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Screening Healthy Infants for Iron Deficiency Using Reticulocyte Hemoglobin Content

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shown to be a poor predictor for iron deficiency. Hematological tests are more widely used to screen for iron deficiency. Hemoglobin is the most commonly used hematological screening test, but it is derived from the entire population of red blood cells, each with a lifespan of about 120 days, and therefore takes some time to be altered by iron deficiency. Consequently, relying on hemoglobin for screening will delay the detection of iron deficiency in infants who are not yet anemic but for whom adverse neurological consequences may have already begun to occur.

With the lifespan of reticulocytes in the circulation being only 24 to 48 hours, reticulocyte-dependent parameters provide a more real-time view of bone marrow iron status. In the initial phases of iron deficiency, before the development of anemia, fluctuations in the iron supply to the bone marrow yield decreased hemoglobin production in reticulocytes, resulting in reticulocytes with less hemoglobin and an overall reduction in reticulocyte hemoglobin content (CHr). A recent study has shown CHr to be a better predictor of iron stores than ferritin, transferrin saturation, or mean corpuscular volume when bone marrow analysis is used as the criterion standard. Unlike biochemical studies, CHr requires no extra tubes of blood to be drawn; CHr is reported as part of the reticulocyte count by the hematology analyzer used in this study and is provided without any additional cost.

A screening approach for iron deficiency based on reticulocyte analysis is appealing for its consistency in various biological states, direct real-time assessment of iron metabolism, and ease of collection. The optimal CHr threshold for predicting iron deficiency in healthy children has not been prospectively determined and CHr has yet to be compared with hemoglobin as a screening tool in the pediatric population. The primary objectives of our study were to establish an optimal CHr threshold for detecting iron deficiency without anemia in 9- to 12-month-old infants and to compare CHr with hemoglobin in screening for iron deficiency in this population. A secondary objective was to explore the association between CHr and subsequent development of anemia. The 9- to 12-month-old age group was chosen because this age group is already routinely screened since it is at particular risk for iron deficiency and its consequences.

**METHODS**

**Study Population**

Healthy 9- to 12-month-old infants presenting to an urban, hospital-based, primary care practice in Boston, Mass, for scheduled well child or nonurgent visits and due for iron deficiency screening as recommended by the American Academy of Pediatrics and the US Centers for Disease Control and Prevention were considered as potential enrollees. To be included, study infants must have been born at 37 or more weeks’ gestation; must not have been diagnosed with otitis media, gastroenteritis, or an upper respiratory tract infection or have had a temperature of more than 38.0°C in the 28 days before the visit; and must not have taken antibiotics for an acute infection or steroids for a 14 or more day course within the past 28 days. In addition, study participants could not have had a known hemoglobinopathy, history of anemia, or have ever received a blood transfusion or iron supplement.

Parents or guardians reported the race/ethnicity of their infant by indicating all that applied to their child: black/African American, Asian, white, Hispanic/Latino, and other/unknown. Race/ethnicity were assessed because values for hemoglobin in African American individuals are 0.5 to 1.0 g/dL lower than values in comparable white populations, although whether this is a racial characteristic or due to a higher frequency of certain genetic traits or nutritional differences is debated. Race/ethnicity data were collected to ensure that representation of these groups did not change between the initial screening visit and the follow-up screening, and to evaluate the generalizability of the study.

Only families whose children met the aforementioned inclusion criteria, as determined by chart review and/or primary care clinician, parent, or guardian contact, were approached by the study research nurses for possible enrollment in the study. Family history and state newborn screen results were not reviewed for the presence of hemoglobinopathy; this more closely simulates real-world pediatrics in which these results do not influence the current clinical practice of screening all 9- to 12-month-old infants.

**Study Design**

This study was approved by the Committee on Clinical Investigation of Children’s Hospital, Boston, Mass. At the initial screening, written informed consent was obtained from the parent or guardian and infant demographics, including height, weight, birth weight, and race/ethnicity, were documented. Five milliliters of blood was obtained by venipuncture for measurement of biochemical (ferritin, iron, total iron-binding capacity, and zinc protoporphyrin) and hematological (mean corpuscular volume, red blood cell distribution width, hemoglobin, reticulocyte count, and CHr) parameters, as well as C-reactive protein. Transferrin saturation (iron-total iron-binding capacity) and mean corpuscular hemoglobin [(hemoglobin × 10)/red blood cell count] were calculated from these measurements. Iron deficiency was defined as transferrin saturation of less than 10%; this is the biochemical parameter that is considered to most accurately reflect the iron available to the bone marrow for erythropoiesis, with a transferrin saturation of less than 16% having been shown to reflect an undersupply of iron to developing erythrocytes. Moreover, this threshold specifically applies to the age range of the infants in our study and is the lower limit of the range of transferrin saturation values widely used clinically to define iron deficiency. Participants with a hemoglobin level of less than 11 g/dL were deemed anemic in accordance with the American Academy of Pediatrics and...
Figure 1. Flow of Study Infants

219 Healthy 9- to 12-Month-Old Infants Enrolled

17 Excluded (Undetermined Hemoglobin, Transferrin Saturation, or Reticulocyte Hemoglobin Content)

202 Included in Initial Analysis of Screening Data

14 Excluded (Hemoglobin <11 g/dL)

188 Eligible for Follow-up Screening During Second Year of Life

41 Excluded

32 Lost to Follow-up

6 Hemoglobin Undetermined

3 Receiving Iron Supplement

147 Included in Analysis of Follow-up Screening Data

Centers for Disease Control and Prevention guidelines and were referred to their primary care clinicians for clinical management (ie, iron supplementation). These infants and those with insufficient samples to determine hemoglobin were excluded from further study. The remaining participants returned for follow-up screening at least 3 months from enrollment but before their second birthday. Enrollees were again required to meet the same inclusion/exclusion criteria at follow-up screening for measurement of the same biochemical and hematological parameters. The original target sample size was 250 participants, chosen to provide 90% power to detect a 0.7-SD difference in the mean CHr level among iron-deficient vs non–iron-deficient patients using a t test with 2-tailed α=0.05 significance level, assuming that the prevalence of iron deficiency would be 10%. The study was closed before meeting the target sample size due to slower than expected accrual.

Laboratory Methods

Erythrocyte and reticulocyte indices were measured with an automated hematology analyzer (ADVIA 120, Bayer Diagnostics, Tarrytown, NY), which quantifies mean values and distributions for cell volume, hemoglobin concentration, and hemoglobin content in both erythrocytes and reticulocytes. Serum iron and total iron-binding capacity (based on a transferrin immunoassay) were measured using a chemistry analyzer (Hitachi 917, Roche Diagnostics, Indianapolis, Ind). C-reactive protein was measured on a BNII nephelometer (Dade-Behring Inc, Deerfield, Ill). Zinc protoporphyrin was measured in whole blood with a hematofluorometer (Aviv Biomedical, Lakewood, NJ) and expressed as μmol/mol of heme.

Statistical Analysis

All data were entered into a Microsoft Excel spreadsheet (Microsoft Corp, Redmond, Wash). Each entry was double-checked and data analysis was performed using SPSS version 11 (SPSS Inc, Chicago, Ill), S-PLUS version 4.5 (Insightful Corp, Seattle, Wash), and Stata version 6 (StataCorp LP, College Station, Tex). All significance testing was 2-tailed and statistical significance was defined as \( P<0.05 \). Any participant with a sample insufficient to determine transferrin saturation or CHr was excluded from analyses for that particular study visit. Characteristics of infants who were included and excluded from analyses were compared using the \( \chi^2 \) test for categorical variables and the t test for continuous variables. Point estimates and exact binomial 95% confidence intervals (CIs) were calculated for the prevalence of iron deficiency (transferrin saturation <10%) and iron-deficiency anemia (transferrin saturation <10% and hemoglobin <11 g/dL) at initial screening.

Box and whisker plots were created to display the distributions of hematological and biochemical marker levels of non–iron-deficient and iron-deficient infants, and median values were compared using the Wilcoxon rank sum test. Receiver operating characteristic (ROC) analysis was used to evaluate the sensitivity and specificity of all possible CHr, hemoglobin, and mean corpuscular hemoglobin thresholds for detecting iron deficiency (using transferrin saturation <10% as the criterion standard). A priori, the minimum requirements of a screening test for iron deficiency were defined to be 80% sensitivity and 50% specificity, and the optimal CHr cutoff was defined to be the one with the highest sensitivity and specificity among all thresholds meeting the minimum requirements. The overall accuracy of CHr, hemoglobin, and mean corpuscular hemoglobin in detecting iron deficiency was summarized using the area under the ROC and compared using a nonparametric test for comparing areas under correlated ROC curves. Ninety-five percent CIs for the sensitivity, specificity, positive predictive value, and negative predictive value were calculated using exact binomial methods.

The association of CHr at initial screening with the incidence of anemia at follow-up screening was estimated by calculating the risk ratio of those above vs below the optimal CHr cutoff at initial screening, with an exact 95% CI. The Fisher exact test was used to assess statistical significance of this association.

RESULTS

Infant Profiles

Two hundred nineteen infants were enrolled between June 2000 and April 2003 (FIGURE 1). Seventeen infants (8%) had insufficient samples to determine hemoglobin, transferrin saturation, or CHr at initial screening and could not be included in data analyses; their demographics did not differ significantly from those of the 202 infants with available data. The baseline characteristics of all infants with available data for initial and follow-up screenings are shown in the TABLE. Ninety-six percent of C-reactive protein measurements were within the normal range at enrollment.

Of those infants with complete data at initial screening, 14 (7%) had anemia and were excluded from further study participation. Of the remaining 188 infants who were eligible for a second screening, 32 (17%) did not re-
turn for follow-up screening and 9 (3%) returned but did not have usable data (3 received iron supplementation by primary care clinician or parental initiative and 6 had insufficient samples to determine hemoglobin). The remaining 147 infants had complete data for both screenings, with a median time interval between screenings of 5.6 months. The baseline characteristics for the 147 infants with complete data for both screenings were similar to the characteristics of the 202 infants at initial screening (Table). With the exception of age (mean, 9.8 vs 10.2 months; \( P = .02 \)), the demographic characteristics of the 55 infants who did not have follow-up screening did not differ significantly from the characteristics of the 147 infants who did have follow-up screening.

Iron Status of Infants at Initial Screening

Of the 202 evaluable infants at initial screening, 23 had iron deficiency (prevalence, 11.4%); 95% CI, 7.4%-16.6%) and 6 had iron deficiency and anemia (prevalence, 3%; 95% CI, 1.1%-6.4%). The mean CHr value was 28.1 pg (SD, 2.3; 95% CI, 27.8-28.5). The mean CHr value was 28.1 pg (SD, 2.3; 95% CI, 27.8-28.5). The area under the ROC curve for CHr was significantly larger than for ferritin. With Initial and Follow-up Screening

Table. Baseline Characteristics of Infants With Initial and Follow-up Screening

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Initial Screening (n = 202)</th>
<th>Initial and Follow-up Screening (n = 147)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Race/ethnicity, No. (%)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Black/African American</td>
<td>85 (42)</td>
<td>63 (43)</td>
</tr>
<tr>
<td>Hispanic/Latino</td>
<td>64 (32)</td>
<td>48 (33)</td>
</tr>
<tr>
<td>White</td>
<td>17 (8)</td>
<td>13 (9)</td>
</tr>
<tr>
<td>Asian</td>
<td>8 (4)</td>
<td>4 (3)</td>
</tr>
<tr>
<td>Other/unknown</td>
<td>28 (14)</td>
<td>19 (13)</td>
</tr>
</tbody>
</table>

*Parents or guardians self-determined the race/ethnicity of their infant. Other/unknown includes any race/ethnicity not represented by any of the aforementioned categories.

Comparison of CHr With Hemoglobin in Screening for Iron Deficiency

Using a hemoglobin level of less than 11 g/dL resulted in a sensitivity of 26% (detected 6 of 23 iron-deficient infants; 95% CI, 10%-48%), a specificity of 93% (95% CI, 91%-98%), a positive predictive value of 43% (95% CI, 18%-71%), and a negative predictive value of 91% (95% CI, 86%-95%). The area under the ROC curve for CHr was significantly larger than for ferritin.
than that for hemoglobin (0.85 vs 0.73, P = .007), indicating that CHr was a more accurate marker overall for the detection of iron deficiency than hemoglobin. For example, a higher hemoglobin cutoff level of 12.3 to 12.4 g/dL would have comparable sensitivity with that of a CHr of less than 27.5 pg (78%-87%), but lower specificity (39%-45%), while a hemoglobin cutoff level of 12.0 g/dL would have comparable specificity with that of a CHr of less than 27.5 pg (73%) but lower sensitivity (61%). Reticulocyte hemoglobin content was more accurate in detecting iron deficiency than mean corpuscular hemoglobin, another parameter derived from the entire population of erythrocytes; the area under the ROC curve for the latter was 0.73, nearly identical to that of hemoglobin. Mean corpuscular hemoglobin and mean corpuscular volume, mean corpuscular hemoglobin, and red blood cell distribution width. CHr was significantly lower than the mean corpuscular hemoglobin, and significantly smaller than that for CHr (P = .006). Using CHr less than mean corpuscular hemoglobin to screen for iron deficiency yielded a specificity of 93% and a sensitivity of 30%.

**Association of CHr at Initial Screening With Incidence of Anemia at Follow-up Screening**

Of the 45 nonanemic infants with CHr of less than 27.5 pg at initial screening who were evaluated at follow-up screening, 4 (9%) developed anemia in contrast, of the 102 nonanemic infants with CHr of at least 27.5 pg at initial screening who were evaluated at follow-up screening, only 1 (1%) developed anemia (risk ratio, 9.1; 95% CI, 1.04-78.9; P = .01).

**COMMENT**

In this prospective study, we have identified a CHr threshold of less than 27.5 pg, with its promising sensitivity and specificity profile, for detecting iron deficiency before anemia in healthy 9- to 12-month-old infants. This threshold also was associated with the development of subsequent anemia. These findings suggest that CHr may prove to be a valuable screening tool for iron deficiency in the ambulatory primary care setting.

Unlike biochemical parameters, hematological parameters, such as CHr and hemoglobin, are advantageous for screening for iron deficiency because they are easily obtained, inexpensive, and free from biological variability that affects iron, total iron-binding capacity, and ferritin measurements. Reticulocyte hemoglobin content reflects the iron availability at the time that today’s reticulocytes were made in the bone marrow. Because of the short duration of the reticulocyte stage in erythropoiesis, evaluation of CHr can reveal states of iron deficiency that are clinically significant but not reflected by parameters derived from the entire red blood cell population. In fact, both hemoglobin and mean corpuscular hemoglobin showed significantly lower accuracy than CHr in detecting iron deficiency.

A decrease in hemoglobin is a late finding in the development of iron deficiency, significantly detracting from its use as a preventive screening tool. Furthermore, as the prevalence of iron-deficiency anemia is declining in children, the value of anemia as a predictor of iron deficiency will diminish further. Like hemoglobin, hematocrit also would not be expected to detect iron deficiency with adequate sensitivity. In 1 study of 321 infants, a hematocrit of 33%, corresponding with a hemoglobin of 11 g/dL, did not detect any infants with iron deficiency, defined as a ferritin level of less than 10 µg/L. We defined iron deficiency as a transferrin saturation of less than 10%. Transferrin saturation is a measure of transported iron and a commonly used biochemical indicator for iron deficiency. A decrease in transferrin saturation can indicate a stage of iron-restricted erythropoiesis that has not yet resulted in frank anemia. Although a difference in the biochemical standard used to define iron deficiency between the aforementioned study and our study likely explains the difference in sensitivities of hemoglobin/hematocrit, both studies highlight that the sensitivity of hemoglobin/hematocrit is insufficient for their use as screening tools for iron deficiency.

A retrospective trial involving an older pediatric population (mean [SD] age, 2.9 [2.0] years) also suggested CHr was the strongest predictor of iron-deficiency anemia in children compared with biochemical parameters used to estimate iron stores (ferritin, soluble transferrin receptor, and zinc protoporphyrin) and hematological parameters (mean corpuscular volume, mean corpuscular hemoglobin, and red blood cell distribution width). In this previous retrospective study, a CHr threshold of 27.5 pg had an almost identical sensitivity (86%) but lower specificity (38%) than in our younger study population.

Our infant population’s mean CHr was significantly lower than the mean CHr reported for healthy adults in 1 study, but not significantly lower than in another study. This may be related to the transient physiologic decreases in mean corpuscular volume and mean corpuscular hemoglobin observed during the first 2 years of life, as well as to differences in instrumentation and methods among the studies. There is little else known about how CHr changes with age, and further work to address this question is needed.

Our study does have some limitations. First, CHr is a sensitive marker that is specific for iron deficiency even in states of inflammation. However, as
a hematological parameter, its specificity is limited by other hematological conditions, such as thalassemia trait and symptomatic thalassemia syndromes, which also cause microcytosis and low hemoglobin content of both reticulocytes and mature erythrocytes. Although we did not evaluate potential participants for thalassemia, several different erythrocyte indices have been described that are helpful in discriminating thalassemia trait from iron deficiency, including the Mentzer formula and the microcytosis-hypochromia ratio. The microcytosis-hypochromia ratio is a feature available on the hematology analyzer used to measure CHr and has been shown to accurately discriminate iron deficiency from thalassemia trait based on the fact that iron-deficient erythropoiesis is characterized by more pronounced hypochromia, whereas in thalassemia trait the erythrocytes are more microcytic. These indices facilitate the distinction of iron deficiency from thalassemia in the setting of microcytosis and a low CHr.

Second, CHr can only be measured by 2 of the major hematology analyzers in use today. A growing number of analyzers can determine reticulocyte cellular indices, including alternatives such as mean reticulocyte volume and RET-Y, a raw reticulocyte measure dependent on size and content of the cell. RET-Y correlates well with CHr and similar to CHr may be useful in the assessment of iron-deficient states. Reticulocyte indices and their possible applications are growing areas of active research, and CHr remains the best characterized of all the indices in use today.

Third, 17% of the infants were lost to follow-up; however, given the characteristics of the infants analyzed at the initial and follow-up screenings, the 2 groups remained comparable. Fourth, the size of the study was relatively small with only 23 iron-deficient infants at initial screening. Fifth, the cohort was mainly black and Hispanic, which may limit the generalizability of this study to all pediatric practices. Finally, the age intervals between initial and follow-up screenings may have influenced the results. This is largely because CHr has not yet been studied in healthy infants and children to allow establishment of age-adjusted normal ranges. These varied age intervals in follow-up were largely a product of the stringent exclusion criteria that were applied to the infants at the follow-up screening. If they were not eligible for follow-up screening due to illness that could interfere with the accuracy of biochemical tests obtained, they were required to wait until they were once again eligible.

A particular strength of our study is that the infants were carefully screened so that those with known possible infection or inflammation were excluded. Although transferrin saturation is considered the criterion standard for iron deficiency, it is derived from iron and transferrin, 2 acute phase reactants that can be altered in states of inflammation. The ability of our criteria to exclude those infants with inflammation is confirmed by the rarity of even minimally increased C-reactive protein in our study infants. Although adult studies have shown that CHr remains an accurate marker of iron status in states of infection and inflammation, infants whose biochemical parameters might not accurately reflect their true iron status were excluded in our study. This is supported by the fact that the prevalence of iron deficiency and iron-deficiency anemia in our population are concordant with prevalence described elsewhere.

In healthy adults, CHr has been shown to be an early indicator of response to therapy in iron-deficiency anemia. Therapy is likely to have the same predictive ability in the pediatric population, further studies are needed to evaluate CHr changes in response to iron therapy. Studies are also needed to evaluate how CHr values change with increasing age and to explore cost/benefit analyses of CHr as a screening tool for iron-deficiency anemia in infants. Further studies are also needed to determine if the use of CHr to detect and treat iron deficiency significantly decreases the subsequent development of anemia and neurocognitive deficits.

The US Department of Health and Human Services initiative Healthy People 2010 aims to decrease the prevalence of iron deficiency. Our study suggests that CHr is a sensitive screening tool for detecting iron deficiency. Although its modestly lower specificity compared with hemoglobin may lead to overtreatment, CHr shows promise in the identification of children with iron deficiency solely on the basis of hematological parameters. Larger multicenter studies will be necessary to determine whether CHr should be the preferred screening tool in the early detection of iron deficiency, bringing us a step closer to reducing the prevalence of this treatable nutritional deficiency.

Author Contributions: Dr Bernstein, as principal investigator of this study, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Wu, Armsby, Brugnara, Bernstein. Acquisition of data: Ullrich, Wu, Armsby, Rieber, Wingert, Bernstein. Analysis and interpretation of data: Ullrich, Armsby, Rieber, Brugnara, Shapiro, Bernstein. Drafting of the manuscript: Ullrich, Rieber, Brugnara, Shapiro, Bernstein. Critical revision of the manuscript for important intellectual content: Ullrich, Wu, Armsby, Rieber, Wingert, Brugnara, Shapiro, Bernstein. Statistical analysis: Ullrich, Rieber, Shapiro. Obtained funding: Wu, Bernstein. Administrative, technical, or material support: Ullrich, Rieber, Brugnara, Bernstein. Study supervision: Brugnara, Bernstein. Financial Disclosures: Dr Brugnara currently has a consulting agreement with Bayer Diagnostics and Dr Bernstein has had a consulting agreement with Bayer Diagnostics in the past.

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