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LEADING EDGE TRANSLATIONAL RESEARCH

Homophilic Interaction Between Transmembrane-JAM-A and Soluble JAM-A Regulates Thrombo-Inflammation

Implications for Coronary Artery Disease

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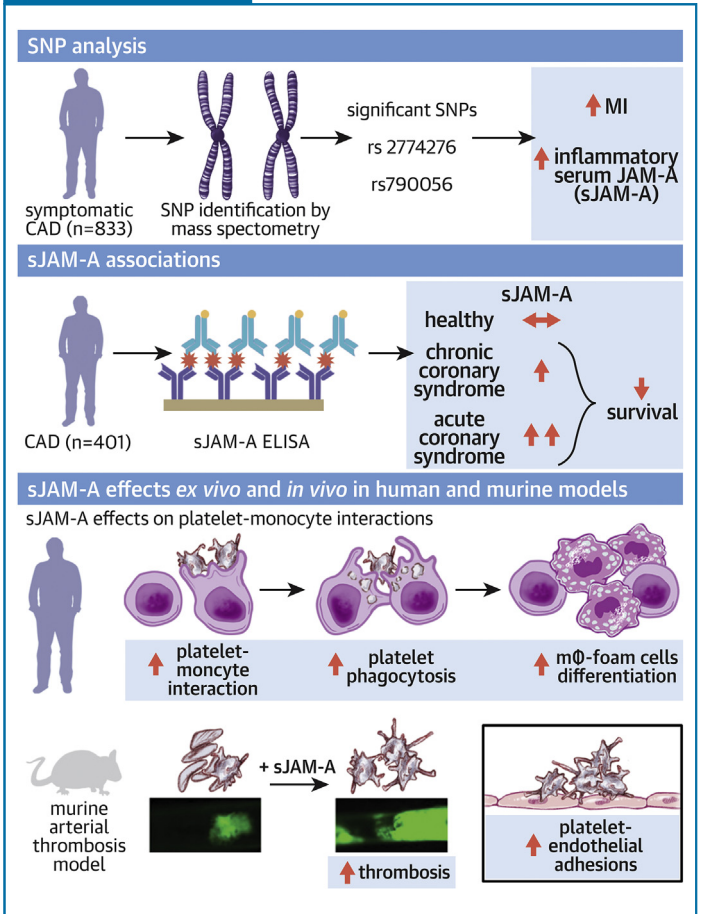
VISUAL ABSTRACT



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HIGHLIGHTS

- **Genetic predisposition through *F11R*-SNVs determine circulatory sJAM-A levels in CAD patients. Homozygous carriers of the minor alleles-(rs2774276, rs790056) show enhanced levels of sJAM-A and worse event-free survival for MI in a 3-year follow-up time period.**
- **Activated platelets shed transmembrane-JAM-A generating sJAM-A. Serum sJAM-A levels are elevated in ACS patients, correlate with peak troponin I, and influence probability of recurrent MI.**
- **Platelet transmembrane-JAM-A and sJAM-A as unique homophilic interaction partners exaggerate thrombotic functions.**
- **sJAM-A-activated apoptotic platelets form aggregates with monocytes and are phagocytosed, which fosters monocyte differentiation into macrophages-(mΦ) and foam cells prompting thrombo-inflammation.**
- **Therapeutic strategies interfering with this homophilic interface between transmembrane-JAM-A and sJAM-A may regulate thrombotic and thrombo-inflammatory platelet response in cardiovascular pathologies where circulatory sJAM-A levels are elevated.**

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ABBREVIATIONS AND ACRONYMS

ACS = acute coronary syndrome

ACM = all-cause mortality

ADP = adenosine diphosphate

CAD = coronary artery disease

CCS = chronic coronary syndrome

CE = combined endpoint

HC = homozygous carriers

IS = ischemic stroke

JAM-A = junctional adhesion molecule-A

MI = myocardial infarction

sJAM-A = soluble junctional adhesion molecule-A

smJAM-A = soluble murine junctional adhesion molecule-A

SNV = single-nucleotide variation

TRAP = thrombin receptor activating peptide

SUMMARY

Genetic predisposition through *F11R*-single-nucleotide variation (SNV) influences circulatory soluble junctional adhesion molecule-A (sJAM-A) levels in coronary artery disease (CAD) patients. Homozygous carriers of the minor alleles (*F11R*-SNVs rs2774276, rs790056) show enhanced levels of thrombo-inflammatory sJAM-A. Both *F11R*-SNVs and sJAM-A are associated with worse prognosis for recurrent myocardial infarction in CAD patients. Platelet surface-associated JAM-A correlate with platelet activation markers in CAD patients. Activated platelets shed transmembrane-JAM-A, generating proinflammatory sJAM-A and JAM-A-bearing microparticles. Platelet transmembrane-JAM-A and sJAM-A as homophilic interaction partners exaggerate thrombotic and thrombo-inflammatory platelet monocyte interactions. Therapeutic strategies interfering with this homophilic interface may regulate thrombotic and thrombo-inflammatory platelet response in cardiovascular pathologies where circulatory sJAM-A levels are elevated.

Inflammatory factors are chronic mediators and therefore therapeutic targets in atherosclerotic cardiovascular complications. Junctional adhesion molecule-A (JAM-A) belonging to the immunoglobulin superfamily exists as a transmembrane adhesion receptor or soluble form (soluble junctional adhesion molecule-A [sJAM-A]), when shed from endothelium, hematopoietic progenitors, and circulatory cells[1] by metalloproteinases ADAM10 and ADAM-17. JAM-A is a proinflammatory mediator in atheroprogression.[2,3] Cytokines up-regulate JAM-A expression in human aortic and venous endothelial cells.[4] Consequently, transmembrane-JAM-A mediates proinflammatory interactions among activated endothelium, monocytes,[5] and platelets[4] through trans-homophilic and heterophilic associations, which facilitates their recruitment to the inflamed endothelium.[1] Enhanced *F11R* transcript and JAM-A protein expression are therefore detected at atherosclerotic plaques2 from cardiovascular disease patients and *Apoe*$^{-/-}$ mice.[1,3] Therefore, therapeutic strategies antagonizing JAM-A are implicated in preventing atherothrombosis.[2,6]

Circulatory sJAM-A levels may depend on the expression status of transmembrane-JAM-A in cellular sources, influenced by single-nucleotide variation (SNV) at the *F11R* locus,[7] and the extent of its release.[1,3,8-10] A meta-analysis of >5,000 peripheral blood samples has shown that minor allele carriers of intronic *F11R*-SNV-rs2774276 and 5′ upstream SNV-rs790056 show significantly enhanced *F11R* gene expression in peripheral blood samples (cis-eQTLs),[11] as well as several other tissues including aorta and the tibial artery.[12] Both SNVs have been associated with low systolic blood pressure, whereas *F11R*-SNVs rs2481084 and rs3737787 have been associated with lower odds of central obesity. Enhanced circulatory sJAM-A levels are observed in end-stage renal failure[10] and hypertension,[8,9] and independently associated with the presence and severity of angiographically defined CAD.[3] However, a link between distinct *F11R*-SNVs and circulatory sJAM-A among CAD patients remained undefined.

F11R was cloned from platelets.[13] Transmembrane-JAM-A regulates platelet activation[14] and signaling through $\alpha_{IIb}\beta_3$, by complex formation with β_3-integrin.[15] Genetic deficiency of *F11r* potentiates

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The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the Author Center.

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TABLE 1 Baseline Characteristics of CAD Patients Stratified According to *F11R*-SNV-rs2774276 and to *F11R*-SNV-rs790056 Major Allele Carriers vs HCs of Minor Allele (Recessive Genetic Model)

	Carriers of Major Allele	HC of Minor Allele	*P* Value
F11R-SNV-rs2774276			
n	777	56	
Age, y	68.4 ± 11.6	70.0 ± 10.0	0.309
Male	549 (70.7)	41 (73.2)	0.716
LVEF, %	51.0 ± 10.7	52.0 ± 10.5	0.528
Risk factors			
Arterial hypertension	649 (83.5)	45 (80.4)	0.926
Hyperlipidemia	454 (58.4)	30 (53.6)	0.621
Diabetes mellitus type II	257 (33.1)	16 (28.6)	0.593
Smoking	319 (41.1)	20 (35.7)	0.525
Medication on admission			
ASA	430 (55.3)	32 (57.1)	0.850
Clopidogrel	94 (12.1)	9 (16.1)	0.400
Prasugrel	15 (1.9)	2 (3.6)	0.418
Ticagrelor	35 (4.5)	2 (3.6)	0.732
ACE inhibitors	332 (42.7)	24 (42.9)	0.983
AT-1 antagonists	139 (17.9)	12 (21.4)	0.522
Calcium-channel blockers	154 (19.8)	14 (25.0)	0.364
Beta-blockers	442 (56.9)	31 (55.4)	0.782
Statins	367 (47.2)	30 (53.6)	0.383
Reason of admission, CCS vs ACS			
ACS	383 (49.3)	28 (50.0)	0.918
Type of coronary intervention			
PCI	658 (84.7)	50 (89.3)	0.352
CABG	8 (1.0)	0 (0.0)	0.445
None	111 (14.3)	6 (10.7)	0.364
Medication at discharge			
ASA	733 (94.3)	52 (92.9)	0.323
Clopidogrel	406 (52.3)	30 (53.6)	0.934
Prasugrel	120 (15.4)	10 (17.9)	0.664
Ticagrelor	145 (18.7)	12 (21.4)	0.646
Simvastatin	591 (76.1)	46 (82.1)	0.388
Atorvastatin	38 (4.8)	2 (3.6)	0.641
Rosuvastatin	24 (3.1)	1 (1.8)	0.571
Pravastatin	2 (0.3)	1 (1.8)	0.068
Fluvastatin	27 (3.5)	2 (3.6)	0.985
Lovastatin	1 (0.1)	0 (0.0)	0.787
F11R-SNV- rs790056			
n	791	40	
Age, y	68.4 ± 11.6	70.4 ± 10.2	0.294
Male	562 (71.0)	28 (70.0)	0.857
LVEF, %	50.9 ± 10.7	53.9 ± 10.0	0.092
Risk factors			
Arterial hypertension	656 (82.9)	35 (87.5)	0.102
Hyperlipidemia	458 (57.9)	24 (60.0)	0.514
Diabetes mellitus type II	260 (32.9)	12 (30.0)	0.888
Smoking	324 (41.0)	14 (35.0)	0.619
Medication on admission			
ASA	436 (55.1)	24 (60.0)	0.586
Clopidogrel	96 (12.1)	7 (17.5)	0.329
Prasugrel	17 (2.1)	0 (0.0)	0.346
Ticagrelor	35 (4.4)	2 (5.0)	0.875
ACE inhibitors	338 (42.7)	17 (42.5)	0.950
AT-1 antagonists	141 (17.8)	10 (25.0)	0.260
Calcium-channel blockers	159 (20.1)	9 (22.5)	0.728
Beta-blockers	450 (56.9)	22 (55.0)	0.779
Statins	373 (47.2)	23 (57.5)	0.215
Reason of admission, CCS vs ACS			
ACS	392 (49.6)	17 (42.5)	0.384
Type of coronary intervention			
PCI	671 (84.8)	35 (87.5)	0.645
CABG	8 (1.0)	0 (0.0)	0.523
None	112 (14.2)	5 (12.5)	0.768
Medication at discharge			
ASA	746 (94.3)	37 (92.5)	0.351
Clopidogrel	413 (52.2)	23 (57.5)	0.574
Prasugrel	124 (15.7)	6 (15.0)	0.880
Ticagrelor	147 (18.6)	8 (20.0)	0.856
Simvastatin	603 (76.2)	32 (72.5)	0.691
Atorvastatin	37 (4.7)	3 (7.5)	0.430
Rosuvastatin	24 (3.0)	1 (2.5)	0.836
Pravastatin	3 (0.4)	0 (0.0)	0.694
Fluvastatin	28 (3.5)	1 (2.5)	0.716
Lovastatin	1 (0.1)	0 (0.0)	0.821

Values are mean ± SD or n (%).

ACE = angiotensin-converting enzyme; ASA = aspirin; ACS = acute coronary syndrome; CAD = coronary artery disease; CCS = chronic coronary syndrome; CE = combined endpoint; HC = homozygous carriers; JAM-A = junctional adhesion molecule-A; SNV = single nucleotide variation

Continued in the next column

thrombotic response in *Jam-A*$^{gt/gt}$ mice.[15-17] Since circulating platelets in CAD patients may shed receptors in their hyperactive state, they might act as a potential novel source of sJAM-A like leukocytes, and the inflamed endothelium, which needed verification. Moreover, as circulatory sJAM-A levels are elevated in hypertensive[8] and CAD patients,[3] its impact on thrombo-inflammatory platelet functions warranted validation. Current investigation uncovered *F11R*-SNVs' influence on circulatory sJAM-A levels in CAD patients and their potential association with long-term cardiovascular prognosis. Moreover, we revealed the novel thrombo inflammatory potential of sJAM-A mediated through homophilic interaction with platelet transmembrane-JAM-A, adding an unexpected dimension to the previously recognized regulatory influence of transmembrane-JAM-A on platelet outside-in-signaling[16,17] and its inflammatory attributes in early stages of atheroprogression.[18,19] The current paper validates the contribution of transmembrane-JAM-A and sJAM-A as a functional duo in influencing thrombotic and thrombo-inflammatory platelet

TABLE 2 Baseline Characteristics of CAD Patients Stratified According to sJAM-A ≤1.71 ng/mL and >1.71 ng/mL

	sJAM-A ≤ Median 1.71 ng/mL (n = 201)	sJAM-A > Median 1.71 ng/mL (n = 200)	*P* Value
Age, y	69.8 ± 11.0	69.0 ± 10.9	0.552
Male	141 (70.1)	148 (74.0)	0.380
LVEF, %	52.3 ± 11.0	49.7 ± 12.4	**0.041**
Risk factors			
Arterial hypertension	164 (81.6)	163 (81.5)	0.826
Hyperlipidemia	115 (57.2)	94 (47.0)	**0.024**
Diabetes mellitus type II	59 (29.4)	65 (32.5)	0.519
Smoking	70 (34.8)	69 (34.5)	0.916
Medication on admission			
ASA	107 (53.2)	102 (51.0)	0.642
Clopidogrel	25 (12.4)	22 (11.0)	0.655
Prasugrel	3 (1.5)	5 (2.5)	0.471
Ticagrelor	9 (4.5)	3 (1.5)	0.096
ACE inhibitors	80 (39.8)	90 (45.0)	0.306
AT-1 antagonists	43 (21.4)	36 (18.0)	0.399
Calcium-channel blockers	40 (19.9)	45 (22.5)	0.517
Beta-blockers	118 (58.7)	112 (56.0)	0.596
Statins	105 (52.2)	91 (45.5)	0.177
Reason of admission, CCS vs ACS			
ACS	98 (48.8)	119 (59.5)	**0.034**
Type of coronary intervention			
PCI	169 (84.1)	174 (87.0)	0.412
CABG	1 (0.5)	1 (0.5)	0.997
None	31 (15.4)	25 (12.5)	0.402
Medication at discharge			
ASA	193 (96.0)	181 (90.5)	0.062
Clopidogrel	104 (51.7)	97 (48.5)	0.575
Prasugrel	26 (12.9)	38 (19.0)	0.096
Ticagrelor	45 (22.4)	40 (20.0)	0.579
Simvastatin	132 (65.7)	130 (65.0)	0.998
Atorvastatin	33 (16.4)	33 (16.5)	0.947
Rosuvastatin	6 (3.0)	6 (3.0)	0.979
Pravastatin	3 (1.5)	2 (1.0)	0.664
Fluvastatin	7 (3.5)	5 (2.5)	0.578
Lovastatin	0 (0.0)	0 (0.0)	–

Values are mean ± SD or n (%). **Bold** values indicate statistical significance.
Abbreviations as in Table 1.

functions, which had not been explored before but is of vital significance in the pathophysiology of CAD.

METHODS

Methodological details for experimental studies are provided in the Supplemental Appendix.

CAD PATIENT COHORT. *F11R*-SNV (rs2774276, rs2481084, rs3737787, rs790056) analysis was performed in consecutive symptomatic CAD[20] patients (n = 833). Chronic coronary syndrome (CCS) and acute coronary syndrome (ACS) were defined as explained previously.[20] All patients were admitted to the Department of Cardiology and Angiology, University Hospital Tübingen, and gave written informed consent. The study was approved by the institutional ethics committee (270/2011BO1, 237/2018BO2), and complied with the Declaration of Helsinki and good clinical practice guidelines. Baseline patient characteristics are given in Table 1.

GENOTYPING OF F11R-SNV VARIANTS IN CAD PATIENTS. Genotyping was performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using the MassARRAY Compact system (Sequenom)[20] Genotype data for all SNVs were in Hardy-Weinberg equilibrium. Study personnel assessing outcome was blinded to the clinical manifestations of patients. Of note, genotyping quality for rs790056 and rs3737787 was too low in 2 and 3 patients, respectively. Hence, rs790056 and rs3737787 were only analyzed in 831 and 830 patients, respectively.

SERUM sJAM-A LEVELS IN CAD PATIENTS. sJAM-A levels were subsequently determined by enzyme-linked immunosorbent assay (ELISA) in 401 CAD patients (Table 2). Among these, 159 (39.7%) were specifically matched patients from the *F11R*-SNV cohort. Because homozygous carriers of the minor alleles (rs2774276, rs790056) showed a significant impact on prognosis, this matched subset was determined by first choosing 51 and 35 of the homozygote minor allele carriers of *F11R*-SNV-rs2774276 and *F11R*-SNV-rs790056, respectively. Corresponding heterozygous as well as homozygous major allele carriers were then selected using library optmatch 0.9-13[21] of statistical software R version 3.5.0 (R Foundation for Statistical Computing)[22] and a 1:1-matching for ACS/CCS and left ventricular ejection fraction class (normal, mild/moderate/severe impairment).

ELISA AND CYTOMETRIC BEAD ARRAY FOR INFLAMMATORY MEDIATORS. Serum samples were used to estimate soluble L-selectin and P-selectin in matched CAD patients (with *F11R-SNVs*) with ELISA (Duo set Kits, R&D System). Similarly, a human proinflammatory chemokine panel (Legendplex, Biolegend) was used to estimate serum levels of inflammatory mediators by cytometric bead array.

SURVIVAL ENDPOINTS AND PROGNOSTIC ASSOCIATION. All patients were followed up for all-cause mortality (ACM), myocardial infarction (MI), and ischemic stroke (IS) for 1,080 days after study enrollment. A combined endpoint (CE) was defined as a composite of ACM and/or MI and/or IS. Further endpoints were defined as single events of ACM and MI. Acute MI and IS were defined as described previously.[20] Follow-up was performed by telephonic interview and/or review

TABLE 3 Event Rates and Incidence Rates/100 Person-Years in the Overall Cohort

Endpoint	Number of Events (Carriers of Major Allele/HC of Minor Allele)	IR/100 Person-y	*P* Value
F11R-SNV-rs2774276 (n = 703/55)			
Combined endpoint	175/16	8.3/9.7	0.49
Myocardial infarction	81/14	3.8/8.5	**0.003**
All-cause mortality	97/6	4.6/3.6	0.547
F11R-SNV-rs790056 (n = 717/39)			
Combined endpoint	179/12	8.3/10.3	0.417
Myocardial infarction	85/10	4.0/8.5	**0.011**
All-cause mortality	97/6	4.5/5.1	0.742

Endpoint	Number of events sJAM-A (≤1.71 ng/mL n = 195/ >1.71 ng/mL n = 196)	IR/100 Person-y	*P* Value
Combined endpoint	33/53	5.6/9.1	**0.012**
Myocardial infarction	13/35	2.2/6.0	**<0.001**
All-cause mortality	14/24	2.4/4.1	0.071

Bold values indicate statistical significance.
IR = incidence rate; other abbreviations as in Table 1.

of patients' charts on readmission by investigators blinded to laboratory results. In the *F11R*-SNV cohort, 75 of 833 (9%) patients were lost to follow-up. In the sJAM-A cohort, 10 of 401 (2.5%) patients were lost to follow-up.

STATISTICAL ANALYSIS. Experimental data were analyzed using GraphPad Prism software with analysis of variance for ≥3 groups and Student's *t*-test for 2 groups. Data represent mean ± SEM.

Clinical cohort. Statistical analyses were performed using SPSS version 27.0 (IBM), GraphPad Prism software, and libraries clusrank_1.0-2, cmprsk_2.2-11, crrSC_1.1, htestClust_0.2.0, and rms_6.1-1 of R statistical software. Data are presented as median with 25th and 75th percentiles (Q1, Q3), mean ± SD, or count and percentage. Chi-square tests, Student's *t*-test, Mann-Whitney *U* tests, clustered Wilcoxon rank sum test using the Rosner-Glynn-Lee method,[23] and logistic regression along with the Huber-White method[24] were applied as appropriate to analyze baseline characteristics. The latter 2 methods were used to correct for the correlation structure in case of matched patients (ie, when analyzing the 401 patients with sJAM-A levels). Correlations of non-normally distributed data were assessed by the usual Spearman's rank correlation coefficient or an analogue for matched data,[25] as appropriate. Clustered Wilcoxon rank sum test was also used to investigate the association between sJAM-A levels and *F11R*-SNV variants in the subset of matched patients. Cox proportional hazard (PH) regression and the Fine-Gray method[26] for competing risks as a secondary method were applied to investigate associations between survival endpoints and *F11R*-SNVs (recessive model) or serum sJAM-A-(≤ median vs > median), using clinical factors as covariables. Here, in the case of matched patients, we applied the Huber-White method (Cox regression) or an extension of the Fine-Gray method for clustered data.[27] The time-dependent covariate method was used to check the proportional hazard assumption of the Cox model. Survival functions were estimated by Kaplan-Meier curves. The log-rank test was applied to compare survival functions between homozygous carriers of the minor allele and carriers of the major allele (ie, recessive model). All statistical tests were 2-tailed, and statistical significance was defined as $P < 0.05$. Where indicated, the Holm-Bonferroni method[28] was applied to adjust *P* values for multiple testing.

RESULTS

PROGNOSTIC ASSOCIATION OF F11R-SNVs IN CAD AND IN INFLUENCING PROINFLAMMATORY sJAM-A LEVELS. Several SNVs of extramyocardial functional significance have been associated with long-term mortality after acute myocardial infarction (AMI), including those affecting platelet responsiveness to therapeutic interventions.[29] Currently, *F11R*-SNV analysis in CAD patients revealed that homozygous carriers (HC) of the minor alleles (rs2774276, rs790056) had significantly worse event-free survival for MI when compared with major allele carriers (unadjusted $P = 0.004$; $P = 0.015$; Holm-adjusted $P = 0.016$; $P = 0.045$, respectively) (Table 3, Figure 1A, i-ii). In multivariable Cox regression analysis, rs2774276 was independently associated with time to MI (Holm-adjusted $P = 0.048$), whereas rs790056 was not significantly correlated after correction for multiple testing (Holm-adjusted $P = 0.075$). However, when applying the Fine-Gray's method with all-cause death as competing risk, rs790056 was significantly associated with MI (Holm-adjusted $P = 0.039$) (Table 4). This implies that rs790056 has a stronger relationship with MI directly than with the underlying etiology of the disease.

Among CCS patients, 25.0% of HC of *F11R*-SNV-rs2774276 minor allele vs 8.9% of major allele carriers experienced MI (log-rank test $P = 0.007$), whereas, in the ACS group, 25.9% of HC of *F11R*-SNV-rs2774276 minor allele and 14.3% of major allele carriers succumbed to recurrent MI (log-rank test $P = 0.127$). There was no significant variation in interventions and medications that the patients received on discharge (Table 1). In Cox PH regression

FIGURE 1 ***F11R*-SNVs May Influence sJAM-A Levels and Be Associated With Prognosis in CAD Patients**

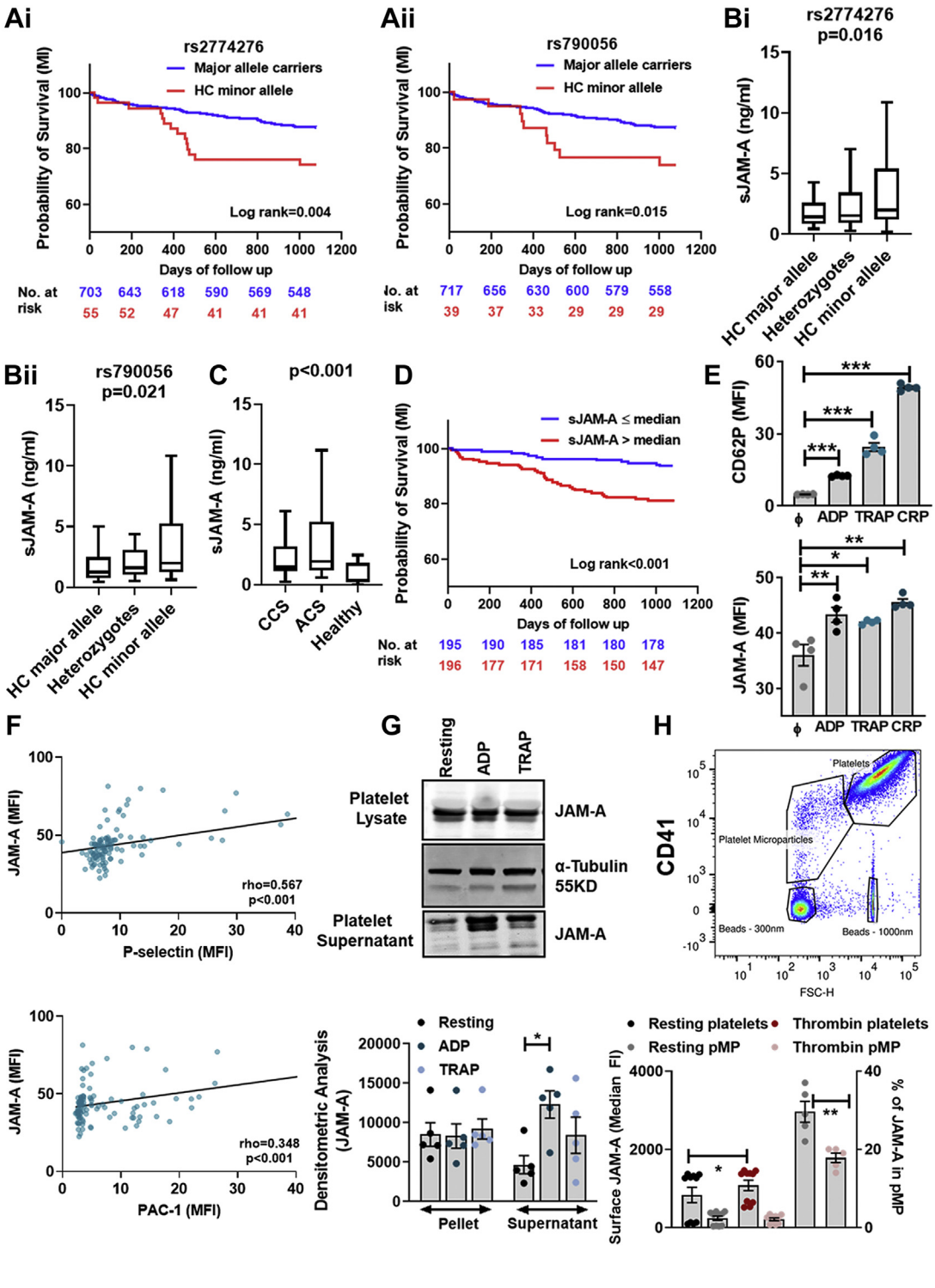

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TABLE 4 Results of Multivariable Cox PH Regression Analyses as Well as Fine and Gray's Proportional Subdistribution Hazards Regression Model for Myocardial Infarction With Clinical Factors[a] as Covariates

	Cox PH Model		Fine-Gray Model	
	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
F11R-SNA-rs2774276 (recessive genetic model)	2.09 (1.18-3.70)	Unadjusted **0.012** Holm-adjusted **0.048**	2.37 (1.32-4.24)	Unadjusted-**0.004** Holm-adjusted-**0.015**
Age	1.05 (1.03-1.07)	**<0.001**	1.04 (1.02-1.06)	**<0.001**
Gender (female/male)	0.72 (0.45-1.17)	0.189	0.72 (0.45-1.16)	0.180
LVEF%	0.79 (0.65-0.96)	**0.020**	0.83 (0.68-1.03)	0.085
Reason of admission (ACS/CCS)	1.67 (1.11-2.52)	**0.016**	1.53 (1.02-2.32)	**0.042**
F11R-SNA-rs790056 (recessive genetic model)	2.13 (1.10-4.11)	Unadjusted **0.025** Holm-adjusted **0.075**	2.38 (1.20-4.70)	Unadjusted-**0.013** Holm-adjusted-**0.039**
Age	1.05 (1.03-1.07)	**<0.001**	1.04 (1.02-1.06)	**<0.001**
Gender (female/male)	0.73 (0.45-1.18)	0.202	0.73 (0.45-1.17)	0.190
LVEF%	0.78 (0.64-0.96)	**0.016**	0.83 (0.68-1.02)	0.073
Reason of admission (ACS/CCS)	1.71 (1.13-2.58)	**0.011**	1.57 (1.04-2.37)	**0.032**
sJAM-A ≤ median vs > median (1.71 ng/mL)	2.95 (1.59-5.46)	Unadjusted **<0.001** Holm-adjusted **0.003**	2.91 (1.57-5.38)	Unadjusted **<0.001** Holm-adjusted **0.003**
Age	1.02 (0.99-1.05)	0.150	1.01 (0.99-1.04)	0.260
Gender (female/male)	0.81 (0.43-1.55)	0.530	0.84 (0.44-1.60)	0.600
LVEF%	0.83 (0.64-1.07)	0.149	0.86 (0.66-1.11)	0.240
Reason of admission (ACS/CCS)	0.96 (0.52-1.75)	0.883	0.94 (0.52-1.70)	0.840

Bold values indicate statistical significance. [a]Clinical variables included into the model: age, gender, LVEF%, and reason of admission (ACS/CCS).
Abbreviations as in Table 1.

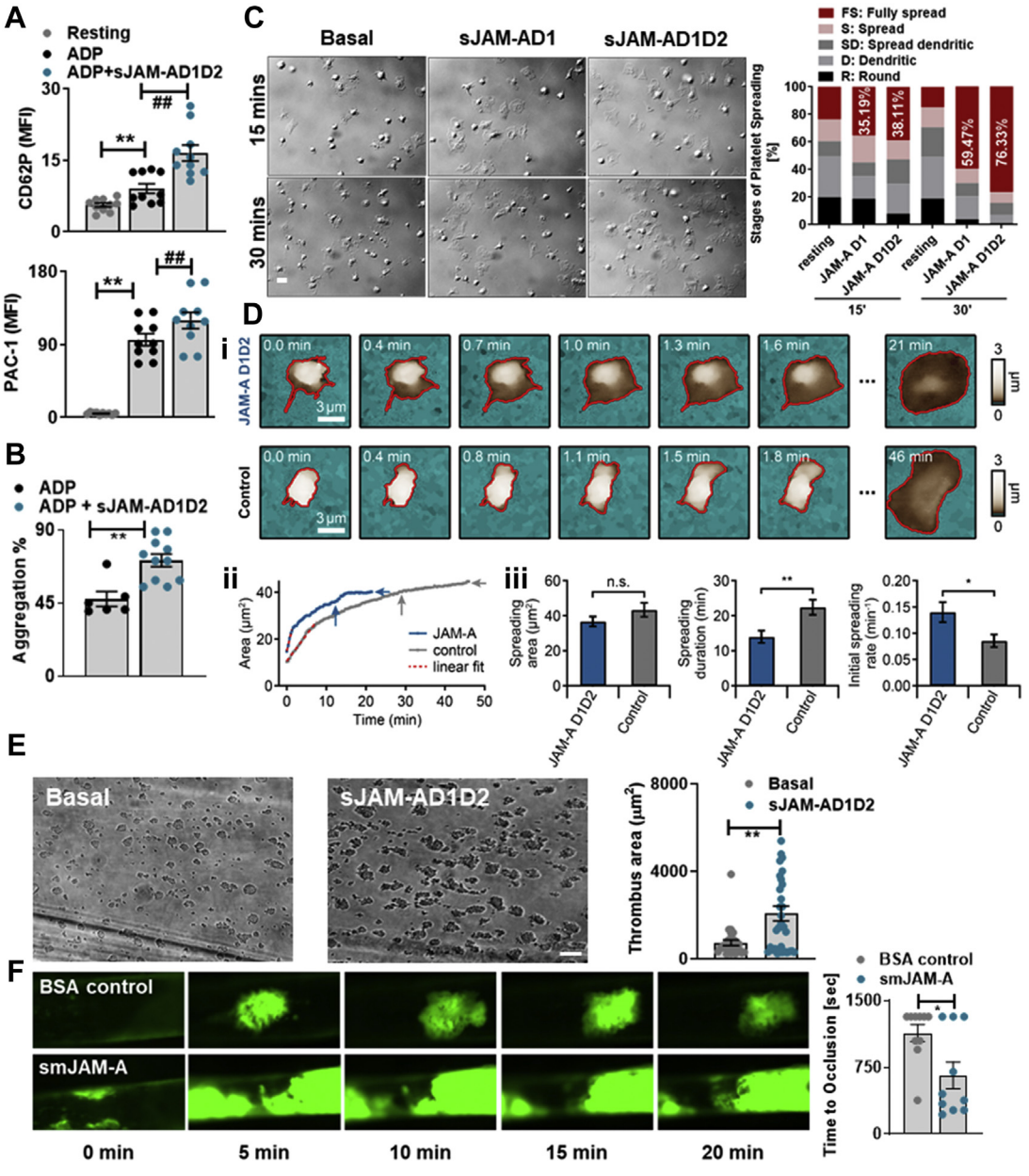
analysis (Supplemental Table 1), none of these treatments were significantly associated with outcome, whereas rs2774276 remained significantly associated with time to MI (Holm-adjusted $P = 0.020$). In contrast, rs790056 closely failed to be independently and significantly associated in Cox regression and Fine-Gray analysis after correction for multiple testing (Holm-adjusted $P = 0.069$ and $P = 0.063$, respectively).

Because *F11R*-SNVs influence JAM-A expression in cells,[11] they may regulate sJAM-A levels arising from shedding of transmembrane-JAM-A from cellular sources. Due to the observed prognostic association of *F11R*-SNV minor alleles, we analyzed serum sJAM-A levels among matched CAD patients. Serum sJAM-A levels were consistently increased with the presence and number of minor alleles For *F11R*-SNV-rs790056, median (Q1, Q3) serum sJAM-A levels in HC of major alleles, heterozygotes, and HC of minor alleles were 1.24 ng/mL (0.75, 2.48 ng/mL), 1.60 ng/mL (1.02, 3.21 ng/mL), and 2.00 ng/mL (1.25, 5.26 ng/mL), respectively (Clustered Wilcoxon rank sum test for HC of minor allele vs major allele carriers: $P = 0.021$). Similarly, for *F11R*-SNV-rs2774276 median (Q1, Q3) serum sJAM-A levels in HC of major alleles, heterozygotes, and HC of minor alleles were 1.45 ng/mL

FIGURE 1 Continued

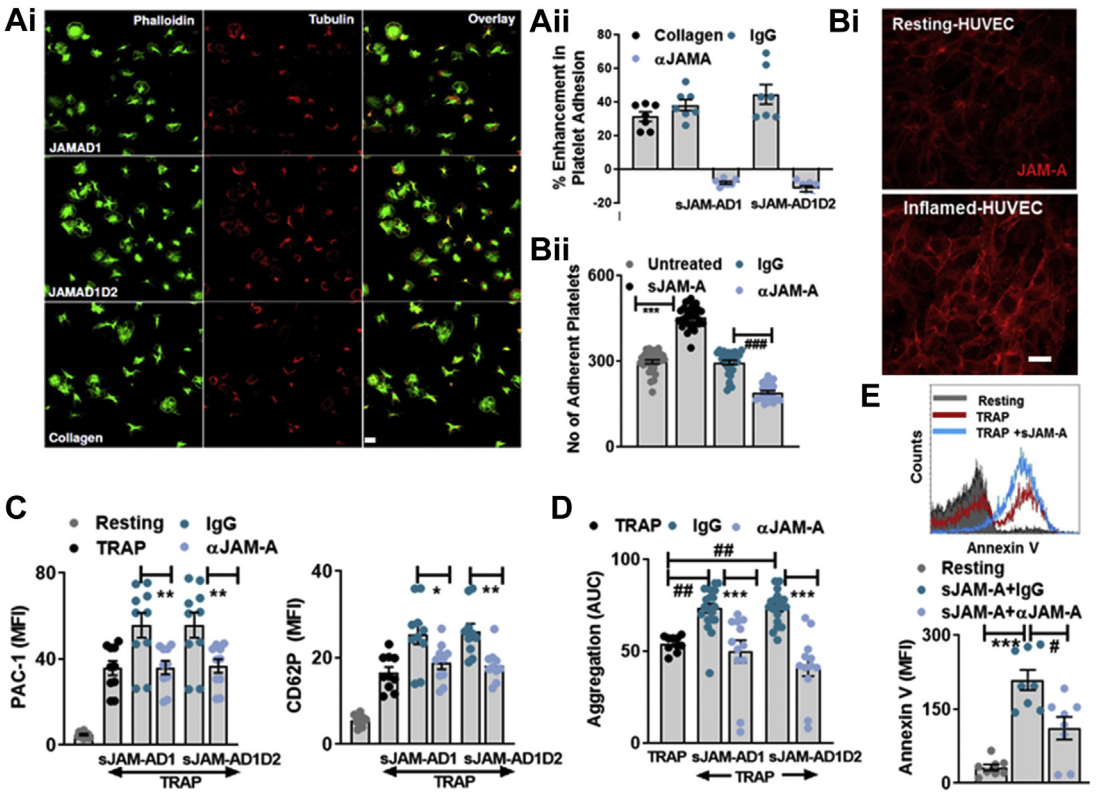
Kaplan-Meier curves showing probability of recurrent MI stratified according to **(Ai)** *F11R*-SNV-rs2774276 genotype and **(Aii)** *F11R*-SNV-rs790056 genotype. **(B)** sJAM-A serum levels in CAD patients (n = 51 each for rs2774276, HC-major, HC-minor, and heterozygotes; n = 35 each for rs790056, HC-major, HC-minor, and heterozygotes), stratified according to *F11R*-SNV genotype. *P* values show differences between HC of minor allele vs major allele carriers. **(C)** Serum sJAM-A levels in CAD (n = 401) patients with CCS (n = 184) vs ACS (n = 217) compared with healthy subjects (n = 24). Between ACS vs CCS and CAD vs healthy: $P < 0.001$. **(D)** Kaplan-Meier curves showing probability of recurrent MI stratified according to sJAM-A ≤ median vs > median (≤1.71 ng/mL vs >1.71 ng/mL) in CAD patients (n = 391). **(E)** Significantly enhanced platelet surface-associated JAM-A upon activation compared with CD62P. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs resting (ϕ) platelets. Correlation among **(F)** P-selectin, PAC-1, and platelet surface-associated JAM-A and/or sJAM-A in CAD (n = 111) patients. **(G)** Western blot and corresponding densitometric analysis for JAM-A in resting and ADP or TRAP-activated platelet lysate and shed sJAM-A detected in activated platelet supernatant. Loading controls for cell lysates as α-tubulin immunoblots. **(H)** Flow cytometry dot plot showing gating strategy for $CD41^+$ platelets and pMPs. Bar diagram shows increased pMP generation in thrombin-activated platelets, and surface-associated JAM-A on platelets and pMPs. *$P < 0.050$; **$P < 0.001$ Data show mean ± SEM for n = 5 donors. ACS = acute coronary syndrome; CAD = coronary artery disease; CCS = chronic coronary syndrome; HC = homozygous carriers; JAM-A = junctional adhesion molecule-A; MI = myocardial infarction; sJAM-A = soluble junctional adhesion molecule-A; SNV = single nucleotide variation; TRAP = thrombin receptor activating peptide.

FIGURE 2 sJAM-A Exerts a Prothrombotic Effect



Synergistic effect of sJAM-AD1D2 (30 μg/mL) on ADP (10 μmol/L) induced platelet **(A)** CD62P surface expression, PAC-1 binding (n = 5). $^{**}P < 0.01$; $^{\#\#}P < 0.01$. **(B)** Aggregation (n = 5 with technical replicates). $^{**}P < 0.01$. **(C)** Relative stages of platelets spreading on fibrinogen (n = 5) over time ±sJAM-AD1 (10 μg/mL) or sJAM-AD1D2 (30 μg/mL). **Bar** = 2 μm. **(D)** Kinetics of platelet spreading on fibrinogen, and sJAM-AD1D2 accelerating the process. **(Di)** SICM topography images of spreading platelets ±sJAM-AD1D2 (30 μg/mL). **Bar** = 3 μm. **(Dii)** The spreading area as a function of time. Spreading was quantified by the final spreading area **(horizontal arrows)** and spreading duration until 90% of final spreading area **(vertical arrows)**. The relative initial spreading rate was determined by fitting the spreading area **(solid traces)** up to 60% of the relative area with a linear fit **(dashed red line)**. **(Diii)** Spreading area, spreading duration, and relative initial spreading rate ±sJAM-AD1D2. Data show mean ± SEM for sJAM-AD1D2-treated (n = 11) and control platelets (n = 8). $^{*}P < 0.05$; $^{**}P < 0.01$ (Student's *t*-test). **(E)** Thrombus coverage (n = 5) ±sJAM-AD1D2 (30 μg/mL). $^{**}P < 0.01$. **Bar** = 50 μm. **(F)** Thrombus (platelets labelled with GP Ibβ-X488 appearing in **green**) build-up over time in smJAM-A (50 μg/mouse) and BSA-control (50 μg/mouse) administered mice (n = 5 mice/group). Bar diagram shows time to vessel occlusion ±smJAM-A. $^{**}P < 0.01$. Data represent mean ± SEM. Abbreviations as in Figure 1.

FIGURE 3 Homophilic Interaction Between Transmembrane-JAM-A and sJAM-A Exerts the Prothrombotic Effect



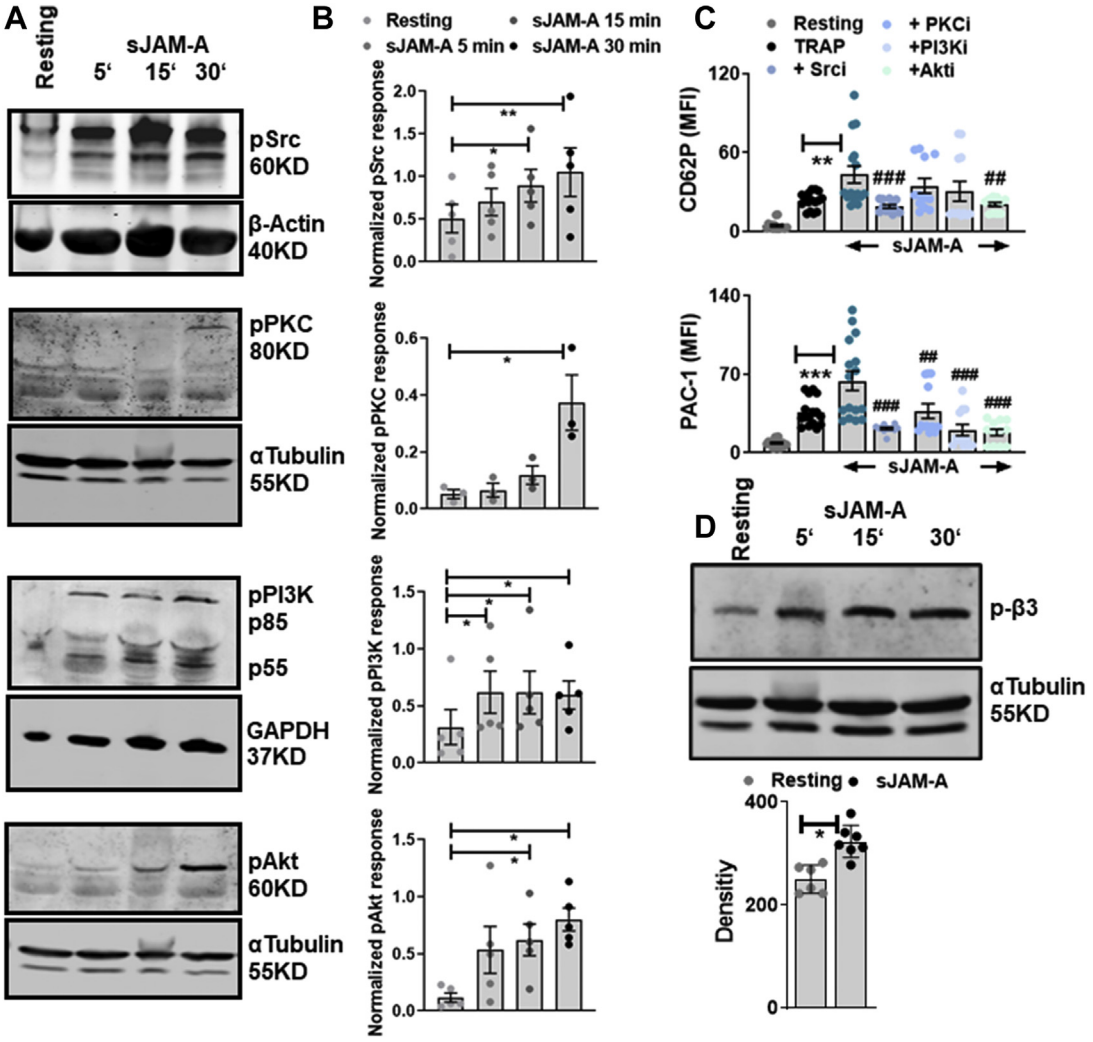
(Ai) Immunofluorescence confocal microscopic images showing phalloidin **(green)** stained platelets adhered to collagen and immobilized recombinant truncated sJAM-AD1 and full-length sJAM-AD1D2. **Bar** = 2 μm. **(Aii)** Fluorescence intensity from platelets adhered to collagen (100 μg/mL), sJAM-AD1 (10 μg/mL), and sJAM-AD1D2 (30 μg/mL), in presence of anti-JAM-A-antibody (10 μg/mL) or IgG control (10 μg/mL) (n = 7). **(Bi)** Immunofluorescence confocal microscopic images showing enhanced expression of JAM-A **(red)** on inflamed endothelial cells. Bar = 5 μm. **(Bii)** sJAM-A (***$P < 0.001$) substantiated platelet adhesion on JAM-A-enriched endothelial surface (n = 5) counteracted by anti-JAM-A-antibody (αJAM-A) (10 μg/mL) (###$P < 0.001$). **(C)** Synergistic effect of sJAM-AD1D2 (30 μg/mL) and sJAM-AD1 (10 μg/mL) on TRAP induced $\alpha_{IIb}\beta_3$-integrin activation (PAC-1) and platelet degranulation (CD62P) ±anti-JAM-A-antibody (αJAM-A) (10 μg/mL) or IgG control (10 μg/mL). *$P < 0.05$; **$P < 0.01$. **(D)** Synergistic effect of sJAM-AD1D2 (30 μg/mL) and sJAM-AD1 (10 μg/mL) on TRAP-induced platelet aggregation is counteracted by anti-JAM-A-antibody (αJAM-A) (10 μg/mL). ##$P < 0.01$; ***$P < 0.001$. **(C and D)** Data represent mean ± SEM from n = 5 experiments with technical replicates. **(E)** Flow cytometric histogram overlay and bar diagram (n = 4 with technical replicates) showing the synergistic effect of sJAM-AD1D2 (30 μg/mL) on TRAP-induced Annexin-V binding ±anti-JAM-A-antibody (αJAM-A) (10 μg/mL). ***$P < 0.001$; #$P < 0.05$. Abbreviations as in Figure 1.

(0.86, 2.60 ng/mL), 1.57 ng/mL (0.95, 3.80 ng/mL), and 1.99 ng/mL (1.19, 5.40 ng/mL), respectively (Clustered Wilcoxon rank sum test for HC of minor allele vs major allele carriers: $P = 0.016$) (Figure 1B).

ASSOCIATION OF sJAM-A WITH THROMBO-ISCHEMIC COMPLICATIONS IN CAD. Encouraged by these findings, we further extended our analysis to explore a potential prognostic association of serum sJAM-A in CAD patients (n = 401). Median sJAM-A levels (Q1, Q3), were significantly ($P < 0.001$) elevated in CAD patients (1.71 ng/mL; 1.18, 4.43 ng/mL) compared with healthy subjects without manifestation of cardiovascular disease (n = 24; 0.37 ng/mL; 0.20, 1.80 ng/mL) (Figure 1C). Moreover, significantly elevated serum sJAM-A levels were observed in ACS compared with CCS patients (1.94 ng/mL; 1.21, 5.21 ng/mL vs 1.51 ng/mL; 1.14, 3.19 ng/mL; $P < 0.001$) (Figure 1C), suggesting a potential link with thrombo-ischemic events. Additionally, peak troponin I correlated moderately and significantly with sJAM-A levels in CAD patients (rho = 0.302; $P < 0.001$).

Elevated (> median, ie, >1.71 ng/mL) sJAM-A levels measured upon admission were significantly associated with CE ($P = 0.008$) but not ACM ($P = 0.058$). Patients with higher (> median, ie, >1.71 ng/mL)

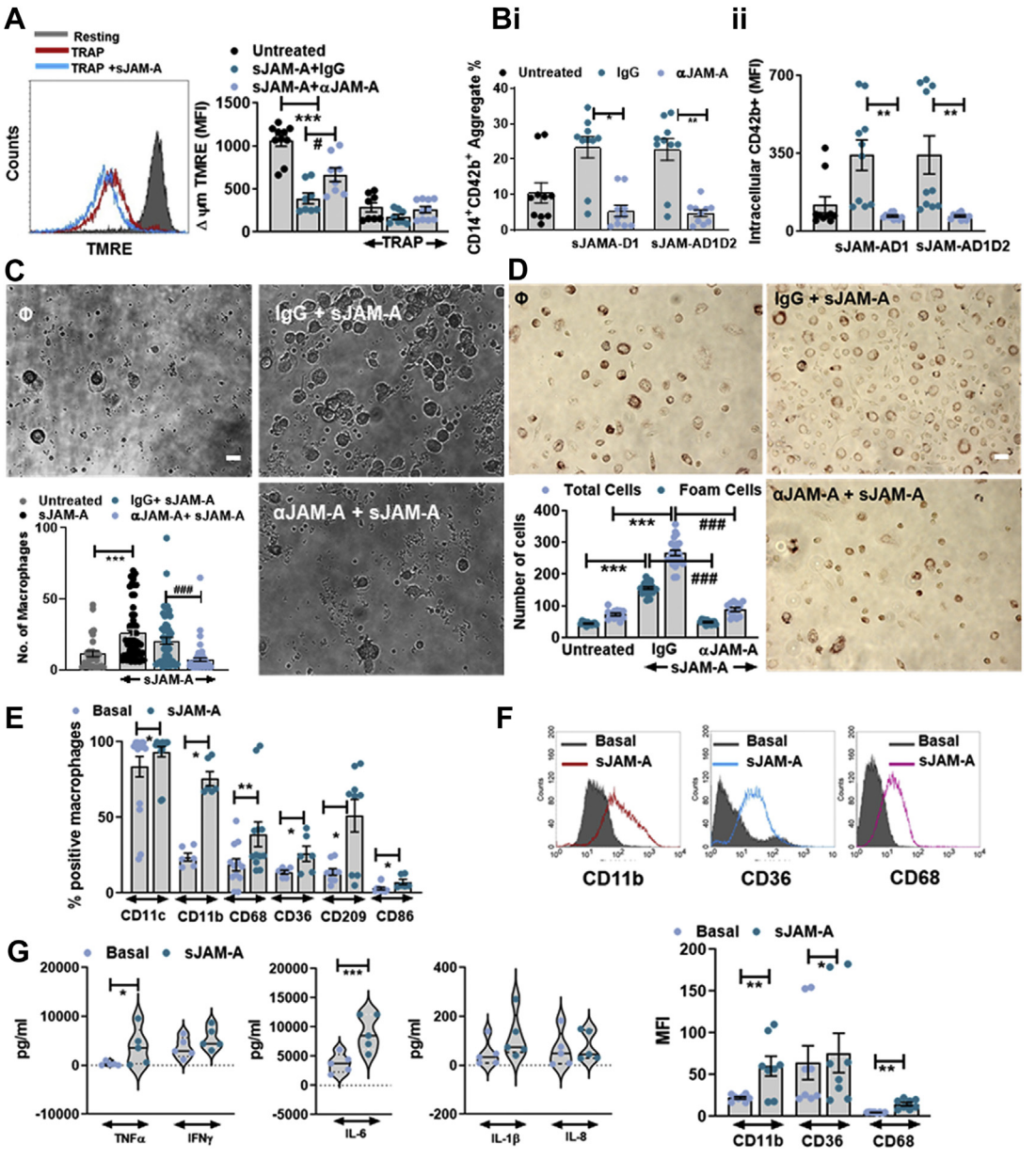
FIGURE 4 sJAM-A Triggers Activatory Signaling Cascade in Platelets



(A) Representative Western blot and **(B)** corresponding densitometric analysis (n = 3-5) for phospho-Src, phospho-PKC, phospho-PI3K, and phospho-Akt in platelets treated with sJAM-AD1D2 (30 μg/mL); GAPDH, β-actin, and α-tubulin immunoblots represent loading controls and used for normalization. *$P < 0.05$; **$P < 0.01$. **(C)** Synergistic effect of sJAM-AD1D2 (30 μg/mL) on TRAP (25 μmol/L)-induced CD62P and PAC-1-binding is decreased in the presence of inhibitors of the following kinases Src (Srci-PP2), PKC (PKCi-Gö6976), PI3K (PI3Ki- Ly29004), and Akt (Akti-). ***$P < 0.001$; **$P < 0.01$; and ###$P < 0.001$; ##$P < 0.01$ compared with sJAM-AD1D2+TRAP. Data are mean ± SEM from n = 6-8 experiments. **(D)** Representative Western blot (n = 6) and corresponding densitometric analysis for phospho-β_3-integrin in platelets treated with sJAM-AD1D2 (30 μg/mL). *$P < 0.05$. Abbreviations as in Figure 1.

sJAM-A levels showed a significantly increased risk for recurrent MI ($P < 0.001$; Holm-adjusted $P = 0.001$, respectively) (Figure 1D). sJAM-A was independently associated with time to MI in multivariable analysis (Table 4, Supplemental Table 1). Notably, we could measure sJAM-A levels only upon admission, which left the potential influence of subsequent antiplatelet therapy to speculation. However, we verified the influence of medication at admission on baseline sJAM-A levels. Although aspirin (ASA), clopidogrel, and prasugrel showed no significant impact, we observed reduced sJAM-A levels (median; Q1, Q3) (0.90 ng/mL; 0.69, 1.82 ng/mL vs 1.72 ng/mL; 1.19, 4.46 ng/mL; $P = 0.014$) in 12 patients with ticagrelor at admission. Because of the association of transmembrane-JAM-A and $\alpha_{IIb}\beta_3$-intergrin in a signaling complex in resting platelets,[17] we compared sJAM-A levels in ACS patients (n = 6) administered with glycoprotein GPIIb/IIIa antagonists in 22 patients after thrombus aspiration who did not receive GPIIbIIIa antagonists. We did

FIGURE 5 sJAM-A Substantiates Thrombo-inflammation



(A) Flow cytometric histogram overlay showing TMRE fluorescence from resting and TRAP (25 μmol/L) activated/apoptotic platelets ±sJAM-A. Mitochondrial transmembrane potential loss (ΔΨm-decreased TMRE fluorescence) induced by sJAM-AD1D2 (30 μg/mL) is counteracted by anti-JAM-A-antibody (αJAM-A) (10 μg/mL). Data mean ± SEM; n = 5 experiments. ***$P < 0.001$; #$P < 0.05$. **(Bi)** Percentage of $CD42b^+CD14^+$ platelet-monocyte aggregates in presence of sJAM-A (D1D2 or D1) ±anti-JAM-A-antibody (αJAM-A) (10 μg/mL). *$P < 0.05$;**$P < 0.01$. **(Bii)** $CD42b^+$ monocytes that have phagocytosed platelets in presence of sJAM-AD1D2 (30 μg/ml) or sJAM-AD1 (10 μg/ml) ± anti-JAM-A-antibody (αJAM-A) (10 μg/mL) of IgG control (10 μg/mL). **$P < 0.01$. **(C)** Images from platelet-monocyte coculture showing differentiation of monocytes into macrophages ±sJAM-AD1D2 (30 μg/mL) and/or ±anti-JAM-A-antibody (αJAM-A) (10 μg/mL). **Bar** = 5 μm. ***$P < 0.0001$; ###$P < 0.001$. **(D)** Monocyte differentiation into oil red positive foam cells ±sJAM-AD1D2 (30 μg/mL) and/or ±anti-JAM-A-antibody (αJAM-A) (10 μg/mL). ***$P < 0.001$; ###$P < 0.001$. **Bar** = 5 μm. **(E)** Relative percentage of $CD11c^+$, $CD11b^+$, $CD68^+$, $CD36^+$, $CD209^+$, $CD86^+$, macrophages in platelet-monocyte coculture ±sJAM-AD1D2 (30 μg/mL). **(F)** Flowcytometric histogram overlay and bar diagram showing relative surface expression of CD11b, CD36, and CD68 ±sJAM-AD1D2 (30 μg/mL) in platelet-monocyte cocultures. **(G)** Presence of proinflammatory mediators in platelet-monocyte cocultures ±sJAM-AD1D2 (30 μg/mL). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. **(E to G)** Data are mean ± SEM from n = 5-6 experiments. Abbreviations as in Figure 1.

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not observe a significant difference in sJAM-A levels (median; Q1, Q3) (4.25 ng/mL; 2.84, 5.82 ng/mL vs 4.63 ng/mL; 1.29, 8.03 ng/mL; $P = 0.740$). Because inflamed endothelium is a primary source of circulatory sJAM-A, we furthermore validated the effect of statins, which reduce endothelial inflammation[30] on sJAM-A levels. Patients receiving statins (n = 196) at admission showed lower sJAM-A levels (median; Q1, Q3) when compared with those without statin treatment (1.56 ng/mL; 1.14, 3.79 ng/mL vs 1.82 ng/mL; 1.21, 4.96 ng/mL; $P = 0.110$). However, this effect was not statistically significant.

ACTIVATED PLATELETS SHED TRANSMEMBRANE-JAM-A TO GENERATE THROMBO-INFLAMMATORY sJAM-A. Among healthy donors, platelet surface-associated JAM-A (cumulative signals from transmembrane-JAM-A + surface-bound circulatory sJAM-A) was significantly increased following in vitro activation for 30 minutes induced by (**Figure 1E**) adenosine diphosphate (ADP) ($P2Y_{12}$), thrombin receptor (PAR-1)-activating peptide (TRAP), and collagen-related peptide-GPVI, although requiring a stronger stimulus than degranulation (CD62P) (Supplemental Figure S1). Moreover, basal platelet surface-associated JAM-A in CAD patients significantly correlated with basal platelet activation markers-CD62P surface expression (rho = 0.567; $P < 0.001$) and PAC-1 binding (rho = 0.348; $P < 0.001$) (**Figure 1F**), revealing an unpredicted association of JAM-A with thrombotic propensity. Upon prolonged activation (60 minutes), activated platelets shed transmembrane-JAM-A, in ADP and TRAP-activated platelet supernatant (**Figure 1G**). Platelet activation with thrombin generates platelet-derived microparticles (pMPs) (**Figure 1H**), which carry thrombo-inflammatory mediators.[31] We detected surface-associated JAM-A on resting and thrombin activated platelets and pMPs (**Figure 1H**), which can engage in thrombo-inflammatory interaction with the endothelium and leukocytes.[1,5]

sJAM-A SUBSTANTIATES THROMBOTIC FUNCTIONS. To assess the pathophysiological relevance of increased sJAM-A levels in CAD patients, we analyzed the effect of recombinant-JAM-A, mimicking circulatory sJAM-A, on thrombotic functions. JAM-A consists of a membrane-proximal-D2 and a membrane-distal-D1 domain.[32] We generated a native recombinant sJAM-A molecule with both domains (sJAM-AD1D2) and a truncated version (sJAM-AD1) with only the D1 domain.[32] Although sJAM-A alone did not induce platelet degranulation (CD62P exposure), or aggregation (Supplemental Figure S2), pretreatment with sJAM-A synergistically enhanced ADP-induced CD62P surface expression, $\alpha_{IIb}\beta_3$-integrin activation (**Figure 2A**), and aggregation (**Figure 2B**). Priming with sJAM-A also enhanced the percentage of fully spread platelets on fibrinogen (**Figure 2C**) and accelerated the kinetics of spreading, deciphered by high-resolution live imaging of single platelet response (**Figure 2D, i-iii**). sJAM-A enhanced thrombus formation (**Figure 2E**) ex vivo. Similarly, administration of soluble murine junctional adhesion molecule-A (smJAM-A) mimicking pathological elevation in circulatory sJAM-A levels enhanced thrombus formation and occluded the injured carotid artery in vivo (**Figure 2F,** Videos 1 and 2) compared with control mice, which mostly did not show complete vessel occlusion. Current results reveal that sJAM-A may exert a unique prothrombotic impact besides its proinflammatory attributes.

sJAM-A EXERTS ITS PROTHROMBOTIC EFFECTS THROUGH HOMOPHILIC INTERACTION WITH PLATELET TRANSMEMBRANE-JAM-A. Transmembrane-JAM-A on platelets engages in trans-homophilic interaction with counterparts in endothelial and inflammatory cells.[1] Homophilic interaction between 2 transmembrane-JAM-A or sJAM-A molecules occurs through the D1-domains.[32] To decipher the molecular features mediating the prothrombotic response, we employed both sJAM-AD1D2 and truncated sJAM-AD1[32] and verified the possibility of a homophilic interaction between D1 domains of monomeric-sJAM-A and platelet transmembrane-JAM-A using an anti-JAM-A blocking antibody.

Platelet adhesion and spreading on immobilized recombinant full form-sJAM-AD1D2 and truncated-sJAM-AD1 under static conditions were comparable, and counteracted by anti-JAM-A-antibody (**Figure 3A, i-ii**). Moreover, presence of sJAM-AD1D2 substantiated platelet adhesion on JAM-A-enriched inflamed endothelial layer under dynamic arterial flow conditions (**Figure 3B, i-ii**), which was also counteracted by anti-JAM-A-antibody, blocking the homophilic interaction. sJAM-AD1D2 and sJAM-AD1 synergistically induced degranulation, $\alpha_{IIb}\beta_3$-integrin activation (**Figure 3C**), aggregation (**Figure 3D**), and thrombus formation (Supplemental Figure S3A) to a similar extent, which were counteracted by anti-JAM-A-antibody compared with control immunoglobulin G (IgG). Therefore, the D1 domain of sJAM-A is sufficient to engage in a homophilic interaction with platelet transmembrane-JAM-A and execute the prothrombotic effects. Transmembrane-JAM-A deficient *Jam-A*$^{gt/gt}$ mice show a prothrombotic disposition, as regulatory breaks on platelet-outside-in signaling are abolished.[16,17] We observed significantly enhanced

basal thrombus formation among megakaryocyte-platelet lineage-specific *Pf4-Cre*$^{+}$*Jama*$^{fl/fl}$ mice ex vivo (Supplemental Figure S3B), confirming these reports. However, unlike control Pf4-Cre^{-}Jama$^{fl/fl}$ littermates, the prothrombotic influence of smJAM-A was absent in *Pf4-Cre*$^{+}$*Jama*$^{fl/fl}$ mice lacking transmembrane-JAM-A on platelets, as the possibility of a homophilic interaction with smJAM-A was prevented (Supplemental Figure S3B).

sJAM-AD1D2-induced priming of platelets prompted surface externalization of thrombogenic phosphatidylserine, counteracted by anti-JAM-A-antibody (Supplemental Figure S3C). sJAM-AD1D2 also substantiated TRAP-induced Annexin V binding (Supplemental Figure S3C). sJAM-AD1D2-induced phosphatidylserine exposure consequently facilitated thrombin generation (Supplemental Figure S3D) and accelerated clot formation in thromboelastographic assay (Supplemental Figure S3E).

sJAM-A TRIGGERS PLATELET ACTIVATORY SIGNALING CASCADE. The endogenous inhibition on platelet outside-in-signaling is dysregulated in the absence of transmembrane-JAM-A in *Jam-A*$^{gt/gt}$ mice.[16,17] However, previous studies did not explore the influence of sJAM-A on platelet response. Currently, priming of platelets with recombinant sJAM-A synergistically induced agonist-driven degranulation, aggregation, and thrombus formation, and accelerated spreading on fibrinogen. Therefore, we explored the influence of sJAM-AD1D2 on platelet activatory signaling cascade. Platelet priming with sJAM-AD1D2 induced phosphorylation of Src, PKC, PI3K, and Akt (**Figures 4A and 4B**). Pharmacological inhibition of downstream kinases Src (PP2), PKC (Gö6976), PI3K (Ly29004), and Akt (SH6) (**Figure 4C**) abolished the synergistic effects of sJAM-AD1D2 on TRAP-induced platelet degranulation (CD62P) and $\alpha_{IIb}\beta_3$-integrin activation (PAC-1). Interestingly we also observed significant β_3-integrin tyrosine (Y733) phosphorylation upon priming of platelets with sJAMA-D1D2 (**Figure 4D**), which could have facilitated spreading of platelets on fibrinogen (**Figure 2D**). Thus, sJAM-A, by engaging in a homophilic interaction with transmembrane-JAM-A, may prime platelets, lowering their activation threshold in cardiovascular pathologies whereby circulatory sJAM-A levels are elevated.

sJAM-A ENHANCES THROMBO-INFLAMMATORY POTENTIAL OF PLATELETS. Because transmembrane-JAM-A is a proinflammatory mediator[1] of transcellular associations between vascular endothelium and circulatory cells, we assessed the impact of sJAM-A on thrombo-inflammatory attributes of platelets.[33] Although serum sJAM-A levels did not show a significant correlation with serum sP-selectin (Supplemental Table 2), platelet surface-associated JAM-A in the current CAD cohort correlated with platelet surface expression of CD62P (P-selectin) (**Figure 1E**), which engages in interaction with leukocyte-associated P-selectin glycoprotein ligand and thereby may substantiate platelet-monocyte aggregate (PMA) formation in patients with advanced atherosclerosis.[34] Recombinant sJAM-A enhanced mitochondrial transmembrane-potential loss ($\Delta\psi$m), like the platelet activating stimulus-TRAP, which was counteracted by anti-JAM-A-antibody (**Figure 5A**). Possibly by enhancing platelet CD62P and the "eat-me" signal phosphatidylserine[33] (**Figure 3G**), presence of sJAM-A facilitated CD14^{+}CD42b^{+}-PMA formation (**Figure 5B, i**) and substantiated phagocytic uptake of phosphatidylserine^{+}-platelets by monocytes (**Figure 5B, ii**). Consequently, sJAM-A supplementation induced phagocytic clearance of platelets by monocytes, fostering their differentiation[32] into macrophages (**Figure 5C**) and foam cells (**Figure 5D**) in platelet-monocyte cocultures. The number of macrophages (**Figure 5C**) and foam cells (**Figure 5D**) in sJAM-A-supplemented cultures were reduced in the presence of anti-JAM-A-antibody. Platelet-monocyte cocultures predominantly yield CD163^{+} regenerative M2 macrophages[33]; however, percentage of CD86^{+} macrophages were increased in the presence of proinflammatory recombinant sJAM-A (**Figure 5E**), as were the relative expression of CD11c, CD209, and CD11b on macrophages (**Figure 5E**). Surface expression of the scavenger receptors CD68 and CD36 were also enhanced in the differentiated macrophages (**Figure 5F**), possibly aiding in foam cell differentiation (**Figure 5D**). Analysis of platelet-monocyte culture supernatant revealed elevated levels of proinflammatory mediators, particularly TNF-α and IL-6, upon recombinant sJAM-A supplementation (**Figure 5G**). These findings taken together with the observation that sJAM-A substantiates platelet adhesion to inflamed endothelium (**Figure 3B**) suggest its thrombo-inflammatory influence. Data from CAD patients showed that serum proinflammatory sJAM-A levels correlated significantly with other platelet- and leukocyte-derived inflammatory mediators, eg, MCP-1 (CCL2), TARC (CCL17), and high-sensitivity C-reactive protein (Supplemental Table 2). A positive correlation between sJAM-A and inflammatory mediators is also suggestive of its involvement in thrombo-inflammatory actions. Whether or not sJAM-A levels are increased as an acute-phase response in CAD patients can only be assessed in longitudinal cohorts.

Whether or not sJAM-A levels are increased in 72 patients without CAD as main reason of admission (Supplemental Table 3) showed that sJAM-A (median;

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Q1, Q3) levels were comparable to those of CAD patients (1.52 ng/mL; 1.31, 4.40 ng/mL in non-CAD vs 1.71 ng/mL; 1.18, 4.43 ng/mL in CAD; $P = 0.769$). A significant number of these non-CAD subjects comprised of elderly, multimorbid patients (myocarditis, hypertensive cardiomyopathy, severe symptomatic aortic stenosis, severe symptomatic mitral regurgitation), explaining high sJAM-A levels possibly associated with a proinflammatory state.

DISCUSSION

The current investigation primarily focused on the thrombotic and thrombo-inflammatory potential of the transmembrane-JAM-A and sJAM-A interaction, revealed the following in CAD patients:

1. *F11R*-SNVs (rs2774276, rs790056) are significantly and independently (rs2774276) associated with recurrent MI, and that genetic disposition through *F11R*-SNVs can influence circulatory sJAM-A levels.
2. sJAM-A levels were significantly increased in ACS compared with CCS patients, thereby confirming a previous report.[3]
3. Higher sJAM-A levels were significantly and independently associated with recurrent MI.
4. Pro-inflammatory[1,18,35] sJAM-A correlated significantly with peak troponin I and thrombo-inflammatory mediators.
5. Moreover, platelet surface-associated JAM-A correlated with platelet activation status in CAD patients.
6. Activated platelets shed transmembrane-JAM-A. Considering the hyperactive status of platelets in circulation, platelet-derived sJAM-A could add to sJAM-A from leukocytes and vascular endothelium, accounting for elevated levels in CAD patients.
7. Moreover, we hereby report a novel homophilic interaction between sJAM-A and platelet transmembrane-JAM-A enhancing thrombotic functions and
8. Fostering thrombo-inflammatory platelet-monocyte interactions .

Although concentrations of recombinant sJAM-A required to significantly induce platelet activation were several folds higher than those observed in serum, much of circulatory JAM-A is present in cell or cell-derived microparticle-associated form. Moreover, recombinant sJAM-A that was mostly present in the dimeric form (dimer to monomer ratio 1.7 under pH 7.0 and 150 mmol/L NaCl)[36] required a higher concentration, because only the monomeric form of sJAM-A with a free D1 domain can engage in a homophilic interaction with the D1-counterpart in transmembrane-JAM-A. However, because activated platelets are a potential source of sJAM-A, enriched levels of this proinflammatory mediator might be achieved locally at sites of vascular inflammation where platelets adhere, aggregate and form thrombi.

Genetic variations may influence platelet functions[29] and their responsiveness to antiplatelet therapies. *F11R*-SNVs were associated with the risk of recurrent MI during a 3-year follow-up in symptomatic CAD patients, possibly caused by their influence in elevating sJAM-A levels. Genome-wide association studies have extensively identified thousands of genetic variants associated with diverse pathophysiological states, many of which are noncoding. Intronic SNVs may regulate gene expression by influencing alternative splicing of the mRNA or by acting as enhancers. How *F11R*-SNVs led to elevated sJAM-A levels in patients with cardiovascular risk factors like hypertension[8] and obesity[7] and in ACS patients needs to be addressed. Further investigations are required to clarify if the observed association of variants is caused by their high linkage with other causative functional *F11R* variants. Therefore, divergent influence of SNVs leading to phenotypic alterations in protein expression, and in turn favoring platelet-centric thrombotic or thrombo-inflammatory events, are possible. Although observational studies have shown a relevant association of platelet hyperreactivity and the occurrence of myocardial infarction,[37,38] whether platelet response influenced by the *F11R*-SNVs and sJAM-A axis acts as causal driver for these events warrants further research.

sJAM-A, as a proinflammatory mediator in atheroprogression,[2,3] might influence adverse thrombo-ischemic outcome in symptomatic CAD patients. A significant correlation of elevated sJAM-A levels in CAD patients with peak troponin-I indicated potential association with infarction size. Higher sJAM-A levels at baseline were significantly associated with both CE and MI. Therefore, a multimodal approach including clinical risk factors, genetic factors like *F11R*-SNVs (rs2774276, and potentially rs790056), and inflammatory mediators like sJAM-A may help in better thrombo-ischemic risk stratification and in tailoring therapies for CAD patients. However, before integrating such approaches into clinical decision making, larger validation cohorts, besides inflammatory and genetic marker-guided interventional trials, are necessary. It also needs to be considered that a significant fraction of circulatory JAM-A is associated with the surface of cellular sources or cell-derived microparticles. Although we have demonstrated the prognostic significance of sJAM-A and that platelet surface-associated JAM-A is significantly enriched in

ACS patients, we have not explored pMP-associated JAM-A in clinical samples from CAD patients, which could also exert a proinflammatory effect. This remains to be addressed in future investigations. Additionally, sJAM-A levels correlated significantly with other atherothrombotic proinflammatory mediators like high-sensitivity C-reactive protein, MCP-1, and TARC. These observations suggest that JAM-A might have potential therapeutic implications in CAD, as previously demonstrated in atherosclerotic mice.[6,35]

Vascular inflammation and inflammatory mediators trigger platelet activation, thrombosis, and thrombo-inflammation, driving atherothrombotic progression leading to CAD.[39] Transmembrane-JAM-A is associated with $\alpha_{IIb}\beta_3$-integrin in resting platelets and acts as an endogenous inhibitor of outside-in-signaling through $\alpha_{IIb}\beta_3$-integrin, thereby regulates platelet hypereactivity[16,17]; but it also mediates inflammatory associations with endothelium and leukocytes.[1] Genetic ablation of *F11r* in murine platelets lowers their activation threshold[18] as transmembrane-JAM-A-imposed regulation on c-Src-dependent outside-in-signaling is abolished, enhancing $\alpha_{IIb}\beta_3$-integrin activation, platelet spreading, thrombus formation, aggregation, and clot retraction, although inside-out-signaling-driven degranulation, thromboxane A2 production, and fibrinogen binding remain unaffected.[16,17] Such gain-of-function in platelets accelerates neointima formation during early stages of atheroprogression and promotes plaque formation in *trJAM-A*$^{-/-}$*Apoe*$^{-/-}$ mice.[18,19] However, transmembrane-JAM-A shed from multiple vascular cells, possibly platelets as shown here, circulate as sJAM-A.[1] Like recombinant sJAM-A, circulatory sJAM-A may engage in a homophilic interaction with platelet transmembrane-JAM-A to trigger platelet activatory signaling involving Src-PKC-PI3K-Akt to easily activate $\alpha_{IIb}\beta_3$-integrin in a synergistic action, and substantiate prothrombotic consequences. Previously, a stimulatory antibody-M.Ab.F11-mediated activation of JAM-A has been shown to increase platelet secretion, and aggregation through dimerizing JAM-A, by promoting its interaction with GPIIb/IIIa and downstream kinase PI3K.[15] Trans-homophilic interaction between the membrane distal domains of transmembrane-JAM-A has been suggested in instigating outside-in-signaling involving β_1-integrin.[40] Currently, observed trans-homophilic interaction between sJAM-A and transmembrane-JAM-A in inducing β_3-integrin phosphorylation resembles this scenario. Recombinant sJAM-A also prompted phosphatidylserine exposure on procoagulant platelets, and thereby increased thrombin generation and accelerated clot formation. Although *F11r* deficiency in mice generates a prothrombotic[16,17] and atherosclerotic[18] phenotype, these mice are immune to the synergistic stimulation from circulatory sJAM-A, in the absence of platelet transmembrane-JAM-A as a homophilic interaction partner.

STUDY LIMITATIONS. Currently, circulatory levels of sJAM-A and platelet surface associated JAM-A were evaluated only at baseline upon admission leaving alternations over time and in response to subsequent anti-thrombotic therapy to speculation. This needs to be addressed in longitudinal studies. Moreover, at present we cannot provide a mechanistic explanation on a causal association between F11R-SNPs and elevated sJAM-A levels in CAD patients which requires further investigation.

CONCLUSIONS

Previous[16-19] and current experimental evidence taken together suggest that transmembrane-JAM-A and sJAM-A may differentially influence platelet functions. Although transmembrane-JAM-A acts as an endogenous inhibitor of platelet outside-in-signaling,[16,17] sJAM-A in homophilic interaction with transmembrane-JAM-A is prothrombotic. Whereas transmembrane-JAM-A-deficient murine platelets exhibit increased interaction with monocytes, neutrophils, and endothelium, mediated through $\alpha_{IIb}\beta_3$-integrin,[18] $\alpha_M\beta_{II}$, or GP Ibα,[19] sJAM-A may instigate thrombo-inflammatory platelet-monocyte interactions, as previously documented for IL-6 and sP-selectin in ACS patients.[41] Therefore, instead of considering their pathophysiological actions separately, transmembrane-JAM-A and sJAM-A might be considered as a functional duo.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Genetic predisposition is a crucial determinant in influencing thrombotic propensity and even response to antiplatelet therapy. Therefore, such information is fundamental to personalized antithrombotic therapy. Genetic disposition through *F11R-SNVs* can elevate circulatory levels of sJAM-A in CAD patients, which in turn might substantiate thrombo-inflammatory consequences through homophilic interaction with platelet transmembrane-JAM-A. The current investigation adds to and substantiates previous clinical and experimental investigations that JAM-A might be considered as a potential therapeutic target to avert atherothrombosis.

TRANSLATIONAL OUTLOOK: Thrombo-inflammation exaggerates cardiovascular complications, necessitating the discovery of novel thrombo-inflammatory mediators that may ascertain disease severity and prognosis, and/or can be utilized as therapeutic targets. In the future, transmembrane-JAM-A and sJAM-A, like IL-1β, might be validated as potential therapeutic targets in CAD patients showing elevated sJAM-A levels. However, evaluation of circulatory sJAM-A, and platelet surface-associated JAM-A being limited to baseline at present, subsequent changes under the influence of antiplatelet therapy should be addressed in longitudinal studies. Recently, an F11R/JAM-A antagonistic peptide was shown to interfere with platelet-inflamed endothelium association and reduce atherosclerotic plaque formation in *Apoe*$^{-/-}$.[6] Interfering with the currently disclosed homophilic interface between transmembrane-JAM-A and sJAM-A may devise therapeutic strategies to regulate thrombotic and thrombo-inflammatory platelet response in cardiovascular pathologies where circulatory sJAM-A levels are elevated.

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