


## RESEARCH PAPER

# Multiparameter platelet function analysis of bleeding patients with a prolonged platelet function analyser closure time

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## Summary

Patients referred for evaluation of bleeding symptoms occasionally have a prolonged platelet function analyser (PFA) closure time, without evidence for von Willebrand disease or impaired platelet aggregation. The aim of this study was to establish a shear-dependent platelet function defect in these patients. Patients were included based on high bleeding score and prior PFA prolongation. Common tests of von Willebrand factor (VWF) and platelet function and exome sequencing were performed. Microfluidic analysis of shear-dependent collagen-induced whole-blood thrombus formation was performed. In 14 PFA-only patients, compared to healthy volunteers, microfluidic tests showed significantly lower platelet adhesion and thrombus formation parameters. This was accompanied by lower integrin activation, phosphatidylserine exposure and P-selectin expression. Principal components analysis indicated VWF as primary explaining variable of PFA prolongation, whereas conventional platelet aggregation primarily explained the reduced thrombus parameters under shear. In five patients with severe microfluidic abnormalities, conventional platelet aggregation was in the lowest range of normal. No causal variants in Mendelian genes known to cause bleeding or platelet disorders were identified. Multiparameter assessment of whole-blood thrombus formation under shear indicates single or combined effects of low-normal VWF and low-normal platelet aggregation in these patients, suggesting a shear-dependent platelet function defect, not detected by static conventional haemostatic tests.

## KEYWORDS

bleeding disorders, diagnostic haematology, flow, genetics of thrombosis and haemostasis, platelet function

## INTRODUCTION

Whole-blood assessment using a platelet function analyser (PFA) is regularly used in the diagnostic work-up of patients suspected of a bleeding disorder. The PFA test has been developed as a proxy measurement of primary haemostasis under conditions of high wall-shear rate.<sup>1–4</sup> However, use of the PFA test in identifying patients with a bleeding disorder has been questioned in single studies and meta-analyses.<sup>2,3,5,6</sup>

As accepted, the PFA is particularly sensitive in the detection of von Willebrand disease (VWD), especially when the level of von Willebrand factor (VWF) is below 30%.<sup>4,7,8</sup> Similarly, in such patients, the PFA is used for therapeutic monitoring of the effects of desmopressin or VWF supplementation.<sup>9–11</sup> Furthermore, the PFA closure time (CT) is sensitive to severe platelet function disorders (PFD), such as Glanzmann's thrombasthenia (GT) and Bernard-Soulier syndrome (BSS).<sup>12–14</sup> However, this test has only limited sensitivity for diseases with milder bleeding phenotype,<sup>15</sup> like primary platelet secretion syndrome, storage pool disease<sup>16–18</sup> or moderately low levels of VWF (30%–50%).<sup>19</sup> Accordingly, in the diagnostic work-up for patients with bleeding symptoms, the finding of a PFA-CT indicates that further diagnostic testing is required.<sup>3,13,20</sup> Often extensive laboratory testing is required before a final diagnosis can be made.

For identification of platelet function disorders, light transmission aggregometry (LTA) is the gold standard, often in combination with adenosine triphosphate (ATP) release or flow cytometry.<sup>21–23</sup> For the diagnosis of VWD, plasma levels of VWF activity and antigen are determined, if indicated in combination with testing of VWF multimer pattern, VWF–collagen binding and VWF–factor VIII binding.<sup>24</sup> Several reports indicate that, even after extensive testing, in a significant proportion of patients with bleeding symptoms, an 'unexplained' prolonged PFA-CT is the only abnormality found.<sup>25,26</sup> These 'PFA-only' patients are, like other patients with bleeding of unknown cause, provided with a treatment plan, e.g. receiving desmopressin or tranexamic acid, to prevent excessive bleeding upon surgery or tooth extraction.<sup>27,28</sup>

An explanation for a prolonged PFA, as single laboratory aberration, is that this is the only test in the diagnostic work-up for bleeding evaluation that relies on high shear forces. In primary haemostasis, initial platelet rolling is regulated by the interaction of platelet GPIb–V–IX complex and VWF bound to collagen. High shear is required to unfold VWF multimers to expose the VWF-A1 binding site for platelet GPIb.<sup>29</sup> Accordingly, shear-dependent defects of both platelets and VWF will contribute to abnormal PFA values, which are likely not detected in static tests of haemostasis. A clear limitation of the PFA, however, is that it only gives the aperture CT as a test result, not providing any information about the cause of a prolonged PFA.

Microfluidic whole-blood flow assays are frequently used to study platelet thrombus formation *in vitro*. As we and others have shown previously, multiparameter measurements of thrombus formation give a wealth of information about

platelet adhesion, activation and aggregation under flow conditions, especially when combined with arrays of microspots in the same flow chamber.<sup>29,30</sup> Thus, when using microspots of collagen-I and collagen-III (both binding plasma VWF), multiple qualitative and quantitative platelet traits have been identified, linked to genetic variation of the collagen receptor GPVI and to inherited bleeding disorders.<sup>31–33</sup> In combination with the use of brightfield and multicolour fluorescence microscopy, the microfluidic assay thus produces multiple platelet-dependent outcome values relevant for flow conditions.<sup>34–36</sup>

For the present study, our aim was to investigate whether and how multiparameter shear-dependent microfluidic testing operating at physiological temperature can help to identify abnormalities in platelet function or VWF that explain the prolonged CTs of PFA-only patients with a bleeding history. Furthermore, patients' DNA was sequenced using targeted high-throughput gene panel testing to identify genetic variants known to cause bleeding and platelet disorders.

## MATERIALS AND METHODS

A detailed description of the methods is available in the Supplementary material.

### Study population

Patients were selected from three observational cohort studies including adult subjects between September 2013 and November 2020. In all cases, a large panel of in-hospital laboratory tests was performed evaluating the blood from: (i) 136 patients referred to the haematologist for bleeding evaluation (ProBe-AHP cohort); (ii) 240 patients examined pre-operatively (PANE cohort); and (iii) 49 patients with a prior diagnosed bleeding disorder of any kind (BEPa cohort). Detailed study designs were described previously.<sup>37</sup>

For the present paper, patients were included if prior PFA results were abnormal (i.e. one or both cartridges showing a prolonged CT) and if this was the only aberration found in the diagnostic work-up. All studies were approved by the local medical ethics committee. All participating patients and healthy individuals gave written informed consent according to the Helsinki declaration.

### Data collection and prospective laboratory evaluation

For all patients, in order to select the PFA-only patients, the following data were retrospectively collected: medical history; family history; medication use; International Society on Thrombosis and Haemostasis bleeding assessment tool (ISTH BAT) score; diagnosis; complete blood counts; PFA results; light transmission aggregometry (LTA) results; VWF activity and antigen; whole-blood rotational

thromboelastography (ROTEM) and plasma thrombin generation. Prospectively, medical history; ISTH BAT score, and medication use were evaluated again. Blood was taken to repeat and extend laboratory tests for VWD (including multimer pattern, VWF-FVIII binding, VWF-collagen binding) and PFD (including ATP release and flow cytometry), as described in the Supplementary material.

## Multiparameter thrombus formation using microfluidics

Multiparameter microfluidic assays were performed using the Maastricht flow chamber, as described previously in detail,<sup>29,33</sup> but adapted to operation at the physiological temperature of 37°C.<sup>38</sup>

Brightfield and tri-colour fluorescence images were taken with an EVOS-FL microscope (Life Technologies Europe, Beringe, Belgium), every minute during an overall time of 7 min. Platelet adhesion and aggregation were assessed using the following parameters (Table 1): platelet surface area coverage: obtained from threshold, representing identified regions of all adhered platelet and thrombus structure (P1); platelet deposition: identified regions of platelet deposition and mono- or multi-layered thrombi (P2); thrombus morphology score (P3); thrombus contraction score (P4) and thrombus multilayer score (P5). Fibrinogen binding was assessed to report on integrin  $\alpha_{IIb}\beta_3$  activation (P6).

**TABLE 1** Overview of microspot surfaces (M), microfluidic parameters (P), and flow cytometry markers (C)

Microspot surface		Platelet receptor involved	
M1	Collagen type I	GP1b, GPVI, $\alpha_2\beta_1$	
M2	Collagen type III	GP1b, GPVI, $\alpha_2\beta_1$	
Brightfield/Fluorescence parameters		Range	Normalized
P1	Platelet surface area coverage (%SAC)	0–100	0–10
P2	Platelet deposition (%SAC)	0–100	0–10
P3	Thrombus morphological score	0–6	0–10
P4	Thrombus contraction score	0–4	0–10
P5	Thrombus multilayer score	0–4	0–10
P6	Integrin $\alpha_{IIb}\beta_3$ activation (%SAC)	0–100	0–10
P7	PS exposure (%SAC)	0–100	0–10
P8	P-selectin expression (%SAC)	0–100	0–10
Flow cytometry markers		Platelet receptor involved	
C1	CD41	GPIIb (integrin $\alpha_{IIb}\beta_3$ )	
C2	CD42a	GPIX	
C3	CD42b	GPIb (integrin $\alpha_2$ )	
C4	CD42d	GPV	
C5	CD61	GPIIIa (integrin $\beta_3$ )	

Abbreviations: GP, glycoprotein; PS, phosphatidylserine; SAC, surface area coverage.

Phosphatidylserine (PS) exposure (P7) was measured as a marker of platelet procoagulant activity. Platelet secretion was assessed by measuring P-selectin expression (P8).

Platelet adhesion and aggregation (P1–5) were assessed at a collagen-I surface (M1) at a wall-shear rate of 1000 s<sup>-1</sup> (S1). Fluorescence parameters (P6–8) were assessed at a collagen-I and a collagen-III surface (M2) at wall-shear rates of 1000 s<sup>-1</sup> and 1600 s<sup>-1</sup> (S2). Figure 1 shows representative images of the results of a healthy individual and a patient with severe (type 1) GT.

## ThromboGenomics high-throughput sequencing (HTS)

Patients were sequenced using the ThromboGenomics HTS panel test of diagnostic-grade genes known to harbour variants associated with rare bleeding, thrombotic, or platelet disorders. The ThromboGenomics HTS test sample preparation, sequencing protocols, tested genes, variant prioritization, interpretation and reporting, were as extensively described before.<sup>39,40</sup>

## RESULTS

### Selection of patients and control subjects

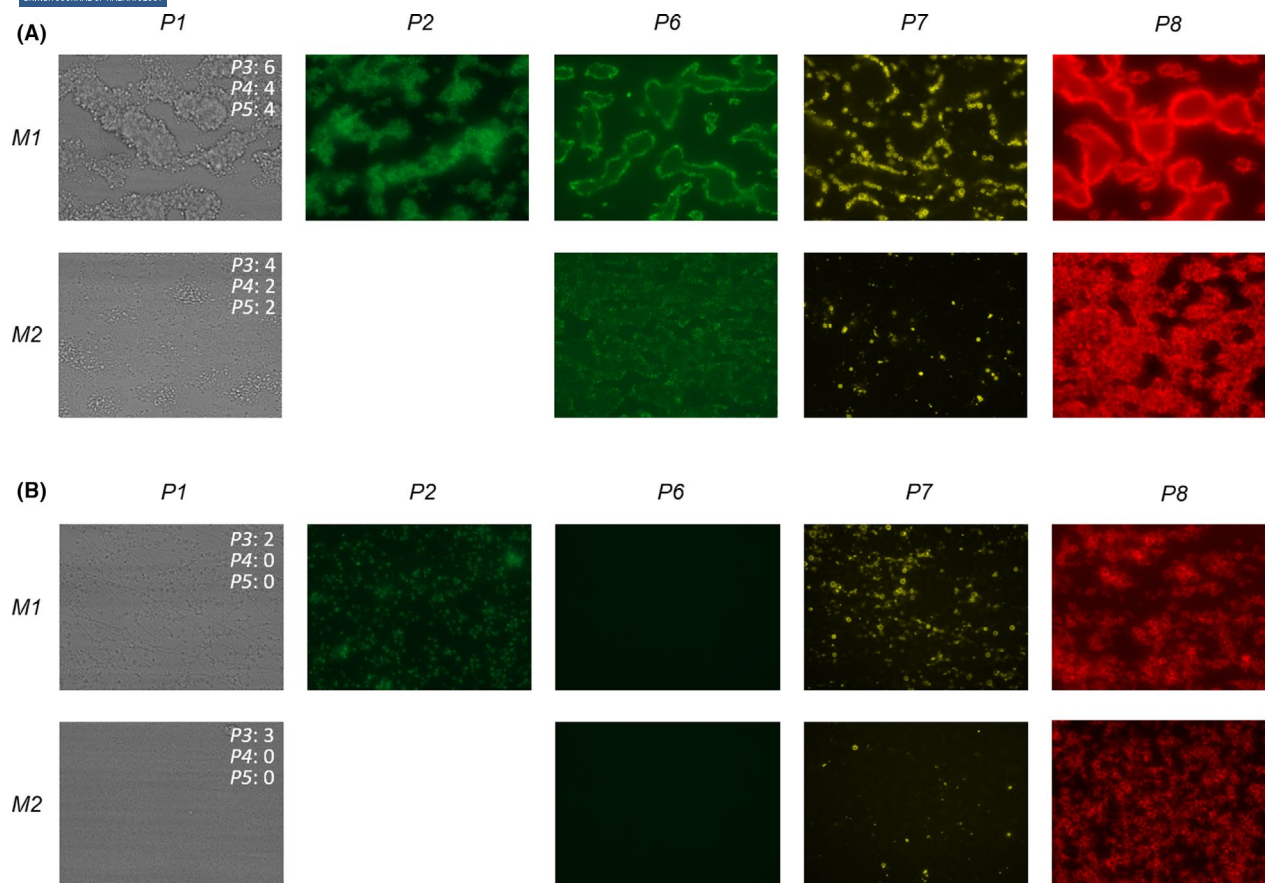
Laboratory data from a total of 425 patients with established or suspected bleeding disorders from three earlier observational studies<sup>37</sup> were evaluated for the identification of 'PFA-only' patients. From the 21 identified PFA-only patients, 14 consented to be included in this study (Figure 2).

Four patients with a known bleeding disorder (VWD or GT) were selected from the BEPA study as positive controls. For the microfluidic assay, blood samples from 50 healthy controls were evaluated, in order to obtain normal values for the sets of thrombus parameters, brightfield microscopic images of shear-dependent thrombus formation at 37°C were recorded for all 50 subjects, while parallel fluorescence images were also recorded for 23 of them and in 15 of those 23 patients, PFA results were obtained as well.

### Baseline characteristics and alterations in PFA, platelet function and VWF in PFA-only patients

Table 2 shows the prospective baseline characteristics of the 14 PFA-only patients and the subgroup of 23 controls with parallel fluorescent images. Baseline characteristics, retrospective and prospective data of PFA-only patients, controls and patients with a bleeding disorder are presented in Tables S1–S4.

Compared to the controls, the PFA-CT results of the PFA-only patients were longer (mean PFA-CT adenosine diphosphate (ADP): 118 ± 27 s vs 94 ± 13 s,  $p = 0.007$ ; mean PFA-CT epinephrine (EPI): 150 ± 42 s vs 111 ± 16 s,  $p = 0.005$ ) and platelet volume was higher [mean platelet volume (MPV): 10.6 ± 1.3 nl vs 9.8 ± 0.8 nl,  $p = 0.016$ ]. Furthermore, the



**FIGURE 1** Microscopic imaging of platelet thrombus formation on two different microspots. Representative images after whole blood flow from a healthy subject (A) and a patient with type 1 Glanzmann thrombasthenia (B) over different microspots (*M1*, *M2*) at shear rate *S1*. For the brightfield images, score values for *P3* (thrombus morphological score), *P4* (thrombus contraction score) and *P5* (thrombus multilayer score) at 7 min are indicated. The definition of all parameters is given in Table 1 [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

patients were significantly older compared to the controls (mean age:  $51 \pm 17$  years vs  $34 \pm 10$  years,  $p = 0.003$ ). The majority of the PFA-only patients was female (86%), had blood type O (64%) and the mean ISTH BAT score was  $10 \pm 4$ . Extensive VWF tests, ATP release and flow cytometry were within the normal ranges in all 14 patients. Prospective LTA results showed two abnormal aggregation curves in one patient (nr. 8), fulfilling the diagnostic criteria of a platelet function disorder.<sup>21,22,41,42</sup> Patient nr. 9 used venlafaxine, a selective serotonin reuptake inhibitor (SSRI), possibly explaining the prolonged PFA-CT.

Whole-blood rotational thromboelastography (ROTEM) showed slightly prolonged clotting time in the INTEM and/or EXTEM in 10/14 PFA-only patients. Plasma thrombin generation was normal in all but one patient, who showed increased thrombin generation (data presented in Tables S5 and S6).

### Altered microfluidic thrombus formation in PFA-only patients

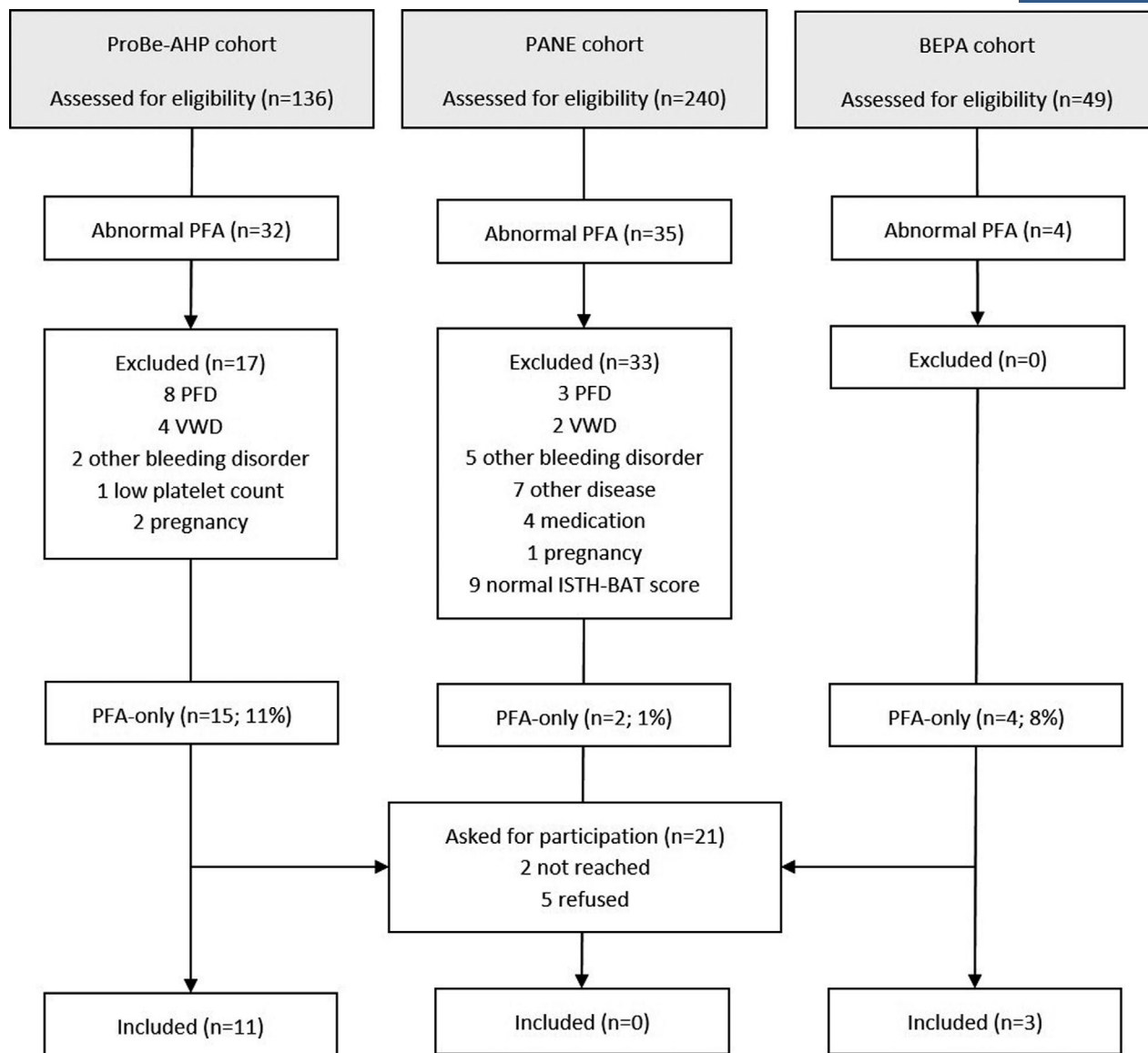
Multiparameter microfluidic assessment of shear-dependent thrombus formation, operating at a shear rate of  $1000 \text{ s}^{-1}$  and  $1600 \text{ s}^{-1}$  and a physiological temperature of  $37^\circ\text{C}$ , was

performed with blood from all patients. Results are shown in Figure 3 (data presented in Table S7).

In the cohort of PFA-only patients, as compared to healthy volunteers, microfluidic test results showed significantly lower platelet surface coverage (*P1*) and platelet deposition (*P2*) at 3 and 7 min (*P1\_7min*: 49% vs 61%,  $p < 0.001$ ; *P2\_7min*: 17 vs 22,  $p < 0.001$ ) and significantly lower thrombus formation scores at all time points ( $p < 0.008$  for all comparisons). Fluorescence markers showed lower integrin activation (*P6*: 20% vs 27%,  $p = 0.001$ ), PS exposure (*P7*: 12% vs 16%,  $p = 0.036$ ) and P-selectin expression (*P8*: 33% vs 41%,  $p = 0.001$ ). No significant difference was seen in the results of PFA-only patients compared to the results of patients with a known bleeding disorder. Thrombus contraction and multilayer score (*P4*, *P5*) were even less in PFA-only patients when compared to patients with a bleeding disorder; however, this was not significant.

In Figure 4, the results of the microfluidic parameters are visualized per individual patient. In all patients abnormalities in microfluidic parameters were found, indicating reduced thrombus formation, compared to the group of controls. In PFA-only patients 8, 9, 12, 13 and 14, severe abnormalities in thrombus formation were found, comparable to patients with VWD or thrombopathy.





**FIGURE 2** Patient Flow Diagram representing patient selection, reasons for exclusion and number of included patients from three observational studies, the ProBe-AHP, PANE and BEPA cohorts. CT, closure time; ISTH BAT, International Society on Thrombosis and Hemostasis bleeding assessment tool; LTA, light transmission aggregometry; *n*, number; PFA, platelet function analyser; PFD, platelet function disorder; VWD, von Willebrand disease; VWF, von Willebrand factor

## Integration of data sets

For a systemic evaluation of the characteristics, correlation analysis were performed for the laboratory results and microfluidic parameters in the 14 PFA-only patients. Integrated results of the correlation coefficients *R* and *p* values are visualized in Figure 5A,B, respectively (results of all subjects are presented in Figure S1). The PFA-CT results correlated moderately with VWF activity ( $R = -0.58$ ,  $p < 0.031$ ) and with PS exposure (*P7*) at *M2*, *S2* ( $R = -0.62$ ,  $p = 0.022$ ) but not with other microfluidic parameters. Moderate to strong correlations were seen between microfluidic parameters and LTA aggregation curves, but not with ATP release. Furthermore, both LTA results

and microfluidic parameters correlated with haematocrit ( $R$  range 0.40–0.77,  $p < 0.05$ ).

Principle components analysis (PCA) was performed to find the gross relationships between the microfluidic parameters at *S1*, *M1* and the subject parameters in the PFA-only patients (Figure 6A,B). Values of all subjects are shown in Figure S2. Markedly, Figure 6A shows the extent of clustering of microfluidic parameters *P1–8* and haematocrit in component 1 (42%). Neither age, sex nor BAT contributed to this component. In component 2 (22%) the other haematology parameters clustered together. Figure 6B shows the extent of clustering of microfluidic parameters, LTA aggregation curves and haematocrit in component 1 (52%), while PFA with VWF and to a lesser extent *P6* clustered in component 2 (18%).

**TABLE 2** Baseline characteristics of PFA-only patients ( $n = 14$ ) and control group ( $n = 23$ )

Characteristics mean $\pm$ SD, $n$ (%)	PFA-only patients	Controls	$p$ (95% CI)
Female	12 (86)	16 (70)	0.273
Age (years)	51 $\pm$ 17	34 $\pm$ 10	0.003 (6.257–26.687)
Blood type O	9 (64)		
ISTH BAT score <sup>a</sup>	10 $\pm$ 4		
Haematocrit (l/l)	0.42 $\pm$ 0.03	0.42 $\pm$ 0.03	0.848 (–0.0221 to 0.018)
Thrombocytes ( $\times 10^9$ /l)	257 $\pm$ 84	279 $\pm$ 68	0.374 (–73.727 to 28.435)
MPV (nl)	10.6 $\pm$ 1.3	9.8 $\pm$ 0.8	0.016 (0.165–1.495)
Leukocytes ( $\times 10^9$ /l)	5.7 $\pm$ 1.1	6.2 $\pm$ 1.6	0.290 (–1.479 to 0.455)
PFA-EPI (s)	150 $\pm$ 42	111 $\pm$ 16	0.005 (13.558–63.728)
PFA-ADP (s)	118 $\pm$ 27	94 $\pm$ 13	0.007 (7.453–41.204)
VWF activity (%)	93 $\pm$ 33		
VWF antigen (%)	91 $\pm$ 32		
VWF ratio	1.0 $\pm$ 0.1		
FVIII (%)	125 $\pm$ 39		
Multimer pattern abnormal	0 (0)		
VWF-collagen binding (%)	82 $\pm$ 31		
VWF-FVIII binding (%)	94 $\pm$ 12		
Flow cytometry abnormal	0 (0)		

Abbreviations: ADP, adenosine diphosphate; CI, confidence interval; EPI, epinephrine; ISTH BAT, International Society on Thrombosis and Hemostasis bleeding assessment tool; MPV, mean platelet volume; PFA, platelet function analyser; SD, standard deviation; VWF, von Willebrand factor.

<sup>a</sup>An ISTH BAT score for males  $\geq 4$  and for females  $\geq 6$  was considered to be abnormal.

To further investigate the parameters in the PFA-only patients, comparative analysis was performed in subgroups stratified for results below respectively above the mean value of all patients, for PFA (total of PFA-CT EPI and ADP), VWF activity, scaled microfluidic results, and haematocrit. Results are shown in Table S8. Patients with more prolonged PFA results (7/14), had less integrin activation ( $P_6$ : 17  $\pm$  3% vs 23  $\pm$  4%,  $p = 0.011$ ) in thrombus formation, and a trend towards a lower VWF activity (76  $\pm$  13% vs 109  $\pm$  39%,  $p = 0.070$ ), compared to patients with higher PFA results. Patients with lower VWF activity (8/14) had significantly prolonged PFA-CT EPI compared to patients with higher VWF (PFA-CT EPI: 169  $\pm$  38 s vs 124  $\pm$  33 s,  $p = 0.038$ ), but no differences were found in microfluidic test results.

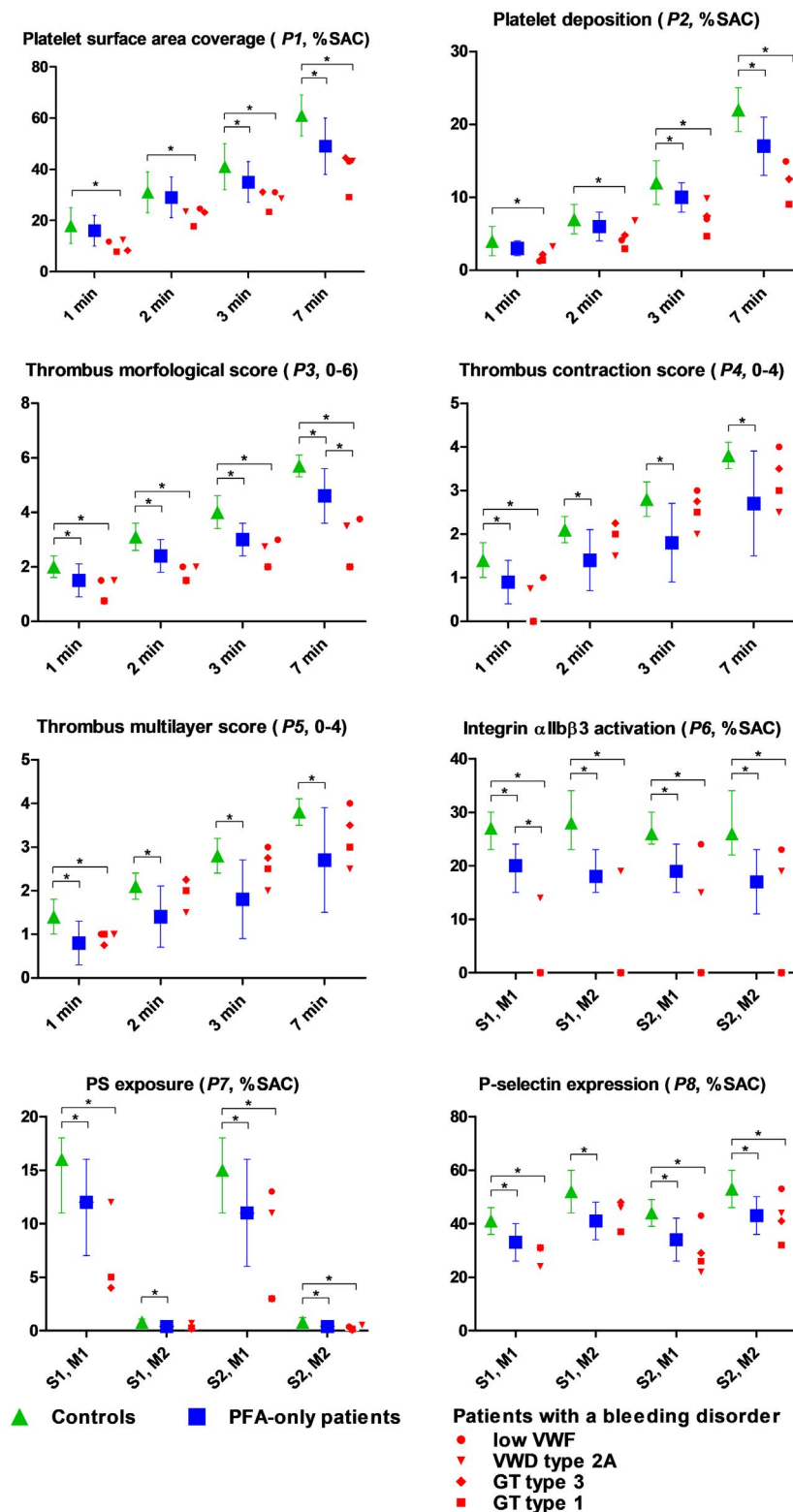
Patients with severe abnormal microfluidic results (5/14), had lower LTA aggregation curves (although not considered as diagnostically relevant) and less ATP release on epinephrine compared to patients with less severe abnormalities [e.g. LTA arachidonic acid (NAAA): 72  $\pm$  7% vs 85  $\pm$  4%,  $p = 0.001$ ; LTA ADP 5: 63  $\pm$  11% vs 81  $\pm$  4%,  $p = 0.001$ ; ATP/EPI: 1.19  $\pm$  0.09 nmol vs 1.54  $\pm$  0.41 nmol,  $p = 0.032$ ], there was no significant difference in VWF activity. Finally, patients with lower haematocrit (6/14) had lower LTA aggregation curves and more abnormal microfluidic test results (e.g. LTA NAAA: 74  $\pm$  8% vs 84  $\pm$  5%,  $p = 0.014$ ; platelet deposition  $P_2$ : 14  $\pm$  3% vs 20  $\pm$  2%,  $p = 0.001$ ).

## Results of platelet addition experiments

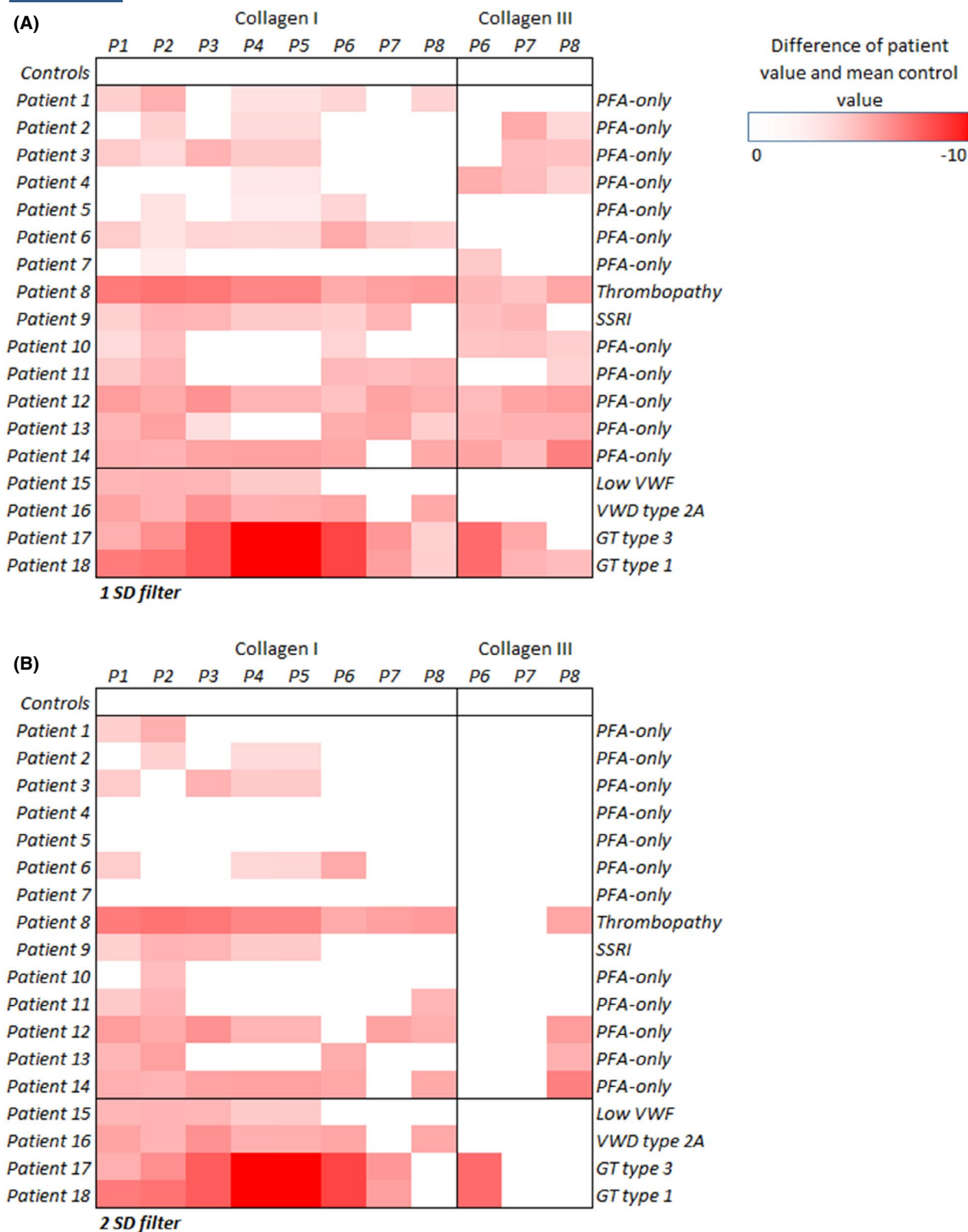
To see if the reduction in thrombus formation could be rescued, blood of patients 12 and 14 as well as two healthy controls was supplemented with either 80  $\mu$ l HEPES buffer pH 7.45 or washed platelets, in order to increase the platelet count of the patients by 20% or 40%. Results displayed in Figure S3 in the Supplementary material, showed that an addition of 20% healthy donor platelets did not increase the amount or size of platelet aggregates formed [end-point multilayer surface area coverage (ML SAC)]. In patient nr. 12, the platelet count was then increased with 40%, which resulted in a significant increase in platelet aggregate size (ML SAC: 3% vs 6%,  $p = 0.010$ ). However, platelet aggregate size remained much lower compared to the healthy control (ML SAC: 22%). A further increase in platelet count was not possible, because this caused too much dilution of the samples.

## Results of high-throughput sequencing of PFA-only patients

The ThromboGenomics HTS gene panel test results were available for all PFA-only patients. The patients were tested with the ThromboGenomics version 3 (TG.V3), including 96 Mendelian genes causing coagulation, thrombotic, or platelet disorders and probes for 10 000 common single-nucleotide variants (SNVs) to estimate relatedness and ancestry.<sup>39</sup> No genetic variants (SNV or structural variants)

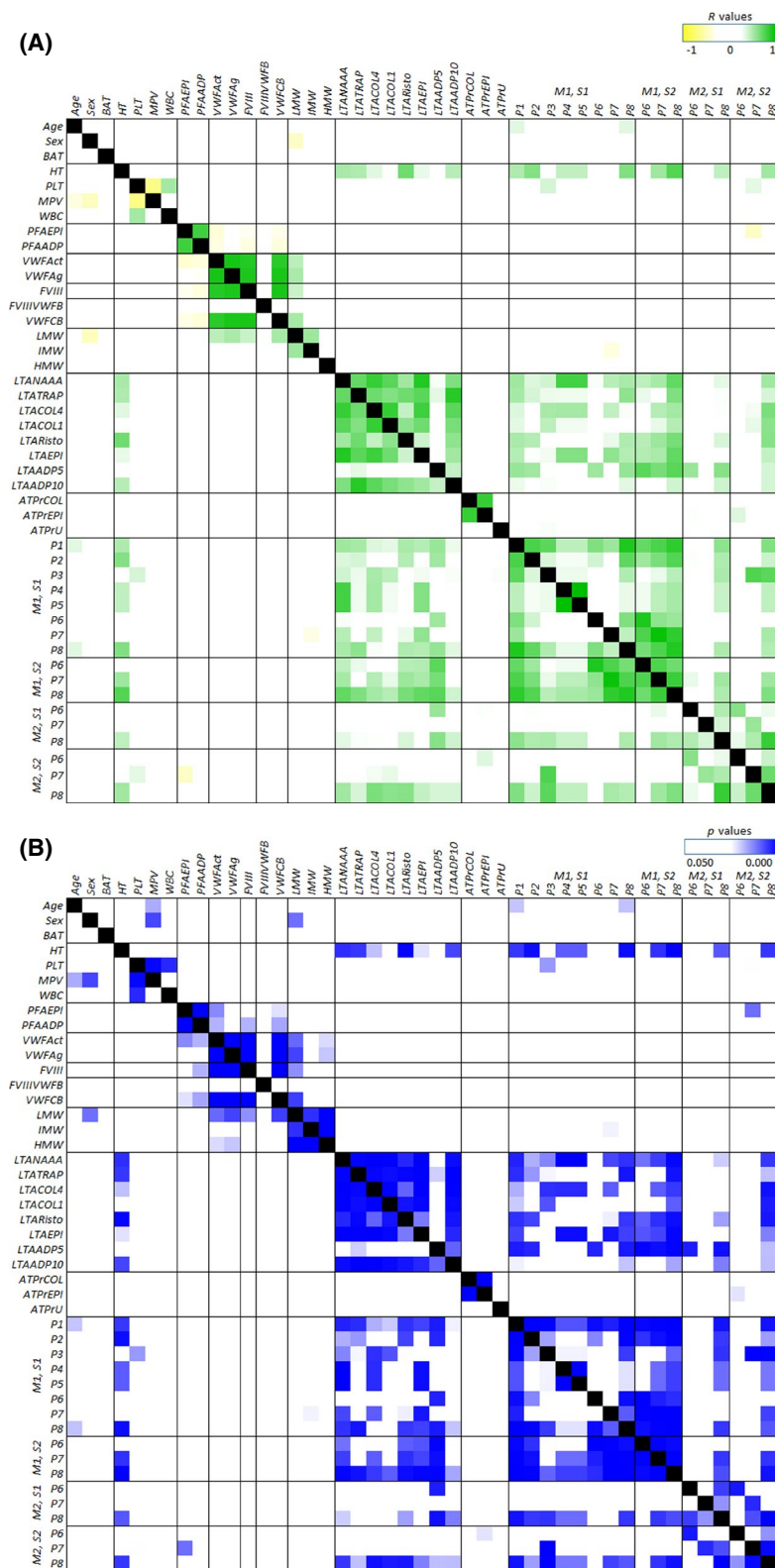


**FIGURE 3** Comparison of microfluidic results of PFA-only patients ( $n = 14$ ), patients with a bleeding disorder ( $n = 4$ ) and control groups ( $n = 50/23$ ). Parameters  $P1-5$  at surface  $M1$  and shear rate  $S1$  are compared between 50 controls (green triangle), PFA-only patients (blue square), and patients with a bleeding disorder (in red, each symbol represents a patient). Parameters  $P6-8$  at surface  $M1$  and  $M2$  and shear rate  $S1$  and  $S2$  are compared between PFA-only patients, patients with a bleeding disorder and 23 controls. \*alpha level of significance =  $p < 0.05$ . GT, Glanzmann thrombasthenia; PFA, platelet function analyser; PS, phosphatidylserine; SAC, surface area coverage; VWD, von Willebrand disease; VWF, von Willebrand factor. For detailed explanation of parameters (P), microspots (M) and shear rates (S) see Table 1 [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

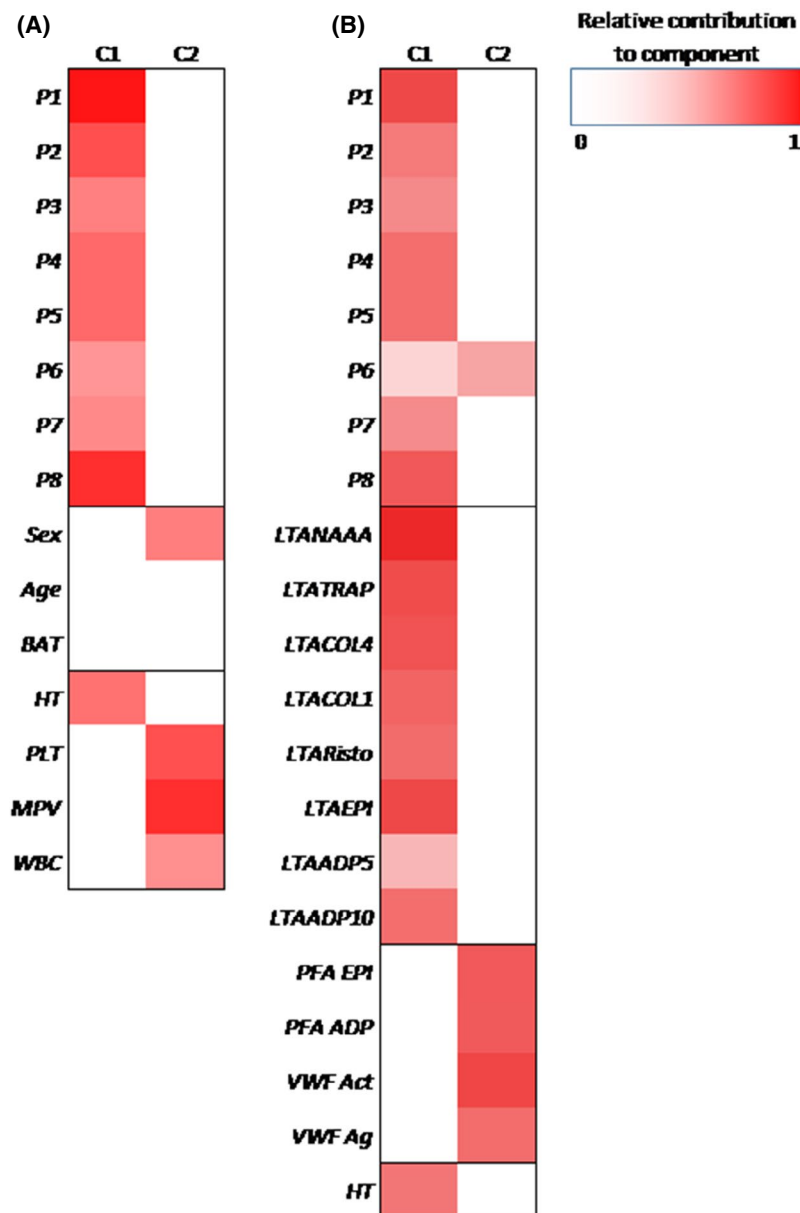


**FIGURE 4** Heatmaps of microfluidic results per individual patient compared to the group of controls with 1 SD (A) and 2 SD filter (B). A darker red colour indicates that the patient's result was lower than the average result of the control group. GT, Glanzmann thrombasthenia; PFA, platelet function analyser; SD, standard deviation; SSRI, selective serotonin re-uptake inhibitor; VWD, von Willebrand disease; VWF, von Willebrand factor [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]





**FIGURE 5** Heatmapped correlations ( $R$ ) with  $p$  values of microfluidic parameters and haematology, PFA, platelet function tests, age, sex, and BAT score in the 14 PFA-only patients. (A) Heatmapped Pearson correlation coefficients  $R$ : a darker colour indicates a stronger negative (yellow) or positive (green) correlation. (B) Heatmapped  $p$  values in which a darker colour (blue) indicates a highly significant correlation (white offset at  $p = 0.05$ ). ADP, adenosine diphosphate; ATP, adenosine triphosphate release; BAT, bleeding assessment tool; COL, collagen; EPI, epinephrine; HT, haematocrit; LTA, light transmission aggregometry; MPV, mean platelet volume; NAAA, arachidonic acid; PFA, platelet function analyser; PLT, platelet count; R, correlation coefficient; Risto, ristocetin; TRAP, thrombin receptor activatable peptide aggregometry; PFA, platelet function analyser; WBC, white blood cell count; VWFact, von Willebrand factor activity; VWFAg, von Willebrand factor antigen. For detailed explanation of parameters (P), microspots (M) and shear rates (S) see Table 1 [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



**FIGURE 6** Principal components analysis of microfluidic parameters *P1–8* at surface *M1* at shear rate *S1* and (A) sex, age, ISTH BAT score, HT, PLT, MPV and WBC score, and (B) LTA aggregation curves, PFA, VWF and HT in PFA-only patients ( $n = 14$ ). Darker (red) colours indicate the parameters that tend to cluster together per component when compared to other sets of parameters. ADP, adenosine diphosphate; BAT, bleeding assessment tool score; COL, collagen; EPI, epinephrine; HT, haematocrit; ISTH BAT, International Society on Thrombosis and Hemostasis bleeding assessment tool; LTA, light transmission aggregometry; MPV, mean platelet volume; NAAA, arachidonic acid; PFA, platelet function analyser; PLT, platelet count; RISTO, ristocetin; TRAP, thrombin receptor activatable peptide aggregometry; WBC, white blood cell count; VWFact, von Willebrand factor activity; VWFag, von Willebrand factor antigen. For detailed coding of parameters (*P*), microspots (*M*) and shear rates (*S*) see Table 1 [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

that could explain the coagulation or platelet abnormality were identified.

## DISCUSSION

In this study we investigated whether and how the multiparameter shear-dependent microfluidic assay could help to identify abnormalities in platelets or VWF that can explain the prolonged closure times of PFA-only patients with a bleeding history.

We found that in PFA-only patients: (i) most microfluidic test parameters were impaired compared to healthy controls. Abnormalities reached to levels seen in patients with established bleeding disorders, such as GT and VWD; (ii) PCA analysis indicated VWF and platelet activation as co-variables of PFA results; (iii) LTA aggregation results and haematocrit were indicated as co-variables of microfluidic parameters; (iv) in patients with the most severe microfluidic abnormalities, LTA aggregation results were in the lowest range of normal; (v) patients with lower haematocrit had more abnormal

microfluidic test results; and (vi) exome sequencing did not reveal a monogenic explanation for the abnormalities found.

Our results show that an abnormal PFA as a single aberrant test in patients referred for bleeding evaluation, has to be considered for further evaluation. We found that PFA-only patients exhibited abnormal multiparameter microfluidic test results, and analysis of the specific microfluidic outcome parameters indicated impaired thrombus formation and less platelet activation in these patients. In some PFA-only patients abnormalities were even comparable to those in patients with previously established VWD or a PFD. PFA prolongation was associated with low-normal VWF, while microfluidic abnormalities were also associated with low-normal LTA aggregation curves and low haematocrit.

Aberrations in microfluidic assays have been described in patients with mild to severe platelet function defects like Gray platelet syndrome, storage pool disease, Bernard-Soulier and Glanzmann thrombasthenia,<sup>29,30</sup> but also in patients with VWD type 1 and low VWF.<sup>30,43</sup> The correlations of microfluidic test results with LTA aggregation results, found in our study, indicate that the abnormalities found in these patients could reflect a shear-dependent platelet activation defect, that is not detected using the current thresholds for normality in the static conventional platelet function tests.

Although addition of healthy platelets, *in vitro*, did not fully rescue the reduction in thrombus formation, an increase in platelet aggregate size was observed when platelet count was increased by 40% by addition of healthy platelets. This result suggests that substantial platelet transfusion will be needed to normalize thrombus formation and to prevent bleeding in PFA-only patients. *In-vivo* experiments, before and after platelet transfusion in PFA-only patients, are needed to investigate this in detail.

Both the PFA-100 and the microfluidic test rely on combined processes of shear-dependent primary (collagen) and secondary (autocrine) platelet activation along with VWF function. Exome sequencing of a large panel of genes associated with coagulation, thrombotic, or platelet disorders, did not reveal a monogenic explanation (e.g., a TIER1 mutation) for the abnormalities found in the PFA-only patients. This does not exclude genetic variations in unknown genes involved in the VWF-platelet interactions, or the accumulative effects of a number of common variants,<sup>39</sup> which might result in the abnormalities found (Tier-2/3 mutations were not tested, since there are no prior data on how these affect platelet traits under high-shear conditions). Furthermore, for the majority of patients we are not aware of a familial history of bleeding, which includes the possibility of an acquired or somatic mutation effect. Some of the patients had low-normal VWF. We hypothesize that the bleeding disorder in PFA-only patients is multifactorial, and includes combinations of low VWF, Tier-2/3 mutations of platelet function genes, reduced autocrine processes and acquired or somatic platelet defects. Furthermore, there may be a

relation with the novel shear-dependent receptor GRP56, but nothing is known about the expression variability of this receptor.<sup>44</sup>

In the ROTEM test the activation and acceleration of coagulation relies on platelet phospholipids, since no phospholipids are added to the samples. Although the shear stress in this method is only low, these results might reflect a problem in the flip-flop mechanism, phospholipid expression and procoagulant activity of the platelets.

In the PFA-only patients, but not in all subjects, haematocrit correlated with the microfluidic test results. Patients with low-normal haematocrit had more abnormal microfluidic test results. Haematocrit is known to influence platelet interaction with the vessel wall, but the effect is limited to levels below 0.25 l/l.<sup>45</sup> A previous study of a microfluidic assay in healthy individuals, found no association between haematocrit and the microfluidic assay results.<sup>46</sup> However, in PFA-only patients, reduced red blood cell-dependent platelet migration towards the vessel wall might aggravate the abnormalities of platelet function found in the microfluidic assay.

Based on the present analysis, we propose that, in all patients with an unexplained abnormal PFA, microfluidic testing should be considered in the diagnostic work-up for mild bleeding tendency as an additional test of shear-dependent VWF or platelet function. Our results also indicate that it would be interesting to perform microfluidic testing in all patients with bleeding of unknown cause (BUC). Only one study investigated the results of a microfluidic assay in a group of ten BUC patients and found no significant differences compared to the healthy controls.<sup>43</sup> However, the microfluidic assay used in this study differed greatly from our assay. This study is limited by the small number of PFA-only patients, of which two patients prospectively had an explanation for the prolongation of PFA-CT. This prevented us from performing a robust regression analysis and therefore, our results should be interpreted with care. However, we still believe that our findings add important information to the discussion about BUC patients with an unexplained abnormal PFA and give directions for further research.

In conclusion, our study showed that an abnormal PFA as a single aberrant test in bleeding patients should be further evaluated. The multiparameter microfluidic test is able to detect abnormalities in these patients, also detecting low-normal LTA aggregation results, suggesting a shear-dependent platelet function defect, not detected by the static conventional platelet function tests. To further develop of this assay, an alpha-version prototype<sup>38</sup> has been placed in the diagnostics laboratory of our hospital, and is currently used to evaluate blood samples from patients with bleeding alongside the routine platelet functions tests. Based on the outcome of this routine analysis, beta versions will be placed in other laboratories.

## CLINICAL TRIAL REGISTRY INFORMATION

Netherlands Trial Register: NL3873 (NTR4070), <https://www.trialregister.nl/trial/3873>

## CONFLICTS OF INTEREST

All authors have read the journal's authorship agreement and policy on disclosure of potential conflicts of interest. NJJ is registered in a joined PhD program of the Universities of Maastricht and Birmingham and received funding from the European Union's Horizon 2020 research and innovation program under Marie Skłodowska-Curie grant agreement TAPAS 766118. PM and JWMH are cofounders and JWMH is share-holder of FlowChamber b.v. FCJIH-M, SLNB, LB, RJHW, PWMV, KM, KD, EAMB and YMCH declare no conflicts of interest.

## AUTHOR CONTRIBUTIONS

FCJIH-M contributed to the concept and study design, included patients, managed the database, performed statistical analysis and wrote the manuscript. SLNB performed statistical analysis and contributed to critical writing, and approved the final version to be published. LH performed laboratory analysis, contributed to critical writing and approved the final version to be published. LB included patients, managed the database, contributed to critical writing and approved the final version to be published. NJJ performed additional laboratory analysis for the revised version of the manuscript and approved the final version to be published. RJHW performed laboratory analysis and contributed to critical writing, and approved the final version to be published. PWMV performed laboratory analysis, contributed to critical writing and approved the final version to be published. PM approved the final version to be published. KM performed and interpreted the ThromboGenomics HTS test, contributed to critical writing and approved the final version to be published. KD performed and interpreted the ThromboGenomics HTS test, contributed to critical writing and approved the final version to be published. JWMH contributed to the concept and study design, contributed to critical writing and revising the intellectual content, and approved the final version to be published. EAMB contributed to the concept and study design, contributed to critical writing and revising the intellectual content, and approved the final version to be published. YMCH contributed to the concept and study design, contributed to critical writing and revising the intellectual content, and approved the final version to be published.

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## SUPPORTING INFORMATION

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