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Full Length Article

Evaluation of a flow cytometer-based functional assay using platelet-rich plasma in the diagnosis of heparin-induced thrombocytopenia

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ABSTRACT

Background: Heparin-induced thrombocytopenia (HIT) is caused by platelet-activating antibodies that recognize platelet factor 4/heparin (PF4/hep)-complexes. The in vitro demonstration of PF4/hep antibodies using functional assays is essential for an optimal management of patients suspected to have HIT. However, conventional functional assays are technically challenging and limited to specialized laboratories. In contrast, flow cytometers are commonly used in routine laboratories. The aim of this study is to investigate the performance characteristics of a commercially available, flow cytometer based assay in the diagnosis of HIT.

Study design: Sera of consecutive patients with suspected HIT were investigated using the Emo-test HIT Confirm® assay and compared to the standard method consisting of an IgG-specific enzyme immunoassay (EIA) for anti-PF4/hep antibodies and the heparin induced platelet aggregation (HIPA) test.

Results: 390 sera were included in the study, 164 sera tested IgG EIA-positive, of which 33 also tested HIPA-positive. No HIPA-positive samples were EIA-negative. In the Emo-test HIT Confirm® assay, 112 sera revealed positive results (%Hepla > 13); however, 51 (45.5%) were EIA-negative. Of the 33 HIPA-positive/EIA-positive HIT sera, 23 tested positive in the Emo-test HIT Confirm® assay, 2 gave ambiguous results, and 8 sera yielded false-negative results. Accordingly, the HIT Confirm® assay showed a sensitivity of 69.7% with a slightly better specificity of 75.4% compared to the EIA (sensitivity 100%, specificity 63.3%). An increase in diagnostic specificity for HIT to 85% was found when positive results were obtained in both the Emo-test HIT Confirm® assay and EIA.

Conclusion: The Emo-Test HIT Confirm® assay may improve the specificity of laboratory investigations of HIT. However, the assay can only be recommended in combination with an immunoassay due to the high rate of false negativity. Our observation indicates a need to establish external quality assessment for functional assays to avoid such clinically relevant pitfalls.

1. Introduction

Heparin-induced thrombocytopenia (HIT) is a prothrombotic, immune-mediated adverse reaction that occurs after exposure to unfractionated heparin (UFH) or low molecular heparin (LMWH) [1]. Platelet-activating antibodies that recognize platelet factor 4/heparin (PF4/hep)-complexes are responsible for the disease. Typical clinical manifestations of HIT are a fall in platelet count by > 50%, beginning between days 4 and 10 of heparin therapy, often complicated by new thrombotic events [2]. However, the clinical diagnosis of HIT is often difficult, especially in patient populations with a high prevalence of thrombocytopenia such as intensive care patients [3]. Thus, the clinical diagnosis must be corroborated by in vitro demonstration of platelet activating PF4/hep antibodies. A rapid functional assay is therefore desirable to identify those patients who can be maintained on heparin to avoid risks associated with alternative anticoagulant agents like bleeding. Although functional assays that utilize “washed platelets”, such as the serotonin release assay (SRA) [4] or the heparin-induced platelet activation assay (HIPA) [5], are considered to be the gold standard in the laboratory diagnosis of HIT, they are technically challenging, require fresh donor platelets and are restricted to specialized laboratories.

Recently, a practical and easy to use flow cytometer based functional assay, the Emo-test HIT Confirm® assay, has been introduced to differentiate between platelet- activating and non-activating PF4/hep antibodies. This assay is thought to have several advantages over

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conventional functional assays. For instance, the assay does not require time consuming preparation of washed platelets and can be performed using platelet rich plasma (PRP) from one donor which allows single sample testing. Therefore, test results can be provided within a relative short turn-around time of approximately 90 min (min). However, avoiding platelet washing could have detrimental consequences, given that this method of platelet preparation is believed to enhance both the sensitivity and specificity for diagnosis of HIT vis-a-vis assays that utilize PRP [6]. In this study, we assessed the performance characteristics of the Emo-test HIT Confirm® assay in the diagnosis of HIT in comparison to an IgG specific EIA and the (washed platelet) HIPA test.

2. Material and methods

2.1. Study design

Blood samples from 394 surgical and medical patients who received UFH or LMWH and in whom HIT was suspected were evaluated in this study. Pretest probability of heparin-induced thrombocytopenia (4Ts score) was used to identify patients with suspected HIT. All patients had 4Ts score higher than 3. These samples were initially tested within 72 h after arrival using an IgG specific EIA and the HIPA test. Thereafter the samples were immediately frozen and stored at −20 °C until further testing using the Emo-test HIT Confirm® assay. HIPA was considered to be the reference test in this study as precise clinical information on the patients was sparse, and the HIPA as an independent “gold standard” for HIT diagnosis has been used in the past [7].

2.1.1. IgG PF4/heparin EIA and heparin-induced platelet activation assay (HIPA)

The commercially available IgG-EIA (Zymutest HIA, IgG, Hyphen BioMed, Neuville-sur-Oise, France) was used according to manufacturers' instructions. A sample was considered positive if the optical density (OD) was higher than 0.3.

The HIPA test was performed as described previously with minor modifications [5]. Briefly, each sample was tested with washed platelets from four different platelet donors in the absence (buffer alone) or presence of heparin (0.2 IU/ml and 100 IU/ml). Reactions were placed in round bottom microtiter wells containing two spheres and stirred at approximately 500 rpm. Wells were examined optically at 5-min intervals for loss of turbidity. A serum was interpreted as reactive (positive), if platelets of at least two donors aggregated within 30 min in the presence of 0.2 IU/ml, but not in the presence of 100 IU/ml heparin. Reading time was 45 min. Each testing included a positive and negative control serum.

2.1.2. Flow based assay Emo-test HIT Confirm® assay

The Emo-test HIT Confirm® assay (Emosis SAS, Illkirch-Graffenstaden, France) was performed according to the manufacturer's instruction. In brief, PRP was obtained from citrated whole blood samples of one healthy donor without use of any medication for 14 days prior to blood collection for each test. After resting for at least 30 min, fresh PRP (< 3 h old) was isolated from collected whole blood samples by centrifugation at 200*g* for 5 min (without brake, at room temperature [RT]). Prior to testing, patient's sera were heat inactivated at 56 °C for 30 min and centrifuged at 12,000*g* for 10 min. 10 µL patient's serum was then incubated with 50 µl donor PRP for 30 min in dark at RT in the presence of 0.3 IU/ml or 100 IU/ml heparin and two platelet markers (CD41-PE and CD62P-FITC, contents of the kit). Maximal platelet activation was defined after incubation with Thrombin Receptor-Activating Peptide (TRAP, 50 µg, content of the kit). The measurement of CD41/CD62P surface expression renders a quantitative result. The percentage of CD62P positive events in the presences of 0.3 IU/ml heparin (H0.3) was subtracted from the percentage of events in the presence of 100 IU/ml heparin (H100) and divided to the increment of CD62P after activation with TRAP. The following equation was used to calculate test results (%HEPLA). % Platelet activation index in the presence of heparin (% HEPLA) = [(%CD62P-H0.3 − %CD62P-H100) / (%CD62P-TRAP − %CD62P-Buffer)] × 100. The cutoff of 13% was determined by the manufacturers based on a pilot study, where plasma samples from 57 heathy volunteers were tested using the presented protocol. Values < 9.6% indicated a negative result. The mean ± 2× standard deviation (SD) was considered for an ambiguous result (%HEPLA: 9.6–13.0%), the mean ± 3× SD was considered for a positive result (%HEPLA: > 13.0%).

2.2. Statistical analysis

The statistical analysis was performed using Prism, Version 7.0 (GraphPad, La Jolla, CA, USA). Performance characteristics were compared using the Receiver-Operating Characteristic (ROC) curve, which is a graph of sensitivity against (1 − specificity). A perfect test would have sensitivity and specificity both equal to 1. The performance characteristic of a diagnostic assay was quantified by calculating the area under the ROC curve (AUROC). The ideal test would have an AUROC of 1, whereas a random guess would have an AUROC of 0.5. Comparison of test characteristics was performed using ANOVA. Comparisons between groups were calculated using one way ANOVA test. Data are given as median (minimal; maximal) and p values < 0.05 were considered statistically significant.

3. Results

3.1. Conventional laboratory investigations

A total of 394 sera were referred to our laboratory between February 2016 and March 2018. 4 samples caused platelet aggregation in the presence of buffer, low and high heparin concentrations in HIPA. These sera were considered indeterminate and excluded from the test evaluation study, as it remained unclear whether these sera contain factors that might overlap heparin dependent antibodies [8]. A total of 390 sera were included in the final assessment, of them 164 (41.9%) tested positive in the IgG EIA (OD median: 0.65, range 0.30–3.78, 95%CI 0.99–1.29), (Table 1).

In the HIPA, heparin-dependent platelet activating antibodies were found in 33 sera (Supplementary Table 1) (8.5%), all of which also tested positive in the IgG EIA. These 33 HIPA-positive sera had

Table 1
Demographic characteristics of the study cohort (EIA = ELISA, OD = optical density).

	All patients (n = 390)	HIT-Confirm® negative (n = 238)	HIT-Confirm® ambiguous (n = 40)	HIT-Confirm® positive (n = 112)
Age (median, [range])	65, [0–92]	64, [0–89]	65, [0–86]	68, [0–92]
Gender (n, [%])	m = 261, [66.9] f = 129, [33.1]	m = 165, [69.3] f = 73, [30.7]	m = 23, [57.5] f = 17, [42.5]	m = 73, [65.2] f = 39, [34.8]
Surgical/medical	271/119	171/67	24/16	76/36
EIA OD (median, [range])	0.235, [0.065–3.739]	0.214, [0.065–3.167]	0.251, [0.075–3.779]	0.331, [0.075–3.739]
4Ts-score (median, (range))	5, [3–8]	4, [3–7]	5, [3–8]	5, [3–8]

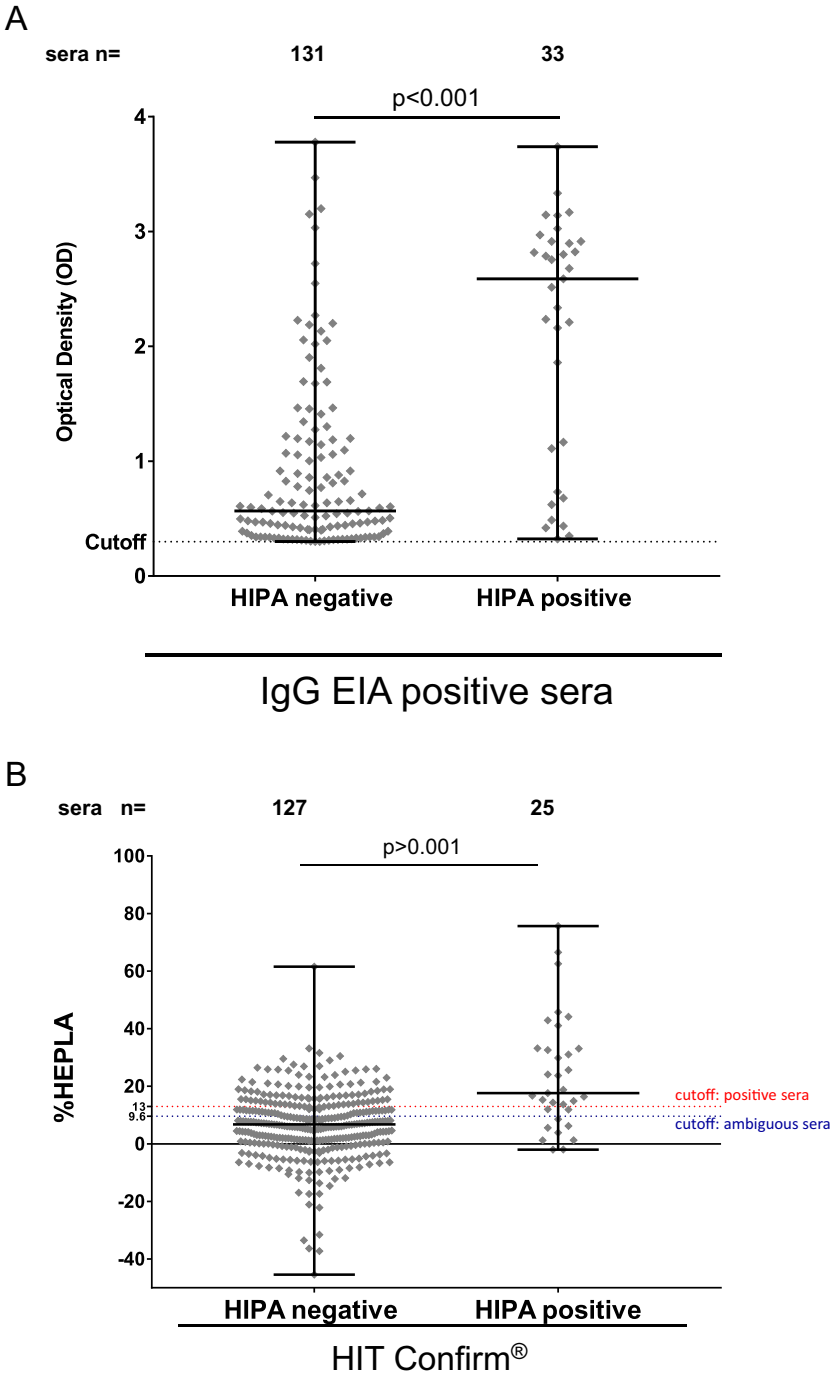

Fig. 1. Functional distribution of Sera tested in the IgG specific immunoassay and the Emo-Test HIT Confirm® assay.
Sera were divided into two groups according to their ability to activate platelets in the heparin-induced platelet activation (HIPA) test: platelet activating (HIPA +) and non-platelet activating antibodies (HIPA −). A) 164 sera that were tested positive in HIA-IgG-EIA, of which 33 were able to activate platelets in the HIPA assay. B) 152 sera had results higher than %HEPLA 9.6 in the Emo-Test HIT Confirm® assay.

significantly higher OD's compared to the 131 IgG EIA positive sera without capacity to activate platelets (median OD: 2.59, range 0.32–3.74, 95%CI 1.75–2.5 vs. 0.57, range 0.3–0.78, 95%CI 0.76–1.02, $p < 0.001$, Fig. 1A). As all HIPA-positive sera tested positive by IgG EIA, the sensitivity and negative predictive value (NPV) of the EIA was 100% (Table 2). However, as additional positive IgG EIA reactions were observed in another 131 sera, the EIA specificity was only 63.3%, with a positive predictive value (PPV) of 20.2% (Table 2).

3.2. Test results of the Emo-test HIT Confirm® assay

112 out of 390 sera (28.7%) revealed clear positive result in the Emo-test HIT Confirm® assay (%HEPLA > 13%, median %HEPLA: 18.3%, range 13.1–75.7%, 95%CI 20.0–24.2%, Fig. 1B). In addition, 40 sera showed ambiguous results (median %HEPLA: 11.5%, range 9.7–12.9%, 95%CI 11.1–11.6%) and 238 sera revealed negative results (median %HEPLA: 2.1%, range −45.0–9.5%, Supplementary Fig. 1). Unlike the findings of the HIPA, where none of the 33 HIPA-positive results were IgG EIA-negative, we found that approximately half of the

Table 2
Summary of test sensitivity, specificity, negative predictive value (NPV), and positive predictive values (PPV) according to the capability of detecting platelet-activating antibodies using two different cutoffs derived from the ROC analyses.

	Sensitivity% (95% CI)	Specificity% (95% CI)	NPV%	PPV%	Likelihood	AUC
EIA (Cutoff: OD 0.300)	100 (89.4–100)	63.3 (58.1–68.3)	100.00	20.2	2.7	0.929
HIT Confirm® assay (Cutoff: 13%)	69.7 (51.3–84.4)	75.4 (70.5–79.7)	96.4	20.7	2.8	0.780
HIT Confirm® assay (Cutoff: 11.89%)	75.8 (57.7–88.9)	70.6 (65.6–75.3)	96.9	19.2	2.6	–
Combined HIT Confirm® assay > 9.6% and EIA > 0.300	75.8 (57.7–88.9)	85.2 (81.0–88.7)	97.4	32.1	5.1	–

Emo-test HIT Confirm® assay-positive samples tested IgG EIA-negative, irrespective of whether the standard cutoff (%HEPLA > 13% was used (51/112 [45.5%] were EIA-negative) or the cutoff was lowered to include also samples yielding indeterminate results (74/152 [48.7%] were EIA-negative). This suggests that factors other than anti-PF4/heparin IgG antibodies can account for positive results in the Emo-test HIT Confirm® assay (Fig. 2).

Only a weak correlation was found between EIA and the Emo-test HIT Confirm® assay (Spearman r: 0.176, 95%CI 0.075–0.273, $p = 0.005$, Supplementary Fig. 2). Of note, while 61 sera revealed positive results according to the manufacturers' recommended cutoffs in both assays, 86 EIA positive sera tested negative in the Emo-test HIT Confirm® (Table 2).

Higher expression of CD62P was observed after incubation of 33 HIPA-positive sera compared to 357 HIPA-negative ones (median % HEPLA: 17.6%, range −2.0–75.7, 95%CI 11.2–0.6% vs. 6.8%, range −45–61.5%, 95%CI 5.2–7.5%, respectively, $p < 0.001$). Nevertheless, 8 of the HIPA positive sera revealed negative results in the Emo-test HIT Confirm® assay.

Since HIT cannot be safely excluded in cases with ambiguous findings, we next evaluated all reactive results in the Emo-test HIT Confirm® assay (%HEPLA > 9.6). 25 out of 152 (16.4%) reactive sera induced platelet activation in the HIPA assay. These sera had significantly higher %HEPLA results compared to HIPA negative sera (median %HEPLA: 25.6%, range 11.9–75.7%, 95%CI 23.2–37.9% vs. % HEPLA 15.5%, range 9.7–61.5, 95%CI 15.8–18.2%, $p < 0.001$, Fig. 1B).

3.3. Performance characteristics of the Emo-test HIT Confirm® assay

Considering HIPA as the reference test, acceptable sensitivity and NPV of the Emo-test HIT Confirm® was found in this study (69.7% and 96.4%, respectively, Table 2). However, 89 of the 112 samples with positive results in the Emo-test HIT Confirm® assay had no platelet activating antibodies according to HIPA resulting in moderate specificity and PPV of the test at the recommended cutoff of %HEPLA > 13% (75.4% and 20.7%, respectively, Table 2). Moreover, 51 of these 89 samples tested negative by IgG EIA (Table 3). The AUROC describes the ability of the test to discriminate between individuals with and without platelet activating antibodies. The AUROC of the IgG-EIA was higher than the Emo-test HIT Confirm® assay, although both assays could be considered as informative assays (AUROC: 0.929 and 0.780, respectively, Fig. 3). The likelihood ratio of having HIT in case of positive result was similar for IgG EIA and Emo-test HIT Confirm® assay (2.725 and 2.788, respectively). The optimal compromise (trade-offs) between sensitivity and specificity of the later assay was identified using the northwest approach as a %HEPLA of 11.9%. However, this modification did not result in significant improvement of the test sensitivity (69.7%

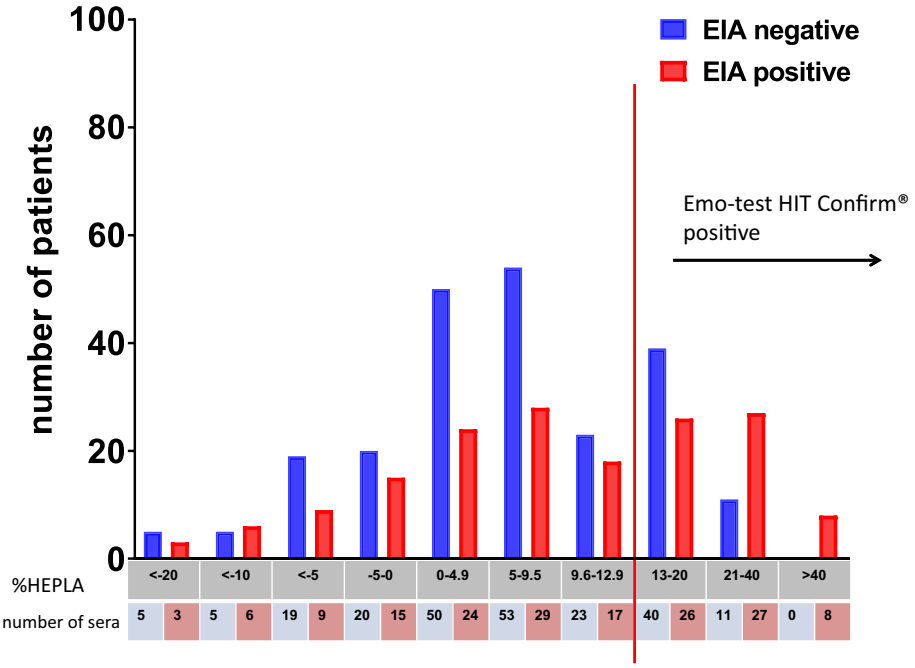

Fig. 2. The distribution of EIA-positive versus EIA-negative status depending on the magnitude of the Emo-test HIT Confirm® assay.
The probability of EIA-positive status increases with a high positive %HEPLA (> 21) but approximately half of the Emo-test HIT Confirm® assay-positive samples tested IgG EIA-negative.

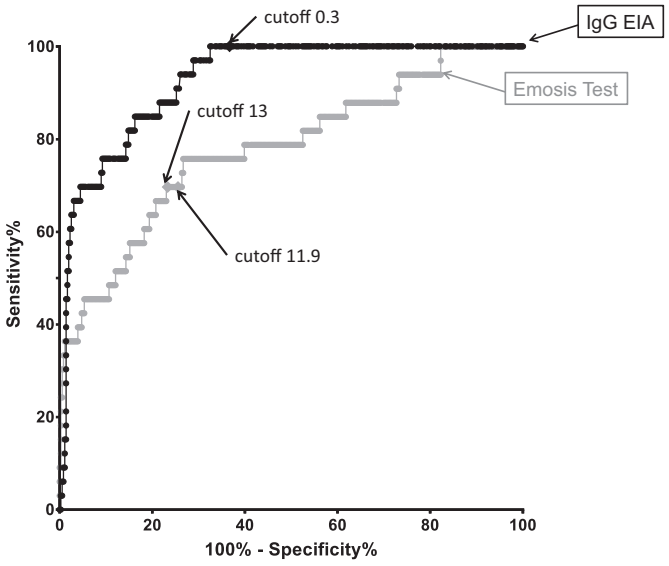

Fig. 3. The performance characteristics of the Emo-test HIT Confirm® assay. A comparison of the operating characteristics for the diagnosis of HIT between the Zymotest IgG-EIA and the Emo-test HIT Confirm® was performed using the receiver-operating characteristics (ROC) curve. 390 sera from patients with suspicion of HIT were investigated for antibodies against PF4/hep complexes in the IgG EIA and for platelet activation ability in the Emo-test HIT Confirm® assay. The diagnostic results of both assays were defined to be true positive or negative depending on the heparin-induced platelet activation (HIPA) test. The larger rhombi represent the cutoffs that are recommended by the manufacturers (OD = 0.3 and %HEPLA = 13%) and the trade cutoff (%HEPLA = 11.9%).

vs. 75.8%) or specificity (75.4% vs. 70.6%). Of note, some HIPA-positive sera induced higher activation in the presence of 100 IU/ml compared to 0.2 IU/ml resulting in %HEPLA below zero (Fig. 4, Supplementary Fig. 3). Such results make the optimisation of test sensitivity using the northwest approach difficult.

Table 3
Results of Emo-test HIT Confirm® assay test in relation to the EIA and HIPA test results.

HIT Confirm® assay	EIA		HIPA	
	Negative (n = 226)	Positive (n = 164)	Negative (n = 357)	Positive (n = 33)
Negative (n = 238)	152	86	230	8
Ambiguous (n = 40)	23	17	38	2
Positive (n = 112)	51	61	89	23

Table 4
Results of a combination of EIA and HIT Confirm® assay.

	HIPA	
	Negative (n = 357)	Positive (n = 33)
Combined positive (n = 78)	53	25
Combined negative (n = 153)	153	0
EIA and HIT Confirm® assay with different results (n = 159)	151	8

3.4. Potential approaches to improve test performance

The use of the Emo-test HIT Confirm® assay is supposed to be associated with an increase in the specificity of laboratory investigations for HIT. Functional assays are normally used in combination with an immunoassay that screens for anti-PF4/hep antibodies. Therefore, we next evaluated the use of a combination between the Emo-test HIT Confirm® assay and the IgG EIA. 78 sera showed reactive results in both assays (EIA > 0.3 and Emo-test HIT Confirm® assay %HEPLA > 9.6), of which 25 (32.1%) tested positive in HIPA (Table 4). When patients were considered to have HIT if the serum revealed positive results in both assays, the specificity of laboratory assessment improved from 63.3% and 75.4% for the IgG EIA to 85.2% for the Emo-test HIT Confirm® assay, respectively. Sensitivity had a slight increase from 69.7% to 75.8% by using the combination of EIA and Emo-test HIT Confirm®

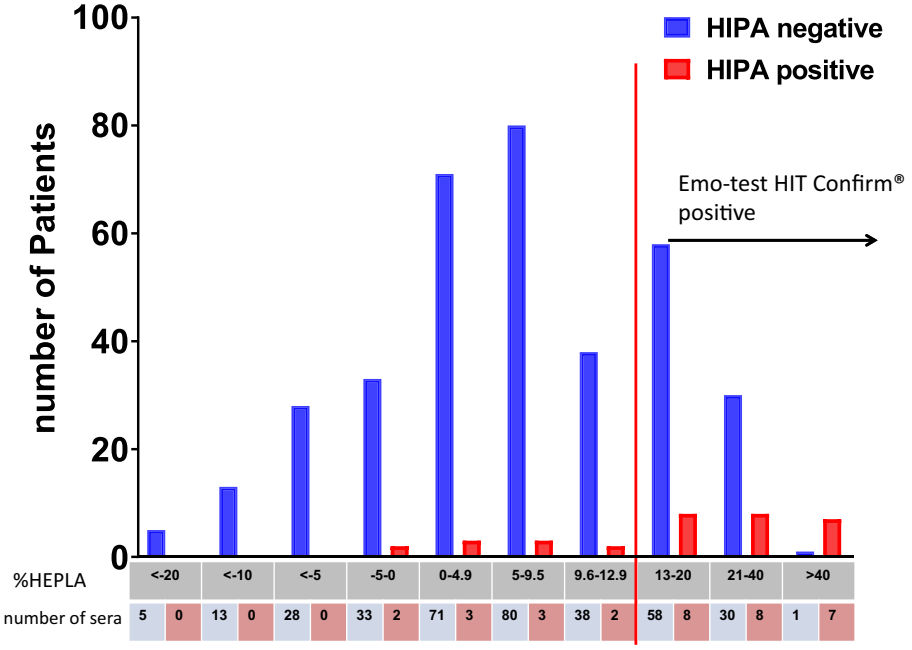

Fig. 4. The distribution of HIT-positive versus HIT-negative status depending on the magnitude of the Emo-test HIT Confirm® assay. Note that the probability of HIT-positive status increases with %HEPLA.

assay but remained lower than in the EIA (100%) (Table 2).

4. Discussion

Immunoassays cannot distinguish between platelet activating and non-platelet activating antibodies [9]. Our study shows that flow cytometer based functional assay, the Emo-test HIT Confirm® assay, could be helpful to reduce the overdiagnosis of HIT that is commonly observed by the immunoassay. However, the use of this assay as a standalone laboratory test had a relatively high rate of false negative results (8/33) in patient samples containing clinically relevant PF4/hep antibodies. A potential approach to overcome this limitation could be the combined use of the Emo-test HIT Confirm® assay and an IgG EIA to confirm HIT. This approach slightly improves the sensitivity (from 69.7% to 75.8%) with a better specificity (from 75.4% to 85.2%). Most importantly, if both tests revealed a positive result then a significantly higher likelihood (5.1) to have HIT was observed.

In our study, the flow cytometer based assay, Emo-test HIT Confirm® assay, was found to have better specificity compared to EIA but lower sensitivity for platelet activating PF4/hep antibodies. Sera from 8 HIT patients revealed negative results in the Emo-test HIT Confirm® assay. Repeated testing with more than one donor showed similar results, excluding donor-related platelet hyporeactivity due to medication intake or Fc gamma receptor IIa non-responsiveness. In addition, titration on HIPA-positive sera was performed to exclude a possible prozone effect which is well described in HLA-antibody testing for example [10]. No positive reactions were detected by testing the diluted sera (data not shown). Of note, some HIPA-positive sera induced strong platelet activation in the presence of 0.3 IU/ml heparin which was not inhibited by higher heparin concentration in the Emo-test HIT Confirm® assay. The heparin-independent platelet activation was still detectable after serum dilution. These observations indicate that the test protocol should be modified to include additional serum incubation in the absence of heparin to verify direct platelet activation by non-IgG serum factors. These false negative results have an important clinical implication. In case of false negative result, a re-exposure or maintaining heparin treatment may cause a rapid onset of HIT or even HIT-induced disseminated intravascular coagulation (DIC) [11]. Therefore, functional assays must have both high sensitivity and specificity to ensure safe anticoagulation treatments. Although the Emo-test HIT Confirm® assay has a better specificity compared to EIA, caution should be considered due to the low test sensitivity. One possibility to avoid false negative results is to use the Emo-test HIT Confirm® assay in combination with EIA and with lower cutoff, i.e. % HEPLA 9.6. The other option for reducing the false negative results might be to test sera with platelets from more than one donor. Finally, a recent study suggests that the addition of exogenous PF4 increases the sensitivity of flow cytometer based assays [12]. The implementation of this step in the commercially available kit could be a potential approach to improve the performance of the Emo-test HIT Confirm® assay. It is well-known that PRP-based assays have frequently false positive reactions as heparin can induce nonspecific aggregation of donor platelets as an effect of proaggregatory factors in the patient serum or plasma [6]. It was suggested that elevated fibrinogen levels or acute-phase reactants in plasma from critically ill patients cause heparin-dependent platelet aggregation especially in functional platelet rich plasma assays. This phenomenon is minimized when using washed platelets [13]. So the high amount of false positive results (89/112) in the Emo-test HIT Confirm® assay underlines this well described phenomenon. Using washing platelets might also increase the specificity of the assay.

Still, these approaches need to be investigated. Recently, a pilot study indicated that external quality assessment for testing of platelet activating antibodies in HIT is feasible [14]. Our study shows an emergent need for eligible evaluation of functional assays using an external quality assessment before commercially launching the test kits.

In conclusion, we show that the Emo-Test HIT Confirm® assay may improve the specificity (versus the EIA) of laboratory investigations of HIT. However, the assay can only be recommended in combination with an immunoassay, and even with a combined approach there remains the issue of a high rate of false negativity. Our observation clearly indicates a need to establish an independent, external quality assessment for commercial diagnostic products to avoid such clinically relevant pitfalls. With regard to the Emo-test HIT Confirm® assay, laboratories should be aware of this issue in order to prevent the risk of false negative results.

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Authorship contributions

K.A. and L.P. performed the experiments and collected the data; L.P., O.H., K.A. and T.B. analyzed the data, interpreted the results and wrote the manuscript. All authors read and approved the manuscript.

Declaration of Competing Interest

This work was partly funded by coachrom diagnostica (Maria Enzersdorf, Austria). All laboratory testing, data analysis and interpretation were performed by the personnel of the Transfusion Medicine of the University of Tuebingen. The sponsor provided comments to the manuscript, but all final decisions regarding manuscript content were made by the authors.

T.B. and K.A. have received lecture honoraria from Aspen Germany, T.B. has also received lecture honoraria from CSL Behring and Stago gGmbH Germany.

All other authors have no relevant conflicts of interest.

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