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High Levels IPF as Possible Predictor Heparin-Induced Thrombocytopenia

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Abstract

Background: Heparin-Induced Thrombocytopenia (HIT) represents a serious complication of heparin treatment. IgG antibodies binding Platelet Factor 4 (PF4) and heparin trigger the clinical manifestations of HIT. A 4T score is used to stratify the selection of patients suitable for examination. However, the selection of suitable patients remains at the discretion of the clinician, who is confronted with determining the cause of thrombocytopenia. The inclusion of the evaluation of the Immature platelet fraction result seems to be a suitable complement to the stratification of patients because we do not climb elevated IPF values when consuming platelets due to their immunization.

Materials and Methods: In a group of 432 thrombocytopenic samples IPF was detected and analyzed in 45 patients with suspected HIT, a 4T score was determined; IPF and HIT functional tests were examined.

IPF was determined by oxazine fluorescent dyeing structures of nucleic acid-containing platelets and fluorescence detection on a Sysmex XN 1000 analyser. To determine HIT, impedance aggregometry using the Multiplate® analyser (MEA) as heparin-induced aggregation techniques. The MEA method uses sensitization of donor platelets with patient plasma in the presence of heparin at a concentration of 0.5IU/mL.

Results: From the results of the test, it is evident that 10 patients from our group of 45 examined showed positivity of HIT, which is a significant number due to the proven occurrence of HIT in patients treated with LMWH and showing thrombocytopenia.

If we evaluate these 10 patients in terms of IPF value, it is evident that 6 of them have an increased value of IPF >10%, which is a 33% positive predictive value and 4 have IPF >30%, when the positive predictive value is even 100%.

In a separate statistical evaluation of the results, a correlation was found between IPF and the result of the MEA test for platelet activation by heparin ($p=0.0233$).

Conclusions: Diagnosis of HIT remains a complicated clinical laboratory issue. However, new diagnostic options provide considerable potential for solving this problem.

The implementation of IPF assays helps us in the diagnosis of HIT on two levels. On the one hand, it provides us with information on platelet consumption in hospitalized patients and thus draws our attention to HIT as one of the options for congestive thrombocytopenia, unless, of course, disseminated intravascular coagulation or thrombotic microangiopathy.

Secondly, its implementation will increase the predictive value of the 4T score in patients at medium risk, which is, however, the vast majority indicated for HIT examination.

Keywords: Heparin-induced thrombocytopenia; Functional test for detection HIT; Immature platelet fraction; Reticulated platelet

Introduction

Heparin-Induced Thrombocytopenia (HIT) is a clinicopathological syndrome that is diagnosed in patients immunized through treatment with both Unfractionated (UFH) and Low-Molecular-Weight (LMWH) heparins [1]. A typical sign for the presence of HIT is the development of antibodies against Platelet Factor (PF) 4/heparin complexes and IgG antibodies activating platelets [2-4].

The biggest problem in diagnosing HIT is the selection of suitable patients. The biggest expansion is the 4T scoring system, which is recommended for the examination of patients in medium and high risk. However, the use of a 4T score is highly demanding and requires

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the attending physician to consider the possibility of developing HIT in patients treated with heparin. Due to its availability, the supplementation of the differential diagnosis scheme with IPF can be performed in a wide group of patients with thrombocytopenia and with current requirements; it is possible to examine them in all patients meeting the criterion for a decrease from the 4T score, i.e. below 50 x 10^9/L or by 30-50 %.

One of the possibilities to supplement the diagnostic balance is the use of reticulated plates or, more recently, immature platelet fraction performed as standard in the examination of thrombocytopenic samples. Reticulated platelets are immature, newly released platelets. They can be distinguished from mature platelets by their RNA content and their larger size. They are suggested to be the platelet analogue of the red cell reticulocytes [2]. They are supposed to reflect bone marrow capacity to produce platelets, and could be a surrogate marker for megakaryocytic activity [3]. A new automated method to reliably quantify reticulated platelets, expressed as the Immature Platelet Fraction (IPF), is available on new generation haemocytometers. IPF is well correlated with reticulated platelets levels [4]. Results are expressed as a percentage of total platelet count (IPF%), or as an absolute number (A-IPF).

Determining the reference range is still a big problem, partly due to various technologies of IPF detection, but also in terms of detecting the causes of thrombocytopenia and its time course, therefore reference range and normal values of IPF among healthy subjects have not been well-defined so far [5-7]. Furthermore, most studies have been conducted on Sysmex XE haemocytometers. There are few data about IPF values on new generation Sysmex XN analyzers, which demonstrated higher sensitivity and specificity for IPF% measurement [8]. One British study, involving 2366 adult patients, aimed to determine reference values for IPF on Sysmex XN-1000 haemocytometers. Median IPF% was 4.4%, with a reference interval of 1.6-10.1%, and median A-IPF was 10.88 x 10^9/L (4.37-23.21). There was a significant difference between male and female; IPF 4.6% (1.8-10.0) for males and 4.3% (1.5-10.1) for females, but without any clinical relevance. There was no significant variation with age among adult patients [9]. Median IPF% was 1.2 point higher and had a wider distribution on Sysmex XN haemocytometers compared to XE analyzers [7]. IPF% also varies with platelet transfusions and with infections [10].

Several studies have suggested a role of IPF% in determining whether thrombocytopenia is due to bone marrow failure or increased peripheral destruction/loss, thus avoiding the need for BM aspiration. It has been shown that IPF% is lower when thrombocytopenia is induced by bone marrow failure. In most published studies, thrombocytopenia is mostly caused by idiopathic aplastic anaemia or by chemotherapy; median IPF% ranges from 1.9 to 8.7 but these values are sometimes >10% [4,6,10,11]. The majority of peripheral thrombocytopenia's are immune thrombocytopenia or caused by Thrombotic Thrombocytopenic Purpura (TTP); median IPF% is higher in those patients, ranging from 7.7 to 17.4%. Two studies have determined cut-off to predict peripheral mechanism, which are 7.3 and 7.7%, with sensitivity of 54 and 87% and a specificity of 92 and 93% respectively [6,11].

However, no work has addressed the use of IPF in consumption thrombocytopenia's, where increased relative IPF levels can be expected, as thrombopoiesis is stimulated in these situations and the number of platelets in the peripheral circulation are reduced [12].

Methods for detecting HIT

Generally, there are two types of laboratory tests to determine HIT. Antigen determination detecting the presence of immunoglobulins that bind antigenic neoepitopes in PF4/heparin complexes [13]. Functional tests are based on detecting the presence of HIT antibodies forming immune complexes that cause platelet activation. Each type of test provides unique and complementary information. None of the laboratory tests should be used alone to confirm or refute the diagnosis of HIT.

Materials and Methods

The group of patients

This study was conducted on a set of 45 blood samples from patients with suspected HIT sent to our laboratory out of 432 thrombocytopenic samples, a 4T score was determined, IPF and HIT functional test were examined.

Whole blood plasma was analyzed immediately using MEA and IPF was measured from K3EDTA plasma. The documentation of patients was determined using the 4T HIT probability scoring system.

Blood collection

Blood sampling was undertaken in a double vacuum tube using a Vacuette® needle (Greiner Bio-One, Vienna, Austria), with a buffered solution containing sodium citrate at a concentration of 0.109mol/L (3.2%) for aggregation test and K3EDTA 5.1mmol/L for detection of IPF. The system ensured the mixing of blood with anticoagulant at a desired ratio of 1:10. Then the blood was carefully mixed in a test tube, with the tube being gently turned upside down several times and transported to the laboratory where the blood was prepared for MEA within two hours and for IPF within four hours.

Impedance aggregometry

To determine the activation of platelet by aggregometry a Multiplate impedance analyser was used (Dynabyte Information system GmbH, Munich, Germany). The determination was performed as follows: to 300µL of citrated blood with donor platelets, 150µL of a heparin solution was added at a final concentration of 0.5 IU/mL, after an incubation of 2 minutes at a temperature of 37°C, the reaction was started by adding 150µL of PPP. Impedance measurement was performed for 20 minutes of continuous stirring of the sample inside the cuvette. The final result is the average of the maximum impedance measured after 20 minutes at both pairs of electrodes.

Confirmation was performed by the same procedure, only the concentration of heparin used for inhibition of platelets was 100IU/mL. Positive results were obtained if the measured impedance was lower than a cut-off of 20AU (aggregation unit) [12].

Donor platelets were obtained directly from a mixture of two whole blood samples collected using Vacuette® (Greiner Bio-One) containing buffered sodium citrate solution at a concentration of 0.109mol/L (3.2%).

IPF

To determine IPF, Sysmex XN 1000 was used. When analysis

was not performed immediately, the samples were stored at a room temperature up to 30 minutes before testing. The internal structures of nucleic acid-containing platelets, such as mitochondria and endoplasmic reticulum, were stained using reagents containing oxazine fluorescent dyes. Two-dimensional scattergrams were plotted based on data obtained *via* flow cytometry using a semiconductor laser, with the X-axis representing the intensity of the Side Fluorescent Light (SFL) and the Y-axis indicating the intensity of the Forward Scattered Light (FSC). The platelets were measured using a platelet-specific channel after staining. Red blood cells typically show weak-to-medium SFL and strong FSC, while normal platelets also show weak-to-medium SFL but weak FSC. The IPF (%) was calculated using the following formula: (particle count in IPF zone/the particle count in the platelet zone) × 100. The AIPC was calculated by multiplying the IPF (%) by the platelet count.

Statistical analysis

For statistical evaluation, Fisher's test of independence and conformity and McNemar's test of frequency were used. The McNemar test only focuses on observation, with repeated measurements providing different results according to the following equation:

$$X^2 = \Sigma \Sigma i<j \, (N_{ij} - N_{ji})^2/N_{ij} + N_{ji}$$

Where N_{ij} is the number of objects, of which the first measurement B_i and the second measurement B_j provided the same or different contrast.

Results and Discussion

Testing for the presence HIT was performed in a group of 45 patients treated 14 days prior with only LMWH not UFH. The examination was performed in a group of patients meeting the clinical criteria for the possible presence of HIT antibodies (known as 4T criteria), relying on the clinical characteristics of the degree and timing of thrombocytopenia in relation to heparin exposure, the presence of new thrombosis, and the exclusion of other etiologies of thrombocytopenia supplemented by an IPF examination.

The group included 13 women (28.9%) and 32 men (71.1%). The average age of the women was 69 years, with a median of 74 years. For men, the average age was 65.8 years, with a median of 68.5 years.

The entire group of patients were assessed by MEA for platelet-activating antibodies. In order to investigate, a modified methodology was used with donor platelets. Positivity samples (cut-off of 20 AU*min) activated by low concentrations of heparin were confirmed by inhibition with high concentrations of heparin determined in 10 patients.

From the results of the test, it is evident that 10 patients from our group of 45 examined showed positivity of HIT, which is a significant number due to the proven occurrence of HIT in patients treated with LMWH and showing thrombocytopenia [1].

If we evaluate these 10 patients in terms of IPF value, it is evident that 6 of them have an increased value of IPF >10%, which is 33% positive predictive value and 4 have IPF >30%, when the positive predictive value is even 100%. Evaluation of cut-off IPF cannot be performed according to the literature found between, as these apply

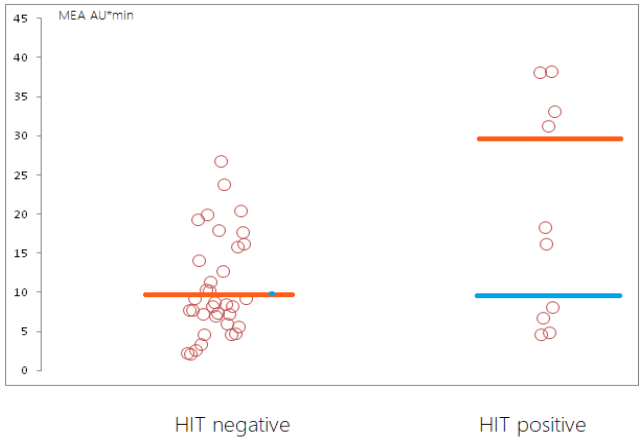

Figure 1: IPF levels in-group HIT positive resp. negative samples.

Table 1: Predictive prognostic value IPF to HIT.

HIT/IPF	IPF <7.2%	IPF >10%	IPF >30%
Negative	35	12	0
Positive	10	6	4
Pre-test PPV	22%		
PPV IPF >10%		33%	
PPV IPF >30%			100%

to patients with other causes of thrombocytopenia.

In a separate statistical evaluation of the results, a correlation was found between IPF and the result of the MEA test for platelet activation by heparin (p=0.0233) (Figure 1 and Table 1).

Diagnosis of HIT remains a complicated clinical laboratory issue. However, new diagnostic options provide a considerable potential for solving this problem.

The implementation of IPF assays helps us in the diagnosis of HIT on two levels. On the one hand, it provides us with information on platelet consumption in hospitalized patients and thus draws our attention to HIT as one of the options for congestive thrombocytopenia, unless, of course, disseminated intravascular coagulation or thrombotic microangiopathy.

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