Reticulocyte Hemoglobin Content
to Diagnose Iron Deficiency in Children

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IRON DEFICIENCY IS ONE OF THE MOST common nutritional deficiencies and is the leading cause of anemia in children and adult women. According to a recent study, 700,000 children aged 1 to 2 years are iron deficient and 240,000 have iron deficiency anemia.1 Although anemia can be reversed with iron supplementation, the alteration in cognitive performance observed in children with iron deficiency may not be fully correctable.2-4 Early recognition of iron deficiency, even before the development of anemia, is therefore crucial to prevent the systemic complications of this disease. Such early diagnosis, by necessity, relies on laboratory testing, a strategy that is expensive and fraught with error.

The diagnosis of simple iron deficiency has been traditionally based on a panel of biochemical indicators of iron metabolism, which includes determination in serum or plasma of iron, transferrin, transferrin saturation (Tfsat), and ferritin. The diagnosis of iron deficiency anemia relies on the presence of anemia with the characteristic morphologic features of iron-deficient erythrocytes (microcytosis, hypochromia) and elevated erythrocyte zinc protoporphyrin (ZPP) in conjunction with the above mentioned biochemical markers of iron metabolism. A large number of articles have been published on the relative merits and weaknesses of these parameters for the diagnosis of iron deficiency in both the adult and pediatric settings.5-9

More recently, measurements of serum circulating transferrin receptor (TfR) and reticulocyte cellular indices have been added to the diagnostic menu for iron deficiency. Several studies have shown that serum circulating TfR is useful in the early identification of mild iron deficiency, and in the distinction of anemia of chronic disease from that due to iron deficiency.10-15

Conclusions  Reticulocyte Hb content level was the strongest predictor of iron deficiency and iron deficiency anemia in children. It holds promise as an alternative to biochemical iron studies in diagnosis. www.jama.com

Context  Early identification of iron deficiency in children is essential to prevent the damaging long-term consequences of this disease. However, it is not clear which indices should be included in a diagnostic panel for iron deficiency and iron deficiency anemia in children.

Objective  To develop an effective approach for the diagnosis of iron deficiency and iron deficiency anemia in young children.

Design and Setting  Retrospective laboratory analysis, carried out over 7 weeks in 1996, using blood samples ordered by pediatricians and sent to a large metropolitan hospital for analysis.

Patients  A total of 210 children (mean [SD] age, 2.9 [2.0] years; 120 were male) who had a lead screening test (complete blood cell count and plasma lead level) ordered by a primary care pediatrician.

Main Outcome Measures  Levels of hemoglobin (Hb), iron, transferrin, transferrin saturation (Tfsat), ferritin, and circulating transferrin receptor and reticulocyte Hb content (CHr) among patients with and without iron deficiency, defined as Tfsat of less than 20%, and iron deficiency anemia, defined as Tfsat of less than 20% and Hb level of less than 110 g/L.

Results  Of the 210 subjects, 43 (20.5%) were iron deficient; 24 of these had iron deficiency anemia. Reticulocyte Hb content and Hb levels were the only significant predictors of iron deficiency (likelihood ratio test [LRT] = 15.96; P < .001 for CHr, and LRT = 6.59; P = .01 for Hb), and CHr was the only significant multivariate predictor of iron deficiency anemia (LRT = 30.43; P < .001). Plasma ferritin level had no predictive value (P = .97). Subjects with CHr of less than 26 pg (optimal cutoff value based on sensitivity/specificity analysis) had lower Hb level, mean corpuscular volume, mean corpuscular Hb level, serum iron level, and Tfsat, and increased red blood cell distribution width vs those with CHr of 26 pg or more (P < .001 for all).

Conclusions  Reticulocyte Hb content level was the strongest predictor of iron deficiency and iron deficiency anemia in children. It holds promise as an alternative to biochemical iron studies in diagnosis.

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demonstrated that reticulocyte hemoglobin content (CHR) is an early indicator of iron-restricted erythropoiesis in healthy subjects receiving recombinant human erythropoietin.16,17 There have been some recent reports on the use of CHR in the identification of functional iron deficiency and monitoring of intravenous iron and recombinant human erythropoietin therapies in dialysis patients.18-20

There is no systematic study that evaluates the performance of these old and new indices of iron deficiency in children and it is not clear which elements should be included in a diagnostic panel for iron deficiency and iron deficiency anemia in children. We present data on the performance of these indicators in a group of children randomly selected from those followed up by general pediatric practices that use our laboratory services.

METHODS
Sample Collection
The study was carried out over 7 weeks in 1996. On the same day of each week (Wednesday), a maximum of 35 samples were selected. Only samples from general pediatric outpatient clinics that had both a complete blood cell count and a lead level ordered were considered. The selection was based on the accession number, which is given at the time the blood is collected. Starting from the lowest accession number of the day, the samples were selected consecutively up to the maximum number of 35. Since this selection took place in the evenings, the samples selected had been collected between 8 AM and 5 PM. Of the 210 samples studied, 94 had been collected before 11 AM. The amount of blood collected for a complete blood cell count is 1.5 mL, and after analysis, approximately 1 mL is leftover in the tube. For lead levels, 1.5 mL of blood is collected in heparin, and after the test is run, there is approximately 400 µL of plasma leftover.

Researchers were blinded to patient identity when they analyzed samples. Therefore, informed consent and institutional review board approval were not required. This is consistent with US Code of Federal Regulations, Part 46, Protection of Human Subjects, under 46.101(b), paragraph 4 and with Children’s Hospital institutional review board guidelines.

Analytical Methods
A complete blood cell count (whole blood collected in EDTA) and plasma lead determination were routinely ordered for all the study subjects. In approximately 40% of the samples, red blood cell ZPP was also ordered by the primary care pediatrician.

The leftover EDTA blood was used on the same day of collection for reticulocyte analysis. The leftover heparinized blood was spun down on the same day of collection. Plasma was collected, aliquoted, and frozen at -70°C for biochemical determinations.

Red blood cell and reticulocyte indices were measured with an automated flow cytometer (Technicon H*3, Bayer Diagnostics, Tarrytown, NY).21-23 This flow cytometry system quantifies the distribution for cellular indices of erythrocytes (mean corpuscular volume [MCV], mean corpuscular hemoglobin [Hb] concentration, mean corpuscular Hb content [MCH], and red blood cell volume distribution width [RDW]) and CHR. Reticulocytes were stained using the dye oxazine 750. Approximately 20,000 red blood cells were counted for each reticulocyte determination.

The ZPP level was measured in whole blood with the Prototfluor-Z hematofluorometer (Helena Laboratories, Beaumont, Tex). Results were expressed as micromoles per mole of heme. Serum iron and transferrin were measured using a Hitachi 911 chemistry analyzer (Roche Diagnostics, Indianapolis, Ind). Ferritin was measured using the Bayer Immuno 1 analyzer (Bayer Diagnostics). Circulating TFR was measured using the Quantikine human TFR immunoassay (R&D Systems Inc, Minneapolis, Minn).

Statistical Analysis
For all 210 patients, 2 clinical outcomes were investigated: iron deficiency and iron deficiency anemia. Iron deficiency was defined as a Tfsat level of less than 20% and iron deficiency anemia as a Tfsat level of less than 20% and Hb level of less than 110 g/L. The 20% cutoff for Tfsat has been used in previous studies,7 and has been shown to have a better diagnostic efficacy than lower cutoff levels.8 Alternative diagnostic criteria were also analyzed based on levels of Tfsat, ferritin, and ZPP. Subgroups were based on these cutoff levels, and mean values of CHR, plasma ferritin, Hb, plasma iron, MCV, MCH, and RDW were compared with 2-sample t tests. The Kolmogorov-Smirnov goodness-of-fit test25 revealed no significant departures from normality for any of the variables. Logistic regression analysis25 was performed to determine the relationship of CHR and ferritin for each outcome. The likelihood-ratio χ² test (LRT) was used to assess the significance of CHR and ferritin. Strength of the relationship was measured by the odds ratio and 95% confidence interval. Slope and y-intercept parameters were used to derive probability curves.26 In addition, multiple stepwise logistic regression analysis was performed to identify the variables independently predictive of each outcome.

Receiver operating characteristic analysis was used to illustrate the diagnostic performance of CHR and ferritin with receiver operating characteristic curves compared by the Wilcoxon statistic.32 A CHR cutoff was established based on the optimal combination of sensitivity and specificity. Values below this cutoff were considered to be abnormal. To validate the CHR cutoff, the patient population was divided into healthy and abnormal subgroups and plasma iron, Hb, MCV, MCH, RDW, ferritin, and Tfsat were compared with 2-sample t tests. Data analysis was conducted using the SPSS software package (version 8.0, SPSS Inc, Chicago, Ill). Areas under receiver operating characteristic curves were compared using GraphROC software (version 2.0, Maxiwatli Oy, Turku, Finland). All statistical tests were 2 sided.

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RESULTS

Mean (SD) age for the 210 study subjects was 2.9 (2.0) years. A total of 90 samples were collected from females (mean [SD] age, 2.7 [2.0] years) and 120 samples from males (mean [SD] age, 3.1 [2.1] years).

Using a cutoff value of 20% for Tfsat, 43 subjects (20.5%) were classified as iron deficient. Twenty-four of these subjects were also anemic, based on an Hb cutoff level of 110 g/L. Of the 210 subjects, 41 (19.5%) had anemia with Tfsat values greater than 20%.

Table 1 compares several hematologic and biochemical variables between iron deficient and healthy subjects. The iron deficient group had significantly lower Hb, MCV, MCH, and increased RDW values (P <.001 for all) compared with the healthy group. Interestingly, no significant differences could be found in plasma ferritin (P = .97). A marked difference was also noted in the values of CHr, which was significantly decreased in the iron deficient group (P <.001). Logistic regression indicated that CHr was a significant predictor of iron deficiency (LRT = 37.28; odds ratio, 0.58 [95% confidence interval, 0.48-0.71]; P <.001).

Hematologic and biochemical characteristics of the 24 subjects in the iron deficiency anemia subgroup are summarized in Table 2. Significant differences in MCV, MCH, and RDW values were detected between the 2 subgroups (P <.001 for all). No differences in serum ferritin could be demonstrated (P = .69), while a marked reduction in CHr was noted in the iron deficient anemic patients (P <.001). Logistic regression indicated that CHr was a significant predictor of iron deficiency anemia among the various indices (LRT = 28.97; odds ratio, 0.57 [95% confidence interval, 0.46-0.70]; P <.001).

The empirical distributions of CHr values among iron deficient and healthy subjects are depicted in the histogram of Figure 1. The superimposed curve illustrates the inverse relationship between CHr and the theoretical probability of iron deficiency. According to this relationship, each unit increase in CHr lowers the risk of iron deficiency by 30%. Similar results were obtained for iron deficiency anemia (data not shown), with each unit increase in CHr lowering the risk of iron deficiency anemia by 42%.

Using cutoff levels from the National Health and Nutrition Examination Survey for Tfsat and ferritin (Tfsat [1] <10%, age 1-2 years, [2] <12%, age 3-5 years, and [3] <14%, age 6-15 years; ferritin level [1] <10 µg/L, age <6 years and [2] <12 µg/L, age ≥6 years), among the 210 subjects in this study population, 18 (8.6%) could be considered iron deficient. Seven of these 18 were also anemic according to National Health and Nutrition Examination Survey Hb criteria. Reticulocyte Hb content emerged as a significant predictor of iron deficiency and iron deficiency anemia (P <.001 for both). The estimated odds of iron deficiency and iron deficiency anemia were reduced by 30% and 45%, respectively, with each unit increase in CHr.

To minimize the effect of diurnal variation in plasma iron levels, a separate statistical analysis was carried out in the 94 samples collected before 11 AM. In this subset, similar findings to those shown in Table 1 were observed for iron deficiency (defined as Tfsat <20%). In addition to the significant predictors of iron deficiency anemia shown in Table 2, circulating Tfr and ferritin become significant predictors (P = .04 and P = .02, respectively) for this outcome in this subset of patients.

Results from the stepwise multiple logistic regression analysis revealed that CHr (LRT = 15.96; P <.001) and Hb (LRT = 6.59; P = .01) were the only significant multivariate predictors of iron deficiency among the indices listed in Table 1. Given that approximately 60%...
of the study population was not tested for ZPP, this index was excluded from the multivariate analysis. The only significant multivariate predictor of iron deficiency anemia among the indices listed in Table 2 was CHr ($LRT = 30.43; P <.001$). Ferritin, MCV, MCH, RDW, and TfR were not significant multivariate predictors of either outcome ($P >.10$ for all).

Receiving operating characteristic curves comparing the performance of CHr and ferritin in the diagnosis of iron deficiency are illustrated in Figure 2. The area under the curve was significantly greater for CHr than for ferritin ($P = .004; P = .02$ for iron deficiency anemia, data not shown). A CHr cutoff of 26 pg had a sensitivity and specificity of 70% and 78%, respectively, in the diagnosis of iron deficiency. For iron deficiency anemia, a cutoff of 26 pg had 83% sensitivity and 75% specificity. For the diagnosis of iron deficiency, CHr cutoffs of 26.5, 27.0, 27.5, and 28.0 pg would increase sensitivity to 74%, 81%, 86%, and 91%, respectively, but specificity would decrease to 63%, 55%, 38%, and 26%, respectively.

TABLE 3 presents the hematologic and biochemical values for patients with CHr levels of less than 26 pg or with CHr levels of 26 pg or more. Differences were found between the 2 groups for Hb, MCV, MCH, RDW, Tfsat, and circulating TfR ($P <.001$ for all). Differences in ZPP were significant ($P <.05$) while ferritin showed no difference ($P = .66$) between the CHr subgroups.

**COMMENT**

In this study of young children we have evaluated 2 relatively new parameters for the diagnosis of iron-deficient states. Circulating TfR and CHr have been shown to be useful parameters for the diagnosis of simple iron deficiency or functional iron deficiency in patients treated with recombinant human erythropoietin.10-20

Our data established that CHr is the strongest predictor of iron deficiency and iron deficiency anemia in children. Ferritin, a parameter that is traditionally used in adults to estimate iron stores, had little or no diagnostic value in children. We have also shown that TfR and ZPP were not as informative as CHr in children. It is also known that serum iron, transferrin, and Tfsat have major limitations based on their biological variability.9,28 Thus, a diagnostic approach based exclusively on hematologic parameters obtained by the complete blood cell count and the reticulocyte analysis is appealing for both its direct assess-
ment of iron metabolism and its potential cost-effectiveness.

There are relatively few conditions that result in reduced CHr. In addition to iron deficiency, α and β thalassemia result in hypochromia and microcytosis for both erythrocytes and reticulocytes. Although several different mathematical indices have been proposed for the differential diagnosis of thalassemia trait and iron deficiency, none of them is superior to MCV alone. Diagnosis of heterozygous β thalassemia (β thalassemia trait) can now be reliably obtained with the determination of the ratio of microcytic to hypochromic red blood cells obtained from the complete blood cell count. This ratio is greater than 0.9 in β thalassemia trait, which is characterized by significant microcytosis and mild hypochromia, and is lower than 0.9 in iron deficiency, which is characterized by marked hypochromia and mild microcytosis. This ratio has a discriminant efficiency of 92.4%, which is the highest among the various formulas described for this kind of analysis. The combination of CHr and the ratio of microcytic to hypochromic red blood cells will allow distinction, in the presence of microcytosis, between thalassemia and iron deficiency. If thalassemia is ruled out by a high ratio, a low CHr can only be due to iron deficiency. The diagnostic value of CHr in more complex settings, such as combined iron deficiency and chronic disease, has not been established.

There is ample evidence to indicate that changes in red blood cell parameters become more apparent late in the development of iron deficiency. Our previous studies have shown that reticulocyte indices provide a real-time evaluation of the bone marrow activity, reflecting the balance between iron and erythropoiesis of the preceding 48 hours. Iron deficiency could be detected at an earlier stage, when red blood cell indicators are still normal but the iron stores are depleted to the point of affecting hematopoiesis and inducing production of a certain percentage of reticulocytes with reduced Hb content, resulting in a progressive reduction of CHr.

There is also experimental evidence that CHr is an early indicator of response to iron therapy in iron deficiency anemia cases. The classic criterion for defining response to iron therapy is based on observing an increase of at least 10 g/L of Hb after 1 month of therapy. None of the biochemical parameters is helpful in defining response to iron therapy. Studies of CHr have shown that a response to oral iron therapy can be identified after 1 or 2 weeks of oral iron supplementation. Further studies in children are necessary to determine the value of this parameter in early identification of responders and nonresponders to iron therapy.

We have not directly compared the performance of complete blood cell count and reticulocyte count panel with the traditional biochemical panel in a clinical setting. Such a study must now be performed to validate this alternative approach. If the value of complete blood cell count and reticulocyte count is confirmed by such a study, it could yield significant reductions in costs. The cost of the current diagnostic panel for iron deficiency, which includes a complete blood cell count and evaluation of iron, transferrin, ferritin, and ZPP, is $154.33 based on typical fees and $20.77 based on Medicare nationally capped fees. Using the published data on the prevalence of iron deficiency in children aged 1 to 2 years, the use of the hematologic panel could result in potential savings of $79.85 million ($41.81 million using nationally capped fees). Since CHr and microcytosis to hypochromic red blood cells ratio are currently provided by only 1 of the 4 major automated hematology analyzers sold in the United States, these potential savings will be attainable only when all manufacturers adopt these 2 parameters. Since all of these measurements can be performed on 1.0 to 1.5 mL of blood in an EDTA tube, use of this panel would also result in a significant reduction in the amount of blood needed for the diagnostic workup and the elimination of the heparin tubes and serum tubes needed for ZPP and biochemical determinations. In children, a simple finger-stick would produce a satisfactory blood sample for this panel.

Our study is limited in the number of subjects and ages investigated. It is also difficult to extrapolate from this data set conclusions that can be readily applicable to the general pediatric population. Future studies should evaluate this parameter in an unselected population of children. The poor diagnostic values of ZPP observed in our study

### Table 3. Comparison of Hematological and Biochemical Indices in the Diagnosis of Iron Deficiency

<table>
<thead>
<tr>
<th>Index</th>
<th>CHr Value</th>
<th>P Value</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>&lt;26 pg (n = 67)</td>
<td>≥26 pg (n = 145)</td>
</tr>
<tr>
<td>Hemoglobin, g/L</td>
<td>107.8 (10.7)</td>
<td>115.7 (6.9)</td>
</tr>
<tr>
<td>MCV, fL</td>
<td>73.6 (4.8)</td>
<td>78.5 (3.4)</td>
</tr>
<tr>
<td>MCH, pg</td>
<td>23.7 (2.3)</td>
<td>26.1 (1.5)</td>
</tr>
<tr>
<td>RDW, %</td>
<td>14.6 (1.4)</td>
<td>13.9 (0.8)</td>
</tr>
<tr>
<td>Transferrin saturation, %</td>
<td>26.8 (14.7)</td>
<td>35.7 (13.7)</td>
</tr>
<tr>
<td>ZPP, µmol/mol of heme†</td>
<td>47.0 (41.7)</td>
<td>32.8 (21.3)</td>
</tr>
<tr>
<td>Transferrin receptor, nmol/L</td>
<td>32.0 (6.6)</td>
<td>28.7 (6.0)</td>
</tr>
<tr>
<td>Ferritin, µg/L‡</td>
<td>35.6 (23.6)</td>
<td>34.0 (22.3)</td>
</tr>
</tbody>
</table>

*Reticulocyte hemoglobin content (CHr) of less than 26 pg was used as the cut-off point. All data are presented as mean (SD). See the asterisk footnote to Table 1 for expansion of abbreviations.†For ZPP, n = 27 for CHr less than 26 pg and n = 54 for CHr of 26 pg or higher.‡For ferritin, n = 59 for CHr less than 26 pg and n = 124 for CHr of 26 pg or higher.
may be due to the limited number of subjects with ZPP values (80/210). Previous studies have shown ZPP is helpful in identifying children who will respond to oral iron therapy. Our data indicate that a panel based on hematologic parameters including CHr may provide an alternative to the traditional hematologic or biochemical panel for the diagnosis of both iron deficiency and iron deficiency anemia in young children. Further studies in larger, unselected groups of children are required to fully validate the general use of these parameters.

REFERENCES