Iron deficiency in childhood
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The value of Ret-Hb and sTfR in the diagnosis of iron depletion in healthy, young children

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ABSTRACT

Introduction
Reticulocyte hemoglobin content (Ret-Hb) and soluble transferrin receptor (sTfR) are described as promising biomarkers in the analysis of iron status. However, the value of Ret-Hb and sTfR in the early detection of iron depletion, as frequently observed in children in high-income countries is unclear. We hypothesized that young children with iron depletion, using the WHO cut-off of ferritin <12 μg/L, would have lower Ret-Hb and higher sTfR compared to children with a ferritin ≥12 μg/L.

Methods
In this cross-sectional study, we analyzed mean concentrations of Ret-Hb and sTfR in 351 healthy children aged 0.5 to 3 years in a high-income country. A Student’s t-test was used to compare Ret-Hb and sTfR concentrations between groups.

Results
We showed that concentrations of Ret-Hb and sTfR are similar in children with and without iron depletion. A decrease in Ret-Hb concentrations was present only when ferritin concentrations were <8 μg/L. sTfR concentrations were similar in children with ferritin concentrations <6 μg/L and ≥12 μg/L.

Conclusions
Our results showed that the discriminative value of Ret-Hb and sTfR for the detection of iron depletion is limited. Our findings suggest that ferritin is the most useful biomarker in the screening of iron depletion in healthy children in high-income countries. However, ideally, reference ranges of iron status biomarkers should be based on studies showing that children with concentrations outside reference ranges have poor neurodevelopmental outcomes.
INTRODUCTION

Young children are at risk of iron deficiency (ID) because of rapid growth with expanding erythroid mass and subsequent high iron requirements. ID occurs in progressive stages, beginning with the depletion of iron stores, the development of iron-deficient erythropoiesis (IDE) and, finally, iron-deficiency anemia (IDA). Iron is essential for neurodevelopment and IDA in infancy is associated with adverse effects on motor, cognitive and social-emotional development later in life. Whether these negative neurodevelopmental effects also apply to ID without anemia is questionable, but there is certain evidence supporting that possibility. Early detection of iron depletion is therefore essential for optimal development.

Ferritin is the most specific biomarker of iron depletion since ferritin concentrations are in direct proportion to body iron stores. Commonly used criteria to define iron depletion in infants younger than 5 years of age are ferritin concentrations <10 μg/L or <12 μg/L. However, the acute-phase response induced by infection or inflammation can elevate ferritin independent of iron stores. Furthermore, once iron stores are depleted, ferritin does not quantitatively reflect further reduction of tissue iron pool. Considering these limitations of ferritin, many studies used additional biomarkers of iron status and defined ID as two or more abnormal concentrations in a set of multiple biomarkers. However, there is no consensus on the best combination of biomarkers and reference ranges of most biomarkers are poorly defined.

Reticulocyte hemoglobin content (Ret-Hb) and soluble transferrin receptor (sTfR) are described as promising biomarkers in the analysis of iron status. Ret-Hb reflects the Hb content in reticulocytes. In adults it provides a real-time view of iron availability for erythropoiesis. sTfR is expressed by iron-requiring cells and reflects cellular iron demands and erythropoietic activity. In populations with a high prevalence of infection/inflammation and concomitant severe IDA, sTfR is less affected by an acute-phase response than ferritin, and provides more information on the extent of iron deficiency once iron stores are depleted.

The value of Ret-Hb and sTfR in the detection of iron depletion, as frequently observed in children in high-income countries with a low prevalence of severe IDA, is still unclear. We hypothesized that children with a ferritin <12 μg/L would have lower Ret-Hb and higher sTfR compared to children with a ferritin ≥12 μg/L. We therefore measured Ret-Hb and sTfR along with other iron status indicators in healthy young children in a high-income country, to establish the extent to which iron stores must be depleted to cause a significant change in Ret-Hb.
and sTfR. We also analyzed the associations between ferritin and Ret-Hb, sTfR, mean corpuscular volume (MCV) and hemoglobin (Hb).

Subjects and methods
Data were obtained from children who participated in an observational study on iron status in the Netherlands between August 2011 and May 2012. Healthy children aged 0.5 – 3 years undergoing general anesthesia because of simple elective surgery or diagnostic procedure, were included in the study. During preoperative screening, an anesthesiologist or an experienced pediatric resident performed an extensive medical history and physical examination according to a standard assessment protocol, in order to include only healthy children. Exclusion criteria were known infection in the last four weeks or an elevated C-reactive protein (CRP) >5 mg/L, use of iron supplementation within the last six weeks, blood transfusion within the last six months, preterm birth before 32 weeks gestational age (GA), known hemoglobinopathies, oncologic disorders, multiple congenital malformations, and metabolic diseases. Demographic characteristics and dietary data are described elsewhere in detail. Concisely, our study population was representative for the Dutch population considering socioeconomic status and educational level. Ethnicity of our study population was representative for the multiethnic population living in the urbanized, Southwestern region of the Netherlands. The study was approved by the Medical Ethics Committee of South-West Holland. All parents of the participating children gave written informed consent.

Study design
All children received a peripheral venous catheter to administer anesthetics. During insertion of the catheter, venous blood was collected with Ethylene Diamine Triacetic Acid (EDTA) and analyzed for Hb, MCV and Ret-Hb. Samples were centrifuged and plasma was aliquoted and frozen at -80°C until measurement of C-reactive protein (CRP), ferritin and sTfR. CRP was measured to detect an infection, which usually is accompanied by an increase in ferritin. Children with an elevated CRP (>5 mg/L) were excluded from the study. We did not include the measurement of serum iron and transferrin saturation in our study design because of their pronounced diurnal variation and reduced specificity with regard to ID. We defined iron depletion as a ferritin <12 μg/L and IDA as a ferritin <12 μg/L in combination with a Hb <110 g/L (<6.8 mmol/L). When iron depletion was established, children were treated with iron supplementation (oral ferrous fumarate 2.9 mg/kg/day iron). After two months of treatment, a control blood sample was taken. If iron depletion persisted after
iron supplementation or anemia persisted with a normal iron status, other causes of iron depletion and/or anemia were investigated. Children who were found to have other causes of iron depletion or anemia were excluded from the study.

**Biochemical analysis of biomarkers**
Hb, MCV and Ret-Hb were determined using Sysmex XE-2100 or XE 5000 (Sysmex Corporation, Kobe, Japan) automated hematology analyzers. Ferritin was determined using a Unicel Dxi 800 immunochemistry analyzer (Beckman Coulter, Fullerton, CA, USA). STfR was determined by ELISA (Ramco, Houston, TX, USA). CRP was determined using the Unicel DxC 800 clinical chemistry analyzer (Beckman Coulter, Fullerton, CA, USA) or Modular P (F. Hoffmann-La Roche Ltd, Basel, Switzerland).

**Statistical analysis**
The original study aim was to establish the prevalence of ID in healthy young infants, and to identify risk factors for ID in the Netherlands. The sample size of the study was based on prevalence of ID in other European countries (7.2%)\(^1\), and an interim analysis was proposed after half of the children were included. Using this data in a power-analysis, a number of 800 children would be sufficient to have 95% confidence limits of ± 2%. Results of the planned interim analysis conducted after inclusion of 400 children showed a 2.5-fold higher prevalence of ID than expected, and therefore we reached sufficient power to answer the study’s aim. SPSS (version 18.0; SPSS Inc., Chicago, IL) was used for statistical analysis. Before analysis, data were checked for normality using histograms and Kolmogorov-Smirnov test. Because ferritin and sTfR were skewed, these values were log transformed for all statistical calculations. Both biomarkers were normally distributed on a logarithmic scale. For interpretation, the values were converted back to the original units as geometric means and standard deviations (SD). Median and 2.5\(^{th}\) and 97.5\(^{th}\) percentiles (P2.5 and P97.5) were calculated from original untransformed values\(^1\).

To analyze the extent to which iron stores must be depleted to cause a significant decrease in the iron available for erythropoiesis or cellular iron content, mean concentrations of Ret-Hb, sTfR, MCV and Hb were analyzed in children with a ferritin concentration ≥12 μg/L, <12 μg/L, <10 μg/L, <8 μg/L, and <6 μg/L. A Student’s t-test was used for comparison of means between groups. Associations between ferritin and Ret-Hb, sTfR, MCV, and Hb were analyzed by calculation of the Pearson's correlation coefficient. Statistical significance was defined as \(P < 0.05\).
RESULTS

The study included 400 children. Six children with underlying causes for anemia, and 43 children with elevated CRP concentrations (>5 mg/L) were excluded. Iron depletion and IDA were present in 66 (18.8%) and 30 (8.5%) of the 351 remaining children respectively. The prevalence of iron depletion and IDA, and mean concentrations of iron status indicators were similar in boys and girls (data not shown). Ret-Hb and sTfR were available in 286 and 350 children, respectively. Mean (SD) and median (P2.5 – P97.5) concentrations of ferritin, Hb, MCV, Ret-Hb, sTfR are shown in Table 1. We found no differences in concentrations of Ret-Hb and sTfR between children aged 0.5 to 1, 1 and 2 years respectively (data not shown).

Table 1: Mean (SD) and median (P2.5 – P97.5) concentrations of ferritin (μg/L), MCV (fL), Ret-Hb (pg), Hb (g/L), and sTfR (mg/L) in 351 healthy children aged 0.5 to 3 years

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
<th>Median</th>
<th>P2.5 – P97.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferritin (μg/L)</td>
<td>19.2</td>
<td>1.7</td>
<td>19.0</td>
<td>5.7 – 56.8</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>76.9</td>
<td>3.7</td>
<td>77.0</td>
<td>68.8 – 84.0</td>
</tr>
<tr>
<td>Ret-Hb (pg)</td>
<td>29.1</td>
<td>2.7</td>
<td>29.5</td>
<td>22.7 – 33.7</td>
</tr>
<tr>
<td>Hb (g/L)</td>
<td>110.2</td>
<td>9.5</td>
<td>111.2</td>
<td>91.5 – 128.9</td>
</tr>
<tr>
<td>sTfR (mg/L)</td>
<td>5.6</td>
<td>1.5</td>
<td>5.5</td>
<td>3.0 – 15.2</td>
</tr>
</tbody>
</table>

*Geometric mean (SD) concentrations of ferritin and sTfR

Abbreviations: Hb – hemoglobin, MCV – mean corpuscular volume, Ret-Hb – reticulocyte hemoglobin content, sTfR – soluble transferrin receptor.

We found no significant differences in mean concentrations of Ret-Hb, sTfR, MCV and Hb between children with a ferritin ≥12 μg/L and <12 μg/L (Table 2). Compared with children with ferritin ≥12 μg/L, mean MCV was significantly lower in children with a ferritin concentration of <10 μg/L (mean difference 1.8 (95% CI 0.6-3.0), P<0.001), mean Ret-Hb was significantly lower in children with a ferritin concentration <8 μg/L (mean difference 0.03 (95% CI 0.01-0.17) P 0.03) and mean Hb was significantly lower in children with a ferritin concentration <6 μg/L (mean difference 8.1 (95% CI 1.9-14.3) P<0.01) (Table 2). Although there was a trend towards higher sTfR concentrations in children with a ferritin concentration <6 μg/L (Table 2), the difference compared to children with a ferritin concentration ≥12 μg/L was not statistically different (P 0.20).
Mean Ret-Hb was significantly lower in children with IDA compared to those without IDA (28.1 pg (SD 2.9) versus 29.2 pg (SD 2.7), P 0.04). However, no differences were observed in mean concentrations of MCV or sTfR between children with and those without IDA (data not shown).

**Table 2:** Mean (SD) of MCV (fL), Ret-Hb (pg), Hb (g/L) and sTfR (mg/L) concentrations in children with ferritin concentrations ≥12 μg/L, <12 μg/L, <10 μg/L, <8 μg/L, and <6 μg/L, respectively.

<table>
<thead>
<tr>
<th>Ferritin</th>
<th>MCV (fL)</th>
<th>Ret-Hb (pg)</th>
<th>Hb (g/L)</th>
<th>sTfR (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥12 μg/L</td>
<td>77.0 (3.4)</td>
<td>29.2 (2.7)</td>
<td>110.2 (9.3)</td>
<td>5.6 (1.4)</td>
</tr>
<tr>
<td>n=285</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;12 μg/L</td>
<td>76.1 (4.6)</td>
<td>29.0 (2.5)</td>
<td>110.5 (10.5)</td>
<td>5.7 (1.5)</td>
</tr>
<tr>
<td>n=66</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;10 μg/L</td>
<td>75.2 (4.9)</td>
<td>28.4 (2.3)</td>
<td>110.8 (10.7)</td>
<td>6.2 (1.5)</td>
</tr>
<tr>
<td>n=40</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;8 μg/L</td>
<td>74.7 (5.4)</td>
<td>27.8 (2.5)</td>
<td>108.6 (11.3)</td>
<td>6.5 (1.7)</td>
</tr>
<tr>
<td>n=19</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;6 μg/L</td>
<td>74.0 (3.8)</td>
<td>26.7 (1.7)</td>
<td>102.1 (10.3)</td>
<td>6.7 (1.4)</td>
</tr>
<tr>
<td>n=9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Significantly different from ferritin concentrations ≥12 at level 0.05. † Significantly different from ferritin concentrations ≥12 at level 0.01. * Abbreviations: Hb – hemoglobin, MCV – mean corpuscular volume, Ret-Hb – reticulocyte hemoglobin content, sTfR – soluble transferrin receptor.

In children with ferritin concentrations <12 μg/L, ferritin was positively associated with Ret-Hb, MCV and Hb and a negative association was found between ferritin and sTfR (table 3). In children with ferritin concentrations ≥12 μg/L no associations were found between ferritin and other iron status indicators (data not shown).

**Table 3:** Results of Pearson’s correlations between ferritin (μg/L), MCV (fL), Ret-Hb (pg), Hb (g/L) and sTfR (mg/L) in 66 children aged 0.5 – 3 years with iron depletion (ferritin <12 μg/L)

<table>
<thead>
<tr>
<th>Ferritin (μg/L)</th>
<th>MCV (fL)</th>
<th>Ret-Hb (pg)</th>
<th>Hb (g/L)</th>
<th>sTfR (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferritin (μg/L)</td>
<td></td>
<td>0.320*</td>
<td>0.417*</td>
<td>0.262*</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>0.635*</td>
<td></td>
<td>0.218</td>
<td>-0.245</td>
</tr>
<tr>
<td>Ret-Hb (pg)</td>
<td>0.350*</td>
<td></td>
<td></td>
<td>-0.297*</td>
</tr>
<tr>
<td>Hb (g/L)</td>
<td></td>
<td></td>
<td>0.016</td>
<td></td>
</tr>
</tbody>
</table>

* Significant at level 0.05. † Significant at level 0.01.

Abbreviations: Hb – hemoglobin, MCV – mean corpuscular volume, Ret-Hb – reticulocyte hemoglobin content, sTfR – soluble transferrin receptor.
DISCUSSION

In this study we showed that mean concentrations of Ret-Hb, sTfR, MCV and Hb remain unchanged in children with ferritin concentrations <12 μg/L. A decrease in Ret-Hb concentrations was present only when ferritin concentrations were <8 μg/L, and no change was observed in sTfR concentrations in children with different ferritin concentrations. Our results indicate that the discriminative value of Ret-Hb and sTfR for the detection iron depletion in young healthy children is limited. Moreover, these results suggest that a lower cutoff value for ferritin might be more appropriate to define ID in healthy children aged 0.5 – 3 years.

Reference ranges for iron status indicators have been poorly defined in infants and young children. The WHO describes ID ‘a condition in which there are no mobilizable iron stores and in which signs of a compromised supply of iron to tissues, including the erythron, are noted’. As we observed no significant changes in Ret-Hb, sTfR, MCV and Hb in children with ferritin concentrations <12 μg/L, our results suggest that a lower cutoff value for ferritin may be more appropriate. We observed an effect on iron available for erythropoiesis only when ferritin was <10 μg/L, suggesting this might be a more suitable cutoff for these children. Different reference ranges have been suggested for Ret-Hb. By using a cut-off value of 25 pg, 26 pg and 27.5 pg, ID would be present in 18 (6.3%), 36 (12.6%) and 62 (21.7%) children in our study, respectively. Reported reference ranges for sTfR in studies using the same assay, varied from 11 mg/L in infants aged 6 – 12 months to 8.3 mg/L in adults. Using these reference ranges, increased sTfR concentrations would be present in 15 (4.3%) and 45 (12.9%) children, respectively. However, we observed no differences in ferritin concentrations between children with decreased Ret-Hb or increased sTfR concentrations, and those with ‘normal’ concentrations according to these suggested reference ranges.

As reticulocytes have a life span of 24 – 48 hours, Ret-Hb provides a real-time view of bone marrow iron status as has been shown in adults. It decreases within days after onset of iron-deficient erythropoiesis. Although studies in children have suggested that Ret-Hb might be a more sensitive indicator of ID than Hb, its value in diagnosing iron depletion remains unclear. In some of these studies transferrin saturations <20%, <12% or <10% were used to define ID, respectively. However, transferrin saturation shows a substantial diurnal variation and is less specific to diagnose ID than ferritin. As ferritin concentrations are directly proportional to body iron stores, it is thought to...
be the most specific biomarker of ID. Using ferritin as an indicator of iron stores, this study shows that Ret-Hb is more sensitive than Hb, but less sensitive than MCV to detect iron depletion. Similar results were described in a recent study in adult blood donors that used low ferritin concentration as an indicator of iron depletion. This study showed that Ret-Hb was no better than MCV in the detection of iron depletion, and suggested that Ret-Hb is best at identifying more severe iron-deficient erythropoiesis and IDA32.

In the present study, we showed that the sensitivity of sTfR to detect iron depletion was poor. These results are in accordance with other studies, suggesting that sTfR remains unchanged in iron depletion and begins to change only when tissue iron deficiency develops33,34. The joint World Health Organization/Centers for Disease Control and Prevention (WHO/CDC) concluded that measurement of both ferritin and sTfR provides the best approach for estimating the iron status in populations9. The sTfR might be valuable in the diagnosis of ID in populations with a high prevalence of infection/inflammation and concomitant severe IDA, as sTfR is less affected by an acute-phase response than ferritin, and provides information on the extent of ID once iron stores are depleted21. Actually, several studies have shown a negative association between sTfR and ferritin in populations with a high prevalence of severe IDA16,21,28,35. However, in populations with a low prevalence of severe IDA, such as our population, the value of sTfR in the diagnosis of iron depletion in populations seems to be limited17,36.

There is no consensus on the diagnostic criteria for ID and various iron status indicators have been used to define ID. However, our results illustrate that different iron status indicators represent different aspects of iron status. The diagnostic value of these biomarkers differs between populations, depending on the severity of iron depletion and the presence of concomitant infection or inflammation. This should be considered when deciding which biomarkers to use in the assessment of iron status.

Ideally, iron status should be measured with as less biomarkers as possible, that provide as much as possible information, in order to minimize the required amount of blood and money. By defining ID as two or more abnormal concentrations in a set of multiple iron status indicators, only children with severe, longstanding IDA will be classified as ID, even if Ret-Hb and sTfR are add to the set of biomarkers. The use of this method in a population with a low prevalence of severe IDA and concomitant infection or inflammation is therefore questionable. We suggest that, in the absence of infection or inflammation, ferritin remains
the most useful biomarker in the screening of iron depletion in healthy children in high-income countries with a low prevalence of severe IDA.

This study shows that ferritin concentrations in young, healthy children were not associated with Ret-Hb, sTfR, MCV and Hb unless iron stores are severely depleted. Studies in adults, have suggested that ferritin is the first biomarker to be affected in iron depletion. With persisting insufficient iron supply, iron appears to be preferentially channeled to erythropoiesis while iron stores become gradually reduced. Only after depletion of iron stores does the iron available for erythropoiesis becomes a limiting factor, explaining the association between ferritin concentrations and Ret-Hb, sTfR, MCV and Hb that we have found only in children with ferritin concentrations <12 μg/L. However, these associations seem to be of a limited clinical relevance since concentrations of Ret-Hb, sTfR, MCV and Hb are affected only in severe, long-standing IDA, which is uncommon in the Netherlands.

The strength of this study is that we analyzed the value of multiple iron status indicators in the diagnosis of iron depletion, in a well-defined population of healthy children in a high-income country. Although mild depletion of iron stores was frequently observed, severe and longstanding IDA was uncommon in this population. Consequently, the number of children with ferritin concentrations <8 μg/L and <6 μg/L was small, which limits the statistical power to detect differences between these groups. A limitation of the study is its cross-sectional design that does not enable to study the sequence of changes of different iron status indicators responding to changes in iron stores. However, longitudinal analysis of the effect of increasing severity of iron depletion on iron status parameters is unethical considering the negative developmental effects of ID that have been described in children.

**CONCLUSION**

In this study, we showed that concentrations of Ret-Hb and sTfR are similar in children with and without iron depletion, using the WHO cut-off of ferritin <12 μg/L. Our results showed that the discriminative value of Ret-Hb and sTfR in the detection of depleted iron stores in children aged 0.5 to 3 years is limited. Ferritin was not associated with MCV, Ret-Hb, Hb and sTfR unless iron stores were depleted. We suggest that ferritin is the most useful biomarker in the screening of iron depletion in healthy children in high-income countries with a low prevalence.
of severe IDA. As our data showed an effect on iron available for erythropoiesis only when ferritin is <10 μg/L, this might be a more appropriate cutoff to define ID in healthy children aged 0.5 – 3 years. However, ideally, reference ranges of ID and IDA should be based on studies showing that children with biomarkers outside reference ranges have poor neurodevelopmental outcomes and more follow-up studies on developmental outcomes in children with low iron stores without anemia are therefore urgently needed in order to better define criteria for ID in young children.

**REFERENCE LIST**


