Haemoglobin and red blood cell reference intervals during infancy

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ABSTRACT

Objectives There is a need for updated haematological reference data in infancy. This study aimed to define intervals for haemoglobin and red blood cell biomarkers based on data from a large cohort of longitudinally followed Swedish infants.

Design Longitudinal cohort study.

Setting Two Swedish study centres.

Participants Three community-based populations including 442 presumably healthy infants born at term and with umbilical cord clamping delayed to 30s or more after birth.

Methods Blood samples were collected from umbilical cord blood (a), at 48–118 hours (b), at 4 months (c) and at 12 months (d). Reference intervals as the 2.5th and 97.5th percentiles were calculated in coherence with Clinical and Laboratory Standards Institute guidelines.

Results Reference intervals for haemoglobin (g/L) were: (a) 116–189, (b) 147–218, (c) 99–130, (d) 104–134, and for mean cell volume (fL): (a) 71–85, (c) 71–85, (d) 70–83. Reference intervals for erythrocyte counts, reticulocyte counts, reticulocyte haemoglobin, mean cell haemoglobin and mean cell haemoglobin concentration were also estimated. According to the WHO definition of anaemia, a haemoglobin value less than 110 g/L, 16% of this presumably healthy cohort could be classified as anaemic at 12 months.

Conclusion We found mainly narrower reference intervals compared with previously published studies. The reference intervals for each parameter varied according to the infants’ age, demonstrating the necessity of age definitions when presenting infant reference intervals. The discrepancy with the WHO classification for anaemia at 12 months, despite favourable conditions in infancy, needs future investigation.

INTRODUCTION

Childhood anaemia is a global health problem that affects young children in both low-income and high-income countries and is associated with cognitive deficits and suboptimal development. The currently used threshold for anaemia, as defined by the WHO, originates from studies published more than 60 years ago and has remained.1 2 When the initial recommendations were published, the WHO reported that the threshold for anaemia, a haemoglobin (Hb) level below 110 g/L, was rather arbitrarily chosen. Data were mainly based on a study that included 96 infants, 6 months old, published in 1959.3 However, later studies indicate that the level defined as anaemia, that is, an Hb value below the 2.5th percentile, is lower than the currently recommended WHO threshold.4–7 This important knowledge gap needs further investigation. But, creating reference data in average childhood populations is associated with ethical and practical difficulties. Recently, Zierk et al added important insights to the continuous changes in haematology reference intervals observed during the rapid development after birth. Their calculations were based on laboratory information systems in 10 German centres and included data from 358 292 paediatric patients.5 The researchers were unable to compare the results from their new hospital data-based approach, with modern reference limits based on established methodology from healthy infant data.

The timing of umbilical cord clamping affects Hb levels during the first months of life. During the last decade, the preventive effects of delaying umbilical cord clamping have again received increased attention as a method for preventing infant anaemia. It is well known that delayed clamping is associated with an increase in blood volume by placental fusion,8 promoting more favourable haematological...
conditions in infancy. Also, other infant characteristics may also need to be considered, since especially the indices mean cell volume (MCV) and mean cell Hb concentration (MCHC) are influenced by an infant’s gestational age at birth. The influence of the timing of cord clamping on reference limits is rarely accounted for in infant studies, although in the study on which the WHO recommendation was mainly based, it was noted that the umbilical cord was not clamped 'until all pulsations had ceased'.

The difficulties in establishing high-quality paediatric reference intervals have been recognised by the International Federation of Clinical Chemistry. The technical development of analytical instruments changes their performance and reference intervals need to be methodology specific. Infant reference intervals have been difficult to establish due to the rapid physiological changes. Reference interval quality is also affected by sensitivity to pre-analytical conditions and the need for prompt transportation to the laboratory.

Infant reference intervals present a challenge and the importance of establishing accurate intervals specific also to the neonatal period has recently been underlined. Therefore, the aim of our study was to define new reference intervals for red blood cell (RBC) biomarkers at birth (umbilical cord), postnatal age 48–118 hours, 4 months and 12 months, based on data from a large well-defined cohort of longitudinally followed and presumably healthy Swedish infants.

### MATERIALS AND METHODS

#### Study population

All infants were born after uncomplicated pregnancies and uneventful perinatal circumstances with a gestational age of 37+0–41+6 weeks. Eligibility criteria of the mothers were non-smoking and healthy (no haemolytic disease, no treatment with any of the following drugs; anticonvulsants, antidepressants, thyroid hormone, insulin, chemotheraphy, cortisone) and normal term, singleton pregnancy (no pre-eclampsia, no diabetes, no prolonged rupture of membranes or sign of infection). Exclusion criteria were serious congenital malformations, syndromes or other congenital diseases. Data on reported illness, medication, parity, weight, height, body mass index, smoking habits, blood group Rhesus factor status and Hb concentration at the time of admission to antenatal care were collected from healthcare records. Data comprised of in total three studies assessing effects of timing of umbilical cord clamping and were collected between 2008 and 2015. The two first studies, from the County Hospital of Halland in Sweden, included children born in vaginal births (n=400) and children born by elective caesarean section (n=64). Data from the Halland cohorts were combined with a study population of infants born in vaginal births (n=200) at Karolinska University Hospital in Huddinge, Stockholm, Sweden. Data from children with umbilical cord clamping <30s were excluded, as were results with corresponding C-reactive protein (CRP) >10 mg/L.

#### Table 1: Study population characteristics

| Time point | Clinical information | Number of results and exclusions | Growth data (g cm) mean (min–max) | Ferritin concentrations (µg/L) median (min–max) |
|------------|----------------------|----------------------------------|-----------------------------------|-----------------------------------------------|
| 1–6 hours  | Assessed at 1 hour after birth and 6 hours after birth by the midwife. Record of feeding and respiratory symptoms. | Umbilical cord clamping ≥30s, N=442 Excluded due to CRP >10 mg/L, N=4 Excluded from reference interval calculations due to corresponding CRP result missing, N=8 Reference interval calculations based on N=430 individuals (boys N=205, girls N=225) | Birth weight: 3616 (2335–5420) Karolinska 3603 (2450–4950) Halland 3620 (2335–5420) Birth length: 51 (45–57) Karolinska 51 (45–56) Halland 51 (46–57) | 202 (25–1046) |
| 72 hours   | Examination by physician in accordance with clinical routines. | | | |
| 48–118 hours | Blood sampling in connection to metabolic screening. | Umbilical cord clamping ≥30s, N=252 Excluded from reference interval calculations due to corresponding CRP result missing, N=49 Results missing due to sampling or instrumental issues (eg, clot or similar), N=13 Reference interval calculations based on N=152 individuals (boys N=66, girls N=86) | | 312 (33–1151) Data from County Hospital of Halland cohort only |
| Follow-up visit 4 months | Blood sampling, weight and length measurement. Median number of days from birth: 122 (109–154) days. | | | 112 (16–880) |
| Follow-up visit 12 months (County Hospital of Halland cohort only) | Blood sampling, weight and length measurement. Median number of days from birth: 363 (350–396) days. | Results from blood sampling of umbilical cord with clamping ≥30s, N=252 Excluded from reference interval calculations due to CRP >10 mg/L, N=23 Results missing due to sampling or instrumental issues (eg, clot or similar), N=3 1 result missing reticulocyte data | Weight: 10098 (8130–13 600) Length: 76 (70–83) | 34 (8–281) |

CRP: C-reactive protein.
protein (CRP) >10 mg/L, or lacking CRP result. Study population characteristics are listed in table 1.

Specimen collection and handling
Samples (County Hospital of Halland) were collected from umbilical cord blood and by venipuncture at metabolic screening (48–118 hours), at 4 months and at 12 months. Blood was collected in EDTA tubes and in tubes with serum separator (BD Vacutainer, Plymouth, UK).

Samples (Karolinska University Hospital) were taken at birth (umbilical cord blood), by venipuncture in conjunction with metabolic screening (48–118 hours) and at 4 months. Blood was collected in EDTA tubes and serum tubes with serum separator (Sarstedt AG & Co, Nümbrecht, Germany).

Before the venous blood sampling at 4 and 12 months, a local dermal analgesia with lidocaine 2.5% and prilocaine 2.5% (EMLA, AstraZeneca) was applied.

Laboratory analysis
The blood samples were analysed at the hospital laboratories within 2 hours after sampling. All blood samples from umbilical cord and at 4 months were analysed for: Hb, haematocrit, erythrocyte particle counts (RBC), reticulocyte particle counts (RET) and reticulocyte Hb equivalent (Ret-He), while the indices MCV and MCHC were calculated by the instrument.

At sampling time point 48–118 hours, analysis schemes differed between the study sites. In County Hospital of Halland, all the listed analyses were performed, while at Karolinska University Hospital, only Hb and haematocrit were analysed. RBC biomarkers were analysed on Sysmex instruments (Sysmex, Kobe, Japan), in Halland model XE-2100 and at Karolinska University Hospital model XE-5000. CRP was analysed on the Cobas 6000 instrument platform (Roche Diagnostics, Basel, Switzerland), in Halland. Both laboratories were accredited.

Calculations and statistical analysis
The 2.5th and 97.5th percentiles were calculated using a non-parametric method with a 90% CI at the lower and upper values, as recommended by the Clinical and Laboratory Standards Institute (CLSI) guidelines. 14 Statistical analyses were conducted using Analyse-it for Microsoft Excel V.4.90.4 from Analyse-it Software (Leeds, UK). No exclusions based on post-analysis assessment of haematological or iron status data were made. Reference interval widths were calculated by subtraction of the 2.5th percentile from the 97.5th percentile. Non-overlapping CIs were considered statistically significantly different.

The group of infants with Hb <110 g/L and the group with Hb ≥110 g/L were compared at 12 months regarding iron status parameters: ferritin, iron, transferrin and soluble transferrin receptor (sTfR) and CRP as a measure of acute phase response. The Mann-Whitney U test was used. The concurrent measurement of these iron status parameters in the cohort has previously been described. 20–23

RESULTS
The 2.5th percentiles and 97.5th percentiles, with 90% CIs for the RBC biomarkers Hb, haematocrit, MCV, RBC, MCH, MCHC, RET and Ret-He from this cohort of presumably healthy Swedish infants are presented in table 3. The total data population is visualised by bee-swarm box-whisker plots in figure 1. The percentage of infants with corresponding cord clamping times can be found in table 3.

In brief, the Hb concentration was higher at 48–118 hours than in the umbilical cord. It was followed by a decline until 4 months. A similar pattern was observed for haematocrit, MCHC and RBC. The high MCV observed in the umbilical cord sample decreased continuously during the first year.

Between 4 and 12 months, there was a minor increase in Hb and haematocrit for both lower and upper reference limits. For MCH, the lower reference limit was constant, while a very slight decrease was observed for the upper reference limit. MCHC decreased significantly for the lower as well as the upper reference limit, as indicated by non-overlapping CIs.

The red cell biomarkers showed decreasing reference interval widths with increasing infant age. At 12 months, the reference interval widths of Hb and haematocrit were approximately half of that at birth.

The RET decreased rapidly during the first 4 months and thereafter the upper level remained constant while there was a small, but non-significant, decrease of the lower reference limit. Regarding Ret-He, both the 2.5th and the 97.5th percentiles showed a decreasing trend from birth (umbilical cord blood) to 4 months of age. Ret-He CIs were overlapping between 4 months and 12 months.

The iron status parameters ferritin, transferrin and sTfR at 12 months in infants with Hb <110 g/L did not differ significantly from infants with Hb ≥110 g/L (table 4). Yet, 12-month-old infants with Hb <110 g/L had significantly lower serum iron concentrations than infants with Hb ≥110 g/L. The groups did not differ with respect to CRP.

DISCUSSION
This Swedish community-based study reports reference intervals for RBC biomarkers in term-born infants at four different time points during the first year of life. We compared the results with other sources of reference intervals: studies including presumably healthy individuals, 24–30 studies from large-scale data mining, 31 and reference intervals listed in textbooks as shown in table 2.

Despite the seemingly ample amount of published studies, reference data from large, well-defined and presumably healthy infant populations are scarce. In a recent initiative, reference intervals for Swedish children >6 months of age with blood samples analysed on Siemens/Bayer ADVIA 2120 were presented, but data included only few individuals aged 6–12 months. 32 Interestingly, our results for 12-month-old infants are mainly in agreement with these data.

Several studies calculate reference intervals based on patient data from laboratory database searches. When comparing these studies with the tool of reference interval widths, to assess possible influence of different populations, 33 we found that these methodologies generally defined broader intervals than cohort studies. As healthy children are seldom subjected to blood sampling, data mining techniques risk inclusion of non-healthy subjects.

The third source of reference intervals is textbooks. Textbook reference intervals should be interpreted with care, as these might have been reprinted from one edition to the next and may not be transferable to results from modern analytical instruments. 34 For instance, in table 2, we include reference intervals from a haematology textbook, edition from 2017. These reference intervals did refer to a book section from 1977. 3 Also, textbook reference intervals were generally broader than intervals calculated in cohort studies.
### Table 2  Red cell biomarkers

| Sampling time point | Lower reference limit (90% CI) | Upper reference limit (90% CI) | RIW 95% | N | Comparison with other published data lower–upper value (RIW) |
|---------------------|---------------------------------|---------------------------------|---------|---|----------------------------------------------------------|
| **Hb (g/L)**        |                                 |                                 |         |   |                                                          |
| Umbilical cord      | 116 (115 to 121)                | 189 (184 to 202)                | 73      | 391 | 146–189 (43) | 135–195 (60) | 24 |
| 48–118 hours        | 147 (139 to 157)                | 218 (213 to 234)                | 71      | 152 | 145–225 (80) capillary | 125–210 (85) | 11 |
| 4 months            | 99 (97 to 101)                  | 130 (128 to 132)                | 31      | 352 | 103–141 (38) | 97–133 (36) | 5 |
| 12 months           | 104 (99 to 106)                 | 134 (131 to 139)                | 30      | 188 | 113–141 (28) | 107–134 (27) | 4 |
| Adult RI            | Women 117–153 (36), men 134–170 (36) |                                 |         |   |                                                          |
| **Haematocrit (L/L)** |                                 |                                 |         |   |                                                          |
| Umbilical cord      | 0.36 (0.35 to 0.37)             | 0.57 (0.56 to 0.59)             | 0.21    | 391 | 0.40–0.58 (0.14) | 0.42–0.60 (0.18) | 24 |
| 48–118 hours        | 0.42 (0.39 to 0.44)             | 0.62 (0.60 to 0.65)             | 0.20    | 152 | 0.45–0.67 (0.22) capillary | 0.35–0.60 (0.25) | 11 |
| 4 months            | 0.29 (0.28 to 0.29)             | 0.37 (0.37 to 0.38)             | 0.08    | 347 | 0.32–0.44 (0.12) | 0.28–0.40 (0.12) | 5 |
| 12 months           | 0.31 (0.30 to 0.31)             | 0.39 (0.39 to 0.41)             | 0.08    | 188 | 0.33–0.41 (0.08) | 0.30–0.39 (0.09) | 4 |
| Adult RI            | Women 0.35–0.46 (0.11), men 0.39–0.50 (0.11) |                                 |         |   |                                                          |
| **MCV (fL)**        |                                 |                                 |         |   |                                                          |
| Umbilical cord      | 97 (95 to 98)                   | 118 (117 to 120)                | 21      | 391 | 102–118 (16) | 98–118 (20) | 24 |
| 48–118 hours        | 91 (85 to 92)                   | 107 (105 to 115)                | 16      | 152 | 101–119 (18) | 91–116 (25) | 5 |
| 4 months            | 71 (69 to 72)                   | 85 (84 to 86)                   | 14      | 347 | 76–97 (21) | 70–90 (20) | 5 |
| 12 months           | 70 (69 to 71)                   | 83 (82 to 84)                   | 13      | 188 | 71–85 (14) | 72–85 (13) | 4 |
| Adult RI            | 82–98 (16) |                                 |         |   |                                                          |
| **MCHC (g/L)**      |                                 |                                 |         |   |                                                          |
| Umbilical cord      | 303 (300 to 310)                | 352 (350 to 354)                | 49      | 391 | 306–342 (36) | 300–360 (60) | 24 |
| 48–118 hours        | 343 (339 to 345)                | 372 (367 to 373)                | 29      | 152 | 305–378 (73) | 290–370 (80) capillary | 5 |
| 4 months            | 328 (327 to 331)                | 363 (360 to 365)                | 35      | 347 | 312–372 (60) | 300–360 (60) | 5 |
| 12 months           | 323 (323 to 326)                | 353 (352 to 355)                | 30      | 188 | 321–365 (44) | 330–380 (50) | 4 |
| Adult RI            | 317–357 (40) |                                 |         |   |                                                          |
| **MCH (pg)**        |                                 |                                 |         |   |                                                          |
| Umbilical cord      | 32 (31 to 33)                   | 39 (38 to 39)                   | 7       | 391 | 33–38 (5) | 31–37 (6) | 6 |

Continued
We found that in particular, MCHC had broad reference interval widths which can be explained by the fact that the MCHC parameter is especially sensitive for pre-analytics as well as analytical instrumentation.17

Broad reference interval widths do have implications in clinical practice as these may reduce diagnostic power and even small to modest overestimation may result in higher false negative rates.33

Of note is that reference intervals specifically for Retic-Hb, using data from the County Hospital of Halland cohort, have already been previously published,28 but with different reference interval methodology (a parametric approach). Since these calculations are less robust to population skewness, we did further analyses and harmonised calculations with the other RBC biomarkers. The slightly different values obtained with the parametric and non-parametric approach are presented in table 2.

### Table 2: Continued

| Sampling time point | Lower reference limit (90% CI) | Upper reference limit (90% CI) | RIW 95% | N  | Comparison with other published data lower–upper value (RIW) |
|---------------------|--------------------------------|--------------------------------|----------|----|-------------------------------------------------------------|
| 48–118 hours        | 32 (31 to 33)                  | 38 (37 to 40)                  | 6        | 152| 34–40 (6)                                                   |
|                     |                                |                                |          |    | 32–38 (6)                                                   |
|                     |                                |                                |          |    | 31–37 (6)                                                   |
| 4 months            | 24 (23 to 25)                  | 29 (29 to 30)                  | 5        | 347| 26–30 (4)                                                   |
|                     |                                |                                |          |    | 24–30 (6)                                                   |
|                     |                                |                                |          |    | 25–35 (10)                                                  |
| 12 months           | 23 (23 to 24)                  | 28 (28 to 29)                  | 5        | 188| 24–30 (6)                                                   |
|                     |                                |                                |          |    | 25–31 (6)                                                   |
|                     |                                |                                |          |    | 23–29 (6)                                                   |
|                     |                                |                                |          |    | 23–31 (8)                                                   |
| Adult RI            |                                |                                |          |    | 27–33 (6)                                                   |
| RET (×10⁹/L)        |                                |                                |          |    |                                                             |
| Umbilical cord      | 99 (91 to 106)                 | 240 (229 to 267)               | 141      | 391| 99–228 (129)                                                |
| 48–118 hours        | 79 (67 to 95)                  | 275 (250 to 300)               | 196      | 152| 24–124 (100)                                                |
|                     |                                |                                |          |    | 97–316 (219)                                                |
|                     |                                |                                |          |    | 218–419 (201)                                               |
| 4 months            | 20 (17 to 22)                  | 65 (62 to 68)                  | 45       | 347| 34–83 (49)                                                  |
|                     |                                |                                |          |    | 25–82 (57)                                                  |
| 12 months           | 18 (11 to 20)                  | 66 (59 to 95)                  | 48       | 187| 34–83 (49)                                                  |
|                     |                                |                                |          |    | 27–98 (71)                                                  |
|                     |                                |                                |          |    | 18–65 (47)                                                  |
| Adult RI            |                                |                                |          |    | 28–120 (92)                                                 |
| RBC (×10¹²/L)       |                                |                                |          |    |                                                             |
| Umbilical cord      | 3.4 (3.1 to 3.5)               | 5.4 (5.3 to 5.4)               | 2.0      | 391| 3.9–5.5 (1.6)                                               |
| 48–118 hours        | 4.2 (3.7 to 4.4)               | 6.2 (6.1 to 6.9)               | 2.0      | 152| 4.1–7.1 (3.0)                                               |
|                     |                                |                                |          |    | 3.9–6.2 (2.3)                                               |
|                     |                                |                                |          |    | 4.0–6.6 (2.6) capillary                                     |
| 4 months            | 3.6 (3.4 to 3.7)               | 4.9 (4.8 to 5.0)               | 1.3      | 347| 3.5–5.1 (1.6)                                               |
|                     |                                |                                |          |    | 3.3–5.0 (1.7)                                               |
|                     |                                |                                |          |    | 3.1–4.5 (1.4)                                               |
| 12 months           | 3.9 (3.9 to 4.0)               | 5.3 (5.1 to 5.4)               | 1.4      | 188| 4.1–5.3 (1.2)                                               |
|                     |                                |                                |          |    | 4.0–5.6 (1.6)                                               |
|                     |                                |                                |          |    | 3.9–5.2 (1.3)                                               |
|                     |                                |                                |          |    | 3.7–5.3 (1.6)                                               |
| Adult RI            |                                |                                |          |    | Women 3.9–5.2 (1.3), men 4.2–5.7 (1.5)                      |
| Ret-Hb (pg)         | Data for Ret-Hb for the County Hospital of Halland cohort have already been published\(^2\) but using different reference interval methodology. Compared with the previous study, we here use alternative exclusion criteria, include the Karolinska University Hospital cohort, harmonise calculations using robust statistical methodology and add calculations of 90% CIs to cohere with current guidelines. |
| Umbilical cord      | 28 (27 to 28)                  | 39 (38 to 40)                  | 11       | 388| 27–36 (9)                                                   |
| 48–118 hours        | 28 (25 to 29)                  | 38 (37 to 41)                  | 10       | 152| 31–37 (6)                                                   |
|                     |                                |                                |          |    | 28–38 (10)                                                  |
| 4 months            | 25 (22 to 26)                  | 34 (33 to 34)                  | 9        | 346| 27–32 (5)                                                   |
|                     |                                |                                |          |    | 26–33 (8)                                                   |
| 12 months           | 24 (21 to 25)                  | 33 (33 to 36)                  | 9        | 187| 26–33 (8)                                                   |
|                     |                                |                                |          |    | 25–34 (9)                                                   |
|                     |                                |                                |          |    | 28–33 (6)                                                   |
| Adult RI            |                                |                                |          |    | 28–35 (7)                                                   |

Lower and upper reference limits calculated as the 2.5th and 97.5th percentiles from N individuals. RIWs, reference limits published elsewhere and values for adults are shown for comparison.

Hb, haemoglobin; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; MCV, mean cell volume; RBC, red blood cell; RET, reticulocyte count; Ret-He, reticulocyte haemoglobin equivalent; RIW, reference interval width.
The major strengths of the present study are the number of children included, the longitudinal study design and the well-defined characteristics with blood sampling ages within precise ranges. The CLSI recommendation regarding number of observations for each group was met. We included information on gestational age, birth weight and timing of cord clamping—a combination that to the best of our knowledge has not been considered in previous infant reference interval studies using modern analytical instrumentation.

A limitation of our study is that the analysers, Sysmex XE-2100 and Sysmex XE-5000, have now been replaced by a new model, Sysmex XN. Even though published studies show excellent agreement for RBC biomarkers between XE-5000 and XN series, the transferability between the instrument lines should be verified at the local laboratories.

We did not perform post-analysis exclusions based on test results from iron status or RBC biomarkers in this study. A consensus on diagnostic exclusion criteria for iron deficiency in infants is lacking. Our concern was that arbitrary exclusion criteria would lead to subjectivity, and the risk of erroneously

| Table 3 Number of infants with corresponding time from birth to clamping |
|-----------------------------|------|
| Time from birth to clamping (s) | Infants |
| 30–59                        | 84 (19%) |
| 60–119                       | 192 (43%) |
| 120–179                      | 3 (1%) |
| >180                         | 163 (37%) |

| Table 4 Median concentration for the iron status biomarkers and CRP for the group of infants with Hb <110 g/L and the group with Hb ≥110 g/L at the age of 12 months |
|-----------------|------|------|
|                 | Hb <110 g/L | Hb ≥110 g/L | P value |
| Ferritin (µg/L) | 37   | 34   | 0.35  |
| Transferrin (g/L) | 2.6  | 2.7  | 0.08  |
| sTfR (mg/L)     | 4.3  | 4.3  | 0.37  |
| Iron (µmol/L)   | 8    | 10   | <0.001|
| CRP (mg/L)      | 1    | 1    | 0.32  |

Difference in distribution tested with Mann-Whitney U test. CRP, C-reactive protein; Hb, haemoglobin; sTfR, soluble transferrin receptor.
trimmed data. However, we applied criteria to ascertain that the infants would represent healthy children in a high-resource setting and with a clamping of the cord after ≥30s. Although we did not apply exclusion criteria for iron status biomarkers, our calculations resulted in reference interval widths that were mainly narrower than other published data.

The cut-off for timing of umbilical cord clamping (>30 s) was based on the American College of Obstetricians and Gynecologists committee opinion,² but the WHO recommendation is to delay cord clamping and cutting for 1–3 min for term infants.³ Most infants in our study (81%) had a delayed cord clamping time of ≥1 min.

It is reasonable to assume that the limited number of larger reference interval studies can have implications on what is considered as normative Hb concentration data. In our cohort, 32 of 195 (16%) of the infants at 12 months of age were classified as having mild anaemia with the WHO guidelines, with Hb below 110 g/L.⁴ A similar discordance to WHO classification is indicated by the 2.5th reference limits found in other studies listed in table 2. This might imply that the WHO threshold can lead to flagging of healthy infant analysis results. The lower reference limit for Hb at 4–12 months in this study is similar to that recommended by the ESPGHAN in this age range (105 g/L).¹⁶ As a group, the infants with Hb < 110 g/L did not have significantly lower iron stores measured as ferritin. Neither did they have upregulated iron need as estimated by transferrin and sTfR concentrations compared with the group with higher Hb. They did have lower serum iron levels and this observation may require further investigation, even though serum iron is usually not regarded as a reliable marker of iron status due to its strong diurnal variation. Future work needs to define Hb thresholds based on clinical needs.

In conclusion, we have defined RBC biomarker reference intervals at four time points during infancy. The reference interval widths are mainly narrower than previously reported for these biomarkers. Based on this comparably large longitudinal cohort study, we suggest that Hb thresholds for infant anaemia between the ages 6 months and 12 months are reevaluated.

### Acknowledgements

The authors thank the staff at the Department of Obstetrics and Gynaecology, Hospital of Halland, Halmstad; the Department of Paediatrics at Hospital of Halland, Halmstad and the delivery ward at Karolinska University Hospital, Huddinge. We also thank the staff at the Clinical Chemistry Departments. We are most grateful to the parents and infants who participated in the study.

**Contributors**

LH-W, MD, UA, CG and OA designed the original cord clamping studies. SML, AH and OA conceptualised this reference interval study. LH-W, MD, UA, CG and OA collected and curated the data. SML carried out the analyses and drafted the initial manuscript. LH-W, AH, OA, PKA, MD, UA and CG revised and reviewed the manuscript for important intellectual content. OA is the guarantor of the study.

**Funding**

This study was supported by grants from the Regional Scientific Council of Halland and funds for development and research from the Swedish Southern Healthcare Region.

**Competing interests**

None declared.

**Patient consent for publication**

Not required.

**Ethics approval**

The studies were approved by the regional research ethics committee at Lund University (41/2008, 344/2009) and by the regional ethical review board in Stockholm (2011/2/142-31/3).

**Provenance and peer review**

Not commissioned; externally peer reviewed.

**Data availability statement**

Data are available upon reasonable request. The data are not publicly available due to privacy or ethical reasons.

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