Platelet function testing and prediction of procedural bleeding risk

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Summary
The essential role of platelets in haemostasis underlies the relationship between platelet function and spontaneous or procedure-related bleeding, which has important prognostic implications. Although not routinely undertaken, platelet function testing offers the potential to tailor antiplatelet therapy for individual patients. However, uncertainties remain about how well platelet function testing may predict haemostasis and guide management of bleeding risk. Studies of aspirin, P2Y12 inhibitors and other antiplatelet drugs clearly demonstrate how inhibition of platelet function increases bleeding risk. More potent antiplatelet drugs are associated with higher bleeding rates, consistent with the levels of platelet inhibition achieved by these drugs. Studies of patients treated with clopidogrel, which is associated with wide inter-individual variation in antiplatelet effect, suggest that platelet function testing may predict bleeding risk related to coronary artery bypass grafting (CABG) surgery and potentially guide the timing of surgery following discontinuation of clopidogrel. Similarly, some studies have demonstrated a relationship between clopidogrel response and bleeding in patients undergoing percutaneous coronary intervention (PCI), although other studies have not supported this. Carriage of the *17 allele of cytochrome P450 2C19, which is associated with gain of function and enhanced response to clopidogrel, seems to be associated with increased bleeding risk, although studies showing lack of apparent effect of loss-of-function alleles provide contradictory evidence. Further large studies are needed to guide best practice in the application of platelet function testing in the clinical management of patients treated with antiplatelet drugs in order to optimise individual care.

Keywords
ADP receptors, antiplatelet agents, clinical trials, antiplatelet drugs, haemostasis, platelet pharmacology

Introduction
The consequences of inherited or acquired platelet dysfunction have been well characterised and reflect the essential role of platelets in maintaining haemostasis. A typical example is Glanzmann's thrombasthenia, which is related to various defects in genes for the glycoprotein (GP) IIb/IIIa receptor complex (otherwise known as integrin αIIbβ3), which represents the final common pathway in the process of platelet activation and aggregation through binding of fibrinogen and other ligands that cross-link platelets (Figure 1) (1). This condition is associated with defective platelet aggregation, manifested by poor agonist-induced responses on platelet aggregometry and various clinical bleeding complications, including spontaneous mucocutaneous bleeding and potentially life-threatening gastrointestinal bleeding (2). An increasing number of genetic disorders of platelet function are being catalogued that further strengthen the appreciation of how different degrees of platelet dysfunction increase the risk of haemorrhage. Acknowledging the pivotal role of platelets in arterial thromboembolism has led to increased use of antiplatelet drugs, which essentially lead to acquired platelet dysfunction, and this has also accumulated knowledge on the relationship between extent of platelet dysfunction and bleeding risk. However, questions still remain about how methods that assess platelet function can predict the risk of bleeding in patients undergoing interventional or surgical procedures and this review will examine current evidence in this area.

Aspirin and bleeding risk
Aspirin, or more formally acetylsalicylic acid, irreversibly acetylates cyclo-oxygenase 1 (COX-1) in platelets and effectively inhibits the conversion of arachidonic acid to the potent platelet agonist thromboxane (TX) A2, which binds to thromboxane (TP) receptors on the platelet surface to induce platelet activation, degranulation and aggregation (Figure 1). This action of aspirin leads to high levels of COX-1 inhibition and a predictable antiplatelet effect in cardiovascular disease patients who are compliant and avoid negative drug interactions with other non-steroidal anti-inflammatory drugs (3, 4). Aspirin's acetylating properties may also have more far-reaching effects including effects on clotting
Figure 1: Numerous receptors on the platelet surface initiate platelet activation. Thrombin acts via protease-activated receptor (PAR)1 and 4, thromboxane A2 via TP receptors, collagen via glycoprotein (GP)VI, and adenosine diphosphatase (ADP) via the P2Y1 receptor. ADP also binds to the P2Y12 receptor, which acts as a powerful amplification pathway. GPVI activation preferentially leads to thromboxane A2 formation, which is blocked by aspirin. Various drugs act on the P2Y12 receptor with differing mechanisms of action. Platelet activation leads to (1) procoagulant changes in the platelet surface membrane that catalyse thrombin generation, (2) release of pro-inflammatory and pro-thrombotic α-granule contents, (3) release of dense granule contents including ADP, and (4) activation of glycoprotein IIb/IIIa (αIIbβ3), which binds fibrinogen leading to cross-linking of platelets as well as outside-in signalling that further amplifies platelet aggregation. Nitric oxide (NO) and prostacyclin (PGI2) released from healthy endothelium act as circulating inhibitors of platelet activation. Adapted with permission from reference 9.
tion and consequent bleeding risk, and this is of particular concern in patients treated with P2Y<sub>12</sub> receptor inhibitors.

The role of the P2Y<sub>12</sub> receptor in haemostasis

The platelet P2Y<sub>12</sub> receptor acts as a major amplification pathway in platelet activation, underpinned by the agonist-induced release of adenosine diphosphate (ADP) from platelet dense granules that then binds to this receptor and amplifies the responses to numerous agonists (Figure 1) (8). Inhibition or genetic deficiency of the platelet P2Y<sub>12</sub> receptor therefore impairs the platelet responses to multiple platelet agonists and this has a more dramatic effect on platelet function, and consequently on thrombosis and haemostasis, compared with COX-1 inhibition by aspirin (9-11). Importantly, combination of a P2Y<sub>12</sub> inhibitor and aspirin leads to additive effects on platelet function and haemostasis (9, 12). There is a linear relationship between extent of P2Y<sub>12</sub> receptor blockade and inhibition of ADP-induced platelet aggregation (13), and this supports the evidence from large clinical studies that increasing extent of P2Y<sub>12</sub> inhibition is associated with increasing compromise to haemostasis and associated bleeding complications (14-18).

Methods for assessing platelet function

Numerous methods are available for assessing the platelet responses to aspirin and P2Y<sub>12</sub> inhibitors with varying strengths and limitations with regard to their clinical utility, as recently reviewed elsewhere (19). The gold standard for assessing the platelet response to aspirin is TXB<sub>2</sub> levels in serum derived from whole blood samples that have been left to clot, since this specifically assesses COX-1-dependent platelet release of TXA<sub>2</sub>. Alternatives include various methods of platelet aggregometry using arachidonic acid (AA) as agonist, although AA-induced platelet aggregation also relies to some extent on P2Y<sub>12</sub> activation and may therefore be sensitive to P2Y<sub>12</sub> inhibitors as well as aspirin (20). Different methods may give different impressions about whether the relationship between COX-1 inhibition and inhibition of platelet aggregation by aspirin is non-linear or linear, which may be important in deciding the optimal dose of aspirin (3, 21).

The gold standard for assessing P2Y<sub>12</sub> inhibition is currently less clear. The vasodilator-stimulated phosphoprotein (VASP) phosphorylation assay is performed by flow cytometry and is widely used to assess the platelet inhibition achieved by P2Y<sub>12</sub> inhibitors. VASP is an intracellular protein that exists in a dephosphorylated and a phosphorylated state. The level of VASP phosphorylation is proportional to the extent of P2Y<sub>12</sub> inhibition, and the VASP index, expressed as a mean percentage of platelet reactivity, correlates inversely with platelet reactivity. This test has the advantage of specifically measuring P2Y<sub>12</sub> inhibition independent of functional responses that may be affected by other antiplatelet drugs but has the potential disadvantages of being insensitive to lower levels of P2Y<sub>12</sub> inhibition and requiring specialised equipment (flow cytometer) (13, 19). Platelet aggregometry measuring the response to ADP is generally a reliable way to assess the extent of P2Y<sub>12</sub> inhibition, as long as a GPIIb/IIIa antagonist has not been used as well, and various point-of-care systems are available to facilitate this, such as the VerifyNow P2Y12 assay and Multiplate system. These tests are based on whole blood measurements and have several advantages compared to conventional time-consuming platelet aggregometry performed in platelet-rich plasma (19). The feasibility and wide availability of these methods have allowed more evidence to emerge regarding the relationships between platelet inhibition and bleeding complications. Notwithstanding the choice of platelet function test, clinical trials investigating the association between platelet function and bleeding should standardise the timing between ingestion of antiplatelet drugs and blood sampling. In these trials, most patients are treated with aspirin monotherapy or as a part of dual antiplatelet therapy. It has previously been shown that the number of non-aspirinated platelets differs between the early and late parts of the usual 24-hour dosing interval (22), and the importance of standardising this time interval was further stressed in a study investigating the time-dependent efficacy of aspirin (23).

There are several gaps in knowledge on the association between platelet function and bleeding, including a need for cut-offs to guide clinical decisions on timing of surgery, dosages, combinations and treatment length, e.g. in patients who have indications for anticoagulant as well as dual antiplatelet treatment. Several studies support the association between platelet function and recurrent ischaemic events, whereas less robust data associate platelet function testing with bleeding. This is likely attributed to the fact that, although platelet function has a pivotal role in primary haemostasis after vascular injury, platelets play a less prominent role in bleeding, compared to arterial thrombosis. Moreover, major bleeding events occur less frequently than ischaemic events, thus limiting the possibility of clinical studies to provide firm conclusions.

Platelet function and surgical bleeding

The relationship between platelet function and the incidence of clinically significant bleeding related to surgical procedures can be either inferred from well-characterised pharmacokinetic and pharmacodynamic properties of different antiplatelet drugs or studied directly by determining the association between platelet function measurements at the time of surgery and bleeding. One may speculate that platelet function testing might enable individualised timing of cessation of antiplatelet drugs prior to surgery with the potential of reducing preoperative ischaemic events without an increased risk of perioperative bleeding.

Preoperative treatment with aspirin is associated with more postoperative bleeding in patients undergoing elective CABG as shown in a recent randomised double-blind trial, which also indicated that preoperative aspirin may reduce the hazard of major cardiac events (24). Clopidogrel compared to placebo in patients with acute coronary syndromes (ACS) was associated with more bleeding in those undergoing CABG surgery and the more potent
thienopyridine prasugrel was associated with more CABG-related bleeding than clopidogrel (14, 25). Aspirin and thienopyridines have an irreversible inhibitory effect on platelets and it takes 7-10 days for inhibited platelets to be replaced in the circulation, whereas ticagrelor binds reversibly to the P2Y12 receptor such that recovery of platelet function is associated with a fall in plasma levels (26). This explains why evidence supports discontinuation of thienopyridines at 5 days (for clopidogrel) or 7 days (for prasugrel) prior to surgery, when feasible (27), and this approach is supported by guidelines (28). However, the antiplatelet effect of clopidogrel is widely variable meaning that low-to-moderate responders recover normal levels of platelet function more rapidly following cessation of clopidogrel than high responders (29, 30). The irreversible vs reversible platelet inhibition by clopidogrel and ticagrelor may also explain why there were more bleeding-related deaths with clopidogrel compared to ticagrelor on detailed analysis of the cause of death in ACS patients undergoing CABG surgery within 7 days of this medication (31, 32). Moreover, there are preclinical data suggesting that reversible P2Y12 receptor blockade might be associated with a wider therapeutic window than irreversible platelet function inhibitors (33).

Platelet function testing can predict both thrombotic and bleeding events after cardiac surgery. Moreover, the extent of platelet inhibition by clopidogrel may predict the requirement for transfusion following CABG surgery (34-38). A study assessing the utility of platelet function testing to guide timing of surgery after clopidogrel cessation has suggested that this approach is safe and that patients with poor responses to clopidogrel can proceed fastest to surgery after treatment cessation (39). Larger randomised studies would further help to guide the optimum timing of surgery after discontinuation of antiplatelet drugs.

The variable use of haemostatic agents such as tranexamic acid or aprotinin at the time of CABG surgery further complicates the interpretation of observational studies investigating the relationship between antiplatelet therapy management and perioperative bleeding. Aprotinin effectively reduces post-operative bleeding in patients undergoing CABG surgery within 5 days of receiving clopidogrel (40). A small study compared the two strategies of either (i) continuing aspirin and clopidogrel up to the time of surgery after discontinuation of antiplatelet drugs.

The recently published 2012 Update to the Society of Thoracic Surgeons (STS) Guideline on Use of Antiplatelet Drugs in Patients

Table 1: Studies showing or not showing a direct relationship between platelet function or genotype and bleeding in PCI or ACS patients.

<table>
<thead>
<tr>
<th>Studies</th>
<th>Size (N)</th>
<th>Relationship</th>
<th>Assay/assays used</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Studies showing a relationship between platelet function/genotype and bleeding</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sibbing et al. (52)</td>
<td>2,533</td>
<td>Incidence of bleeding higher in patients with an enhanced response to clopidogrel: adjusted OR 3.5 (95% CI: 1.6-7.3; p=0.001)</td>
<td>Multiplate ADP test</td>
</tr>
<tr>
<td>Sibbing et al. (53)</td>
<td>1,524</td>
<td>*17 variant of CYP 2C19 associated with increased response to clopidogrel (p&lt;0.001) and a higher risk of bleeding (p=0.006).</td>
<td>TaqMan assay; Multiplate ADP test</td>
</tr>
<tr>
<td>Mokhtar et al. (57)</td>
<td>346</td>
<td>Greater P2Y12 inhibition associated with increased non-CABG related TIMI major bleeding in clopidogrel-treated PCI patients</td>
<td>VASP</td>
</tr>
<tr>
<td>Harmsze et al. (56)</td>
<td>820</td>
<td>CYP2C19 *17 associated with increased P2Y12 inhibition and increased risk of TIMI major bleeding events after PCI</td>
<td>LTA; VerifyNow</td>
</tr>
<tr>
<td>Wallentin et al. (63)</td>
<td>10,285</td>
<td>CYP2C19 *17 associated with increased risk of bleeding in clopidogrel-treated ACS patients</td>
<td>TaqMan assay</td>
</tr>
<tr>
<td>Patti et al. (58)</td>
<td>310</td>
<td>Enhanced response to clopidogrel associated with major bleeding following PCI (OR 4.5; 95% CI: 1.9 to 25.9).</td>
<td>VerifyNow</td>
</tr>
<tr>
<td>Cuisset et al. (55)</td>
<td>597</td>
<td>Enhanced response to clopidogrel associated with major bleeding in non-ST-elevation ACS</td>
<td>LTA, VASP</td>
</tr>
<tr>
<td><strong>Studies not showing a relationship between platelet function/genotype and bleeding</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breet et al. (62)</td>
<td>1,069</td>
<td>Various measures of P2Y12 inhibition did not predict risk of bleeding following PCI</td>
<td>LTA, VerifyNow, Plateletworks, IMPACT-R, PFA-100 P2Y12</td>
</tr>
<tr>
<td>Wallentin et al. (63)</td>
<td>10,285</td>
<td>Loss-of-function CYP2C19 alleles not associated with reduced bleeding risk in clopidogrel-treated ACS patients</td>
<td>TaqMan assay</td>
</tr>
<tr>
<td>Cayla et al. (64)</td>
<td>444</td>
<td>No association between P2Y12 inhibition and bleeding outcomes following PCI</td>
<td>VASP, LTA, VerifyNow</td>
</tr>
<tr>
<td>Serebruany et al. (18)</td>
<td>363</td>
<td>No association between P2Y12 inhibition and major bleeding</td>
<td>LTA</td>
</tr>
</tbody>
</table>
Having Cardiac and Noncardiac Operations reflects increased emphasis on platelet function testing as a tool to manage perioperative antiplatelet treatment (43). This update includes a Class IIa (Level of evidence B) recommendation for patients who require urgent surgery and are on dual antiplatelet therapy. In these patients, it is considered reasonable to make decisions about surgical delay based on tests of platelet inhibition rather than arbitrary use of a specified period of surgical delay. The update also includes a Class IIb (B) recommendation for platelet function testing to assess bleeding risk and potentially identify patients who have high residual platelet reactivity after usual doses of antiplatelet drugs and can undergo operation without elevated bleeding risk. Similarly, a Class IIb (B) recommendation support the use of perioperative platelet function testing that may limit blood transfusion as discussed above.

**Platelet function and bleeding related to PCI**

As with surgical bleeding, the effects of platelet inhibition on PCI-related bleeding can be either inferred by comparing antiplatelet drug characteristics or studied by platelet function assessments at the time of PCI. Clopidogrel compared to placebo and double-dose clopidogrel, prasugrel or ticagrelor compared to standard-dose clopidogrel are associated with more bleeding including PCI-related bleeding (14, 44-46). The findings of the latter comparisons can be attributed to the greater mean levels of P2Y<sub>12</sub> inhibition achieved by double-dose clopidogrel, prasugrel or ticagrelor compared to standard-dose clopidogrel in patients with coronary artery disease (26, 47-50). This indirectly links platelet function and the extent of P2Y<sub>12</sub> receptor blockade with the risk of PCI-related bleeding complications. More evidence of such a link comes from platelet function studies. Thus, in a study of 2533 patients undergoing PCI, Sibbing et al. found that the 38% of patients with a higher antiplatelet response to clopidogrel had significantly higher rates of bleeding compared to the rest of the cohort with lesser antiplatelet effect according to the Multiplate assay of ADP-induced platelet aggregation (adjusted odds ratio [OR] 3.5, 95% confidence interval [CI] 1.6-7.3) (51). The same group also found that the *17 variant of cytochrome P450 (CYP) 2C19, a gain-of-function variant that may enhance clopidogrel active metabolite formation and consequent P2Y<sub>12</sub> inhibition, was independently associated with an increased risk of bleeding with an OR of 1.85 (95% CI 1.19-2.86) (52). These findings, combined with observed increased risk of stent thrombosis in those with a poor response to clopidogrel, led to the proposal that a therapeutic window of P2Y<sub>12</sub> inhibition exists in patients undergoing PCI (53). Such a window is conceptually similar to the international normalised ratio (INR) used for the tailoring of treatment with vitamin K antagonists. In another study of 597 patients with non-ST-elevation ACS, the quartile of patients with the highest response to clopidogrel according to platelet aggregometry and VASP assay had higher rates of bleeding compared to the other quartiles (54). Another study suggested higher rates of bleeding following PCI associated with enhanced response to clopidogrel in carriers of the CYP2C19 *17 allele (55). A further study used propensity matching to suggest that the VASP assay may predict bleeding risk in clopidogrel-treated patients undergoing PCI (56), and a third study suggested the VerifyNow P2Y12 assay may predict bleeding risk in PCI patients (57). Finally, the potential benefit of platelet function testing to tailor the use of intravenous glycoprotein inhibitors in PCI patients with low platelet responses to oral antiplatelet drugs has been tested. Cuisset et al. evaluated the addition of abciximab to dual antiplatelet therapy in patients referred for elective PCI, who were clopidogrel low-responders (n =149) defined by light transmittance aggregometry (58). Tailored antiplatelet treatment with addition of abciximab significantly reduced cardiovascular events at one month with no differences in bleeding events. Similarly, the 3T/2R trial randomised low-responders to aspirin or clopidogrel, assessed by the VerifyNow, to treatment with tirofiban or placebo.
(59). Although this randomised, double blind, placebo-controlled study (n = 263) included 10 European sites, it was not powered to evaluate the risk of bleeding events, which did not differ between groups. The increasing use of new P2Y$_{12}$ receptor antagonists in patients with ACS will reduce the benefit and use of glycoprotein inhibitors (60) and, therefore, it is unlikely that large clinical trials will be undertaken to further explore the potential role of platelet function testing to tailor the use of glycoprotein inhibitors.

Set against the above-mentioned studies suggesting a clear relationship between clopidogrel response and bleeding risk are contradictory findings from other studies (Table 1). The POPULAR study included 1,069 clopidogrel-treated patients following elective PCI and found no evidence that various measures of P2Y$_{12}$ inhibition predicted the risk of bleeding (61). In the large PLATO genetic sub-study (n = 10,285), clopidogrel-treated patients who were carriers of the gain-of-function CYP2C19*17 allele had a higher frequency of major bleeding (11.9%) than did those without any gain-of-function or loss-of-function alleles (9.5%; p = 0.022) (62). A study of 444 ACS patients on prasugrel following PCI found no evidence that VASP assay results predicted the risk of bleeding although the number of patients with high platelet reactivity, measured by several different assays, was low (63). However, it is most likely that large studies including several thousand clopidogrel-treated patients are required to demonstrate an independent influence of extent of P2Y$_{12}$ inhibition on PCI-related bleeding in contrast to the relatively smaller numbers required to show an impact of extent of P2Y$_{12}$ inhibition on stent thrombosis risk, which appears more sensitive to this (Figure 2).

Conclusions

It is clear from current evidence and predictable from knowledge of the relationship between platelet function and haemostasis that, in patients with cardiovascular disease treated with antiplatelet drugs, increasing levels of platelet inhibition are associated with increased bleeding risk. The combination and dosage of antiplatelet and anticoagulant drugs are important determinants of bleeding complications, and only in recent years has platelet function testing been evaluated as a potential clinical tool to predict and reduce bleeding complications. Many platelet function tests are sufficiently reliable to provide an estimate of in vivo platelet reactivity and current evidence supports a role for some of these tests in guiding the management of patients undergoing CABG surgery following discontinuation of clopidogrel. However, more studies are needed to investigate the role, if any, of platelet function measurements in patients treated with the new and potent P2Y$_{12}$-receptor inhibitors. Perhaps better predictive models of ischaemic and bleeding events can be developed by integrating platelet function testing with genotyping. Such a strategy was pursued in the recently published GIPT (Genotype Information and Functional Testing) study, which reported the pharmacodynamic effect of clopidogrel after PCI but was not powered to evaluate clinical outcomes (64). Alternatively, combining measurements of platelet function with biomarkers, fibrinogen, von Willebrand factor or coagulation tests may improve the ability to predict bleeding events. In the future, evaluation of platelet function may challenge the tenet that one size fits all. In theory, platelet function testing may guide clinical decisions in patients treated with one or several antithrombotic drugs, particular prior to surgery or interventional procedures. Whilst there is evidence of a potential therapeutic window of P2Y$_{12}$ inhibition in patients undergoing PCI, this remains controversial and large prospective studies are needed to further explore the clinical role of platelet function testing.

Conflicts of interest

ELG has received lecture fees from AstraZeneca, Bayer, Boehringer-Ingelheim and Pfizer and serves on advisory boards for AstraZeneca and Bristol-Myers-Squibb. RH has no conflicts of interest to declare. RFS has received research grants from AstraZeneca, Eli Lilly/Daiichi Sankyo and Merck, research support from Accumetrics, honoraria from AstraZeneca, Eli Lilly/Daiichi Sankyo, Merck, Novartis, The Medicines Company, Iroko, Sanofi Aventis/BMS, Accumetrics, Medscape and Eisai, and consultancy fees from AstraZeneca, Merck, Novartis, Accumetrics, Roche and Eisai.

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