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Platelet CD40 ligand and bleeding during P2Y12 inhibitor treatment in acute coronary syndrome

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Abstract

Antiplatelet therapy through inhibition of the adenosine diphosphate (ADP)/P2Y12 pathway is commonly used in the treatment of acute coronary syndrome (ACS). Although efficient in preventing platelet activation and thrombus formation, it increases the risk of bleeding complications. In patients with ACS receiving platelet aggregation inhibitors, that is, P2Y12 blockers (n = 923), we investigated the relationship between plasma and platelet-associated CD40L levels and bleeding events (n = 71). Treatment with P2Y12 inhibitors in patients with ACS did not affect plasma-soluble CD40L levels, but decreased platelet CD40L surface expression (pCD40L) and platelet-released CD40L (rCD40L) levels in response to stimulation as compared to healthy controls. In vitro inhibition of the ADP pathway in healthy control platelets reduced both pCD40L and rCD40L levels. In a multivariable analysis, the reduced pCD40L level observed in ACS patients was significantly associated with the risk of bleeding occurrence (adjusted odds ratio = 0.15; 95% confidence interval = 0.034-0.67). P2Y12 inhibitor-treated (ticagrelor) mice exhibited a 2.5-fold increase in tail bleeding duration compared with controls. A significant reduction in bleeding duration was observed on $CD40L^{+/+}$ but not $CD40L^{-/-}$ platelet infusion. In addition, CD40L blockade in P2Y12 inhibitor–treated blood samples from a healthy human reduced thrombus growth over immobilized collagen under arterial flow. In conclusion, measurement of pCD40L may offer a novel approach to assessing bleeding risk in patients with ACS who are being treated with P2Y12 inhibitors.

KEYWORDS

acute coronary syndrome, blood platelets, CD40 ligand, hemorrhage, platelet aggregation inhibitors

Essentials

- Increased levels of platelet-associated CD40L are detected during acute coronary syndrome (ACS).
- Reduced platelet CD40L surface expression (pCD40L) is associated with increased bleeding risk in platelet aggregation inhibitor-treated ACS patients.
- P2Y12 inhibition controls bleeding and thrombus growth partially through platelet-associated CD40L.
- pCD40L levels may help to assess bleeding risk in patients with ACS treated with P2Y12 inhibitors.

1 | INTRODUCTION

Platelets perform their hemostatic functions through the expression of a set of adhesive and signaling receptors associated with the plasma membrane and/or the secretory granules. They also express a wide variety of other molecules that provide them with inflammatory, immune, healing, and angiogenic properties (eg, P-selectin [CD62P], CD40L [CD154], interleukin-1β, transforming growth factor-β, and thrombospondin-1). Interestingly, some of these factors encroach on the hemostatic role of platelets. This is particularly true for the costimulatory substance CD40L, which is stored in alpha granules in resting platelets, translocates to the surface upon activation,[1,2] and is then cleaved and released into the circulation.[3–8] CD40L affects platelet function, promoting activation and aggregation and driving thrombus formation, growth, and stability.[9–11] CD40L has further been suggested to play a role in bleeding, as mice lacking CD40L demonstrate prolonged bleeding[12] that cannot be corrected with angiotensin II.[13] Also, platelets expressing CD40L are required to prevent the induction of lethal hemorrhagic diathesis during lymphocytic choriomeningitis viral infection.[14] In humans, platelets are the main source of circulating soluble CD40L (sCD40L).[10] An elevated serum level of CD40L, which may reflect an increase in platelet-associated CD40L, is independently associated with intracranial hemorrhage severity.[15]

A dual antiplatelet strategy (aspirin and the platelet aggregation inhibitors, P2Y12 blockers) is the gold standard therapy for patients with acute coronary syndrome (ACS).[16] However, bleeding complications remain an important issue.[17–20] In patients with ACS, P2Y12 antagonist treatments result in impaired platelet-associated CD40L levels.[21–24] For sCD40L, the effect of P2Y12 inhibition remains unclear.[25–30] We hypothesized that a decrease in platelet CD40L levels triggered by P2Y12 antagonists could increase bleeding risk in the setting of ACS. We investigated whether platelet-associated CD40L (membrane-expressed CD40L [pCD40L] and released CD40L [rCD40L]) was linked to the occurrence of bleeding events in patients with ACS treated with aspirin and the platelet aggregation inhibitor clopidogrel or prasugrel. We went on to assess whether platelet-associated CD40L played a role in thrombus formation under arterial flow from human blood and in bleeding duration in mice treated with a P2Y12 inhibitor.

2 | MATERIALS AND METHODS

2.1 | Study population

Patients admitted for ACS from June 2011 to March 2013 at the Timone Teaching Hospital, Marseille, France (CHU Timone) were enrolled in this study as described by Cuisset et al[31] (n = 923, of which 584 underwent sCD40L level determination, 244 cytometry analysis of pCD40L, and 95 rCD40L; the 3 populations did not overlap). All the patients received a loading dose of clopidogrel (600 mg) or prasugrel (60 mg) and were then treated at discharge with clopidogrel (75 or 150 mg/day, n = 713) or prasugrel (10 mg/day, n = 210) in combination with aspirin (75 mg/day). Clinical follow-up was performed for all patients at 1 month after discharge from the hospital with a clinic visit, plasma and platelet-associated CD40L level measurements, and platelet P2Y12 inhibition testing. Bleeding and ischemic events were recorded over the follow-up period (Figure 1A). For patients who experienced significant bleeding complications, bleeding symptoms were classified according to the Bleeding Academic Research Consensus (BARC) definition as type 1, 2, 3, or 5 (type 4 was not considered, as no patient had planned coronary artery bypass grafting). Ischemic complications were also recorded.[32] The ethics committee of our institution approved the study protocol, and patients gave their written informed consent for participation in the study. To establish reference intervals for CD40L expression levels, we collected blood samples from 24 healthy control individuals (average age = 41.7 ± 15.8 years and 77% females). Only persons without known disease and not taking any medication were enrolled.

2.2 | Evaluation of P2Y12 inhibition

Phosphorylated vasodilator-stimulated phosphoprotein (VASP) levels were determined on the whole studied population (n = 923 ACS patients and n = 24 healthy controls) using a standardized flow cytometric assay (platelet VASP; Diagnostica Stago/Biocytex, Asnières, France), and the platelet reactivity index–vasodilator-stimulated phosphoprotein (PRI-VASP) was calculated as previously described.[33]

2.3 | CD40L assessment

2.3.1 | Reagents

Monoclonal antibodies against CD40L (clone TRAP-1) and matched isotype controls were purchased from Beckman Coulter (Marseille, France). SFLLRNPNDKYEPF (thrombin receptor-activating peptide [TRAP-14]) was obtained from PolyPeptide Group (Strasbourg, France). Apyrase (adenosine phosphate [ADP] inhibitor), 2MeSAMP (P2Y12 inhibitor), and A3P5PS (P2Y1 inhibitor) were purchased from Sigma-Aldrich (Paris, France). CD40L ELISA was purchased from R&D Systems (Minneapolis, MN).

2.3.2 | Flow cytometry analysis of pCD40L

Platelet-rich plasma (PRP), isolated from citrated whole blood collected from 244 patients, was mixed with modified Tyrode's buffer (137 mmol/L NaCl, 0.3 mmol/L Na_2HPO_4, 2 mmol/L KCl, 12 mmol/L $NaHCO_3$, 5 mmol/L *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid and 5 mmol/L glucose; pH 7.4) alone or containing TRAP-14 (100 µmol/L) for 1 hour. Samples in the resting state (basal pCD40L) and after TRAP-14 stimulation were incubated with phycoerythrin (PE)-conjugated anti-CD40L antibodies or isotype-matched IgG1-PE and analyzed using a FC-500 cytometer (Beckman-Coulter, Paris, France). pCD40L was defined as the percentage of CD40L-positive cells.

2.3.3 | Release of rCD40L

PRP samples isolated from blood collected from 95 enrolled individuals were incubated with TRAP-14 (100 µmol/L) or modified Tyrode's buffer for 1 hour. Supernatants were obtained by centrifugation and then stored at −20°C prior to CD40L ELISA, according to the manufacturer's recommendations (R&D Systems). rCD40L is defined as the rCD40L concentration measured on TRAP-14 stimulation after subtracting the concentration obtained in the resting state.

2.3.4 | sCD40L in circulating plasma

Anticoagulated whole blood collected from a total of 584 patients was centrifuged twice at 2000 × *g* for 15 minutes and 2500 × *g* for 10 minutes to obtain plasma (with residual platelet count <10 g/L). Plasma samples were stored at −80°C prior to assessment of sCD40L levels via ELISA (R&D Systems).

2.4 | Hematologic and biochemical profiles

All parameters were determined by standard laboratory methods at the Department of Hematology and Biochemistry, CHU Timone (Marseille, France). Biochemical parameters were measured in blood samples using ADVIA 120 hematology systems (Bayer Diagnostics, New York, NY) for hemoglobin and platelet counts, Beckman DX 800 (Beckman Coulter, Inc., Fullerton, CA) for creatinine and CRP, Variant II Turbo (Bio-Rad Laboratories, Hercules, CA) for hemoglobin A_{1c}, and COBAS (Roche Diagnostics, Burgess Hill, West Sussex, UK) for insulin. Total serum cholesterol was measured by the reaction of cholesterol esterase/cholesterol oxidase/peroxidase, using a Hitachi 747 analyzer (Hitachi, Bohemia, NY). High-density lipoprotein cholesterol (HDL-C) was quantified after precipitation with polyethylene glycol at room temperature. Low-density lipoprotein cholesterol was calculated using the Friedewald formula. Total serum triglycerides were measured by the reaction of glycerol/phosphate/oxidase and peroxidase.

2.5 | In vitro evaluation of platelet-associated CD40L and CD62P

PRPs from healthy individuals were incubated with apyrase, 2MesAMP, A3P5PS, or vehicle (phosphate-buffered saline) for 10 minutes and then activated with TRAP-14 (10 µmol/L) for 10 minutes. Surface expression levels of CD40L and CD62P were analyzed after activation on a C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ) using the fluorescein isothiocyanate–coupled monoclonal antibodies against CD40L (clone TRAP-1) and the PE-labeled anti-CD62P (clone AK-4, Thermo Fisher Scientific, Waltham, MA). rCD40L was measured by ELISA as described above in supernatant from apyrase-, 2MesAMP-, A3P5PS-, and vehicle-treated PRPs activated with TRAP-14 for 1 hour.

2.6 | In vitro thrombus formation

Thrombus formation was studied as previously described.[34] Briefly, platelets were labeled with 1 µg/mL calcein-AM for 30 minutes and then incubated with the anti-FcγRIIa antibody IV.3 (Stemcell Technologies, Grenoble, France) in the presence or absence of

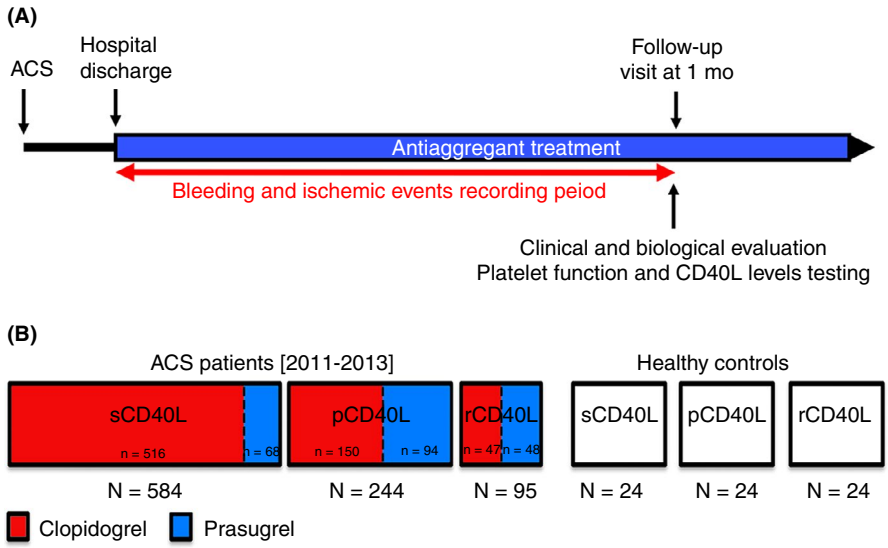

FIGURE 1 Flowchart depicting the study design (A) and populations (B); n equals the number of individuals in each group. In the 3 ACS populations, red boxes represent the clopidogrel-treated patients, blue boxes the prasugrel-treated ones. ACS, acute coronary syndrome; pCD40L, platelet surface CD40L; rCD40L, platelet-released CD40L; sCD40L, plasma-soluble CD40L.

2MesAMP (100 μmol/L). When required, reconstituted whole blood was pre-incubated for 5 minutes at 37°C with recombinant human CD40L-Fc (rhCD40-Fc) chimera protein (50 μg/mL, from R&D Systems) and injected into fibrillar Horm collagen-coated (100 μg/mL) flow chambers (Vena8 Fluoro+ biochips; Cellix, Dublin, Ireland) at an arterial shear rate of 50 dynes/cm^2. Images recorded over 300 seconds were analyzed using ImageJ software. The surface covered (%) by fluorescent platelets and the areas of thrombi were determined.

2.7 | Mouse-tail bleeding duration assay

Ten- to 14-week-old C57BL/6J, CD40L$^{-/-}$ and CD40L$^{+/+}$ mice were purchased from Jackson Laboratories (Bar Harbor, ME). All the mice were housed in the local animal facility before the experiments. All the experiments were conducted in strict compliance with the European animal protection law and with good animal practice as defined by the Federation of Laboratory Animal Science Associations (www.felasa.eu) and the German animal welfare body GV-SOLAS (www.gv-solas.de). The examinations conducted in this study were approved by the federal authorities in Freiburg and the Institutional Review Board with animal experiment permission G-13/024.

2.8 | Tail bleeding duration determination

Wild-type mice received ticagrelor (100 mg/kg) by oral gavage 18 and 6 hours prior to experiments. Platelets from CD40L$^{+/+}$ and CD40L$^{-/-}$ animals were washed in modified Tyrode's buffer. Ticagrelor-treated animals received recombinant murine sCD40L (80 μg) or 2 × 10^7 platelets in 80 μL or vehicle by intraperitoneal injection. Five minutes after transfusion, tail bleeding duration was determined by removing 2 mm of the distal mouse tail and immersing it immediately in saline solution at 37°C. The bleeding duration end point was defined as the point when bleeding stopped for >120 seconds.

2.9 | Mouse platelet aggregation

Wild-type mice received ticagrelor (100 mg/kg) by oral gavage 18 and 6 hours before experiments. Mouse PRPs were obtained from citrate anticoagulated mouse blood and diluted (1/7) into Tyrode's buffer. Light transmission aggregation was performed on 96-well plates using the EnSight multimode plate reader (PerkinElmer, Waltham, MA).

2.10 | Statistical analysis

Statistical analysis was performed using PASW Statistics version 17.0 and SPSS software for Windows (version 20.0; SPSS Inc., Chicago, IL). Continuous variables were reported as the mean ± standard error of the mean. Categorical variables were reported as counts or percentages. Standard 2-sided tests were used to compare continuous characteristics (Student *t* or Mann-Whitney *U* test) or categorical characteristics (chi-square or Fisher exact test) among patient groups. One-way ANOVA was used for comparison between different groups. Acute coronary syndrome patient data were adjusted for age and sex prior to statistical analysis. Correlations were assayed using Pearson's method. The stepwise method for multivariable linear regression models was used to calculate the linear association between CD40L parameters and cardiovascular risk factors. Logistic regression was performed to identify independent predictors of bleeding, using standardized values (the increment was 1 standard deviation). Age and variables showing a $P < 0.2$ were introduced in a stepwise procedure multivariable analysis. The number of variables included in the model was selected to avoid overfitting.

Statistical significance was defined as $P \leq .05$.

The manuscript was prepared according to the STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) observational cohort study reporting guidelines.[35]

3 | RESULTS

A total of 923 patients undergoing percutaneous coronary intervention with coronary stenting for ACS and discharged were included in the study according to the experimental design depicted in Figure 1A. All the patients received aspirin (75 mg) in combination with a P2Y12 inhibitor. A total of 713 were treated with clopidogrel (75 or 150 mg), and 210 received prasugrel (10 mg) (Figure 1B). The clinical characteristics of the ACS patient populations in which sCD40L, pCD40L, or rCD40L was measured are summarized in Table 1.

3.1 | Plasma and platelet CD40L levels correlated poorly with cardiovascular risk factors

sCD40L levels were assessed in plasmas obtained from an initial population of 584 patients. Owing to limitations in blood sample volume and restrictive experimental platelet isolation, activation, and analysis procedures, pCD40L levels were obtained from 244 other enrolled individuals and rCD40L levels measured from 95 other patients. The cardiovascular risk factors indicated in Table 1 did not correlate with sCD40L, basal pCD40L, TRAP-pCD40L or rCD40L, with the exception of a weak but significant positive linear correlation between rCD40L and age ($r = 0.24$; $P = .02$) and HDL levels ($r = 0.34$; $P = .001$).

3.2 | pCD40L, but not sCD40L, was affected by P2Y12 inhibitor treatment

We investigated the effect of P2Y12 inhibitor treatment on CD40L expression and release. PRI-VASP, pCD40L (basal and TRAP stimulated), and rCD40L levels were significantly reduced during P2Y12 inhibitor therapy (Figure 2A, B, and C, respectively), and these differences remain significant after data adjustment for the key confounders age and sex (Table 2). By contrast, P2Y12 inhibitor treatment did not affect sCD40L levels (Figure 2D). In line with these observations,

TABLE 1 Basal characteristics of the ACS patient populations in which sCD40L, pCD40L, or rCD40L were measured (these 3 populations did not overlap)

Mean ± SD, %	sCD40L (n = 584)	pCD40L (n = 244)	rCD40L (n = 95)
Females (n, %)	115 (20%)	46 (19%)	14 (15%)
Age (y)	63.1 ± 12.2	64.9 ± 11.8	63.1 ± 11.8
BMI (kg/m^2)	26.9 ± 4.4	26.6 ± 4.1	26.0 ± 3.2
Hypertension (n, %)	308 (53%)	136 (56%)	39 (41%)
Type 2 diabetes (n, %)	163 (28%)	52 (21%)	21 (22%)
Dyslipidemia (n, %)	322 (55%)	120 (49%)	39 (41%)
Current smoker (n, %)	229 (39%)	79 (32%)	34 (36%)
EF (%)	54.9 ± 7.8	56.2 ± 7.4	56.0 ± 9.3
STEMI (n, %)	200 (34%)	66 (27%)	28 (30%)
NSTEMI (n, %)	384 (66%)	178 (73%)	67 (71%)
Bleeding events (n, %)	50 (9%)	18 (7%)	3 (3%)
Ischemic events (n, %)	13 (2%)	3 (1%)	1 (1%)
Insulinemia (mUI/L)	12.8 ± 12.1	13.4 ± 17.5	13.7 ± 10.0
HbA_{1c} (%)	6.2 ± 1.0	6.2 ± 1.0	6.0 ± 1.0
CRP (mg/dL)	0.41 ± 1	0.53 ± 1.03	0.49 ± 0.74
Triglycerides (mg/dL)	120 ± 70	130 ± 80	130 ± 80
Cholesterol (mg/dL)	150 ± 40	150 ± 40	160 ± 40
HDL (mg/dL)	40 ± 10	40 ± 10	50 ± 10
LDL (mg/dL)	80 ± 30	90 ± 30	80 ± 40
Hemoglobin (g/dL)	13.9 ± 4.5	13.9 ± 1.5	14.1 ± 1.5
Creatinine (mg/dL)	0.97 ± 0.30	0.99 ± 0.27	0.99 ± 0.28
Platelets (10^9/L)	256 ± 72	248 ± 71	245 ± 60
Clopidogrel (n, %)	518 (89%)	150 (62%)	51 (54%)
Prasugrel (n, %)	67 (11%)	94 (39%)	44 (46%)
PRI-VASP (%)	39.3 ± 18.4	37.5 ± 18.9	37.0 ± 16.9
Beta-blocker (n, %)	427 (73%)	162 (66%)	55 (58%)
Statin (n, %)	532 (91%)	224 (92%)	89 (94%)
$GP_{IIbIIIa}$ inhibitor (n, %)	228 (39%)	74 (30%)	30 (32%)
ACE inhibitor (n, %)	436 (75%)	156 (64%)	54 (57%)
Calcium blocker (n, %)	61 (10%)	41 (17%)	6 (6%)

ACE, angiotensin-converting enzyme; BMI, body mass index; CAD, coronary artery disease; CRP, C-reactive protein; EF, ejection fraction; GP, platelet glycoprotein; HDL, high-density lipoprotein; HbA_{1C}, glycosylated hemoglobin; LDL, low-density lipoprotein; SD, standard deviation; NSTEMI, non–ST-elevation myocardial infarction; pCD40L, platelet surface CD40L; PRI-VASP, platelet reactivity index–vasodilator-stimulated phosphoprotein; rCD40L, platelet-released CD40L; sCD40L, plasma-soluble CD40L; STEMI, ST-elevation myocardial infarction.

TRAP-pCD40L, and rCD40L levels correlated positively with PRI-VASP levels (r = 0.13, P = .05 and r = 0.25, P = .02, respectively), whereas sCD40L levels did not. Prasugrel showed a stronger inhibitory effect on PRI-VASP than clopidogrel. By contrast, the 2 P2Y12 inhibitors had similar effects on pCD40L, rCD40L (basal and TRAP stimulated), and sCD40L levels.

3.3 | Bleeding events were associated with low expression levels of platelet surface CD40L

Of the 923 individuals followed, 71 (ie, 8% of the total population) experienced bleeding complications within 1 month of the discharge. Platelets from patients with bleeding complications displayed significantly lower PRI-VASP levels (Figure 3 and Table 3) and decreased expression levels of basal pCD40L and TRAP pCD40L, whereas sCD40L and rCD40L levels did not (sCD40L, 1.0 ± 0.1 vs 0.9 ± 0.2 ng/mL, P = .68; rCD40L, 21.5 ± 2.4 vs 13.2 ± 11.3 ng/mL, P = 0.48 in nonbleeders and bleeders, respectively). In the 244 patients for whom pCD40L levels were determined, we observed 18 cases of bleeding complications (7% of the total population), including 13 BARC 1 (72.2%), 4 BARC 2 (22.2%), and 1 BARC 3 (5.5%) bleeding events. Univariate analysis revealed that sex, ischemic events, hemoglobin, basal pCD40L and TRAP pCD40L level were associated with bleeding risk (Table 4). In a multivariable analysis also adjusted for age, basal pCD40L levels remained

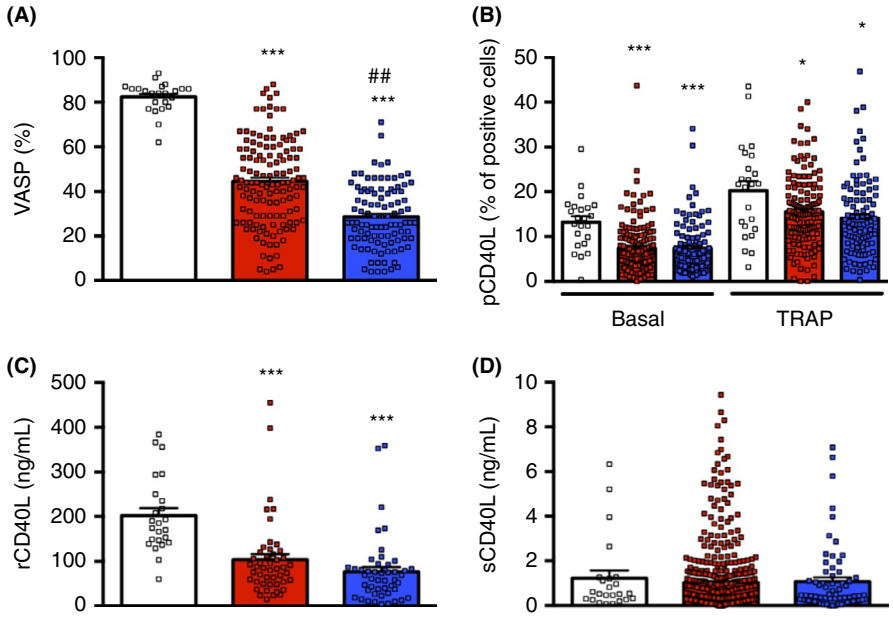

FIGURE 2 PRI-VASP (A), pCD40L (B), rCD40L (C), and sCD40L (D) levels in ACS patients treated with clopidogrel (red bar) and prasugrel (blue bar) compared with untreated healthy controls (white bars). *P < .05; ***P < .001 vs controls; ##P < .05 vs clopidogrel. The data are given as mean ± standard error of the mean adjusted for age and sex. pCD40L is defined as the percentage of CD40L-positive cells in the resting state (basal pCD40L) and after TRAP-14 stimulation (TRAP pCD40L). rCD40L is defined as the rCD40L concentrations measured on TRAP-14 stimulation after subtracting the concentration obtained in the resting state without TRAP-14. ACS, acute coronary syndrome; pCD40L, platelet surface CD40L; PRI-VASP, platelet reactivity index–vasodilator-stimulated phosphoprotein; rCD40L, platelet-released CD40L; sCD40L, plasma-soluble CD40L; TRAP, thrombin receptor-activating peptide

TABLE 2 PRI-VASP, basal and TRAP-induced pCD40L, rCD40L, and sCD40L levels adjusted for age and sex in patients with ACS treated with clopidogrel and prasugrel compared with untreated healthy controls

	Control	Clopidogrel	Prasugrel
VASP	85.7 ± 3.1	43.8 ± 1.5****	28.3 ± 1.6$^{****,\#\#\#\#}$
Basal pCD40L	13.2 ± 1.4	7.3 ± 0.5***	7.3 ± 0.6***
TRAP CD40L	20.6 ± 2.3	15.4 ± 0.8*	14.8 ± 0.9*
rCD40L	218.4 ± 21.1	94.6 ± 13.4***	76.8 ± 11.8***
sCD40L	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.2

Abbreviations: pCD40L, platelet surface CD40L; rCD40L, platelet-released CD40L; sCD40L, plasma-soluble CD40L; TRAP, thrombin receptor-activating peptide; VASP, vasodilator-stimulated phosphoprotein.
The data are given as mean ± standard error of the mean (*P < .05; ***P < .001; ****P < .0001 vs controls; $^{\#\#\#\#}P$ < .0001 vs clopidogrel).

significantly associated with bleeding risk (adjusted odds ratio [aOR], 0.15; 95% confidence interval [CI], 0.03-0.67), independently of PRI-VASP levels (aOR, 0.17; 95% CI, 0.06-0.48), with an increment equal to 1 (Table 4). As a consequence, lower PRI-VASP levels were significantly associated with higher risk of bleeding.

3.4 | Platelet CD40L surface expression and release is dependent on the ADP/P2Y12 signaling pathway

To test the importance of ADP signaling pathways on platelet CD40L bioavailability, the ADP-degrading enzyme apyrase, a specific P2Y12 inhibitor (2MeSAMP) and a specific P2Y1 inhibitor (A3P5PS), were incubated with platelets from healthy individuals before stimulation with TRAP-14. ADP degradation and P2Y12 inhibition reduced pCD40L levels (47% using apyrase and 66% with 2MeSAMP) (Figure 4A) and almost entirely diminished TRAP-induced rCD40L levels (Figure 4B). By contrast, inhibition of P2Y1 had no effect on either TRAP-induced CD40L expression or release. Furthermore, inhibition of neither cyclooxygenase-1 (indomethacin, 10 μmol/L) nor $\alpha_{IIb}\beta_3$ integrin activation (tirofiban, 50 ng/mL) decreased CD40L surface expression on platelet upon stimulation with TRAP (10 μmol/L) (data not shown). To evaluate whether inhibition of the ADP/P2Y12 signaling pathways differentially affected CD40L expression and other platelet responses, we determined the percentage of 2MesAMP-induced inhibition of platelet-associated CD40L and CD62P on agonist stimulation. The presence of 2MesAMP resulted in the reduction of both CD40L- and CD62P-expressing platelets on TRAP

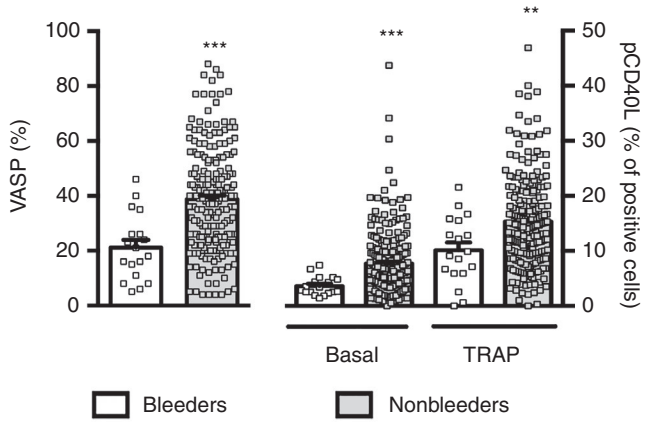

FIGURE 3 PRI-VASP levels together with basal and TRAP-induced pCD40L levels in ACS patients with and without bleeding (**P < .01, ***P < .001). Data are expressed as mean ± standard error of the mean adjusted for age and sex; n = 244. ACS, acute coronary syndrome; pCD40L, platelet surface CD40L; PRI-VASP, platelet reactivity index–vasodilator-stimulated phosphoprotein; TRAP, thrombin receptor-activating peptide

TABLE 3 PRI-VASP, basal and TRAP-induced pCD40L levels adjusted for age and sex in patients with ACS with and without bleeding

	Bleeders	Nonbleeders
VASP	38.8 ± 1.2	20.9 ± 4.5***
Basal pCD40L	7.7 ± 0.4	3.7 ± 1.4**
TRAP CD40L	15.6 ± 0.6	10.0 ± 2.2*

Abbreviations: pCD40L, platelet surface CD40L; TRAP, thrombin receptor-activating peptide; VASP, vasodilator-stimulated phosphoprotein. Data are expressed as mean ± standard error of the mean (*P < .05, **P < .01, ***P < .001).

stimulation (Figure 4C). However, CD40L surface expression was more markedly affected by P2Y12 inhibition compared to CD62P (45% ± 16% vs 14% ± 6% inhibition, respectively). CD40L and CD62P expression kinetics obtained from TRAP-activated platelets (Figure S1) show that the percentage of CD62P-expressing platelets had reached a maximum by 1 minute after TRAP stimulation but required 5 minutes for CD40L. This suggests that CD62P and CD40L do not share identical expression kinetics on platelet surface on TRAP-6 stimulation, which may explain the different sensitivity to P2Y12 inhibition observed.

3.5 | CD40L influences arterial thrombus formation on P2Y12 blockade

The importance of CD40L in hemostasis on platelet P2Y12 inhibition was evaluated by measuring thrombus formation from human whole blood treated with 2MesAMP flowed at arterial shear rate (50 dynes/cm^2) over collagen fibrils in the presence of a CD40L blocker, rhCD40-Fc (Figure 5). Experiments were performed in the presence of a FcγRIIa (CD32a) blocking antibody (clone IV.3) to avoid any platelet activation induced by the IgG1-Fc part of the rhCD40-Fc.

TABLE 4 Odds ratios of bleeding by factors studied in the ACS patient population in which pCD40L was measured

	Univariate	Multivariable
Independent variable	OR (95% CI)	aOR (95% CI)
Females	**0.43 (0.15-1.21)**	
Age	**0.98 (0.54-1.77)**	
BMI	1.21 (0.75-1.95)	
Hypertension	0.61 (0.23-1.61)	
Type 2 diabetes	0.44 (0.10-1.98)	
Current smoker	1.75 (0.66-4.61)	
EF	1.04 (0.63-1.69)	
STEMI	1.04 (0.36-3.04)	
NSTEMI	1.69 (0.58-4.90)	
Ischemic events	**6.59 (0.57-76.39)**	
Insulinemia	0.87 (0.40-1.87)	
HbA_{1c}	**0.60 (0.31-1.19)**	
CRP	0.74 (0.31-1.77)	
Triglycerides	0.91 (0.54-1.55)	
Cholesterol	1.10 (0.63-1.63)	
HDL	1.13 (0.71-1.80)	
LDL	1.01 (0.63-1.63)	
Hemoglobin	**0.65 (0.43-0.99)**	
Creatinine	0.85 (0.38-1.86)	
Platelets	0.79 (0.48-1.30)	
Clopidogrel	0.77 (0.29-2.02)	
Prasugrel	1.30 (0.49-3.43)	
PRI-VASP	**0.17 (0.06-0.45)**	**0.17 (0.06-0.48)**
Basal pCD40L	**0.17 (0.05-0.58)**	**0.15 (0.034-0.067)**
TRAP pCD40L	**0.38 (0.18-0.79)**	
Beta-blocker	1.84 (0.59-5.79)	
$GP_{IIbIIIa}$ inhibitor	0.87 (0.30-2.55)	
ACE inhibitor	2.07 (0.66-6.50)	
Calcium blocker	0.27 (0.03-2.11)	

Note: Data for all tested continuous parameters were standardized. Data in bold in the univariate analysis were used for the multivariable logistic regression. Robustness test of the multivariable analysis—Hosmer-Lemeshow test: χ^2 = 6.728, P = .566. Correlation coefficient bleeding events/VASP (Spearman test) = −0.261 (P < .0001). Logistic regression, dependent variable: bleeding events.
ACE, angiotensin-converting enzyme; aOR, adjusted odds ratio; BMI, body mass index; CAD, coronary artery disease; CI, confidence interval; CRP, C-reactive protein; EF, ejection fraction; GP, platelet glycoprotein; HDL, high-density lipoprotein; HbA_{1C}, glycosylated hemoglobin; LDL, low-density lipoprotein; NSTEMI, non–ST-elevation myocardial infarction; OR, odds ratio; pCD40L, platelet surface CD40L; PRI-VASP, platelet reactivity index–vasodilator-stimulated phosphoprotein; STEMI, ST-elevation myocardial infarction; TRAP, thrombin receptor-activating peptide.

As expected, 2MesAMP reduced thrombus formation. We observed that rhCD40-Fc did not modify the area covered by platelets after 5 minutes of perfusion but led to a delayed thrombus growth.

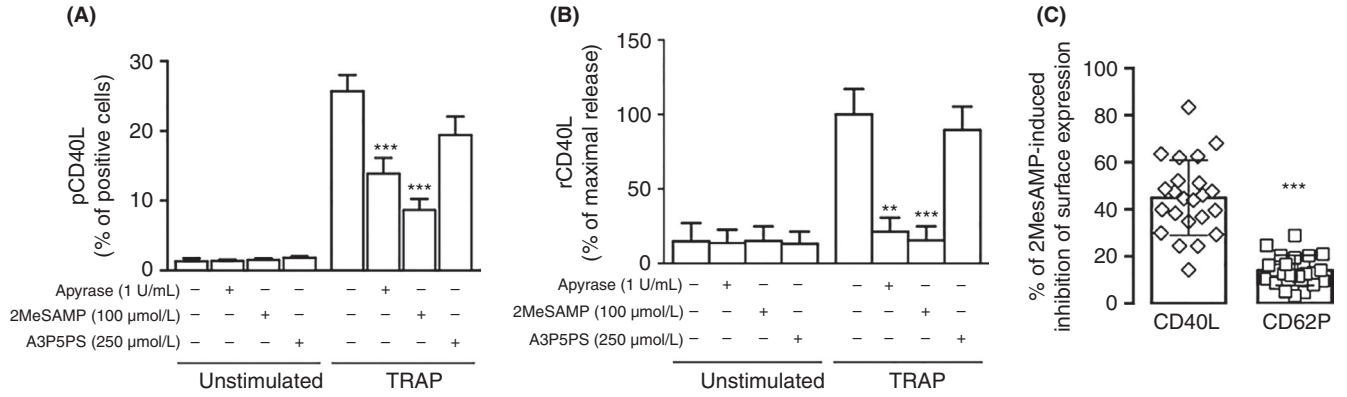

FIGURE 4 Effect of ADP degradation, P2Y12 inhibition and P2Y1 inhibition on pCD40L levels (platelet surface expression) (A) and rCD40L levels (released) (B) from healthy human platelets: Washed platelets from nontreated healthy volunteers were pretreated with either 1 U/mL of apyrase, 100 µmol/L 2MeSAMP, 250 µmol/L A3P5PS, or vehicle for 10 minutes. CD40L surface expression levels (pCD40L) were analyzed by flow cytometry, based on percentage of positive cells before (unstimulated) and after TRAP-14 stimulation (TRAP). Released CD40L (rCD40L) levels in the supernatant were assessed. The 100% rCD40L corresponds to the concentration of CD40L in the supernatant of platelets stimulated with 10 µmol/L TRAP-14 in the absence of inhibitors (vehicle). Data are expressed as mean ± standard error of the mean (SEM) for n = 5 (**P < .01; ***P < .001 vs control stimulated platelets). (C) Effect of 2MesAMP on healthy human platelet surface expression of CD40L and CD62P. Results are represented in a scatter plot with bars for the percentage of 2MesAMP-induced inhibition of CD40L and CD62P positive cells. Bars are expressed as mean ± SEM for n = 23 (***P < .001). ADP, adenosine diphosphate; TRAP, thrombin receptor-activating peptide

Remarkably, addition of rhCD40-Fc to 2MesAMP-treated samples further reduced the extent of platelet coverage compared to 2MesAMP treatment alone, arguing for a role for CD40L in thrombus formation on P2Y12 inhibition.

3.6 | Platelet-associated CD40L influenced hemostasis in mice treated with P2Y12 inhibitor

To test whether platelet-associated CD40L played a role in the regulation of bleeding, tail bleeding duration was assessed in wild-type mice treated with the P2Y12 inhibitor, ticagrelor (administered orally), and injected with sCD40L or platelets isolated from either $CD40L^{+/+}$ or $CD40L^{-/-}$ mice. Ticagrelor was shown to significantly prolong bleeding time and increase blood loss.[36] Thus, 6 hours after the second ticagrelor administration, we noted a significant lengthening of bleeding duration in all the tested animals (from 200 ± 8 to 470 ± 79 seconds; Figure 6A) and an inhibition of platelet aggregation induced by low doses agonists (ADP, 10 µmol/L; and AYPGKF, 100 µmol/L; Figure 6B). Injection of 80 µg sCD40L did not influence the bleeding duration in ticagrelor-treated animals. Interestingly, transfusion with 2 × 10^7 $CD40L^{+/+}$ platelets (1.25% of the total amount of circulating mouse platelets) resulted in a significant 30% reduction in bleeding duration (327 ± 41 seconds, P < .05). By contrast, transfusion of $CD40L^{-/-}$ platelets had no effect on bleeding duration, indicating that platelet-associated CD40L may play a protective role in bleeding in mice treated with ticagrelor.

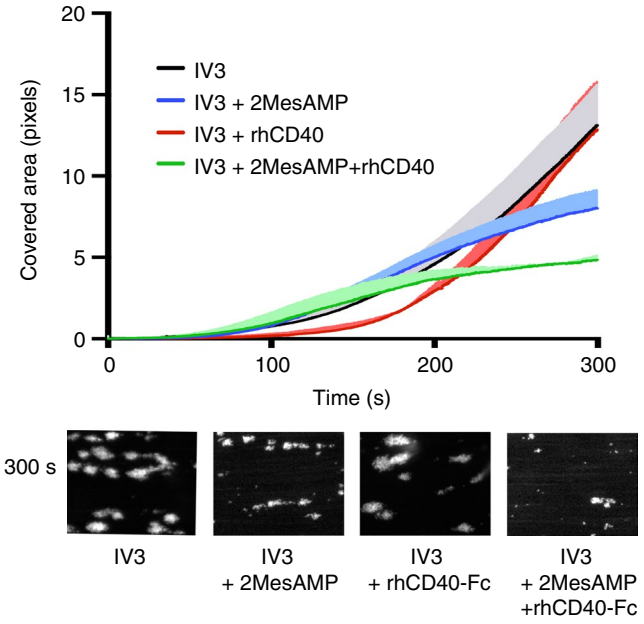

FIGURE 5 Adhesion under flow (50 dynes/cm^2) on collagen of calcein-AM–labeled healthy human platelets from healthy controls treated with the anti-FcγRIIa antibody (IV3, black line), IV3 + 100 µmol/L 2MesAMP (blue line), IV3 + 50 µg/mL rhCD40-Fc (red line), IV3 + 2MesAMP + rhCD40-Fc (green line). Percentage of covered area was assessed over 300 seconds. Results are expressed as mean (dark colors) and standard error of the mean (light colors, above) for n = 3. Below are representative images of the area covered by platelets at 300 seconds. rhCD40-Fc, recombinant human CD40-Fc

4 | DISCUSSION

Our study shows a correlation between platelet CD40L surface expression and release with the ADP/P2Y12 signaling pathway both in vitro and in vivo. We demonstrate that P2Y12 blockade markedly diminished the ability of platelets from ACS patients to express and release CD40L on activation as compared to healthy controls. These

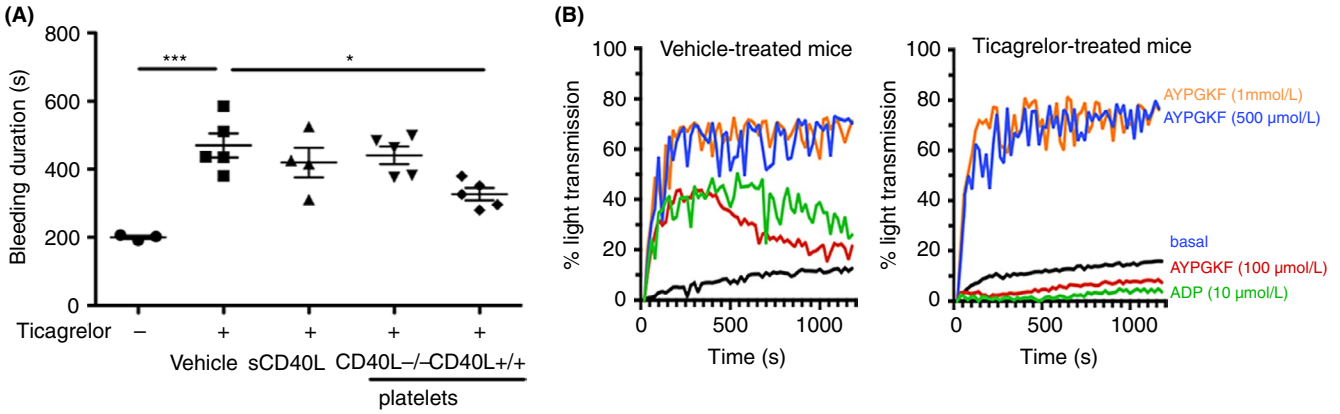

FIGURE 6 (A) Bleeding time assay from C57BL/6J wild-type mice were treated with ticagrelor (100 mg/kg per os) or water per os for 18 and 6 h prior to experimentation. Ticagrelor-treated mice were injected with sCD40L or transfused with the indicated platelet preparations (2×10^7 platelets/mouse). Tail bleeding duration was measured 5 min after platelet transfusion or rmCD40L injection. Data are expressed as mean ± standard error of the mean (*$P < .05$, ***$P < .001$). (B) aggregation analysis on platelets isolated from C57BL/6J wild-type mice treated with ticagrelor or water per os for 18 and 6 h prior to experimentation. Traces are representative of data obtained from 3 mice treated with vehicle and 3 receiving ticagrelor). rmCD40L, recombinant murine CD40L; sCD40L, plasma-soluble CD40L

new findings are important given the role of both platelet-associated and cleaved CD40L in controlling thrombus formation as demonstrated in atherosclerotic mice.[37]

Nevertheless our study has some limitations. The reduced pCD40L and rCD40L levels measured in the ACS patients compared to healthy individuals may have been due to age and sex differences between the control and case patients. However, in earlier studies of healthy populations, these 2 factors did not influence circulating levels of CD40L,[38,39] and we showed that adjusting data for age and sex still led to significantly reduced pCD40L and rCD40L levels in ACS patients. Also, residual confounding may be present if there were relevant covariates that were not considered or were imprecisely measured. Additionally, not all biomarkers (including sCD40L, pCD40L, and rCD40L) were measured in all participants, so precision was limited for some analyses. In particular, sCD40L was not measured in the populations where pCD40L and rCD40L were evaluated due to the constraints related to the experimental conditions for obtaining platelet solutions.

Our findings are evidence that P2Y12 inhibitors may preclude secondary thrombotic events by preventing platelet aggregation and reducing pCD40L and rCD40L levels. The importance of pCD40L and/or locally released rCD40L still remains to be clearly determined. Our data showing that blocking CD40L reduces arterial thrombus from healthy human blood samples treated or not treated with a P2Y12 inhibitor support this hypothesis. Additionally, CD40L deficiency reduces arterial thrombus formation in vivo,[10] impairs formation of large multilayered platelet aggregate atherogenic material,[37] and prevents microvascular thrombosis in mice challenged with lipopolysaccharides.[40]

Interestingly, pCD40L expression on healthy human platelets appears to be more sensitive to ADP/P2Y12 inhibition than CD62P expression. This suggests that although both are cargo proteins of alpha granules, CD62P and CD40L do not share the same expression kinetics on platelet surface upon TRAP stimulation. Accordingly, differential expression of CD40L and CD62P has been suggested in response to various agonist stimulations with probable separated cellular sublocalizations.[5,41] These observations and ours could reflect (a) differential packaging of CD40L and CD62P in morphologically distinct granules as demonstrated for 2 of the main alpha granule cargoes, fibrinogen and von Willebrand factor[42]; or (b) the existence in platelets of kinetically distinct classes of granule release events.[43] In this study, CD40L and CD62P secretions were found to belong to 2 kinetically distinct classes of secretion events. Further work is now needed to determine the exact mechanisms controlling CD40L and CD62P differential expression in platelets.

Compared with nonbleeders, patients with ACS who experienced bleeding events during P2Y12 antagonist therapy displayed significantly lower levels of pCD40L. Moreover, platelet-associated CD40L controlled tail bleeding in mice treated with ticagrelor and arterial thrombus formation upon P2Y12 inhibition. These results emphasize the potential of platelet-associated CD40L in hemostasis control mechanisms. Thus, the decrease in CD40L expression levels on platelets from ACS patients treated with P2Y12 inhibitor may represent an additional risk factor for bleeding complications. The underlying mechanism of platelet CD40L-mediated control of bleeding remains to be elucidated. Platelet-associated and platelet-released CD40L may directly affect platelet function, as they enhance collagen-induced platelet $\alpha_{IIb}\beta_3$ activation, secretion, and thrombus growth.[37] Platelet-associated CD40L also induces a procoagulant and proinflammatory state in the endothelium.[16,44–46] Furthermore, the function of platelet-associated CD40L may extend beyond the endothelium and platelets, as it is a key component of platelet-leukocyte crosstalk, potentiating the recruitment of leukocytes to the site of vascular injury.[47]

In conclusion, platelet CD40L may represent a key element of bleeding control mechanisms and an independent marker of bleeding risk in patients receiving P2Y12 inhibitor treatment. Evaluating platelet CD40L expression levels may help risk-stratify such patients threatened by bleeding events.

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RELATIONSHIP DISCLOSURE

The authors report nothing to disclose.

AUTHOR CONTRIBUTIONS

MC, CG, and DB performed research, helped design the research study, analyzed data, and helped write the paper. PD, KDB, MP, and DB performed research, analyzed data, and helped write the paper. MG performed the statistical analysis of the data and helped write the paper. DW, DD, TC, and M-CA helped design the research study, analyzed data, and helped write the paper.

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SUPPORTING INFORMATION

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