Evaluating Heparin-Induced Thrombocytopenia: The Old and the New

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Abstract

Heparin-induced thrombocytopenia (HIT) is a rare but potentially serious complication of heparin use. Prompt diagnosis is crucial and requires the integration of clinical assessment and laboratory testing. Pretest clinical scoring systems (i.e., 4 Ts) have been established. Immunoassays can detect the presence of antibodies directed toward heparin-platelet factor 4 (H-PF4) complexes, but provide no information about their ability to activate platelets. A low clinical score, when combined with a negative immunoassay result obviates the need for further testing. However, immunoassays and 4 Ts scores have only modest specificity. Functional testing (serotonin release assay or heparin-induced platelet activation) remain important in confirming the presence of pathogenic H-PF4 antibodies, but are technically demanding to perform and limited in guiding clinical decisions in the acute setting. This review evaluates current immuno-and functional assays available in the laboratory diagnosis of HIT, and describes recent attempts to improve the specificity of enzyme immunoassays, including adopting an immunoglobulin G-specific assay and raising the optical density value cutoff for a positive result. The importance of donor selection and newer functional assays including flow cytometry-based assays, are also discussed. A current approach to integrating clinical scoring, immunoassays, and functional testing for HIT is also outlined.

Keywords
► heparin-induced thrombocytopenia
► heparin-platelet factor 4 enzyme immunoassays
► heparin-platelet factor 4 particle-gel immunoassay
► functional assays
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► clinical scoring

Heparin-induced thrombocytopenia (HIT) remains an important clinical condition.1–3 Non-heparin anticoagulants are used to prevent potentially life-threatening thrombosis but such treatments are costly and carry a significant risk of major bleeding.4–6 HIT is a clinicopathological syndrome, and hence diagnosis requires both clinical assessment and laboratory testing demonstrating the presence of antibodies, usually of immunoglobulin G (IgG) class, directed toward heparin-platelet factor 4 (H-PF4) complexes.7–9 The “iceberg” model of HIT emphasizes that only a minority of H-PF4 antibodies are pathogenic and able to trigger platelet activation and subsequent thrombosis.10

Clinical Assessment in HIT

More accurate clinical assessment has been facilitated by the establishment of pretest clinical scores, most notably the 4 Ts score.11 The 4 Ts score is obtained by assessing the degree and timing of thrombocytopenia, the presence of thrombosis, and the possibility of other causes accounting for thrombocytopenia. A score of 0 to 3 represents low probability of HIT, a score of 4 or 5 an intermediate probability, whereas a score of 6 to 8 suggests high clinical probability of HIT. The 4 Ts score correlates well with HIT laboratory testing, and has high sensitivity and negative predictive value (NPV).11–13 However, it is limited by a low positive predictive value (PPV) of 9 to 17% and moderate interobserver variability.2,11–15 Various...
The utility of the PaGIA was enhanced when combined with the 4 Ts score. In the same study by Pouplard et al., the probability of HIT in intermediate risk patients based solely on the 4 Ts score was reduced from 10.9 to 0.6% when combined with a negative PaGIA result. Hence the combination of a 4 Ts score and PaGIA appears to be a suitable strategy to rule out HIT.

The PaGIA would appear to have high utility in peripheral laboratories. However, the issue of false-negative results is critically important for assessing a screening test, and several prospective studies have assessed the performance of the PaGIA. In the study by Bakchoul et al., the PaGIA had a lower PPV (37 vs. 41%) and NPV (99.5 vs. 100%) compared with the IgG-specific EIA and did not detect two patients with both an intermediate/high 4 Ts score and a positive functional assay (heparin-induced platelet activation [HIPA] test). A patient that was positive for serotonin release assay (SRA) and EIA was negative for the PaGIA in a study conducted by Pouplard et al. Thus, the PaGIA may fail to detect some patients with HIT, with potentially major ramifications. Schneiter et al reported a dramatic variability of PaGIA results that was dependent on the reagent lot, with false-negative results as high as 82%. Concerns regarding the false-negative results led to a worldwide recall of the PaGIA in 2008. The PaGIA has been reintroduced and prospective data of the new assay are awaited with great interest. Until such data are available, we recommend that initial screening assessment of the likelihood of HIT based on the PaGIA is performed in conjunction with a clinical score such as 4 Ts.

Another rapid EIA, the particle immunofiltration assay (PIFA; Akers Biosciences, Inc., Thorofare, NJ) operates in a similar fashion to the PaGIA, with the degree of agglutination of dyed particles coated with PF4 influencing the migration of such particles through a filter. However, the test has low sensitivity and specificity, with results not correlating with EIAs or HIPA.

EIAs
At present three main solid phase EIAs are available for use, supplied by either Genetics Testing Institute (GTI, Waukesha, WI), Diagnostica Stago (Asnieres Sur Seine, France), or Hyphen Biomed (Neuville-Sur-Oise, France). Commercial kits supplied by these companies can detect IgG specifically or antibodies of IgG, IgM, or IgA classes (polyspecific EIA) and provide semiquantitative information concerning the presence of HIT antibodies. A fluid-phase PF4/EIA is also available, and has the advantage of minimizing nonspecific binding that can occur in the solid phase. However, it has not been widely used and is mostly for research applications.

The EIAs use H-PF4 (Stago) or polyvinylsulfate-PF4 (GTI) as target antigens, which are immobilized on microtiter plates. The Zymutest kits developed by Hyphen Biomed differ from other EIAs in that unfractionated heparin (UFH) without PF4 is immobilized at the bottom of microwells. The addition of a platelet lysate to the wells supplies the necessary PF4.
EIAs are technically simple to perform, and can be easily integrated into automated platforms in routine laboratories, measuring large numbers of samples simultaneously.\textsuperscript{9,20,28} EIAs are also highly sensitive, with sensitivities and NPV of 100\% reported in various studies.\textsuperscript{18,31,33} In view of this, it has been reported that a negative EIA, in combination with a low 4 Ts clinical score, effectively rules out clinical HIT.\textsuperscript{21,23} However, EIAs have limited specificity, with high rates of false positives.\textsuperscript{23–25,34,35} EIAs are also often false positive in patients with antiphospholipid syndrome.\textsuperscript{36,37} This leads to the possibility of considerable overdiagnosis of HIT, particularly in clinical settings such as cardiac surgery, which result in a high incidence of H-PF4 antibodies that may not be pathogenic.\textsuperscript{23,24,38,39} False-positive EIAs may lead to unnecessary commencement of non-heparin anticoagulants with significant risk of major bleeding.\textsuperscript{40}

**IgG-Specific EIA Improves Specificity**

One method of increasing the specificity is to use IgG-specific EIAs.\textsuperscript{18,23,28,31,33,41} IgG H-PF4 antibodies are the only class of antibodies able to activate platelets via the IgG-Fc receptor (FcγRIIA). Studies have shown that H-PF4 antibodies of IgM or IgA class do not bind and cross-link FcγRIIA on platelets and are of minor clinical and pathophysiological relevance.\textsuperscript{23,28,34} Bakchoul et al reported superior specificity of the IgG-specific EIA to the polyspecific EIA with identical sensitivity (100\%).\textsuperscript{18} Similarly, Warkentin et al found greater specificity of the GTI-IgG assay to detect platelet-activating antibodies over the corresponding polyspecific assay at two SRA cutoffs (\( \geq 20\% \) release: 0.986 vs. 0.857 \((p = 0.0077)\); \( \geq 50\% \) release: 0.921 vs. 0.789 \((p < 0.005)\)), without significantly altering the sensitivity.\textsuperscript{31} Pouplard et al reported a specificity of 90\% for the Zymutest HIA IgG compared with 77\% in the corresponding polyspecific test.\textsuperscript{31} IgG-specific assays were also superior to their polyspecific counterparts in a recent study by Morel-Kopp et al.\textsuperscript{42}

**High Optical Density (OD) Associated with Better PPV**

Despite these encouraging results, the PPV of IgG-specific assays in studies comparing such assays with functional tests such as the HIPA remain modest (40 to 55\%).\textsuperscript{13,43} Another method of increasing the specificity of the IgG-specific assays is to raise the OD cutoffs for positive results.\textsuperscript{18,21,23,25,33,34,38,43,44} Current manufacturer’s cutoffs for the three main EIAs range from 0.4 to 0.5. Zwicker et al reported that mean EIA OD measurements were significantly higher in HIT patients with thrombosis, and patients with an initial OD reading of 1.0 had a sixfold increase in risk of future thrombotic events compared with patients with OD results between 0.4 and 0.99.\textsuperscript{44} Lo et al reported good correlation of strong EIA–GTI reactivity (>1.20 OD) with positivity for SRA and intermediate or high clinical pretest probability for HIT.\textsuperscript{24} Warkentin et al calculated the likelihood of a pathogenic HIT antibody based on SRA results being greater than 50\% with an OD result \( \geq 1.40 \) units.\textsuperscript{25} Higher OD values also correlated well with clinical outcomes of HIT in another study.\textsuperscript{33} There is increasing acceptance that the higher the OD value, the greater the likelihood of clinical HIT for a given pretest probability. Efforts to standardize the reporting of OD results for EIA in ranges to predict likelihood of pathogenic HIT antibodies have commenced.\textsuperscript{43}

**High-Dose Heparin Confirmatory Step May Improve EIA Specificity**

The addition of a confirmatory step using high-dose heparin may also improve the utility of EIAs. Whitlatch et al performed an extra confirmatory step by adding high-dose heparin (100 IU/mL) to samples which were EIA (GTI) positive and compared outcomes with assessment of HIT as per the clinical criteria outlined by the American College of Chest Physicians guidelines.\textsuperscript{38} 98\% of EIA-positive samples were also confirmatory test positive. Importantly, patients who were positive for the confirmatory test were more likely to be diagnosed with clinical HIT than patients who were negative (72 vs. 18\%, \( p < 0.001 \)). A further study by the same authors also emphasized the utility of the confirmatory test.\textsuperscript{45} However, in another study of cardiac surgery patients, the confirmatory step did not improve diagnostic specificity of the EIA.\textsuperscript{46} A further study, also in the cardiac surgery setting, showed that only 50\% of HIPA-positive samples were positive for the EIA confirmatory test.\textsuperscript{47} In the same study, one of three patients who unequivocally satisfied clinical criteria for HIT was negative for the confirmatory test. In another study, the additional confirmatory step improved specificity at the expense of sensitivity.\textsuperscript{33} More recently, Althaus et al reported that use of high-dose heparin (100 IU/mL) increased the specificity of EIAs with weak OD (0.5 to \( \leq 1.0 \) units) but not strong OD (>1.0 units) results, with a significant risk of false negatives (16\%) associated with testing of strongly positive ODs.\textsuperscript{48} It is also possible that the confirmatory test can inhibit true pathogenic antibodies and hence not add further information to the one-step EIA.\textsuperscript{7} More prospective information is needed to confirm the utility of the confirmatory step, and also whether its utility varies according to the clinical setting.

Despite IgG-specific assays and higher diagnostic cutoffs for OD, the specificity of EIAs remains modest at best. If diagnosis is based solely on a positive EIA result, \( \sim 50\% \) of patients will be mislabeled as being positive for HIT, with attendant bleeding risks associated with the use of non-heparin anticoagulants.\textsuperscript{24,43} It has been suggested that the OD for the EIA be further raised to \( \geq 2.0 \) in the surgical intensive care unit setting to improve its utility.\textsuperscript{15} Hence functional assays, which detect the presence of pathogenic antibodies by their ability to induce platelet activation and have high specificity, continue to play a major role in the diagnosis of HIT.

**Functional Assays**

The two most widely used functional assays are the SRA and the HIPA test.\textsuperscript{49,50} Functional assays play a crucial role in the overall assessment of the likelihood of HIT, particularly in equivocal cases (i.e., intermediate 4 Ts score, positive EIA with “weak” OD [0.4 to 1.0]). The results of functional assays can
guide decisions on whether to continue non-heparin anticoagulants in patients with suspected HIT.

**Donor Selection**

It has been reported since 1989 that some antiplatelet monoclonal antibodies can activate platelets via the FcγRIIa receptor.51–53 The Arg/His131 polymorphism on FcγRIIa receptor and its importance for platelet response to activating monoclonal antibodies was first published in the early 1990s.54–56 Another FcγRIIa polymorphism, Gln/Lys137, was later described.57

Bachelot-Loza et al showed that using a high responder donor (Arg131/Arg131) for HIT investigation with functional assays improves functional test sensitivity.58 Positive HIT samples may be misdiagnosed as HIT negative when tested using His131/His131 platelets.59 Most American and Canadian laboratories use selected donors as recommended,20,60 but this is not standard practice elsewhere.61,62 where use of random healthy donors can lead to interlaboratory variability.

**Light Transmission Aggregometry (LTA)**

The first functional assay described to aid diagnosis of HIT was LTA (platelet aggregation test, PAT), described by Fratantoni et al and further modified by Chong et al.63,64 With the LTA, an increase in light transmission with platelet aggregation occurs when low concentration heparin is added to a patient’s sample containing H-PF4 antibodies. This is confirmed with inhibition of platelet aggregation with high-dose heparin. However, many technical factors influence its utility, including platelet count and hematocrit.1,65–67 Additional manipulation required to generate platelet rich plasma (PRP) results in increased artifactual platelet activation. There is also a lack of standardization of test performance and interpretation of results.

**SRA**

SRA, first described by Sheridan et al in 1986, is now widely recognized as the gold standard test in diagnosis of HIT.49 The SRA has been reported to be the most sensitive and specific of the functional assays.20 In this assay, donor platelets are washed and incubated with 14C-labeled serotonin. In the presence of platelet activating H-PF4 antibodies, serotonin is released from the dense granules of platelets and test results are positive if there is >20% release at therapeutic heparin levels (0.1 to 0.3 IU/mL) and <20% release at supratherapeutic heparin levels (10 to 100 IU/mL).49 The utility of the assay is enhanced by (1) the selection of suitable platelet donors, (2) the use of apyrase, an adenosine diphosphate scavenger, in the washing step, (3) additional testing involving the usage of a FcγRIIa monoclonal antibody directed against the FcγRIIa receptor (which suppresses the reactivity of HIT-positive serum),58 and (4) the addition of low-molecular-weight heparin (LMWH), which may give better reactivity for weak samples (Warkentin TD, personal communication, ISTH Kyoto 2011).

However, the SRA includes radioactive isotopes, is labor- and time-intensive, and requires specialized personnel and equipment, thus limiting its use to major reference laboratories.5,20 The SRA is performed very infrequently in Australia, with results only available retrospectively. Hence the value of the SRA on decision-making in the acute setting is limited, although this may be a lesser issue in countries with established referral laboratories (i.e., United States and Canada).

**HIPA Assay**

The HIPA test, first described by Greinacher et al in 1991, is the most widely used functional assay in Europe and obviates the need for radioisotope tracers.50 Washed platelets prepared from citrated blood obtained from four random healthy donors (reactivity to HIT-positive plasma is unknown) are incubated with both therapeutic heparin (0.1 to 1.0 IU/mL) and supratherapeutic heparin (100 IU/mL) and patient serum, with buffers acting as negative controls.7,69 A positive test is demonstrated with transparency of the suspension (visual evaluation) at therapeutic but not high-dose heparin in at least two of the four healthy donors.

The HIPA has similar sensitivity and specificity to the SRA, and has the advantages of a more rapid turnaround time of results compared with the SRA (results reported within 24 hours in Germany) without the use of radioactivity.35 However, it remains technically challenging, particularly in peripheral laboratories, and is not amenable to automation, unlike the EIAs. Furthermore, there is a possibility that the PRP of all four healthy donors used in HIPA testing do not react well to HIT antibodies (low responders), raising the possibility of false negatives.28

**Whole Blood Impedance Aggregometry (WBIA)**

Concerns regarding the preanalytical and analytical variables that can impact on the utility of LTA have lead to the development of WBIA. Originally designed to monitor the effectiveness of antiplatelet therapy, WBIA can now be used to assist in the diagnosis of HIT.59,70 The Multiplate® analyser (Verum Diagnostica, Munich, Germany) is one such instrument, in which platelet aggregation as a result of the presence of pathogenic H-PF4 antibodies is detected as an increase in impedance across paired electrodes. The advantages of the WBIA include a rapid turnaround time, and it is easy to perform. As it is a whole blood assay, there is no requirement for sample manipulation to generate PRP, platelet-poor plasma, or washed platelets, minimizing artifactual platelet activation. Morel-Kopp et al reported on the efficacy of WBIA in diagnosing HIT using a known reactive platelet donor, with superior sensitivity to LTA, and comparable results to SRA.59

**Other Functional Assays**

**Flow Cytometry**

Functional assays using the capabilities of flow cytometry have also been developed. Khairy et al described a whole blood flow cytometry assay measuring the presence of leucocyte-platelet aggregates (defined as events positive for both the presence of CD45 and platelet glycoprotein IIb) as a marker of platelet activation with different heparin concentrations, using plasma from HIT-positive patients.71
Leucocyte-platelet aggregates were found in 75% of HIT-positive patients, and correlated with levels of anti H-PF4 antibodies as determined by EIA. The advantages of this assay are the option of analyzing the sample up to 24 hours from collection with usage of the fixative, paraformaldehyde, and the usage of whole blood, hence requiring lesser volumes. Platelet microparticles are thought to contribute to the procoagulant effects of HIT-positive sera and the use of flow cytometry to measure HIT antibody-induced microparticle generation was first described by Warkentin and colleagues. More recently, Mullier et al reported on a pilot study examining the utility of a flow cytometry-based platelet microparticle generation assay as a marker of platelet activation in HIT. Platelet microparticles ranged in size from 0.5 to 1.0 µm, and were also defined by the expression of platelet glycoprotein Ib, CD41, and annexin V in this assay. While this was a pilot study, the assay showed good correlation with the SRA (specificity, 0.96; NPV, 1) and also reversal of reactivity with high-dose heparin (500 IU/mL) compared with the therapeutic dose (1 IU/mL).

A flow cytometry-based functional assay measuring CD62P was first described by Jy et al and later modified by Denys and colleagues. It has been reported that CD62P is a more reliable marker of platelet activation as a result of the presence of pathogenic H-PF4 antibodies than the procoagulant phospholipid annexin V.

**Thrombin Generation**

Tardy-Poncet et al measured thrombin generation in PRP as a marker of the hypercoagulable state induced by HIT as a result of generation of procoagulant platelet microparticles. The resultant calibrated automated thrombogram (CAT) with or without heparin was then compared between HIT positive and HIT negative patients.

While the above results of newer functional assays appear promising, more prospective studies are required to validate these tests. Furthermore, these assays are currently limited to research laboratories. Assays which measure thrombin generation may have uncertain specificity as other causes of thrombocytopenia in patients suspected of HIT can also reflect hypercoagulable states, such as DIC and sepsis. Some publications have shown that H-PF4 antibodies are able to activate platelets in the absence of heparin during SRA and HIPA testing. The spontaneous platelet activation observed was not eliminated by the addition of heparinase or via chromatography in both functional assays. Hence, the immune response of HIT is similar to that of an autoimmune process and may explain the clinical manifestations of delayed-onset HIT. While such antibodies are unlikely to affect the results of the SRA or HIPA (as these assays do not always include a no-heparin step), they may influence the utility of the newer assays such as the measurement of thrombin generation via the CAT where baseline thrombin generation is already maximal due to the platelet-activating properties of such antibodies independent of heparin.

**Conclusions**

HIT is a clinicopathological syndrome and hence both clinical and laboratory assessments are essential for accurate diagnosis. A wide range of immunoassays and functional tests are now available. In a recent report on the clinical and laboratory management of suspected HIT in current practice, the authors commented about the lack of clinical information provided to laboratories performing HIT testing. The authors commented that use of UFH at therapeutic doses and the presence of thrombosis appear to be excellent predictors of HIT. This is supported by the fact that in a recent meta-analysis, the absolute risk of HIT associated with use of LMWH at prophylactic doses was very low (0.2%). Hence, it is imperative that cooperation between clinicians and laboratories exists to ensure relaying of relevant clinical data to aid laboratory diagnosis.

Such clinical information may be conveyed in the form of a validated scoring system, such as the 4 Ts. While the 4 Ts score has a high NPV, reporting varies between centers and may be more difficult in medical and critical care settings. A new score, the HEP score may correlate better

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<th>Table 1 Summary of HIT Laboratory Investigation Assays, the Old versus the New</th>
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HIT, heparin-induced thrombocytopenia; H-PF4, heparin-platelet factor 4; IgGAM, immunoglobulins G, A, and M; EIA, enzyme immunoassay; PaGIA, particle gel Immunoassay; G, immunoglobulin G; PF4, platelet factor 4; OD, optical density; LTA, light transmission aggregometry; PAT, platelet aggregation test; SRA, serotonin release assay; HIPA, heparin-induced platelet activation; WBIA, whole blood impedance aggregometry.
Further studies validating this score are awaited.

The current reality is that pretest clinical scoring results are often not available to laboratories where further testing has been requested. EIAs are highly sensitive, with rapid turnaround time and the capability for concurrent sampling of large numbers of samples. It is likely to be accessible to most laboratories, especially in peripheral centers or in developing countries. Hence, we recommend the EIA, in particular an IgG-specific EIA, as the initial screening laboratory investigation for HIT, conforming to the increasing belief that pathogenic platelet-activating H-PF4 antibodies are of the IgG subtype. The magnitude of the OD has been shown to correlate well with clinical outcomes of HIT and efforts are now underway to standardize the reporting of OD values derived from EIAs. Further information regarding the use of high-dose heparin as a confirmatory step in EIA testing is awaited. The PaGIA is also available as a rapid screening tool and has recently been reintroduced into the market. Prospective studies are required to assess the utility of the new PaGIA.

The biggest drawback of the EIAs is that its PPV remains modest. EIAs are therefore useful in ruling out, but not in confirming HIT. Hence, functional assays, which have high specificity, play an important role in the assessment of HIT. We recommend that the combination of a clinical score such as the 4T and an immunoassay be used as initial screening for HIT, with results guiding further testing by a functional assay as outlined in ►Fig. 1. The clinician may consider omitting further testing in samples with low 4Ts score. Functional testing (SRA, HIPA, or WBIA) is recommended as a confirmatory test for positive EIA results with equivocal or strong ODs, and should occur after cessation of heparin.

Functional assays are technically demanding to perform, and are less practical to assist clinical decision-making in the acute setting. However, new methods are emerging that may address this issue. Data regarding the use of WBIA appear promising, and various flow cytometry assays measuring a range of markers of platelet activation have the advantages of rapid turnaround time, require less sample volume and show good correlation with other functional assays in initial studies. Future prospective studies validating such methods are awaited.

In summary, accurate HIT diagnosis requires clinical judgment and a sensitive screening test initially, followed by a functional assay to confirm suspected HIT.

In North America, samples are referred to major reference laboratories for SRA for confirmatory testing, with results available within 1 or 2 days. Similarly, in Germany, a network of laboratories with capability of performing the HIPA has been established, enabling nationwide access to a functional assay, with results generally reported within 24 hours. This is a model which we believe should be adopted in any consensus approach to HIT diagnosis. In Australia, a large study was recently completed, with samples sent to a reference laboratory for functional testing by WBIA and SRA and results have confirmed the utility of the WBIA as an alternative functional assay for HIT.

Figure 1 An algorithm on the indications of heparin-induced thrombocytopenia (HIT) functional assay based on pretest clinical scoring and immunoassay results. FA, functional assay; H/PF4 Ab; heparin/platelet factor 4 antibody assay.
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