

## The Diagnostic Criteria for Iron Deficiency in Infants Should Be Reevaluated<sup>1</sup>

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**ABSTRACT** Diagnostic criteria for iron deficiency (ID) and iron deficiency anemia (IDA) in infants are poorly defined. Our aim was to establish appropriate cut-off values for hemoglobin (Hb), plasma ferritin, erythrocyte mean cell volume (MCV), zinc protoporphyrin (ZPP) and soluble transferrin receptors (TfR) in infancy. Exclusively breast-fed infants ( $n = 263$ ) in Honduras and Sweden were randomly assigned to receive iron supplementation or placebo, and blood samples were obtained at 4, 6 and 9 mo of age. Reference ranges were determined using three different approaches for defining iron-replete infants. The usefulness of several variables for predicting the Hb response to iron was evaluated. We found the following 2 SD cut-off values in iron-replete infants: Hb <105 g/L at 4–6 mo and <100 g/L at 9 mo; ZPP >75  $\mu\text{mol/mol}$  heme at 4–6 mo and >90  $\mu\text{mol/mol}$  heme at 9 mo; ferritin <20  $\mu\text{g/L}$  at 4 mo, <9  $\mu\text{g/L}$  at 6 mo and <5  $\mu\text{g/L}$  at 9 mo; and TfR >11 mg/L at 4–9 mo. The Hb response to iron was not a useful definition of IDA at 4 mo of age. Hb, MCV and ZPP at 6 mo as well as growth variables predicted the Hb response at 6–9 mo, but ferritin and TfR at 6 mo did not. We conclude that there is need for a reevaluation of the definitions of ID and IDA in infants. *J. Nutr.* 132: 3680–3686, 2002.

**KEY WORDS:** • iron deficiency anemia • reference values • hemoglobin • ferritin • transferrin receptors • infants

Infancy is a period of rapid growth and, consequently, high iron requirements. Iron deficiency anemia (IDA)<sup>3</sup> is a major public health problem in infants and young children, with an estimated prevalence of 3–80% in different populations, and is associated with impaired neurodevelopment in infants and children (1–4). Laboratory criteria for IDA include anemia, i.e., low hemoglobin (Hb), together with other signs of iron deficiency (ID) such as low erythrocyte mean cell volume (MCV), low serum ferritin, high zinc protoporphyrin (ZPP) and/or high soluble transferrin receptors (TfR). However, there is no agreement on the specific laboratory criteria for ID. The usual requirement is either a low serum ferritin (5) or a combination of multiple criteria, e.g., abnormal values for any two of three iron status variables (6). Even though the latter model is most commonly used, there is no consensus concerning whether to use single or multiple criteria, or which iron status variables to use in the multiple criteria model (7). The diagnosis of IDA in adults can be confirmed by the observation

of an increase in Hb of a certain magnitude (e.g., >10 g/L) after at least 1 mo of iron supplementation (7–9). Older reference methods, such as iron staining of bone marrow and quantitative phlebotomy, are rarely used in adults, and are not appropriate for use in infants.

Reference values for iron status variables in infants are not well developed. In clinical practice as well as in research, commonly used cut-off values for identifying ID and IDA at 6–12 mo of age are Hb <110 g/L (10,11) and serum ferritin <10–12  $\mu\text{g/L}$  (12,13), but these values are extrapolated from older age groups and may not be appropriate for infants (14). Cut-off values for other measures of iron status such as MCV, ZPP and TfR are even less well defined for infants (15). Furthermore, no reference values based on breast-fed infants have been published for any iron status variables, even though exclusive breast-feeding is generally recommended for the first 6 mo of life (16,17).

We recently performed a randomized, controlled trial of iron supplementation that included Honduran and Swedish infants ( $n = 263$ ; 4–9 mo old). The primary aim was to study the effects of iron supplementation on iron status (18). All infants were exclusively or nearly exclusively breast-fed until 6 mo and partially breast-fed until 9 mo of age. The dual site design ensured a wide range of iron status. Using the same cohort, the aim of the analyses presented here was to identify appropriate reference values for evaluating iron status, based on the “biological norm” of breast-fed infants. To determine reference values, different methods for defining the normative

<sup>1</sup> Supported by the United States Department of Agriculture, the Thrasher Research Fund, Stiftelsen Oskarfonden, Swedish Nutrition Foundation, Stiftelsen Samariten and the Swedish Medical Research Council (05708).

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<sup>3</sup> Abbreviations used: AUC, area under the curve; Fe 4–9, group receiving iron from 4–9 mo; Fe 6–9, group receiving iron from 6–9 mo; Hb, hemoglobin; HbA, adult Hb; HbF, fetal Hb; ID, iron deficiency; IDA, iron deficiency anemia; MCV, erythrocyte mean cell volume; P 4–9, group receiving placebo from 4–9 mo; ROC, receiver operating characteristics; TfR, soluble transferrin receptors; ZPP, zinc protoporphyrin.

population were used. The Hb response to iron supplementation was evaluated as an alternative definition of IDA.

## SUBJECTS AND METHODS

**Subjects and study design.** Healthy, term ( $\geq 37$  gestational wk) Honduran and Swedish infants with adequate birth weight ( $>2500$  g) were recruited at 0–3 mo of age as previously described (18). The study was approved by the Ethical Committee, Faculty of Medicine and Odontology, Umeå University, Sweden and the Human Subjects Review Committees of the University of California, Davis, CA. All participating mothers/parents gave written, informed consent.

Subjects were stratified by study site and sex, and randomly assigned to three intervention groups: 1) iron supplement from 4 to 9 mo of age (Fe 4–9); 2) placebo from 4 to 6 mo and iron from 6 to 9 mo (Fe 6–9); and 3) placebo from 4 to 9 mo (P 4–9). Infants were given a daily dose of placebo or ferrous sulfate mixture (Fer-In-Sol, Mead Johnson, Evansville, IN) corresponding to 1 mg/kg body of elemental iron. The investigators as well as the parents were unaware of the nature of the intervention.

Between 4 and 6 mo, the mothers were discouraged from giving any other foods or fluids than breast milk, except for “taste portions” [ $\leq 1$  tablespoon (15 mL/d)] of foods with little or no iron. Between 6 and 9 mo, the mothers continued breast-feeding and were encouraged to give complementary food at their own discretion.

Venous blood ( $\sim 5$  mL) was obtained at 4, 6 and 9 mo of age and analyzed for Hb, MCV, ZPP, ferritin and TfR as previously described (18). If the infant had a febrile infection, the blood sampling was postponed 1 wk. Infant weight and length were measured monthly. Compliance was monitored by mothers’ daily checklist of administering the drops, as well as measurement of remaining fluid in returned bottles. Subjects were considered noncompliant if they received the study drops  $< 75\%$  of the days according to either of those two indicators (18).

**Statistical methods.** As explained elsewhere, the target sample size was 60 infants per treatment group, or 30 per group at each site, based on detecting a difference in hemoglobin of 5 g/L among treatment groups (power 80%,  $\alpha 0.05$ ) (18). Infants who remained in the study at 6 mo of age were included in the statistical analyses.

All statistical analyses were performed using SPSS software version 10.0 (SPSS, Chicago, IL). Statistical methods used were Fisher’s exact test for comparing proportions, *t* test for comparing means and receiver operating characteristics (ROC) curves analysis for evaluating sensitivity and specificity of diagnostic tests based on the Hb response (19).

Because distributions for ferritin and ZPP were skewed, these values were log transformed for all statistical calculations. In the presentation, the values were converted back to the original units as geometric means and SD.

**Normative population methods.** Several different methods have been used to establish cut-off values for iron status variables. The most common approach is the normative population method in which the reference range is calculated for a “healthy” population sample. The basic approach is to sample a population likely to have a low prevalence of ID, but without further selection of iron-sufficient subjects within the population sample (14). Below, we will call this the “unselected” normative population approach. Beyond this, there are at least two possible approaches to minimize the risk of including iron-deficient individuals in the normative population. One is to exclude individuals with possible ID according to conventional cut-off values for iron status variables other than the one under study (13,20). We will call this the “iron-replete” normative population approach. Finally, ID can be excluded by prophylactic iron supplementation of the population sample (20,21). We will call this the “iron-supplemented” normative population approach.

We applied the unselected, iron-replete and iron-supplemented normative population methods to our population sample. To illustrate the differences between the approaches, they were not combined. Therefore, iron-supplemented infants were excluded when using the “unselected” and “iron-replete” approaches. To avoid using adult reference values in infants, we decided to modify the iron-replete normative population method in the following way. First, we

assumed that the Swedish healthy, breast-fed infants were iron sufficient at 4 mo of age, which is a reasonable assumption because normal infants without risk factors for IDA are generally believed to have sufficient iron stores to prevent ID during at least the first 4 mo of life (9,22). Second, because unsupplemented infants with borderline ID would be expected to become gradually more iron deficient with time (assuming no change in dietary patterns), we decided to apply at 6 and 9 mo the 2 SD cut-off values for the iron-replete normative samples from the previous time point (4 and 6 mo, respectively) to exclude those who had developed ID. For example, to identify the iron-replete normative population sample for Hb at 6 mo, we excluded any infant who at 6 mo demonstrated possible ID in any of the other iron status variables (i.e., MCV, ZPP, ferritin or TfR) according to the 2 SD cut-off values for the Swedish infants at 4 mo. After having established reference ranges in this way for all variables at 6 mo, we used the resulting 2 SD cut-off values from all iron-replete infants at 6 mo to similarly exclude infants with possible ID from the population sample at 9 mo.

**Hb response to iron.** A quite different approach to establish cut-off values is the “Hb response” method in which individuals with IDA are identified by studying the Hb response after iron treatment. Sensitivity and specificity for different cut-off levels, for various iron status variables and other variables can then be tested against this “gold standard.” Because the optimal Hb response in infants is not known, we tested three response levels:  $>5$ ,  $>10$  and  $>15$  g/L. Noncompliers and infants with any missing iron status variable during the time period (4–6 or 6–9 mo) were excluded from the analyses. Receiver operating characteristics (ROC) curves were constructed and area under the curve (AUC) calculated as an estimate of the performance of each variable as a diagnostic test for IDA. Theoretically, AUC varies between 0.5 and 1. An AUC of 1 means 100% sensitivity and specificity. The null hypothesis (AUC = 0.5) is that the diagnostic test is no better than chance.

## RESULTS

**Subjects.** Subject characteristics, background data, recruitment procedures, randomization and dropouts are described in detail elsewhere (18). Briefly, 263 infants (142 in Honduras, 121 in Sweden) were randomly assigned to three groups at 4 mo of age. At 6 mo, 232 infants (131 in Honduras, 101 in Sweden) remained, and 214 infants (118 in Honduras, 96 in Sweden) completed the study at 9 mo. Compliance from 4 to 9 mo was 86% in Honduras and 92% in Sweden.

**Normative population method.** After the exclusion of 17 infants who had at least one missing iron status variable at 4, 6 or 9 mo, 197 cases remained to be analyzed. At each of the three ages, the following subpopulations were defined: 1) “Unselected” ( $n = 197$ , 134 and 71 at 4, 6 and 9 mo, respectively), defined as all unsupplemented infants. 2) “Iron-replete” ( $n = 83$ , 54–62 and 34–38 at 4, 6 and 9 mo, respectively), defined as those of the unsupplemented infants who fulfilled certain criteria for iron sufficiency (described above). 3) “Iron supplemented” [ $n = 50$  at 6 mo,  $n = 61$  (Fe 6–9) and  $n = 47$  (Fe 4–9) at 9 mo], defined as all iron-supplemented, compliant infants.

At 4 mo, there were significant differences between sites in all variables except TfR (Table 1). All differences indicated more ID in the Honduran infants. However, in iron-replete infants at 9 mo, Hb, ZPP and ferritin means did not differ between sites (Table 1). In contrast, the site difference in MCV observed at 4 mo remained significant at 9 mo in the iron-replete group. A similar site difference in MCV was also observed in the iron-supplemented groups at 9 mo (not shown). Due to this relatively large ( $\sim 2$  SD) site-dependent and iron-independent site difference, reference ranges for MCV were calculated separately for Sweden and Honduras.

At 9 mo, the Fe 4–9 and Fe 6–9 groups were considered separately. The graphs (Fig. 1 and Figs. 3–5) show the mean

TABLE 1

Differences in iron status variables between infants in Honduras and Sweden at 4 and 9 mo of age<sup>1</sup>

|                                 | 4 mo       |            |        | 9 mo, iron-replete <sup>2</sup> |                    |        |
|---------------------------------|------------|------------|--------|---------------------------------|--------------------|--------|
|                                 | Honduras   | Sweden     | P      | Honduras                        | Sweden             | P      |
| n                               | 114        | 83         |        | 11–16 <sup>3</sup>              | 21–24 <sup>3</sup> |        |
| Hb, <sup>4</sup> g/L            | 112 ± 8    | 119 ± 7    | <0.001 | 113 ± 5                         | 114 ± 7            | 0.448  |
| MCV, <sup>4</sup> fL            | 74.5 ± 4.7 | 79.7 ± 3.5 | <0.001 | 71.5 ± 3.1                      | 77.8 ± 3.3         | <0.001 |
| ZPP, <sup>5</sup> μmol/mol heme | 51 ± 1.71  | 46 ± 1.66  | 0.011  | 56 ± 1.33                       | 48 ± 1.31          | 0.109  |
| Ferritin, <sup>5</sup> μmol/L   | 69 ± 1.84  | 105 ± 2.02 | 0.001  | 32 ± 2.52                       | 26 ± 2.52          | 0.535  |
| TfR, <sup>4</sup> mg/L          | 6.5 ± 2.0  | 6.9 ± 2.3  | 0.185  | 7.2 ± 2.2                       | 7.7 ± 1.6          | 0.501  |

<sup>1</sup> Hb, Hemoglobin; MCV, Erythrocyte mean cell volume; ZPP, zinc protoporphyrin; TfR, soluble transferrin receptors.

<sup>2</sup> Iron-replete: unsupplemented infants (P 4–9 group) with possible iron deficiency excluded (see Subjects and Methods).

<sup>3</sup> Minimum and maximum number of infants available for the different iron status indicators.

<sup>4</sup> Mean ± SD.

<sup>5</sup> Geometric mean ± SD. Note: the arithmetic SD is added (subtracted) to the mean, whereas the geometric SD is multiplied (divided) by the geometric mean.

and the 95% reference range ( $\pm 2$  SD) for each group at each age. For Hb, MCV and ferritin, the  $-2$  SD cut-off value is relevant for the diagnosis of ID, whereas for ZPP and TfR, the  $+2$  SD cut-off value is relevant because the latter two variables are expected to increase during ID. The interindividual variation in the plasma variables, ferritin and TfR (Figs. 5–6), was considerably larger than the variation in the erythrocyte variables, Hb, MCV and ZPP (Fig. 1 and Figs. 3, 4).

In unselected, unsupplemented infants, Hb, MCV and ferritin means decreased, whereas ZPP and TfR means increased from 4 to 9 mo. This was paralleled by increasing SD, possibly suggesting an increasing proportion of iron-deficient infants. The only exception was Hb, for which the SD did not increase. This may suggest that the slight decrease in Hb from 4 to 9 mo was physiologic and that the proportion of infants who developed anemia between 4 and 9 mo of age was low.

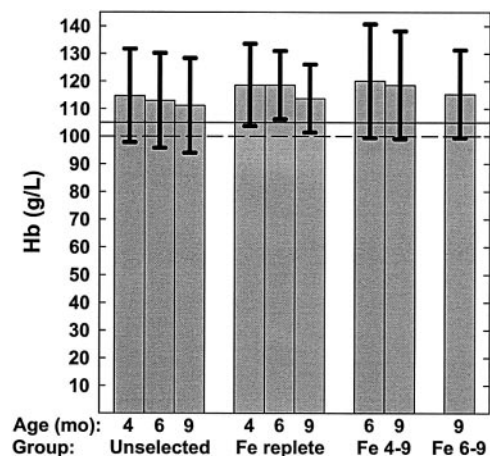
**Hemoglobin.** Among iron-replete infants, mean Hb decreased from 6 to 9 mo, probably reflecting the same physiologic decrease observed in unselected infants (Fig. 1). The reference range for iron-replete infants at all ages was smaller than for unselected infants ( $P = 0.007$  at 6 mo), suggesting that there might exist a certain proportion of anemic infants in the unselected group. The range for iron-replete infants was also smaller than for iron-supplemented infants ( $P < 0.001$  at 6 mo,  $P = 0.035$  at 9 mo compared with the Fe 4–9 group), possibly reflecting a more complete exclusion of anemic infants in the iron-replete group as well as “supernormal” Hb in some iron-supplemented infants (see also Fig. 2, showing the actual frequency distribution of Hb at 6 mo in iron replete and iron-supplemented infants). At 6 and 9 mo, mean Hb was higher in the Fe 4–9 group than in any other group, consistent with the uniform Hb response to iron at 4–6 mo (see below). Despite different means, the  $-2$  SD cut-off values at 9 mo were very close to 100 g/L in iron-replete and both groups of iron-supplemented infants.

**Mean cell volume.** At both sites, a distinct drop in mean MCV, from the “unselected” level at 4 mo, was observed at 6 mo in iron-replete as well as iron-supplemented infants, probably reflecting a continuation of the normal developmental decrease in MCV during the neonatal period (Fig. 3a, b). In Honduran iron-replete and iron-supplemented infants (Fig. 3a), the  $-2$  SD cut-off values at 6 and 9 mo were all close to 64 fL; the only exception was the Fe 6–9 group for which the cut-off value was 62 fL at 9 mo, possibly reflecting a small proportion of ID in this group (which received iron supple-

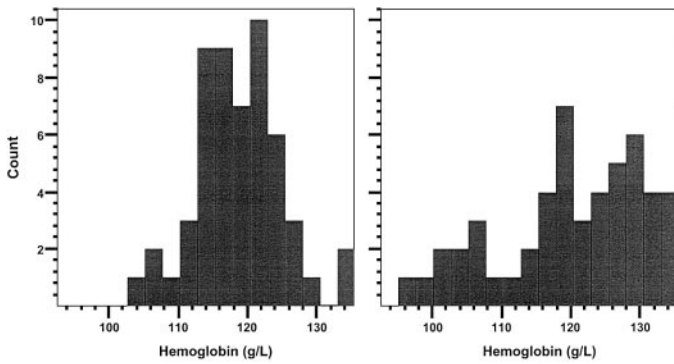
ments for a shorter period). In Swedish infants at 4 mo (assumed to be iron replete), the  $-2$  SD cut-off value was 73 fL, and in iron-replete Swedish infants at 6 and 9 mo, the cut-off value was 71 fL (Fig. 3b). The levels were slightly higher in the Fe 4–9 group, which was consistent with the higher mean Hb in this group.

**Zinc protoporphyrin.** At 4 mo, the  $+2$  SD cut-off value in iron-replete infants was 76 μmol/mol heme (Fig. 4). At 6 mo, in iron-replete and iron-supplemented infants, this cut-off value was 69 and 82, respectively. At 9 mo, the  $+2$  SD cut-off values in the iron-replete and Fe 4–9 groups were both 90, whereas a slightly higher cut-off value of 101 μmol/mol heme was observed in the Fe 6–9 group.

**Ferritin.** Mean serum ferritin decreased profoundly in unselected and iron-replete infants from 4 to 9 mo, whereas



**FIGURE 1** Means and reference ranges ( $\pm 2$  SD) for hemoglobin (Hb) at 4, 6 and 9 mo in “unselected,” “iron-replete” and iron-supplemented infants. “Unselected” are unsupplemented infants [placebo (P) 4–9 group] without further selection. “Iron-replete” are unsupplemented infants (P 4–9 group), excluding those with possible iron deficiency according to iron status variables except the variable studied. Iron-supplemented infants are divided into those supplemented from 4 to 9 mo (Fe 4–9) and those supplemented from 6 to 9 mo (Fe 6–9). The number of infants for each bar in the graph (from left to right) is 197, 134, 71, 83, 54, 34, 61, 50 and 47. Horizontal lines are the suggested  $-2$  SD cut-off values at 4–6 mo (105 g/L, solid line) and 9 mo (100 g/L, dashed line).

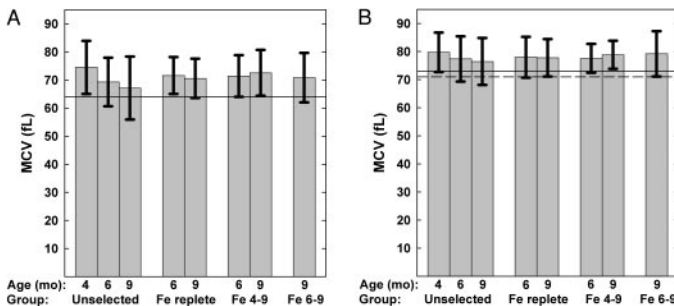


**FIGURE 2** Frequency distributions of hemoglobin (Hb) concentrations at 6 mo in iron-replete (left panel,  $n = 54$ ) and iron-supplemented (right panel,  $n = 50$ ) infants.

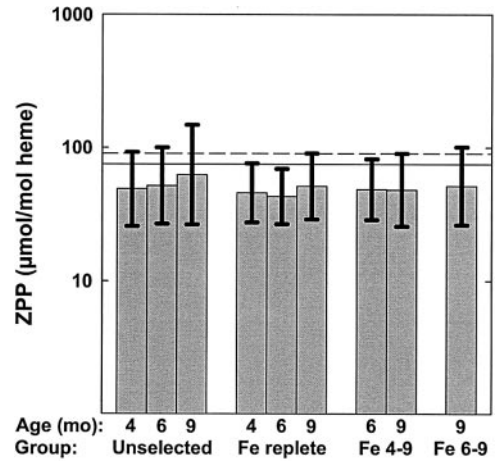
ranges were constant, except for a slight increase from 4 to 6 mo of age (Fig. 5). This is likely to be a continuation of the physiologic decline seen from birth to 4 mo. Ferritin concentrations in iron-replete infants were significantly higher than in unselected infants, but lower than in iron-supplemented infants.

**Transferrin receptors.** With the exception of unselected infants at 9 mo, all groups had mean TfR between 6.3 and 7.5 mg/L; the median of the group means was 6.7 mg/L (Fig. 6). Furthermore, with the same exception, all groups had +2 SD cut-off values between 9.4 and 11.5, and the median of these was 10.9 mg/L.

**Hb response to iron.** There were significant negative correlations between Hb change and initial Hb in the placebo groups at 4–6 mo ( $r = -0.23$ ,  $P = 0.004$ ) and 6–9 mo ( $r = -0.29$ ,  $P = 0.011$ ), corresponding to regression toward the mean (values tend to decrease in those with high initial Hb, and to increase in those with low initial Hb). To correct the Hb response in the iron groups for the expected change in the absence of iron supplementation, the predicted value from the regression equation for the placebo group was calculated for each individual in the iron group and then subtracted from the observed value. There was no correlation between the resulting corrected Hb response and initial Hb at 4–6 mo ( $r = -0.05$ ,  $P = 0.676$ ) but there was a negative correlation at



**FIGURE 3** Means and reference ranges ( $\pm 2$  SD) for erythrocyte mean cell volume (MCV) at 4, 6 and 9 mo in Honduran (a) and Swedish (b) unselected, iron-replete and iron-supplemented (Fe 4–9 and Fe 6–9) infants. (a) The number of infants for each bar in the graph is 114, 76, 40, 38, 18, 35, 28 and 27. Horizontal line is the suggested  $-2$  SD cut-off value at 4–9 mo (64 fL). (b) The number of infants for each bar in the graph is 83, 58, 31, 48, 21, 26, 22 and 20. Horizontal lines are the suggested  $-2$  SD cut-off values at 4 mo (73 fL, solid line) and 6–9 mo (71 fL, dashed line).

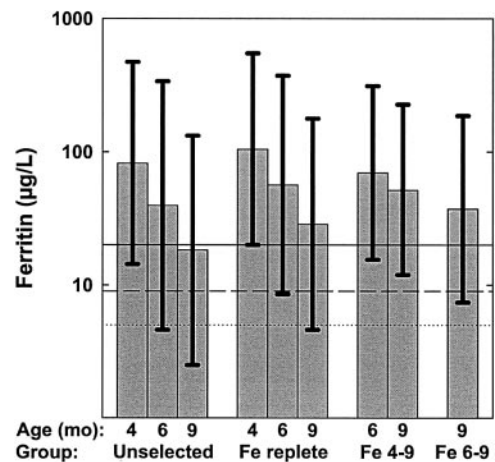


**FIGURE 4** Geometric means and reference ranges ( $\pm 2$  SD) for zinc protoporphyrin (ZPP) at 4, 6 and 9 mo in unselected, iron-replete and iron-supplemented (Fe 4–9 and Fe 6–9) infants. The number of infants for each bar in the graph is 197, 134, 71, 83, 55, 38, 61, 50 and 47. Horizontal lines are the suggested +2 SD cut-off values at 4–6 mo (75  $\mu\text{mol/mol}$  heme, solid line) and 9 mo (90  $\mu\text{mol/mol}$  heme, dashed line).

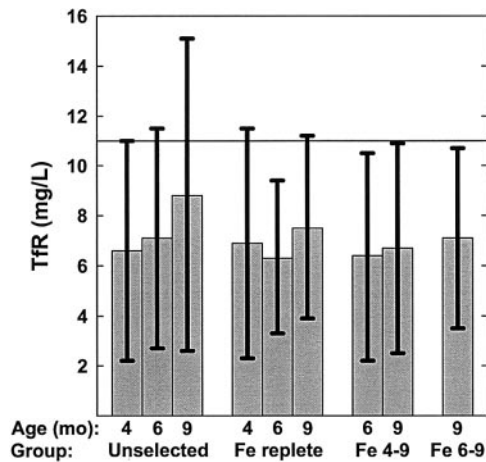
6–9 mo ( $r = -0.36$ ,  $P = 0.003$ ). No inflection point was found in this regression plot (not shown), suggesting that there was no cut-off point for Hb below which the Hb response increased sharply.

The ROC curves and AUC at both time intervals were similar whether the corrected or the uncorrected Hb response was used. For the sake of simplicity, only the uncorrected Hb response values are shown in the analyses below.

**Response for 4–6 mo.** The Hb response from 4 to 6 mo was studied in iron-supplemented infants (Fe 4–9 group) after exclusion of noncompliers and infants with any missing iron status variable in this age interval. The proportions of Honduran and Swedish infants showing Hb responses of different magnitudes are shown in Table 2. ROC analyses performed for the two lower response levels showed that Hb, MCV, ZPP, ferritin and TfR at 4 mo had no significant predictive value for



**FIGURE 5** Geometric means and reference ranges ( $\pm 2$  SD) for plasma ferritin at 4, 6 and 9 mo in unselected, iron-replete and iron-supplemented (Fe 4–9 and Fe 6–9) infants. The number of infants for each bar in the graph is 197, 134, 71, 83, 62, 35, 61, 50 and 47. Horizontal lines are the suggested  $-2$  SD cut-off values at 4 mo (20  $\mu\text{g/L}$ , solid line), 6 mo (9  $\mu\text{g/L}$ , dashed line) and 9 mo (5  $\mu\text{g/L}$ , dotted line).



**FIGURE 6** Means and reference ranges ( $\pm 2$  SD) for soluble transferrin receptors (TfR) at 4, 6 and 9 mo in unselected, iron-replete and iron-supplemented (Fe 4–9 and Fe 6–9) infants. The number of infants for each bar in the graph is 197, 134, 71, 83, 54, 38, 61, 50 and 47. Horizontal line is the suggested +2 SD cut-off value at 4–9 mo (11 mg/L).

the Hb response to iron, nor did birth weight or postnatal weight gain (not shown). ROC analyses were not performed for the response level of  $>15$  g/L because there were only 4 responders out of 56 iron-supplemented infants.

Response for 6–9 mo. Similarly, the Hb response from 6 to 9 mo was studied in iron-supplemented infants (Fe 6–9 group) after exclusion of noncompliers and cases with any missing iron status variables. The proportions of Honduran and Swedish infants showing Hb responses of different magnitudes are shown in Table 2. ROC analyses performed for the two lower response levels showed that Hb, MCV and ZPP at 6 mo, as well as birth weight and postnatal weight gain, were significant predictors of the Hb response, whereas ferritin and TfR at 6 mo were not (Table 3). When cut-off values with specificities of  $>90\%$  were determined for these variables, maximal sensitivities were all low; the highest were for Hb (cut-off value  $<108$  g/L, specificity 92%, sensitivity 56%) and ZPP (cut-off value  $>59$   $\mu\text{mol/mol}$  heme, specificity 92%, sensitivity 60%) for the response level of  $>5$  g/L. ROC analyses were not performed for the response level of  $>15$  g/L because there were only 2 responders out of 63.

Due to the site difference in MCV, ROC curves were

constructed separately for Honduran infants. In Honduran infants, MCV at 6 mo was not a significant predictor of the Hb response at any level. ROC curves for Swedish infants were not constructed because there were only 3 Hb responders ( $>5$  g/L) at this site. When ROC curves for Hb and ZPP were constructed using only the Honduran subpopulation and the Hb response level of  $>5$  g/L, the AUC (not shown) were significant ( $P = 0.003$  and  $0.009$ , respectively).

## DISCUSSION

This is the first study reporting reference values for iron status variables in exclusively breast-fed infants at 4 and 6 mo of age. The results have widespread implications because exclusive breast-feeding is generally recommended during the first 6 mo of life (16,17). The sample size in this study is smaller than in most other studies on reference values for other age groups, which would imply relatively wide confidence intervals around the suggested cut-off values. However, the advantage of our dataset is the strict definition of breast-feeding, the presence of an iron-supplemented group of infants as well as an unsupplemented group, and the wide range in iron status resulting from the two-country design, all of which are essential for evaluating iron status cut-off values during infancy.

As expected, reference values at 6 and 9 mo were different depending on the approach used. The “unselected” approach may theoretically include individuals with ID and thus yield lower reference values (e.g., for Hb). On the other hand, the iron supplementation approach may include infants who have absorbed excessive amounts of iron, resulting in “supernormal” iron status and higher reference values (Fig. 2). This would be supported by our recent observation that iron absorption in infants at 6 mo of age did not decrease with higher serum ferritin concentrations or previous iron supplementation (23). The “true” normative population would thus have reference values somewhere in between the unselected and the iron-supplemented populations. Theoretically, this would apply to the iron-replete normative population because this approach would not include any individuals with ID, nor would it be biased by excessive iron intake. Therefore, we suggest that “iron-replete” infants best represent healthy infants. Suggested 2 SD cut-off values for iron-replete infants at 4, 6 and 9 mo are presented in Table 4. These were close to the 2 SD cut-off values of the iron-supplemented infants for all variables except ferritin, for which iron-supplemented infants would yield considerably higher cut-off values. This does not necessarily mean

**TABLE 2**

Proportions of iron-supplemented Honduran and Swedish infants with a hemoglobin (Hb) response to iron  $>5$ ,  $>10$  or  $>15$  g/L

|             | 4–6 mo <sup>1</sup> |          |        |                       | 6–9 mo <sup>2</sup> |          |        |                       |
|-------------|---------------------|----------|--------|-----------------------|---------------------|----------|--------|-----------------------|
|             | All                 | Honduras | Sweden | <i>P</i> <sup>3</sup> | All                 | Honduras | Sweden | <i>P</i> <sup>3</sup> |
| <i>n</i>    | 56                  | 31       | 25     |                       | 63                  | 35       | 28     |                       |
|             |                     | %        |        |                       |                     | %        |        |                       |
| Hb response |                     |          |        |                       |                     |          |        |                       |
| $>5$ g/L    | 48                  | 45       | 52     | 0.788                 | 40                  | 63       | 11     | $<0.001$              |
| $>10$ g/L   | 21                  | 16       | 28     | 0.338                 | 19                  | 34       | 0      | 0.001                 |
| $>15$ g/L   | 7                   | 10       | 4      | 0.620                 | 3                   | 6        | 0      | 0.498                 |

<sup>1</sup> Infants iron supplemented from 4 to 6 mo (Fe 4–9 group).

<sup>2</sup> Infants iron supplemented from 6 to 9 mo (Fe 6–9 group).

<sup>3</sup> *P*-value for difference between sites (Fisher's exact test).

**TABLE 3**

ROC analyses of the performance of different variables in predicting infants' hemoglobin (Hb) response to iron at 6–9 mo<sup>1</sup>

| Variable                 | Hb response >5 g/L <sup>2</sup> |        | Hb response >10 g/L <sup>3</sup> |       |
|--------------------------|---------------------------------|--------|----------------------------------|-------|
|                          | AUC                             | P      | AUC                              | P     |
| Hb <sup>4</sup>          | 0.875                           | <0.001 | 0.779                            | 0.003 |
| MCV <sup>4</sup>         | 0.765                           | <0.001 | 0.724                            | 0.016 |
| ZPP <sup>4</sup>         | 0.797                           | <0.001 | 0.772                            | 0.004 |
| Ferritin <sup>4</sup>    | 0.609                           | 0.144  | 0.542                            | 0.649 |
| TfR <sup>4</sup>         | 0.547                           | 0.527  | 0.551                            | 0.587 |
| Birth weight             | 0.701                           | 0.007  | 0.714                            | 0.022 |
| Weight gain <sup>5</sup> | 0.693                           | 0.010  | 0.683                            | 0.050 |

<sup>1</sup> ROC, receiver operating characteristics; AUC, area under the curve; MCV, erythrocyte mean cell volume; ZPP, zinc protoporphyrin; TfR, soluble transferrin receptors.

<sup>2</sup>  $n = 63$  (25 responders).

<sup>3</sup>  $n = 63$  (12 responders).

<sup>4</sup> At 6 mo of age.

<sup>5</sup> Weight gain from birth to 6 mo, expressed as percent of birth weight.

that iron-supplemented infants have a more "normal" ferritin than iron-replete infants. Because iron absorption in infants cannot be down-regulated effectively (23) and because plasma ferritin is not homeostatically regulated, it is not surprising that iron-supplemented infants had higher ferritin concentrations.

For Hb, our suggested  $-2$  SD cut-off values of 105–100 g/L (Table 4) are lower than the commonly used cut-off value of 110 g/L at 6–12 mo of age. Our results are supported by a study of 1175 unselected British 8-mo-old infants whose mean  $\pm$  SD Hb was  $117 \pm 11$  g/L, giving a  $-2$  SD cut-off value of 95 g/L (14). This is close to our suggested level of 100 g/L at 9 mo, but our level may be more appropriate because we excluded infants with possible ID.

For MCV, our suggested  $-2$  SD cut-off values of 73–71 fL (Table 4), based on Swedish infants, are similar to those reported in a study of Finnish infants fed iron-fortified formula ( $n = 52$ –56 after excluding individuals with ferritin  $<10$   $\mu$ g/L or transferrin saturation  $<10\%$ ) whose  $-2$  SD cut-off value was 76 at 4 mo and 68–70 at 6–9 mo (20). However, SD were larger than in our study, suggesting that some of these infants may have been iron deficient. The difference in MCV between Honduran and Swedish infants may be due to genetic or environmental factors that we have not identified. A methodological problem cannot be completely excluded because MCV was the only variable that was analyzed with different methods at the two sites (18). We are not aware of any previous reports on geographical variations in MCV, but regardless of the reason, this may be important to consider in future studies.

For ZPP, our suggested  $+2$  SD cut-off values of 75–90  $\mu$ mol/mol heme (Table 4) are similar to the cut-off value of 80  $\mu$ mol/mol heme suggested for children of various ages (24).

For ferritin, our suggested  $-2$  SD cut-off value of 5  $\mu$ g/L at 9 mo (Table 4) is considerably lower than the commonly used cut-off values of 10–12  $\mu$ g/L. In a study of Finnish infants fed iron-fortified formula ( $n = 36$ –43 after excluding individuals with Hb  $<110$  g/L, MCV  $<71$  fL or transferrin saturation  $<10\%$ ), the  $-2$  SD cut-off values were 37, 19 and 14  $\mu$ g/L at 4,

6 and 9 mo, respectively (25). These values are higher than ours, probably due to the iron fortification.

For TfR, our suggested  $+2$  SD cut-off value of 11 mg/L (Table 4) is similar to the  $+2$  SD cut-off value of 11.1 mg/L previously reported for 1-y-old infants, using the same assay (26). In a study of 144 blood samples from Chilean infants between the ages of 8 and 15 mo, the corresponding cut-off value was 12.9 mg/L using a different TfR assay (27). Unfortunately, different assays for TfR do not show absolute comparability (28).

We recently found evidence of sex differences in iron status in infants (29). The number of infants in the current study is too small to allow for accurate calculation of separate reference values for boys and girls, but there was no sex difference in mean Hb in iron-replete infants ( $P = 0.907$ ).

Our results suggest that the Hb response to iron given from 4 to 6 mo of age is not a relevant criterion for ID. For all three response levels (5, 10 and 15 g/L), a larger proportion of responders was observed at 4–6 mo than at 6–9 mo. If the Hb response were a valid criterion for IDA at this age, this outcome would be unlikely because ID can be assumed to increase with time in these breast-fed infants. Furthermore, for the two response levels for which ROC curves could be constructed, no iron status variable at 4 mo (not even Hb itself) was predictive of the Hb response, nor were birth weight or postnatal growth rate, even though these growth variables are associated with risk of ID (30). This is supported by our observation that there was no correlation between the corrected Hb response and initial Hb at 4–6 mo.

One possible explanation for these results may be the persistence of fetal Hb (HbF) production. After birth, the production of HbF is gradually switched to adult Hb (HbA), but at 4 mo of age,  $> 5\%$  of the newly synthesized Hb is still HbF (31). HbF synthesis may not be regulated in the same way as HbA. We have also suggested that the regulation of intestinal iron absorption is not yet mature at 4–6 mo of age (18,23).

In contrast to the results found in the younger age group, the Hb response ( $> 5$  or  $>10$  g/L) to iron given from 6 to 9 mo of age may be a relevant criterion for IDA because low Hb, low MCV and high ZPP at 6 mo as well as low birth weight and large postnatal weight gain were all predictive of the Hb response. The magnitude of the Hb response likely reflects the initial degree of ID. Because very few infants had a response of  $>15$  g/L, and only a single infant had a response of  $>20$  g/L, these two higher response levels were not useful for statistical purposes, but we conclude from this that severe IDA was rare in our study population.

The most useful predictors of the Hb response from 6 to 9 mo were Hb and ZPP at 6 mo. However, when specificity was

**TABLE 4**

Suggested 2 SD cut-off values for iron status variables at 4, 6 and 9 mo of age, based on iron-replete, breast-fed infants<sup>1</sup>

|                         | 4 mo | 6 mo | 9 mo |
|-------------------------|------|------|------|
| Hb, g/L                 | <105 | <105 | <100 |
| MCV, fL <sup>2</sup>    | <73  | <71  | <71  |
| ZPP, $\mu$ mol/mol heme | >75  | >75  | >90  |
| Ferritin, $\mu$ g/L     | <20  | <9   | <5   |
| TfR, mg/L               | >11  | >11  | >11  |

<sup>1</sup> Hb, hemoglobin; MCV, erythrocyte mean cell volume; ZPP, zinc protoporphyrin; TfR, soluble transferrin receptors.

<sup>2</sup> Based on Swedish infants.

set to >90%, sensitivity was low for all variables, even for Hb (cut-off value <108 g/L, specificity 92%, sensitivity 56%). This suggests that there is a considerable overlap between the distribution of Hb in anemic (as defined by the Hb response) and nonanemic (as defined by lack of Hb response) infants. Different combinations of variables did not perform better than individual variables in predicting the Hb response to iron (data not shown).

Table 3 shows that the erythrocyte variables (Hb, MCV and ZPP) were significant predictors of the Hb response at 6–9 mo, whereas the two plasma variables (ferritin and TfR) were not. Assuming that the Hb response is a valid definition of IDA at this age, this suggests either that ferritin and TfR at 6 mo are poor measures of iron stores or, alternatively, that the size of iron stores in the growing 6-mo-old infant is poorly related to subsequent Hb synthesis. In the unselected, un-supplemented population, TfR did not increase until 9 mo, which may suggest that TfR (and maybe ferritin) are not useful for diagnosis of ID until that age.

It was not surprising to find that growth variables were useful predictors of IDA at 6 mo, as defined by the Hb response to iron. Because weight measurements are much less expensive than laboratory analyses, this information may be useful for targeting interventions.

We conclude that there is need to reevaluate the laboratory criteria for ID and IDA in infants. Furthermore, our results strongly suggest that the Hb response to iron is not useful as a criterion of ID in infants <6 mo of age, at least not in infants without severe anemia. This is an important observation, which should lead to a more cautious interpretation of Hb outcome in any study in which iron supplementation is given to infants <6 mo of age. Even more important is to clarify the relationship between laboratory criteria for ID and IDA and clinical outcomes, such as impaired neurological development in infants, which should form the basis for the disease definitions.

## ACKNOWLEDGMENTS

We thank the following persons for their dedicated field work and help with blood samplings and on-site laboratory analyses: Margareta Bäckman and Margareta Henriksson in Umeå and Leonardo Landa Rivera and the field research team in San Pedro Sula. We also thank Shannon Kelleher and Michael Crane in Davis for laboratory analyses, Jan Peerson (Davis) and Hans Stenlund (Umeå) for statistical advice and Angie Lee-Ow (Davis) for making the placebo solution. We also express our gratitude to Karin Dahllöf, HemoCue AB, Sweden for supplying equipment, Robert A. Burns, Mead Johnson for providing the iron drops and Catharina Tennefors, Semper AB, Sweden for providing food data tables.

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