Tissue Factor in Patients With Acute Coronary Syndromes
Expression in Platelets, Leukocytes, and Platelet-Leukocyte Aggregates

Marta Brambilla, Marina Camera, Deborah Colnago, Giancarlo Marenzi, Monica De Metrio, Peter L. Giesen, Alessandra Balduini, Fabrizio Veglia, Karl Gertow, Paolo Bigioli, Elena Tremoli

Objective—Activated platelets and circulating platelet-leukocyte aggregates (PLA) are significantly higher in patients with unstable angina than in those with stable angina (SA). Platelets from healthy subjects express TF on activation. The aim of this study was to investigate the expression of TF in PLA, in platelets, and in monocytes of acute coronary syndrome (ACS) patients compared to SA patients and healthy subjects (Controls).

Methods and Results—We enrolled 26 consecutive patients with ACS, 29 patients with SA, and 25 Controls. A significantly greater number of total and TF positive platelet-monocyte aggregates was found by flow cytometry in blood of ACS patients than in either SA patients (3-fold) or Controls (5-fold). ACS patients also had a significantly higher amount of TF-positive platelets than SA or Controls (>3-fold) and significantly higher thrombin generation capacity. TF mRNA expression in platelets was significantly higher in ACS patients than in SA or Controls.

Conclusions—In ACS patients the greater expression of TF in platelets and PLA strengthens the link between platelet activation, blood coagulation, and thrombus formation and may further contribute to the hypercoagulability associated with the disease. (Arterioscler Thromb Vasc Biol 2008;28:947-953)

Key Words: platelets ■ platelet-leukocyte aggregates ■ tissue factor ■ acute coronary syndrome ■ thrombosis

The acute coronary syndrome (ACS) is a clinical state induced by the thrombosis consequent on the rupture of an unstable atherosclerotic plaque. In this syndrome the procoagulant content of complex plaques triggers both platelet activation and coagulation pathways.1

Activated platelets and circulating platelet-leukocyte aggregates (PLA), a sensitive marker of in vivo platelet activation,2 plaque instability, ongoing vascular thrombosis, and inflammation,3 have been found to be significantly higher in patients with unstable angina than in those with stable angina (SA).4,5 It is widely recognized that the interaction between platelets and leukocytes leads to phenotypic changes in both cell types and to the secretion of a variety of bioactive compounds.6 The contribution, however, of platelet-leukocyte interactions to atheromatous plaque instability and to acute progression of ACS is still under investigation.

Tissue Factor (TF) is the main cellular initiator of blood coagulation, and it is also currently considered the protein that links proinflammatory and prothrombotic mechanisms in the progression of atherosclerosis.7 Expression of TF by leukocytes8 or by PLA9 may trigger the extrinsic coagulation cascade; the thrombin thus generated can activate platelets and leads to the formation of a platelet-fibrin thrombus. Our group and others have recently shown that platelets not only express TF protein.10-12 but they also contain its specific mRNA which has been shown to be translated into protein.13,14 In addition, platelet reactivity to classical agonists results in the expression of functional TF on the platelet surface.10

Previous studies have shown that plaque-associated TF, TF plasma levels, as well as TF expressing monocytes are higher in ACS than in SA.15,16 However, no information is available on the expression of TF in platelets or in PLA in ACS.

To gain further insight into the inflammatory/prothrombotic phenotype of ACS patients, we studied the expression of TF in PLA, in platelets, and in monocytes of ACS patients compared to SA patients and healthy subjects.

Materials and Methods

Patient Population

Twenty-six consecutive patients with non-ST elevation ACS (mean age 65±10 years; 21 men), and 29 patients with SA (mean age 64±10 years; 21 men) were included in the study. Twenty-five clinically healthy subjects (mean age 52±8 years; 16 men) recruited from the health-care staff served as Controls (see Table 1). All

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Table 1. Characteristics of the Study Groups

<table>
<thead>
<tr>
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<th>ACS (n=26)</th>
<th>SA (n=29)</th>
<th>Controls (n=25)</th>
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<tr>
<td>Age, yr</td>
<td>65±10‡</td>
<td>64±10‡</td>
<td>52±8</td>
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<tr>
<td>Male, n (%)</td>
<td>21 (81)‡</td>
<td>21 (72)†</td>
<td>16 (64)</td>
</tr>
<tr>
<td>Platelet count ×10^9/µL</td>
<td>237±67</td>
<td>231±85</td>
<td>222±67</td>
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<tr>
<td>WBC count ×10^9/µL</td>
<td>7.03±2.19†</td>
<td>6.91±1.91†</td>
<td>5.85±1.14</td>
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<td>Risk factors, n (%)</td>
<td></td>
<td></td>
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<tr>
<td>Hypertension</td>
<td>11 (42)</td>
<td>16 (55)</td>
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<tr>
<td>Smoking</td>
<td>5 (19)</td>
<td>8 (28)</td>
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<tr>
<td>Diabetes</td>
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<tr>
<td>No. of coronary vessels with stenosis&gt;75% (%)</td>
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<td>0</td>
<td>3 (12)</td>
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<td>3</td>
<td>7 (27)</td>
<td>11 (38)</td>
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<tr>
<td>Previous MI, n (%)</td>
<td>4 (15)</td>
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<td>Previous PTCA, n (%)</td>
<td>5 (21)§</td>
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<td>Previous CABPG, n (%)</td>
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<td>2 (7)</td>
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<td>0.38±0.15</td>
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<tr>
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<td>7.7±9#</td>
<td>3.6±4.3‡</td>
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<tr>
<td>TF, pg/ml</td>
<td>110±10.6‡</td>
<td>84.2±6‡</td>
<td>69.7±5.6</td>
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<td>Medication, n (%)**</td>
<td>6 (23)§</td>
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<td>13 (45)</td>
<td>0</td>
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<tr>
<td>Nitrates</td>
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<td>13 (45)</td>
<td>0</td>
</tr>
<tr>
<td>Calcium antagonists</td>
<td>9 (35)§</td>
<td>23 (79)</td>
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<tr>
<td>Aspirin</td>
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<td>4 (14)</td>
<td>0</td>
</tr>
<tr>
<td>Statins</td>
<td>2 (8)</td>
<td>6 (21)</td>
<td>0</td>
</tr>
</tbody>
</table>

Data are presented as numbers (percentages) and mean±SD. WBC indicates white blood cells; PTCA, percutaneous transluminal coronary angioplasty; MI, myocardial infarction; CABPG, coronary artery bypass graft surgery; Tnp, troponin I; CRP, C-reactive protein; TF, Tissue Factor.

*at hospital admission.

**for ACS patients medication refers at hospital admission.

†P≤0.05 vs Controls; †P≤0.01 vs Controls; ‡P≤0.05 vs SA; §P≤0.01 vs SA.

participants gave written informed consent and the study protocol was approved by the Ethical Committee of the Institution. For details please see supplemental materials, available online at http://atvb.ahajournals.org.

Blood Sampling
Please see supplemental materials.

Flow Cytometry Analysis
Whole blood (WB) analysis of PLA as well as of PLA-, platelet-, and monocyte-associated TF was performed by 3-color flow cytometry with a fluorescence-activated-cell sort (FACS) Calibur (Becton Dickinson) equipped with a 15-mW, air-cooled, 488-nm argon-ion laser. The sensitivity of fluorescence detectors was set and monitored using CaliBRITE beads (Becton Dickinson) according to the manufacturer’s instructions.

For PLA and monocyte analysis the procedure was as follows: 100 µL of WB was incubated for 15 minutes at room temperature (RT) in the dark with saturating concentration of the following mouse anti human monoclonal antibodies (MoAbs): CD45-PerCp (Becton Dick-
P<0.05 was regarded as statistically significant. All analyses were performed using the SAS statistical package v8.02 (SAS Institute).

Results

Table 1 summarizes the demographic, clinical, and angiographic characteristics of the patients enrolled in the study. The two groups of patients were similar with respect to age, gender, risk factor profile, and platelet and WBC counts. As expected, CRP and TF plasma levels were significantly higher in ACS patients. All patients were using various combinations of drugs such as beta blockers, nitrates, etc. None of the Controls was taking any therapeutic drugs.

Markers of Platelet Activation Are Elevated in ACS Patients

Plasma levels of P-selectin and the percentage of PLA circulating in peripheral blood were measured as markers of platelet activation. As expected, plasma P-selectin was twice as high in ACS (72±27.3 ng/mL, P=0.003 versus Controls) and 1.5 times in SA patients (58.5±18.5 ng/mL, P=0.02 versus Controls) as in Controls (36.9±2.9 ng/mL). By flow cytometry, a significantly greater number of platelet-monocyte aggregates was found in peripheral blood from ACS patients than from either SA or Controls (2 and 3 times, respectively) (Figure 1A). Platelet-granulocyte aggregates were also significantly higher in ACS patients than in Controls (Figure 1B).

TF Expression in PLA, Monocytes and Platelets Is Elevated in ACS Patients

The percentage of TF-positive platelet-monocyte aggregates was significantly higher in ACS than in SA patients (more than 3-fold higher) and in Controls (5-fold higher) (Figure 1C). TF-positive platelet-granulocyte aggregates showed a trend similar to that observed for TF-positive platelet-monocyte aggregates, being higher in ACS patients than in the other groups; the scanty number of events occurring in each subject, however, does not allow a proper statistical evaluation (Figure 1D).

Because, as previously mentioned, both activated platelets and monocytes can express TF on their surface, it would have been of interest to determine how much TF is expressed by each type of cell in the aggregates. Flow cytometry, however, does
not allow to assign TF measured within the aggregate to either cell type. Thus, we further characterize the pattern of TF expression in whole blood measuring TF associated with pure monocyte (CD45+/H11001+CD41+/H11002+) and platelet (CD41+/H11001+/CD45+/H11002+) populations.

Blood from ACS patients contains a significantly higher number of monocytes expressing TF than that from SA patients and Controls (%UR: 4.85±0.55; 2.46±0.46; 1.76±0.62 respectively, _P_ =0.009 ACS versus Controls and _P_ =0.04 ACS versus SA). Further, a significant greater amount of TF-positive platelets was also found in ACS patients than in SA patients or Controls (more than 3-fold in both cases) (Figure 2A). Not only was the percentage of TF expressing platelets higher in ACS, but the TF-specific MFI was also significantly higher in ACS than in SA patients and Controls (roughly twice in both cases), indicating that the number of TF molecules expressed on each platelet is significantly higher in ACS than in SA patients and Controls.

The Platelet-Associated Thrombin Generation Capacity Is Elevated in ACS Patients

The functional activity of platelet-associated TF was determined by measuring the thrombin generation capacity with the Calibrated Automated Thrombography in washed platelet lysates (1.0×10⁶/mL). The endogenous thrombin potential (ETP; black bars) and the peak height (dashed bars) were both significantly higher in ACS than SA patients and Controls. FAU = Fluorescence Arbitrary Units.

Figure 2. Platelet-associated TF expression and thrombin generation capacity is higher in ACS patients. A, WB flow cytometry analysis of TF expression in platelets from ACS (n=26), SA (n=29) patients, and Controls (n=25). Data are expressed as %UR (TP=0.005 ACS vs Controls; #P=0.004 ACS versus SA) as well as MFI (^P=0.04 ACS vs Controls; §P=0.01 ACS vs SA) as indicated. B, The functional activity of platelet-associated TF in ACS and SA patients and Controls (n=10 for each group) was determined by measuring the thrombin generation capacity with the Calibrated Automated Thrombography in washed platelet lysates (1.0×10⁶/mL). The endogenous thrombin potential (ETP; black bars) and the peak height (dashed bars) were both significantly higher in ACS than SA patients and Controls.
Table 2. TF-Positive Cells and Aggregates in ACS, SA, and Controls in 1-Microliter Blood Volume

<table>
<thead>
<tr>
<th></th>
<th>ACS</th>
<th>SA</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelets</td>
<td>20952±6190</td>
<td>6047±1163</td>
<td>5556±889</td>
</tr>
<tr>
<td>Monocytes</td>
<td>14±1.5</td>
<td>7±1.3</td>
<td>4±1.4</td>
</tr>
<tr>
<td>Platelet-monocyte aggregates</td>
<td>8±1.7</td>
<td>2±0.4</td>
<td>1±0.02</td>
</tr>
</tbody>
</table>

Values represent the mean±SD of TF-positive cells determined by using the flow cytometry data and the platelet and WBC counts.

and the peak height were both significantly higher in ACS patients than SA (P=0.01 and 0.02, respectively) and Controls (P=0.004 and 0.03, respectively) (Figure 2B).

Experiments performed in the presence of a specific anti-TF antibody or an anti—tissue factor pathway inhibitor (TFPI) antibody confirmed the partial contribution of platelet-associated TF to thrombin generation (40% reduction and 30% increase respectively; online Figure I).

Increased TF mRNA Expression in Platelets and Monocytes From ACS Patients

We have previously shown that platelets from healthy subjects contain TF mRNA which can even be translated into protein.10,13,14 Platelets are anucleated cells and derive their RNA content from megakaryocytes. Indeed, we report here that human megakaryocytes isolated from cord blood contain consistent amount of TF mRNA (22±1.8 relative expression compared to TF mRNA expressed in platelets from healthy donors).

Measurement of TF mRNA expression in leukocyte-free platelets (online Figure II) by real-time PCR showed significantly higher expression of the transcript in platelets from ACS patients (3.11±0.51 relative expression, P=0.03 versus Controls) compared to SA and to Controls (1.88±0.78 and 0.7±0.1 relative expression, respectively; Figure 3A).

![Figure 3](Figure 3. Expression of TF mRNA is higher in platelets from ACS patients. TF mRNA levels in platelets of ACS (n=11), SA (n=13) patients, and Controls (n=10) were determined by real-time PCR. Amplification of GAPDH and β-actin mRNA was used to normalize for differences in RNA extractions and amplifications. Histograms represent the TF mRNA relative expression (±SEM) calculated on a standard curve. Samples were run in triplicate and amplifications were confirmed 3 times.)

![Figure 4](Figure 4. Expression of TF mRNA in monocytes is higher in ACS patients. TF mRNA levels in monocytes of ACS (n=11), SA (n=13) patients, and Controls (n=10) were determined by real-time PCR. Amplification of GAPDH and β-actin mRNA was used to normalize for differences in RNA extractions and amplifications. Histograms represent the TF mRNA relative expression (±SEM) calculated on a standard curve. Samples were run in triplicate and amplifications were confirmed 3 times. See Methods for details.)

Of interest, TF mRNA levels in ACS significantly correlated with CRP levels (r=0.61, P=0.035).

It is known that platelet turnover may be higher in ACS patients, and newly synthesized platelets contain more mRNA than old platelets.17 Mean platelet volume, an index of newly released platelets, did not differ significantly among ACS, SA, and Controls (7.3±0.8 fl in ACS, 6.6±0.7 in SA, 6.8±0.4 in Controls), and fully concordant data were obtained with the evaluation of reticulated platelets (RP) by thiazole orange staining (% RP: Controls: 6.9±0.7; SA: 7.6±0.6; ACS: 7.3±0.5).

TF mRNA expression in monocytes showed a pattern similar to that observed in platelets within the groups being significantly higher in ACS patients compared to Controls (Figure 4).

Discussion

In this study we show for the first time that the numbers of TF-positive platelets and TF-positive platelet-monocyte aggregates circulating in ACS patients are significantly higher than those in SA patients and in healthy subjects, providing a further explanation of the increased cell thrombogenicity in ACS.

Patients with coronary artery disease (CAD) have long been reported to have circulating activated platelets, platelet-derived microparticles, platelet-monocyte aggregates, and increased platelet reactivity.5,18 Platelets are nowadays recognized as playing a major role in inflammation as well as in hemostasis and thrombosis, being the source of inflammatory mediators and able to both produce and respond to chemoattractant cytokines.19,20 The binding of platelets or platelet-derived microparticles to monocytes (it is not possible to discriminate between platelet and platelet microparticles bound to leukocytes by flow cytometry) in ACS is one of the clues to the interaction of inflammation and thrombosis:
when inflammation begets local thrombosis, this in turn exacerbates inflammation, resulting in a vicious circle.\textsuperscript{1,21}

We now provide evidence that in ACS patients more than 3 times as many circulating platelets expressing TF on their surface as are found in SA patients and in Controls. Not only is the number of TF-positive platelets higher in ACS, but each platelet expresses twice the number of TF molecules than in the other groups studied, so that the total capacity to generate thrombin is greater. Thus, TF-bearing platelets as well as PLA might be responsible not only for the increase in thrombus formation triggered by unstable plaque rupture, but also may provide potential distal sites for the generation of new thrombi.

Although further studies are needed to determine in vivo the relative contribution of platelet-associated TF to thrombotic events, we believe that this finding is of particular importance especially if we consider the absolute number of circulating platelets and that of circulating monocytes in an adult subject. In \(1\ \mu\text{L blood volume there are roughly 200\times10^3\) platelets and 240 monocytes (considering monocytes 4\% of total leukocytes). The percentage of TF positive platelets and monocytes in ACS patients accounts for 8.8 and 4.8\%, respectively, and this results in almost a 2000-fold higher amount of TF positive platelets circulating in blood compared to TF positive monocytes (TF positive platelets: \(\approx21\times10^9\); TF positive monocytes: 14 in 1 \(\mu\text{L blood volume; see also Table 2).\)

We believe that such a big difference has to be carefully considered. We have not performed in the present study a direct comparison of TF activity between platelets and monocytes, and this may be a limitation of the study. Certainly, the TF activity of a single monocyte is higher than that of a single platelet, but if we consider the number of platelets present in a thrombus we might not underestimate their contribution. Further studies, perhaps in animal models, may help solving this issue.

Another major finding of the present study is the observation that the levels of the specific TF mRNA in platelet devoid of leukocyte contamination from ACS patients are higher compared to that found in SA patients and in Controls. Quantitative differences in selected mRNA levels between acute and stable CAD patients have been also recently observed in platelet transcriptome studies.\textsuperscript{22}

Data published in the past few years have shown that platelets possess megakaryocyte-derived mRNAs that can be translated into proteins because young platelets also contain rough endoplasmic reticulum and polyribosomes.\textsuperscript{23} Activated platelets synthesize inflammatory proteins such as Bcl-3\textsuperscript{24} and interleukin (IL)-1,\textsuperscript{25} enzymes,\textsuperscript{26} receptors,\textsuperscript{27,28} and recently the synthesis of TF has also been reported.\textsuperscript{13,14} We have previously shown that platelets from healthy individuals contain TF mRNA,\textsuperscript{10} and in the present study we further provide evidence for the presence of TF mRNA in human megakaryocytes.

Several mechanisms may be responsible for the increased TF mRNA levels observed in ACS patients. The presence, as previously described,\textsuperscript{13,14} of TF pre-mRNA which could be spliced more efficiently to mature message in platelets from ACS patients could account for this finding. Further studies however are needed to test this hypothesis because in our experimental conditions we did not detect TF pre-mRNA in platelet nor in megakaryocytes.

The positive correlation found between TF mRNA and CRP levels may suggest that systemic inflammatory reaction present in ACS patients might affect the TF content in megakaryocytes and consequently in platelets. Finally, an increased stability of TF mRNA through mechanisms involving RNA-binding proteins such as HuR could also be assumed. Appropriate studies however are required to test these hypothesis. Irrespective of the mechanisms involved, the higher amount of TF mRNA found in platelets from ACS patients further strengthen their prothrombotic potential, especially in view of the platelet biosynthetic potential. Of note, we did not find correlation between platelet-associated TF protein expression and TF mRNA levels. This may suggest that other mechanisms, such as a TF-positive microparticle transfer, may also contribute to the TF protein content in platelets.\textsuperscript{29}

The platelet-derived TF may contribute to fibrin formation and to the propagation and stabilization of a thrombus but can also participate, as recently shown, in several cellular processes that stimulate atherogenesis such as angiogenesis and cell migration, both of which are associated with plaque growth and, under certain circumstances, plaque weakening leading to destabilization of the lesion.\textsuperscript{30} All together these observations are of particular relevance also in the light of the finding that activated platelets and platelet-leukocyte aggregates exacerbate atherosclerosis.\textsuperscript{31}

Antiplatelet therapy is reasonably effective in the treatment of ACS, but present forms of antiplatelet therapy fail to prevent coronary events in a substantial proportion of patients.\textsuperscript{32,33} This suggests that the complex mechanisms involved in platelet activation and thrombus formation are not completely understood. While aspirin and calcium antagonists treatments were found to be associated with reduced levels of circulating PLA, TF-positive PLA, and TF-positive monocytes (in agreement with previous studies\textsuperscript{34,35}), the association with TF expression in platelets was not observed. We have previously shown that aspirin has no effect on agonist-induced membrane exposure of TF,\textsuperscript{10} whereas \(\text{P}_2\text{Y}_{12}\) receptor antagonists completely prevent its expression\textsuperscript{36} (MaRina Camera, unpublished data, 2005) and GPIIb/IIIa antagonists, paradoxically, increase the stimulatory effect of adenosine 5'-diphosphate.\textsuperscript{37} None of the patients in this study was receiving ticlopidine/elopidogrel or any GPIIb/IIIa antagonists. As this was an observational study, the associations we have observed should not be interpreted as cause and effect. Randomized studies are in progress to assess the effect of antiplatelet drugs on platelet TF expression in ACS patients.

In conclusion, the present study further extends the proinflammatory/prothrombotic phenotype of ACS patients showing that new players on the scene, ie, TF-positive platelets and TF-positive PLA, may be seen to contribute to the higher procoagulant potential that is characteristic of these patients.

\textbf{Acknowledgments}  
We thank Franco Moro for excellent technical assistance.
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Disclosures
None.

References
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Online Figure I

A

![Graph A: ETP (FAU x min)](image1)

- **ETP (FAU x min)**
- **Controls SA ACS**
- **no Ab**
- **αTFAb**
- **αTFPIAb**

B

![Graph B: Peak height (FAU)](image2)

- **Peak height (FAU)**
- **Controls SA ACS**
- **no Ab**
- **αTFAb**
- **αTFPIAb**
Online Figure II

A

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B

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Figure I: The functional activity of platelet-associated TF in ACS and SA patients and Controls (n=10 for each group) was determined by Calibrated Automated Thrombography in washed platelet lysates (1.0x10^8/mL). The effect of neutralizing anti-TF and anti-TFPI antibodies (10 μg/ml) was used to assess the platelet-associated TF contribution to thrombin generation measured as (A) endogenous thrombin potential (ETP) and (B) peak height.

Figure II: Leukocyte contamination of the platelet RNA preparations was assessed by amplification of CD2 (A) and CD20 (B) by RT-PCR. A representative experiment where 4 different samples for each group of patients analyzed is shown.
Patient population

Twenty-six consecutive patients with non-ST elevation ACS (mean age 65±10 years; 21 men), and 29 patients with SA (mean age 64±10 years; 21 men) were included in the study. Twenty-five clinically healthy subjects (mean age 52±8 years; 16 men) recruited from the health-care staff served as Controls (see Table 1).

ACS was defined as chest pain at rest with documented transient ST segment depression or T-wave inversion in at least two contiguous electrocardiographic leads, without pathological Q waves, and with enzymatic evidence of myocardial necrosis (non-Q-wave myocardial infarction; n=18) or without it (unstable angina; n=8). The last spontaneous episode of chest pain had to have occurred within 24 hours as a condition of entry into the study.

Patients with SA had typical chest pain on exertion associated with ST segment depression >1.0 mm on an exercise test. In all ACS and SA patients coronary angiography confirmed the presence of significant coronary artery stenosis. Exclusion criteria were: age > 80 years, recent myocardial infarction (<6 months), angina precipitated by correctable factors such as valvular heart disease, atrial fibrillation, thyrotoxicosis, and hypertension (>180/110 mmHg) at admission, coronary angioplasty within 6 months, history of hemorrhagic diathesis, platelet disorder or thrombocytopenia, anticoagulation therapy, malignancies, severe liver disease and renal insufficiency. Patients who were receiving thrombolytics or heparin were also excluded.

All participants gave written informed consent and the study protocol was approved by the Ethical Committee of the Institution.
Blood sampling

Venous blood samples were obtained by venipuncture of the antecubital vein with a 19G needle without venous stasis. After the first 4 mL had been discarded, 40 ml blood was drawn into Vacutainer® tubes containing citrate (1/10 vol sodium citrate 0.129 mol/L, Becton Dickinson). In ACS patients blood samples were drawn at hospital admission before administration of drugs; in SA patients blood was withdrawn during hospitalization. Blood samples were processed within 15 minutes of blood drawn.

Plasma samples prepared by centrifugation at 1700xg for 10 minutes at 4°C were frozen and stored at –80°C until assayed.

Preparation of mononuclear cells

Mononuclear cells were separated from venous blood using Ficoll-Paque density gradient and monocytes were isolated by selective adherence (90 min) to tissue culture dishes. Cell preparations were >90% monocytes, as determined by non-specific esterase staining. Total RNA was then extracted as described.

Preparation of megakaryocytes

Cells were obtained from normal human cord blood (CB) doomed to discard after approval of the Institutional Review Board and processed as previously described. Mononuclear cells were separated by layering CB onto Lympholyte (<1077 g/ml, Cedarlane, Hornby, Canada) and centrifuging for 30 min at 1500 rpm (425 x g) at 20°C. The resulting nucleated cells were washed twice in phosphate-buffered saline (PBS) and suspended in Stem Span medium (Stem-Cell Technologies, Vancouver, Canada).

Liquid cultures were initiated by plating 1 x 10⁶/ml low-density cells in Stem Span medium containing 10 ng/ml thrombopoietin (TPO), interleukin (IL)6, IL11, Flt3-L (all from PeproTech EC
Ltd., London, UK). Cultures were maintained for up to 12 days at 37°C in a humidified atmosphere of 5% CO₂. CD41⁺ cells were separated after 12 days of culture using a FACS VantageSE (Becton Dickinson Immunocytometry Systems, San José, CA) and processed for RNA extraction. Sorted cell population was 98% CD41-positive, as determined by flow cytometry and microscopy analysis (data not shown).

**Thiazole orange labeling of reticulated platelets (RP)**

For RP measurement 50μl of fixed whole blood was incubated with monoclonal PE-anti GPIIb/IIIa for 15 minutes and then stained with TO (20ng/ml final concentration in PBS containing 2mM EDTA) at room temperature for 1 hour in the dark. As control of aspecific binding, RNAse- and thrombin receptor activating peptide (TRAP)- treated samples were used. Fixed whole blood was treated with 50μg/ml RNase for 5 min at room temperature before TO incubation. For platelet degranulation, unfixed blood was stimulated with 35μM TRAP for 15 min at room temperature and subsequently processed for TO labeling as described above. Measurement of TO-positive platelets was performed by flow cytometry. Platelets were identified by size and positive PE fluorescence; 10,000 events were acquired per sample and data were reported as percentage of RP.

**Measurement of TF, P-selectin and CRP**

Plasma TF and P-selectin levels were determined by ELISA (American Diagnostica and R&D System, respectively) according to the manufacturer’s instructions. CRP levels were determined using an immunoturbidimetric method applied on the Olympus AU2700 biochemistry analyzer. For all the assays the intra- and inter-assay coefficients of variation were <6%. 
**Generation of thrombin**

The Calibrated Automated Thrombogram (Thrombinoscope BV, The Netherlands) was measured as previously described. Platelet-free plasma (PPP) collected on citrate (0.129 mol/L) and 18 μg/mL corn trypsin inhibitor (Enzyme Laboratories, USA) was added to samples of washed platelets (1.0x10^8/mL) and phospholipid vesicles (4 μmol/L of 20%DOPS-20%DOPE-60%DOPC). The addition of phospholipids was necessary in order to make the assay TF-specific and not phospholipid dependent. Fluorogenic substrate, Z-Gly-Gly-Arg-AMC, was obtained from Bachem (Bubendorf, Switzerland). Upon splitting by thrombin, it releases the fluorescent AMC (7-amino-4-methylcoumarin), which is measured by a 390-nm-excitation and a 460-nm-emission filter set against time in a 96-well plate fluorimeter (Thermo Scientific, Finland). The curves were analyzed by dedicated software (Thrombinoscope, Maastricht, The Nettherlands). Peak height (fluorescence arbitrary units, FAU), time-to-peak (minutes) and the area under the curve (FAU x minutes, i.e. endogenous thrombin potential, ETP) were used as the main parameters determining thrombin generation curves. Control experiments performed in PPP without platelet, but with phospholipids vesicle addition showed no thrombin generation. The effect of neutralizing anti-TF and anti-TFPI antibodies (cat no. 4509 and cat no. 4904, respectively American Diagnostica; 10 μg/ml) was used to assess the platelet-associated TF contribution to thrombin generation. The intra- and inter-assay coefficients of variation were <9%.

**RNA extraction and Real-time PCR**

Megakaryocyte, platelet and monocyte total RNA was isolated by the guanidinium isothiocyanate procedure as previously described. Platelet RNA (100 ng) was then amplified using *in vitro* transcription (MessageAmp II aRNA amplification kit, Ambion).
500 ng of total or amplified RNA was reverse-transcribed using 20U of Stratascript Reverse Transcriptase (Stratagene) and 0.5 µg of random primer together with the manufacturer's buffer, 500 nM dNTP, and 40U of RNase inhibitor. Incubation was at 42°C for 60 min.

Real-time quantitative PCR was carried out in two steps (denaturation at 95°C for 15 sec and annealing/extension at 60°C for 60 sec, 50 cycles) on iCycler Optical System (Bio-Rad Laboratories) as previously described.² cDNA (25 ng) was incubated in 25 µL IQ SYBR® Green Supermix containing specific primers (200 nM) as listed in Table I. The presence of TF pre-mRNA in platelets was also evaluated. Amplifications of two housekeeping genes, GAPDH and β-actin, were used to normalize each sample for fluctuations in input RNA levels. In order to prove that PCR products were RT-dependent and not due to contamination with genomic DNA, RNA was reverse-transcribed in the presence or absence of retro-transcriptase. Samples were run in triplicate and amplifications were confirmed three times.

A 5-step dilution series of cDNA was used to obtain a linear equation in order to calculate the amplification efficiency ($E = 10^{-1/slope}$) of each reaction and used to calculate the amount of mRNAs (relative expression) by Gene Expression Macro™ analysis (Bio-Rad Laboratories).

Analysis of the melting curves obtained by stepwise increase of the temperature from 55°C to 95°C was used to verify the specificity of amplified products. All the amplicons generated a single peak, which reflects the specificity of the primers.
Table I. Primers used in real time-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’→3’)</th>
<th>Exon</th>
<th>Amplicon length</th>
<th>Concentration (nM)</th>
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<td>5</td>
<td>93 bp</td>
<td>500</td>
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<td></td>
<td>Rev tctacggggtgtctgactcttt</td>
<td>5</td>
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<tr>
<td>TF**</td>
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<td>802 bp (pre-mRNA)</td>
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<tr>
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<td>198 bp (mRNA)</td>
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<tr>
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<tr>
<td></td>
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<td>Rev tcgcctctggaagatggtg</td>
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** primers used for pre-mRNA evaluation.

REFERENCES