Reference Ranges of Reticulocyte Haemoglobin Content in Preterm and Term Infants: A Retrospective Analysis

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Key Words
Iron deficiency · Premature infant · Reticulocyte haemoglobin content · Gestational age-specific reference ranges

Abstract

Background: Despite iron supplementation, some preterm infants develop iron deficiency (ID). The optimal iron status parameter for early detection of ID has yet to be determined. Objective: To establish reference ranges for reticulocyte haemoglobin content (Ret-He) in preterm and term infants and to identify confounding factors. Methods: Retrospective analyses of Ret-He and complete blood count in infants with a clinically indicated blood sample obtained within 24 h after birth. Results: Mean (SD) Ret-He was 30.7 (3.0) pg in very preterm infants with a gestational age (GA) of <30 weeks (n = 55), 31.2 (2.6) pg in moderately preterm infants (GA 30–36 weeks, n = 241) and 32.0 (3.2) pg in term infants (GA ≥37 weeks, n = 216). The 2.5th percentile of Ret-He across all GA groups was 25 pg, with a weak correlation between Ret-He and GA (r = 0.18). Moreover, only weak/no correlations were found between Ret-He and C-reactive protein (r = 0.18), interleukin 6 (IL-6) (r = 0.03) and umbilical artery pH (r = -0.07). There was a slight variation in Ret-He with mode of delivery [normal vaginal delivery: 32.3 (3.2) pg, secondary caesarean section (CS): 31.4 (3.0) pg, instrumental delivery: 31.3 (2.7) pg and elective CS: 31.2 (2.8) pg]. Conclusion: GA at birth has a negligible impact on Ret-He, and the lower limit of the normal reference range in newborns within 24 h after birth can be set to 25 pg. Moreover, Ret-He seems to be a robust parameter which is not influenced by perinatal factors within the first 24 h after birth.

Background

Iron deficiency (ID) in preterm infants is caused by low iron stores at birth [1], high postnatal growth velocity [2] and uncompensated iatrogenic blood and hence iron loss [3]. To prevent ID [4], it is common practice to start early [5] enteral iron supplementation in low-birthweight infants [6]. Long-term, yet underpowered outcome data following a randomized controlled trial of enteral iron supplementation suggested impaired neurological outcome if iron supplementation is started late [7]. Despite iron supplementation, some preterm infants...
still develop ID and early detection of ID would be ideal. Several iron status parameters have already been investigated [8], but the majority are either confounded by the physiological shift from foetal to adult erythropoiesis or by inflammatory conditions [1] limiting their applicability during neonatal intensive care. Furthermore, measurements of most iron status parameters require a substantial amount of blood, which further aggravates the ID. Reticulocyte haemoglobin content, however, which is routinely generated during reticulocyte count by the majority of haematological analysers without additional blood loss or relevant financial costs, seems to hold promise. Unlike erythrocytes, reticulocytes have a shorter lifespan and thus their haemoglobin content better reflects current iron availability for haematopoiesis [9, 10].

The aim of this study was to establish gestational age (GA)-specific reference ranges for reticulocyte haemoglobin content in preterm and term infants and to identify any possible confounding factors.

Methods

Study Population

This retrospective analysis was carried out at a tertiary hospital evaluating laboratory data obtained as part of routine admission procedures. Exclusion criteria were haematological diseases, congenital anomalies or the lack of a complete blood count (CBC) within 24 h after birth. At our institution, institutional review board approval and informed consent are not required for retrospective analyses of anonymised data.

Venous blood samples (0.5 ml) were drawn for analysis of CBC, reticulocyte parameters, C-reactive protein (CRP) and interleukin 6 (IL-6). GA was determined by obstetrical assignment. Perinatal data, i.e. birth weight and length, head circumference and umbilical artery pH, was obtained from a neonatal database.

Laboratory Methods

Reticulocyte haemoglobin content, defined as reticulocyte haemoglobin equivalent (Ret-He hereafter), were measured with a Sysmex XE-2100 (Sysmex GmbH, Norderstedt, Germany) haematological analyser. Normoblasts (nucleated red blood cells) are detected by fluorescent flow cytometry after membrane lysis of red blood cells (Sysmex XE-2100). The Reticulocyte Production Index (RPI), assessing the appropriate production of reticulocytes as a bone marrow response to anaemia [11], was calculated as previously described [12]. High-sensitivity wide-range CRP and IL-6 were measured with ADVIA1200 clinical chemistry analyser and Immulite 2000 XPI immunoanalyzer (Siemens, Eschborn, Germany). For IL-6 measurements, aliquots were diluted 1:4. Detection limits were <0.01 mg/dl for CRP and <16 ng/l for IL-6. CRP and IL-6 values below these limits were assigned values of 0.01 and 1, respectively, for subsequent statistical analyses.

Results

In total, 1,257 infants were admitted to the neonatal intensive and special care unit between December 2012 and January 2014; 745 were excluded from the study (fig. 1), leaving 512 with a GA of between 23 +5/7 and 42 +0/7 weeks for further analyses. Demographic data and haematological parameters are shown in table 1.

Two hundred and ninety-six infants (57.5%) were male; there were 375 (73.2%) singletons, 116 (22.7%) twins and 21 (4.1%) triplets. One hundred and fifteen infants (22.3%) were delivered via normal vaginal delivery (NVD), 55 (10.7%) via instrumental vaginal delivery (vacuum or forceps), 179 (34.8%) via elective caesarean section (ECS), and 162 (31.4%) via secondary CS (SCS). In 4 infants (0.8%), the mode of delivery was not documented.

Haematological Parameters and GA

There was a weak positive correlation between GA and Ret-He (r = 0.18, n = 512, p < 0.001; fig. 2) and a statistically significant difference in Ret-He across the 3 groups; GA <30 weeks (n = 55): mean (SD) Ret-He 30.7 (3.0) pg, GA 30–36 weeks (n = 241): Ret-He 31.2 (2.6) pg and GA ≥37 weeks (n = 216): Ret-He 32.0 (3.2) pg; p < 0.005. There was no significant difference in Ret-He between very preterm and moderately preterm infants (p = 0.51), but there was between moderately preterm and term infants (p < 0.05). Percentiles of Ret-He are shown in table 2.

There were weak positive correlations between GA and mean corpuscular haemoglobin concentration (MCHC; r = 0.22, n = 511, p < 0.0001) and red blood cell
Infants admitted to neonatal intensive and special care unit (n = 1,257)  
Excluded (n = 745)  
• No CBC on Sysmex within 24 h (n = 430)  
• More than 1 admission (n = 200)  
• Cardiac anomalies (n = 27)  
• Gastrointestinal anomalies (n = 21)  
• Intraperitoneal blood loss (n = 17)  
• Syndromal or chromosomal anomalies (n = 16)  
• Cerebral anomalies (n = 9)  
• Urogenital anomalies (n = 7)  
• Pulmonary anomalies (n = 6)  
• Skeletal anomalies (n = 5)  
• Haematological disorders (n = 3)  
• Metabolic disorders (n = 3)  
• Congenital viral infection (n = 1)  

Analysed (n = 512)

Table 1. Demographic data and haematological parameters of 512 infants

<table>
<thead>
<tr>
<th>Demographic data</th>
<th>GA &lt;30 weeks (n = 55)</th>
<th>GA 30–36 weeks (n = 241)</th>
<th>GA ≥37 weeks (n = 216)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA, weeks</td>
<td>28 2/7 (26 2/7–29 5/7)</td>
<td>34 2/7 (32 2/7–35 5/7)</td>
<td>39 2/7 (38 2/7–40 5/7)</td>
</tr>
<tr>
<td>Birth weight, g</td>
<td>964 (316)</td>
<td>2,030 (500)</td>
<td>3,250 (532)</td>
</tr>
<tr>
<td>SDS_BW</td>
<td>−0.9 (1.3)</td>
<td>−0.5 (1.1)</td>
<td>−0.3 (1.0)</td>
</tr>
<tr>
<td>Length, cm</td>
<td>35.0 (4.2)</td>
<td>43.8 (3.4)</td>
<td>50.4 (2.6)</td>
</tr>
<tr>
<td>HC, cm</td>
<td>24.9 (2.6)</td>
<td>30.9 (2.3)</td>
<td>34.7 (1.6)</td>
</tr>
<tr>
<td>Umbilical artery pH</td>
<td>7.3 (0.1)</td>
<td>7.3 (0.1)</td>
<td>7.2 (0.1)</td>
</tr>
</tbody>
</table>

Haematological data

<table>
<thead>
<tr>
<th></th>
<th>Hb, mg/dl</th>
<th>Hct, %</th>
<th>RBC count, ×10^6/μl</th>
<th>MCV, fl</th>
<th>MCH, pg</th>
<th>MCHC, g/dl</th>
<th>RETI, ×10^3/μl</th>
<th>Ret-He, pg</th>
<th>RPI, %</th>
<th>Normoblasts, ×10^3/μl</th>
<th>CRP, mg/dl</th>
<th>IL-6, ng/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA &lt;30 weeks</td>
<td>16.0 (2.5)</td>
<td>51.4 (7.7)</td>
<td>4.2 (0.6)</td>
<td>122.9 (9.5)</td>
<td>38.2 (2.7)</td>
<td>31.1 (1.5)</td>
<td>271.7 (83.4)</td>
<td>30.7 (3.0)</td>
<td>1.3 (0.7)</td>
<td>2.0 (1.0–3.9)</td>
<td>0.01 (0.01–0.01)</td>
<td>1 (1–22.0)</td>
</tr>
<tr>
<td>GA 30–36 weeks</td>
<td>17.2 (2.0)</td>
<td>53.1 (6.3)</td>
<td>4.7 (0.5)</td>
<td>112.5 (7.6)</td>
<td>36.3 (2.1)</td>
<td>32.3 (1.4)</td>
<td>228.0 (60.7)</td>
<td>31.2 (2.6)</td>
<td>1.1 (0.5)</td>
<td>1.2 (0.6–2.0)</td>
<td>0.01 (0.01–0.01)</td>
<td>1 (1–23.2)</td>
</tr>
<tr>
<td>GA ≥37 weeks</td>
<td>17.0 (2.3)</td>
<td>52.4 (6.7)</td>
<td>4.9 (0.7)</td>
<td>107.2 (5.5)</td>
<td>34.8 (1.6)</td>
<td>32.5 (1.5)</td>
<td>178.3 (39.6)</td>
<td>32.0 (3.2)</td>
<td>0.8 (0.5)</td>
<td>0.7 (0.2–1.7)</td>
<td>0.01 (0.01–0.02)</td>
<td>22.4 (1–63.2)</td>
</tr>
</tbody>
</table>

Values are expressed as mean (SD) or median (IQR). GA = Gestational age; SDS_bw = standard deviation scores for birth weight; HC = head circumference; Hb = haemoglobin; Hct = haematocrit; RBC = red blood cells; MCV = mean corpuscular volume (Hct/RBC); MCH = mean corpuscular haemoglobin (Hb/RBC); MCHC = mean corpuscular haemoglobin concentration (MCH/MCV); RETI = absolute reticulocyte count; Ret-He = reticulocyte haemoglobin content; RPI = reticulocyte production index; CRP = C-reactive protein; IL-6 = interleukin 6.

Table 2. Reference ranges of Ret-He in venous blood samples shortly after birth

<table>
<thead>
<tr>
<th>GA, weeks</th>
<th>n</th>
<th>Ret-He, pg (by percentile)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5th</td>
<td>10th</td>
</tr>
<tr>
<td>24–29</td>
<td>55</td>
<td>21.1</td>
</tr>
<tr>
<td>30–36</td>
<td>241</td>
<td>25.1</td>
</tr>
<tr>
<td>37–42</td>
<td>216</td>
<td>25.5</td>
</tr>
<tr>
<td>24–42</td>
<td>512</td>
<td>25.2</td>
</tr>
</tbody>
</table>

Fig. 1. Flow chart of infants included in and excluded from the study.
counts ($r = 0.26, n = 512, p < 0.0001$). Moreover, there were negative correlations between GA and mean corpuscular volume (MCV; $r = -0.56, n = 512, p < 0.0001$), mean corpuscular haemoglobin (MCH; $r = -0.48, n = 511, p < 0.0001$), normoblasts ($r = -0.34, n = 512, p < 0.0001$), reticulocytes ($r = -0.53, n = 512, p < 0.0001$) and RPI ($r = -0.38, n = 199, p < 0.0001$). No correlation was found between GA and haemoglobin ($r = 0.03, n = 511, p = 0.42$).

### Ret-He Compared with Other Haematological Parameters

A strong positive correlation between Ret-He and MCHC ($r = 0.61, n = 511, p < 0.0001$; fig. 3a) and weak positive correlations between Ret-He and haemoglobin ($r = 0.20, n = 511, p < 0.0001$) and MCH ($r = 0.24, n = 511, p < 0.0001$; fig. 3b) were observed. Moreover, there were weak negative correlations between Ret-He and MCV ($r = -0.21, n = 512, p < 0.0001$; fig. 3c) and normoblasts ($r = -0.23, n = 512, p < 0.0001$) and no correlation between Ret-He and reticulocytes ($r = 0.04, n = 512, p = 0.42$).

### Potential Perinatal Confounders

There was no difference in Ret-He (mean difference 0.02 pg, 95% CI –0.5 to 0.5, $p = 0.98$) between males [mean (SD) 31.5 (3.1) pg, $n = 296$] and females [31.5 (2.8) pg, $n = 219$], or between singletons [31.6 (3.1) pg], twins [31.2 (1.7) pg] and triplets [31.2 (3.1) pg] ($p = 0.38$).

A significant variation was observed in mean Ret-He across the 4 modes of delivery; NVD [mean (SD) 32.3 (3.2) pg], SCS [31.4 (3.0) pg], instrumental delivery [31.3 (2.7) pg] and ECS [31.2 (2.8) pg], $p < 0.05$. However, multiple comparison analysis showed a significant difference only between NVD and ECS (mean difference = 1.1 pg, 95% CI 0.4 to 1.7, $p < 0.05$).

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Fig. 2. Correlation between GA at birth and Ret-He in 512 preterm and term infants.

Fig. 3. Correlation between Ret-He and MCHC (a), MCH (b) and MCV(c).
A weak correlation was found between Ret-He and CRP \((r = 0.18, n = 477, p < 0.001)\), but not between Ret-He and IL-6 \((r = 0.03, n = 454, p = 0.58)\) or umbilical artery pH \((r = -0.07, n = 502, p = 0.08)\). There was also no difference in Ret-He (mean difference −0.4 pg, 95% CI −1.3 to 0.6, \(p = 0.44\)) between SGA infants [mean (SD) 31.2 (3.0) pg, \(n = 42\)] and non-SGA infants [31.5 (3.0) pg, \(n = 470, p = 0.44\)] and no correlation between Ret-He and SDS\(_{BW}\) \((r = 0.07, n = 512, p = 0.09)\).

**Discussion**

In this study, we aimed to provide reference ranges for Ret-He in term and preterm infants.

Previously reported reticulocyte haemoglobin content values vary between 31.7 and 35.7 pg (mean/median) and between 25.2 and 33.1 pg for the 25th percentile \([16–19]\). This variability might be caused by variations in the proportion of very preterm infants, time of sampling, sample size and device used. Term infants seem to have higher values \([19, 20]\) than preterm infants \([16]\), although our results suggest that the influence of GA may be subordinate. Furthermore, Ret-He drops within the first days after birth \([17, 18]\). Therefore, cord blood values are higher \([20]\) than values in very early postnatal samples (day of birth \([18]\)) or more delayed samples (within 24 h, as in this study or 1.9–4.9 days \([19]\)). Some researchers also report data on only 26 infants \([16]\), whereas others determine reference intervals based on 6,632 blood samples obtained in 9 laboratories \([18]\). Finally, Ret-He using ADVIA \([17, 20]\) seems to be somewhat higher than that using Sysmex (this study and \([16, 18, 19]\)), despite the fact that other studies showed a good agreement between the 2 devices in paediatric and adult patients \([21, 22]\).

In this study, Ret-He was measured using Sysmex XE-2100, which has been replaced by the newer-generation Sysmex XN. For generalizability of our results, we compared both devices using a different set of blood samples and found an excellent agreement for Ret-He (Spearman’s correlation \(r = 0.91, p < 0.0001, n = 50\)). Our results are comparable with unpublished data from a larger cohort \((n = 304)\) previously reported to the Food and Drug Administration by the manufacturer. The reproducibility of Ret-He measurements is accurate (coefficient variation ≤5% if reticulocytes are >20,000/μl) However, if reticulocyte counts are very low, Ret-He may become less reproducible (manufacturer information).

The perinatal transition from foetal to adult haemoglobin is a genetically programmed and post-conceptional age-dependent switch \([23]\) which greatly influences MCV and MCH. Therefore, GA-specific reference ranges are essential when determining MCV and MCH in preterm infants \([24]\). Moreover, erythropoietic activity decreases with increasing GA, affecting normoblasts, reticulocyte counts and RPI, making these parameters difficult to use perinatally too. In contrast, we observed only a weak correlation between GA and Ret-He. Even though there was a statistically significant overall variation in Ret-He between preterm and term infants, the differences were very small and hardly of clinical relevance, as also shown by others \([16]\). In addition, Ret-He is not confounded by gender, perinatal stress (using umbilical artery pH as a surrogate parameter) or infection/inflammation (assessed by IL-6 level). Even though mode of delivery and CRP resulted in statistically significant differences in Ret-He, these differences also seemed to be clinically negligible. In contrast, other iron parameters such as hepcidin or ferritin are more vulnerable to perinatal confounding variables \([15]\) or infection \([25]\).

It is known that ferritin is lower in SGA infants \([26]\) than in non-SGA infants. However, similar to previous studies \([16]\), we did not find a significant difference in Ret-He between SGA and non-SGA infants.

Our study has some limitations. Firstly, it was a retrospective study performed in a single centre. The quantity of analysed blood samples \((n = 512)\) is a strength, but the sample size of the most immature infants (<30 weeks) was relatively small \((n = 55)\). Secondly, reference ranges in hospitalised infants can always be biased by other pathological conditions leading to hospitalisation (or preterm birth). To get as close as possible to a healthy study population, we excluded infants with known congenital or perinatal anomalies and tried to account for the most common confounding factors. Finally, we used blood samples drawn within 24 h of birth, which are not affected by postnatal iron supplementation, transfusion or blood loss.

Ret-He is a promising parameter for detecting early ID in preterm infants, since it requires less blood than other iron status parameters and seems to be only marginally affected by GA, perinatal stress and inflammation – at least within the first 24 h after birth. However, assessing its diagnostic accuracy is a challenge because a gold standard for ID is missing. Although we provide reliable neonatal reference ranges for the use of Ret-He within 24 h of birth, more liberal Ret-He threshold values could be considered since iron supplementation does not seem to have adverse effects in preterm infants \([7]\).
Ret-He seems to be a robust haematological parameter in preterm and term infants, only marginally affected by common confounding factors within the first 24 h after birth. In a clinical setting, reference ranges for Ret-He do not need to be adjusted for GA. Further studies are warranted to evaluate its use in the diagnosis of ID, which could lead to individualised iron supplementation and prevent ID anaemia and long-term neurocognitive deficits in preterm infants.

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Acknowledgements

The authors thank Sarah Bauer for data collection and Dr. Ingo Rettig for help with interpretation of the laboratory data. The work was supported by an AKF grant No. 283-0-0 from the Faculty of Medicine, University of Tübingen and a Research Fellowship from the German Research Society for Dr. Laila Lorenz (DFG No. LO 2162/1-1).

Disclosure Statement

There were no competing interests.