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Original Article

Flow Cytometry-based Functional Assay is a Valuable Diagnostic Approach for Confirmation of Heparin-Induced Thrombocytopenia

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Abstract

Background: Heparin-induced thrombocytopenia (HIT) is a serious immunological adverse drug reaction that rarely occurs in patients receiving heparin. The heparin-induced platelet activation (HIPA) test, a gold-standard assay for HIT, is time-consuming, challenging, and produces qualitative results. We aimed to compare the performance properties of a flow cytometry-based functional assay for HIT diagnosis with HIPA assay.

Materials and Methods: This research was carried out on HIT-suspected patients referred to the Iranian Blood Transfusion Organization between 2021 and 2023. After clinical evaluation and 4Ts scores calculation, anti-PF4 screening and HIPA test were conducted. Thirty HIPA-positive and 30 HIPA-negative samples were selected. Subsequently, a flow cytometry-based functional assay, Emo-Test HIT confirm, was performed, and the sensitivity and specificity for HIT diagnosis were measured.

Results: Among the 30 samples with negative HIPA results, one was positive with the Emo-test HIT Confirm® assay, and the remaining were negative. Among 30 positive HIPA samples, the result of one sample was inconclusive, two samples were negative with flowcytometry Emo-test and the others were positive. The sensitivity and specificity of this flow cytometry-based functional assay were 90% (95% CI: 79.3-100) and 96.6% (95% CI:90.2-100). The negative predictive value and positive predictive value were 93.5% and 96.4% respectively.

Conclusion: Flow cytometry-based functional assay has a good sensitivity and specificity for HIT diagnosis confirmation, indicating that it may be a promising approach in the clinical setting.

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1. INTRODUCTION

Heparin-induced thrombocytopenia (HIT) is an uncommon but serious immunological adverse drug reaction that rarely arises in patients receiving unfractionated heparin (UFH) or, less commonly, low-molecular-weight heparin (LMWH) and leads to thrombocytopenia and potentially devastating thrombosis (1-3). This disorder is caused by the generation of IgG antibodies against PF4-Heparin complexes, leading to a prothrombotic condition by binding these immune complexes to Fc receptors (FcγR) on platelets, neutrophils, and monocytes (4).

An accurate and timely diagnosis of HIT is essential to guarantee prompt administration of a non-heparin anticoagulant (5, 6). A daily risk of thrombosis, amputation, or death is 5-10% higher in people with undiagnosed HIT (7). On the other hand, HIT overdiagnosis may lead to inappropriate heparin cessation, the prescription of alternative anticoagulants at higher expense, and life-threatening hemorrhage caused by the exposure of patients with thrombocytopenia to other anticoagulants (8, 9).

HIT diagnosis combines clinical scoring and laboratory testing, including immunological and functional assays (10, 11). The 4Ts score is a clinical scoring method for HIT, evaluating disease probability (12). Laboratory tests for diagnosing HIT include immunological screening and functional assays (13, 14). Immunological assays are generally available, easy to use, and inexpensive. In contrast to their specificity, they have a substantial negative predictive value due to their high sensitivity. Therefore, these techniques help to exclude HIT but could produce over-diagnosis, especially in patients with critical conditions (5, 6). Functional assays such as serotonin-release assay (SRA) and heparin-induced platelet activation (HIPA) test reveal higher specificity than immunological assays (5, 15-18). Even though SRA and HIPA are considered gold-standard diagnostic assays for HIT (20, 21), they are time-consuming, demand washed platelets from at least four donors, need expert technicians to achieve reliable results because of subjective analysis, and produce qualitative results.

However, flow cytometry-based assays have opened a new avenue for HIT diagnosis over the last years, yielding reliable quantitative results in a short period. The Emo-test HIT Confirm® is a functional method based on flow cytometry, which is utilized for the detection of the antibodies against PF4-Heparin complexes through evaluating the release of P-selectin (CD62P) surface marker from donor platelets following exposure to patients' sera and exogenous heparin (**Figure 1**). This method demands less time and a lower quantity of donor platelets compared to traditional functional methods and produces quantitative results. Few studies have evaluated the diagnostic value of this test, but the results are almost contradictory (19, 20). This study sought to compare the specificity and sensitivity of the HIPA test and the Emo-test HIT Confirm® assay for HIT diagnosis.

2. MATERIALS AND METHODS

2.1 Study design and population

In this study, patients who received UFH or LMWH and in whom HIT was suspected and referred to the coagulation reference laboratory of the Iranian Blood Transfusion Organization (IBTO) between 2021 and 2023 were enrolled. Clinical evaluation including thrombocytopenia level, platelet count declining time, thrombotic events, and other causes of thrombocytopenia, was conducted by an expert physician. These results were recorded for subsequent 4Ts score calculation based on a previously described algorithm to determine the probability of heparin-induced thrombocytopenia (21). Calculated 4Ts scores are grouped as follows (low risk: 0-3; intermediate risk: 4-5; high risk: 6-8). Also, the primary characteristics of patients, such as age, sex, the date of receiving the first dose, and history of prior exposure to heparin were recorded. Afterward, an anti-PF4 screening test (STic Expert), and then a HIPA functional test, were conducted for each sample. HIPA assay was considered a reference gold standard and 30 HIPA-positive and 30 HIPA-negative samples were selected for this study.

2.2 Specimen Collection

Serum samples in tubes without anticoagulant were collected, and centrifuged at 1500 rpm for 10 minutes at room temperature. STic Expert screening test was performed on the samples before freezing, then the samples were stored at -20 °C until subsequent analysis (HIPA and flow cytometry).

2.3 Anti PF4 assay (STic Expert assay)

The presence of anti-heparin/PF4 antibodies in all serum samples was screened by STic Expert HIT kit (Stago, France), a lateral flow immunoassay, according to the manufacturer's instructions. Briefly, the patient's serum and, then, buffer were added to the port coated with PF4-Heparin complex. If there are HIT antibodies in the sample, they bind to the complex. After 10 minutes of incubation at room temperature, a positive result is indicated if a color comparable to or darker than the control appears in the test strip.

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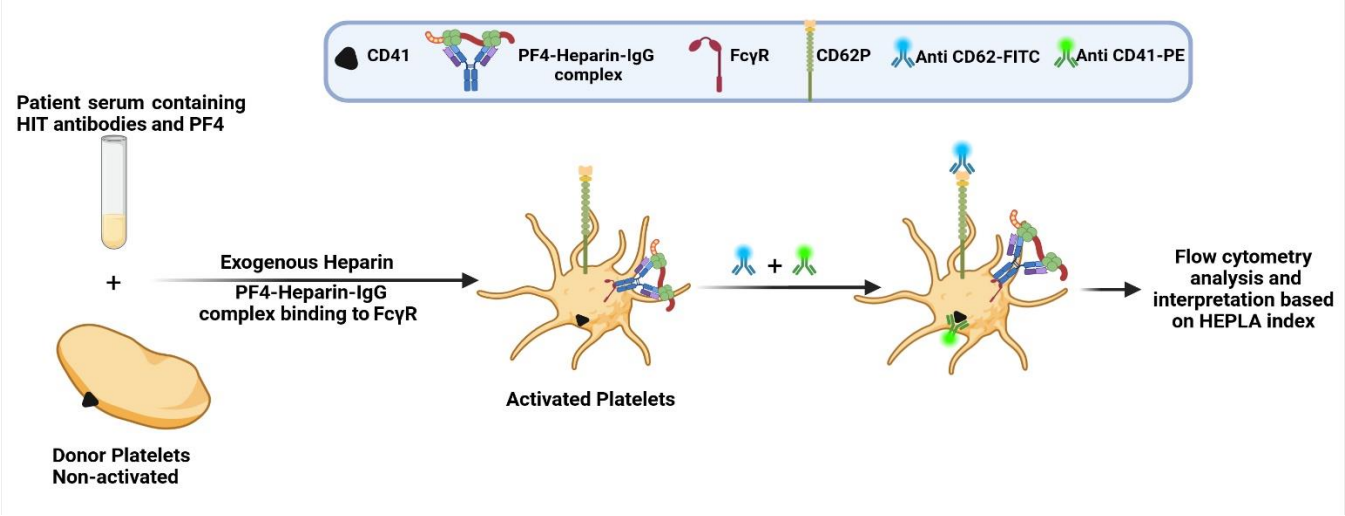

Figure 1. The Summary of the HIT confirmation mechanism by flow cytometry-based functional assays. HIT IgG antibodies and PF4 in the patient's serum bind to exogenous Heparin, forming PF4-Heparin-IgG immune complexes. These complexes interact with Fcγ receptors on the surface of non-activated donor platelets, triggering platelet activation. This activation leads to the expression of CD62P on the platelet surface. Fluorophore-conjugated anti-CD62P and anti-CD41 antibodies are then added to detect the CD62P and CD41 expression. The results of the flow cytometry are used to calculate the HEPLA percentage for each sample and HIT confirmation is performed based on the HEPLA diagnostic algorithm. HIT: Heparin-induced thrombocytopenia; PF4: platelet factor 4.

2.4 Platelet-rich plasma (PRP) preparation

PRP was prepared from citrated whole blood collected from healthy volunteers who had not used any medication for two weeks prior to sampling. The PRP samples were obtained within a maximum of 6 hours after blood collection. The whole blood samples were allowed to rest for at least 30 minutes before being centrifuged at 200 g for 5 minutes at room temperature. The prepared PRP samples were used within 3 hours after preparation. Platelet counts for the PRP samples were standardized to 300,000 platelets/µL using platelet-poor plasma (PPP).

2.5 Heparin-induced platelet aggregation assay (HIPA assay)

As a reference test, the HIPA assay was carried out for all the serum samples. Briefly, each serum sample was assessed with six different PRP samples from platelet donors in the presence of two different concentrations of heparin (0.2 IU/mL and 100 IU/mL). The reactions were performed in a 96-microwell plate with transparent round ends. Each plate included a positive control (collagen) and a negative control (buffer). The test plate was incubated on a magnetic stirrer and the formation of platelet aggregations was visually checked every 5 minutes. The patient sample was considered positive if at least two platelet suspension aggregates (changing from turbid to clear) in the presence of 0.2 IU/mL, but not 100 IU/mL, within 30 minutes (22, 23). Each plate included a diluted HIT patient sample as a weak-positive control and a sample from healthy donors as a weak-negative control.

2.6 Flow cytometry-based assay

The Emo-test HIT Confirm® assay (Emosis SAS, IllkirchGraffenstaden, France) was conducted on serum samples of patients in accordance with the manufacturer's instructions as illustrated in **Figure 2** (19). The forward/side scatter dot plot was used to determine the platelet population. On a single-parameter FL2 histogram, the platelet population (CD41+) was gated. The intersection of CD41-FITC histograms (platelet population) and CD62P-PE (activated platelet population) was defined as the activation threshold.

The HIT Confirm results (Percentage of CD62P expression) were expressed using the HEPLA index (%H0.3-%H100/ (% positive control - % negative control) × 100) which indicates platelet activation. The interpretation of the HEPLA index is illustrated in **Figure 3**.

2.7 Statistical analysis

To summarize the data, the median and range (minimum – maximum) for continuous variables and frequency along

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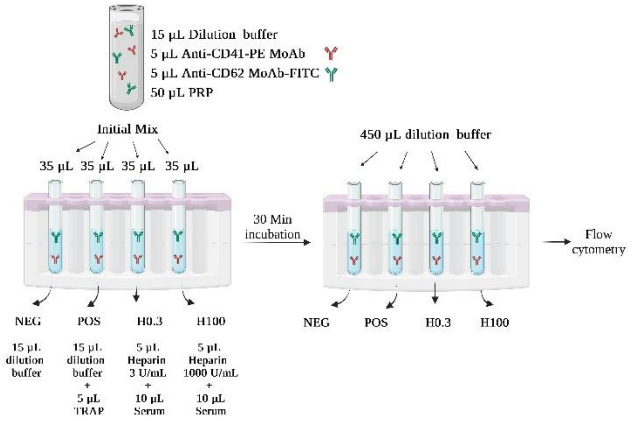

Figure 2. Emo-test HIT Confirm® assay schematic protocol. After preparation of the initial mix and aliquoting in four separate tubes, the patient's serum sample was added to two of them and incubated in the presence of heparin (0.3 IU/mL or 100 IU/mL). Negative control (buffer), and positive control (thrombin receptor activating-peptide (TRAP)) were also included. Then dilution buffer was added to hinder stimulation. The immediate readout was performed by Flow Cytometer. PRP=Platelet-rich plasma, NEG= negative control, POS= positive control, H0.3= 0.3 IU/mL Heparin concentration, H100= 100 IU/mL Heparin concentration, TRAP= thrombin receptor activating-peptide.

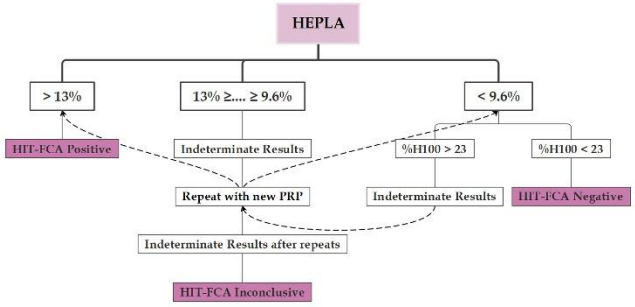

Figure 3. Schematic representation of the HEPLA index interpretation based on the Emo-test HIT Confirm® assay results (37). H100= 100 IU/mL Heparin concentration, PRP=Platelet-rich plasma.

with the percentage for categorical variables are used. Based on the results of the Shapiro-Wilks of normality, an independent t-test or Mann-Whitney test was used to compare two continuous variables. The chi-square test was used to assess the association between two categorical variables. The Receiver-Operating Characteristics (ROC) curve was utilized to assess the performance of the flow cytometry method to diagnose HIPA by computing the Area under the curve (AUC) index. The Youden Index was used to find the best cut-off value. Based on the best cut-off value, crude accuracy, sensitivity, specificity, negative predictive value, and positive predictive value indices were calculated and reported. Kappa coefficient was reported to evaluate the agreement between the flow cytometry and the HIPA test results (reference test). In this study, a significance level of 0.05 is considered. All statistical calculations were performed by R software.

3. RESULTS

3.1 Demographic information

The overall median age of study subjects was 67 years (range 24 to 93), 34 individuals (56.7%) were men, and 26 individuals (43.3%) were women. The median 4Ts score was 6 points (range 2-8) in patients with HIPA positive and 3 points (range 0-8) in patients with HIPA negative. The leading cause of patient hospitalization was medical reasons, for which COVID-19 accounted for the largest proportion. Detailed patient characteristics are reported in **Table 1**.

3.2 Anti-PF4 Ab (STic Expert) and HIPA assay results

Thirty patients (50%) were HIPA positive and 30 patients (50%) were HIPA negative. Two cases with HIPA-positive results were tested negative by STic Expert (93.3% sensitivity). Moreover, among 30 negative sera in the HIPA test, 3 were tested positive for STic Expert (90% specificity) (**Table 2**).

3.3 Flow cytometry-based Emo-test HIT Confirm® assay

Out of 60 serum samples, 28 samples (46%) showed positive results in the Emo-test HIT Confirm® assay (%HEPLA > 13%, Mean %HEPLA: 40±19.9, 95%CI 32.2-47.7). Among our HIT-suspected patients, the result of the Emo-test HIT Confirm® remained inconclusive in only one patient. Emo-test HIT Confirm® results were found to be negative for 31 other patients (Mean %HEPLA: 4.3±3.4, 95%CI 3-5.7). As one sample out of 30 samples with a negative HIPA result tested positive in the Emo-test HIT Confirm®, the specificity of the Emo-test HIT Confirm® assay was found to be 96.6 (95%CI:90.2-100). The sensitivity of Emo-test HIT Confirm® was found to be 90 (95%CI:79.3-100) in our study as two out of 30 HIPA-positive sera revealed negative and one sera revealed inconclusive in the Emo-test HIT Confirm® (**Table 2**). The mean of the HEPLA in HIPA negative cases and HIPA positive cases were 5.1±3.3 (95%CI 3.8-6.3) and 37±22.3 (95%CI 28.6-45.2) respectively ($p<0.001$) (**Figure 4**). The crude agreement between HIPA and Flow Cytometry methods was 93.33%. Furthermore, the agreement level between the two methods using the kappa ratio was 0.87 (95% CI: 0.75-0.99). The specificity and sensitivity of the Emo-test HIT Confirm® compared to the HIPA test were found to be 96.6 and 93.3 respectively when the cutoff of the HEPLA was defined as 11%. The

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Table 1. Demographic characteristics of the study subjects.

	All Patients (n=60)	HIPA negative (n=30)	HIPA positive (n=30)	P-value
Age (median [range])	67 [24-93]	71.5 [24-93]	63 [38-83]	0.525
Gender (n [%])	M= 34 [56.7] F= 26 [43.3]	M= 18 [60] F= 12 [40]	M= 16 [53.3] F= 14 [46.7]	0.795
Chief complaint				
Surgical	14 (23.3)	4 (13.3)	10 (33.3)	1.00*
Medical	24 (40)	8 (26.7)	16 (53.3)	
Not Recorded (%)	22 (36.7)	18 (60)	4 (13.3)	
4Ts-score (median (range))	4 (0-8)	3 (0-8)	6 (2-8)	<0.001
Heparin Type (n (%))				
UFH	33 (55)	15 (50)	18 (60)	<0.011
LMWH	13 (21.6)	5 (16.7)	8 (26.7)	
Not Recorded	13 (21.6)	10 (33.3)	3 (10)	
None	1 (1.6)	-	1 (3.3)	

*chi-square test, P-value was computed after excluding the Not Recorded category. UFH= unfractionated heparin, LMWH= Low-molecular-weight heparin

Table 2. The results of the flow cytometer-based assay, Emo-test HIT Confirm and STic expert, in comparison with the HIPA assay as a gold standard.

HIPA	Flow Cytometry (Emo-test HIT Confirm®)			STic Expert		
	Inconclusive (n=1)	Negative (n=31)	Positive (n=28)	Inconclusive (n=0)	Negative (n=29)	Positive (n=31)
Inconclusive (n=0)	0	0	0	0	0	0
Negative (n=30)	0	29	1	0	27	3
Positive (n=30)	1	2	27	0	2	28

negative predictive value (NPV) and positive predictive value (PPV) were found to be 93.5% and 96.4% respectively when the cut-off of the HEPLA was considered 13%. Moreover, the receiver-operating characteristics (ROC) curve for operating characteristics evaluation revealed that Emo-test HIT Confirm® is an informative assay (AUC:0.939) (**Table 3, Figure 5**).

4. DISCUSSION

This study aimed to compare the flow cytometry-based assay, the Emo-test HIT Confirm® assay, with the HIPA test, which is the gold-standard test for HIT diagnosis. We revealed that the Emo-test HIT Confirm® assay exhibits high diagnostic efficacy for HIT antibody detection in terms of both high sensitivity and specificity (90% and 96.6%, respectively). In consistent with our findings, a study by Brodard et al. on 103 HIT cases, showed a specificity of 94.3% for the Emo-test HIT Confirm® assay in comparison with HIPA results. However, they observed a sensitivity of 60% for this test (20). Moreover, K. Althaus et al. showed lower sensitivity and specificity (69.7% and 75.4% respectively) for this test compared to the HIPA assay. However, they found that the sensitivity and specificity increased, reaching 75.8% sensitivity and 85% specificity when this test was used along with the IgG ELISA assay (19).

Discordance of the Emo-test with the HIPA, as a gold standard, were as follows: one patient exhibited negative STic Expert and HIPA results, but the flow cytometry test showed indeterminate results. However, repeating the test with a PRP from a different donor revealed a positive result. This patient has a HEPLA index of 17%; however, the average HEPLA index was 40±19.9. We postulated that patients with borderline HEPLA may produce inconsistent results in the HIPA test because subjective visual assessment in HIPA may lead to interference with the interpretation (14). On the other hand, flow cytometry yields quantitative results, enabling the identification of weak interactions. However, discrepant results due to variations in donor platelets' responses to HIT antibodies from various PRPs and technical errors cannot be excluded (24-26).

According to the results of the Emo-test HIT Confirm® assay, the percentage of platelet activation of another sample at both 0.3 and 100 IU/mL heparin concentrations was

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Table 3. Performance of Emo-test HIT Confirm® assay according to the cutoffs of 11% derived from the ROC analyses.

	Cut off *	Sensitivity	Specificity	NPV %	PPV%	AUC
HELPA %	11*	0.933	0.966	93.5	96.5	0.939
	13	0. 900	0.966	93.5	96.4	-

*Greater and equal to 11 is positive based on the Youden index; NPV= Negative predictive value; PPV= Positive predictive values.

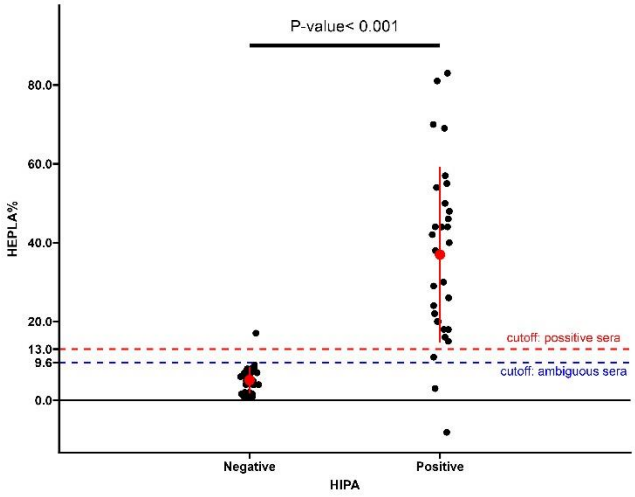

Figure 4. Comparison of HIPA and Emo-test HIT Confirm® assay according to the HEPLA percentage. The cutoff of 13% is considered positive (dashed lines). One sample revealed an ambiguous result in the Emo-test HIT Confirm® assay.

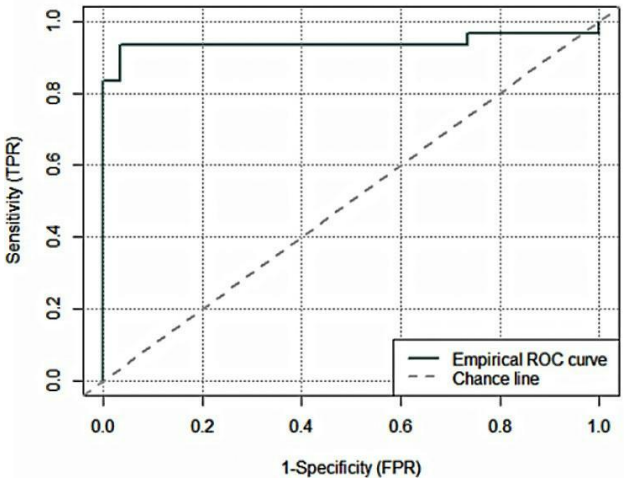

Figure 5. Receiver-operating characteristics (ROC) curve analysis for evaluation of operating characteristics of Emo-test HIT Confirm® flow cytometry-based assay.

almost identical and the HEPLA index remained within the indeterminate range even after the test was repeated with a PRP from two different donors. It was postulated that modifying the test conditions, such as diluting the patient's sample or adjusting the ratio of PRP to the patient's sample, might yield a conclusive result (27). Indeed, the maximum amount of immune complexes are generated at approximately equal concentrations of PF4 and heparin, therefore, optimal antigen-antibody binding occurs in low-dose (therapeutic-dose) heparin but not in excess heparin (28, 29). However, the same inconclusive outcome was achieved after these modifications excluding the prozone effects. As we mentioned before, the interpretation of borderline cases is challenging in HIT functional tests (30). However, factors such as aggregated IgG caused by laboratory conditions like multiple freeze-thaw cycles led to this result (31, 32). In addition, the patient's samples may also contain HIT antibodies that can stimulate platelets even without the presence of heparin. The production of these antibodies is observed in autoimmune HIT syndromes such as delayed-onset HIT, persistent HIT, spontaneous HIT syndrome, and fondaparinux-associated HIT (33). Recent research has classified Vaccine-Induced Thrombotic Thrombocytopenia (VITT) as a contributing factor in autoimmune HIT syndromes (34) in which platelet aggregation was shown to be less dependent on physiological levels of heparin and less sensitive to being inhibited by high-dose heparin than platelet aggregation in patients with typical HIT (35). We hypothesize that conditions like autoimmune HIT syndromes and aggregated IgG due to frequent freeze-thaw may result in a disturbed HEPLA index by changing platelet activation levels. This highlights the necessity of including a buffer control step which consists of the patient sample and the donor PRP without the presence of heparin to detect non-IgG serum factors that activate platelets directly (36).

Two HIT cases in our study had positive results in the HIPA and STic expert assays but were negative for the Emo-test HIT Confirm® assay. The Emo-test HIT Confirm® assay was repeated with a PRP obtained from a different donor but the results did not change. This ruled out hyporeactivity of donor PRP possibly caused by factors such as medication or non-responsive FcγRIIA due to genetic polymorphisms. Moreover, serum titration did not change the results. Consistent with this finding, MALICEV et. al. obtained negative flow cytometry results but HIPA-positive results in three patients (30). Such results may lower the clinical

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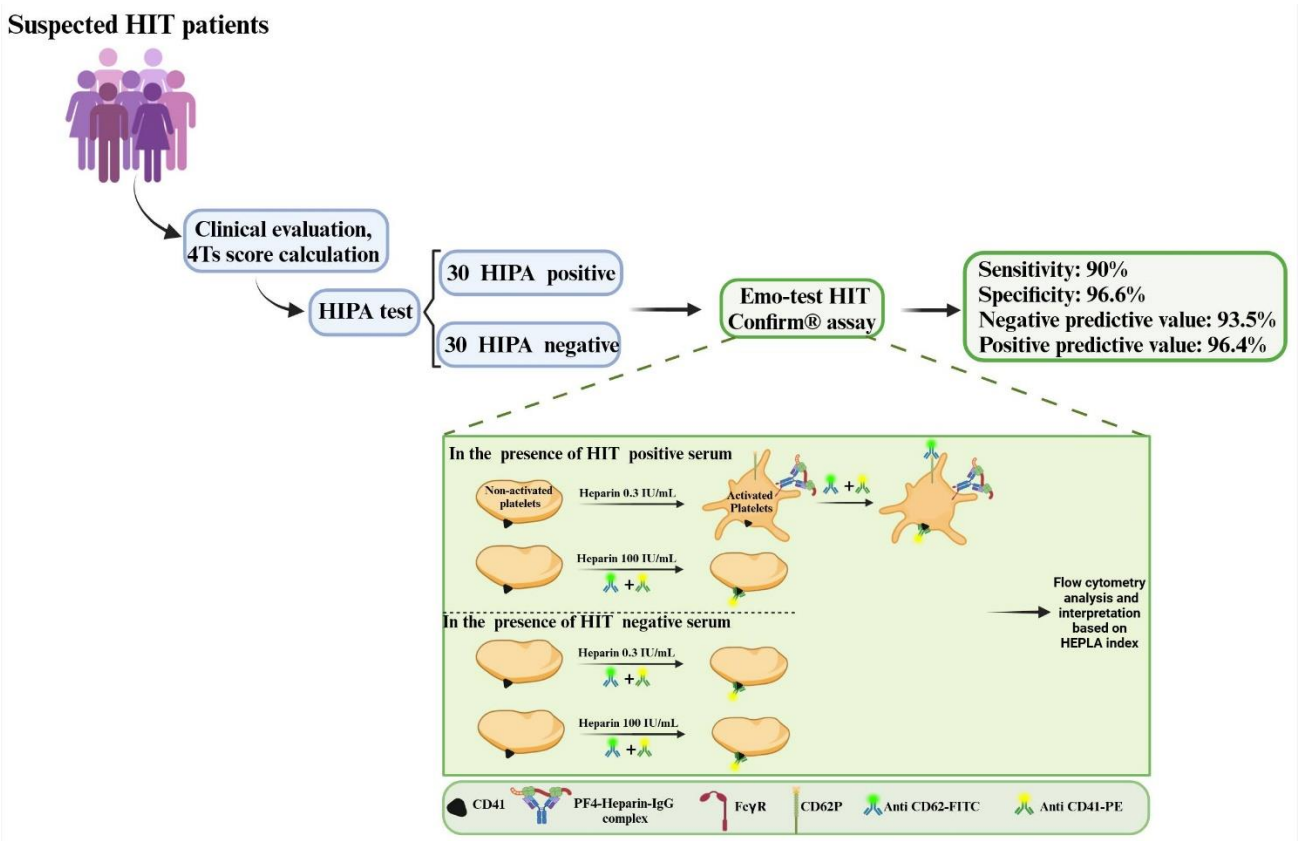

Figure 6. Graphical abstract of Study.

implication of the Emo-test HIT Confirm® assay in comparison with the HIPA assay because undiagnosed or not timely diagnosis of HIT may lead to thrombotic complications, amputation, HIT-induced disseminated intravascular coagulation (DIC) or even death (7, 33). This indicates that it might be better to employ the Emo-test HIT Confirm® assay along with other diagnostic approaches like immunological assays or consider a lower cutoff for the HEPLA index to increase the sensitivity of the test in the clinical context. Immunological assays have a substantial negative predictive value and can help to exclude HIT as well (5, 6).

The strength of this study is that we included well-characterized patients by an expert physician, 4Ts score determination, and conducted STic expert assay along with HIPA assay. Moreover, we included fresh samples for experiments. In addition, we employed modifications and improvement measures to verify the source of discrepancies between the reference test and the Emo-test HIT Confirm® assay. Overall, we found that Emo-test HIT Confirm® yielded a satisfactory performance in diagnosing HIT patients, suggesting its potential utility in confirming the presence of HIT. A graphical representation of study has been provided in **Figure 6**.

5. CONCLUSION

Overall, this study revealed that the flow cytometry-based, Emo-test HIT Confirm® assay, is a valuable functional assay for the detection of HIT antibodies in patients.

Acknowledgment

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Ethical statement

Written informed consent was obtained from all patients, or, in cases where the patient was critically ill, from their legal representatives. The study was approved by the Medical Ethics Committee of IBTO and conducted according to the declaration of Helsinki (IR.PMI.REC.1401.006).

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Conflict of interest

The authors declare that they have no conflict of interest.

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