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Platelet function testing: Update on determinant variables and permissive windows using a platelet-count-based device

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ABSTRACT

While there are various aspects of platelet biology that can be studied in the lab (*i.e.* adhesion, degranulation, integrin activation), the master test for platelet function is that which gives a measure of the platelet aggregation capacity upon stimulation with an agonist. Platelet function testing is necessary for the diagnosis of platelet disorders and the monitoring of patients receiving anti-platelet treatments. Furthermore, it becomes relevant in the quality control of platelet concentrates for transfusion purposes, especially considering the global concern about long term storage, other forms of storage (*i.e.* cryopreservation, lyophilization), and the impact of Pathogen Reduction Treatments (PRTs) on platelet performance upon transfusion. However, it has been acknowledged as technically difficult and demanding, since a fine platelet function test must be carried out under specific conditions. Still, there might be occasions that preclude the platelet function testing abiding to the gold standard requirements, thus, leaving us with the necessity to redefine which variables may condition or limit the analysis of platelet function testing. In the present manuscript, we test different variables (such as the anticoagulant used or the time elapsed since extraction) and the possibility to reconstitute blood prior to platelet function analysis. This study aims to provide windows of action at the diagnostics lab, especially when not all of the recommended procedures and conditions can be followed: for example, when a sample is sent from a long distance, when there is a limitation on blood extraction volume or when certain parameters (platelet count) preclude reliable test results.

1. Introduction

Platelets are the anucleate blood components whose main function is the maintenance of the body haemostasis [1]. In the face of vascular injury, platelets adhere to exposed substrates present at the extracellular matrix or liberated by the damaged endothelial cells. This activation leads to morphological changes, alpha and dense granule release, and the conversion of the fibrinogen receptor or αIIbß3 to a high affinity state, which allows platelet aggregation [2]. In parallel, the coagulation system is activated locally (cell-based model), to stabilize the thrombus with a resulting fibrin mesh [3]. It is increasingly evident that not only platelets and the coagulation system interplay in the maintenance of haemostasis, but also the immune system [4,5]. This interplay seems to be the basis for the other non-negligible roles of platelets in processes such as inflammation or in diseases like cancer[6–13]. Thus, platelet activation, pathway synergy and modes of action (*i.e.* degranulation, conformational changes, aggregation) are essentially the same, however, the physiological context and initial natural ligand activating platelets will determine the extent of the response, and the contribution of platelets to a particular process, *i.e.* endothelial repair, immune response, developmental separation of lymph from blood vessels, *etc.*

Taking all this into consideration, a notion arises, that platelet function testing might be relevant and demanded, not only in the diagnosis of platelet disorders, but also in the management of patients treated with anti-platelet agents, and those suffering from immunothrombosis (as it occurs during sepsis or infections such as COVID-19, due to a disbalanced immune response) [14].

While there are various aspects of platelet biology that can be studied

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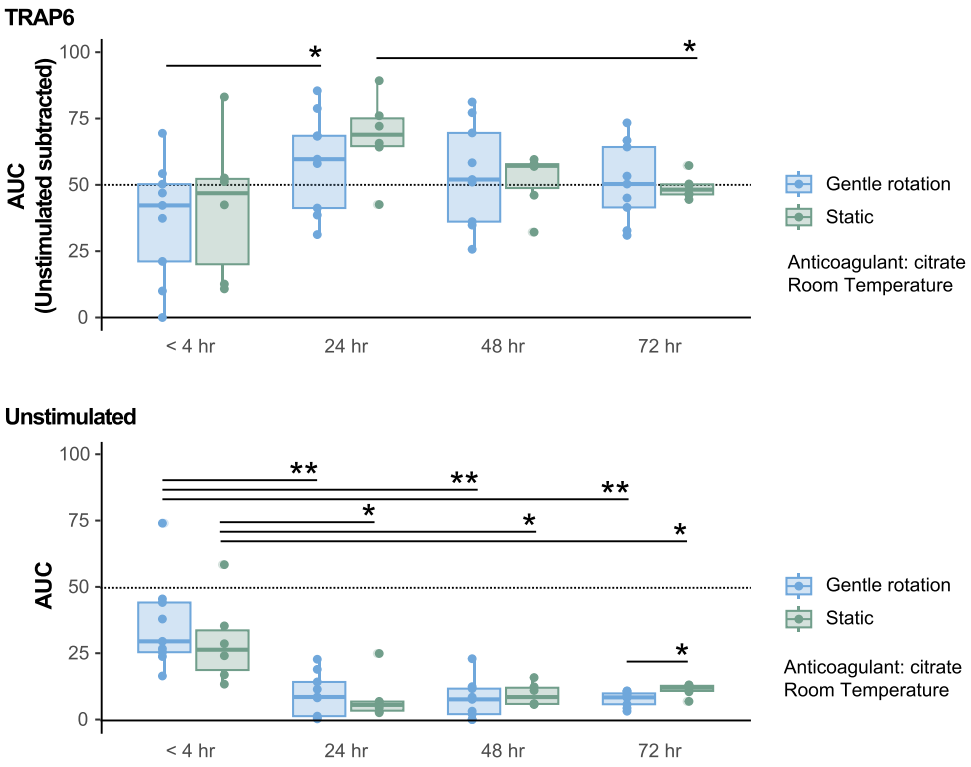

Fig. 1. Effect of the elapsed time and storage motion on platelet function testing. Box plot graphs representing the platelet aggregation capacity upon stimulation with TRAP6 (upper graph), and the basal aggregation without stimulation (lower graph) of citrate-anticoagulated blood samples kept at room temperature either static (in a tube rack) or rotating and analysed at < 4 hr, 24 hr, 48 hr and 72 hr after extraction. In the upper graph, the Area Under the Curve (AUC) of each TRAP6-induced aggregation reaction after subtraction of the AUC of the respective unstimulated control is depicted. * $p < 0.05$; * * $p < 0.01$.

in the lab (*i.e.* adhesion, degranulation, integrin activation), the master test for platelet function is that which gives a measure of the platelet aggregation capacity upon stimulation with an agonist [15,16]. In that regard, several methods have been developed and implemented; however, it has been acknowledged as technically difficult and demanding, since a fine platelet function test must be carried out under specific conditions, and by specialized and trained staff [17]. At the same time, the evolution of platelet function tests is driven to meet simplicity and point-of-care (POC) standards, but all methodologies have their own limitations [18]. Light Transmission Aggregometry (LTA) requires blood processing that might introduce artifacts; furthermore, thrombocytopenic or lipemic samples cannot be tested with this methodology. Whole blood aggregometry with impedance (IA) solved some of the limitations associated with LTA and allowed automatization (*i.e.* Multiple Electrode Aggregometry -MEA-, Multiplate) [19–21]. Flow cytometry-based platelet aggregation methods were developed that allow platelet function testing in plasma and in whole blood. Remarkably, this method, otherwise very laborious, requires minimal sample volumes, as evidenced by the fact that it allows testing platelet aggregation even from mid-term mouse embryos' blood [15,22,23]. Other devices appropriate for POC use, and based on fluidics, are the Platelet Function Analyzer (PFA-100), Any-sis or VerifyNow, being the latter rather popular devices to monitor platelet function in cardiovascular disease patients treated with anti-platelet drugs [24–26]. Of particular interest is a rather recently developed device whose technology is based on the principle that a sequential count drop of platelets in a reaction sample, is inversely proportional to their aggregation capacity [27].

Besides the actual technology employed to assay platelet function, there are some other aspects to consider which have been summarized by Harrison et al. [28]. The method of extraction is crucial, and vacutainer systems are not recommended as they may cause platelet activation. Sodium citrate 3.2% is the recommended collection anticoagulant, while EDTA or heparin are disregarded [19]. The test must always be done as soon as possible after extraction and it is recommended to store the sample at room temperature (RT) or at 37 °C until the test is performed. Some authors suggest maintaining platelets in constant (gentle) agitation until the test is performed (to maintain the sample homogenized, as decantation of blood components might lead to platelet activation at the interphase), while others advice not to [28,29].

Physiological factors might also preclude platelet function testing, such as the platelet count. Impedance and optical methods are less reproducible when platelet counts are below 50×10^9/L. For LTA (that uses PRP), the platelet count should be higher than 150×10^9/L, otherwise results deviate from the normal range, making it impossible to interpret [30]. Finally, we cannot forget one of the most important aspects of platelet function testing, the agonists employed. The most used ones in the clinic are ADP, epinephrine, collagen, arachidonic acid, ristocetin, U46619 (TXA2 analogous), TRAP6 (thrombin receptor activating peptide) and calcium ionophore A23187, which cover either the pathways susceptible to dysfunction in platelet disorders, or the pathways targeted by anti-platelet drugs. In the research laboratory other agonists are used, like snake-venom derived or chemical-based, that may target common or additional pathways [22].

In the present manuscript we test different variables affecting platelet function testing. This study aims to provide readers with windows of action at the diagnostics lab, especially when not all of the recommended procedures and conditions can be followed: for example, when a sample is sent from a long distance, when there is a limitation on blood extraction volume or when certain parameters (platelet count) preclude reliable test results.

2. Material and methods

2.1. Blood samples

This study is part of the PEDIATHROMB project, which obtained

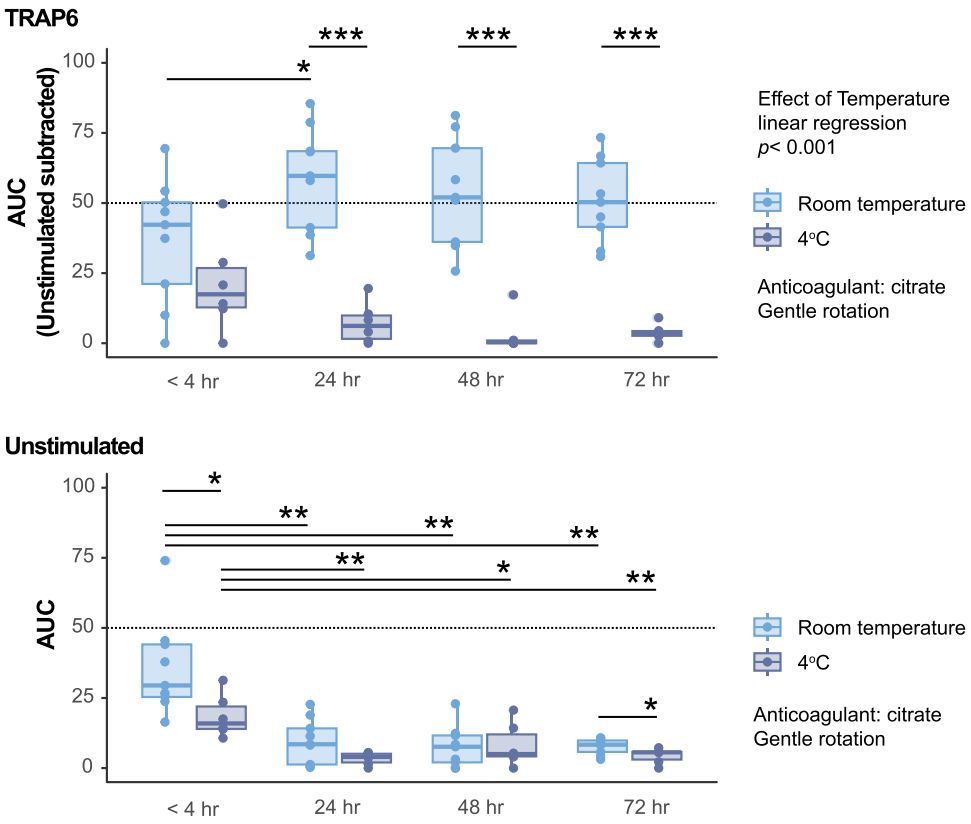

Fig. 2. Effect of storage temperature on platelet function testing. Box plot graphs representing the platelet aggregation capacity upon stimulation with TRAP6 (upper graph), and the basal aggregation without stimulation (lower graph) of citrate-anticoagulated blood samples kept rotating, either at 4 °C or at room temperature, and analysed at < 4 hr, 24 hr, 48 hr and 72 hr after extraction. In the upper graph, the Area Under the Curve (AUC) of each TRAP6-induced aggregation reaction after subtraction of the AUC of the respective unstimulated control is depicted. * $p < 0.05$; * * $p < 0.01$; * ** $p < 0.001$.

approval from the Ethics Committee of the Central University Hospital of Asturias (No. 2021/522). The study was carried out in accordance with the ethical principles of the Declaration of Helsinki and complies with the principles enshrined in the Convention on Human Rights and Biomedicine of the Council of Europe (Bioethics Convention; Oviedo). It was designed to also comply with the ICH Topic E6 Good Clinical Practice Guideline as implemented in Europe (guideline CPMP/ICH/135/95) and incorporated into the respective national laws, accordingly. All samples were obtained after informed consent, and were anonymized.

Four tubes of peripheral venous blood were collected with a vacutainer system from the antecubital vein of healthy volunteers which had not taken any drug that can affect platelet function: 3 were anticoagulated with sodium citrate, and one with EDTA. One of the citrate-anticoagulated tubes was stored static, in a tube rack, at room temperature. The rest of the tubes were kept rotating gently (not more than 250 rpm on a tube roller) at room temperature, except for a citrate-anticoagulated tube that was kept rotating at 4°C. Each series was performed at least in triplicate and samples were taken for platelet function testing at < 4 hr, 24 hr, 48 hr and 72 hr.

2.2. Preparation of thrombocytopenic samples

Citrate-anticoagulated blood was used for these assays. Each sample was divided into two aliquots of 1.5 ml in Eppendorf tubes (#0-thrombocytopenic and #0-control). Both aliquots underwent the same processing, but one aliquot was reconstituted with a reduced number of platelets (approximately 50%, 25% or 10%) to generate a thrombocytopenic sample, while the other aliquot was reconstituted without depleting platelets. In brief, #0 aliquots were centrifuged at 1000 rpm for 5 min to separate the platelet rich plasma (PRP) which was transferred to a clean Eppendorf tube (#1-thombocytopenic and #1-control, respectively). A given volume of PRP (50%, 75% or 90%) was transferred to another Eppendorf (#2-thrombocytopenic and #2-control) and centrifuged at 2000 rpm for 3 min. The supernatant (platelet-depleted plasma) was transferred to the PRP sample of origin (#1-thrombocytopenic), and after gentle mixing was then added to reconstitute the #0-thrombocytopenic blood sample. In parallel, the plasma and platelet pellet fraction of the #2-control tube was gently mixed again, transferred back to the #1-control sample of origin, and used to reconstitute the #0-control blood sample after gentle mixing. Thrombocytopenic samples were customized from healthy donor samples, which served as paired controls of normal platelet counts. All customizations and respective controls were done in triplicate from independent donors.

2.3. Aggregation Assay

Platelet aggregation was analysed using a PL-12 analyser (SINNOWA Co, Nanjing, China). This device measures platelet aggregation applying the method of sequential platelet counting through impedance technology, recording the number of free platelets in the sample (300 µL) before and after adding an agonist with a fixed time interval throughout the reaction (6 measurements, every 75 s approximately). The agonist is added after the second measurement. The platelet counts are then plotted inversely, setting the start to 0, and the Area Under the Curve (AUC) is calculated for every reaction. For every condition and time of analysis we measured the platelet aggregation with several agonists as indicated, and in the absence of agonist (unstimulated). The agonists used were the following: phorbol 12-myristate 13-acetate or PMA (10 µg/ml, Sigma-Aldrich); convulxin or CVX (6,25 ng/µL, Enzo Biochem); ristocetin (10 mg/ml, Sigma-Aldrich); collagen (1 mg/ml, Sigma-

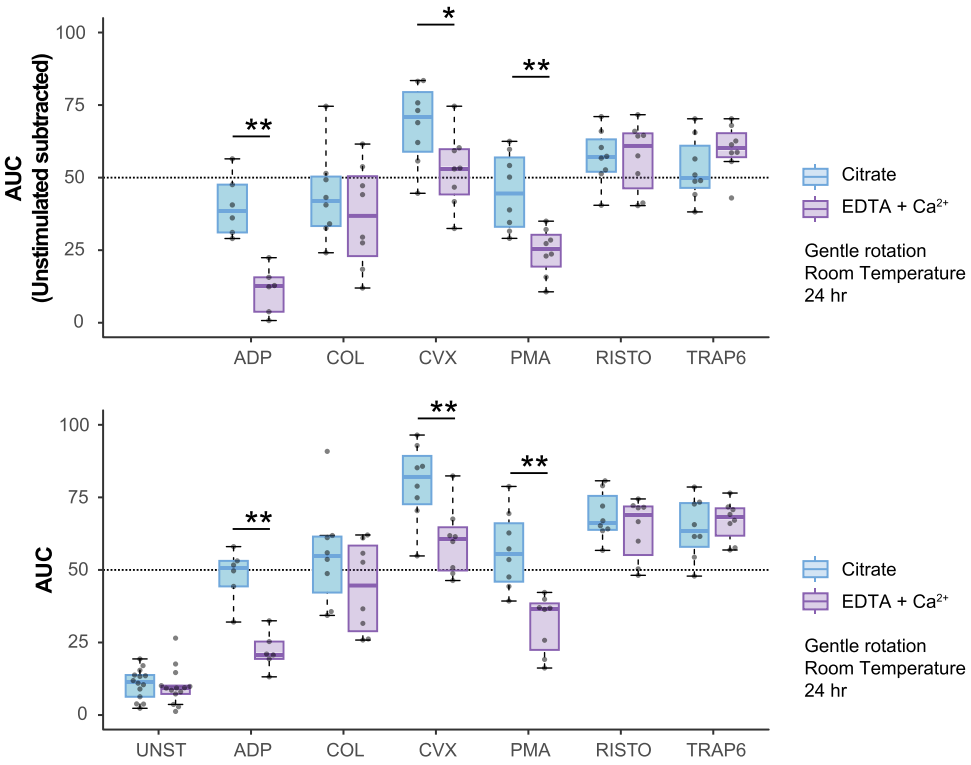

Fig. 3. Effect of the anticoagulant and agonist used on platelet function testing 24 hr after extraction. Box plot graphs representing the platelet aggregation capacity upon stimulation with a battery of agonists of citrate- or EDTA-anticoagulated blood samples kept rotating at room temperature and analysed 24 hr after extraction. In the upper graph, the Area Under the Curve (AUC) of each agonist-induced aggregation reaction after subtraction of the AUC of the respective unstimulated control is depicted. The lower graph shows non-subtracted AUCs. UNST, unstimulated; COL, collagen; CVX, convulxin; RISTO, Ristocetin. Paired T-tests were employed. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Aldrich); Thrombin Receptor Activator Peptide 6 or TRAP6 (10 mM, Sigma-Aldrich); and Adenosine Diphosphate (10μmol/L, Multiplate® ADPtest, Roche). EDTA tubes were reconstituted adding 10 μL of $CaCl_2$ 200 mM prior reaction initiation.

2.4. Statistical analysis

The statistical analysis was performed in RStudio version 4.1.1 and Excel. The AUC of all samples was ranked within a 1–100 scale. Unstimulated samples were analysed and plotted without further processing. Stimulated samples were analysed and plotted after subtraction of respective unstimulated reactions. We employed the Student's t-test (paired when indicated) or multiple linear regression. Box plot graphs were obtained with ggplot2 packages [31,32] and Adobe Illustrator was used for designing the figures.

3. Results

3.1. Effect of the elapsed time and storage motion on platelet function testing

We compared the *in vitro* platelet aggregation capacity in whole blood citrate-anticoagulated samples stimulated with TRAP6 at different timepoints after extraction and stored either static (in a tube rack) or rotating (tube roller, gentle rotation) at room temperature (Fig. 1). While no difference was observed between samples stored either static or in motion at each timepoint measured (<4 hr, 24 hr, 48 hr and 72 hr), the platelet performance upon stimulation with TRAP6 improved when measured 24 hr after extraction, compared to that measured < 4 hr after extraction, on samples that were maintained rotating. Furthermore, on samples maintained static, the 24 hr performance peak declines significantly at 72 hr after extraction, while it is maintained when samples are kept rotating. Of note, the basal aggregation without stimulus is significantly higher in samples measured within 4 hr after extraction, which explains the lower performance at that timepoint (subtracted data). These results support the notion that platelet function testing is possible beyond 4 hr after extraction (having TRAP6 as agonist), and that, while not determinant, maintaining the blood sample homogenised by gentle mixing or rotation, seems beneficial.

3.2. Effect of storage temperature on platelet function testing

Next, we compared the *in vitro* platelet aggregation capacity in whole blood citrate-anticoagulated samples stimulated with TRAP6 at different timepoints after extraction and stored rotating either at 4 °C or at room temperature (Fig. 2). As previously reported and widely known, storage of blood samples at low temperatures activates platelets [33], rendering the platelets unable to respond to TRAP6, something that was evident when the tests were performed > 24 hr after extraction. Although not reaching significance, the negative effect of low temperatures on platelet performance was observed on samples tested within 4 hr after extraction. As shown in Fig. 1, basal aggregation was higher in samples measured < 4 hr after extraction. Interestingly, at this timepoint, samples stored at low temperatures also had reduced basal aggregation. These results support the notion that platelet function testing may not provide reliable results when samples have been kept, stored or transported at low temperatures.

3.3. Effect of the anticoagulant and agonist used on platelet function testing 24 hr after extraction

We next compared the *in vitro* platelet aggregation capacity in whole blood citrate-or EDTA-anticoagulated samples stimulated with a battery of agonists 24 hr after extraction and stored rotating at room

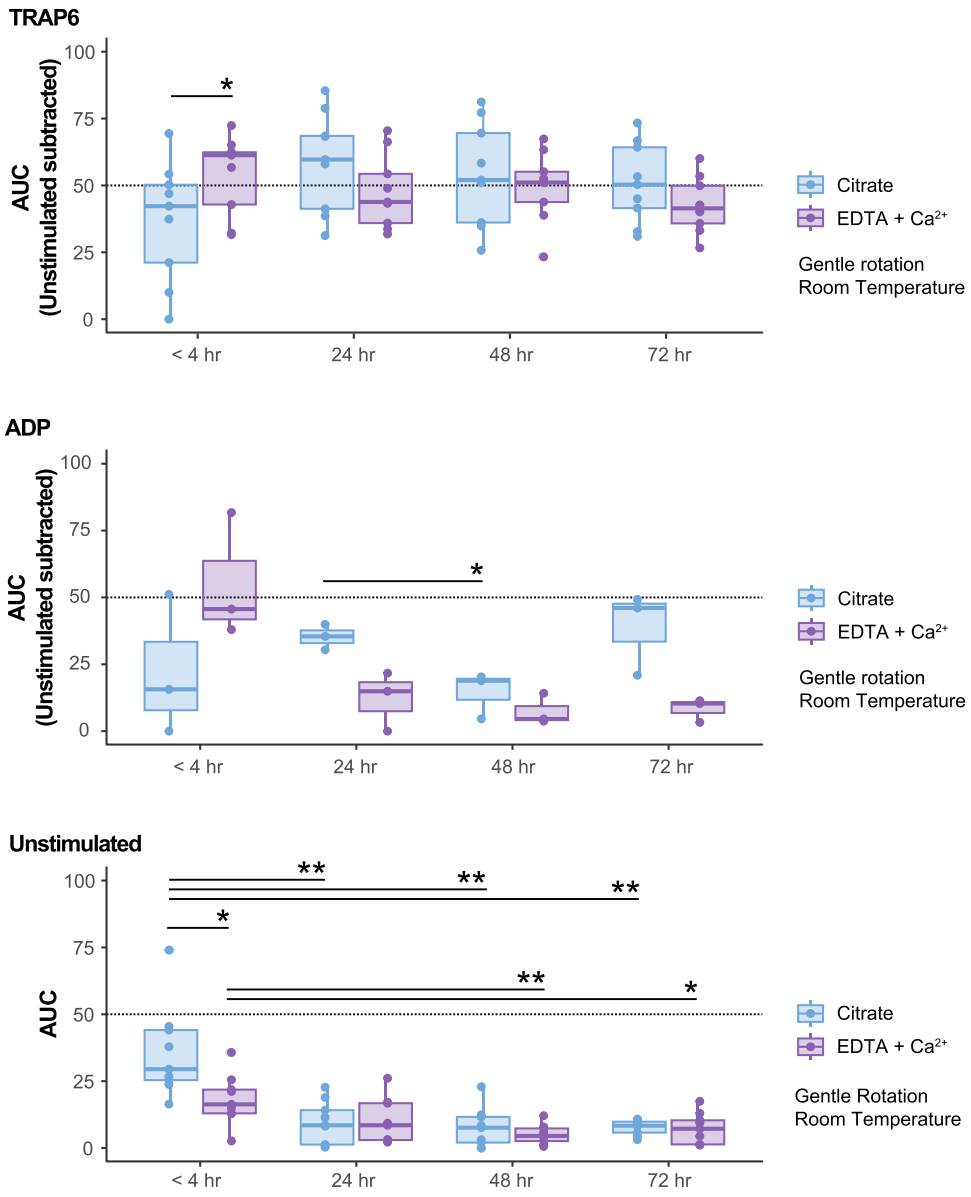

Fig. 4. Effect of the anticoagulant and agonist used on platelet function testing at different timepoints after extraction. Box plot graphs representing the platelet aggregation capacity upon stimulation with TRAP6 or ADP (upper graphs), and the basal aggregation without stimulation (lower graph) of citrate- or EDTA-anticoagulated blood samples kept rotating at room temperature and analysed at < 4 hr, 24 hr, 48 hr and 72 hr after extraction. In the upper graphs, the Area Under the Curve (AUC) of each TRAP6- or ADP-induced aggregation reaction after subtraction of the AUC of the respective unstimulated control is depicted. * $p < 0.05$; ** $p < 0.01$.

temperature (Fig. 3). This timepoint was chosen because the basal aggregation without a given stimulus (agonist) is minimal. EDTA-anticoagulated samples were supplemented with Ca^{2+} just before reaction initiation. The platelet responses upon ADP stimulation were largely affected when using EDTA as anticoagulant, and supplementation with Ca^{2+} at the concentration indicated in the Materials & Methods section was not sufficient to reach maximum platelet aggregation. The responses towards PMA and convulxin stimulation were not as severely affected. On the other hand, the responses towards stimulation with the other agonists used, *i.e.* collagen, ristocetin and TRAP6, were anticoagulant-independent. Our data shows that it is possible to use EDTA-anticoagulated samples, supplemented with Ca^{2+}, for platelet function testing. However, stimulation with ADP, convulxin and PMA might require higher concentrations of Ca^{2+}; caution and optimization should be considered when using certain agonists.

3.4. *Effect of the anticoagulant and agonist used on platelet function testing at different timepoints after extraction*

In order to gain insight on the differences observed with certain agonists, we next compared the *in vitro* platelet aggregation capacity in whole blood citrate-or EDTA-anticoagulated samples stimulated with either TRAP6 or ADP at different timepoints after extraction and stored rotating at room temperature (Fig. 4). As indicated above, EDTA-anticoagulated samples were supplemented with Ca^{2+} just before reaction initiation. Our data shows that platelet performance is maintained at all timepoints measured, with minimal fluctuations, when using TRAP6 as an agonist. Interestingly, the aggregation capacity observed within 4 hr after extraction is higher in samples anticoagulated in EDTA. This is in part due to the fact that basal aggregation without stimulus is better contained (*i.e.* lower) in samples anticoagulated with EDTA. However, and as previously known, other agonists (such as ADP), require rapid testing for reliable results [28]. Importantly, EDTA seems a

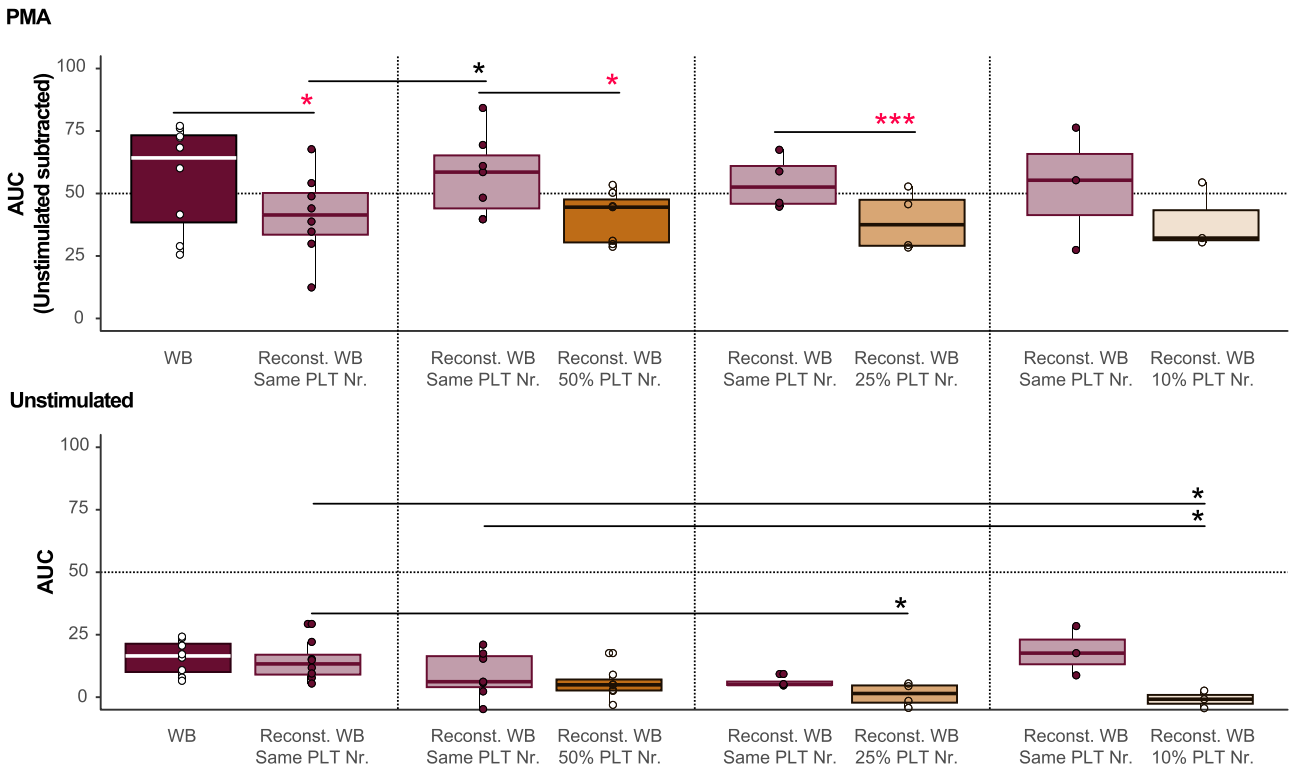

Fig. 5. Effect of whole blood reconstitution and platelet count on platelet function testing. Box plot graphs representing the platelet aggregation capacity upon stimulation with PMA (upper graph), and the basal aggregation without stimulation (lower graph) of citrate-anticoagulated blood samples kept rotating at room temperature and analysed at 24 hr after extraction. In the upper graph, the Area Under the Curve (AUC) of each PMA-induced aggregation reaction after subtraction of the AUC of the respective unstimulated control is depicted. Samples were reconstituted as indicated, either maintaining the same platelet count, or reducing it to 50%, 25% or 10% approximately. Unprocessed whole blood samples serve as reference control. Each reconstitution procedure performed to customize a thrombocytopenic sample (50%, 25% or 10% of original platelet count) has its own paired control, a sample that has followed the same reconstitution process, without depleting platelets. WB, whole blood; Reconst., reconstituted; PLT, platelet; Nr., number. * $p < 0.05$; *** $p < 0.001$. Paired T-tests significant results are indicated in red.

better anticoagulant choice as measured within 4 hr after extraction, with a tendency for better responses upon ADP stimulation. However, higher elapsed times induce a decline in ADP-induced aggregation responses having EDTA as anticoagulant, or unreliable fluctuations having citrate as anticoagulant.

3.5. Effect of whole blood reconstitution and platelet count on platelet function testing

We next aimed at evaluating whether platelet function could be studied in samples after processing, especially when reconstitution is required to customize, for example, a platelet count range. We measured platelet function on citrate-anticoagulated samples that were kept rotating at room temperature for 24 hr. Whole blood reconstitution was done as described in the Materials & Methods section. While there were significant differences comparing reconstituted samples with customized thrombocytopenia and respective controls, those differences were not of great magnitude (Fig. 5). Of note, basal aggregation was lower in customized thrombocytopenic samples. Results suggest that platelet function can be efficiently analysed even in reconstituted samples, and that platelet aggregation is still measurable in samples that were reconstituted with 10% of the original platelet count (approximately), with the methodology employed.

4. Discussion

In the present manuscript, we have studied the effect of several variables on platelet function testing using a platelet-count-based device. There is great concern on how to perform a platelet function test, [16] and a series of recommendations on the ideal conditions for platelet function testing are widely known and assumed by experts in the field [28]. However, the sum of all recommendations might not always be possible to abide, either due to human error, technical issues or particularities in each case, which cannot be controlled from the extraction moment until the sample is processed and/or tested at the diagnostics lab. Therefore, we aimed at revisiting some of the variables that could exert an influence on platelet function testing results.

All samples were obtained using a vacutainer system. We observed basal aggregation without stimulus in all samples measured < 4 hr after extraction, while this phenomenon was not observed 24 hr after extraction and beyond (Figs. 1–2 and 4). The vacutainer extraction method is potentially the reason behind the platelet hyperreactivity observed 4 hr after extraction. Our results indicate that the use of a vacutainer method for extraction is compatible with platelet function testing, however, it is advisable to let the samples rest for a longer period. Surprisingly, there might be fewer agonists than anticipated that should not be considered for testing beyond a day after extraction (such as ADP), as our results suggest that most of the agonists might be employed without compromising results (Figs. 3–4). Still, and as a consequence, certain analyses should be performed close to the extraction moment. On the one hand, monitoring of platelet function on samples from patients receiving anti-platelet drugs might need rapid testing, as the relevant agonists, such as ADP, require platelets as intact as possible. On the other hand, other thrombopathies might be evaluated beyond the day of extraction, which would allow the use of samples that need to travel to the reference diagnostics lab.

Additionally, even in these particular cases, the use of EDTA as anticoagulant seems beneficial for achieving maximum platelet

responses. Basal aggregation without stimulus is lower in EDTA-anticoagulated samples, and platelet responses upon given agonists (TRAP6 and data not shown) is maintained constant through the time-points of analysis, even 3 days after extraction (Figs. 3–4). While there are some Ca^{2+} dependent agonists, and there is room for standardization, the fact that EDTA can be used as anticoagulant for platelet function testing, opens the possibility to reuse samples for distinct analytical purposes (complete blood count and platelet function testing, for example). This would be advantageous in neonates, since the blood extraction volume is often limited [34]. This result is promising since it allows us to break the paradigm that platelet aggregation should only be performed in citrate-anticoagulated samples.

On the other hand, and as globally known, blood samples should never be stored (or transported) cold when a platelet function testing is intended. Low temperatures affect platelet performance, and we have proven this notion with samples stimulated with TRAP6. The idea that warming up samples might "rescue" platelet performance is a debatable argument, and we were not able to observe this phenomenon (data not shown). Of note, we did not observe a deleterious effect of maintaining samples static, related to platelet performance in our aggregation tests. However, our results indicate that maintaining the sample homogenised by gentle rotation might be beneficial.

While this study does not cover all options and combinations of variables that may affect platelet function testing, it provides a new perspective to clinicians and researchers. Platelet function testing might be possible in samples anticoagulated with EDTA, supplementing with Ca^{2+} before the reaction starts, and it can also be performed up to 3 days after blood extraction (with cautiousness on the agonist of choice, and the purpose of the platelet function test). Lastly, tailoring of blood components by reconstituting a sample, is also compatible with platelet function testing, and some methods might allow the study of thrombocytopenic samples with higher sensitivity than others (platelet-count-or flow-cytometry-based *vs* LTA, for example) [22,35]. Future studies will aim at better delineating permissive windows on platelet function testing for facilitating and for adding simplicity to the complexity of platelet function *in vitro* testing, either for clinical or research purposes.

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Author contributions

LG conceived and designed the study and wrote the manuscript. PVF performed experiments, analysed results and wrote the manuscript. SC, SM and ID performed experiments and analysed results. AB coordinated and supervised blood extraction from healthy donors and revised the manuscript.

Declaration of interest

LG is CSO and Co-Founder of Platelet Biotechnologies S.L. (PlaBiTe). The rest of authors declare they have no conflict of interest.

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