Letters to *Blood*

TO THE EDITOR:

High sensitivity and specificity of an automated IgG-specific chemiluminescence immunoassay for diagnosis of HIT

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Heparin-induced thrombocytopenia (HIT) is a prothrombotic disorder caused by platelet-activating antibodies that recognize PF4/heparin complexes.[1-3] Although PF4-dependent enzyme-immunoassays (EIAs) have high diagnostic sensitivity for HIT (~97% to 99%),[4,5] they frequently detect nonpathogenic antibodies in heparin-exposed patients (ie, low specificity), and test results are usually not available for many hours or even several days. Accordingly, it has been recommended (Choosing Wisely[6]) that diagnostic testing for HIT should not be performed in low-clinical-probability settings (eg, 4Ts score of ≤3 points; HIT risk ≤2%).[7,8]

However, if a test for HIT antibodies was both highly sensitive and specific, this could make the test valuable even in certain low-probability situations, especially if the result was quickly available. We used a recently US Food and Drug Administration–cleared, rapid, automated, immunoglobulin G (IgG)–specific chemiluminescence-based immunoassay (CLIA) to evaluate stored blood samples from a previously reported prospective clinical study[8] that evaluated the 4Ts clinical scoring system and an additional 135 HIT-positive samples collected from a local hospital. We compared the CLIA's operating characteristics for HIT antibody detection against a widely used IgG-specific commercial EIA with addition of the "high heparin" inhibition step[9] (thus maximizing EIA specificity).

Stored sera (n = 509) and citrated plasma (n = 429) were available from a prospective evaluation of the 4Ts scoring system.[8] All patients had a 4Ts score, and results from the serotonin-release assay (SRA)[10,11] and a polyspecific EIA that detects anti-PF4/polyvinylsulfonate antibodies of IgG/IgA/IgM classes (LIFECODES PF4 Enhanced assay; Immucor GTI Diagnostics, Waukesha, WI).[12] For this new study, all serum/plasma samples were also tested using an IgG-specific CLIA (HemosIL AcuStar HIT-IgG$_{(PF4-H)}$, Instrumentation Laboratory, Bedford, MA)[13]; per the manufacturer, a result ≥1.00 U/mL was considered positive. In addition, we tested sera in a commercial IgG-specific EIA (LIFECODES PF4 IgG assay; Immucor GTI Diagnostics)[14] using a high-heparin step per the manufacturer's recommendations (reactivity of anti-PF4/heparin antibodies is inhibited at suprapharmacologic heparin concentrations).[9] All testing was performed by laboratory personnel blinded to sample classification.

"HIT-positive" patients were defined as patients whose blood tested positive in both the SRA and polyspecific EIA and whose clinical course was considered consistent with HIT, either by investigators' 4Ts scoring (≥4 points) or (in case of low 4Ts scoring) upon case review. (As previously reported,[8] 4Ts discrepant cases could be explained by incorrect scoring or missing information.) All other patients were classified as "HIT negative." Each patient's HIT status was determined before CLIA testing.

For each test, we calculated sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) (with exact Clopper-Pearson 95% confidence intervals [CIs]), and likelihood ratios (LR$^+$ and LR$^-$; 95% CIs were calculated per Altman et al[15]). We also included 135 consecutive SRA-positive HIT patients from a single hospital (consecutive case series) to aid in calculating test sensitivity. Comparisons between assays were performed using the χ^2 test. Pretest probabilities for 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 participating clinicians); post-test probabilities were calculated according to Bayes theorem by multiplying the pretest odds by the LR$^+$, at the manufacturer's cutoff, and at different stratum-specific likelihood ratios. The Hamilton Integrated Research Ethics Board approved this study (#1288-T).

The 168 HIT-positive patients (33 in the prospective cohort study; 135 in the consecutive case series) comprised 79 males and 89 females, with a median age of 70 years (interquartile range [IQR], 62, 78; range, 29-94). Approximately three-quarters (74.4%) of the patients were surgical. HIT-associated thrombosis occurred in 101 (60.1%) patients. The median platelet count nadir was 59 × 10^9/L (IQR, 33, 82; range, 2-279), and the median percent platelet count fall was 70.1% (IQR, 55.0, 84.1; range, 26.7-97.0).

Table 1 shows the CLIA's operating characteristics (per 509 sera, 4Ts trial). Sensitivity, NPV, and LR$^-$ were similarly high for all immunoassays evaluated, reflecting high assay sensitivity. Specificity was highest for the CLIA, even compared with the IgG-specific EIA with the high-heparin step (98.5% vs 94.1%; $P < .0001$). Similarly, PPV and LR$^+$ were higher for the CLIA than for EIAs.

To obtain a more precise estimate of test sensitivity, we evaluated the CLIA in 135 consecutive HIT-positive patients. Combined with the 33 HIT-positive patients (4Ts trial), the sensitivity of the CLIA using serum was 166 out of 168 (98.8%; 95% CI, 95.8, 99.9%). The IgG-specific CLIA thus has a notably

Table 1. Operating characteristics of the CLIA vs 2 other commercial PF4-dependent EIAs (one performed with the high-heparin step)

	CLIA (95% CI)	EIA-IgG with high-heparin step (95% CI) P value (vs CLIA)	EIA-IgG without high-heparin step (95% CI) P value (vs CLIA)	EIA-IgGAM without high-heparin step (95% CI) P value (vs CLIA)
Se	32/33 = 97.0% (84.2%, 99.9%)	32/33 = 97.0% (84.2%, 99.9%) P = 1.0	33/33 = 100% (89.4%, 100%) P = .3173	33/33 = 100% (89.4%, 100%) P = .3173
Se*	166/168 = 98.8% (95.7%, 99.8%)	162/168 = 96.4% (92.4%, 98.7%) P = 0.1573	167/168 = 99.4% (96.7%, 100.0%) P = .5637	168/168 = 100% (97.83%, 100.1%) P = .1573
Sp	469/476 = 98.5% (97.0%, 99.4%)	448/476 = 94.1% (91.6%, 96.1%) P < .0001	435/476 = 91.4% (88.5%, 93.7%) P < .0001	393/476 = 82.6% (78.8%, 85.9%) P < .0001
PPV	32/39 = 82.1% (66.5%, 92.5)	32/60 = 53.3% (40.0%, 66.3%) P < .0001	33/74 = 44.6% (33.0%, 56.6%) P < .0001	33/116 = 28.5% (20.5%, 37.6%) P < .0001
NPV	469/470 = 99.8% (98.8%, 99.99%)	448/449 = 99.8% (98.8%, 99.99%) P = .9742	435/435 = 100% (99.2%, 100%) P = .3168	393/393 = 100% (99.1%, 100%) P = .3167
LR+	65.9 (31.5, 137.9)	16.5 (11.5, 23.7) P = .0002	11.6 (8.6, 15.6)	5.7 (4.7, 7.0)
LR−	0.031 (0.004, 0.212)	0.032 (0.005, 0.222) P = .9742	0.0 (undefined)	0.0 (undefined)
AUC	0.997 (0.994, 1.000)	0.994 (0.985, 1.000) P = .4016	0.997 (0.994, 1.000) P = .7225	0.992 (0.986, 0.999) P = .1053

The table presents the results of 3 different PF4-dependent immunoassays (including 1 assay, the EIA-IgG, performed with and without the high-heparin step) tested on 509 patients (33 SRA-positive) from a prospective study of the 4Ts scoring system.[8] All samples tested were sera, with the exception of 2 plasma samples tested in the EIA-IgGAM (without high heparin). CLIA results using plasma are as follows: for 429 of the 509 patients, citrated plasma was available for testing in the CLIA, with the following operating characteristics found: (1) sensitivity = 30/31 (96.8%; 95% CI, 83.3%, 99.9%); (2) sensitivity (including 135 consecutive HIT-positive patients from one hospital) = 162/166 (97.6%; 95% CI, 93.9%, 99.3%); (3) specificity = 391/398 (98.2%; 95% CI, 96.4%, 99.3%); (4) PPV = 30/37 (81.1%; 95% CI, 64.8%, 92.0%); (5) NPV = 391/392 (99.7%; 95% CI, 98.6%, 100%); (6) LR+ = 55.0 (95% CI, 26.3, 115.0); and (7) LR− = 0.033 (95% CI, 0.005, 0.226). All comparisons were not significantly different than the results seen with serum (P ≥ .3120 for all 6 comparisons).

AUC, area under the curve; EIA-IgG, enzyme immunoassay (IgG specific); EIA-IgGAM, enzyme immunoassay (polyspecific); Se, sensitivity; Sp, specificity.

*Sensitivity calculations performed with the addition of 135 consecutive HIT-positive patients from one hospital (total HIT-positive patients, n = 168), identified from February 1999 until January 2018, inclusive.
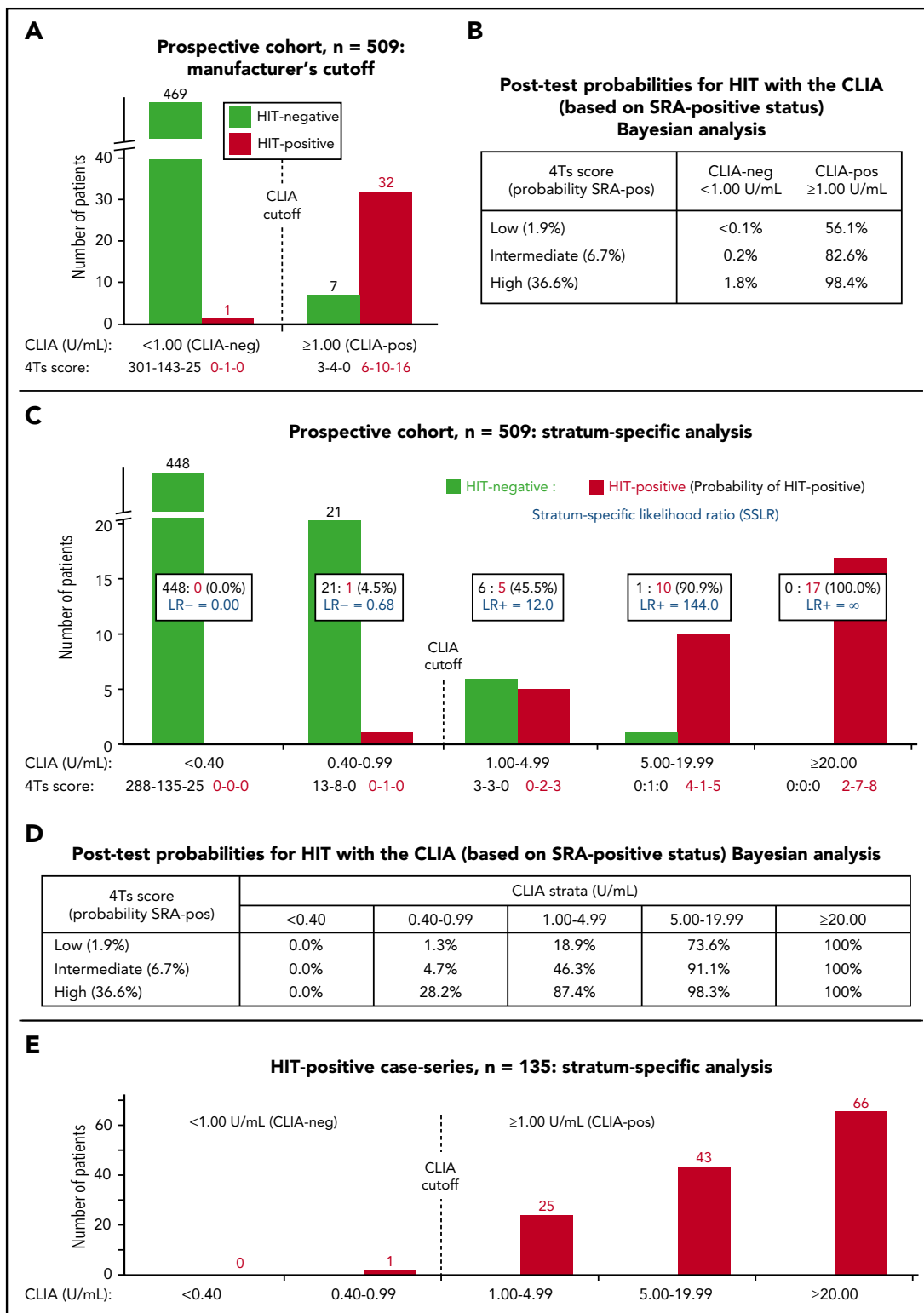
A

Prospective cohort, n = 509: manufacturer's cutoff

B

Post-test probabilities for HIT with the CLIA (based on SRA-positive status) Bayesian analysis

4Ts score (probability SRA-pos)	CLIA-neg <1.00 U/mL	CLIA-pos ≥1.00 U/mL
Low (1.9%)	<0.1%	56.1%
Intermediate (6.7%)	0.2%	82.6%
High (36.6%)	1.8%	98.4%

C

Prospective cohort, n = 509: stratum-specific analysis

D

Post-test probabilities for HIT with the CLIA (based on SRA-positive status) Bayesian analysis

4Ts score (probability SRA-pos)	CLIA strata (U/mL)				
	<0.40	0.40-0.99	1.00-4.99	5.00-19.99	≥20.00
Low (1.9%)	0.0%	1.3%	18.9%	73.6%	100%
Intermediate (6.7%)	0.0%	4.7%	46.3%	91.1%	100%
High (36.6%)	0.0%	28.2%	87.4%	98.3%	100%

E

HIT-positive case-series, n = 135: stratum-specific analysis

Figure 1. CLIA performance for diagnosis of HIT, including Bayesian analysis. (A) Results of the CLIA for the prospective study (n = 509 sera); analysis per manufacturer's recommended cutoff (1.00 U/mL). HIT-positive vs HIT-negative status in relation to CLIA results, CLIA-pos (positive) vs CLIA-neg (negative). The corresponding 4Ts scores, shown as xx-xx-xx for each of the data groupings, correspond to low-intermediate-high (per real-time scoring by the investigators). (B) Post-test probabilities of HIT based upon combining pretest probability of HIT (per investigators' 4Ts score) and CLIA test result at manufacturer's recommended cutoff (Bayesian analysis). For each 4Ts classification (low, intermediate, and high), the probability of an SRA-positive (SRA-pos) test result is indicated. (C) Probability of HIT-positive vs HIT-negative result per strength of CLIA result (stratum-specific analysis). LR$^-$ indicates likelihood ratio for HIT-negative status, whereas LR$^+$ indicates likelihood ratio for HIT-positive status. As above, the corresponding 4Ts scores, shown as xx-xx-xx for each of the data groupings, correspond to low-intermediate-high (per real-time scoring by the investigators). (D) Post-test probabilities of HIT based upon combining pretest probability of HIT (per investigators' 4Ts score) and stratum-specific CLIA test result (Bayesian analysis). The data shown are for the prospective study (n = 509 sera). (E) Distribution of CLIA test results for single-hospital consecutive patients with HIT. The figure shows the results using serum (n = 135). When plasma was used, the corresponding data distribution was similar, as follows: <0.40, n = 0; 0.40-0.99, n = 3; 1.00-4.99, n = 23; 5.00-19.99, n = 50; and ≥ 20.0, n = 59.

high combination of sensitivity and specificity (98.8% and 98.5%, respectively) relative to other HIT immunoassays.[5,16]

Results were similar when 429 available plasmas (HIT positive, n = 31) from the 4Ts trial were tested with the CLIA. When results of the 135 HIT-positive consecutive case-series patient plasmas were combined with 4Ts study patient data, the CLIA's sensitivity for detecting HIT-positive status was 162 out of 166 (97.6%; 95% CI, 93.9%, 99.3%) (see Table 1 legend). Of the 4 false-negative samples, 2 yielded borderline-negative results when using plasma (0.89 and 0.97 U/mL) while the corresponding sera yielded borderline-positive results (1.07 and 1.06, respectively); the other 2 samples tested negative using both plasma and serum. These 4 samples were retested in the SRA and EIA-IgGAM, which confirmed their HIT-positive status. These samples remained negative upon retesting in the CLIA. Overall, we found a strong correlation between the CLIA quantitative results for HIT-positive serum–plasma pairs (r^2 = 0.848; slope = 1.2113; y-intercept = 0.1404; $P <$ .0001).

Figure 1A shows the results of the CLIA for 509 sera (4Ts trial). The overall agreement with the SRA was high (501/509 = 98.4%). Figure 1B shows the corresponding post-test probabilities of HIT for a positive test result (using 1.00 U/mL cutoff), which range from 56.1% (low 4Ts score) to 82.6% (intermediate score) to 98.4% (high score). In comparison with these Bayesian estimates, the actual results were similar, as follows: for the 310 patients with low 4Ts scores, 9 tested CLIA positive, of whom 6 out of 9 (66.7%) tested SRA positive (ie, ~52 patients tested to identify 1 HIT patient). Thus, at a cost of 3 false-positive patients (for whom HIT was ruled out by further testing with the SRA), 6 patients with low 4Ts scores were identified as having HIT (positive SRA) by a positive CLIA. For the 158 patients with intermediate 4Ts scores, 14 tested CLIA positive, of whom 10 (71.4%) tested SRA positive (~16 patients tested to identify 1 HIT patient). For the 41 patients with high 4Ts scores, 16 tested CLIA positive, of whom all 16 (100%) tested SRA positive (~3 patients tested to identify 1 HIT patient). Only 1 of the 470 CLIA-negative patients tested SRA positive; this patient had been classified as 4Ts intermediate.

It is known that a high quantitative optical density value of a positive EIA test is associated with a high likelihood of HIT.[4,17] Figure 1C shows a similar relationship for the CLIA: for a result of ≥5.00 U/mL, the probability of HIT was 96.4% (27/28), whereas for a weak-positive result (1.00-4.99 U/mL), the overall probability of HIT was 45.5% (5/11). Figure 1D summarizes the approximate post-test probabilities of HIT for various strata of positive and negative results (Bayesian analysis). Finally, the distribution of positive results in the 4Ts trial (Figure 1C) was similar to that of the consecutive HIT-positive patients (Figure 1E).

In conclusion, our evaluation of the CLIA shows a high sensitivity/specificity tradeoff for a PF4-dependent immunoassay. Particularly given its rapid, on-demand test capabilities (results available within 30 minutes[13,16] following preparation of test serum or plasma), the clinical usefulness of diagnostic testing for HIT even in some low-probability situations requires further consideration.

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Authorship

Contribution: T.E.W. designed the study, determined 4Ts scores for the consecutive patient series, and wrote the first draft of the manuscript; J.-A.I.S. performed all of the assays; T.E.W. and J-A.I.S. performed data analyses; L.-A.L. was responsible for the 4Ts trial and oversaw the Bayesian analyses in the current study; D.M.A. and I.N. oversaw the laboratory investigations and edited the manuscript; and all authors reviewed and approved the final version of the manuscript.

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TO THE EDITOR:

Homozygous OB-fold variants in telomere protein TPP1 are associated with dyskeratosis congenita–like phenotypes

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Dyskeratosis congenita (DC) and its severe form, Hoyeraal-Hreidarsson syndrome (HHS), are rare and have life-threatening failure of hematopoiesis. Typically, DC patients present with disease features such as nail dystrophy, oral leukoplakia, and abnormal skin pigmentation along with peripheral pancytopenia and marrow hypoplasia with strong predisposition to cancer.[1] In DC, hematopoietic failure occurs due to critical shortening of telomeres,[2,3] which enhances the DNA damage response[4,5] and leads to premature senescence of hematopoietic stem cells.[6]

Telomeres are lengthened by the enzyme telomerase, which constitutes RNA template (TERC) and the catalytic reverse transcriptase (TERT). Variants affecting telomerase complex (*TERT* and *TERC*), telomerase stability (*DKC1*, *NOP10*, *NHP2*, *PARN*, and *NAF1*), telomerase trafficking (*WRAP53*), and telomere replication (*CTC1* and *RTEL1*) have been identified in majority of DC and HHS cases. The shelterin complex proteins (TRF1, TRF2, RAP1, TIN2, POT1, and TPP1) that serve to protect telomeres are critical for telomerase function.[7] The first DC variants to be described in a shelterin component were found in *TINF2*.[8] Variants in *ACD* encoding TPP1 were later described in 2 independent families.[9,10] The first was in a family with a history of aplastic anemia, in which the index case had pancytopenia at 8 years of age and was heterozygous for the *ACD* variant c.499-501del; p.K170 del (K170Δ) that segregated as an autosomal-dominant trait.[9] The proband's mother presented with thrombocytopenia in her 20s and was later diagnosed with myelodysplasia. Her grandmother had mild macrocytic anemia associated with hypocellular bone marrow. The second case, reported by Kocak et al in 2014,[10] had several features of HHS, had the same K170Δ variant on one allele along with c.1471 C>T; p.P491T on the other, suggesting an autosomal-recessive inheritance.[10] It is of interest that in this family, the proband's father, who carried the K170Δ variant, had short telomeres but lacked any disease features. Structural and functional studies of K170Δ have not demonstrated a dominant-negative effect on TPP1 function, but instead it is suggested that dosage of TEL patch (patch of amino acids involved in telomerase binding on the surface of TPP1) is responsible for telomere shortening.[11,12]

The evidence in support of causality in the bone marrow failure (BMF) patients is largely derived from functional analysis of the variants themselves rather than being statistically derived or based on the strength of a significant allelic series.[9-11] It remains to be established as to how loss-of-function (LOF) variants in *ACD* (frameshift, splice donor/acceptor, or stop gain) can have a significant heterozygous frequency in the Genome aggregation database (gnomAD), where 57 of these variants are described (representing ~6 in 10 000 individuals). Given this frequency of heterozygous LOF *ACD* variants in the control population, together with the variable pattern of inheritance and very different clinical presentations in the only 2 families described to date, the case for *ACD* as a proven disease-causing locus remains uncertain.

By whole-exome sequencing in a series of genetically uncharacterized patients (n = 228) presenting with DC or constitutional BMF from our DC registry, we identified nonsynonymous *ACD* variants in 5 unrelated cases (supplemental Table 1, available on the *Blood* Web site). In two of these (index cases of families 1 and 2; Figure 1A) the *ACD* variants were homozygous (supplemental Table 1). The index case of family 1 harbors the homozygous variant c.280C>T; p.V94I, which has been reported once in the homozygous state and 191/270022 alleles in heterozygous state on gnomAD. This index case (age 38 years) had thrombocytopenia, short stature, pulmonary abnormalities, and LSCD (supplemental Table 2; supplemental Figure 1A-B). His older