haematologica
Journal of the Ferrata Storti Foundation

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

by Claudia Ghali, Antonella Fioretti, Mariangela Scavone, Elena Bossi, Bianca Clerici, Simone Birocchi, Evgeny Popov, Marco Centola, Gianmarco Podda and Marco Cattaneo

Received: February 27, 2025.
Accepted: August 6, 2025.

Citation: Claudia Ghali, Antonella Fioretti, Mariangela Scavone, Elena Bossi, Bianca Clerici, Simone Birocchi, Evgeny Popov, Marco Centola, Gianmarco Podda and Marco Cattaneo. Variables influencing the in vitro measurement of spontaneous aggregation of human platelets.
Haematologica. 2025 Aug 14. doi: 10.3324/haematol.2025.287601 [Epub ahead of print]

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

Claudia Ghali[1#], Antonella Fioretti[1#], Mariangela Scavone[1], Elena Bossi[1], Bianca Clerici[1], Simone Birocchi[1], Evgeny Popov[2], Marco Centola[3], Gianmarco Podda[1], Marco Cattaneo[4]

[1]Divisione di Medicina Generale II, ASST Santi Paolo e Carlo, Dipartimento di Scienze della Salute, Università degli Studi di Milano, Milano, Italy

[2]National Medical Research Center of Cardiology, Ministry of Health of the Russian Federation, Moscow

[3]Division of Cardiology, Pio XI Hospital, ASST Brianza, Desio, Italy

[4]Fondazione Arianna Anticoagulazione, Bologna, Italy

[#]*These authors contributed equally to this manuscript*

Running head

Variables affecting SPA

Keywords

Spontaneous platelet aggregation, citrate anticoagulant, hirudin anticoagulant, light transmission aggregometry, optical density fluctuation aggregometer

Correspondence

Gian Marco Podda, gmpodda@gmail.com

Funding

This work was supported by a grant from Ricerca Finalizzata del Ministero della Salute Italiana, RF-2018-12367710, CUP C58D18000190005.

Contribution

CG, AF, MS and EB contributed to the study design, performed the laboratory analysis, analysed 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, coordinated 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.

Disclosures

No conflicts of interest to disclose

Data sharing

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

Acknowledgements

The authors are thankful to all the subjects who accepted to participate to the study and to the medical/nursing staff of the Presidio Ospedaliero San Paolo, ASST Santi Paolo e Carlo, whose cooperation was essential for the collection of samples for this study.

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 subjects[1] 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 samples handling could be responsible for platelet hyper-reactivity, favouring 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) (**Supplementary Table 1)** 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 10 days and from smoking for at least 30 minutes 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 minutes before processing. Samples were then centrifuged at 200×g at RT for 10 minutes to obtain PRP, which

was allowed to rest for 15 minutes before testing.[10, 11] Platelet-poor plasma (PPP) was prepared by further centrifugation at 1400×g for 15 minutes. 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 minutes before testing.

PPP (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 minutes without stirring and then stirred at 1,000 rpm for 6 minutes 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.

SPA 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 (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 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 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-PRP[8] 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 limited availability in current 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.

References

1. Breddin HK, Lippold R, Bittner M, Kirchmaier CM, Krzywanek HJ, Michaelis J. Spontaneous platelet aggregation as a predictive risk factor for vascular occlusions in healthy volunteers? Results of the HAPARG Study. Haemostatic parameters as risk factors in healthy volunteers. Atherosclerosis. 1999;144(1):211-219.
2. Trip MD, Cats VM, van Capelle FJ, Vreeken J. Platelet hyperreactivity and prognosis in survivors of myocardial infarction. N Engl J Med. 1990;322(22):1549-1554.
3. Hara K, Omori K, Sumioka Y, Aso Y. Spontaneous platelet aggregation evaluated by laser light scatter in patients with type 2 diabetes: effects of short-term improved glycemic control and adiponectin. Transl Res. 2012;159(1):15-24.
4. Toshima H, Sugihara H, Hamano H, et al. Spontaneous platelet aggregation in normal subject assessed by a laser light scattering method: an attempt at standardization. Platelets. 2008;19(4):293-299.
5. Yee DL, Sun CW, Bergeron AL, Dong JF, Bray PF. Aggregometry detects platelet hyperreactivity in healthy individuals. Blood. 2005;106(8):2723-2729.
6. Suzuki S, Kudo H, Koyama T. Assessment of spontaneous platelet aggregation using laser light scattering in healthy subjects: an attempt to standardize. Int J Lab Hematol. 2014;36(6):676-685.
7. Packham MA, Kinlough-Rathbone RL, Mustard JF. Thromboxane A2 causes feedback amplification involving extensive thromboxane A2 formation on close contact of human platelets in media with a low concentration of ionized calcium. Blood. 1987;70(3):647-651.
8. Lalau Keraly C, Kinlough-Rathbone RL, Packham MA, Suzuki H, Mustard JF. Conditions affecting the responses of human platelets to epinephrine. Thromb Haemost. 1988;60(2):209-216.
9. Kuhnla A, Reinthaler M, Braune S, et al. Spontaneous and induced platelet aggregation in apparently healthy subjects in relation to age. Clin Hemorheol Microcirc. 2019;71(4):425-435.
10. Cattaneo M, Cerletti C, Harrison P, et al. Recommendations for the Standardization of Light Transmission Aggregometry: A Consensus of the Working Party from the Platelet Physiology Subcommittee of SSC/ISTH. J Thromb Haemost. 2013;11(6):1183-1189.
11. Femia EA, Pugliano M, Podda G, Cattaneo M. Comparison of different procedures to prepare platelet-rich plasma for studies of platelet aggregation by light transmission aggregometry. Platelets. 2012;23(1):7-10.
12. Cattaneo M, Lecchi A, Zighetti ML, Lussana F. Platelet aggregation studies: autologous platelet-poor plasma inhibits platelet aggregation when added to platelet-rich plasma to normalize platelet count. Haematologica. 2007;92(5):694-697.
13. Kelton JG, Powers P, Julian J, et al. Sex-related differences in platelet aggregation: influence of the hematocrit. Blood. 1980;56(1):38-41.
14. Bell DN, Spain S, Goldsmith HL. Extracellular-free Ca++ accounts for the sex difference in the aggregation of human platelets in citrated platelet-rich plasma. Thromb Res. 1990;58(1):47-60.
15. Biino G, Santimone I, Minelli C, et al. Age- and sex-related variations in platelet count in Italy: a proposal of reference ranges based on 40987 subjects' data. PLoS One. 2013;8(1):e54289.

Figure legends

Figure 1. ***Comparison of spontaneous platelet aggregation (SPA) measurements by light transmission aggregometry (LTA) and optical density fluctuation aggregometry (ODFA) in citrate-platelet-rich plasma (PRP) and hirudin-PRP***

PRP samples from 100 (citrate-PRP) and 87 (hirudin-PRP) subjects were incubated in the aggregometer (LTA and ODFA) at 37°C for 3 minutes without stirring and then stirred at 1,000 rpm for 6 minutes without added agonists. Increases in light transmission were recorded and interpreted as 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 (SPA) in citrate-platelet-rich plasma (PRP)***

Citrate-PRP samples from 100 subjects were pre-incubated with 15 µL of saline or aspirin (100 µM) at RT for 10 minutes and then processed in the aggregometer to measure 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.

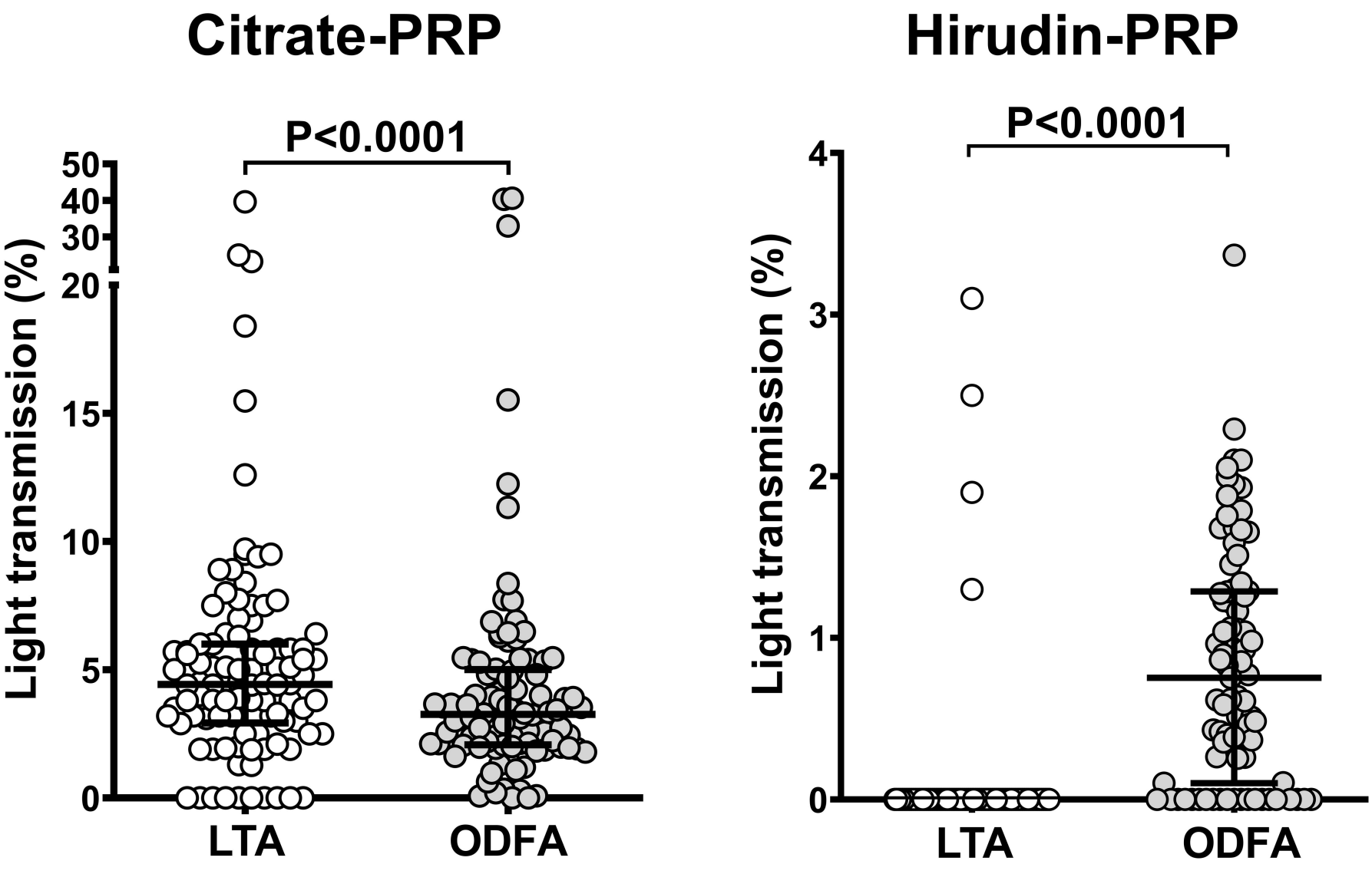
Figure 3. ***Influence of sex, age and platelet count on spontaneous platelet aggregation (SPA)***

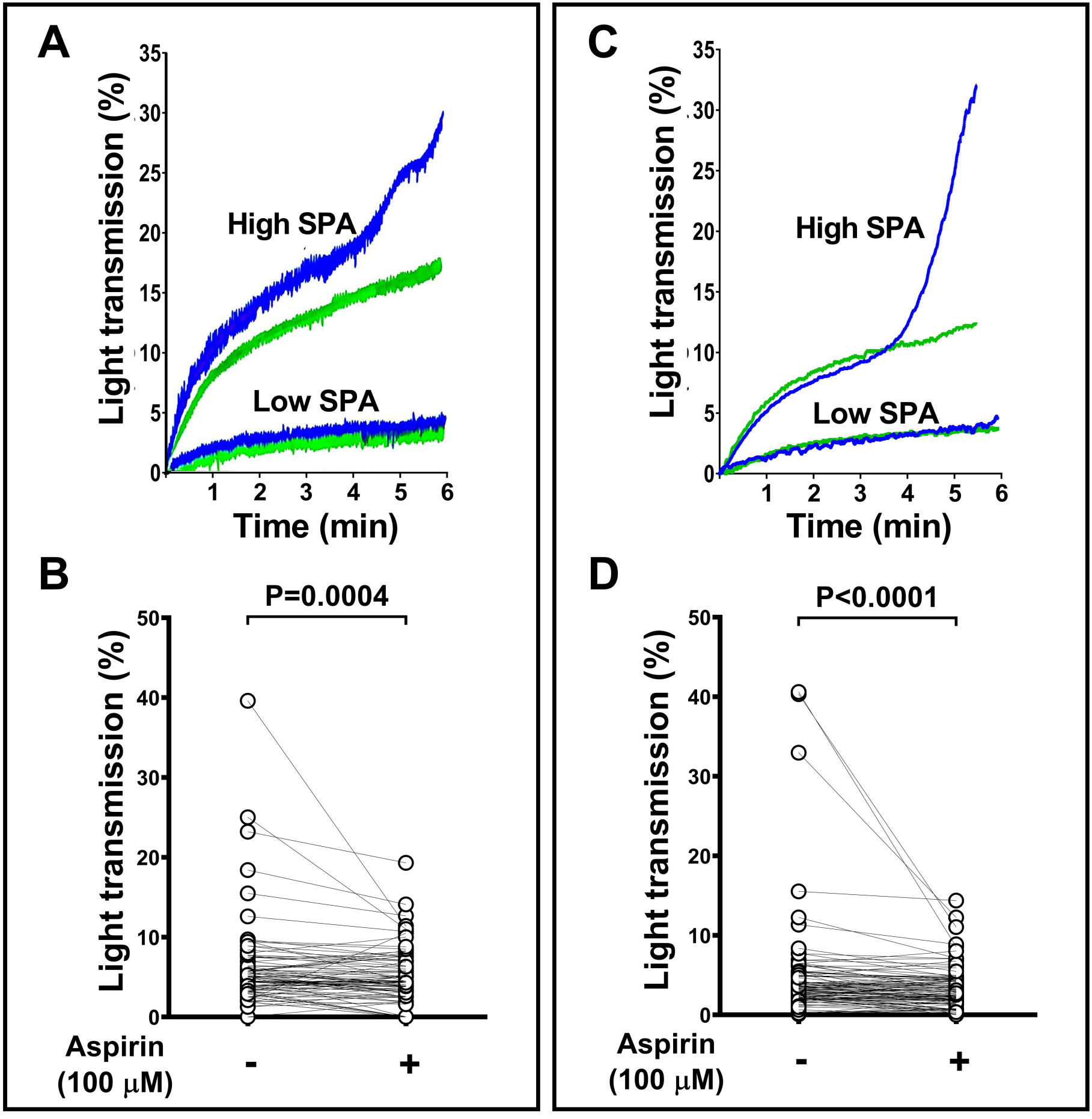
Panel 1: influence of sex. 1) Citrate-platelet-rich plasma (PRP) in light transmission aggregometry (LTA): males 4.4% (2.5-5.7), females 4.6% (3.2-7.5), P=0.1435. 2) Citrate-PRP plus aspirin in LTA: males 3.8% (1.4-5.0), females 4.5% (3.0-7.2), P=0.0105. 3) Citrate-PRP in optical density fluctuation 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.

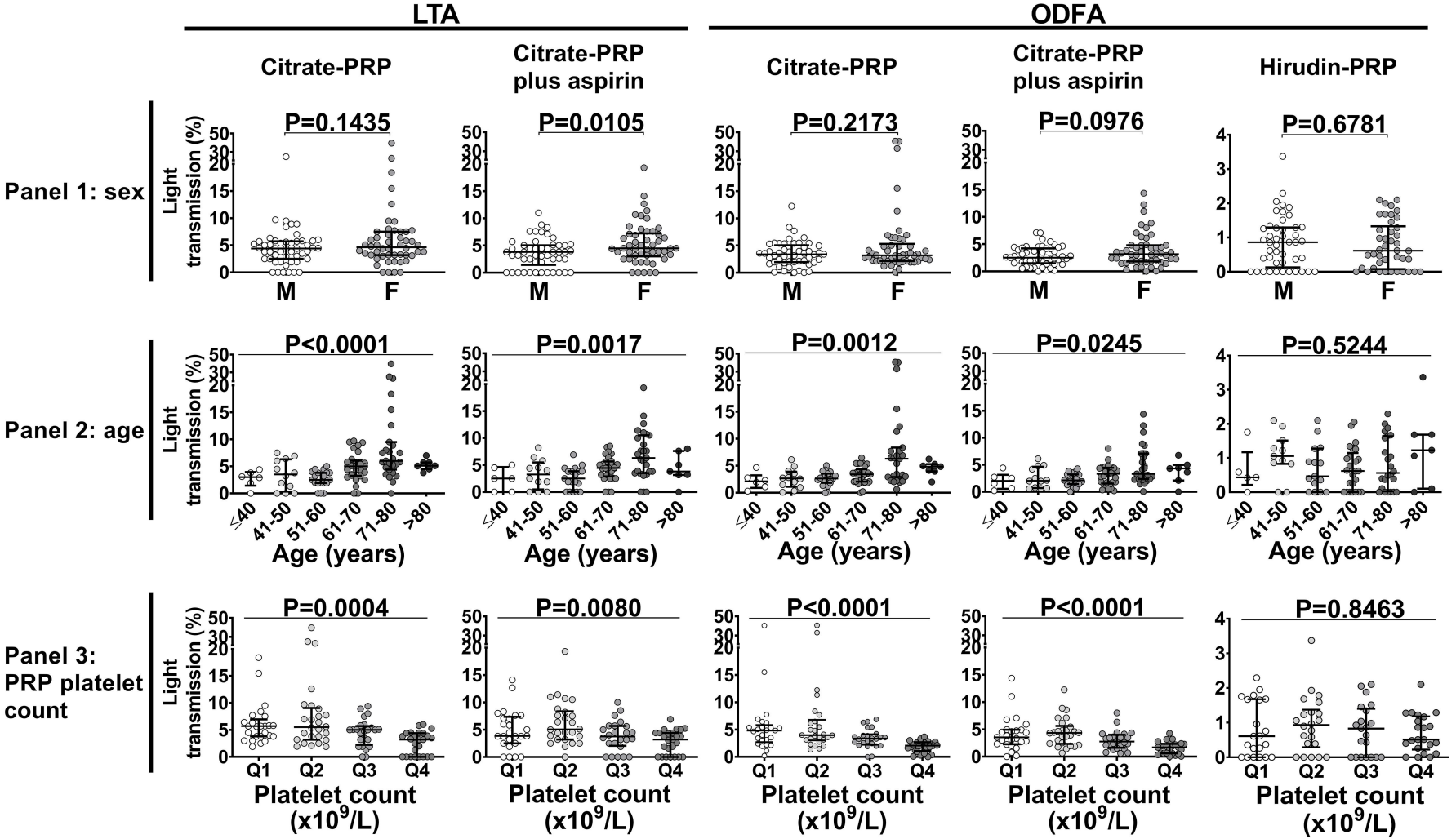
Panel 2: influence of age. 1) Citrate-PRP in LTA: ≤40 years 3.0% (1.4-4.0), 41-50 years 3.5% (0.3-6.3), 51-60 years 2.5% (1.9-3.8), 61-70 years 5.0% (3.3-6.1), 71-80 years 6.0% (4.4-9.5), >80 years 5.1% (4.5-5.7), P<0.0001. 2) Citrate-PRP plus aspirin in LTA: ≤40 years 2.5% (0.0-4.6), 41-50 years 3.3% (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 SPA percentages with interquartile ranges.


Citrate-PRP
P<0.0001
Light transmission (%)
50
40
30
20
15
10
5
0
LTA
ODFA
Hirudin-PRP
P<0.0001
Light transmission (%)
4
3
2
1
0
LTA
ODFA


A
Light transmission (%)
High SPA
Low SPA
Time (min)
C
Light transmission (%)
High SPA
Low SPA
Time (min)
B
P=0.0004
Light transmission (%)
Aspirin
(100 μM)
-
+
D
P<0.0001
Light transmission (%)
Aspirin
(100 μM)
-
+


LTA
ODFA
Citrate-PRP
Citrate-PRP plus aspirin
Citrate-PRP
Citrate-PRP plus aspirin
Hirudin-PRP
Panel 1: sex
Panel 2: age
Panel 3: PRP platelet count
Light transmission (%)
P=0.1435
P=0.0105
P=0.2173
P=0.0976
P=0.6781
M
F
P<0.0001
P=0.0017
P=0.0012
P=0.0245
P=0.5244
<40
41-50
51-60
61-70
71-80
>80
Age (years)
P=0.0004
P=0.0080
P<0.0001
P<0.0001
P=0.8463
Q1
Q2
Q3
Q4
Platelet count (x10^9/L)

SUPPLEMENTARY

Table 1. Demographic, clinical and laboratory characteristics of the study subjects

	Median (IQR) or n (%)
Demographics	
Sex (females)	50 (50%)
Age (years)	66 (53-76)
Clinical characteristics	
Current smokers	13 (13%)
Hypertension	34 (34%)
Dyslipidaemia	16 (16%)
Type 1 and 2 diabetes	0 (0%)
Cardiovascular disease	0 (0%)
Cerebrovascular disease	0 (0%)
Platelet dysfunction	0 (0%)
Myeloproliferative neoplasms	0 (0%)
Thrombocytopenia	0 (0%)
Chronic obstructive pulmonary disease	2 (2%)
Chronic kidney disease	5 (5%)
Treatments	
ACE inibitors or angiotensin receptor blockers	32 (32%)
Beta blockers	24 (24%)
Statins	16 (16%)
Laboratory values	
Platelets ($\times 10^9$/L)	201 (168-240)