and marine (n-3) fatty acids intakes did not significantly change after one year. However, consumption of monounsaturated, polyunsaturated and α-linolenic fatty acids increased, indicating good compliance with the dietary instructions. No differences were observed in adherence to the MedDiet or in the changes in energy and nutrient intakes after one year intervention between no-CVE and CVE patients (data not shown).

Supplemental Table 3. Nutrient intake at baseline and after 1 year of dietary intervention with a Mediterranean diet supplemented with extra-virgin olive oil of 50 study subjects.

	Baseline	**One year**	
Nutrient	mean ± sd	mean ± sd	***P***
Energy (kJ/d)	2219.56 ± 536.45	2248.06 ± 566.51	0.396
Proteins (g/day)	91.75 ± 18.43	91.89 ± 22.14	0.624
Carbohydrates (g/day)	230.34 ± 62.82	216.14 ± 69.51	0.371
Total fat (g/day)	96.02 ± 33.22	103.26 ± 23.73	0.175
SFA (g/day)	25.41 ± 10.14	24.85 ± 8.09	0.992
MUFA (g/day)	47.80 ± 15.77	54.69 ± 11.99	0.005
PUFA (g/day)	14.45 ± 8.21	15.28 ± 5.56	0.044
α-linolenic acid (g/day)	1.28 ± 0.65	1.51 ± 0.89	0.042
Marine (n-3) fatty acids (g/day)	0.78 ± 0.38	0.75 ± 0.38	0.831

Results are expressed as mean ± sd. *P* value for the comparison between baseline and one year (*t* test for related samples). SFA denotes saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

In addition, Supplemental Table 4 shows that no significant changes in anthropometric, biochemical or hematological parameters were observed between the two groups after one year of follow-up.

Supplemental Table 4. Differences in anthropometric, biochemical and hematological parameters after 1 year of dietary intervention in the two groups of study subjects.

	No-CVE mean (95% CI)	**CVE** mean (95% CI)	***P***
Anthropometric variables			
Body weight (Kg)	-0.08 (-2.36, 2.21)	-0.01 (-1.22, 1.21)	0.958
Body mass index (Kg/m^2)	-0.07 (-0.98, 0.84)	0.01 (-0.44, 0.46)	0.874
Systolic blood pressure (mmHg)	-3.59 (-8.77, 1.60)	-5.24 (-16.93, 6.46)	0.787
Diastolic blood pressure (mmHg)	-5.88 (-11.39, 0.37)	-3.29 (-10.39, 3.80)	0.546
Biochemical parameters (mg/dL)			
Fasting glucose	0.43 (-18.73, 19.59)	-6.39 (-22.29, 9.50)	0.570
Triglycerides	6.88 (-52.69, 66.44)	-6.01 (-42.31, 30.29)	0.685
Total cholesterol	4.38 (-12.09, 20.85)	-6.60 (-22.95, 9.75)	0.330
LDL cholesterol	0.61 (-17.87, 19.1)	-8.11 (-24.65, 8.43)	0.463
HDL cholesterol	-0.38 (-6.45, 5.68)	2.57 (-1.38, 6.52)	0.396
Hematological parameters			
Leukocytes (x10^9/L)	0.24 (-0.37, 0.86)	-0.34 (-0.81, 0.14)	0.123
Neutrophils (%)	1.51 (-2.01, 5.03)	0.60 (-2.86, 4.05)	0.697
Lymphocytes (%)	-0.97 (-4.22, 2.28)	-0.94 (-4.12, 2.23)	0.990
Monocytes (%)	0.22 (-0.64, 1.08)	0.12 (-0.66, 0.90)	0.859
Eosinophils (%)	-0.25 (-0.94, 0.44)	0.37 (-0.18, 0.91)	0.139
Basophils (%)	0.01 (-0.30, 0.33)	-0.07 (-0.30, 0.15)	0.618

Results are expressed as mean (95% Confidence Interval) of the differences between values after one year of intervention and values at baseline. *P* value for the comparison between no-CVE and CVE patients (*t* test for unpaired samples). no-CVE indicates patients without cardiovascular disease; CVE, patients who will suffer a cardiovascular event; HDL, high density lipoprotein; LDL, low density lipoprotein.

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