Liquid Biopsy of Extracellular Microvesicles Predicts Future Major Ischemic Events in Genetically Characterized Familial Hypercholesterolemia Patients

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Objective—Circulating microvesicles (cMVs) exert regulatory roles in atherothrombosis. Patients with familial hypercholesterolemia (FH) that are at high risk for premature cardiovascular events (CVEs) have previously shown high levels of cMVs related to disease severity. However, much remains unknown about their value as markers of CVE. We sought to investigate the prognostic cMV signature for future major CVE presentation in patients with FH.

Approach and Results—Liquid biopsies from genetically characterized patients with FH from the SAFEHEART (Spanish Familial Hypercholesterolemia Cohort Study)-cohort without clinical manifestation of disease at entry that were going to suffer a CVE within a mean period of 3.3±2.6 years postsampling (CVE, N=92) and from age/cardiovascular risk factor/treatment-matched patients with FH that did not suffer an event within the same time-period (non-CVE, N=48) were investigated. cMVs were phenotyped by flow cytometry to identify activated parental cells. Patients with CVE had higher number of overall procoagulant annexin V^+-cMVs than non-CVE (P<0.05). Pan-leukocyte-derived and neutrophil-derived cMVs, as well as activated platelet-derived cMVs, were significantly higher in patients with CVE. Baseline number of cMVs derived from lymphocytes, neutrophils, and activated platelets were positively associated with mortality at follow-up (P<0.05). Patient-risk calculated by classical cardiovascular risk-factor scores did not correlate with cMVs. Inclusion of the cMV signature into the SAFEHEART risk model for patients with FH for the prediction of ischemic events increased the area under the curve from 0.603±0.050 to 0.768±0.042 (P<0.005).

Conclusions—Patients with FH who are going to suffer a CVE within a mean period of 3.3 years, despite being treated according to guidelines, have ongoing innate immune cell and platelet activation. The proposed cMV signature is a prognostic marker for accelerated atherosclerosis and clinical event presentation in patients with FH.

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Key Words: biomarkers ■ leukocytes ■ mortality ■ platelets ■ prognosis.

Atherosclerosis is a multifactorial disease that is the leading cause of morbidity and mortality worldwide.[1] One of the main challenges of cardiology is the identification of individuals at risk of developing a major ischemic cardiovascular event (CVE).[2] Specifically, heterozygous familial hypercholesterolemia (FH) patients, characterized by high LDL (low-density lipoprotein)-cholesterol plasma levels, show premature development of atherothrombotic events.[3] Indeed, there is an increased risk of acute CVE in patients with FH.[4,5] Recent efforts have aimed to investigate novel biomarkers to identify those patients with the highest risk of developing a major CVE (MACE). Presently, in the context of dyslipidemia, current laboratory assessment of cardiovascular risk mainly relies on lipoproteins.[6] Liquid biopsies try to identify cell markers of disease that, in addition to the classical risk factors, may have clinical utility to detect those patients who can suffer an unpredictable CVE.[7] These findings in a CVE prone patient population, as FH, may be later on extended to all patients who need new predictive biomarkers to improve their management.[8,9] Although the atherosclerotic risk is 3- to 13-fold higher in patients with FH than in those without this genetic disease, the impact of acute ischemic events is highly variable among patients with FH,[10] implying the need for better biomarkers for this heterogeneous high-risk population. Besides, FH remains widely underdiagnosed and undertreated worldwide.[11] Thus, FH offers a good clinical model to study new biomarkers derived from cells directly involved in the disease process that may accurately report on disease progression.

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Nonstandard Abbreviations and Acronyms

a-pMVs	activated platelet-derived microvesicles
AUC	area under the curve
AV	annexin V
cMVs	circulating microvesicles
CVE	cardiovascular event
FH	familial hypercholesterolemia
FRS	Framingham risk score
hs-CRP	high-sensitivity C-reactive protein
LDL	low-density lipoprotein
LLT	lipid-lowering therapy
lMVs	lymphocyte-derived microvesicles
LMVs	leukocyte-derived microvesicles
mMVs	monocyte-derived microvesicles
nCVE	non-CVE
nMVs	neutrophil-derived microvesicles
PFP	platelet-free plasma
pMVs	platelet-derived microvesicles
ROC	receiver operating characteristic
SAFEHEART	Spanish Familial Hypercholesterolemia Cohort Study
TSP-1	thrombospondin-1

Circulating microvesicles (cMVs) are small phospholipid extracellular vesicles shed by stressed, activated, senescence, and apoptotic cells. cMVs play a pivotal role on atherosclerosis, inflammation, and thrombosis by means of cell signaling and intercellular crosstalk.[12–16] Increased levels of cMVs have been found in a wide variety of atherothrombotic disorders in association with the disease severity.[17–20] Patients with FH had previously shown to have high levels of phenotypically characterized cMVs even when their LDL levels were controlled by lipid-lowering therapy (LLT).[21,22] However, their prognostic value as markers of major cardiovascular disease remains poorly defined.

Differential expression levels of cell-specific cMVs may lead to improve our understanding of pathophysiological mechanisms related to clinical outcomes and help us to predict its occurrence. Profiling of cMVs shed by injured cardiovascular cells may provide information on ongoing disease and, hence, be markers of future adverse ischemic CVE. To the best of our knowledge, no previous studies have addressed whether there is a specific cMV signature in patients with FH at high cardiovascular risk of developing a MACE. This study aims to investigate whether MV level and phenotype in asymptomatic patients with FH are able to predict an adverse cardiovascular outcome.

Materials and Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Study Design and Population

The present study comprised patients with clinical and genetic diagnosis of heterozygous FH from the SAFEHEART (Spanish Familial Hypercholesterolemia Cohort Study),[21,23] a multicenter, nationwide, long-term, prospective cohort study of a molecularly defined patients with heterozygous FH. None of the patients with FH participating in the present study (N=140) had suffered a clinical event before entering the SAFEHEART cohort. Sixty-six percent of the patients with FH presented a MACE such as unstable angina, acute myocardial infarction, sudden death, or ischemic stroke (CVE, N=92) within a mean period of 3.31±2.6 years postsampling as compared to FH patients without a CVE within the same follow-up period of time (8 years; non-CVE [nCVE], N=48; Table I in the online-only Data Supplement). The Coordinating Center was responsible for managing the patient follow-up, and patients were contacted annually by telephone by trained staff using a standardized phone call protocol.[23]

The CVE and nCVE groups were matched by age, sex, and demographics as well as LLT and cardiovascular comorbidities (Table I in the online-only Data Supplement, Graphic Abstract [the Graphic Abstract shows the schematic overview of the study design]). Clinical data (sociodemographic, lifestyle, medical, and therapeutic) were obtained from all subjects using a standardized report form at the inclusion (Table I in the online-only Data Supplement). Data related to LLT included statin type, dose, time, and compliance. All selected subjects had been prescribed an LLT according to current guidelines.[4,24–26] Maximum statin dose (simvastatin 40 mg/d, fluvastatin 80 mg/d, atorvastatin 80 mg/d, and rosuvastatin 20–40 mg/d), as well as maximum combined therapy, and maximum LLT were defined as previously reported.[27,28] Adherence to LLT was assessed by an indirect method with a single question.[29] No patient presented pregnancy or had past history of cancer because these conditions are known to independently impair cMV number.

The study was approved by the Local Ethics Committee of the Investigación Clínica Fundación Jimenez Diaz (protocol's number: 01/09) was conducted according to the Declaration of Helsinki, and a written informed consent was obtained from all participants before the study. The results of the study are presented in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology guidelines[30] as well as Transparent Reporting of a Multivariable Prediction Model for Individual Prognosis or Diagnosis statement.[31]

Blood Sampling, Laboratory Measurements, Genetic Analysis, and cMV Isolation

Venous blood samples were collected after a 10 to 14 hours of fasting from the cubital vein without tourniquet using a 20-gauge needle into serum, EDTA, and 3.8% sodium citrate tubes, for biochemical, genotyping, and microvesicle analysis, respectively.[32] All samples were processed identically and within the first 2 hours after extraction. For lipid profile, total cholesterol, triglycerides, and HDL (high-density lipoprotein)-cholesterol were measured by standardized enzymatic methods; serum LDL-cholesterol concentration was calculated using the Friedewäld formula[33]; and lipoprotein (a) levels were measured using a turbidimetric method. Genetic diagnosis of FH was made using a DNA-microarray (LIPOCHIP).[34] Mutations were classified as receptor negative or receptor defective, depending on their functional class. Platelet-free plasma (PFP) for analysis of the cMV fraction was prepared by 2-step high-speed centrifugation (1258×*g*, 20 minutes at room temperature) to ensure the complete removal of cells, as previously described.[22] PFP samples were tested with a cell counter for the absence of residual cells. PFP aliquots were snap-frozen in liquid nitrogen and immediately stored, for an identical time interval, at −80°C until flow cytometric studies.[35]

cMV Isolation

Frozen PFP aliquots (250 µL) were thawed on melting ice for 1 hour and centrifuged at 20000×*g* 30 minutes to pellet cMVs. The cMV-enriched pellets were washed once with citrate-PBS solution (citrate-PBS; 1.4 mmol/L phosphate, 154 NaCl, 10.9 mmol/L trisodium citrate, and pH 7.4) before a second equal centrifugation step was made. Finally, the remaining cMV pellets were resuspended in citrate-PBS to a final volume of 100 µL.

Flow Cytometry Analysis

Triple-label flow cytometric analysis was performed as previously described.[19,21,22,36] Briefly, washed cMV suspensions were incubated with combinations of AV (annexin V) with 2 cell surface-specific

monoclonal antibodies, or the isotype-matched control antibodies (Table I in the online-only Data Supplement). Samples were diluted before being immediately analyzed on a FACSCantoII Becton Dickinson flow cytometer. Acquisition was performed for 1 minute per sample at low-flow rate. cMVs were identified and quantified based on their FSC/SSC (forward scatter/side scatter) characteristics (obtained with gain settings in the logarithmic scale) according to their size, binding to AV, and reactivity to cell-specific monoclonal antibody. Gate limits were established as previously described using a Flow-Check Size Range Calibration Kit (Polysciences) and an in vitro platelet-derived microvesicle population as positive control (Figure I in the online-only Data Supplement). The lower detection limit was placed as a threshold above the electronic background noise of the flow cytometer and a threshold was set at SSC parameter. Data were analyzed with FACSDiva software (version 6.1.3, Becton Dickinson). The concentration (number of cMVs per μL of plasma) was determined according to Nieuwland procedure,[19,21,22] based on sample's volume, flow cytometer's flow rate, and the number of fluorescence-positive events. Intraassay cardiovascular of cMV counts was 3.1%, while interassay cardiovascular was 5.4%.

Statistical Analysis

Results are reported as median±interquartile range except for continuous variables of patient characteristics that are given as mean (±SEM). Normal sample distribution was determined via Kolmogorov-Smirnov test. Statistical analysis was performed by the nonparametric Mann-Whitney. *P* values were adjusted by the false discovery rate using the graphically sharpened method described by Benjamin and Hochberg.[37] The new calculated adjusted *Q* values (false discovery rate adjusted) indicate the probability of false positives for variables considered to be significant. In the present study, it refers to *Q* values <0.05, which represents >95% truly positives for differentially expressed cMV subtypes. Strength of the association between continuous variables was calculated by Spearman correlation. Risk estimations based on the Framingham Risk Score (FRS),[24] Systematic Coronary Risk Evaluation,[38] Registre Gironí del Cor,[39] and SAFEHEART-CVEs risk estimator[8] equations were performed for each individual. Binary logistic regression models were performed to estimate predicted probabilities for CVE with combination of cMVs, as well as cardiovascular risk factor models. To avoid overfitting in the multivariable model, we combined cMV biomarkers by using binary logistic regression as a single score. Cox proportional hazards regression (time-to-event) analysis was performed using the predicted probabilities for combination of cMVs and individual values for the CVD risk scores. The performance of the models was evaluated with respect to their discrimination and calibration ability on the basis of the Hosmer-Lemeshow type χ^2 goodness of fit statistic test. An associated receiver operating characteristic (ROC) curve analysis was calculated to evaluate cMVs as independent prognostic biomarkers. Then, the areas under the dependent ROC curves (AUC) from each model were compared using the DeLong method.[40] StatView 5.0.1 (Abacus Concepts) and SPSS statistics 22.0.0 (SPSS) were used for all statistical tests. A $P<0.05$ was considered statistically significant and all reported *P* values were 2-tailed.

Results

Characteristics of the Patient Population

Baseline clinical characteristics of the studied population are given in Table I in the online-only Data Supplement. Between CVE and nCVE groups, there were no significant differences in the clinical, sociodemographic, and laboratory characteristics, as well as in cardiovascular risks factors. The mean age of patients without CVE was 60, whereas the mean age was 58 in patients with CVE. There were more men than women in both CVE and nCVE groups, specifically, 70% and 58%, respectively. All patients were at high cardiovascular risk because they presented genetically identified and clinically diagnosed heterozygous FH. All patients were under LLT according to current guidelines at the moment of inclusion,[4,24–26] and there was no significant difference on either the type or the duration of the treatment between CVE and nCVE groups. There were also no differences in the usage of other cardiovascular drugs. Patients with CVE had a nonsignificant trend to higher ApoB levels and clinical presentation of xanthomas compared with patients without CVE. Patients with CVE predominantly presented an AMI (N=41, 45%) while unstable angina occurred in 21 patients (23%), sudden death in 16 (17%), and ischemic stroke in 14 (15%) during follow-up. Only the first event during follow-up was considered.

Liquid Biopsies: cMV Profile in Patients With and Without CVE

Cell origin and cell activation markers were analyzed to identify the cMV signature of patients with CVE at follow-up. Figure 1 illustrates the pattern of cMVs according to their parental cell marker. The total number of AV-positive cMVs (AV^+-cMVs) was significantly higher in patients with CVE than in patients without CVE ($P<0.05$, Figure 1A). While levels of total circulating endothelial-derived ($CD62E^+$-eMVs; Figure 1C), red blood cell-derived ($CD235a^+$-erythrocyte-derived microvesicles; Figure 1D), and platelet-derived microvesicles ($CD61^+$- and $CD41^+$-pMVs; Figure 1E) as well as bearing tissue factor ($CD142^+$; Figure 1F) did not significantly change, there were significant increases in white cell type-derived (Figure 1B) and activated platelet-derived cMVs (Figure 1E) between patients with and without CVE. Indeed, pan-leukocyte-derived cMVs ($CD45^+$-LMVs) were significantly higher in patients with CVE as compared to patients without CVE. About leukocyte-specific subsets of the innate immunity cell-derived cMVs, patients with CVE presented higher numbers of neutrophil-derived cMVs ($CD15^+$-nMVs), while no alteration in lymphocyte-derived ($CD3^+$-lMVs) and monocyte-derived ($CD14^+$-mMVs) cMVs (Figure 1B). These derangements of white blood cell-derived cMVs suggest a proinflammatory profile in patients prone to develop a MACE. Numbers of activated monocytes ($CD14^+/11b^+$-a-mMVs) cMVs were significantly reduced in patients with CVE as compared to patients without CVE. Furthermore, cMVs carrying platelet activation epitopes (activated $\alpha_{IIB}\beta_3$ [$PAC1^+$], thrombospondin 1 [$TSP1^+$] and P-selectin [$CD62P^+$]-a-pMVs) were significantly increased in patients with CVE suggesting a higher level of platelet reactivity despite of similar levels of total pMVs between both groups (Figure 1E). When we stratify our population by sex, there is a similar cMV profile associated to CVE (Table III in the online-only Data Supplement). Among patients with CVE, a correlation was found between type of mutation (null/defective) and pMVs bearing TSP-1 (thrombospondin-1; Figure II in the online-only Data Supplement), showing that those patients with a lifetime null LDL-receptor function have higher platelet activation.

cMVs and Mortality in Patients With High-Risk FH

Specific populations of cMVs were related to mortality at follow-up in the high-risk FH patient population. Similar to the CVE-driven cMV signature, cMVs derived from white blood cells (Figure 2A) and activated platelets (Figure 2B) were associated with mortality at follow-up. Concerning cMVs derived from leukocyte lineage cells, baseline numbers of cMVs derived

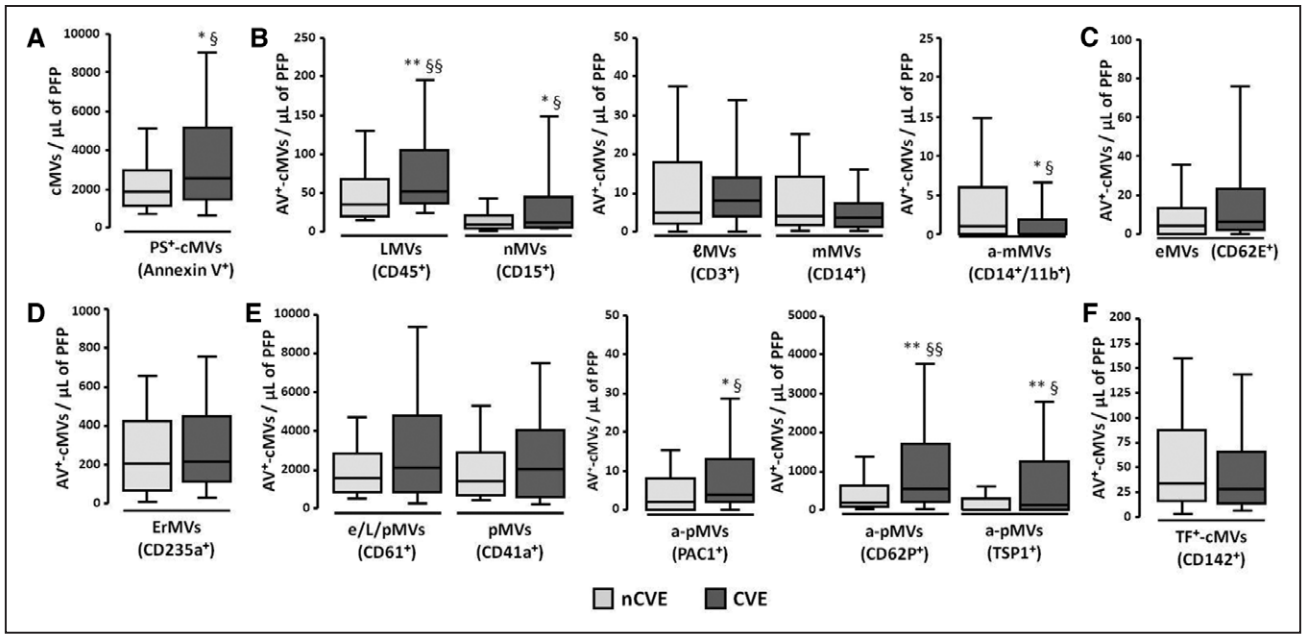

Figure 1. Changes in total, white blood cell, red blood cell, endothelial cell, and platelet-derived circulating microvesicles (cMVs) between cardiovascular event (CVE) and non-CVE (nCVE) high-risk familial hypercholesterolemic (FH) patients. Box and whisker plots showing levels (per µL of platelet-free plasma [PFP]) of (**A**) total annexin V-positive (phosphatidylserine-rich [PS]+), (**B**) specific white blood cell-derived cMVs from leukocytes (LMVs), neutrophils (nMVs), lymphocytes (lMVs), monocytes (mMVs), and activated monocytes (a-mMVs), (**C**) endothelial cell-derived microvesicles (eMVs), (**D**) erythrocyte-derived microvesicles (ErMVs), (**E**) platelet-derived microvesicles (pMVs) bearing parental cell markers (PECAM-1 [CD31+], glycoprotein IIIa [CD61+], and glycoprotein IIb/IIIa [CD41a+]) and platelet activation (a-pMVs) epitopes (activated $\alpha_{IIB}\beta_3$ [PAC1+], P-Selectin [CD62P+] and thrombospondin-1 [TSP-1+]), and (**F**) cMVs bearing tissue factor [CD142+] (TF+-cMVs) in patients with CVE (N=92) compared with patients with nCVE (N=48). Data are expressed as median (interquartile range). **P*<0.05 and ***P*<0.01 for difference using Mann-Whitney *U* nonparametric test. §*Q* value <0.05 and §§*Q* value <0.01 after false discovery rate (FDR)-adjustment.

from lymphocytes (CD3+-lMVs) and neutrophils (CD15+-nMVs) were positively associated with mortality at follow-up (Figure 2A; *P*<0.05), while low levels of mMVs (CD14+-mMVs) were significantly found in patients who died at follow-up. Besides, numbers of survivors and nonsurvivors at follow-up also directly correlated with activated platelet-derived cMVs bearing TSP-1 epitope (TSP1+-a-pMVs, *P*<0.05; Figure 2B). Interestingly, white blood cell- and activated platelet-derived cMVs were also associated with mortality at follow-up in both sexes (Table III in the online-only Data Supplement). Altogether these data indicate a higher proinflammatory and prothrombotic state in those patients prone to die within 3 years.

cMVs and CRP in Patients With High-Risk FH

To further investigate whether systemic inflammation of patients with high-risk FH was related to cMV phenotype, cMV data were analyzed according to the level of hs-CRP (high-sensitivity C-reactive protein; below and above 1.5 mg/L). Although levels of hs-CRP in patients with CVE (2.68 mg/L) and patients without CVE (1.90 mg/L) were nonsignificantly (Table I in the

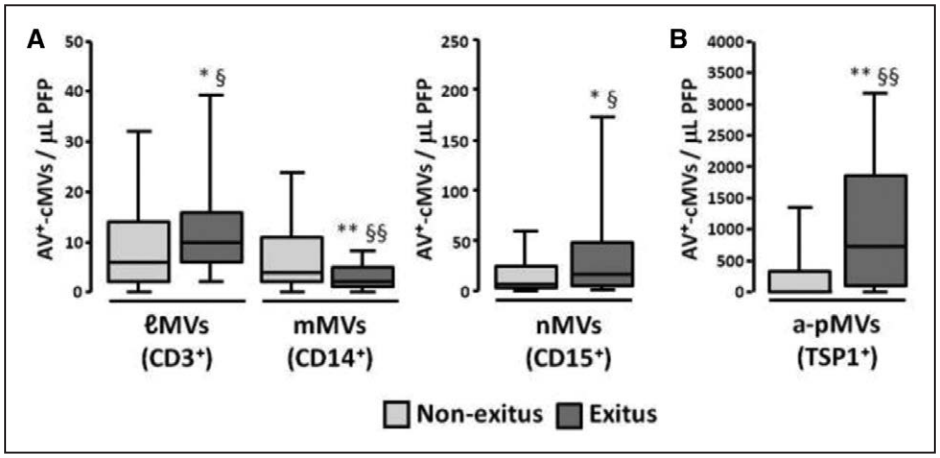

Figure 2. Specific circulating microvesicles (cMVs) associate with exitus. Box and whisker plots showing levels (per µL of platelet-free plasma [PFP]) of (**A**) white blood cell-derived cMVs from lymphocytes (lMVs), monocytes (mMVs), and neutrophils (nMVs), and (**B**) microvesicles derived from activated platelets (a-pMVs) carrying thrombospondin-1 [TSP-1+] marker in non-exitus (N=102) and exitus (N=38) patients. Data are expressed as median (interquartile range). AV indicates Annexin V. **P*<0.05 and ***P*<0.01 for difference using Mann-Whitney *U* nonparametric test. §*Q* value <0.05 and §§*Q* value <0.01 after false discovery rate (FDR)-adjustment.

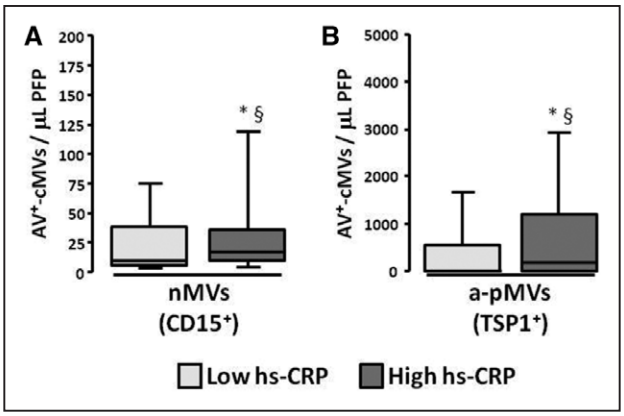

Figure 3. Circulating microvesicles (cMVs) and hs-CRP (high-sensitivity C-reactive protein). Box and whisker plots showing levels (per µL of platelet-free plasma [PFP]) of (**A**) neutrophil-derived microvesicles (nMVs) and (**B**) microvesicles derived from activated platelets (a-pMVs) carrying thrombospondin-1 [TSP-1+] marker in patients classified by low (N=73) and high (N=62) hs-CRP levels. Data are expressed as median (interquartile range). AV indicates Annexin V. *P<0.05 for difference using Mann-Whitney U nonparametric test. §Q value <0.05 after false discovery rate (FDR)-adjustment.

online-only Data Supplement), in those patients with higher levels of hs-CRP there were positive and significant correlations with the degree of cell activation and cMV shedding (Figure 3) likely reflecting the proinflammatory state of high-risk FH patient population. In line with previous data, cMVs derived from neutrophils (CD15+-nMVs; Figure 3A) and cMVs carrying the platelet activation marker TSP-1 (TSP1+-a-pMVs; Figure 3B) were higher in patients with higher levels of hs-CRP (P<0.05). This trend was also found when sex was taken into account (Table III in the online-only Data Supplement).

Cardiovascular Risk Scores Are Poor Predictors of Future CVE in Patients With High-Risk FH

Using ROC curve analysis distinct models of cardiovascular risk score estimators were investigated for their accuracy in identifying those patients prone to develop a MACE. FRS for coronary heart disease (% of risk at 10 years) was calculated using the high-risk Framingham Heart Study equations of the NCEP (National Cholesterol Education Program).[24] Patients were stratified according to their FRS and there was no significant difference between patients with and without CVE. Total numbers of AV+-cMVs did not show a significant correlation with FRS (Figure 4A). In a similar fashion, primary prevention cardiovascular risk estimators Systematic Coronary Risk Evaluation (SCORE; Figure 4B) and Registre Gironí del Cor (Regicor; Figure 4C) showed also poor discriminatory power for future CVE presentation. As expected, both SAFEHEART-CVE risk estimator at 5- and 10-year showed a predictive value for future MACE in our population slightly below the significance threshold (Figure 4D), suggesting that other contributing factors such as cMVs might help to improve the prediction of CVE in these patients.

Prognostic Value of Liquid Biopsy cMVs in Patients With FH

ROC curve analysis was also used to assess the accuracy of altered cMV signature in predicting future MACE in the high-risk FH patient cohort. C statistics analysis for combined predicted probabilities of LMVs (CD45+), nMVs (CD15+), pMVs (CD31+ and CD41a+/61+), and a-pMVs (CD62P+) showed the highest AUC value: 0.745±0.043 (P<0.001; 95% CI, 0.661–0.830; Figure 5A), indicating that clustering these cMVs provides a significant prognostic value for future CVE in patients with high-risk FH. We also tested the added predictive ability of cMVs by examining the improvement in global AUC obtained when adding the combined probability for the leukocyte- and activated platelet-derived cMV (L/p-cMV) signature to the best cardiovascular risk factor models for our specific high-risk FH population: FRS for coronary heart disease (% of risk at 10 years) and SAFEHEART-CVE 10-year risk estimator. To avoid overfitting in the multivariable model, the cMV biomarkers were combined in a single score equation by using logistic regression for binary outcome. Specifically, the Hosmer-Lemeshow type χ^2 goodness of fit test was of $\chi^2(8)=6.28$ (probability $>\chi^2=0.615$) for cMV model, $\chi^2(8)=4.49$ (probability $>\chi^2=0.810$) for FRS-cMV model, of $\chi^2(8)=10.81$ (probability $>\chi^2=0.213$) for cMVs and SAFEHEART 5-year risk model and of $\chi^2(8)=7.28$ (probability $>\chi^2=0.507$) for cMVs and SAFEHEART 10-year risk model. Interestingly, the inclusion of this cMV signature into the FRS-CVFR-model for the prediction of major ischemic events increased the AUC from 0.574±0.051 (P=0.150, [95% CI, 0.475–0.674]) with only classical risk factors-FRS model to 0.760±0.042 (P<0.001, [95% CI, 0.677–0.843]; P<0.005, Figure 5B). Similarly, the L/p-cMV signature enhanced substantially the discrimination power of the SAFEHEART 10-year risk model for the prediction of CVE with an improvement in AUC from 0.603±0.050 (P=0.045, [95% CI, 0.505–0.701]) to 0.771±0.042 (P<0.001, [95% CI, 0.688–0.853]; P<0.005; Figure 5C). Results about the prognosis of patients based on cMVs and FRS model as well as cMVs and SAFEHEART 5- and 10-year CVE risk estimator models are shown in Table IV in the online-only Data Supplement. Multiple linear regression analysis was performed to rule out multicolinearity between cMV variables (Table V in the online-only Data Supplement). Similarly, the prognostic value of cMVs for future MACE was also investigated in males and females. C statistics analyses for the L/p-cMV signature showed significant and high AUC values in both sexes (Figure III in the online-only Data Supplement). Adding this cMV signature into the cardiovascular risk factor models (FRS and SAFEHEART 10-year risk) for the prediction of major ischemic events increased the AUCs in all cases. Of note, the L/p-cMV signature enhanced substantially the discrimination power of the cardiovascular risk factor models in the female subgroup (Figure IIIB in the online-only Data Supplement).

Discussion

FH is a monogenic disorder associated with lifelong exposure to very-high LDL-cholesterol plasma levels and increased risk (up to 20 times) of early atherothrombotic disease.[3,4] Coronary artery disease is the main cause of morbidity and mortality in individuals with FH.[5] We have previously reported that patients with FH at high risk for premature ischemic CVE have high levels of circulating MVs related to atherosclerotic plaque burden (measured by CMR) even when their LDL-cholesterol levels were controlled by LLT.[21,22] This is the first study to demonstrate that cMVs are significant prognostic indicators of major

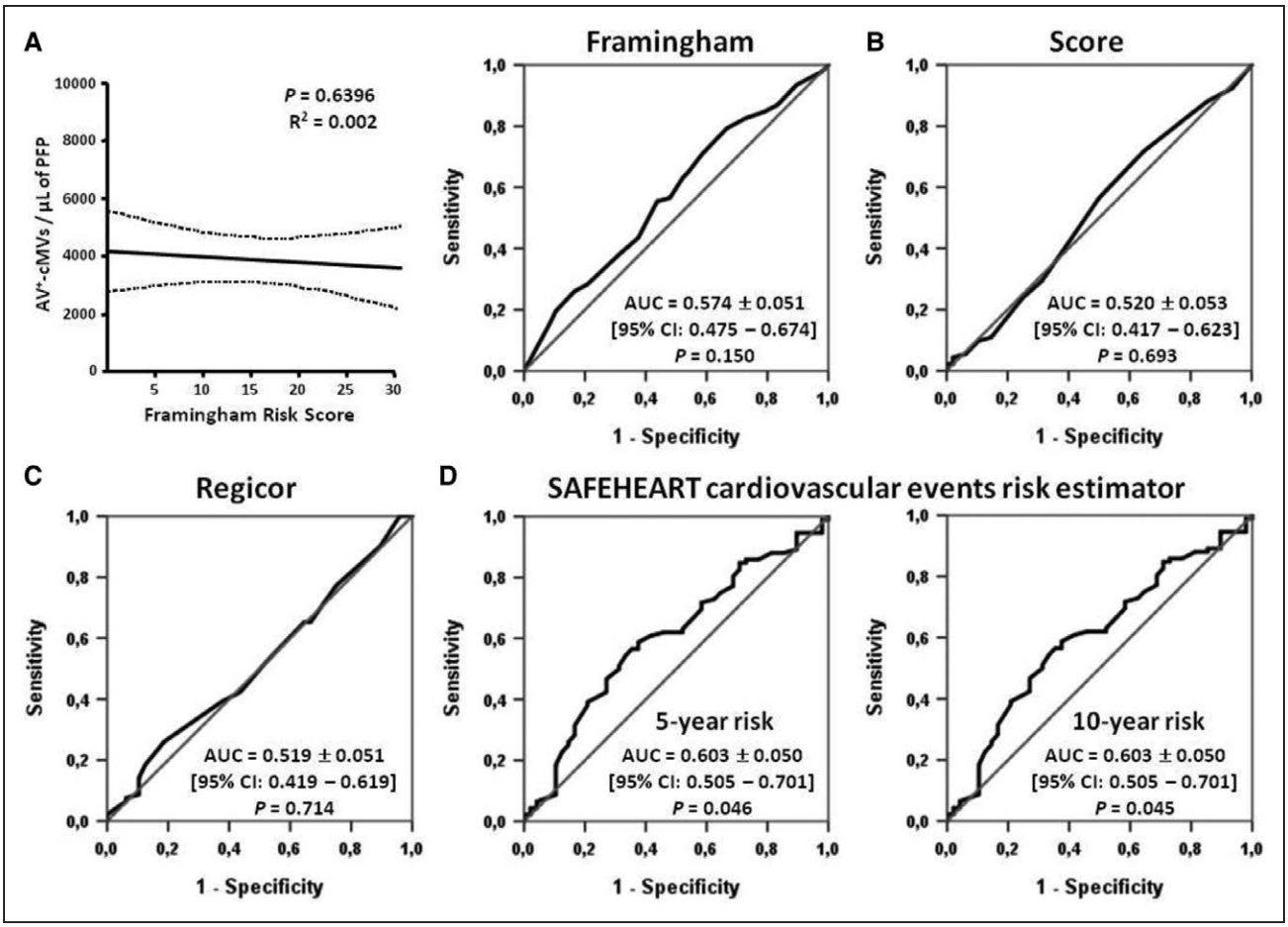

Figure 4. Discriminatory performance of cardiovascular risk scores in the prediction of future major cardiovascular events (CVEs). **A**, Correlation between total AV+- circulating microvesicles (cMVs) with individual Framingham Risk Score for each individual (N=140) by Spearman correlation nonparametric test. **A**–**D**, Receiver operating characteristic (ROC) curve analyses (C statistics) of distinct cardiovascular risk factor models: Framingham Risk Score [FRS] (**A**), Systematic Coronary Risk Evaluation (SCORE; **B**) and Registre Gironí del Cor (Regicor; **C**); SAFEHEART (Spanish Familial Hypercholesterolemia Cohort Study) CVEs risk estimator (**D**), for the prediction of major CVE presentation in high cardiovascular risk familial hypercholesterolemic (FH) patients (N=140). AV+-cMVs, annexin V-positive cMVs. AUC indicates area under the curve; and AV, Annexin V.

ischemic events and the combination of these cMV-based biomarkers have a high predictive value in high-risk asymptomatic patients with FH.

Interactions between blood and vascular cells are key features in atherothrombosis progression. A growing body of evidence suggests that blood cell-derived MVs might play an important role in vascular function by means of intercellular communication,[41] predominantly on inflammatory,[42] and thrombotic processes,[43] which are crucial in the development of CVE. Accordingly, our findings strongly indicate that cMV shedding directly relates to the progression of atherosclerotic lesion leading to adverse cardiovascular outcomes in both men and women. In particular, we described increased levels of cMVs originated from pan-leukocytes, neutrophils, and activated platelets in a molecularly defined population of patients with FH that are going to suffer a MACE within the next few years. And, consistently, white blood cell- and platelet-derived cMV phenotype was also directly related to mortality at follow-up. Few studies have addressed the potential link between cMVs and CVE presentation in primary prevention and none of them in asymptomatic high-risk patients with FH without previous cardiovascular disease.

Nozaki et al[44] reported that increased levels of CD144+-cMVs were independent predictors of acute coronary syndromes and cardiovascular death in a population with coronary artery disease. Besides, Sinning et al[45] found elevated levels of CD31+/AV+-cMVs also in patients with stable coronary artery disease, assessed by coronary angiography, which later develop a major adverse CVE. Albeit risk stratification in patients with coronary artery disease is highly relevant, the latter association was highly influenced by diabetes mellitus, a condition known to present high circulating levels of CD31+/AV+-MVs.[46] Interestingly, those subjects from PREDIMED trial (Prevention With Mediterranean Diet) who despite following a Mediterranean diet supplemented with extra virgin olive oil still suffered a CVE during follow-up, presented increased levels of lMV,[47] extending our previous findings in which CD3+/CD45+-lMVs were able to accurately differentiate between lipid-rich and fibrous atherosclerotic plaques in asymptomatic patients with FH.[21] Indeed, the prevalence of subclinical atherosclerotic burden of patients with FH has been shown by aortic magnetic resonance imaging[48] and coronary computed tomographic angiography.[5] In secondary prevention, contradictory results exist. In this regard, high levels of CD14+/11b+-mMVs were shown to be predictors of future

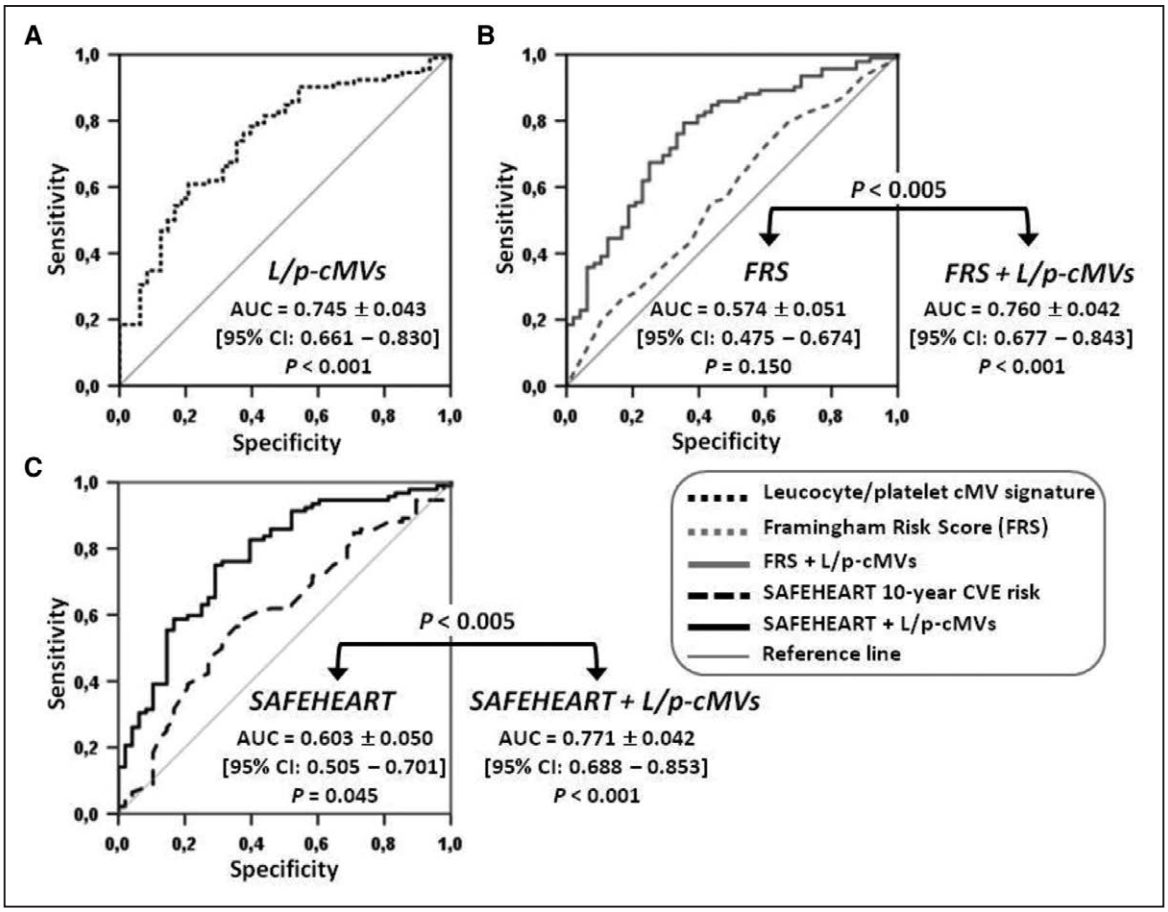

Figure 5. Circulating microvesicles (cMVs) as prognostic markers of future major cardiovascular events (CVEs). Discriminatory power of (**A**) cMV signature in comparison with (**B**) Framingham Risk Score (FRS) and (**C**) SAFEHEART (Spanish Familial Hypercholesterolemia Cohort Study) CVEs risk estimator for the prediction of major CVE presentation in high cardiovascular risk familial hypercholesterolemic (FH) patients (N=140). Prognostic value (*P<0.05) was determined by receiver operating characteristic (ROC) curve analyses (C statistics) and the comparison of the ROC-derived area under the curves (AUCs) by the DeLong method. L/p-cMVs indicates leukocyte- and activated platelet-derived cMVs.

MACE in patients with ST-segment–elevation myocardial infarction.[49] However, low levels of CD11b$^+$-LMVs were associated with both high-risk atherosclerotic lesions and early recurrence of CVE in patients with acute coronary syndromes after coronary stenting.[50] In agreement, in the present study, mMVs were significantly decreased in patients with CVE and negatively associated to cardiovascular mortality. Analysis of CD14$^+$-mMVs might reflect the heterogeneity of blood monocytes and in-depth analyses of the differential subset-specific mMV contribution to atherothrombosis disease deserves further examination.

Our data reflects ongoing activation of proinflammatory cells in the evolution of atherogenesis even in the absence of overt systemic inflammation as measured by hs-CRP. High numbers of cMVs originated from the white blood cell lineage, specifically pan-leukocytes (CD45$^+$-LMVs) and neutrophils (CD15$^+$-nMVs), were associated with enhanced cardiovascular complications in high-risk patients with FH. Accordingly, LMVs were found to be the main pool of MVs within atherosclerotic plaques[51] and patients with high-grade carotid stenosis[17] and asymptomatic subjects with subclinical atherosclerotic burden[52] presented also with high levels of LMVs, reinforcing the idea of LMV contribution to atherosclerotic plaque progression. Of note, an acute release of nMVs occurs in the coronary circulation of acute coronary syndrome patients after percutaneous coronary intervention.[53] Recently, CD15$^+$-MVs derived from neutrophils increased inflammatory response and mortality in a mouse model of sepsis.[54] Indeed, Lewis X Antigen (CD15) is known to act as an adhesive substrate for endothelium, a monocyte activator as well as a signaling initiator of proinflammatory effects in monocytes.[55] nMVs were previously shown to induce myeloperoxidase-mediated damage of vascular endothelium.[56] Similarly, nMVs showed inflammatory and adhesive properties (specifically with monocytes and ECs).[57] In addition, proteolytically active transmembrane proteases carried by nMVs promote accelerated risk of human abdominal aortic aneurysm in smokers.[58]

Here, in our study, nMVs were raised in those patients with high hs-CRP levels, as a surrogate marker of systemic inflammation in patients with FH. Consistently, it has been found that low hs-CRP is associated with reduced risk of incident stroke, coronary heart disease, and death in high cardiovascular risk patients.[59] Thus, altogether our results support the relevance of the inflammatory process in the pathogenesis of CVD. Although statins downregulate inflammatory mediators, as shown in the hallmark JUPITER trial (Justification for the Use of Statins in Prevention: an Intervention Trial

Evaluating Rosuvastatin),[60] patients with FH constitute a special population with a lifetime exposure of the vasculature to high LDL-cholesterol levels and, consequently, they are characterized by chronic vascular cell activation and ensuing inflammation, in line with the emerging concept of residual inflammatory risk as shown in the CANTOS trial (Canakinumab Antiinflammatory Thrombosis Outcome Study).[61]

Not only proinflammatory but also prothrombotic MVs are found as bioeffectors of CVE in patients with FH. cMVs from activated platelets (TSP1$^+$- and CD62P$^+$-a-pMVs) correlated with CVE presentation and mortality at follow-up as well as with high levels of hs-CRP, suggesting that MV shedding from activated platelets reflect a deleterious environment in the vascular compartment of patients with FH predisposing to adverse cardiovascular outcomes. Despite it is well known that pMVs exert key roles in all pathophysiological stages of atherothrombotic disease worsening and exacerbating its progression,[13] no previous study has pointed out circulating pMVs as predictive markers of MACE.

It is well known that lipid infiltration in the vessel wall induces tissue factor release from vascular resident cells.[62] We and others have previously reported increased numbers of TF-rich cMVs in patients with FH.[22,63] In this study, all patients are FH and the numbers of TF$^+$-cMVs in those patients with FH prone to develop a MACE are not significantly different from those not suffering an event. Intriguingly, FH patients with a similar degree of cardiovascular risk and atherosclerotic plaque burden can evolve distinctly in terms of CVE presentation. Our results seem to unveil a fine-tuning regulation of all cells present in the vascular compartment in the atherosclerotic process.

Prevention strategies with state-of-the-art LLT and cardiovascular risk factor control have shown to be effective in reducing cardiovascular outcomes. However, a high number of patients still suffer unexpectedly from CVE. Because of a lack of genetic testing, FH remains underdiagnosed[11] posting asymptomatic FH population at the highest risk of major adverse CVE. Current risk scores for primary prevention are not appropriate for high-risk patients with FH and long-term exposure to LDL-cholesterol plasma levels likely because cardiovascular risk might be underestimated. Indeed, no risk score achieved significance in the prediction of future CVE in our patient group. Although a recent study has suggested that pool cohort equations substantially overestimate atherosclerotic cardiovascular risk in a middle-aged adult population,[64] better existing risk prediction tools to accurately reclassify and stratify patients in the clinical management of FH are urgently needed. This is particularly important because of the new potent therapeutic options now available to reduce LDL as the PCSK9 (proprotein convertase subtilisin/kexin 9) inhibitors. Hence, we investigated the added value of detrimental cMV signature on top of main cardiovascular risk scores for primary prevention namely Systematic Coronary Risk Evaluation and Registre Gironí del Cor and for high-risk patients including FRS for hard coronary events and the SAFEHEART risk estimator. As indicated by the C statistics, the COX Proportional Hazards Regression Model combining cMVs profiles provides high discriminatory power between patients with and without CVE. An important finding of the present study is that L/p-cMV signature beyond being associated to cardiovascular outcomes improves risk stratification of the before mentioned classical risk factor models by a substantial increase in the AUC of ROC curve analysis, as indicative of the significant and sensitive predictive ability of cMVs in this context.

There are well-described differences between men and women in the clinical presentation, pathophysiology, and response to treatment of CVD that might be explained by both biological and environmental processes. This is also true for patients with cardiovascular risk factors such as dyslipidemia. Because women show a lower perception of cardiovascular risk and are less keen to seek medical care when experiencing symptoms, their prognosis is worse than in men. In addition, sex differences in the field of cardiovascular risk prediction remain to be further elucidated. Thus, here, we have investigated cMVs as prognostic factors of future MACE by stratifying our data by sex and showing that sex condition does not influence the cardiovascular risk based on cMV profile.

The current study has some caveats and limitations that warrant discussion. The main caveats are that measurements of circulating MVs in daily clinical practice are still limited because of inherent complex technology needs and that there is a lack of standardization. A second aspect is the relatively small sample size that does not represent a more general population of patients with FH. However, the study evaluates a highly detailed group of patients with molecularly confirmed FH diagnosis and documented cardiovascular ischemic event presentation, a rather unique characteristic that confers a remarkable strength to a study in the field of circulating MVs, usually centered on a case-control approach. Further larger studies are warranted to assess the tentative extension of these findings to patients without FH but also to wider asymptomatic populations and contexts.

Taken together, our data shed light into a cMV-mediated modulation of pathophysiological mechanisms in atherothrombosis progression indicating that leukocytes and platelets are activated in some patients with FH, although treated as per guidelines. A specific cMV signature directly predicts a detrimental vascular milieu that will trigger CVE and cardiovascular death. Early prediction of vascular injury may allow better risk stratification and improved prognosis.

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Disclosures

None.

References

1. Sanz J, Moreno PR, Fuster V. The year in atherothrombosis. *J Am Coll Cardiol.* 2013;62:1131–1143. doi: 10.1016/j.jacc.2013.06.045
2. Fuster V, Kovacic JC. Acute coronary syndromes: pathology, diagnosis, genetics, prevention, and treatment. *Circ Res.* 2014;114:1847–1851. doi: 10.1161/CIRCRESAHA.114.302806
3. Berberich AJ, Hegele RA. The complex molecular genetics of familial hypercholesterolaemia. *Nat Rev Cardiol.* 2019;16:9–20. doi: 10.1038/s41569-018-0052-6
4. Nordestgaard BG, Chapman MJ, Humphries SE, et al; European Atherosclerosis Society Consensus Panel. Familial hypercholesterolaemia is underdiagnosed and undertreated in the general population: guidance for clinicians to prevent coronary heart disease: consensus statement of the European Atherosclerosis Society. *Eur Heart J.* 2013;34:3478a–3490a. doi: 10.1093/eurheartj/eht273
5. Perez de Isla L, Alonso R, Mata N, et al. Coronary heart disease, peripheral arterial disease, and stroke in familial hypercholesterolaemia: insights from the safeheart registry (spanish familial hypercholesterolaemia cohort study). *Arterioscler Thromb Vasc Biol*. 2016;36:2004–2010.
6. Hoefer IE, Steffens S, Ala-Korpela M, et al; ESC Working Group Atherosclerosis and Vascular Biology. Novel methodologies for biomarker discovery in atherosclerosis. *Eur Heart J.* 2015;36:2635–2642. doi: 10.1093/eurheartj/ehv236
7. Khot UN, Khot MB, Bajzer CT, Sapp SK, Ohman EM, Brener SJ, Ellis SG, Lincoff AM, Topol EJ. Prevalence of conventional risk factors in patients with coronary heart disease. *JAMA.* 2003;290:898–904. doi: 10.1001/jama.290.7.898
8. Perez de Isla L, Alonso R, Mata N, et al. Predicting cardiovascular events in familial hypercholesterolemia: the safeheart registry (spanish familial hypercholesterolemia cohort study). *Circulation*. 2017;135:2133–2144.
9. Villa G, Wong B, Kutikova L, Ray KK, Mata P, Bruckert E. Prediction of cardiovascular risk in patients with familial hypercholesterolaemia. *Eur Heart J Qual Care Clin Outcomes.* 2017;3:274–280. doi: 10.1093/ehjqcco/qcx011
10. Khera AV, Won HH, Peloso GM, et al. Diagnostic yield and clinical utility of sequencing familial hypercholesterolemia genes in patients with severe hypercholesterolemia. *J Am Coll Cardiol.* 2016;67:2578–2589. doi: 10.1016/j.jacc.2016.03.520
11. Sturm AC, Knowles JW, Gidding SS, et al; Convened by the Familial Hypercholesterolemia Foundation. Clinical genetic testing for familial hypercholesterolemia: JACC scientific expert panel. *J Am Coll Cardiol.* 2018;72:662–680. doi: 10.1016/j.jacc.2018.05.044
12. Badimon L, Suades R, Arderiu G, Peña E, Chiva-Blanch G, Padró T. Microvesicles in atherosclerosis and angiogenesis: from bench to bedside and reverse. *Front Cardiovasc Med.* 2017;4:77. doi: 10.3389/fcvm.2017.00077
13. Badimon L, Suades R, Fuentes E, Palomo I, Padró T. Role of platelet-derived Microvesicles as crosstalk mediators in atherothrombosis and future pharmacology targets: a link between inflammation, atherosclerosis, and thrombosis. *Front Pharmacol.* 2016;7:293. doi: 10.3389/fphar.2016.00293
14. Ridger VC, Boulanger CM, Angelillo-Scherrer A, et al. Microvesicles in vascular homeostasis and diseases. *Thromb Haemost.* 2017;117:1296–1316. doi: 10.1160/TH16-12-0943
15. Suades R, Padró T, Badimon L. The role of blood-borne microparticles in inflammation and hemostasis. *Semin Thromb Hemost.* 2015;41:590–606. doi: 10.1055/s-0035-1556591
16. Boulanger CM, Loyer X, Rautou PE, Amabile N. Extracellular vesicles in coronary artery disease. *Nat Rev Cardiol.* 2017;14:259–272. doi: 10.1038/nrcardio.2017.7
17. Sarlon-Bartoli G, Bennis Y, Lacroix R, Piercecchi-Marti MD, Bartoli MA, Arnaud L, Mancini J, Boudes A, Sarlon E, Thevenin B, Leroyer AS, Squarcioni C, Magnan PE, Dignat-George F, Sabatier F. Plasmatic level of leukocyte-derived microparticles is associated with unstable plaque in asymptomatic patients with high-grade carotid stenosis. *J Am Coll Cardiol.* 2013;62:1436–1441. doi: 10.1016/j.jacc.2013.03.078
18. Sionis A, Suades R, Sans-Roselló J, Sánchez-Martínez M, Crespo J, Padró T, Cubedo J, Ferrero-Gregori A, Vila-Perales M, Duran-Cambra A, Badimon L. Circulating microparticles are associated with clinical severity of persistent ST-segment elevation myocardial infarction complicated with cardiogenic shock. *Int J Cardiol.* 2018;258:249–256. doi: 10.1016/j.ijcard.2017.10.044
19. Suades R, Padró T, Crespo J, Ramaiola I, Martin-Yuste V, Sabaté M, Sans-Roselló J, Sionis A, Badimon L. Circulating microparticle signature in coronary and peripheral blood of ST elevation myocardial infarction patients in relation to pain-to-PCI elapsed time. *Int J Cardiol.* 2016;202:378–387. doi: 10.1016/j.ijcard.2015.09.011
20. Suades R, Padró T, Vilahur G, Martin-Yuste V, Sabaté M, Sans-Roselló J, Sionis A, Badimon L. Growing thrombi release increased levels of CD235a(+) microparticles and decreased levels of activated platelet-derived microparticles. Validation in ST-elevation myocardial infarction patients. *J Thromb Haemost.* 2015;13:1776–1786. doi: 10.1111/jth.13065
21. Suades R, Padró T, Alonso R, López-Miranda J, Mata P, Badimon L. Circulating CD45+/CD3+ lymphocyte-derived microparticles map lipid-rich atherosclerotic plaques in familial hypercholesterolaemia patients. *Thromb Haemost.* 2014;111:111–121. doi: 10.1160/TH13-07-0612
22. Suades R, Padró T, Alonso R, Mata P, Badimon L. High levels of TSP1+/CD142+ platelet-derived microparticles characterise young patients with high cardiovascular risk and subclinical atherosclerosis. *Thromb Haemost.* 2015;114:1310–1321. doi: 10.1160/TH15-04-0325
23. Mata N, Alonso R, Badimón L, et al. Clinical characteristics and evaluation of LDL-cholesterol treatment of the Spanish Familial Hypercholesterolemia Longitudinal Cohort Study (SAFEHEART). *Lipids Health Dis.* 2011;10:94. doi: 10.1186/1476-511X-10-94
24. National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). Third report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel iii) final report. *Circulation*. 2002;106:3143–3421.
25. Wierzbicki AS, Humphries SE, Minhas R; Guideline Development Group. Familial hypercholesterolaemia: summary of NICE guidance. *BMJ.* 2008;337:a1095. doi: 10.1136/bmj.a1095
26. Reiner Z, Catapano AL, De Backer G, et al. Esc/eas guidelines for the management of dyslipidaemias: the task force for the management of dyslipidaemias of the european society of cardiology (esc) and the european atherosclerosis society (eas). *Eur Heart J*. 2011;32:1769–1818.
27. Perez de Isla L, Alonso R, Watts GF, et al. Attainment of ldl-cholesterol treatment goals in patients with familial hypercholesterolemia: 5-year safeheart registry follow-up. *J Am Coll Cardiol*. 2016;67:1278–1285.
28. Pijlman AH, Huijgen R, Verhagen SN, Imholz BP, Liem AH, Kastelein JJ, Abbink EJ, Stalenhoef AF, Visseren FL. Evaluation of cholesterol lowering treatment of patients with familial hypercholesterolemia: a large cross-sectional study in The Netherlands. *Atherosclerosis.* 2010;209:189–194. doi: 10.1016/j.atherosclerosis.2009.09.014
29. Gehi AK, Ali S, Na B, Whooley MA. Self-reported medication adherence and cardiovascular events in patients with stable coronary heart disease: the heart and soul study. *Arch Intern Med.* 2007;167:1798–1803. doi: 10.1001/archinte.167.16.1798
30. von Elm E, Altman DG, Egger M, Pocock SJ, Gøtzsche PC, Vandenbroucke JP; STROBE Initiative. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement: guidelines for reporting observational studies. *PLoS Med.* 2007;4:e296. doi: 10.1371/journal.pmed.0040296
31. Collins GS, Reitsma JB, Altman DG, Moons KG; TRIPOD Group. Transparent reporting of a multivariable prediction model for individual prognosis or diagnosis (TRIPOD): the TRIPOD statement. The TRIPOD Group. *Circulation.* 2015;131:211–219. doi: 10.1161/CIRCULATIONAHA.114.014508
32. Kong F, Zhang L, Wang H, Yuan G, Guo A, Li Q, Chen Z. Impact of collection, isolation and storage methodology of circulating microvesicles on flow cytometric analysis. *Exp Ther Med.* 2015;10:2093–2101. doi: 10.3892/etm.2015.2780
33. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem.* 1972;18:499–502.
34. Alonso R, Defesche JC, Tejedor D, Castillo S, Stef M, Mata N, Gomez-Enterria P, Martinez-Faedo C, Forga L, Mata P. Genetic diagnosis of familial hypercholesterolemia using a DNA-array based platform. *Clin Biochem.* 2009;42:899–903. doi: 10.1016/j.clinbiochem.2009.01.017
35. Yuana Y, Bertina RM, Osanto S. Pre-analytical and analytical issues in the analysis of blood microparticles. *Thromb Haemost.* 2011;105:396–408. doi: 10.1160/TH10-09-0595
36. Suades R, Padró T, Alonso R, Mata P, Badimon L. Lipid-lowering therapy with statins reduces microparticle shedding from endothelium, platelets and inflammatory cells. *Thromb Haemost.* 2013;110:366–377. doi: 10.1160/TH13-03-0238

37. Benjamini Y, Hochberg Y. On the adaptive control of the false discovery fate in multiple testing with independent statistics. *J Educ Behav Stat*. 2000;25:60–83
38. Perk J, De Backer G, Gohlke H, et al; European Association for Cardiovascular Prevention & Rehabilitation (EACPR); ESC Committee for Practice Guidelines (CPG). European Guidelines on cardiovascular disease prevention in clinical practice (version 2012). The Fifth Joint Task Force of the European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice (constituted by representatives of nine societies and by invited experts). *Eur Heart J*. 2012;33:1635–1701. doi: 10.1093/eurheartj/ehs092
39. Marrugat J, Solanas P, D'Agostino R, Sullivan L, Ordovas J, Cordón F, Ramos R, Sala J, Masià R, Rohlfs I, Elosua R, Kannel WB. [Coronary risk estimation in spain using a calibrated framingham function]. *Rev Esp Cardiol*. 2003;56:253–261.
40. DeLong ER, DeLong DM, Clarke-Pearson DL. Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. *Biometrics*. 1988;44:837–845.
41. Mause SF, Weber C. Microparticles: protagonists of a novel communication network for intercellular information exchange. *Circ Res*. 2010;107:1047–1057. doi: 10.1161/CIRCRESAHA.110.226456
42. Hoyer FF, Giesen MK, Nunes França C, Lütjohann D, Nickenig G, Werner N. Monocytic microparticles promote atherogenesis by modulating inflammatory cells in mice. *J Cell Mol Med*. 2012;16:2777–2788. doi: 10.1111/j.1582-4934.2012.01595.x
43. Suades R, Padró T, Vilahur G, Badimon L. Circulating and platelet-derived microparticles in human blood enhance thrombosis on atherosclerotic plaques. *Thromb Haemost*. 2012;108:1208–1219. doi: 10.1160/TH12-07-0486
44. Nozaki T, Sugiyama S, Koga H, Sugamura K, Ohba K, Matsuzawa Y, Sumida H, Matsui K, Jinnouchi H, Ogawa H. Significance of a multiple biomarkers strategy including endothelial dysfunction to improve risk stratification for cardiovascular events in patients at high risk for coronary heart disease. *J Am Coll Cardiol*. 2009;54:601–608. doi: 10.1016/j.jacc.2009.05.022
45. Sinning JM, Losch J, Walenta K, Böhm M, Nickenig G, Werner N. Circulating CD31+/Annexin V+ microparticles correlate with cardiovascular outcomes. *Eur Heart J*. 2011;32:2034–2041. doi: 10.1093/eurheartj/ehq478
46. Berezin AE, Kremzer AA, Berezina TA, Martovitskaya YV. The pattern of circulating microparticles in patients with diabetes mellitus with asymptomatic atherosclerosis. *Acta Clin Belg*. 2016;71:38–45. doi: 10.1080/17843286.2015.1110894
47. Chiva-Blanch G, Suades R, Crespo J, Vilahur G, Arderiu G, Padró T, Corella D, Salas-Salvadó J, Arós F, Martínez-González MA, Ros E, Fitó M, Estruch R, Badimon L. CD3(+)/CD45(+) and SMA-α(+) circulating microparticles are increased in individuals at high cardiovascular risk who will develop a major cardiovascular event. *Int J Cardiol*. 2016;208:147–149. doi: 10.1016/j.ijcard.2016.01.211
48. Caballero P, Alonso R, Rosado P, Mata N, Fernández-Friera L, Jiménez-Borreguero LJ, Badimon L, Mata P. Detection of subclinical atherosclerosis in familial hypercholesterolemia using non-invasive imaging modalities. *Atherosclerosis*. 2012;222:468–472. doi: 10.1016/j.atherosclerosis.2012.02.043
49. Chiva-Blanch G, Bratseth V, Ritschel V, Andersen GØ, Halvorsen S, Eritsland J, Arnesen H, Badimon L, Seljeflot I. Monocyte-derived circulating microparticles (CD14+, CD14+/CD11b+ and CD14+/CD142+) are related to long-term prognosis for cardiovascular mortality in STEMI patients. *Int J Cardiol*. 2017;227:876–881. doi: 10.1016/j.ijcard.2016.11.302
50. Faille D, Frere C, Cuisset T, Quilici J, Moro PJ, Morange PE, Bonnet JL, Alessi MC. CD11b+ leukocyte microparticles are associated with high-risk angiographic lesions and recurrent cardiovascular events in acute coronary syndromes. *J Thromb Haemost*. 2011;9:1870–1873. doi: 10.1111/j.1538-7836.2011.04418.x
51. Leroyer AS, Isobe H, Lesèche G, Castier Y, Wassef M, Mallat Z, Binder BR, Tedgui A, Boulanger CM. Cellular origins and thrombogenic activity of microparticles isolated from human atherosclerotic plaques. *J Am Coll Cardiol*. 2007;49:772–777. doi: 10.1016/j.jacc.2006.10.053
52. Chironi G, Simon A, Hugel B, Del Pino M, Gariepy J, Freyssinet JM, Tedgui A. Circulating leukocyte-derived microparticles predict subclinical atherosclerosis burden in asymptomatic subjects. *Arterioscler Thromb Vasc Biol*. 2006;26:2775–2780. doi: 10.1161/01.ATV.0000249639.36915.04
53. Martinez GJ, Barraclough JY, Nakhla S, Kienzle V, Robertson S, Mallat Z, Celermajer DS, Patel S. Neutrophil-derived microparticles are released into the coronary circulation following percutaneous coronary intervention in acute coronary syndrome patients. *Biosci Rep*. 2017;37:BSR20160430.
54. Johnson BL 3rd, Midura EF, Prakash PS, Rice TC, Kunz N, Kalies K, Caldwell CC. Neutrophil derived microparticles increase mortality and the counter-inflammatory response in a murine model of sepsis. *Biochim Biophys Acta Mol Basis Dis*. 2017;1863(10 pt B):2554–2563. doi: 10.1016/j.bbadis.2017.01.012
55. Lo SK, Golenbock DT, Sass PM, Maskati A, Xu H, Silverstein RL. Engagement of the Lewis X antigen (CD15) results in monocyte activation. *Blood*. 1997;89:307–314.
56. Pitanga TN, de Aragão França L, Rocha VC, Meirelles T, Borges VM, Gonçalves MS, Pontes-de-Carvalho LC, Noronha-Dutra AA, dos-Santos WL. Neutrophil-derived microparticles induce myeloperoxidase-mediated damage of vascular endothelial cells. *BMC Cell Biol*. 2014;15:21. doi: 10.1186/1471-2121-15-21
57. Gasser O, Hess C, Miot S, Deon C, Sanchez JC, Schifferli JA. Characterisation and properties of ectosomes released by human polymorphonuclear neutrophils. *Exp Cell Res*. 2003;285:243–257.
58. Folkesson M, Li C, Frebelius S, Swedenborg J, Wågsäter D, Williams KJ, Eriksson P, Roy J, Liu ML. Proteolytically active ADAM10 and ADAM17 carried on membrane microvesicles in human abdominal aortic aneurysms. *Thromb Haemost*. 2015;114:1165–1174. doi: 10.1160/TH14-10-0899
59. Penson PE, Long DL, Howard G, Toth PP, Muntner P, Howard VJ, Safford MM, Jones SR, Martin SS, Mazidi M, Catapano AL, Banach M. Associations between very low concentrations of low density lipoprotein cholesterol, high sensitivity C-reactive protein, and health outcomes in the Reasons for Geographical and Racial Differences in Stroke (REGARDS) study. *Eur Heart J*. 2018;39:3641–3653. doi: 10.1093/eurheartj/ehy533
60. Ridker PM, Danielson E, Fonseca FA, Genest J, Gotto AM Jr, Kastelein JJ, Koenig W, Libby P, Lorenzatti AJ, MacFadyen JG, Nordestgaard BG, Shepherd J, Willerson JT, Glynn RJ; JUPITER Study Group. Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. *N Engl J Med*. 2008;359:2195–2207. doi: 10.1056/NEJMoa0807646
61. Ridker PM, Everett BM, Thuren T, et al; CANTOS Trial Group. Antiinflammatory therapy with canakinumab for atherosclerotic disease. *N Engl J Med*. 2017;377:1119–1131. doi: 10.1056/NEJMoa1707914
62. Llorente-Cortés V, Otero-Viñas M, Camino-López S, Llampayas O, Badimon L. Aggregated low-density lipoprotein uptake induces membrane tissue factor procoagulant activity and microparticle release in human vascular smooth muscle cells. *Circulation*. 2004;110:452–459. doi: 10.1161/01.CIR.0000136032.40666.3D
63. Nielsen MH, Irvine H, Vedel S, Raungaard B, Beck-Nielsen H, Handberg A. The Impact of lipoprotein-associated oxidative stress on cell-specific microvesicle release in patients with familial hypercholesterolemia. *Oxid Med Cell Longev*. 2016;2016:2492858. doi: 10.1155/2016/2492858
64. Emdin CA, Khera AV, Natarajan P, Klarin D, Baber U, Mehran R, Rader DJ, Fuster V, Kathiresan S. Evaluation of the pooled cohort equations for prediction of cardiovascular risk in a contemporary prospective cohort. *Am J Cardiol*. 2017;119:881–885. doi: 10.1016/j.amjcard.2016.11.042

Highlights

- Overall levels of circulating microvesicles that exert key regulatory roles in atherothrombosis are found to be significantly elevated in patients with high-risk familial hypercholesterolemic 3 years before cardiovascular event onset.
- Pan-leukocyte-derived and activated neutrophil-derived circulating microvesicles as well as circulating microvesicles bearing markers of platelet activation (P-selectin and thrombospondin-1) are significantly increased in patients about to suffer an ischemic event.
- A leucocyte and platelet activation-based circulating microvesicle signature emerge as an accurate prognostic marker that is able to improve the prediction of ischemic events in patients with asymptomatic high cardiovascular risk familial hypercholesterolemia.