Hyper high haemoglobin content in red blood cells and erythropoietic transitions postnataely in infants of 22 to 26 weeks’ gestation: a prospective cohort study

Sara Marie Larsson 1,2, Tommy Ulinder1,3, Alexander Rakow4, Mireille Vanpee4, Dirk Wackernagel5,6, Karin Sävman7, Ingrid Hansen-Pupp1,3, Ann Hellström8, David Ley1,3, Ola Andersson1,3

ABSTRACT
Objective Blood cell populations, including red blood cells (RBC) unique to the extremely preterm (EPT) infant, are potentially lost due to frequent clinical blood sampling during neonatal intensive care. Currently, neonatal RBC population heterogeneity is not described by measurement of total haemoglobin or haematocrit. We therefore aimed to describe a subpopulation of large RBCs with hyper high haemoglobin content, >49 pg (Hyper-He) following EPT birth.

Design Prospective observational cohort study.

Setting Two Swedish study centres.

Participants Infants (n=62) born between gestational weeks 22+0 to 26+6.

Methods Prospective data (n=280) were collected from March 2020 to September 2022 as part of an ongoing randomised controlled trial. Blood was sampled from the umbilical cord, at postnatal day 1–14, 1 month, 40 weeks’ postmenstrual age and at 3 months’ corrected age.

Results At birth, there was a considerable inter-individual variation; Hyper-He ranging from 1.5% to 24.9% (median 7.0%). An inverse association with birth weight and gestational age was observed; Spearman’s rho (CI) −0.38 (−0.63 to −0.07) and −0.39 (−0.65 to −0.05), respectively. Overall, Hyper-He rapidly decreased, only 0.6%–5.0% (median 2.2%) remaining 2 weeks postnatally. Adult levels (<1%) were reached at corresponding term age.

Conclusion Our results point to gestational age and birth weight-dependent properties of the RBC population. Future work needs to verify results by different measurement techniques and elucidate the potential role of differing properties between endogenous and transfused RBCs in relation to neonatal morbidities during this important time frame of child development.

Trial registration number NCT04239690.

INTRODUCTION
Globally, preterm birth affects 15 million newborns each year.2 Although survival rates increase with modern neonatal care, major challenges remain, particularly for the most preterm born infants.3–4 During initial care, these infants have one of the highest transfusion requirements within hospital settings, as the majority develop anaemia that could compromise oxygenation of vital organs. The aetiology of this neonatal anaemia is multi-factorial, but explanatory models often address decreasing erythropoietin levels following transition from a relatively hypoxic intrauterine environment to more oxygen-rich conditions after birth.5–6 Also well-known is a considerable blood volume loss related to frequent clinical blood sampling resulting from the potential depletion of unique endogenous red blood cells (RBCs).7–9

In contrast to the RBCs from the adult definitive erythroid lineage, originating from bone marrow and haematopoietic stem cells (HSC), the liver is the main erythropoietic organ during the gestational period ascribed to extremely preterm (EPT) birth.10–12 Until recently, it was assumed that HSC drive fetal liver erythropoiesis already from 10th to 12th gestational weeks onwards.11 As new lineage-tracing experiments in murine models however suggest HSC-independent pathways to be present as well as the continuing contribution of erythromyeloid progenitors (EMP) to liver erythropoiesis...
during fetal life, these are results that may have implications also on the general understanding of human fetal and infant erythropoiesis.13 14

A gradual process where the HSC definitive erythroid lineage transitions to the more protected environment in the bone marrow is initiated at 16th to 18th week of gestation.15 During later gestational weeks, corresponding to the period of EPT birth, the actual extent of different possible erythropoietic contributions remains to be clarified.16–18

With potentially different physiological, microenvironmental and cellular origins, the resulting peripheral RBC population may not be homogeneous, potentially having different properties between subpopulations. The mean cell haemoglobin concentration-index (MCHC) after EPT birth is similar to adult values,19 but the RBC count expected at these gestational weeks is essentially lower.20 This implies that either each RBC transports more haemoglobin or that haemoglobin is unevenly distributed in heterogeneous subpopulations of red cells, such as large red cells with hyper high haemoglobin content (Hyper-He). The proportion of cells with a particularly high haemoglobin content in adults can be estimated based on the measurement of high-angle forward scatter on Sysmex XN haematology instruments (Hyper-He, >49 pg/cell). While Hyper-He is low in healthy adult blood, <1%,21 little is known about proportions in newborns.

Our objective of this study was to describe proportions of Hyper-He following EPT birth as a potential biomarker of an erythropoietic transitional process. We also aimed to investigate the influence of gestational age, birth weight and fetal growth on early proportions of Hyper-He.

METHODS
Study population
At birth, the infants had a gestational age of 22+0 to 26+6 weeks. Clinical data were obtained from clinical records. Fetal growth restriction (FGR) was diagnosed in cases with abnormal fetal Doppler velocimetry combined with estimated fetal weight deviation22 and a birth weight SD score below –2 SD calculated according to Niklasson and Albertsson-Wikland.23 Exclusion criteria was major malformations. Data were collected during March 2020 to September 2022 as part of an ongoing two-armed multicentre randomised controlled intervention trial. The aim of the main trial is to investigate the relationship between preservation of neonatal blood factors and neonatal morbidity using blood sampling micro-methods. Our ancillary observational cohort study was carried out at the neonatal intensive care units at the Southern Region University Hospital, Lund, and Karolinska University Hospital, Stockholm, in Sweden.

Specimen collection and handling
Blood samples were collected at birth (umbilical cord blood) and thereafter through an umbilical catheter, peripheral arterial catheter or peripheral venous puncture. During postnatal day 1–14, only blood from clinical sampling was used to analyse the RBC data presented in this study thereby not causing any additional blood loss, essential for the main study design. Blood (230 µL) was collected in microtainer EDTA tubes (BD Vacutainer, Plymouth, UK). In addition to the clinical samples, study samples were obtained at postnatal age 1 month (median age 27 days, IQR 26–29 days, min 21, max 41 days), at postmenstrual age week 40 and at 3 months corrected age. All blood samples were promptly sent to the hospital laboratory for analysis to avoid RBC swelling. Samples were analysed for Hyper-He (proportion of cells with haemoglobin >49 pg/cell), haemoglobin concentration (Hb), mean cell haemoglobin content (MCH), mean cell volume (MCV) and reticulocyte counts (RET).

Laboratory analysis
Blood was analysed on Sysmex XN (Sysmex, Kobe, Japan) at the clinical laboratories at Lund University Hospital and at Karolinska University Hospital. The instrument uses laser to measure forward and side scatter light on a cell-by-cell basis. Measurements of RBC count are based on impedance. Total haemoglobin concentration is measured by an absorption photometric method. The index MCH is derived by the ratio haemoglobin/RBC count and MCV by the ratio haematocrit/RBC count. The instruments were under regular quality control for commonly used haematological parameters and laboratories were accredited according to ISO15189.

Calculations and statistical analysis
Data are presented as mean (±SD), median (IQR), range (min – max) or number (%) where appropriate. Correlations were estimated using Spearman’s rho where p values <0.05 were considered statistically significant. SPSS V.27.0 (IBM, SPSS) was used for data analysis.

RESULTS
Study cohort
Blood measurements (n=280) were available from 62 of 73 infants enrolled in the randomised controlled trial at the two neonatal intensive care units during the present time period. The mean (SD) birth weight of the 62 infants was 727 g (171 g) and median gestational age at birth 24+4. Gestational ages and clinical characteristics are presented in table 1. Distribution in number of measurements, with regard to sampling time point and gestational ages, is shown in figure 1.

RBCs with Hyper-He during the first 14 postnatal days
Umbilical cord samples (n=13) were analysed. One sample with an outlier Hb result (57 g/L) was excluded due to suspected measurement interference. In the remaining samples (n=14), there was a large inter-individual variability in Hyper-He ranging from 1.5% to 24.9% (median 7.0%). Association between Hyper-He and RET in the umbilical cord blood was not statistically significant but pointed towards a possible weak inverse association, Spearman’s rho = −0.47 (CI −0.81 to 0.10), p<0.09. Overall, the median proportion of Hyper-He rapidly decreased with increasing postnatal age, at postnatal day 14 reaching 2.2% (0.6–5.0%) and by term age adult levels <1%. The corresponding total haemoglobin concentration in cord samples was

---

**Table 1** Participant characteristics

<table>
<thead>
<tr>
<th>Gestational age at birth (weeks+days)</th>
<th>Infants (% of total n=62)</th>
</tr>
</thead>
<tbody>
<tr>
<td>22+0 to 22+6</td>
<td>3 (5)</td>
</tr>
<tr>
<td>23+0 to 23+6</td>
<td>12 (19)</td>
</tr>
<tr>
<td>24+0 to 24+6</td>
<td>13 (21)</td>
</tr>
<tr>
<td>25+0 to 25+6</td>
<td>16 (26)</td>
</tr>
<tr>
<td>26+0 to 26+6</td>
<td>18 (29)</td>
</tr>
<tr>
<td>Antenatal steroid treatment</td>
<td>60 (97)</td>
</tr>
<tr>
<td>Preeclampsia</td>
<td>4 (6)</td>
</tr>
<tr>
<td>Chorioamnionitis</td>
<td>17 (27)</td>
</tr>
<tr>
<td>Fetal growth restriction</td>
<td>10 (16)</td>
</tr>
<tr>
<td>Male</td>
<td>33 (53)</td>
</tr>
<tr>
<td>Multiple gestation</td>
<td>22 (35)</td>
</tr>
</tbody>
</table>

---


F613
Original research

Figure 1  Number of measurements for each sampling time point and proportion of gestational ages below and above 25 weeks, respectively.

Figure 2  Box-plots showing longitudinal trends of the proportion of Hyper-He (A) and haemoglobin concentration (B) in the blood of extremely preterm born infants (n=62) cord blood, postnatal day 1–14, at 1 month postnatal age, postmenstrual age week 40 and at corrected age 3 months.

median 133 g/L (115–146 g/L), slightly decreasing to 127 g/L (95–146 g/L) at postnatal day 14. The lowest median Hb concentration was observed at term age, 104 g/L (91–136 g/L). Figure 2 presents box-plots for Hyper-He and haemoglobin at each time point.

Association between Hyper-He and the indices MCH and MCV

Strong associations were found between the proportion of Hyper-He (optically measured) and the mean haemoglobin content of the red cells, MCH (derived from photometrical measurement of Hb divided by RBC count measured by impedance) as well as MCV (derived from haematocrit and RBC count). As for MCH, Spearman’s rho was 0.87 (CI 0.77 to 0.93), p<0.001 (figure 3). The association of Hyper-He with MCV was similar in magnitude, Spearman’s rho 0.86 (CI 0.75 to 0.93), p<0.001.

As by ratio, a large MCH indicates higher total haemoglobin distributed over a lower number of RBC counts, this could mean that infants having a high proportion of Hyper-He at birth also overall have haemoglobin accumulated in fewer, larger cells. The 2.5th percentile and 97.5th percentile for MCH were 32 and 47 pg, respectively. Corresponding percentiles for MCV were 99 and 139 fL.

Postmenstrual age, birth weight and FGR in relation to the proportion of Hyper-He at birth

For 40 infants, Hyper-He was measured within 48 hours. There was a statistically significant inverse association between birth weight and Hyper-He, Spearman’s rho −0.38 (CI −0.63 to −0.07), p=0.02. Also, if the infants with FGR (abnormal fetal Doppler velocimetry combined with estimated fetal weight deviation and a birth weight SD score, in total n=6) were excluded (n=34), we found an association of similar magnitude with regard to gestational age, Spearman’s rho −0.39 (CI −0.65 to −0.05), p=0.02, figure 4. The infants with FGR had median Hyper-He; 11.8% (IQR 8.9%–14.6%) compared with 5.5% (IQR 3.1%–8.3%) for infants without FGR.

DISCUSSION

We report the measurement of a subpopulation of RBCs with a particularly high content of haemoglobin (>49 pg) in EPT infants. Early after birth, the proportion of these cells is inversely associated with birth weight as well as gestational age and varies substantially between individuals. Also, a rapid decrease postnataally is observed.

Development of anaemia after EPT birth has so far mainly been attributed to the decreasing erythropoietin levels following birth. This study adds further aspects to this model.

First, as individual variation during the first postnatal days was considerable (2%–25%), we hypothesise that infants with a high proportion of Hyper-He at birth are more at risk for severe nadirs in Hb, as this cell population mainly seems to be lost reflected by the considerable decrease during the first 14 postnatal days. Avoiding loss of large endogenous RBCs by...
fewer larger cells with higher Hb content in infants with a high proportion of Hyper-He.

With regard to fetal growth, we observed that infants born after FGR seemed to have a high proportion of Hyper-He early after birth, suggesting a positive relationship between chronic fetal hypoxia and proportion of Hyper-He cells. Reibel et al.\textsuperscript{29} described higher transfusion rates during the first week of life in EPT born neonates following FGR with initially similar haemoglobin concentrations. This could indicate the potential dependency on fewer high-loaded Hb cells for oxygen transport as well as presence of stress erythropoiesis. High Epo concentrations have previously been reported in the cord blood of preterm newborns subject to chronic fetal stress, but Epo levels have not been related to Hb or haematocrit.\textsuperscript{30} Neither have Epo concentrations been correlated to a response in RET.\textsuperscript{31} The results from our study therefore suggest that the erythropoietic response to Epo stimulation could include an increase in a subpopulation of few but large red cells with increased Hb content. This hypothesis however needs to be investigated in future studies.

Strengths of our current study include utilisation of the blood from clinical sampling for measurement of the RBC population, thereby requiring no extra phlebotomies day 1–14. Samples were promptly transported to the laboratory and thus RBC swelling was avoided. This is also a prospectively studied, longitudinally followed cohort.

We also identify several weaknesses, and our results do need verification by other methodologies. Mainly, these instruments and algorithms are developed for adults and for children at older ages than our cohort. The extent to which they are verified for the blood from the most preterm infants is currently unknown, thereby introducing analytical uncertainty. The risk for interference or misclassification of cells cannot be ruled out. Yet, we observed excellent repeatability as well as a strong association with MCH, an index being derived from different measurement techniques in the instrument. An additional drawback to this study is a bias towards more critically ill infants during postnatal day 1–14 due to the blood sampling scheme. Neither does the current study differentiate between a possible dilution effect resulting from pRBC transfusions to an actual loss of these cells due to intrinsic or extrinsic factors. This also needs to be addressed in future studies.

Further, with regard to antenatal steroids, these may have influenced the results stimulating erythropoiesis.\textsuperscript{32} Of the infants, 97% were born after antenatal steroid treatment which limited the possibilities to investigate this variable further. The size of our study population was also too small to study the impact of chorioamnionitis on Hyper-He.

Endogenous neonatal RBCs could hypothetically play an important role during this critical time frame for future development. Haematopoietic observations that differ from adults, that historically have been described as ‘immature’ are now beginning to be more widely understood as intricate pathways unique to fetuses and neonates allowing to populate the bone marrow and concurrently meet the needs for increasing blood volumes following rapid growth.\textsuperscript{31} It can be hypothesised that preserving large high haemoglobin content cells in circulation during the transition to an established definitive infant erythropoiesis could impact haemodynamics; gas diffusion properties may act in concert with fetal haemoglobin. These aspects related to oxygenation need further investigation.

In conclusion, our hypothesis generating study implies a possible heterogeneity in the endogenous RBC population after EPT birth where high proportions of peripheral large hyper haemoglobin-loaded RBCs might be indicative of an
erythropoietic transitional process. Future work needs to verify results by different measurement techniques. Further investigations regarding endogenous RBCs in relation to sampling-related blood loss, development of anaemia, transfusions as well as neonatal morbidities are warranted.

Acknowledgements We are most grateful to all the infants and parents participating in the study. We also thank the staffs at the Neonatal Intensive Care Units, the Clinical laboratories at Lund University Hospital and at Karolinska University Hospital and the Department of Biobank and Study Support at the Karolinska University Hospital. In particular, we express our appreciation to the outstanding work of: research nurses Linda Nilsson, Margareta Gebka, Therése Kjellin, Lena Legnevall, Camilla Halzius, study coordinator Claes Ekström as well as the most excellent laboratory skills and study support from Maria Jönsson, Christina Jönsson, Åsa Dikvall and Gunilla Dahlifers.

Contributors DL had primary responsibility for the main trial design, execution and for protocol development and enrolment. SML, TU, AR, MV, DW, KS, IHP and DL had responsibility for study management and recruitment in this observational study. All authors critically revised and approved the final manuscript as submitted. DL and OA are guarantors of the study.

Funding The randomised controlled trial on which this ancillary study is based is funded by the Swedish Research Council. The work in this observational study was supported by grants from the Regional Scientific Council of Region Halland and funds for development and research from the Swedish Southern Healthcare Region.

Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by Swedish Ethical Review Authority (2019-01786). Written informed consent was obtained from both parents.

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 restrictions.

Open access This is an open access article distributed in accordance with the Creative Commons Attribution 4.0 Unported (CC BY 4.0) license, which permits others to copy, redistribute, remix, transform and build upon this work for any purpose, provided the original work is properly cited, a link to the licence is given, and indication of whether changes were made. See: https://creativecommons.org/licenses/by/4.0/.

ORCID iDs
Sara Marie Larsson http://orcid.org/0000-0002-2562-080X
Ann Hellström http://orcid.org/0000-0002-9259-1244

REFERENCES