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TRANSLATIONAL SCIENCES

Cell Surface Platelet Tissue Factor Expression: Regulation by $P2Y_{12}$ and Link to Residual Platelet Reactivity

Marta Brambilla, Alessia Becchetti, Gian Enrico Rovati, Nicola Cosentino, Maria Conti, Paola Canzano, Peter L.A. Giesen, Alessia Loffreda, Alice Bonomi, Marco Cattaneo, Erica De Candia, Gian Marco Podda, Daniela Trabattoni, Pablo Josè Werba, Jeness Campodonico, Christian Pinna, Giancarlo Marenzi, Elena Tremoli, Marina Camera

BACKGROUND: ADP-induced platelet activation leads to cell surface expression of several proteins, including TF (tissue factor). The role of ADP receptors in platelet TF modulation is still unknown. We aimed to assess the (1) involvement of $P2Y_1$ and $P2Y_{12}$ receptors in ADP-induced TF exposure; (2) modulation of TF^{pos}-platelets in anti-$P2Y_{12}$–treated patients with coronary artery disease. Based on the obtained results, we revisited the intracellular localization of TF in platelets.

METHODS: The effects of $P2Y_1$ or $P2Y_{12}$ antagonists on ADP-induced TF expression and activity were analyzed in vitro by flow cytometry and thrombin generation assay in blood from healthy subjects, $P2Y_{12}^{-/-}$, and patients with gray platelet syndrome.

Ex vivo, $P2Y_{12}$ inhibition of TF expression by clopidogrel/prasugrel/ticagrelor, assessed by VASP (vasodilator-stimulated phosphoprotein) platelet reactivity index, was investigated in coronary artery disease (n=238). Inhibition of open canalicular system externalization and electron microscopy (TEM) were used for TF localization.

RESULTS: In blood from healthy subjects, stimulated in vitro by ADP, the percentage of TF^{pos}-platelets (17.3±5.5%) was significantly reduced in a concentration-dependent manner by $P2Y_{12}$ inhibition only (–81.7±9.5% with 100 nM AR-C69931MX). In coronary artery disease, inhibition of $P2Y_{12}$ is paralleled by reduction of ADP-induced platelet TF expression (VASP platelet reactivity index: 17.9±11%, 20.9±11.3%, 40.3±13%; TF^{pos}-platelets: 10.5±4.8%, 9.8±5.9%, 13.6±6.3%, in prasugrel/ticagrelor/clopidogrel-treated patients, respectively). Despite this, 15% of clopidogrel good responders had a level of TF^{pos}-platelets similar to the poor-responder group. Indeed, a stronger $P2Y_{12}$ inhibition (130-fold) is required to inhibit TF than VASP. Thus, a VASP platelet reactivity index <20% (as in prasugrel/ticagrelor-treated patients) identifies patients with TF^{pos}-platelets <20% (92% sensitivity). Finally, colchicine impaired in vitro ADP-induced TF expression but not α-granule release, suggesting that TF is open canalicular system stored as confirmed by TEM and platelet analysis of patients with gray platelet syndrome.

CONCLUSIONS: Data show that TF expression is regulated by $P2Y_{12}$ and not $P2Y_1$; $P2Y_{12}$ antagonists downregulate the percentage of TF^{pos}-platelets. In clopidogrel good-responder patients, assessment of TF^{pos}-platelets highlights those with residual platelet reactivity. TF is stored in open canalicular system, and its membrane exposure upon activation is prevented by colchicine.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: blood platelets ■ coronary artery disease ■ $P2Y_{12}$ antagonists ■ $P2Y_{12}$ receptor ■ thromboplastin

The prothrombotic properties of platelets are the result of their ability to expose phosphatidylserine, which promotes the assembly of coagulation factors and TF (tissue factor). TF is the key protein triggering the coagulation cascade mostly localized at perivascular level where it provides the hemostatic envelope to prevent

Correspondence to: Prof Marina Camera, Department of Pharmaceutical Sciences, Università degli Studi di Milano, via Balzaretti, 9, Milan 20133, Italy. Email marina.camera@unimi.it

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Nonstandard Abbreviations and Acronyms

aGPIIbIIIa	activated glycoprotein IIbIIIa
CAD	coronary artery disease
GPIIbIIIa	glycoprotein IIbIIIa
GPS	gray platelet syndrome
HS	healthy subject
OCS	open canalicular system
PDI	protein disulphide isomerase
PI3	phosphoinositide 3
PLC	phospholipase C
PRI	platelet reactivity index
TF	tissue factor
TG	thrombin generation
VASP	vasodilator-stimulated phosphoprotein

Highlights

- ADP-induced TF (tissue factor) expression on the platelet surface is regulated by $P2Y_{12}$ receptor activation only.
- In patients with coronary artery disease, $P2Y_{12}$ antagonists hamper ADP-induced cell surface exposure of open canalicular system–stored TF to a different extent (prasugrel=ticagrelor>clopidogrel).
- Reduction of ADP-induced platelet-associated TF exposure occurs with a $P2Y_{12}$ inhibition much greater than that required to reduce VASP (vasodilator-stimulated phosphoprotein) platelet reactivity index, P-selectin, and GPIIbIIIa (glycoprotein IIbIIIa).
- Cell surface platelet TF expression identifies patients with coronary artery disease who, despite a good clopidogrel response, show a residual platelet reactivity in terms of prothrombotic potential.

bleeding.[1] Upon activation with ADP, TXA_2 (thromboxane A2), or thrombin, platelets expose on their surface a functionally active TF, which binds factor VIIa thereby promoting generation of subnanomolar concentration of thrombin.[2] Thrombin in turn causes further platelet activation via protease-activated receptors that amplifies thrombin formation to achieve hemostasis.[3,4]

Over the years, increase in the number of prothrombotic TF^{pos}-platelets in different thrombophilic conditions has been extensively described,[5–9] including in SARS-CoV-2 infection, where we recently showed that platelet-associated TF expression was controlled by antiplatelet drugs, especially clopidogrel.[10]

Clopidogrel, prasugrel, and ticagrelor, all used in the treatment of patients with coronary artery disease (CAD), antagonize ADP, one of the several platelet agonists. ADP is stored at high concentrations in platelet dense granules, and, when secreted, it enables platelet activation and aggregation by binding to 2 purinergic receptors on the platelet surface: $P2Y_1$, which triggers platelet activation, and $P2Y_{12}$, which amplifies the process potentiating secretion of molecules stored in platelet granules such as P-selectin.[11,12] Since secreted ADP enhances platelet responses to many physiological agonists including TXA_2, collagen, and thrombin, mainly through $P2Y_{12}$,[13] this receptor has become a major pharmacological target, together with cyclooxygenase-1, for the management of diseases associated with arterial thrombosis.[14]

Interestingly, unlike the regulation of platelet adhesion and aggregation, the 2 P2Y receptors are differently involved in the prothrombotic activity of platelets. Indeed, inhibition of $P2Y_{12}$ only, and not of $P2Y_1$, significantly reduces phosphatidylserine exposure.[15] Whether this occurs also for platelet-associated TF is currently unknown. Since previous studies showing that TF is localized both in α-granules and in the open canalicular system (OCS) of resting platelets,[16,17] it can be hypothesized that both $P2Y_1$ and $P2Y_{12}$ receptors regulate TF expression similarly to the α-granule–stored P-selectin.[15] To date, however, this evidence is lacking.

Based on this rationale, the aim of the study was to assess the involvement of $P2Y_1$ and $P2Y_{12}$ receptors in ADP-induced TF expression on the platelet surface. For this purpose, we also took advantage of the analysis of platelets from a $P2Y_{12}$-deficient patient.

Since our data showed the unique involvement of $P2Y_{12}$ in platelet-associated TF expression, 2 additional aims were pursued in this study. First, we assessed in vivo TF by $P2Y_{12}$ antagonists in platelets of patients with CAD, establishing the relationship, if any, between TF expression and the residual platelet reactivity, a condition known to be significantly associated with recurrent cardiovascular events.[18] Second, we reanalyzed the cytoplasmic localization of TF to shed light on how a protein reported to be stored both in the α-granules and in OCS could be regulated by $P2Y_{12}$ only. This was accomplished by means of a pharmacological approach using cytoskeleton blockers such as cytochalasin D and colchicine and taking advantage from analysis of platelets from patients with gray platelet syndrome (GPS). TF intracellular localization was finally evaluated by immune gold transmission electron microscopy.

METHODS

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

Study Population

In vitro experiments were performed on 10 healthy subjects (HS; n=5 men and n=5 women; mean age, 39±6 years) who did not take antiplatelet drugs within 10 days before blood

withdrawal. The effect of clopidogrel on platelet-associated TF expression was assessed on 193 patients with CAD with non–ST-segment–elevation myocardial infarction admitted to Centro Cardiologico Monzino IRCCS from January 2017 to December 2019. Clinical characteristics of the enrolled patients are reported in Table S1. All patients were on aspirin (100 mg/die) and clopidogrel (75 mg/die) and blood was withdrawn between 4 and 30 days from the index event. Exclusion criteria were age >80 years, valvular heart disease, atrial fibrillation, thyrotoxicosis, history of hemorrhagic diathesis, platelet disorder or thrombocytopenia, and thrombolytic treatment. Consecutive patients on prasugrel treatment (n=34; 10 mg/die for at least 4 days) or on ticagrelor treatment (n=31; 90 mg/bis in die for at least 4 days) were recruited for comparison. Patients with CAD not treated with $P2Y_{12}$ antagonists (n=39) were enrolled as controls. Two patients with GPS (males, aged 31 and 15 years), previously confirmed by mutational screening of NBEAL2,[19,20] and a subject with inherited severe $P2Y_{12}$ deficiency (male, aged 65 years)[21] were also studied.

The study was approved by the ethical committee of the institution, and written informed consent was obtained from all participants according to the principles of the Declaration of Helsinki.

Blood Collection and Platelet Isolation

Whole blood was drawn with a 19-gauge needle without venous stasis into sodium citrate (0.129 M, 1/10 volume/volume)–containing tubes (Vacutainer, catalog number 367704; Becton Dickinson), discarding the first 4 mL. Blood samples were processed within 15 minutes of blood drawing. Please see the Supplemental Material for extended experimental procedures.

Flow Cytometry Analysis

TF, P-selectin, activated GPIIbIIIa (glycoprotein IIbIIIa; aGPIIbIIIa), CD36, CD40L, $P2Y_{12}$, and $P2Y_1$ expression was evaluated by whole blood flow cytometry. Please see the Supplemental Material for extended experimental procedures.

Thrombin Generation and FXa Generation

TF activity was assessed in thrombin generation (TG) assay, performed by calibrated automated thrombogram. TG was run without adding any exogenous TF but using the platelet-associated TF as the only trigger, as described previously.[22] FXa (factor Xa) generation was also assessed. Please see the Supplemental Appendix for extended experimental procedures.

Electron Microscopy

Platelet TF intracellular localization was analyzed by immunogold electron microscopy. Please see the Supplemental Material for extended experimental procedures.

In Vitro Studies

Whole blood was incubated with AR-C69931MX (Cangrelor, 1 pM to 100 nM, catalog number 5720/1; Bio-Techne srl) or MRS-2500 (1 pM to 100 nM, catalog number 2159/1; Bio-Techne srl) for 30 minutes at room temperature (timing that, in preliminary time course experiments, provided maximal and repeatable inhibition). Platelets (1×10^6) were then stimulated with ADP (10 µM, catalog number A2754; Sigma-Aldrich), U46619 (1 µM, catalog number 538944; Sigma-Aldrich), or TRAP-6 (thrombin receptor-activating peptide; 10 µM, catalog number 4017752; Bachem) for 15 minutes at room temperature. Concentration-response curves of TF, P-selectin, and aGPIIbIIIa expression and VASP (vasodilator-stimulated phosphoprotein) platelet reactivity index (PRI) were obtained by flow cytometry analysis after stimulation of human platelets with ADP (10^{-8}–10^{4} M, for 15 minutes), with or without a 30-minute preincubation with AR-C69931MX (10^{-12}–10^{-5} M).

To assess the involvement of actin and tubulin polymerization in TF and P-selectin exposure, platelets were preincubated for 15 minutes at 37 °C with cytochalasin D (10 µM, catalog number C8273; Sigma-Aldrich) and colchicine (20 nM to 100 µM, catalog number C9754; Sigma-Aldrich), respectively, and then stimulated for 15 minutes at room temperature with ADP (10 µM). Treatment of platelets with *Bordetella* pertussis toxin (5 µg/mL for 30 minutes, catalog number P7208; Sigma-Aldrich) or with the specific PLC (phospholipase C) inhibitor U73122 (1 mM for 30 minutes, catalog number U6756; Sigma-Aldrich) was performed to characterize the Gi signaling involved in ADP-induced TF and P-selectin exposure. Preincubation with LY294002 (10 µM for 30 minutes, catalog number 440202; Sigma-Aldrich) was used to assess PI3 (phosphoinositide 3) kinase pathway involvement.

Statistical Analysis

Sample Size Calculation

Assuming that patients with CAD have a mean value of TF^{pos}-platelets of 19.4±4.5% and that the ratio between good responder and poor responder to clopidogrel treatment is 4:1, a sample of 190 patients (152 good responders and 38 poor responders) was estimated to provide 80% power to deem as significant (α=0.05) a group difference of 2.3% of TF^{pos}-platelets, with an SD of 4.5. The sample size of prasugrel- and ticagrelor-treated patients' groups was estimated based on the mean difference value of platelet-associated TF expression in patients treated or not with clopidogrel. A sample of 65 patients was estimated to provide 90% power to deem as significant (α=0.05) a difference between groups. Thus, 34 consecutive patients treated with prasugrel and 31 patients treated with ticagrelor were enrolled.

Statistical Analysis

Results are expressed as mean±SD and were analyzed by the Student paired *t* test or Mann-Whitney *U* test. Differences between patient groups were performed using 1-way ANOVA followed by Dunnet post hoc analysis. The receiver operating characteristic curve was used to determine the optimal cut-off value of VASP-PRI to detect a percentage of TF-positive platelets ≤20%. A *P* value of 0.05 was considered statistically significant. Analyses were performed using SPSS statistical package (v9.4). Concentration-response curves were evaluated using GraphPad Prism 8. pA2s were calculated accordingly to the equation set as described in Prism 8. Parameter errors are expressed as percentage coefficient of variation and calculated by analysis of 4 to 11 different independent experiments. All curves shown are computer generated.

RESULTS

$P2Y_{12}$ Receptor Is Critical for ADP-Induced Platelet-Associated TF Expression and TG

To assess the contribution of the 2 purinergic receptors to the surface expression of TF, whole blood from HS was stimulated in vitro with ADP in the presence or absence of a selective $P2Y_1$ (MRS-2500) or $P2Y_{12}$ (AR-C69931MX) antagonist. ADP induced an almost 7-fold increase in the number of TF-positive platelets (Figure 1A) and a 2-fold increase in the expression of the protein/platelet (mean fluorescence intensity; Figure 1B).

Platelet treatment with MRS-2500, at any tested concentration, did not affect either TF exposure (Figure 1A and 1B) or its activity measured as TG capacity (Table 1), indicating that $P2Y_1$ is not involved in the regulation of platelet-associated TF expression. Conversely, AR-C69931MX concentration dependently prevented ADP-induced TF expression, both in terms of the number of TF-positive cells and mean protein expression, with a 60% inhibition with 10 nM becoming almost complete with 100 nM (Figure 1C and 1D). Similarly, the $P2Y_{12}$ antagonist concentration dependently delayed ADP-induced TG time, which was significantly impaired by 10 and 100 nM AR-C69931MX (Figure 1E). To demonstrate the direct contribution of platelet TF to TG, the test was performed after preincubation of platelets with a neutralizing αTF antibody. Under these conditions, thrombin generated by platelets was impaired. Of note, the effect of the antibody was the greatest in ADP-stimulated samples, and thus in the presence of a greater number of TF^{pos}-platelets, and was progressively reduced in a concentration-dependent manner in the presence of AR-C69931MX, as the expression of TF decreased (Figure S1).

Of note, the $P2Y_{12}$ antagonism did not significantly affect the amount of thrombin produced (endogenous thrombin potential; Table 1) when assessed in the presence of an excess of exogenously added phospholipids—to make TF the only rate-limiting contributor of thrombin formation—thus supporting the role of TF primarily in the early kinetic phase of the TG. Conversely, when platelet TG was assessed in a more physiological setting, that is, without any addition of phospholipids—thus making it dependent on both the cell surface platelet TF and the phospholipid surface—inhibition of $P2Y_{12}$ receptor resulted in both a decrease in the kinetic of thrombin formation (lag time: 31.28±2.43 versus 35.22±3.66 minutes, in control and AR-C69931MX–treated samples, respectively; P=0.05) and a reduction in the concentration of generated thrombin (peak: 76.31±11.7 versus 59.13±5.21 nmol/L and endogenous thrombin potential: 1402±93 versus 1312±40.6 nMxmin, in control and AR-C69931MX–treated samples, respectively; P=0.03 for both; Figure S2). FXa generation experiments, performed as an alternative approach to evaluate the modulation of platelet TF by AR-C69931MX, confirmed the reduction of TF activity when platelets were treated with the $P2Y_{12}$ antagonist but not with the $P2Y_1$ inhibitor MRS-2500 (−19.5% and +1%, with the $P2Y_{12}$ and $P2Y_1$ antagonists, respectively). The specific contribution of TF to FXa generation was evaluated performing the assay in the presence of a neutralizing αTF antibody. Under these conditions, FXa generation was reduced by 20%.

Modulation of TF expression can occur through multiple pathways, such as those induced by thrombin and thromboxane, as shown previously.[2,23] On this regard, TF expression induced by TRAP-6 (+12-fold) and U46619 (+13-fold) was also partially, although significantly, affected by AR-C69931MX, highlighting the enhancing effect of ADP on the platelet response to PAR-1 agonist peptide thrombin and the thromboxane analog U46619 (Figure 1F and 1G), as previously reported.[22]

To confirm the exclusive involvement of $P2Y_{12}$ in TF expression, we analyzed the blood of a $P2Y_{12}$-deficient patient.[21] As expected, $P2Y_{12}$ expression, assessed by flow cytometry analysis, was lower in the proband than in HS, while $P2Y_1$ expression was comparable (Figure 1H and 1I). Of note, upon stimulation with ADP, no increase of TF expression on the platelet surface was observed in the $P2Y_{12}$-deficient patient (Figure 1J and 1K). Conversely, it was readily detectable after stimulation with TRAP-6 (+5-fold) or U46619 (+4-fold). As expected, based on the in vitro data, inhibition of the $P2Y_1$ receptor, which unlike $P2Y_{12}$ is normally expressed in this subject's platelets, did not affect TF expression by any of the tested agonists (Figure 1J and 1K). Finally, intraplatelet TF levels were similar in the $P2Y_{12}$-deficient patient and in HS (Figure 1L and 1M) sustaining a comparable TG (Figure 1N). The delay exerted by the neutralizing anti-TF antibody was also comparable between samples from the $P2Y_{12}$-deficient patient and HS (+3.9 and +3.8±2.7 minutes, respectively).

Altogether, these results highlight that $P2Y_{12}$, and not $P2Y_1$, is involved in the regulation of ADP-induced TF exposure on the platelet surface, and this event is prevented by selective pharmacological inhibition of the $P2Y_{12}$ receptor.

$P2Y_{12}$ Antagonists Reduce Platelet-Associated TF Expression in Patients With CAD

Since the in vitro data showed that AR-C69931MX concentration dependently inhibited TF expression, it can be expected that a similar inhibition of cell surface platelet-associated TF expression in vivo is displayed by anti-$P2Y_{12}$ drugs that are used in the clinical setting. To test this hypothesis, we first analyzed the association between cell surface platelet-associated TF expression and the degree of $P2Y_{12}$ inhibition, measured by the VASP phosphorylation assay, in clopidogrel-treated

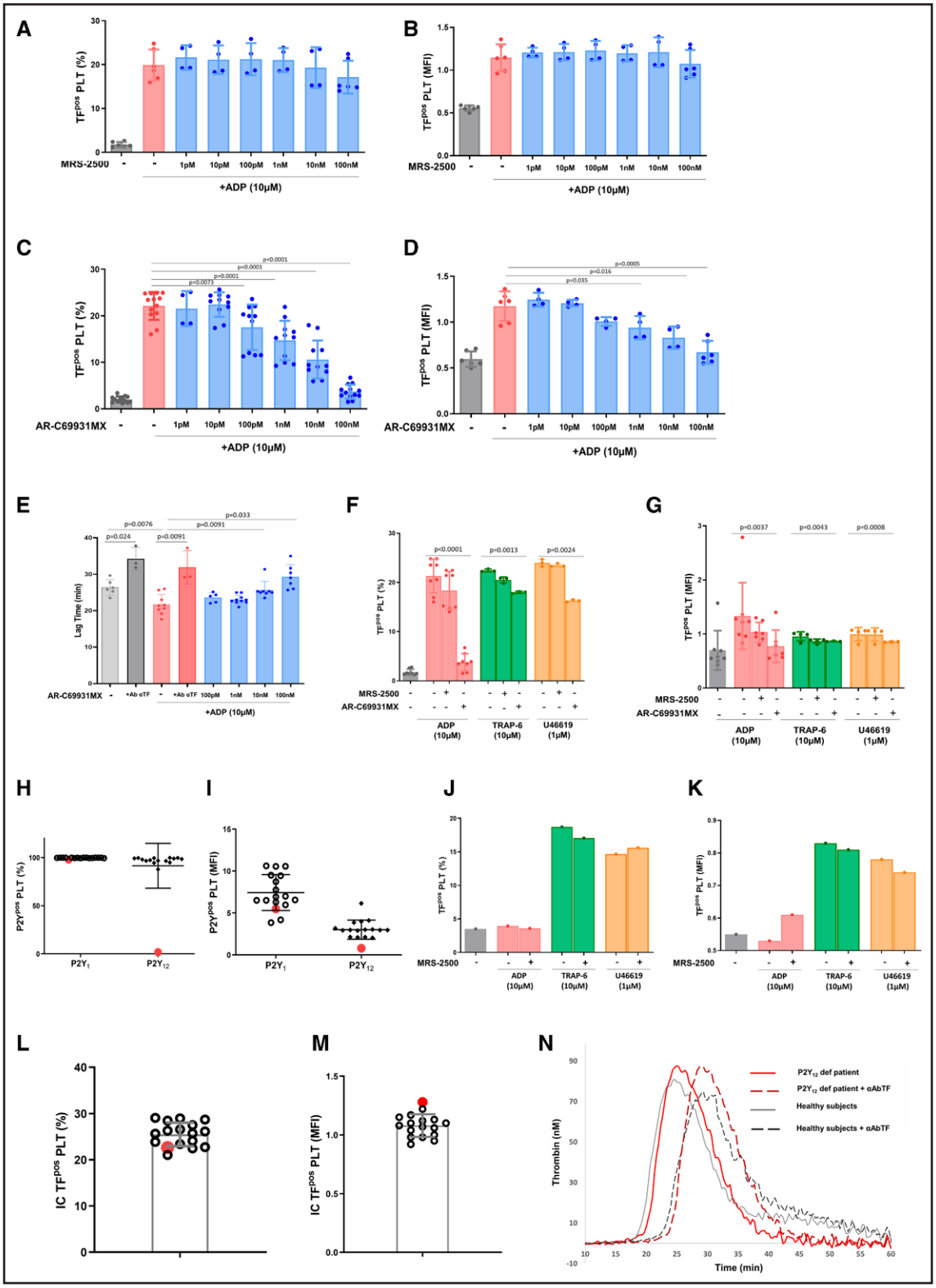

Figure 1. Effect of P2Y receptor inhibition on platelet-associated TF (tissue factor) expression and activity in healthy subjects (HS) and in a P2Y$_{12}$-deficient patient.

Platelet-associated TF expression was analyzed by whole blood flow cytometry while TF-dependent thrombin generation was measured in isolated platelets by calibrated automated thrombogram (CAT) assay, in HS (**A**–**G**) and in a P2Y12-deficient patient (**H**–**N**). Effect of the P2Y$_1$ antagonist MRS-2500 and of P2Y$_{12}$ antagonist AR-C69931MX (blue bars), incubated 30 minutes before ADP stimulation, on platelet-associated TF expression (**A** and **C**, percentage of positive platelets; **B** and **D**, mean fluorescence intensity [MFI]) and on the lag time of thrombin generation (**E**) in HS (n=6). The specificity of TF contribution to thrombin generation was evaluated by preincubating resting and ADP-stimulated platelets with a neutralizing anti-TF antibody. AR-C69931MX and MRS-2500 effects on TF expression were (*Continued*)

patients with CAD. The degree of VASP phosphorylation is indeed associated with levels of clopidogrel active metabolite,[24] allowing the classification of patients as good responders when the platelet reactivity index is <60%.[25] Results show that the percentage of TF-positive platelets was significantly inhibited (–30% versus ADP-stimulated samples) in good responders only, whereas in the poor responders, it was similar to that measured in patients with CAD not on clopidogrel (Figure 2A and 2B; Table S2). Comparable results were obtained when phosphatidylserine exposure on the platelet membrane was assessed under resting conditions in a subgroup of patients (Figure S3). Interestingly, while inhibition of aGPIIbIIIa showed a trend similar to that of TF, with a ≈40% inhibition in good responder (Figure 2C; Table S2), clopidogrel significantly inhibited P-selectin expression to the same extent in all patients with CAD (–55%), irrespective of the degree of VASP phosphorylation (Figure 2D; Table S2).

These results are consistent with the evidence that the estimated in vitro AR-C69931MX potency (pA2) for inhibition of P-selectin expression is ≈130× greater than that needed to inhibit TF. Inhibition of aGPIIbIIIa and VASP occurs at the same potency, which is 4-fold smaller than that for P-selectin and 40-fold greater than that for TF (8.6±0.3% CV for TF, 10.2±0.1% CV for aGPIIbIIIa, 10.3±0.3% CV for VASP, and 10.8±0.3% CV for P-selectin; Figure 2E through 2H).

Thus, to inhibit cell surface TF expression and, therefore, the platelet prothrombotic potential, drug concentration has to be much higher than that needed to inhibit the exposure of the classical platelet activation markers such as P-selectin and aGPIIbIIIa, as well as VASP.

Of interest, 23 of 144 good-responder patients, accounting for 15% of them, had a percentage of TF-positive platelets greater than the mean value (20.4±8.4%) measured in the poor-responder group (Figure 2A, green dots). This percentage even doubles (reaching 38%) if the mean amount of protein expressed on the platelet surface is considered (Figure 2B, green dots). This finding highlights the presence of a subgroup of patients with a potentially increased TG capacity despite an apparent good pharmacological response to clopidogrel according to the VASP-PRI (although it was significantly higher in the subgroup compared with the good responder patients, 45.9±10.9% and 39.0±13.1%, respectively; P=0.0192) and P-selectin and aGPIIbIIIa expression (Figure S4).

To gain insight into this finding, we evaluated the expression of cell surface platelet-associated TF in patients treated with prasugrel, an irreversible $P2Y_{12}$ receptor inhibitor, or with ticagrelor, a reversibly binding $P2Y_{12}$ antagonist. All enrolled patients were good responders with a mean VASP-PRI value significantly lower than that of clopidogrel good-responder patients (17.9±11%, 20.9±11.3% versus 40.3±13%, in prasugrel-, ticagrelor-, and clopidogrel-treated patients, respectively; Figure 3A). After stimulation with ADP, the percentage of TF^{pos}-platelets was significantly lower than that measured in patients treated with clopidogrel (10.5±4.8%, 9.8±5.9%, and 13.6±6.3% in prasugrel-, ticagrelor-, and clopidogrel-treated patients, respectively) and, unlike what was observed in the clopidogrel group, no subject showed levels of TF^{pos}-platelets higher than the mean value (20.4±8.4%) of the clopidogrel poor-responder group. In addition, significantly lower levels of P-selectin and aGPIIbIIIa were also observed in these patients compared with those of clopidogrel-treated patients (Figure 3B through 3D), highlighting an overall higher degree of inhibition of platelet activation by prasugrel and ticagrelor than by clopidogrel.

Based on these observations, receiver operating characteristic curve was used to determine the optimal cutoff value of VASP-PRI to detect a percentage of TF^{pos}-platelets <20%, the mean value of TF^{pos}-platelets measured in poor-responder patients. This relationship was evaluated both on platelets treated in vitro with AR-C69931MX and on platelets from clopidogrel-treated patients. While in vitro a VASP-PRI of 60% was identified as the matched point to have TF^{pos}-platelet levels lower than 20% (area under the curve, 0.986 [95% CI, 0.959–1.000]; P<0.0001; sensitivity, 95%; specificity, 95%), in vivo only a VASP-PRI <20% identifies with a 92% sensitivity (and 23% specificity) patients with a TF^{pos}-platelet level <20%. As observed in the enrolled patients, such a low VASP-PRI value is better obtained with ticagrelor- and prasugrel-treated patients.

TF Is Localized in the OCS

The finding that $P2Y_{12}$ only is involved in the regulation of ADP-induced TF exposure on the platelet surface, together with the evidence of the different degrees of inhibition of P-selectin and TF expression by $P2Y_{12}$ inhibition, led us to hypothesize that different pathways or cellular localization are involved in the membrane

Figure 1 Continued. evaluated also upon TRAP-6 (thrombin receptor-activating peptide) and U46619 stimulation (**F** and **G**; n=3). Platelet purinergic receptor expression was analyzed by flow cytometry in a $P2Y_{12}$-deficient patient (red dot) in comparison with a reference group of HS (**H**: percentage of positive platelets; **I**, MFI). Platelet-associated TF expression upon stimulation with ADP, TRAP-6, and U46619 in the presence or absence of the $P2Y_1$ receptor antagonist MRS-2500 in $P2Y_{12}$-deficient patient (**J** and **K**). TF intracellular expression (**L**, percentage of positive platelets; **M**, MFI; red dot, $P2Y_{12}$-deficient patient). Thrombin generation of untreated platelets from the $P2Y_{12}$-deficient patient (red lines) was assessed in comparison to that of n=3 HS (**N**). Curves generated by preincubating platelets with a neutralizing αTF antibody to evaluate TF-dependent thrombin formation are also reported. Data are expressed as mean±SD from the indicated numbers of independent experiments and were analyzed by Student paired *t* test or Mann-Whitney *U* test as appropriate. IC indicates intracellular; and PLT, platelets.

Table 1. Effect of AR-C69931MX and MRS-2500 on ADP-Induced Thrombin Generation by Platelets

ADP	–	+	+	+
MRS-2500	–	–	+	–
AR-C69931MX	–	–	–	+
Lag time, min	27.9±9.3	24.4±6.5*	18.1±1	32.4±8.8†
ETP, nM·min	1182.5±267.8	1263.7±303.1	1579.7±101.2	1025.9±343.2
ttPeak, min	33.6±8.7	27.9±7.9‡	26.9±1.7	40.5±7.4§
Velocity index, nM/min	15.1±2.2	17.8±4.5	12.7±2.1	13.1±7.1

Values are mean±SD of 5 independent experiments performed in triplicate. ETP indicates endogenous thrombin potential; and ttPeak, time to peak.
*P=0.049 vs resting platelets;
†P=0.0073 vs ADP-stimulated platelets;
‡P=0.050 vs resting platelets;
§P=0.0067 vs ADP-stimulated platelets.

exposure of the 2 proteins. Indeed, unlike what was observed for TF, the membrane exposure of P-selectin, a protein stored in platelet α-granules, was significantly modulated by both $P2Y_1$ and $P2Y_{12}$ (Figure S5). Engagement of $P2Y_{12}$ receptor implies the activation of Gi-mediated intracellular signaling pathways. Treatment with *Bordetella* pertussis toxin, by inhibiting the Giα-mediated signaling (Figure S6A and S6B), or with LY294002, affecting PI3 kinase signaling, downstream Giβγ activation (Figure S6E and S6F), reduced ADP-dependent TF expression. Under these experimental settings, P-selectin expression was not affected (Figure S6C, S6D, S6G, and S6H), further highlighting $P2Y_1$ involvement. As expected, U73112-mediated inhibition of PLC, the common enzyme to both the $P2Y_1$ and $P2Y_{12,}$ triggered signal mediating intracellular calcium increase, led to a reduction of both TF and P-selectin exposure in response to ADP stimulation (Figure S6I through S6L). Altogether, these results confirm that the mechanism underpinning the membrane expression of TF and P-selectin, upon platelet activation, operates through different signaling pathways according to the receptors involved; they also suggest that TF is not stored in the α-granules.

We tested this hypothesis in patients with GPS, a disease characterized by large platelets with reduced number of α-granules. As expected, indeed, flow cytometry analysis showed that levels of P-selectin, exposed on the platelet surface after stimulation with ADP, TRAP-6, or U46619, as well as its intracellular levels, were considerably reduced in patients with GPS compared with HS (Figure 4A and 4B). As a direct functional consequence, the number of platelet-monocyte and platelet-granulocyte aggregates was also reduced in these patients compared with HS (Figure 4C and 4D). Conversely, of note, cell surface expression of platelet-associated TF in patients with GPS, both in resting conditions and upon stimulation with classical platelet agonists, was comparable, despite the α-granule defect, to that of HS. Not only the number of platelets exposing TF on the cell surface (Figure 4E) but also the number of TF-positive platelets by intracellular staining (Figure 4F) was similar in patients with GPS and in HS. Moreover, cell surface TF expression was also regulated by the same $P2Y_{12}$ downstream pathway, being the preincubation with pertussis toxin and LY294002 able to prevent ADP-induced TF expression (Figure 4G and 4H) while not affecting the already low P-selectin expression (Figure 4I and 4J).

This compelling evidence that TF is not stored in α-granules led us to verify its association with OCS, a further proposed intracellular platelet storage site. Immunogold labeling and electron microscopy analysis confirmed that in resting platelets from HS, TF is associated with the OCS rather than with α-granules (Figure 5). Of note, cell surface TF expression upon platelet stimulation is the result of OCS externalization. Preincubation of platelets with cytochalasin D, which blocks actin polymerization, resulted indeed in a statistically significant reduction of ADP-induced TF expression (Figure 6A and 6B), without affecting its intracellular levels (Figure 6C and 6D). Similar data were obtained upon stimulation of platelets by other stimuli, such as TRAP-6 or U46619 (data not shown). Of note, cytochalasin D treatment did not prevent P-selectin (Figure 6E and 6F) and CD36 (Figure 6G and 6H) exposure, which are stored within αgranules, nor that of CD40L, a cytoplasmic protein[26] (Figure 6I and 6J), while it reduced the surface recruitment of aGPIIbIIIa (Figure 6K and 6L) as published previously by Addo et al.[27] Also microtubule disruption by colchicine concentration dependently prevented ADP-induced TF externalization (Figure 7A and 7B). Similarly to what observed using cytochalasin D, colchicine did not affect P-selectin exposure, inhibiting α-granule release only at the highest concentration tested (Figure 7C and 7D), while reducing aGPIIbIIIa membrane expression (Figure 7E and 7F). The same results were also obtained when we analyzed ADP-stimulated platelets from patients with GPS (Figure S7) or TRAP-stimulated platelets from $P2Y_{12}$-deficient patients (Figure S8).

Altogether, these results suggest that in our experimental conditions, cell surface TF expression is not associated with platelet degranulation but rather with OCS externalization.

DISCUSSION

The present study, by using in vitro and ex vivo experiments combining a cell biology, pharmacological, and electron microscopy approach, provides new insights into the regulation and cellular localization of platelet-associated TF expression, thus expanding the characterization of the platelet prothrombotic properties. These findings, relevant also for patients with CAD treated with $P2Y_{12}$ antagonists, were supported by 2 human models of platelet defects such as $P2Y_{12}$ deficiency and GPS. Results show for the first time that ADP-induced TF

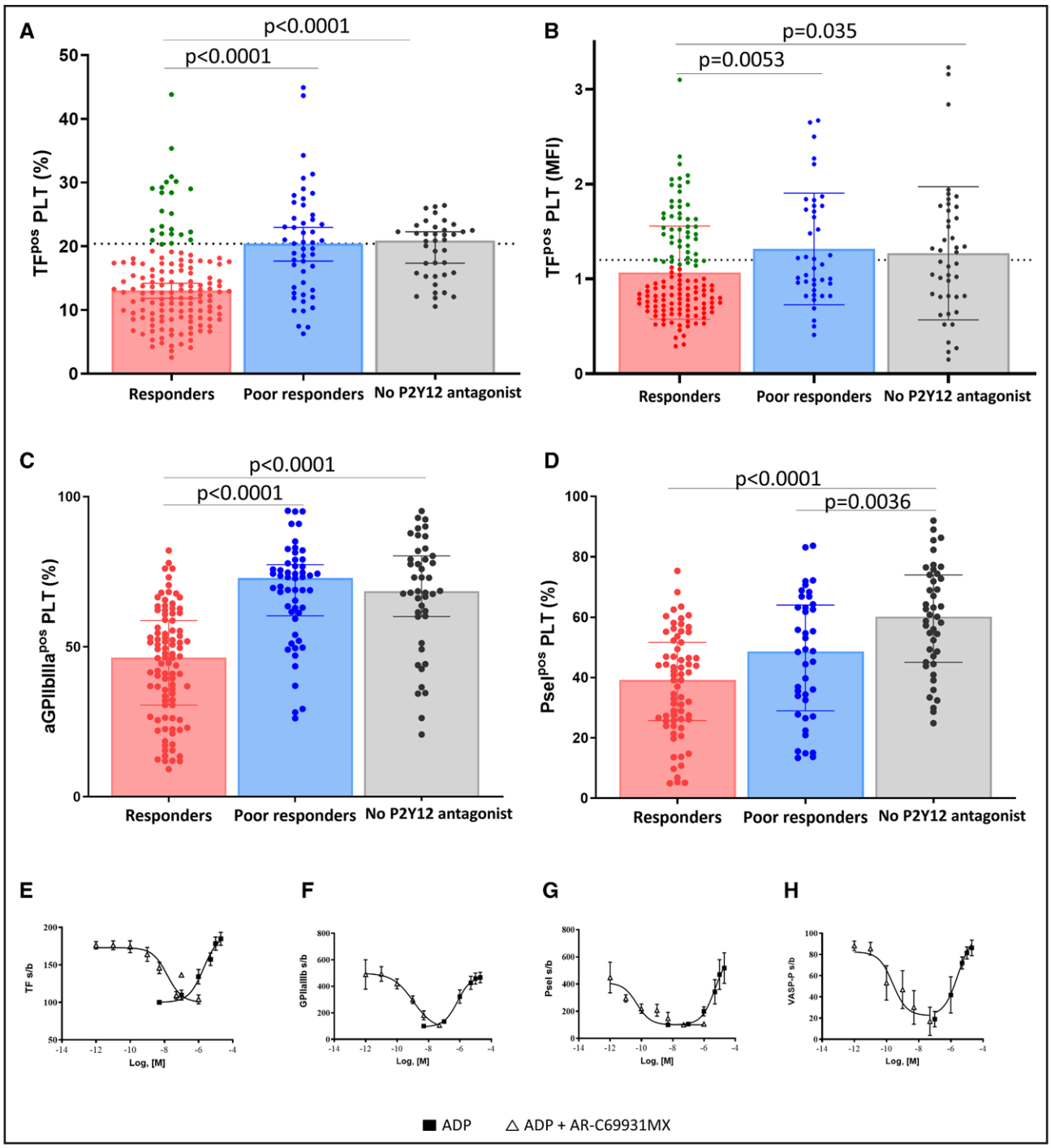

Figure 2. Platelet activation marker expression in clopidogrel-treated patients with coronary artery disease (CAD). Clopidogrel (75 mg/die)-treated patients were divided according to their platelet reactivity index (PRI) measured by VASP (vasodilator-stimulated phosphoprotein) phosphorylation assay by flow cytometry: patients with PRI <60% were defined as responders (n=144; red bars), and those with PRI >60% were defined as poor responders (n=49; blue bars). Flow cytometry analysis of platelet (PLT)-associated TF (tissue factor) expression (**A**, percentage of positive platelets; **B**, mean fluorescence intensity [MFI]), activated GPIIbIIIa (glycoprotein IIbIIIa; aGPIIbIIIa; **C**), and P-selectin (**D**) assessment upon ADP stimulation (10 μM, 15 min) was reported for the 2 groups of patients and compared with a group of CAD patients (n=39) free of anti $P2Y_{12}$ treatment (no $P2Y_{12}$ antagonist; gray bar). Green dots highlight patients who, despite a good clopidogrel response, show a percentage of TF^{pos}-platelets higher than the median value measured in the poor responder group. Dot line in **A** and **B** indicates the mean value measured in healthy subject as reference. Results are shown as mean±SD of the percentage and MFI of TF^{pos}-platelets and as percentage of aGPIIbIIIa-positive and P-selectin–positive platelets. Data were analyzed by 1-way ANOVA followed by Dunnet post hoc analysis. Concentration-response curves of platelet TF (**E**), platelet-aGPIIbIIIa (**F**), platelet-Psel (**G**) and VASP (**H**) induced by ADP (black squares) and upon AR-C69931MX treatment (open triangles). Agonist EC_{50} was calculated using a 4-parameter logistic model while antagonist pA2s were calculated as described in Materials and Methods. Results are presented as percentage of protein expression over baseline (s/b; n=4–11). All curves shown were computer generated.

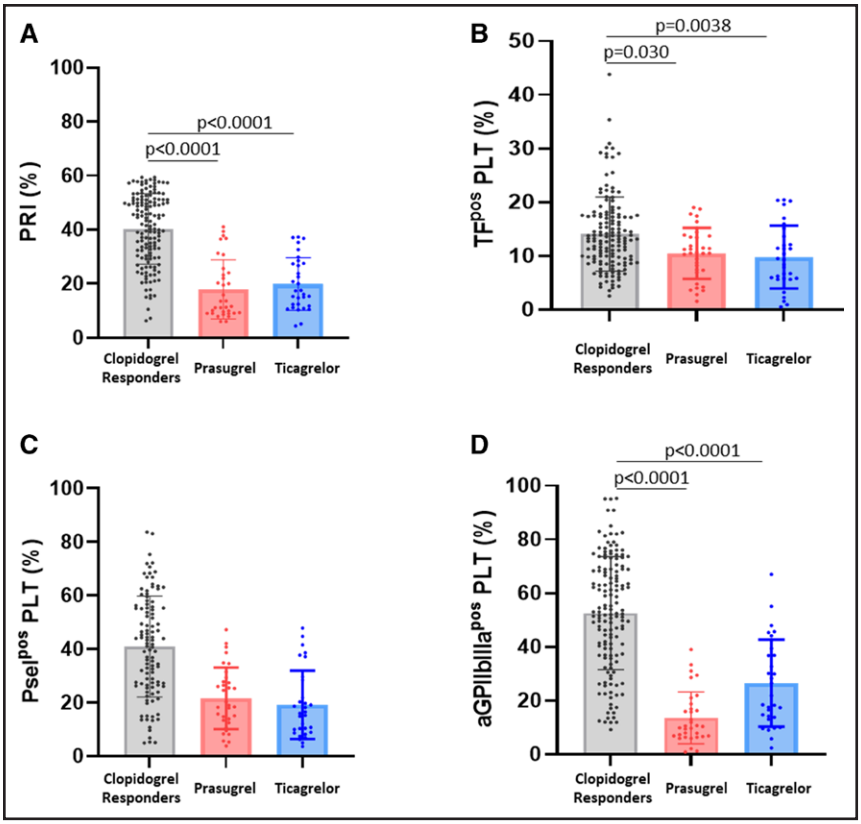

Figure 3. TF (tissue factor) expression in prasugrel-, ticagrelor-, and clopidogrel-treated patients with coronary artery disease (CAD). Flow cytometry analysis of VASP (vasodilator-stimulated phosphoprotein) platelet reactivity index (PRI; **A**) and platelet-associated TF (**B**), PSel (P-selectin; **C**), and activated GPIIbIIIa (glycoprotein IIbIIIa; aGPIIbIIIa; **D**) expression upon ADP stimulation (10 μM, 15 min) was analyzed in prasugrel- (n=34) and ticagrelor-treated (n=31) patients and compared with that measured in clopidogrel-treated patients. Results are shown as mean±SD of the percentage of antigen-positive platelets (PLT) and were analyzed by 1-way ANOVA followed by Dunnet post hoc analysis.

expression on the platelet surface is regulated by $P2Y_{12}$ receptor activation only, being $P2Y_1$ engagement effectless. Indeed, only $P2Y_{12}$ antagonism prevented in a concentration-dependent manner TF exposure on the cell surface, in turn affecting the kinetic of TG. This effect was observed both in in vitro experiments and ex vivo in platelets from clopidogrel-, prasugrel-, and ticagrelor-treated patients with CAD.

This study also shows for the first time that downregulation of ADP-stimulated platelet-associated TF exposure occurs with a $P2Y_{12}$ inhibition much greater—requiring a higher drug concentration—than that needed to reduce aGPIIbIIIa, VASP, and P-selectin expression. From a clinical point of view, this finding is of relevance since assessment of TF levels and activity allows to highlight patients who, despite a good clopidogrel response according to the VASP-PRI, the gold standard method for the assessment of $P2Y_{12}$ antagonism, show a residual platelet reactivity in terms of prothrombotic potential.

Finally, we also provide the evidence that TF is stored in the platelet OCS; its appearance on the cell membrane upon activation occurs following OCS externalization, and this event is prevented by cytoskeleton blockers, such as cytochalasin D and, more clinically relevant, colchicine.

$P2Y_1$ and $P2Y_{12}$ are the 2 ADP receptors that mediate the amplification of platelet response within the hemostatic and thrombotic process. It is well established that platelet activation (ie, granule secretion and cell aggregation) requires concomitant activation of $P2Y_1$ and $P2Y_{12}$ signaling.[28] Conversely, regulation of the prothrombotic properties of platelets, essential in secondary hemostasis, appears to be a $P2Y_{12}$ prerogative. Indeed, it has been previously shown that inhibition of $P2Y_{12}$ only, and not of $P2Y_1$, is able to modify the platelet prothrombotic phenotype by decreasing platelet phosphatidylserine exposure.[15] Furthermore, $P2Y_{12}$ antagonism strongly delayed the kinetic of thrombin formation in PRP (platelet-rich plasma), a process that is mainly TF dependent.[15,29] Our data complement these findings by showing that TF expression on the platelet membrane following ADP stimulation is also a $P2Y_{12}$-dependent mechanism since (1) only the $P2Y_{12}$ antagonist, and not the $P2Y_1$ antagonist, was able in vitro to inhibit it and (2) no increase of TF expression on the platelet surface was observed in a $P2Y_{12}$-deficient patient upon ADP activation, whereas the protein was detectable following TRAP-6 or U46619 stimulation. Within this context, our findings add new insights into the regulation of the prothrombotic activity of platelets highlighting that both phosphatidylserine and TF exposure are $P2Y_{12}$-dependent events. To further complement this scenario, it is worth mentioning that the secretion of platelet PDI (protein disulphide isomerase)—a key enzyme involved in the regulation of TF prothrombotic activity[30]—is also controlled by $P2Y_{12}$ thus emphasizing an overall consistent and uniform regulation of the prothrombotic activity of platelets.[31] Clearly, the platelet-associated prothrombotic response may be elicited through multiple pathways including, for instance, activation of the

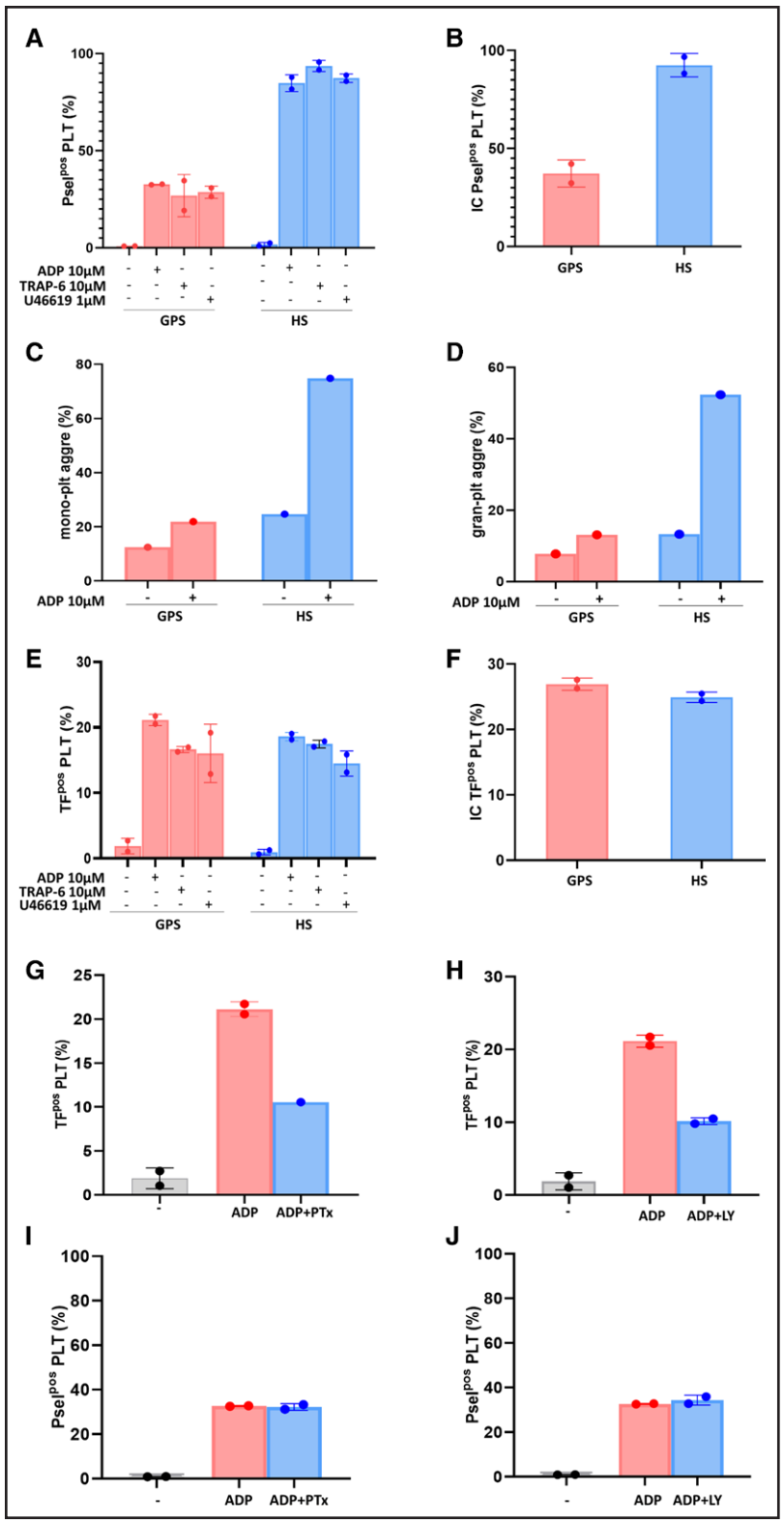

Figure 4. Platelet activation marker expression in patients with gray platelet syndrome (GPS).
Platelet-Psel (P-selectin; **A** and **B**) and TF (tissue factor; **E** and **F**) expression under resting conditions and upon ADP (10 µM, 15 min), TRAP-6 (thrombin receptor-activating peptide; 10 µM, 15 min), and U46619 (1 µM, 15 min) stimulation was assessed by flow cytometry and reported as surface (**A** and **E**) and intracellular (IC; **B** and **F**) percentage of positive cells in 2 patients with GPS and in 2 healthy subjects studied in parallel. Percentage of monocyte-platelet aggregates (mon-plt aggre; **C**) and of granulocyte-platelet aggregates (gran-plt; **D**) was also reported. The effect of the Gαi inhibitor *Bordetella* pertussis toxin (PTx, 5 µg/mL; **G** and **I**) and the PI3 (phosphoinositide 3) kinase inhibitor LY294002 (10 µM; **H** and **J**) was analyzed upon ADP (10 µM) stimulation as percentage of TF-positive and Psel-positive platelets. Data are expressed as mean±SD.

thrombin receptors PAR-1 and PAR-4.[32,33] The possible regulation of platelet-associated TF expression by these other signaling pathways was not analyzed being out of the scope of the study.

Increased levels of TF^{pos}-platelets have been reported in several pathological conditions characterized by a prothrombotic phenotype, including thrombocythemia,[5] cancer,[7] antiphospholipid syndrome,[9] viral infections,[8,10] and cardiovascular diseases.[6,34] The clinical relevance of these findings lays on the significant correlation between platelet-associated TF expression and its functional activity, measured as TG capacity, to residual cardiovascular

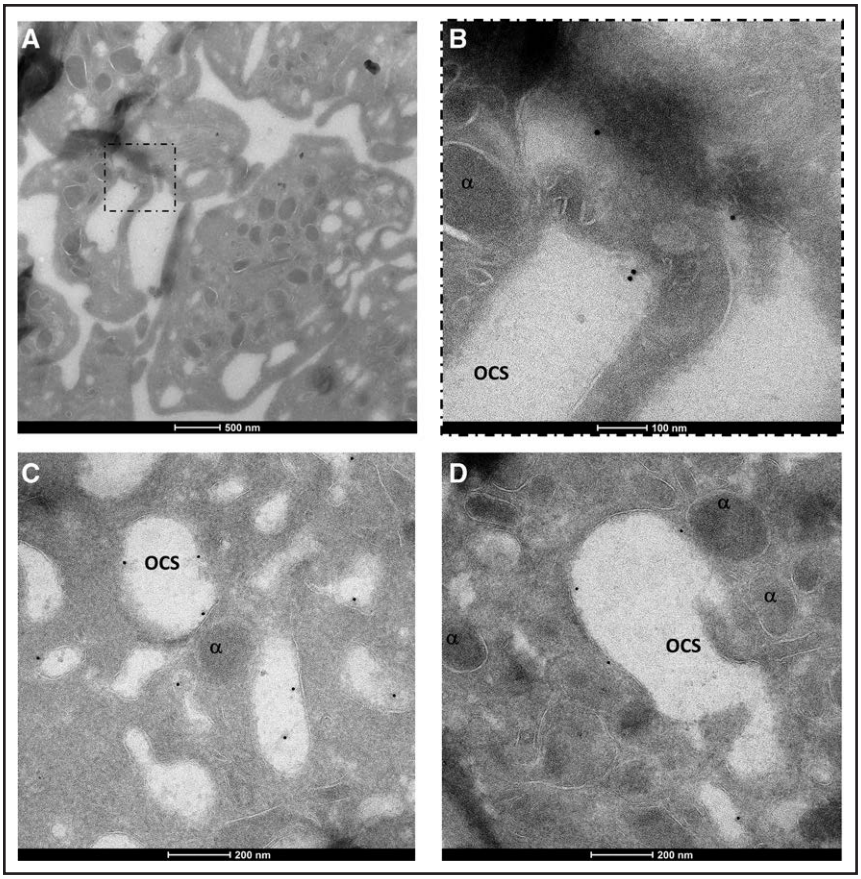

Figure 5. Localization of TF (tissue factor) in human platelets.
Ultrathin Tokuyasu cryosections were single immunolabeled for TF (10 nm gold). Only a fraction of the platelet population expresses TF (**A**); when expressed, TF localizes in the open canalicular system (OCS; **B**–**D**).

risk.[10,22] Indeed, the translational implications of TF regulation by P2Y$_{12}$ receptor are extremely relevant in this setting, considering that treatment with P2Y$_{12}$ antagonists is, together with aspirin, the gold standard therapy for secondary prevention of cardiovascular diseases.[35]

We report here that in CAD patients good responders to P2Y$_{12}$ antagonists, based on VASP-PRI <60%, the highest is P2Y$_{12}$ inhibition, as occurs in prasugrel- and ticagrelor-treated patients, and the lowest is the cell surface TF expression. Interestingly indeed, the receiver operating characteristic analysis showed that in vivo levels of TF^{pos}-platelet <20% (ie, below the levels found in poor-responder patients) are predicted by VASP-PRI ≤20%, value that is consistently obtained in prasugrel- and ticagrelor-treated patients only and not in clopidogrel-treated ones.

Several data in the literature, also supported by our present results, have already established that prasugrel and ticagrelor are able, compared with clopidogrel, to achieve a more pronounced inhibition of platelet activation in terms of P-selectin or aGPIIbIIIa expression and release of microvesicles.[36–38] Interestingly, Braun et al[39] reported that prasugrel treatment also results in a better inhibition of prothrombotic anionic phospholipids exposure than clopidogrel. In agreement, our study shows that the platelet prothrombotic potential, measured as percentage of circulating TF^{pos}-platelets, was significantly lower in patients receiving prasugrel or ticagrelor compared with clopidogrel-treated ones. This overall greater inhibition of platelet prothrombotic capacity could, therefore, account for the observed reduction in the incidence of MACE (major adverse cardiovascular events) in patients with ACS (acute coronary syndrome) who received prasugrel or ticagrelor compared with clopidogrel.[40] Our findings may also help to explain why a positive impact of a personalized antiplatelet therapy has been observed only in trials where patients with high-on-treatment platelet reactivity on clopidogrel (based on aggregometry-based tests) were shifted, to achieve a better platelet inhibition, to ticagrelor or prasugrel and not to high-dose clopidogrel.[41–47] Indeed, a clinically relevant aspect highlighted in this study is the finding that 15% of clopidogrel good-responder patients show levels of cell surface TF^{pos}-platelets similar to those found in poor-responder patients, thus carrying a residual prothrombotic potential. This finding led us to calculate the potency of AR-C69931MX in inhibiting TF and VASP, as well as other classical markers of platelet activation such as P-selectin and aGPIIbIIIa. Results showed that the degree of P2Y$_{12}$ inhibition needed to downregulate ADP-induced TF expression is greater than that needed to inhibit VASP and aGPIIbIIIa (40-fold). Furthermore, the potency of the P2Y$_{12}$ antagonists to inhibit P-selectin exposure is even greater. This accounts for our evidence

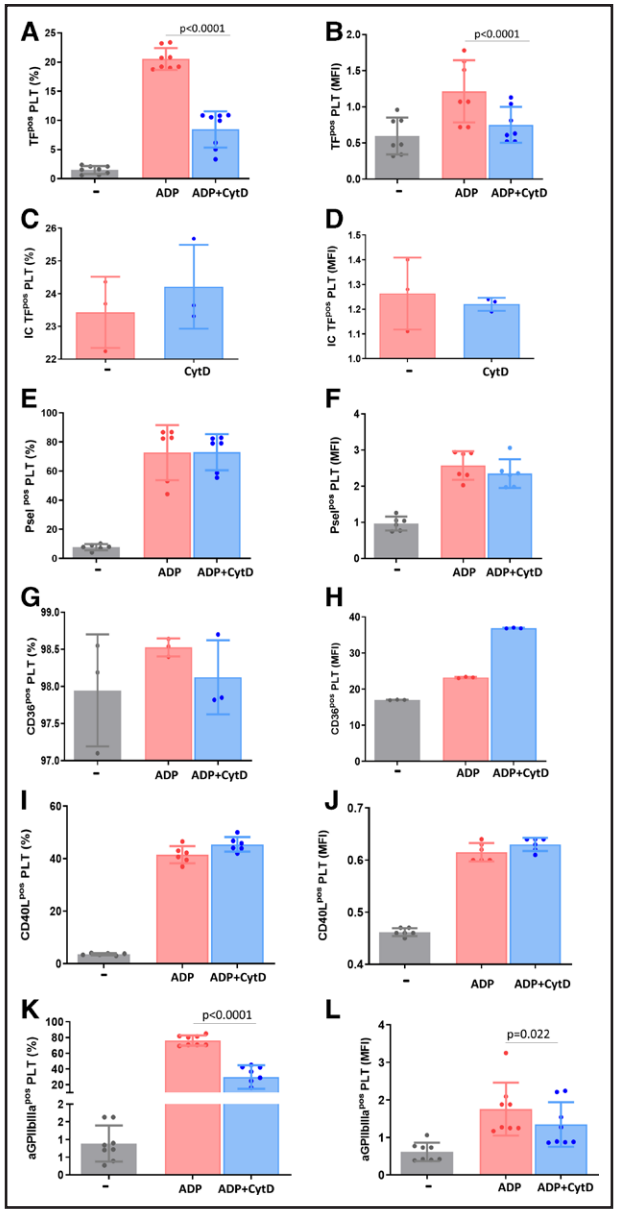

Figure 6. Involvement of actin polymerization in platelet activation marker expression.
Whole blood was incubated with cytochalasin D (CytD; 10 µmol/L, 15 min). Surface expression of platelet-associated TF (tissue factor; **A** and **B**), P-selectin (**E** and **F**), CD36 (**G** and **H**), CD40L (**I** and **J**) and activated GPIIbIIIa (glycoprotein IIbIIIa; aGPIIbIIIa; **K** and **L**) was measured by flow cytometry upon stimulation with ADP (10 µM, 15 min). Intracellular (IC) expression of platelet-associated TF was also assessed (**C** and **D**). Data are expressed as mean±SD (n=3–8) of percentage of positive platelets (PLT; **A**, **C**, **E**, **G**, **I**, and **K**) or mean fluorescence intensity (MFI; **B**, **D**, **F**, **H**, **J**, and **L**). Results were analyzed by Student paired *t* test or Mann-Whitney *U* test as appropriate.

that P-selectin does not indeed differ between good and poor responders, in agreement with published data showing that ADP-induced P-selectin inhibition occurs already at low levels of the P2Y$_{12}$ blockade.[48] Although aGPIIbIIIa evaluation seems to better distinguish the responsiveness to clopidogrel, aggregation-based tests fail to provide an accurate stratification of patients' thrombotic risk. Indeed, data from different clinical studies, such as the ARCTIC and GRAVITAS trials, showed that, depending on the cutoff value used to define platelet hyperreactivity, the degree of aGPIIbIIIa inhibition might not be able to fully predict major adverse cardiovascular events in stable patients with CAD treated with clopidogrel.[44,49–51] By contrast, a subanalysis of the PREPARE POST-STENTING study reported that, in a subgroup of patients with post-PCI (percutaneous coronary intervention) ischemic events—despite optimal inhibition by aspirin and clopidogrel—high-platelet fibrin clot strength and prothrombotic potential, involving elevated TF expression phenotype, are driving factors for ischemic events.[52,53]

Since recurrent thrombotic events occur in about 1 to 10 patients in the first year after CAD despite treatment with aspirin and the most potent P2Y$_{12}$ antagonists,[54] the efficacy of the use of anticoagulants on top of antiplatelet treatment has been explored in the clinical setting. Interestingly, the ATLAS ACS and the COMPASS study reported the benefit of very-low-dose rivaroxaban in reducing MACE when combined with antiplatelet therapy.[55,56] A remaining challenge is the identification of the biomarkers of platelet-dependent TG that can help to identify patients who would have a clinical benefit from this dual pathway inhibition. In this context, platelet-associated TF could be a good candidate because it might be a potential driver toward adverse cardiovascular outcomes. The predictive value of platelet-associated TF is indeed matter of our currently ongoing investigations. Although <5% of total thrombin is formed during the initiation step,[3] it is important to consider that this is critical in the development of the prothrombotic response.[57] In the absence of platelet contribution to thrombin formation, thrombin indeed would be rapidly inhibited by circulating plasma antithrombin in <1 minute.[58] By contrast, the TF-dependent initiation phase could support the continuous generation of thrombin that can persist for months after the index event.[59]

A further important clinical implication of this study results from the evidence that in resting platelets, the pool of intracellular TF is associated with OCS rather than with α-granules, as proposed previously.[16,17] This evidence, highlighted by pharmacological data and by immune gold labeling and transmission electron microscopy images, was confirmed by the finding that patients with GPS, deficient in α-granules, have normal levels of platelet-associated TF. Cytochalasin D or colchicine treatment—inhibiting actin or tubulin polymerization, respectively, and thus OCS externalization—prevented TF and GPIIbIIIa exposure on the cell membrane. In this view, in resting platelets, OCS appears to be a cryptic site where proteins such as TF and GPIIbIIIa are not accessible to their physiological agonists (ie, FVIIa [factor VIIa] to promote TG and fibrinogen to sustain platelet aggregation, respectively). It could be speculated that in this compartment, TF can

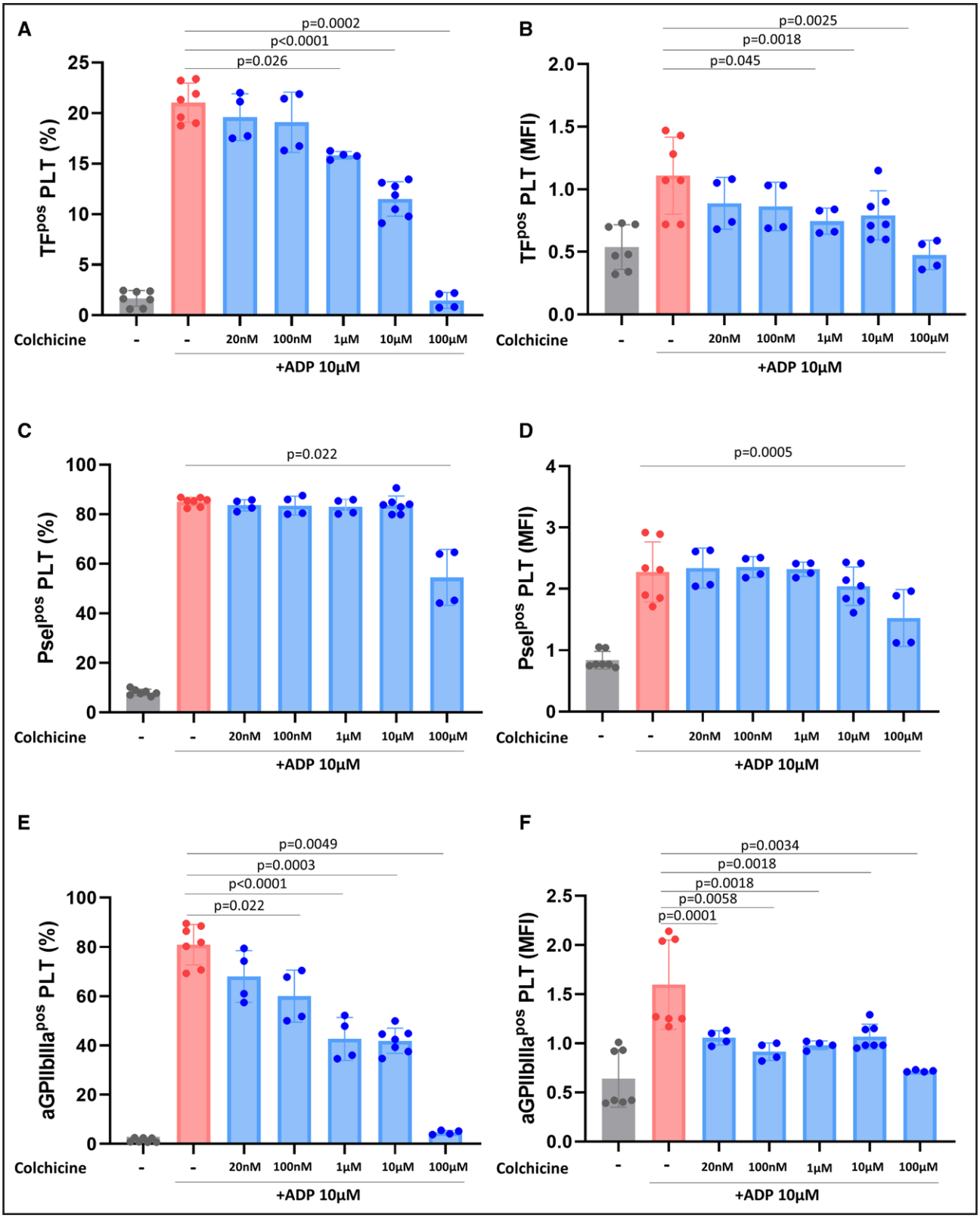

Figure 7. Involvement of microtubule disruption in platelet activation marker expression.
Whole blood was incubated with colchicine (20 nM to 100 µM, 15 min) and platelet-associated TF (tissue factor; **A** and **B**), PSel (P-selectin; **C** and **D**), and activated GPIIbIIIa (glycoprotein IIbIIIa; aGPIIbIIIa; **E** and **F**) expression was measured by flow cytometry upon stimulation with ADP (10 µM, 15 min). Data are expressed as mean±SD (n=4–7) of percentage of positive cells (**A**, **C**, and **E**) or mean fluorescence intensity (MFI; **B**, **D**, and **F**) and were analyzed by Student paired *t* test or Mann-Whitney *U* test as appropriate.

be retained in its inactive form. Upon platelet activation, rapid events including platelet shape change, cytoskeleton reorganization, and OCS externalization—bringing these proteins to the cell membrane—occur. Then they can switch to their active form due to the redox enzymatic activity of PDI and become competent for triggering coagulation and platelet plug formation.[30]

Interestingly, low-dose colchicine on top of standard antiplatelet therapy has been shown to reduce major adverse cardiovascular events either in stable patients[60,61] or, albeit with less consistent results,[62,63] in patients with acute cardiovascular disease.[64–66] Cimmino et al[67] speculated that colchicine, by modulating platelet aggregation, may be effective in reducing the risk of developing future acute cardiovascular events. Although some evidences indicates that colchicine in the therapeutical range, as that used in this study, is not able to inhibit in vivo platelet aggregation,[68,69] it could prevent TF exposure on cell surface. Thus, the effect of colchicine, besides its well-established anti-inflammatory properties, could be also related to inhibition of the platelet prothrombotic potential.

CONCLUSIONS

Overall, our findings provide a step forward in the characterization of platelet-associated TF regulation and its pharmacological modulation and support the hypothesis that the therapeutic benefits of the antiplatelet therapy with $P2Y_{12}$ antagonists are also the result of the inhibition of the cell surface TF expression. Of note, active drug concentrations that adequately inhibit the most studied platelet activation markers may not completely turn off the residual platelet reactivity, and this is particularly relevant in clopidogrel-treated patients. This may explain the adverse events recorded in trials despite an adequate inhibition of platelet aggregation. Within this context, concomitant assessment of both VASP-PRI and TF in $P2Y_{12}$ antagonist–treated patients could improve the evaluation of the residual platelet TF-dependent prothrombotic potential.

ARTICLE INFORMATION

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Affiliations

Centro Cardiologico Monzino IRCCS, Milan, Italy (M.B., A. Becchetti, N.C., M. Conti, P.C., A. Bonomi, D.T., P.J.W., J.C., G.M., M. Camera). Department of Pharmaceutical Sciences (G.E.R., C.P., M. Camera) and Unità di Medicina II, ASST Santi Paolo e Carlo, Department of Scienze della Salute (M. Cattaneo, G.M.P.), Università degli Studi di Milano, Italy. CoagScope BV, Maastricht, the Netherlands (P.L.A.G.). Experimental Imaging Center, San Raffaele Scientific Institute, Milan, Italy (A.L.). Department of Translational Medicine and Surgery, Università Cattolica del Sacro Cuore, Rome, Italy (E.D.C.). Maria Cecilia Hospital, Cotignola, Italy (E.T.).

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Disclosures

M. Camera, M. Brambilla, and P. Canzano are inventors of a patent application covering the work described in the study. The other authors report no conflicts.

Supplemental Material

Expanded Materials & Methods
Tables S1 and S2
Figures S1–S8

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