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Figure 1

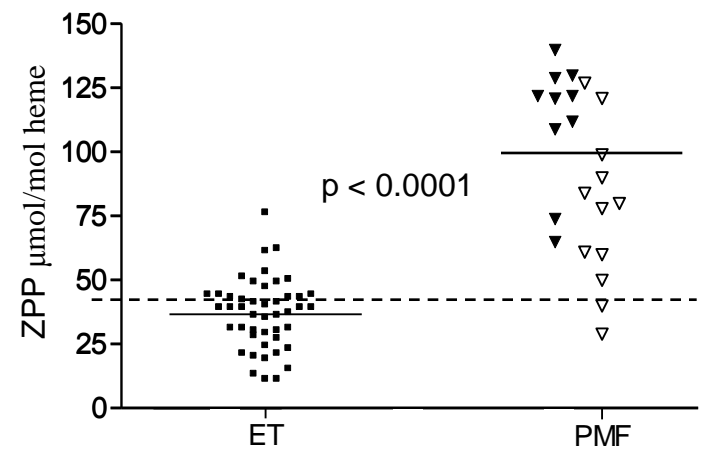
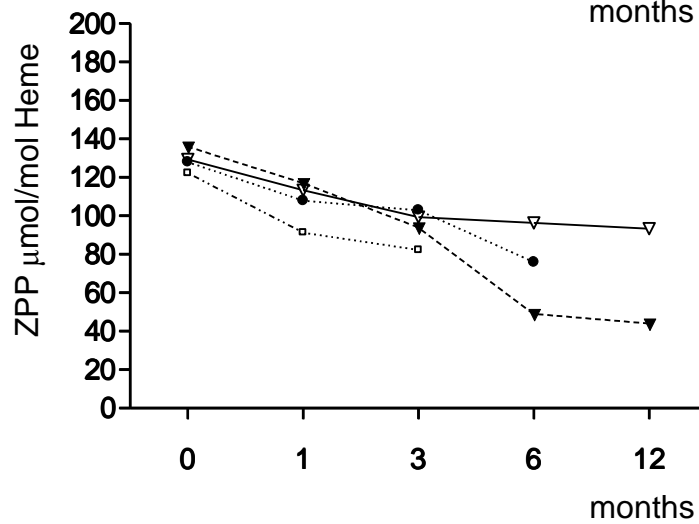
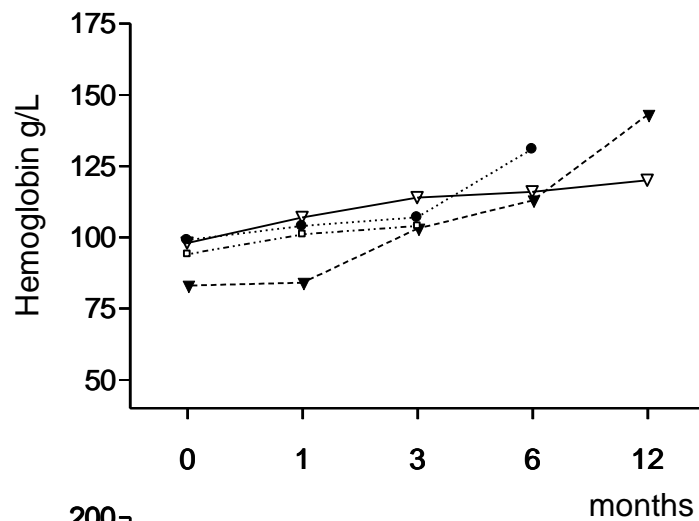


Figure 2



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The role of zinc protoporphyrin measurement in the differentiation between primary myelofibrosis and essential thrombocythaemia

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Key words: Zinc protoporphyrin; primary myelofibrosis; essential thrombocythaemia

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Abstract

1
2 The differentiation between primary myelofibrosis (PMF) and essential thrombocythaemia (ET) may
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4 be difficult especially in early stage disease. In PMF, increased levels of inflammatory cytokines
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6 induce impaired iron-utilization and disturbed erythropoiesis. In conditions with impaired iron support
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8 to erythropoiesis, zinc protoporphyrin (ZPP) is produced instead of heme. Here we investigate,
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10 whether ZPP concentration can be useful in the differentiation between PMF and ET.

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13 Seventy newly diagnosed patients (PMF, n=24; ET, n=46) were analysed. Intraerythrocytic ZPP
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15 concentration (normal: ≤ 40 $\mu\text{mol/mol}$ heme) was measured by Aviv front-face hematofluorometer. In
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17 PMF, ZPP concentrations were significantly increased when compared to ET (99 ± 37 $\mu\text{mol/mol}$ heme
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19 vs. 36 ± 13 $\mu\text{mol/mol}$ heme, $p < 0.0001$). There was also a significant difference between early stage
20
21 PMF and advanced disease (77 ± 30 $\mu\text{mol/mol}$ heme vs. 122 ± 31 $\mu\text{mol/mol}$ heme, $p = 0.003$). ZPP
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23 > 76 $\mu\text{mol/mol}$ heme as observed in 71% of PMF-patients were not seen in ET. In PMF-patients
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25 responding to immunosuppressive treatment (n=4), the increase of hemoglobin was accompanied by
26
27 declining ZPP. In summary, by detecting the disturbed iron metabolism common in PMF, ZPP may
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29 assist in the differentiation between PMF and ET. Concentrations > 60 $\mu\text{mol/mol}$ heme are unlikely in
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31 ET if iron deficiency is excluded. ZPP determination is also useful for monitoring the effect of therapy
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Introduction

Primary myelofibrosis (PMF) is a clonal proliferative neoplasm of the hematopoietic stem cell which is clinically characterized by pancytopenia, splenomegaly and extramedullar hematopoiesis [1,2]. The diagnosis of PMF is based on clinical features, laboratory findings and bone marrow biopsy [3]. The latter typically reveals megakaryocytic proliferation, accompanied by a reactive deposition of fibrous connective tissue which results in a characteristic picture in advanced disease. However, at early stages of the disease and especially in the prefibrotic phase, the accurate morphological differentiation between PMF and other chronic myeloproliferative neoplasms poses a diagnostic challenge [4]. This is in particular the case for the essential thrombocythaemia (ET), as marked thrombocytosis is often the only finding in both entities and subtype-specific markers are absent. The histological examination of the bone marrow biopsy plays a pivotal role in distinguishing these two entities. The entire histological pattern, ranging from cellularity to granulopoiesis, erythropoiesis and especially the megakaryopoiesis, has to be considered in these cases. Apart from the new diagnostic criteria of the revised WHO classification, the precise differentiation of PMF and ET strongly depends on investigators experience and remains a problem in daily clinical practice.

The outstanding finding in PMF is the reticulin or collagen fibrosis, which is regularly present in more advanced disease. One major pathogenetic mechanism involved in the development of myelofibrosis represents the increase of inflammatory cytokines such as platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and transforming growth factor β -1 (TGF β -1) [5]. These cytokines mediate essential changes in the bone marrow homeostasis. These changes result in shortened survival of red blood cells, suppression of erythroid progenitor cells, inadequate erythropoietin production, impaired iron utilization and finally in increased production of connective tissue [5-7]. Therefore, the PMF displays changes typical for anaemia of chronic diseases (ACD). The latter develops in the course of chronic disorders and is also associated with impaired iron metabolism caused by an increase of inflammatory cytokines.

1 In a previous study we have shown that zinc protoporphyrin (ZPP) reflects the extent of the impaired
2 iron utilisation in ACD [8], being produced instead of heme when iron is not available. By qualitative
3 and quantitative assessment of the impaired iron supplementation to the erythropoiesis, measurement
4 of the ZPP concentration does not only support the diagnosis of ACD but also the supervision of the
5 course of the disease [8].
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10 Supposing similar effects on iron metabolism in PMF and ACD and the lack of iron disturbance in ET,
11 we evaluated whether the ZPP measurement could be useful for the discrimination between PMF
12 and ET and for the management of PMF.
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Patients and Methods

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2 The study was a single center analysis at III. Department of Medicine, University Medical Centre
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4 Mannheim, University of Heidelberg, Germany. Untreated patients at first diagnosis of ET or PMF
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6 were selected between January 2000 and July 2009. Altogether, 70 patients (median age 65 years,
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8 range 25-91 years) diagnosed by the revised WHO criteria [9] as ET or PMF were enrolled into the
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10 study and analysed. The laboratory data obtained from these patients are summarised in Table 1.
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12 Individual ZPP concentrations of patients at diagnosis are depicted in Figure 1. Only previously
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14 untreated, non-transfused individuals diagnosed with PMF or ET were analysed. The diagnosis was
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16 performed according to the criteria of the revised WHO classification [9], including bone marrow
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18 biopsy, cytogenetic and molecular genetic examinations of BCR-ABL in all individuals.
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22 All patients were diagnosed and regularly followed-up in our institution. Patients without stainable
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24 iron in bone marrow were not enrolled into this analysis.
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29 The complete blood counts and tests of iron metabolism including ferritin, serum iron, transferrin,
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31 transferrin saturation, ZPP and the serum concentration of the soluble transferrin receptor (sTfR) were
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33 determined at baseline in all enrolled patients.
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36 The measurements of hemoglobin and the other parameters of the blood count were performed by an
37
38 automatic cell counter (Model CC-180, Sysmex, Kobe, Japan). Serum ferritin (normal value: 35–310
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40 µg/L for men, 25–210 µg/L for women) was determined by the Access Ferritin immunoassay
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42 (Beckmann-Coulter, Krefeld, Germany). Serum iron, transferrin and transferrin saturation were
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44 determined by ELISA. ZPP concentration (normal ≤ 40 µmol/mol heme) was measured with the Aviv
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46 front-face hematofluorometer (Aviv Biomedical Company, Lakewood, New Jersey, USA) on washed
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48 erythrocytes [10].
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51 For *JAK2V617F* mutation analysis the genomic DNA was extracted from the peripheral blood using
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53 QIAampDNA Kit (Qiagen, Hilden, Germany). The stored DNA samples were tested for the
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55 *JAK2V617F* mutation by tetra ARMS PCR Assay as described [11] in 55 patients.
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Statistical analyses were performed using the GraphPad Prism statistical package for Windows (Version 4.0, GraphPad Software Inc., San Diego, CA, USA). The non-parametric two-tailed Mann-Whitney test was used to determine significances between groups. Correlation coefficients were determined by Pearson`s correlation coefficient test. Differences between frequencies were proved for significance by the Chi-square Test, a p-value less than 0.05 was considered as statistically significant. The diagnostic accuracy was defined as the ratio of “true positives” + “true negatives” divided by the sum of “true positives” + “true negatives” + “false positives” + “false negatives”.

Ethics: The study was conducted in accordance with the Declaration of Helsinki 1975, as revised in 1983 and approved by the institutional review board. The study did not influence in any way the management of the patients, as all the tests used in the study including the bone marrow examinations were routinely done in all patients with ET and PMF at time of diagnosis. All patients gave written informed consent for testing and analysis of their data.

Results

Forty-six patients (19 males and 27 females, median age 62 years, range 25-78) were diagnosed as ET (Table 1). The mean ferritin concentration was 111 ± 80 $\mu\text{g/L}$ (range 25-452) and the transferrin saturation 26 ± 7 % (range 18-45). The mean ZPP concentration at diagnosis was 36 ± 13 $\mu\text{mol/mol}$ heme (range 11-76) (Figure 1). Twenty-eight of the 46 patients (61%) with ET had a ZPP concentration below the upper normal value of 40 $\mu\text{mol/mol}$ heme. Eighteen ET patients (39%) showed an increased ZPP, the concentration was, however, only marginally elevated. Forty ET patients (87%) had a ZPP concentration < 50 $\mu\text{mol/mol}$ heme, 43 patients (93%) < 60 $\mu\text{mol/mol}$ heme. Only three patients revealed higher ZPP concentrations (61, 62 and 76 $\mu\text{mol/mol}$ heme, respectively). ZPP concentrations > 76 $\mu\text{mol/mol}$ heme were not observed in ET.

Twenty-four patients (15 males and 9 females, median age 71 years, range 52-86) were classified to have PMF (Table 1). The mean ferritin concentration (480 ± 653 $\mu\text{g/L}$, range 36-1792) was significantly higher ($p=0.0004$) compared to ET. There was no difference in transferrin saturation between PMF and ET patients observed.

The mean ZPP concentration at the diagnosis of PMF was 99 ± 37 $\mu\text{mol/mol}$ heme (range 29-178), which was significantly higher compared to patients diagnosed with ET ($p<0.0001$). The individual ZPP concentrations were above the upper normal value of 40 $\mu\text{mol/mol}$ heme in 92% (22/24) of cases. ZPP concentrations > 50 $\mu\text{mol/mol}$ heme were exceeded by 21 of the 24 PMF patients (88%), concentrations > 60 $\mu\text{mol/mol}$ heme by 20 (83%) patients. Comparing these data with the distribution of ZPP concentrations obtained in ET patients, there was a significant difference in all three groups (ZPP > 40 $\mu\text{mol/mol}$ heme, > 50 $\mu\text{mol/mol}$ heme and > 60 $\mu\text{mol/mol}$ heme, respectively) observed ($p<0.0001$; chi-square test). ZPP concentrations >76 $\mu\text{mol/mol}$ heme, which were not observed in ET, were exceeded by 17 of the 24 PMF patients (71%).

The two PMF patients with normal ZPP concentrations (29 and 40 $\mu\text{mol/mol}$ heme) showed remarkable high platelet counts ($1,700 \times 10^9/\text{L}$ and $808 \times 10^9/\text{L}$), an enlarged spleen (22 cm and 12 cm)

1 and were tested positive for the *JAK2V617F* mutation. The clinical course was much more comparable
2 to the course of ET.
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4 When subdividing the PMF patient in prefibrotic stage and fibrotic stage according to the bone
5 marrow examination, 12 PMF patients were in the hypercellular prefibrotic stage and 12 in the fibrotic
6 phase at the time of the diagnosis. Comparing these groups, mean ZPP concentrations were
7 significantly higher in fibrotic stage of the disease ($122 \pm 31 \mu\text{mol/mol heme}$, range 65-178) than in
8 early stage PMF ($77 \pm 30 \mu\text{mol/mol heme}$, range 29-127; $p=0.003$). In fibrotic stage, hemoglobin
9 values ($96 \pm 11 \text{ g/L}$, range 79-130) were lower than in the prefibrotic phase (121 ± 26 , range 75-160; p
10 $= 0.02$). Patients with fibrosis showed higher erythropoietin levels ($84 \pm 80 \text{ U/L}$, range 14-245) than
11 patients in prefibrotic stage of PMF ($19 \pm 23 \text{ U/L}$, range 3-79; $p=0.01$). There were no significant
12 differences observed in mean WBC count, platelet count, transferrin saturation, sTfR and ferritin.
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27 The *JAK2V617F* mutation was found in 26 of all patients analysed (26/55, 47%). Seventeen of the ET
28 patients (17/38, 45%) and 9 of the PMF patients (9/17, 53%) were *JAK2V617F* positive. The mean
29 ZPP, ferritin and sTfR concentration did not differ in both entities between patients with mutated and
30 non-mutated *JAK2*. In ET with non-mutated *JAK2* the mean platelet count was significantly higher
31 ($1027 \pm 333 \times 10^9/\text{L}$; range 547-1740), than in *JAK2*-mutated ET (761 ± 460 , range 460-1370;
32 $p=0.008$).
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43 Four of the PMF patients responded to immunosuppressive treatment (lenalidomide/prednisone, $n=1$;
44 thalidomide/prednisone, $n=1$; cyclophosphamide, $n=1$; cyclophosphamid/mycophenolate mofetil,
45 $n=1$, UPN 2) administered for 3 – 12 months. In these patients ZPP concentration decreased
46 successively (Figure 2). The drop in ZPP concentration was accompanied by an improvement of
47 hemoglobin levels. One case (UPN 2) showed a ZPP reduction from $136 \mu\text{mol/mol heme}$ to normal
48 values with a significant reduction of bone marrow fibrosis accompanied by a complete normalisation
49 of the peripheral blood cell counts for now more than 4 years without further treatment.
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58 In summary, 20 of 24 (83%) PMF patients and 3 of 46 (7%) ET patients had a ZPP concentration > 60
59 $\mu\text{mol/mol heme}$ at diagnosis of the disease. Accordingly, the diagnostic accuracy of a ZPP
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concentration > 60 $\mu\text{mol/mol}$ heme to identify PMF in a PMF/ET patients group was 90%, with a sensitivity of 83% and a specificity of 93%.

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Discussion

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2 In the present study, we could show that the measurement of the ZPP concentration provides a useful
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4 contribution to differentiate PMF from ET. ZPP concentration further allows a monitoring of the
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6 therapeutic measures in PMF.
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10 The intraerythrocytic ZPP concentration observed in our PMF patients at diagnosis was significantly
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12 higher when compared to the patients diagnosed with ET. Whereas 61% of the ET patients had normal
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14 ZPP, the concentrations were elevated in 92% of the PMF patients. The only two PMF patients with a
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16 normal ZPP concentration had markedly elevated platelet counts and normal hemoglobin levels. They
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18 were diagnosed as PMF due to bone marrow morphology in which clusters of atypical
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20 megakaryocytes were present. The marrow specimens, however, revealed no relevant marrow fibrosis
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22 and the clinical appearance of these patients corresponded better to ET.
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26 In contrast to PMF, the elevation of the ZPP concentration observed in ET individuals was only
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28 minimal. ZPP concentrations $> 60 \mu\text{mol/mol}$ heme, as detected in 83% of PMF patients, were
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30 observed only in 7% of the ET individuals ($p < 0.0001$; chi-square test). Therefore, the ZPP
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32 concentration of $60 \mu\text{mol/mol}$ heme seems to be a suitable cut-off value to separate ET and PMF.
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38 The diagnostic potential of the ZPP determination to separate PMF from ET patients is obviously
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40 based on the disturbance of iron utilization in PMF [6,12], whereas iron deficient erythropoiesis is
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42 uncommon in ET. The impaired iron bioavailability observed in PMF seems to be a consequence of
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44 the overexpression of inflammatory cytokines such as interleukin-1 and transforming growth factor-
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46 beta (TGF- β) which contributes to the underlying pathogenetic mechanism finally leading to fibrosis
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48 [13]. This aspect of the PMF pathogenesis strongly resembles features known from the anaemia of
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50 chronic diseases (ACD). In ACD there is also a cytokines-mediated impairment of iron bioavailability,
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52 mainly caused by tumor necrosis factor- α (TNF- α), interleukin-1 and interferon γ [14]. The impaired
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54 iron supply to the bone marrow leads to an iron-deficient erythropoiesis with decreased hemoglobin
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56 production finally resulting in anaemia [15-17]. The impaired bioavailability in ACD is regarded to be
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58 an unspecific defence mechanism to suppress the inflammatory process [18;19]. It is characterized by
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1 a redistribution of iron to storage sites, which is expressed by an increase of ferritin and low levels of
2 iron and saturation of transferrin in serum. In this condition, as soon as the iron concentration gets
3 insufficient to satisfy the need of the erythropoiesis, zinc is increasingly incorporated into
4 protoporphyrin IX and ZPP is produced [15-17]. Similar to ACD, the sustained inflammatory process
5 in patients with PMF obviously leads to a shifting of iron to storage sites, resulting in increase of
6 stainable iron in bone marrow and serum ferritin, as also observed in our study. The bone marrow
7 specimens of the PMF patients showed increased amounts of stainable iron and the serum ferritin
8 concentration was significantly elevated when compared to the ET group. However, due to the wide
9 range of serum ferritin levels in both disorders a reliable discrimination between ET and IMF by the
10 determination of the serum ferritin concentration was not feasible.
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24 The extent of the ZPP elevation depends on the severity and duration of the inflammatory process. Our
25 results indicate that the increased inflammatory activity caused by overexpressed cytokines is
26 obviously present already in the prefibrotic phase [20]. Despite the lack of significant marrow fibrosis,
27 there was a significant increase of ZPP observed in patients with hypercellular prefibrotic stage PMF
28 comparing to both normal population and ET group.
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35 The ZPP concentration directly reflects the amount of impaired iron bioavailability, which depends
36 directly on the inflammatory activity of the underlying disease [21-23]. By quantitative assessment of
37 the impaired iron bioavailability, follow-up measurements of ZPP allow a reliable monitoring of
38 response of the underlying disease to therapy [21-23]. Thus, the goal of the treatment in such a clinical
39 situation must be the complete normalisation of this parameter, which verifies the positive effect of the
40 therapy on the underlying pathophysiological process. The clinical value of the ZPP measurement for
41 supervising the success of the therapy was clearly demonstrated in ACD [21]. The four presented PMF
42 patients who responded to immunosuppressive therapy illustrate that follow-up measurements of ZPP
43 provide useful information for treatment monitoring also in PMF. The response, visible by the increase
44 of the hemoglobin concentration was associated by a substantial decrease of ZPP (Figure 2). One of
45 the patients (UPN 2) who presented with a hemoglobin value of 83 g/L and fibrotic marrow at
46 diagnosis reached a complete normalisation of the peripheral blood counts and regression of the
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1 marrow fibrosis. The response was accompanied by a decrease of ZPP from 136 $\mu\text{mol/mol}$ heme, at
2 the beginning of treatment to normal levels during normalisation of peripheral blood counts.
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6 It has to be pointed out that the use of ZPP in patients with regular red blood cell transfusions is
7 limited, as it is not the iron metabolism of the patient but rather the iron metabolism of the blood donor
8 which is reflected by the ZPP determination. This is especially true for patients who have received
9 numerous blood transfusions within 4 weeks prior to testing, as the life span of transfused red cells is
10 supposed to be about 30 days [24].
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19 In summary, while further studies are needed, based on the impaired iron bioavailability common in
20 PMF, the ZPP measurement may provide valuable additional information which aids in the differential
21 diagnosis between PMF and ET. A ZPP concentration of 60 $\mu\text{mol/mol}$ heme seems to be the
22 appropriate cut-off value for this purpose. ZPP concentrations $> 60 \mu\text{mol/mol}$ heme are uncommon in
23 ET if iron deficiency is excluded.
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Table 1 Characteristics of all patients.

	PMF			ET	P-values ET vs. PMF
	total	prefibrotic stage	fibrotic stage		
N	24	12	12	46	
male / female	15 / 9	8 / 4	7 / 5	19 / 27	
ZPP [≤ 40 $\mu\text{mol/mol}$ heme]	99 \pm 37 (29 – 178)	¹ 77 \pm 30 (29 – 127)	122 \pm 31 (65 – 178)	36 \pm 13 (11 – 76)	< 0.0001
Ferritin [male: 35 – 310 $\mu\text{g/L}$ [female: 25 – 210 $\mu\text{g/L}$]	480 \pm 653 (36 – 1792)	324 \pm 366 (80 – 1369)	654 \pm 848 (36 – 1792)	111 \pm 80 (25 – 452)	0.0004
sTfR [0.81 – 1.75 mg/L]	2.67 \pm 1.50 (0.96 – 6.80)	2.15 \pm 2.79 (0.96 – 6.80)	2.27 \pm 1.08 (1.03 – 5.17)	1.43 \pm 0.42 (0.82 – 2.67)	< 0.0001
Transferrin saturation [16 - 45%]	33 \pm 18 (17 – 72)	33 \pm 22 (17 – 72)	33 \pm 13 (20 – 57)	26 \pm 7 (18 – 45)	n.s.
Platelet count [male: 145–348 x 10 ⁹ /L [women: 165–387 x 10 ⁹ /L]	363 \pm 276 (31 – 1700)	468 \pm 317 (106 – 1700)	259 \pm 189 (31 – 675)	941 \pm 327 (460 – 1956)	< 0.0001
WBC [4.2–10.2 x 10 ⁹ /L]	13.0 \pm 14.5 (9.9– 60.4)	15.6 \pm 12.2 (18.0– 48.4)	10.3 \pm 16.9 (9.9– 60.4)	8.8 \pm 3.4 (4.0 – 17.1)	n.s.
Hb [male: 130 – 170 g/L [female: 120 – 160 g/L]	109 \pm 23 (75 – 160)	¹ 121 \pm 26 (75 – 160)	96 \pm 11 (79 – 130)	114 \pm 14 (111 – 168)	< 0.0001
Erythropoetin [6 – 25 U/L]	45 \pm 60 (3 – 245)	¹ 19 \pm 23 (3 – 79)	84 \pm 80 (14 – 245)	12 \pm 12 (2 – 67)	0.02
JAK2V617F positive (N, %)	9/17 53%	4/8 50%	5/9 56%	17/38 45%	n.s.

Patients with PMF were subdivided in prefibrotic and fibrotic stage PMF. The values are mean \pm standard deviation (and range). Values in square brackets are reference values. Abbreviations were notified as PMF, primary myelofibrosis; ET, essential thrombocythaemia; Hb, hemoglobin; sTfR, soluble transferrin receptor; WBC, white blood cell; ZPP, zinc protoporphyrin. ¹The differences between prefibrotic and fibrotic PMF group were statistically significant.

Legends

Figure 1

Individual ZPP concentrations and median ZPP concentration (–) of all ET and PMF patients at time of diagnosis.

In PMF individual ZPP concentrations were above the upper normal value of 40 $\mu\text{mol/mol}$ heme in 92% (22/24). The two PMF patients with normal ZPP concentrations (29 and 40 $\mu\text{mol/mol}$ heme) showed remarkable high platelet counts ($1,700 \times 10^9/\text{L}$ and $880 \times 10^9/\text{L}$) and much more resembled the clinically course of ET. Patient in fibrotic stage of PMF (\blacktriangledown) had significantly higher ZPP concentrations as patients in prefibrotic phase (∇) ($p=0.003$). The dotted line represents the normal upper ZPP concentration.

Figure 2

Individual ZPP concentrations of four patients with PMF and response to immunosuppressive treatment.

Increased ZPP concentrations in PMF decreased successively under the treatment allowing a monitoring to therapy efficacy. The decline of ZPP was accompanied by an improvement of hemoglobin level. In one case (UPN 2, dashed line, follow-up for more than 4 years) bone marrow examination showed also a significant reduction of fibrosis.

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