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### Platelet microparticle generation assay

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## Regular Article

## Platelet microparticle generation assay: A valuable test for immune heparin-induced thrombocytopenia diagnosis

F. Mullier<sup>a,b,c,\*</sup>, V. Minet<sup>b</sup>, N. Bailly<sup>a</sup>, B. Devalet<sup>c,d</sup>, J. Douxfils<sup>b,c</sup>, C. Chatelain<sup>c,d</sup>, I. Elalamy<sup>e</sup>, J.M. Dogné<sup>b,c,1</sup>, B. Chatelain<sup>a,c,1</sup><sup>a</sup> Hematology Laboratory- Namur Research Institute for Life Sciences (NARILIS), CHU Dinant-Godinne UCL Namur, Yvoir, Belgium<sup>b</sup> Department of Pharmacy- Namur Research Institute for Life Sciences (NARILIS), University of Namur, Namur, Belgium<sup>c</sup> Namur Thrombosis and Hemostasis Center (NTHC), Namur, Belgium<sup>d</sup> Hematology Department- Namur Research Institute for Life Sciences (NARILIS), CHU Dinant-Godinne UCL Namur, Yvoir, Belgium<sup>e</sup> Hematology Department, Hôpital Tenon, Paris, France

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## ABSTRACT

**Background:** Early diagnosis of immune heparin-induced thrombocytopenia (HIT) is essential to improve clinical outcome but remains challenging. The release of platelet microparticles (PMPs) is considered of major pathophysiological significance.

**Objectives:** The aim of this study was to evaluate performances of PMP generation assay (PMPGA) compared to clinical outcome to diagnose HIT. The second objective was to compare PMPGA with performances of <sup>14</sup>C-serotonin release assay (SRA) on the same series of patients.

**Methods:** Sera of 53 HIT-suspected patients were retrospectively incubated with citrated-whole blood from healthy donors with 1 IU and 500 IU/ml of unfractionated heparin (UH). PMPGA was performed using FACSARIA® flow cytometer. The clinical diagnosis was established by two blinded independent investigators analysing in a standardized manner the patient's medical records. Performances of PMPGA and SRA (n = 53) were evaluated using ROC curve analysis with clinical outcome as reference.

**Results:** In positive HIT patients, PMPs expressing phosphatidylserine are generated with low UH concentration whereas PMP rate decreases significantly in presence of high UH concentration. Using clinical outcome as reference, sensitivity and specificity of PMPGA reached 88.9% (95% CI: 50.7–99.4) and 100.0% (95% CI: 90.0–100.0). Sensitivity and specificity of <sup>14</sup>C-SRA were 88.9% (95% CI: 50.7–99.4) and 95.5% (95% CI: 83.3–99.2).

**Conclusions:** PMPGA is a rapid and reliable assay for HIT diagnosis. PMPGA showed good correlation with <sup>14</sup>C-SRA performances and predominately with clinical outcome.

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## Introduction

Immune heparin-induced thrombocytopenia (HIT) is a severe immune-mediated adverse effect of heparin treatment that can result in potentially life-threatening conditions such as venous or arterial thrombosis. Venous thrombosis in HIT patients is four-fold more common than arterial thrombosis [1]. HIT consists of an immune response leading to platelet activation, platelet aggregation, production and release of procoagulant platelet microparticles (PMPs), activation of monocytes, endothelial cells and finally to thrombin generation. Platelet activation by pathogenic anti-platelet factor 4 (PF4)-heparin antibodies generates PMPs. Moreover, PMPs serve as a catalytic surface for enhanced thrombin generation, considered as a major component of

this reaction [2,3]. These PMPs are characterised by a size ranging from 0.1 µm to 1.0 µm, and by membrane expression of glycoprotein Ib (GPIb, CD42b) and integrin αIIbβ3 (GPIIb-IIIa, CD41/CD61) [4].

Early diagnosis of HIT is essential to improve clinical outcomes. However, this diagnosis remains challenging. The current diagnostic approach consists of the combination of the clinical scoring system (“4Ts score”) with immunoassays and functional tests [2,5,6]. Immunoassays [polyspecific antigen assays (IgG/A/M) and the IgG-specific enzyme-immunoassay (EIA)] are acceptable to rule out HIT [2] but are still lacking specificity [7] and need standardization of optical density ranges [8]. Heparin-induced platelet aggregation (HIPA) and <sup>14</sup>C-serotonin release assay (<sup>14</sup>C-SRA) are considered as reference functional assays [9,10]. However, <sup>14</sup>C-SRA is time-consuming, technically demanding and requires radioactivity. In addition, this assay is not easily available in routine clinical laboratories and is therefore seldom available to clinicians in real time. Inter-laboratory variability and lack of standardization are also of concern [11,12]. A previous study reported approaches to perform quality control of the SRA [13].

\* Corresponding author at: Hematology Laboratory-NARILIS, CHU Dinant-Godinne UCL Namur, 1, avenue Gaston Therasse, B5530 Yvoir, Belgium. Tel.: +32 81424986; fax: +32 81423204.

E-mail address: [mullierfrancois@gmail.com](mailto:mullierfrancois@gmail.com) (F. Mullier).

<sup>1</sup> Contributed equally.

The validation of a new gold standard assay would be useful to avoid misdiagnosis [11] and overdiagnosis [14].

We have recently developed a PMP generation assay (PMPGA) in whole blood that could be routinely used for diagnosis of HIT [15]. This assay was not compared with clinical outcome. In the present larger study, we compared PMPGA with SRA and clinical outcome. The ultimate goal is to provide a validated easy to use and rapid functional test with similar or better performances than the standard reference <sup>14</sup>C-SRA.

## Subjects and methods

### Healthy subjects

Healthy platelet donors did not take any drug potentially affecting the platelet function for 10 days before the blood sampling.

### Patients

After approval by the local ethical committee, 57 patients with suspected diagnosis of HIT at CHU Dinant-Godinne UCL Namur were included in this study. HIT was suspected because of a rapidly decreasing platelet count occurring in hospitalised patients under heparin therapy.

### 4Ts score and clinical diagnosis

Following HIT suspicion, the “4Ts score” was calculated (based on four criteria: the severity of the thrombocytopenia and its timing, the occurrence of a thrombosis and the exclusion of other causes of thrombocytopenia) [16]. Clinical data were recorded in real time in the hospital medical database. The following information was taken into consideration: patient’s medical history, types (fractionated vs. unfractionated) and doses of heparin administered, thrombotic complications, alternative diagnoses, therapeutic attitude, clinical and platelet count evolution, co-suspected medications, and physician’s diagnoses [6,17].

Complete compression ultrasonography and multidetector spiral computed tomography were performed for suspected thrombosis.

Patients were classified as positive or negative HIT according to clinical outcome. Clinical outcome were retrospectively and independently confirmed by two investigators (VM and FM), not aware of the results of the laboratory assays. Several clinical criteria have to be fulfilled for the confirmation of clinical HIT diagnosis. Criteria from the ACCP (American College of Chest Physicians) guidelines were used to make the clinical diagnosis of HIT: (i) Thrombocytopenia, defined as at least a 30% decline in the platelet count, with a platelet count increase after heparin cessation; (ii) Timing of platelet count fall after the initiation of heparin occurring between 4 and 14 days, or occurring within 24 to 48 hours (in case of prior heparin exposure within 30 days); and (iii) lack of other, predominant causes of thrombocytopenia [18]. Other causes of thrombocytopenia analysed in this study were: neoplasia, current pregnancy or postpartum, autoimmune disease, sepsis, disseminated intravascular coagulation, intra-aortic balloon pump counterpulsation, multitransfusion, multi-trauma, shock syndrome and drug-induced thrombocytopenia (quinolone,  $\beta$ -lactam, vancomycin, teicoplanin, rifampicin, isoniazid, amphotericin, fluconazole, chemotherapy, anti-GPIIb IIIa; furosemide and proton pump inhibitor). All those 3 clinical criteria have to be fulfilled for the confirmation of clinical HIT diagnosis. Clinical diagnoses made by the 2 local investigators were 100% concordant among them and with conclusions of the medical database.

### Blood sampling and handling

Briefly, blood was collected with a 20 gauge needle via atraumatic antecubital venipuncture into polyethylene tubes terephthalate Venosafe®

(Terumo Europe, Leuven, Belgium) containing buffered sodium citrate (109 mM, nine parts blood to one part sodium citrate solution). A discard tube was used to avoid thromboplastin contamination.

### Laboratory testing

PMPGA and <sup>14</sup>C-SRA were performed retrospectively on frozen (–80 °C for maximum 18 months) sera.

### Platelet microparticle generation assay (PMPGA)

The PMPGA was performed on the 53 HIT-suspected patients who completed the clinical follow-up.

Briefly, 150  $\mu$ l of sera of HIT-suspected patients were first incubated 20 minutes at 37 °C with 165  $\mu$ l of citrated 109 mM whole blood from one appropriate healthy donor (group O Rh + or isogroup ABO and Rh) with 1 IU unfractionated heparin (UH)/ml and 500 IU UH/ml. Platelet microparticles (PMPs) are positive for antiCD41-PE. PMPs negative for annexin-V FITC (phosphatidylserine (PS)) (Fig. 1, Q1) and PMPs positive for annexin-V FITC (Fig. 1, Q2), were quantified on a BDIS FACS Aria® flow cytometer (BD Biosciences, San Jose, CA, USA). The gating strategy involves the following gates: the size of the MPs was defined using a blend of monodisperse fluorescent beads (Megamix, BioCyteX, Marseille, France) of three diameters (0.5, 0.9 and 3  $\mu$ m) according to a previously described protocol [19,20]. The threshold was set on the forward scatter according to ISTH recommendations [19]. In addition, the threshold on side scatter (SSC) was set at the lower limit (i.e. 200 AU). Then, the CD41-Annexin V gate was applied on the MP area for detecting platelet MPs expressing PS (PMPs PS +). After dividing the PE/FITC plot into four quadrants, CD41/PS + MPs would appear in the upper right quadrant. The acquisition started only after one minute to ensure fluidics stability. Flow rate and acquisition time were recorded to calculate the PMP concentration in the samples.

PMP concentrations were measured with 1 IU UH/ml and with 500 IU UH/ml to determine, respectively, PMP concentration generated by HIT antibodies and to check the specificity. Results of PMPGA are expressed as the ratio between PMP annexin V positive (Q2) concentration generated with 1 IU UH/ml and 500 IU UH/ml (rule 1) and as the concentration of PMPs annexin V positive (Q2) generated at 1 IU UH/ml (rule 2).

The flow rate was determined by recording during 10 minutes a known number of beads included in a TruCount® tube (BD biosciences). This tube contains a mix of serum and whole blood of a healthy subject (in proportions mentioned above). The aim was to have a similar viscosity index that in the test sample. The measurement was performed each 60 sec until 10 minutes with a coefficient of variation lower than 10%.

### <sup>14</sup>C-serotonin release assay

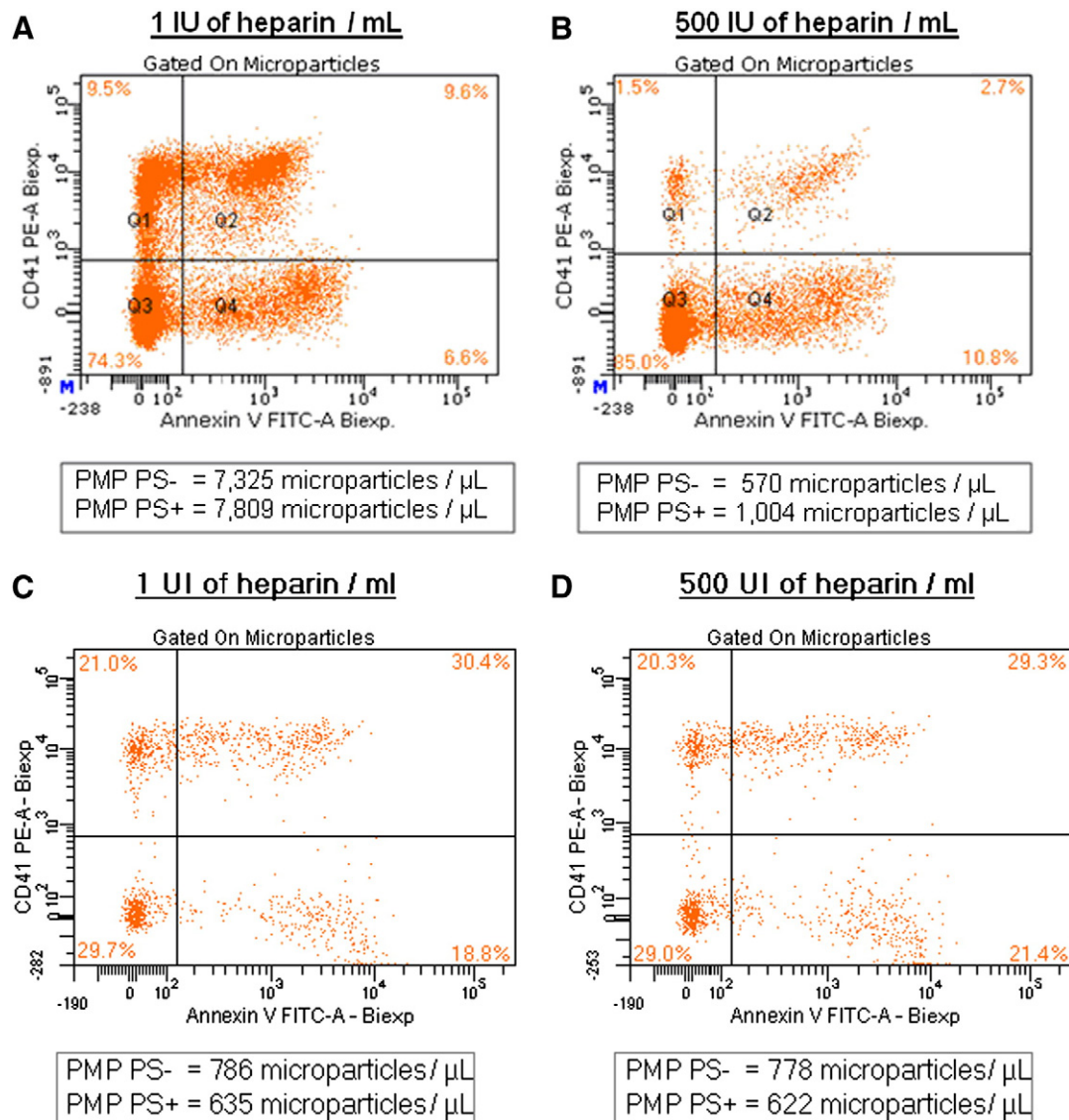
The <sup>14</sup>C-serotonin release assay was carried out according to previously published protocols on the 53 HIT-suspected patients who completed the clinical follow-up [6,10].

### Data analysis

Statistical analysis was performed using Medcalc software (version 10–4.8) (Gent, Belgium).

ROC Curves were performed to determine the optimal cut-offs of PMPGA for rule 1 and rule 2 compared to clinical outcome. When indicated, comparison of ROC Curves was also performed.

Area under the curve, sensitivity and specificity of PMPGA (rule 1, rule 2, rule 1 + 2), <sup>14</sup>C-SRA and their 95% CI were calculated with clinical outcome as reference.



**Fig. 1. Comparison of PMP concentration generated with 1 IU UH/ml and 500 IU UH/ml.** A, B) HIT patient: ratio between PMP annexin V positive concentration generated with 1 IU heparin/ml and 500 IU heparin/ml = 7.8. C, D) Non HIT patient: ratio between PMP annexin V positive concentration generated with 1 IU heparin/ml and 500 IU heparin/ml = 1.0.

**Footnote:**

MPs: Microparticles  
PMPs: Platelet microparticles  
PS: Phosphatidylserine  
Pos: Positive  
Neg: Negative.

**Results**

Among the 57 patients, 53 completed the clinical follow-up and 4 patients were excluded of the study because of lack of clinical data. 35 males and 18 females aged from 24 to 97 years were included in this study (mean: 65 years, median: 66 years) (53 inpatients and 0 outpatients; 31 surgical and 22 medical patients). Nine (17%) were diagnosed HIT by clinical diagnosis. According to the 4Ts score, the 53 patients included in the study were classified as low ( $n = 24$ ; 45%), medium ( $n = 22$ ; 42%), and high pre-test probability (PTP) ( $n = 7$ ; 13%).

Platelet activation by immune complexes IgG-PF4-heparin generates PMPs expressing phosphatidylserine during PMPGA. As shown in Fig. 1, the PMPGA assay is based on the ratio between PMP annexin V positive concentration generated with 1 IU UH/ml and 500 IU UH/ml (rule 1: PMPs PS + ratio) and the concentration of PMPs annexin V positive generated at 1 IU UH/ml (rule 2: PMPs PS + concentration).

*Comparison of PMPGA to the clinical outcome ( $n = 53$  including 9 positive HIT patients)*

The optimal cut-off for rule 1 and rule 2 induced by 1 IU heparin/ml were 2.4 and 4,835 MPs/ $\mu$ L, respectively. Within positive patients, the mean MPs concentration measured with 1 IU heparin/ml was 10,756 MPs/ $\mu$ L (range: 2,246–22,610 MPs/ $\mu$ L) and the mean specificity ratio was 10.1 (range: 3.5–32.0). In the negative patient group, the mean MPs concentration measured with 1 IU heparin/ml was 2,204 MPs/ $\mu$ L (range: 434–12,628 MPs/ $\mu$ L) and the mean specificity ratio was 1.1 (range: 0.5–2.4).

When rule 1 is taken into account, AUC, sensitivity and specificity were 0.989 (95% CI: 0.911–0.995), 100.0% (95% CI: 62.9–100.0) and 97.7% (95% CI: 86.5–99.9), respectively. When rule 2 is taken into account, AUC, sensitivity and specificity were 0.933 (95% CI: 82.9–98.3), 88.9% (95% CI: 51.7–98.2) and 97.7% (95% CI: 86.5–99.9), respectively.

**Table 1**  
Comparison of PMPGA (rule 1, rule 2, rule 1 + rule 2), <sup>14</sup>C-SRA with the clinical outcome. Only data from patient with available PMPGA, <sup>14</sup>C-SRA and clinical outcome, were considered (n = 53).

		Clinical outcome		Sensitivity, % (95% CI)	Specificity, % (95% CI)	PPV, % (95% CI)	NPV, % (95% CI)
		Negative	Positive				
PMPGA	Negative	43	0	100.0	97.7	90.0	100.0
rule 1	Positive	1	9	(62.9–100.0)	(86.5–99.9)	(54.1–99.5)	(89.8–100.0)
PMPGA	Negative	43	1	88.9	97.7	88.9	97.7
rule 2	Positive	1	8	(51.7–98.2)	(86.5–99.9)	(50.7–99.4)	(86.5–99.9)
PMPGA	Negative	44	1	88.9	100.0	100.0	97.8
rule 1 + 2	Positive	0	8	(50.7–99.4)	(90.0–100.0)	(59.8–100.0)	(86.8–99.9)
<sup>14</sup> C-SRA	Negative	42	1	88.9	95.5	80.0	97.7
	Positive	2	8	(50.7–99.4)	(83.3–99.2)	(44.2–96.5)	(86.2–99.9)

When both rules are considered, AUC, sensitivity and specificity were 0.944 (95% CI: 0.845–0.988), 88.9% (95% CI: 50.7–99.4) and 100.0% (95% CI: 90.0–100.0%), respectively. (Table 1).

When comparing ROC Curves for “rule 1”, “rule 2” and “rule 1 + rule 2”, the AUC were not statistically different between “rule 1” and “rule 2” (p-value: 0.142) and “rule 1” and “rule 1 + rule 2” (p-value: 0.436). However, the AUC was significantly higher for “rule 1 + rule 2” in comparison to “rule 2” (p-value: 0.038).

#### Comparison of SRA to the clinical outcome (n = 53 including 9 positive HIT patients)

AUC, sensitivity and specificity of SRA were 0.922 (95% CI: 81.4 – 97.7), 88.9% (95% CI: 50.7–99.4) and 95.5% (95% CI: 83.3–99.2).

#### Comparison of PMPGA and <sup>14</sup>C-SRA

PMPGA (rule 1 and 2) presented 1 false negative. SRA presented 2 false positives and 1 false negative. Four discordant cases between PMPGA and SRA are shown in Table 2 (patient 1–4).

Patient 1 had a 4Ts score of 3 with negative immunoassay and light transmission aggregometry (LTA). Patient 2 had a 4Ts score of 4 with negative immunoassay and positive LTA. She received low-molecular-weight heparin for more than 3 months and thrombocytopenia worsed after switching to danaparoid and hirudin. Platelet count normalized after treatment of pneumonia *Klebsiella oxytoca* pneumonia by ceftazidime. Patient 3 had a 4Ts score of 6 with positive immunoassay and positive LTA. She developed thrombosis after heparin administration and her platelet count normalized within 5 days after heparin cessation. Finally, patient 4 had a 4Ts score of 5 with positive immunoassay (optical density = 1.5) and positive LTA. After heparin cessation, his platelet count normalized within one week.

## Discussion

In this study, we compared the performances of the PMPGA and <sup>14</sup>C-SRA to the clinical outcome to diagnose HIT. As a surface area

unit of PMP has approximately 50- to 100-fold higher procoagulant properties than an identical surface area unit of an activated platelet [21], PMPs is a more relevant biomarker than activated platelets [22,23]. Other flow cytometry tests mainly based on the detection of activated platelets [22,23] were already proposed for the diagnosis of HIT. A test based on PMPs was described in 1996 [24]. However, it was limited by several issues: i) it was performed on the FACScan, an old generation FCMr non validated for large MP analysis [19], ii) the size of MPs was not calibrated making impossible the distinction between platelets and MPs [25] and thus the accurate MP quantification, and iii) EDTA was added before the assay whereas this is not recommended for MP analysis since EDTA chelates calcium, a key actor in the MP synthesis [26]. In addition, EDTA dissociates GpIIb-IIIa complex [27] and is known to induce a P-selectin-dependent platelet activation process [28] that may result in pseudo-thrombopenia and platelet aggregates on blood smears. Finally, EDTA tubes contain extremely high concentration of potassium [29], whose impact on vesiculation remains unknown. An advantage of the study of Lee *et al.* is that they used washed platelets, giving potentially less false-negative results than if it was performed in whole blood or in platelet-rich-plasma (PRP) [5].

During the incubation of a HIT patient's serum with citrated 109 mM whole blood from a healthy donor, PMPs expressing phosphatidylserine are generated at low heparin concentration (1 IU UH/ml) due to the formation of immune complexes (i.e. IgG-PF4-heparin). On the contrary, PMP rate decreases in presence of higher UH concentration (500 IU UH/ml). This high concentration leads to a dissociation of the complex Ig-PF4-Heparin and is therefore used to enhance the specificity of all functional tests [2]. Consequently, we used a combination of ratio between PMP annexin V positive concentration generated with 1 IU UH/ml and 500 IU UH/ml (rule 1: ratio PMP PS +) and the concentration of PMPs annexin V positive generated at 1 IU UH/ml (rule 2: PMP PS + concentration) to define one positive HIT (Fig. 1).

The 9 patients with clinical HIT were detected with the rule 1 of PMPGA.

**Table 2**  
Clinical and laboratory data for the 9 patients with a positive diagnosis of HIT and the 2 non HIT patients with discordant PMPGA and SRA results.

Patient	Laboratory assays		Clinical data					Surgical or medical patient	
	PMPGA rule 1 + 2	SRA	4Ts score	Clinical evolution from the HIT suspicion to the discharge from hospital			Clinical diagnosis		
				Stop heparin	Platelet count increase	Thrombosis			
1	-	+	3	-	-	-	No HIT	medical	
2	-	+	4	-	-	-	No HIT	medical	
3	+	-	6	+	+	+	(arterial)	HIT	medical
4	-	+	5	+	+	-		HIT	surgical
5	+	+	5	+	ND (death)	+	(venous and arterial)	HIT	surgical
6	+	+	7	+	+	+	(venous)	HIT	medical
7	+	+	6	+	+	-		HIT	medical
8	+	+	6	+	+	+	(venous)	HIT	medical
9	+	+	8	+	+	+	(arterial)	HIT	medical
10	+	+	4	+	+	+	(venous)	HIT	medical
11	+	+	6	+	+	+	(venous)	HIT	medical

Consequently, the PMPGA is sufficient sensitive to detect clinically important HIT antibodies. Whereas AUC for “rule 1” and “rule 1 + rule 2” were not statistically different, the addition of rule 2 to rule 1 allowed to suppress 1 false positive result in our series but led to one false negative. A larger prospective study should be undertaken to better characterize the usefulness of these 2 rules.

It is well recognized that MP determination is affected by a variety of pre-analytical and analytical variables [30–32]. We have previously shown within-assay and between-assay variations of 18.6% and 30.5%, respectively [15].

A limitation for performing of PMPGA is the immediate availability of a healthy compatible blood group donor. Moreover, the choice of the healthy subject influence the MP counts in the mix. Consequently, each laboratory should theoretically determine its own cut-offs. To overcome this difficulty, we proposed the use of ratios which are independent of the MP counts of the healthy subject.

A PMP ratio between buffer (absence of heparin) and low heparin concentration should be included in future investigations. To try to increase the specificity of the study, blockade of Fc-receptor can also be included.

By using a whole blood procedure, our test simulates the *in vivo* HIT reaction and can be considered as easy to perform by avoiding platelet washing. Consequently, PMPGA is a rapid assay with a turnaround time (from sampling to final result) of maximum 2 hours. But a disadvantage of whole blood as PRP-based procedures compared to washed platelet assay, is the risk of suboptimal sensitivity for detecting HIT antibodies [5].

Among patients with clinical HIT, there is 2 HIT without thrombosis, 5 with venous thrombosis and 2 with arterial thrombosis. The ability of PMPGA to detect venous and arterial thrombosis was not addressed in this study and may be an interesting perspective in the future.

The strengths of our study are the use of i) standardized clinical outcomes to assess the performances of different assays [17], ii) FACS Aria I, a validated instrument for large MV analysis [19] with a highly stable flow rate (between assay variation lower than 4%), and iii) calibrated beads according to an international protocol for the standardization and the validation of MPs analysis using FCM [19,20]. We have also shown that no more than 2% of the PLT overlapped with the MPs gate defined on FSC with Mgx [25]. Nevertheless, it remains uncertain whether the small-sized material seen in the flow cytometric analysis represents single or a swarm of PMPs [33], activated platelets or immune complexes [34].

However, some limitations of the present study need to be highlighted. First, the size of the cohort of patients is limited. The second limitation in our study is the absence of a weak-positive control [5]. Multiple positive controls are described in the literature (Polyclonal antibodies to PF4 (Hyphen), positive plasma from a known confirmed HIT patient [35], confirmed anti-PF4-H platelet activating antibodies [36]. However, no international recommendation is currently available for the use of positive controls.

We showed that PMPGA presented at least similar performances than <sup>14</sup>C-SRA. As flow cytometry is more available and less time-consuming than <sup>14</sup>C-SRA and as it does not require radioactive material contrary to <sup>14</sup>C-SRA, PMPGA can be implemented in routine clinical laboratories and may become a new promising biological reference to diagnose HIT.

## Conclusion

PMPGA is a rapid and reliable assay mimicking *in vivo* HIT reaction. In our sample of patients, PMPGA showed good correlation with <sup>14</sup>C-SRA performances and predominately with clinical outcome. A prospective study on a large cohort of suspected HIT patients would be valuable to confirm the use of PMPGA as a new promising biological reference assay.

## Conflict of Interest Statement

The authors declare no competing financial interests.

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## Authorship

F.M., V.M., I.E., B.D., C.C, J-M.D and B.C conceived the idea and designed the study protocol; F.M, V.M., N.B and J.D performed the research. F.M, V.M., N.B, J.D, I.E., J-C.O, B.C and J-M.D interpreted the data; F.M performed statistical analysis; F.M, B.D., B.C, C.C and J-M.D wrote the manuscript; and all authors reviewed and approved the manuscript.

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