Heparin-induced Thrombocytopenia Diagnosis: A Retrospective Study Comparing Heparin-induced Platelet Activation Test to ^{14}C-serotonin Release Assay

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Abstract

Laboratory confirmation of heparin-induced thrombocytopenia (HIT) is of crucial importance and remains challenging and relies on platelet functional assays highlighting the presence of heparin-dependent platelet-activating antibodies in patient serum or plasma. Platelet functional assays using washed platelets include the ^{14}C-serotonin release assay (SRA), usually described as the gold standard, and the heparin-induced platelet activation assay (HIPA). Since its first comparison with SRA there has been no additional published study regarding HIPA diagnostic performances compared with SRA. Aim of our retrospective study was to compare the concordance between HIPA and SRA in HIT suspected-patients with positive anti-PF4/heparin antibodies between October 2010 and October 2015. Fifty-five HIT-suspected patients who beneficiated from both HIPA and SRA were included. Positive and negative percent agreements were 83.8% (95% CI 68.0–93.8%) and 66.7% (95% CI 41.0–86.7%), respectively. Overall percent agreement was 78.2% (95% CI 65.0–92.2%). Agreement was higher in patients who underwent cardiopulmonary bypass with extracorporeal circulation circuit for cardiac surgery. We also confirm that the use of a minimum of 2 platelet donors to establish positive HIT diagnosis and 4 platelet donors to exclude HIT diagnosis allows

Keywords
- heparin-induced thrombocytopenia
- heparin induced platelet activation test
- ^{14}C-serotonin release assay
- PF4/heparin antibodies
- laboratory diagnosis

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obtaining a good agreement with SRA. Although HIPA and SRA were performed with different platelet donors and in different laboratories, HIPA had a good positive agreement with SRA for HIT diagnosis, showing that HIPA is a useful functional assay that does not require radioactivity and could be developed worldwide to improve HIT diagnosis.

Introduction

Heparin-induced thrombocytopenia (HIT) is a life-threatening thrombotic complication of heparin therapy.[1,2] This adverse drug reaction is mediated by transient platelet-activating IgG antibodies against macromolecular complexes of the cationic tetrameric platelet factor 4 (PF4) and heparin.[3] In a limited number of cases the resulting immune complexes cross-link FcγRIIa receptors on platelets enhancing platelet activation and aggregation but also endothelial and leucocyte activation. This prothrombotic process can lead to venous and/or arterial thromboembolic complications that occur in ~50% of patients.[1] HIT diagnosis is based on clinical and biological features[4] and is characterized by an unexpected decrease in platelet count of at least 50% occurring 5 to 21 days after heparin initiation. Beyond clinical suspicion of HIT, laboratory confirmation of HIT is mandatory.[5] Immunoassays detect anti-PF4/heparin (anti-PF4/H) antibodies with a high sensitivity (varying between 96.5% and 98.9%, depending on the kit used)[6] and are performed as first line assays. As only a subset of anti-PF4/H antibodies is able to activate platelets and cause clinical HIT, platelet functional assays that investigate the ability of antibodies to activate platelets from healthy donors in the presence of heparin are required to confirm HIT diagnosis.[7] Among those functional tests, ^{14}C-serotonine release assay (SRA) is considered as the "gold standard." However, this assay is not suitable for routine testing as it requires radioactive tracers and is restricted to specialized laboratories.[8,9] In this context, heparin-induced platelet activation assay (HIPA) could be a good alternative[10] since it does not require radioactivity. Twenty years after its first comparison with SRA in a small retrospective study,[9] there has been no additional published data regarding HIPA diagnostic performances. Our study aimed to evaluate the agreement between HIPA and SRA in a retrospective cohort of patients with suspected HIT.

Material and Methods

We retrospectively analyzed the medical records of 55 consecutive patients who were suspected for HIT and had positive anti-PF4/H antibodies in Bichat – Claude Bernard Hospital (Paris, France) between October 2010 and October 2015. After this period, only patients with strong clinical suspicion of HIT and high anti-PF4/H levels and negative or indeterminate HIPA were tested for confirmatory SRA. Therefore, HIT-suspected patients after 2015 were not included in this study to avoid patient selection bias. Some of these patients had been included in the international, obser-

vational study on HIT score (NCT00748839). The study was performed in accordance with the Declaration of Helsinki. The institutional review board of our center approved the study and anonymous clinical and biological data collection from medical records was declared to the appropriate authorities.

Heparin-induced Thrombocytopenia Suspicion

HIT was clinically suspected in case of any significant thrombocytopenia or fall in platelet count occurring 5 to 21 days after initiation of heparin in the absence of another evident etiology and/or in case of any thrombotic event occurring during heparin treatment. HIT antibody testing was performed on citrated plasma samples using a commercial enzyme-linked immunosorbent assay specific for IgG PF4/H antibodies (anti-PF4/H IgG, Zymutest HIA IgG, Hyphen BioMed, Neuville sur Oise, France). Results were expressed in optical density (OD) units and values >0.5 were reported positive in concordance with the manufacturer's established ranges. Then, plasmas were heated at 56°C for 30 minutes to inactivate traces of thrombin and were stored at −80°C for both functional tests as recommended.[11] Plasma was preferred over serum for platelet functional assays in the present study as suggested by others[7,12]

Heparin-induced Platelet-activation Assay (HIPA)

HIPA was performed as previously reported[13] with some modifications in Bichat – Claude Bernard Hospital (Paris, France). Platelet donors, who were known to be "good responders," i.e., donors whose platelets are known to be sensitive to HIT antibodies as previously suggested,[10] and were free of aspirin and non-steroidal anti-inflammatory drugs for at least 10 days were carefully selected (two donors at a time). Whole blood samples were drawn into 15% (v/v) trisodium citrate acid–citric–dextrose (ACD, Vacutainer system, Beckton Dickinson, Le Pont-de-Clais, France) and washed platelets were prepared as previously described.[13] Briefly, platelet-rich plasma (PRP), prepared by centrifugation 10 minute at 200 g was acidified to pH 6.5 by addition of 100 µL ACD per mL[14] and 2 µL apyrase (150 IU/ml, Sigma Aldrich) and 1 µL prostaglandin E1 0.1 mM (PGE1, Sigma Aldrich) were added per mL of PRP. After centrifugation (15 minute, 1200 g), the supernatant was discarded and platelets were carefully resuspended in washing buffer (NaCl 103 mM, citric acid 36 mM, glucose 5 mM, KCl 5 mM, $MgCl_2$ 1 mM, bovine serum albumin 3.5 mg/mL, apyrase 0.3 IU/mL and PGE1 0.1 µM, adjusted to pH 6.5). Platelets were washed twice and resuspended in reaction buffer (NaCl

137 mM, Hepes 5 mM, glucose 5.5 mM, KCl 1 mM, MgCl$_2$ 2 mM, NaH$_2$PO$_4$ 0.3 mM, NaHCO$_3$ 12 mM, CaCl$_2$ 2 mM, bovine serum albumin 3 mg/mL, adjusted to pH 7.2). The suspension was adjusted to 300,000–400,000 platelets/mL in reaction buffer. 10 µL of UFH solution (final concentrations 0.2, 0.5 or 48 IU/mL) or saline control buffer (to detect any spontaneous aggregation), 20 µL of heat-inactivated patient plasma and 75 µL of platelet suspension were dispensed with one steel sphere (Diagnostica Stago, Asnieres-Sur-Seine, France) in a microtiter plate that was incubated 30 minute at 37°C on a plate stirrer (1000 rpm). The transparency of the suspension was assessed using an indirect light source. The sample was considered positive for HIT if: (i) the suspension became transparent due to platelet aggregation with UFH 0.2 IU/mL and/or 0.5 IU/ml but not with UFH 48 IU/ml, (ii) positive results were obtained with at least 2 platelet donors within 25 minutes. If platelet activation occurred in the presence of high UFH concentration (48 IU/ml), the sample was considered as indeterminate due to non-specific activation. The sample was considered negative for HIT if negative results were obtained with 4 donors. Results that did not fulfill these criteria were considered incomplete. Positive and negative control plasma were run in parallel in each series.

[14]C-serotonin Release Assay (SRA)

SRA was performed by 3 hematology laboratories in France: Georges Pompidou European Hospital (AP-HP, Paris, France),[15] University hospital of Tours (France) and Robert Debré University Hospital (Reims, France). Washed platelets of selected healthy donors (one donor at a time) who were free of aspirin and non-steroidal anti-inflammatory drugs for at least 10 days and known to react well in the SRA as previously suggested,[10] were used for the assays. The result was considered positive for HIT if all the following criteria were met with at least one platelet donor[1]: 20% or greater serotonin release at 0.1 IU/mL and/ or 0.5 IU/mL UFH[2]; at least 50% decrease of serotonin release at high UFH concentrations (10 to 100 IU/mL). If the release was more than 20% in the presence of high UFH concentration, the result was considered as indeterminate. The result was considered negative if serotonin release was <20% with at least 3 platelet donors. In all other cases, results were considered incomplete. Saline control buffer was used instead of UFH to detect any spontaneous activation. Positive and negative control plasma were run in parallel in each series. All assays were performed and interpreted according to the recommendations of the SSC of ISTH[8] and to local practice.

Statistical Analysis

Categorical and continuous data were compared according to results of the HIPA assay with Fisher exact of Wilcoxon nonparametric tests, as appropriate.

As the SRA assay alone is considered as a non-standard reference,[16] we computed the positive percent agreement (PPA) and negative percent agreement (NPA), as well as the overall percent agreement (OPA) of HIPA and SRA, and their 95% confidence intervals (CI). Indeterminate and incomplete results from HIPA or SRA were not analyzed in the present analysis.

All analyses were performed using the R statistical software v3.5.0.[17] Continuous data were expressed as median (interquartile range, IQR), and categorical data are reported as frequencies and percentages.

Results and Discussion

During a 5-year period study, a total of 55 patients with clinical suspicion of HIT, IgG anti-PF4/H antibody titer >0.5 and available results from both HIPA and SRA were included. Baseline demographic, clinical and biological characteristics of patients are presented in ►Table 1. Briefly, median age of patients was 62 years (IQR 48–73) and 56.4% of them were males. Patients were mainly from cardiovascular surgical units (74.6%). Remaining patients were from intensive care (10.9%) or medical units (14.5%). Median OD value of anti-PF4/H IgG was 2.12 (IQR 1.41–2.65). HIPA was considered positive in 37 patients (tested with 2 to 4 platelet donors) and negative in 18 (tested with 4 platelet donors, ►Table 2). SRA was positive in 37 patients and negative in 18. Among the 37 patients with positive HIPA, 31 were also positive for SRA. Among the 18 patients with negative HIPA, 12 were also negative in SRA. The PPA of HIPA and SRA was 83.8% (95% CI 68.0–93.8%), the NPA was 66.7% (95% CI 41.0–86.7%) and the OPA was 78.2% (95% CI 65.0–92.2%, ►Table 3). Higher positive and overall agreements of HIPA compared with SRA were observed in the subgroup of patients who underwent cardiopulmonary bypass with extracorporeal circulation circuit for cardiac surgery (PPA 88.9%, 95% CI 70.8–97.6% and OPA 80.0%, 95% CI 64.3–90.9%) but negative agreement was lower (NPA 61.5%, 95% CI 31.6–86.1%).

Initial diagnosis of HIT is based on an estimation of clinical probability and identification of elevated titer of anti-PF4/H antibodies. Immunoassays have a high negative predictive value (>95%).[6] However, only 28% to 40% of patients with positive anti-PF4/H antibodies have platelet-activating antibodies capable of causing HIT, depending on the clinical setting and on antibody titers.[18] Risk of false positive diagnosis of HIT is mainly significant in the context of cardiac surgery and exposes to costly parenteral non-heparin anticoagulants and their associated risk of major bleeding. Thus, functional tests have a higher positive predictive value. In North America, SRA is the most common functional assay and is considered as the gold standard for HIT diagnosis. One of the most important inconveniences is the use of ^{14}C-serotonin that requires specific authorization for radioactivity manipulation. Alternatively, HIPA which is the most widely used functional assay in Germany,[19] does not require radioactivity, is less time consuming than SRA and enables to test many patients and different types of heparins and heparinoids in the same time without being limited by the short stability of platelet donors.[7] The major difference between HIPA and SRA tests is that HIPA is a semiquantitative assay evaluated visually that might be a source of inter-operator variability. HIPA performances was compared with the SRA in only few studies performed by the same team.[9,20] Furthermore, there is a growing interest currently to confirm HIPA performances because other hematology laboratory

Table 1 Demographic, clinical and biological characteristics of patients with heparin-induced thrombocytopenia suspicion

	Patients with HIT suspicion			p-value
	Overall (n = 55)	Negative HIPA result(n = 18)	Positive HIPA result(n = 37)	
Age - years, median (IQR)	62 (48–73)	58 (52–66)	66 (48–75)	0.27
Male sex – n (%)	31 (56.4)	10 (55.6)	21 (56.8)	>0.99
Treatment – n (%)				
UFH	44 (81.5)	17 (94.4)	27 (75.0)	0.14
LMWH	10 (18.5)	1 (5.6)	9 (25.0)	
prophylactic anticoagulation – n (%)	12 (21.8)	3 (16.7)	9 (24.3)	0.73
Therapeutic anticoagulation – n (%)	43 (78.2)	15 (83.3)	28 (75.7)	
Prior heparin therapy in the last 3 months	7 (12.7)	2 (11.1)	5 (13.5)	>0.99
Delay after heparin initiation - days, median (IQR)	12 (8–13)	13 (10–17)	11 (8–13)	0.055
Anti-PF4/H antibodies - OD - median (IQR)	2.12 (1.41–2/65)	1.77 (1.06–2.26)	2.33 (1.87–2.78)	0.02
Hemoglobin nadir - g/L - median (IQR)*	98 (85–108)	96 (85–108)	98 (85–109)	0.96
Platelet count nadir - G/L - median (IQR)*	66 (42–92)	86 (29–126)	64 (46–86)	0.14
Day of platelet count nadir - median (IQR)*	10 (8–12)	12 (9–16)	10 (8–11)	0.04
Department – n (%)				
Cardiac surgery	41 (74.6)	12 (66.7)	29 (78.4)	0.57
Intensive care unit	6 (10.9)	3 (16.7)	3 (8.11)	
Medicine	8 (14.5)	3 (16.7)	5 (13.5)	
Extracorporeal circulation – n (%)	40 (72.7)	11 (61.1)	29 (78.4)	0.21
Thrombosis – n (%)	16 (29.1)	7 (38.9)	9 (24.3)	0.35
Thrombosis localisation				
Venous thrombosis – n (%)**	5 (9.1)	1 (5.6)	4 (10.8)	>0.99
Arterial thrombosis – n (%)**	4 (7.3)	2 (11.1)	2 (5.4)	0.59
Material thrombosis – n (%)**/***	4 (7.3)	2 (11.1)	2 (5.4)	0.59
Cutaneous symptom – n (%)	2 (3.6)	0 (0)	2 (5.4)	>0.99
Substitutive treatment – n (%)	52 (94.6)	17 (94.4)	35 (94.6)	>0.99
Danaparoid	47 (90.4)	14 (82.4)	33 (94.3)	0.1
Argatroban	4 (7.7)	3 (17.7)	1 (2.9)	
Fondaparinux	1 (1.9)	0 (0)	1 (2.9)	
Outcomes				
Bleeding – n (%)	13 (23.6)	4 (22.2)	9 (24.3)	>0.99
Recurrent thrombosis – n (%)	1 (1.8)	1 (5.6)	0 (0)	0.33
Death – n (%)**	6 (10.9)	1 (5.6)	5 (13.5)	0.65

Abbreviations: HIPA, heparin-induced platelet activation; HIT, heparin-induced thrombocytopenia; IQR, interquartile range; LMWH, low-molecular-weight heparin; OD, optical density; UFH, unfractionated heparin; VKA, vitamin K antagonist.
*After heparin initiation.
**Patients may have more than one type of thrombosis.
*** Material thrombosis: 3 prosthetic valve thrombosis and 1 circuit thrombosis of extracorporeal membrane oxygenation.

Table 2 Comparison of HIPA and SRA results

	HIPA positive	HIPA negative
SRA positive	31	6
SRA negative	6	12

Abbreviations: HIPA, heparin-induced platelet activation; SRA, ^{14}C-serotonin release assay.

could confirm more easily diagnosis of the new syndrome called vaccine-induced thrombotic thrombocytopenia.[21]

Herein, we confirm that HIPA and SRA both performed on heat-inactivated plasma are concordant functional tests for HIT diagnosis, especially in cardiac surgery and critically ill patients but also in medical settings. We also confirm that the use of a minimum of 2 platelet donors to establish

Table 3 Agreement between HIPA and SRA results

	PPA		NPA		OPA	
HIPA (≥2 positive results/≥ 4 negative results)	(%)	95% CI	(%)	95% CI	(%)	95% CI
Overall (n = 55)	83.8	[68.0–93.8]	66.7	[41.0–86.7]	78.2	[65.0–88.2]
Patients with extracorporeal circulation (n = 40)	88.9	[70.8–97.6]	61.5	[31.6–86.1]	80.0	[64.3–90.9]

Abbreviations: CI, confidential interval; HIPA, heparin-induced platelet activation; NPA, negative percent agreement; OPA, overall percent agreement; PPA, positive percent agreement; SRA, ^{14}C-serotonin release assay.

positive HIT diagnosis and 4 platelet donors to exclude HIT diagnosis allows obtaining a good agreement with SRA.

Our study has some limitations. As HIPA and SRA were not performed in the same laboratory, although carefully selected as "good responders," platelet donors were different between both assays. This probably results in a lower overall agreement than expected.[9] Overall, results from HIPA and SRA were discordant for 12 patients with strong HIT clinical probability. Among those 12 patients, 8 had a cardiac surgery, 5 had a thrombotic event while treated by heparin and all patients, except one who was under extracorporeal membrane oxygenation, had an increase in platelet count after heparin replacement. Median (IQR) anti-PF4/H antibodies OD of these samples was high 2.07 (1.36–2.50) and only one sample had a borderline OD of 0.59. Serotonin release intensity was not different between HIPA-positive and -negative samples either at UFH 0.1 UI/ml [62.0% (36.0–84.0) versus 65.0% (33.0–76.0)] or 0.5 UI/mL [54.0% (26.5–92.0) versus 60.5% (55.0–76.0)]. Among the 12 discordant samples only 2 had a spontaneous activation (1 in SRA and 1 in HIPA), but platelet activation was inhibited with high UFH concentration for both samples, ruling out non-specific activation. These discrepant results might be related to the different selected platelet donors used for each assay. Indeed, it is well known that platelet sensitivity to HIT antibodies varies among donors. This inherent variability can usually be overcome by selecting donors. There is no general consensus on the number of donors to be tested and one difference between the 2 assays is that HIPA was considered positive if aggregation is observed with 2 platelet donors whereas SRA was considered positive from only 1 donor. This could explain some "false positive" conclusions obtained with SRA. Among the 6 patients with positive SRA and negative HIPA, 4 patients were tested SRA positive with only 1 donor.

Moreover SRA was performed in 3 different French laboratories according to their own local protocol. Although they all have an important expertise in the field and trained personnel who performed the assays, this can also underestimate the overall agreement of both functional tests. However, we would like to emphasize that these conditions better reflect the real life settings in which laboratories are often required to exchange patient plasma samples to conclude on HIT diagnosis.

To conclude, we demonstrate that HIPA has a good positive agreement with SRA for HIT diagnosis, more specifically in cardiac surgery patients in whom HIT diagnosis is especially challenging. This test, more accessible than SRA, could be developed worldwide to improve HIT diagnosis.

Author Contributions
Cİ, NG, MCB, MAG, CP, BT, JS, DF and NA performed the research
CG, DF and NA designed the research study
NG, PE, CB, VG, DF and NA analyzed the data
NG, VG, DF and NA wrote the paper.

Disclosures
Authors do not have any conflict of interest to declare.

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