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Association of Microvesicles With Graft Patency in Patients Undergoing CABG Surgery

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ABSTRACT

BACKGROUND Graft patency is one of the major determinants of long-term outcome following coronary artery bypass graft surgery (CABG). Biomarkers, if indicative of the underlying pathophysiological mechanisms, would suggest strategies to limit graft failure. The prognostic value of microvesicles (MVs) for midterm graft patency has never been tested.

OBJECTIVES The aim of this study was to evaluate whether MV pre-operative signature (number, cellular origin, procoagulant phenotype) could predict midterm graft failure and to investigate potential functional role of MVs in graft occlusion.

METHODS This was a nested case-control substudy of the CAGE (CoronAry bypass grafting: factors related to late events and Graft patency) study that enrolled 330 patients undergoing elective CABG. Of these, 179 underwent coronary computed tomography angiography 18 months post-surgery showing 24% graft occlusion. Flow cytometry MV analysis was performed in 60 patients (30 per group with occluded [cases] and patent [control subjects] grafts) on plasma samples collected the day before surgery and at follow-up.

RESULTS Before surgery, cases had 2- and 4-fold more activated platelet-derived and tissue-factor positive MVs respectively than control subjects. The MV procoagulant capacity was also significantly greater. Altogether this MV signature properly classified graft occlusion (area under the curve 0.897 [95% confidence interval: 0.81 to 0.98]; $p < 0.0001$). By using an MV score (0 to 6), the odds ratio for occlusion for a score above 3 was 16.3 (95% confidence interval: 4.1 to 65.3; $p < 0.0001$).

CONCLUSIONS The pre-operative signature of MVs is independently associated with midterm graft occlusion in CABG patients and a cumulative MV score stratifies patients' risk. Because the MV signature mirrors platelet activation, patients with a high MV score could benefit from a personalized antiplatelet therapy. (J Am Coll Cardiol 2020;75:2819-32)

Coronary artery bypass graft (CABG) surgery is still the standard treatment of coronary revascularization for patients with severe coronary artery disease (CAD) (1). Graft patency, together with completeness of revascularization, is a major determinant of long-term outcome following CABG. The surgical procedure elicits a persistent systemic inflammatory response associated with the

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ABBREVIATIONS AND ACRONYMS

CABG = coronary artery bypass graft

CAD = coronary artery disease

CTA = computed tomography angiography

DAPT = dual antiplatelet therapy

MVs = microvesicles

PCI = percutaneous coronary intervention

TF = tissue factor

activation of the hemostatic system leading to perturbation of endothelial and vascular function and activation of platelets and leukocytes (2). All of these events are the main players responsible for the early and late graft failure in a significant percentage of patients (1).

The availability of biomarkers able to predict graft occlusion would possibly suggest strategies to limit graft failure. Several studies aimed to identify predictors of early-term graft occlusion after CABG focusing on the presence of conventional risk factors, genetic markers, features of coronary targets, or technical aspects. Other studies focused on plasmatic biomarkers, such as perioperative inflammatory and hemostatic factors (reviewed in Parolari et al. [3]). We recently identified D-dimer as a biomarker associated with medium-term graft occlusion (4). The molecular mechanisms underlying the modulation of these biomarkers are, however, often unclear, thus limiting possible interventions to improve the graft patency and hard outcomes.

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Circulating microvesicles (MVs) have received increasing attention during the last years as novel players in cardiovascular disease (5,6). MVs are small membrane vesicles involved in cell-to-cell communication acting as biological messengers. MVs of different origin are present in the circulation of healthy subjects, and their number increases in several pathological conditions contributing to the development, progression, and clinical outcome of diseases. They have been proposed as biomarkers of thrombosis, vascular injury, and inflammation in atherothrombosis and myocardial infarction, where elevated levels have been correlated with disease severity (7). Among the circulating MVs, those expressing phosphatidylserine are defined as procoagulant MVs (8). A subgroup of procoagulant MVs also express tissue factor (TF) (9), the key activator of the blood coagulation cascade. These procoagulant MVs have a role in the prediction of cardiovascular events (10,11) and are able to identify patients at high recurrence risk (12). Thus far, many studies have generated compelling data on the sensitivity of circulating MVs as biomarkers of cardiovascular disease progression and events. The usefulness of MVs in patients undergoing CABG, however, has only been tested in 1 study that highlighted their importance in surgical hemostasis (13). No information is so far available on the association between the amount or pattern of circulating MVs and CABG outcome.

Thus, we carried out this study to: 1) elucidate whether graft occlusion, evaluated 18 months after CABG, associates with a specific signature of circulating MVs in terms of number, cellular origin, and procoagulant phenotype; 2) assess what MV signature analyzed before surgery could identify those patients who will experience graft occlusion; and 3) investigate potential functional role of MVs in graft occlusion based on their protein profile as well as their procoagulant potential.

METHODS

STUDY DESIGN FOR MV ANALYSIS. The study took advantage of an existing biobank of plasma samples prepared from a cohort of 330 consecutive patients enrolled for elective surgical myocardial revascularization between November 2006 and February 2010 at Centro Cardiologico Monzino IRCCS (NCT00755248) (Supplemental Figure 1) (5). The study was approved by the Ethical Committee of Centro Cardiologico Monzino IRCCS and was conducted according to the Declaration of Helsinki. A written informed consent was obtained from all the participants.

At 18-month follow-up, coronary computed tomography angiography (CTA) performed on 179 patients showed the presence of at least 1 occluded graft in 43 subjects. Clinical outcomes at 52-month follow-up was assessed by telephone interview. A nested case-control study comparing age- and sex-matched patients was designed to analyze MVs. Suitable samples were available from 30 of 43 patients with occluded grafts (cases) at follow-up (n = 6 plasma were hemolyzed, n = 2 plasma had fibrin clots, n = 5 were missing), and were compared with 30 patients with patent grafts (control subjects).

STATISTICAL ANALYSIS. Quantitative variables were reported as mean ± SD or median (interquartile range [IQR]). Spearman's correlation was used to find monotonic association between variables. Categorical variables were compared between the 2 groups by the chi-square test, and quantitative variables by the Wilcoxon rank-sum test. Multivariable logistic regression was used to assess whether the MV levels, measured at baseline, were independently associated with future graft occlusion, after adjustment for the variables significantly differing between cases and control subjects. To summarize the overall potential

predictive ability of the 6 MV classes, a score was constructed adding 1 point for each MV class with levels above its median.

The ability of individual MV classes and of the score to discriminate between patent and occluded grafts at 18 months was assessed by receiver-operating characteristic (ROC) curve analysis. SAS statistical software version 9.4 (SAS Institute, Cary, North Carolina) was used for all analyses, and a p value <0.05 was considered statistically significant. For the main analyses (logistic regression and ROC curve analysis), the p value threshold for significance was set at 0.0083, accounting for Bonferroni correction for 6 independent tests.

For a complete Methods section, including Supplemental Tables 1 and 2, please see the Supplemental Appendix.

RESULTS

PATIENT CHARACTERISTICS. Patients were divided into 2 groups according to graft patency (controls = patent graft; cases = occluded graft; n = 30 per group) assessed by coronary CTA 18 months after surgery. Their baseline characteristics are summarized in **Table 1**. There were no significant differences in age, sex, blood cell counts, risk factors, and medications between the 2 groups as well as in the number of diseased coronary vessels and the vessels used for bypass grafting (**Table 1**). The rate of occlusion between vein and artery grafts was also similar (Supplemental Table 3).

At 18-month follow-up, all patients were on antiplatelet therapy, cholesterol-lowering medications, and beta-blockers (Supplemental Table 4), and no difference in clinical outcomes was observed between the 2 groups. Conversely, a significant higher occurrence of major adverse cardiovascular or cerebrovascular events was observed in cases at 52-month follow-up (Supplemental Table 5).

CHARACTERIZATION OF MICROVESICLE PHENOTYPE AT 18-MONTH FOLLOW-UP. Circulating MVs were studied taking into account: 1) their total number; 2) their cell origin, focusing on those derived from platelets, granulocytes, monocytes, and endothelium; and 3) the expression of platelet activation markers (CD62P and CD40L) and TF.

The relative amount of platelet-, leukocyte-, and endothelium-derived MVs was similar in the 2 groups of patients (~60%, 25%, 7%, and 8% of the total amount of MVs for platelet-, granulocyte-, monocyte-, and endothelium-derived MVs, respectively). Despite this, cases had a 2-fold higher number of total circulating MVs compared with control subjects (1,658 MVs/μl [IQR: 1,034 to 3,022 MVs/μl] vs. 705 MVs/μl [IQR: 389 to 863 MVs/μl], respectively; p = 0.008) (**Figure 1A**). MVs derived from platelets were the most abundant and they were twice as abundant in cases than in control subjects (p = 0.019) (**Table 2**). A similar difference was also observed in the number of granulocyte- and monocyte-derived MVs, but not in endothelium-derived MVs (**Table 2**).

Interestingly, the number of MVs shed from activated platelets ($CD62P^+/CD41^+$ or $CD40L^+/CD41^+$) was 3 times greater in cases compared with control subjects (p = 0.003 for $CD62P^+/CD41^+$ and p = 0.022 for $CD40L^+/CD41^+$ MVs). Similarly, the number of

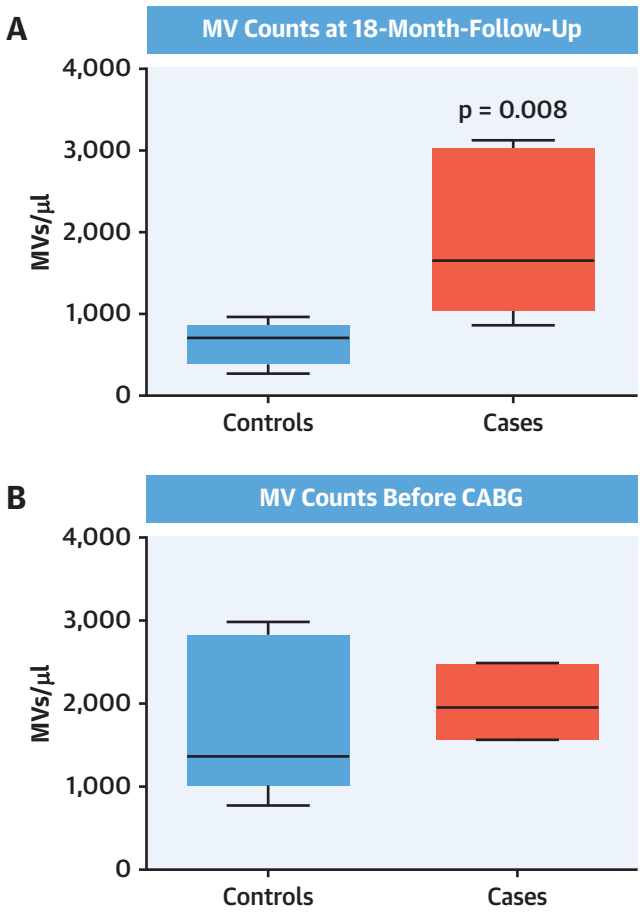
TABLE 1 Baseline Characteristics of the Patients Included in the Study

	Cases (n = 30)	Control Subjects (n = 30)	p Value
Age, yrs	63 ± 8	64 ± 8	0.53
Male	27 (88)	26 (86)	0.70
Body mass index, kg/m^2	25.4 ± 2.4	27.5 ± 3.6	0.005
Blood cell counts			
Platelet count	260 ± 72	236 ± 59	0.15
Monocyte count	0.40 ± 0.18	0.53 ± 0.48	0.29
Granulocyte count	4.80 ± 1.77	4.55 ± 1.77	0.57
Lymphocyte count	2.00 ± 0.63	2.14 ± 0.45	0.41
Risk factors			
Diabetes mellitus	7 (23)	9 (30)	0.56
Hypertension	25 (83)	24 (80)	0.95
Hyperlipidemia	24 (80)	23 (77)	0.76
Current smoker	7 (23)	4 (13)	0.39
Medications			
Antiplatelet	19 (63)	16 (53)	0.40
Hypoglycemic	5 (17)	6 (20)	0.95
Antihypertensive	26 (86)	25 (83)	0.70
Antiarrhythmic	1 (3)	0 (2)	0.75
Hypolipidemic	20 (66)	18 (60)	0.50
Surgical parameters			
Diseased coronary vessels	2.74 ± 0.52	2.84 ± 0.58	0.28
Great saphenous vein use	30 (100)	30 (100)	1.00
LIMA use	30 (100)	29 (97)	0.41
RIMA use	9 (30)	4 (13)	0.06
Radial artery use	2 (7)	0 (0)	0.07
Echocardiographic EF, %	54.7 ± 11.5	58.1 ± 7.9	0.15
Additive EuroSCORE	2.8 ± 2.5	2.6 ± 2.1	0.71
Logistic EuroSCORE	2.8 ± 3.4	2.3 ± 2.2	0.50
Surgery time, h	4.5 ± 0.8	4.1 ± 0.8	0.07
ECC time, min	109.8 ± 39.1	100.2 ± 25.9	0.23
Clamp time, min	77.0 ± 31.3	69.1 ± 19.0	0.20

Values are mean ± SD or n (%).

ECC = extracorporeal circulation; EF = ejection fraction; LIMA = left internal mammary artery; RIMA = right internal mammary artery.

FIGURE 1 Quantification of Circulating MVs in Patients Undergoing Elective CABG



Microvesicle (MV) counts were evaluated by flow cytometry in platelet-free-plasma collected at 18-month follow-up **(A)** and the day before coronary artery bypass graft (CABG) surgery **(B)**. Data are presented as a box plot, with median and 25th to 75th percentiles **(box)** and min-max values **(whiskers)**.

TABLE 2 Levels of Circulating MVs Measured 18 Months After CABG Surgery

MV Cell Origin	Cases (MVs/μl)	Control Subjects (MVs/μl)	p Value
$CD41^+$	939 (483–1,740)	460 (330–672)	0.019
$CD66^+$	489 (476–879)	178 (93–259)	0.021
$CD14^+$	103 (72–195)	79 (42–133)	0.034
$CD31^+/CD41^-$	104 (61–129)	98 (49–186)	0.904
Activated platelet-derived MVs			
$CD62P^+/CD41^+$	108 (77–437)	32 (21–62)	0.003
$CD40L^+/CD41^+$	73 (23–128)	29 (21–45)	0.022
TF^+ MVs			
Total TF^+	204 (128–569)	97 (39–168)	0.0004
$TF^+/CD41^+$	33 (19–50)	21 (7–29)	0.014
$TF^+/CD66^+$	128 (101–220)	47 (25–115)	0.119
$TF^+/CD14^+$	31 (12–40)	11 (5–21)	0.009
$TF^+/CD31^+/CD41^-$	33 (5–70)	12 (9–19)	0.326

Values are median (interquartile range). Controls are patients who would have had patent graft at follow-up; cases are patients who would have had occluded graft at follow-up.

CABG = coronary artery bypass graft; $CD14^+$ = monocyte-derived; $CD31^+/CD41$ = endothelium-derived; CD40L = CD40 ligand; $CD41^+$ = platelet-derived; CD62P = P-selectin; $CD66^+$ = granulocyte-derived;. MVs = microvesicles; TF = tissue factor.

total TF^+ MVs was also higher (2-fold; p = 0.0004) in cases compared with control subjects (Table 2).

Overall, these data suggest that in patients with occluded grafts, the number of activated platelet-derived MVs and of TF^+ MVs is significantly greater compared with patients with a patent graft.

CHARACTERIZATION OF MICROVESICLE PHENOTYPE BEFORE CABG SURGERY. We then assessed whether an association exists between circulating MV composition before surgery and bypass graft occlusion at follow-up. Before surgery, cases showed a trend toward higher levels of total MVs compared with controls, although the difference was not statistically significant (Figure 1B).

In terms of cell origin, no differences were observed in the number of leukocyte- and endothelium-derived MVs between the 2 cohorts of patients (Table 3). Conversely, a 2-fold higher number of platelet-shed MVs was observed in cases (p = 0.020), similarly to that reported at follow-up. Of interest, no correlation was found between platelet blood counts and platelet-derived MV levels (r = 0.10; p = 0.500), suggesting a higher MV release per cell and, therefore, a higher cell activation state. Indeed, the number of $CD62P^+/CD41^+$ MVs, which correlated with that of $CD40L^+/CD41^+$ MVs (r = 0.53; p = 0.0004), was higher in cases (p = 0.042 and p = 0.026, respectively). Moreover, before CABG, cases also had 4 times more TF^+ MVs compared with control subjects (p = 0.05), and those derived from platelets were 3-fold higher (p = 0.003) (Table 3).

All together these results support the evidence that a significantly higher platelet activation state characterizes patients who will experience graft failure at follow-up.

PLASMA PROTEIN PROFILE AND FUNCTIONAL ANNOTATION ANALYSIS. The higher platelet activation state highlighted in cases through MV analysis was further supported by a global plasma protein profile performed on the same samples. Pre-surgery levels of 92 cardiovascular disease-related

biomarkers showed that 20 proteins were associated with graft failure after adjustment for multiple comparisons. In particular, 18 proteins were overexpressed before surgery in plasma of cases (Table 4).

Analysis of the biological pathways in which these proteins are mainly involved (Table 5) indicated that 7 proteins are associated with the hemostatic/thrombotic process, in particular, platelet activation (5 of 20; $p = 0.028$) and degranulation (3 of 20; $p = 0.05$), whereas 5 proteins are involved in the inflammatory response ($p = 0.042$). Processes such as positive regulation of cell migration and proliferation ($p = 0.045$) and cell division ($p = 0.042$) are also involved.

Taken together, these data confirm that processes strictly related to the progression of atherosclerosis, such as platelet activation, inflammation, cell migration, and proliferation, are more activated in patients who will experience bypass graft failure compared with those who will have patent graft at follow-up.

THROMBIN GENERATION CAPACITY OF MVs BEFORE CABG SURGERY. Thrombin plays an important role not only in coagulation, but also in processes such as inflammation and cell proliferation, mechanisms involved in graft failure. Thus, we analyzed the pre-surgery thrombin generation potential of MVs from cases and control subjects.

MVs from cases generated a higher amount of thrombin compared with control subjects (peak: 323.8 ± 102.8 nmol/l vs. 245.0 ± 106.9 nmol/l; $p = 0.04$, respectively) with a faster kinetic rate (velocity index: 134.3 ± 68.2 nmol/l/min vs. 87.1 ± 57.7 nmol/l/min; $p = 0.05$, respectively) (Figure 2). Interestingly, the endogenous thrombin potential (ETP) correlated with the number of procoagulant annexin V^+ (AnV^+)/TF^+ MVs ($r = 0.40$; $p = 0.03$).

Flow cytometric enumeration of procoagulant MVs showed that cases had a double number of AnV^+/TF^+ MVs (Figure 3), which derived mainly from platelets and granulocytes. However, although the number of AnV^+/TF^+ MVs released from granulocytes was not different between cases and control subjects, the number of procoagulant AnV^+/TF^+ MVs released from platelets was significantly higher in cases compared with control subjects ($p = 0.001$) accounting for 72 ± 12% and 48 ± 18% of the total AnV^+/TF^+ MVs, respectively (Figure 3).

These data, underscoring the greater prothrombotic profile of MVs found in patients who will experience graft failure at follow-up, provide insights into the potential mechanisms involved in the loss of patency.

TABLE 3 Levels of Circulating MVs Measured Before CABG Surgery

MV Cell Origin	Cases (MVs/μl)	Control Subjects (MVs/μl)	p Value
$CD41^+$	1,171 (493–3,008)	750 (158–1,788)	0.020
$CD66^+$	591 (372–600)	324 (134–492)	0.290
$CD14^+$	140 (59–343)	120 (41–186)	0.070
$CD31^+/CD41^-$	62 (42–102)	145 (56–233)	0.133
Activated platelet-derived MVs			
$CD62P^+/CD41^+$	263 (167–720)	160 (99–310)	0.042
$CD40L^+/CD41^+$	143 (71–405)	79 (24–103)	0.026
TF^+ MVs			
Total TF^+	422 (171–804)	106 (56–446)	0.050
$TF^+/CD41^+$	48 (27–70)	17 (10–28)	0.003
$TF^+/CD66^+$	187 (114–258)	53 (38–69)	0.192
$TF^+/CD14^+$	33 (18–69)	17 (9–41)	0.038
$TF^+/CD31^+/CD41^-$	13 (12–29)	36 (11–63)	0.236

Values are median (interquartile range).
Abbreviations as in Table 2.

ASSOCIATION OF BASELINE MVs WITH CABG OCCLUSION. Among the 13 MV classes analyzed before CABG, 6 of them—including those derived from activated platelets ($CD40L^+/CD41^+$, $CD62P^+/CD41^+$, $TF^+/CD41^+$) and the procoagulant MVs (TF^+, AnV^+/TF^+, and platelet-derived AnV^+/TF^+ MVs)—significantly discriminated between cases and control subjects in ROC curve analysis, with areas under the curve

TABLE 4 Pre-Surgery Levels of Plasma Proteins Differentially Expressed in Cases and Control Subjects

Protein Symbol	ID Uniprot	Protein Name	Log_2 FC	p Value
CXCL1	P09341	C-X-C motif chemokine 1	0.282	0.0011
HB-EGF	Q99075	Heparin-binding EGF-like growth factor	0.124	0.0015
HSP 27	P04792	Heat shock 27 kDa protein	0.577	0.0033
Dkk-1	O94907	Dickkopf-related protein 1	0.224	0.0033
VEGF-A	P15692	Vascular endothelial growth factor A	0.046	0.0051
PDGF Subunit B	P01127	Platelet-derived growth factor subunit B	0.326	0.0058
CD40	P25942	Tumor necrosis factor receptor superfamily member 5	0.089	0.0062
CASP-8	Q14790	Caspase-8	0.664	0.0062
SIRT2	Q8IXJ6	SIR2-like protein 2	0.593	0.0086
CD40-L	P29965	CD40 ligand	0.285	0.0092
PAR-1	P25116	Proteinase-activated receptor 1	0.095	0.0105
MMP-1	P03956	Matrix metalloproteinase-1	0.627	0.0105
EGF	P01133	Epidermal growth factor	0.406	0.0112
TNFSF14	O43557	Tumor necrosis factor ligand superfamily member 14	0.213	0.0112
NEMO	Q9Y6K9	NF-kappa-B essential modulator	0.357	0.0127
PAPPA	Q13219	Pappalysin-1	-0.271	0.0172
U-PAR	Q03405	Urokinase plasminogen activator surface receptor	0.017	0.0245
AGRP	O00253	Agouti-related protein	0.070	0.0307

Differentially expressed protein are reported. Data are expressed as fold changes (log_2 FC) (see Methods section).

TABLE 5 Functional Annotation Analysis

Biological Process	Adjusted p Value (BH)	ID Uniprot	Protein Name
Platelet activation	0.028	C369965	CD40LG
		P25942	CD40 molecule
		P12931	SRC proto-oncogene, nonreceptor tyrosine kinase
		P25116	F2R
		P01133	EGF
Platelet degranulation	0.05	P16284	PECAM1
		P01127	PDGFB
		P15692	VEGFA
Inflammatory response	0.042	P09341	CXCL1
		P29965	CD40LG
		P25942	CD40 molecule
		P25116	F2R
		Q9Y6K9	IKBKG
Positive regulation of cell migration	0.045	P25116	F2R
		Q99075	HBEGF
		P01127	PDGFB
		P15692	VEGFA
Positive regulation of cell proliferation	0.045	P25116	F2R
		P01133	EGF
		Q99075	HBEGF
		P01127	PDGFB
		P15692	VEGFA
Positive regulation of cell division	0.042	P01127	PDGFB
		Q8IXJ6	SIRT2
		P15692	VEGFA
Negative regulation of apoptotic process	0.044	P29965	CD40LG
		P12931	SRC proto-oncogene, nonreceptor tyrosine kinase
		P04792	HSPB1
		Q03405	PLAUR
		P15692	VEGFA
Immune response	0.04	P09341	CXCL1
		P29965	CD40LG
		P25942	CD40 molecule
		Q9Y6K9	IKBKG
		O43557	TNFSF14

Proteins are grouped according to the biological process in which they are involved.

CD40LG = CD40 ligand; CXCL1 = C-X-C motif chemokine ligand 1; EGF = epidermal growth factor; F2R = coagulation factor II thrombin receptor; HBEGF = heparin-binding EGF-like growth factor; HSPB1 = heat shock protein family B (small) member 1; IKBKG = inhibitor of kappa light polypeptide gene enhancer in B cells, kinase gamma; PDGFB = platelet-derived growth factor subunit B; PECAM1 = platelet and endothelial cell adhesion molecule 1; PLAUR = plasminogen activator, urokinase receptor; SIRT2 = Sirtuin 2; TNFSF14 = tumor necrosis factor superfamily member 14; VEGFA = vascular endothelial growth factor A.

(AUCs) ranging from 0.74 to 0.81 (**Figure 4**). When the discriminant ability of these MVs was evaluated on top of body mass index (the only baseline variable significantly different between the 2 groups) the AUC increase remained significant for all MV subtypes, but not for $CD62P^+/CD41^+$ ($p = 0.085$) (Supplemental Figure 2, Supplemental Table 6).

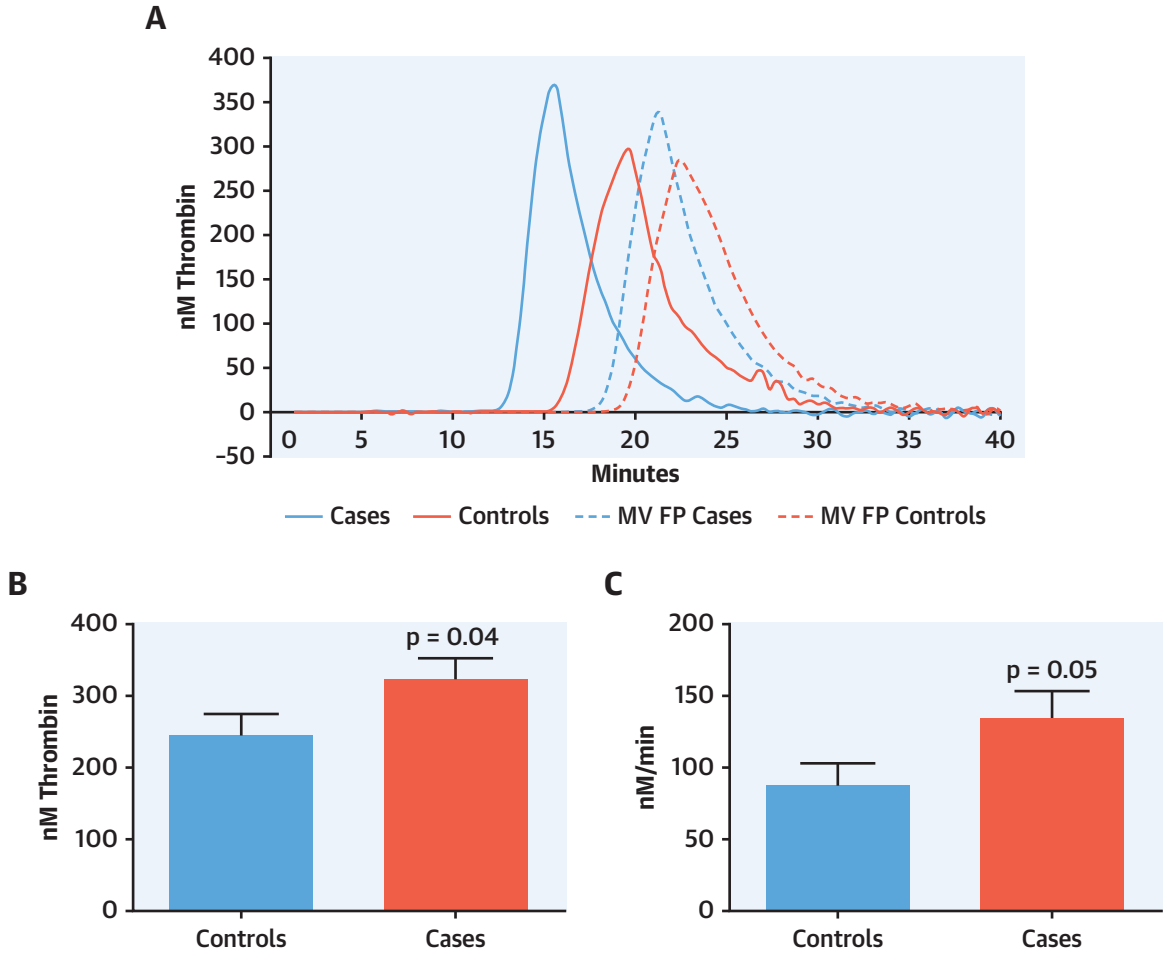
To summarize the overall MV discriminating ability, the 6 MV classes mentioned previously (procoagulant and activated platelet-derived MVs) were combined in the MV score (see Methods section for details). Results showed that all patients with a score <2 would have had a patent graft after 18 months, whereas all patients with a score of 6 would have had an occluded graft (Supplemental Figure 3). The median MV score was indeed 1 (IQR: 0 to 3) and 4.5 (IQR: 4 to 5) in control subjects and cases, respectively ($p < 0.0001$). For a score above the overall median value of 3, the adjusted OR for occlusion was 16.3 (95% confidence interval [CI]: 4.1 to 65.3; $p < 0.0001$). Using the same cutoff, the MV score was able to correctly classify cases and control subjects with a sensitivity of 77.3% and a specificity of 82.8%. Of interest, the AUC for the MV score was higher than the AUC for any single MV class (AUC 0.897; 95% CI: 0.81 to 0.98) (**Figure 5**).

The discriminant capability of MVs and of MV score was compared with that of D-dimer, a recently demonstrated independent predictor of midterm graft occlusion (4). The AUCs of the single MVs were not significantly different from that of D-dimer (all $p > 0.24$). When the discriminant ability of MVs was analyzed on top of D-dimer, the procoagulant MVs increased the AUC (**Table 6**), but did not reach the Bonferroni statistical significance. Of note, AUC for the MV score added on top of D-dimer levels was significantly higher than that of D-dimer alone (+0.18; 95% CI: +0.05 to +0.31; $p = 0.007$) but similar to that of the MV score alone (+0.01; 95% CI: −0.02 to +0.04; $p = 0.38$) (**Figure 5**).

DISCUSSION

The present study provides, for the first time, the evidence that the pre-surgical signature of circulating MVs is independently associated with midterm graft occlusion in patients undergoing CABG (**Central Illustration**). Levels of platelet-derived and procoagulant MVs are significantly greater in patients who will experience graft failure compared with patients with patent grafts, and a cumulative MV score, based on the MV phenotypic characterization, stratifies patients' risk. Because the MV signature mirrors the activated state of circulating platelets, patients with a high pre-surgery MV score could benefit from additional

FIGURE 2 Thrombin Generation Capacity of MVs



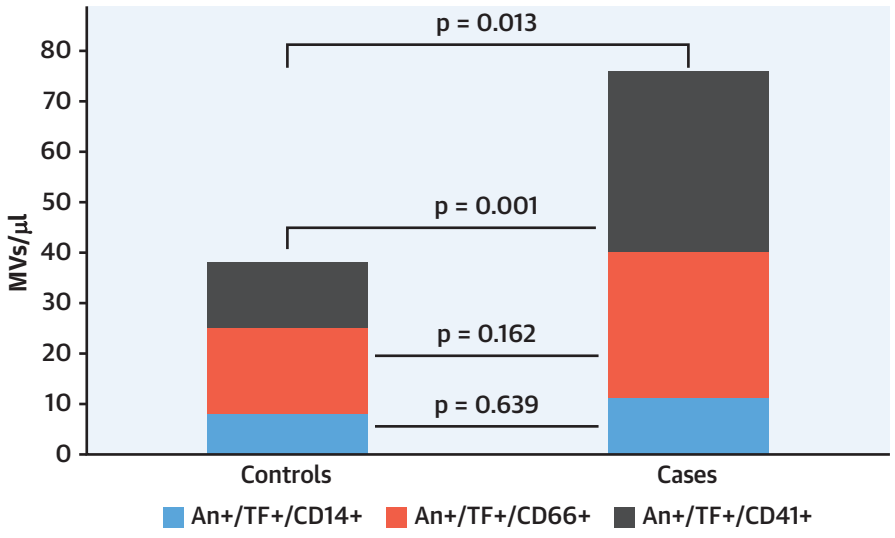
Prothrombotic potential of microvesicles (MVs) from cases and control subjects was analyzed by thrombin generation assay (calibrated automated thrombogram [CAT]). **(A)** Curves obtained in a representative experiment. Curves generated in the absence of MVs (MV free plasma [FP], **dotted lines**) are shown for comparison. **(B)** Peak height (maximum concentration of generated thrombin) and **(C)** velocity index (velocity of thrombin formation) were used as main parameters describing thrombin generation. Data reported in the histograms are expressed as mean ± SD.

therapies aimed at reducing platelet activation and thrombin generation.

Graft occlusion 1 year after surgery affects a consistent number of patients with a 11% rate of saphenous vein graft occlusion (14) despite the best pharmacological treatment as per guidelines (15,16). The identification of patients who are at risk of graft failure continues to be challenging. Several biomarkers correlate with graft occlusion. Among plasma biomarkers, pre-operative levels of C-reactive protein, interleukin-6, F_{1+2}, tissue plasminogen activator, and FVIII predict early graft occlusion (3), whereas D-dimer has a prognostic value in midterm graft failure (4).

The role of biomarkers in outcome prediction of CABG is, however, still controversial due to several limitations in the published studies (3). The "ideal" biomarkers should not only be useful in assessing the risk of negative outcome, but should also prompt the adoption of (pharmacological) strategies to limit graft failure. To this aim, however, biomarkers should provide the link between their changes and the

FIGURE 3 Analysis of Procoagulant AnV$^+$/TF$^+$ MVs



Number of AnV$^+$/tissue factor (TF)$^+$ microvesicles (MVs) was measured in control subjects and cases before coronary artery bypass graft (CABG). The amount of procoagulant MVs derived from monocytes (CD14$^+$, **blue**), granulocytes (CD66$^+$, **red**), and platelets (CD41$^+$, **black**) is reported.

underlying pathophysiological mechanisms. Determination of the previously mentioned plasmatic biomarkers does not identify the molecular mechanisms and/or the dysfunctional cell population involved. Conversely, MV signature provides a direct link between the MV's phenotype and the parental cell/cells.

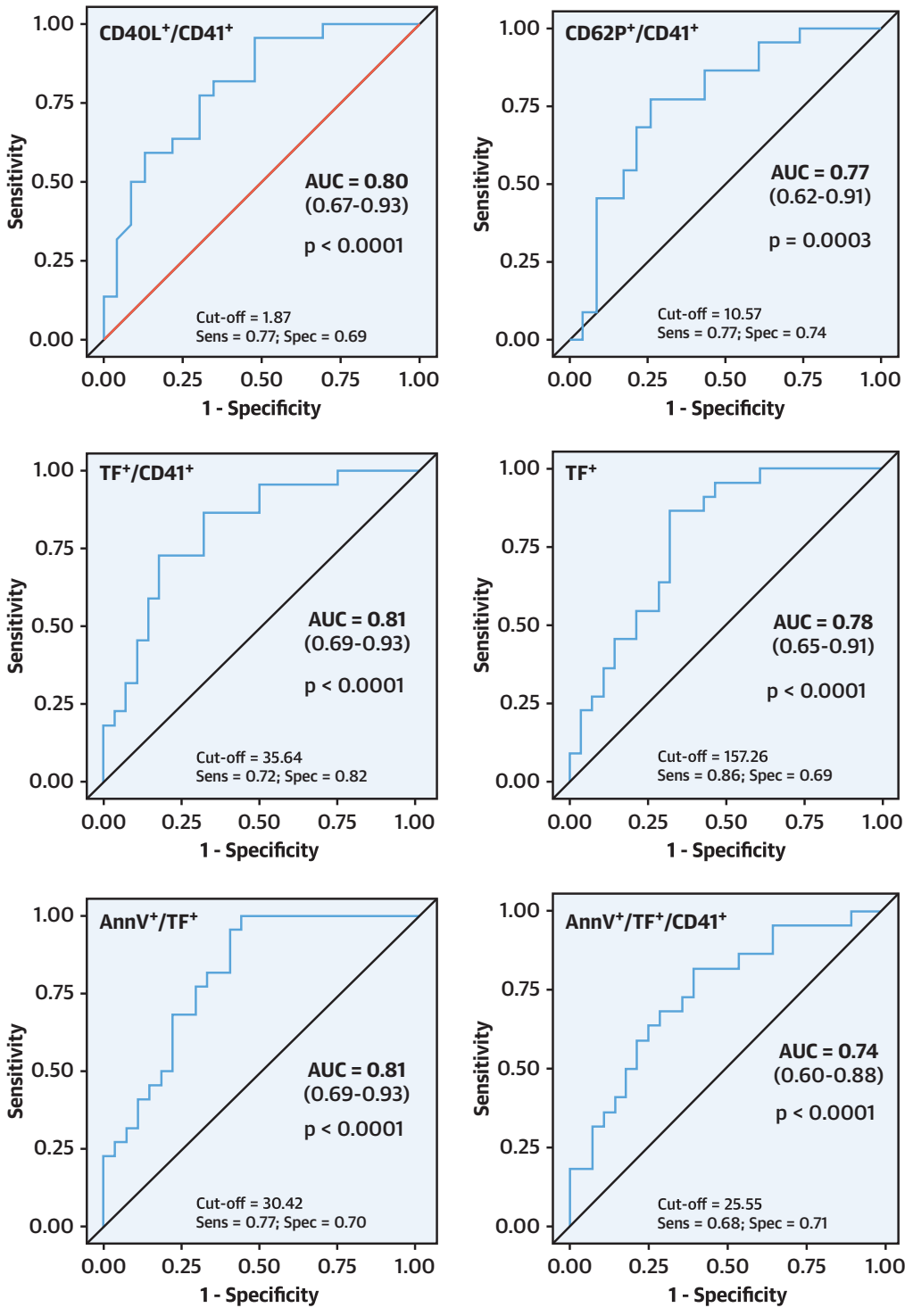
Circulating MVs shed from cells of the vascular compartment indeed reflect the presence of activated cells in vivo, as documented in several pathophysiological conditions, including cardiovascular disease (17). Mallat et al. (18) in 2000 showed that patients with acute coronary syndrome have a greater number of procoagulant MVs compared with patients with stable angina or other non-CAD control subjects (18). They also first proposed that levels of circulating MVs could be a prognostic marker of the recurrence of ischemic events.

Over the years, other studies confirmed that the higher MV levels found in patients with CAD correlated with the severity of disease (6,19). Interestingly, it has been reported that in patients with myocardial infarction, the amount of procoagulant and platelet-derived MVs were significantly reduced after successful revascularization with percutaneous coronary intervention (PCI) (20). We observed the same trend in our study. Patients with patent grafts 18 months after CABG had a significantly lower number of circulating MVs compared with patients with occluded grafts, in whom MVs were as high as the pre-surgery levels. Interestingly, before CABG, the number of MVs was similar in cases and control subjects, indicating that the total count of MVs per se does not have a prognostic value.

Only the detailed phenotypic pre-surgery signature provided the information needed to discriminate between cases and control subjects and to stratify the patient's risk at follow-up. Patients with graft failure have twice the number of activated platelet-derived MVs (CD40L$^+$/CD41$^+$, CD62P$^+$/CD41$^+$, TF$^+$/CD41$^+$MVs) and 4-fold more procoagulant MVs (TF$^+$, AnV$^+$/TF$^+$, and platelet-derived AnV$^+$/TF$^+$ MVs) compared with patients with patent graft.

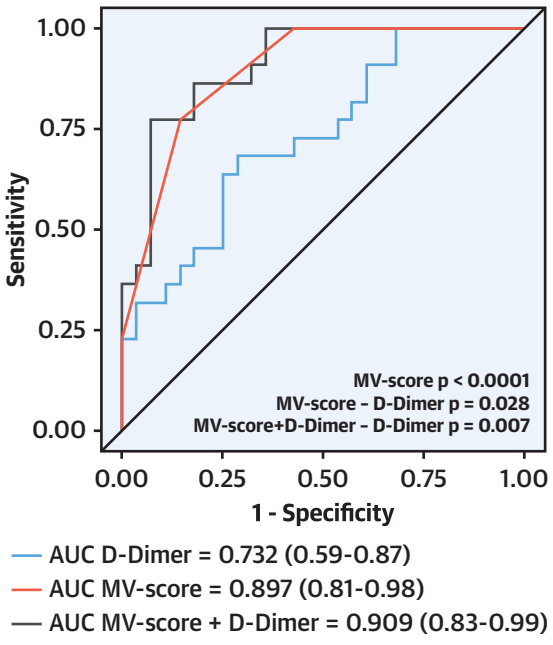
No differences were observed in the number of leukocyte- and endothelium-derived MVs between the 2 cohorts of patients. Thus, the MV signature found in cases clearly reflected a platelet activation status that was also confirmed by a global plasma protein profile. In cases, we found a pre-surgery up-regulation of 18 proteins involved not only in platelet activation, but also in inflammatory response and in

FIGURE 4 ROC Curve Analysis of MVs Versus Future Graft Occlusion



Receiver-operating characteristic (ROC) curve analysis was used to evaluate the potential prognostic value of the baseline number of activated platelet-derived MVs ($CD40L^+/CD41^+$, $CD62P^+/CD41^+$, and $TF^+/CD41^+$ MVs) and procoagulant MVs (TF^+, AnV^+/TF^+, and $CD41^+/TF^+/AnV^+$ MVs) for CABG occlusion. Area under the curve (AUCs), p value for AUC differences, cut-off value, sensitivity, and specificity are reported. Abbreviations as in Figure 3.

FIGURE 5 ROC Curve Analysis of MV Score Versus Future Graft Occlusion



An MV score was elaborated to summarize the overall association of the 6 MV classes ($CD40L^+/CD41^+$, $CD62P^+/CD41^+$, $TF^+/CD41^+$, TF^+, AnV^+/TF^+, and $AnV^+/CD41^+/TF^+$ MVs) with future graft occlusion (see Methods section for details). Results of ROC curve analysis, comparing the AUC of the MV score with that of D-dimer levels, are shown. AUC and p value for AUC differences are reported. Abbreviations as in Figures 3 and 4.

cell proliferation and migration. These processes are well known to be sustained by activated platelets in the setting of atherothrombosis (21) and graft occlusion (22,23). Several studies have also shown that platelet-derived MVs, thanks to their capacity to transfer proteins, lipids, and nucleic acids to recipient cells, can actively participate in the same processes (24). In addition, they can promote thrombin formation that, besides its central role in hemostasis and thrombosis, is involved in cell proliferation, angiogenesis, and inflammation (25) and is implicated in post-angioplasty restenosis through induction of VSMC proliferation (22).

Thus, to analyze one of the potential functional roles of MVs in graft occlusion, we assessed the capacity of MVs from cases and control subjects to generate thrombin. Patients with occluded grafts showed a significantly higher pre-surgical thrombin generation capacity, which correlated with the amount of procoagulant Ann^+/TF^+ MVs, compared with that of control subjects.

These findings bolster the increased levels of D-dimer, marker of activation of coagulation, that we previously reported in cases (4) and highlight that a significantly higher prothrombotic status characterizes patients who will experience graft failure. This has been attributed to acute thrombosis within the first month, intimal hyperplasia up to 1 year, and atherosclerosis beyond 1 year (23). Although the coronary CTA scan used to assess graft patency at follow-up could not provide information whether a thrombotic or stenotic process was responsible for the graft occlusion, the overall data of the present study are certainly indicative of a pathophysiological status that could sustain both processes.

Remarkably, D-dimer pre-surgery levels were significantly associated with loss of graft patency discriminating cases and control subjects with a specificity and sensitivity comparable to that of each of the 6 MV classes. However, a significantly higher potential predictive ability was observed when MV classes were considered together in a cumulative MV score that provided the best prognostic value with an area under the ROC curve of 0.897 ($p < 0.0001$). Moreover, the MV score (ranging from 0 to 6 based on the classes of MVs present in the plasma samples) allowed patients' risk stratification showing a 16-fold higher risk of graft occlusion for a score higher than 3.

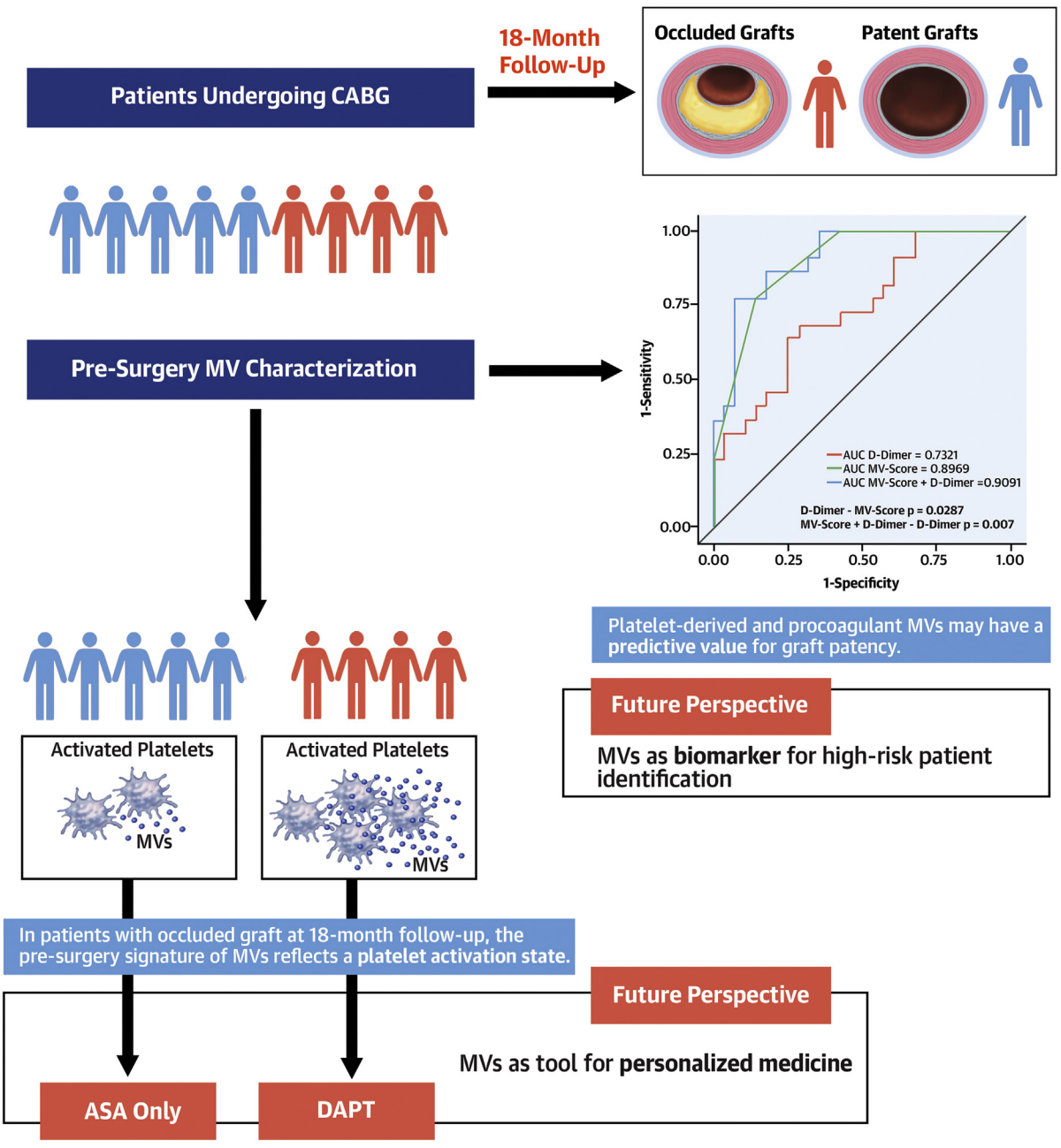
Interestingly, Suades et al. (26) recently reported the additive value of the multipanel approach, as the one used in this study, in ST-segment elevation myocardial infarction prediction. The combination of distinct MV subsets was indeed significantly superior in predicting ST-segment elevation myocardial infarction than 1 type of MV alone (26). Our

TABLE 6 Comparison of the Discriminant Ability of MVs With That of D-Dimer

MVs		AUC (95% CI)	p Value vs. D-Dimer
D-dimer		0.72 (0.58–0.86)	
$CD40L^+/CD41^+$		0.80 (0.67–0.93)	0.548
$CD40L^+/CD41^+$	on top of D-dimer	0.83 (0.71–0.96)	0.129
$CD62P^+/CD41^+$		0.77 (0.62–0.91)	0.948
$CD62P^+/CD41^+$	on top of D-dimer	0.82 (0.69–0.95)	0.237
$TF^+/CD41^+$		0.81 (0.69–0.93)	0.333
$TF^+/CD41^+$	on top of D-dimer	0.86 (0.76–0.97)	**0.027**
TF^+		0.78 (0.65–0.91)	0.443
TF^+	on top of D-dimer	0.85 (0.75–0.96)	**0.039**
AnV^+/TF^+		0.81 (0.69–0.93)	0.241
AnV^+/TF^+	on top of D-dimer	0.86 (0.77–0.96)	**0.015**
$AnV^+/CD41^+/TF^+$		0.74 (0.60–0.88)	0.795
$AnV^+/CD41^+/TF^+$	on top of D-dimer	0.85 (0.75–0.95)	**0.026**

Bold p values are statistically significant. AUC = area under the curve; CI = confidence interval; other abbreviations as in Table 2.

CENTRAL ILLUSTRATION **Capacity of Pre-Coronary Artery Bypass Graft Microvesicle Signature to Detect Midterm Graft Occlusion**



Camera, M. et al. J Am Coll Cardiol. 2020;75(22):2819–32.

Microvesicle (MV) signature was assessed in pre-surgery plasma samples of patients undergoing coronary artery bypass grafting (CABG). In patients with graft occlusion 18 months after surgery (in **red**), the MV signature highlighted a higher platelet activation state compared with patients with patent grafts in the follow-up (in **blue**). Interestingly, the pre-surgery MV signature was independently associated with midterm graft occlusion, thus suggesting that circulating MVs might represent a useful biomarker to implement stratification of high-risk patients. The identification of platelet activation status, documented before surgery, may help the clinician to tailor a personalized antiplatelet treatment. This future perspective has to be addressed in an ad hoc designed clinical trial. ASA = aspirin; AUC = area under the curve; DAPT = dual antiplatelet therapy.

study, providing for the first time data in patients undergoing CABG, adds new evidence that MVs are independent markers of increased risk of cardiovascular events in high-risk patients (11,26). Indeed, cases showed a higher occurrence of major adverse cardiovascular or cerebrovascular events at 52-month follow-up. Moreover, the MV signature has a 2-fold clinical utility in this setting: by both providing information in risk assessment and highlighting platelet activation as the underlying pathophysiological mechanism, it could help in the evaluation of efficacy of different strategies to limit graft failure, such as a more intensive antiplatelet therapy.

Guidelines recommend aspirin monotherapy after CABG to maintain graft patency and prevent atherothrombotic complications (15,16). Dual antiplatelet therapy (DAPT) consisting of aspirin and a $P2Y_{12}$ receptor inhibitor in the setting of CABG is a controversial issue. It is recommended only in patients undergoing CABG after acute coronary syndrome, whereas there is currently no evidence of a survival benefit or a reduction of thromboembolic complications with DAPT in patients with stable CAD undergoing CABG (15).

The pharmacological approach used after the 2 main myocardial revascularization procedures, CABG and PCI, deserves some consideration. Although they both elicit a sustained platelet activation that deeply affects the outcome, DAPT represents the cornerstone of treatment in patients undergoing elective PCI, whereas its effect in the setting of CABG has not been definitely confirmed yet (15). Several reasons may account for the inconsistency in previous trials' data, including small sample sizes, heterogeneous populations, and post-hoc analysis with low statistical power. The most recent data, however, suggest that DAPT may have a role in preventing graft occlusion. Results of meta-analyses, carried out to compare graft patency in patients treated with aspirin alone or aspirin + clopidogrel after CABG, suggest that DAPT was associated with a significant reduction in saphenous vein graft occlusions (27–29). This finding has been recently confirmed by Zhao et al. (30) in a multicenter open-label clinical trial on 500 elective CABG patients carried out to compare the effect of ticagrelor + aspirin versus monotherapy with either aspirin or ticagrelor. The results showed that DAPT was superior in maintaining saphenous vein graft patency for up to 1 year in both patients with ACS and stable CAD (30).

In the era of precision medicine and of a continuous effort to identify patients with the greatest cardiovascular risk, availability of biomarkers with high predictive value is of paramount importance (31). If the results of the present study will be confirmed in a larger population, it is tempting to speculate that the MV signature could help physician to tailor the antiplatelet therapy accordingly.

STUDY LIMITATIONS. Our findings should be interpreted in the context of their limitations. First, being based on a relatively small sample size and a single dataset, our results should be considered hypothesis-generating: undoubtedly, is most likely that, in the absence of a validation dataset, our results may have a considerable bias, with a substantial overestimation of true AUCs. Second, the thrombin generation capacity of MVs has been assessed on the whole plasma and not on isolated MVs. Because cells release not only MVs but also exosomes (32), we cannot exclude that these vesicles also participate in the process. Finally, assessment of graft patency by coronary CTA scan has been performed only at 18-month follow-up; thus, we cannot rule out the possibility that graft failure occurred even before that time.

CONCLUSIONS

We report that CABG-treated patients who will experience mid-term graft occlusion are characterized by a pre-surgery MV signature indicative of a platelet activation status and supporting a greater thrombin generation capacity compared to that of patients with patent grafts. Thrombin, in addition to its role in coagulation, can sustain inflammatory and cell proliferation processes that lead to graft failure. The pre-operative signature of MVs is an independent predictor of midterm graft occlusion in CABG patients and a cumulative MV score stratifies patients' risk. Because the MV signature mirrors an ongoing platelet activation, patients with a high MV score would benefit from a personalized antiplatelet therapy.

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PERSPECTIVES

COMPETENCY IN PATIENT CARE AND PROCEDURAL SKILLS: In patients undergoing CABG surgery, pre-operative circulating microvesicles are associated with platelet activation and risk of subsequent graft occlusion.

TRANSLATIONAL OUTLOOK: Future studies should address whether risk stratification based on microvesicle characterization can be employed to guide the intensity of antiplatelet therapy and improve long-term clinical outcomes in patients undergoing CABG surgery.

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KEY WORDS circulating microvesicles, coronary artery bypass graft, graft occlusion, platelets, thrombin generation

APPENDIX For an expanded Methods section as well as supplemental tables and figures, please see the online version of this paper.