High on-treatment platelet reactivity – definition and measurement

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Summary
In the last decade, several studies revealed inter-patient response variability to antiplatelet agents: patients who display negligible or no responses to these drugs are considered poor responders, or “resistant” to treatment. In order to identify poor responders to an antiplatelet drug, laboratory tests of platelet function that specifically explore the platelet activation pathway that is targeted by the drug should be utilised. In addition, they should be performed both at baseline and during treatment: however, most studies explored platelet function during antiplatelet treatment, in order to identify those patients with “high on-treatment platelet reactivity” (HPR), which exposes them to increased risk of major adverse cardiovascular events (MACE). Many tests of platelet function have been used, most of which are able to identify patients at risk of MACE. Unfortunately, universal cut-off values for HPR have not been clearly established yet. In addition, the concordance among different tests in the identification of patients at risk is very poor and the most effective and safe treatment for patients at risk is still unknown.

Keywords
Antiplatelet agents, platelet pharmacology, arterial thrombosis, clopidogrel, aspirin

Introduction
Acetylsalicylic acid (aspirin) and inhibitors of the platelet P2Y\textsubscript{12} receptor for adenosine diphosphate (ADP) are antiplatelet drugs that decrease the risk of major adverse cardiovascular events (MACE). They are used during coronary interventions, in the medical management of acute coronary syndromes, and in long-term secondary prevention of cardiovascular and cerebrovascular events.

Both aspirin and P2Y\textsubscript{12} antagonists selectively inhibit a single pathway of platelet activation: aspirin affects the thromboxane A\textsubscript{2} (TxA\textsubscript{2}) pathway by irreversibly inhibiting cyclo-oxygenase-1 (COX-1), while P2Y\textsubscript{12} antagonists affect the ADP pathway, by antagonizing one of the two platelet ADP receptors irreversibly (thienopyridines) or reversibly (ticagrelor) (1, 2). The good anti-thrombotic efficacy of these drugs, despite their selective mechanism of action, is explained by the fact that both the TxA\textsubscript{2} pathway and the ADP pathway contribute to the amplification of platelet activation and are essential for the full aggregation response of platelets (1).

Since their introduction in clinical practice, antiplatelet drugs have been administered to patients at standard doses, without monitoring their pharmacological effects by means of laboratory tests. However, in the last few years several studies revealed inter-patient response variability to aspirin and the thienopyridine drug clopidogrel: patients who display no or negligible responses to these drugs have been considered poor responders, or “resistant” to treatment.

Resistance (or poor responsiveness) to antiplatelet agents versus high on-treatment platelet reactivity (HPR)

The term “resistance” to a drug should be used when a drug is unable to hit its pharmacological target, due to inability to reach it (as a consequence of reduced bioavailability, in vivo inactivation, negative interaction with other substances) or to alterations of the target (1). Based on this definition, resistance to aspirin should be limited to situations in which aspirin is unable to inhibit COX-1-dependent TxA\textsubscript{2} production (and, consequently, TxA\textsubscript{2}-dependent platelet functions), while resistance to clopidogrel should be limited to situations in which the drug is unable to inhibit the platelet P2Y\textsubscript{12} receptor (and, consequently, the P2Y\textsubscript{12}-dependent platelet functions).

Although it can be easily predicted that patients who fail to show a pharmacological response to an effective antithrombotic drug are not adequately protected from MACE, it is incorrect to consider “resistant” to antiplatelet agents those patients who experience MACE, despite being compliant with the therapy. This phenomenon has been called “clinical antiplatelet resistance”, but it should be termed “treatment failure” (1): it can be observed with any kind of treatment and is expected to be particularly frequent for drugs, like all anti-thrombotic agents, that are used to prevent multi-factorial diseases, such as those associated with vascular occlusions. As previously mentioned, aspirin and clopidogrel inhibit only one pathway of platelet aggregation; platelet aggregation is...
only one mechanism regulating thrombus formation; thrombus formation is the most common, but not the only mechanism leading to vascular occlusion; vascular occlusion causes clinical events that can be widely different in terms of severity, ranging from asymptomatic in some patients to lethal in others. Given this picture, it would be unreasonable to expect an antiplatelet agent, or any anti-thrombotic drug, to prevent clinical events in all patients at risk. Therefore, the definition of “antiplatelet drug resistance” that is based on clinical outcomes is certainly unacceptable (1).

Because both the TxA2 and the ADP pathways contribute to the amplification of platelet activation and are essential for full aggregation responses of platelets, inhibition of each of these pathways will negatively affect not only thrombus formation in vivo, but also platelet function in vitro. Therefore, many studies used various techniques to measure platelet function in vitro in order to evaluate the degree of its inhibition by anti-platelet treatment and, in some instances, to predict the risk of atherothrombotic events. Although this approach is justified and rational, one should be aware that the relative importance of the TxA2 and P2Y12 pathways in platelet activation varies considerably among different subjects and with the type of laboratory test used (1). Two important issues should be taken in consideration when measuring platelet function in vitro in patients on treatment with antiplatelet agents: a) the specificity of the laboratory tests for the platelet activation pathway targeted by the drug; b) whether or not the laboratory test is performed both before and after the administration of the drug.

**Specificity of the laboratory test**

Because antiplatelet drugs target one single pathway of platelet activation, in order to measure the pharmacological response to an antiplatelet agent, tests that are very specific for the platelet activation pathway that is targeted by the drug should be used (1, 3, 4). The use of unspecific, global tests of haemostasis, which are sensitive to several pathways that lead to the formation of platelet aggregates, may be very marginally affected by inhibition of the pathway targeted by the drug, and therefore are not adequate for this purpose (1, 3, 4). However, because all pathways of platelet activation are expected to be involved in thrombus formation in vivo, global tests of hemostasis are theoretically more useful than specific tests to predict the risk of MACE (3).

**“Response to treatment” versus “On-treatment platelet reactivity”**

In order to measure the pharmacological response to an antiplatelet agent one should compare the results obtained before and after the ingestion of the drug (1). As a matter of fact, due to the high baseline inter-individual variability, high platelet reactivity measured during antiplatelet treatment could result either from insufficient inhibition of “normal” baseline platelet reactivity or from adequate inhibition of platelets that were hyper-reactive at baseline (1). To distinguish between these two scenarios, it would be necessary to measure platelet reactivity both before and after treatment. However, measurement of baseline platelet reactivity would be impractical or even impossible in many cases of patients with acute coronary syndromes, who need to be treated with antiplatelet agents under emergency conditions. Therefore, it has become common practice to measure platelet reactivity during antiplatelet treatment (“on treatment platelet reactivity”), without having measured a reference, baseline value. Although measurement of on-treatment platelet reactivity is a valuable parameter to consider when the aim of platelet function testing is to predict the risk of clinical events that are dependent on platelet activation/aggregation (3), it will fail to provide information on the individual pharmacological response to the drug. Knowing whether or not a high level of on-treatment platelet reactivity is caused by failure to respond to the drug is clinically relevant: treatment could be changed increasing the dose of the drug or switching to a different drug of the same family, in case of poor responsiveness, while it would not be changed in case of platelet hyper-reactivity despite good responsiveness to the drug, although additional treatments, if available, might be taken in consideration by the treating physician.

While on-treatment platelet reactivity was initially studied mostly with unspecific or global tests of platelet function, in the last years it has been preferentially measured using specific tests. Therefore, the most recent and the ongoing studies that use specific tests aim at identifying patients with HPR that is likely due to poor responsiveness to the drug. The cut-off values for “HPR” must be identified, based on its ability to discriminate between patients who are at increased risk of future MACE from those who are not: this goal can be accomplished uniquely based on the results of large-scale, well-designed, ad hoc studies.

**Laboratory tests of platelet function that have been used to measure platelet reactivity during antiplatelet treatment**

Platelet function in vivo has been measured by the bleeding time, while platelet function in vitro has been measured by several tests, many of which have been criticised because they do not reproduce the physiological conditions that characterise the development of platelet aggregates in vivo. However, this potential drawback does not necessarily decrease their accuracy in measuring the efficacy of an anti-platelet drug to hit its pharmacological target (3). In addition, it is likely that in the future platelet tests will also focus on platelet functions that do not seem strictly related to haemostasis (5).

Finally, it must be emphasised that the results of platelet function is affected by many variables, including the circadian rhythm, comorbidities, physical exercise, the type and quantity of food and drinks, etc. As a consequence, the inter-assay coefficient of variation of platelet function tests tends to be high, although this has been only very rarely reported in published studies. For a list of laboratory tests of platelet function that have been used to measure platelet reactivity during antiplatelet treatment see Table 1.
Bleeding time

The bleeding time is an invasive technique, which is poorly reproducible, even when carried out by experienced personnel. In addition to these drawbacks, the technique is influenced by several variables, which include platelet function, platelet count, plasma factors, red blood cells, the vessel wall and the skin trophism. For this reason, it displays low sensitivity to mild/moderate abnormalities of inherited and acquired (including drug-induced) defects of platelet function and should not be used to evaluate the effects of anti-platelet agents (1).

Light transmission aggregometry (LTA)

LTA measures the increase in light transmission through a platelet suspension that occurs when platelets aggregate in response to an agonist. It is not ideal for testing platelet sensitivity to aspirin or thienopyridines for several reasons:

- It is time-consuming and should be performed in highly specialised laboratories only.
- Many pre-analytical and analytical variables affect the results: even when all of them are controlled for, the accuracy and reproducibility of the technique are very poor.
- The results obtained within one lab can hardly be compared to those obtained in a different lab due to lack of standardisation (6); therefore, any attempt to define universal cut-off values of platelet aggregation to identify non responders to anti-platelet therapies would be pointless.
- Depending on the type and concentration of agonist used, and the type of anticoagulant used for blood collection, the aggregation response is only partially and variably modulated by TxA₂, synthesised from arachidonic acid in the platelet membrane, or ADP, released from platelet granules. Even when the two most specific platelet agonists are used, arachidonic acid for monitoring aspirin, and ADP for monitoring P2Y₁₂ inhibitors, the results obtained with this technique may over-estimate the prevalence of resistance to anti-platelet agents (1, 3).

The inadequacy of LTA for measuring the pharmacological effect of aspirin was demonstrated by Ohmori et al., who showed that, although platelet COX-1 activity seemed to be uniformly inhibited in all studied patients, LTA studies showed great inter-individual differences (7). Despite its limitations, the extent of maximal and/or late platelet aggregation measured by LTA was linked to clinical outcomes after percutaneous coronary interventions (PCI) in some studies (8-15).

Impedance aggregometry

Impedance aggregometry measures the change in electrical impedance that occurs when platelets form an aggregate on electrodes, which are immersed in a sample of diluted whole blood, after the addition of a platelet agonist. The instrument Multiplate® (Verum Diagnostica, Munich, Germany) is the impedance aggregometer that has been most widely used in studies that measured the platelet aggregation in patients on treatment with clopidogrel. The technique is less technical demanding and time consuming than LTA; however, some drawbacks that have been listed for LTA, also apply to this technique. In particular, pre-analytical variables (correctness of blood sampling and handling, for instance) will affect the aggregation response of platelets, independently of the instrument used for its measurement. According to some studies, the technique is able not only to identify patients at risk of MACE, but also those at risk of bleeding (16-19).

Whole blood platelet aggregation measured by platelet counting

This is an easy and extremely sensitive method to study platelet aggregation. It is based on the principle that single platelet counts in whole blood stimulated with a platelet agonist decrease as a function of platelet aggregate formation. It is more sensitive than LTA, because it can detect very small aggregates, formed by 2-3 aggregated platelets. Very limited experience with this technique is available for assessment of the effects of clopidogrel on platelet aggregation (20).

The PFA-100® system

PFA-100® (Dade Behring, Marburg, Germany) could be considered an in vitro bleeding time: a sample of anticoagulated blood is aspirated through a capillary (mimicking the resistance of a small artery) and a 150 μm aperture in a membrane (mimicking the injured part of the vessel wall) coated with collagen plus ADP (C-ADP) or collagen plus epinephrine (C-EPI). A platelet plug forms that gradually occludes the aperture; as a consequence, the blood flow through the aperture gradually decreases and eventually stops. The time needed till blood flow interruption (“closure time”) is recorded (1, 21). The system is very easy to use, automated, quick and requires a small volume of whole blood. It is sensitive to von Willebrand Disease and to severe abnormalities of platelet function, for example Glanzmann Thrombasthenia and Bernard-Soulier Syndrome (22). In contrast, its sensitivity to mild/
moderate, inherited and drug-induced defects of platelet function is low (22): closure times (CT) of the C-EPI cartridge may be prolonged in some, but not all patients on aspirin treatment, while variable but often normal CT have been observed on both C-ADP and C-EPI cartridges in samples from patients receiving clopidogrel therapy (1, 22-27). The low sensitivity of the method to mild abnormalities of platelet function can be accounted for by the fact that it is sensitive to many variables including platelet count, red blood cells, platelet reactivity to collagen and, above all, plasma von Willebrand factor (28, 29), the effects of which on platelet aggregate formation can easily outweigh the effects of mild inhibition of platelet function (1). As a matter of fact, it has been shown that both individuals with short and individuals with prolonged C-EPI closure times on aspirin treatment had very low levels of serum thromboxane B₂ (TXB₂), indicating that aspirin inhibited COX-1 to the same extent in the two groups of patients (30, 31). For the above reasons the PFA-100® system is not an adequate method to measure platelet inhibition by aspirin or P2Y12 inhibitors.

A novel cartridge (Innovance PFA* P2Y, Siemens, Erlangen, Germany), which is more sensitive to P2Y12, is under clinical evaluation (32).

### Ultegra Rapid Platelet Function Assay (RPFA)-VerifyNow®

The Ultegra Rapid Platelet Function Assay (RPFA)-VerifyNow® (Accumetrics, San Diego, CA, USA) is a simple point-of-care whole blood test that measures the agglutination of fibrinogen-coated beads by platelets stimulated by an agonist in citrated whole blood. It was initially developed to measure the anti-platelet effects of glycoprotein (GP)IIb-IIIa antagonists. Later, it was slightly modified to become more sensitive and specific for the two most widely used anti-platelet treatments: RPFA-VerifyNow® ASA, for monitoring aspirin treatment (24, 33) and RPFA-VerifyNow® P2Y12 (34), which is a rather specific test for monitoring clopidogrel treatment (35, 36). Results are expressed as P2Y₁₂ reaction units (PRU), with higher values being associated with higher platelet reactivity. In the last years, VerifyNow® P2Y₁₂ has become the most widely used test for monitoring clopidogrel treatment. PRU values have been correlated with MACE in many studies, involving a large number of patients (37-41). Despite its widespread use, the cut-off value for predicting MACE is still a matter of debate (42). VerifyNow has been chosen as the laboratory test to measure platelet function in trials that were designed to test the safety and efficacy of tailored treatment with clopidogrel (37, 43), based on platelet function testing, which so far have given disappointing results. The main drawback of the assay is its very high cost.

### Cone-and-Plate(let) Analyzer (CPA)

The Cone-and-Plate(let) Analyzer (CPA) is a cone-and-plate viscometer that measures platelet adhesion and aggregation under laminar flow with uniform high shear. It was shown to be accurate for detection of von Willebrand Disease and for monitoring treatment with GPIIb/IIIa antagonists. In a small study, it could detect poor responders to clopidogrel (44). Its clinical development has been recently interrupted.

### Flow cytometry

Flow cytometry allows the evaluation of platelet reactivity to agonists in vitro and the presence of circulating activated platelets, platelet-leukocyte aggregates and platelet-derived microparticles. Although it requires a sophisticated and expensive instrument, which is not available to every institution, it is simple to perform and requires very small volumes of whole blood samples. It has been used to measure in vitro platelet activation on anti-platelet treatment (45).

A flow cytometry-based method, the Platelet VASP®, (Biocytex, Diagnostica Stago, Asnieres, France) measures the inhibition by ADP of phosphorylation of vasodilator-stimulated phosphoprotein (VASP) (46), which is mediated by P2Y₁₂ through the inhibition of adenyl cyclase. The ratio of dephosphorylated and phosphorylated VASP is a specific measure of P2Y₁₂ activity, which is expressed as “platelet reactivity index” (PRI). Therefore, it represents a most specific available assay for measuring the effects of thienopyridines and other drugs inhibiting the platelet P2Y₁₂ receptor (23, 47, 48): good inhibition of P2Y₁₂ is reflected by low PRI values. Unfortunately, the technique is not sensitive to mild abnormalities of the receptor (49, 50). There was a good correlation between ADP-induced platelet aggregation and VASP-phosphorylation assay in detecting P2Y₁₂ inhibition (23, 51), although occasional individuals on clopidogrel treatment who appeared low responders with LTA were good responders on the more specific VASP-phosphorylation assay (23). An important advantage of Platelet VASP® is that its endpoint is not based on platelet aggregation and is therefore absolutely independent of GPIIb/IIIa function: this property allows its use also in patients on treatment with GPIIb/IIIa antagonists, which are used in some categories of ACS patients undergoing PCI. Several studies showed that high PRI values in patients on treatment with clopidogrel (52-55), or even prasugrel in one case (56), correlate well with the risk of MACE. However, the correct cut-off value has not been clearly identified yet (55, 57), as it significantly depends on the method of calculation, the time-to-assay as well as on the lag time after processing (38). Recently, an ELISA-based kit became available, which might simplify the measurement of VASP phosphorylation in some circumstances.

### Thromboelastography (TEG)

The method measures the viscoelastic changes that occur during clot formation in whole blood. It is influenced by platelet function, coagulation factors, natural inhibitors of coagulation and fibrinolysis. Some studies showed that TEG is potentially useful for studying patients on antiplatelet treatment (59-66).
Serum TxB<sub>2</sub> levels

Serum TxB<sub>2</sub> reflects the total capacity of platelets to synthesise thromboxane A<sub>2</sub>, of which it is a stable metabolite, and is therefore the most specific test to measure the pharmacological effect of aspirin (1). In studies of 680 patients undergoing cardiac catheterisation (45) and 96 healthy subjects (31) on aspirin treatment, only about 1-2% had evidence of incomplete response to aspirin, based on serum TxB<sub>2</sub> levels, which was either due to under-dosing or non-compliance (45), with the exception of one healthy subject who really appeared resistant to aspirin (31). Frelinger et al. reported that a direct measure (serum TxB<sub>2</sub> < 3.1 ng/ml) but not indirect measures of residual platelet COX-1 function are associated with subsequent MACE in aspirin-treated patients. However, given a potential for bias based on the method used to define the cut-off for high residual serum TxB<sub>2</sub>, the need to adjust for covariables to show a significant association between serum TxB<sub>2</sub> and subsequent MACE, and the failure of indirect assays of residual platelet COX-1 function to confirm an association with MACE, the link between residual platelet COX-1 function as reported by serum TxB<sub>2</sub> < 3.1 ng/ml and MACE should be further verified (63).

The ability of platelets to synthesize TxB<sub>2</sub> after in vitro stimulation with an agonist, such as collagen, can also be considered a specific test for measuring the effects of aspirin, albeit probably less sensitive and more time consuming than serum TxB<sub>2</sub> (7).

Urinary levels of the TxB<sub>2</sub> metabolite, 11-dehydrothromboxane B<sub>2</sub>

Urinary levels of 11-dehydro-TxB<sub>2</sub> represent a time-integrated index of TxA<sub>2</sub> biosynthesis in vivo (64). Because it is not formed in the kidney, detection of this TxA<sub>2</sub> metabolite in the urine reflects systemic TxA<sub>2</sub> formation, which largely, although not exclusively, occurs in the platelets. It has been calculated that about 30% of the urinary metabolite derives from extra-platelet sources (65); however, in pathological conditions, such as in inflammatory diseases, the contribution of extra-platelet sources may increase. Since atherosclerosis is an inflammatory condition, high levels of urinary 11-dehydro-TxB<sub>2</sub> may be considered a marker of inflammation, rather than a marker of poor responsiveness to aspirin (66).

Comparison of different laboratory tests of platelet function in the assessment of the pharmacological responses to aspirin or P2Y<sub>12</sub> inhibitors

Comparison of different laboratory methods in patients on aspirin treatment (arachidonic acid-induced aggregation with LTA, PFA-100®, Ultegra VerifyNow® ASA, serum TxB<sub>2</sub>, agonist-induced TxB<sub>2</sub> production, urinary 11-dehydrothromboxane B<sub>2</sub>) usually showed very weak or no correlation in published studies (24, 31, 67, 68), indicating that they are sensitive to different parameters. Usually, the number of individuals with residual, significant TxB<sub>2</sub> production on aspirin was extremely low, while the prevalence of individuals with no inhibition of platelet function measured by other tests tended to be much higher.

Many studies compared the performance of different laboratory tests for evaluating the pharmacological effects of clopidogrel. All studies demonstrated that the degree of agreement among different tests is unacceptably low (69-77): the same patients could be considered poor responders by one test and good responders by another test, and vice-versa. Therefore, because no single laboratory test has been shown to be more accurate than others in measuring the degree of inhibition of ADP/P2Y<sub>12</sub>-dependent platelet function by clopidogrel, the ideal test for monitoring clopidogrel therapy has not been identified yet.

Conclusion

In conclusion, a clear distinction should be made between monitoring patients on anti-platelet treatment to identify “poor responders” and to identify patients with HPR. For practical reasons, the vast majority of studies that have been performed so far in patients on antiplatelet therapy measured on-treatment platelet reactivity. Many good tests of platelet function have been used, most of which were shown to be able to identify patients at risk of MACE. Unfortunately, universal cut-off values for high on-treatment platelet reactivity have not been clearly established yet. In addition, the concordance among different tests in the identification of patients at risk is very poor, and the most effective and safe treatment for patients at risk is still unknown. Therefore, many studies need to be performed to answer basic questions on its clinical utility and cost-effectiveness, before anti-platelet monitoring can be recommended in the clinical practice. While waiting for the results of some of these studies that are ongoing, monitoring of anti-platelet therapy should be done for investigational purposes only.

Conflicts of interest

M. Cattaneo has received honoraria and research funds from AstraZeneca, Eli Lilly and Daiichi Sankyo.

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