Clinical Chemistry Trainee Council
Pearls of Laboratory Medicine
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TITLE: Testing for Non-responders of Antiplatelet Therapy

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Hello, my name is Zsuzsa Bagoly. I am an assistant professor at the University of Debrecen, Clinical Research Center in Debrecen, Hungary. Welcome to this Pearl of Laboratory Medicine on "Testing for Non-responders of Antiplatelet therapy."

Slide 2:
Platelet activation and aggregation play a key role in the pathogenesis of atherothrombosis. Antiplatelet agents are widely used and constitute a cornerstone therapy for those at high risk for atherothrombotic events. Major classes of antiplatelet drugs include aspirin, platelet $P2Y_{12}$ ADP receptor inhibitors and platelet glycoprotein IIb-IIIa inhibitors. Depending on the clinical setting, these agents might be used as monotherapy or they can be combined to achieve greater platelet inhibition. As the monitoring of GPIIb-IIIa antagonist therapy is currently not clinically recommended, in this presentation I will focus on the first two classes of platelet inhibitors.

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Aspirin has been used as an antiplatelet agent for over 30 years and has proven to be highly effective in the secondary prevention of ischemic events. This effect is achieved through the permanent inhibition of cyclooxygenase-1 (COX-1) in platelets. By acetylating a serine residue at position 529 of the COX-1 enzyme, the access of arachidonic acid to the active center of the enzyme is blocked, which prevents the generation of thromboxane A_2 (TXA_2) for the lifespan of the platelet. TXA_2 provides an amplifying signal during platelet activation in response to various platelet agonists. This means, that by inhibiting COX-1, aspirin effectively inhibits multiple pathways of platelet activation.

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Before considering the mechanisms of aspirin resistance, it is important to define the terms linked to this phenomenon. Unfortunately, in the past years "aspirin resistance" has not been unequivocally defined, which could lead to misinterpretation of laboratory test results.

The term “aspirin resistance” in general, covers three different, although not quite unrelated definitions.

1. Chemical –or true– aspirin resistance means the lack of acetylation of Ser529 in platelet COX-1 by aspirin
2. Laboratory resistance or non-responsiveness could be defined as poor response to aspirin as measured by certain laboratory tests (which may or may not be specific to COX-1 inhibition)
3. The terms: "clinical resistance", "ineffectivity", or "treatment failure" are used for cases when aspirin fails to protect the patient from acute vascular events. These terms can only be established retrospectively.

Due to the different criteria and various methods used, the reported frequency of aspirin resistance varies widely, ranging from 0 to 57% in the literature.

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Today, there is no single reliable and specific laboratory assay uniformly accepted for testing the effect of aspirin. Laboratory methods could be divided to COX-1 specific and COX-1 non-specific methods. As the degree of COX-1 blockade by aspirin are best assessed by the COX-1 specific methods, these could be recommended for the laboratory assessment of aspirin’s effect.

COX-1 specific methods are:
The measurement of thromboxane B_2 (TXB_2) from serum or from platelet rich plasma after arachidonic acid induced platelet aggregation. These methods are the most specific to indicate COX-1 activity; however, it is to be noted that serum TXB_2 measurements may be affected by non-platelet sources (mainly leukocytes). Arachidonic acid induced platelet aggregation or agglutination tests are also COX-1 specific. Light transmittance aggregometry is considered as gold standard when performing aggregation studies. If light transmittance aggregometry is not available, the Multiplate analyzer can be used, which is an impedance aggregometer and the sample is whole blood. The VerifyNow Aspirin assay is a whole blood point-of-care test, which measures arachidonic acid induced agglutination of platelets to fibrinogen-coated beads.

COX-1 non-specific methods include platelet aggregation by any agonists other than arachidonic acid: most commonly ADP, collagen, or epinephrine, irrespective of the instrument used. For the same reason, PFA-100 closure time using either collagen/ADP or collagen/epinephrine cartridge is considered non-specific to COX-1 inhibition by aspirin. Urinary 11-dehydro TXB_2 excretion measurements represent the whole body TXA_2 production and are strongly influenced by non-platelet sources as compared to TXB_2 measurements from serum or from platelet rich plasma after arachidonic acid stimulation.
It is to be noted that even if COX-1 is optimally inhibited by aspirin, the COX-1 non-specific assays could indicate high platelet reactivity. When measured by adequate COX-1 specific methods, the reported prevalence of aspirin non-responders is extremely low-or in some studies-even non-existent.

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Although “true” aspirin resistance today is thought to be a rare phenomenon, aspirin does not prevent acute vascular events in every patient. What are the possible mechanisms of aspirin “resistance” leading to aspirin treatment failure? Lack of compliance is probably the largest contributing factor of insufficient inhibition of COX-1 by aspirin. Low absorption due to drug interactions, such as proton pump inhibitors, or alternative aspirin preparations are now not believed to be important contributing factors.

Another important cause of resistance could be the interference by non-steroid anti-inflammatory drugs (e.g. ibuprofen). By reversibly binding to platelet COX-1, NSAIDs could prevent the access of aspirin to its binding site. In theory, other, probably less prominent causes of aspirin resistance could be identified: high platelet turnover in some disease states could lead to a more rapid recovery of COX-1 dependent platelet function. The expression of COX-2, which is not inhibited by low dose of aspirin, in certain pathological conditions (e.g. inflammation) might also be responsible for a diminished suppression of platelet TXB_2 production by aspirin. These mechanisms combined with a number of additional factors, independent of aspirin's effect could lead to atherothrombotic events that will be recognized as aspirin treatment failure. Additional factors could be the following: highly reactive platelets, non-platelet TXA_2 production (which might be significant in certain pathological conditions, such as inflammation) and other, platelet independent atheroembolic events.

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So what is the current opinion of aspirin resistance and what is the role of clinical laboratories when it comes to the question of testing patients for the effect of aspirin? In the past, numerous studies using different assays have demonstrated that aspirin "resistance" is associated with worse clinical outcomes. Today it is becoming evident that when tested by adequate methods and when non-compliance is ruled out, the prevalence of aspirin non-responders is very low. The so-called chemical, or "true" aspirin resistance, if it exists, is a rarity. Except for research trials, the main role of laboratories when performing aspirin resistance tests is to detect non-compliance and possible NSAID drug interference. This should be done by reliable (COX-1 dependent) methods. It should be noted that no published studies address the clinical effectiveness of altering therapy based on laboratory finding; therefore, today, it is not recommended to change therapy based only on results of laboratory tests.

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ADP plays a key role in mediating platelet function. It exerts its function through two major ADP receptors, namely $P2Y_1$ and $P2Y_{12}$, both of which are G-protein coupled receptors. $P2Y_1$ receptor is responsible for platelet shape change and the initiation of platelet aggregation, while the $P2Y_{12}$ receptor potentiates dense granule secretion and stabilizes platelet aggregation. The latter makes $P2Y_{12}$ receptor an attractive antiplatelet target. The first $P2Y_{12}$ ADP receptor inhibitors on the market were ticlopidine and clopidogrel. As ticlopidine was reported to increase the risk of thrombotic thrombocytopenic purpura and neutropenia, use of clopidogrel became more widespread. The limitations of clopidogrel lead to the development of the new-generation $P2Y_{12}$ inhibitors, namely prasugrel, ticagrelor, elinogrel, and cangrelor.

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Clopidogrel is currently the most widely used $P2Y_{12}$ ADP receptor inhibitor. It is a pro-drug, most of the absorbed clopidogrel (approx. 85-90%) is hydrolyzed to inactive metabolites, whereas the remaining 10-15% needs to be metabolized by the hepatic cytochrome P450 (CYP) system in a two-step process in order to be converted to the active metabolite. The active metabolite is an irreversible inhibitor of the $P2Y_{12}$ ADP receptor, it binds to an extracellular cysteine residue of the receptor covalently.

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How to test the effectiveness of clopidogrel? Just like in the case of aspirin, there is no single recommended assay. Tests could be divided to $P2Y_{12}$ specific and $P2Y_{12}$ non-specific methods. A common feature of $P2Y_{12}$ specific methods is that the agonist in the test is ADP in the presence of prostaglandin E_1 (PGE_1). The presence of PGE_1 eliminates the effect of $P2Y_1$ ADP receptor in the assay result. The vasodilator associated stimulated phosphoprotein (VASP) is an intracellular platelet protein. The VASP phosphorylation assay is generally performed by flow cytometry, although ELISA tests are available as well. The test is based on the fact that in the presence of PGE_1 and ADP, the extent of VASP phosphorylation is proportional to the inhibition of platelets by clopidogrel. There are a number of $P2Y_{12}$ specific assays based on platelet aggregation/agglutination as well. These include light transmittance aggregometry or if not available, the Multiplate analyzer using ADP in PGE_1 pretreated platelets. Among point-of care tests, the most widely used is the VerifyNow P2Y12 test. There is less experience with the Innovance PFA-100 P2Y test. $P2Y_{12}$ specific assays are commonly used in patients on dual antiplatelet therapy and although it is suspected that aspirin does not have an influence on the test results, studies are awaited regarding this interference. For the time being, although a $P2Y_{12}$ non-specific method, platelet aggregation induced by ADP is still considered as gold standard for testing of clopidogrel's effect. However, a major drawback of this method is that aspirin influences results, which has to be taken into consideration when the patient is on dual antiplatelet therapy. Flow cytometric methods for the measurement of GPIIb-IIIa activation or P-selectin expression and other non-$P2Y_{12}$ specific methods, e.g. thromboelastometry are not suggested for the routine testing of clopidogrel's effect. Unlike in the case of aspirin, the reported prevalence of clopidogrel non-responders is high, irrespective of the methods used.

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What are the main reasons for variable platelet inhibition by clopidogrel (or so-called high on-treatment platelet reactivity=HPR)? Of course, just as in the case of aspirin, one has to consider non-compliance as an important and frequent cause. Limited intestinal absorption, associated with a common polymorphism of the ABCB1 (P-glycoprotein) gene could also lead to diminished effect. Several lines of evidence strongly suggest that variability in the active metabolite generation is the primary explanation for clopidogrel antiplatelet response variability. Genetic variability of CYP isoenzymes, especially that of CYP2C19 leads to a low degree of active metabolite production. Interactions with other drugs that are involved in the same metabolic pathway (e.g. statins, proton pump inhibitors) could also account for low generation of active metabolite concentrations and thus poor platelet inhibition by clopidogrel.

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CYP2C19 is the predominant isoenzyme in the transformation of clopidogrel to its active metabolite. The *2 allele is the most common loss-of-function allele associated with diminished CYP2C19 activity. The *17 variant is a gain of function variant–its relation to the antiplatelet response of clopidogrel is inconclusive at this time. Carriers of the loss-of-function alleles have: lower concentrations of active metabolite while on clopidogrel therapy, less potent platelet inhibition, and elevated risk for thrombotic events. Recognizing the diminished effectiveness of clopidogrel in carriers of the loss-of-function alleles, the FDA added a warning to the drug's label in 2008. The drug label recommends considering alternative treatment or treatment strategies in patients identified as CYP2C19 poor metabolizers. It should be noted, however, that it is unclear at this time whether genotyping itself provides risk assessment

independent of its influence on platelet reactivity. For this reason, genotyping might help to determine treatment strategies in clopidogrel-naïve patients, but in those patients already on clopidogrel therapy, genotyping is suggested to be performed only in conjunction with platelet function tests.

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In the past few years, two major strategies have emerged to overcome clopidogrel resistance. Before more potent $P2Y_{12}$ inhibitors were available, intensified clopidogrel treatment seemed a solution to overcome resistance. However, clinical studies showed that simply doubling the dose is not a solution for every patient to rule out resistance and tailored treatment is far from ideal as it is cumbersome, time consuming, and most importantly, it is still ineffective in many patients despite multiple dose adjustments. It has been shown that switching to new generation $P2Y_{12}$ inhibitors is a better choice for patients with high on-treatment platelet reactivity (HPR) and especially for carriers of loss-of-function CYP2C19 alleles as compared to clopidogrel dose adjustment.

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Why are new generation $P2Y_{12}$ inhibitors more potent than clopidogrel? This table summarizes the main features of these new agents. The active metabolite of prasugrel is formed by a single-step conversion in the liver with a less rigorous dependence on CYP isoenzymes. This provides the basis of a faster, more profound, and less variable inhibition of platelet function. The direct and reversible $P2Y_{12}$ antagonists, ticagrelor, elinogrel, and cangrelor do not require metabolic activation by the liver and are associated with rapid onset and offset of platelet inhibition. Most clopidogrel resistant patients show significant platelet inhibition by prasugrel or ticagrelor; however, it has to be noted that bleeding risk might be increased with new generation agents. This is very likely to be related to the good inhibition of platelet function rather than the type of drug used.

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To conclude, in contrast to aspirin, “true” clopidogrel resistance is relatively frequent. Recent European guidelines recommend prasugrel or ticagrelor for all acute coronary syndrome patients instead of clopidogrel. For patients on clopidogrel, platelet function testing (and/or genotyping) could be recommended if the results of testing may alter management. Unfortunately, there is no clear guidance yet with respect to methodology or interpretation of results (cut-offs, etc.). According to current literature, the best available tests for $P2Y_{12}$ inhibitors are the VASP phosphorylation assay (flow cytometry), the VerifyNow $P2Y_{12}$ test, and the ADP induced platelet aggregation (or Multiplate assay).

Slide 16: References

Slide 17: Disclosures

Slide 18: Thank You from www.TraineeCouncil.org

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