Evaluation of flow cytometric HIT assays in relation to an IgG-specific immunoassay and clinical outcome

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Abstract

Background: Heparin-induced thrombocytopenia (HIT) is a severe side effect of heparin treatment caused by platelet activating IgG antibodies generated against the platelet factor 4 (PF4)-heparin complex. Thrombocytopenia and thrombosis are the leading clinical symptoms of HIT.

Methods: The clinical pretest probability of HIT was evaluated by the 4T score system. Laboratory testing of HIT was performed by immunological detection of antibodies against PF4-heparin complex (EIA) and two functional assays. Heparin-dependent activation of donor platelets by patient plasma was detected by flow cytometry. Increased binding of Annexin-V to platelets and elevated number of platelet-derived microparticles (PMP) were the indicators of platelet activation.

Results: EIA for IgG isotype HIT antibodies was performed in 405 suspected HIT patients. Based on negative EIA results HIT was excluded in 365 (90%) of cases. In 40 patients with positive EIA test result functional tests were performed. Platelet activating antibodies were detected in 17 cases by Annexin V binding. PMP count analysis provided nearly identical results. The probability of a positive flow cytometric assay result was higher in patients with elevated antibody titer. 71% of patients with positive EIA and functional assay had thrombosis.

Conclusions: EIA is an important first line laboratory test in the diagnosis of HIT, however, HIT must be confirmed by a functional test. Annexin V binding and PMP assays using flow cytometry are functional HIT tests convenient in a clinical diagnostic laboratory. The positive results of functional assays may predict the onset of thrombosis.

INTRODUCTION

Heparin-induced thrombocytopenia (HIT) is an immune response-mediated severe adverse effect of anticoagulant therapy by unfractionated (UFH) or low-molecular weight heparin (LMWH). Major clinical symptoms of HIT are thrombocytopenia and thrombosis due to the generation of IgG isotype HIT antibodies that recognize platelet factor 4 (PF4) bound to heparin. It causes heparin-dependent platelet activation through cross-linking FcγIIa receptors leading to the release of prothrombotic platelet-derived microparticles (PMPs) with platelet consumption and severe thrombocytopenia (1,2). Due to the severity of HIT, early and accurate diagnosis is essential to initiate a proper treatment, i.e. the cessation of heparin with simultaneous application of an alternative anticoagulant therapy in order to reduce the risk of thrombosis, organ failure, amputation or death (3).

The laboratory evaluation for clinically suspected HIT should focus on the detection of heparin-dependent platelet activation induced by HIT antibodies (4). Since testing is expensive and time-consuming, first the calculation of the 4T score based on four criteria (thrombocytopenia, timing of platelet count fall, thrombosis, and other causes for thrombocytopenia) needs to precede laboratory testing for HIT (5). The 4T score system calculates the pretest probability of HIT, it has a high negative predictive value. HIT is unlikely in patients with a low pretest probability of HIT, however, suspected HIT has to be validated by laboratory tests.

Two types of laboratory approaches are used for the diagnosis of HIT: (i) immunoassays for the detection of antibodies against PF4-heparin complex, and (ii) functional assays to detect the platelet activating potential of HIT antibodies (6). Immunoassays, especially the IgG specific solid-phase enzyme immunoassay (EIA), have a high negative predictive value, since negative antibody result excludes HIT (1,7,8). Previous studies have suggested that the degree of positivity of the immunoassay should be taken into consideration: the higher the antibody titer, the higher the probability of thrombosis (9-11).

In case of a positive immunoassay result, a functional test must be performed to detect the platelet activating HIT antibodies. The gold standard method for this assay is the serotonin release assay (SRA) that measures ^{14}C-serotonin released from activated ^{14}C-serotonin loaded washed donor platelets (12). The other frequently used functional assay is the heparin induced platelet activation test (HIPA), which detects washed donor platelet aggregation in the presence of patient plasma containing HIT antibody (13). Several years ago a sensitive and specific flow cytometric functional assay was described for HIT testing, in which HIT

antibodies in the presence of therapeutic concentration of heparin activated healthy donor's platelet rich plasma and the translocation of phosphatidylserine to the surface of activated platelets could be observed by using fluorescently labeled Annexin V (14). The elevated number of PMPs generated during platelet activation was also suggested as a surrogate marker for identifying HIT (15, 16).

Since nowadays flow cytometers are usually available in larger clinical diagnostic laboratories we thought to take advantage of the detection of Annexin V positivity and PMP generation by activated platelets as a complementary approach to diagnose functional HIT antibodies. The aim of this study was to evaluate the performance of this flow cytometric functional assay in relation to the 4T score system, optical density (OD) results of EIA and the development of thrombosis. This way the study intended to provide additional data for the assessment of this functional assay in the diagnosis of HIT.

MATERIALS AND METHODS

Study design, Patients and Samples

Between January 2009 and December 2014 plasma samples of 405 patients with physician-suspected HIT were evaluated in order to confirm or exclude the clinical diagnosis. HIT was suspected if there was a decrease in platelet count after the initiation of UFH or LMWH therapy in these hospitalized patients. We draw blood from healthy controls (n=4) and from patients receiving heparin treatment without suspected HIT (n=4) in order to validate our HIT assays. Plasma samples were obtained from 0.105 M trisodium citrate-anticoagulated whole blood in Vacutainer tubes (Becton Dickinson, San Jose, CA, USA) by the standard centrifugation procedure (1500 *g*, 15 min, 22^{o}C).

4T score

In order to calculate the 4T score value for patients, the clinical pathologist contacted the referring physician, and prior to the laboratory tests each patient was evaluated according to the four suggested criteria as published by Lo *et al.* to establish the clinical pretest probability of HIT (17). Clinical 4T scores were grouped as follows: 0–3: low probability; 4–5: intermediate probability; 6–8: high probability of HIT.

Solid-phase enzyme immunoassay (EIA)

To detect the IgG isotype anti-PF4/heparin antibodies the commercially available ZYMUTEST HIA IgG (Hyphen BioMed, Neuville-sur-Oise, France) kit was used according to the manufacturer's instructions. The measured OD value was directly proportional to the amount of IgG isotype heparin-dependent antibodies present in the patient's plasma. According to the OD values the result of the assay was graded as negative ($Abs_{450} \leq 0.5$, including the Abs_{450} values between 0.3-0.5 as grey zone), or positive ($Abs_{450} > 0.5$). The EIA test was carried out for all patients (n=405) independently from the 4T score values.

Flow cytometric functional assay

The functional test was carried out in borderline patients (OD=0.3-0.5; n=11) and patients with positive EIA results (n=40). In order to validate the flow cytometric assay, the test was also carried out in 40 randomly chosen patients with negative EIA results in addition to the healthy controls (n=4) and patients receiving heparin treatment without suspected HIT (n=4).

The Annexin V-binding assay described by Tomer *et al.* (14) was performed with some minor modifications. Platelet rich plasma (PRP) was obtained from trisodium citrate-anticoagulated blood of healthy donors having O blood type. To avoid donor platelet variation, during our tests PRP was obtained from the same selected high-responder donors as described previously (18). Donor PRP was separated by centrifugation (150 *g*, 5 min, 22^{o}C). Ten μL PRP was added to 10 μL patient plasma. The mixture was incubated in the presence of therapeutic (0.3 IU/mL) and excessive dose (100 IU/mL) of UFH in a final volume of 50 μL adjusted by PBS. HIT antibodies induced platelet activation only in the presence of therapeutic concentration of heparin, while excess of heparin disrupted antigen-antibody complexes, thus attenuated platelet activation (19). As positive controls, in each series of measurements we used patient plasma, which was previously found to be positive in our functional assay. After a 30 min incubation at 27^{o}C, 5 μL aliquots were removed from each tube and incubated in the presence of 5 μL phycoerythrin (PE)-labeled 20-fold diluted anti-CD41 antibody (Dako, Glostrup Denmark) for platelet identification and 1 μL Annexin V-fluorescein-isothiocyanate (FITC) (Becton Dickinson) for the detection of activated platelets. The samples were adjusted to a final volume of 50 μL with 0.02 M HEPES buffer (pH 7.3) containing 2.2 mM Ca^{2+}. After 15 min incubation at room temperature in the dark, samples were supplemented with 400 μL Ca^{2+}-containing HEPES buffer (pH 7.3) and analyzed by FACScan flow cytometer (Becton Dickinson); platelet acquisition time was 1 min with high flow rate. Platelets were identified by the immunofluorescence of the PE-labeled anti-CD41 antibody. In negative control samples, PRP without patient plasma but with 0.3 IU/mL UFH, less than 5% of platelets demonstrated Annexin V-binding. According to the report of Poley and Mempel in which Annexin V-binding flow cytometric test was compared to HIPA test, the result of the Annexin V-binding assay was considered positive if: (i) at least 11% of platelets were Annexin V positive after treatment with 0.3 IU/mL heparin, (ii) and the ratio of the percentage of Annexin V positive platelets generated in the presence of 0.3 IU/mL and that of 100 IU/mL of heparin was more than 1.5 (20). PMPs produced by activated platelets were identified by size-selection based on their forward (FSC) and side scatter (SSC) properties. Latex spheres were used to test the correctness of PMP gating. The result of the PMP assay was expressed as the ratio between the number of PMPs generated at 0.3 IU/mL and 100 IU/mL of heparin. Positive result was considered when the PMP ratio was 1.5 or higher.

Statistical analysis

Mean values of the heparin-dependent EIA OD parameters of patient groups with either positive or negative Annexin V-binding functional assay results were compared by unpaired Student's *t*-test. Logistic regression analysis was used to estimate the effect of EIA OD units (continuous explanatory variable) on the outcome of the flow cytometric functional assays and on the risk of thrombosis (dichotomous dependent variables). Odds ratio with 95% confidence intervals was calculated using STATA 9.0 software, a P value <0.05 was considered statistically significant.

RESULTS

Patient characteristics and laboratory test results in HIT-suspected patients

Out of the 405 IgG-specific EIA tests carried out on suspected HIT patients, 365 proved to be negative (354 patients with OD=<0.3 and 11 patients with borderline result, OD=0.3-0.5). On the basis of these negative immunoassay results HIT was excluded in 90% of all enrolled patients (365/405). Plasma samples of the remaining 40 patients contained heparin-dependent IgG isotype antibody (EIA OD range: 0.589–3.382). The functional assays were performed in samples of all EIA positive patients (n=40) in addition to 40 randomly chosen EIA negative patients, 11 borderline/grey zone cases, 4 healthy controls (EIA OD=<0.3) and 4 patients receiving heparin treatment without suspected HIT (EIA OD=<0.3). Out of the 40 EIA positive patients the Annexin V-binding assay was positive in 17, while the PMP assay was positive in 14 cases. In two cases (one EIA positive and one EIA borderline patient) PMP results were not available. Similarly to controls, patients with borderline or negative EIA results had negative functional assay results confirming HIT negativity.

Representative results of the two functional flow cytometric assays are depicted in Fig. 1. HIT negative control plasma did not activate donor platelets, however, plasma sample of a HIT patient stimulated donor platelets in the presence of pharmacological heparin concentration (0.3 IU/mL). The fact that in the presence of excessive amount of heparin (100 IU/mL) platelets were not activated - or significantly less activated - proves the strict heparin concentration dependency of platelet activation (19). The clinical and laboratory data of the 17 patients with positive Annexin V-binding functional assay results are shown in Table 1. The majority of the patients (n=11) were surgical cases, the remaining 6 patients were medical cases. Almost 2/3rd of these patients were female (64.7%), which was in agreement with a previous report (21). In all 17 HIT patients, heparin administration was discontinued and 14 patients were switched to alternative anticoagulants (lepirudin or rivaroxaban). After the cessation of heparin treatment, platelet count recovered which was a clear indicator of HIT except for 3 patients who subsequently died. In Table 2 we summarized the clinical and laboratory data of EIA positive but functional test negative (n= 23) patients, including 10 medical and 13 surgical cases. Out of these 23 cases 18 patients were not treated with an alternative anticoagulant. Seven of these 18 patients died from causes other than thrombosis, their death were due to multiorgan failure, in 7 patients the platelet count recovered spontaneously. In the remaining 4 cases, platelet count did not recover, however thrombocytopenia had causes other than HIT. Five patients were given alternative

anticoagulants, in 2 of them the platelet count remained low, in 3 cases (1 medical, 2 cardiac surgery cases) the platelet count recovered. The medical and one of the surgical patients had gastrointestinal bleeding and they died one month after the laboratory testing for HIT due to hypoxia and septicemia, respectively. It is to be noted that none of the Annexin V positive patients had low pretest probability of HIT based on their 4T score, their mean 4T score value was 5.71. The mean 4T score of EIA positive but functional assays negative patients was 4.22 while that of EIA negative patients was 3.63.

Association of heparin-dependent antibody level with functional test positivity

In patients with positive (n=40) and borderline (n=11) EIA results the functional assays were also carried out. First, the association between the IgG isotype EIA OD values with the outcome of the Annexin V-binding assay (Fig. 2A) and with that of PMP assay (Fig. 2B) were analyzed, only those patients are shown for whom both assay results are available (n=49). There were two Annexin V-binding positive cases in which negative results were obtained with the PMP assay (Table 1). There was a positive association between the EIA OD values and the functional assay positivity: the higher the IgG isotype EIA OD value was measured, the higher the probability of a positive functional assay result was found.

In patients with a negative Annexin V-binding assay result and a positive EIA test (n=23) the average EIA OD value was 1.330. However, in those with positive functional assay results (n=17), the average EIA OD value was 2.544, which was significantly higher than the OD values in the former group ($P<0.0001$).

We used a logistic regression analysis to calculate the strength of the association between IgG isotype EIA OD values and the dichotomous outcome of the two flow cytometric functional assays. The quantitative OD results of the EIA test were significantly associated with the positive outcome of the Annexin V-binding assay and PMP assay. The odds of positive Annexin V-binding assay result was elevated for each unit increment in the corresponding independent variable (OD unit) as follows: OR: 6.38 (95%CI, 2.58–15.78; $P= 0.0001$); for the PMP assay these values were: OR: 4.64 (95%CI, 2.08–10.35; $P= 0.0002$).

Association of heparin-dependent antibody level and functional test positivity with thrombosis

Since the most severe adverse event associated with HIT is thrombosis, we statistically analyzed the relationship between heparin-dependent antibody levels and the frequency of thrombotic events. Fig. 2C demonstrates that higher level of heparin-dependent antibody was

associated with increased probability of thrombosis. Among subjects with positive EIA OD values (n=40) the overall rate of thrombotic events was 30% (12/40). The logistic regression analysis showed that the OD values of the EIA test were significantly associated with the presence of thrombosis in patients. The odds for having thrombosis was raised for each unit elevation in the OD value: OR: 3.05 (95%CI, 1.51–6.18; *P*= 0.002).

The positive flow cytometric assay results detecting platelet activation also showed a good agreement with the presence of thrombosis in our group of patients (Table 1, Figs. 2A and B). 70.6% (12/17) of patients with both EIA and Annexin V test positivity showed thrombosis (Table 1). The majority of PMP positive cases, 10 out of 14, also suffered from thrombosis.

Association of clinical pretest probability of HIT and functional test positivity

We also assessed the pretest probability of HIT by applying the 4T score system in our patient cohort. Based on these score values, EIA positive patients were classified as groups with low (n=5), intermediate (n=26) and high (n=9) pretest probability of HIT. In individuals with low pretest probability of HIT, the functional assay results were negative, showing that platelet activating antibodies were not present in these samples even in cases with high antibody titer (Fig. 3). At intermediate pretest probability the antibody titer was higher in patients possessing platelet-activating antibodies, and the functional tests were positive for all patients with an OD≥2.633. With a single exception, all patients with high pretest probability of HIT had positive Annexin V binding assay result, as well. There was a single patient with high 4T score and thrombosis in whom a relatively low EIA OD value (OD=0.589) was associated with positive Annexin V-binding and negative PMP assay result.

DISCUSSION

In our single center study, we assessed the performance of the Annexin V-binding flow cytometric assay in the diagnosis of HIT. The results of this functional assay were analyzed in comparison with the OD values of EIA assay to determine the association between the antibody titer and platelet activating potential of the plasma sample. Functional test results and EIA OD values were also compared to the 4T score values and the occurrence of thrombosis.

So far only a limited number of studies have been published on this flow cytometric functional assay. Our intention was to provide additional data to assess the clinical usefulness of this assay in the laboratory diagnosis of HIT. Moreover, this is the first study, which compares the flow cytometric functional assay results with the distribution of EIA OD values. We found a significant positive association between the OD values and the positivity of the functional assay result. Warkentin *et al.* also reported a positive association between the degree of the EIA OD positivity and the positive outcome of the SRA test (10). The fact that the magnitude of the EIA OD value was positively associated with the platelet-activating potential of the HIT antibody emphasized the importance of the exact EIA OD value in the interpretation of the immunoassay results (4,9,22).

During our six-year long study, 405 suspected HIT patients were investigated. In 365 patients representing 90% of all suspected cases, HIT was excluded based on the low EIA OD values (OD<0.5). Similar finding has been published previously (1). On the other hand, the combined use of EIA and Annexin V-binding test confirmed HIT in 4.2% of all cases (17/405). In a previous study Greinacher et al. reported that immunoassay and washed platelet activation assay, HIPA verified the diagnosis of HIT in almost 6% of suspected HIT patients (1). Among our EIA positive patients 42.5% (17/40) had positive results with Annexin V-binding flow cytometric functional assay, while the same ratio was higher, 48.6% (17/35) among the EIA positive and clinically suspected HIT patients (4T score>3). According to previous publications platelet activating antibodies are present in only one third to one half of EIA positive patients (1,23,24). The actual value strongly depends on the patient population and the antibody class specificity of the EIA assay used for diagnosis. Our result is in good agreement with these data. The results suggest that the disease might be considerably over-diagnosed using only EIA assay without a functional test; EIA may detect non-platelet activating antibodies as well (22). The PMP region analysis is a convenient flow assay to predict the platelet activating ability of the patient plasma, however, it proved to be somewhat less sensitive for predicting thrombosis at lower antibody OD values.

We carried out the laboratory investigation for all physician-suspected HIT patients, and the results supported the recommendation that in patients with a low pretest probability score (4T score ≤ 3) HIT could be confidently excluded without laboratory testing. None of our EIA positive patients with low 4T score had positive functional assay result and thrombosis in this large cohort of HIT suspected patients.

According to the literature 69% of the EIA and SRA positive patients suffered from thrombosis (25). In our study 71% (12/17) of patients with EIA and Annexin V-binding functional test positivity had thrombosis, which means that 30% (12/40) of the immunoassay positive cases had thrombotic events. It is noteworthy that these patients had high OD values in their EIA test with a single exception. We also found that higher heparin-dependent antibody titer (expressed as OD value) was significantly associated with an increased probability of thrombosis.

A limitation of our study is that we did not compare flow cytometric results with that of an established reference test, like SRA or HIPA. SRA is a sensitive test for laboratory confirmation of HIT, however, it is not suitable for routine testing since it uses radioactive substances [^{14}C-serotonin]. It is a technically demanding, time-consuming test (12, 26) hence, the test is not available in most clinical routine laboratories restricting its use for clinicians. The other washed platelet assay, HIPA is also a specific and sensitive test to detect clinically significant HIT antibodies. However, the appropriate donor platelet selection is essential for correct results in HIPA and the critically important washing steps also represent a crucial handling problem (27). Indeed, in our preliminary experiments in a series of trisodium citrated samples, whole blood displayed 1.6% of mean platelet P-selectin positivity that became only slightly elevated in PRP (3.5%), but was significantly higher in washed platelets (30-41%) (data not shown). This means that washed platelets used for both SRA and HIPA became considerably activated already during the isolation procedures that may bias further functional responses.

The Annexin V-binding flow cytometric assay was shown to be an accurate test for the diagnosis of HIT. Previously Tomer then Poley and Mempel compared its sensitivity and specificity to these of the gold standard SRA and HIPA tests. The sensitivity of the flow cytometric assay was found to be 95% and 100% when it was compared to SRA and HIPA respectively, the specificity of the assay was 100% in both comparisons (14, 20).

Even in early reports about the laboratory diagnosis of HIT the authors suggested that a flow cytometric assay can serve as a fast, accurate and cost effective test for this sometimes life-threating state, but as they described a special skill is needed to run a flow cytometer (20). Even in recent publications about the diagnosis of HIT, flow cytometry is described as one of the future possibilities (16,18,28). In the past decade, flow cytometry has become an everyday practice in clinical laboratories by the introduction of novel compact size flow cytometers with user friendly softwares and it is gradually gaining space in confirming the

diagnosis of HIT (24, 29-33). Based on our current results it is concluded, that flow cytometry reliably predicts clinical outcome and is a useful aid in avoiding the over-diagnosis of HIT.

CONFLICT OF INTEREST DECLARATION

The authors declare that they have no conflicting interests.

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REFERENCES

1. Greinacher A, Juhl D, Strobel U, Wessel A, Lubenow N, Selleng K, Eighler P, Warkentin TE. Heparin-induced trombocytopenia: a prospective study on the incidence, platelet – activating capacity and clinical significance of antiplatelet factor4/heparin antibodies of the IgG, IgM and IgA classes. J Thromb Haemost 2007;5:1666-1673.

2. Warkentin TE. Heparin-Induced thrombocytopenia: IgG-Mediated platelet activation, platelet microparticle generation, and altered procoagulant/anticoagulant balance in the pathogenesis of thrombosis and venous limb gangrene complicating heparin-Induced thrombocytopenia. Transfus Med Rev 1996;10:249–258.

3. Wilke T, Tesch S, Scholz A, Kohlmann T, Greinacher A. The costs of heparin-induced thrombocytopenia: a patient-based cost of illness analysis. J Thromb Haemost 2009;7:766-773.

4. Warkentin TE, Greinacher A, Gruel Y, Aster RH, Chong BH. Laboratory testing for heparin-induced thrombocytopenia: a conceptual framework and implications for diagnosis. J Thromb Haemost 2011;9:2498-2500.

5. Cuker A, Gimotty PA, Crowther MA, Warkentin TE. Predictive value of the 4T scoring system for heparin-induced thrombocytopenia: a systematic review and meta-analysis. Blood 2012;120:4160-4167.

6. Greinacher A, Althaus K, Krauel K, Selleng S. Heparin-induced thrombocytopenia. Hamostaseologie 2010;30:17-28.

7. Meyer O, Salama A, Pittet N, Schwind P. Rapid detection of heparin-induced platelet antibodies with particle gel immunoassay (ID-HPF4). Lancet 1999;354:1525-1526.

8. Warkentin TE, Sheppard JA, Moore JC, Moore KM, Sigouin CS, Kelton JG. Laboratory testing for the antibodies that cause heparin-induced thrombocytopenia: how much class do we need? J Lab Clin Med 2005;146:341-346.

9. Baroletti S, Hurwitz S, Conti NAS, Famikos J, Piazza G, Goldhaber SZ. Thrombosis in suspected heparin-induced thrombocytopenia occurs with high antibody levels. Am J Med 2012;125:44-49.

10. Warkentin TE, Sheppard JI, Moore JC, Sigouin CS, Kelton G. Quantitative interpretation of optical density measurements using PF4-dependent enzyme-immunoassays. J Thromb Haemost 2008;6:1304-1312.

11. Kim SY, Kim HK, Han KS, Kim I, Yoon S, Park S, Kim BK. Utility of EIA Optical Density Values and Clinical Scores for the Diagnosis of and thrombosis prediction in Heparin-induced thrombocytopenia. Korean J Lab Med 2011;31:1-8.

12. Sheridan D, Carter C, Kelton JG. A diagnostic test for heparin- induced thrombocytopenia. Blood 1986; 67:27–30.

13. Greinacher A, Michels I, Kiefel V, Mueller-Eckhardt C. A rapid and sensitive test for diagnosing heparin-associated thrombocytopenia. Thromb Haemost 1991;66:734-736.

14. Tomer A. A sensitive and specific functional flow cytometric assay for the diagnosis of heparin-induced thrombocytopenia. Brit J Haematol 1997;98:648-656.

15. Lee DH, Warkentin TE, Denomme GA, Hayward CPM, Kelton JG. A diagnostic test for heparin- induced thrombocytopenia: detection of platelet microparticles using flow cytometry. Br J Haematol 1996; 95:724–731.

16. Mullier F, Minet V, Bailly N, Devalet B, Douxfils J, Chatelain C, Elalamy I, Dogne JM, Chatelain B. Platelet microparticle generation assay: A valuable test for immune heparin-induced thrombocytopenia diagnosis. Thromb Res 2014;133:1068-1073.

17. Lo GK, Juhl D, Warkentin TE, Sigouin CS, Eichler P, Greinacher A. Evaluation of pretest clinical score (4T's) for the diagnosis of heparin-induced thrombocytopenia in two clinical settings. J Thromb Haemost 2006;4:759-765.

18. Bakchoul T, Zollner H, Greinacher A. Current insights into the laboratory diagnosis of HIT. Int J Lab Haematol 2014;36:296-305.

19. Greinacher A, Michels I, Mueller-Eckhardt C. Heparin-Associated Thrombocytopenia – the antibody is not heparin specific. Thromb Haemost 1992;67:545-549.

20. Poley S, Mempel W. Laboratory diagnosis of heparin-induced thrombocytopenia: advantages of a functional flow cytometric test in comparison to the heparin-induced platelet-activation test. Eur J Haematol 2001;66:253-262.

21. Warkentin TE, Sheppard JI, Sigouin, CS, Kohlmann T, Eichler P, Greinacher A. Gender imbalance and risk factor interactions in heparin-induced thrombocytopenia. Blood 2006;108:2937-2941.

22. Warkentin TE. How I Diagnose and Manage HIT. Hematology Am Soc Hematol Educ Program. 2011;2011:143-149.

23. Warkentin TE, Sheppard JI, Horsewood P, Simpson PJ, Moore JC, Kelton JG. Impact of the patient population on the risk for heparin-induced thrombocytopenia. Blood 2000;96:1703-1708.

24. Vitale M, Tazzari P, Ricci F,3 Mazza MA, Zauli G, Martini G, Caimi L, Manzoli FA, Conte R. Comparison Between Different Laboratory Tests for the Detection and Prevention of Heparin-Induced Thrombocytopenia. Cytometry (Communications in Clinical Cytometry) 2001;46:290–295.

25. Lo GK, Sigouin CS, Warkentin TE. What is the potential for overdiagnosis of heparin-induced thrombocytopenia? Am J Hematol 2007;82:1037-1043.

26. Price EA, Hayward CP, Moffat KA, Moore JC, Warkentin TE, Zender JL. Laboratory testing for heparin-induced thrombocytopenia is inconsistent in North America: a survey of North American specialized coagulation laboratories. Thromb Haemost 2007;98:1357-1361.

27. Eichler P, Budde U, Haas S, Kroll H, Loreth RM, Meyer O, Pachmann U, Potzsch B, Schabel A, Albrecht D, Greinacher A. First Workshop for detection of heparin-induced antibodies: validation of the heparin-induced platelet-activation test (HIPA) in comparison with a PF4/heparin ELISA. Thromb Haemost 1999;81:625-629.

28. Warkentin TE. Platelet microparticle generation assay for detection of HIT antibodies: advance, retreat, or too soon to tell? Thromb Res 2014;133:957-958.

29. Denys B, Stove V, Philippe J, Devreese K. A clinical-laboratory approach contributing to a rapid and reliable diagnosis of heparin-induced thrombocytopenia. Thromb Res 2008;123:137-145.

30. Denys B, Devreese K. A clinical-laboratory approach contributing to a rapid and reliable diagnosis of heparin-induced thrombocytopenia: an update. Thromb Res 2009;124:642-643.

31. Solano C, Mutsando H, Self M, Morell-Kopp MC, Mollee P. Using HitAlert flow cytomerty to detect heparin-induced thrombocytopenia antibodies in a tertiary care hospital. Blood Coagul Fibrinolysis 2013;24:365-370.

32. Olah Z, Kerenyi A, Kappelmayer J, Schlammadinger A, Razso K, Boda Z. Rapid-onset heparin-induced thrombocytopenia without previous heparin exposure. Platelets 2012;23(6):495-498.

33. Gobbi G, Mirandola P, Tazzari PL, Talarico E, Caimi L, Martini G, Papa S, Conte R, Manzoli FA, Vitale M. New Laboratory Test in Flow Cytometry for the Combined Analysis of Serologic and Cellular Parameters in the Diagnosis of Heparin-Induced Thrombocytopenia. Cytometry Part B (Clinical Cytometry) 2004;58B:32–38.

Figure Legends

Fig. 1. Flow cytometric functional assays for HIT. Results with a representative HIT negative control and a HIT positive patient's sample. Donor platelet-rich plasma was incubated with control plasma or with plasma from a HIT patient in the presence of pharmacological concentration (0.3 IU/mL) or excessive dose (100 IU/mL) of heparin. (A) Platelet-derived microparticles were identified by size-selection, the number of generated microparticles is provided in the lower left rectangle. (B) Platelet activation induced by HIT plasma in the presence of 0.3 IU/mL of heparin was detected by the elevated percentage of Annexin V positive platelets (shown in the upper right quadrant).

Fig. 2. Association between the IgG-specific anti-PF4/heparin antibody (EIA-IgG) titer with (A) the result of the flow cytometric assay detecting Annexin V-binding, (B) the results of PMP determination, and (C) thrombotic outcome.

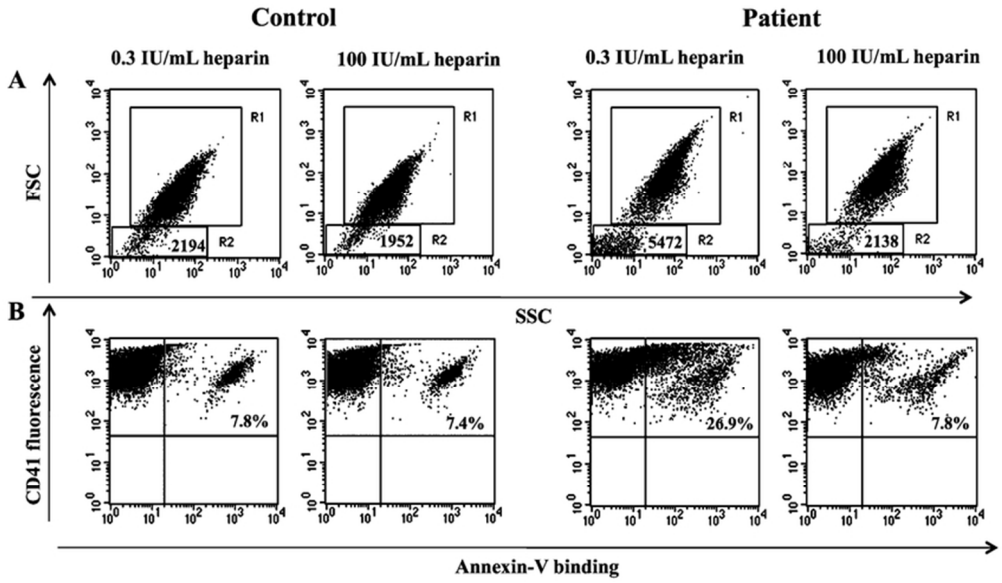
Fig. 3. Distribution of the platelet activating and non-activating IgG-specific antibody titer (EIA-IgG OD units) according to the 4T score. Open circle: negative for Annexin V binding and platelet microparticle (PMP) assay; solid circle: positive for both assays; half filled circle: Annexin V assay positive, PMP assay negative; solid square: Annexin V binding positive, PMP was not investigated.

Table 1. Laboratory and clinical data of HIT patients with positive Annexin V-binding assay result

Patient		Laboratory results			Clinical data			Follow up	
	Male (M)/ Female (F)	EIA (OD)	Annexin V binding assay	Platelet MP assay	4T score	Thrombosis	Surgical or medical patient	Alternative anticoagulant	Platelet count recovery
1	M	3.382	+	+	5	+	surgical	+	+
2	M	3.273	+	+	7	+	surgical	+	+
3	F	3.262	+	+	5	-	surgical	+	+
4	F	3.206	+	+	6	+	surgical	-	death
5	M	3.028	+	+	4	-	medical	+	+
6	F	3.010	+	+	5	+	surgical	-	death
7	F	2.967	+	not determined	5	-	surgical	+	+
8	F	2.920	+	+	5	+	surgical	+	+
9	F	2.898	+	+	6	+	medical	+	+
10	F	2.798	+	+	5	-	surgical	+	+
11	F	2.633	+	+	5	-	surgical	+	+
12	M	2.441	+	+	7	+	medical	+	death
13	M	2.341	+	+	7	+	surgical	-	+
14	M	1.801	+	+	7	+	medical	+	+
15	F	1.405	+	+	7	+	surgical	+	+
16	F	1.303	+	-	5	+	medical	+	+
17	F	0.589	+	-	6	+	medical	+	+

Table 2. Laboratory and clinical data of suspected HIT patients with negative Annexin V-binding assay result

Patient		Laboratory results			Clinical data			Follow up	
	Male (M)/ Female (F)	EIA (OD)	Annexin V binding assay	Platelet MP assay	4T score	Thrombosis	Surgical or medical patient	Alternative anticoagulant	Platelet count recovery
1	F	2.968	-	-	3	-	medical	-	+
2	M	2.664	-	-	3	-	surgical	+	-
3	M	2.450	-	-	5	-	medical	-	+
4	M	2.350	-	-	4	-	surgical	-	death
5	F	2.218	-	-	3	-	medical	-	death
6	F	2.031	-	-	5	-	surgical	+	+
7	M	1.381	-	-	4	-	surgical	-	+
8	F	1.316	-	-	4	-	medical	-	-
9	F	1.292	-	-	5	-	surgical	-	-
10	F	1.238	-	-	5	-	surgical	+	-
11	F	1.200	-	-	4	-	surgical	-	death
12	F	0.988	-	-	5	-	medical	+	+
13	M	0.939	-	-	3	-	surgical	-	-
14	F	0.903	-	-	5	-	medical	-	+
15	M	0.897	-	-	4	-	medical	-	death
16	F	0.865	-	-	5	-	surgical	+	+
17	M	0.809	-	-	4	-	surgical	-	-
18	M	0.775	-	-	6	-	surgical	-	death
19	F	0.697	-	-	3	-	surgical	-	death
20	F	0.694	-	-	4	-	medical	-	+
21	F	0.666	-	-	4	-	medical	-	+
22	M	0.636	-	-	4	-	medical	-	death
23	M	0.615	-	-	5	-	surgical	-	+



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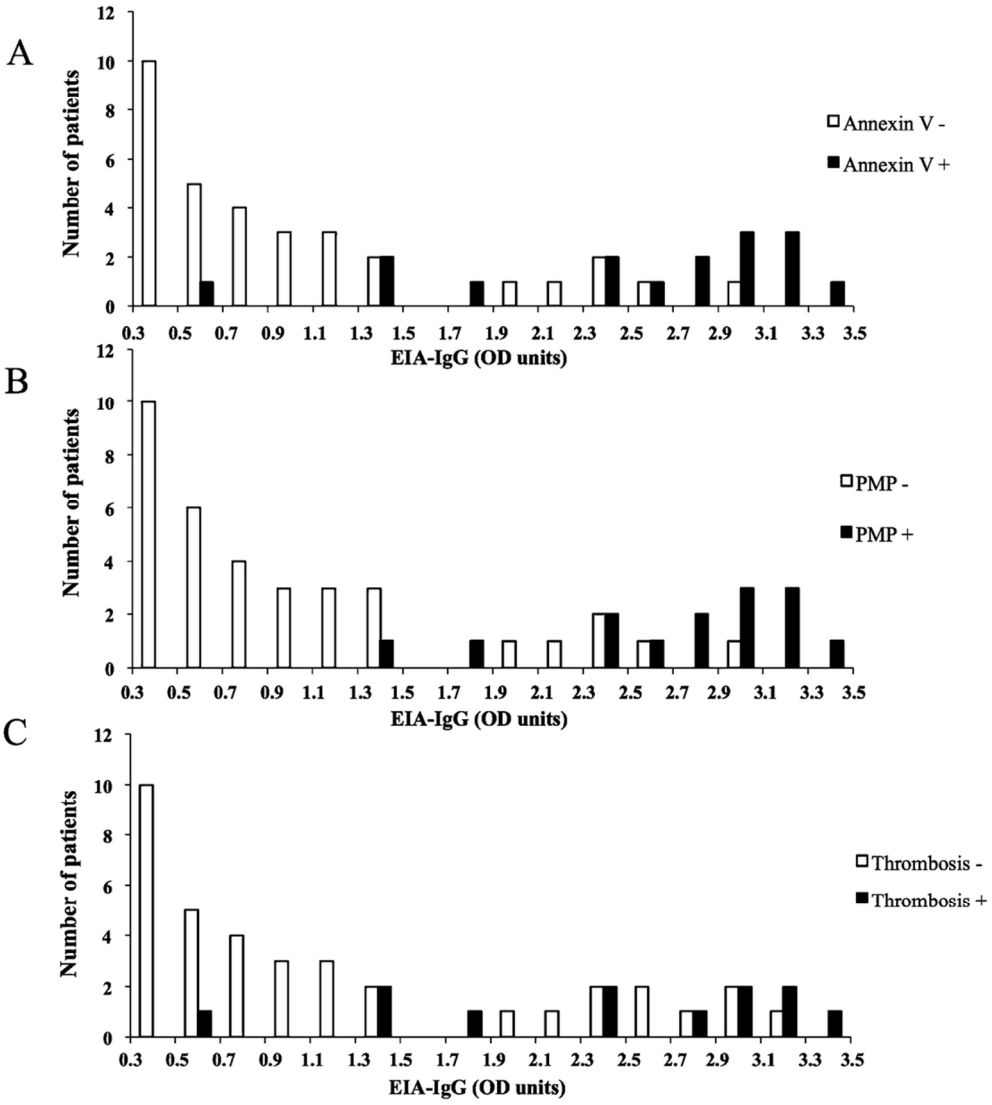

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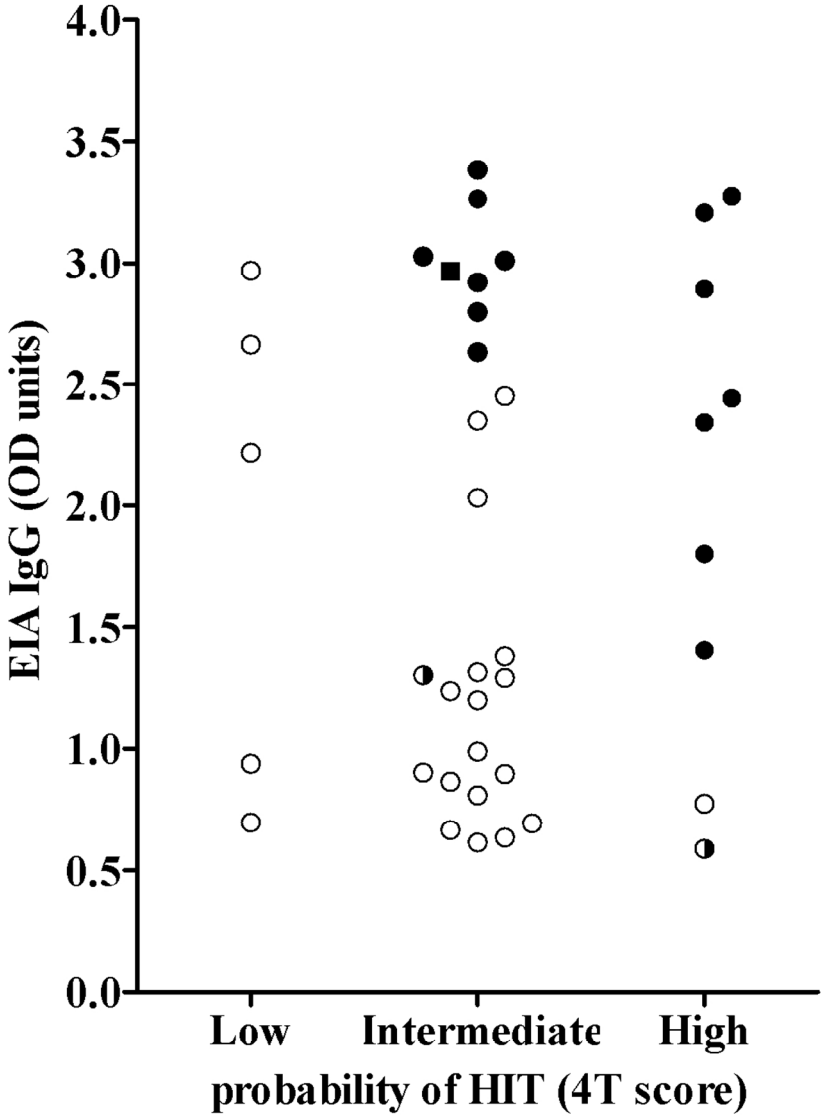

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