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TITLE PAGE

Factors which modulate platelet reactivity as measured by five assay platforms in 3,429 individuals

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ESSENTIALS

- Platelet function testing in large populations is rare.
- We performed 5 platelet test types on 3,429 participants of the Framingham Heart Study population.
- Different tests are unique and so one cannot be substituted for another.
- Sex, age and aspirin significantly affect results so care must be used in interpreting reference ranges.

ABSTRACT

Background Assessment of platelet function is key in diagnosing bleeding disorders and evaluating anti-platelet drug efficacy. However, there is a prevailing “one-size fits all” approach in the interpretation of measures of platelet reactivity, with arbitrary cut-offs often derived from healthy volunteer responses.

Objectives Our aim was to compare well-used platelet reactivity assays.

Methods Blood and platelet-rich plasma obtained from the Framingham Heart Study (N=3,429) were assayed using a range of agonists in five platelet assays: light transmission aggregometry (LTA), Optimul aggregometry, Multiplate impedance aggregometry, Total Thrombus-formation Analysis System and flow cytometry. Using linear mixed-effect models, we determined the contribution of pre-analytical and technical factors that modulated platelet reactivity traits.

Results A strong intra-assay correlation of platelet traits was seen in all assays, particularly Multiplate velocity ($r=0.740$; ristocetin vs arachidonic acid [AA]). In contrast, only moderate inter-assay correlations were observed ($r=0.375$; adenosine diphosphate [ADP] Optimul eMax vs LTA high AUC). As expected, anti-platelet drugs strongly reduced platelet responses with aspirin-use primarily targeting AA-induced aggregation and explained substantial variance ($\beta=-1.735$, $P=4.59E-780$, variance proportion [VP]=46.2%) and P2Y₁₂ antagonists blocking ADP responses ($\beta=-1.612$, $P=6.75E-27$, VP=2.1%). Notably, female sex and older age were associated with enhanced platelet reactivity. Fasting status and deviations from standard venipuncture practices did not alter platelet reactivity significantly. Finally, the agonist batch, phlebotomist,

and assay technician (more so for assays which require additional sample manipulation) had a moderate to large effect on measured platelet reactivity.

Conclusions Caution must be exercised extrapolating findings between assays and that the use of standard ranges must be medication-specific and sex-specific at a minimum. Researchers should also consider pre-analytical and technical variables when designing experiments and interpreting platelet reactivity measures.

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INTRODUCTION

Cardiovascular diseases (CVD) are the primary cause of mortality worldwide^{1,2}. It is therefore critical to understand their pathophysiology in order to prevent cardiovascular events, including myocardial infarction and stroke³. Platelets are the lynchpin of hemostasis and thrombosis, and platelet activation is known to be caused by a myriad of factors^{4,5}. However, the assessment of platelet reactivity using the gold standard light transmission aggregometry (LTA) is generally time-consuming, requires fresh blood samples, experienced personnel and dedicated equipment to generate reproducible data⁶.

As a result, though there are many studies assessing platelet reactivity in patients, large-scale population studies are rare⁷⁻¹². Amongst the largest studies with platelet reactivity data are the Framingham Heart Study (FHS) Generation 2 cohort (N=2,604) in which our group previously identified genetic variants related to increased platelet function, which was replicated in the GeneSTAR participants with European ancestry (N=1,060)^{13,14}. An additional study in 825 African Americans of GeneSTAR cohort similarly identified novel loci associated with platelet aggregation which were replicated in 1,221 European Americans¹⁵. However, these studies were limited to LTA using a narrow concentration range of particular platelet agonists (arachidonic acid [AA], adenosine diphosphate [ADP], collagen and epinephrine) in platelet-rich plasma (PRP)¹⁶. Indeed, despite a recent study of LTA and Optimul aggregometry which was performed in 338 elderly participants of the Bruneck Study¹⁷, there is still a paucity of even

small-scale comparative studies on platelet assays, with most focused on the effects of aspirin and P2Y₁₂ receptor antagonists in patients^{9,18,19}.

Here, we describe a comprehensive study of platelet reactivity in participants of the FHS Third Generation, New Offspring Spouse and Omni 2 cohorts using five different platelet function assays in a range of agonists. These included traditional LTA, Optimul aggregometry²⁰, Multiplate[®] impedance aggregometry (MP), thrombus formation under flow (T-TAS[®])²¹ and flow cytometry²². We report standard ranges, assay comparisons and discuss pre-analytic and technical aspects which moderate these platelet reactivity assays, in some cases explaining large variances in the assay outputs.

METHODS

Study participants

FHS is a community-based, prospective, longitudinal study following three generations of participants²³. The participants of the sample population studied here represent the third exam of the Third Generation cohort and New Offspring Spouse (NOS) cohorts of primarily European ancestry (N=3,140) and the Omni 2 cohort of African, Hispanic, Asian and mixed ancestry (N=289) with examinations conducted between 2016-2019. Self-reported race and ethnicity questionnaires based on the commonly used race/ethnicity forms of the respective time periods were administered at FHS exams between 1994–2011. The participants in the present study were given the questionnaires at examinations 1 (2002-2005) and/or 2 (2008-2011). Self-reported race and ethnicity in this study was based on a cumulative race and ethnicity dataset created to record participants' responses to race and ethnicity questions over time. Participants were allowed to select more than one race and could vary their response over time.

Participants attended a detailed ~4.5 hour examination with detailed concurrent and historic anthropometric measurements, disease event surveillance, and other clinical and sub-clinical measures were collected. Participants stated medication use at time of blood draw and were also asked to bring medications used to the examination. Formulations were noted and categorized with the World Health Organization's Anatomical Therapeutic Chemical classifications. This study was approved by the Boston University Medical Center institutional review board and participants gave written informed consent²⁴.

Blood processing

Participants fasted overnight and blood was drawn the following morning in a supine position from the antecubital vein with a 21-gauge butterfly collection set with the aid of a tourniquet^{25,26}. Blood was collected into glass sodium citrate (3.2%; Becton Dickinson, San Jose, CA) and hirudin (whole blood [WB]; Roche Diagnostics, Diapharma, West Chester, OH) vacutainers. Additional EDTA and serum vacutainers were collected for standard laboratory measures (see Supplemental Material). The workflow is summarized in Figure 1.

Any deviations from this standard phlebotomy procedures were noted, as well as other relevant factors including self-reported last anti-platelet medications, known diabetes and time of last meal. Participants were considered fasting after a minimum 10-hour fast.

Blood samples were processed at room temperature for platelet function testing in accordance with ISTH guidelines²⁷. Briefly, blood was left to stand for >15 mins after blood taking before being processed. Sodium citrate tubes were centrifuged (200 x g, 10 min; Sorvall ST8 centrifuge, Thermo Sci 75003181 rotor) to isolate platelet rich plasma (PRP) and pooled. Samples were further centrifuged (1,500 x g, 15 min) for isolation of platelet poor plasma (PPP).

Platelet function testing

Platelet function tests were conducted using the MP system (Roche Diagnostics) and T-TAS[®] automated microchip flow chamber (PL-chip; Zacros, Fujimori Kogyo, Tokyo, Japan) in whole

blood, LTA (PAP-8E; Bio/Data Corporation, Horsham, PA) and Optimul aggregometry in PRP, and flow cytometry (Accuri C6; BD Biosciences, San Jose, CA) in both whole blood and PRP.

All agonists, except AA/ASPItest (Roche Diagnostics), U46619 (Cayman Chemical, Ann Arbor, MI) and the agonists in a limited number of Optimul plates, were obtained from Bio/Data Corporation in large, dedicated batches (~1 batch/year) to minimize variation in this study. Further assay testing details can be found in the Supplemental materials.

Aspirin use was defined as a final aggregation response of <40% to AA (500 µg/mL) in LTA. If this was not available, it was determined by MP AUC of <40 units to ASPItest. Using this definition, our study consisted of 736 aspirin users.

Exclusion criteria

Data was excluded from 92 participants who did not have platelet assays (total N=3429/3521). In addition, participant data from PRP assays (LTA, Optimul, PRP flow cytometry) was excluded due to hemolytic (N=5) and lipidemic samples (N=25) but retained for the WB assays (MP, T-TAS, WB flow cytometry). Due to instrument downtime, reagent availability and the addition of T-TAS later in the exam, the sample size was variable for each instrument (Supplemental Figure 1).

Statistical analysis

All platelet traits and technical factors were treated as continuous except for sex, aspirin use, P2Y₁₂ antagonist use (determined by prescribed medications brought to the exam), fasting status, deviation in blood draw, short blood draw, multiple venipuncture, tourniquet use, phlebotomist, assay technician and assay batch which were categorical.

Correlation coefficients were calculated using the Pearson's correlation method. Linearity was not tested prior to calculating.

Inverse normal transformation was applied to platelet traits and linear mixed-effect models were used to determine the association between these transformed traits (dependent variables) and technical factors (independent variables) including age, sex and aspirin²⁸. All models accounted for family relatedness and for each trait were specifically tailored for sample preparation (PRP or WB), assays and agonists used (Supplemental Table 1). A p-value was computed for a global test for those technical factors with 3 or more levels such as agonist batch, technician or phlebotomist. This tests whether there is association between a platelet trait and a technical factor with 3 or more levels, that is, H_0 : all levels have the same platelet mean versus H_1 : at least one level has different platelet trait mean from other(s). Differences were considered significant at $P < 0.0000467$ after Bonferroni correction for multiple testing for 63 platelet traits and 17 technical factors.

All analyses and figures were conducted or produced using the R language and environment²⁹.

RESULTS

Study Participants

The study consisted of 3,429 participants who had at least one platelet function measure. Of these, 53.7% (N=1,842) were female, the mean age was 54.4±9.3 years and the average BMI was 28.5±5.8 kg/m². Overall, 351 individuals were diagnosed with diabetes (10.2%) and 995 with hypertension (29.0%; Table 1). The Third Generation and NOS cohorts were of primarily European ancestry and the Omni 2 cohort included other ancestries (White 0.7%, Black 18.0%, Hispanic 9.7%, Asian 23.9%, Mixed or Other 47.8%: where 57.2% of Mixed or Other were Hispanic-White and Other; Supplemental Table 2).

We observed 19.9% (N=683) self-reported aspirin use, which was superseded by the 21.5% (N=736) which we identified as aspirin-takers using our criteria. There was good agreement between self-report and LTA (kappa=0.68) and MP (kappa=0.64, whilst agreement between self-report and Optimul (kappa=0.3) and T-TAS (kappa=0.28) was fair (Supplemental Table 3). In addition, there was a clear separation of LTA responses to AA in the aspirin (7.0±6.1%) group vs the non-aspirin group (74.9±8.0%; P<0.0001).

Since sex and aspirin status can be easily determined and have a large effect on platelet reactivity, reference ranges for each assay were derived based on aspirin use and sex (Table 2A-E).

Correlation between Assays

We constructed correlation matrices for the five platelet assays in all participants included in the study (Figure 2A). In particular, AA-mediated responses in LTA and the MP aspirin tests were strongly correlated ($r=0.793$, $P=4.34E-687$). Since these responses were principally driven by aspirin users ($N=736$), we repeated these correlations in the individuals not on aspirin and found that most of these correlations were significantly reduced (Figure 2B). We found intra-assay correlations, particularly with LTA and MP (ristocetin vs AA velocity; $r=0.740$, $P=9.84E-561$) even using different agonists.

There was varying inter-assay correlation across the same agonist in different assays with weak to moderate correlations between PRP and WB assays. For example, Optimul and LTA were weakly correlated in shared agonists (e.g. ristocetin LTA final aggregation vs Optimul eMax, $r=0.378$, $P=7.46E-82$) and collagen MP AUC and T-TAS AUC responses were moderately correlated ($r=0.467$, $P=1.84E-50$), as well as ADP measures across all assays in the non-aspirin sample (ADP LTA AUC vs Optimul eMax; $r=0.375$, $P=1.68E-80$). However, there was no correlation between LTA and MP aggregation after stimulation with ristocetin or TRAP-6 amide (ristocetin: $r=0.037$, $P=0.06$; TRAP-6: $r=-0.007$, $P=0.73$).

P-values and correlation coefficients for each assay in the presence and absence of aspirin, as defined by our criteria, are provided in the Supplemental Materials (Supplemental Table 4A-D).

Technical Factors

We used the significance (Figure 3A) in multivariable regression models as well as the proportion of explained variance (Figure 3B) to determine the contribution of each technical, pre-analytic or medication factor to each platelet trait (all results shown in Supplemental Table 5). In the text below we highlight results for those factors that account for the highest explained variance proportion (VP) in platelet reactivity traits.

Age and sex

Age was directly associated with ADP (0.95 $\mu\text{mol/L}$, VP=2.9%; 1.82 $\mu\text{mol/L}$, VP=0.8%) and ristocetin (VP=0.6%)-induced platelet final aggregation in LTA. Similarly, ADP responses in Optimul are increased with age, as well as epinephrine-induced reactivity. Interestingly, platelet responses are counter-directional in MP with reduced reactivity to collagen with increased age. Age did not contribute to >0.5% explained variance in T-TAS or flow assays but, generally, greater age was associated with a reduction in MP traits and an increase in flow cytometry related traits.

All assays except T-TAS were profoundly influenced by sex, though even T-TAS platelet thrombus formation trended to be higher in women. Female sex was associated with an increase in platelet reactivity in all agonists studied in the MP WB assay. Lower concentrations of ADP were associated with increased reactivity in females in LTA (0.95 $\mu\text{mol/L}$, VP=0.7%; 1.82 $\mu\text{mol/L}$, VP=1.0%). In addition, epinephrine (AUC, VP=0.6%) and U46619 (AUC, VP=0.8%) responses in Optimul were elevated in females. ADP-stimulation in PRP (P-selectin, VP=2.0%;

PAC-1 binding, VP=0.8%; double positivity, VP=1.0%) and WB (P-selectin, VP=3.9%; PAC-1 binding, VP=1.8%; double positivity, VP=3.0%) samples also resulted in greater expression of activation markers in flow cytometry in females. Furthermore, females had a higher estimated platelet count than males in both WB (mean $368,770 \pm 130,037/\mu\text{L}$ vs. $330,272 \pm 119,164/\mu\text{L}$) and PRP ($521,022 \pm 179,432/\mu\text{L}$ vs. $510,310 \pm 192,079/\mu\text{L}$) as estimated by flow cytometry direct volume counting.

Once centrifuged, the PRP volume from each tube of citrated WB was noted ($0.422 \pm 0.069\text{mL}$ citrate per mL WB; range: 0.178-0.741mL citrate per mL WB). Greater PRP volume (per mL WB), which is linked to hematocrit and by extension being female, enhanced platelet reactivity as measured in PRP-based assays.

Aspirin and P2Y₁₂ antagonist use

As expected, aspirin strongly attenuated AA traits in the LTA (VP=46.2%) and Optimul (VP=30.4%) assays. Additionally, aspirin reduced platelet reactivity to several other agonists in PRP assays, including collagen (LTA, VP=28.8%; Optimul, VP=9.1%), epinephrine (LTA, VP=34.3%; Optimul, VP=7.0%) and ristocetin (LTA, VP=0.7%; Optimul, VP=11.1%; Figure 3A and 3B, Supplemental Table 5). Interestingly, aspirin also significantly dampened ADP responses in these assays (LTA, VP=18.7%; Optimul, VP=0.7%). Similarly, since T-TAS uses collagen as an agonist, these responses were blunted with aspirin use (VP=13.9%). MP responses to ASPItest (VP=38.7%), collagen (VP=9.8%) and ristocetin (VP=23.2%) were attenuated. Aspirin use had no

effect on thromboxane receptor agonism by the thromboxane (Tx) A₂ mimetic U46619, and very little effect on TRAP-6 amide (thrombin PAR-1 agonist) assays.

P2Y₁₂ antagonists, as noted from medications being taken by participants, elicited an expected reduction in ADP aggregation traits in the LTA (VP=2.1%) and Optimul (VP=2.0%) assays. Of note, P2Y₁₂ antagonist use was also associated with reduced epinephrine, TRAP-6 amide and U46619 responses in the Optimul assay as well as a reduced T-TAS. Similarly, P2Y₁₂ drug use associated with lower expression of platelet activation markers in flow cytometry after the addition of ADP. Multiplate ADP responses were blunted with P2Y₁₂ antagonist use but unaffected by aspirin (P>0.05, Supplemental Table 5).

Timing

Day in the year did not associate with platelet reactivity in LTA or T-TAS. However, as the time of year advanced, collagen responses were attenuated in the Multiplate assay and ADP-stimulated PAC-1 binding and P-selectin were reduced in flow cytometry. Conversely, responses in the Optimul assay to collagen were enhanced (Supplemental Table 5).

The majority of blood draws (93.5%) were completed between 7am-10am, with only N=14 conducted after 11am. Mean draw time was 8:48 am±54 min (SD). Interestingly, later draw times in the day significantly blunted LTA responses to AA, collagen and ristocetin and P-selectin expression in flow. Conversely, a longer time from blood draw to test blunted responses in only the MP assay.

The time in the morning in which blood was centrifuged to obtain PRP (PRP spin time) was inversely correlated with ADP, collagen, ristocetin and TRAP-6-induced aggregation in LTA and a later PRP spin time associated with increased P-selectin expression and enhanced PAC-1 binding. Conversely, a later PPP spin time was associated with the opposite effect (Supplemental Table 5).

When we defined the time of year as seasons: spring (March 1 - May 31), summer (June 1 – August 31), fall (September 1 – November 30) and winter (December 1 – February 28), we saw a reduction in PAC-1 expression in summer and fall (Supplemental Figure 2).

Fasting status and draw issues

Fasting status (defined as >10hrs fasting) had no effect on platelet traits. Fasting time in individuals using this criteria (N=3317) ranged from 600-1470 min (775 ± 89 min), with 93% of fasting participants fasting between 10-15hrs. Similarly, any deviations from the protocol regarding blood draw such as a short draw (a less than complete number of tubes drawn due to early discontinuation of phlebotomy), more than one attempt at obtaining blood or the requirement of the tourniquet throughout the draw did not seem to affect platelet traits (N=443).

Individual phlebotomist, however, did have a moderately significant bearing on platelet reactivity induced by collagen and TRAP-6 in LTA. It is important to note, however, that certain

highly experienced phlebotomists were called upon for difficult draws and older participants which may have skewed results.

Agonist batch

We identified a major effect of the place of manufacture (London, UK or Framingham, MA USA) for the Optimul plates. This likely primarily results from different agonist origin, but it is also possible there are effects due to different technicians manufacturing plates, different freeze-driers and other equipment, or changes in agonists with the extra transportation involved. Although efforts were made to reduce the number of agonist batches across the entire exam, batch was associated with a few other variables, particularly in LTA (ADP, collagen, TRAP-6 amide), Multiplate (AA, collagen, TRAP-6 amide) and flow cytometry (ADP) (Figure 3, Supplemental Table 5).

Technicians

The assay technician influenced some of the tests. In particular, the Optimul tests, T-TAS and flow cytometry traits were most strongly associated with technician differences. Technician differences for these assays typically explained around 1-3% of variance. However, it was as high as 6.4% for Optimul ristocetin AUC, 5.2% for PRP 20 $\mu\text{mol/L}$ ADP P-selectin response, ~3% for Optimul ADP, collagen and epinephrine assays (Figure 3; Supplemental Table 5). It is worth noting that there may have been some confounding factors such as certain technicians working primarily in earlier periods of the 3-year exam when Optimul plates came from the UK, or technicians that tended to run samples earlier or later in the day.

DISCUSSION

Overview of the Study

In the present study, we used the FHS cohort to provide a previously unprecedented scale of population-level data on platelet function using five assay platforms, including the ubiquitous LTA and newer Optimul PRP assays as well as the whole blood MP assay, flow cytometric analysis of platelet activation markers and finally the recently developed T-TAS assay that flows platelets under shear stress over collagen-coated microvessels²⁵. By directly comparing our assay outputs, we were able to determine that there are strong intra-assay correlations and that consistency across different assay modalities is relatively weak. In addition, we identified previously unexplored technical contributions to platelet reactivity measures.

Assay Correlations

The agonist is usually considered the driving factor of platelet reactivity, with 'stronger', primary agonists such as collagen or thrombin eliciting greater aggregation. Although we observed inter-assay correlations for some agonists, they were not overwhelming and somewhat tied to sample preparation (e.g., WB or PRP)^{30,31}. Indeed, TRAP-6 amide LTA and Optimul responses correlated well with each other though differences between these assays have been discussed previously^{32,33}. Interestingly, TRAP-6 amide LTA responses did not correlate with MP responses at all. Previous studies have shown only a fair correlation of LTA and MP responses with an overall 85% concordance and other studies concluded that MP, or

impedance aggregometry generally, is less sensitive than LTA in detecting mild platelet function disorders^{7,11,34,35}. We did show, however, that LTA and Optimul responses to AA corresponded strongly with MP ASPItest, confirming its application as a point-of-care test to detect aspirin use, though likely with less discriminative capacity than PRP-based measurements³⁶. In line, ADP-stimulated responses in LTA and P-selectin expression in flow cytometry were moderately correlated which have been demonstrated in patients with suspected platelet function defects and in the assessment of dual antiplatelet therapy efficacy^{37,38}.

Technical factors

A unique aspect of our study is the addition of an analysis of the technical aspects of blood collection and performance of each assay using well statistically powered multivariable models. Though there have been efforts to standardize blood collection and analysis of platelets using LTA or flow cytometry, to our knowledge there are no large-scale studies which have systematically analyzed platelet function testing readouts with technical factors^{27,39}. Here, we have confirmed previous factors which influence platelet reactivity, and identified new ones which must be considered when interpreting results.

Antiplatelet drugs

Although there was good concordance with self-reported aspirin use and aspirin use defined according to our criteria of AA response in LTA, followed by second-line use of MP ASPItest, it was not perfect due to possible significant platelet turnover since last aspirin dosing, or reporting errors in participant recall or knowledge of over-the-counter drug formulations⁴⁰.

Even after correcting for aspirin use, aspirin contributed to ~50% of the variation in the AA LTA aggregation response. Our study shows that aspirin also targets collagen and epinephrine-induced platelet reactivity, indicating that TxA₂ is likely involved in the amplification of these responses. We replicated findings that aspirin attenuates collagen and AA MP responses in patients, including those with diabetes^{19,41,42}. Aspirin also had a large effect on the collagen-based T-TAS assay which demonstrates its ability to be used to discriminate the effects of this drug⁴³. In concert, we showed that ADP responses in all assays were blunted in the presence of P2Y₁₂ receptor antagonists which has also been demonstrated in healthy volunteers and patients^{18,44}. Studies have assessed high-on platelet reactivity in the presence of P2Y₁₂ inhibitors in patients using LTA and MP stimulated by ADP but 15% of patients were still misclassified compared to compared to vasodilator-stimulated protein expression in flow cytometry⁴⁵. Our findings underline the importance to any platelet study of having strict accounting of antiplatelet medications by questionnaire, and also reliable assays to determine this. Otherwise, unaccounted medications could produce very strong outlier effects in nearly any study design format. Unfortunately, we were unable to discern the effects of aspirin (N=714) or P2Y₁₂ antagonist (N=11) monotherapy vs dual anti-platelet therapy (N=22) due to low P2Y₁₂ antagonist use in this general middle-aged population though we accounted for P2Y₁₂ antagonist use in our multivariable models.

Age and sex

Reference ranges for assays are generally provided by the manufacturer or in-house lab measures but tend to be inferred from a limited number of healthy volunteers and not

separated by demographic groups, in particular sex, which has a large effect on platelet reactivity. Here, we provided population reference ranges according to sex and aspirin-use for each assay and have performed multivariable regression analyses between technical factors and platelet reactivity outcome measurements that support major group differences (Tables 2A-E). In addition, we report that both age and sex affect platelet reactivity traits, with sex strongly affecting MP and flow cytometry traits, and age dominating ADP and epinephrine PRP traits. We confirm the findings of others that female sex and increased age are associated with greater platelet reactivity in LTA^{42,45,46}. This is in contrast to our findings in a population over 65 years old which showed no difference in platelet reactivity in LTA. However, in the present study we assayed a broad range of ages from 32-93 years¹⁷. Furthermore, we saw strong effects of PRP volume per citrate tube. Lower PRP volume, which tends to be in males due to a higher hematocrit, was associated with lower platelet reactivity. Indeed, elevated hematocrit has been implicated in thrombosis which may contribute to the elevated cardiovascular risk seen in males, and could be due to increased platelet margination by increased red cells in central flow^{47,48}.

Other factors

In addition to the major effects of aspirin, age and sex, we identified several other factors which affected platelet reactivity measures. We saw a large batch effect in Optimul due to differences in origin of agonists (Supplemental Material) on plates which were manufactured in the UK vs in-house which was due to early startup phases in the US or equipment downtime. Smaller but significant batch variations were seen in ADP batch used in flow cytometry, MP and LTA. In

agreement, P-selectin expression over time with the same donor has been shown to be consistent when the same batch of ADP was used⁴⁹. Further, we noticed a reduction in PAC-1 expression in the summer and fall which is likely linked to surface marker distribution since seasonal effects have been described where platelet counts are lowest in the summer and mean platelet volume highest in the spring^{50,51}. A later morning draw time moderately corresponded with decreased platelet function⁵²⁻⁵⁴. The early morning rise in platelet function may have adaptive origins and has been suggested as one factor that may account for higher prevalence of thrombosis in those hours⁵⁵. Nearly all (99%) of all our assays were conducted within 4 hrs which falls in line with the recommendations by various guidelines^{27,56,57} and the time to assay did not greatly effect platelet function. With this, we conclude that recording draw time is likely more important than time from draw to test for most assays. However, calculating time until assay may still be a useful factor that can easily be acquired, and may be most relevant in impedance aggregometry.

Finally, personnel accounted for some variation in assays. There were 4 phlebotomists used through the exam and 2 performed 76% of all blood draws. Our results indicate the phlebotomist had a moderate effect on some LTA traits (collagen, TRAP-6), accounting for 0.78-0.87% of the variance in these traits. This may have been due to the fact that some phlebotomists are called for on more difficult blood draws (older participants, co-morbidities) which may have skewed results. In addition, deviation in blood draw and fasting status did not effect platelet traits. The greatest variations seen in technician was demonstrated in assays with more complex sample manipulation. In particular, in flow cytometry there are multiple

centrifugation and liquid handling steps, including discarding supernatant around a cell pellet, which can be subjective and result in increased variability.

Limitations/future studies

Though we described the first demonstration of in-depth platelet function across a variety of agonists and assays in a large population, this is still by no means comprehensive. Indeed, other agonists, combinations of agonists, concentrations and assays, such as PFA-100/200, VASP and VerifyNow point of care tests are utilized by researchers in the field. However, we were limited in time, cost and blood volume available⁵⁸. In addition, we did not directly measure platelet count by a traditional approach and, therefore, did not exclude participants with thrombocytopenia from our analysis⁵⁹. Furthermore, since this was a population sample study, we did not exclude participants who reported common bleeding disorders including Von Willebrand disease and immune thrombocytopenic purpura to reflect the prevalence of these in the population. Finally, reports of platelet reactivity are mostly limited to European ancestry, though some small studies have demonstrated attenuation of responses in African ancestries, particularly to ristocetin^{45,60}. The influence of ethnicity on platelet reactivity is ill-explored and the diversity of this cohort allows the exploration of this, which we hope to address in future studies⁶¹.

Conclusions

In this study, we have identified several technical factors which should be considered when interpreting platelet reactivity outcomes. Though, ideally, we would recommend that these sources of variabilities be limited, the practicalities of platelet reactivity testing often preclude this. As such, we encourage researchers to note these covariates and, where possible, match or exclude these variables when designing their studies. At the very least, age, sex and aspirin use should be considered when interpreting platelet reactivity data rather than a “one-size fits all” approach. Here, we provide a road map of factors which researchers should mitigate if they have chosen a particular assay (Supplemental Table 5). By sorting according to their assay of choice, they can then identify which technical factors significantly modulate the data. For example, if they are already using Optimul, they should take care to note batches or perform all their experiments within one batch. Conversely, they should not be overly concerned if a blood draw is performed in the early vs late morning, or a fasting period is within a precisely defined window as long as they exclude lipidemic samples in LTA. If they are choosing which assay would be the best for a long-term study, they could use LTA as it offers assay flexibility, reaction-time outputs and appears less reliant on effects like seasonality and technician variation. We recognize that each study, however, is highly context-dependent and the dynamics of each assay as well as the sample preparation need to be reviewed as well as the agonist. In addition, which assays are chosen must balance with the availability of equipment, blood volume and the research question to be addressed.

We emphasize that platelet assays are not interchangeable and that one assay cannot be considered a surrogate for another due to different technologies and platelet preparations. Indeed, in order to provide more comprehensive studies of platelet function dynamics, researchers will be in a best position if they apply several testing modalities, agonists and concentrations.

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AUTHORSHIP DETAILS

M. V. Chan contributed to the concept and design, collected, analyzed and interpreted the data and drafted the manuscript. M-H. Chen, F. Thibord, B. B. Nkambule, J Grech and J.E. Huffman analyzed and interpreted the data. A.R. Lachapelle, Z. E Schneider and C. Wallace de Melendez collected the data. M. A. Hayman, H.E. Allan, P. C. Armstrong and T. D. Warner contributed to the design and revised the intellectual content. A. D. Johnson contributed to the overall concept and design, analysis and interpretation of the data. All authors approved the version to be published.

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DISCLOSURES

None.

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FIGURE LEGENDS

Figure 1. Daily workflow by **(A)** assay and **(B)** timeline. For all experiments, blood was drawn and either kept as whole blood or processed by centrifugation for platelet rich plasma (PRP) and platelet poor plasma (PPP). Light transmission aggregometry (LTA), Optimul aggregometry, Multiplate impedance aggregometry, Total Thrombus-formation System (T-TAS) and flow assays were then conducted as soon as possible and within a 4-hour timeframe. AUC: area under the curve; P-Sel: P-selectin; PAC-1: procaspase activating compound-1.

Figure 2. A Correlation matrices of the five platelet assays in **(A)** all FHS participants (N=3,429) and in **(B)** participants without aspirin-use (N=2,693). Darker red shows high positive correlation, darker blue shows high negative correlation and white shows no correlation.

Figure 3. Graphical results of technical factor analysis against platelet traits of **(A)** P-values and **(B)** explanation of variance in all assays. In A, the data were binned according to P-value, with a smaller P-value corresponding with a larger circle. Positive (blue) and negative (orange) effect direction was determined by the calculated beta from analyses. No effect direction (grey) was reported when a global test was performed in 3 or more levels. In B, variance percentages were binned and colored red. Therefore, larger, red circles represent a large portion of the trait variance is attributed to this factor.

TABLES

Table 1. Participant demographics summary of the Framingham Heart Study (FHS) samples in Generation 3, NOS and Omni 2 cohorts. Data are presented as mean±s.d. unless stated.

	No aspirin (Male)	No aspirin (Female)	Aspirin (Male)	Aspirin (Female)
Number, N (%)	1141	1552	446	290
Age, years	52.76±8.85 (32-87)	53.47±9.13 (32-93)	59.44±8.72 (33-89)	58.34±8.94 (34-87)
Body mass index (BMI), kg/m ²	29.29±5.08 (17.29-51.13)	27.52±6.24 (15.45-54.64)	30.17±5.11 (18.52-53.01)	28.67±6.5 (17.01-59.22)
Total cholesterol, mg/dL	187.13±33.97 (91-380)	195.64±35.97 (101-386)	171.07±39.28 (68-382)	191.85±35.34 (108-333)
High-density lipoprotein (HDL), mg/dL	50.6±15.18 (11-175)	67.86±19.29 (7-161)	49.68±14.78 (13-107)	64.64±19.21 (18-134)
Current smoker, N (%)	80 (7)	104 (6.7)	21 (4.7)	19 (6.6)
Diagnosed hypertension, N (%)	311 (27.3)	321 (20.7)	240 (53.9)	123 (42.6)
Diagnosed diabetes, N (%)	101 (8.9)	114 (7.3)	95 (21.3)	41 (14.2)
Fasting at blood draw, N (%)	1101 (96.5)	1515 (97.6)	420 (94.2)	281 (96.9)
Self-reported aspirin use at time of blood draw, N (%)	82 (7.2)	69 (4.4)	349 (78.3)	183 (63.1)
P2Y ₁₂ antagonist use determined by medications brought to exam, N (%)	4 (0.4)	7 (0.5)	18 (4)	4 (1.4)
Light transmission aggregometry (LTA), N (%)	1105 (96.8)	1517 (97.7)	431 (96.6)	288 (99.3)
Optimul aggregometry, N (%)	1056 (92.6)	1424 (91.8)	414 (92.8)	275 (94.8)
Multiplate impedance aggregometry, N (%)	1137 (99.6)	1547 (99.7)	446 (100)	290 (100)
Total Thrombus-formation Analysis System (T-TAS), N (%)	384 (33.7)	530 (34.1)	126 (28.3)	89 (30.7)
Flow cytometry, N (%)	986 (86.4)	1266 (81.6)	373 (83.6)	237 (81.7)

Table 2. Reference means and ranges for (A) LTA, (B) Optimul, (C) Multiplate, (D) T-TAS and (E) flow cytometry assays performed in the FHS participants by aspirin use and sex.**(A) Light transmission aggregometry (LTA)**

Agonist (concentration)	Measurement	No aspirin (Male)		No aspirin (Female)		Aspirin (Male)		Aspirin (Female)	
		Mean±SD (range)	N	Mean±SD (range)	N	Mean±SD (range)	N	Mean±SD (range)	N
AA (500 µg/mL)	AUC	0.647±0.083 (0.24-0.97)	1101	0.665±0.083 (0.17-0.96)	1511	0.069±0.047 (0-0.3)	429	0.082±0.051 (0-0.39)	288
Collagen (0.19 mg/mL)	AUC	0.54±0.075 (0- 0.87)	1104	0.561±0.064 (0.29-0.85)	1517	0.393±0.1 (0.01-0.63)	429	0.428±0.085 (0.03-0.67)	287
ADP (0.95 µmol/L)	AUC	0.168±0.17 (0- 0.9)	1047	0.267±0.22 (0-0.92)	1400	0.108±0.083 (0-0.7)	417	0.124±0.076 (0.01-0.46)	269
ADP (1.82 µmol/L)	AUC	0.413±0.232 (0-0.98)	1102	0.544±0.209 (0.02-0.97)	1510	0.246±0.142 (0-0.84)	431	0.294±0.131 (0.03-0.77)	288
ADP (5.71 µmol/L)	AUC	0.667±0.104 (0.08-0.98)	1102	0.698±0.087 (0.14-0.97)	1511	0.525±0.143 (0.01-0.97)	429	0.579±0.112 (0.1-0.95)	287
Epinephrine (0.1 mmol/L)	AUC	0.54±0.139 (0.01-0.97)	1102	0.576±0.126 (0.01-0.94)	1516	0.249±0.133 (0-0.73)	431	0.266±0.128 (0.02-0.7)	288
Ristocetin (1.5 mg/mL)	AUC	0.73±0.078 (0.03-0.98)	1090	0.747±0.073 (0.01-0.97)	1494	0.715±0.1 (0.03-0.97)	430	0.736±0.087 (0.21-0.96)	286
TRAP-6 amide (670 µmol/L)	AUC	0.723±0.078 (0.42-1)	1103	0.737±0.076 (0.37-0.99)	1517	0.72±0.085 (0.37-0.98)	429	0.738±0.073 (0.5-0.97)	287
AA (500 µg/mL)	Final aggregation (%)	74.136±8.085 (40-100)	1102	75.498±7.962 (43-100)	1511	6.415±5.922 (0-36)	431	7.938±6.224 (0-36)	288
Collagen (0.19 mg/mL)	Final aggregation (%)	73.47±8.863 (0-100)	1105	74.729±7.845 (45-100)	1517	68.406±11.6 66 (0-100)	431	70.573±8.412 (11-100)	288
ADP (0.95 µmol/L)	Final aggregation (%)	21.89±26.079 (0-100)	1047	33.886±31.25 8 (0-100)	1400	9.662±9.122 (0-78)	417	10.309±7.824 (0-51)	269
ADP (1.82 µmol/L)	Final aggregation (%)	47.45±30.876 (0-100)	1102	62.079±27.47 (0-100)	1510	19.93±15.18 9 (0-95)	431	23.142±14.03 9 (0-70)	288
ADP (5.71 µmol/L)	Final aggregation (%)	73.73±12.997 (0-100)	1103	76.317±10.81 6 (4-100)	1512	48.83±18.4 21 (0-100)	429	54.767±15.29 5 (5-100)	287
Epinephrine (0.1 mmol/L)	Final aggregation (%)	71.064±14.404 (4-100)	1103	73.637±13.13 2 (0-100)	1517	33.619±19.6 39 (0-100)	431	35.542±18.82 5 (0-93)	288
Ristocetin (1.5 mg/mL)	Final aggregation (%)	78.481±8.36 (0-100)	1091	80.248±7.9 (0-100)	1494	78.667±10.8 69 (4-100)	430	80.64±8.981 (14-100)	286
TRAP-6 amide (670 µmol/L)	Final aggregation (%)	78.514±8.517 (46-100)	1103	79.933±8.295 (42-100)	1517	77.54±9.009 (40-100)	430	79.39±7.91 (54-100)	287
AA (500 µg/mL)	Lag time	4.24±10.066 (0-79)	1102	3.014±9.042 (0-79)	1511	30.053±22.8 13 (0-187)	431	28.219±18.80 4 (0-105)	288
Collagen (0.19 mg/mL)	Lag time	58.056±21.837 (0-291)	1105	53.075±19.42 4 (0-125)	1517	99.148±37.1 16 (0-301)	431	91.885±32.55 7 (0-218)	287
ADP (0.95 µmol/L)	Disaggregation (%)	3.945±4.379 (0-25)	1047	4.544±5.653 (0-38)	1400	7.482±4.646 (0-22)	417	10.74±5.348 (0-26)	269
ADP (1.82 µmol/L)	Disaggregation (%)	5.287±7.312 (0-34)	1102	3.957±8.078 (0-43)	1510	15.659±7.09 (0-38)	431	18.26±8.223 (0-47)	288
ADP (5.71 µmol/L)	Disaggregation (%)	0.995±3.78 (0- 33)	1103	0.855±3.367 (0-35)	1512	11.925±8.60 6 (0-49)	429	10.948±7.732 (0-37)	287

(B) Optimul aggregometry

Agonist	Measurement	No aspirin (Male)		No aspirin (Female)		Aspirin (Male)		Aspirin (Female)	
		Mean±SD (range)	N	Mean±SD (range)	N	Mean±SD (range)	N	Mean±SD (range)	N
AA	AUC	0.75±0.28 (0.1-1.5)	963	0.81±0.29 (0.1-1.5)	1335	0.28±0.14 (0-0.8)	323	0.27±0.15 (0-1.1)	222
ADP	AUC	1.58±0.36 (0.5-3.4)	1010	1.69±0.38 (0.7-3.6)	1384	1.53±0.31 (0.3-2.7)	398	1.6±0.31 (0.8-2.7)	271
Collagen	AUC	1.3±0.54 (0.2-3.2)	997	1.45±0.59 (0.3-3.2)	1373	1.03±0.47 (0.1-2.5)	388	1.07±0.49 (0.3-2.4)	263
Epinephrine	AUC	1.9±0.55 (0.4-3.9)	1018	2±0.55 (0.2-3.8)	1391	1.53±0.46 (0.2-3.3)	393	1.64±0.45 (0.6-2.7)	267
Ristocetin	AUC	0.81±0.17 (0.1-1.4)	988	0.82±0.17 (0.2-1.3)	1377	0.69±0.12 (0.2-1.2)	384	0.68±0.11 (0.3-1.2)	265
TRAP-6 amide	AUC	2.68±0.16 (0.6-3.1)	941	2.7±0.14 (0.4-3.1)	1222	2.65±0.25 (0-3)	361	2.69±0.1 (2-3.1)	238
U46619	AUC	2.18±0.39 (1.3-3.7)	980	2.27±0.42 (0.6-3.5)	1393	2.11±0.37 (0.4-3.2)	386	2.2±0.38 (1-3.2)	272
AA	E _{max}	85.7±11.06 (10.5-100)	982	87.31±7.41 (20-100)	1356	51.98±21.82 (6.9-100)	339	50.43±20.25 (4.6-98.7)	228
ADP	E _{max}	87.57±4.43 (19.8-100)	1020	88.17±2.96 (66.3-100)	1385	87.02±6.16 (25.5-99.9)	399	88.01±4.06 (48.8-99.9)	271
Collagen	E _{max}	84.48±7.06 (13-100)	1008	85.13±5.64 (32.7-100)	1378	77.46±13.03 (18.1-99)	392	78.95±10.8 (25.7-98.7)	263
Epinephrine	E _{max}	85.16±8.09 (14.6-100)	1027	86.19±7.29 (33.3-99.9)	1394	77.44±13.37 (15.5-99.9)	397	80.45±11.27 (26.2-97.8)	269
Ristocetin	E _{max}	90.77±3.27 (52.1-100)	1004	90.87±2.76 (55.6-100)	1387	90.07±5.09 (29.5-100)	389	90.38±3.61 (48.5-100)	266
TRAP-6 amide	E _{max}	89.94±3.52 (38.8-100)	941	90.06±2.7 (54.1-100)	1222	89.74±4.35 (27.2-99.3)	361	90.09±2.21 (80.4-99.9)	238
U46619	E _{max}	89.77±3.65 (41.4-100)	998	89.6±3.46 (30.1-100)	1398	89.5±5.1 (38.2-100)	393	89.78±2.82 (65.6-99.7)	272

(C) Multiplate impedance aggregometry (MP)

Agonist (concentration)	Measurement	No aspirin (Male)		No aspirin (Female)		Aspirin (Male)		Aspirin (Female)	
		Mean±SD (range)	N	Mean±SD (range)	N	Mean±SD (range)	N	Mean±SD (range)	N
AA (0.5 mmol/L)	AUC	87.52±21.91 (10.2-162.8)	1098	91.51±23.92 (1.1-164.8)	1467	26.49±16.41 (1.3-118.3)	434	23.9±15.2 (2.3-135.6)	274
ADP (3.19 µmol/L)	AUC	66.19±18.52 (22.2-141.8)	1137	77.17±18.84 (18.9-164.9)	1542	64.82±22.28 (10.2-160)	445	77.06±22.19 (9.1-142.2)	289
Collagen (0.061mg/mL)	AUC	55.06±13.97 (7.7-112.2)	1137	57.52±14.74 (9.9-116.1)	1545	42.9±14.4 (4.9-104.5)	444	44.3±14.28 (6-101)	289
Ristocetin (1.15 mg/mL)	AUC	102.37±25.71 (32.9-259.8)	1126	117±28.24 (24.6-236.6)	1519	73.1±26.68 (3.3-162.3)	444	82.28±29.88 (9.1-178.7)	286
TRAP-6 amide (216 µmol/L)	AUC	114.69±21.51 (13.2-207.1)	1137	117.91±21.06 (31-187.1)	1545	113.25±23.63 (26-183.1)	444	122.35±24.37 (33.4-201.7)	288
AA (0.5 mmol/L)	Aggregation (%)	163.38±34.65 (22.1-287.2)	1098	166.37±37.25 (4.7-297.4)	1467	60.24±34.29 (5.1-235.1)	434	54.49±32.53 (5.8-238.9)	274
ADP (3.19 µmol/L)	Aggregation (%)	127.78±34.72 (41.6-255.6)	1137	143.02±33.27 (37.7-308.1)	1542	123.88±41.5 (22.7-277.3)	445	143.01±40.56 (20.4-265.9)	289
Collagen (0.061mg/mL)	Aggregation (%)	142.46±26.99 (39.9-239.2)	1137	145.87±27.67 (27.2-261.2)	1545	116.77±29.31 (13.3-215.2)	444	121.33±30.21 (36.6-229.5)	289
Ristocetin (1.15 mg/mL)	Aggregation (%)	207.82±44.99 (67.3-381)	1126	230.17±49.66 (63.4-423)	1519	161.45±58 (12.8-334.7)	444	177.98±65.99 (29.6-358.9)	286
TRAP-6 amide (216 µmol/L)	Aggregation (%)	196.34±35.41 (26.8-329.6)	1137	198.51±33.96 (53.6-300.6)	1545	192.37±38.76 (48.8-303.2)	444	204.21±39.11 (59.1-320.4)	288
AA (0.5 mmol/L)	Velocity	19.17±4.75 (3.1-45.5)	1098	20.83±5.44 (1.6-44.3)	1467	7.03±3.44 (1.9-23.9)	434	6.68±3.47 (2- 30.1)	274
ADP (3.19 µmol/L)	Velocity	14.04±3.7 (5.3-35.4)	1137	16.51±3.9 (5.3-36.2)	1542	13.87±4.45 (3.6-42)	445	16±4.07 (4- 26.2)	289
Collagen (0.061mg/mL)	Velocity	16.37±2.98 (5.5-32)	1137	17.26±3.22 (4.4-30.4)	1545	13.46±2.97 (3.3-27.9)	444	14.33±3.19 (4.9-27.4)	289
Ristocetin (1.15 mg/mL)	Velocity	32.34±9.24 (7.6-88.2)	1126	36.89±10.31 (8.4-80.4)	1519	18.59±7.85 (3.2-57.2)	444	20.82±8.5 (4.9-46.4)	286
TRAP-6 amide (216 µmol/L)	Velocity	25.81±5.37 (4-51.4)	1137	27.68±5.65 (6.9-48.8)	1545	25.42±5.82 (6.3-45.9)	444	28.2±6.27 (8.4-47.8)	288

(D) Total Thrombus-formation Analysis System (T-TAS)

Agonist (concentration)	Measurement	No aspirin (Male)		No aspirin (Female)		Aspirin (Male)		Aspirin (Female)	
		Mean±SD (range)	N	Mean±SD (range)	N	Mean±SD (range)	N	Mean±SD (range)	N
Collagen (PL chip)	AUC	307.35±82.73 (7.4-458.5)	384	312.61±93.34 (2.7-481.9)	530	174.34±102.94 (3.2-395.3)	126	202.69±99.11 (5.2-447.1)	89

(E) Flow cytometry

Agonist (concentration)	Measurement	No aspirin (Male)		No aspirin (Female)		Aspirin (Male)		Aspirin (Female)	
		Mean±SD (range)	N	Mean±SD (range)	N	Mean±SD (range)	N	Mean±SD (range)	N
ADP (20 µmol/L)	PAC1 % positivity in whole blood	20.25±15.22 (0-87.1)	983	23.85±16.41 (0-87)	1277	18.63±14.98 (0-71.6)	371	23.65±16.84 (0-76.2)	237
ADP (20 µmol/L)	P-selectin % positivity in whole blood	9.45±6.44 (0-44.9)	983	12.8±8.45 (0-54.3)	1277	9.32±7.1 (0- 54.7)	371	12.4±8 (0- 36.4)	237
ADP (20 µmol/L)	PAC1 and P- selectin % double- positivity in whole blood	9.02±6.81 (0-47.1)	983	11.97±8.78 (0-61.3)	1277	9.01±7.6 (0- 42.9)	371	11.63±8.5 (0-45.1)	237
ADP (20 µmol/L)	PAC1 % positivity in PRP	14.38±12.46 (0-69.7)	974	15.8±13.59 (0-73.5)	1254	13.61±12.87 (0-61.6)	365	15.38±13.71 (0-57.2)	238
ADP (20 µmol/L)	P-selectin % positivity in PRP	31.27±12.54 (0.9-71.9)	974	35.74±13.12 (0-75.2)	1254	31.04±12.68 (0-60.7)	365	33.77±13.17 (0-66.2)	238
ADP (20 µmol/L)	PAC1 and P- selectin % double- positivity in PRP	10.39±8.55 (0-46.3)	974	11.87±9.83 (0-51.7)	1254	9.95±8.68 (0-39.8)	365	11.49±9.75 (0-41.2)	238

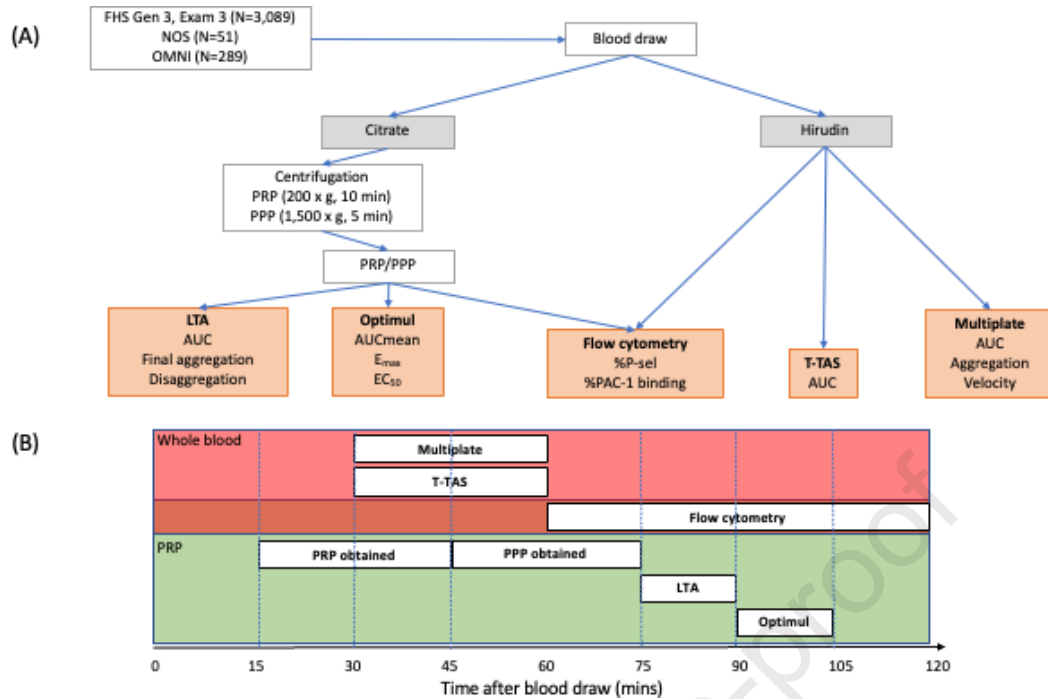


Figure 1. Daily workflow by (A) assay and (B) timeline. For all experiments, blood was drawn and either kept as whole blood or processed by centrifugation for platelet rich plasma (PRP) and platelet poor plasma (PPP). Light transmission aggregometry (LTA), Optimul aggregometry, Multiplate impedance aggregometry, Total Thrombus-formation Analysis System (T-TAS) and flow assays were then conducted as soon as possible and within a 4 hour timeframe. AUC: area under the curve; P-Sel: P-selectin; PAC-1: procaspase activating compound-1.

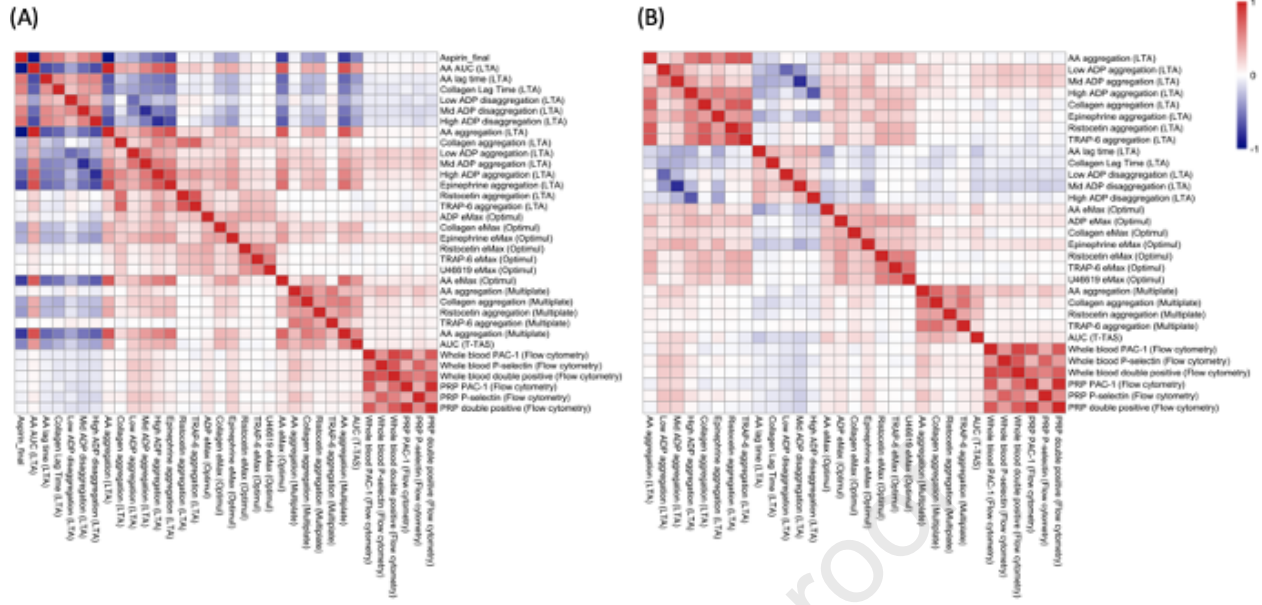


Figure 2. A Correlation matrices of the five platelet assays in (A) all FHS participants (N=3,429) and in (B) participants without aspirin-use (N=2,693). Darker red shows high positive correlation, darker blue shows high negative correlation and white shows no correlation.

