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Performance characteristics of an automated latex immunoturbidimetric assay [HemosIL® HIT-Ab$_{(PF4-H)}$] for the diagnosis of immune heparin-induced thrombocytopenia

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ABSTRACT

Background: Heparin-induced thrombocytopenia (HIT) is a prothrombotic drug reaction caused by platelet-activating anti-PF4/heparin antibodies. Given time-sensitive treatment considerations, a rapid and accurate laboratory test for HIT antibodies is needed.
Aims: To determine operating characteristics for the HemosIL® HIT-Ab$_{(PF4/H)}$, a rapid, on-demand, fully-automated, latex immunoturbidimetric assay (LIA), for diagnosis of HIT.
Methods: We evaluated LIA sensitivity, specificity, negative (NPV) and positive predictive value (PPV), negative (LR−) and positive likelihood ratio (LR+), using citrated-plasma from 429 patients (prospective cohort study of 4Ts scoring; HIT, $n = 31$), and from consecutive HIT patients ($n = 125$), using reference standard serotonin-release assay (SRA). Comparators included two PF4-dependent enzyme-immunoassays (EIAs). We used stratum-specific likelihood ratios (SSLRs) to determine how differing magnitudes of LIA-positivity influenced post-test probability of HIT.
Results: LIA operating characteristics were: sensitivity = 97.4% (152/156); specificity = 94.0% (374/398); PPV = 55.6% (30/54); and NPV = 99.7% (374/375). At manufacturers' cutoffs, LIA specificity and PPV were superior to the EIAs. Although a negative LIA pointed strongly against HIT (LR−, 0.034), the post-test probability was ~2% with high 4Ts score. The LIA's LR+ was high (16.0), with SSLRs rising substantially with greater LIA-positivity: 5.7 (1.0–4.9 U/mL), 31 (5.0–15.9 U/mL), and 128 (≥16 U/mL). A LIA-positive result (at 1.0 cutoff) indicated at least 24% HIT probability (low 4Ts score), rising to 90% with high 4Ts score.
Conclusions: Although approximately 1 in 40 SRA-positive patients tested LIA-negative, the LIA's high NPV and PPV indicate that this rapid assay is useful for the diagnostic evaluation of HIT, including in low pre-test situations.

1. Introduction

Heparin-induced thrombocytopenia (HIT) is an antibody-mediated prothrombotic drug reaction viewed as a clinical-pathological disorder, i.e., the diagnosis is based upon a compatible clinical picture in a patient whose blood contains platelet-activating antibodies that recognize complexes of platelet factor 4 (PF4) bound to heparin or certain other polyanions [1–3]. PF4-dependent immunoassays, especially enzyme-immunoassays (EIAs), are widely used for laboratory detection of anti-PF4/heparin antibodies [4,5]. Advantages of EIAs include their commercial availability, high sensitivity (98–99%) for detecting pathological (platelet-activating) HIT antibodies [5], and semi-quantitative performance characteristics, i.e., higher optical densities (ODs) predict greater likelihood of HIT antibodies being present [6,7]. Disadvantages of EIAs include their relatively low diagnostic specificity (compared with platelet activation assays) [8,9] and usual testing of samples in batches, frequently resulting in a 6 to 24 h (or longer) delay in obtaining test results.

1.1. Latex immunoturbidimetric assay

Recently, an instrumentation-based immunoassay, HemosIL® HIT-Ab$_{(PF4-H)}$ (Instrumentation Laboratory, Bedford, MA), was cleared by the United States Food and Drug Administration for detection of HIT antibodies in citrated plasma (not serum). This is the first fully-automated assay developed for HIT diagnosis using a commercially-available

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coagulation instrument (hemostasis testing system). Classified as a latex immunoturbidimetric assay (LIA), patient samples are tested on-demand, with a result available within 20 min [10,11]. As this assay is fully-automated and standardized with a monoclonal antibody calibrator, the LIA offers the prospect for comparable test results to be obtained in different laboratories.

Although the LIA is classified as an immunoassay, it differs from most other immunoassays, such as EIAs, in that the presence of PF4/heparin-reactive antibodies within patient plasma results in inhibition of particle agglutination through competition with a HIT-mimicking monoclonal antibody. Accordingly, the LIA has been called a "functionalized immunoassay" [12], which in theory suggests that it might provide diagnostic specificity that is intermediate between the highly specific functional assays (e.g., serotonin-release assay [SRA]) and the less specific PF4-dependent EIAs, a conjecture which our study aimed to address.

1.2. Study aims

The purpose of our study was to determine the performance characteristics of the LIA using plasma samples from a previously published prospective cohort study [13] and a large case-series of consecutive HIT-positive patients, with all diagnoses of HIT confirmed by SRA. We were particularly interested in assessing whether differences in the magnitude of LIA-positive results influenced probability of HIT, as well as its severity (frequency of HIT-associated thrombosis, platelet count nadirs).

2. Materials and methods

2.1. Latex immunoturbidimetric assay (LIA)

The LIA (Fig. 1) makes use of a HIT-mimicking (platelet-activating) anti-PF4/heparin murine monoclonal antibody (MAb), KKO [14], which is coated onto polystyrene latex nanoparticles; the particles agglutinate when mixed with complexes of PF4/polyvinylsulfonate (PVS), which results in higher absorbance (lower light transmission). Addition of negative (non-HIT antibody-containing) plasma has no (or minimal) inhibitory effect on particle agglutination (Fig. 1, upper-third). In contrast, inhibition of particle agglutination occurs when free KKO itself (positive calibrator or positive control) is added along with PF4/PVS complexes (Fig. 1, middle-third). Similarly, addition of patient plasma containing PF4/heparin-reactive antibodies (of any immunoglobulin class) that bind to the same (or similar) antigen sites, inhibits particle agglutination (Fig. 1, lower-third). Thus, no or minimal increase in absorbance by patient sample indicates a positive test result (Fig. 1, lower-third). The instrument automatically calculates the inhibitory antibody concentration by interpolation in the calibration curve where the concentration of antibodies within the plasma sample is inversely proportional to the increase in absorbance. The concentration is expressed in arbitrary units per milliliter (U/mL), with values equal to or >1.0 U/mL considered positive. This cutoff was determined by the manufacturer through expected value studies with healthy donors and heparin-exposed patients, as well as receiver operating characteristic (ROC) curve analysis versus SRA.

The LIA [HemosIL® HIT-Ab$_{(PF4-H)}$] was performed using the ACL TOP® CTS 500 instrument (Instrumentation Laboratory, Bedford, MA) following the manufacturer's recommendations. The test range of the LIA is from 0 to 5.7 U/mL; when positive results occur above this range, the instrument automatically reruns the test, after making an on-board 1/4 dilution, and correcting the result for the dilution factor. This expands the measuring range to 16.0 U/mL when the automated on-board dilution is run (note: 16.0 U/mL, rather than 22.8 U/mL [i.e., 4 × 5.7 U/mL]) is used, to better meet the linearity expectations). However, for this study, our instrument was set up to perform additional automated on-board dilutions (two-fold, up to 1/32), which allowed for a

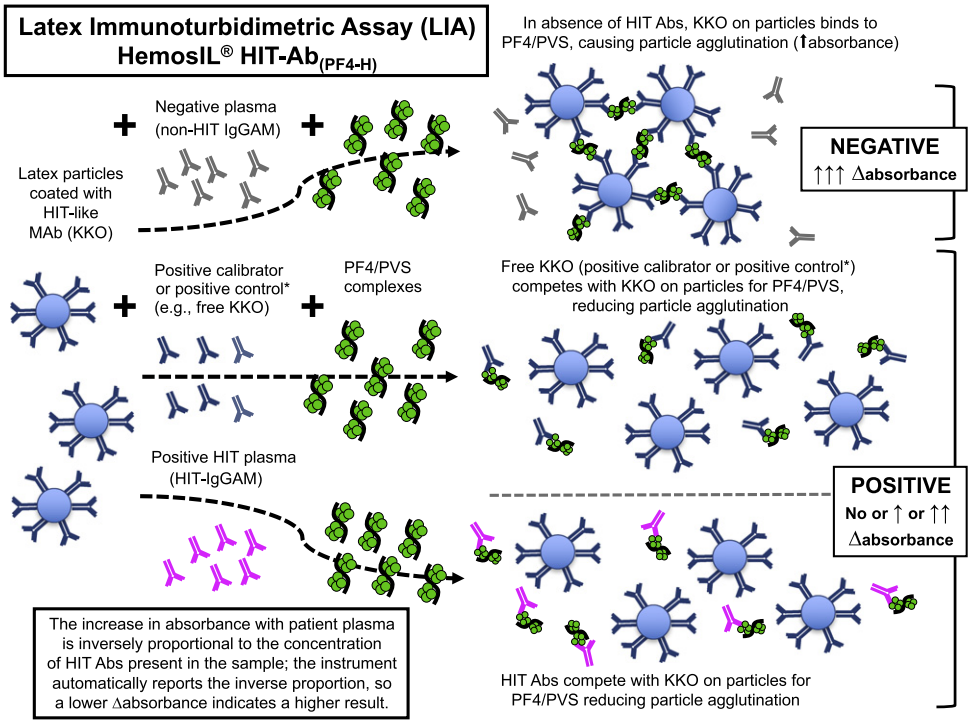

Fig. 1. Schematic representation of the latex immunoturbidimetric assay (LIA) for detection of HIT antibodies. Latex nanoparticles coated with a HIT-like monoclonal antibody (KKO), upon addition of PF4/PVS complexes, will become maximally agglutinated if tested with a blood sample that contains no PF4/heparin-reactive antibodies (upper-third of figure). In contrast, inhibition of particle agglutination occurs if free KKO (positive calibrator [used to define the calibration curve, expressed in arbitrary U/mL] or positive control) is added before PF4/PVS (middle-third). HIT antibodies within patient plasma will also inhibit (to varying degrees) particle agglutination triggered by PF4/PVS (lower-third). The instrument automatically interpolates delta (Δ) absorbance with the calibration curve where the concentration of antibodies in the sample is inversely proportional to the degree of agglutination. *Positive control can be KKO or another HIT-like monoclonal antibody. Abbr.: Δ, delta (change); HIT, heparin-induced thrombocytopenia; HIT Abs, HIT antibodies; IgGAM, antibodies of any of the immunoglobulin classes, IgG, IgA, and IgM; KKO, name of HIT-like monoclonal antibody used in the LIA; MAb, monoclonal antibody; PF4/PVS, platelet factor 4/polyvinylsulfonate.

(calculated) positive result as high as 182.4 U/mL. For samples that underwent automated on-board dilutions, and which therefore yielded two results (e.g., readable results at 1/8 and 1/16 dilutions), the result obtained at the higher dilution was accepted, since (per the manufacturer) this result corresponds better to the acceptable linear range.

2.2. Plasma samples tested

Anti-PF4/heparin antibodies are stable during long-term storage at −70 °C, which allows for frozen serum/plasma from well-characterized heparin-exposed patients to be used in the assessment of novel HIT assays [15]. For evaluation of the LIA, we used plasma samples available from two sources:

(a) $n = 429$ residual plasma samples from a recently completed prospective cohort study [13] that evaluated the 4Ts pretest scoring system [16,17] together with a rapid assay for HIT antibodies, the particle gel immunoassay (PaGIA; DiaMed GmbH, Cressier, Switzerland) [18,19]. The original trial reported on 526 patients, of whom 429 patients had plasma available for testing in the LIA. Of the 429 plasma samples available, 31 patients were SRA-positive and 398 patients were SRA-negative.
(b) $n = 125$ residual plasma samples (case-series) from consecutive patients (other than those enrolled in the prospective cohort study) diagnosed as having HIT at a single centre (Hamilton General Hospital), from February 1999 to September 2016. All 125 patients were SRA-positive and had a 4Ts score of greater than or equal to 4 points, as determined by one of the investigators (T.E.W.) based on information available in the medical record.

2.3. Additional assays for HIT antibodies

For all plasma samples evaluated, results were available for the following tests: (a) SRA [20,21]; (b) in-house IgG-specific anti-PF4/heparin EIA [22], hereafter called EIA-IgG; (c) commercial polyspecific anti-PF4/PVS EIA (LIFECODES PF4 Enhanced®, Immucor GTI Diagnostics, Inc., Waukesha, WI, USA), hereafter called EIA-IgGAM [15]; for the 429 plasma samples evaluated in the prospective cohort study, PaGIA results were also available [13].

2.4. Definition of HIT-positive patients

We regarded as "HIT-positive" those patients with at least one blood sample that met the following serological criteria for HIT antibodies [2]: (a) SRA-positive (defined as ≥50% serotonin-release at the mean of two pharmacologic heparin concentrations (0.1 and 0.3 IU/mL unfractionated heparin [UFH]), and with control positive and negative reactions working as expected; and (b) EIA-IgG-positive (≥0.45 OD units). SRA control conditions included: inhibition at high heparin (100 U/mL) and in presence of 0.1 or 0.3 IU/mL UFH and Fc receptor-blocking monoclonal antibody, IV.3. "HIT-negative" referred to all other reaction patterns.

For the 125 consecutive patients with HIT from the Hamilton General Hospital, all patients had a 4Ts score of at least 4 points (scored by T.E.W.), which together with the requirement for a positive SRA, supported their designation as "HIT-positive". Further, and consistent with our definition used for the 4Ts trial [13], all 31 SRA-positive patients from that prospective cohort study were also classified as "HIT-positive", even though a few of the SRA-positive patients in that trial had been scored in real-time as low probability for HIT. However, as previously reported [13], these patients were either incorrectly scored or had mitigating factors that made scoring difficult (e.g., outpatients with few available platelet counts, concomitant non-HIT thrombocytopenic disorders). Accordingly, we believe that all 31 SRA-positive patients did have a clinical course consistent with a diagnosis of HIT, i.e., we did not identify any false-positive SRA results among the 31 patients judged "HIT-positive" in the 4Ts trial for whom plasma was available for inclusion in our current study evaluating the LIA. Accordingly, in this report, "SRA-positive" status is synonymous with "HIT-positive" status.

2.5. Definitions: thrombocytopenia and HIT-associated thrombosis

For each patient, the lowest platelet count recorded during the episode of HIT was identified, and called the "nadir" value. The percent platelet count fall was calculated based on the peak platelet count that preceded the HIT-related platelet count decline. HIT-associated thrombosis included any objectively-proven thrombosis that occurred during or within 30 days of an episode of HIT (specifically, any thrombosis that preceded HIT was *not* included in evaluation of HIT-related thrombosis) [23].

2.6. Further evaluation of samples yielding unexpected LIA-negative results

For plasma samples that were classified as HIT-positive but which unexpectedly tested LIA-negative (<1.0 U/mL), we performed the following systematic evaluation:

(a) Visual check of the plasma for sample integrity (e.g., hemolysis, icterus, turbidity, clots);
(b) Retest of the original plasma aliquot in the EIA-IgG, EIA-IgGAM, and SRA, to determine whether the sample still reacted as expected in the standard HIT assays;
(c) Performed the LIA on other SRA-positive plasma samples obtained on different days from the same patient, to examine whether the unexpected LIA-negative status was a consistent patient-related phenomenon, or whether preceding or subsequent samples for a given patient became positive (e.g., potentially because of changing antibody levels or other factors). For one of the HIT-positive patients with (unexpected) LIA-negative results, we repeated the LIA by testing the patient plasma with manual dilutions (made using normal plasma), 1/5, 1/10, 1/50, and 1/100, which we performed in case a false-negative LIA result could have resulted from unusually high-titer HIT antibodies; and,
(d) We "deheparinized" the plasma samples, as follows: 500 μL plasma was incubated with 35 mg of heparin adsorbent (Sigma Diagnostics, St. Louis, MO) for 10 min; following centrifugation, the deheparinized plasma was separated from the adsorbent for testing. This was performed to evaluate whether residual heparin might cause false-negative LIA results. As a positive control, we used a HIT plasma sample that tested LIA-positive.

2.7. Evaluation of a semiquantitative role for LIA-positive results: stratum-specific likelihood ratios

It is known that increasing positivity of the EIAs (per OD values) predicts for a greater likelihood of a positive SRA and, hence, HIT-positive status [6,7]. To determine whether this relationship also holds for the LIA, we analyzed various ranges ("strata") of a positive LIA result, grouped as follows: negative (<1.0 U/mL) and three different ranges of positive results: weak-positive (1.0–4.9 U/mL); moderate-positive (5.0–15.9 U/mL), and strong-positive (≥16.0 U/mL). These groups were chosen for several reasons, including: (i) the instrument performs an automatic on-board dilution for results >5.7 U/mL (i.e., marginally higher than our first cutoff of 5.0 U/mL); (ii) the standard 1/4 automated on-board dilution permits an acceptable linear range up to 16.0 U/mL [11]; and (iii) each of the three groups contained approximately one-third of the LIA-positive/HIT-positive subjects identified in our study. For each group, we determined the stratum-specific likelihood ratios (SSLRs), as described [24].

2.8. Analysis plan and statistics

The operating characteristics for the LIA, PaGIA, EIA-IgG, and EIA-IgGAM were determined using the 429-patient plasma set, as this study reflected assessment of heparin-exposed patients with clinically-suspected HIT, and therefore includes "background" antibody positivity without necessarily having clinical HIT. Assay operating characteristics that we evaluated included: sensitivity, specificity, PPV, and NPV (with 95% CIs shown as exact Clopper-Pearson 95% CIs), and LR+ and LR− (with 95% CIs calculated per Altman et al. [25]). We also determined test sensitivity by including the 125 consecutive SRA-positive patients (case-series). Comparisons between assays were performed using the Chi-square test, with Delong's test used to compare the area-under-the-curve (AUC) for the ROC curves. Pretest probabilities for the samples from the prospective cohort study were determined by the frequency of SRA-positive status within each 4Ts score category (as assessed in real time by the clinicians participating in the 4Ts trial); post-test probabilities were calculated according to Bayes' theorem by multiplying the pretest odds by the LR+ (at the manufacturer's single cutoff) as well at different SSLRs determined in the current study for the LIA.

The Kruskal-Wallis rank sum test was used to compare platelet count median values among different assay-positive subgroups, with the Wilcoxon rank sum test used for comparing 2 groups. Correlations between two platelet count indicators of HIT severity (platelet count nadirs, percent platelet count falls) and quantitative assay results were examined using Spearman's rank correlation.

2.9. Role of sponsor

The study was funded by Instrumentation Laboratory. However, the sponsor had no role in the design of the study, performance of the assays, the analysis of the results obtained, or in the writing of the paper.

3. Results

3.1. Characteristics of the HIT-positive patients

The 156 HIT-positive patients (31 in the prospective cohort study; 125 in the single-hospital consecutive case-series patient cohort) comprised 74 males and 82 females, of median age 70 years (IQR, 62, 78; range, 29–94). One-quarter ($n = 39$) of the patients were classified as medical, and the remaining 117 (75.0%) were surgical (there were no pediatric or obstetrical patient cases). HIT-associated thrombosis was diagnosed in 61.5% (96/156) of the patients. The median platelet count nadir was 60×10^9/L (IQR, 32, 84; range, 2, 279), and the median percent platelet count fall (from the pre-HIT peak platelet count) was 70.8% (IQR, 56.5, 84.8, range, 26.7, 97.0). The proportion of females in the HIT-positive group was 52.6% versus 47.4% in the non-HIT population ($p = 0.30$).

3.2. Test performance at manufacturer's cutoff: LIA vs other immunoassays

3.2.1. Prospective cohort study samples

The proportion of patients who were HIT-positive (i.e., SRA-positive) for negative and positive results by LIA, at the manufacturer's cutoff (1.0 U/mL), as well as for the 3 other immunoassays, are summarized in Table 1. Shown are six parameters: sensitivity (Se), specificity (Sp), PPV, NPV, LR+, and LR−. Fig. 2 [see also Graphical Abstract] shows the distribution of positive and negative results, for both the LIA and the commercial polyspecific EIA, at the manufacturers' cutoffs, for the 31 HIT-positive and 398 HIT-negative samples, showing also how the LR− and LR+ values were calculated. In addition, Fig. 3 shows the ROC curves for the LIA and for the two EIAs (EIA-IgG, EIA-IgGAM), including the data point corresponding to the sensitivity/specificity tradeoff at the standard (manufacturer's) cutoff; for a fourth assay (PaGIA), the single data point corresponding to the sensitivity/specificity tradeoff is shown (triangle), without a corresponding ROC curve (as the PaGIA was interpreted as either "positive" or "negative" using a single undiluted sample specimen [13]). As summarized in Table 1, at the manufacturers' cutoffs, the specificity and PPV were highest for the LIA (94.0% and 55.6%, respectively) versus the corresponding values for the PaGIA (89.4% and 42.5%, respectively), the EIA-IgG (85.2% and 34.4%, respectively) and for the EIA-IgGAM (83.2% and 31.6%, respectively). However, sensitivity for the LIA appeared lower for the LIA versus the 3 other immunoassays (96.8% vs 100%; $p = 0.3193$).

Table 1
Operating characteristics of the LIA and 3 other immunoassays at manufacturers' cutoff.

	LIA (95% CI) --	PaGIA[a] (95% CI) *p* value (vs. LIA)	EIA-IgG (95% CI) *p* value (vs. LIA)	EIA-IgGAM (95% CI) *p* value (vs. LIA)
Se	30/31 = 96.8% (83.3%, 99.9%) --	31/31 = 100% (88.8%, 100%) $p = 0.3193$	31/31 = 100% (88.8%, 100%) $p = 0.3193$	31/31 = 100% (88.8%, 100%) $p = 0.3193$
Sp	374/398 = 94.0% (91.2%, 96.1%) --	354/396 = 89.4% (85.9%, 92.3%) $p = 0.0189$	339/398 = 85.2% (81.3%, 88.5%) $p < 0.0001$	331/398 = 83.2% (78.9%, 86.5%) $p < 0.0001$
PPV	30/54 = 55.6% (41.4%, 69.1%) --	31/73 = 42.5% (31.0%, 54.6%) $p = 0.1457$	31/90 = 34.4% (24.7%, 45.2%) $p = 0.013$	31/98 = 31.6% (23.3%, 41.4%) $p = 0.004$
NPV	374/375 = 99.7% (98.5%, 100%) --	354/354 = 100% (99.0%, 100%) $p = 0.3027$	339/339 = 100% (98.9%, 100%) $p = 0.3132$	331/331 = 100% (98.9%, 100%) $p = 0.3190$
LR+	16.05 (10.8, 23.8)	9.43 (7.1, 12.6)	6.75 (5.3, 8.5)	5.94 (4.7, 7.3)
LR−	0.034 (0.005, 0.24)	0.0 (undefined)	0.0 (undefined)	0.0 (undefined)
AUC	98.6% (97.0%, 99.5%) --	Not applicable	99.5% (98.3%, 99.9%) $p = 0.0576$	99.1% (97.6%, 99.7%) $p = 0.3617$

The table presents the results of 4 different PF4-dependent immunoassays, tested on 429 patients (31 SRA-positive) from a prospective study of the 4Ts scoring system [13].
Abbr.: AUC, area-under-the-curve; 95% CI, 95% confidence interval; EIA-IgG, enzyme-immunoassay (IgG-specific); EIA-IgGAM, enzyme-immunoassay (polyspecific); LIA, latex immunoturbidimetric assay; LR+, positive likelihood ratio; LR−, negative likelihood ratio; NPV, negative predictive value; PaGIA, particle gel immunoassay; PPV, positive predictive value; Se, sensitivity; Sp, specificity.

[a] 2 PaGIA "indeterminate" test results were not included in the analysis.

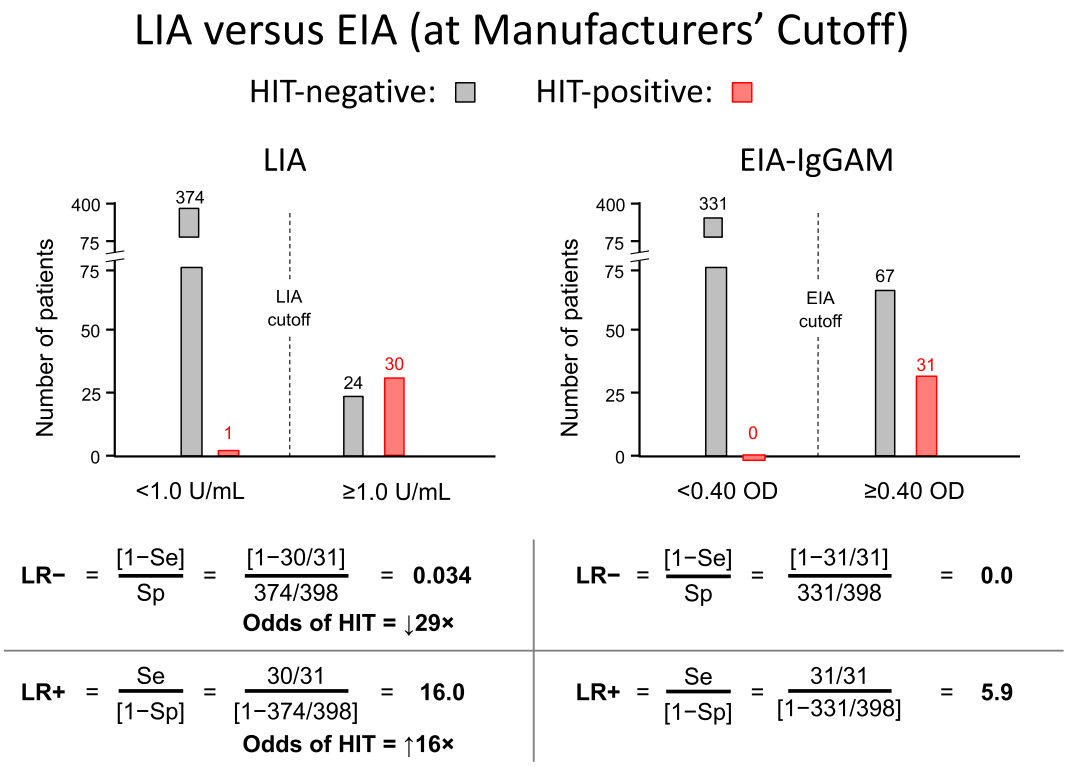

Fig. 2. Distribution of HIT-positive versus HIT-negative patients, with corresponding LR− and LR+ values, for negative and positive LIA (Left-panel) and EIA-IgGAM (Right-panel) test results (at manufacturers' cutoffs) in the 429-patient cohort. The calculations for the LR− and LR+ values are shown. Thus, for the LIA, a negative test result indicates an approximate 29× lower odds of HIT (LR− = 0.034) whereas a positive test result indicates an approximate 16× higher odds of HIT.

3.2.2. Consecutive case-series of HIT-positive plasma samples: LIA sensitivity

When the consecutive cases of HIT-positive samples were analyzed using the LIA, 122/125 patients tested positive. Combining these data with the 31 patients identified in the prospective cohort study, the LIA sensitivity was 152/156 = 97.4% (95% CI, 93.6%–99.3%).

3.3. Further evaluation of false-negative LIA test results

A total of 4 HIT-positive patients tested LIA-negative. No interfering substance was evident by visual inspection of the samples. Repeat testing by LIA showed persistent negative results. Table 2 summarizes the 4

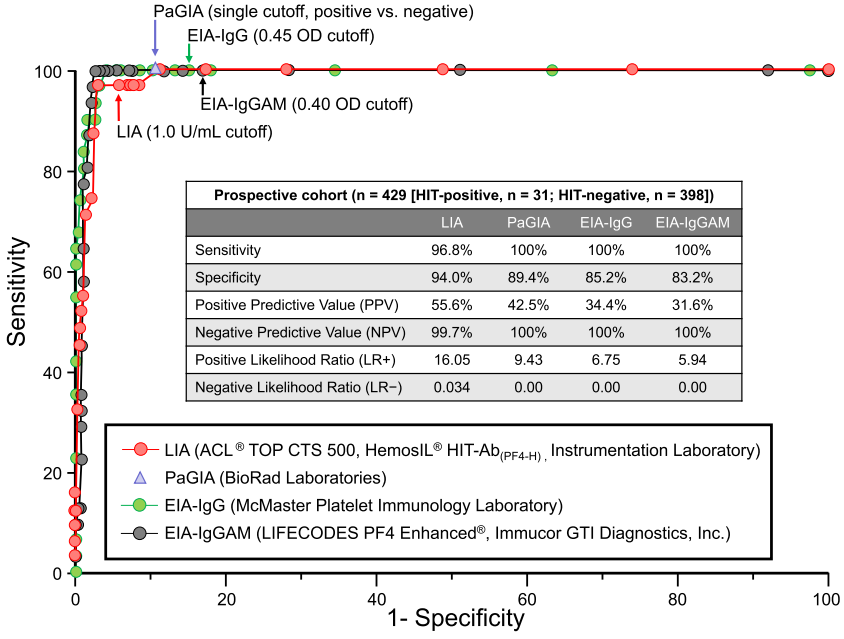

Fig. 3. Receiver operating characteristic (ROC) curves for 4 assays that detect HIT antibodies: LIA, EIA-IgG, EIA-IgGAM, and PaGIA. The 4 arrows indicate for each of the 4 assays the sensitivity-specificity profile at the manufacturer's recommended cutoff. The Table inset compares the 4 assays with respect to 6 parameters (at the manufacturers' cutoffs): sensitivity, specificity, PPV, NPV, LR+, and LR−. Among the 4 assays, the LIA has the highest specificity and PPV. Abbr.: EIA-IgG, enzyme-immunoassay (IgG-specific); EIA-IgGAM, enzyme-immunoassay (polyspecific); HIT, heparin-induced thrombocytopenia; LIA, latex immunoturbidimetric assay; OD, optical density; PaGIA, particle gel immunoassay; U, units.

Table 2
Further investigations of 4 false-negative LIA test results.

Patient (age, sex)	4Ts	LIA (U/mL)	LIA-D (U/mL)	Repeat results in standard HIT assays			Number of other plasma samples tested in the LIA: days before (−) and days after (+) date of original plasma sample tested (LIA results of additional plasma samples tested)
				EIA-IgG (OD units)	EIA-IgGAM (OD units)	SRA	
62, F	8	0.5	NSQ	2.05	2.12	Weak	n = 8: −1 to +7 (LIA, 0.3–0.9 U/mL)
80, M	8	0.0[a]	0.0	2.66	2.24	Strong	n = 7: +2 to +64 (median, +22); (LIA, all 0.0 U/mL)
62, M	8	0.5	0.3	1.97	1.75	Strong	n = 2: +4, +8 (LIA, 0.5, 0.4 U/mL)
74, F	5	0.9	0.7	1.73	1.49	Weak	n = 2: −8, +6 (LIA, 0.6, 0.3 U/mL)

In this table, a "weak" SRA refers to a patient sample that tested variably in the SRA with different donors; however, percent serotonin-release of >50% (mean at 0.1 and 0.3 IU/ml UFH) was seen with at least one platelet donor.
Abbr.: 4Ts, Four T's scoring system; D, deheparinized: EIA, enzyme-immunoassay; F, female; LIA, latex immunoturbidimetric assay; M, male; neg, negative; NSQ, not sufficient quantity; OD, optical density; SRA, serotonin-release assay; U, units.
[a] For the patient, we also tested manually-diluted plasma (diluted using normal plasma), 1/5, 1/10, 1/50, and 1/100, all of which a result in the LIA of 0.0 U/mL.

patient cases, including results of repeat testing in the standard HIT assays. Of note, 19 additional SRA-positive plasma samples obtained from these 4 patients also tested LIA-negative, suggesting that lack of LIA reactivity may be an inherent feature of occasional HIT-positive plasma samples. Residual heparin did not appear to be an explanation for false-negative LIA, as "deheparinization" did not convert LIA-negative to LIA-positive status (note: deheparinization of the positive control yielded the identical LIA-positive result).

3.4. Stratum-specific likelihood ratio: assessing the magnitude of a positive LIA result

Fig. 4 shows the distribution of negative and positive LIA results (expressed in U/mL) for the 429 patient prospective cohort (top-panel), as well as for the 125 HIT-positive case-series patients (bottom-panel), for the following four LIA test result groups: <1.0 (negative); 1.0 to 4.9 U/mL (weak-positive); 5.0 to 15.9 U/mL (moderate-positive); and ≥16.0 U/mL (strong-positive). Fig. 5 shows the corresponding EIA-IgGAM results, showing the manufacturer's cutoff (0.40 OD units), as well as other cutoffs (1.0, 2.0) typically used in assessing EIA operating characteristics [6]. Both Figs. 4 and 5 show that the proportion of HIT-positive patients increased with stronger LIA and EIA-IgGAM results.

Table 3 shows the stratum-specific likelihood ratios (SSLRs) for negative results and for the three positive strata; for both the LIA and the EIA-IgGAM, the magnitude of a positive test result influences the likelihood ratio, as indicated for three different strata of positive results.

3.5. LIA quantitative test results, thrombosis risk, and magnitude of thrombocytopenia

Table 4 compares frequency of HIT-associated thrombosis and the severity of thrombocytopenia (judged by nadir values) for 3 different groups of LIA-positivity: 1.0–4.9 U/mL (weak-positive); 5.0–15.9 U/mL (moderate-positive); and ≥16.0 U/mL (strong-positive). The table also shows the corresponding data for patients classified by strength of reactivity in the EIA-IgGAM, with three OD ranges chosen (1.0–2.09; 2.10–2.69; and ≥2.70) to create group sizes approximately the same as those determined by LIA reactivity (note: no HIT-positive plasma yielded an OD value < 1.0).

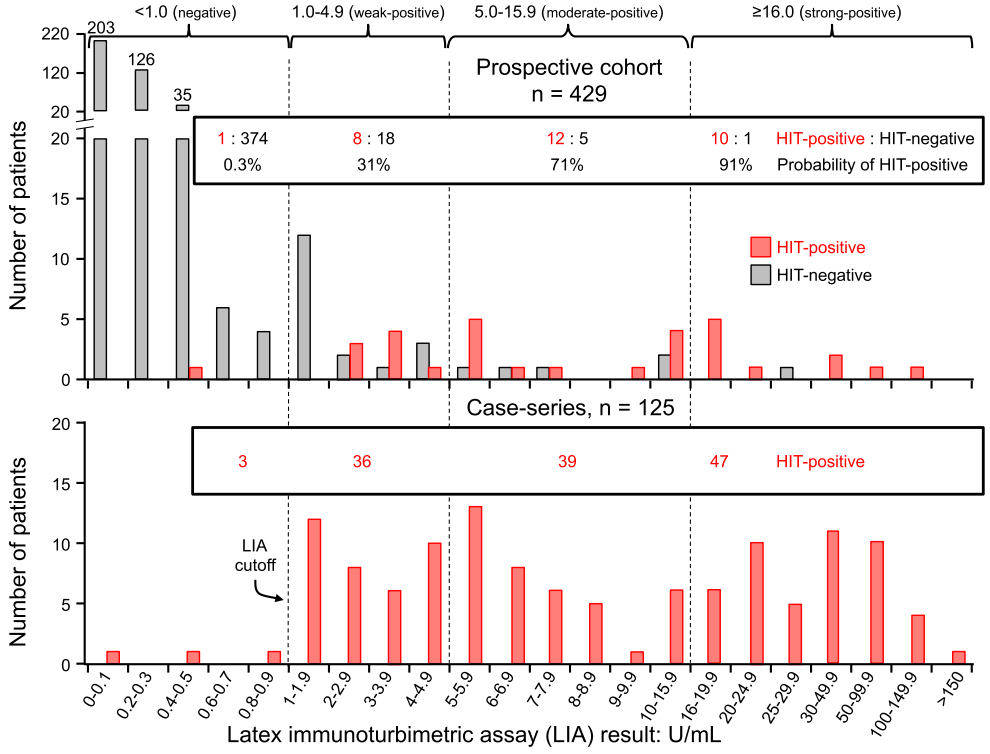

Fig. 4. Probability of HIT-positive versus HIT-negative status per magnitude of LIA-positive result. Upper-panel. For the prospective cohort ($n = 429$), increasing magnitude of a LIA-positive result increased the probability of HIT-positive status. Lower-panel. For the HIT case-series, the distribution of LIA results is shown. Note: the mean OD (±SD) in the EIA-IgGAM for the 10 LIA-negative samples with high LIA-negative result (0.6–0.9 U/mL) was significantly greater than for the remaining 364 LIA-negative samples (≤0.5 U/mL): 0.374 ± 0.202 vs. 0.248 ± 0.197 units ($p = 0.048$). Abbr.: HIT, heparin-induced thrombocytopenia; U, units.

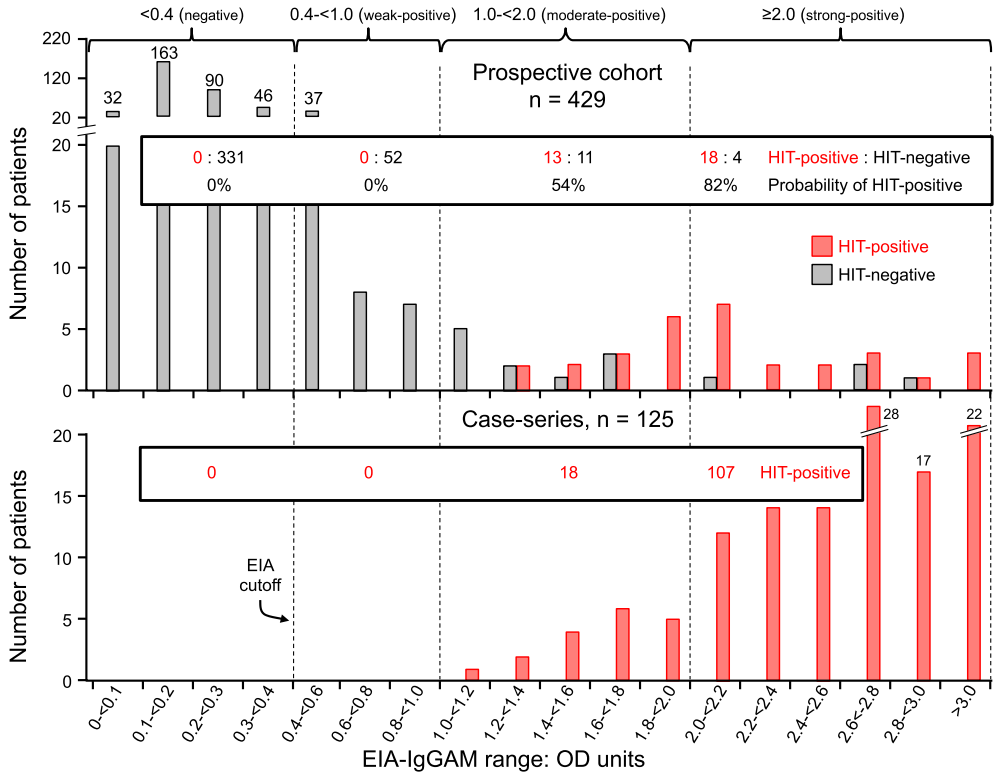

Fig. 5. Probability of HIT-positive versus HIT-negative status per magnitude of the EIA-IgGAM result. Upper-panel. For the prospective cohort ($n = 429$), increasing magnitude of the EIA-IgGAM result increased the probability of HIT-positive status. Lower-panel. For the HIT case-series, the distribution of the EIA-IgGAM results is shown. Abbr.: EIA, enzyme-immunoassay (polyspecific); HIT, heparin-induced thrombocytopenia; OD, optical density.

We found no difference in frequency of HIT-associated thrombosis between the three patient groups, either for the LIA or for the EIA-IgGAM. The overall frequency of HIT-associated thrombosis (61.2%) in the LIA-positive HIT patients was similar whether the LIA-positive result was weak-, moderate- or strong-positive (52.3%, 70.6%, and 59.6%, respectively). Similar findings were seen with the EIA-IgGAM) (Table 4). Thus, at least 50% of patients in each group developed HIT-associated thrombosis, irrespective of how weak or strong the positive LIA or EIA-IgGAM result was.

In contrast, the median platelet count nadir values differed significantly among the 3 LIA-positive groups; in particular, the platelet count nadir was significantly lower for patients with moderate- or strong-positive LIA results versus patients with weak-positive results: median platelet count nadir $= 51 \times 10^9$/L (IQR, 28, 79) versus 74×10^9/L (IQR, 56, 98), respectively ($p = 0.0017$). Overall, a quantitatively higher LIA result correlated with a lower platelet count nadir (correlation estimate, −0.24; $p = 0.0023$), with no significant correlation for EIA-IgGAM values (correlation estimate, −0.12; $p = 0.122$). In parallel, a quantitatively higher LIA result correlated with a greater percent platelet count fall (correlation estimate, 0.17; $p = 0.0381$), whereas higher EIA-IgGAM results did not (correlation estimate, 0.12; $p = 0.1409$).

Table 3
Stratum-specific likelihood ratios (SSLRs): analysis of 429-patient cohort.

LIA				
Result stratum	<1.0 U/mL (negative)	1.0–4.9 U/mL (weak-positive)	5.0–15.9 U/mL (moderate-positive)	≥16.0 U/mL (strong-positive)
HIT-positive, n	1	8	12	10
HIT-negative, n	374	18	5	1
SSLR[a]	0.034	5.7	31	128
EIA-IgGAM				
Result stratum	<0.40 (negative)	0.40–0.99 (weak-positive)	1.00–1.99 (moderate-positive)	≥2.00 (strong-positive)
HIT-positive, *n*	0	0	13	18
HIT-negative, *n*	331	52	11	4
SSLR[a]	0	0	15	58

[a] Stratum-specific likelihood ratio (SSLR) is calculated using the formula [24]:

$$\frac{\textit{Number of SRA-positive patients within the test result stratum}}{\textit{Total number of SRA-positive patients in the study}} \div \frac{\textit{Number of SRA-negative patients within the test result stratum}}{\textit{Total number of SRA-negative patients in the study}}$$

Table 4
Quantitative test results, thrombosis risk, and platelet count nadirs for HIT-positive patients.

	LIA (U/mL)[a]			EIA-IgGAM (OD units)		
	1.0–4.9	5.0–15.9	≥16.0	1.00 -2.09	2.10 -2.69	≥2.70
	$n = 44$ (29%)	$n = 51$ (34%)	$n = 57$ (37%)	$n = 42$ (27%)	$n = 52$ (33%)	$n = 62$ (40%)
HIT-T, frequency (%)	23/44 (52.3%)	36/51 (70.6%)	34/57 (59.6%)	25/42 (59.5%)	31/52 (59.6%)	40/62 (64.5%)
p[b]		$p = 0.5287$			$p = 0.8280$	
Platelet nadir: Median (Q1, Q3)	74 (56, 98)	49 (28, 77)	53 (28, 80)	71 (41, 89)	59 (33, 83)	53 (28, 80)
p[c]		$p = 0.0064$			$p = 0.3741$	

Abbr.: EIA-IgGAM, enzyme-immunoassay (polyspecific); HIT, heparin-induced thrombocytopenia; HIT-T, HIT-associated thrombosis; LIA, latex immunoturbidimetric assay; Q1, value separating first and second quartiles; Q3, value separating third and fourth quartiles; U, units.

[a] The 4 LIA-negative, HIT-positive patients were excluded from the analysis (as they did not fall into any of the 3 LIA-positive groups).

[b] The thrombosis risk was no different among patients with higher values (U/mL) in the LIA (odds ratio = 0.997 [95% CI, 0.988, 1.006]) or with higher OD values in the EIA-IgGAM (odds ratio = 1.06 [95% CI, 0.64, 1.78] by logistic regression).

[c] Difference between median values among 3 groups (Kruskal-Wallis rank sum test); further, there was a significant difference between the weak-positive group (1.0–4.9 U/mL) versus the combined moderate-/strong-positive LIA (≥5.0 U/mL) groups ($p = 0.0017$ using the Wilcoxon rank sum test).

3.6. Bayesian analysis: incorporating LIA results with pretest probability

Likelihood ratios can be used to either increase or decrease the post-test probability of a disease [26]. In the prospective cohort, the probability of SRA-positive status (and, hence, a probable diagnosis of HIT) was associated with the 4Ts score, as follows: low 4Ts (0 to 3 points), 1.9%; intermediate 4Ts score (4 or 5 points), 6.7%; and high 4Ts score (6 to 8 points), 36.6% [13]. To show how a LIA-negative result, or a LIA-positive result (classified per strength of LIA-positive result, as weak-, moderate-, or strong-positive, defined above), changes the estimated pretest probability of HIT to a post-test probability, is shown in Table 5. The data show that a negative LIA is associated with a post-test probability of HIT of <2%, even in a patient with a high 4Ts score. In contrast, even a weak-positive LIA result (with a low 4Ts score) is associated with at least a 10% probability of a positive SRA, and hence a likely diagnosis of HIT, rising to 29% and 77%, respectively, for patients with intermediate and high 4Ts scores. Of note, a strong-positive LIA was associated with a substantial risk of a positive SRA (at least 70%) irrespective of the 4Ts score.

4. Discussion

This study showed that the LIA, a "functionalized immunoassay" that detects HIT antibodies based on their ability to competitively inhibit agglutination of HIT-like MAb-bearing particles, has diagnostic specificity and PPV that is higher than that of two EIAs and the PaGIA, at the manufacturers' recommended cutoffs. The PPV of 55.6% for a positive LIA result obtained in a patient with clinically suspected HIT indicates an approximate 50% probability of the patient having a true diagnosis of HIT. In contrast, the PPV of a commercially available polyspecific EIA at the manufacturer's recommended cutoff was only 31.6% ($p = 0.004$). However, the ROC curves of the 3 immunoassays we evaluated (LIA, EIA-IgG, EIA-IgGAM) were very similar, which adds to the data from previous studies supporting the use of higher OD cutoffs for the EIAs [26–28].

The overall sensitivity of the LIA, based upon the 156 HIT-positive sample set, was relatively high, at 97.4% (152/156). We observed higher sensitivities (156/156; 100%) of both PF4-dependent EIAs, although this may in part represent incorporation bias, as the definition of "HIT-positive" status included a positive EIA-IgG. Nevertheless, four HIT-positive plasma samples were LIA-negative. This appears to be a patient-dependent feature, since testing serial plasmas from these patients showed consistent LIA-negative results. However, as we used frozen plasma samples, we cannot exclude the possibility that these LIA-negative samples may have tested LIA-positive if they had been tested fresh. Future large-scale studies using real-time sample testing can better address this question. Alternatively (and perhaps more plausibly), occasional HIT antibodies could bind to antigen sites distinct from those recognized by the HIT-mimicking monoclonal antibody (KKO) used in the LIA, accounting for false-negative results. Indeed, another HIT assay based on KKO inhibition observed some false-negative results versus an SRA standard [29].

We chose to use frozen plasma samples because this approach permitted us to rapidly assess the operating characteristics of the LIA [15]. Thus, our approach does not allow us to evaluate the potential value of "real-time" diagnosis. However, a recent study by Caton et al. [30] suggests that the on-demand, rapid-turnaround characteristics of the LIA has the potential to improve treatment by allowing the clinician to better evaluate the probability of HIT in real time, thus reducing use of expensive anticoagulants in patients who test LIA-negative, in whom such use may have occurred when waiting for results of slower turnaround assays.

It is known that the magnitude of a positive EIA predicts greater probability of a positive SRA, and hence a probable diagnosis of HIT [6, 7]. We showed that a similar relationship exists for the LIA. As shown in Fig. 4, the probability of HIT-positive status progressively increases as the LIA result increases quantitatively. Table 3 expresses the

Table 5
Post-test probabilities for HIT (SRA-positive status) with the LIA.

4Ts score	Pretest probability of HIT[a]	LIA-negative (<1.0 U/mL) LR = 0.034	LIA-positive (any pos result, i.e., ≥1.0 U/mL) LR = 16.0	LIA-positive (weak) (1.0–4.9 U/mL) LR = 5.7	LIA-positive (moderate) (5.0–15.9 U/mL) LR = 31	LIA-positive (strong) (≥16.0 U/mL) LR = 128
Low	1.9%	<0.1%	24.2%	10.0%	37.5%	71.3%
Intermediate	6.7%	0.2%	53.5%	29.0%	69.0%	90.2%
High	36.6%	1.9%	90.2%	76.7%	94.7%	98.7%

Abbr.: 4Ts, Four T's scoring system; HIT, heparin-induced thrombocytopenia; LIA, latex immunoturbidimetric assay; LR, likelihood ratio; U, units.

[a] The pretest probabilities shown are based upon the frequency of SRA-positive status for the three 4Ts scoring groups (low, intermediate, high), per the clinician who performed the score at the time of enrollment into the 4Ts clinical trial.

relationship by SSLR values, which can be used to determine post-test probabilities of HIT in the context of pretest probability [26]. As shown in Table 5, a LIA-negative result indicates a ~2.0% chance of HIT if the pretest probability was judged to be high. In contrast, a positive LIA indicates a probability of HIT of at least 10%, even in a patient judged to have a low pretest probability of HIT, with the post-test probability increasing to approximately 29% and 77%, respectively, for patients with moderate- and strong-positive LIA results. These findings suggest that a rapidly available test, such as the LIA, might be a reasonable option even in patients judged to have a "low probability" of HIT. This strategy is in contrast to the "Choosing Wisely" recommendation [31] that states that patients with low probability 4Ts score should not undergo laboratory testing for HIT. It is possible that such a recommendation could be reconsidered in clinical settings where a rapid assay with relatively high specificity is readily available.

4.1. Comparisons with previous studies of the LIA

To our knowledge, there are only 3 previous studies that have examined the LIA [32–34]. Davidson and colleagues [32] evaluated thawed, previously-frozen plasma aliquots from 414 HIT-suspected patients in 3 medical centers, and compared the results in the LIA with a polyspecific commercial EIA, the Asserachrom HPIA (Diagnostica Stago, Parsippany, NJ). The authors reported (versus the EIA) a copositivity of 60.2%, conegativity of 94.6%, and overall agreement of 87.7%. However, this study did not include an independent reference standard assay such as an SRA.

Althaus and coworkers [33] evaluated the LIA using 119 plasmas from patients with suspected HIT. The reference standard was a positive heparin-induced platelet activation (HIPA) test [35]. They found that 44 (40.0%) of 119 plasma samples tested LIA-positive, of which 20 also tested HIPA-positive; the corresponding PPV (20/44 = 45.5% [95% CI, 29.6%–60.0%]) is similar to what we observed (30/54 = 55.6% [95% CI, 41.4%–69.1%] in our study. Although Althaus et al. did not identify any false-negative LIA results, they only tested 20 HIPA-positive samples versus the 156 SRA-positive samples we evaluated.

Finally, Jourdy and colleagues [34] compared the LIA with a commercial PF4-dependent EIA (Zymutest HIA IgG) in 100 consecutive plasmas (from patients with 4Ts ≥ 4 points) referred for diagnostic testing for HIT; the HIPA was the reference standard. Nine study patients (9%) were HIPA-positive and thus considered HIT-positive; the LIA was positive in 17/100 samples, including all 9 HIPA-positive patients. Thus, LIA sensitivity was 9/9 (100%; 95% CI, 66.4%–100%), specificity 83/91 (91.2%; 95% CI, 83.4%–96.1%), and PPV = 52.9% (95% CI, 27.8%–77.0%), results similar to ours. The Zymutest EIA was positive in 18/100 samples (including all 9 HIPA-positive patients), and both the LIA and Zymutest EIA gave nearly identical ROCs.

4.2. Limitations

One limitation of our study was the use of frozen rather than fresh samples. It is possible that the operating characteristics of the assay might have been even better with fresh plasma samples. Additionally, the retrospective design of this study limits conclusions about its performance in real-time. Ideally, a prospective clinical management study, with clinically relevant outcomes, would be required to establish whether LIA results obtained in real-time would have a beneficial impact on timely HIT diagnosis and treatment.

4.3. Conclusions

The LIA is a rapid, on-demand, fully-automated laboratory assay for detection of HIT antibodies. It has specificity and PPV superior to that of EIAs (at manufacturers' recommended cutoffs) while retaining high diagnostic sensitivity. Moreover, substantially higher SSLRs at quantitatively greater LIA-positive results (expressed in standardized U/mL) will help clinicians better estimate post-test probability of HIT among LIA-positive patients. The high NPV and PPV of this assay, along with a turnaround time of 20 min, make the LIA an ideal rapid HIT assay.

Conflicts of interest

This work was funded by Instrumentation Laboratory, Bedford, MA, the manufacturers of the HemosIL HIT-Ab$_{(PF4-H)}$ assay. All laboratory testing, data analysis and interpretation were performed by McMaster personnel. The first draft of the manuscript was written by T.E.W. J.I.S. and L.A.L., with subsequent input from the other coauthors. The sponsor provided comments to the manuscript, but all final decisions regarding manuscript content were made by the authors.

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D.M. Arnold has no relevant conflicts of interest.

I. Nazy has no relevant conflicts of interest.

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