Platelet Function Tests and Resistance to Antiplatelet Therapy

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SUMMARY

The clinical efficacy of antiplatelet therapy (aspirin, P2Y12 and glycoprotein IIb/IIIa receptor antagonists) to prevent occlusive arterial events in patients with atherothrombotic disease is well established. Despite the proven benefits of antiplatelet therapy, many patients continue to experience arterial events. Many factors may influence the response of platelets to antiplatelet therapy and some patients with adequate compliance to the treatment may exhibit failure of platelet inhibition as determined by ex vivo laboratory tests, a phenomenon termed "resistance"to antiplatelet therapy. Platelet function can be measured by numerous platelet function tests, with which various parameters of platelet activation, secretion, adhesion and aggregation can be determined. These tests include light transmission (optical) and whole blood aggregometry, point-of-care devices, such as platelet function analyzers PFA-100®, and VerifyNow®, flow cytometry, serum thromboxane B2 and urinary levels of the thromboxane B2 metabolite 11-dehyro-thromboxane B2. Other tests, such as whole blood platelet aggregation measured by platelet counting, thrombelastography and devices such as the cone and plate(let) analyzer, Plateletworks and thrombotic status analyzer have also been used to determine platelet inhibition by antiplatelet drugs, but their use is not widespread and therefore experience is limited. Further studies need to be carried out to answer basic questions on the clinical utility and cost-effectiveness of laboratory monitoring of antiplatelet therapy before it can be recommended in clinical practice.

Keywords: antiplatelet therapy; tests; resistance

INTRODUCTION

The clinical efficacy of antiplatelet therapy to prevent occlusive arterial events in patients with atherothrombotic disease is well established. The balance of benefits and risks of antiplatelet drugs in various clinical settings has been evaluated over the past decade in large-scale randomized trials [1]. However, for the absolute benefit of an individual patient, it may become useful to monitor the individual's response to antiplatelet therapy so that either the dosage and/or the type of drug(s) administered can be titrated or optimized to help control and minimize the risk of either thrombosis or bleeding.

There are three families of antiplatelet drugs with proven clinical efficacy: (i) cyclooxygenase-1 inhibitors such as aspirin; (ii) adenosine 5-diphosphate (ADP) inhibitors, such as the thienopyridine compounds prasugrel, clopidogrel and ticlopidine; and (iii) glycoprotein IIb/IIIa (GPIIb/IIIa) receptor antagonists, such as abciximab, tirofiban and eptifibatide. Both aspirin and thienopyridines selectively inhibit a single pathway of platelet activation: aspirin affects the thromboxane A2 pathway by irreversibly inhibiting cyclooxygenase-1, while thienopyridines affect the ADP pathway by irreversibly antagonizing one of the two platelet ADP receptors - P2Y12 [2]. Good antithrombotic efficacy of these drugs, despite their selective mechanism of action, is explained by the fact that both the thromboxaneA2 pathway and the ADP pathway contribute to the amplification of platelet activation and are essential for the full aggregation response of platelets. The identification of GPIIb/IIIa complex importance in mediating platelet aggregation suggests that this receptor could be an attractive target for antiplatelet therapy. The GPIIb/IIIa antagonists have now become an important class of antiplatelet agents that are widely used for the prevention of thrombotic complications in patients undergoing percutaneous coronary interventions or presenting with acute coronary syndromes [3].

LABORATORY TESTS USED TO MEASURE PLATELET FUNCTION DURING ANTIPLATELET THERAPY

New and existing platelet function tests are increasingly being used for monitoring the efficacy of antiplatelet drugs and some of these tests have been shown to predict clinical outcomes after antiplatelet therapy. The development of new, simpler tests and point-of-care instruments has resulted in an increasing tendency for platelet function testing to be performed in and away from specialized haemostasis clinical or research laboratories, where the more traditional and complex tests are still performed.

Platelet function can be measured *in vivo* by the bleeding time and *in vitro* by numerous platelet function tests, with which various parameters of platelet activation, secretion, adhesion and aggregation can be determined. Some traditional and new platelet function tests used to monitor antiplatelet therapies are listed in Table 1. These tests include light transmission (optical) and whole blood aggregometry, point-of-care devices such as the platelet function analyzers PFA-100® and VerifyNow®, flow cytometry, serum thromboxane B2 and urinary levels of thromboxane B2 metabolite 11-dehydrothromboxane B2 [4]. Besides, there are a number of other tests such whole blood

Table 1. Advantages, limitations and sensitivity to antiplatelet drugs of some traditional and new platelet function tests. Modified from [4, 11, 22]

Test	Principle	Advantages	Limitations	Sensitivity to antiplatelet drugs
Bleeding time	In vivo cessation of blood flow	Widely available Simple Physiological	Highly operator dependent Limited reproducibility Invasive	Low sensitivity
Light transmission (optical) platelet aggregometry	Low shear platelet-to-platelet aggregation in response to classical agonists	A panel of agonists provides information about different aspects of platelet function Correlated with clinical events	Labour intensive, large volume of blood needed Many pre- and analytical variables affect the result Restricted to specialized laboratories Low shear stress rate	Aspirin (agonist arachidonic acid) Clopidogrel (agonist ADP) GPIIb/IIIa
Impendance platelet aggregometry	Monitors changes in impedance in response to classical agonists	Whole blood	Insensitive Restricted to specialized laboratories Time-consuming electrode cleaning	Aspirin (agonist arachidonic acid) Clopidogrel (agonist ADP) GPIIb/IIIa
Semi-automated platelet function test PFA-100®	High shear platelet adhesion and aggregation during formation of a platelet plug	Whole blood Widely available, rapid and simple Rapidly interpreted outside tertiary institutions Reproducible High shear stress rate	No instrument adjustment Inflexible Dependent on von Willebrand factor, platelet count and haematocrit	Aspirin (collagen/epinephrine cartridge) Insensitive to thienopyridines (clopidogrel cartridge in development) GPIIb/IIIa (collagen/ADP cartridge)
Semi-automated platelet function test VerifyNow®	Fully automated platelet aggregometer to measure antiplatelet therapy	Whole blood Widely available, rapid and simple Rapidly interpreted outside tertiary institutions Reproducible	No instrument adjustment Cartridges can only be used for single purpose Does not reproduce high shear stress	Aspirin (aspirin cartridge) Clopidogrel (P2Y12 cartridge) GPIIb/IIIa (GPIIb/IIIa cartridge)
Flow cytometry	Measurement of platelet glycoproteins and activation markers by fluorescence	Whole blood Small blood sample Correlated with function tests Quantification of free and/or occupied sites	Restricted to specialized laboratories Expensive equipment Not widely standardized	Aspirin (arachidonic acid stimulation) Clopidogrel (measurement of VASP phosphorylation, analysis of activation-dependent markers) GPIIb/IIIa
Thromboxane generation	Immunoassay for serum thromboxane B2 or urinary 11-dehydro-thromboxane B2	Highly dependent upon cyclo-oxygenase-1 Correlated with clinical events	Indirect test Urinary levels not platelet specific Uncertain reproducibility Not widely evaluated Restricted to specialized laboratories	Aspirin

VASP – vasodilator-stimulated phosphoprotein

platelet aggregation measured by platelet counting, thromboelastography and devices such as the cone and plate(let) analyzer, and the plateletworks and thrombotic status analyzer which have also been used to determine platelet inhibition by antiplatelet drugs, but their use is not widespread and therefore experience is limited [5].

The bleeding time – this *in vivo* test of platelet function is highly operator-dependent and poorly reproducible, even when carried out by experienced personnel. It has been utilized to detect the efficacy of aspirin therapy [6], although, due to its limitations, it is not recommended for monitoring antiplatelet drugs [2].

Light transmission (optical) platelet aggregometry according to Born [7] is considered as the gold standard for platelet function testing [8]. In this test the increase in light transmission is determined after a platelet stimulating substance (usually collagen, ADP or arachidonic acid) has triggered platelet activation and aggregation in platelet-rich plasma. The test is widely used, although it requires a relatively large amount of blood, it is highly dependent on sample preparation and technical procedure, it is time-consuming and the results between different laboratories are poorly comparable [9]. Furthermore, light transmission platelet aggregometry does not take into account the role of red blood cells and shear stress. In spite of these limitations, this test remains most widely used in the evaluation of platelet function.

Whole blood impedance aggregometry measures the change in electrical impedance between two electrodes that occurs when platelets are aggregated by an agonist [10]. The use of whole blood circumvents the need for preparation of platelet-rich plasma. Pre-analytical restrictions are the same as those for transmission aggregometry and in some instruments the time-consuming cleaning of the electrodes is an obstacle to its wide-spread application. A new five-channel computerized instrument (Multiple Platelet Function Analyzer or Multiplate®) has disposable cuvettes/electrodes with a range of different agonists for the diagnosis and monitoring of antiplatelet therapy [11].

Due to the limitations of light transmission platelet aggregometry, point-of-care devices, such as the platelet function analyzer PFA-100® have been introduced for the

detection of platelet function [12]. The PFA-100 measures platelet adhesion and aggregation in whole blood under the conditions of high shear in an attempt to simulate the primary haemostatic mechanisms that are encountered *in vivo*. The device is easy to use, automated, quick and reproducible [13]. The use of commercially available cartridges facilitates the comparability of results coming from different laboratories. Because the PFA-100® measures platelet function under flow conditions which are characterized by high shear stress, plasma von Willebrand factor is the major determinant of the results (closure times). Classically, under aspirin treatment the closure time in a collagen/epinephrine coated cartridge is prolonged, but not with the collagen/ADP coated cartridge. The PFA-100®is not an appropriate test for monitoring thienopyridines [13], while GPIIb/IIIa antagonists prolong closure times in collagen/ADP cartridges in a dose dependent manner [14].

The VerifyNow® is a simple, fully automated point-of-care device that measures agglutination of fibrinogen coated beads in response to a platelet agonist [15]. In the cartridge platelets in whole blood are activated by an agonist; activated platelets agglutinate fibrinogen coated beads, which results in an increase in light transmission. The test has been originally used to monitor antiGPIIb/IIIa therapy [4, 15]. To evaluate the effect of platelet inhibition during aspirin and clopidogrel therapy, cartridges with arachidonic acid [16] or ADP [17] as agonists, respectively, have been developed. The VerifyNow® is reported to give results in correlation with aggregometry [18]. However, as for aggregometry, this measuring system does not take into account the shear conditions which exist in vivo.

Flow cytometry is a powerful laboratory tool for the assessment of platelet activation and function. It can be used to measure platelet reactivity, circulating activated platelets, leukocyte-platelet aggregates, and procoagulant platelet-derived microparticles [19]. Although flow cytometry requires sophisticated equipment and reagents and is not widely standardized, it has several advantages. It can be performed on a very small sample of whole blood and the test can be also done in thrombocytopenia. The flow cytometry based method, measures the inhibition by ADP of phosphorylation of intracellular platelet protein, a vasodilator-stimulated phosphoprotein (VASP), which is closely correlated to the inhibition of GPIIb/IIIa and P2Y12 ADP receptors [20].

Serum thromboxane B2, a stable metabolite of thromboxane A2, reflects the total capacity of platelets to synthesize thromboxane A2, and is therefore the most specific test to measure the pharmacological effect of aspirin [2]. Urinary levels of 11-dehydrothromboxane B2, a thromboxane B2 metabolite, also reflect the extent of aspirin-mediated inhibition of thromboxane A2 generation [21], but the sensitivity and the specificity of this test need further evaluation, because the urinary 11-dehydrothromboxane B2 level can be influenced by recent acute thrombotic events and by non-cyclooxygenase-1-mediated pathways of thromboxane A2 synthesis.

The comparison of different laboratory methods for the detection of the effects of aspirin (arachidonic acid-induced light transmission platelet aggregation, PFA-100®, VerifyNow® aspirin cartridge, serum thromboxane B2, agonist-induced thromboxane B2 production, and urinary 11-dehydrothromboxane B_2 usually showed very weak or no correlation, indicating that they are sensitive to different parameters. Usually, the number of individuals on aspirin with residual, significant thromboxane B2 production is extremely low, while the incidence of individuals with no inhibition of platelet function measured by other tests tends to be much higher [2] (Table 1).

RESISTANCE TO ASPIRIN AND CLOPIDOGREL

Despite the proven benefits of antiplatelet therapy, many patients continue to experience thrombotic events. Many factors may influence the response of platelets to antiplatelet therapy and some patients with adequate compliance to the treatment may exhibit failure of platelet inhibition determined by *ex vivo* laboratory tests, a phenomenon termed "resistance" to antiplatelet therapy. This still poorly defined phenomenon of "drug resistance" has led to an explosion of interest, research and availability of a variety of tests that can potentially monitor an individual's response to antiplatelet therapy. The question remains as to whether these tests are clinically useful in the prediction of bleeding and/or thrombosis [23].

The incidence of true "aspirin resistance" in compliant patients is extremely low (probably <1%) [24, 25] when using specific methods, such as arachidonic acid stimulation in light transmission aggregometry or measurement of serum thromboxane B2. On the other hand, non-specific methods, such as collagen- or ADP-stimulated aggregometry, the PFA-100®, or urinary thromboxane metabolite determination yield much higher frequencies of "aspirin resistance", namely 20-30%, or even up to 65% [24, 26]. It has been observed that a significant variation of the results of light transmission platelet aggregometry and the PTA-100® exists and is presumed to have a major effect on the determination of aspirin resistance [27]. Comparison of light transmission platelet aggregometry with the VerifyNow® and PFA-100® demonstrated that aspirin non-responsiveness was not only higher in both point-of-care tests but that agreement between the tests was poor and a few patients were non-responsive by all three tests [28].

It seems that "aspirin resistance" may thus reflect poor compliance, non-specific and variable measurements of platelet function, too long an interval since the last dose and/or too low a dose, rather than inability of the drug to inhibit platelet cyclooxygenase-1 in the studied patient [29].

In contrast to aspirin, studies that used specific tests to measure the pharmacological effect of thienopyridines (e.g. VASP) showed a wide variability of responses to these drugs, with significant proportions of subjects (15-30%) being very poor responders. Inter-individual differences in the extent of metabolism of thienopyridines to their active metabolites is the most plausible mechanism for the observed inter-individual variability in platelet inhibition [24]. The proportion of "resistant" patients also varies owing to differences in the platelet function tests used and the definitions of "resistance". Studies using

ADP-induced platelet aggregation and a cut-off point at 10% inhibition have found 5-44% "resistant" patients [30] The dosage and time since dosing are important determinants, and increasing the clopidogrel maintenance dose in "low responders" has been advocated, despite the lack of clinical documentation [30, 31].

Many studies still need to be carried out to identify the ideal laboratory test to detect "resistance" to antiplatelet drugs and to answer basic questions on their clinical utility and cost-effectiveness, before monitoring of antiplatelet therapy can be recommended in clinical practice. Until then, monitoring of antiplatelet therapy should be considered for investigational purposes only [24].

CONCLUSION

Many tests of platelet function are now available for clinical use, and some of these tests have been shown to predict clinical outcomes after antiplatelet therapy. Comparison of different platelet function tests utilized for the detection of the effects of antiplatelet therapy usually show very weak or no correlation, indicating that they are sensitive to different parameters of platelet function. More studies need to be carried out to answer basic questions on the clinical utility and cost-effectiveness of laboratory monitoring of antiplatelet therapy before it can be recommended in clinical practice.

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Тестови за испитивање функције тромбоцита и резистенције на антитромбоцитну терапију

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КРАТАК САДРЖАЈ

Веома је добро утврђена клиничка ефикасност антитромбоцитне терапије (ацетилсалицилна киселина, *P2Y12* и антагонист рецептора гликопротеина *IIb/IIIa*) у спречавању артеријских догађаја код болесника с атеротромбозним обољењем. Упркос доказаним позитивним својствима антитромбоцитне терапије, многи болесници и даље доживљавају артеријске проблеме. Много чинилаца утиче на тромбоцитну реакцију након примене антитромбоцитне терапије, док се код неких болесника који добро реагују на лечење може развити смањење инхибиције тромбоцита, што се потврђује лабораторијским испитивањима *ex vivo* – феноменом званим „резистенција" на антитромбоцитну терапију. Функција тромбоцита може да се испита многим тестовима помоћу којих се одређују разни параметри активирања, излучивања, адхезије и агрегације тромбоцита. Ови тестови обухватају: светлосну трансмисију (оптичку) и агрометрију целокупне крви, дијагностичке апарате, као што су анализатори функције тромбоцита (*PFA-100®* и *VerifyNow®*), проточну цитометрију, тромбоксан *B2* у серуму и 11-дехидро-тромбоксан *B2*, метаболит тромбоксана *B2*, у урину. Остала испитивања, као што је број тромбоцита одређиван агрегацијом тромбоцита из целокупне крви, тромбоеластографија и апарати попут конусног плателетног анализатора (енгл. *cone and plate(let) analyzer*), *Platelworks* (за брзо одређивање функције тромбоцита) и анализатора стања тромбоцита, такође су коришћени у одређивању инхибиције тромбоцита антитромбоцитним лековима. Међутим, они нису у широј примени, због чега су и искуства ограничена. Потребно је извести даља истраживања како би се добили одговори на основна питања о клиничкој употреби и трошковима лабораторијског праћења антитромбоцитне терапије пре давања препоруке о њеној примени у клиничкој пракси.

Кључне речи: антитромбоцитна терапија; тестови; резистентност

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