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Estimating short- and long-term reference change values and index of individuality for tests of platelet function

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ABSTRACT

Background: In order to manage risks of bleeding and thrombosis after some surgical procedures, platelet function is often measured repeatedly over days or weeks using laboratory tests of platelet function. To interpret test results in the perioperative period, it is necessary to understand analytical, biological and between-person variation.

Methods: We collected three separate blood specimens from 16 healthy volunteers on the first study day, and one additional specimen from each volunteer 1, 2, and 3 months later. Arachidonic acid-induced and adenosine diphosphate (ADP)-induced platelet function were measured in duplicate by whole blood impedance aggregometry using Multiplate (ASPI/ADP tests) and VerifyNow (Aspirin Reaction Units [ARU] and P2Y12 Reaction Units [PRU]). The analytical variation (CV_A), within-subject variation (CV_I), between-subject variation (CV_G), index of individuality (II), and reference change values (RCV) were calculated.

Results: VerifyNow ARU demonstrated the smallest short-term and long-term variability (CV_A, CV_I, and CV_G ~1%), resulting in short- and long-term RCV values < 5%. II was also higher (1.92) for VerifyNow ARU than other platelet function tests. Multiplate ASPI and ADP tests had the highest RCV both short-(19.0% and 25.2%, respectively) and long-term (32.1% and 39.6%, respectively) due to increased CV_A (> 5%) and CV_I (3.9–13.1%). VerifyNow PRU had a lower RCV than Multiplate ADP; but was the only test with II < 0.6.

Conclusions: VerifyNow ARU results can be interpreted relative to a fixed cut-off or population-based reference interval; or relative to small changes in an individual's previous values. VerifyNow PRU and Multiplate ASPI and ADP tests should only be interpreted based upon relative change; and can only distinguish relatively large (> 23%) changes over several weeks.

1. Introduction

Long-term mechanical circulatory support (MCS) is used as a bridge to cardiac transplant, a bridge to recovery in patients with myocardial damage, and a destination therapy for end-stage heart failure patients who are not candidates for transplant [1]. These patients are both prothrombotic and exhibit a tendency to bleed. During the perioperative period, up to 60% of patients experience excess bleeding; while historically 5–20% have experienced thromboembolism or stroke [1]. Although some newer continuous flow LVAD devices demonstrate lower rates of systemic thromboembolism and bleeding, device thrombosis continues to be a problem [2].

To balance the risk of perioperative bleeding with device thrombosis and thromboembolism, protocols for MCS placement call for titration of antiplatelet agents using laboratory tests of platelet function. Tests of arachidonic-induced platelet function are used to titrate aspirin and adenosine diphosphate (ADP)-induced platelet function to titrate agents such as dipyrimidole and clopidogrel [3–5]. However there is little evidence to suggest that platelet function testing (PFT) is precise or reliable enough to allow for monitoring of platelet function over short periods of time.

Thromboembolic complications are also the most common cause of

Abbreviations: ADP, adenosine diphosphate; ARU, aspirin response units; PRU, P2Y12 reaction units; CV_A, analytic coefficient of variation; CV_I, biologic (within-subject) coefficient of variation; CV_G, between-subject coefficient of variation; II, index of individuality; RCV, relative change value; MCS, mechanical circulatory support; PFT, platelet function testing; PED, pipeline embolization device; AUC, area under the curve

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morbidity after neurointerventional procedures, especially with use of intra-arterial flow diverters such as the pipeline embolization device (PED). Dual antiplatelet therapy with aspirin and clopidogrel is nearly universal before and after PED placement; with protocols to monitor response with PFT increasingly common [6–9]. Some studies demonstrated that monitoring platelet function and tailoring antiplatelet agents using PFT results improved outcomes after PED procedures [6–9]. One investigator has also demonstrated wide dynamic variability in individual response to clopidogrel after PED placement, requiring repeat measurement and titration to remain in desired therapeutic ranges [7,9]. However other studies have found that PED outcomes in centers that did not monitor PFT were better than those that did [10]; or that risk of bleeding or thrombosis was not higher in patients with higher on-treatment platelet function [11].

In these and other critical care settings, PFT may be monitored over days or weeks to determine whether a patient's risk for coagulopathy/bleeding has increased, or to determine whether antiplatelet medications have affected platelet function. In order to use PFT in this context, it is necessary to know whether these tests can be interpreted relative to a fixed clinical cut-off or reference interval; or whether only "delta" values (changes relative to a patient's baseline value) should be used to interpret results. Traditionally, this question can be answered by studying biological variation (CV_I) and calculating parameters such as index of individuality (II) and reference change value (RCV) [12]. While biological variability studies are performed on healthy individuals, the data obtained allow both definition and comparison (between tests) of the minimum detectable changes in defining disease states or changes in patient condition (e.g. the troponin RCV or "delta" used to suspect acute coronary syndrome). In this study, we used a repeated measurement design [13,14] in order to estimate short- and long-term RCV and II for VerifyNow and Multiplate tests of arachidonic acid-induced and ADP-induced platelet function. The results help determine how changes in PFT should be interpreted over days and months.

2. Materials and methods

2.1. Subjects and samples

Sixteen apparently healthy volunteers (8 male and 8 female) who denied taking aspirin-containing or nonsteroidal anti-inflammatory products for at least 10 days, and had no history of abnormal bruising or bleeding were recruited for the study. Blood samples were collected by venipuncture into 2.0 mL 3.2% sodium citrate tubes (Greiner Bio-One) for VerifyNow testing and Multiplate 3.0 mL hirudin tubes for testing on the Multiplate instrument. Study volunteers had blood drawn on 3 occasions on the day of study enrollment (with each draw occurring at least one hour apart), and returned for a single blood draw 1, 2, and 3 months after the initial measurements. Study volunteers were asked to refrain from taking aspirin or nonsteroidal medications for at least 10 days before each blood draw. All measurements were performed in duplicate within two hours of each blood draw on the same VerifyNow or Multiplate instrument by two testing personnel, for a total of 96 measurement pairs. All VerifyNow cartridges and Multiplate cuvettes were from the same lot and received as a single shipment. The study protocol was reviewed and approved by the Mayo Clinic Institutional Review Board.

2.2. Platelet function assays

Multiplate impedance aggregometry (Dynabyte, Munich Germany) is a whole blood impedance aggregation system that records platelet aggregation in a single cell using a dual sensor unit and Teflon-coated stirring magnet. Electrical resistance between sensing wires is continuously recorded, with platelet activation resulting in increased electrical resistance. Multiplate utilizes whole blood collected into hirudin blood collection tubes, with separate measuring cells for the ASPI test (0.5 mmol/L arachidonic acid-induced platelet activation) or ADP test (6.5 umol/L ADP-dependent platelet activation). Results are expressed in area under the curve (AUC) arbitrary units reflecting the area under the aggregation curve over 6 min [15].

VerifyNow (Accumetrics, San Diego CA) is a whole blood optical aggregation system that utilizes fibrinogen-coated beads to perform a turbidimetric optical aggregation measurement in a disposable cartridge. Whole blood collected into Greiner 3.2% citrate tubes is automatically dispensed from the blood collection tube into the cartridge by the instrument. One cartridge is available to measure arachidonic acid-induced platelet function (VerifyNow aspirin assay); using arachidonic acid to activate platelets and measuring the increase in light transmission as activated platelets bind fibrinogen-coated beads, with the results expressed in Aspirin Reaction Units (ARU). Another cartridge (VerifyNow P2Y12 assay) uses the same measurement principle to measure ADP-induced platelet function and reports in P2Y12 Reaction Units (PRU) [15].

2.3. Statistical analysis

A repeated measures design was used to assess both short-term and long-term variability in arachidonic acid-induced and ADP-induced platelet function. The study design, including number of healthy donors included and statistical approach [16–21], was similar to that described for biological variation studies of clot-based coagulation tests and chemical analytes such as glycated hemoglobin. First, the data for each volunteer was assessed to determine if it was sufficiently Gaussian by using the Shapiro-Wilk test (with $p < 0.05$ indicating a deviation from normality) and calculating the percentage of tests violating the normality assumption. Based upon previous guidelines and studies, sufficient normality was defined as over 50% of patients demonstrating normality for all analytes [21]. The means of the volunteers' data was also assessed using the Shapiro-Wilk test. After assuring normality, the analytic variation was assessed for VerifyNow and Multiplate tests by using all 12 observations per donor in a mixed linear model with dependent variable being the measured platelet function and random effects parameters of donor and draw nested within donor. All 12 observations were used because platelet function tests cannot be performed on frozen samples and the analytical variation should not change over this study timeframe. Draw is defined as each specimen taken from the donor, so there are 6 draws per donor, with each draw measured twice. The residual variance from this model, after removing the effects of donor and draw within donor, is the analytic variance for these tests, denoted as SD_A. The analytic coefficient of variation, CV_A, is this variance divided by the overall mean for each platelet function test. Next, short-term variation was assessed for VerifyNow and Multiplate tests using the 6 measurements per patient collected on the first study day. Mixed linear models with the dependent variable being the measured platelet function and random effects parameters of donor and replicate within the first day nested within donor were used to estimate the within-person (biological) and between-person variances, denoted by SD_I and SD_G, respectively. Dividing these variances by the overall mean for each measure resulted in the appropriate coefficient of variations (CVs). Long-term variation was assessed for each of the measurements using the first duplicate pair from the first study day, along with the data from the 1-, 2- and 3-month assessments. Only the first duplicate pair was used to assess the long term variation because of the slight possibility that the subsequent sampling would have activated the platelet aggregation process, impacting the test results. Mixed linear models with the dependent variable being the measure of platelet function and random effects parameters of donor and day nested within donor were used to estimate the long-term within-person (biological) and between-person variances, expressed as CV_I and CV_G, respectively. The relative change value (RCV), traditionally referred to as the 'critical difference', was calculated as:

$$RCV = 2^{1/2} * Z * [(CV_A^2 + CV_I^2)]^{1/2},$$

with z = 1.965, representing a two-directional 95% probability. To assess the utility of population-based reference intervals, we calculated the index of individuality (II) as:

$$II = ([(CV_A^2 + CV_I^2)]/CV_G)^{1/2}.$$

To test for significant differences in CV_A, CV_I, and CV_G among the four platelet function tests, we used an asymptotic test for the equality of coefficients of variation from k populations and the modified signed-likelihood ratio test (SLRT) for equality of CVs, implemented with the R package cvequality (Version 0.1.1) [18,19]. SAS version 9.4 was used for all other analyses presented.

3. Results

Each donor in the study had 6 blood collections, assayed in duplicate on both testing platforms yielding 12 analytical results per platelet function test per person. Three specimens were collected on the first study day, and were used to estimate the short-term biologic variability and RCV. More than 56% of volunteers' data followed a Gaussian distribution with no transformations needed, and the means of volunteers' data were also sufficiently normal (all $p > 0.11$). Analytic precision of the VerifyNow ARU test was significantly better (CV 1.2%) than the other PFT (all 5–6%, both p-values < 0.00001, Table 1). Biologic (within-subject) variability was also smaller for VerifyNow ARU than VerifyNow PRU or Multiplate ASPI or ADP tests (both p-values < 0.00001, Table 1). The result was a very small short-term RCV estimate of 4.1%, such that changes in VerifyNow ARU of > 4.1% would represent a change in patient condition (effect of aspirin or change in platelet function). In contrast, short-term RCV estimates for the other PFT ranged from 16 to 25% (Table 1).

Long-term biological and between-subject variability demonstrated similar trends. Both within- and between-subject long-term variability for VerifyNow ARU were very low (< 1%), much smaller than the other PFT (all p-values < 0.00001). The result was an RCV estimate < 5% even over 3 months (Table 2). In contrast, higher biological and between-person variability resulted in a much higher RCV of 32% for the Multiplate ASPI test, the other arachidonic acid-induced PFT for assessing aspirin response.

Both VerifyNow and Multiplate tests for ADP-induced aggregation had analytic precision in the 5–6% CV range and higher between-person variability of 15–19%. Lower biological variation for the VerifyNow PRU test led to a lower RCV compared to the Multiplate ADP test (Table 2). Among PFT studied only VerifyNow ARU had an II > 1.4, indicating that interpretation relative to fixed population-based reference intervals is appropriate (Table 2). Figs. 1 and 2 show the individual median values and range of results obtained for arachidonic acid-induced and ADP-induced platelet function testing, respectively.

4. Discussion

In order to balance the risk of bleeding and thrombosis, protocols for both MCS and neurointerventional procedures recommend monitoring platelet function frequently and titrating antiplatelet agents based upon these results [3–9]. However in order to effectively utilize these tests in these settings, it is important to understand how changes in platelet function over a given period of time should be interpreted—relative to a fixed decision limit or population-based reference interval; or relative to a "delta" or change from an individual patient's previous value. This information is typically derived from studies of biological variation. Relative change value (RCV) is a function of analytic and biologic variability, and defines the percent change or "delta"

Table 1
Short-term (1 day) analytical and biological (within-person) variation, and estimate of short-term RCV for VerifyNow and Multiplate tests.

Analyte	Mean	CV_A, %	CV_I, %	CV_G, %	RCV, %
VerifyNow® ARU[a]	657 ARU	1.2	0.9	0.6	4.1
MultiPlate® ASPI[a]	109 U	5.7	3.9	17.8	19.0
VerifyNow® PRU[b]	269 PRU	4.8	3.2	14.6	16.0
Multiplate® ADP[b]	93 U	5.6	7.2	21.1	25.2

[a] arachidonic acid-induced platelet function,
[b] ADP-induced platelet function.

Table 2
Long-term (3 months) analytical and biological (within-person) variation, and estimate of long-term RCV and Index of Individuality, for VerifyNow and Multiplate tests.

Analyte	Mean	CV_A, %	CV_I, %	CV_G, %	II	RCV, %
VerifyNow® ARU[a]	655 ARU	1.2	0.9	0.8	1.92	4.1
MultiPlate® ASPI[a]	107 U	5.7	10.1	14.2	0.82	32.1
VerifyNow® PRU[b]	269 PRU	4.8	6.8	14.6	0.57	23.1
Multiplate® ADP[b]	92 U	5.6	13.1	19.0	0.75	39.6

[a] arachidonic acid-induced platelet function,
[b] ADP-induced platelet function.

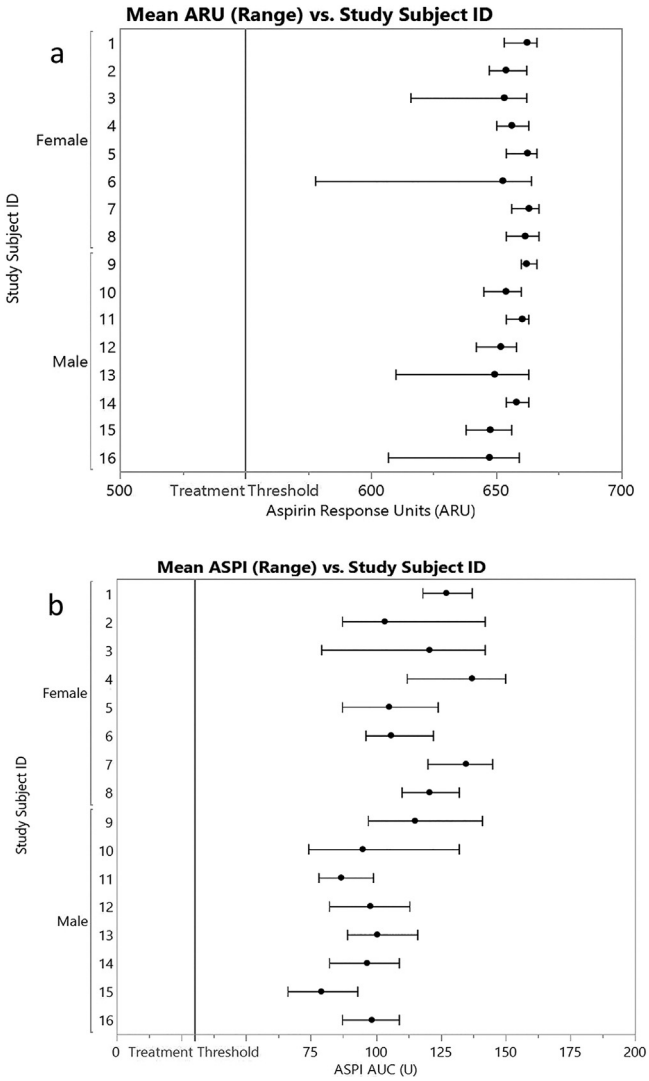

Fig. 1. Mean value, and range of values observed in 16 healthy volunteers over 3 months, for arachidonic acid-induced platelet function as measured by VerifyNow (Fig. 1a) and Multiplate (Fig. 1b). The solid line on each figure represents published or commonly used thresholds to define aspirin response.

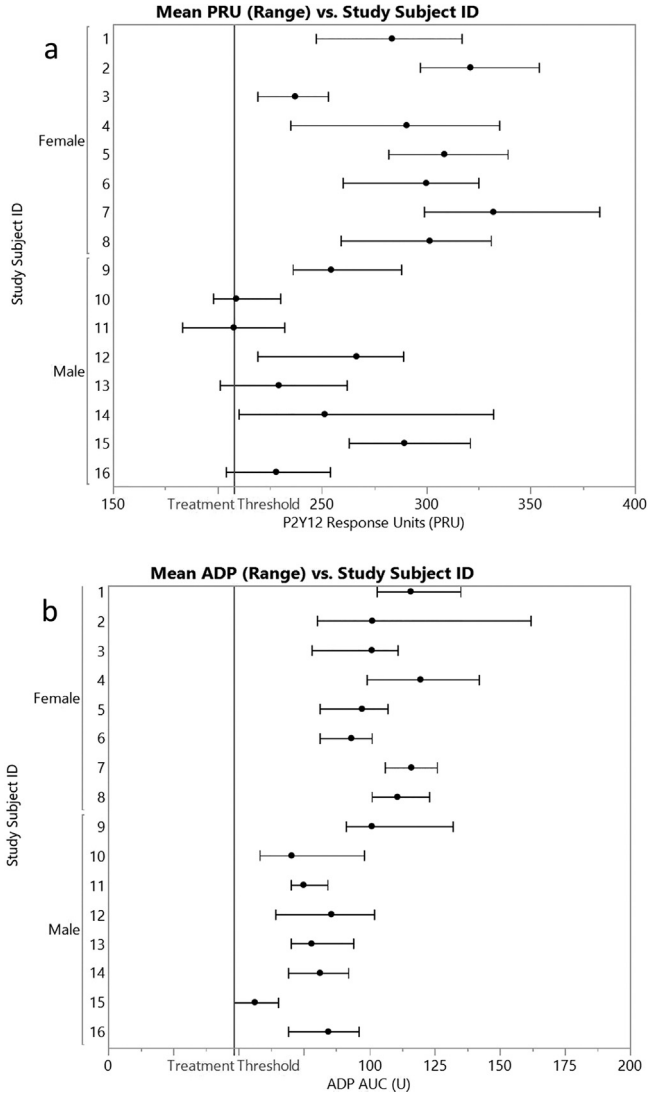

Fig. 2. Mean value, and range of values observed in 16 healthy volunteers over 3 months, for ADP-induced platelet function as measured by VerifyNow (Fig. 1a) and Multiplate (Fig. 1b). The solid line on each figure represents published or commonly used thresholds to define clopidogrel response.

that represents a change in physiologic state of a patient [12].

For troponin studies, investigators have defined a short-term RCV to interpret changes over hours for diagnosis of acute cardiac events; and a long-term RCV to interpret troponin changes over weeks or months [22]. The II is used to evaluate the utility of conventional population-based reference values through an objective calculation that incorporates analytic variability (CV_A), biological variation (CV_I), and between-subject variation (CV_G). Conceptually, II is the ratio of biological (within-subject) to between-subject variability. When the II is high, e.g. > 1.4, tests can be interpreted according to a population-based reference interval or fixed clinical decision limit. However, when II is low, < 0.6, reference intervals are of less value because between-person variability may cause individual values in healthy and disease (or treated and untreated) populations to overlap [12].

Both analytical and biological variation are difficult to measure for PFT due to limited sample stability and platelet activation occurring during blood sampling and processing [23–26]. One previous study did evaluate biologic variability (CV_I), analytic variability (CV_A), and between individual (CV_G) for PFT by measuring PFT once per week over four weeks in healthy dogs. This study used 3 PFT, of which only the Multiplate ASPI and ADP tests were included in our study. In this study, between-subject variability (CV_G) was much higher than biologic (CV_I) variability for all but one method studied. This resulted in II < 0.6 for Multiplate ASPI and ADP methods. The authors concluded that Multiplate results should not be interpreted using reference intervals, as values between healthy and diseased populations could overlap due to large between-subject variability [27].

In our study, we found that II was > 0.6, but less than the 1.4, for both Multiplate ASPI and ADP tests. Compared to results in the study of healthy dogs, we did observe higher biological variation in humans over 3 months than observed in dogs over one month. This resulted in slightly higher II values. Whereas RCV values of 1–20% over one month were obtained in the study of healthy dogs, we obtained RCV values of 30–40% over 3 months in healthy humans. It is unclear whether the time period, study design, or difference in platelet function between humans and dogs is responsible for the differences in study findings. Another study in humans found lower biological and between-person variability for arachidonic acid-induced and ADP-induced platelet function by light transmission aggregometry, but this study used only two samples collected on consecutive days and did not perform measurement in duplicate to assess analytical variability [28].

The VerifyNow ARU test had very low analytical, biological and between-person variation over 3 months. The result was an II > 1.4, indicating that this test can be interpreted relative to a population-based reference interval or fixed clinical decision limit. Fig. 2a demonstrates that all values obtained in all subjects over 3 months were well above the cut-off of 550 ARU commonly used to define aspirin response [29]. Therefore a patient on aspirin with ARU < 550 ARU can be considered to have platelet function that differs from that expected in a healthy population. In addition, changes in VerifyNow ARU as small as 5% can be interpreted to represent changes in platelet function (whether induced by antiplatelet agent or disease state) rather than effects of analytical and biological variation. While the II for Multiplate ASPI was in the intermediate range of 0.6–1.4, all healthy volunteers had values > 30 U, the treatment threshold for aspirin effect determined in previous studies [30].

Fig. 2b demonstrates that for VerifyNow PRU results, between-subject variability greatly exceeds biologic variability. In fact, between-subject variability is more than 2-fold greater than biologic variability for VerifyNow PRU (Table 2). The result is that some healthy individuals have VerifyNow PRU values that are centered near the commonly used clinical cut-off of 208 PRU used to define clopidogrel response [31], while others have values centered around 300 PRU (Fig. 2b). Multiplate ADP demonstrated an intermediate II between 0.6 and 1.4, but all volunteers had values above 46 U, the commonly used threshold for clopidogrel response [30].

Index of individuality for VerifyNow PRU is < 0.6, suggesting that this test should not be interpreted relative to a population-based reference interval or fixed decision clinical decision limit. One study demonstrated that intrinsic (pre-treatment with clopidogrel) platelet function is the strongest predictor of on-treatment response [32]. Thus while our study was limited to healthy volunteers not taking antiplatelet agent therapy, available evidence suggests between-person variability observed in healthy adults presents a limitation to use of these tests as predictors of antiplatelet agent response. Large between-subject variability, resulting in a low estimated index of individuality, suggests that VerifyNow PRU and Multiplate ADP should not be interpreted with respect to a fixed clinical decision threshold or population-based reference interval. Despite this, clinical guidelines do suggest interpreting PFT values using a fixed treatment threshold for patients on clopidogrel [31,33].

While the study was not powered to calculate sex-specific variability, PRU and ADP values in male subjects trended lower than in females and only male subjects had PRU or ADP values that overlapped with common treatment thresholds (Fig. 2). Two previous studies found that female sex was associated with higher on-treatment platelet reactivity when VerifyNow was used to assess clopidogrel response

[34,35]. In contrast, one of these studies found that females exhibited lower platelet reactivity by Multiplate [35]. Effects of gender on platelet function and response to antiplatelet agent therapy merit further study. Studies have found that clopidogrel response as measured by VerifyNow is also affected by diabetes and smoking status [36,37]. Whatever the causes of between-subject variability, it is clear that large between-subject variation observed among healthy individuals confounds interpretation of ADP-induced platelet function using both VerifyNow and Multiplate. RCV can be used to interpret changes from an individual patient's previous values, with changes of 23% or more (for VerifyNow) representing a change in platelet function for an individual patient that cannot be explained by analytical and biological variability.

The primary methodologic limitation to our study was the inability for PFT to measure all samples from one patient on a single day or with a single analytical run. However, all VerifyNow cartridges and Multiplate cuvettes were from the same lot and received as a single shipment to the laboratory. Two study personnel performed all testing on one VerifyNow and one Multiplate instrument to minimize analytical variation over time. As both VerifyNow and Multiplate testing relies upon single use disposable measurement cells, conceptually no greater analytical variation is introduced by testing over 3 months as opposed to testing in one day. Analytical variation may be greater if testing is performed in different laboratories, by more testing personnel, or on multiple instruments. Analogous to biological variation studies for troponin and hemoglobin A1c, measurement of RCV does not define optimal treatment response. The RCV obtained from biological variation studies defines the minimum change that can be measured over days or weeks to assess for changes in patient condition, for patients starting with normal platelet function. Future studies are needed to determine whether patients on antiplatelet agent therapy have similar biological variation.

5. Conclusion

For VerifyNow ARU, very low biologic and between-subject variability, combined with low analytic imprecision, allows interpretation of results relative to a fixed cut-off or population-based reference interval. Very small (less than 5%) changes in VerifyNow ARU represent changes in platelet function for an individual patient. In contrast, VerifyNow PRU and Multiplate ASPI and ADP tests demonstrated greater between-subject variability; resulting in II < 1.4 and confounding interpretation of results relative to a fixed clinical decision limit or reference interval. Relative change value for VerifyNow PRU was smaller (23%) than for Multiplate ADP (40%), suggesting that VerifyNow may be more effective than Multiplate for monitoring both arachidonic acid and ADP-induced platelet function over days or weeks.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clinbiochem.2019.10.001.

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