Biomarkers of platelet activation in acute coronary syndromes

Patrizia Ferroni1; Silvia Riondino1; Natale Vazzana2; Nicole Santoro2; Fiorella Guadagni1; Giovanni Davì2
1Department of Advanced Biotechnologies and Bioimaging, IRCCS San Raffaele Pisana, Rome, Italy; 2Internal Medicine and Center of Excellence on Aging, "G. D’Annunzio" University Foundation, Chieti, Italy

Summary
The most convincing evidence for the participation of platelets in arterial thrombosis in humans comes from studies of platelet activation in patients with acute coronary syndromes (ACS) and from trials of antiplatelet drugs. Both strongly support the concept that repeated episodes of platelet activation over the thrombogenic surface of a vulnerable plaque may contribute to the risk of death from coronary causes. However, the relation of in vivo platelet activation and adverse clinical events to results of platelet function tests remains largely unknown. A valuable marker of in vivo platelet activation should be specific, unaltered by pre-analytical artefacts and reproducibly measured by easily performed methods. This article describes current biomarkers of platelet activation in ACS, reviews their advantages and disadvantages, discusses their potential pitfalls, and demonstrates emerging data supporting the positive clinical implications of monitoring in vivo platelet activation in the setting of ACS.

Keywords
Acute coronary syndromes, platelet-derived biomarkers, thromboxane metabolites, P-selectin, CD40L

Platelet involvement in ACS
Acute coronary syndromes (ACS) represent clinical conditions that vary in gravity from unstable angina (UA) to myocardial infarction (MI), either non-ST-segment elevated (NSTEMI) or ST-segment elevated (STEMI). The involvement of platelets in atherothrombotic disease is unquestioned and platelet activation in course of ACS is an important determinant (Fig. 1) for clinical outcome (1) as demonstrated by several prospective studies (2–4). Platelet function is increased under high shear stress in patients with ACS and contributes to endothelial activation and modulation of inflammatory responses, thus favouring the formation of atherosclerotic lesions and their subsequent thrombotic complications (5). Indeed, following vascular damage, the consequent exposition of the highly procoagulant contents of the core constituents to circulating platelets, causes platelet deposition, that, depending on the type and nature of lesions and the composition of the atherosclerotic plaque (6), culminates in intracoronary thrombus formation with severe stenosis.

The first step in thrombus formation entails the classical phases of platelet activation, namely adhesion, activation and aggregation that, however, sees the involvement of a complex network of signalling triggered by activation of inflammatory and endothelial cells and the consequent outfit of released mediators (7). The mechanism through which platelets adhere to damaged subendothelium involves the classical multistep of tethering, followed by rolling and subsequent firm adhesion, although transient platelet interactions with morphologically intact endothelium is currently acknowledged as an important step in the initiation and progression of atherosclerosis (8). Many inflammatory conditions are associated with endothelial activation, and release and exposure of P-selectin on the surface of endothelium may promote platelet adhesion by binding to glycoprotein Ib/V/IX (GPIb/V/IX) (9). GPIb/V/IX interacts also with vessel wall von Willebrand factor (VWF) (10) and, in conditions of high shear rates such as those encountered in ACS vessels, VWF substitutes for fibrinogen as a bridge molecule between fibrinogen receptor integrin αIIbβ3 for platelet aggregation which functions in all circulatory conditions of low shear (11).

Platelet activation, through the signalling events evoked by engagement of integrin αIIbβ3, leads also to de novo protein synthesis through rapid and highly regulated translation of pre-existing megakaryocyte-derived mRNAs (12). Of interest, it has been recently suggested that platelets in ACS are potentially preconditioned to a higher degree of reactivity on the transcriptional level and that changes of the mRNA pool might mediate an increased platelet prothrombotic potential in this setting (13). Indeed, NSTEMI patients proved to have enhanced baseline platelet function than patients with UA and normal subjects (14). Accordingly, Tantly et al. (15), by analysing the relation of platelet reactivity, hypercoagulability and inflammation in various stages of coronary artery disease (CAD) acuity, observed an overall increase in all measurements from a clinically stable to an unstable disease state, and highlighted the role of platelets in plaque destabilisation.

In this scenario, it is widely recognised that the cornerstone of antithrombotic therapy is represented by platelet antagonists.

Correspondence to:
Giovanni Davì
Center of Excellence on Aging
"G. D’Annunzio" University Foundation
Via Colle dell’Ara, 66013 Chieti, Italy
Tel.: +39 0871 541312, Fax: +39 0871 541261
E-mail: gdavi@unich.it

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There is, therefore, great interest in the identification of a reliable platelet biomarker. An ideal marker, especially in the emergency setting represented by ACS, is one in which there is a specific, easily measurable increase that clearly orientates the physician towards a diagnosis or a predictable clinical outcome in such a way that it guides antiplatelet therapy.

Platelet-derived biomarkers: a translational approach

The biomarkers currently used in ACS diagnosis and risk stratification include molecules – such as troponins and natriuretic peptides – that are released only after cardiomyocyte necrosis. Although clinically useful, they do not actually reflect the pathobiology of early phases of atherothrombosis. In addition, a significant proportion of patients have unstable coronary disease and myocardial ischaemia without infarction, which cannot be easily detected using current diagnostic strategies. Thus, it has been proposed that markers of inflammation, endothelial dysfunction or platelet activation can be used to identify disease activity even before myocardial necrosis occurs. These biomarkers may also provide important supplementary information for early diagnosis, risk stratification and therapy monitoring.

The ideal hypothetical biochemical marker should represent a laboratory measurement exhibiting high clinical sensitivity and specificity, a predictable release profile with variations in its rise and fall pattern and should be assayed with a rapid and simple technique (16). A surrogate marker is a laboratory measurement or physical sign that can be used in therapeutic trials as a substitute for a clinically meaningful endpoint that represents a direct measure of how a patient feels, functions, or survives and that is expected to predict the effect of a therapy (17). Both biomarkers and surrogate endpoints use could be hampered by poor reproducibility of measurement techniques (18).

Methods such as platelet aggregation quantify in vitro platelet activation in response to exogenous agonists, whereas the measurement of platelet release products in the plasma quantifies in vivo platelet activation (19). The ex vivo measurement of platelet responses to various agonists provides an index of the functional capacity of platelets, but such measurements could not reflect the extent of platelet activation in vivo (6).

A valuable plasma marker of in vivo platelet activation should be specific, unaltered by pre-analytical artefacts and reproducibly measured by easily performed methods. A wide variety of techniques have been proposed to assess in vivo platelet activation, including flow cytometry evaluation of activation-dependent changes or biochemical immunoassays for measurement of platelet-derived or released substances or for molecules firstly exposed and then shed (Fig. 2 and Table 1) from the platelet surface (20). At present, however, since platelet activation involves more than one signalling pathway and due to complex preanalytic factors, reduced specificity, and poor reproducibility, no single biomarker may be indicated as the “gold standard” predictive of in vivo platelet activation.

An over-didactic division of the platelet molecules that could represent possible candidate biomarkers considers: a) circulating biomarkers, b) membrane-associated markers, c) urinary biomarkers, and d) a multimarker approach (Table 1) (16).

Figure 1: The role of platelets in acute coronary syndromes (ACS). Platelet activation plays a pivotal role in the pathophysiology of ACS. In fact, several molecules, released by platelets after activation, may directly induce cardiac arrhythmias and myocardial dysfunction. In addition, activated platelets are a source of potent vasoconstrictors. Finally, platelet activation is critically involved in coronary plaque rupture and subsequent atherothrombosis. MMPs, matrix metalloproteinase; ROS: reactive oxidant species; VSMC, vascular smooth muscle cell.
**Methodological pitfalls**

Pre-activation due to methodological pitfalls is one of the major risks when analysing platelet-derived biomarkers, in that pre-analytical and analytical interferences may confound interpretation of analyte measurements (3, 21–23). While the attention should always be high when handling results obtained by analysing platelet samples, this is of particularly meaningful in acute settings, such as ACS (24).

Indeed, studies on the most widely used platelet biomarkers recovery have demonstrated that their dosage can be influenced by several factors, including blood sampling, anticoagulation, centrifugation protocols, storage and/or thawing. Recent guidelines drawn up by the British Committee for Standards in Haematology highlight the importance of all the phases of blood withdrawal, from patient’s resting and fasting, venipuncture, choice of anticoagulant, blood sampling and processing, among the most common preanalytical variables that can cause artifactual platelet activation (25).

Moreover, the authors strongly recommend to register a detailed personal history recording all drugs and even food ingested by the patients, that can cause acquired platelet dysfunction (25).

Among the techniques employed to assess platelet-derived biomarkers, flow cytometry appears as an effective technique and is currently used to measure glycoprotein activation-dependent changes, exposure of granule membrane proteins, platelet-leukocyte aggregates, or procoagulant platelet-derived microparticles in a number of clinical settings, including ACS. Major disadvantage of flow cytometry, despite the unquestioned advantages that this technique has provided in reducing artifactual activation, is that it requires specialist equipment and experienced technicians and, most importantly, fresh blood samples that can be hardly stored for large epidemiological studies.

Presently, among all the available techniques developed for the study of platelet function, none embodies the best test, in that the right test should be chosen according to the purpose of testing. Since, however, the description of current clinical test of platelet function is beyond the aim of this review, we recommend referring to more pertaining studies (26–28).

**Circulating biomarkers: the platelet releasate and the platelet sheddome**

Platelets possess an outfit of preformed internal granules, namely dense, alpha, and lysosomes that, upon platelet activation, release their content through a process known as secretion. The first, most evident, biological role of platelet secretion is a paracrine role, since released ADP recruits and activates other platelets in the surrounding area, serotonin acts on smooth muscle cells and fibrinogen and VWF trigger wound repair. However, there are many other chemokines, cytokines, adhesive proteins, cytoskeletal proteins and growth factors that, due to their heterogeneity, are involved in a wide variety of functions. Moreover, platelets contain distinct populations of α-granules that can undergo differential release in vitro and might even exert opposite effects.

![Figure 2: Platelet-derived biomarkers. Biomarkers of platelet activation include 1) cleaved surface molecules: soluble (s)CD40L, sP-selectin, sGPVI, sGPV; 2) platelet surface molecules: CD40L, P-selectin, activated GPIb/IIa, phosphatidylserine (PS); 3) molecules released by platelet after activation: TXA2 and its metabolites, in particular urinary 11-dehydro-TXB2, matrix metalloproteinases (MMPs), β-thromboglobulin (β-TG), platelet factor (PF)-4, thrombospondin (TSP)-1, myeloid related protein (MRP) 8/14; (3) P-selectin- or tissue factor (TF)- bearing platelet-derived microparticles; 4) platelet-leukocyte aggregates and 5) platelet transcript profile.](image-url)
example stems from the study by Italiano et al. (29), who demonstrated that pro- and anti-angiogenic proteins are separated in distinct subpopulations of α-granules in platelets and megakaryocytes and may be regulated by differential G-protein-mediated signalling pathways. Similarly, it has been observed that thrombin receptor PAR1, ADP receptors (P2Y12 and P2Y1) and GPVI stimulation favours platelet release of pro-angiogenic factors, while thrombin receptor PAR4 stimulation selectively induces platelet secretion of anti-angiogenic factors. The differential release in response to distinct agonists might be mediated by different SNARE (Soluble NSF Attachment Protein Receptors) proteins (30).

The exocytosis of platelet granules causes the redistribution of membrane proteins, some of which, upon activation, become externalised, while others, normally constituent of the outer platelet membrane, become internalised or proteolytically shed in membrane-derived microparticles (MPs). For instance, a novel platelet receptor for phosphatidylserine (PS) and oxidised lipoprotein, SR-PSOX/CXCL16 (CXCL16) has been identified, which upon

### Table 1: Biomarkers of platelet activation in acute coronary syndrome (ACS): advantages, limitations and clinical significance.

<table>
<thead>
<tr>
<th>Class</th>
<th>Biomarker</th>
<th>Assay</th>
<th>Pros</th>
<th>Cons</th>
<th>Clinical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Circulating molecules</td>
<td></td>
<td></td>
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<tr>
<td>Shed</td>
<td>sP-selectin</td>
<td>ELISA*</td>
<td>Limited ex vivo generation</td>
<td>Extraplatelet sources</td>
<td>May help to identify patients at increased risk of adverse events after ACS (44, 46)</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td>May be useful in risk stratification of patients presenting with chest pain (50–52)</td>
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<td></td>
<td>sCD40L</td>
<td></td>
<td>Extensively studied in ACS &gt;95% platelet-derived</td>
<td>Investigates the link between inflammation and thrombosis</td>
<td>May be influenced by underlining inflammation, genetic polymorphisms</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Methodological issues (ex vivo shedding, circadian variations)</td>
</tr>
<tr>
<td></td>
<td>sGPVI</td>
<td>Platelet-specific marker</td>
<td>May be influenced by genetic polymorphisms</td>
<td>Extraplatelet sources</td>
<td>May help to identify ACS patients at highest risk for subsequent cardiovascular events and those who may benefit from a more aggressive antiplatelet treatment (63)</td>
</tr>
<tr>
<td></td>
<td>sGPV</td>
<td>Platelet-specific marker</td>
<td>May be influenced by cigarette smoking</td>
<td></td>
<td>Controversial results (endogenous protective factor?) (109, 180, 181)</td>
</tr>
<tr>
<td>Released/secreted</td>
<td>TSP-1, PF4 and β-TG</td>
<td></td>
<td>Reflect α-granule secretion</td>
<td>Methodological issues (anticoagulant, centrifugation setting, ex vivo platelet activation)</td>
<td>Limited data available (77)</td>
</tr>
<tr>
<td></td>
<td>SCUBE1</td>
<td></td>
<td>High specificity (undetectable in healthy controls and stable CAD patients)</td>
<td>Slow kinetics, limited sensitivity, extraplatelet sources</td>
<td>Preliminary data showed increased levels in ACS patients, concomitant with the increase in plasma sCD40L (80)</td>
</tr>
<tr>
<td></td>
<td>MMPs</td>
<td></td>
<td>Directly involved in plaque destabilisation and rupture</td>
<td>Extraplatelet sources</td>
<td>MMP-2 is an independent predictor of all-cause mortality post-ACS (183)</td>
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<tr>
<td></td>
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<td></td>
<td>MMP-9 is an independent predictor of cardiovascular mortality in patients with CAD (87)</td>
</tr>
</tbody>
</table>

| B. Membrane-associated markers | | | | | |
| Surface receptors | P-selectin | Flow-cytometry | Expressed only on activated platelets |Need of fresh whole blood | Limited and controversial results; poor correlation with plasma sP-selectin levels (97) |
| | CD40L | | Investigates the link between inflammation and thrombosis | Surface expression is influenced by receptor shedding and/or internalisation | Tight correlation between platelet CD40L expression and plasma sCD40L (101) |
| | GPIIb/IIIa | | | High costs | Increased expression from a clinically stable to an unstable phenotype (15) |
| | | | | Limited availability | |
| | GPVI | Platelet-specific marker | | Need of experienced personnel | |
| | | | | Need of fresh whole blood | |
| | | | * The ELISA kit cannot discriminate between particles of different sizes (e.g. fragmentary membranes) | | |
| | CX3CR1 | | Investigates the link between inflammation and thrombosis | | May help in the early identification of an imminent MI in patients with suspected ACS (105, 106) |
| | | | | | Surface CX3CR1 expression is elevated after platelet activation (184) |
| | Platelet-derived microparticles (PDMP) | Flow-cytometry ELISA* | Directly involved in thrombogenesis | | PDMP are significantly higher in ACS patients (126) and in UA requiring coronary intervention (127) |
stimulation is up-regulated on platelets and released into the extracellular space (31). Enhanced surface expression of CXCL16 has been shown in patients with ACS compared to patients with stable angina (SA), correlating with the extent of platelet activation and systemic inflammation (31).

Membrane releasate and shedding are involved in many processes such as modulation of adhesive interactions or receptor binding during inflammation and wound healing (32) being responsible for attracting atherogenic leukocytes, activating endothelial cells and triggering vascular cell proliferation, migration, and inflammation by infiltrating the vessel wall (33).

By applying proteomic techniques to the detection of platelet biomarkers in ACS, Parguiña et al. found 40 differentially regulated proteins corresponding to cytoskeletal, signalling, secretion or secretion trafficking pathway, and highlighted proteins involved in αIIbβ3 and GPVI signalling as differentially regulated in NSTEMI and in STEMI patients, in which the number of differentially regulated proteins raised up to 56 (34, 35). In the latter group of patients the active form of the kinase Src (phosphorylated in Tyr418) was also up-regulated, as well as GPVI signalling, suggesting a potential target for antiplatelet treatment. By studying platelet proteome profile of a group of CAD patients, Banfi and colleagues detected six altered proteins including some related to energy metabolism (LDH, OGDH), cytoskeleton processes (lambda actin, coronin 1B, pleckstrin) and protein degradation (PSB8) (36). Similarly, expression of proteins involved in platelet cytoskeleton, glycolysis pathway, and cellular-re
luted antioxidant system, which might predispose platelets to an easier activation were demonstrated in CAD patients (37). For an extensive review, please refer to (38).

Although platelet secretome is characterised by a huge number of molecules, only few of them have been proposed as platelet activation markers.

**Soluble P-selectin**

P-selectin is an adhesion molecule that can be found in the α-granules of platelets and the Weibel-Palade bodies of endothelial cells (39). Although its origin in the circulation is still debated, platelets are currently considered as the major source of circulating soluble (s)P-selectin in healthy individuals (40). Following platelet activation, P-selectin is expressed on the surface membrane (41) and then shed by cleavage (42). This particular mechanism of P-selectin expression and cleavage following activation makes this molecule apparently resistant to ex vivo activation, provided that plasma is immediately separated from the cellular component (42). Furthermore, circulating levels of sP-selectin do not seem to be influenced by different anticoagulants and/or varying methods of plasma preparation, indicating that sP-selectin can be used as a reliable marker of in vivo platelet activation (43).

Increased levels of plasma sP-selectin have been found in patients with acute MI (AMI) (44, 45) or UA (46). High sP-selectin levels are also associated with increased risk of future MI, stroke, coronary revascularisation, and cardiovascular death (47). There was a significant reduction of sP-selectin levels after coronary revascularisation (48). Interestingly, increased sP-selectin levels after coronary angioplasty were associated with high risk of restenosis (49). Moreover, it has been suggested that elevated sP-selectin levels may predict early adverse events in patients with chest pain presumably caused by myocardial ischaemia (50). In the emergency department, sP-selectin may be useful in risk stratification for patients presenting with chest pain (51). In this respect, an interesting challenge recently proposed is to consider low levels of sP-selectin as likely indicative of not having a heart attack in people presenting with a chest pain (52). However, data linking sP-selectin levels with ACS outcomes are limited.

**Soluble CD40L**

CD40L is a type II transmembrane protein and a member of the tumour necrosis factor (TNF) family that can be found on several vascular cells, including platelets (53). Indeed, CD40L, cryptic in unstimulated platelets, becomes quickly exposed on the platelet surface after stimulation (54). The surface-expressed CD40L is then rapidly cleaved from the platelet membrane generating a soluble fragment (sCD40L).

The first clinical evidence that platelet-released sCD40L may play a pathogenetic role in the atherosclerotic process dates back to 1999, when Aukrust et al. hypothesised that platelets are a major contributor to increased circulating sCD40L in the setting of ACS, and that sCD40L may be a contributor rather than the consequence of ACS, being responsible for the progressive plaque instability and eventually plaque rupture (55).

Since then, a growing body of evidence has supported the association between circulating sCD40L and ACS clinical outcomes. Indeed, elevated circulating sCD40L levels have been shown in patients with AMI (56) or UA (57). In the latter group of patients elevated sCD40L levels identified those who were at higher risk for death or non-fatal MI and might benefit from a more aggressive antiplatelet treatment (2, 58, 59).

A gradual increase in sCD40L levels has been demonstrated with ACS progression (55, 60) exhibiting an early peak just 9 hours (h) after onset of AMI or UA (61, 62). Indeed, it has been proposed that the combined assessment of sCD40L and troponin-T levels may have a better predictive value regarding MI risk (59, 63). A small prospective study suggested that high sCD40L levels also predict restenosis in patients who undergo coronary angioplasty (64).

The demonstration of a positive association between sCD40L levels and circulating monocyte-platelet aggregates, as an index of platelet activation, in ACS patients, further corroborates its reliability as a biomarker (2, 53, 54). According, the use of antithrombocyte agents, such as aspirin, has been associated with a significant reduction of plasma sCD40L (23, 65). The saturaability of the effect of aspirin and the time-dependent pattern of recovery of plasma sCD40L upon aspirin withdrawal support the likelihood of a platelet cyclooxygenase (COX)-1-dependent sCD40L release during platelet activation (65).

However, a close relationship between increased sCD40L levels and in vivo platelet activation has been demonstrated not only in patients with clinically overt atherosclerotic disease, but also in patients with atherosclerotic risk factors without complicated atherosclerosis, namely type 2 diabetes (65), hypercholesterolaemia (66), hypertension (67), or obesity (68), suggesting that these risk factors might stimulate platelet release of sCD40L. In addition, genetic polymorphisms of CD40L and GPIa may significantly affect sCD40L levels and their increase after endothelial injury (59, 69). Thus, the complex regulatory network of sCD40L levels may limit the clinical usefulness of this biomarker (63).

**PF4, β-thromboglobulin and thrombospondin-1**

Platelet factor 4 (PF4) and β-thromboglobulin (β-TG) are platelet-specific CXC chemokines, stored in platelet α-granules, and released extracellularly upon activation. Although elevated levels of these cytokines were considered to reflect in vivo platelet activation of prethrombotic and thrombotic patients (70), nowadays are mainly considered as an index of in vitro platelet activation. Indeed, the measurement of released platelet materials might indicate an inappropriate in vitro release (71). Their different release ratio and clearance also suggest that both markers should be measured simultaneously in order to discern between in vivo pla-
Platelet activation and in vitro artefact (71). In fact, while PF4 is rapidly cleared from the circulation, by binding to endothelial cells, β-TG remains elevated for a longer time. The finding of elevated levels of both cytokines is strongly suggestive of artefactual in vitro release (71).

Similar considerations can be drawn for thrombospondin-1 (TSP-1), a trimeric glycoprotein secreted from platelet α-granules upon activation and involved in platelet aggregation, by stabilisation of fibrinogen binding to platelets and multimer size control of VWF (72). Once released, the full-length 200-kDa TSP-1 is processed by proteolysis and cleaved into protein fragments which vary in size from 140–25 kDa (73). Although the 200-kDa TSP-1 is rapidly cleared from circulation by cell surface binding and internalisation (74), elevated TSP-1 levels are detectable in blood for several hours after platelet activation (75). Nonetheless, in vivo TSP-1 release is represented by an increase in the more stable 140-kDa fragment in circulation, whereas the immediate (in vitro) activation of platelets results only in a rise of the full-length 200-kDa molecule (76). Thus, the ratio of TSP-1 isoforms might represent a valuable tool to exclude in vitro artefacts in monitoring platelet activation.

Despite initial observations from basic science supporting the role of these molecules as promising markers of platelet activation, only few studies evaluated the clinical usefulness of TSP-1, PF4 and/or β-TG in the ACS setting, leading to inconclusive results (77).

**SCUBE1**

Recently, a novel family of secreted, surface-anchored proteins harbouring an array of signal peptide, complement proteins C1r/C1s, Uegf, and Bmp1 (CUB), and epidermal growth factor (EGF)-like domains (SCUBEs) have been identified in human endothelial cells and in platelets (78, 79). These proteins, normally stored in platelet α-granules and released upon platelet activation, are incorporated into the growing thrombus (79). SCUBE1 has recently been proposed as a novel platelet-endothelial adhesion molecule and a marker of platelet activation since particularly elevated levels have been shown in ACS and acute large-vessel atherothrombotic stroke (80).

**Matrix metalloproteinases**

Another interesting class of markers in the acute setting is represented by matrix metalloproteinases (MMPs) which are part of a larger family of zinc- and calcium-dependent endopeptidases, involved in the remodelling of extracellular matrix, one of the prominent steps of the atherosclerotic process. Platelets express several MMPs as well as their endogenous inhibitors TIMP (81). The most active are MMP-2 which amplifies agonist-induced platelet aggregation through phosphatidylinositol 3-kinase (PI3-K) activation and MMP-9 that counteracts the pro-aggregatory effect of MMP-2 (82). Moreover, MMPs positively modulate the release of sCD40L from platelets (83).

MMPs are involved in many steps of plaque formation and rupture (84) and have been found to be released across the coronary bed during ACS (85). It has been demonstrated that the intra-coronary release of MMP-2 is, to a significant extent, related to intracoronary platelet activation (86) and that elevated values of MMP-9 had a prognostic significance related to future cardiovascular death in patients with cardiovascular disorders (87).

**GPV**

GPV is a transmembrane constituent of platelets and megakaryocytes, and is non-covalently linked to the GPIb-IX complex to form a receptor both for VWF and for thrombin (88). Following platelet activation by thrombin, GPV is cleaved and a smaller, soluble fragment is formed. Soluble (s)GPV is considered an indirect although highly sensitive marker of thrombin generation accompanying platelet activation (89). High levels of sGPV have been detected in patients with two major manifestations of atherosclerosis (peripheral arterial disease and CAD) (90), in patients with occluded infarct arteries (91) and in AMI (92), although it seems to be influenced by smoking status and blood pressure (90). However, because sGPV has been suggested to be involved in the first steps of platelet activation by thrombin, it might be not only an early marker of platelet activation, but also a valuable tool in detecting storage-dependent platelet activation in that platelet chilling modifies the (GPIbα9)3V complex (93).

**Membrane-associated markers**

These are mostly represented by adhesion receptors that mediate haemostasis and thrombosis and might represent hypothetically ideal markers, being unquestionably involved in atherothrombotic disease. The expression of these molecules on platelet surface is usually assessed by flow-cytometry (Table 1).

**P-selectin**

Flow cytometry analysis of platelet P-selectin expression (CD62P), alone or in combination with other platelet antigens, is commonly used as a reliable technique to assess in vivo platelet activation. However, contradictory data have been reported on the percentages of circulating activated platelets in cardiovascular patients (94), and CD62P expressing platelets may not be found in clinical conditions in which platelet activation is expected to occur. Indeed, both animal (95) and clinical studies (96) demonstrated a loss of platelet surface CD62P expression soon after an activating
event, followed by a concomitant increase of plasma soluble (s)P-selectin levels. These findings led to hypothesise that flow cytometry analysis of CD62P cannot be considered an ideal marker for platelet activation in clinical settings where a chronic stimulus is generally present, but should be limited to the acute setting (97).

CD40-CD40L system

CD40L expression on platelets, alone or in combination with its receptor CD40, is significantly increased in patients with ACS (98–100). In addition, patients with UA who needed coronary angioplasty or who had recurrence of angina were characterised by the highest CD40L expression on platelets (98). A significant positive correlation has been found between platelet CD40L expression, endothelial dysfunction and circulating levels of oxidised low-density lipoproteins, C-reactive protein and fibrinogen (99). As surface expression of CD40L is tightly related to circulating soluble CD40L levels (101), the latter assay has been more extensively used in the clinical setting because of its advantages as compared to flow-cytometry, including limited costs and higher degree of standardisation.

GPIIb/IIIa

Platelet activation leads to conformational changes of the fibrinogen receptor, GPIIb/IIIa, on platelet surface. Activated GP IIb-IIIa binds fibrinogen or VWF, which forms molecular bridges between aggregating platelets. The monoclonal antibodies PAC1, anti-LIBS1 and anti-RIBS bind to conformation-dependent epitopes on GPIIb/IIIa complexes expressed as a primary response of platelets to physiologic agonist on activated and not on resting platelets (39). The test is performed by flow cytometry and has revealed capable to detect in vivo platelet activation due to shear stress when used with proper testing procedures. Platelet GPIIb/IIIa expression has been found increased in CAD patients (102). Moreover, in the Thrombotic Risk Progression (TRIP) study, an increased platelet GPIIb/IIIa expression from a clinically stable to an unstable disease state was observed (15). These findings corroborate the evidence of the critical role played by platelets in plaque destabilisation (103).

GPVI

GPVI is a 62-kDa type I transmembrane collagen receptor of the immunoglobulin superfamily exclusively expressed in megakaryocytes and platelets. Collagen binding to GPVI triggers platelet activation following vascular injury (104). However, GPVI has also been described to be the major receptor mediating platelet adhesion on the surface of VWF and aggregation on human atherosclerotic plaque tissues (104). GPVI expression is increased in ACS, reflecting platelet activation. GPVI is significantly increased in ACS patients compared with patients with SA and may also be helpful as a biomarker for the early identification of an imminent MI in patients with suspected ACS (105, 106). Moreover GPVI may be used to identify patients with transient ischaemic attack or ischaemic stroke (107, 108). Despite its promising results, the assessment of GPVI shows a rather low diagnostic sensitivity and specificity. Moreover, as for the majority of flow-cytometry assays, its availability is limited to a few academic health centres. In order to overcome this limitation, a sandwich immunoassay has been recently developed to measure platelet-released plasma soluble (s)GPVI (109). Interestingly, a poor but inverse relationship has been found between soluble and membrane bound GPVI. In addition, sGPVI was negatively associated with the development of ACS in the very early stage of disease at time of hospital admission, suggesting the hypothesis that GPVI shedding may act as an inhibitor of atherothrombosis (109).

Fractalkine receptor (CX3CR1)

Chemokines (chemotactic cytokines) are a large family of small proteins inducing chemotaxis of cells, involved in immune surveillance and atherogenesis. Apart from the established role of PF4 (or CLCX4) in platelet activation, it has been demonstrated not only that other chemokines induce platelet activation (110, 111), but also that their respective receptors (CCR1, CCR3, CCR4, and CXCR4) are expressed on platelets (112). The presence of a functional fractalkine receptor (CX3CR1) on human and rat platelets, provides experimental evidence for a functional role of fractalkine in platelet activation and adhesion (113). Fractalkine, which exists both as a membrane-anchored and a soluble chemokine, triggers platelet exposure of P-selectin in a G-protein-dependent manner and promotes GPIIb/IIIa-dependent platelet adhesion to fibrinogen (113). Interestingly, fractalkine has proven capable to, at least partially, substitute for ADP-mediated platelet activation despite pharmacological inhibition of the P2Y12 receptor in patients with CAD. Indeed, elevated fractalkine levels were recently observed in patients with impaired clopidogrel responsiveness (114). Moreover, CX3CR1 I249 polymorphism in the fractalkine receptor is now considered as a genetic risk factor for CAD (115).

Platelet-derived microparticles

An emerging role in the search of a reliable platelet activation marker is played by platelet-derived microparticles (PMPs). These vesicles are the product of exocytic budding and consist of cytoplasmic components and phospholipids. MPs are smaller than 1 μm, expose the anionic phospholipid PS on the outer leaflet of their membrane, and carry surface membrane antigens reflecting their cellular origin, allowing to distinguish between specific sub-
populations (83). Indeed, the release of MPs from platelets has been associated with the secretive response (95) and the numbers of circulating PMPs may reflect in vivo platelet activation in several settings (116–119). Although both MPs from monocytes and platelets exhibit unique procoagulant activities, monocyte-derived MPs trigger coagulation predominantly via TF (120), while PMPs promote thrombus propagation not only by exposing PS but also by initiating thrombin generation independently of TF in a FXII-dependent manner (120, 121). However, due to the lack of uniform consensus regarding the definition and detection of MPs, their levels highly depend on the employed detection technique. This could explain the low number of comparable studies and the different results reached so far, highlighting the need to optimise and standardise the detection methods to correctly define MPs and to avoid falsely high or low quantification (122). Due to the procoagulant activity of platelets, which is linked to increased exposure of PS on the outer leaflet of platelet membrane (123), although not all PMPs expose PS, annexin V is commonly used as a probe to detect activated platelet shedding MPs exhibiting procoagulant activity (124), thus measuring the total number of PMPs, by flow cytometry. The binding of annexin V to PMPs is influenced by the calcium concentration and the membrane PS content, and this should be taken into account when preparing the buffers. However, by using annexin V-labeling, it has been demonstrated that PMPs are independent markers of increased risk of cardiovascular events in high-risk patients (125) and that they are significantly higher in ACS patients (126) and in the setting of UA requiring coronary intervention (127).

**Platelet-leukocyte aggregates**

Activated platelets rapidly bind to blood leukocytes through the interaction of adhesion molecules present on platelet and leukocyte surfaces (128). The interaction of platelet P-selectin with its counter-receptor on leukocytes, P-selectin glycoprotein ligand-1 (PSGL-1), triggers platelet adhesion resulting in leukocyte activation and in stable platelet-leukocyte aggregate (PLA) formation (129). This initial association causes an increased expression of CD11b/CD18 (Mac-1) on leukocytes (130) further supporting and maintaining interactions with platelets. The in vivo half-life of detectable circulating monocyte-platelet aggregates is longer (30 minutes [min]) than both the in vivo half-life of neutrophil-platelet aggregates (5 min) and the platelet surface P-selectin (that is rapidly cleared) (131). These findings suggest that measurement of circulating monocyte-platelet aggregates may be a more sensitive indicator of in vivo platelet activation than either circulating neutrophil-platelet aggregates or circulating, P-selectin-positive, non-aggregated platelets.

In some inflammatory states, platelet adhesion to endothelium depends largely on platelet-leukocyte adhesive interactions, through a mechanisms mediated by PSGL-1 (132, 133). Accordingly, in ACS, PLA might provide a good picture of plaque instability and associated vascular thrombosis and inflammation. Consistent with this hypothesis, PLA have been found to be significantly higher in patients with UA than in those with SA (134, 135).

A significantly greater number of TF positive platelet-monocyte aggregates was also found in ACS patients than in either SA patients or control subjects (136). Expression of TF by leukocytes or by PLA may trigger the extrinsic coagulation cascade; thrombin thus generated can activate platelets leading to formation of a platelet-fibrin thrombus (136). Thus, the greater expression of TF in platelets and PLA strengthens the link between platelet activation, blood coagulation, and thrombus formation in ACS (136).

**Mean platelet volume**

During the last decade, mean platelet volume (MPV) has started to be regarded as an indicator of platelet activation (137) in that increased platelet size reflects increased platelet reactivity due to the presence of more adhesive receptors, granules and metabolically and enzymatically active mediators. The contribution of these mediators to inflammation and atherogenesis might explain the association between MPV and severity of coronary atherosclerosis (138). Elevated MPV has been also identified as an indicator of CAD-related complications (139) and an independent risk factor for MI in patients with coronary heart disease and for death or recurrent vascular events (140, 141). Moreover, increased MPV was observed in patients with UA as compared to patients with stable disease (142) and was associated with adverse microvascular outcomes in AMI (143). Due to the simplicity and reliability of the method, it has been recently suggested to add MPV to the conventional risk factors for CAD (144).

**Reticulated/immature platelets**

In ACS patients, the acute event is able to elicit an inflammatory response with the release of a variety of proinflammatory cytokines, that may influence platelet turnover with the subsequent mobilisation and release of newly formed large, reticulated, reactive platelets from the bone marrow (145). These immature platelets contain high amounts of mRNA and are termed “reticulated platelets” (RP) because of the staining patterns produced by the cytoplasmic mRNA distribution (146). A higher number of these circulating RP is thus considered as an indicator of accelerated platelet turnover. RP are larger and more reactive platelets, as platelet size correlates with greater platelet reactivity, measured by aggregation and total release of granular content. They contain denser granules, secrete more serotonin and β-TG, and produce more thromboxane (TXA), than smaller platelets. Thus, the increased potential for aggregation of such platelets lowers their threshold for activation, and may contribute to the increased incidence of acute cardiovascular events.

RP may be evaluated by flow-cytometry, measuring the percentage of thiazole orange-positive platelets, which represent the
newly-released, mRNA-positive platelets (147). The recent advances in automated cell counting methods with no need for any preanalytical step such as type and concentration of mRNA stain, incubation time, temperature, centrifugation and resuspension, allow an easier detection of the RP and of the immature platelet fraction (IPF), that is well correlated with RP measurement (148).

In the ACS setting, a significant increase in RP has been demonstrated in patients with AMI (NSTEMI or STEMI) and UA compared with patients with SA and a difference was also observed in patients with AMI compared with UA (149). Similarly, IPF is significantly elevated in patients with STEMI (150).

The growing interest on RP and IPF resides in the hypothesis that an increased platelet turnover may cause "resistance" to antiplatelet drugs (151). Indeed, due to the accelerated platelet turnover, platelets unaffected by drugs are introduced into the blood stream especially in the late phase of drug kinetic, thus causing the overall platelet inhibition to be insufficient (150).

This hypothesis is strengthened by the findings obtained on diabetic patients, whose platelets are characterised by an accelerated platelet turnover, as indicated by the presence of a higher number of circulating RP. Moreover, both the mRNA-positive platelet fraction and the MPV both positively correlate with the rate of recovery of platelet cyclooxygenase activity in aspirin-treated diabetic patients (152).

Finally, the percentage of IPF or RP appears to be a determinant of residual platelet reactivity in CAD patients treated with aspirin or dual antiplatelet therapy, suggesting that platelets are in a hyper-reactive state that may further contribute to the variable response to antiplatelet therapy (153–155).

**Urinary enzymatic thromboxane metabolites**

TXA₂ is a potent agonist released by platelets in response to a variety of stimuli. Most of vascular actions of TXA₂ results from the activation of TX receptor (TP) expressed on platelets, endothelial and vascular smooth muscle cells. TXA₂-dependent platelet activation can mediate or amplify, at least in part, the short-term occlusive consequences of acute vascular lesions (plaque rupture) in the coronary circulation. This is likely to reflect a localised (both in time and space) stimulus to platelet activation, such as that provided by collagen and lipids in the fissured atherosclerotic plaque (6). Increased TX biosynthesis has been also associated with several clinical settings characterised by increased cardiovascular risk (65, 156, 157).

TXA₂ is hydrolysed to TXB₂ that is enzymatically metabolised and excreted in urine (Fig. 3). The maximum capacity of platelets to synthesise TXA₂ in vitro following clot formation, as reflected by serum TXB₂, is approximately 5,000 times the basal rate of TX biosynthesis in vivo (6), and only a fraction of this biosynthetic capacity appears to contribute to platelet activation, as reflected by excretion of TX metabolites (6). In patients treated with low-dose aspirin, serum TXB₂ levels have been shown to reflect the adequacy of platelet COX-1 inhibition and its duration (6) rather than a reliable index of in vivo platelet TXA₂ production, due to its short half-life and artifacts due to ex vivo platelet activation (6). Conversely, urinary levels of 11-dehydro-TXB₂ and 2,3-dinor-TXB₂, the most abundant TXB₂ metabolites, have been both regarded as potential biomarkers of platelet activation (6). 11-dehydro-TXB₂ being excreted at a higher rate than others, with a longer plasma half-life (158) could represent the marker of choice. Moreover, a linear correlation between levels of exogenous administered TXB₂ and urinary 11-dehydro-TXB₂ excretion has been reported (156, 159) suggesting that urinary 11-dehydro-TXB₂ may provide a time-integrated index of endogenous systemic TXA₂ biosynthesis, allowing evaluation of ongoing platelet activation (160). However, given the systemic nature of TX metabolite excretion, involving both platelet and extraplatelet sources, urinary 11-dehydro-TXB₂ may reflect either platelet COX-1-dependent TX generation or COX-2-dependent biosynthesis by inflammatory cells and/or platelets, or a combination of the two, especially in clinical settings characterised by low-grade inflammation (161) or enhanced platelet turnover (162).

Episodic increases in the excretion rate of TX enzymatic metabolites have been detected in UA (6, 163–165) in relation to episodes of chest pain (163), or associated with ST-segment changes in UA (165). In contrast, metabolite excretion was normal in patients with stable coronary disease, both at rest and after exercise-induced myocardial ischaemia. Thus, enhanced TXA₂ production occurs episodically in the vast majority of patients, although the number of such episodes varies greatly among studies. That a similar dynamic thrombotic process occurs during the early phase (that is, within 24 h after the onset of symptoms) of AMI is suggested by measurements of urinary 2,3 dinor-TXB₂ and plasma 11-dehydro-TXB₂ in this setting (163) and by the results of the ISIS-2 trial (166).

**Figure 3: Biomarkers of thromboxane biosynthesis.** Thromboxane (TX)A₂ generated from platelet cyclooxygenase (COX)-1 is rapidly hydrolysed to TXB₂. This, in turn, is metabolised within minutes into several enzymatic metabolites that are mainly excreted in urine. In particular, 11-dehydro-TXB₂ is cleared by the kidney and can be assayed in the urine as an in vivo marker of platelet activation.
Measurement of urinary 11-dehydro-TXB₂ levels has proved to represent a useful marker to investigate the effects on platelet activation of various cardioprotective interventions, including lipid-lowering therapy, improved glycemic control, weight loss, and antiplatelet agents (6).

Finally, it has been recently reported that urinary 11-dehydro-TXB₂ predicts the future risk of AMI or cardiovascular death in aspirin-treated patients (167, 168). Nevertheless, whether urinary 11-dehydro-TXB₂ levels are independent predictors of future vascular events in untreated patients still remains controversial.

**Multimarker approach: platelet transcriptomic and genomic**

The application of genomic and proteomic technologies to the study of platelets promises to represent a helpful tool in the search for biomarkers, not only in the clinical setting of arterial thrombosis, where they might even represent potential drug targets, but also in rare platelet-related disorders.

Despite being anucleate, platelets retain megakaryocyte-derived mRNAs and are capable of protein synthesis. The whole output of mRNAs constitutes the platelet transcriptome. Platelet mRNA profile is significantly different between patients with ACS as compared to stable CAD patients. In particular, the pro-inflammatory myeloid related protein (MRP) 8/14 is significantly increased in ischaemic patients, both at intra-platelet transcript and circulating protein levels (169) indicating that, although highly expressed in leukocytes, platelets and megakaryocytes function as additional sources of MRP-8/14 (86). Increases in MRP-14 expression before STEMI and increasing plasma concentrations of MRP-8/14 among healthy individuals, predict the risk of future cardiovascular events (86). It should be underlined that platelets from healthy subjects display similar RNA-seq expression patterns as compared to diseased subjects and, among the latter, there is evidence that molecular RNA-seq may be changed in disease conditions that alter platelet function at the molecular level (170). In this scenario, transcriptome profiling might even identify transcripts that may contribute indirectly to phenotypic differences in platelets from knockout animals, and guide the design of future studies (170). Some platelet-derived proinflammatory transcripts – in particular those involved in the nuclear factor-kB pathway – are also significantly associated with cardiovascular risk factors (171).

Circulating microRNAs (miRNAs) are short, non-coding RNAs that are emerging as novel biomarkers for cardiovascular disease (172). It is well known that some miRNAs are expressed in a cell type– and tissue-specific manner. Platelets possess diverse assortment of miRNAs, which act as natural antisense molecules that negatively regulate the transcription of target mRNAs, thus exerting important regulatory functions (173). The most abundant miRNA in platelets is miR-223 followed by miR-126 (174). The miR-96, miR-200b, miR-495, miR-107 and miR-223 are critically involved in platelet reactivity, aggregation, secretion and adhesion (174).

Circulating levels of miR-223 were modestly, though non-significantly, increased in the aorta of patients with ACS compared with stable CAD patients (175). In a subsequent study, two other platelet-derived miRNAs (miR624* and miR340*) have been found to be significantly up-regulated in patients with CAD as compared to healthy controls (176). Taken together, these preliminary data suggest a promising role for miRNAs as biomarkers of platelet activation in ACS and warrant further investigations.

**Conclusions**

An ideal and reliable platelet activation biomarker, in the emergency setting represented by ACS, should be specific, easily measurable in order to orientate the physician towards a diagnosis or a predictable outcome in such a way that it guides therapy. Unfortunately, unlike myocardial injury markers like troponin, and despite the fact that several biomarkers of *in vivo* platelet activation have been suggested, none offers ideal diagnostic properties.

On the other hand, since management of ACS patients includes the use of antiplatelet agents (mainly aspirin and clopidogrel) administered either alone or in combination, one application of platelet biomarker assessment, is represented by the response to antiplatelet regimen (177). Indeed, the observation that some patients experience adverse cardiac events despite such antiplatelet treatment corroborates the current intense controversy surrounding the clinical importance of non-responsiveness to antiplatelet drugs (5). Nonetheless, current platelet function tests *in vitro* as well as point-of-care testing, even though they may have some usefulness in monitoring antiplatelet therapy, especially clopidogrel (26), cannot be considered as reliable measures of *in vivo* platelet activation (147). In addition, their predictive value for adverse cardiovascular events is limited (178).

In conclusion, increasing evidence suggests that the combined use of biomarkers reflecting distinct pathophysiological features of ACS such as cell necrosis, vascular inflammation, oxidative stress and platelet activation will significantly improve in near future our ability to identify patients who are at high risk for adverse cardiovascular events (179). In addition, platelet activation markers may be of particular value in tailoring strategies for risk reduction in patients presenting post-treatment platelet reactivity. However, larger clinical trials are needed to investigate both the prognostic and diagnostic utility of these biomarkers in the ACS setting.

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**Conflicts of interest**

None declared.
References


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