

Variables influencing the *in vitro* measurement of spontaneous aggregation of human platelets

Spontaneous platelet aggregation (SPA), defined as the ability of platelets to aggregate *in vitro* in an aggregometer in the absence of exogenous agonists, has been associated with an increased risk of cardiovascular events among healthy subjects¹ and a poor prognosis in acute coronary syndrome (ACS) patients,² suggesting its potential as an easily available marker of platelet hyper-reactivity that might predispose individuals to cardiovascular events.^{1,3-6} However, its clinical application has been limited by concerns about its dependency on the following potential artefacts: 1) untoward *in vitro* platelet activation during inaccurate blood sample handling could be responsible for platelet hyper-reactivity, favoring the formation of spontaneous platelet aggregates when samples are stirred in the aggregometer; 2) SPA is only observed in citrate-anticoagulated blood samples and not in thrombin inhibiting anticoagulants (such as hirudin), suggesting that SPA depends on the known platelet hyper-reactivity at low Ca^{2+} ,^{7,8} and/or platelet stimulation by trace amounts of thrombin formed in citrate-anticoagulated samples.^{7,8}

We measured SPA using both the traditional light transmission aggregometry (LTA: Chrono-log 560) and the more sensitive optical density fluctuation aggregometry (ODFA: ALAT-2, Biola Scientific), which can detect microaggregates of 2-3 platelets. We recruited 100 participants (median age: 66 years [range: 18-94]; 50 females) (*Online Supplementary Table S1*) without overt cardiovascular, metabolic, or inflammatory disease. This sample size, consistent with previous published studies on the same topic,⁹ provided adequate statistical power to detect significant differences in SPA across anticoagulants, measurement methods, and participant characteristics such as age, sex, and platelet count. Participants were allowed to consume a light breakfast at home, and abstained from medications affecting platelet function for at least ten days and from smoking for at least 30 minutes (min) prior to sampling.¹⁰ The study received ethical approval and was conducted in accordance with the Declaration of Helsinki.

Blood collection and platelet-rich plasma (PRP) preparation followed the International Society on Thrombosis and Haemostasis (ISTH) recommendations.¹⁰ Briefly, venous blood samples were collected using a 21-gauge butterfly needle without tourniquet, between 9:30 and 10:30 a.m. to minimize circadian variability in platelet function. The first 4 mL of blood were discarded and subsequent samples were drawn into tubes containing either trisodium citrate (109 mmol/L, 1:9; v:v) or hirudin (525 ATU/mL, final concentration; Sartsted). In a subset of 17 subjects, additional samples were collected using a mixture of both anticoagulants in the same tube. Samples

were mixed and allowed to rest at room temperature (RT) for 15 min before processing. Samples were then centrifuged at 200×g at RT for 10 min to obtain PRP, which was allowed to rest for 15 min before testing.^{10,11} Platelet-poor plasma (PPP) was prepared by further centrifugation at 1400×g for 15 min. Platelet counts in PRP were not adjusted to a standardized value using autologous PPP to avoid potential artefactual effects on platelet aggregation.^{10,12} To test the effects of preventing TxA_2 synthesis at low plasma Ca^{2+} levels,⁷ 15 μL of saline or aspirin (100 μM final concentration; Flectadol) was added to 985 μL of citrate-PRP and incubated at RT for 10 min before testing.

Platelet-poor plasma (for LTA) or distilled water (for ODFA, as per manufacturer's instructions) was used to set 100% light transmission (LT). PRP (250 μL for LTA, 300 μL for ODFA) was incubated in the aggregometer at 37°C for 3 min without stirring and then stirred at 1,000 rpm for 6 min without added agonists. SPA was expressed as the percentage of maximum LT. ODFA additionally provided the mean aggregate size in relative units (RU). All tests were completed within 2 hours of blood collection.

Spontaneous platelet aggregation was detected in the majority of citrate-PRP samples by both LTA and ODFA (Figure 1). The median extent of LT was significantly higher with LTA compared to ODFA, likely due to the fact that, instead of PPP, the more light-transparent water was used to set 100% LT in ODFA. LTA failed to detect SPA in hirudin-PRP samples, except for 4 subjects, while ODFA detected SPA in most hirudin-PRP samples (Figure 1). Comparable SPA extents were observed in PRP anticoagulated with citrate alone and with citrate plus hirudin (2.1% [1.5-4.3] vs. 2.0% [1.1-4.1], $P>0.05$), suggesting that the formation of trace amounts of thrombin in citrate samples^{7,8} are very unlikely to be responsible for SPA. The size of platelet aggregates formed in ODFA was significantly higher in citrate-PRP than in hirudin-PRP samples (1.8 RU [1.3-2.2] vs. 1.1 RU [1.0-1.2], $P<0.0001$). The platelet aggregation inhibitor prostaglandin E_1 (10 μM) prevented the occurrence of SPA under all tested experimental conditions (*data not shown*).

In some citrate-PRP samples, the primary wave of SPA was followed by a secondary aggregation wave, which was clearly manifest when the extent of the primary wave exceeded a high threshold (Figure 2A-C). Aspirin prevented the formation of the secondary wave without affecting the primary wave of SPA (Figure 2A-C), demonstrating that cyclooxygenase-1-dependent platelet TxA_2 production is responsible for the well-known amplification of platelet aggregation generated by close platelet-to-platelet contact at low Ca^{2+} levels,⁷ but has no causal relationship with the primary wave of SPA. Due

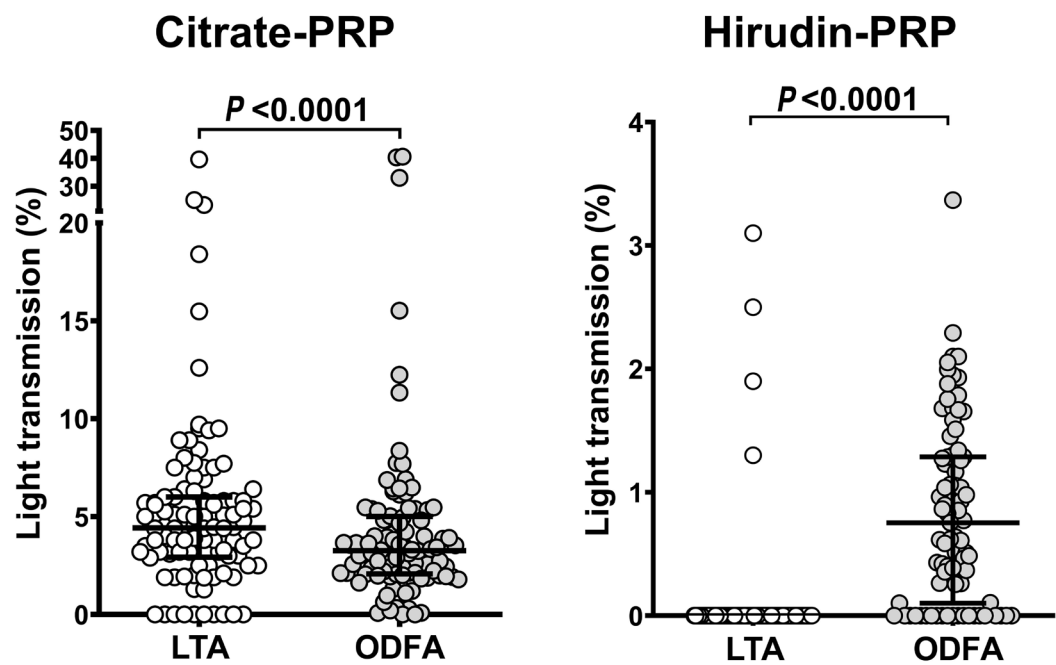


Figure 1. Comparison of spontaneous platelet aggregation measurements by light transmission aggregometry and optical density fluctuation aggregometry in citrate- and hirudin-platelet rich plasma. Platelet-rich plasma (PRP) samples from 100 (citrate-PRP) and 87 (hirudin-PRP) subjects were incubated in the aggregometer (light transmission aggregometry [LTA] and optical density fluctuation aggregometry [ODFA]) at 37°C for 3 minutes (min) without stirring and then stirred at 1,000 rpm for 6 min without added agonists. Increases in light transmission were recorded and interpreted as spontaneous platelet aggregation (SPA). Median SPA values (expressed as the percentage increase in light transmission) measured by LTA were significantly higher than those measured by ODFA (4.4% [3.0–6.0] vs. 3.3% [2.1–5.0], $P<0.0001$). In contrast, SPA was undetectable in hirudin-PRP samples tested with LTA, except in four subjects, whereas it was detectable in most subjects when measured by ODFA (0.0% [0.0–0.0] vs. 0.8% [0.1–1.3], $P<0.0001$). Data are presented as medians with interquartile ranges. Statistical analysis was performed using the Wilcoxon test.

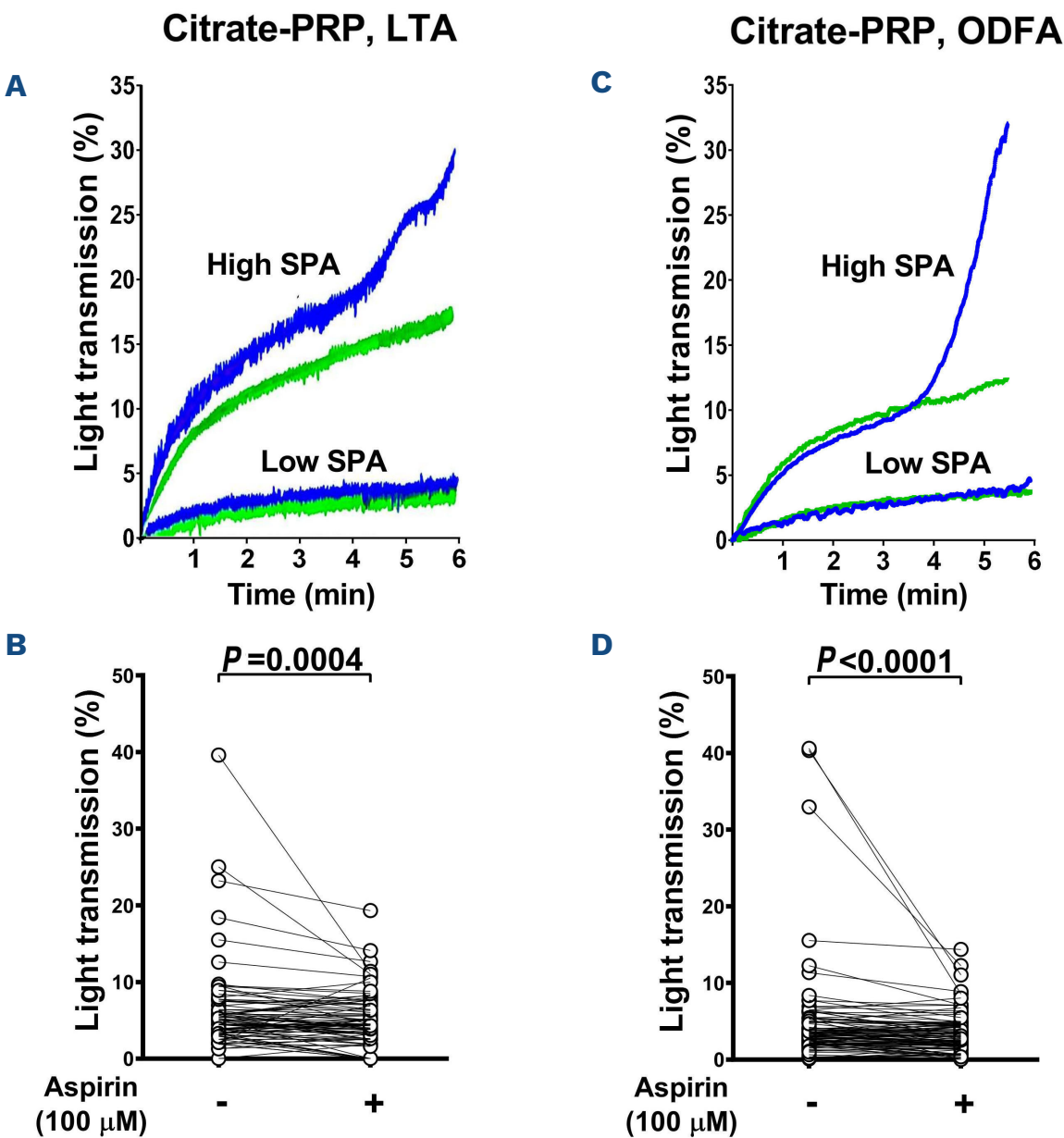


Figure 2. Influence of aspirin on spontaneous platelet aggregation in citrate-platelet-rich plasma. Citrate-platelet-rich plasma (PRP) samples from 100 subjects were pre-incubated with 15 μ L of saline or aspirin (100 μ M) at RT for 10 minutes (min) and then processed in the aggregometer to measure spontaneous platelet aggregation (SPA). Representative tracings of high and low SPA extents, with (green line) and without (blue line) aspirin, are shown for (A) light transmission aggregometry (LTA) and (C) optical density fluctuation aggregometry (ODFA). Individual variations of SPA induced by the *in vitro* addition of aspirin to PRP were measured in 100 subjects by (B) LTA (no aspirin 4.4% [2.9–6.0] vs. aspirin 4.0% [2.5–6.2], $P=0.0004$) and (D) ODFA (no aspirin 3.3% [2.1–5.0] vs. aspirin 2.8% [1.7–4.4], $P<0.0001$). Statistical analysis was performed using the Wilcoxon test.

to its inhibition of the secondary aggregation wave, aspirin marginally reduced the median extent of SPA in both LTA (4.4% [2.9-6.0] vs. 4.0% [2.5-6.2], $P=0.0004$) (Figure 2B) and ODFA (3.3% [2.1-5.0] vs. 2.8% [1.7-4.4], $P<0.0001$) (Figure 2D). In citrate-PRP, no statistically significant sex-related differences in SPA were observed when tested by both LTA and ODFA (Figure 3). However, when experiments were performed in the presence of aspirin, which abolished the confounding effects of TxA_2 -dependent secondary wave of platelet aggregation, SPA was (LTA) or tended to be (ODFA) significantly higher in females than in males (Figure 3). Moreover, SPA in citrate-PRP increased with age, when

measured both by LTA and ODFA, both in presence and absence of aspirin (Figure 3). In contrast, when SPA was measured in hirudin-PRP, no sex- or age-related differences were observed (Figure 3). The discrepancy between the two anticoagulants is compatible with the observation that women and elderly subjects display lower hematocrit levels, which increase the volume of citrate distribution in blood samples, resulting in higher Ca^{2+} plasma concentrations and increased platelet aggregability *in vitro*.^{13,14} Finally, we found an inverse relationship between the PRP platelet count and the extent of SPA, as measured in citrate-PRP by both LTA and ODFA, independently of the presence of

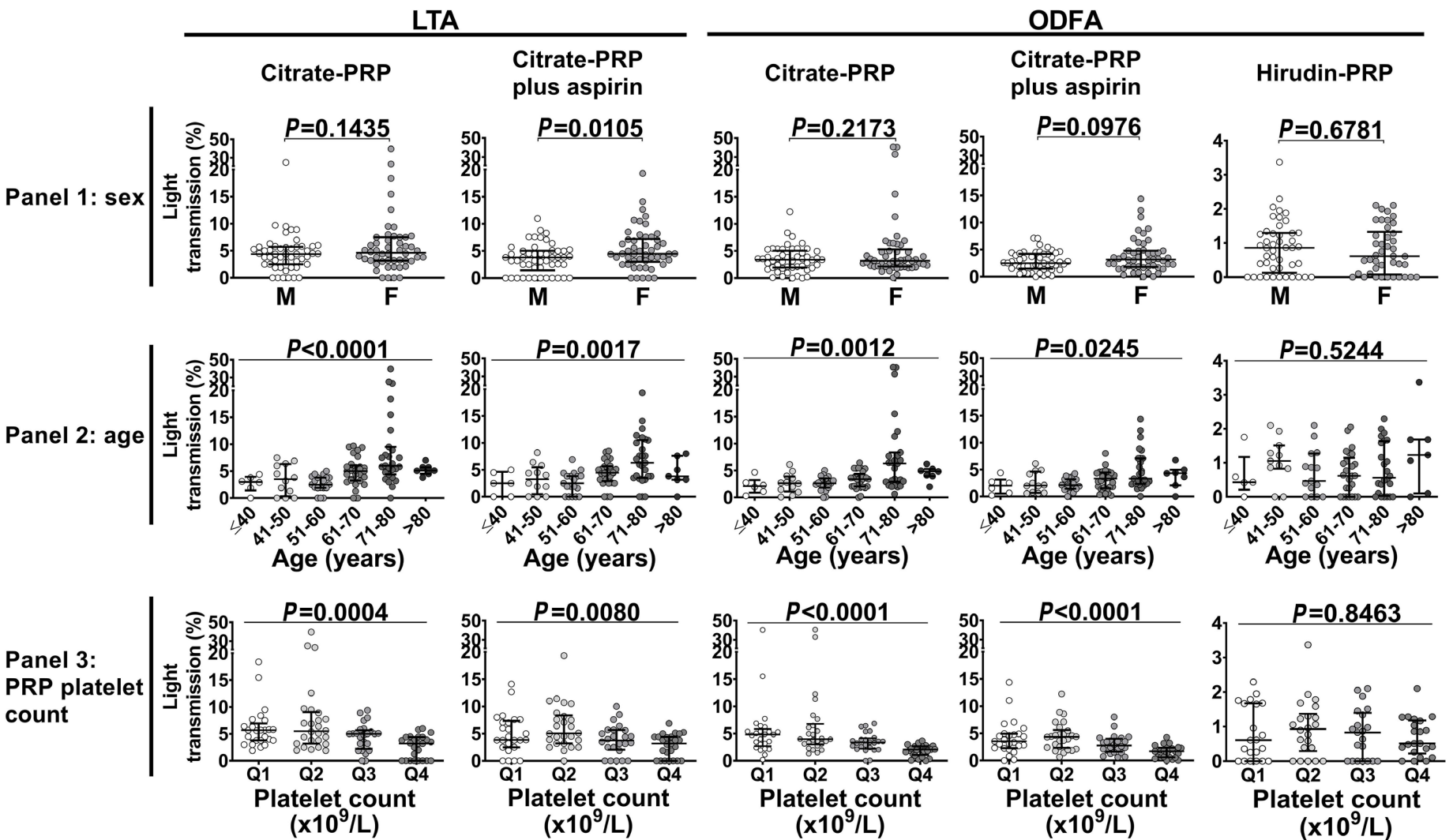


Figure 3. Influence of sex, age and platelet count on spontaneous platelet aggregation. Panel 1: influence of sex. 1) Citrate-platelet-rich plasma (PRP) in light transmission aggregometry (LTA): males (M) 4.4% (2.5-5.7), females (F) 4.6% (3.2-7.5), $P=0.1435$. 2) Citrate-PRP plus aspirin in LTA: males 3.8% (1.4-5.0), females 4.5% (3.0-7.2), $P=0.0105$. 3) Citrate-PRP in optical density fluctuation aggregometry (ODFA): males 3.4% (1.9-5.0), females 3.2% (2.1-5.3), $P=0.2173$. 4) Citrate-PRP plus aspirin in ODFA: males 2.5% (1.5-4.2), females 3.2% (1.8-4.8), $P=0.0976$. 5) Hirudin-PRP in ODFA: males 0.9% (0.1-1.3), females 0.6% (0.1-1.3), $P=0.6781$. Panel 2: influence of age. 1) Citrate-PRP in LTA: ≤40 years 3.0% (1.4-4.0), 41-50 years 3.5% (0.3-6.3), 51-60 years 2.5% (1.9-3.8), 61-70 years 5.0% (3.3-6.1), 71-80 years 6.0% (4.4-9.5), >80 years 5.1% (4.5-5.7), $P<0.0001$. 2) Citrate-PRP plus aspirin in LTA: ≤40 years 2.5% (0.0-4.6), 41-50 years 3.3% (0.5-5.5), 51-60 years 2.5% (0.0-3.9), 61-70 years 4.5% (2.9-5.7), 71-80 years 6.3% (3.5-10.5), >80 years 3.8% (3.2-7.6), $P=0.0017$. 3) Citrate-PRP in ODFA: ≤40 years 2.1% (0.9-3.2), 41-50 years 2.6% (1.1-3.9), 51-60 years 2.6% (1.8-3.5), 61-70 years 3.4% (2.1-4.4), 71-80 years 6.3% (2.9-8.4), >80 years 4.8% (3.9-5.3), $P=0.0012$. 4) Citrate-PRP plus aspirin in ODFA: ≤40 years 2.0% (0.5-3.1), 41-50 years 2.0% (0.7-4.6), 51-60 years 2.1% (1.4-3.2), 61-70 years 3.3% (1.5-4.5), 71-80 years 3.3% (2.4-7.1), >80 years 4.3% (2.1-5.0), $P=0.0245$. 5) Hirudin-PRP in ODFA: ≤40 years 0.4% (0.2-1.2), 41-50 years 1.1% (0.8-1.5), 51-60 years 0.5% (0.0-1.3), 61-70 years 0.6% (0.0-1.1), 71-80 years 0.6% (0.0-1.6), >80 years 1.2% (0.1-1.7), $P=0.5244$. Panel 3: influence of PRP platelet count. 1) Citrate-PRP in LTA: Q1 5.7% (3.8-7.0), Q2 5.5% (3.2-9.1), Q3 5.0% (2.2-5.7), Q4 3.2% (0.0-4.4), $P=0.0004$. 2) Citrate-PRP plus aspirin in LTA: Q1 3.9% (2.5-7.4), Q2 5.1% (3.2-8.4), Q3 3.8% (2.1-5.7), Q4 3.2% (0.0-4.4), $P=0.0080$. 3) Citrate-PRP in ODFA: Q1 4.8% (2.7-5.8), Q2 4.0% (3.0-6.8), Q3 3.3% (2.2-4.1), Q4 2.1% (1.1-2.7), $P<0.0001$. 4) Citrate-PRP plus aspirin in ODFA: Q1 3.5% (2.3-4.9), Q2 4.4% (2.3-5.6), Q3 2.8% (1.6-4.0), Q4 1.7% (0.6-2.4), $P<0.0001$. 5) Hirudin-PRP in ODFA: Q1 0.6% (0.0-1.7), Q2 0.9% (0.3-1.4), Q3 0.8% (0.0-1.4), Q4 0.5% (0.2-1.2), $P=0.8463$. Statistical significance was evaluated using the Mann-Whitney test for comparisons between two groups, and the Kruskal-Wallis test. Data are shown as median spontaneous platelet aggregation (SPA) percentages with interquartile ranges.

aspirin (Figure 3). However, this observation is compatible with the known physiological decrease in platelet count with age.¹⁵ Indeed, we found that there was a negative correlation between age and platelet count in our study population ($r=-0.2540$; $P=0.0108$), with younger subjects displaying higher platelet counts than elderly subjects. Therefore, in citrate PRP, the lower extent of SPA in samples with high platelet counts reflects its inverse relationship with age; this interpretation is supported by the observed lack of relationship between SPA in hirudin-PRP and the platelet count (Figure 3).

In conclusion, our study shows that SPA is detectable, albeit to various degrees, in the majority of citrate-PRP samples from subjects without overt cardiovascular, metabolic or inflammatory disease, which were prepared following the ISTH recommendations to limit untoward *in vitro* platelet activation, that could predispose platelets to aggregate spontaneously when stirred in an aggregometer. As previously shown, we found that SPA is detectable by LTA in citrate-PRP but not in hirudin-PRP samples (except in 4/100 subjects). The observation that similar degrees of SPA were observed in PRP anticoagulated with citrate or a mixture of citrate plus hirudin disproves the hypothesis that the formation of trace amounts of thrombin in citrate-PRP⁸ is responsible for SPA. It is likely that the known increased platelet aggregability in citrate-PRP^{7,8} is responsible for the generation of SPA, and that SPA might also occur in hirudin-PRP, albeit to a lesser extent, potentially missed by traditional, relatively insensitive LTA. Consistent with this hypothesis is our demonstration that SPA of low extent was detectable in hirudin-PRP by the very sensitive ODFA. Therefore, it appears that SPA is a real phenomenon, not caused by laboratory artefacts, which needs a very sensitive instrument, such as ODFA, to be detected also in PRP anticoagulated in non- Ca^{2+} -chelating anticoagulants.

Both LTA and ODFA display advantages and disadvantages for measuring SPA. LTA is widely available in laboratories, but it can only detect SPA in citrate-PRP samples. This brings about the potential disadvantage of having to treat PRP with aspirin in order to prevent the confounding effect of TxA_2 -dependent formation of a secondary wave of platelet aggregation, caused by close platelet-to-platelet contact at low Ca^{2+} levels.⁷ ODFA allows SPA to be studied in hirudin-PRP (i.e., under conditions that are closer to physiological compared to citrate-PRP) and its results are not artefactually affected by the sex and age of the study population, but it has the obvious disadvantage of its current limited availability in laboratories. The association of SPA with cardiovascular events has not been tested yet in either aspirin-treated citrate-PRP or in hirudin-PRP by ODFA. For this reason, we are currently comparing SPA measured in parallel by both LTA and ODFA in PRP samples anticoagulated with citrate (with and without aspirin) or hirudin in a case-control study of subjects with and without previous cardiovascular events.

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Disclosures

No conflicts of interest to disclose.

Contributions

CG, AF, MS and EB contributed to the study design, performed the laboratory analysis, analyzed the data, and drafted the manuscript. BC and SB contributed to the literature search and subject recruitment. EGP optimized the optical density fluctuation aggregometer protocols. MCe, GP and MCa designed the study, co-ordinated the group, contributed to the interpretation of the data analysis, and drafted and revised the manuscript. All authors read and approved the final version of the manuscript.

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Data-sharing statement

Questions regarding data sharing should be addressed to the corresponding author.

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