Review

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Influence of different methods and anticoagulants on platelet parameter measurement

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Abstract: Platelets are the smallest and perhaps the most versatile components of human blood. Besides their role in coagulation and the maintenance of vascular integrity, they are involved in many physiological processes, ranging from immune response and leukocyte recruitment to the production of antimicrobial peptides and immune-suppressive factors like TGF- β . These versatile abilities make platelets interesting for researchers from different disciplines. However, beside profound investigation into platelets' physiological role, there is a need for correct, standardized and thus reproducible quantification of platelet parameters. Mean platelet volume (MPV) is a widespread prognostic marker for several conditions, such as, acute coronary syndrome, chronic kidney disease and liver cirrhosis. Platelet activation is regarded as a marker for inflammatory processes, for example in autoimmune diseases such as type-1 diabetes, systemic lupus erythematosus and rheumatoid arthritis. The monitoring of platelet function is relevant for patients receiving antiplatelet medication. Platelet parameter measurement is affected by the choice of *in vitro* anticoagulant, the measurement technology and the time delay after sampling. This review focuses on the pre-analytical variability that arises as a result of the use of different *in vitro* anticoagulants and analyzer technologies when determining platelet parameters, since, even approximately 180 years after the discovery of platelets, there is still no standardized procedure.

Keywords: *in vitro* anticoagulation; mean platelet volume; measurement technology; platelet activation; platelet counting; platelet function.

Introduction

From “blood granules” and “colorless beads”

It is not clear to whom the initial description of the platelet and its function is actually attributable. In 1836, Hermann Nasse described his microscopic blood observation, stating that in addition to the “blood globules,” there are also “blood granules,” which “combine to form irregular, elongated piles (flakes)” [1].

In 1865, Max Schultze described in his work *Ein heizbarer Objecttisch und seine Verwendung bei Untersuchungen des Blutes* “lumps of colorless beads” and referred to preliminary studies by his colleague Schulten from 1858, who found “white bodies in large numbers and of very different size so that the smallest had scarcely half the size of the larger blood disks, whereas the largest had probably double and three times the same” [2, 3].

In contrast to Schulten, who attributed his observation to the “puerperal fever” of his examined patient, Schultze assumed a “normal form-factor” of human blood.

In 1882, Julius Bizzozero's work *Ueber einen neuen Formbestandtheil des Blutes und dessen Rolle bei der Thrombose und der Blutgerinnung* dealt with the observations made by his colleagues and their different methods of microscopic blood examination. He was the first to recognize the role of platelets as a functional component of blood clotting, and he investigated the various substances he and his colleagues used experimentally to prevent coagulation. Some of his medical colleagues, such as Riess, rejected the idea that platelets are involved in coagulation, since one can indeed see them even before the same. Rather, they are decay products of white blood cells [4]. It was not until 1897 that William Duke recognized in the case of a young man who suffered from thrombocytopenia a correlation of measured platelet count and bleeding time. He observed that the increased platelet count after a transfusion led to a shortening of the duration of bleeding and thus realized that the platelets were apparently necessary for hemostasis. He concluded from his observations that

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thrombocytopenia is the cause of a purpura [5]. In the same year, James Homer Wright, in the context of staining experiments and microscopic observations, investigated the origin of platelets and came to the conclusion that platelets emerge by detaching from megakaryocytes of the bone marrow. He published the results of his investigations in his 1910 work “The Histogenesis of the Blood Platelets,” in which he critically examines his colleagues’ theories of platelet development [6, 7].

After their detachment from megakaryocytes, platelets are the smallest cellular constituent of human blood. Under physiological conditions, their number is about 150,000 to 450,000 per μL of blood, and the mean platelet volume (MPV) is between 5 fL and 13 fL, depending on the applied method of measurement, the *in vitro* anticoagulant and the time delay between blood sampling and measurement [6, 8–10].

***In vitro* anticoagulation**

The choice of an appropriate *in vitro* anticoagulant is a prerequisite for the correct measurement of hematological analytes and must be taken into consideration when interpreting the results. The requirements for an appropriate anticoagulant include the correct representation of the *in vivo* situation and the time-dependent stability of parameters, as there are undefined or not always controlled time periods between blood collection and measurement.

The anticoagulant of choice for blood cell counting is ethylene-diamine-tetraacetate (EDTA). Its anticoagulant properties (chelation of divalent cations, such as Ca^{2+} -ions) were already investigated in the 1940s [11], and, after its introduction to the hematological laboratory in 1951 [12], EDTA became an established anticoagulant. Since the early 1990s, EDTA has been the standard anticoagulant for the determination and counting of blood cells [13]. There are several formulations available, namely Na_2EDTA , K_2EDTA and K_3EDTA [14, 15] and although the International Council for Standardization in Hematology (ICSH) recommends the usage of K_2EDTA [13], the K_3EDTA [14, 15] and Na_2EDTA formulations (in combination with sodium fluoride for the measurement of blood glucose) [16] are still in use. Well-known phenomena are the loss of discoid shape and the time-dependent swelling of platelets, resulting in an increase of the MPV in EDTA anti-coagulated blood samples [15, 17, 18]. Thompson et al. found that the K_3EDTA formulation causes greater platelet swelling over time than the Na_2EDTA formulation [19]. Electron microscopic investigations of platelet ultrastructure revealed that platelets thereby undergo a shape

change and form pseudopods [18]. Besides the impact on MPV, another rare but relevant shortcoming of EDTA is the phenomenon known as “EDTA-dependent pseudo-thrombocytopenia” (PTCP). The first description of platelet agglutination by a serum factor in EDTA anti-coagulated blood dates to 1969 [20]. The prevalence of this *in vitro* phenomenon is reported in the literature as 0.1 to 0.21% [14, 21]. Conversely, 1.25 to 15.3% of all thrombocytopenic patients are pseudo-thrombocytopenic cases [15]. Failure to detect the presence of PTCP can lead to misdiagnosis and incorrect treatment decisions. A large number of such cases has been documented [22–24], and diagnosis is further complicated by the fact that in some cases the phenomenon is transient [25–28]. The basis of an EDTA-dependent PTCP is auto-antibodies directed against the platelet fibrinogen receptor GpIIb/IIIa , which is unmasked by EDTA [14]. Since such a phenomenon has also been described for other *in vitro* anticoagulants [29], partial combined incompatibilities occur [30–33], and the *in vitro* agglutination does not necessarily lead to a platelet count that defines as thrombocytopenia [34], the correct term should be “anticoagulant-induced platelet agglutination.”

A reliable anticoagulant for the correct determination of platelet counts in cases of anticoagulant-induced platelet agglutination is magnesium sulfate (MgSO_4) [30, 34].

Bizzozzero had already described MgSO_4 as “the best anticoagulant salt” in 1882, and it had previously been used experimentally by Hayem (1878) and Hoffman (1881) to microscopically examine blood samples on glass slides [4]. In 1909, Anton Fonio anti-coagulated capillary blood for a microscopic platelet count with a 14% MgSO_4 solution [35].

In 1986, the Japanese working group of Nakamoto successfully used MgSO_4 as an *in vitro* anticoagulant to determine the actual platelet count in cases of EDTA-induced PTCP [36]. Since the results of this study were solely published in a Japanese journal, they remained widely unrecognized. In addition, a study published in 2002 on hematological examinations of MgSO_4 anti-coagulated blood by Kondo and colleagues did not lead to a widespread use of MgSO_4 as an anticoagulant in the hematological laboratory [37]. An article by Schuff-Werner and colleagues published in 2013 that proved the correction of platelet counts in cases of EDTA-induced PTCP using MgSO_4 anti-coagulated blood collection systems [34] finally resulted in the commercial introduction of MgSO_4 as an *in vitro* anticoagulant into the laboratory routine. The diverse effects of magnesium salts on coagulation, both at the cellular and plasmatic levels, have been described in several studies [38–40]. A dose-dependent inhibition of platelet-derived thromboxane A_2 synthesis [40] and

inhibition of ADP and arachidonic acid inducible platelet aggregation [34] by Mg^{2+} were demonstrated.

Investigations of the plasmatic coagulation are routinely performed with citrate anti-coagulated blood samples [41]. As early as 1891, Pekelharing found that the effect of Ca^{2+} on the course of blood clotting can be neutralized by the addition of sodium citrate [42]. Citrate is the salt of citric acid that binds with free Ca^{2+} ions and thus removes them from the coagulation cascade. The anticoagulant effect of citrate is reversible by the addition of Ca^{2+} to the sample material [43]. When determining absolute cell counts from citrate anti-coagulated blood sampling devices, the dilution effect from the addition of liquid citrate has to be considered and the results corrected by the factor 1.1 [44]. Due to osmotic effects, a reduced platelet and erythrocyte volume in citrate anti-coagulated blood samples is observed which is not the case with EDTA anti-coagulated blood [19]. In addition to laboratory procedures, citrate anticoagulation is also used in renal replacement therapy [45].

The first description of heparin dates to 1918, when William Henry Howell and Emmett Holt devoted themselves to the identification and characterization of coagulation factors. Just two years earlier, Howell's colleague Jay McLean had extracted substances from animal livers that showed *in vitro* anticoagulant activity [46]. Howell and Holt reopened McLean's preliminary studies and succeeded in extracting a phosphatide, which was henceforth referred to as heparin because of its high content in the liver [47]. Heparin acts as an anticoagulant by multiplying the activation of antithrombin III (AT III) [48]. In laboratory medicine, heparin is used in the formulations lithium heparinate or sodium heparinate for the investigation of clinical chemistry parameters and electrolytes in blood plasma [49]. Heparin is not recommended for microscopic investigations of blood cells because it interferes with the cells' staining properties [44, 50].

In 1884, John Haycraft described in his work "On the action of a secretion obtained from medicinal leech on the coagulation of the blood" experiments that dealt with the anticoagulant properties of the saliva of leeches [51]. Hirudin is derived in particular from the leech *Hirudo medicinalis*, and acts as anticoagulant by the inhibition of thrombin. It binds with high affinity, thereby preventing the thrombin-induced conversion of fibrinogen to fibrin [52]. Since the composition and concentration of the electrolytes in the sample material are not affected, hirudin anti-coagulated blood collection systems are suitable for the investigation of platelet function by impedance aggregometry [53].

Impact of *in vitro* anticoagulation on platelet count and MPV

Since the addition of the platelet count to the automated full blood count in the 1970s, several studies have focused on the comparability and time-dependent stability of platelet counts and MPV in differently anti-coagulated whole blood samples [19, 54–58]. These studies show that platelet counts as well as MPV are affected by the choice of *in vitro* anticoagulant. Perrotta and colleagues investigated sodium citrate as an alternative to EDTA in order to enhance efficiency in hematological analysis. They concluded that citrate is suitable for blood cell counting if the dilution is corrected by a factor of 1.1. The MPV as measured in citrate samples is about 1.4 fL lower than in respective EDTA samples and the platelet counts about $10 \times 10^9/\text{L}$ less. However, they did not investigate the time-dependent stability of platelet counts and MPV [44]. Thompson and colleagues concluded that sodium citrate and heparin proved unreliable for MPV measurements due to a decrease of MPV after 8 h, whereas, in Na_2EDTA and K_3EDTA samples, after an initial increase during the first 2 h, the MPV remained stable up to 8 h. However, the MPV variations over time of all investigated anticoagulants were not more than 5–7%. The platelet counts, except in the heparin anti-coagulated samples, were similar, but, in contrast to the Na_2EDTA and K_3EDTA samples, the platelet counts in the citrate samples tended to decrease over time in some samples [19]. McShine and colleagues state that the MPV is around 6–13% lower in citrated samples as compared to the respective EDTA samples [55], which is in accordance with the observation of Dastjerdi et al., who show that the mean MPV measured from citrated samples was 0.66 fL (9%) lower than from the respective EDTA samples when measured within 1 h after sampling [56], and Mannuß et al., who found a difference of 1.32 fL (13.5%) after 180 min [8]. The observations of McShine, Dastjerdi and Mannuß are also consistent regarding the platelet count. McShine and colleagues state that in EDTA samples the platelet count became stable after 1 h, whereas the platelet count in citrate samples continued to drop about 18–30% for 4–6 h [55]. Dastjerdi and colleagues state that the mean platelet count measured from citrate samples was 36% less than from the respective EDTA samples within one hour after sampling [56] and Mannuß et al. state that the immediately measured platelet count in citrate samples is 15% lower than in the respective EDTA samples. After 180 min, the difference amounts to 26%. The platelet count in MgSO_4 samples was intermediate and remained most stable for 180 min [8].

O'Malley et al. investigated a variety of antiplatelet mixtures based on EDTA and citrate. Their results indicate that in the commercially available citrate formulation (3.2% trisodium citrate) the MPV is lower in comparison with EDTA and also increases markedly less over time [58]. Bath and colleagues show that interpreting the results of MPV measurements is further complicated by different citrate concentrations that are commercially available and cause more or less pronounced platelet swelling [54]. Taking that into account for EDTA and citrate, one can see that, besides the choice, the formulation and concentration of the respective anticoagulant also affect platelet parameters.

The time-dependent changes of MPV in EDTA and citrate anti-coagulated blood must be taken into account because elevated and decreased MPV levels are associated with a great number of pro-inflammatory diseases [59] and are therefore used as prognostic markers [60–62]. The time delay between sampling and measurement of samples can produce an artificially altered MPV, which misrepresents the *in vivo* situation. Lance and colleagues therefore recommend the MPV measurement in EDTA samples after 120 min and in citrate samples after 60 min, respectively [57].

However, standardized pre-analytic conditions for studies that evaluate MPV as potential marker are not established. Beyan and Beyan reviewed 181 studies on MPV and found that 47.5% did not state the time-delay between blood sampling and measurement and that 33.1% did not even mention the type of anticoagulant used [63]. Lancé and colleagues state that from 61 reviewed studies, only two were sufficiently standardized by stating the anti-coagulation, the time delay after sampling and the measurement method [17]. These shortcomings led to new editorial policy that proposed minimum requirements for manuscripts focusing on the association of MPV with certain conditions [64].

Alternative *in vitro* anticoagulants such as hirudin seem to be much less of a matter of interest for the measurement of platelet counts and MPV, as there are not many studies dealing with this topic. Kumura and colleagues compared EDTA and hirudin anti-coagulated blood for hematology tests and found that hirudin proved unreliable for delayed measurement of platelet counts due to complete coagulation of the samples after 4 h. If the platelet count is measured within 2 h after sampling, there is no difference in the values of the respective EDTA samples [65]. However, they used a solution containing 2 µg/mL, which is much lower than the concentration of 25 µg/mL in commercially available blood sampling devices [66]. Instead of the concentration, some authors state the antithrombotic power of the hirudin formulations in antithrombotic units (ATU). Menssen et al. investigated the

hirudin concentration required for anticoagulation and showed that a hirudin concentration of at least 200 ATU/mL is sufficient for automated blood cell counting and 300 ATU/mL prevents blood clotting for at least 24 h [67]. In samples containing 1000 ATU/mL, the difference in platelet counts between hirudinized blood and EDTA blood was, although statistically significant, small in absolute terms and thus not clinically relevant. Microscopic investigations revealed that platelet micro aggregates (PMA) occur more frequently in blood films of hirudinized blood than in the respective EDTA blood and a hirudin concentration of 400 ATU/mL and above is required to minimize this phenomenon. However, in some cases, even high concentrations up to 2000 ATU/mL could not totally prevent PMA formation [67]. These findings are confirmed by a recent communication from Chapman and Favaloro, who describe a decline of platelet count in manufacturer recommended hirudin devices (up to 60%) within 4 h after sampling and the emerging of PMA in 50% of the samples. Although respective citrate samples also show the trend to decreasing platelet counts (up to 40%), the emerging of PMA is less frequent. No PMA formation can be observed in EDTA tubes and the platelet count varied <10% over 4 h [68]. These findings and the high costs of hirudin contradict the routine application of hirudin anti-coagulated blood sampling devices for platelet counting.

Table 1 summarizes the properties and limitations of anticoagulants that are routinely applied for the measurement of platelet parameters.

Technologies for the measurement of platelet parameters

At the time when platelets were described by Bizzozero as an independent blood constituent [4], the physiological platelet count was not well-known. Bizzozero's colleague Hayem, who considered platelets to be precursors of erythrocytes and therefore called them "hematoblasts", had already made microscopic counts of neonatal, pediatric and adult blood samples, and found a physiological "hematoblast number" of, depending on the age group, 200,000 to 346,000 per µL [69]. Microscopy, or chamber counting, was the only means of platelet counting until the development of the impedance measurement method by Wallace Coulter in 1953 [70]. The automated measurement of cell counts and cell volumes is now part of the laboratory routine. For specialized questions, such as the measurement of functional parameters and the degree of activation, various methods are available, which are described in the following section.

Microscopic platelet counting according to Fonio

When platelets are counted according to Fonio, capillary blood is taken up in a drop of 14% MgSO₄ solution and a dried preparation is produced therefrom, which is then dyed according to Jenner or Giemsa. The next step is to count how many platelets are detected by the microscopic counting of 1,000 erythrocytes. The ratio of erythrocyte count to platelet count as determined by this approach allows the derivation of the platelet count based on the total number of erythrocytes [35].

Direct microscopic platelet counting according to Brecher

An advance in the microscopic platelet count (counting chamber method) was achieved in 1953 by the introduction of phase-contrast microscopy, as this method allows platelets to be more clearly distinguished from cell debris [71, 72]. Brecher’s approach to direct microscopic counting has been used since 1988 as a reference method for the manual determination of the platelet count [73]. In this case, the blood is mixed with a hypotonic ammonium oxalate solution, resulting in a lysis of the interfering erythrocytes and allowing the microscopic platelet count in a Neubauer counting chamber.

Automated platelet counting

Impedance method (Coulter principle)

The impedance measurement method developed by Wallace Coulter in 1953 [70] is based on the fact that blood cells cause a measurable increase in electrical resistance when

they pass through a measuring chamber to which an electrical voltage is applied. The extent of the increase in resistance is proportional to the volume of the passing particles. As a result, this measuring principle not only detects the number of cells but also provides volume information concerning the detected cells and thus allows a distinction of platelets from erythrocytes [71].

The device software of the XE 2100, the XE 5000 and the XN series (Sysmex, Norderstedt, Germany) sums up the results of the impedance measurement to create a histogram, which is limited by two discriminators that adapt flexibly to the position of the histogram. The lower discriminator flexibly captures particles between 2 and 6 fL, whereas the upper discriminator covers those in the range of 12–30 fL. By adapting the discriminators to the location of the platelet distribution curve, erythrocyte fragments at the upper end or cell debris at the lower end of the histogram are excluded [71, 74].

The DxH 800 (Beckman Coulter, Krefeld, Germany) identifies particles between 2 and 20 fL as platelets. The pulses are generated by three measuring cells, from each of which 64-channel histograms are created. The device software identifies the two lowest points and the highest point within the distribution curves and places a logarithmic normal distribution curve over these points. From the curve generated, covering the range from 0–70 fL, the platelet count is deduced by assigning cells with a volume of 2–20 fL to the platelet fraction [71, 75].

Scattered light optical method

An alternative approach for the identification and counting of cells is the scattered light optical method, as utilized by the Advia 120 (Siemens, Eschborn, Germany). In a first step, the blood cells are brought into a spherical shape by means of an iso-osmotic reagent

Table 1: Properties of different routinely applied *in vitro* anticoagulants.

Anticoagulant	Anticoagulant properties	Application	Positive features	Negative features
EDTA	Chelation of divalent cations, e.g. Ca ²⁺	Routine hematology and blood cell counting	Good preservation of cellular components and morphology of blood cells	Time-dependent increase of MPV and induction of platelet activation
Heparin	Activation of antithrombin III	Measuring of clinical chemistry parameters	Suitable for several platelet function tests	Interference with the staining properties of cells
Citrate	Binding of Ca ²⁺ ions	Testing of plasmatic coagulation	Applicable for multiple test settings	Lower platelet counts (up to 36%) than in EDTA
Hirudin	Specific inhibition of thrombin	Impedance aggregometry	No affection of the samples’ electrolyte composition	Time-dependent formation of PMA
MgSO ₄	Inhibition of fibrinogen-mediated aggregation	Platelet counting in cases of PTCP	Reliable platelet counts in cases of PTCP from multiple anticoagulants	Not suitable for impedance aggregometry

containing an additive called “surfactant,” and a laser diode-generated monochromatic light beam is then directed to the flow cell [75, 76]. Through the application of the Mie theory of scattered light properties, which states that the intensity of monochromatic scattered light measured at a certain angle depends on the volume of a cell and the difference in the refractive indices of cell and medium, information about the illuminated particle can be derived [76, 77]. The scattered light generated at the cell is measured by photodiodes, which are positioned at two different angles. The signal of the low-angle light scattering at 2–3° is amplified 30 times and gives information about the volume of the cell (MCV or MPV). The signal of the high-angle light scattering at 5° to 15° is amplified 12 times and reveals the refractive index (RI) of a cell and allows determining its density [78]. Thus, due to the refraction, cell fragments are distinguished from platelets [71]. Using these two parameters, the device software generates a scattergram in which the platelets are identified by their volume (1–30 fL) and their RI (1.35–1.40) [78, 79]. The information is converted into cell volume and density using the Mie theory [77]. The ability to measure even platelets of small size that would otherwise be excluded by the lower discriminators of the impedance method is an advantage of the scattered-light method. In contrast, large platelets, which are identified as erythrocytes in the impedance method corresponding to the upper discrimination, can be correctly assigned to the platelet fraction on the basis of their lower RI [71].

The CELL-DYN Sapphire (Abbott, Wiesbaden, Germany) utilizes, additional to an impedance method, the proprietary MAPSS™ technology, which is also a scattered light optical method but the technical approach differs from the Advia system. Platelets are identified by scattered light intensity at 7° and 90° angles and a scattergram is produced from this information, discriminating platelets from nonplatelet particles by floating thresholds [80].

The recently launched Alinity hq analyzer (Abbott, Wiesbaden, Germany) exclusively utilizes an improved scattered light optical MAPSS™ technology. The information from seven unique light detectors, detecting the polarized and depolarized side scatter, axial light loss and four intermediate angles of light scatter are combined with fluorescence detection and therefrom a unique signal signature of each cell is generated [81].

Flow cytometry

The utilization of flow cytometry allows the rapid and automated identification, counting and sorting of different

cell populations. The cells, suspended in a hydrodynamically focused liquid flow, pass a measuring chamber and are thereby illuminated by a monochromatic light beam (laser). Depending on their size and cell structure, the laser light is scattered at certain angles and detected by differently arranged photocells. Different cells are identified by a change in the scattering angle of the detected forward and side scattered light, respectively. These changes allow the identification of the passing cells. By further modifications of this principle, for example the use of fluorescent dyes and fluorochrome-labeled antibodies, one can gain additional information, such as the RNA/DNA content of the cell or the expression of surface markers [82, 83].

Analyzers of the XE-series, as well as the CELL-DYN Sapphire feature a fluorescence-optical counting method (PLT-O). It is activated when the analyzer runs in the “reticulocyte mode” [84, 85]. The fluorescence-optical method is based on the staining of the cellular nucleic acids with a fluorescent dye after prior partial lysis of the cell membrane. In the flow cell, the different cell populations can be differentiated by their forward scattered light and the fluorescence intensity, which is dependent on the nucleic acid content [85, 86]. Due to their larger amount of RNA, immature platelets (reticulated platelets) can be distinguished from mature platelets by this method. Since the life span of immature platelets is about 24–36 h, their proportion reflects the recent state of thrombopoiesis, allowing the distinction of decreased platelet production from increased peripheral platelet destruction [87, 88]. For the automated quantification of reticulated platelets, the analyzers of the XE-series report the immature platelet fraction (IPF) [89], whereas the Cell-Dyn Sapphire reports “reticulated platelets” (retPLT) as proportion of the respective total optical platelet count [85]. However, IPF and retPLT are essentially different methods and cannot be used interchangeably [88]. This is in accordance with the observations of Meintker and colleagues, who state that the IPF and retPLT methods show only a moderate inter-instrument correlation and have different reference ranges (retPLT: 1.0–3.8%; IPF: 0.8–7.9%). They further state that the separation of patient groups with high platelet turnover like immune thrombocytopenia from control groups is better achieved with the retPLT method [90]. IPF was shown to be useful for the evaluation of platelet recovery after chemotherapy [91], stem cell transplantation [92] and dengue [93]. Additionally, IPF was investigated as a marker for the prognosis of myelodysplastic syndromes [94] and sepsis [95]. Although reticulated platelets are routinely measured from EDTA anti-coagulated blood, Nishiyama and colleagues recommend the application of CTAD anti-coagulated blood from patients with chronic ITP [96].

However, the PLT-O method does not allow the determination of the platelet volume. The analyzer utilizes a switching algorithm to report the apparently correct platelet count. If the algorithm detects an affected platelet distribution curve (e.g., due to fragmented red blood cells) the analyzer switches to report the platelet count as measured by the fluorescence-optical method. If the fluorescence-optical method is affected (e.g., due to fragmented leukocytes), the platelet count as derived from the impedance method is reported [84]. Briggs and colleagues also report that it is somehow “widespread practice” to override the switching algorithm and solely rely on the fluorescence-optical derived platelet count. This is a critical issue, as Mannuß and colleagues demonstrated that, depending on the applied *in vitro* anticoagulant, the platelet counts of both methods may differ significantly [8].

The analyzers of the XN series (Sysmex, Norderstedt, Germany) are equipped with a fluorescence-optical channel (PLT-F), in which platelets are stained with a fluorescent RNA dye and are irradiated with a semiconductor laser beam. The resulting forward scattered light and side fluorescence intensities of each platelet are detected and the information plotted on a 2D scattergram. The PLT-F method is regarded as preferable to the PLT-O method due to its ability to discriminate apoptotic leukocytes from platelets and its more accurate estimation of platelets in thrombocytopenic samples [74, 97–100].

Since 2001, the flow cytometric approach using the monoclonal antibodies CD41 and CD61 has been the reference method for platelet counting. The so determined platelet count is set in relation to the number of erythrocytes as measured by impedance in EDTA anti-coagulated whole blood samples (RBC/Platelet Ratio Method) [101].

The CELL-DYN Sapphire is equipped with the CD61-immunoplatelet method, which is based on a fluorescein isothiocyanate conjugated monoclonal antibody directed against the platelet glycoprotein IIIa. Platelets are identified by the fluorescence channel FL1 and scattered light detection at 7° and 90° [80]. Trabuio and colleagues compared the platelet counts as measured from the CD61-immunoplatelet method with the respective counts from the CD41/CD61 reference method and found that the results of both methods correlated well [102].

Table 2 sums up the different technical approaches for the automated determination of platelet counts.

Since activated platelets show an increased expression of P-selectin (CD62p) and granulophysin (CD63) to mediate the interaction with endothelial cells and leukocytes [103, 104], reliable flow cytometric approaches for the determination of platelet activation status are the measurement of the proportion of CD62p- and CD63-positive platelets as

well as the actual formation of platelet-leukocyte complexes (as measured by platelet antigen-bearing leukocytes) [105].

Impact of analyzer technology on platelet count and MPV

As early as 1985, Towbridge and colleagues compared MPV and platelet count, as measured with an impedance-based analyzer (Coulter S Plus) and a light scattering-based analyzer (Technicon H6000) in K2-EDTA anti-coagulated blood samples. Besides the time-dependent increase of MPV by 17% (during 39 h of sample storage), as measured by impedance, they also observed a decrease in the MPV by 22% when measured by light-scattering. However, the concomitant platelet counts in both systems changed by less than 4%. Towbridge et al. concluded that as long as the procedures of MPV measurement are not standardized, the clinical value of MPV cannot be assessed [106]. Interestingly, three decades later, an approach to MPV measurement has still not been standardized [63].

In 2012, Latger-Cannard and colleagues compared impedance-based analyzers (XE 2100 and LH 750) and a light scattering-based analyzer (Advia 2120) with regard to the standardization of MPV measurement with K2-EDTA anti-coagulated samples. In accordance with Towbridge et al. [106] and Mannuß et al. [107], they report lower MPV values from light scattering-based analyzers but the difference between the MPV values as obtained by both impedance-based methods is even larger [75].

Mannuß and colleagues not only compared EDTA anti-coagulated samples but also MgSO₄ anti-coagulated samples using impedance-based (XE 5000 and DxH 800) and a light-scattering-based analyzer (Advia 120) regarding platelet count and MPV measurement. The results of their investigation show that, in EDTA anti-coagulated samples, a significantly higher MPV is measured on the XE 5000 than on the DxH 800 and the Advia 120. As mentioned previously, this phenomenon had already been reported for the previous model XE 2100D from Sysmex and also for the LH 750 from Beckman Coulter [75]. In MgSO₄ anti-coagulated samples, a lower MPV is measured than in EDTA anti-coagulated samples when performed by impedance method. However, if the measurement is performed using the scattered light method (Advia 120), the MPV measured from MgSO₄ samples is almost 1 fl higher than that from EDTA samples. This outcome is most likely due to the measuring principle of the Advia 120. The hypothesis that MgSO₄ has an impact on cell volume and

Table 2: Different methods of automated platelet counting.

Method	Technological approach	Positive features	Negative features	Analyzer
Impedance method	Particles cause an increase in electrical resistance, which is proportional to their respective volumes	Simultaneous generation of cell count and cell volume	Interference from cell debris may occur	XE series XN series LH 750, DxH 800 CELL-DYN series
Fluorescence optical method	Staining of nucleic acids / platelet granules with fluorescent dyes	Detection of giant platelets and exclusion of nonplatelet particles from the count	The measurement of platelet volumes is not possible	XE series XN series
Scattered light optical method	Detection of scattered light after irradiation of cells with monochromatic light	Exclusion from cell fragments and detection of giant platelets	Altered refractive indices may lead to wrong cell assignment	Advia series CELL-DYN series Alinity hq
Immunological method	Labeling of platelets with anti-CD61	Strong correlation with the CD41/CD61 reference method	Inappropriate in cases of Glanzmann thrombasthenia	CELL-DYN series

refractive index is supported by the observation of Francois and colleagues [108], who, when measured in MgSO_4 anti-coagulated blood, found very small ungranulated particles in the platelet diffraction pattern outside the platelet gate.

Within 24 h, the mean MPV of the EDTA samples as measured with the Advia 120 increases by 1.9 fL and by 0.7 fL, in MgSO_4 anti-coagulated samples, respectively. In contrast, when measured with the XE 5000 and DxH 800, low volume increases in the range of 0.2–0.4 fL in the EDTA samples and 0.1 fL to 0.2 fL in the MgSO_4 samples are measured. From this perspective, the impedance technology proves superior to scattered light technology for the measurement of platelet count and MPV after delayed sample processing.

The assumption that the impedance method of the XE 5000 fails to fully detect platelets in MgSO_4 anti-coagulated samples although they are obviously present is supported by the fact that the fluorescence-optical method of the XE 5000 measures significantly higher platelet counts, which differ only insignificantly from the values of the EDTA anti-coagulated samples. The situation is different with comparative measurements of platelets in citrate anti-coagulated blood. Although higher values can be measured using the fluorescence-optical method than with the impedance method, the difference to the fluorescence-optic measured values of the EDTA and MgSO_4 samples is still significant [8]. Possible reasons for this are either the dilution effect, which is not completely corrected by the factor of 1.1, or poorer staining properties of the fluorescent RET-SEARCH reagent in citrate anti-coagulated blood. However, it cannot be ruled out that platelets in citrated blood vanish

in a time-dependent manner or form smaller aggregates, as Shimizu and colleagues, who investigated the blood samples of patients with a suspected hyperaggregable state of platelets, found aggregate formation in citrate anti-coagulated samples, which was not demonstrated in the respective EDTA anti-coagulated samples [109]. It is conceivable that during sample storage, the calcium reversibly bound by citrate is released, and the aggregate formation is thus favored.

The DxH 800 shows the smallest difference in platelet counts as measured in EDTA and MgSO_4 samples, although the difference in MPV values is greatest [107]. The fact that the DxH 800 measures significantly lower MPV values in both EDTA and MgSO_4 anti-coagulated blood than the XE 5000, which is also based on the impedance measurement method, is likely due to the fact that the DxH 800 can detect platelets that are as small as 2 fL on its logarithmic normal distribution curve [110]. Furthermore, the platelet counts on the XE 5000 and DxH 800 devices remain stable over 24 h in both EDTA and MgSO_4 anti-coagulated samples, whereas the measurement on the Advia 120 device shows a marked decrease in the mean platelet counts of the tested population (around $40 \times 10^9/\text{L}$) for both anticoagulants [107].

As mentioned with regard to the observation of Francois and colleagues [108], it is quite conceivable that time-dependent alterations of the refractive indices lead to an incorrect assignment of the cells by the Advia 120 device software. Specially customized software to correct the altered refractive indices such as that used by Ahnadi and colleagues for citrate anti-coagulated samples [111] might also be relevant for MgSO_4 anti-coagulated samples.

These time-dependent alterations of the refractive index may also affect the scattered light optical platelet measurement of the CELL-DYN Sapphire and Alinity hq. Hedberg and Lehto state that the CBC parameters, as measured from EDTA anti-coagulated blood with the CELL-DYN Sapphire, proved to be stable for 24 h, except the optical platelet count [112]. For samples aged over 24 h the impedance method provided more reliable results. However, the direct comparison of the platelet counting methods that are available on the CELL-DYN Sapphire using thrombocytopenic samples revealed that the impedance method overestimates platelet counts and in some cases even fails to provide any result [80].

The novel hematology analyzer Alinity hq is not equipped with impedance technology and solely utilizes an improved optical MAPSS™ method for platelet counting [113]. In contrast to the MAPSS™ technology of the CELL-DYN Sapphire, there are three additional low angle scatter detectors for an improved separation of platelets from erythrocytes [81]. Initial performance evaluations showed strong agreement and correlation of platelet counts as measured by the optical method of the Alinity hq and the immunological method of the CELL-DYN Sapphire [81, 113]. Kwang-Sook and colleagues compared the Alinity hq with the Sysmex XN-9000 and confirm the good analytical performance but also state that all parameters were stable up to 36 h at room temperature except MPV [114]. Nevertheless, further comparisons of MAPSS™ technology with impedance methods, regarding long-term stability and the impact of different *in vitro* anticoagulants on platelet parameter measurement are required. Taken together, the results of the abovementioned studies reveal that, depending on the analyzer technique and the *in vitro* anticoagulation, the MPV measurements yield highly variable results. When considering these variables in addition to time-dependent changes, the mean MPV from a group of 61 individuals can amount to 7.7 fL or 10.8 fL [107].

Since MPV measurement is influenced by the type of anticoagulation [56], the preanalytical period [57] and the method of measurement [107] but has broadly been used as a prognostic biomarker in a growing number of studies [61, 62, 115–120], a standardized method must be found. Such standardization, in addition to the use of the same *in vitro* anticoagulant and the same measuring method, also encompasses the uniform pre-analytical treatment of the sample; that is, there should be the same time delay after the blood was taken. In their meta-analysis of such MPV studies, Lancé [17] and Beyan [63] demonstrated that such standardized conditions are generally not met, although Threatte (1984) and Towbridge (1985) had previously stated that there is a need

for a standard reference method to obtain reliable results from MPV measurements [106, 121].

Platelet activation parameters

Besides their role in coagulation, platelets are involved in inflammatory processes [122–124], for example in recruiting leukocytes to inflammatory sites at the vascular defects [125, 126]. Such recruitment is mainly realized by the formation of platelet-leukocyte aggregates (PLA) after previous platelet activation [127]. Platelet activation can be monitored by either measuring CD62p and CD63 expression or PLA formation. Increased levels of platelet activation markers and PLA are associated with liver cirrhosis [128], acute myocardial infarction [129], type 1 diabetes [130], rheumatoid arthritis [131] and systemic lupus erythematosus [132].

Several studies indicate that the time-dependent increase of platelet volume in EDTA anti-coagulated blood samples is accompanied by an increased expression of platelet activation markers P-selectin (CD62p) and granulophysin (CD63) [50, 133]. Initial experiments by Stenberg and colleagues showed that CD62p is present in unstimulated platelets in the α -granule membrane and subsequently expressed in the surface-connected open canalicular system (SCS) after thrombin stimulation during degranulation [134]. *In vivo*, CD62p mediates the binding of activated platelets to leukocytes and the vascular endothelium via its Ca^{2+} -dependent lectin domain [104, 135], thus supporting the recruitment of leukocytes to the endothelium defect [136, 137] and the formation of stable platelet aggregates [138].

Granulophysin (CD63) is a protein in the tetraspanin group and is found in, among others, the dense granules and the α -granules of platelets [139, 140], as well as the azurophilic granules of neutrophils [141] and the Weibel-Palade bodies of endothelial cells [142]. The interaction of CD63 with neutrophils leads to their activation and expression of CD11b/18 [103]. *In vivo*, binding of CD11b/18 with endothelial ICAM-1 leads to adhesion of neutrophils to the endothelial defect [143]. The fact that spontaneous platelet activation occurs in EDTA anti-coagulated blood has been known for almost 25 years [32]. In addition to its Ca^{2+} -binding properties, EDTA affects membrane fluidity and the glycoprotein structure, in particular that of Gp IIb/IIIa [144, 145]. The morphological changes of platelets as induced by EDTA are similar to those caused by the binding of fibrinogen to the Gp IIb/IIIa receptor, which was demonstrated by experiments with platelets of thrombasthenia patients in citrated plasma [144, 146]. Since the application of heparin

and EDTA anti-coagulated blood leads to spontaneous CD62p-expression, these anticoagulants are not suitable for measuring platelet activation [144]. For flow cytometric examinations of platelet activation, citrate anti-coagulated sample material and the combination of EDTA and citrate theophylline adenosine dipyridamole (EDTA-CTAD) are mainly used, as this combination causes significantly lower spontaneous platelet activation [50, 76, 111].

Macey and colleagues investigated, whether the parameter “mean platelet component” (MPC) as reported from the Advia analyzers could be a useful marker of platelet activation. MPC is mathematically derived by subtracting the RI of water (1.333) from the RI of platelets and dividing the difference by the average RI increment (0.0018 dL/g) [76]. The assumption of MPC as marker of platelet activation is, that a degranulation and subsequent surface expression of P-selectin leads to a decrease of platelet density, thus resulting in a lower MPC. Several studies showed that P-selectin expression and MPC are inversely correlated and a reduction of MPC indicates anticoagulant and thrombin induced platelet activation [50, 76, 111, 133]. Therefore, MPC as an automated approach that can be determined from routinely measured blood samples is a time-saving alternative to the flow cytometric P-selectin measurement.

Until the 2000s, the measurement of P-selectin was considered a gold standard for detecting platelet activation, but since platelets lose P-selectin after degranulation and P-selectin-expressing platelets rapidly adhere to leukocytes via PSGL-1, the measurement of PLA is a more reliable marker of platelet activation [105, 147]. In order to apply PLA as a prognostic marker, standardized and reproducible procedures are required. Several authors have proposed protocols for PLA measurement [105, 148–150], but they differ in terms of choice of anticoagulation, activation markers, sample preparation and data evaluation. For example, Harding and colleagues recommend measuring platelet-monocyte aggregates as the proportion of CD14- and CD42a-bearing cells using a direct thrombin inhibitor as the anticoagulant [149]. Nagasawa and colleagues applied citrate anti-coagulated blood samples and measured the proportion of CD41-positive monocytes, neutrophils and lymphocytes after their separation based on CD45 expression and side scatter properties [148]. Pearson et al. applied CTAD tubes and defined platelet-monocyte aggregates as cells positive for CD14 and CD61 [105]. For further insights regarding methodology and clinical implications of measuring PLA, the recent review by Finsterbusch and colleagues is recommended [151]. Bournazos and colleagues state that citrate anti-coagulation only results in partial Ca^{2+} complexation, and,

in contrast to EDTA, other divalent cations (e.g. Mg^{2+}) are not complexed. The authors observed significantly lower percentages of platelets binding to monocytes in citrate anti-coagulated samples compared with hirudin, heparin or PPACK anti-coagulated samples, and, irrespective of the anticoagulant used, the incubation of the blood sample with EDTA resulted in a decrease of PLA [152].

The interaction between platelet P-selectin and leukocyte PSGL-1 is strictly Ca^{2+} -dependent [153] and can only initiate after platelet activation and consequent granular expression of P-selectin [127, 128, 137, 154]. Several authors state in common [149, 150] that EDTA anti-coagulated samples are not suitable for measuring PLA due to Ca^{2+} chelation. Experiments with hirudin anti-coagulated blood showed that the subsequent addition of EDTA even leads to the dissociation of the platelet-leukocyte complexes [155]. This is supported by the observation that EDTA causes high levels of CD62p but low levels of PLA. However, some authors have different views and state that the Ca^{2+} -chelating properties of EDTA prevent artificial formation of PLA [151].

MgSO_4 anticoagulation does not lead to cation complexation, and the Ca^{2+} effect on P-selectin-mediated platelet binding to leukocytes [135] is supported. In addition, Mannuß and colleagues showed that MgSO_4 anticoagulation avoids *in vitro* platelet activation as effectively as citrate anticoagulation [8]. Given these findings, MgSO_4 anticoagulation could be an alternative to the conventional use of citrate for flow cytometric studies on platelet activation and platelet-leukocyte interaction.

Platelet function parameters

Light transmission aggregometry (LTA) according to Born

In 1962, Gustav Victor Rudolf Born developed a turbidimetric method to measure platelet aggregation. This approach to measurement is based on the increase in light transmission through citrated platelet-rich plasma (PRP), which occurs due to the formation of platelet aggregates after platelet stimulation with ADP, ristocetin, arachidonic acid, epinephrine or collagen [156, 157]. For further technical insights concerning LTA and additional platelet function tests, the review by Paniccia and colleagues is recommended [158]. Over the years, several protocols for the preparation of PRP have been proposed [159, 160], but the guidelines of the ISTH recommend the centrifugation of citrated blood with 200 g for 10 min [161].

Impedance aggregometry

The measuring principle of an impedance aggregometer is based on the fact that platelets in hirudinized blood are activated and aggregate on the surface of sensor electrodes after stimulation by physiological agonists such as ADP, arachidonic acid, ristocetin, thrombin receptor-activating peptide-6 (TRAP-6) or collagen. After *in vitro* activation by one of these agonists, platelets express the glycoprotein receptor GpIIb/IIIa, which enables the platelet to attach to the sensor electrodes in the measuring cell. The aggregation of platelets on the surface of the two sensor electrodes causes an increase of electrical resistance (impedance) between them. This increase of resistance is measured continuously over 6 min and reported as the dimensionless unit AUC (area under the curve) [53, 162].

The recommendations for the standardization of LTA from the SSC/ISTH state that LTA studies should be performed using sodium citrate anti-coagulated samples, buffered, at concentrations of 109 mM or 129 mM and the studies should be completed within 4 h after blood sampling [161]. Nevertheless, the application of citrated blood for platelet function tests is controversial since the Ca^{2+} -binding properties of citrate create a non-physiological milieu in which the platelets are suspended [163, 164]. Direct comparisons of citrate and heparin anti-coagulated samples using LTA as well as impedance aggregometry revealed that, as measured with both methods, the platelet function was impaired in citrate anti-coagulated samples after 24 h, whereas platelet function in heparin anti-coagulated blood was predominantly preserved [165]. However, other authors state that heparin is unsatisfactory due to *in vitro* platelet clumping and platelet adherence to the sampling device, resulting in low platelet numbers in PRP [166]. Since the Ca^{2+} -binding is regarded as disadvantageous, alternative PRP preparations for LTA have been investigated. As demonstrated by Mani and colleagues, the arachidonic acid-induced platelet aggregation was stable up to 24 h after sampling in hirudin and BAPA (benzylsulfonyl-D-Arg-Pro-4-amidinobenzylamide), whereas a significantly reduced platelet response was already observed after 12 h in citrate anti-coagulated blood [164]. BAPA, a dual inhibitor of factor Xa (FXa) and thrombin, is known to also be superior to hirudin in inhibiting thrombin generation and therefore providing a better sample stability over 48 h [167]. The problem of incomplete inhibition of thrombin formation by hirudin was confirmed by Chapman and Favaloro's observation that in some cases there is a time-dependent reduction in platelet aggregation, as measured with impedance

aggregometry, and the formation of platelet clumps in hirudin anti-coagulated whole blood occurs even within the recommended 3 h test window [68].

Kalb and colleagues compared platelet function from blood samples that were anti-coagulated with direct thrombin inhibitors (melagatran, lepirudin and argatroban), citrate or heparin using impedance aggregometry. There were no significant differences in aggregability between the direct thrombin inhibitor samples and heparin samples, whereas the aggregation in citrated samples after stimulation from most of the agonists was impaired [168]. This finding is in accordance with several studies and supports the recommendation to apply hirudin in preference to citrate anti-coagulated samples [53, 165, 169].

Kaiser and colleagues investigated the influence of *in vitro* anticoagulation, storage time and storage temperature on platelet function as measured by impedance aggregometry. They compared six *in vitro* anticoagulants and found that besides the hirudin anticoagulation, sodium heparin guarantees the longest storage time (up to 12 h for ADP tests and up to 24 h for arachidonic acid tests). Cold (4 °C) and warm (37 °C) storage temperatures had a negative effect on sample stability regardless of the applied *in vitro* anticoagulant. Therefore, the storage in sodium heparin or hirudin (concentration: 45 µg/mL) anti-coagulated samples at room temperature for no longer than 24 h is recommended [170].

When Mannuß and colleagues investigated platelet activation in MgSO_4 anti-coagulated blood, they demonstrated that the stimulation with ADP and arachidonic acid leads to a similarly high state of platelet activation (proportion of CD62p- and CD63-positive platelets) as in citrate anti-coagulated blood, but the aggregation as induced by several agonists (ADP, arachidonic acid, ristocetin, collagen and TRAP) was lower in MgSO_4 anti-coagulated blood than in the respective citrate or hirudin anti-coagulated samples [171]. This observation suggests that MgSO_4 anticoagulation has more far-reaching effects on platelets than citrate anticoagulation, which works primarily via Ca^{2+} withdrawal. Hwang and Ravn's observation that Mg^{2+} inhibits platelet aggregation in a dose-dependent manner [40, 172] was confirmed by Mannuß et al., who focused on the effects of increasing MgSO_4 concentrations in hirudin anti-coagulated blood sampling devices on impedance aggregometry [171]. The activated fibrinogen receptor Gp IIb/IIIa plays an important role in the formation of a stable platelet aggregate by recruiting further platelets via fibrinogen binding and ensuring the formation of a stable aggregate. Gawaz and colleagues showed that Mg^{2+} inhibits fibrinogen-mediated platelet aggregation

regardless of the applied agonist and reduces platelet adhesion to immobilized fibrinogen [173].

Summary and outlook

Throughout the history of platelet measurement, several *in vitro* anticoagulants have been proposed and different measurement technologies established. This review notes the pre-analytical variability that arises due to the choice of *in vitro* anticoagulation and measurement technology for the determination of platelet parameters.

It is broadly recognized that *in vitro* anticoagulation affects blood cells (e.g., platelets) in terms of structural properties [18], state of activation [174] and intracellular signaling [40]. For example, EDTA, the standard anticoagulant for platelet counting, causes a time-dependent platelet shape change with consecutive spontaneous platelet activation, and, in rare cases, the EDTA-dependent PTCP can lead to misdiagnosis. Since this phenomenon has also been described for citrate and heparin [29, 30, 32], a reliable alternative for the correct estimation of platelet counts in suspected PTCP is MgSO_4 [34].

As mentioned previously, automated hematology analyzers apply different measuring principles and algorithms to define a detected signal as a platelet or to sort it to another cell population. Several studies report that, when measuring from EDTA anti-coagulated blood, the impedance methods measure higher MPV values than the scatter-light method. However, the difference between the MPV values measured with different impedance methods may be even larger [75]. Therefore, studies on MPV and platelet counts that were performed with different analyzer technologies are poorly comparable, even if the same *in vitro* anticoagulant was applied. When performing measurements at different times after sampling from differently anti-coagulated blood samples, the results may vary even more. In order to improve the comparability of studies on MPV and platelet count measurement, a standardized study design must state the applied *in vitro* anticoagulant, the time delay after sampling and whether the measurements were performed on impedance, fluorescence optical or scatter light optical-based analyzers. Although the need for standardization of MPV measurements was identified in the early 1980s [106, 121], recent meta-analyses have revealed that the majority of studies on MPV as a biomarker lack standardized study designs [17, 63]. This led to a new editorial policy defining minimum requirements for manuscripts addressing MPV as a prognostic marker in certain conditions [64].

For measurements of platelet activation by accessing the expression of activation markers (e.g. CD62p and CD63), it is necessary to take blood samples in anticoagulants that do not cause an increased spontaneous expression of platelet activation markers but mainly leave the platelets unaffected. Therefore, the application of heparin and EDTA anti-coagulated blood is not suitable for the measurement of platelet activation [144], and the application of citrate, MgSO_4 or EDTA-CTAD anti-coagulated blood is recommended [8, 76, 111]. When choosing the approach by which to measure PLA formation, it is important to keep in mind that the formation is mainly mediated via PSGL-CD62p binding and is thus strictly Ca^{2+} -dependent. Nevertheless, the proposed protocols for the measurement of PLA formation vary in the choice of *in vitro* anticoagulant, activation markers, sample preparation and data evaluation [105, 148–150].

As for MPV studies, there is a need for a standardized study design that states the applied *in vitro* anticoagulant, flow cytometry protocol and time delay after sampling in order to facilitate the comparison of acquired data.

Although there are reports that platelet response is better maintained over 24 h in heparin anti-coagulated blood [165] and PRP from hirudin anti-coagulated blood is more sensitive to show the effects of aspirin [175, 176], the recommendation of the SSC/ISTH is to perform the measurement of platelet function by LTA from citrate anti-coagulated blood [161]. Citrate anti-coagulated blood is also applied for the measurement of platelet function using impedance aggregometry [177, 178], but, according to several studies [53, 169, 170] and the manufacturer's recommendation, hirudin anti-coagulated blood is preferable.

For all studies on platelet parameters, it is a prerequisite to adhere to recommended standardizations, prepare the study design accordingly and clearly state the study design.

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