



Early Release Paper

## Rapid exclusion or confirmation of heparin-induced thrombocytopenia: a single-centre experience with 1291 patients

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*Haematologica* 2011 [Epub ahead of print]

Citation: Nellen V, Sulzer I, Barizzi G, Lammler B, and Alberio L. Rapid exclusion or confirmation of heparin-induced thrombocytopenia: a single-centre experience with 1291 patients. *Haematologica*. 2011; 96:xxx  
doi:10.3324/haematol.2011.048074

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**Rapid exclusion or confirmation of heparin-induced thrombocytopenia: a single-centre experience with 1291 patients**

***Running title: Rapid HIT diagnosis***

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## Abstract

### Background

The current gold-standard for diagnosing heparin-induced thrombocytopenia is the detection of platelet-activating antibodies by means of functional assays which, being time consuming and not widely available, are not suited for guiding acute treatment decisions. The objective of our study was to assess the ability of more rapid immunoassays to predict the presence of functionally relevant anti-platelet factor 4/heparin-antibodies.

### Design and Methods

We analysed 1291 out of 1383 (93.4%) patients consecutively evaluated for suspected heparin-induced thrombocytopenia at our institution. Clinical pre-test probability was defined by the 4T-score. Anti-platelet factor 4/heparin-antibodies were measured with three immunoassays (ID-H/PF4-PaGIA, Asserachrom-HPIA, and GTI-PF4) and their functional relevance was assessed by a two-point heparin-induced platelet aggregation-test. The performance of the immunoassays was evaluated by receiver operating characteristic analysis.

### Results

Among 1291 patients, 96 (7.4%) had a positive heparin-induced platelet aggregation-test: 7/859 (0.8%) with a low, 50/358 (14.0%) with an intermediate, and 39/74 (52.7%) with a high 4T-score. Receiver operating characteristics analysis indicated that best immunoassays' thresholds for predicting a positive platelet aggregation-test were: Titer  $\geq 4$  (ID-H/PF4-PaGIA), optical density  $>0.943$  (Asserachrom-HPIA) and  $>1.367$  (GTI-PF4). A 100% negative predictive value was observed at following thresholds: Titer  $\leq 1$  (ID-H/PF4-PaGIA), optical density  $<0.300$  (Asserachrom-HPIA) and  $<0.870$

(GTI-PF4). A 100% positive predictive value was reached only by ID-H/PF4-PaGIA, at titers  $\geq 32$ . Positive and negative likelihood ratios were calculated for results between the thresholds with 100% negative or positive predictive value.

## Conclusions

We show that: 1. Negative and weak positive results of immunoassays detecting anti-platelet factor 4/heparin-antibodies exclude heparin-induced thrombocytopenia; 2. Anti-platelet factor 4/heparin-antibody titres  $\geq 32$  (ID-H/PF4-PaGIA) have a 100% positive predictive value for functionally relevant antibodies; 3. Combining the pre-test probability with the likelihood ratio of intermediate immunoassay results allows to assess post-test probability for heparin-induced thrombocytopenia in individual patients.

**Key words:** heparin-induced thrombocytopenia, diagnosis, quantitative immunoassay, predictive value, likelihood ratio, Bayes' theorem.

## Introduction

Heparin-induced thrombocytopenia (HIT) is a drug-induced, antibody-mediated condition characterised by a highly procoagulant state.<sup>1, 2</sup> HIT is usually caused by IgG antibodies directed against heparin-bound platelet factor 4 (PF4). Macromolecular ternary complexes – formed by HIT antibodies, PF4-tetramers and heparin chains – are able to activate platelets, endothelial cells and monocytes, leading to excessive *in vivo* thrombin generation.<sup>3</sup> If unrecognized and left untreated, HIT can lead to severe venous and arterial thromboembolic complications threatening patients' limbs and lives.

The diagnosis of HIT is based on clinical features, which can be employed to determine the 4T pre-test probability score,<sup>4-6</sup> and laboratory documentation of heparin-dependent antibodies.<sup>7</sup> Recent studies have shown that a low clinical probability assessed by the 4T scoring system has a high negative predictive value for the presence of HIT.<sup>6, 8-12</sup> However, these publications also indicate that a high 4T probability score is not strongly predictive for HIT and a relevant proportion of the investigated patients turn out to have an intermediate pre-test probability.<sup>8-12</sup> These results support the concept that identification of patients with HIT cannot be made on a clinical basis only but requires laboratory demonstration of relevant HIT antibodies. The turn-around time of these assays has clinical implications, because of the ensuing treatment decisions. In fact, continuing heparin, or even stopping it without starting an alternative anticoagulant drug in a patient with unrecognized HIT carries a high thrombotic risk;<sup>13</sup> on the other hand, initiating danaparoid or a direct thrombin inhibitor (argatroban, lepirudin) in patients without HIT exposes them to an unnecessary high bleeding risk and is expensive.<sup>14, 15</sup> Therefore, a case can be made for the necessity of rapid laboratory HIT diagnosis, in order to guide treatment decisions.<sup>16</sup>

Up to now, the laboratory gold-standard for the diagnosis of HIT is the demonstration of in vitro platelet-activating HIT antibodies.<sup>7</sup> Unfortunately, these functional assays are time consuming and not widely available, making them unsuitable for helping clinicians facing a patient with suspected HIT.<sup>17</sup> More rapid laboratory evidence of anti-PF4/heparin antibodies can be achieved by immunoassays, either enzyme-linked immuno-sorbent assays (ELISA)<sup>18, 19</sup> or particle-gel immune assays (PaGIA).<sup>20</sup>

The primary aim of the present work was to assess the ability of three commercial immunoassays for anti-PF4/heparin antibodies to predict the presence of HIT antibodies activating platelets in vitro. The second objective of our work was to evaluate the prevalence of functionally relevant antibodies according to the pre-test clinical probability for HIT, as assessed by the 4T score.<sup>4, 5</sup> We show that with these information it is possible to apply Bayes' theorem to HIT diagnostic work-up of individual patients.

## Design and Methods

### *Patients*

Between May 1995 and December 2009 we recorded 1383 patients evaluated for clinically suspected HIT at our institution. After exclusion of patients with incomplete information concerning the 4T score or without plasma samples in order to complete laboratory assays (see below), we could evaluate 1291 (93.3%) patients. Clinical categories were as follows: 691 (53.5%) medical, 303 (23.5%) surgical, 259 (20.1%) intensive care, 15 (1.2%) obstetrics/gynecology, 7 (0.5%) pediatrics, and 16 (1.2%) unknown. Among the surgical patients 154 (50.8%) were general or abdominal surgery, 95 (31.4%) cardio-vascular surgery, 43 (14.2%), orthopaedics, and 11 (3.6%) neurosurgery. Overall, median age was 67.9 years, ranging from 1.5 to 106.4 years (interquartile range: 56.7–76.2 years). There was no age difference between women (n=566; median 67.4 years) and men (n=725; median 68.3; p=0.7125). The study was performed in accordance with local regulations for diagnostic-laboratory studies (Kantonale Ethikkommission Bern, [www.kek-bern.ch](http://www.kek-bern.ch)).

### *Historical note*

The founding document of the Swiss Confederation, named Federal Charter or Letter of Alliance, was written in August 1291.

### *Pre-test clinical probability for HIT (4T score)*

Starting in January 2004 the clinical probability for HIT was routinely assessed according to the 4T score<sup>4, 5</sup> by the consulting hematologist (n=1021). For 19 HIT patients treated with lepirudin between 2001 and 2003 the 4T score had been retrospectively calculated in the context of a previous study.<sup>21</sup> The 4T

score for the remaining 251 patients (19.4%) was retrospectively assessed by the first author in a blinded fashion and verified by the corresponding author.

### *Plasma samples*

Blood was drawn into 10 ml plastic syringes (Monovette<sup>®</sup>, Sarstedt, Nümbrecht, Germany) containing 1 ml 0.106 mol/l trisodium citrate. Plasma was prepared by double centrifugation at 1500 x g for 10 minutes each at room temperature. Plasma aliquots were stored in polypropylene tubes at -70°C.

### *Detection of anti-PF4/heparin antibodies by immunoassays*

Anti-PF4/heparin antibodies were detected by the ID-H/PF4-PaGIA (DiaMed SA, Cressier sur Morat, Switzerland).<sup>20</sup> Specifically, 10 µl of plasma were pipetted into the reaction chamber of the test ID-card followed by 50 µl of polymer particles (red high-density polystyrene beads coated with heparin/PF4 complexes). After incubation at room temperature for 5 minutes, the ID-card was centrifuged for 10 minutes in the dedicated ID-centrifuge (DiaMed SA). In the absence of a significant level of anti-PF4/heparin antibodies in the test sample, the particles sank to the bottom of the gel chamber. If anti-PF4/heparin antibodies were present, the red polymer particles were cross-linked and remained on the top of the gel chamber. In case of an indeterminate (defined as: “Neither a clear agglutinate nor a full sedimentation of the particles”) or positive test with the undiluted sample, we repeated the assay with serially diluted plasma (up to 1:1024) until the result was negative. Thus, for a 1:2 dilution, 50 µl of plasma were mixed with 50 µl of Diluent II (DiaMed SA), and subsequently dilutions were obtained by pipetting 50 µl of the preceding one with 50 µl of Diluent II. The reported titre is the last positive detection followed by either indeterminate or negative results, as previously



described.<sup>22</sup> For the 130 patients evaluated before July 2001, the ID-H/PF4-PaGIA was performed in the context of our initial study.<sup>22</sup> Since July 2001 we routinely perform the ID-H/PF4-PaGIA at the moment of evaluation for suspected HIT. The ID-H/PF4-PaGIA results reported in this paper were obtained with carefully evaluated polymer lots, thus excluding false negative results.<sup>23</sup>

Anti-PF4/heparin antibodies were also detected with two commercially available enzyme-linked immunosorbent assays (ELISAs; GTI-PF4 Enhanced, Genetic Testing Institute, Waukesha, WI, USA and Asserachrom HPIA, Diagnostica Stago, Asnières sur Seine, France) and measured at 405 (GTI-PF4) or 450 (Asserachrom HPIA) nm with a microtiter plate reader (Anthos ht III, Hemotec, Gelterkinden, Switzerland). As previously published we did not notice different results when using serum instead of plasma.<sup>23</sup>

#### *Heparin-induced Platelet Aggregation Test (PAT)*

This assay was performed in a light transmission aggregometer (models PAP-4, Bio/Data, Hatboro, Pennsylvania, USA until 2005 and thereafter APACT 4A and APACT 4004, LABiTec, Ahrensburg, Germany) as previously described.<sup>24</sup> Briefly, 4 separate mixtures each containing 100  $\mu$ l patient's platelet poor plasma and 100  $\mu$ l platelet rich plasma from one of 4 different selected donors known to have platelets reactive to HIT antibodies. The samples are initially stirred for 4 minutes at 37°C in order to detect spontaneous heparin-independent aggregation. Thereafter, 10  $\mu$ l of a solution of unfractionated heparin (Liquemin®) and – in a subsequent run – low molecular weight heparin (the preparation administered to the specific patient) are added and light transmission is recorded for up to 15 minutes. A positive test is defined by: 1. At least 2 out of 4 samples reaching  $\geq 50\%$  aggregation with 0.5 U/ml heparin

and 2. Abrogation of the reaction by 100 U/ml heparin.<sup>24</sup> A two-point PAT with these two final heparin concentrations has been shown to reach a 100% specificity,<sup>24, 25</sup> with a sensitivity of about 80% if reactive platelets from selected donors were employed.<sup>25</sup> As an internal control we always test a plasma sample from a previously positive patient at the beginning and at the end of each PAT series.

### *Statistics*

Quantitative data were analysed by SigmaStat software (version 3.5; Systat Software Inc., San Jose, CA, USA) and are expressed as median, range and interquartile range. The 95% confidence interval (95%CI) for the proportions (p) reported on table 1 was calculated by following equation: " $p \pm 1.96 \times$  standard error (S.E.)", where the S.E. was estimated by the quadratic square of  $[p(1-p)/n]$  (n = sample size). Receiver operating characteristic (ROC) analysis was performed with MedCalc software (version 11.1; Mariakerke, Belgium).<sup>26</sup> Comparison of ROC curves obtained with the different immunoassays was performed by the non-parametric method of DeLong<sup>27</sup> (MedCalc software). Significance was set at the 5% level. The interested reader is referred to the Supplemental Material online "Brief tutorial on ROC analysis and clinical application of Bayes' theorem".

## Results

### *Prevalence of in vitro platelet-activating heparin-dependent antibodies in patients evaluated for suspected HIT*

Among the 1291 patients of our original Swiss cohort, 96 (7.4%) had a positive heparin-induced platelet aggregation test (PAT), demonstrating the presence of platelet-activating HIT antibodies. Table 1a shows that among the patients evaluated in Bern, 7 out of 859 (0.8%) with a low 4T score (0-3),<sup>4, 5</sup> 50/358 (14.0%) with an intermediate 4T score (4-5), and 39/74 (52.7%) with a high 4T score (6-8) had functionally relevant HIT antibodies. Laboratory data of the 7 patients with low 4T score and positive PAT are summarized in Table 1b. We consider that these 7 patients had *bona fide* heparin-dependent platelet-activating anti-PF4/heparin antibodies because: a. PAT excluded spontaneous platelet aggregation and demonstrated inhibition of aggregation with heparin excess (see Design and Methods section); b. Plasma samples contained high-titer anti-PF4/heparin antibodies (Table 1b); c. The combination of a positive PAT with a positive ELISA has been shown to have a 100% positive predictive value for HIT;<sup>28</sup> and d. Plasma samples did not test positive for antiphospholipid antibodies<sup>29</sup> (Table 1b).

### *Performance of immunoassays in identifying patients with functionally relevant HIT antibodies*

Figure 1 shows that the ID-H/PF4-PaGIA giving a negative/positive result according to the manufacturer's instruction<sup>30</sup> was significantly less informative than both ELISAs, with an area under the ROC curve (AUROC) of 0.943 compared to 0.990 for the Asserachrom HPIA ( $p < 0.0001$ ) and 0.985 for the GTI-PF4 ( $p < 0.0001$ ). However, when a positive result of the ID-H/PF4-PaGIA was semi-quantitated by titer<sup>3, 22</sup> the performance of this assay, with an AUROC of 0.992, was at least as good as that of both ELISAs ( $p = 0.4683$

versus HPIA and  $p=0.0174$  versus GTI-PF4). According to ROC analysis, the cut-offs with the best compromise between sensitivity and specificity for detecting functionally relevant HIT antibodies were a titer of  $\geq 4$  (ID-H/PF4-PaGIA), an optical density (OD)  $> 0.943$  for the Asserachrom HPIA and an OD  $> 1.367$  for the GTI-PF4 (Table 2).

*Negative predictive values (NPV) and negative likelihood ratios (LR -) of immunoassays for functionally relevant HIT antibodies*

From a clinical point of view, the most useful characteristic of a diagnostic test result is its ability to exclude or confirm a given disease. This information is conveyed by the predictive value and likelihood ratio. Specifically, the negative predictive value (NPV) represents the proportion of individuals with a negative test result who do not have the disease and excludes a disease when it reaches a value of 100%. Table 3 shows that all three assays were able to exclude the presence of functionally relevant HIT antibodies, at following thresholds: titer  $\leq 1$  (ID-H/PF4-PaGIA), OD  $< 0.300$  (Asserachrom HPIA), OD  $< 0.870$  (GTI-PF4). Of note, these thresholds differ from those suggested by the manufacturers, being a negative result for the ID-H/PF4-PaGIA, an OD of about 0.450 for the Asserachrom HPIA and an OD of 0.400 for the GTI-PF4. For immunoassay results above the threshold with a 100% NPV but below the cut-off values with the best compromise between sensitivity and specificity, and therefore with a NPV less than 100%, it is possible to calculate the negative likelihood ratio (LR -). This ratio represents the probability of an individual *with* the disease having a negative test divided by the probability of an individual *without* the disease having a negative test result, and can be employed in order to modify the individual clinical pre-test probability for a given disease (see below). Negative likelihood ratios (i.e.  $< 1.0$ ) of the three immunoassays are displayed in table 5.

### *Positive predictive values (PPV) and positive likelihood ratios (LR +) of immunoassays for functionally relevant HIT antibodies*

Similarly to the ability of a negative or low quantitative assay result to exclude a disease, the magnitude of a positive test result can be employed in order to confirm the presence of a given disease. This is expressed by the positive predictive value (PPV), which represents the proportion of individuals with a positive test result who do have the disease. Among the three immunoassays investigated, only the ID-H/PF4-PaGIA reached, at titers  $\geq 32$ , a 100% PPV for a positive PAT, and is therefore able to predict with certainty the presence of functionally relevant HIT antibodies. Of note, in our cohort 49 out of the 96 (51%) patients with a positive PAT had an anti-PF4/heparin antibody titer  $\geq 32$ . For test results lying above the cut-off value with the best compromise between sensitivity and specificity, but lower than the threshold with a 100% PPV, it is possible to calculate the positive likelihood ratio (LR +). This ratio expresses the probability of an individual *with* the disease having a positive test divided by the probability of an individual *without* the disease having a positive test result. Positive likelihood ratios (i.e.  $> 1.0$ ) of the three immunoassays are displayed in table 5.

### *Applying Bayes' theorem to HIT diagnostic work-up*

PPV and NPV strongly depend on the disease prevalence in the patients being tested and can be misleading. Likelihood ratios are clinically more useful, because they provide an information on how many times more (or less) likely patients *with* the disease are to have a particular test result than patients *without* the disease. Therefore, likelihood ratios can be used to calculate the probability of disease for individual patients.<sup>31, 32</sup> Specifically, the LR of a quantitative test result can be combined with the clinical pre-test probability for a given disease in order to reach a higher or lower post-test probability. This

can be performed either by a mathematical calculation (see Supplemental Material online) or visually by the aid of Fagan's nomogram.<sup>32, 33</sup>

The 4T scoring system allows defining pre-test probability for the presence of functionally relevant HIT antibodies. Table 1a summarizes the published results of several groups around the world and shows that for patients with a low 4T score (0-3) the probability of having a positive functional assay for HIT antibodies is less than 1% (0.5%; 95%CI: 0.2 – 0.9). For patients with an intermediate 4T score (4-5) this probability is about 10% (10.8; 95%CI: 8.8 – 12.8) and for patients with a high 4T score (6-8) it is about 50% (47.8%; 95%CI: 41.1 – 54.7). Figure 2A illustrates the application of Bayes' theorem to HIT diagnosis for an hypothetical patient with an intermediate 4T score. For this patient the pre-test clinical probability of having in vitro platelet-activating HIT antibodies is about 10% (Table 1a), corresponding to a pre-test odds ratio of 0.11 (see Supplemental Material online).

In case of a positive ID-H/PF4-PaGIA with a titer of 2, which is below the optimal cut-off but does not have a 100% NPV (in our cohort 1 out of 35 patients with a titer of 2 had a positive PAT), we can employ its LR – (0.366; Table 5) in order to decrease the post-test probability of HIT. By plotting both variables on the Fagan's nomogram (Figure 2A) we derive a probability for functionally relevant HIT antibodies of about 4% (by mathematical calculation:  $\text{post-test odds} = 0.11 \times 0.366 = 0.04026$ , corresponding to a post-test probability of 3.9%). This probability argues against the presence of HIT. On the other hand, in case of a positive ID-H/PF4-PaGIA with a titer of 4, which is above the optimal cut-off without reaching a 100% PPV, we can employ its LR + (6.224; Table 5) in order to estimate post-test probability of HIT. With the Fagan's nomogram (Figure 2A) we derive a probability for a positive PAT of about 40% ( $\text{post-test odds} = 0.11 \times 6.224 = 0.68464$ , corresponding to a post-

test probability of 40.6%). Finally, the same patient with a titer of 8 (LR + 16.597; Table 5) reaches a post test-probability for HIT of about 65% (post-test odds= $0.11 \times 16.597 = 1.82567$ , corresponding to a post-test probability of 64.6%). Both these probabilities argue in favor of HIT and are high enough to justify acute treatment as potential HIT, until further testing will confirm the diagnosis.

Similarly, for an imaginary patient with a high 4T score (Figure 2B) and therefore a clinical pre-test probability for a positive PAT of about 50% (Table 1a), the same test results would either decrease post-test probability to about 25% (titer of 2: post-test odds= $1.0 \times 0.366 = 0.366$ , i.e. post-test probability 26.8%) or increase it to about 85% (titer of 4: post-test odds= $1.0 \times 6.224 = 6.224$ , i.e. post-test probability 86.2%) and about 95% (titer of 8: post-test odds= $1.0 \times 16.597 = 16.597$ , i.e. post-test probability 94.3%).

It is important to underscore that the diverging clinical implications of a titer of 4 compared to a titer of 2 derive from the fact that these values lay at opposite sides of the optimal cut-offs (Table 2). As a consequence (and similarly to categorical, yes/no cut-offs) a careful interpretation is required for quantitative immunoassay results close to the cut-off threshold, taking into account the coefficient of variation of the assay employed.

## Discussion

In our Swiss cohort of 1291 patients evaluated for suspected HIT we found a prevalence of platelet-activating antibodies of 0.8% among those with a low 4T score,<sup>4,5</sup> 14.4% among those with an intermediate 4T score, and 52.7% among patients with a high 4T score. These numbers are consistent with the prevalence published by various groups around the world (Table 1a). Overall, the diagnosis of HIT, as defined by a positive functional assay for heparin-dependent antibodies, could be confirmed in a small proportion (less than 10%) of patients and therefore the suggestion of switching to an alternative anticoagulant drug as soon as HIT is suspected is questionable. In order to guide treatment decisions it would be very helpful to achieve rapid laboratory diagnosis of HIT.<sup>16</sup> The aim of the present work was to evaluate the ability of immunoassays to predict the presence of platelet-activating HIT antibodies which are usually assessed by more time-consuming functional assays.

We compared the operating characteristics of three commercial immunoassays for anti-PF4/heparin antibodies: Asserachrom HPIA, GTI-PF4 and ID-H/PF4-PaGIA. Figure 1 shows that the ID-H/PF4-PaGIA giving a categorical (negative/positive) result according to the manufacturer's instruction<sup>30</sup> was significantly less informative than both ELISAs. However, when a positive result was semi-quantitated by titer<sup>22</sup> the ID-H/PF4-PaGIA performed at least as well as both quantitative ELISAs (Figure 1). Of note, the optimal cut-off value of 1.367 identified by ROC analysis for the GTI-PF4 ELISA (Table 2) is well in line with threshold OD values published by other groups: 1.400 described by the group of Warkentin<sup>34, 35</sup> and 1.185 described by Bakchoul et al.<sup>12</sup> ROC analysis also confirms an ideal cut-off of  $\geq 4$  for the ID-H/PF4-PaGIA, as previously published.<sup>3, 22</sup> Besides identifying optimal cut-off thresholds for the three immunoassays, ROC analysis allows to define



likelihood ratios for all possible results (Table 5). This information is clinically very useful because, applying Bayes' theorem, it allows to transform pre-test clinical probability for HIT into a post-test probability when evaluating individual patients.<sup>31, 32</sup>

#### *Applying Bayes' theorem to HIT diagnostic work-up*

*Ruling out HIT.* In centers where a low 4T score has been shown to exclude HIT with a 100% NPV (Table 1a), one may rely on clinical judgment. In our cohort we found 7 out 859 (0.8%) patients with a low 4T score and *bona fide* platelet activating anti-PF4/heparin antibodies (Table 1b). Therefore, we still prefer to employ the ID-H/PF4-PaGIA in order to safely exclude HIT.<sup>16, 36</sup> This concept has been confirmed by four groups.<sup>9-12</sup> For instance, Pouplard et al. also employing a Bayesian approach have demonstrated that in patients with an intermediate 4T score, a negative ID-H/PF4-PaGIA significantly decreased the probability of HIT from 10.9% to 0.6%.<sup>9</sup> Of note, in that study a negative PaGIA did not reach a 100% NPV for platelet-activating HIT antibodies because of 1/22 false negative result,<sup>9</sup> probably due to a faulty polymer lot.<sup>23</sup> The present work, indicating negative likelihood ratios for different values results below the optimal cut-offs according to ROC analysis (Table 5), expands this diagnostic concept and allows to employ also low positive results in order to exclude HIT. In fact, as stated by Warkentin et al.,<sup>35</sup> a weak positive immunoassay for anti-PF4/heparin antibodies is paradoxically a strong argument against the presence of HIT. According to our data, OD values  $\leq 0.943$  with the Asserachrom HPIA,  $\leq 1.367$  with the GTI-PF4, and a titer  $< 4$  with the ID-H/PF4-PaGIA significantly decrease the post-test probability for HIT. In our experience, this approach can also be employed for patients with an intermediate or even high pre-test clinical probability for HIT.

*Ruling in HIT.* The present work supports the possibility to rapidly confirm HIT employing the quantitative result of an immunoassay for anti-PF4/heparin antibodies. Several groups have suggested that the magnitude of a positive ELISA is clinically relevant,<sup>34, 37-41</sup> and concerning the ID-H/PF4-PaGIA we had already demonstrated the clinical utility of this approach.<sup>3, 22</sup> In the present study, ROC analysis confirms a cut-off titer of  $\geq 4$ <sup>3, 22</sup> as providing the best compromise between sensitivity and specificity, and shows that titers  $\geq 32$  have a PPV of 100% for the presence of in vitro platelet-activating HIT antibodies. Additionally, we show that the magnitude of a quantitative immunoassay result above the ideal cut-off as indicated by ROC analysis, can be employed to modify the clinical pre-test probability of HIT in individual patients (Figure 2).

In conclusion, we suggest that combining pre-test clinical probability for HIT as assessed by the 4T score with the quantitative result of an immunoassay for antibodies directed against the PF4/heparin complex can be employed not only for excluding but also for confirming HIT. On one hand, we show that a negative and a weak positive immunoassay result can be employed to exclude HIT. On the other hand, we demonstrate that a quantitative positive result above the ideal cut-off value (as defined by ROC analysis) increases the post-test probability for HIT as a function of its magnitude. Of particular note, a positive ID-H/PF4-PaGIA with a titer  $\geq 32$  has a PPV of 100% for functionally relevant HIT antibodies. In Figure 3 we illustrate the diagnostic algorithm employed at our institution.

We think that our results should be clinically relevant and widely applicable because: a. We present data on 1291 out of 1383 (93.4%) patients evaluated for suspected HIT over a period of 15 years; b. The patient population is mixed (53.5 % medical, 23.5 % surgical, 20.5% intensive care) representing real life

in a general hospital; c. The majority of the 4T scores (1021/1291) was prospectively assessed by the consulting haematologist; d. The majority of assays for HIT-antibodies were also prospectively performed at the time of diagnostic work-up (see Design and Methods). Moreover, employing as gold-standard for platelet activating HIT antibodies a two-point PAT, which has been shown to have a high specificity and an acceptable sensitivity,<sup>25</sup> we avoid the risk of overestimating the performance of immunoassays.

This is the first report showing – on a large cohort of over 1200 patients – that a quantitative result of an immunoassay above a given threshold is equivalent to a positive functional assay for HIT antibodies. In addition, we show that applying Bayes' theorem to HIT diagnostic work-up allows for rapid exclusion or confirmation within a few hours after HIT has been suspected, thus enhancing the rationale for individualized treatment decisions.

## **Acknowledgments**

We would like to thank all the physicians of our Department of Hematology who were involved in care of HIT patients over the years and the technicians of the Central Hematology Laboratory who perform routine diagnostic HIT assays. We also thank Therese Jost for her efficient technical assistance.

## **Funding**

Lorenzo Alberio is supported by a grant from the Swiss National Science Foundation (Grant Nr. 3200-065337.01).

## **Authorship and Disclosures**

VN collected and analysed the data, calculated missing 4T scores, wrote the first drafts of the manuscript, and corrected the final manuscript (MD-thesis); IS performed research and corrected the final manuscript; GB performed research and corrected the final manuscript; BL helped designing research and corrected the final manuscript; LA conceived the study, collected and analysed the data, and wrote the final draft of the manuscript.

The authors declare that there are no competing financial interests in relation to the work described.

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**Table 1a. Pre-test probability for in vitro platelet-activating HIT antibodies according to the 4T score**

4T score		Hamilton <sup>8</sup>	Greifswald <sup>8</sup>	Tours <sup>9</sup>	Sidney <sup>11</sup>	Ghent <sup>10</sup>	Giessen <sup>12</sup>	Bern	Total	Probability	
										%	95% CI
<b>Low (0-3)</b>	n/N	<b>1/64</b>	<b>0/55</b>	<b>0/74</b>	<b>0/142</b>	<b>0/31</b>	<b>0/316</b>	<b>7(*)/859</b>	<b>8/1541</b>	<b>0.5</b>	<b>0.2 – 0.9</b>
	%	1.6	0	0	0	0	0	0.8			
<b>Int. (4-5)</b>	n/N	<b>8/28</b>	<b>11/139</b>	<b>14/129</b>	<b>5/92</b>	<b>4/62</b>	<b>9/130</b>	<b>50/358</b>	<b>101/938</b>	<b>10.8</b>	<b>8.8 – 12.8</b>
	%	28.6	7.9	10.9	5.4	6.5	6.9	14.0			
<b>High (6-8)</b>	n/N	<b>8/8</b>	<b>9/42</b>	<b>8/10</b>	<b>4/12</b>	<b>6/9</b>	<b>26/54</b>	<b>39/74</b>	<b>100/209</b>	<b>47.8</b>	<b>41.1 – 54.7</b>
	%	100	21.4	80.0	33.3	66.7	48.1	52.7			
<b>All</b>	n/N	<b>17/100</b>	<b>20/236</b>	<b>22/213</b>	<b>9/246</b>	<b>10/102</b>	<b>35/500</b>	<b>96/1291</b>	<b>209/2688</b>	<b>7.8</b>	<b>6.8 – 8.8</b>
	%	17.0	8.5	10.3	3.7	9.8	7.0	7.4			

n = Patients with a positive functional assay for HIT antibodies; N = All patients evaluated for suspected HIT; (\*) = See table 1b

**Table 1b. Characteristics of the 7 patients with low 4T score and positive PAT (see table 1a)**

	Age years	Sex	4T	PAT	H/PF4- PaGIA Titer	HPIA O.D.	GTI- PF4 O.D.	LA	aCL (IgG)	aCL (IgM)
1	72.5	F	3	positive	32	3.000	3.000	1.17	1.3	4.6
2	69.4	M	3	positive	128	2.867	2.763	1.14	2.3	3.2
3	60.6	F	3	positive	256	2.880	2.801	1.31	5.6	7.2
4	69.4	M	3	positive	16	3.000	1.948	1.29	22.5	3.3
5	75.0	M	3	positive	16	2.435	3.000	1.20	4.4	0.6
6	63.7	M	2	positive	4	1.365	1.616	1.32	0.9	0.8
7	60.1	M	3	positive	8	1.566	3.000	n/a	2.4	3.1

PAT = Heparin-induced platelet aggregation test

H/PF4-PaGIA = ID-H/PF4 particle gel immunoassay

HPIA = Asserachrom HPIA

LA = Confirmation assay for lupus anticoagulant (cut-off for positivity:  $\geq 1.40$ )<sup>42</sup>

aCL = anticardiolipin antibody (cut-off for positivity: IgG > 15 GPL; IgM > 15 MPL)

n/a = not available

**Table 2. Sensitivity and specificity for optimal cut-offs of immunoassays for in vitro platelet-activating HIT antibodies**

<b>Immunoassay</b>	<b>optimal cut-off</b>	<b>sensitivity</b>	<b>95% CI</b>	<b>specificity</b>	<b>95% CI</b>
<b>ID-HPF4-PaGIA</b>	≥ 4 (titer)	<b>98.96</b>	94.3 – 100	<b>95.90</b>	94.6 – 97.0
<b>Asserachrom HPIA</b>	> 0.943 (OD)	<b>98.96</b>	94.3 – 100	<b>93.64</b>	92.1 – 95.0
<b>GTI-PF4</b>	> 1.367 (OD)	<b>97.92</b>	92.7 – 99.7	<b>94.39</b>	92.9 – 95.6

**Table 3. Cut-off negative predictive values (NPV) of different quantitative immunoassays for in vitro platelet-activating HIT antibodies**

<b>Immunoassay</b>	<b>result</b>	<b>NPV</b>	<b>95% CI</b>
<b>ID-HPF4-PaGIA</b>	(*) Negative	<b>100</b>	99.7 – 100
	≤ 1	<b>100</b>	99.7 – 100
	(†) ≤ 2	<b>99.9</b>	99.5 – 100
<b>Asserachrom HPIA</b>	< 0.300	<b>100</b>	99.6 – 100
	< 0.350	<b>99.9</b>	99.4 – 100
	(*) < 0.450	<b>99.9</b>	99.4 – 100
	< 0.700	<b>99.9</b>	99.5 – 100
	(†) ≤ 0.943	<b>99.9</b>	99.5 – 100
<b>GTI-PF4</b>	(*) < 0.400	<b>100</b>	99.6 – 100
	< 0.870	<b>100</b>	99.7 – 100
	< 1.000	<b>99.9</b>	99.5 – 100
	< 1.200	<b>99.8</b>	99.4 – 100
	(†) ≤ 1.367	<b>99.8</b>	99.4 – 100

(\*) = Manufacturer's cut-off value; (†) = Optimal cut-off value according to ROC-analysis

**Table 4. Cut-off positive predictive values (PPV) of different quantitative immunoassays for in vitro platelet-activating HIT antibodies**

<b>Immunoassay</b>	<b>result</b>	<b>PPV</b>	<b>95% CI</b>
<b>ID-HPF4-PaGIA</b>	(†) $\geq 4$	<b>66.0</b>	57.6 – 73.7
	$\geq 8$	<b>75.7</b>	66.6 – 83.3
	$\geq 16$	<b>81.9</b>	72.0 – 89.5
	$\geq 32$	<b>100</b>	99.7 – 100
<b>Asserachrom HPIA</b>	(†) $> 0.943$	<b>55.6</b>	47.8 – 63.1
	$> 1.500$	<b>66.4</b>	57.8 – 74.3
	$> 2.000$	<b>82.2</b>	73.3 – 89.1
	$> 2.500$	<b>92.3</b>	81.5 – 97.9
	$> 3.000$	<b>96.0</b>	79.6 – 99.9
<b>GTI-PF4</b>	(†) $> 1.367$	<b>58.4</b>	50.4 – 66.1
	$> 1.500$	<b>59.4</b>	51.2 – 67.2
	$> 2.000$	<b>69.8</b>	60.6 – 78.0
	$> 2.500$	<b>78.3</b>	67.9 – 86.6
	$> 3.000$	<b>83.3</b>	68.6 – 93.0

(†) = Optimal cut-off value according to ROC-analysis

**Table 5. Interval likelihood ratios (LR) of quantitative immunoassays for in vitro platelet-activating HIT antibodies**

<b>Immunoassay</b>	<b>result</b>	<b>LR</b>	<b>95%CI</b>
<b>ID-HPF4-PaGIA</b>	Negative	<b>0.000</b>	0.000 – 0.093
	1	<b>0.000</b>	0.000 – 1.852
	2	<b>0.366</b>	0.051 – 2.646
	(†) 4	<b>6.224</b>	3.112 – 12.449
	8	<b>16.597</b>	8.087 – 34.065
	16	<b>15.767</b>	8.279 – 30.030
	≥ 32	<b>∞</b>	31.89 – ∞
	<b>Asserachrom HPIA</b>	0.000 – 0.500	<b>0.012</b>
0.500 – 0.943		<b>0.143</b>	0.0201 – 1.016
(†) 0.943 – 1.500		<b>2.409</b>	1.031 – 5.632
1.500 – 2.000		<b>2.766</b>	1.171 – 6.536
2.000 – 2.500		<b>31.12</b>	17.36 – 55.80
2.500 – 3.000		<b>99.58</b>	30.54 – 324.8
3.000 – 3.500		<b>298.8</b>	40.86 – 2185
<b>GTI-PF4</b>		0.000 – 0.500	<b>0.000</b>
	0.500 – 1.000	<b>0.113</b>	0.160 – 0.802
	1.000 – 1.367	<b>0.319</b>	0.044 – 2.298
	(†) 1.367 – 2.000	<b>4.668</b>	2.486 – 8.865
	2.000 – 2.500	<b>13.226</b>	6.903 – 25.34
	2.500 – 3.000	<b>31.120</b>	16.47 – 58.80
	3.000 – 3.500	<b>70.538</b>	30.37 – 163.8

(†) = Optimal cut-off value according to ROC-analysis

## Figure legend

### Figure 1. ROC curves of three immunoassays for anti-PF4/heparin antibodies.

The result of the immunoassays were defined to be true positive or negative depending on whether the respective plasma sample was able to activate donor platelets in a heparin-induced platelet aggregation test (PAT). **Panel A:** ID-H/PF4-PaGIA with qualitative result. **Panel B:** ID-H/PF4-PaGIA with quantitative result. **Panel C:** Asserachrom HPIA. **Panel D:** GTI-PF4. The dotted lines represent the 95%CI of the ROC curve. Note the significantly lower performance of the ID-H/PF4-PaGIA with a categorical (negative/positive) result (Panel A) as compared to a semi-quantitated result by titer (Panel B). See text and Supplemental Material online for details.

### Figure 2. Applying Bayes' theorem to HIT diagnostic work-up.

**Panel A.** Fagan's nomogram<sup>32, 33</sup> illustrating how a titer of 2 (dotted line), 4 (interrupted line) or 8 (straight line) modify post-test probability for HIT, as defined by a positive heparin-induced platelet aggregation test (PAT), in a patient with an intermediate 4T score. **Panel B.** Fagan's nomogram<sup>32, 33</sup> illustrating how a titer of 2 (dotted line), 4 (interrupted line) or 8 (straight line) modify post-test probability for HIT, as defined by a positive heparin-induced platelet aggregation test (PAT), in a patient with a high 4T score. See text for details.

### Figure 3. Diagnostic algorithm for patients with suspected HIT at our institution.

We first assess pre-test probability for a positive PAT calculating the 4T score. We then consider the magnitude of the immunoassay for anti-PF4/heparin antibodies (ID-HPF4-PaGIA) and combine both information as detailed under "Diagnostic Reasoning". As a note of caution, immunoassay results close to the optimal cut-off value should be interpreted carefully, considering the in house coefficient of variation of the assay employed.

APS = Look for clinical or laboratory clues for an antiphospholipid syndrome<sup>29</sup>

D-dimers = Low D-dimers represent a strong argument against the presence of HIT<sup>3</sup>

Figure 1A

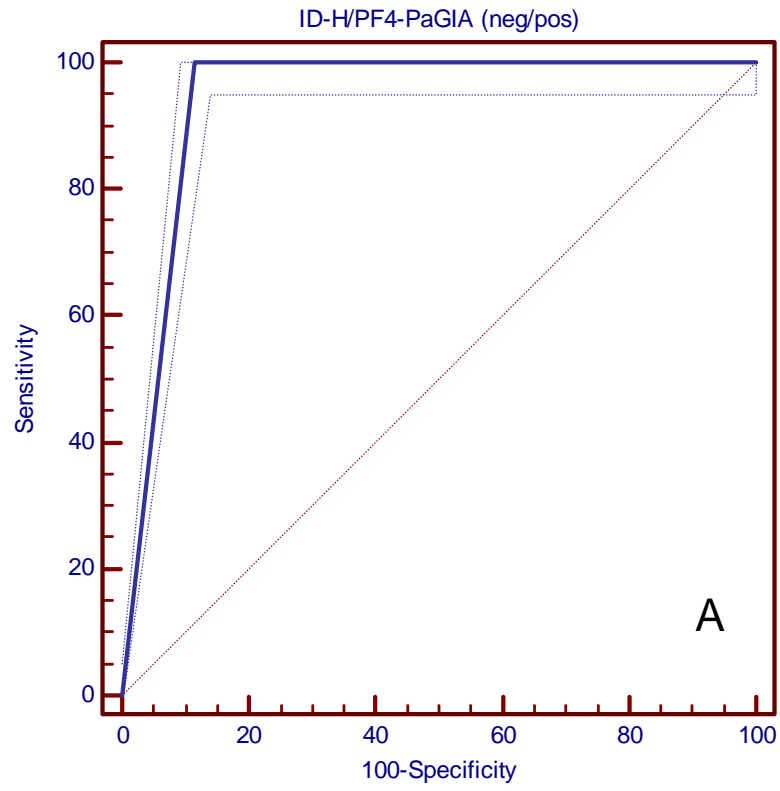




Figure 1B

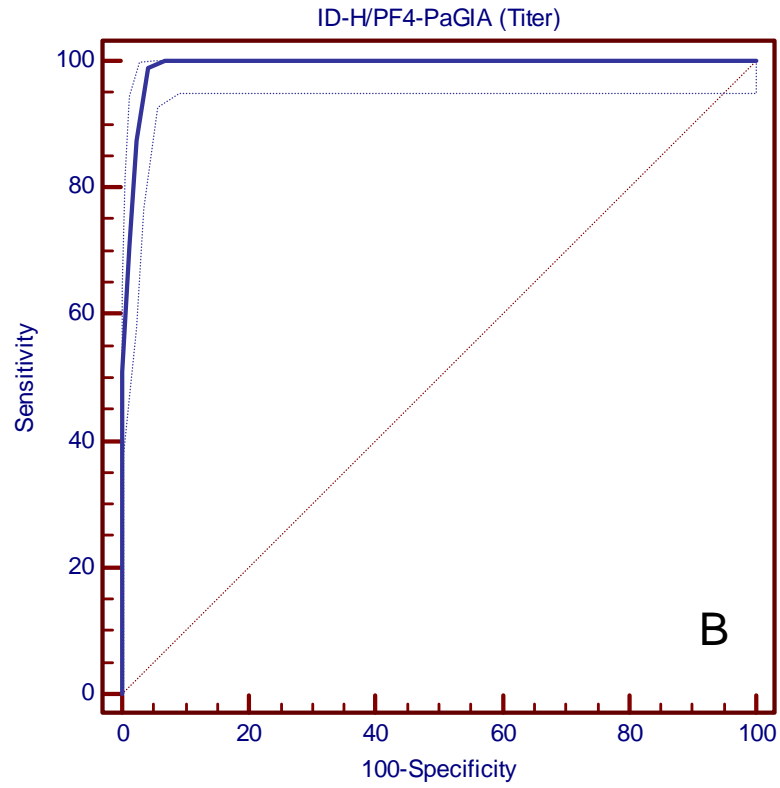


Figure 1C

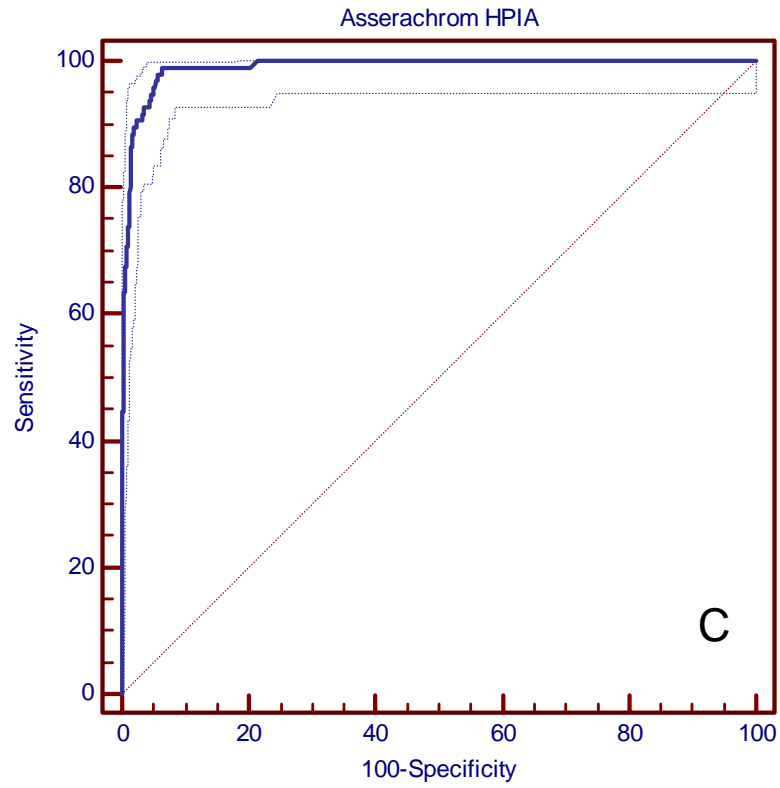


Figure 1D

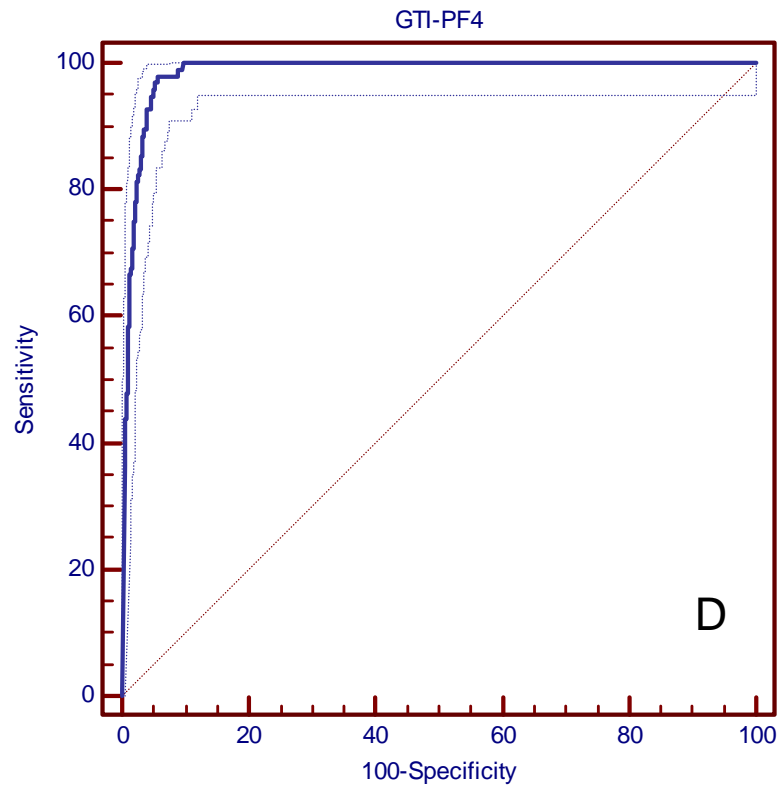


Figure 2. Panel A.

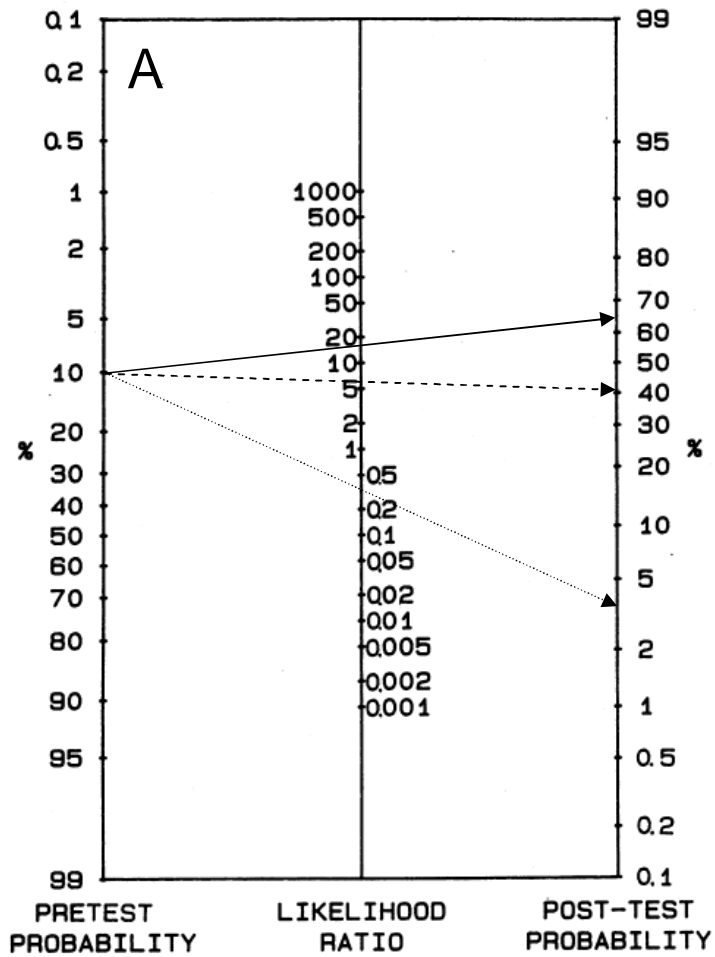
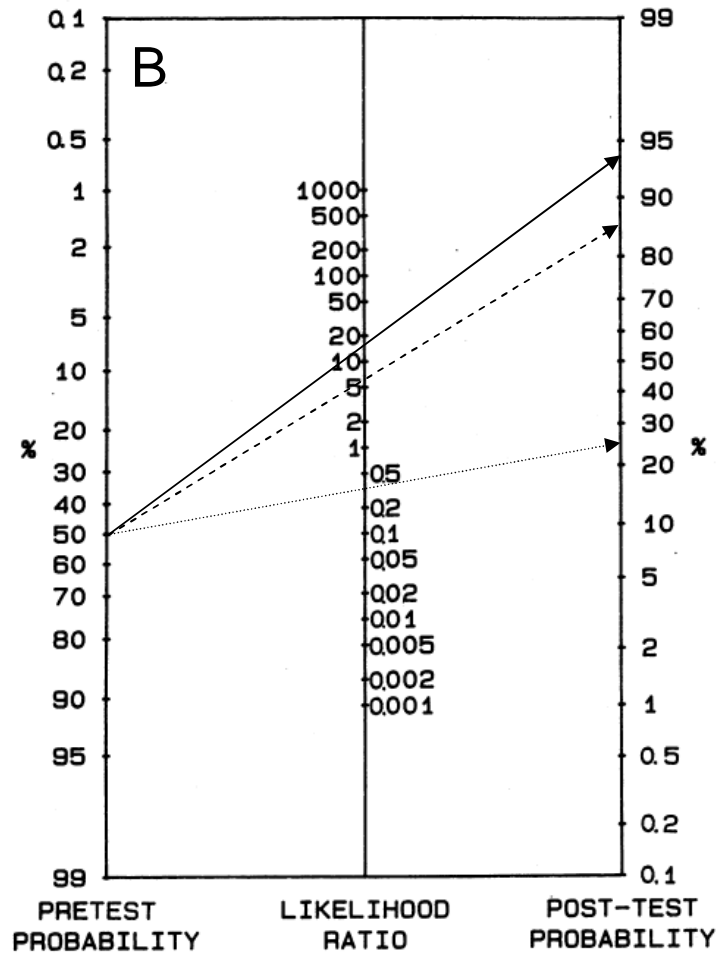
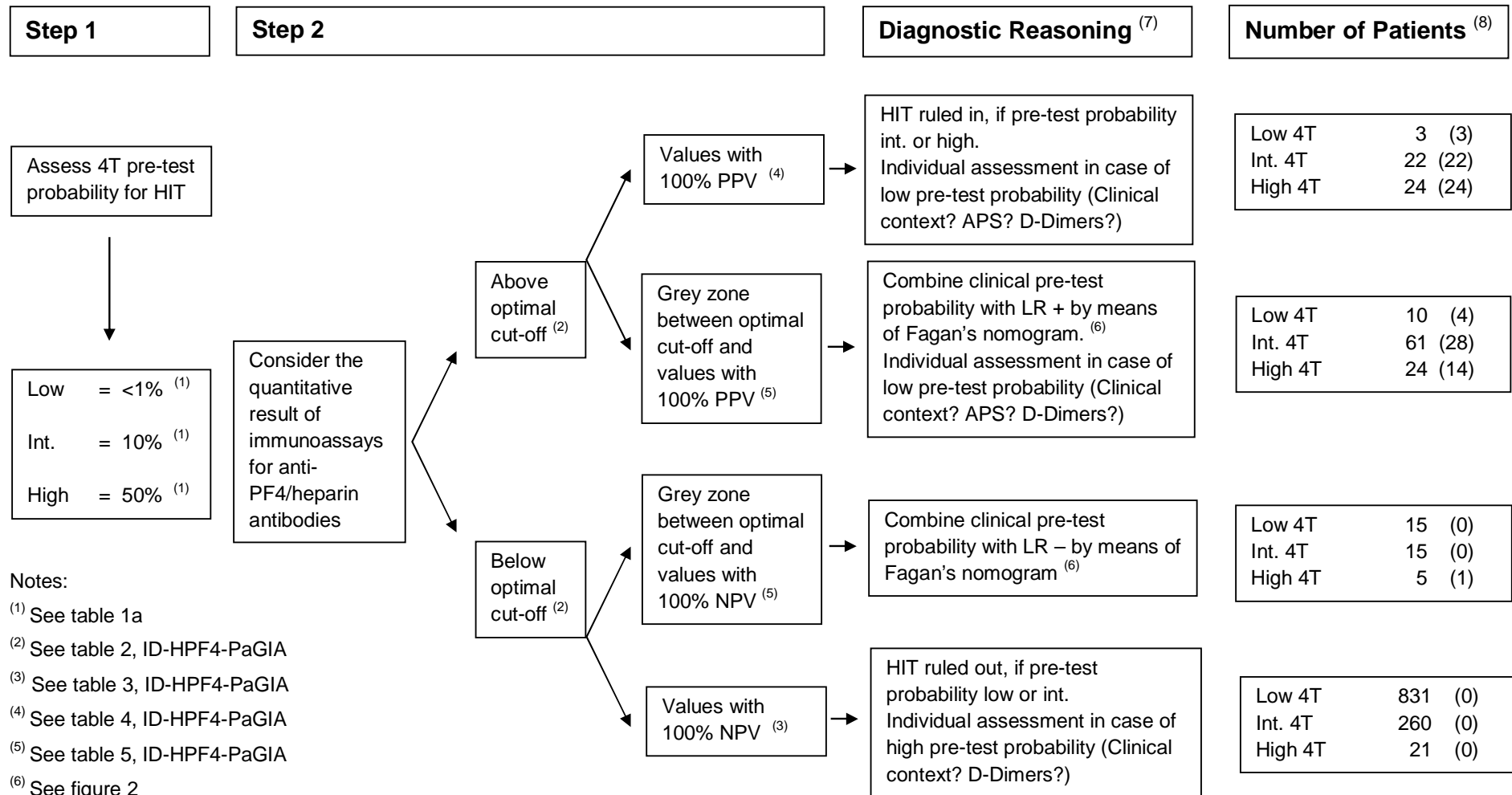


Figure 2. Panel B.



**Figure 3. Diagnostic algorithm for patients with suspected HIT at our institution****Notes:**<sup>(1)</sup> See table 1a<sup>(2)</sup> See table 2, ID-HPF4-PaGIA<sup>(3)</sup> See table 3, ID-HPF4-PaGIA<sup>(4)</sup> See table 4, ID-HPF4-PaGIA<sup>(5)</sup> See table 5, ID-HPF4-PaGIA<sup>(6)</sup> See figure 2<sup>(7)</sup> The performance of this diagnostic algorithm based on the combination of pre-test probability and magnitude of rapid testing for anti-PF4/heparin-antibodies by ID-HPF4-PaGIA, is controlled by routinely performing a functional in-house two-point platelet aggregation assay<sup>(8)</sup> Number of patients classified according to the diagnostic algorithm (total 1291) and, in brackets, the number of those with a positive PAT (total 96)

## Supplementary Appendix

### ***Brief tutorial on ROC analysis and clinical application of Bayes' theorem***

A Receiver operating characteristic (ROC) curve is obtained by calculating the sensitivity and specificity of every observed test value and plotting sensitivity (true positive results; ordinate) against 1–specificity (false positive results; abscissa). A test that perfectly discriminates between two groups would yield a *curve* coinciding with the left and top sides of the plot and having an area under the ROC curve (AUROC) of 1.0. A test that is completely useless would give a straight line from the bottom left corner to the top right corner and have an AUROC of 0.5.<sup>1</sup> In addition, ROC analysis calculates sensitivity, specificity, likelihood ratios, and positive and negative predictive values for all possible threshold values of a given assay, and identifies the cut-off point with the best compromise between sensitivity and specificity.<sup>2</sup> This is the cut-off which creates the smallest total number of false positive plus false negative results.<sup>3</sup>

The positive likelihood ratio (LR +) of a test result is defined as “the probability of an individual *with* disease having a positive test” (true positive) divided by “the probability of an individual *without* disease having a positive test” (false positive) and is calculated as follows: sensitivity / (1–specificity). The negative likelihood ratio (LR –) is defined as “the probability of an individual *with* disease having a negative test” (false negative) divided by “the probability of an individual *without* the disease having a negative test” (true negative), and is calculated as follows: (1 – sensitivity) / specificity.<sup>2</sup> The greater a LR + is, the more useful is the given test result to detect patients *with* the target disease. On the other hand, the smaller the LR – is, the more useful is the given test result to exclude the disease. By convention, LR + above 10 and LR – below 0.1 are considered to provide strong evidence to rule in or rule out diagnosis in most circumstances.<sup>4</sup>

Applying Bayes' theorem, likelihood ratios can be used to calculate the probability of disease for individual patients.<sup>4,5</sup> This is accomplished by combining the pre-test probability of disease with the likelihood ratio of the test result, either by direct mathematical calculation or by using the Fagan's nomogram.<sup>5, 6</sup> The mathematical calculation makes use of the odds rather than probability according to the equation: "pre-test odds x likelihood ratio = post-test odds". The pre-test probability  $p_1$  can be converted to the pre-test odds with the formula: " $p_1 / (1 - p_1) = \text{pre-test odds}$ ". After calculation, the post-test odds  $o_2$  can be converted back to a post-test probability with the equation: " $o_2 / (1 + o_2) = \text{post-test probability}$ ".<sup>4, 5</sup>

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