Central Role of Zinc Protoporphyrin in Staging Iron Deficiency

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In iron deficiency, zinc protoporphyrin (ZPP) is produced instead of heme, and the ZPP concentration in erythrocytes is increased (normal ≤40 μmol/mol heme). We investigated the relevance of ZPP for staging iron deficiency. ZPP was determined by hematofluorometry in samples from 103 patients. Nineteen patients with iron depletion showed decreased serum ferritin (12.1 ± 4.4 μg/L) with normal ZPP and hemoglobin (Hb). Twelve patients with iron-deficient erythropoiesis had decreased ferritin (10.4 ± 2.4 μg/L), increased ZPP (72 ± 9 μmol/mol heme), and normal Hb concentrations. In 72 patients with iron-deficiency anemia, ferritin was <12 μg/L. In mild anemia (Hb between 100 and 120 g/L), and normal erythrocyte indices, ZPP was 100 ± 16 μmol/mol heme. In severe anemia (Hb <100 g/L, decreased erythrocyte indices), ZPP values were significantly higher (265 ± 109 μmol/mol heme). We conclude that measurements of ZPP, ferritin, and Hb can reliably be combined to classify the degree of iron deficiency.

Indexing Terms: heme, ferritin, hemoglobin, erythropoiesis, anemia

Iron deficiency probably represents the most common deficiency disease in humans (1)—not only in developing countries but also in the industrial countries, where the prevalence of iron deficiency in premenopausal women is estimated to be ~20% (2). This widespread occurrence, the result of socioeconomic and physiological factors, is promoted by the lack of a reliable, inexpensive measure of iron deficiency and by the inability to estimate severity of iron deficiency without an extensive diagnostic workup.

Iron deficiency, defined as a diminished total body iron content, has been grouped according to three stages of severity (3). A negative iron balance leads first to iron depletion, wherein total body iron is decreased but synthesis of hemoglobin is not affected. When the iron supply to the erythropoietic marrow is inadequate, the second phase of iron deficiency, iron-deficient erythropoiesis, occurs.5 When finally the iron supply is insufficient to maintain a normal hemoglobin concentration, the most severe phase of iron deficiency results, iron-deficiency anemia.

Staging patients according to their degree of iron deficiency is of clinical importance. Whereas iron depletion can easily be diagnosed by decreased ferritin and iron-deficiency anemia by low hemoglobin values, the diagnosis of iron-deficient erythropoiesis requires extensive diagnostic evaluation. This stage of iron deficiency, which is demonstrated only by decreased sideroblast counts in bone marrow, is assumed to be present when transferrin saturation is <16% (4).

Hematofluorometric determination of zinc protoporphyrin (ZPP) is used as a screening method to detect iron deficiency in blood donors (5–7). The pathophysiological background of this method is the increased incorporation of zinc instead of iron into protoporphyrin IX in iron deficiency, which results in increased production of ZPP instead of heme (8–16). In normal persons without iron deficiency, ZPP is ≤40 μmol/mol heme (17).

Hematofluorometric measurement of ZPP in a drop of whole blood seemed to be an ideal screening marker of iron deficiency but came into general use only for screening blood donors (5–7). Thus far, ZPP has not been used widely in clinical practice, because it often has not correlated with concentrations of ferritin, the main analyte used to assess iron metabolism. As we showed previously, however, the nonspecificity ascribed to ZPP is chiefly caused by plasma interferences and can be avoided by a simple washing procedure (17).

In the present study we investigated the relevance of ZPP in estimating the different stages of iron deficiency.

Patients and Methods

The study was carried out during 1988–1993 in the III. Medizinische Klinik in Mannheim. The procedures followed the Helsinki Declaration of 1975, as revised in 1983.

To examine ZPP as a way to classify patients with iron deficiency, we first classified the patients by established laboratory tests according to the following criteria:

Iron depletion (grade I)—no marrow hemosiderin and normal sideroblast count, or ferritin <20 μg/L and normal transferrin saturation. Patients with stainable marrow hemosiderin or ferritin ≥20 μg/L were excluded.

Iron-deficient erythropoiesis (grade II)—no marrow hemosiderin and decreased sideroblast count or ferritin <20 μg/L and transferrin saturation <16%; hemoglobin, mean corpuscular volume (MCV), and mean corpuscular hemoglobin (MCH) values within the normal range.

Mild iron-deficiency anemia (grade III)—no marrow hemosiderin and decreased sideroblast count, or ferritin

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5 This term is meant to be synonymous with the anemia of chronic disease.
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8 Nonstandard abbreviations: ZPP, zinc protoporphyrin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; 5-ALA, 5-aminolevulinic acid.
<12 μg/L and transferrin saturation <16%; hemoglobin between 100 and 120 g/L and normal erythrocyte indices (MCV ≥80 fL, MCH ≥28 pg).

Severe iron-deficiency anemia (also grade III)—no marrow hemosiderin and decreased sideroblast count, or ferritin <12 μg/L and transferrin saturation <16%; hemoglobin <100 g/L, MCV <80 fL, and MCH <28 pg.

We excluded 86 patients who could not be classified according to these criteria or who had received blood transfusions during the preceding 4 months.

To investigate the changes of these markers in situations of continuous iron loss, we also examined patients undergoing repeated therapeutic phlebotomy: one with polycythemia vera, one with hemochromatosis.

In all patients we determined ZPP, hemoglobin, MCV, MCH, and serum concentrations of iron, ferritin, and transferrin. The transferrin saturation (normal reference interval 25–50%) was calculated (18). Iron concentrations (reference interval 10.7–32.2 μmol/L) were determined with a SMAC II automated analyzer (Technicon, Tarrytown, NY); transferrin (2–4 g/L) by nephelometry; and ferritin (34–310 μg/L for men, 25–210 μg/L for women) by ELISA. Hemoglobin (≥130 g/L for men, ≥120 g/L for women), MCV (80–96 fL) and MCH (28–34 pg) were measured with an automatic cell counter (Model CC-180; Sysmex, Kobe, Japan). δ-Aminolevulinic acid in urine (δ-ALA; <5.5 mg/24 h) was measured by ion-exchange chromatography, using disposable chromatographic columns (Bio-Rad, Munich, Germany).

Blood samples for ZPP measurement were obtained by venipuncture and anticoagulated with EDTA. Samples that were not analyzed on the day of collection were stored in the refrigerator at 4°C for not longer than 2 days.

We measured ZPP in washed erythrocytes with a front-face hematofluorometer (Aviv Biomedical Co., Lakewood, NJ), as described elsewhere (17).

Bone marrow aspirates were obtained from the sternum or the posterior iliac crest, stained with Prussian blue, and counterstained with hematoxylin to reveal the presence of iron. Erythrocyte precursors were evaluated for the presence of iron granules. Sideroblast count was determined as the percent of erythroblasts (reference interval 30–50%). Iron stores were assessed by a semiquantitative scale of storage iron: 0, iron absent; 1, iron decreased; 2, normal amount; 3, iron increased; 4, iron markedly increased; 5, iron massively increased.

In all, we classified 103 patients with iron deficiency according to the criteria described above.

Results

Iron Depletion

Grade I iron deficiency was diagnosed in 19 female patients (Table 1). All 19 showed decreased ferritin values (12.1 ± 4.4 μg/L; range 1–19), but normal transferrin saturation (35.6% ± 4.8%) and normal values for hemoglobin (132 ± 9 g/L) and erythrocyte indices (MCV 90.5 ± 3.6 fL; MCH 30 ± 1.9 pg). ZPP concentrations were within the normal range in all 19 (30 ± 6 μmol/mol heme; range 19–39). Four of these patients underwent bone marrow examination; in none was hemosiderin detectable, but sideroblast counts were not decreased. This indicates that the iron supply to the erythroid marrow was still sufficient in these patients.

One patient developed completely exhausted iron stores by hypermenorrhagia (ferritin 1 μg/L, serum iron 14 μmol/L, hemoglobin 142 g/L, MCV 92 fL, MCH 32 pg, ZPP 31 μmol/mol heme). At the time of testing, she had already been treated by a peroral substitution of 100 mg of Fe3+ (Eryfer®; Castella-med, Köln, Germany) daily for 8 weeks. This therapy was sufficient to prevent iron-deficient erythropoiesis, as shown by ZPP within the normal range. It was, however, insufficient to repair the iron depletion because the ferritin was still low. After intravenous iron substitution (62.5 mg of Fe3+ for 10 days; Ferrlecit®; Nottermann, Köln, Germany), her ferritin increased to 71 μg/L.

Iron-Deficient Erythropoiesis

Grade II iron deficiency was diagnosed in 12 female patients. In eight (Table 2, patients 1–8), the impaired iron support to the erythropoietic marrow was proven directly by decreased sideroblast counts (gold standard), despite transferrin saturation >15% in patients 1 and 4. In the remaining four (patients 9–12), the iron-deficient erythropoiesis was demonstrated by transferrin saturation <16%.

All 12 patients had decreased ferritin (10.4 ± 2.4 μg/L; range 7–15), but hemoglobin (126 ± 4 g/L; range 121–134), MCV (86.6 ± 2.6 fL), and MCH (28.9 ± 1.0 pg) were still within their normal reference ranges. In contrast to the situation in iron depletion, ZPP was increased (72 ± 9 μmol/mol heme; range 48–83). After iron substitution therapy, ferritin, transferrin saturation, and ZPP returned to normal values in all 12 patients. Normal urine excretion of δ-ALA (2.3 ± 1.2 mg/24 h, range 1.0–4.9) excluded lead poisoning.

Iron-Deficiency Anemia

Grade III iron deficiency was diagnosed in 72 patients. We examined these patients in two separate groups, according to the severity of anemia.

Mild iron-deficiency anemia (hemoglobin 100–120 g/L, normal erythrocyte indices) was diagnosed in 11
female patients (Table 3). Ferritin (9.5 ± 1.5 μg/L) and transferrin saturation (10.1% ± 2.8%) were below normal. Prussian blue staining of bone marrow, performed in three patients, showed totally exhausted iron stores and decreased sideroblast counts. In all 11 cases, ZPP was moderately increased (100 ± 16 μmol/mol heme, range 77–122).

Severe iron-deficiency anemia (hemoglobin <100 g/L, erythrocyte indices below the normal range) was diagnosed in 61 patients (9 men, 52 women; Table 3). In these patients ferritin concentrations (5.1 ± 3.0 μg/L) were strongly decreased, and transferrin saturation (7.5% ± 2.9%) was below normal. Prussian blue-stained bone marrow, performed in 16 patients, showed totally exhausted iron stores and decreased sideroblast counts. In all 61 cases, ZPP was strongly increased (265 ± 109 μmol/mol heme, range 114–661) and was significantly higher than in patients with mild iron-deficiency anemia ($P < 0.001$).

Continuous Iron Loss

ZPP was then applied to the monitoring of iron deficiency in two patients treated by phlebotomies (Table 4). In the patient with hemochromatosis, ferritin was extremely high (2902 μg/L) at the time of diagnosis; ZPP and hemoglobin were within the normal range (11 μmol/mol heme and 158 g/L, respectively), and transferin saturation was 0.8 ± 0.1 (Table 4).

### Table 2. Characteristics of iron-deficient erythropoiesis in 12 patients.  

<table>
<thead>
<tr>
<th>Patient</th>
<th>ZPP, μmol/mol heme</th>
<th>Hb, g/L</th>
<th>MCV, fl</th>
<th>MCH, pg</th>
<th>Fe, μmol/L</th>
<th>Fer, μg/L</th>
<th>Sadt. transf., %</th>
<th>SBC, %</th>
<th>BM*</th>
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<td>88</td>
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<td>28</td>
<td>5</td>
<td>7 (133)</td>
<td>10 (46)</td>
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<td>13 (43)</td>
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<td>29</td>
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<td>19 (38)</td>
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<td>7</td>
<td>14 (139)</td>
<td>14 (46)</td>
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* Decreased ferritin (Fe) indicates exhausted iron stores. The increased ZPP (normal ≤40 μmol/mol heme) verifies the iron-deficient erythropoiesis (iron deficiency, grade II). Iron-deficient anemia has not yet developed, because hemoglobin concentrations are still within the normal range. ZPP, ferritin, and saturated transferrin (sadt. transf.) values normalized after iron substitution.

** Values before (and after) iron substitution are listed.

* Blood marrow homosiderin (6 point scale, with 0 = no iron detected).

* Not done.

Hb, hemoglobin; SBC, sideroblast count.

### Table 3. Characteristics of mild and severe iron-deficiency anemia.  

<table>
<thead>
<tr>
<th>Measure</th>
<th>Mild (n=11)</th>
<th>Severe (n=61)</th>
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</thead>
<tbody>
<tr>
<td>ZPP, μmol/mol heme</td>
<td>100 ± 16</td>
<td>265 ± 109</td>
</tr>
<tr>
<td>Hemoglobin, g/L</td>
<td>109 ± 5</td>
<td>81 ± 14</td>
</tr>
<tr>
<td>MCV, fl</td>
<td>85.9 ± 3.1</td>
<td>69.7 ± 5.9</td>
</tr>
<tr>
<td>MCH, pg</td>
<td>28.5 ± 0.5</td>
<td>19.5 ± 2.3</td>
</tr>
<tr>
<td>Fe, μmol/L</td>
<td>6.4 ± 2.8</td>
<td>3.9 ± 2.0</td>
</tr>
<tr>
<td>Ferritin, μg/L</td>
<td>9.5 ± 1.5</td>
<td>5.1 ± 3.0</td>
</tr>
<tr>
<td>Sadt. transf., %</td>
<td>10.1 ± 2.8</td>
<td>7.5 ± 2.9</td>
</tr>
<tr>
<td>SBC, %</td>
<td>5 ± 2</td>
<td>2.8 ± 2.3</td>
</tr>
</tbody>
</table>

* Mild = hemoglobin between 100 and 120 g/L, normal erythrocyte indices; severe = hemoglobin <100 g/L, erythrocyte indices below normal. Decreased ferritin demonstrates exhausted iron stores. Increased ZPP indicates iron-deficient erythropoiesis, but low hemoglobin values indicate iron-deficiency anemia.

** No stainable iron was found in any of these specimens.

Abbreviations as in Table 1.

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rin saturation was 100%. During the phlebotomy therapy, ferritin decreased continuously to subnormal values (9 μg/L), while transferrin saturation (37%), hemoglobin (136 g/L), MCV (93 fL), MCH (32 pg), and ZPP (17 μmol/mol heme) remained within the normal range. The phlebotomy led to iron depletion (grade I iron deficiency), whereas the erythropoiesis remained unaffected.

The patient with a polycythemia vera had increased hemoglobin at the time of diagnosis; erythrocyte indices, ferritin, and ZPP were within the normal range. Phlebotomy led first to iron depletion (ferritin decreased, transferrin saturation and ZPP normal), followed by iron-deficient erythropoiesis (ferritin and transferrin saturation decreased, ZPP increased). After further phlebotomies ZPP increased to >100 μmol/mol heme. At this time hemoglobin was still normal, due to the neoplastic disease, but the sideroblast count was decreased and the erythrocytes were hypochromic and microcytic.

Criteria for Exclusion from Classification

Eighty-six patients with iron deficiency who could not be classified according to the criteria described in Patients and Methods were excluded from this analysis:

1. 41 dialysis patients with iron-deficient erythropoiesis as diagnosed by increased ZPP, because their ferritin was within the normal range. Iron substitution led to significant increase of hemoglobin in these patients and their ZPP normalized. The data were published elsewhere (19).

2. 26 patients with iron-deficiency anemia caused by chronic inflammatory or neoplastic diseases—mainly of the gastrointestinal tract. These patients had normal or above-normal ferritin as well (90.9 ± 63.8 μg/L, range 46–324). Their diagnosis was based on their high ZPP concentrations (229 ± 90 μmol/mol heme, range 106–401) and low hemoglobin (92 ± 12 g/L, range 68–110). Also in these patients hemoglobin increased and ZPP decreased after iron substitution.

3. 3 patients with low ferritin (10, 15, and 9 μg/L) and slightly decreased transferrin saturation (21%, 23%, and 24%). ZPP concentrations were high (54, 58, and 48 μmol/mol heme) but normalized after iron substitution. We believe that iron-deficient erythropoiesis developed in these patients, but we excluded them because their transferrin saturation was >15%, so their diagnosis was not proven by the established tests.

4. 16 iron-depleted patients as diagnosed by low ferritin (12.7 ± 3.3 μg/L, range 8–18) and normal ZPP (29 ± 5 μmol/mol heme, range 18–36), whose anemia was caused by other diseases: thalassemia (n = 7), immune hemolysis (n = 5), vitamin B₁₂ deficiency (n = 3), and folic acid deficiency (n = 1).

Discussion

Although Labbé and Rettmer already described ZPP as a marker of iron-deficient erythropoiesis (20), ZPP has generally been used for detecting iron deficiency without consideration of the different stages of iron deficiency. However, ZPP is a reliable tool to classify iron deficiency. Our present data demonstrate that ZPP is not increased in the first stage, simple iron depletion, but only when erythropoiesis is affected by iron deficiency (the second stage). This explains the observation of several investigators that there is no good correlation between ferritin and ZPP (10, 11). Iron-overloaded persons in hemochromatosis with extremely high concentrations of ferritin have normal ZPP values, as do individuals with iron depletion, whose ferritin is below normal, because the iron supply to the erythropoietic marrow is sufficient in both cases. This lack of correlation is the main reason why ZPP has not been used widely in clinical practice. High correlation between ferritin and ZPP is possible only in a group consisting mainly of healthy individuals with normal values and persons with severe iron-deficiency anemia (17) and containing no one with iron depletion and iron overload.

In healthy individuals without iron deficiency, hemoglobin and ferritin concentrations are within the normal range. ZPP in these persons (as measured in washed erythrocytes) is ≤40 μmol/mol heme (17).

The earliest stage of iron deficiency, iron depletion, is defined as ferritin <35 μg/L in men and <23 μg/L in women (21). Transferrin saturation is still within the normal range, indicating that iron supply to the erythropoiesis is still sufficient (4). As our data show, ZPP, hemoglobin, and erythrocyte indices are still normal at this stage of iron deficiency.

The diagnosis of iron-deficient erythropoiesis requires an extensive diagnostic workup, including examination of Prussian blue-stained bone marrow smears and evaluation of the sideroblast count. Because bone marrow examination is not an option in most cases, iron-deficient erythropoiesis generally is diagnosed indirectly, by decreased transferrin saturation, considered the best marker of iron supply to the erythropoietic marrow. Values <16% are regarded as an indication of iron-deficient erythropoiesis (4). As our data show, however, transferrin saturation >15% does not exclude the presence of iron-deficient erythropoiesis; e.g., patients 1 and 4 in Table 2 had iron-deficient erythropoiesis with only moderately decreased transferrin saturation. They were correctly classified by low sideroblast counts and increased ZPP. In contrast to transferrin saturation, ZPP detects iron-deficient erythropoiesis directly, by reflecting the increased incorporation of zinc into protoporphyrin IX when the iron supply to the erythropoietic marrow becomes insufficient. Thus, the second stage of iron deficiency can easily be detected by above-normal concentrations of ZPP. MCV and MCH do not contribute to the detection of iron-deficient erythropoiesis, because they are not affected at this stage of iron deficiency (3). Hemoglobin concentrations are still within the normal range by definition.

An iron-deficient anemia develops when the iron-deficient erythropoiesis persists and the hemoglobin values decrease, to <130 g/L in men or <120 g/L in women. In mild cases, the anemia is normochromic, normocytic. Erythrocyte indices are abnormal only when anemia is
moderate or severe (9). We examined patients with mild anemia and severe anemia. In mild normocytic, normochromic anemia, ZPP concentrations were moderately increased (77–122 μmol/mol heme); in patients with severe hypochromic, microcytic anemia, they were significantly higher (114–661 μmol/mol heme). We could not define an exact cutoff value of ZPP between iron-deficient erythropoiesis and iron-deficiency anemia; there is of course an overlap, given that the hemoglobin value depends on many factors. However, we found that patients with ZPP > 80 μmol/mol heme usually had hemoglobin values below the normal range. Thus, a possible ZPP cutoff value between iron-deficient erythropoiesis and iron-deficiency anemia might be ~80 μmol/mol heme. Accordingly, iron-deficiency anemia can be easily diagnosed by the triad: low ferritin, increased ZPP, and subnormal hemoglobin (Table 5).

The predictive value of an iron-deficiency test cannot be discussed without taking into account the different stages of iron deficiency. It is important to know which stage of iron deficiency the various tests detect. Theoretically, ferritin is frequently considered to be the best marker of iron deficiency, by identifying the early stage, iron depletion. The practical significance of ferritin as a screening test for iron deficiency is, however, restricted by the high costs of the assay and the fact that ferritin is also increased in inflammatory and hepatic diseases (22–31). Moreover, except for hemochromatosis, there is virtually no clinical relevance to knowing whether iron stores are full or only half-full. What is relevant is whether there is a sufficient iron supply to the cells, especially to the erythropoietic marrow. When the iron supply is insufficient, zinc instead of iron is incorporated into protoporphyrin IX to build ZPP; thus, as shown by our data, the beginning ZPP increase describes the moment when the intracellular iron concentration becomes insufficient and iron deficiency becomes a disease. ZPP detects the iron deficiency in a later stage than ferritin, but it always detects iron deficiency of clinical relevance. Despite detecting iron deficiency later than the storage indicator ferritin, ZPP can be the more sensitive indicator—especially in patients with inflammatory or liver diseases. In a preliminary study we have shown that ZPP is more sensitive than ferritin for the detection of iron deficiency in erythropoietin-treated patients with end-stage renal failure (19). And in the patients we had to exclude in the present study because of lack of ferritin sensitivity, we were able to diagnose and classify their iron deficiency on the basis of ZPP concentrations.

Regarding the specificity of ZPP for detecting iron-deficient erythropoiesis, the only possible sources of error recognized thus far appear to be the rare congenital erythropoietic porphyria and protoporphyria. Free erythrocyte protoporphyrin fluoresces at a wavelength close to that of ZPP, resulting in falsely high concentrations of ZPP measured in the Aviv hematofluorometer (12).

Of course, ZPP concentrations are increased not only in iron deficiency, but in all conditions that induce an impaired iron supply to the erythroid marrow. We showed previously that the ZPP pathway works also in the presence of the impaired iron bioavailability in anemias of chronic disorders (32) and in myelodysplastic syndrome (33). Increased ZPP concentration in lead poisoning, caused by the decreased heme production due to ferrochelatase inhibition, is well known (34–36). Supervising the final stage of the heme production, ZPP is not a specific test for iron deficiency, but rather is a screening marker of the whole iron metabolism (32). This does not, however, limit the value of ZPP for detecting iron deficiency. Myelodysplastic syndromes and lead poisoning are rare and can easily be excluded by clinical course or further laboratory tests. High ZPP values are not obligatory in chronic disorders. Increased ZPP is observed only in severe chronic inflammation and indicates more advanced disease (37). Acute inflammatory diseases do not induce an iron-deficient erythropoiesis, and ZPP values are not increased (38).

In conclusion, we suggest that using ferritin, ZPP, and hemoglobin allows an easy staging of iron deficiency (Table 5). Furthermore, ZPP can be used to screen for iron deficiency, with ZPP values <40 μmol/mol heme excluding both iron-deficient erythropoiesis and clinically relevant iron deficiency.

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References