Liquid Biopsy of Extracellular Microvesicles Maps Coronary Calcification and Atherosclerotic Plaque in Asymptomatic Patients With Familial Hypercholesterolemia

A Computed Tomographic Angiography Imaging Study

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Objective—Heterozygous familial hypercholesterolemia (FH) is the most common genetic disorder associated with premature atherosclerotic cardiovascular disease. Circulating microvesicles (cMV) are released when cells are activated. We investigated whether cMV could provide information on coronary calcification and atherosclerosis in FH patients.

Approach and Results—Eighty-two patients (mean of 44±9 years old) with molecular diagnosis of heterozygous FH and asymptomatic cardiovascular disease were investigated. Atherosclerotic plaque characterization was performed by computed tomography angiography, and Agatston coronary calcium score and plaque composition sum were calculated. cMV were quantified by flow cytometry using AV (annexin V) and cell surface-specific antibodies.Of the 82 FH patients, 48 presented atherosclerotic plaque. Patients with atherosclerosis were men and older in a higher percentage than patients without atherosclerotic plaque. FH patients with atherosclerotic plaque showed higher levels of total AV^{+} cMV, cMV AV^{+} from platelet origin, from granulocytes and neutrophils, and cMV $AV^{+/-}$ from endothelial cells than FH-patients without atherosclerotic plaque. Plaque composition sum correlated with platelet- and endothelial-derived cMV, and Agatston coronary calcium score correlated with granulocyte-, platelet-, and endothelial-derived cMV. Receiver operating characteristic curve analyses indicated that the cluster of platelet-, granulocyte-, neutrophil, and endothelial-derived cMV considered together, added significant predictive value to the specific SAFEHEART (Spanish Familial Hypercholesterolaemia Cohort Study) risk equation for plaque presence (area under the curve=0.866, 95% CI, 0.775–0.958; $P<0.0001$, $P=0.030$ for the increment of the area under the curve).

Conclusions—Endothelial-, granulocyte-, neutrophil- and platelet-derived cMV discriminate and map coronary atherosclerotic plaque and calcification in asymptomatic patients with FH. Liquid biopsy of cMV may be a surrogate biomarker of coronary atherosclerotic plaque burden in FH patients.

Visual Overview—An online visual overview is available for this article. **(*Arterioscler Thromb Vasc Biol*. 2019;39:945-955. DOI: 10.1161/ATVBAHA.118.312414.)**

Key Words: atherosclerosis ■ carotid stenosis ■ endothelial cells ■ extracellular vesicles ■ familial hypercholesterolemia ■ neutrophil ■ platelets

Heterozygous familial hypercholesterolemia (FH) is the most common genetic disorder associated with increased LDL (low-density lipoprotein)-cholesterol levels from birth onwards leading to premature atherosclerotic cardiovascular disease, being at up to 13-fold increased risk of coronary artery disease (CAD).[1] Recent studies have shown that the prevalence of clinical FH is about 1/200 to 1/300.[2]

Coronary atherosclerosis is highly prevalent in asymptomatic patients with FH,[3] but varies considerably across cohorts and individual patients treated as per guidelines,[1,4,5] suggesting a significant contribution of additional factors besides LDL-cholesterol to the atherosclerotic burden in these patients.

Circulating microvesicles (cMV) are 0.1 to 1 µm phospholipid-rich blebs released when cells are activated, and are an important part of cell-to-cell communication and signaling machinery. cMV can induce deleterious changes in the expression of substances related to inflammation and oxidative stress, contributing to cardiovascular dysfunction.[6] In addition, cMV contain phosphatidylserine at the outer leaflet, conferring procoagulant activity,[7] thus promoting the development and progression of atherothrombosis. In fact,

Received on: January 15, 2019; final version accepted on: February 26, 2019.

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The online-only Data Supplement is available with this article at https://www.ahajournals.org/doi/suppl/10.1161/ATVBAHA.118.312414.

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Nonstandard Abbreviations and Acronyms	
AUC	area under the curve
AV	annexin V
CAD	coronary artery disease
CCS	coronary calcium score
cMV	circulating microvesicles
CTA	computed tomographic angiography
FH	familial hypercholesterolemia
LDL	low-density lipoprotein
LLT	lipid-lowering therapy
PCS	plaque composition sum

leukocyte-derived (CD11a$^+$/ AV$^+$[annexin V]) cMV correlate with number of atherosclerotic plaques in subjects with evidence of subclinical atherosclerosis in the carotid, abdominal aorta, and femoral arteries.[8] In FH patients, total AV$^+$, CD45$^+$/AV$^+$, and CD3$^+$/CD45$^+$/AV$^+$ cMV were found elevated in those patients with lipid-rich atherosclerotic plaques[9] compared with patients with fibrous plaques. In patients with carotid stenosis, leukocyte-derived MV were increased in those patients with unstable plaque compared with patients with stable plaque.[10] However, there is no information on the relationship between cMV and coronary plaque composition.

Despite intensive lipid-lowering therapy (LLT), FH patients show high MV shedding.[9] FH is associated with early onset and greater intensity of subclinical atherosclerotic cardiovascular disease in coronary, carotid, and peripheral arteries.[11] cMV have shown to map magnetic resonance imaging-detected aortic lipid-rich atherosclerotic plaques[9]; however little is known on the possible effectiveness of cMV to map coronary calcification and atherosclerosis burden. Here, we have hypothesized that cMV will be able to identify the burden of coronary atherosclerosis as imaged by computed tomographic angiography (CTA) in young FH patients; and, additionally, cMV will report on the parental cells involved in the residual risk for atherosclerosis progression in young FH patients treated as per guidelines.

Materials and Methods

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

Patients

Eighty-two patients with clinical and genetic diagnosis of FH were randomly selected from the SAFEHEART cohort (Spanish Familial Hypercholesterolaemia Cohort Study)[12–14] (Materials and methods in the online-only Data Supplement). Local ethics committees approved the study protocol, and all participants gave written consent before participation in the study. The genetic variants investigated were classified as null or defective mutations as previously described.[15,16] Estimated cardiovascular risk at 5-years was obtained by using the SAFEHEART risk equation[17] which estimates the likelihood to occur fatal or nonfatal myocardial infarction, fatal or nonfatal ischemic stroke, coronary revascularization, peripheral artery revascularization, and cardiovascular death (considered as any death related to cardiovascular disease or derived of cardiovascular therapeutic procedures not described in the previous definitions).

Atherosclerotic Plaque Characterization and Stenosis Grade Evaluation

Atherosclerotic plaque characterization was performed by CTA according to a 17-segment American Heart Association classification, as previously described.[3,17] Briefly, using a tomographic scanner, 3 mm thick slices were obtained during a breath-holding protocol, and the Agatston coronary calcium score (CCS) was calculated. Coronary CTA was performed using 64–detector row scanners with prospective or retrospective electrocardiographic gating. Every coronary CTA was analyzed by 2 independent experienced readers, blinded to the clinical characteristics of the subjects, in a central laboratory, using axial, multiplanar reformat, maximum intensity projection, and cross-sectional views. In case of discrepancy, a third reader was consulted. Plaque presence was defined as determined in at least 1 segment of the CTA.

The stenosis severity was visually evaluated. A lesion severity score was defined as follows: 0, no stenosis; 1, mild diameter stenosis (<50%); 2, moderate (50% to 70%); and 3, severe diameter stenosis (>70%). Sum of stenosis severity was defined as the sum of the lesion severity in all segments. Plaque composition was classified as follows, according to a new score system designed for this assessment[3]: 0, no plaque; 1, calcified plaque (highly attenuating tissue for >70% of the plaque volume, which could be clearly separated from the contrast-enhanced coronary lumen); 2, mixed plaque (containing both calcified and noncalcified tissue); and 3, noncalcified plaque (low-attenuating lesions that could be clearly separated from the coronary lumen and the surrounding epicardial fat or myocardium). Plaque composition sum (PCS), defined as the sum of all the plaque composition values in all segments, was calculated for each patient. Vessel segments <1.5 mm in diameter were excluded from analysis.

cMVs Isolation and Quantification

Blood samples were collected from patients at the time of CTA (Materials and Methods in the online-only Data Supplement). Five hundred microliters of frozen plasma aliquots were thawed on melting ice for 1 hour and centrifuged again at 1300×*g*, 10 minutes, at room temperature to guarantee complete cell removal. Then, 250 µL of plasma were transferred to another vial and centrifuged at 20 000×*g* for 30 minutes at room temperature to pellet cMV. The supernatants were discarded, and the cMV-enriched pellet was washed once with citrate-PBS solution before a second equal centrifugation step was made. Finally, the cMV pellets were resuspended in 100 µL citrate-PBS. Afterwards, 5 µL of washed cMV suspensions were diluted in 30 µL PBS buffer containing 2.5 mmol/L $CaCl_2$ (annexin binding buffer). Thereafter, combinations of 5 µL of CF405M-conjugated AV, which has high affinity for phosphatidylserine, with 2 specific monoclonal antibodies (1–5 µL each; Table I in the online-only Data Supplement) labeled with fluorescein isothiocyanate or phycoerythrin, or the isotype-matched control antibodies were added in a final volume of 50 µL annexin binding buffer to label and characterize phosphatidylserine -positive cMV with bioactive and biomarker molecules from their parental cells, according to Table I in the online-only Data Supplement. Samples were incubated 20 minutes at room temperature in the dark and diluted with annexin binding buffer before being immediately analyzed on a FACSCanto II flow cytometer.

Acquisition was performed at 1 minute per sample and flow rate was measured before each experiment. Forward scatter, side scatter and fluorescence data were obtained with the settings in the logarithmic scale. The lower detection limit was placed as a threshold above the electronic noise of the flow cytometer. To identify positive marked events, thresholds 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 MV in a calcium-free buffer PBS. cMV were identified and quantified based on their forward scatter/side scatter characteristics according to their size (Figure I in the online-only Data Supplement), binding or not to AV, and therefore

to phosphatidylserine externalization, and reactivity to cell-specific monoclonal antibodies (Figure II in the online-only Data Supplement). Data were analyzed with FACSDiva software (BD). The cMV concentration (number of cMV per µL of plasma) was determined according to Nieuwland formula.[18] All buffers were prepared on the same day and filtered through 0.2 µm pore size filters under vacuum to reduce background noise.

Statistical Analysis

Statistical analyses were performed using the SPSS Statistical Analysis System (version 23.0). Results are expressed as mean±SD or n (%) when indicated. Discriminate analysis was performed to determine the type of cMV able to classify patients with and without coronary plaque. *t* tests for unpaired samples were used to analyze differences in cMV according to atherosclerotic plaque presence. Receiver operating characteristic (ROC)-curve analyses were performed to identify the cMV able to indicate atherosclerotic plaque presence and calcification, and their corresponding C statistics (area under the curve [AUC] with their 95% CI) were calculated. A cutoff level of cMV was determined with the shortest distance from upper left corner of the ROC curve (where sensitivity=1 and specificity=1), and therefore, minimizing $[(1\text{-sensitivity})^2+(1\text{-specificity})^2]$. Multivariable models for the prediction of plaque presence were performed with a logistic regression model with cMV levels from different cell origins, as well as for the SAFEHEART cardiovascular risk equation by creating predicted probabilities, which then were transferred to the ROC-curve algorithm to estimate the likelihood of plaque presence by calculating the corresponding AUC along with their 95% CI. Please see Materials and methods in the online-only Data Supplement for further details.

A 2 tail *P* value of <0.05 was considered statistically significant.

Results

Patients' Characteristics

Main demographic and clinical characteristics of FH patients are shown in Table 1. Mean age was 44 years old, 55% were men, and most patients were normotensive and normoglycemic. All subjects were on LLT; mean LDL-C was 137.7 mg/dL. Men presented more overweight, higher blood pressure, triglycerides and glucose levels, and lower circulating HDL-cholesterol and ApoAI. In addition, men had twice the cardiovascular risk at 5-years calculated with the SAFEHEART risk equation, and were on more intensive LLT compared with women. In addition, a higher percentage of men presented coronary atherosclerosis (Figure 1) and thus, higher PCS and sum of stenosis severity (Table 1), although no significant differences were observed in CCS between men and women.

There were no differences in the amount of cMV according to age or sex.

Table II in the online-only Data Supplement shows the levels of cMV from different cell origins in the circulating blood of FH patients. No differences in total cMV ($AV^{+/-}$) and cMV AV^+ levels were observed for any type of cMV except for $CD62E^+$, Connexin 43^+, and $CD142^+$ cMV.

Atherosclerotic Plaque, Calcification, and Stenosis Grade

An atherosclerotic plaque compromising the arterial lumen was found in 48 out of 82 patients (58%). Main demographic and clinical characteristics of patients with and without atherosclerotic plaque are presented in Table 2. Patients with atherosclerotic plaque were older, presented more overweight, a higher median 5-year CV risk, quantified with the SAFEHEART risk equation, and were receiving more intensive LLT (defined by maximum statin dose, maximum LLT, and potency of LLT) compared with patients without coronary plaque.

Figure 1 depicts that patients with atherosclerotic plaque were men in a higher percentage. Overall patients with atherosclerotic plaque were a mean of 10 years older, but the differences in age were more pronounced for men with and without CAD (a mean of 14 years of difference, $P<0.0001$) than for women (mean of 6.5-years of difference, $P=0.027$). In fact, men without atherosclerotic plaque were a mean of 4 years younger than women without plaque ($P=0.003$), although no statistical differences were observed in the age of men and women with coronary atherosclerosis. Within patients with or without coronary plaque, no significant differences in the amounts of cMV were observed according to sex.

cMVs and Lipid Lowering Therapy

A negative correlation was observed between years of LLT and the levels of $CD3^+/CD45^+/AV^+$ and $CD3^+/CD45^+/AV^{+/-}$ cMV originated from lymphocytes, leukocyte-derived $CD62L^+/AV^+$ and $CD62L^+/AV^{+/-}$ and $CD62L^+/CD45^+/AV^+$, monocyte-derived $CD14^+/AV^+$ cMV, and monocyte-derived cMV carrying tissue factor $CD142^+/CD14^+/AV^+$ (−0.258 Spearman coefficient, $P=0.019$; −0.226, 0.041; −0.426, <0.0001; −0.261, 0.018; −0.297, 0.007; −0.219, 0.048; and −0.253, 0.022, respectively).

No differences in cMV levels were observed according to the type of mutation. Severity of CAD did not affect cMV.

Discriminative Ability of cMVs for Presence of Atherosclerotic Plaque

Within measured cMV, discriminative analyses revealed that endothelial-derived cMV ($CD62E^+/AV^{+/-}$), total AV^+ cMV, AV^+ cMV from platelet origin ($CD31^+/AV^+$, $CD41a^+/CD31^+/AV^+$), and cMV from granulocytes and neutrophils ($CD66^+/AV^+$ and $CD11b^+/CD66^+/AV^+$) were able to discriminate FH patients with atherosclerotic plaque (Table 3). When these cMV were analyzed as a clustered model by creating predicted probabilities, they were able to properly classify 70.1% of patients according to the presence of atherosclerotic plaque, with a 79.1% sensitivity and 45.8% specificity ($P=0.004$).

cMVs, Atherosclerotic Plaque Burden, Calcification, and Stenosis Grade

Despite receiving a more intensive LLT treatment and for a longer period of time, FH patients with atherosclerotic plaques had significantly higher levels of endothelial-derived cMV ($CD62E^+/AV^{+/-}$), total AV^+ cMV, cMV from platelet origin ($CD41a^+/AV^+$, $CD31^+/AV^+$, $CD41a^+/CD31^+/AV^+$), and cMV from granulocytes and neutrophils ($CD66^+/AV^+$ and $CD11b^+/CD66^+/AV^+$), as depicted in Figure 2. Table III in the online-only Data Supplement shows the correlation between cMV involved in coronary atherosclerosis.

In addition, PCS correlated with endothelial-derived cMV (Spearman correlation coefficient of 0.351, $P=0.002$ for $CD62E^+/AV^{+/-}$), and platelet-derived AV^+ cMV

Table 1. Clinical Characteristics of the 82 Patients With Heterozygous Familial Hypercholesterolemia Included in the Study

	All Patients	Males	Females	*P* Value
Sex, n (%)		45(54.9)	37 (45.1)	0.377
Age, y	44.4±9.5	43.8±10.0	45.1±9.0	0.548
body mass index, Kg/m^2	25.0±3.6	26.5±3.0	23.2±3.4	<0.001
Overweight (body mass index ≥25 Kg/m^2), n (%)	38 (46.3)	29 (64.4)	9 (24.3)	<0.001
Systolic blood pressure, mm Hg	118.2±17.6	123.4±12.5	111.9±20.8	0.003
Diastolic blood pressure, mm Hg	74.2±8.8	76.9±9.3	70.9±7.0	0.002
Total cholesterol, mg/dL	207.1±45.2	212.9±51.8	200.2±34.8	0.206
HDL-cholesterol, mg/dL	50.5±10.4	46.6±9.8	55.3±9.1	<0.001
LDL-cholesterol, mg/dL	137.7±41.9	143.2±47.0	131.1±34.2	0.195
Triglycerides, mg/dL	91.6±50.8	109.2±60.4	70.2±22.2	<0.001
Lipoprotein(a)	47.1±50.8	38.9±42.0	57.0±58.8	0.109
ApoA1	127.2±15.2	123.6±14.3	131.7±15.2	0.015
ApoB	108.4±25.1	111.5±26.9	104.7±22.7	0.223
Glucose, mg/dL	90.7±9.6	94.3±9.6	86.3±7.7	<0.001
C reactive protein	0.12±0.23	0.14±0.27	0.10±0.16	0.392
Hypertension, n (%)	3 (3.7)	3 (6.7)	0 (0)	0.110
Diabetes mellitus, n (%)	1 (1.2)	1 (2.2)	0 (0)	0.362
Current smokers, n (%)	28 (34.1)	11 (24.4)	17 (45.9)	0.120
Family history of premature CVD, n (%)	28 (34.1)	12 (26.7)	16 (43.2)	0.115
Corneal arcus, n (%)	32 (39.0)	16 (35.5)	16 (43.2)	0.478
Xanthoma, n (%)	9 (11.0)	5 (11.1)	4 (10.8)	0.965
CAD severity, n (%)				0.080
Low	29 (35.4)	15 (33.3)	14 (37.8)	
Moderate	13 (15.9)	11 (24.4)	2 (5.4)	
Severe	6 (7.3)	4 (8.9)	2 (5.4)	
CAD type, n (%)				0.100
Obstructive CAD	8 (9.8)	6 (13.3)	2 (5.4)	
Nonobstructive CAD	40 (48.8)	29 (64.4)	11 (29.7)	
Atherosclerotic plaque presence, n (%)	48 (58.5)	31 (68.9)	17 (45.9)	0.036
Agaston coronary calcium score	115.9±317.9	138.5±325.9	89.0±309.5	0.488
Plaque composition sum	3.1±3.9	3.9±4.3	2.2±3.2	0.044
Sum of stenosis severity	2.0±3.3	2.7±3.7	1.2±2.5	0.039
Cardiovascular risk at 5-years (%)	0.96±0.79	1.26±0.91	0.57±0.33	<0.001
Medication, n (%)				
Statins	82 (100)	45 (100)	37 (100)	1.000
Maximum statin dose	22 (26.8)	17 (37.8)	5 (13.5)	0.016
Ezetimibe	15 (18.3)	10 (22.2)	5 (13.5)	0.342
Maximum combined therapy	9 (11.0)	7 (15.6)	2 (5.4)	0.157
Maximum lipid lowering therapy	26 (31.7)	19 (42.2)	7 (18.9)	0.029
Potency of lipid lowering therapy	5.5±1.6	6.0±1.4	5.0±1.6	0.009
Years of lipid-lowering therapy	12.8±7.0	13.4±7.3	12.1±6.6	0.419

Results are expressed as mean±SD or n(%) when indicated. *P* from the comparison between males and females (*t* test and χ^2 test for quantitative and qualitative variables, respectively). Cardiovascular risk was calculated using the specific SAFEHEART risk equation, which estimates the likelihood to occur fatal or nonfatal myocardial infarction, fatal or nonfatal ischemic stroke, coronary revascularization, peripheral artery revascularization, and cardiovascular death (any death related to cardiovascular disease or derived of cardiovascular therapeutic procedures not described in the previous definitions). Maximum statin dose, maximum combined therapy, maximum lipid-lowering therapy, and LLT potency have been calculated according the method described by Pérez de Isla et al.[17] Plaque composition sum, defined as the sum of all the plaque composition values in all segments, was calculated. Plaque composition was classified as follows, according to a new score system designed for this assessment: 0, no plaque; 1, calcified plaque (highly attenuating tissue for >70% of the plaque volume, which could be clearly separated from the contrast-enhanced coronary lumen); 2, mixed plaque (containing both calcified and noncalcified tissue); and 3, noncalcified plaque (low-attenuating lesions that could be clearly separated from the coronary lumen and the surrounding epicardial fat or myocardium). Vessel segments <1.5 mm in diameter were excluded from analysis. Sum of stenosis severity was defined as the sum of the lesion severity in all segments, defined as follows: 0, no stenosis; 1, mild diameter stenosis (<50%); 2, moderate (50% to 70%); and 3, severe diameter stenosis (>70%).

CAD indicates coronary artery disease; CVD, cardiovascular disease; HDL, high-density lipoprotein; and LDL, low-density lipoprotein.

(Spearman correlation coefficient of 0.268, 0.314, and 0.331, and *P*=0.017, 0.005, and 0.003, for CD41a$^+$/AV$^+$, CD31$^+$/AV$^+$ and CD41a$^+$/CD31$^+$/AV$^+$, respectively). Multivariable adjusting did not substantially modify the observed results (Table IV in the online-only Data Supplement). As depicted in Figure 3, when patients were divided by tertiles of PCS (PCS=0–14, n=29; PCS=15–17, n=26; and PCS≥18, n=24), they showed increased levels of these cMV in the upper tertiles.

CCS positively correlated with endothelial-, granulocyte-, and platelet-derived cMV (Spearman correlation coefficient of 0.268, 0.271, 0.251, and 0.263, and *P*=0.023, 0.019, 0.027, and 0.018, for CD62E$^+$/AV$^{+/-}$, CD66$^+$/AV$^+$, CD11b$^+$/CD66$^+$/AV$^+$, and CD41a$^+$/CD31$^+$/AV$^+$, respectively).

However, only CD62E$^+$/AV$^{+/-}$ correlated with sum of stenosis severity (Spearman correlation coefficient of 0.305, *P*=0.041). A marginal correlation was observed between sum

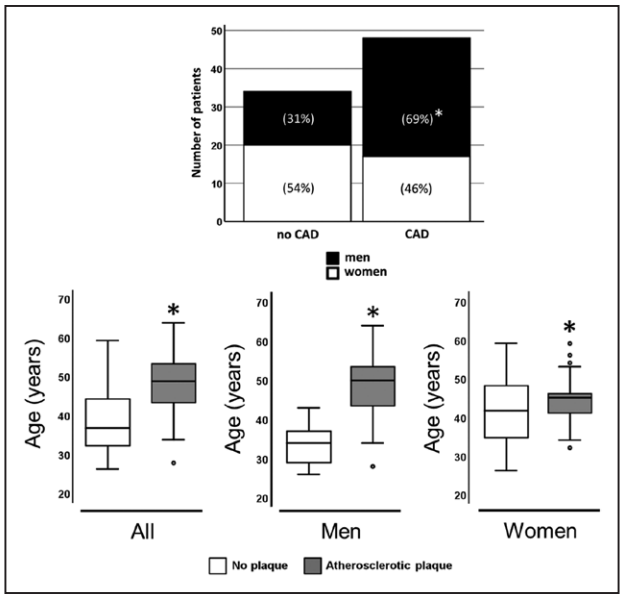

Figure 1. Distribution of age and sex within the 82 familial hypercholesterolemia patients with and without atherosclerotic plaque. *P<0.05 from the comparison between patients with and without plaque presence (t test). CAD indicates coronary artery disease.

of stenosis severity and CD41a$^+$/CD31$^+$/AV$^+$ cMV (Spearman correlation coefficient of 0.220, P=0.050).

Within patients with coronary plaque, cMV did not discriminate for CAD severity or CAD type.

Prognostic Validity of cMVs for the Prediction of Atherosclerotic Plaque

To evaluate the predictive power of cMV for coronary plaque presence and calcification degree, ROC-curve analyses were performed for total AV$^+$ cMV, CD41a$^+$/AV$^+$, CD31$^+$/AV$^+$, CD41a$^+$/CD31$^+$/AV$^+$, CD66$^+$/AV$^+$, CD11b$^+$/CD66$^+$/AV$^+$, and CD62E$^+$/AV$^{+/-}$ cMV levels. All cMV properly predicted coronary plaque presence in FH patients (Figure II in the online-only Data Supplement and Table V in the online-only Data Supplement). As illustrated in Figure 4, ROC-curve analysis indicated that total AV$^+$ cMV, platelet-, granulocyte-, neutrophil-, and endothelial- derived cMV considered together (CD41a$^+$/AV$^+$, CD31$^+$/AV$^+$, CD41a$^+$/CD31$^+$/AV$^+$, CD66$^+$/AV$^+$, CD11b$^+$/CD66$^+$/AV$^+$, and CD62E$^+$/AV$^{+/-}$), predicted the presence of coronary atherosclerotic plaques (AUC=0.804, 95% CI [0.706–0.902], P<0.0001) in FH patients. The prognostic value of the cluster of cMV was higher than that achieved with the specific SAFEHEART risk equation (AUC=0.714, 95% CI [0.591–0.838], P=0.002), although the difference did not reach statistical significance (P=0.092). However, when considering the cluster of cMV and the SAFEHEART risk equation together, the predictive power for the prediction of atherosclerotic plaque significantly increased (AUC=0.866, 95% CI [0.775–0.9580, P<0.0001], in comparison with the SAFEHEART risk equation alone (P=0.030 for the comparison between AUCs).

As depicted in Figure 5, granulocyte-, neutrophil-, and endothelial-derived cMV predicted plaque calcification, determined by a CCS ≥1. CD66$^+$/AV$^+$ cMV at a cutoff point of 7.4 cMV/μL plasma predicted plaque calcification with 63.8 % selectivity and 66.7 % specificity (AUC=0.712, 95% CI [0.590–0.834], P=0.003). CD11b$^+$/CD66$^+$/AV$^+$ cMV at a cutoff point of 1 cMV/μL plasma predicted plaque calcification with 56.3% selectivity and 65.5% specificity (AUC=0.659, 95% CI [0.538–0.779], P=0.020), and CD62E$^+$/AV$^{+/-}$ cMV at a cutoff point of 77 cMV/μL plasma predicted plaque calcification with 66.7% selectivity and 57.1% specificity (AUC=0.672, 95% CI [0.547–0.797], P=0.013). When considered together, the prognostic power for plaque calcification prediction significantly increased (AUC=0.812, 95% CI [0.711–0.913], P<0.0001).

Discussion

In the era of precision medicine, the identification of new biomarkers to better characterize and individualize cardiovascular risk prediction in FH patients remains as one of the major challenges. This emphasizes the importance of clinical prospective registries linked to a biobank such as the SAFEHEART cohort.[19] This study was aimed to investigate if cMV in blood could provide information on coronary calcification and atherosclerosis thus providing new information on the residual CAD risk, independent of classical risk factors and clinical indicators, in a small cohort of genetically and molecularly defined FH without clinical cardiovascular disease. Some studies have investigated the relationship between peripheral artery atherosclerosis and the levels of cMV[9–11]; however, this is the first study investigating whether peripheral blood cMV can provide direct information of coronary atherosclerosis. In FH patients the coronary arteries are the most frequently affected arteries,[20] even though they are under LLT as per guidelines. LLT decreases MV shedding from platelets, endothelial cells and leukocytes.[21] However, FH patients have increased levels of cMV compared non-FH peers,[9,22] indicating that long term exposure to high levels of LDL induces a chronic cell activation, and therefore highlighting the implication of these specific cMV in coronary atherosclerosis despite the more intensive use of statins in these patients. Here we show that the presence of coronary atherosclerotic plaque is associated with higher levels of total AV$^+$ cMV, and cMV originated from platelets (CD41a$^+$/AV$^+$, CD31$^+$/AV$^+$, CD41a$^+$/CD31$^+$/AV$^+$), granulocytes (CD66$^+$/AV$^+$), neutrophils (CD11b$^+$/CD66$^+$/AV$^+$), and endothelial cells (CD62E$^+$/AV$^{+/-}$) in peripheral blood, a direct indication of chronic activation of these cells in the HF patients. Activation of these cell lineages accelerates the atherothrombotic process. From all cMV quantified, endothelial-, platelet-, granulocyte-, and neutrophil-derived cMV are the most related to atherosclerosis progression and plaque calcification in FH patients. In fact, we have previously shown that patients who suffered a myocardial infarction with cardiogenic shock have increased levels of platelet-, granulocyte-, and neutrophil-derived cMV compared with patients with myocardial infarction without cardiogenic shock,[23] and ST-segment–elevation myocardial infarction patients show increased levels of platelet-derived cMV compared with non–ST-segment–elevation myocardial infarction patients, even 2 to 8 weeks after suffering an acute

Table 2. Clinical Characteristics of the 82 Patients With Heterozygous Familial Hypercholesterolemia Included in the Study According to Atherosclerotic Plaque Presence

	No Plaque (n=34)	Atherosclerotic Plaque (n=48)	*P* Value
Males, n (%)	14 (41.2)	31 (64.6)	0.036
Age, y	39.0±9.0	48.0±8.0	<0.0001
Body mass index, Kg/m^2	23.8±3.5	25.7±3.4	0.009
Overweight (body mass index ≥25 Kg/m^2), n (%)	10 (29.4)	28 (58.3)	0.016
Systolic blood pressure, mm Hg	116.0±11.3	120.0±21.1	0.287
Diastolic blood pressure, mm Hg	73.4±8.2	75.1±9.4	0.271
Total cholesterol, mg/dL	199.2±33.3	212.8±51.6	0.180
HDL-cholesterol, mg/dL	51.4±10.5	49.9±10.4	0.505
LDL-cholesterol, mg/dL	131.8±29.4	142.0±48.8	0.283
Triglycerides, mg/dL	79.5±47.6	100.1±51.7	0.070
Lipoprotein(a)	40.5±45.9	51.79±53.9	0.323
ApoA1	127.0±16.1	127.4±14.6	0.901
ApoB	103.8±21.0	111.7±27.5	0.164
Glucose, mg/dL	87.7±6.5	92.8±10.9	0.010
C reactive protein	0.12±0.18	0.12±0.25	0.904
Hypertension, n (%)	0 (0)	3 (6.2)	0.138
Diabetes mellitus, n (%)	0 (0)	1 (2.1)	0.397
Current smokers, n (%)	19 (55.9)	9 (18.7)	0.002
Family history of premature CVD, n (%)	8 (23.5)	20 (41.7)	0.088
Corneal Arcus, n (%)	6 (17.7)	26 (54.2)	0.001
Xanthoma, n (%)	2 (5.9)	7 (14.6)	0.214
CAD severity, n (%)			<0.0001
Low	0 (0)	29 (60.4)	
Moderate	0 (0)	13 (27.1)	
Severe	0 (0)	6 (12.5)	
CAD type, n (%)			<0.0001
Obstructive CAD	0 (0)	8 (16.7)	
Nonobstructive CAD	0 (0)	40 (83.3)	
Agatston coronary calcium score	0±0	199.2±184.1	<0.0001
Plaque composition sum	0±0	5.3±3.8	<0.0001
Sum of stenosis severity	0±0	3.4±3.7	<0.0001
Cardiovascular risk at 5-years (%)	0.71±0.60	1.14±0.86	0.014
Medication, n (%)			
Statins	34 (100)	48 (100)	1.000
Maximum statin dose	5 (14.7)	17 (35.4)	0.024
Ezetimibe	3 (8.8)	12 (25)	0.045
Maximum combined therapy	1 (2.9)	8 (16.7)	0.040
Maximum lipid lowering therapy	6 (17.6)	20 (41.7)	0.012
Potency of lipid-lowering therapy	4.8±1.5	6.0±1.5	0.001
Years of lipid-lowering therapy	10.4±6.7	14.5±6.8	0.034*

Results are expressed as mean±SD or n(%) when indicated. *P* for the comparison between FH patients with and without atherosclerotic plaque (*t* test and χ^2 test for quantitative and qualitative variables, respectively). Cardiovascular risk was calculated using the specific SAFEHEART risk equation, which estimates the likelihood to occur fatal or nonfatal myocardial infarction, fatal or nonfatal ischemic stroke, coronary revascularization, peripheral artery revascularization, and cardiovascular death (any death related to cardiovascular disease or derived of cardiovascular therapeutic procedures not described in the previous definitions). Maximum statin dose, maximum combined therapy, maximum lipid-lowering therapy, and LLT potency have been calculated according the method described by by Pérez de Isla et al.[17] Plaque composition sum, defined as the sum of all the plaque composition values in all segments, was calculated. Plaque composition was classified as follows, according to a new score system designed for this assessment: 0, no plaque; 1, calcified plaque (highly attenuating tissue for >70% of the plaque volume, which could be clearly separated from the contrast-enhanced coronary lumen); 2, mixed plaque (containing both calcified and noncalcified tissue); and 3, noncalcified plaque (low-attenuating lesions that could be clearly separated from the coronary lumen and the surrounding epicardial fat or myocardium). Vessel segments <1.5 mm in diameter were excluded from analysis. Sum of stenosis severity was defined as the sum of the lesion severity in all segments, defined as follows: 0, no stenosis; 1, mild diameter stenosis (<50%); 2, moderate (50%–70%); and 3, severe diameter stenosis (>70%).

*Nonsignificant when adjusted for age.

CVD indicates cardiovascular disease; CAD, coronary artery disease; HDL, high-density lipoprotein; and LDL, low-density lipoprotein.

myocardial infarction.[24] In addition, in ACS patients higher release of MV from neutrophils has been observed after percutaneous coronary intervention, potentially reflecting the importance of these cells in vulnerable lesions.[25] Taking into account these considerations, increased MV shedding may both reflect the cause and effects of CV events.

PCS reflects total atherosclerotic burden and is a surrogate marker of coronary plaque stability.[3] Usually, high PCS, implies higher proportion of noncalcified arteries (which are more prone to rupture), and thus higher the risk of an acute coronary syndrome. However, our patients presented mostly calcified plaques. Nevertheless, FH patients who suffered an event after 0.5-years of the CTA showed higher PCS, CCS, and sum of stenosis severity scores than patients without a CV event after the follow-up.[3] In Japanese FH patients, coronary plaque burden was significantly associated with future coronary events beyond classical risk factors.[26] PCS positively correlated with platelet-derived cMV, indicating the active involvement of platelets in atherothrombogenesis. Platelet-derived CD41$^+$ cMV have been shown to be elevated in postinfarcted patients with atherosclerosis in 3 or more locations compared with patients with atherosclerosis in < 3 locations.[27] Previous findings from our group, showed that these cMV were elevated in FH patients independently of the atherosclerotic burden.[22] However, patients with lipid-rich

Table 3. Discriminative Ability of cMV for Coronary Plaque in the 82 Patients With Heterozygous Familial Hypercholesterolemia Studied

cMV μL Plasma	Sensitivity (%)	Specificity (%)	Patients >Cutoff Value* [n (%)]	Cases Properly Classified (%)	Positive Predictive Value (PPV, %)	Negative Predictive Value, (NPV, %)	*P* Value
Total AV^+	87.5	14.7	50 (61)	59.8	71.4	40.7	0.039
Platelet-derived MV							
$CD31^+/AV^+$	100	0	48 (58.5)	59.8	77.3	46.5	0.042
$CD41a^+/AV^+$			48 (58.5)		67.8	46.3	
$CD41a^+/CD31^+/AV^+$	95.8	2.9	46 (50.1)	58.5	76.9	48.1	0.035
Granulocyte-derived MV							
$CD66^+/AV^+$	87.5	32.4	40 (48.8)	64.6	70.3	47.9	0.029
$CD66^+/CD11b^+/AV^+$	58.3	58.8	38 (46.3)	58.5	80.9	50.9	0.034
Endothelial-derived MV							
$CD62E^+/AV^{+/-}$	75.0	37.8	50 (61)	58.4	68.0	46.15	0.006
Clustered cMV†	79.1	45.8	47 (57.3)	70.1	83.3	56.1	0.004

Sensitivity, specificity and *P* value from the discriminative analysis. Used markers are described in Table I in the online-only Data Supplement.

*Cutoff values for plaque presence from each MV are detailed in Table IV in the online-only Data Supplement. Please note that 48 patients (58.5%) presented coronary atherosclerotic plaque.

†Clustered cMV (AV^+, $CD41a^+/AV^+$, $CD31^+/AV^+$, $CD41a^+/CD31^+/AV^+$, $CD66^+/AV^+$ and $CD11b^+/CD66^+/AV^+$, and $CD62E^+/AV^{+/-}$) were created by predicted probabilities with a logistic regression model.

AV indicates annexin V; and cMV, circulating microvesicles.

plaque at the aorta or the carotid showed increased levels of cMV,[9] and cMV from total ($CD41^+/CD61^+/AV^+$) and activated platelets ($TSP1^+/AV^+$ and $CD62P^+/AV^+$), leukocytes ($CD45^+$ and $CD3^+/CD45^+/AV^+$),[9,10] and cMV carrying tissue factor ($CD142^+/AV^+$).[22] We did not observe differences in activated platelet- or leukocyte-derived cMV levels or profile according to plaque composition in the coronary artery as detected by CTA. On the contrary, Sarlon-Bartoli et al[10] observed that granulocyte-derived cMV ($CD11b^+/CD66b^+/AV^+$ and $CD15^+/AV^+$) were increased in patients with carotid unstable plaque compared with patients with stable plaque, measured by histological analysis after thromboendarterectomy. In patients with coronary plaques we observed the opposite; the higher the calcification degree (and thus, the higher the atherosclerotic burden), the higher the granulocyte-derived cMV levels observed. This may be attributed to the fact that, within patients with atherosclerotic burden, only 5 (10%) patients present lipid-rich plaque in 1 segment and 1 patient in 2 segments of the coronary tree, and oppositely, 95.8% of patients (n=46) present >3 segments with calcified plaque and 20% of patients present >5 segments of calcified plaque. The fact that about 96% FH patients presented calcified coronary plaques could be partially attributed to the statin treatment.[28] This has also been observed in other FH cohorts, with early coronary calcification in patients below 45-years old,[29] and associated to higher risk of coronary heart disease.[30]

In addition, cMV profile may differ according to the vessel that presents atherosclerosis. Platelet-derived cMV have been shown to be decreased in postinfarcted patients with atherosclerosis in coronary, carotid, and peripheral arteries compared with patients with atherosclerosis in 1 or 2 arterial locations.[27] Moreover, in that study, plaque composition was determined by aortic-magnetic resonance imaging. In the present study, plaque composition was quantified by CTA, which may partially explain the dissimilar results found in both studies considering FH patients with similar clinical characteristics.

Noncalcified composition is associated to unstable plaques, and calcified plaques are usually stable plaques unless the calcification is spotty.[31] However, CCS, a calcification burden score, has also been related to increased incidence of CV events,[32,33] and a more advanced stage of atherosclerosis.[34] As discussed above, PCS correlated with endothelial- and platelet-derived cMV, and CCS, correlated mostly to cMV originated granulocytes, neutrophils, and endothelial cells. This is in accordance to Jayachandran et al,[35] who observed increased levels of $CD62E^+$ MV in early menopausal women with coronary artery calcification. Low levels of cMV may be associated with lower degree of vascular inflammation associated to the progression of atherothrombotic disease, as shown in the ROC-curve analysis. In fact, considering all types of cMV related to coronary calcification and atherosclerosis, properly predicted plaque presence in FH patients. In addition, FH patients with higher coronary atherosclerotic burden are at increased risk of CV events, demonstrating that cMV from endothelial cells, platelets and granulocytes are surrogate markers of CV risk. In asymptomatic patients, no significant difference existed in AV^+, platelet-derived and endothelium-derived MV levels between subjects with moderate to high Framingham risk score and those with low Framingham risk score,[8] indicating that cMV levels may be a more accurate predictor of cardiovascular disease than the Framingham risk score in subjects with specific CV burden as previously observed.[36] It is worth mentioning that the Framingham risk score and the SAFEHEART risk equations are based in Cox regression models and the present analyses are based on logistic regression. However, if we take into consideration the specific SAFEHEART risk equation,[17] the cluster of cMV significantly improved the discriminative potency for plaque presence, even taking into consideration that FH patients have high basal levels of cMV,

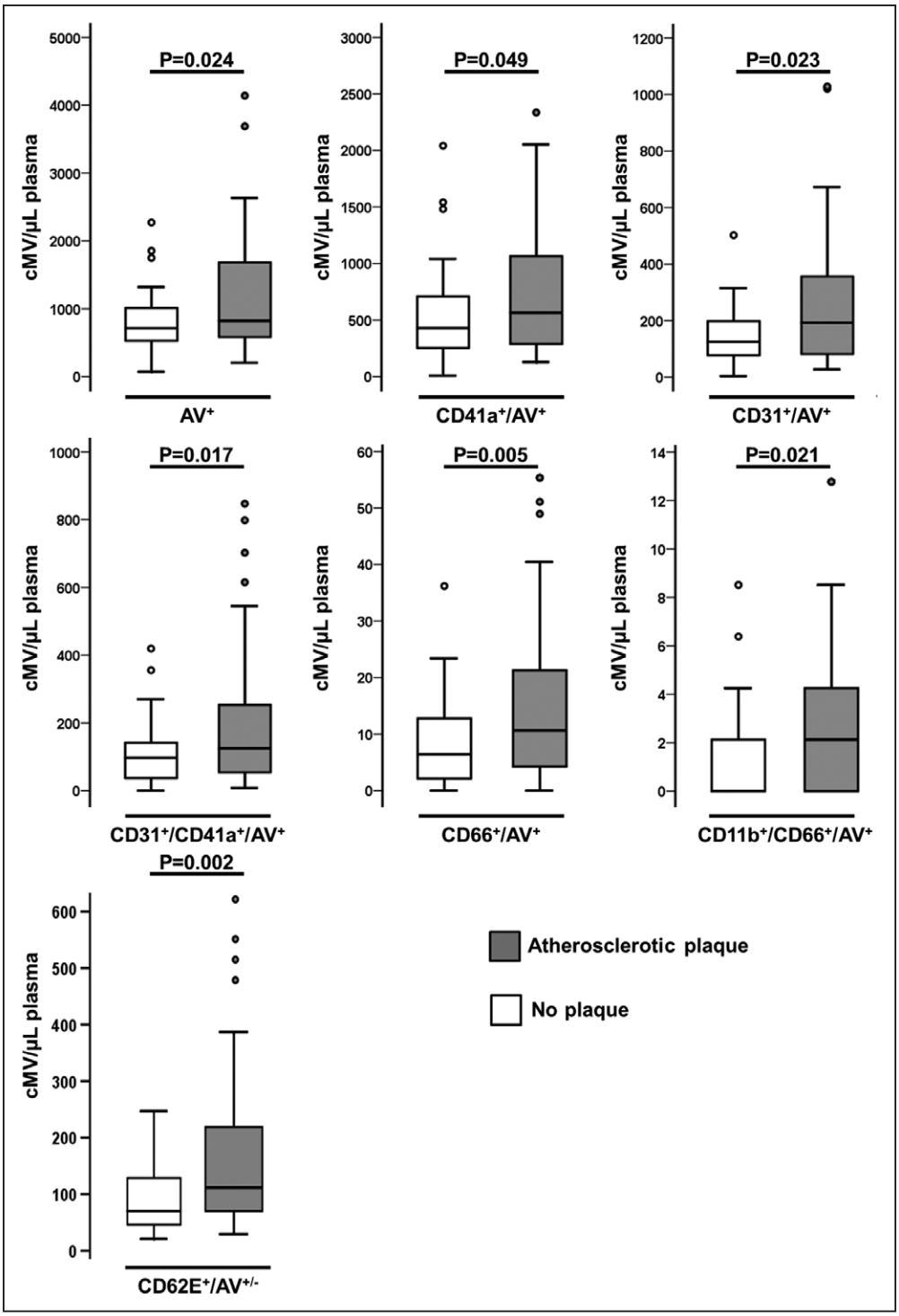

Figure 2. Circulating microvesicle (cMV) levels according to the presence of atherosclerotic plaque in the 82 patients with heterozygous familial hypercholesterolemia studied. CD66 was used to determine granulocyte origin. The other CDs were used as biomarkers of cell activation (Table I in the online-only Data Supplement). 48 heterozygous familial hypercholesterolemia patients presented atherosclerotic plaque and 34 did not. AV indicates annexin V.

and that cMV are highly sensitive but moderately specific as biomarkers of coronary atherosclerosis.

Therefore, increased MV shedding may reflect the burden of integrated and cumulative effects of traditional risk factors leading to a cardiovascular event. This is important in the grounds that among patients with CAD or other atherosclerotic diseases the frequency of FH is significantly higher than in general population, and these patients are at particularly elevated risk of recurrent events,[37,38] and given the fact that intervention strategies at the preclinical stage are more likely to confer CV benefit. cMV in patients with atherosclerosis in the aorta or carotid arteries were previously investigated in FH patients.[9,10,22] In this study, we have investigated whether peripheral blood cMV could also provide direct information of

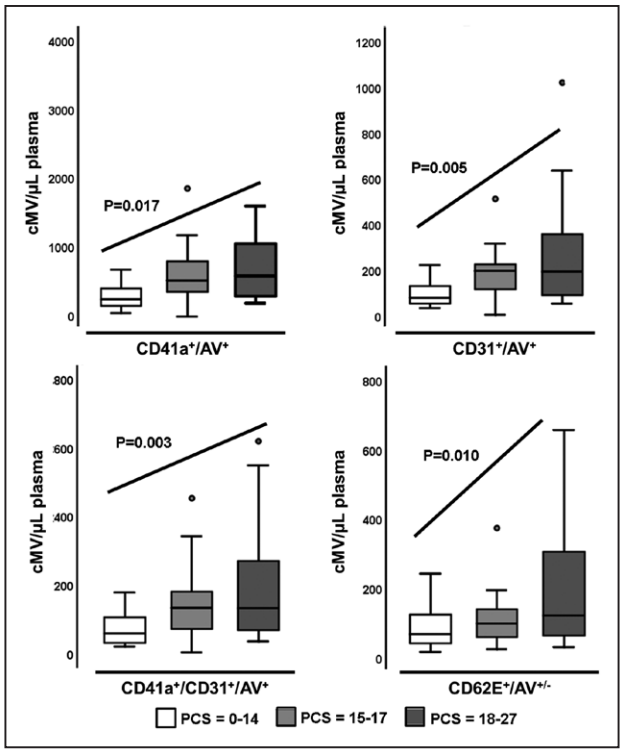

Figure 3. Levels of circulating microvesicle (cMV) in the 82 patients with heterozygous familial hypercholesterolemia studied according to tertiles of plaque composition sum (PCS). 29 heterozygous familial hypercholesterolemia patients were allocated in the tertiles of PCS=0 to 14; 26 in the tertile of PCS=15 to 17; and 24 patients were allocated at PCS≥18. AV indicates annexin V.

coronary atherosclerosis. Interestingly, the liquid biopsy of cMV maps the presence of coronary atherosclerosis in FH patients. Because of the lack of standardization, cMV measurement is yet a research tool. In the near future liquid biopsies on MVs may become a noninvasive quantitative method, with negligible risk for the patient. In contrast, although CTA is able to detect and quantify (somehow subjectively) the calcium in the coronary artery wall and the luminal stenosis, as well as to analyze the plaque composition characteristics, CTA requires contrast injection and radiation exposure, and there are controversies in the criteria of indications, cost-effectiveness, contraindications, risks, and benefits.[39] However, standardization of MV measurement is urgently needed to be able to compare measurements from different laboratories.

This study has some limitations. This is a cross-sectional study and thus of associative nature and lacking on data on hard CV endpoints. The study is performed on a well characterized genetically selected population consisting of 82 FH patients. Therefore, the results cannot be extrapolated as yet to other high CV risk population subsets. In addition, the SAFEHEART risk score of FH patients is still waiting external validation.[17] There was a relatively low number of coronary plaque cases, and patients presented mostly calcified coronary plaques; therefore, we could not compare the cMV profile according to plaque composition. Although flow cytometry is the most widely method used for MV quantification and characterization, the methodology of this new area of research requires further development. At present our quantification limit is 0.1 to 0.3 µm of particle size, which characterizes MV and excludes exosomes; thus, the MV populations below 100 nm are not considered in this study. cMV can carry markers from nonparental cells because it is possible that they suffer remodeling in the circulation and further investigation will be needed to fully characterize microvesicle cargos. Finally, the molecular cargo of these MV was not analyzed in this study and it is presently being investigated.

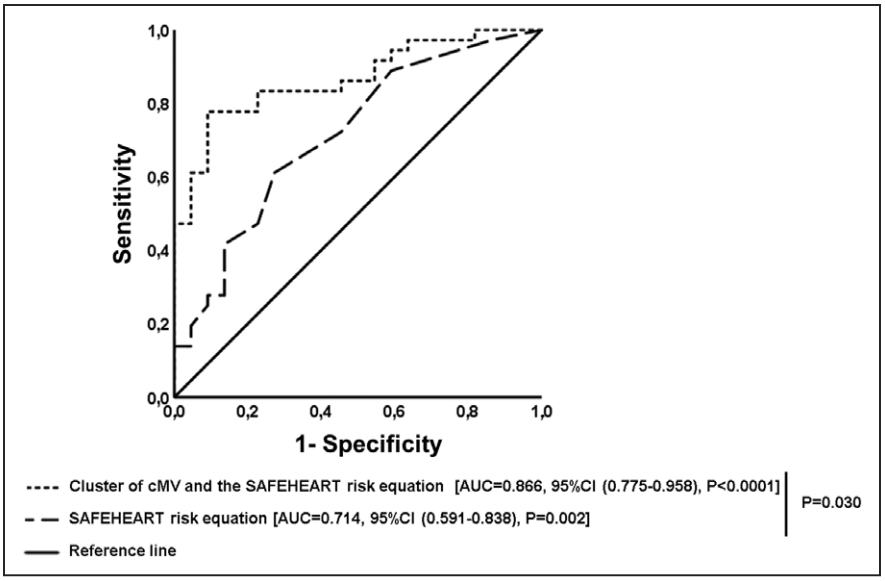

Figure 4. Receiver operating characteristic (ROC)-Curve analysis for atherosclerotic plaque presence prediction. Plaque presence was considered when determined in at least 1 segment of the computed tomographic angiography. ROC-curve analysis was used to determine the capacity of circulating microvesicles (cMV) to predict plaque presence in heterozygous familial hypercholesterolemia patients. Area under the curve (AUC) of the SAFEHEART risk equation, and AUC of the cluster of cMV (annexin V [AV+], CD41a+/AV+, CD31+/AV+, CD41a+/CD31+/AV+, CD66+/AV+ and CD11b+/CD66+/AV+, and CD62E+/AV+/−) and the SAFEHEART risk equation considered together. The cluster of cMV adds predictive value for plaque presence to the specific SAFEHEART risk equation. The SAFEHEART risk equation, estimates the likelihood to occur the first 1 of the following: fatal or nonfatal myocardial infarction, fatal or nonfatal ischemic stroke, coronary revascularization, peripheral artery revascularization, and cardiovascular death (any death related to cardiovascular disease or derived of cardiovascular therapeutic procedures not described in the previous definitions).

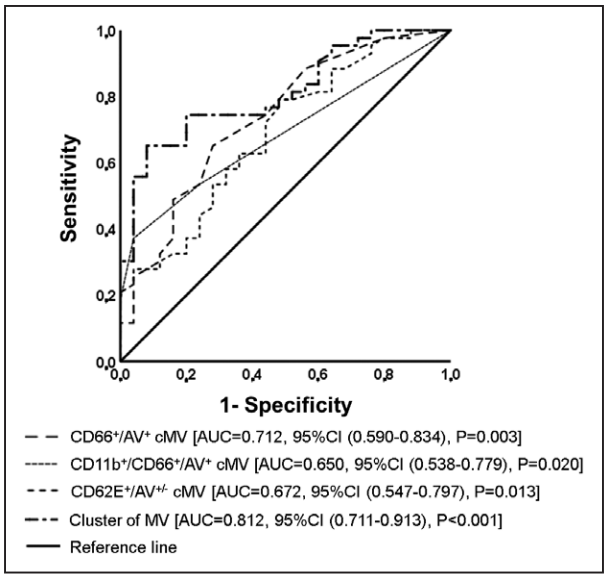

Figure 5. Receiver operating characteristic (ROC)-Curve analysis for atherosclerotic plaque calcification prediction. Plaque calcification was considered when coronary calcium score (CCS) ≥1. ROC-curve analysis was used to determine the capacity of circulating microvesicles (cMV) to predict plaque calcification in the 82 patients with heterozygous familial hypercholesterolemia studied. Area under the curve (AUC) of the cluster of granulocyte-, neutrophil-, and endothelial-derived cMV ($CD66^+/AV^+$ and $CD11b^+/CD66^+/AV^+$, and $CD62E^+/AV^{+/-}$, respectively) and these cMV considered independently. $CD66^+/AV^+$ cMV at a cutoff point of 7.4 cMV/μL plasma predicted plaque calcification with 63.8% selectivity and 66.7% specificity. $CD11b^+/CD66^+/AV^+$ cMV at a cutoff point of 1 cMV/μL plasma predicted plaque calcification with 56.3% selectivity and 65.5% specificity, and $CD62E^+/AV^{+/-}$ cMV at a cutoff point of 77 cMV/μL plasma predicted plaque calcification with 66.7% selectivity and 57.1% specificity.

In conclusion, a high proportion of young asymptomatic FH subjects exhibit early-calcified coronary atherosclerosis. Increased endothelial-, granulocyte-, neutrophil-, and platelet-cMV release characterize coronary atherosclerotic plaque and calcification in statin-treated asymptomatic patients with FH, contributing to the increased risk of a major CV event. The quantification of these cMV may be useful to identify more severe phenotypes of FH patients, potentially allowing the early detection of coronary atherosclerotic plaque in these patients. Monitoring cMV levels, and the development of therapeutic strategies to decrease their release may contribute, together with the LLT, to delay the onset and progression of atherosclerosis in FH patients and improve their prognosis. Finally, these cMV may be surrogate biomarkers of coronary calcification in FH patients.

Acknowledgments

We thank the Spanish Familial Hypercholesterolaemia Foundation (FHF) and physicians that contribute to the FHF-patient cohort SAFEHEART recruitment. We also thank the Fundació d'Investigació Cardiovascular (FIC)-Fundacion Jesús Serra, Barcelona, Spain, for their continuous support. We are indebted to the FH patients and relatives for their valuable contribution and willingness to participate.

Sources of Funding

This work was supported by grants from Spanish Ministry of Economy and Competitiveness of Science (SAF2016-76819-R to L. Badimon); Institute of Health Carlos III, ISCIII (TERCEL—RD16/0011/0018 and CIBERCV CB16/11/0041 to L. Badimon; and FIS PI16/01915 to T. Padró); and FEDER Una Manera de Hacer Europa. G. Chiva-Blanch is a Juan de la Cierva- Incorporación Postdoctoral Fellow (IJCI-2015–26358) from the Spanish Ministry of Economy and Competitiveness (MINECO), Spain.

Disclosures

None.

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Highlights

- Familial hypercholesterolemia (FH) is the most common genetic disorder associated with premature atherosclerotic cardiovascular disease.
- Circulating microvesicles (cMV) are released when cells are activated.
- FH patients with coronary atherosclerotic plaque showed higher levels of total annexin V^+ cMV, cMV annexin V^+ from platelet origin, from granulocytes and neutrophils, and cMV annexin $V^{+/-}$ from endothelial cells than FH-patients without atherosclerotic plaque.
- ROC-curve analyses indicate that the cluster of platelet-, granulocyte-, neutrophil, and endothelial-derived cMV considered together, added significant predictive value to the specific SAFEHEART risk equation for plaque presence in FH patients.
- Endothelial, granulocyte-, neutrophil-, and platelet-derived cMV discriminate and map coronary atherosclerotic plaque and calcification in asymptomatic patients with FH.