Laboratory tests of iron status: correlation or common sense?

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We demonstrate that simple correlation between the various tests of iron status is not sufficient for examining their value in diagnosing iron deficiency (ID). Three degrees of ID are recognized: Iron depletion (ID grade I) is defined by decreased total body iron and normal iron support to erythropoiesis, as diagnosed by decreased storage iron, decreased ferritin, normal sideroblast count, normal zinc protoporphyrin (ZPP), and transferrin saturation >15%. When the iron supply to erythropoiesis becomes insufficient, as diagnosed by transferrin saturation ≤15%, increased ZPP, and decreased sideroblast count, iron-deficient erythropoiesis (ID grade II) occurs. When finally hemoglobin is below its normal range, iron-deficiency anemia (ID grade III) results. The various tests for ID cannot be compared without taking into account the severity of the deficiency. Depending on the grade of ID examined, the correlation of markers seen in our patients' data varied considerably. We conclude that a "best" marker of ID does not exist. However, the different tests efficiently complement each other by detecting different stages and individually show the clinical extent of ID. Ferritin reflects the iron stores. ZPP indicates whether the ID in a given patient is clinically relevant or not. Finally, the extent of a clinically relevant ID can be assessed by the measured ZPP, hemoglobin concentration, and red cell indices.

INDEXING TERMS: iron deficiency • ferritin • zinc protoporphyrin • hemoglobin • erythropoiesis • anemia

Iron deficiency (ID) is one of the most widespread nutritional deficiencies in industrial as well as developing countries.4

Worldwide, the number of people with ID is estimated at −500 million [1, 2].

ID is defined as a diminished total body iron content. Depending on the severity of the deficiency, three degrees of ID are recognized [3]. A negative iron balance leads first to ID with decreased total body iron but unaffected synthesis of hemoglobin (Hb): ID grade I. When the iron supply to the erythropoietic marrow is inadequate, ID grade II occurs, iron-deficient erythropoiesis. When finally the iron supply is no longer sufficient to maintain a normal concentration of Hb, the most severe phase of ID results, ID anemia (grade III).

Several laboratory tests are used to confirm or exclude ID. In the past, many investigators performed studies to investigate the diagnostic value of the individual tests and to identify the best marker of ID (e.g., [4–13]). Some authors found a good correlation between the various markers, but in other studies the correlation was poor.

We observed that these discrepancies as well as other problems are mainly caused by failing to take into account the different stages of ID. In our present study we show that simple correlation is not sufficient to investigate the diagnostic value of a laboratory test in ID.

Materials and Methods

Subjects
The study was carried out from 1988 to 1995 at the III. Medizinische Klinik in Mannheim. To examine whether simple correlation of the laboratory tests of iron status was sufficient to support their diagnostic value in the diagnosis of ID, we compared the various tests in healthy individuals and in patients with different stages of ID. Included in this study were only those individuals and patients who underwent Prussian blue examination of the bone marrow and who fulfilled the following criteria:

- Healthy individuals (H): normal marrow hemosiderin, normal sideroblast count, and normal Hb.
- Patients with iron depletion (I): no marrow hemosiderin, normal sideroblast count, and normal Hb.
• Patients with iron-deficient erythropoiesis (II): no marrow hemosiderin, decreased sideroblast count, and normal Hb.5
• Patients with mild ID anemia (III5): no marrow hemosiderin, decreased sideroblast count, and Hb between 100 and 120 g/L.
• Patients with severe ID anemia (III6): no marrow hemosiderin, decreased sideroblast count, and Hb <100 g/L.

Patients who had received blood transfusions during the last 4 months and who had an inflammatory or neoplastic disease at the time of testing were excluded. All procedures followed the Helsinki Declaration of 1975, as revised in 1983.

METHODS
For all patients included in the study, we examined their Prussian blue-stained bone marrow and determined their zinc protoporphyrin (ZPP), Hb, mean corpuscular volume (MCV), and mean corpuscular Hb (MCH) blood content as well as their serum concentrations of ferritin and transferrin. Their transferrin saturation values (normal: 25–50%) were calculated [14]. Transferrin concentrations (normal: 2–4 g/L) were determined by nephelometry, and ferritin (normal: 34–310 µg/L for men, 25–210 µg/L for women) by ELISA. Hb (normal: >130 g/L for men, >120 g/L for women), MCV (normal: 80–96 fL), and MCH (normal: 28–34 pg) were measured with an automated cell counter (Model CC-180; Sysmex, Kobe, Japan).

Blood samples for ZPP measurement were obtained by venipuncture and anticoagulated with EDTA. Measurements were carried out within 2 days of blood sampling; samples that were not examined on the same day were stored at 4°C. We measured ZPP with the Aviv front-face hematoefluorometer (Aviv Biomedical Co., Lakewood, NJ), using exclusively washed erythrocytes (normal: ≤40 µmol/mol heme) as described elsewhere [15], but with a simplified washing procedure. Using a disposable pipette tip, we added 50 µL of the anticoagulated blood sample to a 1.5-mL centrifuge tube containing 1 mL of 9 g/L NaCl and vortex-mixed briefly. After pelleting the cells by centrifugation for 15 s in a small tabletop centrifuge (Model 3200; Eppendorf, Hamburg, Germany), we removed as much supernate as possible by aspiration with a suction nozzle connected to a small membrane vacuum pump. The pellet was resuspended in the residual liquid by vortex-mixing in a shaker, and 40 µL of the suspension was used for the measurement.

Bone marrow aspirates were obtained from the sternum or the posterior iliac crest. For iron examination the aspirates were stained by the Prussian blue reaction and counterstained with hematoxylin. Erythrocyte precursors were evaluated for the presence of iron granules. Sideroblast counts were determined as a percentage of erythroblasts (normal: 30–50%). The iron stores were assessed by a semiquantitative estimation of storage iron according to a six-point scale: 0 = iron absent; 1 = iron decreased; 2 = normal amount of iron; 3 = iron increased; 4 = iron markedly increased; 5 = iron massively increased.

STATISTICS
The relationship of ferritin to the other ID tests was examined by linear regression analysis, calculating the Pearson's product-moment correlation coefficient (r) and the significance of correlation (P). This analysis was performed separately for results from healthy individuals and for those from patients in different stages of ID (I, II, III5, III6) as well as in the synopsis of the different groups. All statistical analyses were performed with the SPSS statistical package for Windows (SPSS, Chicago, IL).

RESULTS
We classified and investigated 78 subjects according to the criteria described above, 16 healthy and 62 with ID. The data are summarized in Table 1.

Correlation values between the various ID marker tests varied depending on the ID stages. To avoid extensive tables, we present as an example the correlation between ferritin and the other markers (Table 2). Ferritin and MCV, for example, showed good correlation (r = 0.83; P <10^-6) for data from healthy individuals and from patients with severe ID (H + III6):

In healthy individuals both markers have normal values, and in patients with severe ID both are below normal. However, the correlation decreased after addition of data from patients with iron depletion and iron-deficient erythropoiesis, who already had low ferritin but still normal MCV, giving r = 0.29 if the synopsis of all data was examined (H + I + II + III5 + III6).

Patients with severe ID anemia (III6) showed a complete lack of quantitative correlation between ferritin and ZPP (r = -0.01; P <0.95), despite the fact that the individual values of ferritin and ZPP were always pathological and showed the correct diagnosis in all cases (ferritin range: 2–10 µg/L; ZPP range: 127–556 µmol/mol heme). Healthy individuals (H) also showed no correlation between ferritin and ZPP, even though the individual values were always normal (ferritin range: 89–235 µg/L; ZPP range: 19–38 µmol/mol heme). When evaluating both groups simultaneously (H + III6), however, the same data delivered good correlation (r = -0.77; P <10^-6).

The best ferritin/ZPP correlation was obtained with the data synopsis from healthy individuals and patients with mild anemia (H + III6). All the other tests (saturation of transferrin, hemoglobin, MCV, MCH, sideroblast count) showed the best correlation with ferritin for the data synopsis from healthy individuals and patients with severe anemia (H + III6).

Predictive values of the different tests of the iron status as obtained in our study are summarized in Table 3. Because we included only patients with no inflammatory diseases who could be clearly categorized by bone marrow examination, this evaluation of course delivers ideal results. Nevertheless, the results demonstrate that the various tests are sensitive in different stages of ID and that each marker indicates something different in terms of iron status. The prevalence of the different ID stages in relation to the 62 ID patients was 24.2% stage I, 22.6% stage II, and 53.2% stage III.

DISCUSSION
As we have shown here, the diagnostic value of a laboratory test of iron status in the diagnosis of ID cannot be discussed without

5 This term excludes anemia of chronic disorders.
Table 1. Characteristics of healthy individuals and of patients in different ID stages.

<table>
<thead>
<tr>
<th></th>
<th>H</th>
<th>I</th>
<th>II</th>
<th>III^a</th>
<th>III^b</th>
<th>III^c</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (males/females)</td>
<td>16 (5/11)</td>
<td>15 (0/15)</td>
<td>14 (0/14)</td>
<td>9 (2/7)</td>
<td>24 (4/20)</td>
<td></td>
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<tr>
<td>ZPP, µmol/mol heme</td>
<td>29 ± 5</td>
<td>29 ± 7</td>
<td>71 ± 9</td>
<td>105 ± 15</td>
<td>283 ± 101</td>
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<tr>
<td>Hb, g/L</td>
<td>145 ± 7</td>
<td>131 ± 8</td>
<td>127 ± 6</td>
<td>110 ± 5</td>
<td>80 ± 13</td>
<td></td>
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<tr>
<td>MCV, fl</td>
<td>90.2 ± 2.6</td>
<td>89.9 ± 2.4</td>
<td>86.1 ± 2.2</td>
<td>83.4 ± 4.1</td>
<td>69.1 ± 6.0</td>
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<tr>
<td>MCH, pg</td>
<td>30.0 ± 1.2</td>
<td>30.3 ± 1.8</td>
<td>28.5 ± 1.3</td>
<td>27.1 ± 1.4</td>
<td>19.5 ± 2.4</td>
<td></td>
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<tr>
<td>Ferritin, µg/L</td>
<td>147 ± 51</td>
<td>129 ± 4.1</td>
<td>10.4 ± 3.7</td>
<td>6.8 ± 2.4</td>
<td>4.8 ± 2.7</td>
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<tr>
<td>Sat, %</td>
<td>38 ± 5</td>
<td>35 ± 4</td>
<td>14 ± 3</td>
<td>11 ± 4</td>
<td>9 ± 4</td>
<td></td>
</tr>
<tr>
<td>SBC, %</td>
<td>41 ± 4</td>
<td>38 ± 4</td>
<td>13 ± 4</td>
<td>6 ± 2</td>
<td>3 ± 2</td>
<td></td>
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<tr>
<td>(35 – 47)</td>
<td>(32 – 46)</td>
<td>(6 – 19)</td>
<td>(3 – 8)</td>
<td>(0 – 7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BM (scale 0–5)</td>
<td>2 ± 0 (2)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a Characteristics described in text.

*b Values are mean ± SD (end range).

Sat, transferrin saturation; SBC, sideroblast count; BM, bone marrow hemosiderin (2 = normal).

Taking into account the different stages of ID. For efficient use of the various tests in clinical practice, it is important to know which stages of ID the tests detect.

Examination of Prussian blue-stained bone marrow is regarded as the "gold standard" in the diagnosis of ID [3, 9]. This examination allows the detection of the earliest stage of ID, iron depletion. The number of sideroblasts shows further whether the iron support to erythropoiesis is sufficient or not and allows the diagnosis of the second stage of ID, iron-deficient erythropoiesis. The third stage of ID cannot be diagnosed by bone marrow examination alone because, by definition, information on Hb concentration is required.

Serum ferritin concentration correlates well with total body iron stores [4, 16–18]. Therefore, ferritin is a good marker for detecting stage I ID, iron depletion. Ferritin values <12 µg/L prove ID [9]. Ferritin examination alone, however, does not give any information as to the iron support to erythropoiesis and allows no statement concerning the severity of ID. As shown elsewhere [19], even exhausted iron stores with a ferritin value of 1 µg/L are no proof of an iron-deficient erythropoiesis.

ZPP is produced instead of heme when the iron support to erythropoiesis becomes insufficient and zinc instead of iron is incorporated into protoporphyrin IX [19–22]. Thus, ZPP detects stage II ID, iron-deficient erythropoiesis. In iron depletion (stage I), the ZPP values are normal (<40 µmol/mol heme) because the iron support to erythropoiesis is still sufficient [19].

High ZPP values not only are diagnostic for iron-deficient erythropoiesis but also show its severity and clinical relevance. ZPP values of 40–60 µmol/mol heme are associated with mild iron-deficient erythropoiesis with no clinical symptoms or ane-
mia. In our experience, clinical symptoms and anemia usually first appeared when ZPP was >80 μmol/mol heme. In patients with severe ID anemia (Hb <100 g/L), the ZPP concentrations were always >100 μmol/mol heme.

In assessing above-normal ZPP values, one must be aware that increased values are measured not only in real ID, but also in all conditions where iron support for erythropoiesis becomes insufficient: e.g., lead intoxication, anemia of chronic disorders, myelodysplastic syndromes [23–28]. In contrast to cases with real ID, ZPP values in myelodysplastic syndromes are even in the most severe cases <80 μmol/mol heme, usually <60 μmol/mol heme [29]. Disturbances of iron metabolism in chronic disorders are usually associated with ZPP <80 μmol/mol heme; ZPP exceeds 100 μmol/mol heme only in severe cases [25]. Clinically relevant lead intoxication, on the other hand, is associated with very high ZPP, always >1000 μmol/mol heme in our patients.

Transferrin saturation is considered the best marker of the iron supply for erythropoiesis [4]. In clinical practice, however, the saturation obtained supports a definite statement only if the value is very low or very high [9]. Values <15% are regarded as proof of deficient iron delivery to erythropoiesis [4]. Therefore, transferrin saturation is a marker of iron-deficient erythropoiesis.

Hb concentrations <130 g/L in men and <120 g/L in women define the last stage of iron deficiency, ID anemia. Given that many conditions lead to low Hb concentrations, ID anemia cannot be diagnosed by a low Hb only.

The red cell indices MCV and MCH are predictive of only late ID anemia [3, 5, 8–10]. Statements such as “MCV detects iron deficiency in 50%” are misleading, because these markers work only in severe ID anemia and show no conclusive changes in the first two stages of ID. Thus, the “sensitivity” of MCV obtained without ID staging depends primarily on the composition of the groups of patients examined. This explains why different investigators have reached different conclusions about the diagnostic sensitivity of MCV—reasoning that is also valid for the other laboratory tests.

Despite the selective nature of our groups of patients, the predictive values of the various tests at the different stages of ID demonstrate that each of the several iron status markers indicates something different in terms of ID. We conclude that no single “best” test of ID exists. What screening strategy and sequence of tests to use when the iron status is unknown depend on the individual patient, the clinical conditions, and especially the laboratory equipment available. Most clinicians prefer using ferritin as the first-line test of iron status: It is not invasive, it is widely available, and, from a theoretical point of view, it is more sensitive than the other laboratory tests (being a marker of body iron stores). However, the higher sensitivity of ferritin is true only for healthy individuals without inflammatory diseases. For patients with acute-phase response or with neoplastic and hepatic disorders, ferritin frequently is not useful even in cases of severe ID anemia [9, 12, 30–35]. Especially in patients with ID due to occult bleeding caused by a neoplasm—the only patients in whom early detection of decreased body iron stores is of vital interest—we frequently observed falsely normal ferritin values [19]. Of our dialysis patients with ferritin between 50 and 100 μg/L, 93% had ID and did not respond to erythropoietin until they were treated with iron [12]. In both of these groups, ID was reliably detected by the ZPP values.

Given the observed diagnostic uncertainty of ferritin in hospitalized patients, measuring ZPP seems to be a reliable alternative. However, should ZPP be used supplementary to ferritin, or as a first-line test instead of ferritin, if appropriate laboratory equipment is available? We prefer simultaneous measurement of ferritin and ZPP in such at-risk patients as young and pregnant women, blood donors, or persons with disorders of malabsorption—i.e., patients who frequently develop ID and for whom ferritin determinations usually deliver correct results (being not disturbed by inflammatory diseases). We also prefer this strategy in hematologic patients, particularly in those with anemia. Simultaneous determination of ferritin and ZPP is of practical interest in hemochromatosis patients treated by venesection as well, to guarantee iron depletion and prevent the development of iron-deficient erythropoiesis in such patients [19].

In our daily clinical routine, however, we use only ZPP as the first-line test and determine ferritin in a second step, if necessary, to distinguish between real ID and sideroachrestic disorders (Fig. 1). Although this strategy does not detect cases with iron depletion, we find it justifiable because, except for hemochromatosis, whether the iron stores are full or only half full is of little clinical relevance, and, except for a neoplasia (where ferritin values frequently do not work well), early detection of decreased body iron stores is not of vital interest. In clinical practice, physicians want to know whether or not a given patient has clinically relevant ID and whether the symptoms or pathological findings (e.g., anemia) are caused by ID. ZPP always detects an iron-deficient erythropoiesis, the first clinically relevant stage of ID [19]. Normal or only slightly increased ZPP values exclude clinically relevant ID and show the physician that, whatever the pathological findings for the given patient, they are not caused by ID (Fig. 1). High ZPP values indicate an iron-deficient erythropoiesis—which can be caused by real ID or by disturbed iron metabolism in lead poisoning, inflammatory diseases, or myelodysplastic syndromes. Thus, when ZPP is

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**Table 3. Predictive values (%) of the various iron tests in different stages of ID.**

<table>
<thead>
<tr>
<th>ID stage</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
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<tbody>
<tr>
<td>Prevalence, %</td>
<td>24.2</td>
<td>22.6</td>
<td>14.5</td>
<td>38.7</td>
</tr>
<tr>
<td>BM</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Fer</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>SBC</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>ZPP</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>SAT</td>
<td>0</td>
<td>71</td>
<td>78</td>
<td>96</td>
</tr>
<tr>
<td>Hb</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>MCV</td>
<td>0</td>
<td>0</td>
<td>22</td>
<td>100</td>
</tr>
<tr>
<td>MCH</td>
<td>0</td>
<td>0</td>
<td>33</td>
<td>100</td>
</tr>
</tbody>
</table>

* In the total investigated 62 ID patients. Abbreviations as in Table 1.
above the normal range, the physician must ascertain the differential diagnosis by clinical findings and further laboratory tests.

Despite the described advantages of ZPP in diagnosing and managing ID, we nonetheless emphasize that our recommendation of ZPP as the only first-line test is mainly for financial reasons. If diagnostic costs do not play a role, we recommend measuring both ferritin and ZPP to determine a person's iron status.

As we have shown, examining the simple correlation between the various markers of ID as frequently practiced is not appropriate for assessing their diagnostic value. Depending on the composition of the group investigated, that correlation shows considerable variation if the compared tests do not detect the same stage of ID. This is particularly true when the marker of iron storage, ferritin, is compared with tests that relate to iron utilization for erythropoiesis, e.g., ZPP or the red cell indices. Ferritin and MCV, for example, correlate poorly if the group investigated consists of many individuals with iron depletion and ID erythropoiesis, which is not detected by MCV. In groups containing patients with severe ID anemia and healthy individuals (H + III\textsuperscript{P}), the sensitivity advantage of ferritin no longer exists and a good correlation is observed.

Given that bone marrow examination cannot be performed in large epidemiological studies, investigators often use ferritin as the diagnostic "gold standard" of ID [36-40]. The lack of correlation between ferritin and other markers is generally interpreted off-hand as a defect of the latter and that marker is rejected as a valid test of ID. Our results should warn against such uncritical acceptance of statistics and against the use of statistics without careful analysis of the underlying assumptions. The data from patients with severe ID anemia, for example, show no correlation between ferritin and ZPP concentrations, although both markers deliver "correct" results and allow for correct diagnosis in all cases. The reason for this is trivial (Fig. 2): Ferritin, as a marker of the iron stores, shows the same low values in all patients (range: 2–10 μg/L), because the iron stores are exhausted; ZPP, in contrast, varies according to the severity of the iron-deficient erythropoiesis (range 127–536 μmol/mol heme). Thus, in severe ID anemia, ZPP is superior to ferritin, diagnosing not only the ID but also the severity of the iron-deficient erythropoiesis. The lack of correlation observed is not the result of ZPP insensitivity, but rather the fact that the ferritin values can show no further decline in advanced ID (Fig. 2). Healthy individuals also showed poor ferritin/ZPP correlation, even though the individual ferritin and ZPP values were all correctly within the normal range. In these patients ZPP showed minimal variation that was not dependent on the storage marker ferritin, which varied according to the amount of the storage iron.

The simultaneous evaluation of healthy individuals and patients with severe anemia (H + III\textsuperscript{P}) creates the paradoxical situation that the same data do not correlate if examined separately, but yield good correlation if examined simultaneously. Healthy individuals, who have normal values for ferritin and ZPP, represent one extreme in the diagnosis of ID; patients with severe ID anemia, who have low ferritin and high ZPP values, are the other. When these extreme situations are evaluated simultaneously, the measured values account for the endpoints for the regression line (which is not "disturbed" by the intermediate states) and apparently demonstrate a direct dependence between ferritin and ZPP (Fig. 2). This mathematical trick, in which only the endpoint data are used, always produces good correlation between two variables and accounts for the good correlations observed between ferritin and the other tests in our study.

Of course, poor correlation between the measured values of two different markers of ID does not necessarily mean a lack of diagnostic correlation. In patients with severe ID anemia (III\textsuperscript{P}), for example, the ferritin and ZPP values do not correlate; nonetheless, the low ferritin values allow the correct diagnosis of ID just as well as the high ZPP values do. Thus, despite poor correlation between the measured values, the diagnoses themselves correlate well.
Tyrosine (ZPP), hemosiderin (ID), sideroblast (SBC), and ferritin (Fer) assay the iron stores and allow the diagnosis of iron deficiency (ID grade I). Pathological values of sideroblast count (SBC), ZPP, and transferrin saturation (SAT) indicate that an iron-deficient erythropoiesis (ID grade II) has already developed. Finally, Hb below normal values defines ID anemia (ID grade III), which in severe cases is accompanied by low red cell indices.

The diagnosis of ID as currently practiced is mainly based on data from epidemiological studies without consideration of the severity of ID. Under these conditions, however, knowledge of the "ID sensitivity" of various laboratory tests is useless for individual cases. Studies of iron metabolism should not have the goal of determining the "best" marker, for none exists: ID is a dynamic process and cannot be defined by one test only. To understand the clinical severity of ID in a given patient, one must know which stage of ID the individual test detects and by which factors it is disturbed. By detecting different stages, the various markers efficiently complement each other to characterize ID severity in an individual case (Fig. 3).

In conclusion, the iron status of a given patient can be assessed according to the following schema: Low ferritin indicates exhausted iron stores. ZPP determinations show whether or not the diagnosed ID in such a case is of clinical relevance. The extent of the clinically relevant ID can finally be assessed by the measured value of ZPP, the Hb concentration, and the red cell indices.

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References