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Light transmission aggregometry in the diagnosis of thrombocytopathy

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Summary

Platelet function disorders (PFD) are a rare and heterogeneous group of hemorrhagic diathesis. Comprehensive diagnostics of impaired platelet function (PLT) — the so called thrombocytopathy — requires the use of special testing. The gold standard for measurement/diagnostics of platelet function disorders is light transmission aggregometry (LTA). A platelet agonist (ADP, arachidonic acid, collagen, ristocetin, epinephrine) is added at an appropriate concentration to the sample of platelet-rich plasma. The interaction with the agonist leads to platelet activation and subsequent platelet aggregation. Aggregate formation is accompanied by a corresponding increase in light transmission. This review presents the principle of the LTA method, and a discussion of the basic panel of platelet aggregation agonists. In addition, the strengths and limitations of the LTA method are characterized and the method is compared with other alternative methods of PFD diagnostics.

Key words: platelets, thrombocytopathies, agonist, laboratory diagnostics, light transmission aggregometry, LTA

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Introduction

Platelet abnormalities include both quantitative disorders (problems with the number of platelets) as well as the less frequent — disability of the function performed by the PLT in hemostasis thrombocytopathies are a group of bleeding abnormalities caused by congenital or acquired platelet disorders that impair platelet function in the blood coagulation process. Inherited thrombocytopathies may result either from structural abnormalities in platelet granules, receptors and phospholipid membrane or from abnormal PLT activation due to inappropriate signaling. However, acquired thrombocytopathies which result from the use of certain drugs and accompany other chronic diseases (e.g. myeloproliferative neoplasms, cirrhosis, etc.) are diagnosed more frequently. Correct diagnosis of inherited thrombocytopathies is often problematic due to the high complexity of platelet defects. In addition, the diagnosis of this group of disorders is often associated with series of complex laboratory tests, which are not easily accessible and often provide ambiguous information [1].

Comprehensive diagnosis of thrombocytopathy — dysfunctional platelets (thrombocytes) — requires special testing. Depending on their availability, the panel of laboratory tests includes: microscopic evaluation of the peripheral blood smear, measurement of the occlusion time (CT,

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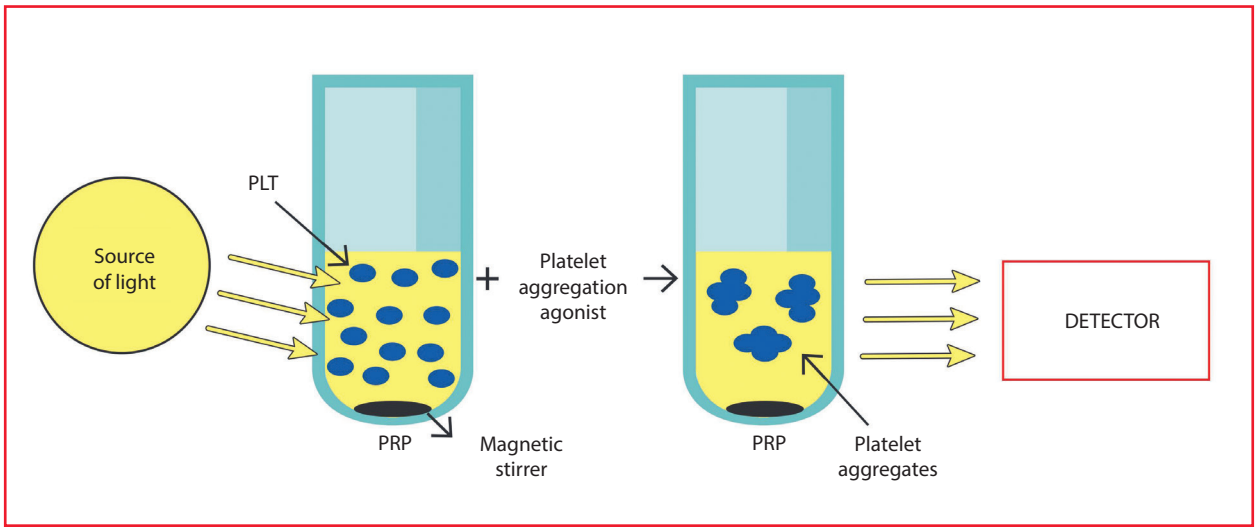

Figure 1. The mechanism of the LTA (description in the text) [6]; PLT — platelets, PRP — platelet-rich plasma

closure time) in the platelet function analyzer (PFA-100/200), analysis of the degree of PLT aggregation in the presence of aggregating factors, measurement of the concentration of intraplatelet proteins and their metabolites, flow cytometry assessing proteins (glycoproteins) on platelet surface as well as genetic tests which are becoming increasingly frequent [1].

Among these special tests, the so-called platelet aggregation tests, are classified as specific for primary hemostasis. The most common assessment method of platelet aggregation and also the gold standard for PFD diagnosis is optical aggregometry based on the measurement of visible light transmission (LTA). The method was first described by Born and O'Brien about 50 years ago, and up to date it has remained a recognized diagnostic tool for identification of platelet dysfunction (both congenital and acquired) and is still used in many specialized hemostasis laboratories [2, 3].

Principle of the light transmission aggregometry method

Light transmission aggregometry (LTA) is based on the measurement of the changes in the transmitted light which passes through two cuvettes, one of which contains autologous standard platelet-poor plasma (100% light transmittance). The other cuvette contains the tested platelet-rich plasma (PRP) sample with agonist (activator) added at a volume not exceeding 10% of the plasma sample. The observed aggregation of platelets (aggregates formation) is induced by the interaction between the agonist added to PRP and the appropriate activated membrane receptor complex (glycoprotein GP) [4]. During platelet aggregation, a number of biochemical and structural changes occur which are induced by the activation of various receptor-associated signaling pathways. Ultimately, irrespective of the mechanism, the fibrinogen receptor (glycoprotein — GPIIb/IIIa) is activated to form a platelet-fibrin network. Platelet aggregates are formed which are visible in platelet-rich plasma (Fig. 1). Aggregation of platelets makes cell suspension less turbid (decreases optical density). A minimum of 3 minutes is needed for an aggregate to form. The process is multi-stage, so the time may be extended to 5 minutes, and in some cases even to 10 minutes. Specific conditions must be ensured during the test; the tested citrate plasma sample must undergo constant mixing with a magnetic stirrer and heated to 37°C to create an environment as similar as possible to the conditions of blood vessels [5]. The test is semi-automated using aggregometers (e.g. Chrono-log Corporation) or performed on fully automated coagulation analyzers (e.g. Atellica COAG 360, Sysmex CS series, Siemens Healthneers).

Platelet aggregation curve

Formation of platelet aggregates is accompanied by higher light transmission which is related to a lower number of light-scattering molecules [1]. The output signal recorded by the detector is proportional to the constantly measured difference in light transmittance between the tested and the

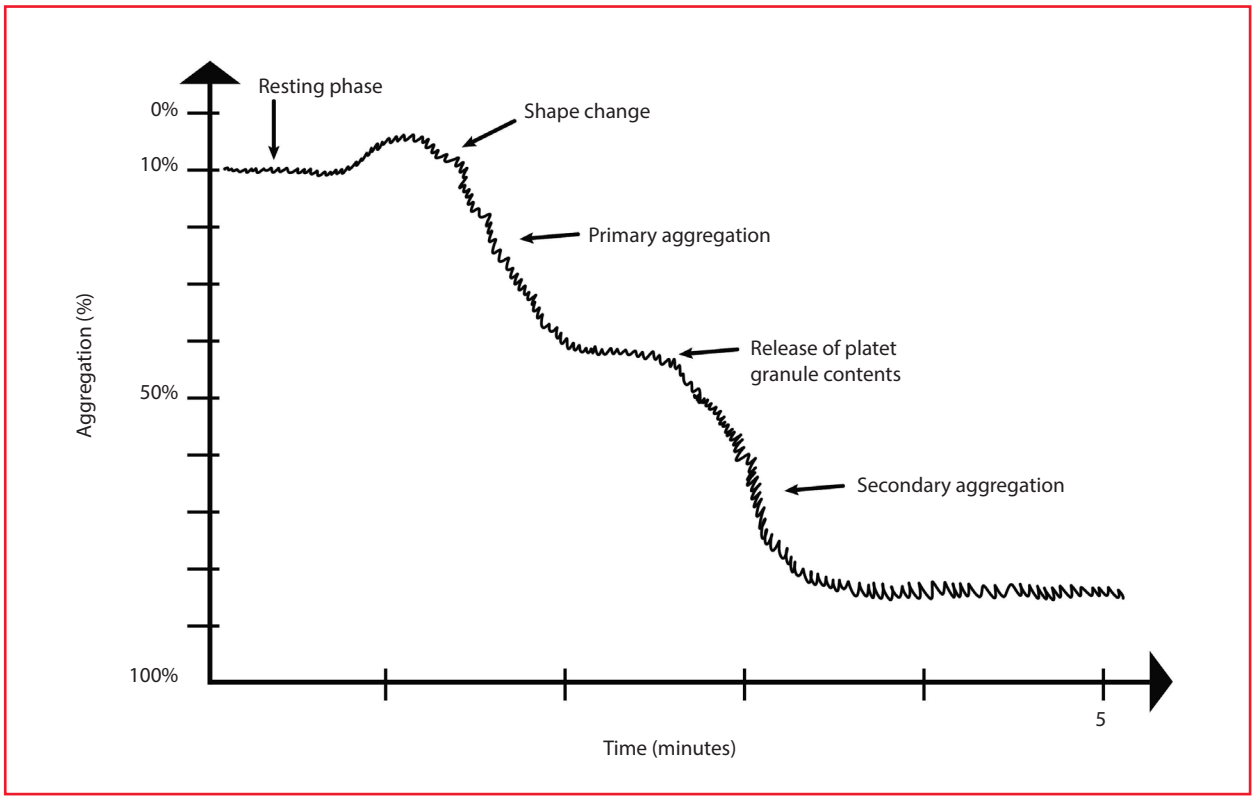

Figure 2. The aggregation curve (description in the text) [8]; PLT — platelets

reference samples. This signal is then expressed as the "aggregation percentage [%]" of PLTs, which is represented by the curve of aggregation intensity versus reaction time (Fig. 2). Reference values are usually in the range 60–90% [7]. The aggregation curve is thus an illustration of the rate of formation and the "quality" of platelet aggregates.

As result of interaction with the agonist changes in the shape of PLT occur, which may be observed in the form of a small peak on the aggregation curve. If the platelet-activating stimulus is strong enough or the process of aggregation is inhibited by the presence of antiplatelet drugs, platelets desegregation may occur. The threshold concentration of any agonist induces a change in the shape of the PLT from discoidal to spiny spheres, which is associated with a transient increase in optical density. Further platelet exposure to the agonist results in platelet adherence and aggregate formation, so more light passes through the platelet-rich plasma sample (primary aggregation). When the stimulus is strong enough, the granular content is released from platelets, and the primary aggregation is strengthened (the so called secondary aggregation occurs). For some strong agonists (e.g. arachidonic acid, thrombin, collagen) the deformation of the curve is not clearly visible, therefore only one aggregation phase is observed [8].

Platelet aggregation agonists

Under physiological conditions, collagen and von Willebrand factor (VWF) are necessary for activation and subsequent platelet adhesion and aggregation. In vitro however, a number of compounds collectively referred to as platelet aggregation agonists can be used for the same purpose. The panel of platelet aggregation agonists typically used in the diagnosis of PFD includes five compounds: ADP (adenosine diphosphate), arachidonic acid (AA), collagen, ristocetin, and epinephrine [9]. For each agonist the process of aggregation is different; the so-called aggregation profile differs as demonstrated on the time-aggregation curve. Apart from the basic panel of agonists, sometimes alternative activators from the extended panel of agonists are used and these include, among others: thrombin receptor activating peptide (TRAP) and thrombin, thromboxane A2 (TXA2), as well as its equivalent U46619 and collagen-related peptide (CRP) [9]. The choice of an agonist requires the determination of its optimal concentration (Table 1) to eliminate the influence of individual variability

Table 1. Optimum concentration values of platelet aggregation agonists [11]

PLT aggregation agonist	Optimum concentration	Concentration range	Platelet receptor
Adenosine diphosphate	2–2.5 μM	0.2–20 μM	P2Y1, P2Y12
Arachidonic acid	1 mM	0.2–2.0 mM	TXA2
Collagen	1–2 μg/ml	1.0–5.0 μg/ml	GPVI
Ristocetin	1.2 mg/ml 0.5 mg/ml	1.0–1.5 mg/ml 0.5–0.7 mg/ml	GPIb
Epinephrine	5 μM	0.5–10 μM	α2A
Extended panel agonists			
Thrombin receptor activating peptide	10 μM	10–100 μM	TXA2
U46619	1 μM	1–5 μM	PAR-1
Collagen related peptide	10 ng/ml	10–1000 ng/ml	GPVI

on the test result. In case of incorrectly selected concentration, aggregation intensity may vary and induce false aggregation profiles [10].

The use of diverse agonists allows to identify PFD of various etiology in one examination. Interpretation of LTA results should take into account the aggregation profile in response to a single activator, as well as a holistic view of the agonist panel because many signaling pathways coupled to PLT surface receptors are interconnected/interrelated. Abnormal platelet aggregation in response to a particular agonist may be suggestive of specific disorders as shown in Table 2.

Ristocetin

Ristocetin was once administered as an antibiotic in staphylococcal infections. Currently, the *in vitro* properties of ristocetin are used to induce platelet agglutination, provided VWF multimers are present in plasma. Thus, ristocetin is used to diagnose deficiencies of both VWF and its receptor — GP Ib/IX/V glycoprotein. The mechanism of ristocetin-induced platelet aggregation (RIPA) test consists in adding ristocetin to platelet-rich plasma at a standard concentration of **1.2 mg/ml.** Low Dose Ristocetin-induced platelet aggregation (LD-RIPA) test at a lower concentration of **0.5–0.7 mg/ml** is used for more accurate diagnosis [13]. Healthy individuals present a normal response to a high concentration of ristocetin and no aggregation in response to a low concentration of ristocetin. In contrast, excessive aggregation following the addition of low concentration-ristocetin to PRP is observed in two cases namely for von Willebrand disease (VWD) type 2B and platelet-type von Willebrand disease (PT-VWD). The difference between these diseases is that in type 2B VWD the defect refers to the von Willebrand factor, while in PT-VWD the abnormalities affect the platelet GPIb receptor, which results in higher affinity of PLT to VWF. In the differential diagnosis of these disorders, it may be helpful to add cryoprecipitate to the tested plasma; the platelets will agreggate only in PT-VWD. As for the other types of VWD (type 1, 2A, 2N, 2M), test with a higher concentration of ristocetin is routinely performed and the result may be either reduced or normal [14]. Moreover, no ristocetin-induced aggregation or its reduction is characteristic for congenital deficiency of the GP Ib/IX/V complex, which is the cause ofthe Bernard-Soulier syndrome (BSS). In Glanzmann's thrombasthenia, no platelet aggregation occurs in response to ristocetin, while normal aggregation with other agonists is maintained.

Collagen (Col)

Glycoprotein VI (GPVI) is the main receptor for the collagen molecule on the platelet surface. Isolated impaired aggregation (absent or reduced) in response to 2 μg/ml collagen added to platelet-rich plasma indicates abnormalities of the GPVI collagen-platelet receptor axis. The degreeof

Table 2. Agonist-related causes of the abnormal platelet response [12]

Agonist (basic panel)	Causes of incorrect PLT aggregation
Adenosine diphosphate	• P2Y12 receptor defect • Gray platelet syndrome • Glanzman's thrombasthenia myelodysplastic syndrome, acute myeloid leukemia • Cytosolic phospholipase A2 deficiency • Storage pool disease (SPD)
Arachidonic ACID	• COX-1 deficiency • Thromboxane receptor defect • Glanzman's thrombasthenia • P2Y12 receptor defect • Storage pool disease (SPD) • Myelodysplastic syndrome
Collagen	• GPVI receptor defect • P2Y12 receptor defect • Storage pool disease (SPD) • Glanzman's thrombasthenia • Cytosolic phospholipase A2 deficiency
Ristocetin	• Platelet type von Willebrand disease • Bernard-Soulier syndrome
Epinephrine	• α2-adrenergic receptor defect • Quebec platelet disorder (QPD) • Glanzman's thrombasthenia • Myelodysplastic syndrome • Storage pool disease (SPD) • Cytosolic phospholipase A2 deficiency

COX-1 — cyclooxygenase-1

collagen-mediated aggregation in congenital or acquired (autoimmune) GPVI deficiency depends on the total number of receptors that remain on platelet surface. It is also possible to extend the diagnostics of the PLT response to collagen by using alternative agonists from the extended panel such as convulxin and CRP [4]. Moreover, the lack of inhibition of aggregation after adding low-concentration collagen to PRP is also indicative of resistance to acetylsalicylic acid.

Epinephrine (EPI)

Platelet activation in response to epinephrine in a concentration of 5–10 μM added to platelet-rich plasma is the result of the interaction of this molecule with its platelet adrenergic receptor — α2A. Diagnosis of the platelet response to epinephrine may prove problematic because healthy individuals are often observed to present no aggregation after epinephrine-stimulation of platelets which may result from individual variability in the number of platelet α2A receptors [15]. An isolated, abnormal result of epinephrine-stimulated PLT aggregation is clinically insignificantunless it is accompanied by other abnormalities or unless another specific disease is suspected. An example of such a disease is the Quebec platelet disorder (QPD) with epinephrine-induced abnormal aggregation profile.

Arachidonic Acid (AA)

Arachidonic acid-induced platelet aggregation is related to its conversion by cyclooxygenase 1 (COX-1) to prostaglandin G2 and H2 and then to thromboxane A2 (a compound which belongs to the family of prostanoids). This AA metabolite has a pro-aggregating effect resulting from platelet TPα and TPβ receptor activation (G protein-coupled receptors). Moreover, platelets activation via stimulation with low concentrations of collagen or ADP is associated with the release of endogenous arachidonic acid in consequence of phospholipase enzyme-A activity, which is metabolized by COX-1 to TXA2. The inhibitor of this metabolic pathway is acetylsalicylic acid, which permanently inhibits the activity of the COX-1 enzyme [16]. The irreversibility of this process is due to the fact that

platelets do not contain a cell nucleus and are incapable of producing new, properly functioning cyclooxygenase-1. Apart from arachidonic acid, which belongs to the basic panel of platelets aggregation agonists, there are also alternative compounds (U46619 or STA2) which canbe useful in case of diagnostic difficulties. No aggregation in response to these compounds is indicative of a TXA2 defect, while normal aggregation in the absence of platelets response to arachidonic acid alone indicates an aspirin defect [1]. Impaired aggregation in response to stimulation with arachidonic acid may be related to impaired conversion of AA to TXA2. In such case, the use of an extended panel of agonists may prove helpful. On the other hand, no platelets aggregation may be due to the mutation in the TPα receptor encoding gene.

Adenosine Disphosphate (ADP)

ADP is a natural platelets aggregation agonist which activates them by interacting with specific purinergic receptors — $P2Y_1$ and $P2Y_{12}$ [17]. ADP-induced platelets stimulation (via $P2Y_1$ receptor) initially increases the concentration of calcium ions (Ca^{2+}) in the cytoplasm and leads to inhibition of adenylyl cyclase (AC). Thus, no synthesis of the cyclic adenosine monophosphate (cAMP) occurs and aggregation is uninhibited. The ADP-induced intracellular increase in Ca^{2+} is responsible for the activation of membrane calcium channels (purinergic $P2X_1$ receptor) and the influx of calcium ions from the external environment into platelets cytoplasm. Abnormal aggregation in response to ADP stimulation may either result from congenital defects of purinergic receptors or from platelets signaling disorders. Due to the fact that ADP is stored in dense granules of PLT, as a result of their activation, the content of granules (including ADP) is released to enhance aggregation and induce the second phase of the aggregation curve. Disruption of the PLT activation pathway by ADP leads to an abnormal response to other agonists.

Strengths and limitations of optical aggregometry

Optical aggregometry is a recognized tool in the diagnosis of platelets dysfunction mainly because it allows to use various groups of aggregation agonists that stimulate different mechanisms leading to abnormal platelet activation and can be detected during one test. Apart from PFD diagnosis, the LTA method has also been used for monitoring the effect of antiplatelets therapy. However further analyses are required to see if the method may be used for purposes other than scientific ones (there is no accurate correlation between the clinical phenotype and test results). Although the LTA method is considered the gold standard for PFD diagnosis, it has numerous limitations. The main disadvantage is no adequate standardization of the procedure as well as problematic quality control of conducted tests [18]. The pre-analytical stage of the LTA is rather complex therefore it is important to properly prepare the patient for the procedure as well as to properly prepare the test samples [5]. Standardization of the technical conditions aims at obtaining the most repeatable and reliable results as well as elimination of factors affecting plateletsactivation. The procedure of optical aggregometry is time-consuming which is another example of its limitations, especially when it comes to the proper preparation of platelet-rich and poor plasma samples. However it should be emphasized, that the use of automated coagulation analyzers significantly improves the procedure. It is also recommended to leave blood and PRP samples at rest for up to 30 minutes prior to centrifugation and testing of PLT-agonist interaction [19, 20]. Thus the entire time of LTA procedure is markedly prolonged but the total testing time should not exceed 4 hours (from blood collection) or 2 hours (from plasma preparation). Prolonged time of LTA procedure may leads to incorrect results — false-low aggregation due to the loss of PLT activity over time. The LTA test requires an adequate amount of plasma which involves the collection of significant volume of blood from the patient (even 20–25 ml) [7]. Furthermore, fresh blood samples are required, with no signs of hemolysis or lipemia [19]. It is not always possible to obtain such volume of blood/so many blood samples, particularly in the case of children. An important aspect of the LTA method is also the proper platelets count in the PRP. Determining the number of PLT in the PRP sample of both the patient plasma and normal plasma is an essential element of the LTA and it is an internal control of the test conditions. The PLT aggregation test is not recommended if platelet count is < 150 G/L. The LTA method cannot therefore be used to diagnose platelet dysfunction with coexistent thrombocytopenia. Too high platelets count remain a debatable issue. Dilution of PRP with autologous PPP to the desired values is an internal control of the test conditions. Standardization of the PLT count to the desired values of 200–300 G/L may result in abnormal platelet response to the agonists and it is not recommended. However, standardization of

Table 3. Comparison of assessment methods for platelet aggregation [6]

Method	Principle of the method	Platelet aggregation agonist	Test material	Test time
LTA	Turbidimetric measurement	AA, ADP, collagen, epinephrine, ristocetin (additionally extended panel agonists)	PRP	1–2 h
PFA-100/200	CT measurement	ADP, collagen, epinephrine	Whole blood	5–10 min
MEA	Impedance measurement	AA, collagen, ADP, TRAP	Whole blood	5–7 min

AA — arachidonic acid, ADP — adenosine diphosphate, CT — closure time, LTA — light transmission aggregometry, MEA — multi-electrode aggregometry, PFA-100/200 — platelet function analyzer 100/200, PRP — platelet-rich plasma, TRAP — thrombin receptor activating peptide

platelets count has been common in the LTA procedure for years [20, 21]. The procedure used in PFD diagnostics also requires proper preparation of the patient for the test, so as to eliminate the factors that may affect PLT activity [19, 20] such as: physical activity, stimulants, medications or meals, as well as the type of food or stimulants consumed [22]. The pre-analytical stage of the LTA method is considered to be the most error-prone. Therefore, only proper preparation of the patient for the examination and proper handling of the collected material will eliminate the impact of pre-analytical variables on the LTA test results. Interpretation of the outcome of platelet aggregation tests may cause difficulties, therefore the laboratory staff who analyzes the aggregation curves must be well skilled and experienced [7].

Other diagnostic methods for platelet dysfunction

Numerous limitations of LTA, have directed attention to the use of complementary diagnostic techniques for assessment of platelet function. The right choice of the diagnostic method allows either to confirm or exclude the preliminary diagnosis in the shortest possible time as well as to implement the appropriate therapy or to continue the diagnostic process.

A common screening method used to assess primary hemostasis disorders is the Closure Time (CT) test in the PFA-100 or PFA-200 analyzer (Platelet Function Analyser-100/200). The principle of the PFA method is to assess the function of platelets in whole blood, which passes through a hole in the membrane of a test cartridge coated with an appropriate aggregation agonist (collagen and epinephrine or collagen and ADP). Agonists trigger platelets activation and aggregation. When platelets aggregate enough to closes the hole and prevent blood from passing through the membrane that's the closure time. The test is extremely useful for the diagnosis of severe platelet dysfunction (Glanzman's thrombasthenia or Bernard-Soulier syndrome) but it is of limited use in the case of milder thrombocytopathies. A normal PFA result allows to exclude — with high probability — serious, congenital platelets dysfunctions [23].

Advancement in the technology of platelet dysfunction diagnosis has also contributed to the development of POCT (Point-of-Care Testing) methods for assessment of platelet aggregation. One of such methods is multiple electrode platelet aggregometry (MEA) based on the measurement of the change in electrical impedance between two electrodes when platelets aggregation is induced by an agonist. On the electrodes immersed in the blood sample, PLT aggregation occurs as a result of interaction with the agonist. The change in electrical resistance (impedance) is directly proportional to the degree of platelet aggregation [24]. Platelet aggregation is stimulated by a basic panel of aggregating factors although at different concentrations [7]. It is worth noting that the platelets response to aggregation agonists is different for LTA and for impedance aggregometry, especially in the presence of ADP and epinephrine. The measurement expressed in ohms (Ω) can be converted to arbitrary aggregation units. Unlike the LTA method, impedance aggregometry requires no time-consuming samples preparation as whole blood is used which markedly shortens the time until the result becomes available. This reduces the risk of PLT activation prior to testing. Also the required volume of blood is smaller than for the LTA method. However the limitation of impedance aggregometry r, is its dependence on the platelets count and hematocrit. The PLT aggregation test in whole blood more closely reflects the in vivo conditions in blood vessels due to the presence of PLT subpopulations and other blood morphotic elements. However, this test does not take into

account the effects of vascular endothelium and shear forces on platelets activity. Table 3 is the comparison of the above methods of assessing the ability of platelets to aggregate in response to stimulation with the appropriate activator. The impedance method is used in hemostasis laboratories less frequently than LTA; in the literature there are no extensive comparative studies with LTA or clinical experience of its usefulness in the diagnosis of platelet function disorders [7].

Another important method of PFD diagnosis is flow cytometry (FCM). The test is primarily performed to quantify (express) glycoprotein receptors, the deficiencies of which are the cause of inherited thrombocytopathies [1]. An unquestioned advantage of this method is that it enables a comprehensive assessment of platelet function, also in thrombocytopenia patients. Moreover, it requires a small volume of blood for testing [25]. Platelet GP cytometry is performed in patients with abnormal aggregation test results and/or a significant clinical history suggestive of thrombocytopathy. Therefore, FCM plays an important role in the diagnosis of Glanzmann's thrombasthenia (deficiency or lack of GPIIb/IIIa) and Bernard-Soulier syndrome (deficiency of GPIb/IX/V) [7].

Molecular tests are also becoming increasingly popular for PFD diagnosis. The use of Next Generation Sequencing (NGS) techniques allow for a comprehensive analysis of genes responsible for platelets dysfunction. So far, more than 80 genes whose mutations are responsible for the occurrence of platelet bleeding disorders have been identified, and a given pathological variant has been correlated with the presented phenotype. Identification of the causative mutation allows to confirm the diagnosis. However, due to the complex nature of thrombocytopathy, the detection of the pathogenic variant responsible for the development of the disease is sometimes problematic. In addition, it is also possible to detect numerous variants of unknown etiology (VUS, Variants Of Uncertain Clinical Significance). Due to the complex nature of platelet bleeding disorders, such variants require further analysis and continuous updating of common mutation databases in order to be considered benign or pathogenic [26]. Few laboratories perform genetic tests for platelet dysfunction, which is a significant limitation of this method. Considering the fact that platelet bleeding disorders are a very heterogeneous group of diseases, molecular tests are mainly performed in the case of well-known platelet disorders such as: Glanzmann's thrombasthenia, Bernard-Soulier syndrome or platelet type of von Willebrand disease. Molecular diagnostics of PFD is not recommended as the test of first choice. However, the exceptions are some rare disorders that are difficult to diagnose in any other way (such as QPD), as well as conditions in which there is a clinical suspicion of a specific PFD [27]. In such situations, the initial DNA analysis can significantly save time in obtaining the correct diagnosis. Therefore, NGS is an effective diagnostic tool for PFD. However, it is necessary to thoroughly understand the molecular basis of platelet bleeding disorders so that this method can be used in routine practice [28].

Summary

The LTA assay is a recognized diagnostic tool for the assessment of platelet dysfunction. However this technique r is susceptible to pre-analytical, analytical and interpretation errors as there is no standardization of the testing procedure and the procedure itself is work and time-consuming. Despite numerous attempts at standardization, the procedure still varies between laboratories, and the interpretation of data requires special know-how. Moreover, the LTA technique is available only to high-tech laboratories of hemostasis. The recent successful attempts at LTA automation using coagulation analyzers (Siemens, Sysmex CS series, Atellica) represent therefore a breakthrough in the availability of these tests. They simplify test performance and enhance the readiness of clinicians to order LTA tests [29]. Comparative studies of these analyzers with standard aggregometers have demonstrated the automated method to be highly repeatable and less time-consuming for samples processing. For better evaluation of the performance and usefulness of the automated method, it is necessary to conduct further research on these coagulation analyzers in a broader spectrum of use with alternative platelets aggregation agonists or in patients with rare congenital platelet disorders [30]. It is also worth emphasizing that the close cooperation of clinicians/hematologists with the laboratory staff is essential for proper interpretation of LTA results and accurate diagnosis of difficult thrombocytopathies.

Conflict of interest: none declared

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