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Assessing prognosis by quantifying FcγRIIa on fixed platelets

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ABSTRACT

Introduction: FcγRIIa amplifies platelet activation and higher platelet FcγRIIa identifies patients at greater risk of subsequent cardiovascular events. We report the accuracy and precision of a modified test to quantify FcγRIIa on previously fixed platelets (pFCG test).

Methods & results: An antibody clone (5G1) was developed after exposure of mice to formaldehyde treated FcγRIIa. Accuracy and precision of the modified test was evaluated with biologic specimens (platelets) and engineered synthetic cells conjugated with FcγRIIa (Slingshot Biosciences). The modified pFCG test on fixed platelets (using 5G1) consistently identified modestly more (~300 molecules) of FcγRIIa on platelets compared with the pFCG test on nonfixed platelets (using clone FL18.26). With biologic specimens, the intra-assay coefficient of variation (CV) was 2.1 $\pm$ 0.1% (standard error of the mean, n = 750). The interassay CV was assessed intraday (4.5 $\pm$ 1%) and interday (up to 5 days after fixation, 6.5 $\pm$ 0.4%, n = 50). The pFCG test performed on Slingshot Synthetic cells conjugated with FcγRIIa demonstrated accuracy, linearity (R^2 = 0.984) and similar interassay CV both intraday (2% $\pm$ 0.6%) and interday (20 nonconsecutive days, 9.9% $\pm$ 2.1%).

Conclusion: In summary, modification of the pFCG test to be performed on fixed platelets allows accurate quantification of pFCG with high precision.

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KEYWORDS
accuracy; biomarker; platelet; precision; prognosis

1. Introduction

FcγRIIa was originally identified as the low-affinity receptor for the fragment constant (Fc) portion of IgG that mediates interaction between platelets and immune complexes [1]. More recently, a new function of FcγRIIa has been delineated. In addition to its function to directly activate platelets, FcγRIIa amplifies platelet activation. During the cytoskeletal rearrangement that accompanies the activation of platelets, FcγRIIa clusters in lipid rafts, is cross-linked and phosphorylated [2,3]. This phosphorylation of FcγRIIa amplifies the activation of platelets. Greater platelet FcγRIIa (pFCG) is associated with increased platelet reactivity [4]. We have found that high pFCG is associated with a ~fourfold greater risk of death, myocardial infarction (MI) and stroke [5]. These results suggest that the pFCG test may be useful to guide individualized therapy.

Platelet function testing that assesses platelet reactivity requires activation of platelets *in vitro* in response to a specific concentration of agonist or group of agonists [6]. Technical aspects of the test including phlebotomy, the anticoagulant used to prevent blood clotting and time elapsed before sample processing influence results [7–9]. Platelet function tests exhibit substantial intraindividual variability during the course of 1 day [10]. Unlike platelet function tests, the pFCG test that quantifies the expression of FcγRIIa on platelets is a test with high precision that is not influenced by anticoagulants, antiplatelet agents, or activation of platelets [11].

Because storage can lead to degradation of platelets, the pFCG test was modified to be performed on fixed platelets. This modification required the generation of an antibody that bound effectively to FcγRIIa on the surface of platelets that had been previously fixed. We compare the accuracy and precision of the pFCG test performed on fixed platelets with the original test that was performed on platelets prior to fixation. The analytic validation was performed with both biologic specimens (fixed platelets) and TruCytes^{TM} synthetic cells conjugated with FcγRIIa (from Slingshot Biosciences Inc, CA USA). Slingshot TruCytes^{TM} are synthetic cells with specific protein markers embedded on the surface to mimic cells of interest. By integrating biochemistry, high-precision manufacturing and polymer chemistry, Slingshot utilizes

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cell printing technology to engineer synthetic cells customized to mimic biological cells.

2. Methods

2.1. Development of an antibody that binds to FcγRIIa on fixed platelets

Purified FcγRIIa (produced by mammalian cells – human embryonic kidney 293 cells) that was demonstrated to be >95% pure and endotoxin free was used for antibody production. Before injection, FcγRIIa in solution was exposed to formaldehyde (2%) for 4 h. Subsequently the formaldehyde treated FcγRIIas was injected into mice at Green Mountain Antibody (Winooski, Vermont). Mouse serum and cell culture media from hybridomas were screened for antibodies that bound to FcγRIIa that had been exposed to formaldehyde. A direct immunoassay with the formaldehyde treated FcγRIIa was used to screen for the presence of antibodies of interest. To identify clones producing antibodies with high binding affinity, competition immunoassays were performed (equal concentrations of FL18.26 and candidate clones were used). Clones whose antibodies competed (effectively with an antibody (FL18.26, BD Biosciences, NJ USA) that we have used in our previous work [5,11] were selected for further evaluation. For these immunoassays, we used FcγRIIa that had not been exposed to formaldehyde. This approach enabled us to identify clones that not only bound 'fixed' FcγRIIa but did so with high affinity.

Candidate clones were evaluated with a series of additional tests. We used Chinese hamster ovary (CHO) cells stably transfected with FcγRIIa to assess binding of the clones with the use of fluorescence microscopy and flow cytometry. In addition, antibodies from candidate clones were used to perform immunoprecipitation with platelet lysates. These experiments confirmed that binding was specific to FcγRIIa (no other proteins were immunoprecipitated). Based on these screening tests, a high binding candidate clone (5G1) was selected.

Western blot analysis was performed on cell lysates of CHO cells, CHO cells stably transfected with FcγRIIa and platelet lysates. CHO cell adhesion was released with trypsin and suspended cells were washed with phosphate buffered saline. Platelets were isolated from platelet rich plasma with the use of gel filtration over Sepharose CL2B. Lysates were prepared by addition of 2× lysis buffer. Protein concentration was determined with a BioRad protein assay and 50 μg of CHO and platelet lysate was loaded on a BioRad mini polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane. After blocking with 5% milk powder, the membrane was incubated with 1 μg/ml of 5G1 or FL18.26 antibody for 1 h. Subsequently a goat anti mouse IRDye700 antibody for 1 h. The membrane was scanned on a Licor Odyssey system.

2.2. Analytic validation of pFCG test

Both biologic specimens (platelets from patients) and TruCytes™ synthetic cells conjugated with FcγRIIa (Slingshot Biosciences) were used to assess the precision and accuracy of the test performed on fixed platelets.

Biologic specimens were obtained from subjects who provided written informed consent to participate in protocols approved by the Institutional Review Board of the University of Vermont (Federalwide Assurance: FWA00000727, study: STUDY00001852) and WCG (a centralized institutional review board, Federalwide Assurance: IRB00000533, study: 20211492). In protocols approved at the University of Vermont subjects were recruited to quantify pFCG. The WCG protocol is a 25-center 800-patient study in patients with MI. This is a prospective observational (noninterventional) study designed to validate the prognostic implications of the pFCG test. The study enrolled patients from January 2022 to September 2023. Enrolled patients had at least 2 of the following: age ≥65, multivessel coronary artery disease, prior MI, chronic kidney disease (defined as glomerular filtration rate <60 ml/min/1.73 m^2), or diabetes mellitus. Both ST elevation MI and non-ST elevation MI were included. Citrate anticoagulated (trisodium citrate, 3.2%) blood was taken at the enrolling hospital, platelets were fixed and samples were shipped to a core laboratory where the pFCG test was performed. Citrate anticoagulated blood was stored at room temperature until fixation that was performed within 24 h. Fixation was performed by exposure of blood samples to 0.5% formaldehyde (final concentration, Medium A diluted with phosphate buffered saline, BD Biosciences). After 15 min, samples were diluted with phosphate buffered saline 1:10 to reduce formaldehyde concentration to 0.05% for storage. After fixation, platelets were stored at 4°C until assays were performed.

TruCytes™ synthetic cells conjugated with FcγRIIa were manufactured by Slingshot Biosciences. The engineered synthetic cells were made by Slingshot Biosciences using a proprietary cell printing technology that can be customized to resemble a range of cell types. Through the combination of biochemistry, high-precision manufacturing and polymer chemistry, the hydrogel-based particles were optimized with respect to their forward and side scatter properties. Additionally, the surface of the particles was chemically modified with purified FcγRIIa at various levels of protein loading, representative of a broad range of protein expression on biological samples. The antibody accessible FcγRIIa molecules were

then quantified in the multilevel cell mimic, using target MESF (Molecules of Equivalent Soluble Fluorochrome) values reflective of those observed on biological samples, providing utility for quantification of FcγRIIa in the pFCG test. The TruCytes™ synthetic cells were stored in a lyophilized format to be ready to use in the pFCG test. Slingshot performed an accelerated stability study and demonstrated that the product exhibited accelerated stability over 18 months when stored in lyophilized form at -20°C. When reconstituted in phosphate buffered saline with 0.2% bovine serum albumen, TruCytes™ were stable for 30 days when stored at 4°C.

2.3. pFCG test

In the pFCG test, platelet FcγRIIa is quantified with the use of flow cytometry. In preliminary experiments, we ensured that saturating concentrations of anti-FcγRIIa antibodies were used. We used 0.5 μg of 5G1 and FL18.26. In the original test, nonfixed platelets in whole blood (3 μl) were added to 50 μl HEPES-Tyrode's buffer plus 20 μl (0.5 μg) of phycoerythin (PE) labeled anti-CD32 (BD Biosciences). After 15 min at room temperature, platelets were fixed and red blood cells lysed by the addition of 100 μl of Optilyse-C (Beckman Coulter). After an additional 15 min incubation at room temperature, samples were diluted in 1.5 ml of HEPES-Tyrode's buffer to enable assessment of surface expression of FcγRIIa on individual platelets. In the pFCG test performed on fixed platelets, red blood cells in the sample were lysed osmotically (Medium B, ThermoFisher, MA USA) and then platelets were washed 3× by centrifugation (1200 g for 2 min). Washed platelets were stained with anti-FcγRIIa - PE (0.5 μg, 5G1) and 2 μl CD42b-PE/CY5 (clone HIP1, BD Biosciences) to identify platelets.

Flow cytometric analysis was performed with the use of a Attune NXT instrument (ThermoFisher). Platelets were identified on the basis of size (forward and side scatter) and expression of CD42b. Quantibrite Beads (BD Biosciences) were used to translate flow cytometry output (mean fluorescence intensity [MFI]) to molecules of FcγRIIa/platelet.

3. Results

Analytic validation assessed accuracy and precision of the modified test. Initial assessment of commercially available clones of anti-FcγRIIa demonstrated that while those clones were capable of binding to FcγRIIa on the surface of fixed platelets, they did so with reduced affinity. For this reason, assessment of accuracy was central to our analytic validation.

3.1. Assessment of accuracy

We identified 5G1 as the candidate clone for use in our revised pFCG test with the use of competition assays. For those assays FcγRIIa was in solution. The focus of the analytic validation was to determine whether 5G1 quantified FcγRIIa on the surface of platelets with high affinity. As noted previously, 5G1 was selected because, in solution, it was able to effectively compete with the commercial antibody used in our initial studies. We quantified pFCG with two approaches – exposing nonfixed platelets to the commercial antibody (FL18.26, BD Biosciences) and exposing fixed platelets to 5G1 (Figure 1). In preliminary studies, we have found that pFCG expression is low (<1000 molecules/platetet) in young healthy subjects. By contrast, we have found a broad range (~600–5000 molecules/platelet) of expression in patients with MI, stable, coronary artery disease and stroke [12,13]. To assess accuracy over a broad range of pFCG expression that includes expression seen in healthy young subjects, analytic studies were performed with platelets from patients with MI. We found that 5G1 consistently identified a modestly greater number of molecules of FcγRIIa on platelets. The absolute increment (~300 molecules/platelet) was consistent across the range of expression (Figure 1). Figure 1 includes results from a western blot demonstrating that 5G1 and FL18.26 have similar specificity, recognizing the same target protein in a platelet lysate. The greater signal intensity associated with 5G1 supports the conclusion that 5G1 has greater affinity.

TruCytes™ synthetic cells conjugated with FcγRIIa were used as a second test of accuracy. Individual synthetic cells encompassing a broad range of FcγRIIa expression were used. The pFCG effectively quantified FcγRIIa protein expression on the synthetic cells (Figure 2). TruCytes™ synthetic cells conjugated with FcγRIIa were used to assess linearity (Figure 2). Linearity was demonstrated ($R^2 = 0.984$) over a range of expression that encompasses that seen in healthy subjects and patients with MI (n = 765 determinations, minimum expression 591, maximum expression 7640).

3.2. Assessment of precision

An advantage of flow cytometry is that pFCG can be quantified in approximately 10,000 platelets in 1 min. Thus, the output, MFI, reflects a large sample size. MFI is translated to molecules/platelet with the use of a standard curve much in the same way as results are quantified with an enzyme linked immunoassay.

Precision was quantified by determining the coefficient of variation (CV, standard deviation/mean). For biologic samples (platelets) the intra-assay CV (Figure 3) and

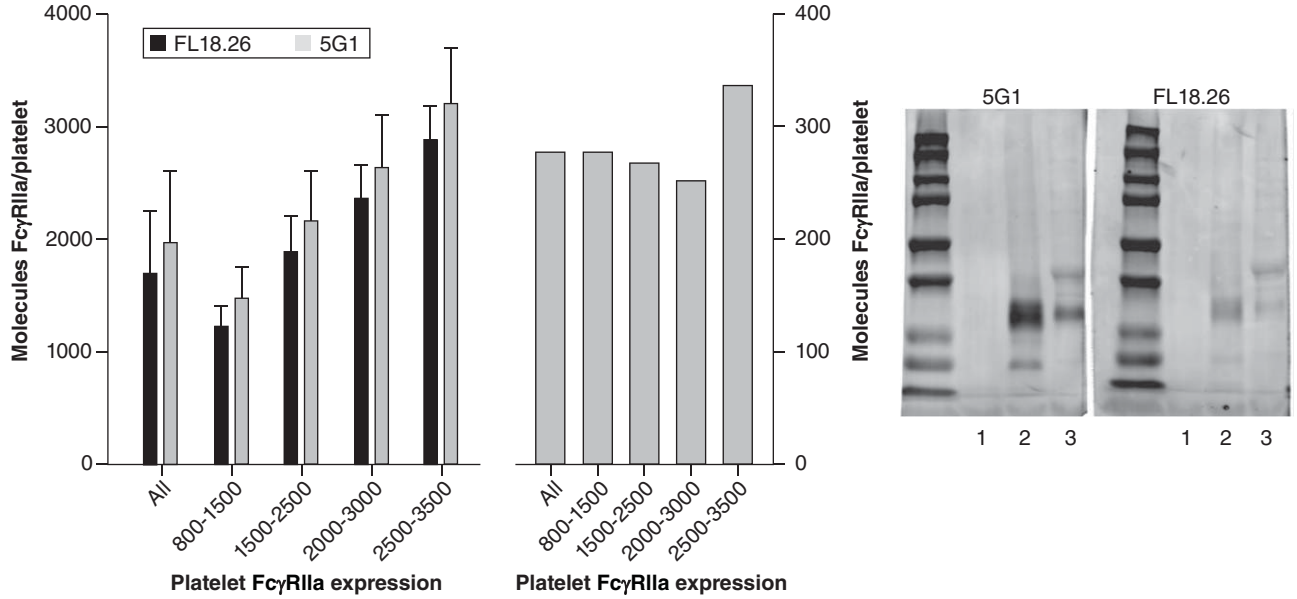

Figure 1. The pFCG test was performed with the addition of clone FL18.26 to nonfixed platelets and the addition of clone 5G1 to fixed platelets (n = 100 patients with MI). Patients with MI were chosen for this analysis because the range of expression (~400->5000 molecules/platelet) is greater than that seen in healthy subjects (<1000 molecules/platelet). The graph on the left shows a comparison of the two methods across a broad range of FcγRIIa expression. The graph in the middle shows the absolute difference between results obtained with the two methods (results with 5G1 minus results with FL18.26) over a broad range of expression. The image on the right shows results from western blot analysis. Lane 1 shows results obtained with probing of the lysate from CHO cells (that do not express FCγRIIa). Lane 2 shows results from CHO cells that have been stably transfected to express FCγRIIa. Lane 3 shows results from a platelet lysate. Similar specificity of 5G1 and FL18.26 is demonstrated. The greater intensity associated with the 5G1 clone is consistent with greater affinity.
CHO: Chines Hamster Ovary; MI: Myocardial infarction; pFCG: Platelet FcγRIIa.

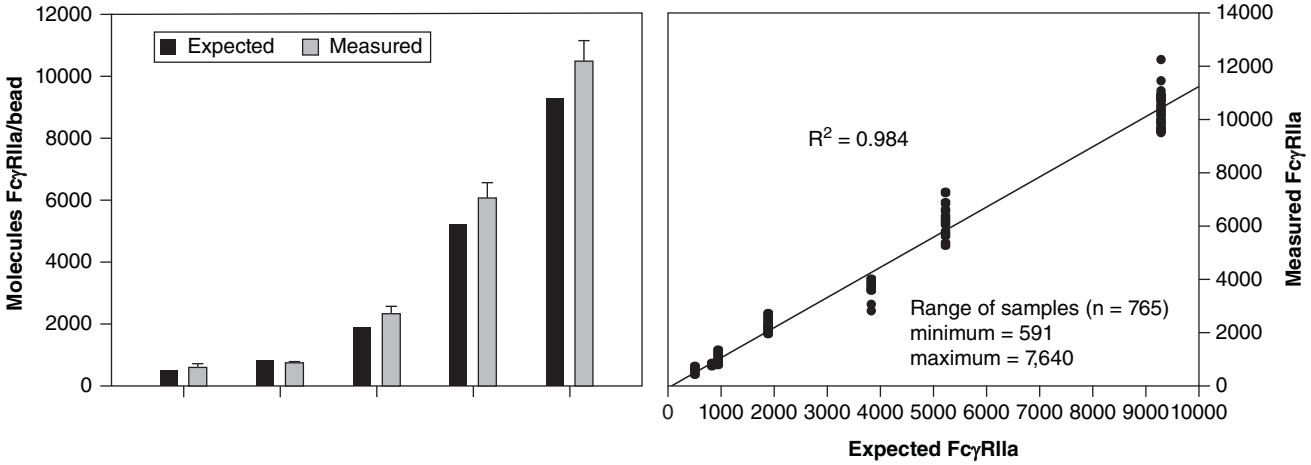

Figure 2. FcγRIIa was conjugated to Slingshot synthetic cells (TruCytes™). The pFCG test was performed and results were compared with the expected results (left). Linearity of the pFCG test (right) was assessed with the use of Slingshot synthetic cells (TruCytes™). The pFCG test was performed (n = 765 determinations) on samples that ranged from 591 to 7640 molecules of FcγRIIa/bead. This range encompasses values seen in healthy subjects and patients with MI. Linear regression demonstrated $R^2 = 0.984$.
MI: Myocardial infarction; pFCG: Platelet FcγRIIa.

interassay CV (Figure 4) were determined. The intra-assay CV was 2.1 ± 0.1% (standard error of the mean, n = 750). The interassay CV was assessed intraday and interday. These tests were performed by 2 technicians. Platelets were stored (at 4°) after fixation and fluorochrome labeled antibodies were added at the time of assay. For intraday assessment, performance of the pFCG test was separated by at least 2 h and the CV was 4.5 ± 1%. The interday tests were performed within 5 days of fixation and the CV was 6.5 ± 0.4%, (n = 50 for intraday and interday).

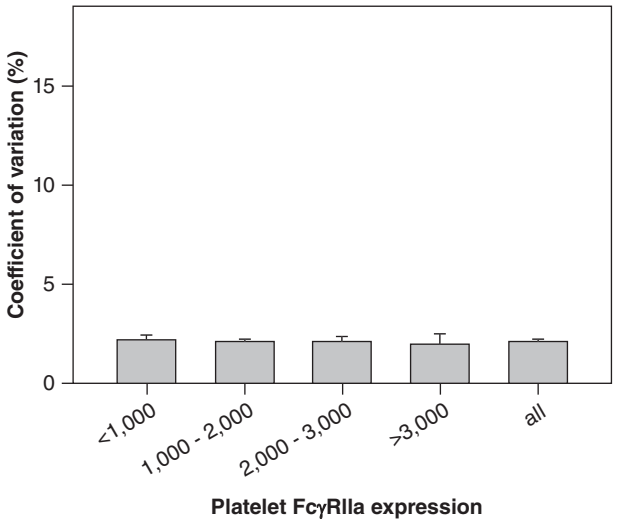

Figure 3. The intra-assay CV was assessed during the performance of the assay on biological specimens (platelets from 750 patients).
CV: Coefficient of variation.

interassay CV was also assessed for TruCytes™ synthetic cells conjugated with FcγRIIa (Figure 5). For intraday assessment, performance of the pFCG test was separated by at least 2 h and the CV was 2% $\pm$ 0.6%. The interday tests were performed on 20 nonconsecutive days and the CV was 9.9% $\pm$ 2.1%.

4. Discussion

Our results demonstrate that the modified pFCG test exhibits high precision and consistently quantifies a modestly higher platelet expression of FcγRIIa compared with the original test. A multicenter trial demonstrated that the modified pFCG test identifies patients at high and low risk of subsequent cardiovascular events [14]. Platelet function tests have consistently demonstrated that patients with increased platelet reactivity have an increased risk of subsequent cardiovascular events [15,16]. The pFCG test quantifies a protein on platelets that amplifies platelet activation [2,3]. Thus, high pFCG identifies subjects whose platelets would be expected to exhibit high reactivity [4]. Unlike platelet function tests that assess platelet activation in response to a selected agonist or group of agonists, high pFCG identifies individuals whose platelets will exhibit increased activation in response to any stimulus [4]. In the pFCG test, FcγRIIa is quantified with the use of flow cytometry that is capable of testing 10,000 platelets in approximately 1 min. The capability of flow cytometry to quantify the average expression of pFCG on 10,000 platelets enhances the accuracy and precision of the pFCG test.

Platelets show evidence of degradation after 1 day of storage [17]. To facilitate widespread use of this test, we sought to perform the pFCG test on fixed platelets to limit degradation of platelets. Formaldehyde fixation can lead to conformational changes of proteins. These conformational changes may impact on the ability of an antibody to bind to a protein. We tested commercially available clones of anti-FcγRIIa antibodies and found that fixation of platelets reduced their detection of FcγRIIa. For this reason, we developed clones of anti-FcγRIIa antibodies that were raised against FcγRIIa that had been exposed to formaldehyde. To select a clone with high affinity, we performed competition assay to identify 5G1 that effectively competed with a commercially available anti-FcγRIIa clone (FL18.26). We tested the accuracy of 5G1 in two ways, with biologic specimens (platelets) as well as with TruCytes™ synthetic cells conjugated with FcγRIIa. For platelets, we compared results obtained from nonfixed platelets exposed to FL18.26 with results obtained when 5G1 was added to previously fixed platelets. We found that 5G1 consistently identified a modestly higher number of FcγRIIa molecules across a broad range of expression. Results obtained with the TruCytes™ synthetic cells conjugated with FcγRIIa confirmed that 5G1 accurately quantified FcγRIIa on particles across a broad range of expression.

Initial studies with 5G1 suggested that results with the pFCG test were highly reproducible for 6–7 days. From a practical perspective, the ability to perform the pFCG test up to 5 days after samples have been fixed should provide adequate flexibility for test performance. As noted, flow cytometry is designed to yield precise results. Calibrating the flow cytometry output (MFI) with Quantibrite beads enhances precision. The intra-assay and interassay variability fall well below guidance documents provided by the US FDA (https://www.fda.gov/regulatory-information/search-fda-guidance-documents/bioanalytical-method-validation-guidance-industry). Based on our initial studies, we confined interday assessment of biological specimens (platelets) to 5 days. An advantage of the TruCytes™ synthetic cells was the ability to assess interday variation over the course of 20 nonconsecutive days. Results with biological specimens and TruCytes™ synthetic cells conjugated with FcγRIIa were consistent and demonstrated that the pFCG test has high precision.

While the focus of these studies was to perform analytic validation, the utility of the test is demonstrated by its ability to assess prognosis. In our initial single center study, all 200 patients enrolled were treated with percutaneous coronary intervention (PCI) and the pFCG test was performed by exposing nonfixed platelets to an anti-FcγRIIa antibody [5,11]. Patients with high compared with low pFCG had greater risk of MI, stroke and death (hazard ratio: 3.95, 95% CI: 1.17–7.69, $p = 0.007$). An interim analysis of our 800 patient multicenter study

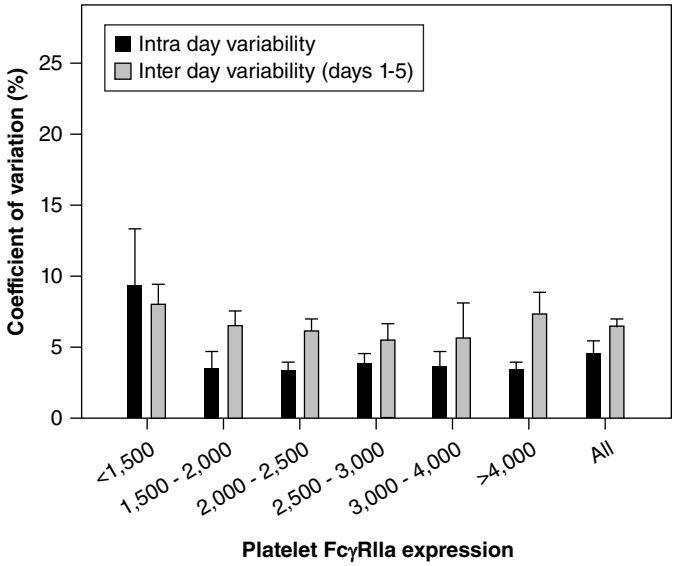

Figure 4. The interassay CV was assessed with biological samples (platelets from 50 patients). Intraday samples were performed on the same day. The second pFCG test was performed at least 2 h after the first test. interday samples were performed on days 1–5 after fixation of samples.
CV: Coefficient of variation.

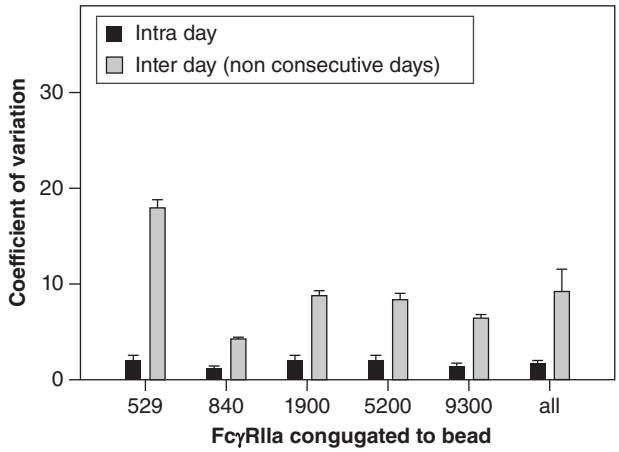

Figure 5. The interassay CV was assessed with TruCytes™ synthetic cells conjugated with FcγRIIa. Intraday samples were performed on the same day. The second pFCG test was performed at least 2 h after the first test. interday samples were performed on 20 nonconsecutive days.
CV: Coefficient of variation.

performed after accrual of 400 patient years of follow-up [14] demonstrated a similar prognosis among patients treated with PCI (hazard ratio: 4.0, 95% CI: 1.34, 12.04, $p = 0.01$).

The consistency of the prognostic implications of the original test and the revised test performed on fixed platelets are striking. In patients with MI treated with PCI, the pFCG test can identify patients at high and low risk of subsequent MI, stroke and death [5,11,14]. The hazard ratio of ~4 identifies this test as a powerful predictor of subsequent risk.

5. Conclusion

Modification of the pFCG test to be performed on fixed platelets allows performance of the test with high precision up to 5 days after platelets are fixed. The antibody developed (5G1), accurately quantifies FcγRIIa on platelets.

Article highlights

- A new antibody (5G1) was developed that binds to FcγRIIa on the surface of platelets that have been previously fixed. The development of this antibody allows for fixation of platelets before quantification of FcγRIIa on platelets. Because platelets can activate and degrade *in vitro*, the revised test was developed to exhibit greater precision.
- Accuracy of the platelet FcγRIIa (pFCG) test was assessed by comparison of results. The pFCG test (using clone 5G1) was performed on previously fixed platelets. Separately, the pFCG test was performed with another anti-FcγRIIa clone (FL18.26) added to nonfixed platelets. The 5G1 clone consistently identified ~300 more molecules of FcγRIIa/platelet across the broad range of expression seen with platelets from patients with myocardial infarction. Western blot analysis demonstrated greater affinity of the 5G1 clone compared with FL18.26 and similar specificity.
- Accuracy was further assessed with the use of Slingshot Synthetic cells (TruCytes™) conjugated with FcγRIIa. Results with TruCytes™ demonstrated accuracy of assessment and linearity across the range of biologic expression ($R^2 = 0.984$).
- With biologic specimens (platelets), the intra-assay coefficient of variation (CV) was 2.1 ± 0.1% (standard error of the mean, n = 750). The interassay CV was assessed intraday (4.5 ± 1%) and interday (up to 5 days after fixation, 6.5 ± 0.4%, n = 50).
- With TruCytes™, the interassay CV was 2% ± 0.6% (intraday) and 9.9% ± 2.1% (interday on 20 nonconsecutive days).

- In summary, modification of the pFCG test to be performed on fixed platelets with the newly developed clone (5G1) allows accurate quantification of pFCG with high precision.

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Competing interests disclosure

DJ Schneider is named inventor on a patent (10,502,737, 11,747,335 B2) that propose the use of FcγRIIa for assaying platelet reactivity and treatment selection and on a pending application (application 63/371,636) that describes quantification of FcγRIIa on fixed platelets. DJ Schneider is co-founder of Prolocor Inc.; PM DiBattiste is co-founder of Prolocor, Inc, J Ohrnberger is employed by Prolocor Inc. KS Pallah, T Shovah and S Biswas are employed by Slingshot Biosciences. The authors have no other competing interests or relevant affiliations with any organization or entity with the subject matter or materials discussed in the manuscript apart from those disclosed.

Writing disclosure

No writing assistance was utilized in the production of this manuscript.

Data availability statement

The authors certify that this manuscript reports original clinical trial data. Deidentified, individual data that underlie the results reported in this article (text, tables, figures and appendices), along with the study protocol will be available indefinitely for anyone who wants access to them. Clinical Trial Registration: NCT05175261

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