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ORIGINAL ARTICLE

Heparin-induced thrombocytopenia: a prospective study on the incidence, platelet-activating capacity and clinical significance of antiplatelet factor 4/heparin antibodies of the IgG, IgM, and IgA classes

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Summary. *Introduction:* Platelet-activating antiplatelet factor 4/heparin (anti-PF4/heparin) antibodies are the major cause of heparin-induced thrombocytopenia (HIT). However, the relative utility of functional (platelet activation) vs. antigen [enzyme-immunoassay (EIA)] assays, and the significance of assay discrepancies remain unresolved. *Methods:* Consecutive patient sera ($n = 1650$) referred for diagnostic HIT testing were screened prospectively by both the heparin-induced platelet activation (HIPA) test and anti-PF4/heparin EIA – including individual classes (IgG, IgA, IgM) – with clinical correlations studied. Platelet microparticle and annexin-V-binding properties of the sera were also investigated. *Results:* Only 205 (12.4%) sera tested positive in either the HIPA and/or EIA: 95 (46.3%) were positive in both, 109 (53.1%) were only EIA-positive, and, notably, only one serum was HIPA-positive/EIA-negative. Of 185 EIA-positive sera, only 17.6% had detectable IgM and/or IgA without detectable IgG. Among sera positive for EIA IgG, optical density values were higher when the sera were HIPA-positive (1.117 vs. 0.768; $P < 0.0001$), with widely overlapping values. Two HIPA-positive but EIA-IgG-negative sera became HIPA-negative following IgG depletion, suggesting platelet-activating antibodies against non-PF4-dependent antigens. Clinical correlations showed that HIPA-negative/EIA-positive patients did not develop thrombosis and had reasons other than HIT to explain thrombocytopenia. IgM/A antibodies did not increase microparticle penetration, but increased annexin-V binding. *Conclusions:* The anti-PF4/heparin EIA has high (~99%) sensitivity for HIT. However, only about half of EIA-positive patients are likely to have HIT. Anti-PF4/heparin antibodies of IgM/A class and non-PF4-dependent antigens have only a minor role in HIT.

Keywords: anti-PF4/heparin EIA, heparin, heparin-induced thrombocytopenia, platelet activation assays, platelet factor 4.

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Introduction

Heparin-induced thrombocytopenia (HIT) is an important adverse drug reaction caused by platelet-activating antibodies reactive against complexes between heparin and chemokine proteins, particularly platelet factor 4 (PF4). Despite its transient, self-limited nature [1,2], many patients with HIT develop life- or limb-threatening thromboembolic complications [3]. Because of the increasing use of low-molecular-weight heparin (LMWH) thromboprophylaxis in elective surgery, with its tenfold lower risk of HIT compared with unfractionated heparin (UFH) [4], HIT is now becoming relatively more common among medical and other severely ill patients, in whom UFH is often preferred [5]. In these patients, thrombocytopenia or new thrombosis can be explained either by HIT or by the underlying medical problem(s) [6]. Laboratory detection of 'HIT antibodies' is thus important to support or refute the diagnosis of HIT.

Two types of serological assays are available for laboratory detection of HIT antibodies [7]. Functional (platelet activation) assays using washed platelets, such as the platelet ^{14}C-serotonin-release assay [8,9] or the heparin-induced platelet activation (HIPA) test [10,11], primarily detect platelet-activating antibodies of the IgG class, whether they are reactive against PF4/heparin or other heparin-dependent antigens [12]. In contrast, enzyme-immunoassays (EIAs) employing PF4/heparin- or PF4/polyvinylsulfonate complexes [13–17] detect antibodies only when PF4 is the heparin-dependent protein; they also detect non-platelet-activating antibodies. These

non-functional antibodies can also be IgG, but are more likely to be of the IgM and IgA classes [18].

Thus, there are two major problems with the EIAs. Firstly, the anti-PF4/heparin antibodies detected may not be platelet-activating, and consequently not pathogenic [19–21]. Secondly, these assays cannot detect antibodies against heparin-dependent antigens other than PF4. Few studies have examined what fraction of EIA-positive sera contain non-functional antibodies, or determined their antibody class composition. Furthermore, it is unclear whether, in addition to PF4/polyanion-based antigen assays, other tests including EIAs for antibodies reactive against other heparin-dependent proteins [e.g. interleukin-8 (IL-8), neutrophil-activating peptide-2 (NAP-2)] would improve laboratory diagnosis of HIT significantly. The purpose of this prospective study was to analyze systematically a large number of samples referred to a reference laboratory for diagnosis of HIT, and to address the following questions.

1 How many patients with a clinical suspicion of HIT tested positive in the anti-PF4/heparin EIA and/or the HIPA test?
2 How many of the positive anti-PF4/heparin EIA test results are caused by IgG antibodies, and how many by IgM/IgA antibodies only?
3 Do anti-PF4/heparin antibodies of IgM/IgA class induce platelet procoagulant changes as assessed by annexin-V binding and/or generation of platelet-derived microparticles?
4 How frequently do thrombotic events occur in patients with anti-PF4/heparin IgM/IgA (but not IgG) antibodies?
5 How frequently are antibodies detected that are negative in the EIA but positive in the HIPA test?

Our hypothesis was that a positive EIA in the absence of platelet-activating antibodies was unlikely to represent clinical HIT. We sought also to identify the frequency and significance of EIA-negative samples that none the less tested positive in the platelet activation assay for heparin-dependent antibodies.

Patients, material and methods

We evaluated sera of 1650 patients referred for diagnostic HIT testing from consecutive patients throughout Germany in whom the treating physician suspected HIT or intended to rule out HIT, during an 18-month period ending July 2004. All sera were screened using both the HIPA test [10,11] and an in-house anti-PF4/heparin EIA [5] that detects all three major immunoglobulin classes [anti-PF4/heparin IgG/A/M, positive test >0.5 optical density (OD) units]. Sera were stored at −70 °C before further investigation by EIA for anti-PF4/heparin antibodies of the individual immunoglobulin classes, IgG, IgM, and IgA, as described [5].

Clinical information was obtained by a standardized questionnaire and by telephone interview of the treating physician.

HIPA test

The HIPA test was performed as described [10,11]. Serum, IgG-depleted serum, and the IgG-fraction were incubated with washed platelets of four normal donors at low (0.2 IU mL^{-1}) and high (100 IU mL^{-1}) heparin concentrations. The activation profile also included the use of platelets after blocking their FcγIIa receptor using the monoclonal antibody (mAb) IV-3 [22]. A known serum containing anti-PF4/heparin IgG and serum of a healthy blood donor served as controls. An 'indeterminate' HIPA test result was defined as platelet activation at both low and high heparin concentrations.

IgG-depletion of HIPA positive sera containing no IgG anti-PF4/heparin antibodies by EIA

One milliliter protein-G-Sepharose (Amersham Biosciences, Freiburg, Germany) was pelleted (0.5 min $120 \times g$), incubated with 500 μL, heat-inactivated (56 °C, 45 min) patient serum [1 h at room temperature (RT)], and centrifuged (0.5 min, $120 \times g$). Protein-G-Sepharose was washed several times in binding buffer (20 mM sodium phosphate buffer, pH 7.0), carried over in a column, and eluted by 1.5 mL 0.1 M glycine buffer, pH 2.7. Eluates were collected in tubes containing 50 μL 1 M Tris–HCl, pH 9.0, dialyzed for at least 2 h at RT against 0.9% NaCl under constant stirring.

Platelet activation by anti-PF4/heparin IgM and IgA antibodies

Anti-PF4/heparin antibody-positive sera containing only IgM ($n = 5$), IgA ($n = 2$), or both ($n = 1$) and of which at least 3 mL were available, were assessed for their capacity to induce annexin-V binding and platelet microparticle generation, compared with anti-PF4/heparin IgG-positive sera (positive control), and the following negative controls: group 1, healthy blood donors ($n = 10$); group 2, patients developing thrombocytopenia after at least five days of heparin treatment, but having negative HIT tests ($n = 20$); group 3, patients with thromboembolic complications during heparin therapy but having negative HIT tests ($n = 20$).

Expression of a procoagulant surface, measured by annexin-V binding For annexin-V binding [23], washed platelets of normal donors were prepared as for the HIPA test and incubated with the respective sera in the presence of 0.2 IU mL^{-1} heparin for 45 min under stirring. The reaction was stopped by 100 μL cold (4 °C) 0.5% EDTA solution. Subsamples (10 μL) with 40 μL phosphate buffered saline (PBS), pH 7.2 were centrifuged (5 min, $200 \times g$); pellets were resuspended in 100 μL HEPES buffer (10 mM HEPES in NaOH, pH 7.4, 140 mM NaCl and 5 mM $CaCl_2$) containing 10 μL phycoerythrin (PE)-conjugated anti-CD42a antibody (Biozol, Eching, Germany) and 2 μL annexin-V-fluorescein-isothiocyanate (FITC, Roche Diagnostics, Penzberg, Germany), incubated (15 min, RT in the dark), diluted with 500 μL HEPES buffer and analyzed immediately by flow cytometry (FACScan; Becton-Dickinson, San Jose, CA, USA). 10 000 events were measured for each sample.

Generation of platelet-derived microparticles For assessment of microparticle generation [24], 20 μL of serum were incubated with washed platelets and heparin 0.2 IU mL^{-1} under stirring (45 min, RT); the reaction was stopped with 100 μL cold 0.5% EDTA. 50 μL of the platelet suspension were incubated with 7.5 μL mouse anti-CD42a mAb (Serotec Ltd, Oxford, UK; 0.5 mg mL^{-1} final concentration; 30 min; 37 °C), diluted with 1 mL FACS flow, centrifuged (5 min; 2000 × *g*), incubated with 7.5 μL FITC-labelled antimouse conjugate (DAKO, Glostrup, Denmark; 30 min in the dark, RT), washed (1 mL FACS flow, 5 min, 2000 × *g*), resuspended in 500 μL FACS flow and analyzed immediately by flow cytometry. At least 5000 events were measured. Platelet microparticles were distinguished from platelets by their characteristic flow cytometric profile for forward scatter vs. FITC fluorescence by two gates. The first gate highlighted platelets and platelet-derived microparticles; the second gate highlighted only microparticles. To quantitate microparticle generation, the percentage of events in the microparticle gate in relation to all events in the first gate was used.

Statistical analysis

For statistical analysis we used SAS, version 9.0 (SAS Institute Inc., Cary, NC, USA). Comparisons were performed using a Wilcoxon's rank-sum test. A *P*-value < 0.05 was considered as statistically significant.

Results

Number of samples testing positive in the anti-PF4/heparin EIA and in the HIPA test

Of 1650 referred patient samples, 205 (12.4%) tested positive in either assay (Table 1). Of these, 95 sera (46.3%) were positive in both the EIA and HIPA, and 100 sera (48.8%) were positive only in the EIA; nine sera (4.4%) were positive in the EIA but showed an indeterminate result in the HIPA test. Remarkably, just one of the 205 sera was positive only in the HIPA test (0.5%).

Of these 204 sera testing positive using the anti-PF4/heparin IgG/A/M EIA, 185 were assessed by single class EIA: 168 (82.4%) contained IgG, 112 (54.9%) IgM, and 57 (27.9%) IgA anti-PF4/heparin antibodies (Table 2).

Of the 168 anti-PF4/heparin IgG-positive sera, 92 (54.8%) were also positive in the HIPA test. The median OD in the IgG EIA of HIPA positive sera was higher than the median OD of HIPA test negative sera (1.117 vs. 0.768; $P < 0.0001$), with a wide range of overlap of OD values (Fig. 1). Fig. 1 also shows that the degree of EIA IgG positivity did not differ whether concomitant IgA or IgM antibodies are present.

Table 1 Results of heparin-induced thrombocytopenia (HIT) antibody tests in the heparin-induced platelet activation (HIPA) test and in the platelet factor 4/heparin (PF4/H) IgG/A/M enzyme-immunoassay (EIA) of 1650 patients referred for diagnosis of HIT

	PF4/heparin IgG/A/M EIA, *n* (%)		
HIPA test	Positive	Negative	Total
Positive	95 (5.8)	1 (0.1)	96 (5.8)
Indeterminate*	9 (0.5)	59 (3.6)	68 (4.1)
Negative	100 (6.1)	1386 (84.0)	1486 (90.1)
Total	204 (12.4)	1446 (87.6)	1650

*Indeterminate is defined as the activation of donor platelets in the presence of both low and high heparin concentrations.

Table 2 Immunoglobulin class distributions of antiplatelet factor 4/heparin (anti-PF4/heparin) antibodies in 205 sera testing positive in any assay

Immunoglobulin classes of heparin-induced thrombocytopenia antibodies	Monospecific PF4/heparin EIA positive, *n* (%)	HIPA positive, *n* (%)
IgG only	55 (26.8)	33 (16.1)
IgG and IgA	14 (6.8)	7 (3.4)
IgG and IgM	63 (30.7)	32 (15.6)
IgG, IgA and IgM	36 (17.6)	20 (9.8)
Any IgG	168 (82.0)	92 (44.9)
IgM only	10 (4.9)	1 (0.5)
IgM and IgA	3 (1.5)	0
IgA only	4 (2.0)	0
Total	185	93
Positive IgG/A/M, but single class EIA not determined	19 (9.3)	2 (1.0)
Negative EIA but positive HIPA	0	1 (0.5)
Total	204 (99.5)	96 (46.8)

EIA, enzyme-immunoassay; HIPA, heparin-induced platelet activation.

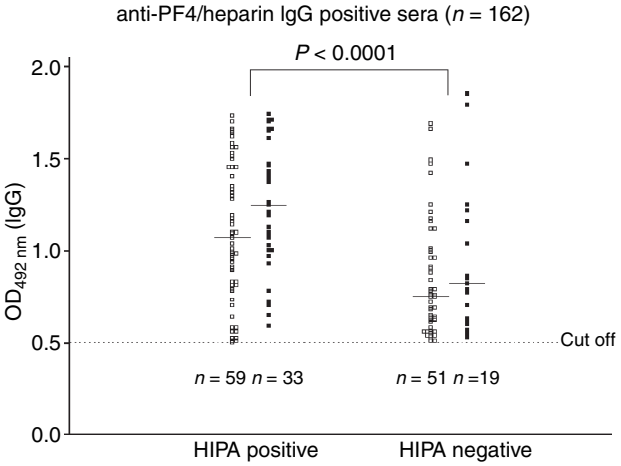

Fig. 1. Optical densities (ODs) of the anti-platelet factor 4/heparin (anti-PF4/heparin) IgG enzyme-immunoassay. The left two columns show the ODs of the heparin-induced platelet activation (HIPA) positive sera and the right two columns show the ODs of the HIPA negative sera. The open symbols give the IgG ODs of sera that also contained anti-PF4/heparin IgM and/or IgA antibodies (median OD HIPA positive 1.070; median OD HIPA negative 0.751). The filled symbols give the ODs of sera containing anti-PF4/heparin IgG only (median OD HIPA positive 1.246; median OD HIPA negative: 0.823). Anti-PF4/heparin IgG positive sera which showed an indeterminate result in the HIPA test ($n = 6$) are not given in the figure.

Among the 205 positive sera, only two showed the unusual reaction profile of a positive HIPA test but a negative IgG EIA. One of these contained anti-PF4/heparin IgM; the other was below the cutoff in all the EIAs. With high 100 IU mL^{-1} heparin, the inhibition of the (below cutoff) IgG EIA OD was < 50%, suggesting that low levels of platelet-activating anti-PF4/heparin IgG are likely not the explanation for the discrepant HIPA/EIA data. However, both sera became negative in the HIPA test after IgG depletion by protein G. The protein G eluates, containing the IgG fractions, again showed activation at low but not high heparin concentration in the HIPA test, which was fully inhibited by coincubation with IV-3, but remained negative in the PF4/heparin IgG EIA. This result suggests that these sera contained IgG antibodies against heparin-dependent antigens other than PF4/heparin.

Annexin-V binding

Figure 2A compares the typical pattern of annexin-V binding to the surface of platelets induced by (a) anti-PF4/heparin IgG sera, (b) normal sera, and (c) anti-PF4/heparin IgM/A sera. Figure 2B gives comparisons between the eight patient sera containing only anti-PF4/heparin IgM/IgA and the positive (HIT IgG) and three negative control groups, presented as box plots showing 25–75% quartiles and the median of the fluorescence intensities (MFI).

Anti-PF4/heparin IgM and/or IgA sera induced much less annexin-V binding than anti-PF4/heparin IgG sera (MFI 60.45 vs. 221.65; $P < 0.0001$) but more than sera of healthy controls: blood donors (group 1: MFI 36.45; $P = 0.0149$) and patient controls; patients with thrombocytopenia and negative HIT test (group 2: $n = 20$; MFI 35.50; $P < 0.0001$); patients with thrombosis and negative HIT test (group 3: $n = 20$; MFI 34.55; $P = 0.0001$).

Microparticle generation

Figure 3A shows the typical pattern of microparticle generation of (a) anti-PF4/heparin IgG sera, (b) normal sera (blood donors, group 1), and (c) anti-PF4/heparin IgM/A sera. Comparisons between groups are given in Fig. 3B. While sera containing anti-PF4/heparin IgG strongly caused generation of microparticles (68.9%), sera containing anti-PF4/heparin IgM/A antibodies induced microparticles (22.0%) in a similar range as controls (Fig. 3B): group 1 (blood donors; $n = 10$; 20.8%; $P = 1.0$); group 2 (patients with thrombocytopenia and negative HIT test; $n = 20$; 17.2%; $P = 0.0228$); group 3 (patients with thrombosis and negative HIT test; $n = 20$; 22.5%; $P = 0.7926$).

Patient characteristics

Of the 205 patients with any positive test, the mean age was 66 years (range 18–92) and 92 (44.9%) were females. 106 (51.7%) were medical patients [internal medicine ($n = 101$; 49.3%), neurology ($n = 5$; 2.4%)] and 78 (38.0%) were surgical patients [general surgery ($n = 17$; 8.3%); cardiac surgery ($n = 23$; 11.2%); orthopaedic surgery ($n = 2$; 0.9%); urology ($n = 2$; 0.9%); gynaecology ($n = 3$; 1.5%); surgical intensive care ($n = 31$; 15.1%)]. 21 (10.2%) of the patients were treated at different departments during the course of the disease.

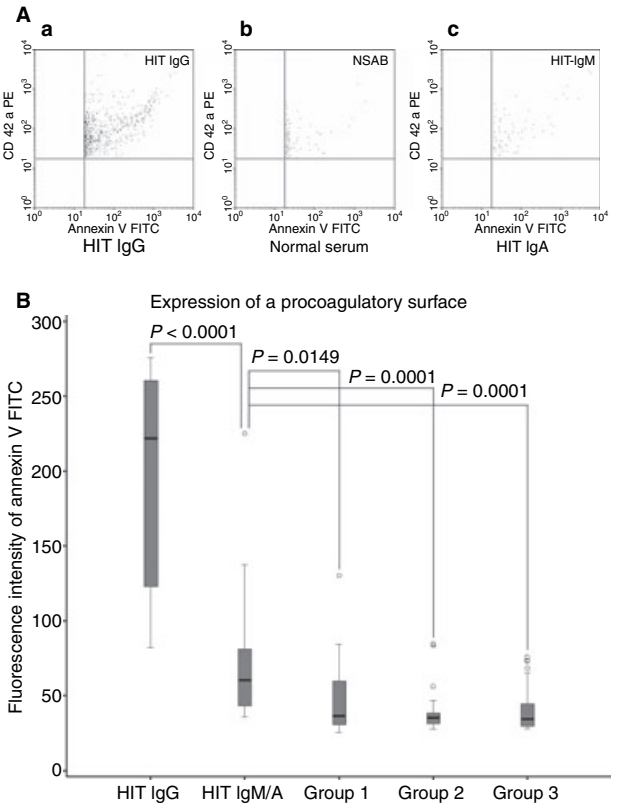

Fig. 2. (A) Representative examples of FACS analysis of annexin-V binding on washed platelets incubated with heparin 0.2 IU mL^{-1} and sera containing (a) antiplatelet factor 4/heparin (anti-PF4/heparin) IgG antibodies, (b) sera of healthy blood donors, and (c) sera containing anti-PF4/heparin IgM/A. (B) Comparison of annexin-V binding on washed platelets incubated with sera containing anti-PF4/heparin IgG ($n = 20$), sera containing anti-PF4/heparin IgM and/or –A ($n = 8$), and sera of the following three control groups: group 1, normal sera (blood donors; $n = 10$); group 2, patients with thrombocytopenia after at least five days of heparin administration but negative heparin-induced thrombocytopenia (HIT) test ($n = 20$); group 3, patients with thromboembolic complications despite heparin administration but negative HIT tests ($n = 20$). Results are given as median and 25–75% quartiles of fluorescence intensities.

Clinical information was available for 184 patients who tested positive in one or both assays (EIA or HIPA; 183 EIA positive patients; only one HIPA only positive patient; Table 3). 17 patients (9.2%) presented with thrombocytopenia and new thrombosis; of these, 13 (76.5%) were positive in the HIPA. 157 (85.3%) had isolated thrombocytopenia; of these, 73 (46.5%) were HIPA positive. Four (2.2%) patients presented with thrombosis without a major decrease in platelet counts. Six (3.3%) patients had neither thrombocytopenia nor new thrombosis.

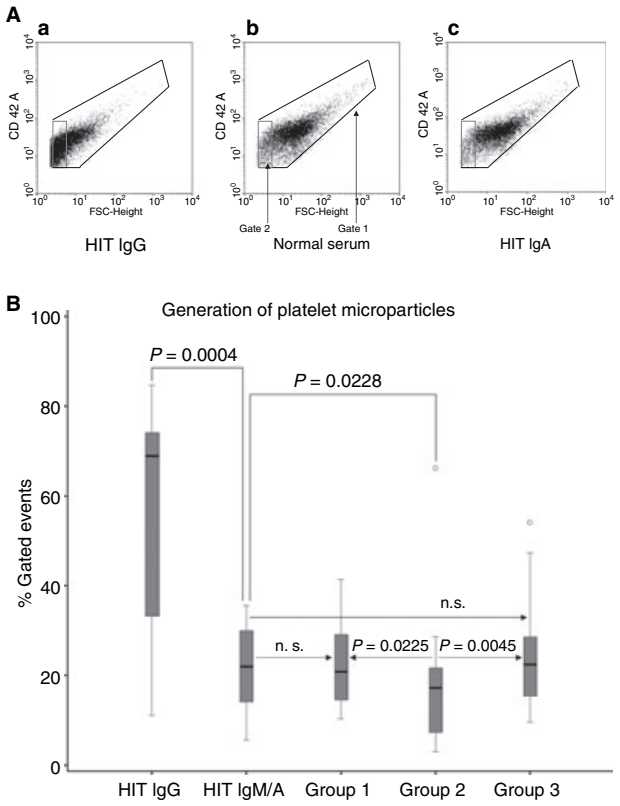

Fig. 3. (A) Representative examples of FACS analysis of microparticle generation by washed platelets incubated with heparin 0.2 IU mL^{-1} and sera containing (a) antiplatelet factor 4/heparin (anti-PF4/heparin) IgG antibodies, (b) sera of healthy blood donors, and (c) sera containing anti-PF4/heparin IgM/A. The large gate includes all platelets and the small gate includes the area for microparticle assessment. (B) Comparison of microparticle generation by washed platelets incubated with sera containing anti-PF4/heparin IgG (n = 20), sera containing anti-PF4/heparin IgM and/or –A (n = 8), and sera of the three control groups (for details caption for Fig. 2B). Results are given as median and 25–75% quartiles of gated events.

Clinical presentation of EIA and/or HIPA positive patients without anti-PF4/heparin IgG antibodies

None of the 13 thrombocytopenic patients with anti-PF4/heparin antibodies of the IgM and/or –A class but negative HIPA test (IgM, n = 6; IgA, n = 4; IgM and IgA, n = 3) developed thrombosis. All had other explanations for the platelet count decrease and/or the clinical course was not typical for HIT, for the following reasons: (i) a plausible other explanation for the thrombocytopenia was present in eight patients (sepsis, n = 6; multi-organ failure, n = 2); (ii) the timing was not typical for HIT in three patients (occurrence of thrombocytopenia >14 days after beginning heparin treatment); (iii) in two patients, the platelet counts increased despite continuing heparin therapy.

Two patients had no PF4/heparin IgG but a positive HIPA test. One of these had anti-PF4/heparin IgM. This female patient with cirrhosis had received one dose of LMWH three weeks earlier, and then presented with mediastinitis/sepsis and concomitant thrombocytopenia. This clinical picture is inconsistent with HIT. The other patient, who was negative in the PF4/heparin EIA but HIPA-positive, had isolated thrombocytopenia (nadir, 80×10^9 L^{-1}) beginning one week after UFH was started for idiopathic pulmonary embolism. Anticoagulation was switched to lepirudin and platelet counts increased to $> 200 \times 10^9$ L^{-1}. This clinical picture is consistent with the possibility of HIT caused by platelet-activating antibodies against a heparin-dependent antigen other than PF4 (we did not test for IL-8 or NAP-2 antibodies, as these techniques are not established in our laboratory).

Discussion

Our study of 1650 consecutive patients referred for laboratory diagnosis of HIT found that only 12.4% of all patients tested positive in any test, and only 5.6% overall (or 44.9% of those

Table 3 Immunoglobulin class distribution of the 184 patients with clinical information

	TEC and thrombocytopenia		TEC without thrombocytopenia		Thrombocytopenia without proven TEC		Neither TEC nor thrombocytopenia	
	n (%)	HIPA pos of these	*n* (%)	HIPA pos of these	*n* (%)	HIPA pos of these	*n* (%)	HIPA pos of these
IgG only	4 (2.2)	4	2 (1.1)	0	45 (24.5)	26	0	0
IgG and IgA	1 (0.5)	1	0	0	11 (6.0)	6	1 (0.5)	0
IgG and IgM	7 (3.8)	5	0	0	48 (26.1)	22	1 (0.5)	0
IgG, IgA and IgM	3 (1.6)	3	0	0	28 (15.2)	16	0	0
All IgG	15 (8.2)	13	2 (1.1)	0	132 (71.7)	70	2 (1.1)	0
IgM only	0	0	0	0	7 (3.8)	1	1 (0.5)	0
IgM and IgA	0	0	0	0	3 (1.6)	0	0	0
IgA only	0	0	0	0	4 (2.2)	0	0	0
Not tested by single class EIA	2 (1.1)	0	2 (1.1)	0	10 (5.4)	2	3 (1.6)	0
Total*	17 (9.2)	13	4 (2.2)	0	156 (84.8)	73	6 (3.3)	0

*One additional patient serum was positive only in the HIPA test (the patient had isolated thrombocytopenia). TEC, thromboembolic complication.

testing positive in any assay) had the typical serological profile of HIT (i.e. positive HIPA and positive EIA IgG). This indicates that almost 95% of patients who undergo laboratory testing for HIT are unlikely to have this diagnosis. One factor could be the characteristics of the patient populations currently being evaluated for HIT: more than 50% were medical patients, and 15.1% were treated in postsurgery intensive care units. In contrast, only two patients developed HIT following orthopedic surgery, which formerly constituted the major at-risk patient group. In medical and intensive care patients, it is more difficult to differentiate between HIT and non-HIT disorders on clinical grounds alone.

Our study corroborates the point of view that the anti-PF4/polyanion EIA is ideal for ruling out HIT. All but one of the HIPA test-positive sera also tested positive in the EIA IgG/A/M; thus, the sensitivity of the EIA is 99.5% for detecting heparin-dependent platelet-activating antibodies. Given this very high test sensitivity, a negative EIA result usually indicates that further assessment with a functional assay is not indicated.

In contrast, the specificity of the EIA for platelet-activating antibodies – and thus for clinical HIT – is relatively low. Only 95/204 (46.6%) anti-PF4/heparin IgG/A/M EIA positive sera were also positive in the HIPA test. One reason is the detection of (non-platelet-activating) IgM and IgA antibodies by the EIA. Based on our study, it appears that approximately 10% of all sera testing positive in the EIA (and 17% of all EIA-positive but HIPA-negative sera) contain only IgM and/or IgA antibodies. Previous reports have suggested that this serological profile could indicate clinical HIT [18,25], a subject that remains controversial. We therefore evaluated the serological and clinical features of these patients to address whether these antibodies were likely to have been clinically relevant. We found several lines of evidence against this contention. None of the IgM and IgA antibodies induced the generation of platelet-derived microparticles. However, they did induce slightly more annexin-V binding than controls, suggesting a minor platelet-activating effect that might contribute to thrombocytopenia by increased platelet clearance [26]. More importantly, none of the 14 patients with detectable anti-PF4/heparin antibodies of IgM and/or IgA class – but no IgG antibodies – developed thrombosis. Clinically, 13 of these patients (without platelet-activating IgG antibodies) had thrombocytopenia with either another explanation for the platelet count decrease, and/or the timing was atypical for HIT, and/or they had platelet count increase despite continuing heparin. However, in the *in vitro* test system we used for assessing platelet activation, the sera were heat-inactivated (otherwise, even normal sera can cause platelet activation because of residual thrombin). Therefore, we cannot rule out the possibility that anti-PF4/heparin IgM antibodies can also enhance binding of complement to the platelet surface *in vivo*, and thereby contribute to some degree of thrombocytopenia. Another uncertainty of our study is that we did not test sera sequentially. It is therefore possible that some patients with non-activating antibodies of the IgA or IgM class seroconvert at a later time to platelet-activating IgG antibodies. This issue requires further study.

Occasionally, other antigens can be involved in HIT pathogenesis, such as IL-8 or NAP-2, two CXC chemokines of the PF4 superfamily with high affinity for heparin [12,27]. We identified two patients whose sera tested positive in the HIPA test but did not contain detectable anti-PF4/heparin antibodies of IgG class. By purifying the IgG fractions, we demonstrated for both sera that their platelet-activating effects were caused by IgG antibodies that acted in a heparin- and FcγRIIa-dependent fashion, but that presumably had a specificity other than PF4/heparin. In view of our experiments, the previous observation of a platelet-activating effect of anti-PF4/heparin IgM and IgA antibodies based on 12 patients with putative clinical HIT in the absence of anti-PF4/heparin IgG [18] might have also been caused by the additional presence of heparin-dependent IgG antibodies of other non-PF4-dependent specificities.

It has been suggested that an anti-PF4/heparin IgG EIA would enhance clinical specificity of the assay without decreasing sensitivity [28]. Based on this study, we estimate that a PF4/heparin IgG EIA still has a very high sensitivity (99.0%).

However, specificity for platelet-activating HIT antibodies is still relatively low (although better than that of the IgG/A/M EIA), as only 54.8% (92/168) of IgG-positive sera were also positive in the HIPA test.

As previously reported [5,28,29], anti-PF4/heparin IgG antibodies testing positive in the functional assay showed an overall higher OD in the EIA than those without platelet-activating capacity ($P < 0.0001$). Increasing the cut-off for the PF4/heparin antigen assays will improve specificity for clinical HIT, but this will also cause some false-negative results (Fig. 1) and will not substitute for the additional information provided by a functional assay.

When combined with our previous study of 755 referred patient samples [5], we have found that only 13.0% of 2405 patients referred to a reference laboratory over three years tested positive for HIT antibodies in any test. More importantly, only about half of these (or about 6.2% overall) showed platelet-activating antibodies, a serological feature regarded as being relatively specific for clinical HIT. Thus, our study indicates that using an antigen assay that recognizes IgG, IgA, and –M antibodies has the potential to overdiagnose HIT by approximately 100%, if any positive EIA is considered 'diagnostic' for HIT irrespective of the clinical situation or the result of a functional assay. In other words, for every two patients investigated serologically for HIT who has a positive EIA, only one really has this diagnosis. A clinically feasible approach would be, in case of clinical suspicion of HIT, to apply the 4 T's scoring system [6] to determine the pretest probability and then to order a polyvalent EIA. If the pretest probability is low and the EIA is negative, HIT is effectively ruled out. In case of a high pretest probability and a positive EIA, the patient should be treated as having HIT and at the same time a test for IgG antibodies and ideally a functional HIT test should be ordered, and only when these are positive should a definitive

diagnosis of HIT be made. The most problematic group comprises patients with an intermediate pretest probability and a positive polyspecific EIA. In these patients the risk of overdiagnosing HIT should be considered when choosing treatment. All alternative non-heparin anticoagulants given in therapeutic dose have significant bleeding risks. In patients with only a low or intermediate likelihood of HIT, the use of an alternative anticoagulant in prophylactic doses might be safer, unless otherwise dictated by the patient's clinical condition, or if a platelet activation test gives a strong positive test result. We have recently given detailed recommendations as to how to handle this problem in patients requiring intensive care treatment [30].

In conclusion, anti-PF4/heparin antigen assays are excellent for ruling out HIT (approximate 99% sensitivity). In most cases, further testing with a platelet activation test is not required if the EIA is negative, but may be appropriate if the EIA is positive. Anti-PF4/heparin antibodies of the IgM and IgA classes and other antigens than PF4/heparin seem to be of minor clinical relevance.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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