HEMOSTASIS AND THROMBOSIS

Original article

Heparin-induced thrombocytopenia: effect of heat inactivation of patient's serum on functional flow cytometric assay results

Klara Železnik[1,2], Manuela Čitar[1], Ivica Marić[1], Gordana Tomac[3], Elvira Maličev[1,4]

[1]*Blood Transfusion Centre of Slovenia, Department for Diagnostic Services, Ljubljana, Slovenia;*
[2]*Medical Faculty, University of Ljubljana, Ljubljana Slovenia;*
[3]*Clinical Department of Transfusion Medicine and Transplantation Biology, University Hospital Centre Zagreb, Zagreb, Croatia;*
[4]*Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia*

Background - Heat inactivation of a patient's sample is not systematically performed in the diagnostics of heparin-induced thrombocytopenia (HIT). Some authors recommend that the patient's sample is heat-inactivated to avoid the effect of thrombin on platelet activation in a functional assay. Others do not find this additional step essential or even advise against it.

Material and methods - An enzyme-linked immunosorbent assay (ELISA) and flow cytometry-based functional assay with CD62P as a marker of platelet activation were performed. Forty-seven patients with suspected HIT and three healthy controls were included in the study. Each serum sample was divided into two aliquots: one was heat-inactivated and the other was not. Both aliquots were tested in parallel using the same donor platelets from four randomly selected individuals. We designed an index of platelet activation for both protocols to assess platelet activation in the assay and to compare the results.

Results - We observed a higher percentage of platelet activation in heat-inactivated compared to non-heat-inactivated sera. This phenomenon was seen in low and high heparin steps, although it did not occur for all samples. There were discrepant results in seven samples, which tested negative in the non-heat-inactivated protocol and positive in the heat-inactivated protocol. There was no case in which the result of a non-heat-inactivated aliquot was positive and the corresponding heat-inactivated aliquot was negative.

Discussion - Due to the higher percentages of donor platelet activation, all seven non-compliant cases met our current criteria for a positive result. However, those results were probably false-positive based on ELISA optical density value and 4T score. Therefore, in the current settings, heat inactivation of serum is not suitable for our flow cytometric functional assay since it can cause an elevated risk of creating false-positive results.

Keywords: *heparin, thrombocytopenia, flow cytometry, heat-inactivated serum, platelet activation.*

Arrived: 1 March 2022
Revision accepted: 9 June 2022
Correspondence: Klara Železnik
e-mail: klara.zeleznik@ztm.si

INTRODUCTION

Laboratory diagnostics of heparin-induced thrombocytopenia (HIT) include immunoassays and functional platelet-activating assays. Performing a functional test in

Blood Transfus 2023; 21: 268-276 doi: 10.2450/2022.0045-22
© SIMTIPRO Srl

All rights reserved - For personal use only
No other use without premission

© SIMTIPRO Srl

the case of a positive immunoassay is strongly needed to reduce over-diagnosis and subsequent mistreatment of patients without HIT[1-5].

Immunoassays are commercially available. On the other hand, functional assays are mostly in-house methods, which differ vastly between laboratories and directly comparing test results between laboratories is difficult. In addition to the platelet activation marker measured, functional assays differ in many other ways: selection of platelet donors, preparation of donor platelets and the patient's sample, heparin concentrations used, quality control applied, the volume of individual components used (platelets, plasma, heparin), time and temperature of incubation, and technologies and methods used[3-6]. All functional assays require a source of fresh platelets, which are incubated with the patient's serum or plasma in the presence of low and high concentrations of heparin. If pathological HIT antibodies are present, they activate platelets at low (pharmacological) heparin concentrations. In a parallel reaction, a high concentration of heparin should disrupt heparin/PF4 complexes and inhibit anti-heparin/PF4 antibody-induced platelet activation[3,4,7]. Testing should be performed using acute patients' samples because HIT antibodies are transient[8]. Residual thrombin may contaminate a patient's sample, especially serum, and cause heparin-independent platelet activation leading to false-positive results (e.g., patient with disseminated intravascular coagulation). In addition, there may be interference due to coagulation in serum caused by complex reciprocal coagulation-complement interactions, including activation and aggregation of platelets, which in turn contribute to the amplification of serum complement. This coagulation-complement interaction justifies heat inactivation of serum complement since it is not required for IgG-dependent platelet activation in HIT[9,10]. If residual thrombin is present in the sample, donor platelet activation progressively decreases from the state with buffer control (without heparin) to the state with low heparin concentration and then to the high heparin concentration[3,4,11]. Since some laboratories only perform "two-point" functional tests (low and high heparin concentrations), such atypical patterns might not be recognized[11]. Therefore, some authors recommend that a patient's sample be first heated at 56°C for 30-45 min to inactivate thrombin and complement[11-14]. After heat-inactivation, fibrin and other precipitates are removed by high-speed centrifugation. However, overheating a patient's sample can generate aggregated IgG causing platelet activation leading to false-positive results[3,5,12,13]. False-negative results can also occur due to reduced free antibodies against heparin/PF4 complexes[5].

Heat inactivation is not systematically performed in HIT functional assays[15]. It is predominantly but not universally used in washed platelet assays, such as the serotonin release assay and heparin-induced platelet activation assay[4,11,13,16]. Flow cytometric functional assays are being increasingly performed, using either heat-inactivated (HI) or non-heat-inactivated (non-HI) samples[17,18]. According to both available commercial functional test manufacturers' instructions, a non-HI serum should be used[19,20]. Moreover, heat-inactivation is not recommended for heparin-induced multiple electrode aggregometry testing because it may decrease antibody titers and affect platelet aggregation results[21].

Confirmation of HIT antibodies is of crucial importance for the appropriate treatment of HIT. However, to our knowledge, no systematic study comparing non-HI and HI serum samples using the same donor platelets has been performed. In the present work, we studied whether heat-inactivation of a patient's serum has an impact on the results of flow cytometric functional assay. The results were compared to non-HI serum testing of the same donor platelets. To assess platelet activation in a flow cytometric assay and standardize the results, we designed an index of platelet activation for both protocols. Calculating an index of platelet activation reduces the impact of donor platelet variability to HIT antibodies.

MATERIALS AND METHODS

Study overview

The study was approved by the National Medical Ethics Committee, Ministry of Health, Slovenia (approval number: 0120-436/2019/7) and by the Medical Ethics Committee of University Hospital Centre Zagreb, Croatia (approval number: 02/21 AG). In total, sera from 47 heparin-treated patients with suspected HIT and three healthy controls were included in our study. The retrospective cohort included patients hospitalized in two countries (Slovenia, 28 patients; Croatia, 19 patients) and from multiple services, including internal medicine

© SIMLIPRO Srl

All rights reserved - For personal use only
No other use without premission

and surgery. Routine testing was performed in the country of hospitalization according to local protocols. After routine testing, sera were frozen at –20°C and then thawed at room temperature for the study, which was conducted in the Blood Transfusion Center of Slovenia. Each sample was divided into two aliquots. The first aliquot was not additionally manipulated (non-HI, which is a routine protocol in the institution), and the other aliquot was HI. In the flow cytometric assay, both aliquots were tested in parallel using the same donor platelets in platelet-rich plasma (PRP). In addition, sera from three healthy controls were obtained from employees of the Blood Transfusion Center of Slovenia and were freshly tested.

Clinical probability of heparin-induced thrombocytopenia

The 4T score evaluates the relative platelet count decrease, the timing of the onset of thrombocytopenia, the presence or absence of thrombosis, and the likelihood of another cause of thrombocytopenia, and is described in detail elsewhere[22].

Detecting heparin/PF4 IgG antibodies with an enzyme-linked immunosorbent assay

All patients' samples were tested with an IgG-specific enzyme-linked immunosorbent assay (ELISA) kit (Immucor GTI Diagnostics, Inc., Waukesha, WI, USA). Assays, in which the target antigen is PF4 complexed to polyvinyl sulfonate (PVS), were performed according to the manufacturer's instructions[23]. A sample was considered positive if the optical density (OD) was 0.4 or above. In addition, heparin inhibition of the antibody binding by 50% or more confirmed the positive result.

Detecting platelet-activating antibodies with a functional flow cytometric assay

This flow cytometric assay is based on detecting two platelet markers: CD61, which is expressed on all platelets, and CD62P (P-selectin), which is expressed only on activated platelets after α-granule exocytosis. Flow cytometric assays with a HI and a non-HI aliquot were performed according to our routine protocol[24]. Both HI and non-HI aliquots were tested in parallel using the same platelets from four randomly selected blood donors with blood group O. Fresh donor PRP was prepared from citrated blood by centrifugation at 120 × *g* for 7 min, and 13.4 µL of PRP were placed in microwells. A sample of the patient's serum, and positive and negative controls (25 µL) were added to the PRP with 1.6 µL of 0.2 or 200 IU/mL heparin and incubated for 1 hour at room temperature. After incubation, 10 µL of each sample were transferred into tubes containing anti-CD61-fluorescein isothiocyanate and anti-CD62P-phycoerythrin monoclonal antibodies (both Becton Dickinson, San Jose, CA, USA). After 15 min of incubation at room temperature in the dark, the samples were diluted in 300 µL 1% formaldehyde and immediately analyzed in the flow cytometer (FACSCalibur, Becton Dickinson) with 100,000 CD61-positive cells counted (software CellQuest [BD Bioscience, Franklin Lakes, NJ, USA]). The results were expressed as a percentage of CD62P-positive events among CD61-positive events. The assay was considered positive if the platelets were >10% CD62P positive at a low concentration of heparin, and ≥50% inhibition of platelet activation was detected with the high heparin concentration. HIT was confirmed if the assay was positive with at least two out of the four-panel platelets.

Heat inactivation

Patient's serum was HI at 56°C for 35 min in a thermoblock. Subsequently, high-speed centrifugation (8,000 × *g* for 8 min) was performed to remove fibrin and other precipitates. Both steps were performed immediately before the flow cytometric assay.

Index of platelet activation

Following the example of the heparin platelet activation index HEPLA[10,20] in a commercial flow cytometric assay, we developed the $Index_{(non\text{-}HI)}$. Due to the differences in control material between the two assays, we had to adjust the formula to our routine testing conditions. As positive controls, we test known HIT-positive sera with different ELISA OD values and platelet activation capacity. As negative controls, we test sera from HIT-negative patients. In contrast, in HEPLA thrombin receptor agonist peptide (TRAP) is used as a positive control, but a negative control serum is not included in the assay and calculation. Later, to compare non-HI and HI serum protocols, we designed an additional index of platelet activation ($Index_{[HI]}$), which is adapted to heat inactivation.

Statistical analysis

The dependencies between variables were calculated using linear regression and correlation with IBM SPSS v.25.

© SIMEIPRO Srl

Blood Transfus 2023; 21: 268-276 doi: 10.2450/2022.0045-22

All rights reserved - For personal use only
No other use without premission

Table I - *Patients' characteristics, results of the IgG enzyme-linked immunosorbent assay, and flow cytometry with calculated indices of platelet activation*

Patient	Gender	4T score	IgG ELISA OD	FC: non-HI serum	Index$_{(non-HI)}$	FC: HI serum	Index$_{(HI)}$
1	F	5	0.03	NEG	0.25	NEG	0.43
2	F	2	0.04	NEG	1.15	NEG	0.81
3	F	/	0.05	NEG	1.06	POS	0.72
4	M	/	0.06	NEG	1.27	POS	0.64
5	M	2	0.06	NEG	0.8	POS	0.8
6	M	4	0.07	NEG	0.52	NEG	0.54
7	M	3	0.1	NEG	0.88	NEG	0.82
8	M	3	0.1	NEG	0.27	NEG	0.29
9	M	4	0.2	NEG	0.86	POS	0.64
10	M	5	0.2	NEG	0.71	NEG	0.23
11	F	/	0.3	NEG	0.45	POS	1.01
12	F	4	0.4	NEG	0.38	NEG	0.35
13	M	4	0.4	NEG	0.36	NEG	0.5
14	M	5	0.4	NEG	0.2	NEG	0.28
15	M	5	0.6	NEG	0.3	NEG	0.27
16	M	2	0.7	NEG	0.56	NEG	0.38
17	M	/	0,7	NEG	0.42	NEG	0.31
18	M	5	0.8	POS	3	POS	1.62
19	M	5	0.8	NEG	0.18	NEG	0.25
20	M	4	0.8	NEG	0.62	POS	1.61
21	M	2	0.8	NEG	0.44	NEG	0.36
22	M	6	0.9	NEG	0.26	NEG	0.31
23	M	4	0.9	NEG	0.43	NEG	0.23
24	M	6	1.0	NEG	0.64	POS	0.61
25	F	5	1.1	POS	1.6	POS	2.0
26	M	5	1.1	POS	2.27	POS	1.72
27	F	4	1.1	POS	1.02	POS	1.49
28	M	5	1.2	POS	1.53	POS	1.37
29	F	5	1.2	POS	1.01	POS	1.25
30	M	3	1.2	NEG	0.26	NEG	0.13
31	M	/	1.3	POS	2.68	POS	1.95
32	M	3	1.6	POS	3.39	POS	1.03
33	M	/	1.6	POS	2.01	POS	1.06
34	M	/	1.7	POS	2.44	POS	0.55
35	F	8	2.1	POS	2.93	POS	2.42
36	F	/	2.2	POS	6.29	POS	4.66
37	M	/	2.2	POS	2.75	POS	2.21
38	M	5	2.2	POS	5.23	POS	2.69
39	F	7	2.3	POS	2.49	POS	2.0
40	F	/	2.7	POS	6.06	POS	2.61
41	M	/	2.8	POS	4.78	POS	3.68
42	M	/	2.9	POS	8.06	POS	3.04
43	M	4	2.9	POS	4.09	POS	2.44
44	F	7	2.9	POS	1.24	POS	0.94
45	M	6	3.0	POS	3.81	POS	3.18
46	F	5	3.2	POS	10.21	POS	4.47
47	M	/	3.3	POS	2.57	POS	1.87
48	F	0	Healthy	NEG	0.62	NEG	0.43
49	M	0	Healthy	NEG	0.88	NEG	0.38
50	F	0	Healthy	NEG	−0.07	NEG	0.33

ELISA: enzyme-linked immunosorbent assay; OD: optical density; M: male; F: female; NEG: negative; POS: positive; FC: flow cytometry; HI: heat-inactivated. Total 4T scores of 0 to 3, 4 to 5, and 6 to 8 correspond to low, intermediate, and high pretest probabilities of heparin-induced thrombocytopenia, respectively.

© SIMTIPRO Srl

All rights reserved - For personal use only
No other use without premission

Data in the analysis are presented as mean and standard deviation, and the statistical comparison between results with the two protocols was performed using paired samples τ-test and the χ^2 test. We considered a p-value of less than 0.05 as statistically significant.

RESULTS

A retrospective cohort consisting of 34 (68%) males and 16 (32%) females was evaluated in this study. Only 34 patients' samples were accompanied by a completed 4T score from the ordering physician. The patients' characteristics, including 4T scores (where available), test results, and calculated indices, are summarized in **Table I**.

There were 11 patients (23%) with a negative ELISA (OD <0.4), and 36 (77%) with a positive ELISA (OD ≥0.4). ELISA OD values ranged from 0.03 to 3.3. In the study, all 11 ELISA-negative patients also had negative flow cytometric assay results when using a non-HI serum. Among 36 ELISA-positive patients, 13 (36%) had negative, and 23 (64%) had positive flow cytometric assay results when using a non-HI serum. A comparison of the functional test results, performed during prior routine testing (22 ELISA-positive samples) and as part of the study (non-HI sera in both cases), showed a match of all but one result (patient n. 22).

We generally observed a higher percentage of platelet activation in HI sera compared to non-HI sera. This phenomenon was seen in low and high heparin steps, although it did not occur for all samples. When comparing flow cytometric assay results of non-HI and HI aliquots, there were discrepancies in five ELISA negative patients (n. 3, 4, 5, 9 and 11) and two ELISA positive patients (n. 20 and 24). All seven discrepant cases tested negative with non-HI sera and positive with HI sera (**Table II**). There was no case where the result of the non-HI aliquot was positive and that of the HI aliquot was negative.

Table II - *Results obtained testing native and heat-inactivated sera*

N = 50		Flow cytometry: non-HI serum	
		Positive	Negative
Flow cytometry: HI serum	Positive	23	7
	Negative	0	20

HI: heat-inactivated.

Calculation of the index of platelet activation

First, we designed the $Index_{(non\text{-}HI)}$, which is adjusted to our testing conditions and assessed based on a retrospective cohort of 148 patients with suspected HIT, who were tested with a flow cytometric assay according to local protocols. The $Index_{(non\text{-}HI)}$ and its components are defined as follows:

$$Index_{(non\text{-}HI)}: (\%A\ H0.2 - \%A\ H200) / 5.4\%$$

where %A H0.2 is the mean donor platelet activation (average of 4) with the patient's serum in the low heparin (0.2 IU/mL) step. %A H200 is the mean donor platelet activation (average of 4) with the patient's serum in the high heparin (200 IU/mL) step, and 5.4% is the mean negative control platelet activation percentage in the low heparin step in non-HI serum protocol. It is calculated based on 538 negative control reactions with 136 different sera. The results are standardized by putting a mean value of multiple negative control platelet activation in the denominator.

Using a logistic regression model (the result of the functional test is a dependent variable, and the $Index_{(non\text{-}HI)}$ is an independent variable), we calculated a coefficient of determination of the proposed model, which is very high (R^2=0.976). Therefore, the predictive model is very reliable. Based on 148 patients with suspected HIT, the $Index_{(non\text{-}HI)}$ has a statistically significant influence on the functional test result. It has a very good negative predictive value at 1.1 or less and a good positive predictive value at 1.6 or more. We also checked the influence of ELISA OD value on the $Index_{(non\text{-}HI)}$, and proved that it is statistically significant with a positive slope of a regression curve (k=1.87) (*data not shown*).

We also designed the $Index_{(HI)}$ to compare the results of our study on non-HI and HI serum protocols. Since the values of negative controls were much higher with HI aliquots, we considered a higher percentage of platelet activation. The $Index_{(HI)}$ is defined as follows:

$$Index_{(HI)}: (\%A\ H0.2 - \%A\ H200) / 11.6\%$$

where 11.6% is the mean negative control platelet activation percentage in the low heparin step in the HI serum protocol. It is calculated based on 102 negative control reactions (29 different sera). For comparison, a mean

© SIMEPRO Srl

Blood Transfus 2023; 21: 268-276 doi: 10.2450/2022.0045-22

All rights reserved - For personal use only
No other use without premission

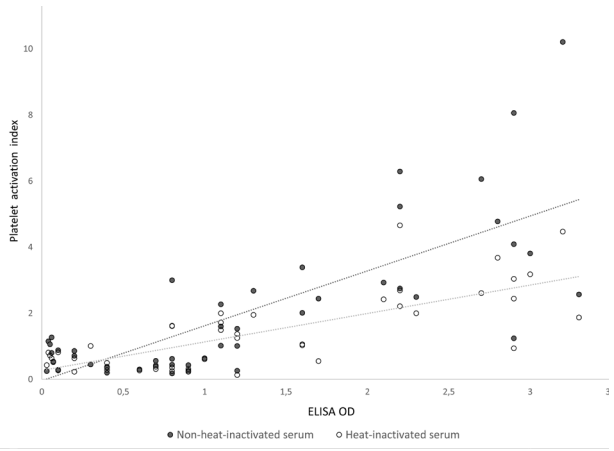


Figure 1- Indices of platelet activation depending on the optical density values of the enzyme-linked immunosorbent assay

The scatter correlation diagram represents the correlation between enzyme-linked immunosorbent assay (ELISA) optical density (OD) values and the $Index_{non-HI}$ (black circles) or $Index_{HI}$ (white circles). There is a statistically significant positive correlation between ELISA OD and $Index_{(non-HI)}$ (r=0.761) as well as between ELISA OD and $Index_{(HI)}$ (r=0.763). The dark grey and the light grey dotted lines represent the regression lines for the non-heat-inactivated protocol with a slope of 1.66 and the heat-inactivated protocol with a slope of 0.86, respectively.

negative control platelet activation with non-HI aliquots is 6.8% for the same cohort of samples.

In the study, the mean values (± standard deviation) of $Index_{(non-HI)}$ and $Index_{(HI)}$ are 1.9±2.2 and 1.3±1.1, respectively. Correlating indices of non-HI and HI serum shows a statistically significant and strong correlation (r=0.89) between the two protocols. There is a statistically significant difference between the two protocols, as shown in the paired samples τ-test and also in the χ^2 test. The ELISA OD value has a statistically significant impact on the indices of both protocols with a positive correlation (r=0.761 in non-HI and r=0.763 in HI serum protocol), and a positive slope of regression curves (k=1.66 in non-HI and k=0.86 in HI serum protocol) (**Figure 1**).

DISCUSSION

Heat inactivation should eliminate the effect of residual thrombin and complement on donor platelet activation in a patient's sample [11-14]. On the other hand, overheating a sample can generate aggregated IgG causing platelet activation leading to false-positive and erroneous results[3,5,12,13,25]. We investigated whether heat inactivation of a patient's serum affects the result of a flow cytometric assay compared to the results obtained with non-HI serum using the same donor platelets.

A retrospective cohort consisting of 47 patients with suspected HIT and three healthy controls was evaluated in this study. Comparing the functional test results, which were obtained during prior routine testing and as part of the study (non-HI sera in both cases), revealed a mismatch in one case. In a patient (n. 22) with a high clinical probability (4T 6) and a low OD value (OD 0.9), the diagnosis of HIT was initially confirmed, but the functional test in the study was negative with low values of $Index_{(non-HI)}$ and $Index_{(HI)}$. The cause of the mismatch in this retrospectively analyzed sample is difficult to identify.

Generally, we observed a higher percentage of platelet activation in HI sera than in non-HI aliquots. Platelet activation was higher with both heparin concentrations. Similar findings were recently reported by another group, which noticed an overall rise in HIT-$IgG_{(PF4-H)}$ levels with HI sera compared to non-HI sera in an AcuStar immunoassay. They observed a statistically significant increase in HIT-$IgG_{(PF4-H)}$ levels after heat inactivation, although on a case-by-case basis, with not all samples showing this effect[26]. Another group reported increased platelet activation with HI sera, independently of added heparin, in a flow cytometric functional test that detects annexin V binding as an activation marker[27]. On the other hand, testing showed no difference in commercial flow cytometric HitAlert assay results between paired non-HI and HI samples, even though the manufacturer recommends using a non-HI serum[28]. A recently published study investigated the effect of heat inactivation on serum. The IgG concentration was upregulated after heat inactivation, and the authors supposed that IgG was released by heat from preexisting immune complexes[29].

Comparing our flow cytometric assay results of non-HI and HI sera, there were discrepant results in five ELISA-negative patients and two ELISA-positive patients. All seven discrepant samples had low ELISA OD values (≤1) and tested negative with non-HI sera and positive after heat inactivation. Unfortunately, due to the insufficient volume of residual sera, the assays could not be completely repeated according to the study protocol. Therefore, two discrepant ELISA-positive samples were retested with platelets obtained from two other donors. In repeated testing, no platelet activation was observed with non-HI

© SIMTIPRO Srl

All rights reserved - For personal use only
No other use without premission

sera, and platelet activation in only one donor was seen for each sample with HI sera. Based on all the results obtained for these two samples, we concluded that the results of HI sera were probably false-positive. Moreover, five discrepant ELISA-negative patients had negative flow cytometric assays with non-HI serum, and platelet activation seen after heat inactivation is most likely independent of anti-heparin/PF4 antibodies. Since the negative predictive value of negative ELISA is very high, these five samples did not undergo a functional test in the previously conducted routine testing[2,5,30,31]. Some authors propose that an immunoassay can be viewed as a "quality control" step that will help to avoid a false-positive functional assay report. Laboratories should, therefore, be cautious in reporting a positive functional assay report if the corresponding PF4-dependent immunoassay is negative or weakly positive. Consequently, there is a high chance that a result is false-positive due to a non-HIT-related platelet-activating factor, such as immune complexes (either circulating or formed *ex vivo* during the heat treatment of samples)[11]. Depending on the 4T score (or other clinical guidance), laboratory testing for HIT antibodies may ensue, typically using an initial immunological screening assay and, only if positive, a secondary functional assay for confirmation[5].

Due to the higher percentages of donor platelet activation observed with the HI sera, all seven non-compliant cases met the criteria (relative to the standard operating procedure of the existing routine protocol), in which the result is interpreted as positive. For the study, we did not change the algorithms for interpreting the results of the HI serum protocol. Adjustment of the cut-off value for platelet activation would be necessary if a HI serum protocol were to be introduced into diagnostics since the average activation of donor platelets in the negative control was >10%. According to our routine protocol, which does not include heat inactivation, the result is considered positive if the platelets are >10% CD62P-positive at the low heparin concentration and ≥50% inhibition of platelet activation is detected at the high heparin concentration[24]. The test's specificity is improved with the high-heparin concentration step as high-dose heparin disrupts the heparin/PF4 multi-molecular complexes and suppresses platelet activation induced by HIT antibodies[3,4,7,12]. Warkentin *et al.* report that platelet activation induced by residual thrombin cannot be reliably distinguished from that caused by HIT antibodies in a functional assay with only two heparin concentrations. The reason is that thrombin has a similar platelet activation pattern as HIT antibodies: its effect is inhibited at high heparin concentrations but not at low, which is why the authors recommended heat inactivation[11,13]. In our study, 23 samples tested positive with non-HI sera, and all were also positive in parallel testing with the HI aliquot. There was no case where the result of the non-HI serum protocol was positive, and the parallel test with HI serum was negative. Heat inactivation in our testing conditions did not improve the specificity, which was even reduced due to false-positive cases (7/50).

To assess platelet activation in a flow cytometric assay and to reduce the impact of donor platelet variability, we designed the $Index_{(non\text{-}HI)}$. Based on 148 cases of suspected HIT, the $Index_{(non\text{-}HI)}$ has a very good negative predictive value at 1.1 or less and a good positive predictive value at 1.6 or more. Therefore, for our study, we also designed the $Index_{(HI)}$ to compare the results of non-HI and HI serum protocols.

In the present study, the mean values of $Index_{(non\text{-}HI)}$ and $Index_{(HI)}$ were 1.9 and 1.3, respectively. $Index_{(HI)}$ values lower than $Index_{(non\text{-}HI)}$ ones are the consequence of higher mean negative control platelet activation observed with HI sera, which is taken into account in the denominator. The ELISA OD value has a statistically significant impact on the indices of both protocols. A higher ELISA OD value represents a higher risk for the presence of platelet-activating antibodies[2,30-32]. A higher percentage of donor platelet activation leads to higher values of both indices, but due to the different denominators in formulas (5.4 *vs* 11.6%), higher ELISA OD values are associated with a greater difference between the corresponding indices. We have shown a significant difference among protocols based on index values and interpreted results.

There are some limitations of the study. In daily practice, we only use serum for the functional assay. However, some authors also use citrated plasma. It would be informative to do some comparative testing with plasma too, since it is less prone to thrombin contamination. Furthermore, we performed the ELISA according to the manufacturer's instructions using a native serum. A rise in HIT-$IgG_{(PF4\text{-}H)}$ levels with HI sera compared to non-HI ones was noted

© SIMTIPRO Srl

All rights reserved - For personal use only
No other use without premission

in the AcuStar immunoassay. Additionally, it would be informative to study the effect of heat inactivation on ELISA OD values.

CONCLUSIONS

Our flow cytometric assay is based on the detection of CD62P as a marker of donor platelet activation. Donor platelets are prepared in PRP, and each patient's sample is tested against four donor platelets. Since we routinely perform the high-heparin concentration step, which also improves the specificity of the assay, in our testing conditions, heat inactivation is not essential. Moreover, in our study, excessive donor platelet activation was observed with HI sera leading to false-positive and erroneous results. In seven of 50 samples in which platelet activation was seen after heat inactivation, this activation was probably independent of anti-heparin/PF4 antibodies. Our current criteria for result interpretation would be inappropriate when using HI sera because of the potentially increased platelet activation. Adjusting the algorithm for result interpretation (raising a cut-off value for a positive result) would be necessary before introducing heat inactivation into diagnostics. False-positive results with HI sera at that stage argue against using heat inactivation in our routine test settings, and argue for more extensive study.

ACKNOWLEDGMENTS

We thank Assistant Professor Branka Golubić Cepulić and Koraljka Gojčeta for their help with providing patients' samples.

AUTHORSHIP CONTRIBUTIONS

KŽ, EM, and MČ designed and conducted the study, KŽ, EM and IM created the indices, KŽ and EM analyzed the data, GT provided data for patients from Croatia, KŽ performed the statistical analysis and prepared the first draft of this manuscript. All authors helped to revise the manuscript and approved the submission.

The Authors declare that they have no conflicts of interest.

REFERENCES

1. Arepally GM. Heparin-induced thrombocytopenia. Blood 2017; 129: 2864-2872. doi: 10.1182/blood-2016-11-709873.
2. Cuker A. Clinical and laboratory diagnosis of heparin-induced thrombocytopenia: an integrated approach. Semin Thromb Hemost 2014; 40: 106-114. doi: 10.1055/s-0033-1363461.
3. Minet V, Dogné JM, Mullier F. Functional assays in the diagnosis of heparin-induced thrombocytopenia: a review. Molecules 2017; 22: 617. doi: 10.3390/molecules22040617.
4. Tardy B, Lecompte T, Mullier F, Vayne C, Pouplard C. Detection of platelet-activating antibodies associated with heparin-induced thrombocytopenia. J Clin Med 2020; 9: 1226. doi: 10.3390/jcm9041226.
5. Favaloro EJ. Laboratory tests for identification or exclusion of heparin induced thrombocytopenia: HIT or miss? Am J Hematol 2018; 93: 308-314. doi: 10.1002/ajh.24979.
6. Warkentin TE. Laboratory diagnosis of heparin-induced thrombocytopenia. Int J Lab Hematol 2019; 41 (Suppl. 1): 15-25. doi: 10.1111/ijlh.12993.
7. Maličev E. The use of flow cytometry in the diagnosis of heparin-induced thrombocytopenia (HIT). Transfus Med Rev 2020; 34: 34-41. doi: 10.1016/j.tmrv.2019.08.001.
8. Warkentin TE, Kelton JG. Temporal aspects of heparin-induced thrombocytopenia. N Engl J Med 2001; 344: 1286-1292. doi: 10.1056/NEJM200104263441704.
9. Warkentin TE, Moore JC. Laboratory evaluation of heparin-induced thrombocytopenia. In: Kitchen S, Olson JD, Preston FE, editors. *Quality in Laboratory Hemostasis and Thrombosis. 2nd ed*. Hoboken, NJ: Wiley-Blackwell; 2009. p. 174-191.
10. Runser A, Schaning C, Allemand F, Amiral J. An optimized and standardized rapid flow cytometry functional method for heparin-induced thrombocytopenia. Biomedicines 2021; 9: 296. doi: 10.3390/biomedicines9030296.
11. Warkentin TE, Arnold DM, Nazi I, Kelton JG. The platelet serotonin-release assay. Am J Hematol 2015; 90: 564-572. doi: 10.1002/ajh.24006.
12. Greinacher A, Warkentin TE, Chong BH. Heparin-induced thrombocytopenia. In: Michelson AD, Cattaneo M, Frelinger A, Newman P, editors. *Platelets. 4th ed*. Amsterdam, NL: Elsevier; 2019. p. 741-767.
13. Warkentin TE, Greinacher A. Laboratory testing for heparin-induced thrombocytopenia. In: Warkentin TE, Greinacher A, editors. *Heparin-Induced Thrombocytopenia.* 5th ed. Boca Raton, FL: CRC Press; 2013. p. 285-327.
14. Sono-Koree NK, Crist RA, Frank EL, Rodgers GM, Smock KJ. A high-performance liquid chromatography method for the serotonin release assay is equivalent to the radioactive method. Int J Lab Hematol 2016; 38: 72-80. doi: 10.1111/ijlh.12442.
15. Favaloro EJ, McCaughan G, Mohammed S, Lau KKE, Gemmell R, Cavanaugh L, et al. HIT or miss? A comprehensive contemporary investigation of laboratory tests for heparin induced thrombocytopenia. Pathology 2018; 50: 426-436. doi: 10.1016/j.pathol.2017.11.089.
16. Nagler M, Bakchoul T. Clinical and laboratory tests for the diagnosis of heparin-induced thrombocytopenia. Thromb Haemost 2016; 116: 823-834. doi: 10.1160/TH16-03-0240.
17. Tardy-Poncet B, Montmartin A, Piot M, Alhenc-Gelas M, Nguyen P, Elalamy I, et al. Functional flow cytometric assay for reliable and convenient heparin-induced thrombocytopenia diagnosis in daily practice. Biomedicines 2021; 9: 332. doi: 10.3390/biomedicines9040332.
18. Brodard J, Benites V, Stalder Zeerleder D, Nagler M. Accuracy of the functional, flow cytometer-based Emo-Test HIT Confirm® for the diagnosis of heparin-induced thrombocytopenia. Thromb Res 2021; 203: 22-26. doi: 10.1016/j.thromres.2021.04.017.
19. HIT*Alert*™ Kit. IQ Products. Available at: https://diapharma.com/product/hemostasis/hemo/iq-hitalert/. Accessed on 05/02/2022.
20. Emo-Test HIT Confirm®. Emosis. Available at: https://www.coachrom.com/fileadmin/docs/IFU_HIT_Confirm_DE.pdf. Accessed on 05/02/2022.
21. Morel-Kopp MC, Mullier F, Gkalea V, Bakchoul T, Minet V, Elalamy I, et al. Heparin-induced multi-electrode aggregometry method for heparin-induced thrombocytopenia testing: communication from the SSC of the ISTH. J Thromb Haemost 2016; 14: 2548-2552. doi: 10.1111/jth.13516.
22. Cuker A, Gimotty PA, Crowther MA, Warkentin TE. Predictive value of the 4Ts scoring system for heparin-induced thrombocytopenia: a systematic review and meta-analysis. Blood 2012; 120: 4160-4167. doi: 10.1182/blood-2012-07-443051.
23. PF4 Enhanced® and PF4 IgG Assay. Immucor. Available at: https://www.immucor.com/en-bnl/product/pf4-enhanced-and-pf4-igg-assay%e2%80%8b/. Accessed on 22/01/2022.

© SIMTIPRO Srl

All rights reserved - For personal use only
No other use without premission

24. Maličev E, Kozak M, Rožman P. Evaluation of a flow cytometric assay for the confirmation of heparin-induced thrombocytopenia. Int J Lab Hematol 2016; 38: 240-245. doi: 10.1111/ijlh.12475.
25. Soltis RD, Hasz D, Morris MJ, Wilson ID. The effect of heat inactivation of serum on aggregation of immunoglobulins. Immunology 1979; 36: 37-45. PMID: 422227.
26. Favaloro EJ, Mohammed S, Donikian D, Kondo M, Hamdam S, Hocker N, et al. Effect of sample heat inactivation on test levels of HIT-$IgG_{(PF4-H)}$ detected by the ACL AcuStar. Thromb Res 2021; 200: 12-15. doi: 10.1016/j.thromres.2021.01.004.
27. Poley S, Mempel W. Laboratory diagnosis of heparin-induced thrombocytopenia: advantages of a functional flow cytometric test in comparison to the heparin-induced platelet-activation test. Eur J Haematol 2001; 66: 253-262. doi: 10.1034/j.1600-0609.2001.066004253.x.
28. Solano C, Mutsando H, Self M, Morel-Kopp MC, Mollee P. Using HitAlert flow cytometry to detect heparin-induced thrombocytopenia antibodies in a tertiary care hospital. Blood Coagul Fibrinolysis 2013; 24: 365-370. doi: 10.1097/MBC.0b013e32835cc17e.
29. Fante MA, Decking SM, Bruss C, Schreml S, Siska PJ, Kreutz M, et al. Heat-inactivation of human serum destroys C1 inhibitor, promotes immune complex formation, and improves human T cell function. Int J Mol Sci 2021; 22: 2646. doi: 10.3390/ijms22052646.
30. Bakchoul T, Zöllner H, Greinacher A. Current insights into the laboratory diagnosis of HIT. Int J Lab Hematol 2014; 36: 296-305. doi: 10.1111/ijlh.12236.
31. Zwicker JI, Uhl L, Huang WY, Shaz BH, Bauer KA. Thrombosis and ELISA optical density values in hospitalized patients with heparin-induced thrombocytopenia. J Thromb Haemost 2004; 2: 2133-2137. doi: 10.1111/j.1538-7836.2004.01039.x.
32. Maličev E, Maček Kvanka M, Klemenc P, Rožman P. The level of heparin-induced antibodies in correlation with the result of the flow cytometric functional assay in the patients with suspected HIT. J Clin Pathol 2017; 70: 1084-1087. doi: 10.1136/jclinpath-2017-204498.

© SIMTIPRO Srl

All rights reserved - For personal use only
No other use without premission