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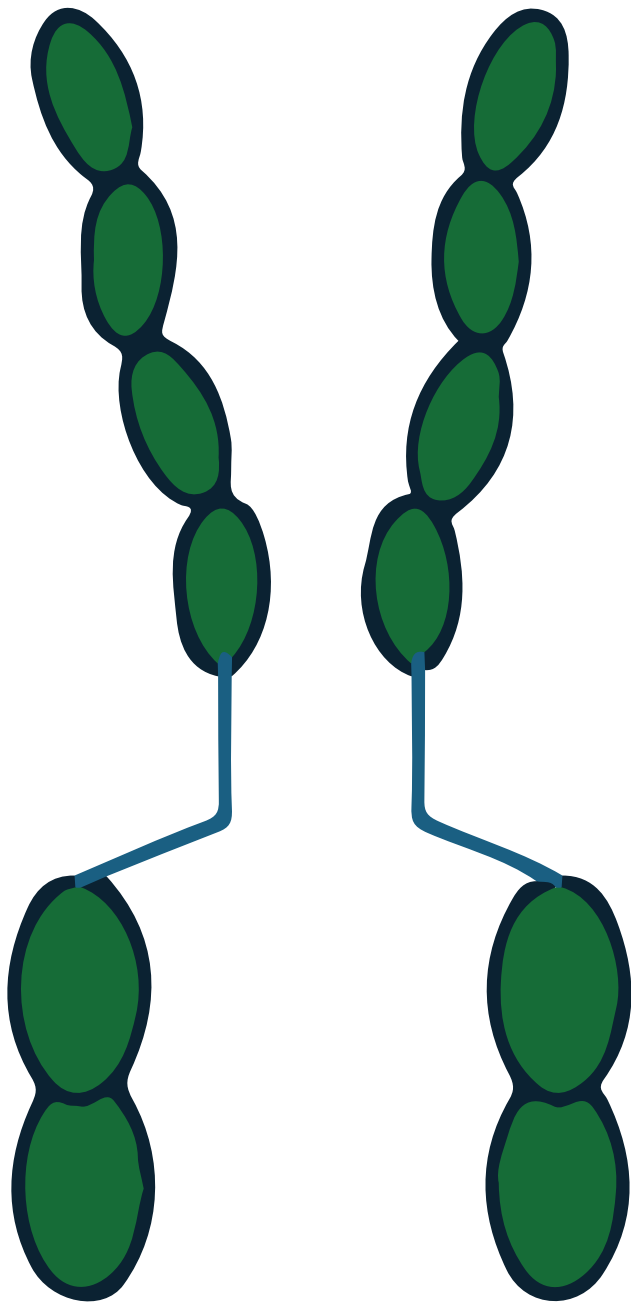
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## CHAPTER 2

### **The diagnostic value of trombopoietin level measurements in thrombocytopenia**

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# The Diagnostic Value of Thrombopoietin level Measurements in Thrombocytopenia

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## Summary

It has been reported that blood thrombopoietin (TPO) levels can discriminate between thrombocytopenia due to increased platelet destruction and decreased platelet production. With our TPO ELISA and a glyocalicin ELISA we analysed a large group of patients in detail and could confirm and amplify the above notion in detail.

TPO levels were determined in plasma from 178 clinically and serologically well-defined thrombocytopenic patients: 72 patients with idiopathic autoimmune thrombocytopenia (AITP), 29 patients with secondary AITP, 5 patients with amegakaryocytic thrombocytopenia and 72 patients who suffered from various diseases (46 in whom megakaryocyte deficiency was not and 26 in whom it was expected). In addition, we measured the level of glyocalicin as a marker of total body mass of platelets.

In all patients with primary AITP and secondary AITP, TPO levels were within the normal range or in some (n=7) cases only slightly increased. The level of glyocalicin was not significantly different from that of the controls (n=95). The patients with amegakaryocytic thrombocytopenia had strongly elevated TPO levels and significantly decreased glyocalicin levels. Similarly, among the 72 thrombocytopenic patients with various disorders, elevated TPO levels were only found in patients in whom platelet production was depressed. The mean level of glyocalicin in these patients was decreased compared to that in controls and patients with AITP, but was not as low as in patients with amegakaryocytic thrombocytopenia.

In conclusion, all patients with depressed platelet production had elevated levels of circulating TPO, whereas the TPO levels in patients with an immune-mediated thrombocytopenia were mostly within the normal range. Therefore, measurement of plasma TPO levels provides valuable diagnostic information for the analysis of thrombocytopenia in general..

Moreover, treatment with TPO may be an option in AITP.

## Introduction

Recent studies indicate that the measurement of the serum level of thrombopoietin (TPO) might be useful to discriminate between patients with thrombocytopenia due to increased platelet destruction, and those with a deficient platelet production.<sup>1-3</sup> TPO levels were found to be not or only mildly increased in patients with autoimmune thrombocytopenia, drug-induced immune thrombocytopenia, post transfusion purpura and X-linked hereditary thrombocytopenia (a variant of Wiskott-Aldrich syndrome).<sup>1-3</sup> In patients with bone-marrow hypoplasia TPO levels were significantly increased.<sup>1-3</sup> However, it should be emphasized that in all these studies only sera were analysed. We found serum TPO levels in normal individuals to be 3.5 times higher than in plasma, due to the release of TPO from platelets during coagulation.<sup>4</sup> Moreover, in the three published studies the number of analysed patients was small and the patient groups were not always clinically and serologically well defined.

To evaluate the value of plasma TPO levels in the differential diagnosis of thrombocytopenia, we analysed a large group of 178 patients in detail. Clinical data were obtained, TPO serum and plasma levels determined and serological tests were performed. Furthermore as a measure of total platelet mass glycofalin levels were determined.<sup>5,6</sup>

Our results show that only in patients with a suppressed platelet production plasma TPO levels were found to be significantly increased. Therefore, the measurement of TPO levels is an important diagnostic tool for the evaluation of thrombocytopenia. Moreover, it could help in selecting those patients who might benefit from TPO therapy.

## Materials and methods

### *Patient samples*

EDTA-anticoagulated blood and serum samples from patients suspected of having autoimmune thrombocytopenia were sent to our laboratory for diagnostic evaluation. Informed consent and clinical data were obtained via the referring physicians by a questionnaire and/or an interview on the telephone. In this way, clinical data were collected from 217 of 377 analysed patients with various forms of thrombocytopenia. Of these 217 patients, 23 pregnant women (mostly with mild thrombocytopenia) were excluded. Gestational thrombocytopenia, thrombocytopenia associated with pregnancy-induced hypertension and the HELLP syndrome account for most of these cases and differentiation from AITP in pregnancy is difficult.<sup>7,8</sup> Only patients with a platelet count of less than  $100 \times 10^9/L$  were included. All together 178 patients were analysed. The male/female

ratio was 0.76, the mean age was  $55 \pm 21$  years (age range from 4 to 91 years). Based on the clinical data, four groups of thrombocytopenic patients were distinguished. Patients with autoimmune thrombocytopenic purpura (AITP) (n=72) were defined, in accordance with the recommendation of the American Society of Hematology (ASH)<sup>11</sup>, by their medical history, physical examination, complete blood count and examination of the peripheral blood smear. Secondary AITP patients (n=29) were defined as patients with isolated thrombocytopenia and an autoimmune disorder frequently associated with autoimmune thrombocytopenia, such as SLE, RA or autoimmune thyroiditis. In 51 AITP and secondary AITP patients bone marrow aspirates were taken and evaluated by the referring physicians. The bone-marrow was normocellular with normal or increased numbers of megakaryocytes was obtained in all patients, which is in agreement with the diagnosis of AITP.

Patients with amegakaryocytic thrombocytopenia (n=5) were AITP patients (as defined by the ASH) but with a severely decreased number of megakaryocytes in the bone marrow.

All other thrombocytopenic patients, suffering from a variety of diseases (table 1), were classified as the miscellaneous group (n=72). Based on clinical data the patients in the miscellaneous group were divided in patients without (A) and with (B) megakaryocyte deficiency (Table 1)

**Table 1 Miscellaneous group**

| <u>miscellaneous group A</u>                            |                         | <u>miscellaneous group B</u> |
|---------------------------------------------------------|-------------------------|------------------------------|
| Neoplastic diseases (n=34)                              |                         |                              |
| CLL                                                     | 4                       | 4 (myelosuppressive drugs)   |
| ALL                                                     | 1                       | 1 (myelosuppressive drugs)   |
| NHL                                                     | 3                       | 5 (myelosuppressive drugs)   |
| Myelodysplasia*                                         | 1                       | 1 (aplasia)                  |
| Breast cancer                                           |                         | 3 (myelosuppressive drugs)   |
| Lung neoplasms                                          |                         | 1 (myelosuppressive drugs)   |
| Intestinal tumors                                       |                         | 2 (myelosuppressive drugs)   |
| Prostatic cancer                                        | 1                       |                              |
| Melanoma                                                | 1                       |                              |
| Urinary tract tumor                                     | 1                       |                              |
| Primary tumor unknown                                   | 1                       |                              |
| Neoplasms of the brain                                  | 1                       | 1 (myelosuppressive drugs)   |
| Multiple myeloma                                        | 1                       |                              |
| Waldenström's macroglobul.                              |                         | 1 (infiltration)             |
| Infections (n=11)                                       |                         |                              |
| viral (n=9)                                             |                         |                              |
|                                                         | HIV                     | 2                            |
|                                                         | EBV                     | 2                            |
|                                                         | HCV                     | 1                            |
|                                                         | CMV                     | 1                            |
|                                                         | unknown**               | 1                            |
| bacterial (n=2)                                         |                         |                              |
|                                                         | Borrelia                | 1                            |
|                                                         | Urinary tract infection | 1                            |
| Drug induced (n=4)                                      |                         |                              |
| peripheral (n=2)                                        |                         |                              |
|                                                         | Salazopyrin             | 1                            |
|                                                         | Fraxiparin              | 1                            |
| myelotoxic (n=2)                                        |                         |                              |
|                                                         | Imuran                  | 1 (myelosuppressive drugs)   |
|                                                         | Methotrexate            | 1 (myelosuppressive drugs)   |
| Liver diseases                                          |                         |                              |
|                                                         | 7                       |                              |
| Cardio-vascular diseases                                |                         |                              |
|                                                         | 5                       |                              |
| Renal disorders                                         |                         |                              |
|                                                         | 2                       |                              |
| Diabetes mellitus                                       |                         |                              |
|                                                         | 2                       |                              |
| Aplastic anemia                                         |                         | 1 (aplasia)                  |
| Bone marrow transplantation                             |                         | 2 (aplasia)                  |
| Alpha-thalassemia                                       | 1                       |                              |
| Pernicious anemia/hypersplenism/<br>portal hypertension | 1                       |                              |
| diffuse intravascular<br>coagulation                    | 1                       |                              |

\* one patient with a normal number of megakaryocytes in the bone marrow

\*\* suspected for viral infection

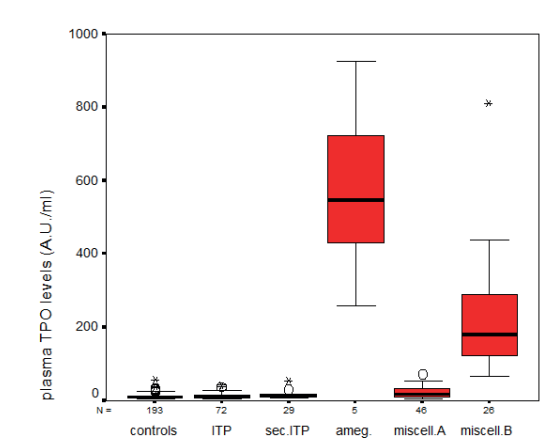


Fig. 1 Plasma TPO levels in different patient groups with thrombocytopenia. AITP, autoimmune thrombocytopenia; SAITP, secondary autoimmune thrombocytopenia; Ameg., amegakaryocytic thrombocytopenia; Miscell.A, miscellaneous thrombocytopenic patients without expected megakaryocyte deficiency; Miscell.B, miscellaneous thrombocytopenic patients with expected megakaryocyte deficiency

## Serological analysis

The direct platelet immunofluorescence test (PIFT), indirect eluate PIFT and direct monoclonal antibody immobilization of platelet antigens assay (MAIPA) were performed as described by von dem Borne et al<sup>9</sup> and Kiefel et al<sup>10</sup>, respectively. The monoclonal antibodies (MoAb) used in the MAIPA were CLBthromb/1 (CD41, anti-GPIIb), MB45 (CD42a, anti-GPIX), SW16 (CD42d, anti-GPV), 10G11 (CD49b, anti-GPIa/IIa) or P58 (CD36, anti-GPIV)). All MoAb were from our institute.

Enough platelets could not be isolated in all cases to perform a MAIPA with all the listed MoAbs. In 90 of 178 (33 of 72 AITP, 16 of 29 SAITP and 41 of 72 miscellaneous) cases the MAIPA was performed.

## TPO ELISA

A solid phase sandwich ELISA for the measurement of plasma TPO concentrations was performed as previously described.<sup>4</sup> Briefly, a mixture of two non-crossreactive MoAb was coated on a microtiter plate. Plates were blocked and washed, after which samples were incubated together with a third biotinylated MoAb. A streptavidin horseradish peroxidase conjugate and a signal amplification system were used for the final colorimetric reaction. A pool of EDTA-anticoagulated plasma derived from thrombocytopenic patients with a high TPO level, was used as a standard. The first dilution of this standard was arbitrarily set at 100 A.U. Normal TPO levels, as determined in a population of 193 healthy individuals, ranged from 4 to 32 A.U. (2.5th- 97.5th percentile). Serum Tpo levels were on average 3.4 times higher.

### Glycocalicin ELISA

MoAb MB45 (CLB, Amsterdam, The Netherlands) was coated overnight on a 96 wells microtiter plate (Nunc Immunoplate Maxisorp, Rockslide Denmark) at a concentration of 2 µg/ml in 100 µl 0.1M carbonate buffer pH9.6. Plates were washed with PBS/0.02% Tween (v/v) and remaining binding sites were blocked for 30 minutes with 150 µl PBS containing 2% pasteurized cows' milk. Subsequently, plates were washed 5 times and a 50 µl sample (or standard) diluted in High Performance Elisabuffer (CLB, Amsterdam, The Netherlands) was incubated for 2 hours together with 50 µl biotinylated MoAb MB15 (CLB, Amsterdam, The Netherlands) (1lg/ml). Again, plates were washed and incubated for 30 minutes with 100 µl streptavidin polyhorseradish peroxidase (1:10.000; CLB, Amsterdam, The Netherlands) in PBS with 2% pasteurized cows' milk. A colorimetric reaction was obtained by addition of 100 µl substrate TMB (0.1mg/ml) in substrate buffer (0.11M NaAc pH5.5 with 0.003% H<sub>2</sub>O<sub>2</sub>) after plates were washed. After 15 minutes the reaction was stopped with 100 µl H<sub>2</sub>SO<sub>4</sub>. The absorbance at 450 nm was measured in a Titertek multiscan Elisa-reader (Flow laboratory, Rockville, MD). All incubations were performed at RT under shaking conditions. Supernatant of a platelet concentrate was used as a standard. Concentrations of glycocalicin (GC) were expressed in Arbitrary Units. Normal plasma GC values as determined in 95 healthy individuals were between 144-444 A.U./ml (mean ± twice the std.).

### Statistical analysis

Statistical analysis was performed in SPSS for Windows, release 6.1.3 (SPSS Inc.). For comparison of groups the Mann-Whitney U - Wilcoxon Rank Sum W Test was used. The correlation between two variables was calculated with Spearman correlation coefficients.

### Results

#### *TPO and glycocalicin levels*

Plasma TPO levels in AITP and SAITP patients were found to be within the normal range in most cases (89%) ( $13 \pm 10$  A.U., mean ± std., range 2 A.U to 54 A.U.) (fig.1). Four patients in the AITP group and three patients in the SAITP group had a slightly increased TPO level (36, 39, 42, 41 A.U./ml and 54, 53, 53 A.U./ml, respectively), whereas in four patients in the AITP group the TPO level was lower than 4 A.U./ml (one 1 A.U./ml and three 3 A.U./ml). As shown in figure 1, the mean level of TPO was slightly higher in the group of AITP patients as well as the SAITP group, as compared to the controls. This difference was statistically significant ( $p=0.03$  and  $p=0.01$ , respectively). There was no correlation between the platelet count and the plasma TPO level in either AITP or SAITP. Serum TPO levels ranged from 6 A.U. to 81 A.U. ( $28 \pm 14$ , mean±std.). The serum/plasma ratio in these two groups of patients was  $2 \pm 0.9$  and  $2 \pm 0.8$  (mean ± std.),

respectively, which is significantly lower than the ratio found in controls ( $3 \pm 0.6$ , mean  $\pm$  std.)( $p < 0.001$ ). There was no correlation between either the serum TPO level or the serum/plasma ratio and the platelet number.

As shown in figure 1, all five patients, classified as amegakaryocytic thrombocytopenia, had strongly elevated TPO levels (range 258-927 A.U./ml). Furthermore in all patients in the miscellaneous B group whose medical data indicated that a decreased hematopoiesis was the cause of their thrombocytopenia, TPO levels were clearly increased ( $231 \pm 160$  A.U./ml, mean  $\pm$  std, range 66-811 A.U./ml,  $n=26$ )(fig.1). These patients either had a malignant infiltration of the bone marrow and/or were receiving myelotoxic therapy ( $n=22$ ) or suffered from bone marrow aplasia ( $n=4$ , one aplastic anemia, two post transplant bone-marrow failure and one myelodysplasia). In contrast the TPO levels of most patients included in the miscellaneous group A, were within the normal range, although the mean TPO level in this group was somewhat higher compared to that in controls ( $21 \pm 16$  A.U./ml, mean  $\pm$  std, range 2-72 A.U./ml).

Figure 2 shows for the patients in the miscellaneous group that analysis of the number of megakaryocytes in bone marrow aspirates, which was performed in 36 of the 72 patients, correlated with the TPO level. In all, but one, decreased numbers of megakaryocytes were counted in the bone-marrow aspirate.

Most AITP and SAITP patients had normal levels of plasma glyocalicin (Fig.3). The level of glyocalicin in the plasma of patients was not significantly different from that in controls. In the amegakaryocytic thrombocytopenia patients the glyocalicin levels were clearly decreased (fig.3). Also in the miscellaneous group with megakaryocyte deficiency (group B), glyocalicin levels were significantly decreased ( $p=0.042$ ). In the miscellaneous group without megakaryocyte deficiency (group A) the glyocalicin levels were mildly increased ( $p=0.046$ ) (fig.3).

### Serological analysis

Autoantibodies were detected by the direct PIFT in 66 of the 101 (65%) AITP and SAITP patients. This was not different in the two AITP groups: 46 of 72 (64%) AITP patients were positive versus 20 of 29 (69%) SAITP patients. The MAIPA was positive in 39% of the patients suffering from AITP. In all but one of the AITP patients with a positive MAIPA the PIFT was also positive. The autoantibodies were directed against the GP IIb/IIIa complex ( $n=5$ ), the GP Ib/IX complex ( $n=2$ ), GP V ( $n=6$ ) and combinations of these three GP complexes ( $n=6$ ) (table 2). In 35% of the patients of the miscellaneous group a positive result with the PIFT was obtained and only in five of 41 (12%) miscellaneous patients a positive MAIPA was found (Table 2).

There was no correlation between the presence or absence of detectable platelet autoantibodies in either the PIFT or the MAIPA, and the TPO level (data not shown).

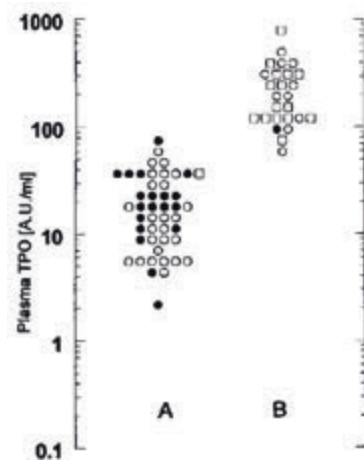


Fig. 2 Plasma TPO levels in patients forming the miscellaneous group. A) without expected megakaryocyte deficiency (n = 46), B) with expected megakaryocyte deficiency (n = 26); O, BM aspiration not performed; •, normal or increased megakaryocyte number in BM aspirate; □, decreased megakaryocyte number in BM aspirate

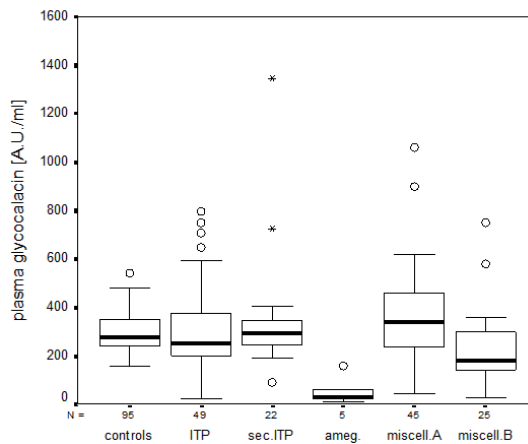


Fig. 3 Plasma glyocalcin levels in different thrombocytopenic patient groups. AITP, autoimmune thrombocytopenia; SAITP, secondary autoimmune thrombocytopenia; Ameg., amegakaryocytic thrombocytopenia; Miscell.A, miscellaneous thrombocytopenic patients without expected megakaryocyte deficiency; Miscell.B, miscellaneous thrombocytopenic patients with expected megakaryocyte deficiency

Table 2: Direct MAIPA results

| Performed*     | AITP<br>31/72 | SAITP<br>16/29 | Miscellaneous<br>26/72 |
|----------------|---------------|----------------|------------------------|
| GPIIb/IIIa     | 4             | 1              |                        |
| GPIb/IX        | 1             | 1              | 1                      |
| GPV            | 1             | 5              | 1                      |
| GPIa/IIa       |               |                | 1                      |
| Combination**  | 6             |                | 2                      |
| Negative       | 19/31 (61%)   | 9/16 (56%)     | 21/26 (81%)            |
| Total positive | 12/31 (39%)   | 7/16 (44%)     | 5/26(19%)              |

\* at least the MAIPA performed for GPIIb/IIIa, GPIb/IX and GPV

\*\* all combinations of GPIIb/IIIa, GPIb/IX and GPV

## Discussion

In this study, we confirm and extend the results of earlier studies<sup>1-3</sup>, that TPO levels in AITP patients are normal or only mildly increased, while they are strongly increased in patients with suppressed megakaryocytopoiesis. In contrast to other published studies, we measured TPO levels in EDTA-anticoagulated plasma samples instead of in serum, because we previously found that serum TPO levels are 3.4 times higher than plasma levels due to TPO release from the platelets during clotting.<sup>4</sup> In our opinion, plasma levels are therefore a more accurate measure for circulating TPO than serum TPO levels.

TPO is the major regulator of platelet production and is normally produced mainly by the liver and the kidney.<sup>12-14</sup> Early hematopoietic progenitor cells, all cells of the megakaryocyte lineage and platelets express the receptor for TPO, MPL.<sup>15</sup> Binding of plasma TPO by circulating platelets has been proposed to be the main regulatory mechanism of plasma TPO level.<sup>16-18</sup> This conclusion was based on static levels of TPO-encoding mRNA in the liver and kidney of mice made thrombocytopenic.<sup>16,17</sup> However, recent data showed that some upregulation of TPO-encoding mRNA in bone-marrow stromal cells may occur in thrombocytopenic mice and humans.<sup>13,14</sup> TPO produced locally, in the bone-marrow environment, may largely account for the increased numbers of megakaryocytes found in 27 of the 51 analyzed patients with AITP in this study (data not shown).

Although thrombocytopenia may induce an elevated production of TPO in the bone marrow, this TPO does not seem to be capable of enhancing platelet

production in AITP patients. Circulating TPO presumably is trapped by the platelets and/or megakaryocytes and is subsequently destroyed in these cells or in the spleen.<sup>18</sup> The decreased serum/plasma TPO ratio's in the AITP groups could be explained by the low platelet counts which results in a lower release of TPO upon coagulation.

It has been agreed that the lifespan of the platelets in AITP patients is shortened. However, mostly platelet production seems to be within normal limits and, platelet production is increased or decreased in only a small percentage of AITP patients.<sup>19-21</sup> This is in accordance with the finding that glycocalicin levels in AITP patients are not significantly different from those in controls, because glycocalicin levels seem to reflect the total body mass of platelets.<sup>5,6</sup> In our study, only in a small percentage of AITP cases (both from the primary and the secondary group), an increased (11 of 71 (15%)) or decreased (5 of 71 (7%)) glycocalicin level was found. All other AITP patients had normal glycocalicin levels and thus a normal platelet production. Severely decreased glycocalicin levels were measured in plasma from patients with amegakaryocytic thrombocytopenia only. The glycocalicin levels were also decreased, although to a lesser extent, in plasma of patients suffering from diseases accompanied by a suppressed megakaryocytopoiesis (miscellaneous group B). Thus, our study confirms the value of glycocalicin measurement as a marker for total platelet mass. However, the levels in individual patients were found to be too widely spread. This makes plasma glycocalicin measurement less useful for diagnostic purposes.

The absence of elevation of circulating TPO levels in immune-mediated thrombocytopenia, can be considered to be a relative endogenous TPO deficiency, and therefore may be of clinical importance. It might indicate that in AITP treatment with TPO is relevant. Hematopoietic growth factors are already used in the treatment of autoimmune mediated blood cell destruction. Granulocyte Colony Stimulating Factor has been successfully used to obtain normal neutrophil counts in several cases of autoimmune neutropenia.<sup>22-28</sup> These patients showed an increase in the number of neutrophils and a decrease in the titer of neutrophil-specific autoantibodies, most probably by endogenous consumption of the autoantibodies.<sup>22-28</sup> The same mechanism might apply in AITP.

The diagnosis of AITP remains a clinical diagnosis. The suggested diagnostic evaluation of AITP, based principally on medical history, physical examination and examination of the peripheral blood is confirmed by the exclusion of other causes of thrombocytopenia.<sup>11</sup> According to the American Society of Hematology guideline bone marrow evaluation is not found to be necessary.

However, cases of amegakaryocytic thrombocytopenia would be missed in this way. We show here that measurement of the TPO level may give additional information in the screening of thrombocytopenic patients, to exclude amegakaryocytosis and a depressed megakaryocyte/platelet formation as the cause of the thrombocytopenia. The sensitivity of serological tests was found to be quite low. Platelet-bound antibodies were detected with the PIFT in 63%, and with the MAIPA in only 39% of clinically well-defined AITP patients. In all cases of AITP we found normal or only mildly increased TPO levels, whereas all amegakaryocytic thrombocytopenia patients and patients suspected of having megakaryocyte deficiency showed strongly increased TPO levels.

In conclusion, our study confirms that measurement of TPO levels is an important diagnostic tool for the evaluation of thrombocytopenic patients. The low TPO levels in AITP patients indicate that AITP patients might benefit from the administration of TPO.

### **Acknowledgement**

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