# Platelets *versus* monocytes as cellular sources of blood-borne tissue factor: a quantitative and functional comparison in healthy subjects and in patients with STEMI

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Dedication: this work is dedicated to the memory of Professor Armando D'Angelo, whose scientific rigor, intellectual generosity, mentorship, and friendship have left a lasting mark on the field of thrombosis and hemostasis and on all those who had the privilege of knowing, working, and collaborating with him.

## ABSTRACT

Monocytes are an established source of blood-borne tissue factor (TF), while platelet has emerged over 2 decades as a functionally and clinically relevant circulating source of TF. Their relative quantitative and functional contributions remain undefined. Surface TF was quantified by whole-blood flow cytometry in platelets and monocytes from 20 healthy subjects (HS) and 20 patients with ST-segment elevation myocardial infarction (STEMI). Calibrated automated thrombography (CAT) compared platelet and monocyte preparations at cell numbers reflecting their relative abundance in a reference blood volume. TF and Tissue Factor Pathway Inhibitor (TFPI) were neutralized with mAbs; platelet TF exposure was assessed under CAT-like conditions. $TF^{pos}$ fractions were similar, but TF-positive platelets per microliter were more than 400-fold more abundant in both groups. Platelets showed shorter lag times and higher thrombin peaks than monocytes in HS and STEMI; endogenous thrombin potential was greater in STEMI and numerically higher in HS. Anti-TF delayed and anti-TFPI accelerated thrombin generation. Platelet-associated TF increased under CAT-like conditions to 21±1.5% at 8 minutes. Accounting for cellular abundance identifies platelets as quantitatively predominant over monocytes, with greater thrombin-generating capacity in HS and STEMI.

**Key words:** tissue factor; platelets; monocytes; thrombin generation; ST-segment elevation myocardial infarction.

## Introduction

Tissue factor (TF) is the principal initiator of the coagulation cascade through formation of the TF–factor VII/VIIa complex and subsequent activation of factors IX and X.[1] Under physiological conditions, TF is largely segregated from circulating blood, whereas cells within the vascular compartment, particularly monocytes and endothelial cells, can be induced to express TF in response to inflammatory or infectious stimuli.[2-4] This inducible expression provided the biological framework for considering circulating cells as potential contributors to intravascular coagulation. The subsequent demonstration of a blood-borne TF pool further broadened the classical concept that coagulation initiation depends predominantly on TF exposed after vascular injury.[5]

Within this evolving paradigm, monocytes became the best characterized inducible cellular source of blood-borne TF. Activated monocytes express functionally active TF and acquire a procoagulant phenotype in several pathological conditions, including acute coronary syndromes.[6-9] In patients with acute myocardial infarction, increased monocyte TF expression was associated with enhanced factor Xa generation and systemic coagulation activation, while cell-associated Tissue Factor Pathway Inhibitor (TFPI) limited this procoagulant activity.[9] Experimental studies further supported an important role for monocyte-derived TF in thrombosis. In an *in vitro* model of stent thrombosis, monocyte depletion markedly reduced thrombus formation and TF deposition. Interestingly, TF neutralization also reduced thrombus formation in leukocyte-reduced platelet-rich plasma, suggesting that additional TF-bearing blood components may contribute to TF-dependent coagulation.[10]

At the same time, platelets emerged as another potential cellular source of blood-borne TF. Platelet activation was shown to induce rapid exposure of functionally active TF at the cell surface,[11] and subsequent studies provided evidence that platelets can synthesize TF from pre-existing mRNA.[12,13] In patients with acute coronary syndromes, we showed increased TF expression not only in monocytes but also in platelets and platelet-leukocyte aggregates, with platelet-associated TF displaying functional procoagulant activity.[14] We later showed that human megakaryocytes transfer a pre-existing TF pool to a subset of newly generated platelets, providing a cellular origin for platelet-associated TF.[15] Thus, while monocyte TF became extensively characterized as an inducible procoagulant pathway, platelet-associated TF emerged as an additional, although more debated, component of circulating TF biology.[16,17]

Despite these two lines of evidence, the relative quantitative and functional contributions of platelets and monocytes to circulating cellular TF have not been directly established under comparable conditions. This comparison cannot be inferred from the percentage of $TF^{pos}$-cells, or from TF content on a per-cell basis alone, because platelets and monocytes differ by several orders of magnitude in their abundance in blood. We therefore directly compared platelets and monocytes from healthy subjects and patients with ST-segment elevation myocardial infarction (STEMI), assessing surface TF expression, the absolute number of $TF^{pos}$-cells in circulating blood, and their capacity to support thrombin generation when tested at cell numbers reflecting their relative abundance in the same reference blood volume. The functional contribution of TF and its regulation by TFPI were examined using neutralizing antibodies, while the dynamics of platelet TF surface exposure were assessed during the early phase of the thrombin generation assay to provide insight into how TF exposure may occur *in vivo*.

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## Materials and methods

### Patient enrollment

We prospectively enrolled volunteer healthy subjects (HS, n=20), who had not taken antiplatelet drugs within the 10 days preceding blood donation, and 20 consecutive patients with STEMI admitted to the Intensive Cardiac Care Unit of Centro Cardiologico Monzino IRCCS, Milan (Italy). Exclusion criteria were age >80 years, history of bleeding diathesis, recent stroke (<3 months), drug or alcohol abuse, severe liver disease, renal insufficiency (creatinine >2.0 mg/dL), prothrombin time >1.5 times control, platelet count <100,000/$mm^3$, and hematocrit <30%.

### Blood collection and cell preparation

Whole blood was drawn from an antecubital vein with a 19-gauge needle, without venous stasis, into citrate tubes [1/10 volume of 0.129 M sodium citrate; Vacutainer (Becton Dickinson, Franklin Lakes, NJ, USA], discarding the first 4 mL. In STEMI patients, sampling was performed approximately 48 hours after primary percutaneous coronary intervention (PCI), while patients were receiving dual antiplatelet therapy with aspirin and a $P2Y_{12}$ receptor antagonist. Platelet-rich plasma was prepared by centrifugation of whole blood at 100 g for 10 minutes at room temperature without brake. $PGE_1$ (4 μM) was added to minimize *ex vivo* platelet activation. Platelet count, mean platelet volume (MPV), platelet distribution width (PDW), platelet recovery and leukocyte contamination were assessed using a Sysmex XE-2100 analyzer (Sysmex, Kobe, Japan). Only platelet preparations without leukocyte contamination and with platelet recovery ≥70% were used for subsequent analyses. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll density-gradient centrifugation as previously described.[18] Monocyte counts within the isolated mononuclear cell fraction were determined using a Sysmex XE-2100 analyzer. The activation status of platelet and monocyte preparations was assessed by flow cytometry analyzing cell-specific activation markers (platelet αIIbβ3 activation and P-selectin expression, and leukocyte CD11b upregulation, respectively). Preparations showing evidence of procedure-related activation were excluded from subsequent flow cytometry and calibrated automated thrombography (CAT) analyses.

## Flow cytometry

Whole blood flow cytometry was performed on a Gallios cytometer (Beckman Coulter, Brea, CA, USA) equipped with four solid-state lasers and enhanced wide-angle forward scatter to optimize separation of small events. Platelets and monocytes were identified according to physical parameters and lineage markers (CD41a for platelets, CD14 for monocytes).

Flow cytometry analysis of TF expression was performed as previously described, with minor modifications.[14,19] For surface TF expression, samples were labeled with saturating concentrations of mouse anti-human TF monoclonal antibody (mAb, clone HTF-1) for 15 minutes at room temperature in the dark. Before use, all antibodies were centrifuged at 17,000g for 5 minutes at 4°C to remove aggregates. Fluorescence-minus-one controls were used to quantify background labeling. A total of 20,000 CD41-positive and 4,000 CD14-positive events per sample were acquired. Flow-check Pro fluorospheres were used daily to monitor cytometer performance. Data were analyzed with Kaluza software 2.1 (Beckman Coulter) and reported as percentage of positive events. Absolute numbers of $TF^{pos}$ cells per microliter of whole blood were calculated by applying the percentage of $TF^{pos}$ events to the corresponding absolute circulating platelet or monocyte count. Imaging flow cytometry was additionally used to confirm the cellular identity of $TF^{pos}$ events and TF-associated surface fluorescence (Supplementary Figure S1). Platelets were identified by morphology, light-scatter properties and CD41 positivity, whereas monocytes were identified by forward- and side-scatter characteristics and a CD45-positive/CD41-negative phenotype. Imaging analysis also allowed exclusion of antibody aggregates, debris, doublets and cell aggregates.

## Thrombin generation assay

Fresh platelet preparations obtained from platelet-rich plasma (PRP) and PBMC preparations were tested for thrombin-generation capacity using CAT.[16,20] Cells were resuspended in microvesicle-free pooled plasma prepared from multi-donor citrated whole blood containing thermostable inhibitor of contact activation (TICA, 37 µg/mL) to prevent *ex vivo* contact pathway activation. No exogenous TF was added to the assay.

The assay was optimized for $40\times10^6$ platelets per well. For each subject, the blood volume containing $40\times10^6$ circulating platelets was calculated from the individual platelet count. Based on the corresponding individual circulating monocyte count, the number of monocytes present in the same blood volume was then calculated. An aliquot of the autologous PBMC preparation containing this number of monocytes was used for CAT. Thus, platelet and monocyte-containing preparations were tested at cell numbers reflecting their relative abundance in the same reference blood volume. The resulting mean numbers of monocytes used in CAT were 89,770±32,901 in HS and 136,042±83,671 in STEMI patients. Triplicate samples were incubated for 10 minutes with microvesicle-free TICA pooled plasma in round-bottom 96-well plates. To assess TF contribution, CAT was performed in the presence of a neutralizing anti-TF mAb (HTF-1 clone, 8 µg/mL). The inhibitory contribution of TFPI present in the assay was assessed by preincubation with a neutralizing anti-TFPI mAb (2H8 clone, 8 µg/mL). To minimize differences due to cell-associated phospholipid availability, CAT experiments were performed with an excess of exogenous phospholipids (4 µM, MP reagent). An irrelevant IgG was used in control experiments and did not affect thrombin generation. Thrombin generation was initiated by addition of $CaCl_2$/fluorogenic substrate mixture (FluCa kit), and fluorescence was read for 90 minutes in a Fluoroskan Ascent reader (Thermo Fisher Scientific, Waltham, MA, USA). Lag time, endogenous thrombin potential (ETP), and peak thrombin were calculated using Thrombinoscope software v5.1 (Diagnostica Stago, Asnières-sur-Seine Cedex, France).

## Cell-surface tissue factor during calibrated automated thrombography

To characterize TF exposure during CAT-like conditions, aliquots of platelets were recovered from dedicated wells at 0, 2, 4 and 8 minutes after FluCa addition and stained for flow cytometry as described above. Sampling remained technically feasible throughout this early time window, before substantial clot formation occurred. Two independent experiments were performed, each with duplicate determinations.

## Statistical analysis

Results are expressed as mean ± standard deviation (SD) or median [interquartile range], as appropriate. Comparisons between HS and STEMI patients were performed using the unpaired *t* test or Mann-Whitney U test, according to data distribution. In Figure 1, within-group comparisons between platelet and monocyte measurements in HS were performed using the Wilcoxon matched-pairs signed-rank test, whereas platelet and monocyte datasets in STEMI patients were compared using the Mann-Whitney U test.

In Figure 2, paired comparisons between platelet and monocyte preparations were performed using the Wilcoxon matched-pairs signed-rank test and included only matched preparations in which thrombin generation was detectable in both samples. Samples without detectable thrombin generation within the 90-minute CAT acquisition period were assigned a lag time of 90 min and values of zero for peak thrombin and ETP for graphical representation only and were excluded from Figure 2 statistical comparisons.

In Figure 3, paired comparisons between untreated and antibody-treated conditions (anti-TF or anti-TFPI) were performed using the Wilcoxon matched-pairs signed-rank test with Holm correction for multiple comparisons. Samples in which no detectable thrombin generation occurred within the 90-minute acquisition period after anti-TF treatment were assigned the assay endpoint of 90 min and retained in the statistical analysis, as absence of detectable thrombin generation represented an effect of treatment.

Figure 4 presents descriptive data from two independent experiments, each performed in duplicate; no inferential statistical analysis was performed. A two-sided p value<0.05 was considered statistically significant. Analyses were performed using GraphPad Prism version 10.

## Results

### Studied population

Demographic and clinical characteristics are reported in Table 1. Platelet count, immature platelet fraction and mean platelet volume were comparable between HS and STEMI patients. As expected in acute myocardial infarction, monocyte and granulocyte counts were significantly higher in STEMI patients than in HS.

### Platelets constitute the predominant circulating tissue factor-positive cellular pool

We first compared surface TF expression on platelets and monocytes by whole-blood flow cytometry in HS and STEMI samples. The percentage of $TF^{pos}$-cells within each population was closely comparable, with TF detected on 1.9±1.1% of platelets and 1.9±0.7% of monocytes in HS, and on 3.1±1.8% of platelets and 2.8±2.0% of monocytes in STEMI patients (Figure 1A).

However, since platelets and monocytes differ by several orders of magnitude in their physiological abundance in circulating blood, we calculated the absolute number of $TF^{pos}$-cells per microliter of whole blood. This analysis markedly changed the quantitative perspective. In HS, $TF^{pos}$-platelets accounted for 4,296±2,624 cells/µL, compared with only 9.35±4.26 $TF^{pos}$-monocytes/µL (p=0.0020). Similarly, in STEMI patients, 7,361±5,978 $TF^{pos}$-platelets/µL were present compared with 17.8±7.2 $TF^{pos}$-monocytes/µL (p<0.0001; Figure 1B). Thus, $TF^{pos}$-platelets were more than 400-fold more abundant than $TF^{pos}$-monocytes in both HS and STEMI samples. These findings show that the percentage of $TF^{pos}$-cells alone does not reflect the numerical contribution of a circulating cell population to the circulating TF-positive cellular pool.

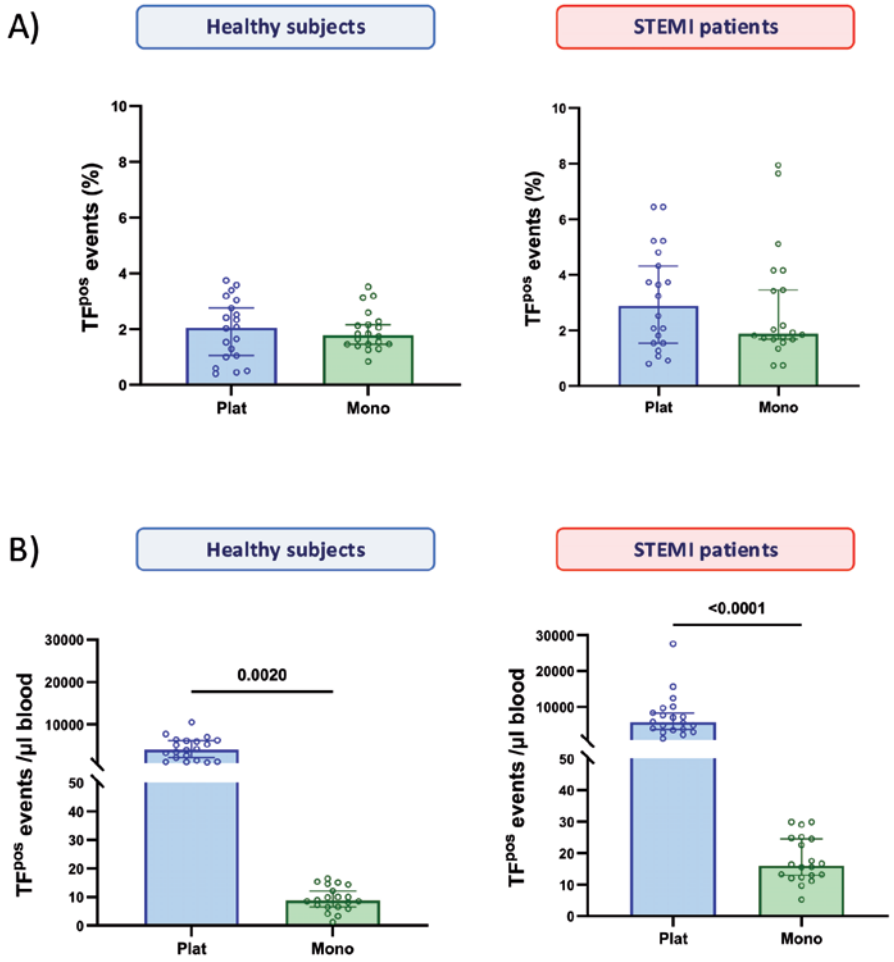



**Figure 1.** Platelets constitute the predominant circulating tissue factor-positive cellular pool. Surface tissue factor (TF) expression was assessed by flow cytometry in platelets and monocytes from healthy subjects (HS; n=20) and patients with ST-segment elevation myocardial infarction (STEMI; n=20). (A) Percentage of $TF^{pos}$-platelets and $TF^{pos}$-monocytes within each cell population. (B) Absolute number of $TF^{pos}$-platelets and $TF^{pos}$-monocytes per microliter of whole blood, calculated by integrating the percentage of $TF^{pos}$ events with the corresponding absolute circulating cell count. Data are presented as median with 95% confidence interval and individual values are shown. In HS, platelet and monocyte measurements were compared using the Wilcoxon matched-pairs signed-rank test, whereas in STEMI patients comparisons were performed using the Mann-Whitney U test. P values are indicated in the figure.

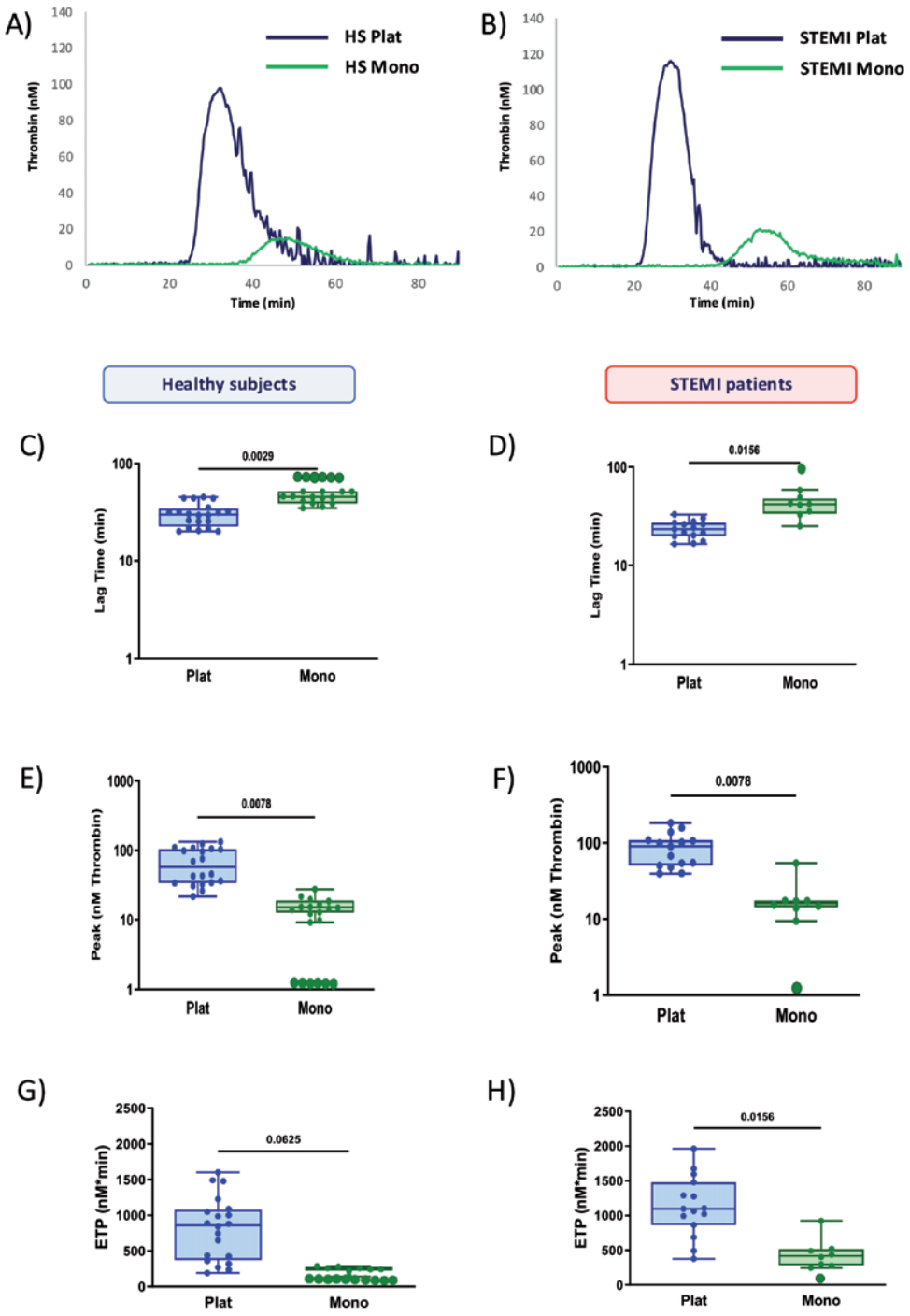



**Figure 2.** Platelets support faster and greater thrombin generation than monocytes at cell numbers reflecting their relative abundance in blood. Thrombin-generating capacity of platelet preparations and monocyte-containing PBMC preparations was assessed by calibrated automated thrombography (CAT), with the two preparations tested at cell numbers reflecting their relative abundance in the reference blood volume containing $40\times10^6$ platelets. (A,B) Representative thrombin generation curves obtained with platelet and monocyte preparations from HS (A) and STEMI patients (B). (C,D) Lag time, (E,F) peak thrombin, and (G,H) endogenous thrombin potential (ETP) in HS (C,E,G; platelets n=20, monocytes n=20) and STEMI patients (D,F,H; platelets n=15, monocytes n=9). Monocyte preparations in which no detectable thrombin generation occurred during the 90-minute acquisition period were assigned a lag time of 90 min and values of zero for peak thrombin and ETP for graphical representation only; in logarithmically scaled panels, zero values are displayed at the lower plotting limit. These samples were excluded from statistical analyses. Paired comparisons were therefore performed only on matched platelet and monocyte preparations with detectable thrombin generation in both samples (HS, n=14 matched pairs; STEMI, n=7 matched pairs), using the Wilcoxon matched-pairs signed-rank test. Boxes indicate the median and interquartile range, and whiskers the minimum and maximum values. Lag time and peak thrombin are displayed on logarithmic scales. P values are indicated in the figure.

Once relative cellular abundance is taken into account, platelets emerge as the quantitatively predominant $TF^{pos}$-population among the 2 cellular compartments examined in both HS and STEMI.

As a secondary between-group observation, the proportion of $TF^{pos}$-platelets was higher in STEMI than in HS (3.1±1.8% *vs* 1.9±1.1%; p=0.021), whereas the approximately 1.7-fold higher absolute $TF^{pos}$-platelet count did not reach statistical significance (p=0.060). $TF^{pos}$-monocyte percentages did not differ between groups, while absolute $TF^{pos}$-monocyte counts were significantly higher in STEMI (p<0.0001).

## Platelets support faster and greater thrombin generation than monocytes at cell numbers reflecting their relative abundance in blood

We next investigated whether the quantitative predominance of $TF^{pos}$-platelets over $TF^{pos}$-monocytes in circulating blood translated into a distinct thrombin-generating capacity. To directly compare the two cell populations under conditions reflecting their relative abundance, CAT was performed using $40\times10^6$ platelets and PBMC preparations containing the number of monocytes present in the same reference blood volume. For

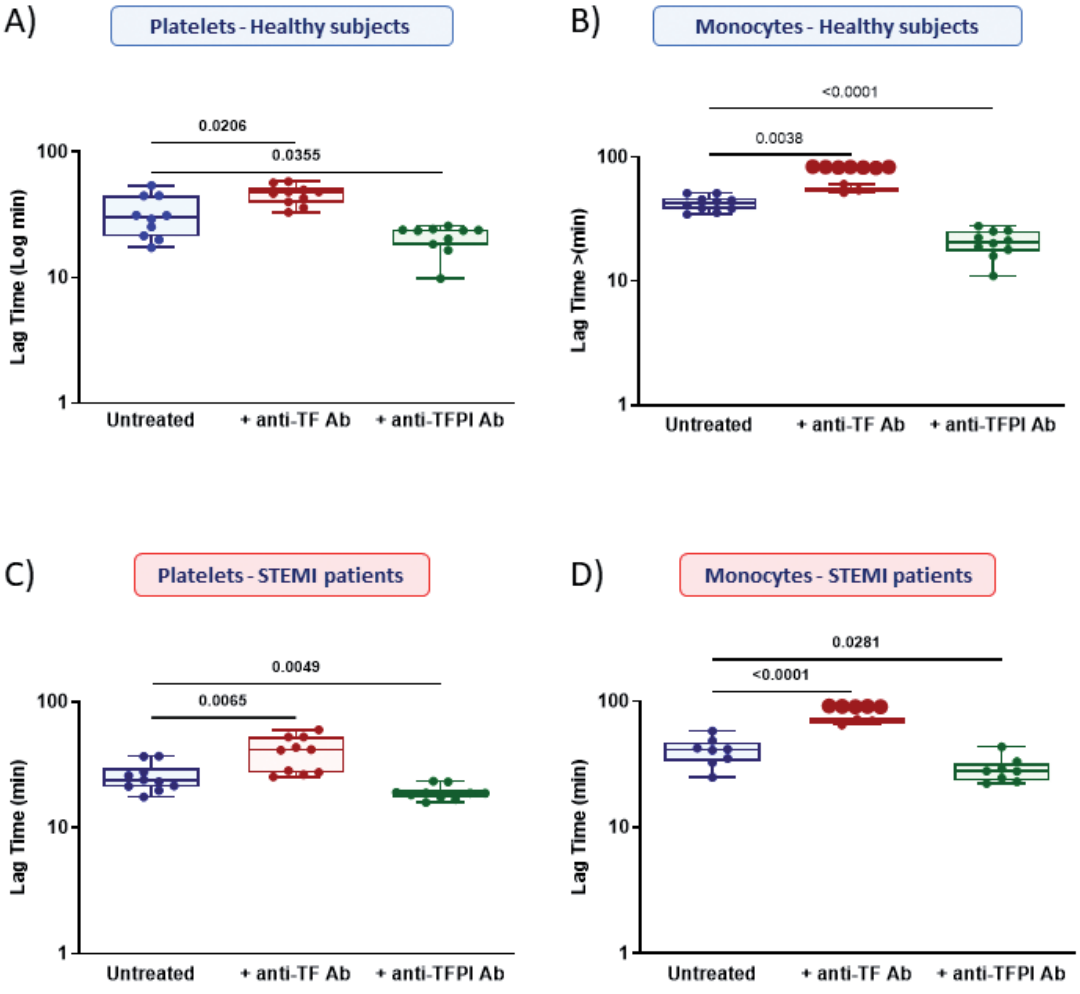



**Figure 3.** Tissue factor and tissue factor pathway inhibitor regulate platelet- and monocyte-associated thrombin generation. The contribution of tissue factor (TF) and tissue factor pathway inhibitor (TFPI) to cell-associated thrombin generation was assessed by CAT in platelet preparations and monocyte-containing PBMC preparations under untreated conditions and after incubation with neutralizing anti-TF or anti-TFPI mAbs. (A) Platelets from HS (n=10 per condition). (B) Monocyte preparations from HS (n=10 per condition). (C) Platelets from STEMI patients (n=10 per condition). (D) Monocyte preparations from STEMI patients (n=8 per condition). Samples in which no detectable thrombin generation occurred within the 90-minute acquisition period after anti-TF treatment were assigned the assay endpoint of 90 min for graphical and statistical analysis, reflecting absence of detectable thrombin generation initiation within the observation window rather than a true lag time value. Box-and-whisker plots with individual values are shown. Lag time is displayed on a logarithmic scale. P values for comparisons between untreated and antibody-treated conditions are indicated in the figure. Statistical comparisons between untreated and antibody-treated conditions were performed using the Wilcoxon matched-pairs signed-rank test with Holm correction for multiple comparisons. Adjusted p values are indicated in the figure.

simplicity, these preparations are hereafter referred to as platelet and monocyte preparations.

In HS, all platelet preparations generated detectable thrombin, whereas monocyte-associated thrombin generation was measurable in 70% of the preparations. Representative thrombin-generation curves differed markedly between the two populations (Figure 2A). Among matched preparations with detectable thrombin generation, platelets initiated thrombin generation significantly earlier than monocytes, as indicated by a shorter lag time (p=0.0029; Figure 2C), and generated higher thrombin peaks (p=0.0078; Figure 2E). ETP was also higher in platelet than in monocyte preparations, although the difference did not reach statistical significance in the paired analysis (p=0.0625; Figure 2G).

A similar functional hierarchy was observed in STEMI patients (Figure 2B). Monocyte-associated thrombin generation was detectable in 8 of 9 preparations (89%), compared with 70% in HS. Among the available matched preparations with detectable thrombin generation, platelets displayed a significantly shorter lag time (p=0.0156; Figure 2D), higher thrombin peaks (p=0.0078; Figure 2F), and greater ETP (p=0.0156; Figure 2H) than monocyte-containing preparations. Thus, when platelet and monocyte preparations were compared at cell numbers reflecting their relative abundance in the same reference blood volume, platelets retained a greater thrombin-generating capacity.

Overall, platelets displayed faster thrombin generation kinetics and higher thrombin peaks than monocytes in both HS and STEMI patients, with significantly greater ETP also observed in STEMI.

## Tissue factor and tissue factor pathway inhibitor regulate platelet- and monocyte-associated thrombin generation

To assess the functional contribution of TF to platelet- and monocyte-associated thrombin generation, cell preparations were incubated with a neutralizing anti-TF mAb or, conversely, with an anti-TFPI mAb to assess the inhibitory contribution of TFPI present in the assay.

In platelets from HS, TF neutralization significantly prolonged thrombin generation lag time compared with untreated samples (p=0.0206; Figure 3A), whereas TFPI neutralization significantly shortened lag time (p=0.0355). A similar reciprocal modulation was observed in platelets from STEMI patients, in whom anti-TF prolonged lag time (p=0.0065), while anti-TFPI significantly shortened it compared with untreated samples (p=0.0049; Figure 3C).

The effect of TF neutralization was particularly evident in monocyte preparations. In HS, anti-TF significantly delayed thrombin generation initiation (p=0.0038; Figure 3B), with several preparations showing no detectable thrombin generation within the 90-minute acquisition period. Conversely, TFPI neutralization markedly shortened lag time compared with untreated samples (p<0.0001). In STEMI monocyte preparations, anti-TF similarly prolonged thrombin-generation initiation (p<0.0001; Figure 3D), with complete suppression of detectable thrombin generation within 90 minutes in several samples, whereas TFPI neutralization significantly shortened lag time (p=0.0281).

Overall, these findings demonstrate that TF contributes

**Table 1.** Baseline demographic characteristics of the subjects enrolled in the study.

| Variable | Healthy subjects (n=20) | STEMI patients (n=20) | p value |
|---|---|---|---|
| Age, years | 54±14 | 63±9.3 | ns |
| Male gender, n (%) | 11 (55) | 10 (50) | ns |
| Blood cell count | | | |
| Platelets (×10$^3$/μL) | 198±45 | 201±88 | ns |
| Immature platelet fraction (%) | 3.7±1.6 | 4±1.6 | ns |
| Mean platelet volume (fL) | 11.4±1.2 | 10.7±1 | ns |
| Monocytes (×10$^3$/μL) | 0.5±0.3 | 0.9±0.4 | 0.01 |
| Granulocytes (×10$^3$/μL) | 4±1.2 | 7.4±2.5 | <0.0001 |
| Lymphocytes (×10$^3$/μL) | 1.8±0.3 | 1.7±0.7 | ns |
| Risk factors | | | |
| Type 2 diabetes, n (%) | - | 6 (30) | - |
| Hypertension, n (%) | - | 12 (60) | - |
| Dyslipidemia, n (%) | - | 8 (40) | - |
| Current smoker, n (%) | - | 7 (35) | - |
| Medications | | | |
| Antiplatelet agents (aspirin+P2Y$_{12}$ antagonists), n (%) | - | 20 (100) | - |
| Hypoglycemic agents, n (%) | - | 6 (30) | - |
| Hypolipidemic agents, n (%) | - | 8 (40) | - |
| Nitrates, n (%) | - | 5 (25) | - |
| Beta-blockers, n (%) | - | 9 (45) | - |
| ACE inhibitors, n (%) | - | 7 (35) | - |
| Proton-pump inhibitors, n (%) | - | 10 (50) | - |

STEMI, ST-segment elevation myocardial infarction. ns, not significant. Data are expressed mean±standard deviation or n (%).

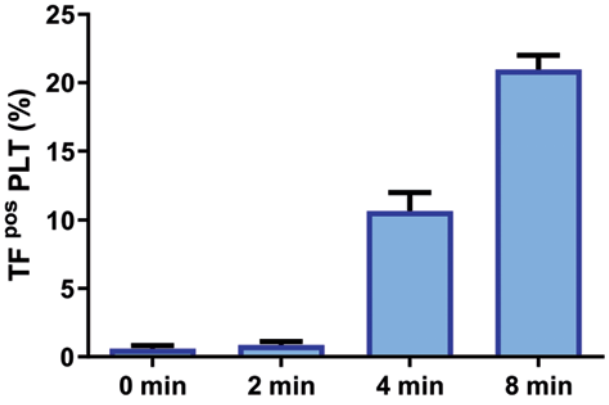



**Figure 4.** Platelet tissue factor is rapidly exposed under CAT-like conditions. Platelets from HS were incubated under conditions reproducing the initial phase of the CAT assay, and surface TF expression was assessed by flow cytometry at 0, 2, 4 and 8 minutes after initiation of the reaction. Data are expressed as mean±SD of two independent experiments, each performed in duplicate.

functionally to thrombin generation initiation in both platelet and monocyte preparations, whereas TFPI present in the assay exerts an opposing inhibitory effect on this process.

### Platelet tissue factor is rapidly exposed under calibrated automated thrombography-like conditions

The relatively low proportion of TF$^{pos}$-platelets detected in unstimulated blood might appear difficult to reconcile with the robust thrombin generation supported by platelets. To investigate whether platelet TF surface exposure increased under the conditions used for thrombin generation, platelet surface TF was monitored during the initial phase of the CAT assay. This experiment was performed using platelets from HS. TF exposure increased progressively after initiation of thrombin generation, reaching 21.0±1.5% TF$^{pos}$-platelets at 8 minutes (Figure 4).

## Discussion

This study directly compares platelets and monocytes as circulating TF-bearing cell populations in healthy subjects and patients with STEMI. Four main findings emerge. First, although TF$^{pos}$ fractions were similar, expressing TF$^{pos}$ cells as absolute circulating counts revealed several hundred-fold more TF$^{pos}$-platelets than TF$^{pos}$-monocytes per microliter. Second, when thrombin generation was assessed at cell numbers reflecting their relative abundance in the same reference blood volume, platelets showed faster thrombin generation kinetics and higher thrombin peaks in both HS and STEMI, with greater ETP also observed in STEMI. Third, thrombin generation in both preparations was delayed by TF neutralization and accelerated by TFPI blockade, demonstrating reciprocal regulation by TF and TFPI. Finally, platelet surface TF increased rapidly under CAT-like conditions, reaching approximately 21% within 8 minutes. Together, these findings identify platelets, among the two cellular compartments examined, as the quantitatively predominant TF$^{pos}$ population with greater thrombin-generating capacity when compared with monocytes at cell numbers reflecting their relative abundance in blood.

This comparison is relevant because TF content at the individual-cell level does not necessarily reflect the contribution of the entire circulating population. Monocytes may carry more TF per cell than platelets, but the numerical excess of platelets can translate modest TF positivity into a much larger circulating TF$^{pos}$ burden, as we previously showed in acute coronary syndromes.[14,21] This study extends this concept by linking relative cellular abundance to thrombin-generating capacity. Thus, assessment of blood-borne TF should consider both TF positivity or content and the abundance of the TF-bearing population.

This perspective does not diminish the established role of monocyte TF. In acute myocardial infarction, increased monocyte TF expression and activity have been linked to enhanced factor Xa generation and systemic coagulation activation.[9] Consistently, monocyte preparations in the present study supported thrombin generation. Palmerini *et al.*[10] showed that monocyte depletion markedly reduced TF deposition and thrombus formation, while TF neutralization also reduced thrombus formation in leukocyte-reduced platelet-rich plasma. Our data provide a quantitative and functional framework for these complementary observations: monocytes remain an important TF-bearing procoagulant compartment, whereas the greater abundance of platelets can translate modest TF surface expression into a substantial circulating TF$^{pos}$ pool with greater thrombin-generating capacity under the conditions tested. Because monocyte TF is highly inducible, the relative contribution of the 2 compartments may vary with inflammatory or thrombotic activation. Moreover, our experimental design assessed platelet and monocyte-containing preparations separately and did not capture platelet-monocyte crosstalk; *in vivo*, activated platelets may enhance monocyte procoagulant activity through P-selectin-dependent induction of monocyte TF expression.[22,23]

Neutralization experiments confirmed the functional involvement of TF in both preparations: anti-TF prolonged lag time, whereas TFPI blockade accelerated thrombin generation, indicating that TFPI present in the assay restrains TF-driven coagulation initiation. This is consistent with studies showing that surface-associated TFPI limits monocyte TF activity and that its blockade enhances thrombin generation.[9,23] Thus, CAT captures the functional TF–TFPI balance rather than TF antigen expression alone. The prolongation of lag time after TF neutralization is consistent with the established sensitivity of CAT lag time to TF-dependent coagulation initiation.[24] Persistence of thrombin generation after anti-TF treatment does not imply absence of TF involvement, as amplification and propagation mechanisms can sustain thrombin generation once coagulation has been initiated.

The low proportion of TF$^{pos}$-platelets in unstimulated blood was reconciled by the rapid increase in platelet surface TF under CAT-like conditions, reaching approximately 21% within 8 minutes. This is consistent with mobilization of the pre-existing

intracellular TF pool and agonist-dependent TF surface exposure.[11,15] Such dynamic exposure may also contribute to the controversy surrounding platelet TF detection, because sample handling, platelet activation, antibody selection, and analytical procedures influence the amount of TF detected at the platelet surface.[16,17]

These findings may have clinical implications. Platelet-associated TF has emerged as a marker of the prothrombotic platelet phenotype, and prospective data showed that circulating $TF^{pos}$-platelets independently predicted all-cause and cardiovascular mortality over five years in patients with coronary artery disease receiving antiplatelet therapy.[25] These findings provide a mechanistic context by showing that even a small $TF^{pos}$-platelets fraction corresponds to a large circulating TF-bearing cell burden with substantial thrombin-generating capacity. Notably, the platelet–monocyte functional hierarchy remained evident in STEMI samples obtained after primary PCI during dual antiplatelet therapy. Although antiplatelet treatment may have influenced the platelet phenotype, the greater thrombin-generating capacity of platelet preparations relative to monocyte preparations was still detectable in this treated clinical setting. Larger prospective studies should determine whether the $TF^{pos}$-platelet phenotype identifies a higher-risk prothrombotic subset of STEMI patients and provides prognostic information on clinical outcomes. This does not establish platelet TF as the sole determinant of thrombotic risk or exclude an important contribution of monocyte TF, but suggests that its biological relevance may be underestimated when platelet abundance and dynamic surface exposure are not considered.

## Strengths and limitations

A major strength of this study is the direct quantitative and functional comparison of platelet- and monocyte-associated TF under standardized experimental conditions, integrating whole-blood flow cytometry, blood-volume-based cell scaling for CAT, neutralization experiments, and dynamic assessment of platelet TF surface exposure. Several limitations should also be acknowledged. First, $TF^{pos}$ cell counts do not provide information on TF molecular density per cell or total TF antigen burden, and the numerical predominance of $TF^{pos}$-platelets should not be interpreted as evidence that individual platelets carry more TF than monocytes. Second, monocytes were tested within PBMC preparations rather than as purified cells, and a contribution of the accompanying lymphocyte fraction cannot be excluded; future studies using purified monocytes should help confirm these findings and exclude lymphocyte-related effects. Third, the relatively small sample size and the smaller number of monocyte-containing preparations available for some CAT analyses may limit the generalizability and statistical power of the study. Fourth, STEMI samples were obtained approximately 48 hours after primary PCI while patients were receiving dual antiplatelet therapy, which may have influenced platelet phenotype and thrombin-generation characteristics. Fifth, excess exogenous phospholipids were used to minimize differences in cell-associated phospholipid availability; the assay therefore preferentially interrogated TF-dependent coagulation initiation rather than the complete cellular procoagulant phenotype. Sixth, the anti-TF experiments were designed to assess the contribution of TF to thrombin-generation initiation rather than exclusive TF dependence of the entire thrombin generation process. Finally, other potential sources of blood-borne TF, including activated polymorphonuclear leukocytes and extracellular vesicle-associated TF,[26,27] were not examined. Accordingly, the term predominant refers only to the two cellular compartments examined.

## Conclusions

In conclusion, accounting for relative cellular abundance identifies platelets, compared with monocytes, as the quantitatively predominant TF-bearing population with greater thrombin-generating capacity in both HS and STEMI. The rapid increase in platelet TF surface exposure under CAT-like conditions further highlights the dynamic nature of platelet-associated TF and provides a mechanistic context for its previously reported prognostic relevance in coronary artery disease,[25] supporting further evaluation of $TF^{pos}$-platelets as a cardiovascular risk biomarker.

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*Online supplementary material:*

*Supplementary Figure S1. Imaging flow cytometry confirms $TF^{pos}$-platelet and monocyte events.*