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ORIGINAL ARTICLE

Multicolor flow cytometry in clinical samples for platelet signaling assessment

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Abstract

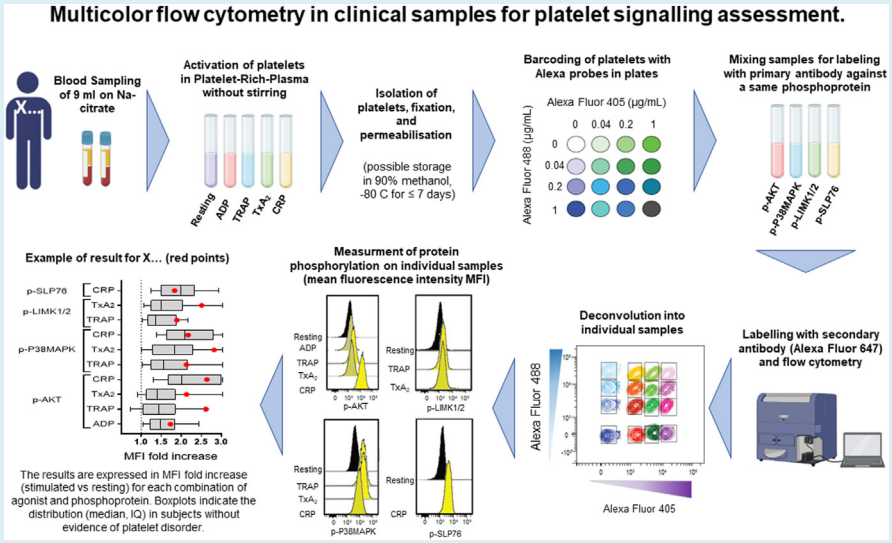
Background: Availability of multichannel cytometers and specific commercial antibodies makes flow cytometry a new option to simultaneously assess multiple intracellular platelet signaling pathways for clinical purposes, in small volume of blood or low platelet count.

Objectives: To describe a multicolor flow cytometry with fluorescent barcoding technique for screening signaling pathways downstream membrane receptors of major platelet agonists (adenosine diphosphate, thrombin, thromboxane, and collagen).

Methods: By comparison with immunoblotting, we first selected the target phosphoproteins, AKT, P38MAPK, LIMK, and SPL76; the times of stimulation; and phosphoflow barcoding conditions. We then performed a clinical study on whole blood of patients without evidence of blood platelet disorder on standard biological screening, consulting for trivial or occasionally provoked bleeds without familial antecedent (bleeding of unknown origin, n = 23) or type-1 von Willebrand disease (n = 9). In addition, we included a small group of patients with definite platelet disorders (Glanzmann thrombasthenia, δ-storage pool deficiency, and immune glycoprotein VI–related disease with granule secretion defect).

Results: The range, kinetics, and distribution of fluorescence intensity were established for each agonist-target protein combination. Principal component analysis indicates a correlation in response to a target phosphoprotein (AKT and P38MAPK) to different agonists but no correlation in the response of different target phosphoproteins to the same agonist. The heterogeneity of individual responses in the whole population displayed was analyzed using clustering algorithm. Patients with platelet storage pool deficiency were positioned as lowest responders on the heatmap.

Conclusion: In complement of functional tests, this study introduces a new approach for rapid platelet signaling profiling in clinical practice.



KEYWORDS
blood platelet disorders, fluorescent barcoding, multicolor flow cytometry, phosphoproteins, platelet signaling

Essentials

- Flow cytometry with fluorescent barcoding can assess simultaneous intracellular signaling.
- We studied platelet response to various agonists in patients with or without platelet disorders.
- Patient heterogeneity based on multiple signaling response is demonstrable.
- The technique is promising for rapid profiling of platelet signaling in clinical practice.

1 | INTRODUCTION

Platelet signaling pathways form a complex regulated network that drives the response to numerous agonists downstream of cognate membrane receptors [1]. Western blotting approach to assess intracellular protein phosphorylation is limited by the large volume of blood sample required, especially when time course of platelet activation by different agonists and multiple signaling proteins are investigated. Flow cytometry analysis (FCA) of intracellular protein phosphorylation using specific antibodies is an ideal tool to test kinase-dependent pathways on a small volume of sample in cells stimulated by several agonists. Such a method, first used in the field of immunologic signaling [2], was developed in platelets for monitoring the response to P2Y12 antagonists through the phosphorylation of vasodilator-stimulated phosphoprotein (VASP) [3] and commercial kits are now available for clinical use in this setting [4]. More recently, FCA of RAC-gamma serine/threonine-protein kinase (AKT) signaling was proposed for testing platelet function in small volumes of blood [5]. For research purposes, FCA has been applied to the analysis of phosphorylation events in mixed platelet subpopulations by combining cellular and signaling markers [6]. However, studying multiple signaling pathways in response to several agonists at different times requires high sample throughput analysis. The multicolor flow cytometry has opened the possibilities of large-scale profiling [7,8] and offers multiple advantages over traditional western blotting [9,10]. It was first applied to platelets for drug screening using p-vasodilator-stimulated phosphoprotein as a marker of platelet inhibition [9]. Here, we report the development of a phosphoflow cytometry technique combined with fluorescent cell barcoding (FBC) for clinical purposes, which provides a tool for the simultaneous assessment of multiple platelet signaling pathways and reduces the time of analytical run. In preliminary work, using washed platelets from healthy volunteers, we assessed the phosphorylation pattern of various signaling proteins in parallel by western blotting and flow cytometry after stimulation by the agonists of major platelet receptors. We used thrombin-related peptide (TRAP), adenosine diphosphate (ADP), thromboxane analog U46619 TxA_2 and the collagen-related peptide (CRP), respective ligands of the protease activated receptor 1, the purinergic receptors P2Y12 and P2Y1, the thromboxane A2 receptor, and the glycoprotein VI complex (GpVI). After selection of the target phosphoproteins, AKT, LIM-domain containing kinase 1 and 2 (LIMK1/2), p38 mitogen-activated protein kinase (P38MAPK), and SH2 domain-containing leukocyte protein of 76 kD (SLP76), we established the conditions for platelet FBC in platelet-rich plasma (PRP). Then, we determined the range and distribution of values and the kinetics of protein phosphorylation in a population of patients without disorder in platelet

function and studied the relationship between the responses of target phosphoproteins to different agonists. We analyzed the heterogeneity of individual responses in this population and, finally, compared these results with those of a small group of patients with definite functional platelet disorder.

2 | METHODS

Materials are described in Supplementary Material (Supplementary Table S1).

2.1 | Preliminary experiments

In a first set of experiments, we validated the choice of target phosphoproteins for phosphoflow cytometry by comparing with immunoblotting data, established the optimal conditions of FBC, determined the kinetics of protein phosphorylation with various agonists, and assessed the stability of phosphorylation in samples stored at −80 °C. This part of the study was performed using blood from healthy volunteers among laboratory staff, who gave consent after complete information and denied taking any medication in the 10 days before blood sampling. Venous blood was collected into vacuum tubes containing 3.2% sodium citrate. Platelets were isolated as described in Supplementary Material. ADP (10 μM), TxA_2 (1 μM), TRAP (50 μM), CRP (9 μg/mL), or none (resting platelets) were gently mixed to platelet suspension for 5 minutes at 37 °C without stirring. When indicated, resting platelets were preincubated for 10 minutes with pharmacologic antagonists before the addition of the respective agonists. Samples were then processed for western blotting and phosphoflow experiments, as described in Supplementary Material. According to the results of these experiments, we selected anti-p-AKT (ser473), anti-p-P38MAPK (thr180/tyr182), anti-p-LIMK1/2 (thr508/505), and anti-p-SLP76 (tyr145) as primary antibodies for bidimensional platelet FBC. Barcoding was obtained by mixing 2 fluorophores, Alexa Fluor 405 and 488 (Supplementary Table S2), which were selected to minimize spillover with the specific fluorescence of the secondary target phosphoprotein antibodies labeled with Alexa Fluor 647, as described in Supplementary Material and Supplementary Figure S1. Results were expressed in median fluorescence intensity (MFI) and MFI-fold increase (MFI-fi). The kinetics of protein phosphorylation was studied in PRP of 3 healthy subjects for over 30 minutes after stimulation by the agonists (Supplementary Figure S1). According to the results, the time periods 5 and 15 minutes were chosen for the clinical study. Storage stability of protein phosphorylation was studied on aliquots of stimulated platelets in PRP, rapidly frozen after fixation and permeabilization, and stored in methanol at −80 °C. After thawing, FBC, phosphoprotein labeling, and subsequent flow cytometry were performed as described above. Compared with the MFI-fi obtained on the aliquot of fresh, unfrozen samples, MFI-fi were not reduced up to 10 days of storage by more than 10% in 4 experiments (not shown). These results are in agreement with those obtained in Jurkat cells [11], indicating storage stability at −20 °C for at least 5 weeks.

2.2 | Clinical study

2.2.1 | Patient selection and blood collection

We constituted a cohort of patients without evidence of platelet disorders as follows. The subjects were referred to the hemostasis clinic (Laboratory A, Academic Hospital of Toulouse) mainly for preoperative or mild/moderate bleeding investigations. Two physicians recorded clinical data. All subjects denied intake of drugs known to influence platelet function for at least 10 days. Biological screening of hemostasis of patients included coagulation testing; measurement of von Willebrand factor (VWF) activity and antigen; platelet count; platelet function analyzer-100 (PFA-100) with collagen/ADP; and light transmission aggregometry in response to ADP (2.5, 5, and 10 μM), arachidonic acid (1 mM), TRAP (25 μM), collagen (3 μg/mL), and ristocetin (0.5-1.5 mg/mL) [12]. Two groups of patients without evidence of platelet dysfunction were eligible for the study if platelet count and function tests were normal on at least 2 separate visits. In the first group, patients reported only trivial, minor, or provoked bleeds (wounds, postoperative, postpartum) or menorrhagia without familial antecedents of bleeding disease and with normal VWF and coagulation tests (hereafter "BUO" for "bleeding of unknown origin," n = 23). In the second group of patients, type-1 von Willebrand disease (VWD; n = 9), diagnosed on typical biological and clinical features according to American society of hematology/International society of thrombosis ans hemostasis guidelines [13], was established as the sole cause of bleeding.

During the study, for comparison with the above cohort, we included 7 patients with long-lasting moderate/severe bleeding history followed in our hemostasis clinic for a definite hereditary or acquired platelet function disorder (described in Supplementary Material).

Interlaboratory comparison was performed in Laboratory B (Academic Hospital of Lyon), which included 9 patients with type-1 VWD based on the same biological screening.

For the research protocol, we collected blood (9 mL) in vacuum tubes containing 3.2% sodium citrate. Patients were tested only once for multiplex phosphoflow cytometry.

The research protocol (NCT01957345) complied with the Declaration of Helsinki and was approved by the Ethic Committee of Toulouse Academic Hospital. All subjects gave written consent after complete information.

2.2.2 | Clinical study: multicolor flow cytometry

Multiplex phosphoflow cytometry followed the protocol established in the preliminary study, with few exceptions (see Supplementary Material and Figure 1). Within 1 hour after sampling, blood was

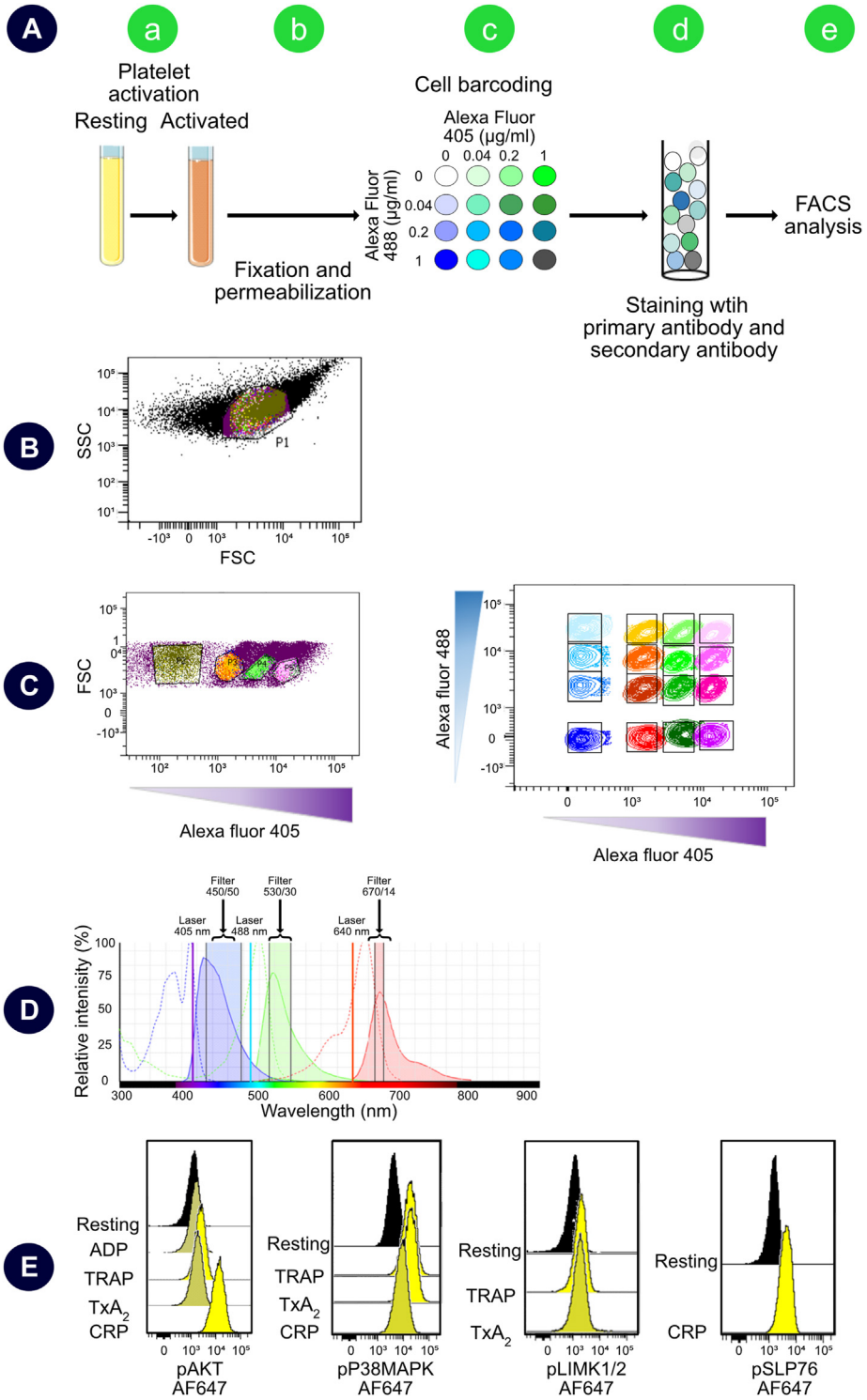


FIGURE 1 Preparation and gating strategy for multiplex cytometry and fluorescent barcoding. (A) Successive steps from sample preparation to flow cytometry analysis. Platelets (washed in preliminary experiments, platelet-rich plasma in clinical study) are activated or not (resting) with agonists (a), fixed with formaldehyde and permeabilized by Triton X-100 and cold methanol (b), and labeled for FBC by mixing various concentrations of Alexa Fluor 405 and Alexa Fluor 488 dyes (c). Barcoded samples are pooled into a single tube for labeling with appropriate primary antibodies (1 phosphospecific antibody per multiplex sample) and then with Alexa Fluor 647–conjugated secondary F(ab)'2 (d), before flow cytometry analysis (e). (B) Platelets are gated forward (FSC) vs side scatter (SSC). (C) Platelets are gated on FSC vs Alexa Fluor 405 intensities (4 populations from the dimmest to the brightest) and then vs Alexa Fluor 488 intensities (4 populations). (D) Each population is exported as an individual FSC file and analyzed for phosphoprotein fluorescence using Alexa Fluor 647 intensity. The figure shows the absence of spillover between grid dyes and phosphoprotein fluorescence. (E) Typical histograms of protein phosphorylation induced by various platelet agonists. ADP; CRP; F(ab); FACS, fluorescence activated cell sorting; FBC, fluorescent bar coding; FSC, forward scatter; SSC, side scatter; TRAP; TxA_2.

centrifuged for 10 minutes at 190× *g*, and after resting for 20 minutes at room temperature, PRP was incubated in polypropylene tubes without (base) or with the same agonists as in the preliminary study for 5 and 15 minutes at 37 °C without stirring. Fixation, permeabilization, and staining were essentially performed according to the optimized techniques described by Spurgeon et al. [10] Platelets were fixed (CellFIX 3×) for 15 minutes, permeabilized with 0.4% Triton X-100 for 15 minutes at 37 °C, washed once in phosphate-buffered saline, and mixed under strong vortexing with cold methanol to reach a final concentration of 90% methanol. After 20 minutes at −20 °C, the samples were stored in methanol at −80 °C for a maximum of 7 days until thawing for FBC, antibody labeling, and FCA, which were performed as described above. Fold-increase of phosphorylation (MFI-fi) was calculated by dividing the MFI of the stimulated sample by that of resting platelets (baseline).

Using the same protocol of cytometry, reagents, and fluorescent antibodies, we performed an interlaboratory comparison with the results obtained in the laboratory of Academic Hospital of Lyon (Laboratory B) for patients with type-1 VWD. For practical reasons, access to a multichannel cytometer was not possible at the time of the study in Laboratory B. The barcoding step was omitted and FCA analysis was performed on a BD-FACSCalibur (Becton Dickinson) cytometer.

2.3 | Statistical analysis

Statistical analysis was performed using GraphPad Prism software. There was no missing value in the data of any patients, and no value was censured. Continuous variables were expressed as mean (±SD), median (interquartile), or number (percentage), as appropriate. Comparisons between groups were performed after log transformation when indicated. Principal component analysis provided a visual overview of the relationships between the results obtained using different combinations of agonists and target phosphoproteins using R statistics software. Heatmap was prepared according to overall response to agonists and dendrogram hierarchy clustering using Displayr software (www.displayr.com).

3 | RESULTS

3.1 | Preliminary study

The results of flow cytometry measurement of platelet phosphoproteins AKT, P38MAPK, LIMK1/2, and SLP76 were concordant with western blotting profiles in most instances (Figure 2A–D). MFIc-fi vs unstimulated platelets ranged between 2 and 6, depending on the agonist and the signaling protein. As expected, signals were efficiently tuned down by appropriate receptor antagonists or a tyrosine kinase inhibitor. The addition of apyrase or indomethacin alleviated agonist-induced phosphorylation signals, indicating a contribution of the secretion of platelet granules or thromboxane A2 synthesis (not shown). The kinetics of phosphorylation over 30 minutes indicated a rapid signal increase following agonist addition, but after 10 minutes, depending on the agonist-phosphoprotein combination, continuous increase or sustained plateau was observed (Supplementary Figure S1).

3.2 | Study in patients without evidence of platelet disorder

3.2.1 | Patient groups

A cohort of 32 patients (6 men and 26 women; mean age, 41 years [range, 18-78]; White) with normal platelet counts (mean ± SD, 248 ± 55×10^9/L; range, 170-357) and normal results to platelet function tests on at least 2 occasions was used to establish the ranges, distribution, and kinetics of phosphorylation in Laboratory A. The cohort comprised 23 patients without abnormality of biological screening (labeled $BUO_{1\text{-}23}$) and 9 patients with type-1 VWD (labeled $VWD_{1\text{-}9}$). Type-1 VWD displayed mild or moderate bleeding (ISTH-Bleeding Assessment Tool (BAT) score, 3-9) and 5 reported familial antecedents of VWD. VWF activity was between 0.30 and 0.60 U/mL with a ratio to VWF antigen ≥0.7. Results of platelet function assays (except agglutination with ristocetin) were normal, and platelet function analyzer-100 closure time was prolonged in 4 patients. Nine patients with type-1 VWD were recruited in Laboratory B using the same criteria.

3.2.2 | Ranges, distribution, and kinetics of phosphorylation and MFI-fi values

As MFI-fi distributions of BUO and type-1 VWD (Laboratory A) were close (Supplementary Table S3), the groups were pooled for further analysis. Figure 3 summarizes the results obtained in the cohort for resting platelets and baseline MFI for each phosphoprotein and for each condition of platelet activation. There was no significant correlation between baseline MFI and MFI-fi (not shown). Depending on the combination of agonist-target phosphoprotein, median MFI-fi values ranged between 1.5 and 3 (Supplementary Table S4), but the distribution of MFI-fi in the study population was skewed toward lower values and 2 BUO displayed values over the 95th percentile at 15 minutes (Supplementary Figure S2). Stimulation by CRP provided the highest MFI-fi. In all combinations, except for CRP-SLP76, a small number of patients displayed no increase in MFI upon stimulation at 5 or 15 minutes (MFI-fi ≤ 1).

Between 5 and 15 minutes, MFI-fi increased significantly for all agonist-target phosphoprotein combinations, except for p-AKT after stimulation by ADP, TxA_2, or CRP, and p-P38MAPK after stimulation by TxA_2 or CRP (Supplementary Table S4). To illustrate the changes at individual patient level, we constructed compact heatmap

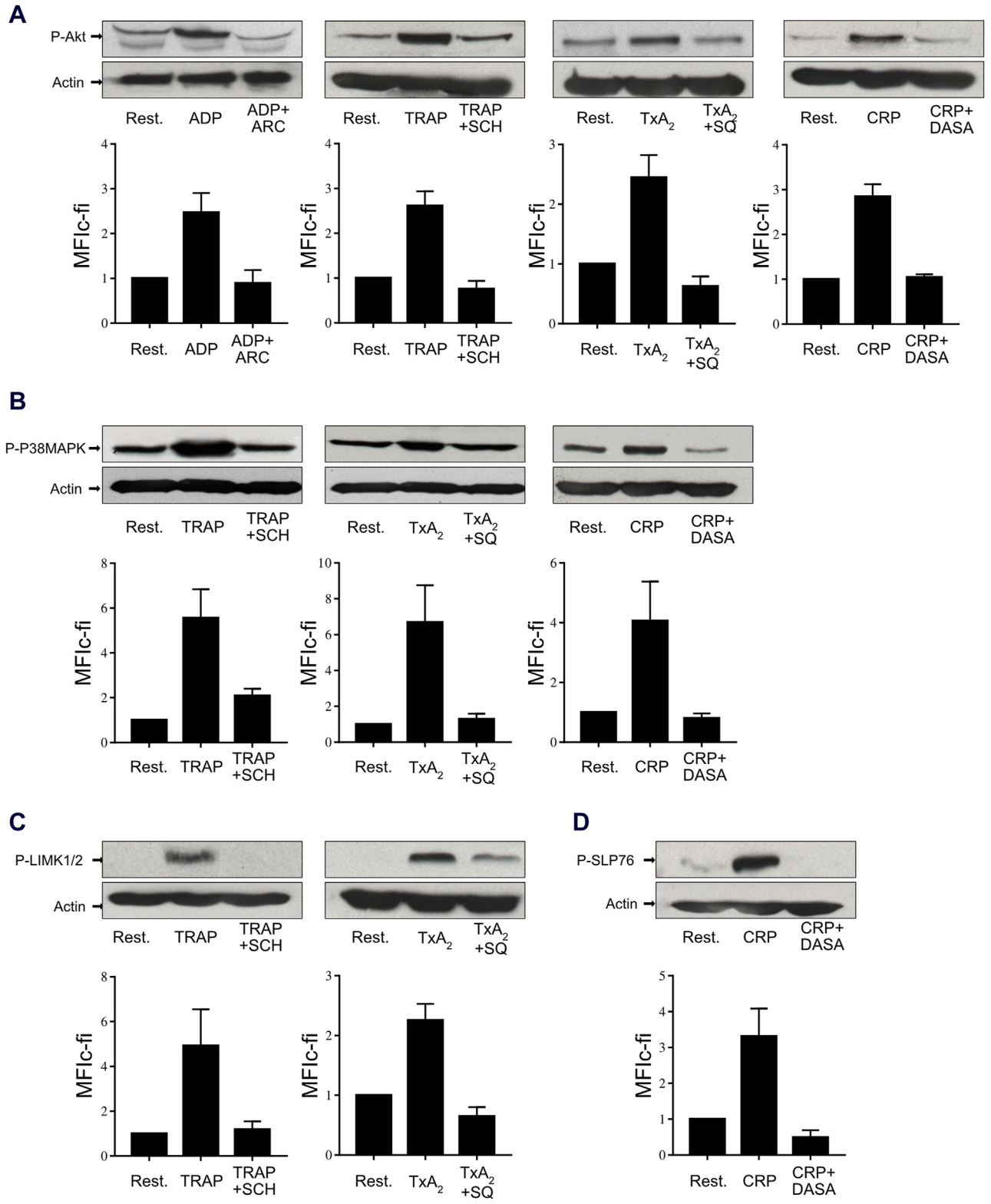


FIGURE 2 Preliminary study: validation of target phosphoproteins. For each condition, a representative picture of Western blot analysis is shown above the flow cytometry results expressed as MFIc-fi (MFIc ratio of stimulated over resting platelets, mean + SD, *n* = 3-5). Panels A, B, C, and D illustrate phosphorylation of AKT, P38MAPK, LIMK1/2, and SLP76, respectively, of washed platelets at rest or stimulated for 5 minutes with ADP, TRAP, TxA_2, or CRP, with or without the respective antagonists of P2Y12 (ARC), PAR1 (SCH), and TPα (SQ) or the inhibitor of Src family kinases (DASA). ADP-induced phosphorylation of AKT was unaffected by MRS-2179, an antagonist of P2Y1 (not shown). Pretreatment of platelets by wortmannin 100 nM, a pan-inhibitor of PI3-kinase, abolished AKT-ser 473 phosphorylation signal in response to all agonists (not shown). ADP; ARC; CRP; DASA; MFIc; MFIc-fi; MRS-2179; PAR1, protease activated receptor 1; SCH; SQ; TPα; TRAP; TxA_2.

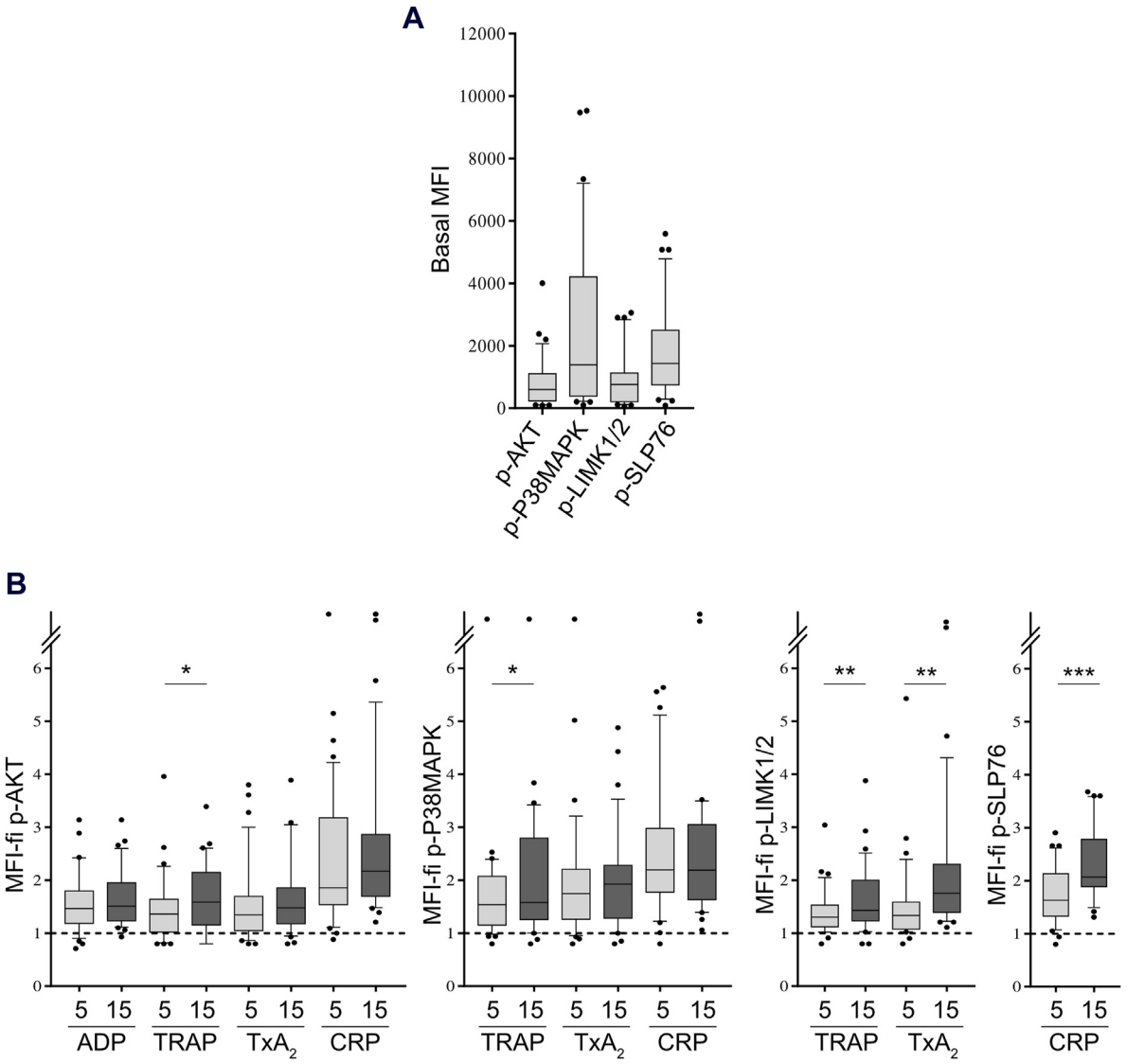


FIGURE 3 Baseline MFI and MFI-fi in the cohort of patients without evidence of platelet disorders. The results are expressed as boxplots (median, interquartile, n = 32) of baseline MFI for each phosphoprotein in resting platelets (A), or MFI-fi for various agonist-target phosphoprotein combinations (B). Individual values of MFI-fi at 5 and 15 minutes of stimulation were compared with the Wilcoxon paired test. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$. ADP; CRP; MFI; MFI-fi; TRAP; TxA_2.

representation, with a color code for each change of quartile of MFI-fi distribution between the 2 time-points (Supplementary Figure S3). Visual inspection suggests that no patient displayed uniform increase or decrease for all agonist-target phosphoprotein combinations. Pearson correlation between the distribution into quartiles at the 2 time-points was significant for all agonist-target phosphoprotein combinations but 1 (CRP/SLP76). These results indicate that the global increase of median MFI-fi with time observed in the cohort was due to a moderate upward shift of MFI-fi with a similar time course in a large majority of subjects.

Unpaired comparison between the results of laboratories A and B in different cohorts of 9 patients with type-1 VWD did not show a significant difference in 20 but 1 agonist/target and phosphoprotein/time combinations (Supplementary Table S5).

3.2.3 | Interrelation between phosphorylation of target proteins and agonists

The agreement between phosphorylation induced by different agonists on the same target phosphoprotein was examined by measuring the correlation between phosphorylation of the same protein (AKT, P38MAPK, or LIMK1/2) upon platelet stimulation by ADP, TRAP, TxA_2, or CRP. As shown in Table (left), with 1 exception, MFI-fi of p-AKT (5 couples of agonists) and p-P38MAPK (3 couples of agonists) was significantly and positively correlated at 1 or both times. The agreement between phosphorylation of different target proteins induced by the same agonist was examined by measuring the correlation between phosphorylation of various couples of target proteins (AKT, P38MAPK, LIMK1/2, and SLP76) upon platelet stimulation by

TABLE Interrelation between phosphorylation of target proteins and agonists by paired analysis.

AKT		
	5 min	15 min
ADP vs TRAP (*P* value)	0.61 (.0002)	0.33 (.07)
ADP vs CRP (*P* value)	0.38 (.0002)	0.09 (.61)
ADP vs TxA_2 (*P* value)	−0.03 (.87)	0.58 (.0005)
TxA_2 vs TRAP (*P* value)	−0.24 (.19)	−0.13 (.46)
TRAP vs CRP (*P* value)	0.39 (.025)	−0.23 (.20)
P38MAPK		
	5 min	**15 min**
TRAP vs TxA_2 (*P* value)	0.01 (.94)	0.64 (.0001)
TRAP vs CRP (*P* value)	0.27 (.13)	0.70 (.0001)
TxA_2 vs CRP (*P* value)	0.43 (.014)	0.56 (.0008)
LIMK1/2		
	5 min	**15 min**
TRAP vs TxA_2 (*P* value)	−0.24 (.19)	−0.07 (.70)

TRAP		
	5 min	15 min
AKT vs LIMK1/2 (*P* value)	0.05 (.79)	0.05 (.76)
AKT vs P38MAPK (*P* value)	−0.06 (.72)	−0.10 (.59)
P38MAPK vs LIMK1/2 (*P* value)	0.01 (.96)	0.59 (.0004)
TxA_2		
	5 min	**15 min**
AKT vs LIMK1/2 (*P* value)	0.53 (.002)	0.05 (.76)
AKT vs P38MAPK (*P* value)	−0.09 (.62)	−0.04 (.84)
P38MAPK vs LIMK1/2 (*P* value)	−0.125 (.49)	0.19 (.29)
CRP		
	5 min	**15 min**
AKT vs SLP76 (*P* value)	0.17 (.35)	0.149 (.41)
AKT vs P38MAPK (*P* value)	0.257 (.15)	0.067 (.71)
P38MAPK vs SLP76 (*P* value)	−0.028 (.87)	−0.143 (.43)

Left column: correlation of phosphorylation of the same target phosphoprotein (AKT, P38MAPK, and LIMK1/2) induced by different couples of agonists. Right column: correlation of phosphorylation of different couples of target phosphoproteins induced by the same agonist (TRAP, TxA_2, and CRP). Pearson correlation between MFI-fi is calculated for each couple of agonists for AKT, P38MAPK, and LIMK1/2 (left) and for each couple of phosphoproteins for TRAP, TxA_2, and CRP (right) at time periods of 5 and 15 minutes.

the same agonist (TRAP, TxA_2, or CRP). As shown in the Table (right), with 2 exceptions, phosphorylation of different target proteins in response to the same agonist was not correlated. Figure 4 provides a visual overview of the relationships between all experimental conditions examined by principal component analysis. Whatever the agonist, the proteins P38MAPK and AKT stayed in distinct and relatively compact sectors. In contrast, irrespective of the target proteins, the agonists were not distributed in close areas. These results, in agreement with those depicted above, point to a relatively good correlation in the response of the same target protein to different agonists, in contrast to the poor correlation, if any, in the response of different target proteins to the same agonist.

3.3 | Study in patients with a definite disorder of platelet function

Figure 5 shows the results of phosphoflow cytometry obtained in these patients. The small number of patients precludes a statistical analysis. MFI-fi observed with δ-storage pool disease (δ-SPD) were in the lower quartile of reference values for most agonist-target phosphoprotein combinations at both times. In GpVI-Related disease (GpVI-RD), as expected, the phosphorylation of all target proteins tested in response to CRP was abolished (red arrow), but the response to most other agonists was usually low, especially at 5 minutes. We had the opportunity to reassess the response of this patient 2 months later. She was still symptomatic and displayed the same profile of platelet secretion defect, and the results of FCA during the second visit were fully consistent with the first one (not shown).

3.4 | Heterogeneity of individual response to agonists/target phosphoprotein combinations

Figure 6 provides a heatmap visualization of the population-level variation in our 3 groups of patients: BUO (column labels 1-23), type-1 VWD (labels VWD_1 to VWD_9), and the 7 patients with definite platelet disorder (labels $GT_{1\text{-}2}$, GpVI-RD, and δ-$SPD_{1\text{-}4}$). For each condition, a 4-color code is associated with the quartile of MFI-fi distribution, from the lowest ($<25^{th}$ percentile) to the highest ($>75^{th}$ percentile) quartile. A hierarchical ranking of the 39 subjects according to the overall intensity of signaling responses at each time generates a dendrogram that identifies subgroups of responders: lowest, low, intermediate-low, intermediate-high, high, and highest (n = 7, 7, 8, 5, 6, and 6, respectively, at 5 minutes). At 15 minutes, the distribution is very close (n = 6, 10, 5, 9, 5, and 4). Between 5 and 15 minutes, 15 of 39 subjects clustered in the same subgroups and 17 of 39 clustered in adjacent subgroups (Pearson chi-squared test, P = .02). This is consistent with the kinetics described above. Clustering is independent of age, sex, platelet counts, and bleeding history in BUO. A majority of the type-1 VWD (5 of 9) cluster as high responders, and all δ-SPD and GpVI-RD cluster as low responders.

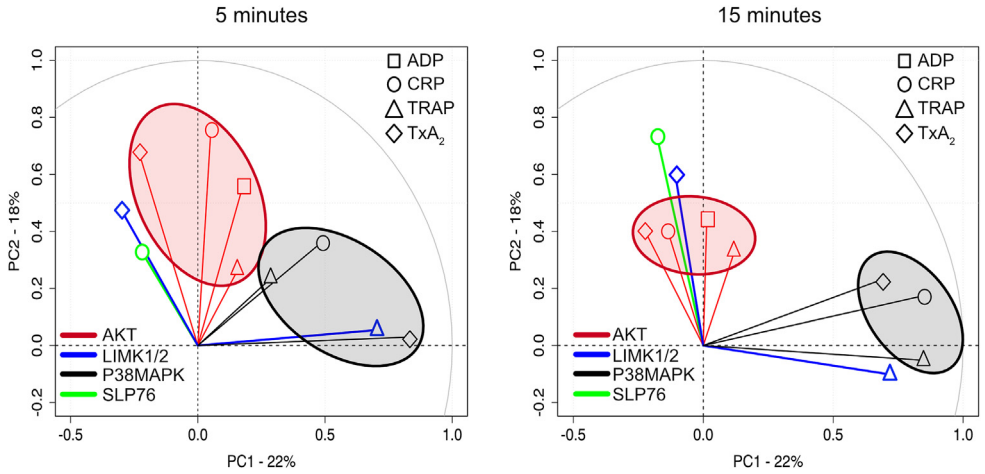


FIGURE 4 Representation, in correlation circle plots, of all agonist-target phosphoprotein combinations considered as variables of the data set submitted to principal component analysis. The data set was reshaped in 32 rows (subjects) × 10 columns (combinations of 1 agonist and 1 protein) for each time. When considering the variable plot, arrows of similar length and pointing in the same direction (red for AKT, black for P38MAPK) display the higher degree of correlation at both times. The first 2 principal components (PC1 and PC2) contribute to 40% (22% and 18%) of the total variability and resolve 2 main trends: PC1 on the horizontal X-axis is mainly driven by P38MAPK (3 black arrows) whereas PC2 on the vertical Y-axis highlights AKT (4 red arrows). ADP; CRP; TRAP; TxA_2.

4 | DISCUSSION

Multiplex methodology of FCA combined with FBC offers multiple advantages over western blotting for the study of platelet signaling [10]. We report our experience of this approach for clinical laboratories. The target phosphoproteins and respective antibodies, timepoints, storage of samples, and conditions of FBC and FCA were selected in prior experiments in healthy subjects. A major difference between the preliminary study on washed platelets and the clinical study performed in PRP should be stressed. In PRP, even under nonaggregating conditions, fibrinogen binding to activated GpIIbIIIa provides outside-in feedback for the activation of numerous signaling pathways, including a mild activation of SLP76 (not shown) [14]. Combined with the contribution of ADP or TxA_2 release, the technique used in the clinical study provides a picture of integrated platelet signaling networks in response to single agonists.

The distributions of baseline MFI and MFI-fi were established in a cohort of 32 patients with a low probability of bleeding disorder from platelet origin on the basis of clinical and biological data: 23 patients with BUO and 9 with type-1 VWD. The interindividual variability of baseline MFI (Figure 3A) was not unexpected. G-protein–coupled receptors display complex allostery [15] and a constitutive activity of P2Y12 and P2Y1 has recently been reported [16,17]. Phosphorylation of SLP76 in resting platelets may be influenced by the activity of tyrosine phosphatases involved in SLP76 dephosphorylation, especially CD148 polymorphism, or by the balance between immunoreceptor tyrosine-based inhibitory motif (ITIM) and immunoreceptor tyrosine-based activation motif (ITAM) systems [18–21]. MFI-fi were asymmetrically distributed (Supplementary Figure S2). The median values of MFI-fi usually range between 1.5 and 2.5 at 5 minutes of stimulation, with a modest increase at 15 minutes (Figure 3B, Supplementary Table S4). The kinetics of phosphorylation show a slow increase in most agonist/target phosphoprotein combinations and in most subjects (Supplementary Figure S3), suggesting that the analysis could be performed at a single time-point without loss of information. MFI-fi values, much lower than those observed for membrane antigens, are in the range of those observed by others with monoclonal antibodies in activated platelets for p-P38MAPK, p-AKT, or p-ERK1/2 [5,6,9]. This may result from technical or biological specificities: 1) antibody access to intracellular targets is more difficult compared with access to surface targets; 2) fixation/permeabilization steps can change the light scattering properties of platelets and increase nonspecific background because methanol alters the lipids and precipitates platelet proteins [22]; 3) upon activation, phosphoproteins cluster with partners, reducing access to antibody; 4) because only the population of singlet platelets is suitable for FCA, the area of platelet selection is narrow, reducing the fluorescence amplitude; and 5) platelets are heterogeneous, and under nonaggregating conditions, only a minority of platelets are strongly activated [23–26].

Multiplex flow cytometry allows study of the relationships in the pattern of phosphorylation of multiple target proteins with multiple agonists (Table and Figure 4). We show a positive relationship between p-AKT response to the 4 agonists tested and, similarly, between p-P38MAPK response to the 3 agonists tested. An explanation could be that the phosphorylation signal, whatever the agonist, is proportional to the level of the nonphosphorylated protein in resting platelets. Alternatively, upstream signaling pathways may be common to multiple agonists [27,28]. In contrast, we did not observe synchronous activation of AKT and P38MAPK in response to TRAP, TxA_2, or CRP. This suggests that the phosphorylation of the target protein varies according to factors that differ between agonists, such as the density of receptor membrane or its downstream effectors.

Although the study was not tailored to provide platelet signaling phenotypes, subgroups between lowest and highest responders were

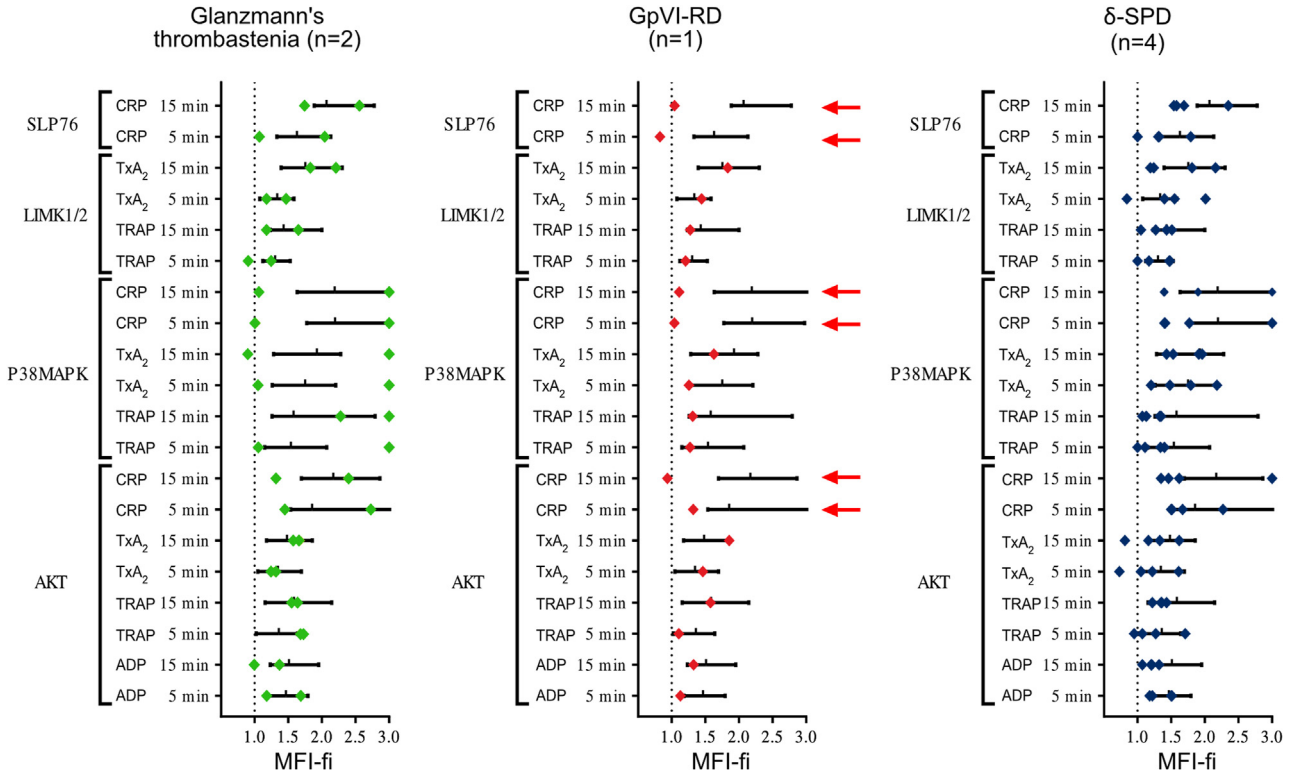


FIGURE 5 MFI-fi in patients with definite platelet disorder. Plots of MFI-fi of individual patients with Glanzmann thrombasthenia (green, n = 2), GpVI-RD (red, n = 1), and δ-SPD (blue, n = 4). Horizontal bars show the median and 25th-75th interquartile for each combination of agonist, target phosphoprotein, and time. Red arrows indicate the loss of response of GpVI-RD platelets to CRP. ADP; CRP; GpVI-RD; MFI-fi; δ-SPD; TRAP; TxA_2.

observed in the cohort of patients without evidence of platelet defect (Figure 6). The results obtained in patients with a definite platelet functional disorder suggest a role of the positive feedback of ADP release from dense granules. This is not surprising as platelet simulation for the multiplex FCA is the same as that for the single channel FCA routinely used to assess the expression of membrane granule markers CD63 and CD62P. δ-SPD platelets cluster in the lower part of distribution (Figure 6). In GpVI-RD, the response of all target proteins to CRP is absent, but in this patient with defective granule secretion, the response to other agonists is low (Figures 5 and 6).

Interlaboratory comparison (Supplementary Table S3) was performed in different subjects with a same well-characterized disease (type1-VWD). Small numerical differences may be due to the small size of the cohorts or the minor differences in the selection area of platelets in sideward and forward scatter. This indicates that local reference values should be established. Alternately, after initial steps of platelet stimulation and cell preparation, the frozen samples can be processed in a central laboratory for FBC, antibody labeling, and acquisition on a flow cytometer facility.

Our study suffers from several limitations. We did not collect reference values in the healthy population. Although BUO could not be viewed as healthy controls, they are frequently encountered in clinical practice and should be distinguished from patients with platelet disorders of unknown cause [29]. Only 1, relatively high, concentration of each agonist was used, precluding the analysis of the sensitivity in response to low stimulation levels. The size of the cohort was not tailored to identify subpopulations of subjects with distinct phenotypes of platelet signaling, as established for platelet function using analysis of high-dimensional data [30]. Reproducibility was not evaluated on repeated experiments on the same patients at different time intervals. We cannot drive firm conclusions from the data of the small group of patients with a definite platelet disorder.

Despite these limitations, the current study introduces a new approach, relatively simple and rapid, in complement with functional tests, for the investigation of signaling disorders in clinical practice. It is not limited by the volume of blood or severe thrombocytopenia, with the exception of macrothrombocytopenia, because large/giant platelets are not fully recovered in the PRP. We only evaluated intracellular markers of platelet signaling because many cell-surface antigens are sensitive to fixation/permeabilization procedures, resulting in loss of recognition by antibodies. However, the use of a reversible permeabilization agent and conjugated epitope-specific antibodies selected for this specific condition should allow study of intracellular signaling in parallel with surface activation markers, receptor densities, or platelet subpopulations. Future studies to ascertain this new method should include the establishment of reference ranges in healthy subjects. The tremendous development of specific antibodies available for intracellular antigens should allow investigation of numerous signaling pathways in bleeding or thrombotic diseases. Finally, the strategy may apply to research on small laboratory

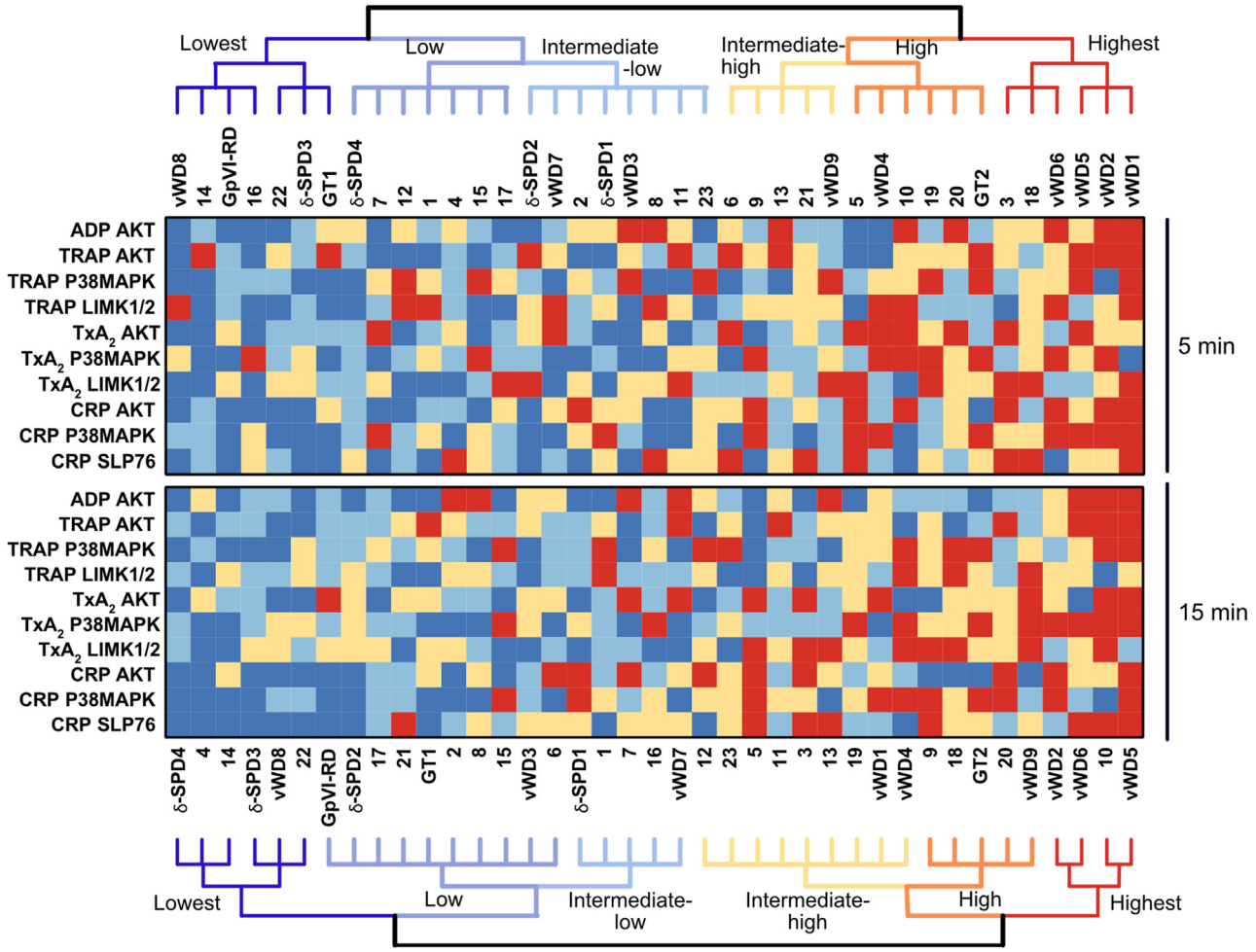


FIGURE 6 Heatmap of individual response to different agonists/target phosphoprotein combination in patients without evidence of platelet disorder (BUO and type 1-VWD) and patients with a definite platelet functional disorder. Each column (n = 39) represents a patient (labeled: 1-23 for BUO, VWD1-9, GT1-2, GpVI-RD, and δ-SPD1-4) and rows depict MFI-fi for each combination of target phosphoprotein and agonist. According to the quartile of distribution of MFI-fi, each square is colored as follows: 1st quartile (lowest values, blue), 2nd quartile (intermediate low, light blue), 3rd quartile (intermediate-high, accent yellow), and 4th quartile (highest values, red). According to the overall response to all conditions at each time, the patients are ranked in clusters from "lowest" on the left, to "highest" responders on the right. Heatmap and dendrogram hierarchy clustering were constructed using Display R. Upper and lower heatmaps refer to time periods of 5 and 15 minutes, respectively.

animals or as a surrogate model of signaling alterations in non-hematological diseases [31].

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ETHICS STATEMENT

The research protocol (NCT01957345) complied with the Declaration of Helsinki and was approved by the ethics committee of Toulouse Academic Hospital. All subjects gave written consent after complete information.

AUTHOR CONTRIBUTIONS

B.P. and P.S. conceived and designed the research. C.G. performed preliminary experiments. S.V., A.R., and L.R. recruited the patients. J.S., S.C., and J.C.B. performed the main experiments. S.D. and N.S. provided statistical support. C.G. and P.S. assembled and analyzed the data and wrote the manuscript. All authors revised and gave final approval of the manuscript.

RELATIONSHIP DISCLOSURE

There are no competing interests to disclose.

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SUPPLEMENTARY MATERIAL

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