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LUND
UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on October 6, 2023, at 13:00 in Segerfalksalen, BMC A10, Sölvegatan 17, Lund, Sweden

Faculty opponent
Professor Enrico Lopriore, MD, PhD
Leiden University Medical Center, Leiden, The Netherlands

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...inget framåtskridande har hittills skett utan en blick i backspegeln.

(Kristoffer Hellsing, Klinisk kemi i Norden, Nummer 4, volym 9, 1997)

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Abstract

Red blood cell (RBC) and iron status biomarkers are commonly used in clinical diagnostics. However, interpretations of infant test results are inherently challenging. Modern well-defined comparative data based on presumably healthy term-born infants have mainly been lacking and interpretations could be further complicated by interference from frequent infections. Moreover, the trends that follow with transition from fetal life and rapid growth are dynamic. The processes, which are also dependent on gestational age at birth, need to be better understood.

This dissertation includes three retrospective reference interval studies of term-born presumably healthy infants (Papers I, II, III) followed by one prospective study investigating RBC dynamics in infants after extremely preterm birth (Paper IV).

The first three studies defined reference intervals at four time points during the first year of life. Paper I investigated ferritin as a biomarker of iron stores and presented reference intervals divided into subgroups by age and sex. In Paper II the biomarker soluble transferrin receptor (sTfR) was studied. Age dynamics in the first year of life were established, where the upper reference limits were higher compared with adults even in iron-replete infants. Influence from the acute phase response was not demonstrated as sTfR concentrations did not co-vary with CRP concentrations except in samples 48-96 hours after birth. This association could hypothetically be due to the triggering of transferrin receptor expression following the fluctuations in oxygenation during normal labor. In Paper III, hemoglobin (Hb) and RBC biomarkers were studied. The reference interval widths found were mainly narrower compared with other studies. In addition, adherence to the World Health Organization threshold of Hb <110 g/L would classify 16% of the presumably healthy infants as anemic at 12 months. In Paper IV, the RBC dynamics of extremely preterm infants were investigated. The proportions of a subpopulation of large RBCs with hyper high Hb content (>49 pg/cell) rapidly decreased during the first weeks in life.

In conclusion, this work presents opportunities for improvements to the interpretation of infant test results. As reference data are also required for epidemiological studies, the disagreement with the WHO classification for Hb despite seemingly favorable conditions in infancy needs further investigation. The insights from studying the RBC dynamics after extremely preterm birth underline the unique characteristics of their peripheral RBC population and further reveal a potential biomarker, Hyper-He, for studying postnatal erythropoietic transitions. Future research is needed to investigate the potential role of the loss of these endogenous RBCs and the development of infant morbidities after extremely preterm birth.

Populärvetenskaplig sammanfattning

Som ett stöd i diagnostiken inom sjukvården vill man ofta kunna mäta mängden röda blodkroppar, deras innehåll av hemoglobin och kroppens förråd av järn. Under första levnadsåret kommer de här biomarkörerna att variera mycket över tid och resultaten skilja sig från äldre barn och vuxna. Detta beror bland annat på att blodets syretransport håller på att anpassa sig till nya förutsättningar, från att barnet andas genom mammans moderkaka till att barnet andas genom lungorna. Hur den här anpassningen ser ut skiljer sig bland annat beroende på om man föds efter en full graviditetstid eller om man föds för tidigt. Biomarkörerna kan också påverkas av att barnet växer.

För att bedöma ett provresultat behöver man jämförande siffror. Dessa jämförande siffror kan till exempel komma från studier som visar hur provresultat varierar hos friska individer (referensintervall). Referensintervall är mycket svåra att definiera för små barn. Dels ändrar sig siffrorna fort när barnet växer, vilket gör att man behöver sticka väldigt många barn för att få pålitliga siffror. Dels vill man av etiska skäl inte orsaka små friska barn smärta eller ta ifrån dem deras eget blod. Det här har gjort att grundligt framtagna jämförande siffror länge har saknats. Man har främst använt referensintervall etablerade före utvecklingen av modern analysteknik och dagens etiska ramverk. I nyare studier har man ofta använt siffror från patientdata eller mestadels äldre barn.

Forskningen som presenteras i det här arbetet har tagit fram nya referensintervall genom att titta på hur röda blodkroppar och järnstatus ändrar sig under första levnadsåret hos friska barn födda efter okomplicerade graviditeter. Det har varit möjligt genom att samarbeta och använda redan insamlade forskningsdata.

Det första arbetet beskriver hur de järnförråd i levern som barnen samlat under sista delen av graviditeten, gradvis minskar första levnadsåret. Det visar också på att det är skillnader mellan flickor och pojkar. Det andra arbetet visar hur järnupptag för tillverkning av röda blodkroppar hos många barn har reglerats upp, även hos barn som har tillräckliga järnförråd. I det tredje arbetet undersöks dynamiken hos de röda blodkropparna första året i livet.

Denna kunskap har sedan använts vidare för att ta fram en ny studie. Detta fjärde arbete har studerat hur de röda blodkropparna förändras hos barn som har fötts mer än tre månader för tidigt. En del av de här extremt för tidigt födda barnen verkar strax efter födelsen ha en stor andel av speciella röda blodkroppar i blodet som innehåller avsevärda mängder hemoglobin, särskilt de barn som fötts efter fetal tillväxthämning. De här speciella röda blodkropparna försvinner sedan ganska snabbt de första levnadsveckorna. Man skulle kunna tänka sig att de här blodkropparna kan fungera som potentiella långsamma jättefraktare av syre. Vi vet dock inte vilken roll de spelar för barnets utveckling eller om man skulle kunna hålla kvar de här cellerna längre i blodbanan. De här frågorna behöver undersökas vidare i framtida forskning.

List of papers

Paper I

Larsson SM., Hillarp A., Hellström-Westas L., Domellöf M., Lundahl T., Andersson O. When age really matters; ferritin reference intervals during infancy revisited. Scand J Clin Lab Invest. 2019;79(8):590-594.

Paper II

Larsson SM., Hillarp A., Karlsland Åkeson P., Hellström-Westas L., Domellöf M., Askelöf U., Götherström C., Andersson O. Soluble Transferrin Receptor during infancy and reference intervals for the Roche Cobas platform. Int J Lab Hematol. 2021;43:378–386.

Paper III

Larsson SM., Hellström-Westas L., Hillarp A., Karlsland Åkeson P., Domellöf M., Askelöf U., Götherström C., Andersson O. Haemoglobin and red blood cell reference intervals during infancy. Arch Dis Child. 2022;107(4):351-358.

Paper IV

Larsson SM., Ulinder T., Rakow A., Vanpee M., Wackernagel D., Sävman K., Hansen Pupp I., Hellström A., Ley D., Andersson O. Hyper high haemoglobin content of red blood cells and erythropoietic transitions: a prospective cohort study in infants of 22 to 26 weeks' gestation. Arch Dis Child - Fetal and Neonatal Ed. Published Online First 11 May 2023.

Abbreviations

CI	Confidence intervals
CLSI	Clinical and Laboratory Standards Institute
CRP	C-reactive protein
EDTA	ethylenediaminetetraacetic acid
EMP	erythroid-myeloid progenitor
FPN	ferroportin
Hb	hemoglobin
Hct	hematocrit
HSC	hematopoietic stem cell
ICSH	International Council for Standardization in Hematology
IFCC	International Federation of Clinical Chemistry
MCH	erythrocyte mean cell hemoglobin content
MCHC	mean cell hemoglobin concentration
MCV	mean cell volume
NIBSC	National Institute for Biological Standards and Control
pRBC	packed red blood cells
RBC	red blood cell
RET	reticulocyte counts
Ret-He	reticulocyte mean cell hemoglobin content
RI	reference interval
SD	standard deviation
sTfR	soluble transferrin receptor
STB	syncytiotrophoblasts
WHO	World Health Organization

Preface

Being puzzled, I studied the widely differing numbers. This was early on in 2018 and a between-laboratory survey of infant ferritin reference intervals in the Nordic countries. Unarguably, bias between the commercial ferritin measurement systems at that time was well known and something I was highly aware of. But this was something else. Why were there such extraordinarily large differences between the infant reference intervals, even between laboratories that used the same assay?

Now it is time to sum up the years of research that this confused moment sparked. Initially this work was perhaps more aimed towards the nutritional and neurodevelopmental aspects of early life iron deficiency. But as the work progressed, my aim and the theoretical framework took a turn towards the red blood cells.

I think I have always had a fascination for these impressively super strong, yet fragile cells. In the beginning of the 1990s, I spent a week in a small hospital laboratory. It was part of an opportunity in school to explore possible future career choices. Unfortunately, I cannot remember her name, but the biomedical scientist who introduced me to her work exclaimed enthusiastically in front of her microscope *"Detta med blod är så spännande!"* (English translation: I find blood just so fascinating!) and one could not miss out on her true dedication for her work.

Years later, in 2001, I finished my MSc studies in Chemistry and again met the red blood cells but from a completely different perspective. I worked in the computational lab of Prof. Dieter Cremer at the University of Gothenburg with density functional theory calculations on the iron – oxygen interactions and hemoglobin. But back then I was so eager to work with other aspects of chemistry and particularly analytical chemistry, so I left the subject there. Little did I know that some years later, in 2007, I would find my way to the Department of Clinical Chemistry at the Halland's hospitals.

At the end of 2017, through the initiation of a ferritin project by Dr. Andreas Hillarp who at that time point was head of the laboratories, I had the great privilege of meeting neonatologist Dr. Ola Andersson and the project developed into these cross-disciplinary PhD studies together with Dr. Ola Andersson (main supervisor) and Dr. Andreas Hillarp and Dr. Pia Karlsland Åkeson (co-supervisors).

I further became aware of the urgency for updated knowledge concerning the red blood cell reference intervals. In current use were reference intervals that seemed to be based on studies performed decades ago on outdated technology, even for a

commonly used biomarker as hemoglobin. More recently obtained findings in the literature were most often based on mathematically trimmed patient data.

In addition, I was intrigued by the iron regulation and transitional red blood cell dynamics after preterm birth. Through my main supervisor, I had the excellent privilege of meeting Prof. David Ley. This opened up for very stimulating discussions and the opportunity to plan the fourth project of these PhD studies within a prospective clinical trial.

Last year I read some newly published inspiring research papers from pre-clinical studies, challenging our current understanding of the fetal erythropoiesis. This was the spark for Paper IV in this dissertation. Could the peripheral red blood cells present during the gestational ages that correspond to extremely preterm birth have developed from erythroid cell lineages unique to the fetus? How and what do we actually know about the niches and microenvironments? How heterogenous is the resulting red cell population? And most importantly, do these characteristic cells and the potential loss of them, play a role for infant development?

My hope is that a future better understanding of the fetal and infant erythropoietic processes will enable development of clinical approaches that can optimize support to the transitional erythropoiesis after extremely preterm birth.

2023-08-22
Marie Larsson

Funding
This research was funded by the Regional Scientific Council of Halland and funds from the Swedish Southern Healthcare Region. The randomized controlled trial on which the data in Paper IV is based, is funded by the Swedish Research Council.

Dissertation at a glance

The dynamics of red blood cells and iron status during infancy

Retrospective reference interval studies.

Term-born infants first year of life.

Paper 1 (2019)

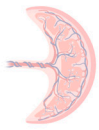
Iron stores (ferritin)

Paper 2 (2021)

Iron uptake (sTfR)

Paper 3 (2022)

Hemoglobin and red blood cells



A prospective study.

Red blood cell dynamics in infants born gestational week 22+0 to 26+6.

Paper 4 (2023)

Hemoglobin and red blood cell dynamics after extremely preterm birth

Introduction

At no other time in human life are there such rapid growth and dynamic transitions than during fetal development and the year following birth. Thus, biomarkers require considerations far beyond what is normative for older children and adults.

A long-standing problem has been the lack of well-defined reference intervals (RI), comparative biomarker data representing results from healthy term-born infants for some widely used biomarkers during infancy: hemoglobin, red blood cells (RBC) as well as the iron status biomarkers ferritin and soluble transferrin receptor (sTfR).

Further challenges have emerged. For the optimal management of infants born extremely preterm, laboratory medicine developments need to keep pace with the advances in modern neonatal care. The total blood volume of an infant born extremely preterm with a birth weight of 450 g could be estimated at volumes as little as 45 mL (not more than three tablespoons). Loss of endogenous cell populations such as potentially unique RBCs to frequent blood sampling, could hypothetically be causal in development of morbidities. For this particularly vulnerable population, research efforts are required that seek to maximize clinical laboratory test information on minimized blood sampling (1).

This work aims to investigate and clarify aspects of red blood cell and iron status dynamics during infancy by defining RIs based on healthy term-born infants. Potential improvements are sought that can support the interpretation of infant test results. The knowledge is thereafter expanded upon to gain further insights into Hb and RBC dynamics of infants born extremely preterm and a potential new biomarker Hyper-He is investigated.

Laboratory results and comparative data

In clinical medicine, laboratory tests are used for diagnosis, screening, confirmation, monitoring, exclusion and an estimation of probabilities (2, 3). The measured results from patient laboratory testing are usually interpreted by comparison or combinations of comparisons in the specific clinical context.

These comparisons can be based on data from:

Previous results from the individual
or
Data from presumably healthy individuals (reference intervals)
or
Limits established to decide risk for disease (decision limits, also sometimes called cut-offs or thresholds)

RIs are generally defined as the lower and upper limit comprising 95% of the measured results in a presumably healthy population. Thereby, within that particular population they provide information on the expected variation.
Until the 1960s, laboratories often worked in isolation and developed their own comparative data. *Reference intervals* as a concept was introduced in 1969 (4). Further work (5-9) led to common recommendations and in 1992, the Clinical and Laboratory Standards Institute (CLSI) published the first edition of their international guidelines on the establishment of RIs (10).

These current guidelines (CLSI EP28-A3c) recommend that the population used for establishing RIs is selected using well-defined criteria. Statistical methodology should be appropriate for the non-gaussian behavior of the data populations for most biomarkers. With these statistical approaches follow the recommendation of at least 120 individuals per group (9-11).

The RIs are a part of everyday medical practice, but not without difficulties. There is usually an overlap between the healthy and the pathological results (**Figure 1A-C**). The RIs are also influenced by the measuring system used, as well as by many biological variables. Clinical decisions are nevertheless usually faced with turning interpretations of laboratory test results (on continuous scales) into binary decisions.

On the contrary to RIs, the medical *decision limits* (**Figure 1D**) separate patients into clinical categories or outcomes. These limits vary according to clinical contexts and are medical action thresholds. Generally, decision limits are based on agreements in consensus groups. The designs for deriving decision limits differ from RI studies, e.g. epidemiological approaches are used (2).

In the literature as well as in current practice, the historical term "*normal values*" (referring to RIs) is commonly encountered. This potentially misleading terminology is outdated and not supported by international guidelines (10). *Normal* can be interpreted in many ways (3, 12). Results outside defined reference limits are not necessarily associated with a certain disease; they are data that are less likely to

be found within a presumably healthy population. By definition, five of 100 results for different individuals for a specific biomarker will be found outside the reference limits. Similarly, results within the RIs do not necessarily rule out a disease. *Normality* can also be confused with the statistical terminology referring to the data distribution as being Gaussian.

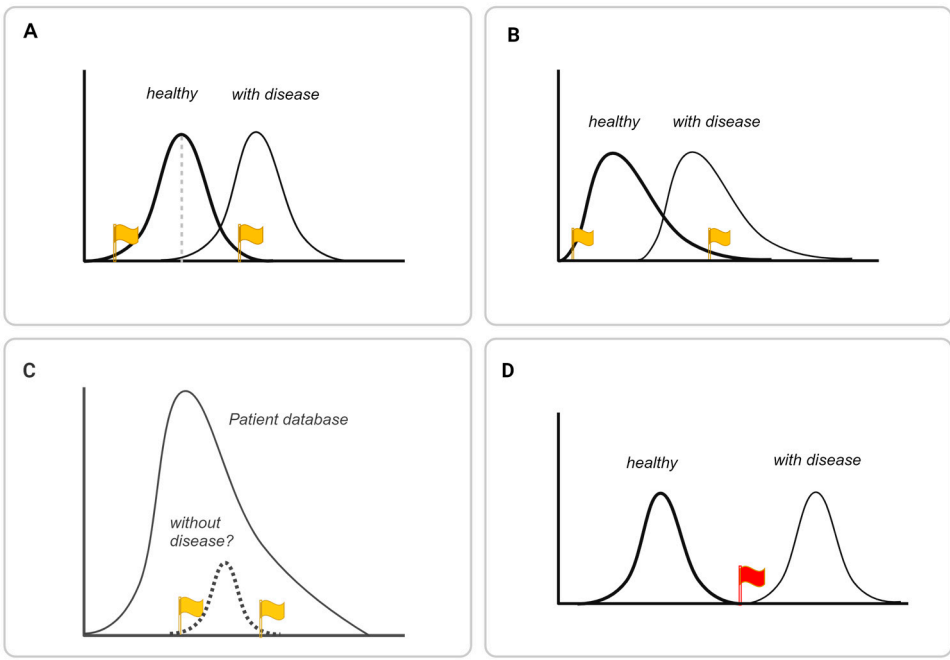


Figure 1. A schematic presentation of biomarker distribution and approaches to derive comparative data. A) Biomarker with two populations (healthy and with disease). Both have the ideal gaussian distributions. The reference interval (RI) calculations can therefore be based on a parametric approach using mean ±2 standard deviations. There is usually an overlap between results from healthy individuals and results from individuals with the disease. B) The gaussian distribution is uncommon for biomarkers, most distributions are skewed. Therefore non-parametric or transformed parametric reference interval methodologies are required. C) There are methodologies to estimate reference intervals using patient results. These RIs are based on data exclusions, transformations and mathematical approaches. D) A schematic drawing of an ideal biomarker with a decision limit separating the population with the disease from a population without the disease.

Infant reference intervals

For the definition of infant RIs, adhering to the CLSI EP28-A3c guidelines is difficult. Following the rapid physiological changes, age subgroups should comprise short time intervals. For some age intervals and biomarkers, division into months or weeks is sufficient. But for other biomarkers, weeks, days or even minutes would

be more appropriate. In addition, further division into subgroups could be necessary for other variables than postnatal age, e.g., gestational age or sex. Still, optimally at least 120 individuals are required for each subgroup.

Consequently, the CLSI guidelines have been questioned for their usefulness in pediatrics (13, 14). Fulfilment of the criteria has been described as impossible (15, 16). To meet the existing requirements for infant RIs in clinical practice, the most commonly used approach is infant RIs based on mathematically trimmed patient data (**Figure 1 C**). These methodologies are called *indirect* in contrast to the *direct* which use data from presumably healthy populations. The basic assumption of the indirect RI methodology is that a representative RI population can be found within the database of patient results. The indirect methodology delivers a possibility to base RIs on an exceedingly large number of patient results, dividing age subgroups into days. These studies thus provide information about the dynamic changes occurring during the first year of life. Several of the RIs provided in the well-known textbook edited by Soldin et al. are derived from indirect methodologies (16). Other examples of initiatives using these techniques include the studies from the Intermountain Healthcare Systems, United States (17-19) or the German PEDREF studies (20).

Nevertheless, the major issue inherent to the indirect methodologies is the limitation in their basic assumption. Finding a representative healthy population within the infant hospital data is problematic since routine laboratory testing of infants is uncommon and blood samples are not taken from infants seeking health care for minor conditions. As a general practice this is avoided due to a potentially painful procedure, loss of endogenous blood factors and the risk of causing blood-sampling related anemia.

The limited possibility to compare the results with published reference limits based on healthy infants, further increases the uncertainties with RIs derived from indirect techniques. The expected widths of the infant reference intervals require particular consideration. Broad subgroup partitioning with regard to infant age, or unknown gestational ages at birth, could result in wide RIs. As reported by Horn and Pesce for adult RIs, even small shifts towards wider intervals can result in a substantial increase in false-negative rates (21).

Development of erythropoiesis

During development, fetal and infant erythropoiesis is subject to the most remarkable expansion. This is in sharp contrast to adult erythropoiesis where RBC mass in health is maintained at a steady state. Fetal life is accompanied by an increase in RBCs and blood volume that need to follow the more than sixfold weight gain between gestational week 22 and 40 (22) as well as for a term-born infant, a threefold weight gain during the first year of life (23, 24).

Fetal life

The traditional model of developmental hematopoiesis is usually described as three waves, where only the two early initial waves are independent of hematopoietic stem cells (HSC) (25). This is illustrated in **Figure 2**.

The first RBCs originate as primitive erythroblasts from the hemangioblasts in the yolk sac. Hemangioblasts are multipotent precursor cells that can differentiate both into endothelial as well as hematopoietic cells (26). The resulting RBCs which can be observed 4 to 5 weeks post conception, are large 10-15 μm, nucleated and carriers of abundant Hb (27). Compared with RBCs later in the development, these cells are characterized by increased oxygen-carrying as well as gas exchanging capacity (28, 29). They do enucleate, but first several days after entering the bloodstream (30).

Following the first wave of RBC originating from the hemangioblasts, is the second wave derived from the erythroid-myeloid-progenitors (EMP). The EMPs form in an endothelial-to-hematopoietic transitional process and migrate to the fetal liver where they further differentiate. By 6 to 8 weeks' gestation, this liver-sited erythropoiesis has replaced the yolk sac.

In the third wave, HSCs enter the liver after migrating from the major arteries. So far this HSC derived erythropoiesis has been regarded as taking over after the transient production of erythroid cells from the EMPs and it was until recently believed that HSC-derived erythropoiesis predominates during the remainder of the pregnancy.

The traditional model has now been challenged by pre-clinical lineage-tracing and cloning experiments (31-35). These new experiments have suggested that there are only minor contributions from HSCs to the lineage committed progenitors, even towards end gestation. Worth of notice, compared with the HSC erythroid lineage, EMP derived erythroid progenitors seem to substantially differ in their response to erythropoietin. Requirements for proliferation have been estimated to be 10-fold lower (35).

As for the site of erythropoiesis and its transition from the liver to the bone marrow, colonization of the bone marrow by HSCs is initiated from the 15th to 16th postmenstrual weeks (36, 37). But the actual contribution from the bone marrow to the peripheral RBC population in the following weeks seems mainly to be uninvestigated, particularly between gestational weeks 22+0 to 26+6 i.e. extremely preterm birth. An approximate 50/50 relationship has been suggested (38, 39). In addition, there could be contributions to the peripheral RBC population from the placenta (40, 41).

In the perspective of extremely preterm birth "stress" erythropoiesis is an additional process that need consideration. In adults, "stress" erythropoiesis describes an erythroid response that follows a critically increased demand of RBCs, due to for example hemolysis or blood loss. It is known to occur extramedullary and results in large cells. The larger RBCs arise from erythroid flexibility in the number

and rate of divisions ("skipped divisions") and rate of enucleation. A stress erythropoietic pathway has also been shown to be activated by inflammatory signals such as bone morphogenetic protein 4, BMP4 (42). Nevertheless, little seems to be described with regard to the detailed characteristics of the stress related erythropoietic response in the fetus or the extremely preterm born.

The rapidly evolving erythroid landscape so far described at the gestational ages of extremely preterm birth could point towards heterogenous populations of RBCs to be present. Clinical measurements describing the RBC population have so far mainly been limited to mean indices or morphological assessments. In fetuses gestational weeks 22 to 23, the RBC mean concentration of Hb (MCHC) has been estimated to approximately 330 g/L, thereby similar in magnitude to both term-born infants and adults (43-45). However, a lower RBC count is reported in fetuses; 3.0×10^{12}/L (45) compared with ca 4.5×10^{12}/L for a term born infant (43) or ca 5.0×10^{12}/L found in adults (44). A similar mean concentration of Hb distributed in fewer cells suggest that either each fetal RBC carries more Hb per cell compared with the RBCs of older children and adults, and/or that there could be a fetal subpopulation of particularly dense cells.

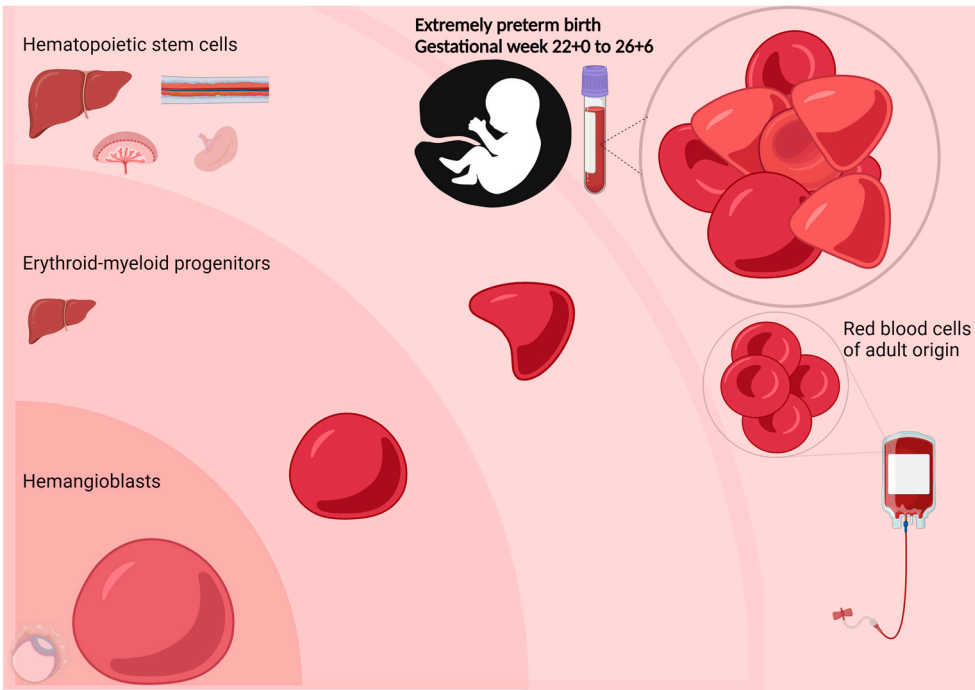


Figure 2. The peripheral red blood cell population at the timing of extremely preterm birth could originate from several erythropietic cell lineages and niches. Frequent blood sampling risk depriving the infant from the unique endogenous cells, and transfusion replace them with a homogenous population of red blood cells with adult red cell characteristics.

Postnatally and first year of life

The Hb concentration of infants changes rapidly after birth. Among the first to describe these dynamics was Dr. Helen Mackay in East London in the 1920s (46, 47). At that time, the increased risk of iron deficiency during infancy was as yet unrecognized. Instead, a common treatment for infant anemia was light therapy (48).

In her records, Dr. Mackay first described high Hb concentrations at birth, followed by a decrease until around 2 months. Thereafter, she observed Hb concentrations in her infants that generally remained steady. Nevertheless, for some the Hb concentrations further decreased from 6 months onwards. This was more commonly observed in non-breastfed than in breast-fed infants. In her clinic, Dr. Mackay was successful in preventing this second decrease in Hb concentration by additionally offering treatment for infections and iron supplementation.

In the 1950s, details were added to the knowledge of RBC dynamics by studies e.g. involving radioactively labelled iron (49) and bone marrow sampling (50, 51). Gairdner et al. observed that the high Hb concentrations around birth usually persist only approximately 4 to 8 days and that it was caused by the fluid redistribution from the vascular space after birth (52). Hb concentrations thereafter return to the values observed in the umbilical cord.

Following postnatal day 9, Gairdner et al. further divided the decrease in Hb into two phases: the first month rapid and thereafter followed by a more gradual decrease until a nadir was reached after about 2 months. These trends were explained as a bone marrow response to extra-uterine oxygen levels and an adaptation phase. First with a suggested nadir of 110 g/L in Hb concentration, the bone marrow seemed to be able to sense a decreased oxygen-carrying capacity (50, 51).

In their studies of infant erythropoiesis, the authors also described the Hb dynamics after preterm birth. For a preterm born infant, the decrease in Hb concentrations will be faster, it will reach lower levels and the recovery will be slower (53). Preterm birth also predisposes to iron deficiency, as iron stores are mainly endowed during the last trimester (54).

Extremely preterm birth

According to current Swedish guidelines issued in 2016, antenatal steroid treatment and active resuscitation of the newborn infants are considered from gestational week 22+0 and are recommended from gestational week 23+0.

The postnatal anemia that develops after extremely preterm birth is most pronounced. To prevent a potentially impaired oxygenation, the infants commonly receive transfusions with packed RBCs (pRBC). These RBCs are of adult origin. The clinical decision for transfusion is usually based on algorithms with combined information on respiratory support and Hb concentration (55, 56).

The Hb decision limit for transfusion included in these algorithms is debated. A recent survey across Europe showed that transfusion thresholds in neonatal

intensive care practice vary widely (57). In 2020, two large randomized controlled trials, compared liberal to more restrictive transfusions (using high or low Hb decision limits). They coherently found no differences between groups in neurodevelopment, morbidities or mortality (58, 59).

Apart from the main findings of the randomized controlled trials they might further point towards limitations of total Hb as a biomarker, as measured today. Generally, in clinical practice, Hb is measured spectrophotometrically as a total concentration (60). Methodology is usually based on hemolysis of the RBCs and conversion of Hb. Thereby, the amount of Hb in each cell, proportions of fetal hemoglobin (HbF) with differing oxygen affinity compared with adult hemoglobin HbA, or other differences on a cellular level such as cell size, are not described. In addition, methods based on spectrophotometric measurements could be susceptible to interference from turbidity from parenteral emulsions (61).

The correlation between Hb and oxygen-related variables during early gestational ages has been reported as poor (62). Further details describing the characteristics of the infant RBC population, could hypothetically be of potential value in the future development of transfusion decision algorithms.

Iron requirements for erythropoiesis

Erythroid iron acquisition in vivo is facilitated through the transferrin cycle (63, 64). Iron-bound transferrin docks to transferrin receptors (TfR) on the cell surface whereafter the iron-transferrin complex is engulfed by endocytosis. Although this is the route by which all dividing cells in the body acquire iron, iron uptake predominates in the erythroid committed lineage (65).

Most iron in the erythropoiesis is recycled from senescent RBCs. However, if the recycled iron is not sufficient to meet the iron requirements, the transferrin cycle is readily supported by the liver iron stores. This is unless infection or inflammation blocks iron exit from the storage, through the hepcidin–ferroportin axis (66).

Iron stores

Iron stores are mainly gained the last trimester and a term-born infant holds in total approximately 300 mg body iron at birth, mostly present in the form of Hb in the RBCs (54). With a seemingly large inter-individual variation, only about 1/8 of the total body iron is stored in the liver (54, 67).

Therefore, due to the low iron content of human milk, the breastfed infant is mainly dependent on the slow release of iron from senescent RBCs. The iron stores in the liver support the physiological requirements until a more iron rich complementary nutrition is established. This limited source of iron (ca 38 mg) is estimated at to be sufficient for a production of 11 g of Hb, or 22 g/L assuming a

total blood volume of 500 mL for a 4 to 6 months old infant (24) but it is also needed to support the other physiological needs and the growth of the tissues.

It is generally accepted that liver iron stores are reflected by the ferritin concentration in plasma or serum, in the absence of inflammation or infection. Thus, ferritin concentrations can be expected to decrease during the first year of life also for a healthy term-born infant with adequate complementary feeding after 6 months of age.

Currently, a ferritin decision limit (12 µg/L) is recommended by the current WHO guidelines for definition of iron deficiency, independent of infant age (0 to 23 months) (68, 69). This approach could have limitations. Firstly, due to the low comparability between the ferritin measurement systems, a common decision limit is currently not supported (70, 71). Secondly, the decision limit does not reflect the different phases of the physiological development from birth and onwards. Thus, extrapolation of data from older age groups may not be applicable (69). Thirdly, it can be argued that a well-defined decision limit should be based on a clinical outcome. However, the design of a well-defined study with this aim, is hindered by substantial ethical as well as practical obstacles.

Contrary to decision limits, RIs do not claim association with an outcome, but is rather comparative data providing information on what to expect in a population with a low risk of iron deficiency. Flagging a result as lower than what is usually observed for low-risk infants of similar age, could allow for early correction.

Moreover, the potential differences in liver iron storage between girls and boys could present difficulties when defining a common ferritin decision limit. That iron storage in part could be controlled by gonadal steroid hormones was investigated in animal models as early as 1948 by Widdowson and McCance (72). Differences between the sexes have later also been confirmed by studies of the livers of human newborns (67) as well as infants in large observational clinical studies (73-75).

Iron uptake

In sensing decreasing intra-cellular iron levels through iron responsive elements, cells upregulate the expression of TfRs in their membranes. TfRs are transmembrane receptors, but in the erythroid lineage they are released through vesicles when they are no longer needed (76, 77). In this process, a fragment called soluble Transferrin Receptor (sTfR) is proteolytically cleaved (78). The concentration of sTfR can be measured in circulation. As the relationship between tissue TfRs and sTfR has been reported to be constant, an increase in sTfR concentrations is generally regarded to reflect an increase in the iron requirements (79).

During fetal life, the syncytiotrophoblasts of the placenta express TfRs on the maternal side to facilitate the increased demands from placenta and fetus. From these cells, the iron is transferred to the fetus through ferroportin. Since the syncytiotrophoblasts only express ferroportin on the fetal side, the potential for ferroportin regulation is only possible through hepcidin expressed by the fetus.

Regardless, Kämmerer et al. have shown that the fetal hepcidin does not seem to regulate the placenta iron export appreciably, but instead rather regulates ferroportin on the cell membranes of the hepatocytes, **Figure 3** (80), a mechanism suggested to protect the fetal liver erythropoiesis (81).

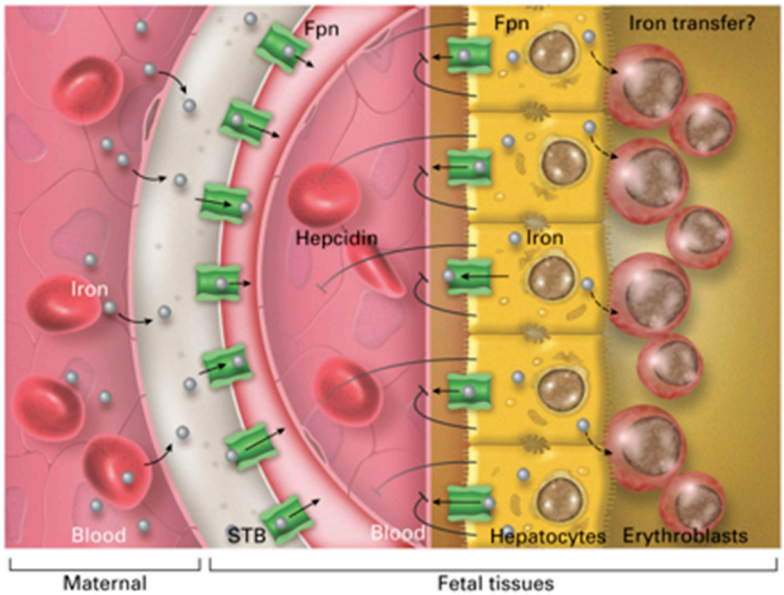


Figure 3. The syncytiotrophoblasts (STB) express ferroportin (Fpn) only on the fetal side. The regulation of iron export by this route is thereby excerted through fetal hepcidin. Studies suggest minor hepcidin induced regulation by this route, instead being more pronounced in the fetal liver than the placenta. Reproduced with permission from Thomas Ganz, The role of hepcidin in fetal iron homeostasis, Blood, American Society of Hematology 2020 (81). Professional illustration by Somersault 18:24.

The vast majority of TfRs are expressed in the erythroid lineage with a peak at the erythroblast stage (82). Due to the changes in erythropoietic activity during infancy (50, 51), the sTfR concentrations and the corresponding RIs, can be expected to vary dynamically during the first year of life.

Reports on RIs describing these potential dynamics of sTfR are conflicting. Some reports define RIs as constant throughout the first year, some define decreasing concentrations from birth and onwards, while others on the contrary describe increasing concentrations (75, 83-90).

As a biomarker, sTfR has been suggested to distinguish iron deficiency anemia (sTfR increased) from anemia due to infection or inflammation (sTfR unchanged) (91). During infancy the reports on the influence from the acute phase response on the sTfR results disagree (87, 92, 93).

Moreover, differences between the sexes during infancy, similarly to ferritin, have been reported (73), but the results remain to be confirmed by other studies.

Aim

The overall aim was to investigate and clarify aspects of red blood cell and iron status dynamics during infancy. This knowledge was expanded upon with the aim to provide further insights in red blood cell dynamics for infants born extremely preterm.

The specific aims were:

1. To describe the dynamics of iron stores in girls and boys during first year of life by defining reference intervals for ferritin at four time points in a large cohort of presumably healthy infants *(Paper I)*.
2. To review what is known about the soluble transferrin receptor during infancy and describe reference intervals based on a large cohort of presumably healthy infants *(Paper II)*.
3. To investigate whether soluble transferrin receptor in term-born infants is associated with the acute phase response as measured by C-reactive protein *(Paper II)*.
4. To study red blood cell biomarkers: Hb, Hct, MCV, MCHC, MCH, RET, RBC count and Ret-He in a large cohort of presumably healthy infants and present updated reference intervals at four different time points in the first year of life, based on modern analytical instrumentation *(Paper III)*.
5. To describe the dynamic changes of the hemoglobin content of red blood cells in extremely preterm born infants by the exploration of a potential biomarker Hyper-He, the proportion of red blood cells with a content of more than 49 pg /cell *(Paper IV)*.
6. To investigate if gestational age, birth weight and fetal growth are associated with the proportions of Hyper-He in extremely preterm born infants soon after birth *(Paper IV)*.

Methods

This dissertation firstly comprises three retrospective studies (Papers I, II, III). These address the aims of studying ferritin, sTfR and RBC dynamics in term-born infants. While the original papers present the defined RI data, the focus of this dissertation is to summarize the findings with a descriptive narrative review based on the observed trends. Furthermore, the work comprises one prospective observational cohort study that investigates the dynamic changes of the Hb content of RBCs after extremely preterm birth with a descriptive aim (Paper IV).

Participants

Reference intervals (Papers I, II, III)

Overall, the RI studies included data collected during seven years. From the county of Halland, two data sets were combined. The first study collected data between April 2008 and September 2009 in a randomized controlled trial (94-97). This study included only infants from vaginal births. The second study collected data between June 2010 and February 2012 in a cohort study. (98). Infants born after elective Caesarean sections were included.

All the included infants were born of non-smoking and healthy women. The criteria for healthy were: no hemolytic disease, no treatment with anticonvulsants, antidepressants, thyroid hormone, insulin, chemotherapy or cortisone. The pregnancies were normal (criteria: no diabetes, no preeclampsia, no signs of infection, no prolonged rupture of membranes). They were also singleton and term (gestational age 37+0 to 41+6). All infants were examined within the first 72 hours by a physician. At the 4-month visit, the parents declared subjective well-being of their children by a questionnaire for morbidities.

The data sets from the county of Halland were combined with additional data from a cohort study performed at Karolinska University Hospital, Stockholm. These data were acquired from infants, vaginally born in April 2012 to May 2015 (99). The inclusion criteria in the study performed at Karolinska University Hospital were coherent with the studies performed in the county of Halland.

Exclusion criteria in the RI studies were applied based on the corresponding C-reactive protein (CRP) measurement in each sample. The particularly restrictive

CRP limit applied in the ferritin RI study were based on the findings of interferences from the acute phase response, already at very low concentrations as reported in the BRINDA study (100). The exclusion criteria for CRP were therefore based on the 97.5th percentile for CRP reported by the CALIPER study in 2016 (101). In the calculation of RIs for ferritin in the umbilical cord or at 48–72 h, data were excluded from further analysis if CRP concentration was >5 mg/L. At 4 months or 12 months data were excluded if the corresponding CRP was >1 mg/L. As for sTfR and RBC, the corresponding CRP exclusion criteria was >10 mg/L, as in the original studies (94, 96, 98, 99).

While the ferritin RI study was based on data from infants with both early and delayed clamping of the cord, the sTfR and RBC studies aimed to include only infants subjected to delayed cord clamping based on the American College of Obstetricians and Gynecologists opinion (≥30 sec) (102).

Red blood cell dynamics after extremely preterm birth (Paper IV)

This prospective study collected data from March 2020 to September 2022 from infants born between 22+0 to 26+6 gestational weeks. The observational cohort study, ancillary to a two-armed multicenter randomized controlled intervention trial (NCT04239690) was carried out at two specialized tertiary level units (Skåne University Hospital, Lund, Sweden and Karolinska University Hospital, Stockholm, Sweden). Exclusion criteria were major malformations. Data were obtained from clinical records. Fetal growth restriction was diagnosed in cases of a birth weight SD score below -2SD and an abnormal Doppler velocimetry (23, 103, 104).
The main trial, which at this date is still ongoing, investigates the relationship between preservation of neonatal blood factors and neonatal morbidity.

Blood samples and analytical methodology

Reference interval studies (Papers I, II, III)

In the studies upon which the RIs were based, blood was sampled according to **Table 1**. The ethylenediaminetetraacetic acid (EDTA) blood samples were promptly transported to the clinical chemistry laboratory where analyses were performed. In total, a maximum of 2.5 mL of blood was collected at each sampling time point. At 4 and 12 months, blood sampling was performed after application of a local dermal analgesia with lidocaine 2.5% and prilocaine 2.5% (EMLA, AstraZeneca).

Table 1. Blood samples in the reference interval studies.

	Red blood cell biomarkers		Ferritin, sTfR and CRP	
	Halland	Karolinska	Halland	Karolinska
Test tubes	EDTA tubes (BD Vacutainer®, Plymouth, UK)	EDTA tubes (Sarstedt AG & Co, Nümbrecht, Germany)	Serum separator tubes (BD Vacutainer®, Plymouth, UK)	Serum separator tubes (Sarstedt AG & Co, Nümbrecht, Germany)
Analytical instrumentation	Sysmex XE-2100 (Sysmex, Kobe, Japan)	Sysmex XE-5000 (Sysmex, Kobe, Japan)	Cobas 6000 (Roche Diagnostics, Basel, Switzerland)	Cobas 6000 (Roche Diagnostics, Basel, Switzerland)
Laboratory and accreditation	Halmstad SE-EN ISO/IEC 17025:2005	Huddinge SE-EN ISO/IEC 15189:2007	Halmstad SE-EN ISO/IEC 17025:2005	Halmstad SE-EN ISO/IEC 17025:2005
Sampling time points	Umbilical cord	Umbilical cord	Umbilical cord	Umbilical cord
	48-118 h*	48-118 h*	48-96 h*	48-96 h*
	4 months	4 months	4 months	4 months
	12 months		12 months	

*In connection with metabolic screening.

Red blood cell dynamics after extremely preterm birth (Paper IV)

EDTA blood (250 µL) was collected at birth (umbilical cord blood), from day 1 to 14 (blood from clinical sampling only), at 1 month (infants cared for in the NICU tertiary centers only), term age (postmenstrual age week 40) and at the corrected age of 3 months. The blood samples were promptly transported to the laboratory for analysis on Sysmex XN (Sysmex, Kobe, Japan).

The instrument uses laser to measure forward and side scatters. Hb concentration is measured by the SLS-method. RBC counts are measured by impedance.

A limitation is recognized regarding the measurement technique for Hyper-He. As the algorithms are mainly developed for individuals of older ages, the risk for misclassification of cells cannot be ruled out. The results reported therefore need verification by different measurement techniques.

Measurement systems and traceability

Diagnostic assays are complex measurement systems combining multiple components such as reagents, sample preparation solutions and calibrators, in addition to the advanced technical instrumental set-ups. All parts are influenced by variables such as lot-lot variations, operators, laboratory settings, wearing (for many instruments on a 24/7 basis), maintenance etc. The optimal function of all these components is important for the measured result to be consistent over time with a minimal measurement uncertainty.

The assay manufacturers provide a calibration valid for the specific assay. Usually, this calibration is standardized to an internationally accepted reference system. Locally, the laboratory calibrates the assay on a regular basis to adjust for lot-lot variations and the continuous shifts in various conditions.

For comparative data to be valid across different diagnostic assays, a traceability chain to the internationally accepted reference system is essential. For the RBC biomarkers such as RBC counts, Hb and Hct, harmonization activities have been successful and they are today measured with high reproducibility and accuracy (105-108). Method performance between different commercial assays for ferritin is, however in spite of three generations of WHO internal standards, currently less satisfactory (70, 71). Although the manufacturer claims traceability to the 1st international standard (NIBSC 80/602) and agreement to the 3rd international standard (94/572) with reference to Blackmore et al. (109), harmonization is lacking (70, 71). Therefore, the numerical findings for ferritin RIs defined in Paper I are applicable for transferability to the Roche assay only. A fourth international standard has very recently been introduced (110). To the best of my knowledge manufacturers have not yet recalibrated to this new reference material, but establishment of traceability could likely be expected in the coming years.

As for sTfR, the long-standing issue with the lack of an international standardization is well known (111). The initiative to improve the situation in 2010 (112) was never implemented in commercial assays. The RI values reported in this study are hence traceable to a Roche internal reference material and applicable for transference investigations to the Roche measurement system.

Statistical methodology

Statistical methodology for the RI studies was chosen based on guidance from CLSI EP28-A3c, from the data simulations performed by Daly et al. (113) and visual assessments of the data population distributions. In Papers I and II, a transformed parametric method (Box-Cox) (114) was used, except for the umbilical cord data for girls in Paper I where transformation was instead carried out according to Manly (115). Success of transformation was approved after the hypothesis test of

Anderson–Darling and the inspection of Q-Q plots. The 90% confidence intervals (CI) were estimated by 500 bootstrap samples.

The transformed parametric method was preferred as it avoids potential information loss that follows with ranking methods. The difference in reference limits arising from the choice of statistical methodology, non-parametric or transformed parametric, was illustrated in Paper I by a method comparison. For example, the lower limit of ferritin in the umbilical cord blood for a girl was 60 µg/L if calculated non-parametrically but 44 µg/L with the transformed parametric.

As for Paper III, since transformation did not provide satisfactory Gaussian distributions for the RBC biomarkers, the non-parametric quantile methodology was used.

For Papers I, II and III partitioning into subgroups between the sexes was based on visual inspection of bi-histograms and the assessment of overlapping 90% CIs.

In Paper III, groups were compared above and below Hb cut-off 110 g/L by the non-parametric Mann-Whitney U-test.

The potential association of sTfR to CRP in Paper II was investigated non-parametrically using Kendall rank correlation.

Paper IV had a descriptive aim: visual presentation was combined with non-parametrical associations using Spearman's rho with 90% CIs. Further statistical analyses comparing groups were not possible as data were limited at this time point for trial interim analysis.

Ethical considerations

This research is for the most vulnerable group of human beings. The benefit is for the infants only and can not be replaced with research based on adults, CIOMS Guideline 14 (116). The benefits of well-defined comparative data could be: reduced rates of repeated blood sampling, reduced iatrogenic blood loss, less parental worries and increased diagnostic power of the biomarkers. Alternative approaches have tried to fill the RI knowledge gaps by the use of results from infants seeking healthcare. Although computational studies are non-invasive and thereby attractive from an ethical perspective, they have an inherent risk of establishing comparative data based on pathological values. Still in use in clinical practice, are also since long technically outdated data. These data were mainly established before the ethical framework of today had developed (117).

Infants can not make an active decision as to whether they want to participate in research or not. All infants in these studies were included after informed parental consent. Parents could withdraw participation at any time.

The well-being of the infants is priority (Declaration of Helsinki 2013). In this perspective, blood sampling volume is an essential aspect to consider. Small volumes for an adult, can result in the loss of a considerable proportion of the total blood volume of an infant (118, 119) and preventive measures to minimize iatrogenic blood loss is of great importance (120). Even though most state-of-the-art instrumentation use very small aspiration volumes, sometimes only a few microliters, most blood sampling tubes and measurement systems today require substantially larger blood volumes. Sample homogeneity and reproducibility are important aspects that need consideration in technical developments.

The blood sampling in the prospective study involving extremely preterm infants uses sampling volumes minimized to what can be practically reproducibly accomplished with currently available pre-analytics, logistics and technology. Blood that was already drawn for required clinical sampling has been used whenever possible.

The concentration ranges where methods need to perform optimally, sometimes differ substantially between infants and adults. Most diagnostic measurement systems are mainly developed with focus on the adult population. This can lead to inequities in access to optimized biomarker performance.

As for iron deficiency, why just not implement broad iron supplementation programs? This approach was studied in the well-known Pemba trial that was early ended (121). The infants in the intervention group supplemented with iron suffered

increased morbidity and mortality. Researchers became aware that making iron unavailable to infecting organisms has evolved as an important part of our defense against infection (122).

Considering that iron deficiency is the most common nutritional deficiency worldwide, research contributing to better diagnostics, to determine which individuals are helped by supplementation or not, could be regarded as beneficial from a broad society and health perspective.

The first three studies were approved by the Regional Ethics Committee at Lund University (41/2008, 344/2009) and the Regional Ethical Review Committee in Stockholm (2011/2142-31/3). The fourth study was approved by the Swedish Ethical Review Authority (2019-01786).

Results

RIs for RBC biomarkers were calculated based on data from in total 442 term-born presumably healthy infants (Paper III). Correspondingly for iron status biomarkers, RIs were based on 456 infants for ferritin (Paper I) and 451 infants for sTfR (Paper II). As for Paper IV, a total of 280 measurements from 62 extremely preterm born infants were analyzed. Graphical summaries of the four papers are presented in the end of this chapter.

Reference intervals

In the following section the findings of the dynamics of iron stores (ferritin), iron uptake (sTfR), hemoglobin and the RBC biomarkers from Papers I, II and III are reported.

Umbilical cord blood compared with early after birth

Reference limits for most biomarkers differed between the umbilical cord blood sample and the second sampling time point (**Table 2**). Hb, hematocrit (Hct), RBC counts as well as mean cell hemoglobin concentration (MCHC) increased. The mean cell volume (MCV) decreased slightly at both the lower and the upper limit. The increase observed for ferritin between these sampling time points was substantial despite the exclusions based on CRP, and more pronounced at the lower limit. For sTfR, the observations at the lower and upper limits were inconsistent in direction, increasing at the lower limit and decreasing at the upper limit. MCH and Ret-He remained mainly constant.

Table 2. Umbilical cord blood compared with postnatal age 48-118 hours
The lower and upper reference limits defined in Papers I, II and III in relation to the corresponding percentual change between the time points.

		Lower reference limit			Upper reference limit		
Biomarker	Unit	Umbilical cord	48-118 h	**Change (%)**	Umbilical cord	48-118 h	**Change (%)**
Ferritin (Girls)	µg/L	44	109	148	609	735	21
Ferritin (Boys)	µg/L	36	83	131	505	699	38
sTfR	mg/L	2.4	2,9	21	9.5	8.4	-12
Hb	g/L	116	147	27	189	218	15
Hct	L/L	0.36	0.42	17	0.57	0.62	9
MCV	fL	97	91	-6	118	107	-9
MCHC	g/L	303	343	13	352	372	6
MCH	pg	32	32	0	39	38	-3
RET	$\times 10^9$/L	99	79	-20	240	275	15
RBC counts	$\times 10^{12}$/L	3.4	4.2	24	5.4	6.2	15
Ret-He	pg	28	28	0	39	38	-3

From umbilical cord to 4 months

Compared with the initial results obtained from the umbilical cord blood samples, most biomarker reference limits decreased during the first 4 months (**Table 3**). For Hb and Hct decreases were more than 30% at the upper limit. MCV was reduced with an approximate decrease of 30 fL and MCH decreased with approximately 10 pg. Ret-He decreased with 3 pg at the lower reference limit and with 5 pg at the upper reference limit. RBC counts at the upper limit had almost 10% lower values at the upper limit but increased somewhat at the lower limit. Ferritin concentrations were almost half compared with what was observed in the umbilical cord, at both the upper and the lower limits. The reduction in sTfR was 40% at the upper limit while a tendency towards upregulation was observed at the lower limit.

Table 3. From umbilical cord blood to 4 months

The lower and upper reference limits in Papers I, II and III in relation to the corresponding percentual change observed between the time points.

		Lower reference limit			Upper reference limit		
Biomarker	Unit	**Umbilical cord**	**4 months**	**Change (%)**	**Umbilical cord**	**4 months**	**Change (%)**
Ferritin (Girls)	µg/L	44	21	**-52**	609	441	**-28**
Ferritin (Boys)	µg/L	36	16	**-56**	505	274	**-46**
sTfR	mg/L	2.4	2.6	**8**	9.5	5.7	**-40**
Hb	g/L	116	99	**-15**	189	130	**-31**
Hct	L/L	0.36	0.29	**-19**	0.57	0.37	**-35**
MCV	fL	97	71	**-27**	118	85	**-28**
MCHC	g/L	303	328	**8**	352	363	**3**
MCH	pg	32	24	**-25**	39	29	**-26**
RET	$\times 10^9$/L	99	20	**-80**	240	65	**-73**
RBC	$\times 10^{12}$/L	3.4	3.6	**6**	5.4	4.9	**-9**
Ret-He	pg	28	25	**-11**	39	34	**-13**

From 4 to 12 months

Only minor changes were observed between 4 and 12 months (**Table 4**). Hb, Hct, RBC counts and sTfR concentrations increased, indicating a growing RBC mass in relation to the blood volume. A further reduction of ferritin concentrations was observed at both the upper and lower reference limits, but the percentual change was substantially larger at the upper limit. Reticulocyte counts were mainly maintained while their Hb content decreased somewhat further with 1 pg both at the lower and the upper reference limits.

Table 4. From 4 to 12 months

The lower and upper reference limits defined in Papers I, II and III in relation to the corresponding percentual change observed between the time points.

		Lower reference limit			Upper reference limit		
Biomarker	Unit	**4 months**	**12 months**	**Change (%)**	**4 months**	**12 months**	**Change (%)**
Ferritin (Girls)	µg/L	21	13	**-38**	441	124	**-72**
Ferritin (Boys)	µg/L	16	12	**-25**	274	70	**-74**
sTfR	mg/L	2.6	3.0	**15**	5.7	6.3	**11**
Hb	g/L	99	104	**5**	130	134	**3**
Hct	L/L	0.29	0.31	**7**	0.37	0.39	**5**
MCV	fL	71	70	**-1**	85	83	**-2**
MCHC	g/L	328	323	**-2**	363	353	**-3**
MCH	pg	24	23	**-4**	29	28	**-3**
RET	$\times 10^9$/L	20	18	**-10**	65	66	**2**
RBC counts	$\times 10^{12}$/L	3.6	3.9	**8**	4.9	5.3	**8**
Ret-He	pg	25	24	**-4**	34	33	**-3**

Reference interval widths

The dynamics of iron stores (ferritin), iron uptake (sTfR), hemoglobin and the RBC biomarkers in the cohort were further defined by investigating the reference interval widths, **Table 5**. Early after birth, the biomarker variation within the population was generally larger than later during infancy. For the iron status biomarkers ferritin and sTfR there was a large decrease in the width of the RIs while only minor changes were demonstrated for MCH and Ret-He.

Table 5. Varation within the reference interval population as defined by the widths of the 95% reference intervals.

Biomarker	Unit	Umbilical cord	48-118 h	4 months	12 monts
Ferritin (Girls)	µg/L	565	626	420	111
Ferritin (Boys)	µg/L	469	616	258	58
sTfR	mg/L	7.1	5.5	3.1	3.3
Hb	g/L	73	71	31	30
Hct	L/L	0.21	0.20	0.08	0.08
MCV	fL	21	16	14	13
MCHC	g/L	49	29	35	30
MCH	pg	7	6	5	5
RET	$\times 10^9$/L	141	196	45	48
RBC	$\times 10^{12}$/L	2.0	2.0	1.3	1.4
Ret-He	pg	11	10	9	9

Differences between the sexes

RIs partitioned into subgroups by girls and boys were presented for ferritin (Paper I), but not for the other biomarkers investigated in this dissertation (Papers II and III).

For ferritin, the differences between the sexes were generally small at the lower reference limits but more pronounced at the upper limits, **Tables 2, 3 and 4**. The differences peaked at the upper reference limit at 12 months. At this time point, the boys' upper 97.5th percentile was 56% compared with the girls (70 µg/L vs 124 µg/L respectively).

As for sTfR the numerical findings in subgroups by sex are presented in **Table 5**. At the upper limits the 48–96 hours and 12 months sampling time points pointed to no differences between the sexes as the 90% CIs were almost completely overlapping. In umbilical cord blood, the lower reference limit was slightly higher in boys than in girls with non-overlapping 90% CIs while the upper reference limit had overlapping CIs. At 4 months the CIs did not overlap at the lower limit and only

marginally overlapped at the upper reference limit. Statistically non-significant, this could nevertheless point towards possible differences between the sexes for sTfR.

Table 5. Differences between the sexes for sTfR (mg/L) at the four different sampling time points.

	Lower reference limit		Upper reference limit	
Sampling time point	Girls	Boys	Girls	Boys
Umbilical cord	2.3 (2.0 – 2.4)	2.5 (2.5 – 2.7)	9.1 (8.4 –10.2)	9.4 (9.4 – 10.9)
48-96 hours	2.9 (2.7 – 3.1)	2.8 (2.6 – 3.0)	8.5 (7.8 – 9.1)	8.2 (7.2 – 9.1)
4 months	2.5 (2.4 – 2.5)	2.8 (2.7 – 2.9)	5.4 (5.0 – 5.7)	6.0 (5.7 – 6.3)
12 months	2.8 (2.7 – 3.0)	3.1 (3.0 – 3.3)	6.3 (5.8 – 6.7)	6.3 (6.0 – 6.6)

The hemoglobin and RBC RIs all had overlapping 90% CIs. Common RIs for the sexes were defined (Paper III).

Interference from the acute phase response and sTfR concentrations

In Paper II the association between the sTfR concentrations and CRP was investigated. This revealed a weak but statistically significant correlation at the sampling time point 48 to 96 hours, τ=0.20 (0.09 to 0.30), $p<0.0001$. No statistically significant associations were found in the blood from the umbilical cord, at 4 months or at 12 months.

Red blood cell dynamics after extremely preterm birth

Paper IV investigated RBC dynamics in infants from birth (gestational week 22+0 to 26+6) until corrected age 3 months. Median gestational age at birth was 24+4 and mean birth weight (SD) was 727 g (171 g). Participant characteristics are listed in **Table 6**. The distribution of available blood samples for each sampling time point and the proportion of infants born below or above gestational week 25 is shown in **Figure 4**.

Table 6. Participant characteristcs (Paper IV). Reprinted from Larsson et al. Arch Dis Child Fetal Neonatal Ed (123) under a CC BY 4.0 license, (http://creativecommons.org/licenses/by/4.0/).

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

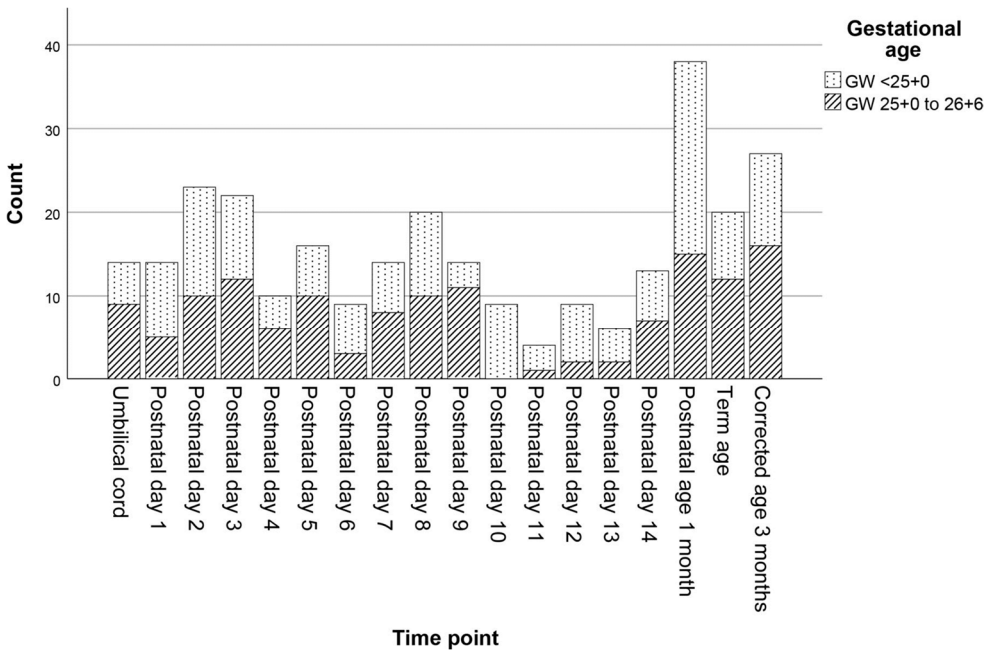


Figure 4. The number of measurements performed at each sampling time point (in total n=280). The proportion of infants born below and above gestational week 25 is shown for each sampling time point. Reprinted from Larsson et al. Arch Dis Child Fetal Neonatal Ed (123) under a CC BY 4.0 license, (http://creativecommons.org/licenses/by/4.0/).

Hyper-He in the umbilical cord blood showed a large inter-individual variability ranging from 1.5% to 24.9% (median 6.8%). The proportion rapidly decreased after birth and postnatal day 14, only a median proportion of 2.2% (0.6-5.5%) remained. At postmenstrual age 40 weeks, Hyper-He proportions had further decreased and

were <1% for all infants. Concurrently, median Hb in the umbilical cord blood was 133 g/L ranging from 115-146 g/L. At postnatal day 14, the median Hb concentration was 127 g/L (95-146 g/L) and at term age 104 g/L (91-136 g/L). **Figure 5** shows the decrease in Hyper-He proportions and the total Hb concentration for each sampling time point.

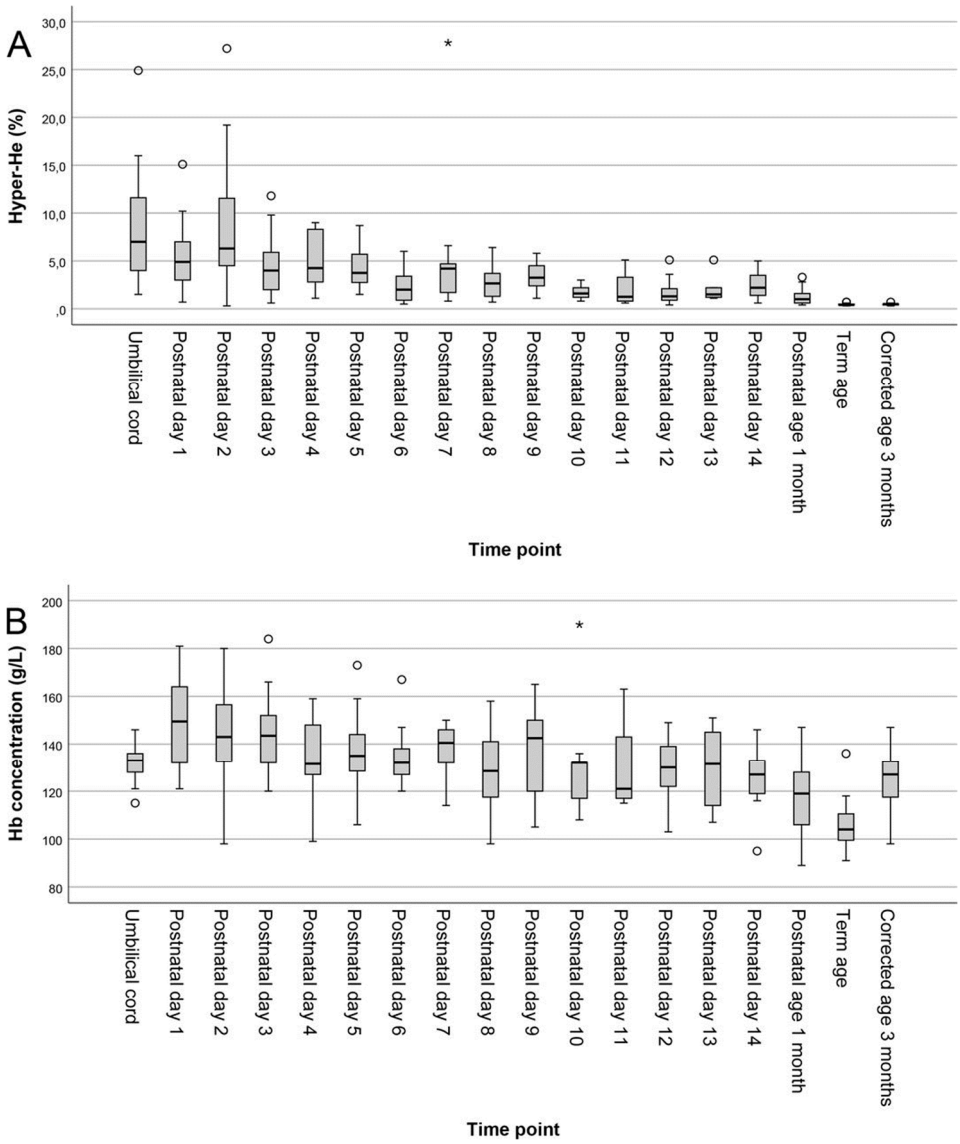


Figure 5. The decreasing proportions of Hyper-He (A) and the corresponding measurments of total Hb concentrations (B). Reprinted from Larsson et al. Arch Dis Child Fetal Neonatal Ed (123) under a CC BY 4.0 license, (http://creativecommons.org/licenses/by/4.0/).

Infants born after fetal growth restriction (FGR) (n=6) had early after birth (measured within 48 hours) a median Hyper-He of 11.8% (IQR 8.9-14.6%) compared with 5.5% (IQR 3.1-8.3%) in infants without FGR. While there was a statistically significant association between Hyper-He and birth weight; Spearman´s rho (CI) -0.38 (-0.63 to -0.07), a similar association between gestational age and Hyper-He was observed only after the removal of the data points from the infants born after FGR, Spearman´s rho -0.39 (-0.65 to -0.05). Scatter plots are shown in **Figure 6**.

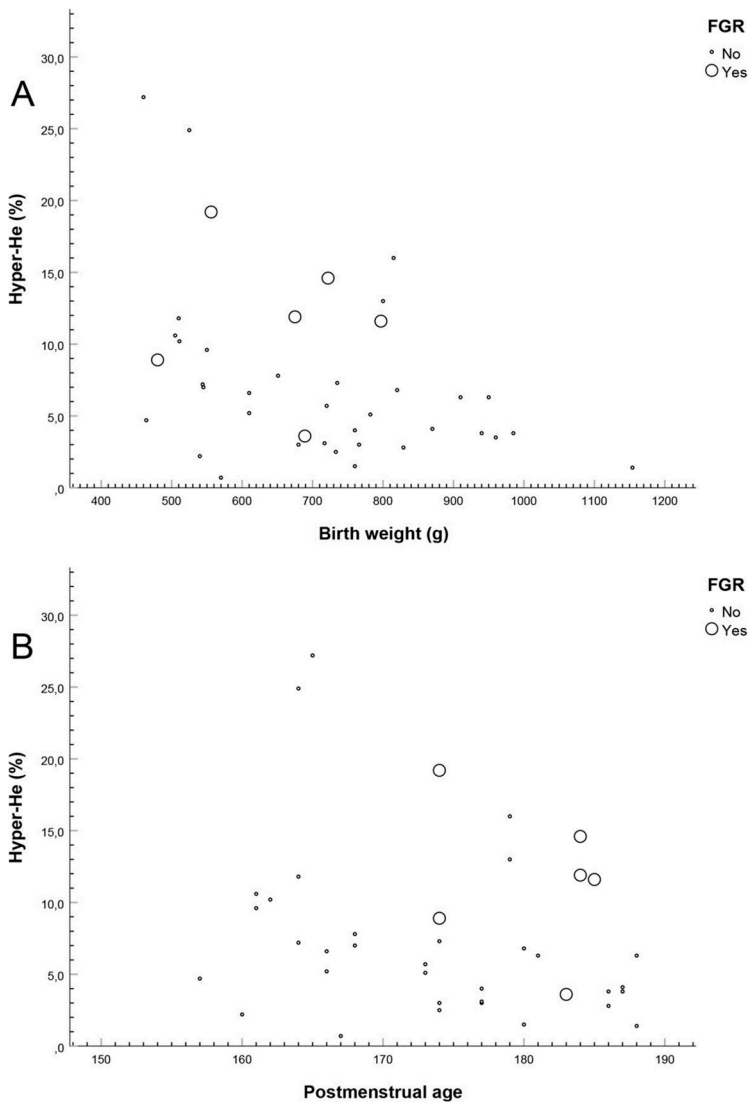
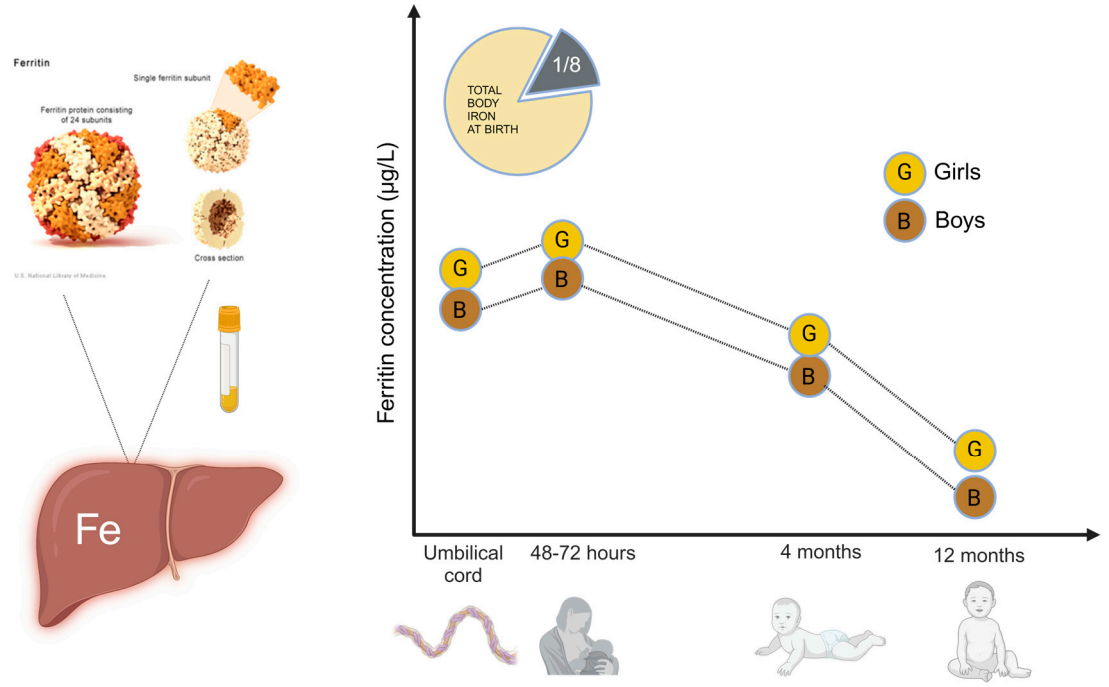


Figure 6. Scatter plots showing the assocation between Hyper-He and birth weight (A) and postmenstrual age (B) respectively. Reprinted from Larsson et al. Arch Dis Child Fetal Neonatal Ed (123) under a CC BY 4.0 license, (http://creativecommons.org/licenses/by/4.0/).

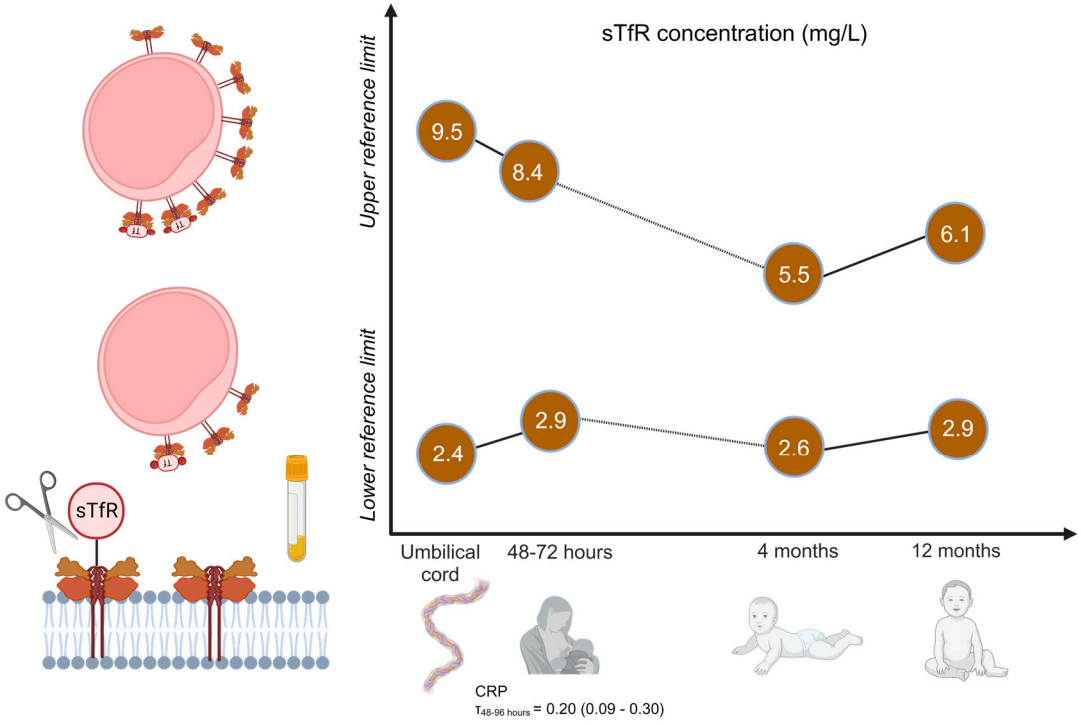
For term-born infants, the reference interval width for MCH was 7 pg (Paper III). For the extremely preterm born infants, an approximately two times higher variation (15 pg) was observed within the studied population. The 2.5th percentile was similar in term and extremely preterm born infants (32 pg) but the 97.5th percentile differed. This was 39 pg for term-born whereas for the extremely preterm born infants it was 47 pg. For the extremely preterm born infants, a high MCH did also correlate with a high proportion of Hyper-He early after birth, Spearman's rho 0.87 (CI 0.77-0.93), $p<0.001$. A similar correlation was observed for MCV, Spearman's rho 0.86 (CI 0.75-0.93).

Graphical summaries of the papers

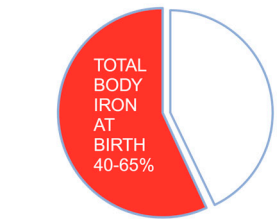
Paper I



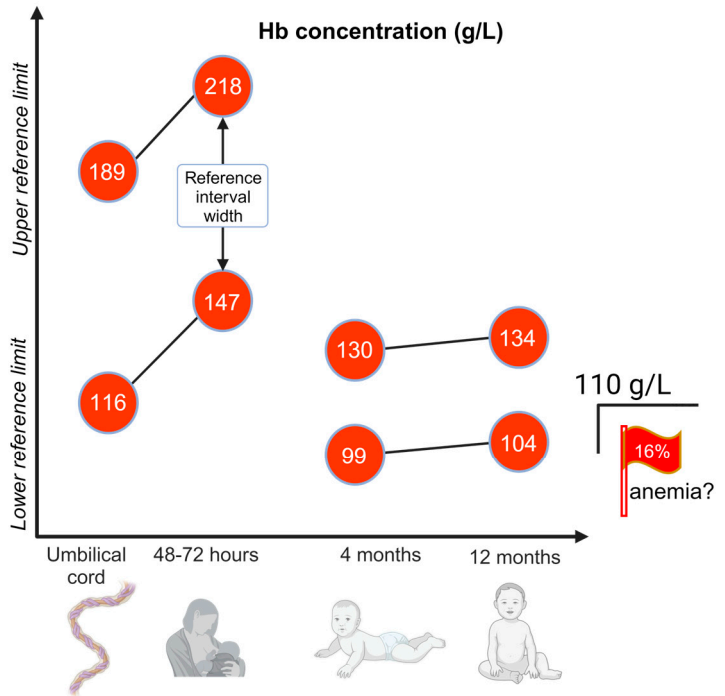
Paper II


sTfR
sTfR concentration (mg/L)
Upper reference limit
Lower reference limit
9.5
8.4
5.5
6.1
2.4
2.9
2.6
2.9
Umbilical cord
48-72 hours
4 months
12 months
CRP
T48-96 hours = 0.20 (0.09 - 0.30)

Paper III



Hb, Hct, MCV, MCHC,
MCH, RET, RBC, RET-HE



Paper IV

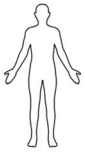
Mean Cell Hemoglobin Content (MCH)
27-33 pg

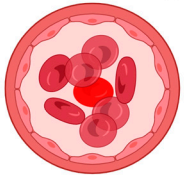
Mean Cell Volume
82-98 fL

Mean Cell Hemoglobin Content (MCH)
32-47 pg

Mean Cell Volume
99-139 fL

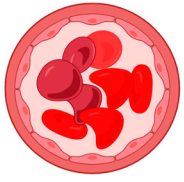
Proportion of Hyper-He (>49 pg/cell)





Adult blood

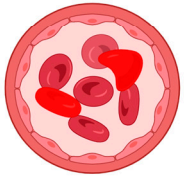
<1%



At birth

<25%

Fetal growth restriction
Low gestational age



Postnatal age 14 days

<5%

Discussion

Papers I, II and III address the aim of defining RIs based on a large cohort of presumably healthy, term-born infants, meeting the need for improved and updated comparative data. Paper IV describes RBC dynamics after extremely preterm birth and reveals a potential biomarker (Hyper-He) associated with gestational age and fetal growth.

Reference intervals

A major strength of the RI studies in this dissertation is the adherence to current international guidelines CLSI EP28-A3c (10). A key step is the definition of an RI population. As the RI studies used already collected data, this allowed RIs to be defined from a large cohort of presumably healthy infants. The recommended 120 individuals were exceeded and allowed for further investigation of partitioning boys and girls into subgroups.

Several of the variables included in the design of the original studies upon which the RIs are based, contribute to the combined cohorts' strengths as an RI population.

First, information on the health of the mothers and the pregnancies was included. Women were non-smoking, presumably healthy and the pregnancies were uncomplicated. The detrimental effects of tobacco use during pregnancy is well known and, in some countries recent reports estimate prevalence up to 10% (124). Regarding the pregnancies, fetal exposure prenatally to non-optimal conditions intra-uterine is known to have long-term effects on the health of the individual (125-127). Compared with the inclusion criteria of other recent RI studies (CALIPER, HAPPI Kids and PRINCE), uncomplicated pregnancy was applied only in the PRINCE study.

Second, the timing of clamping of the cord is a variable that affects iron and hematological status (128, 129). Acknowledged in older studies such as the study by Sturgeon et al. in 1959 (130), this information has mainly been lacking in modern RI studies.

Third, the infants were longitudinally followed from birth and onwards, and this allowed for infant growth to be assessed. On the contrary, the HAPPI Kids and CALIPER studies were based on cross-sectional designs and did not report data on infant growth.

Apart from these variables, the original studies were also conducted in socioeconomically strong settings with a low risk of infant iron deficiency. A food intake diary was recorded at 4 and 12 months, and neurodevelopment was assessed. These data were however not used as inclusion or exclusion criteria or further analyzed in these RI studies.

A further strength of the studies is the accuracy of the specified time points for blood sampling, thereby reducing the risk for false-negative flags due to broad age groups (21).

As reported by several authors, the prospective collection of infant data for the purpose of defining RIs has been associated with substantial difficulties (15, 131-133). In 2019, the Australian initiative (HAPPI Kids) (134) aimed to address current gaps by the inclusion of 20 neonates (birth to 72 hours) and 20 infants 30 to 365 days (attending hospital for minor elective surgery requiring general anesthetic). But even with this quite modestly set target, the initiative was restricted in its continuation due to the low consent rates. They reported inclusion rates of only 16%, making it impossible to define RIs without extrapolation from children of older ages (15). Other studies, such as the Canadian CALIPER initiative, used selected data collected from neonatal wards and outpatient clinics. Despite this, less than 50 infants representing this age group were included (135). The limitation of including to few individuals was acknowledged by the CALIPER project already in 2017 and a project particularly focusing on the neonatal and infant period was initiated (136). Nevertheless, to the best of my knowledge, no reports are as of this day yet published from this initiative.

Additionally, despite the initial intention to include neonates as well as infants, the recent initiative in China (PRINCE) published in 2022 did not include children younger than 3 months in their calculations. They were unable to collect the sufficient number of samples and also reported a high variability of biomarker results postnatally (131, 137). The PRINCE study therefore partitioned ages below 12 months into three broader age groups: 0.25 years, 0.5 years and 1 year. The high neonatal variability observed in their study, agrees with the findings reported in Paper III. For example, the reference interval width for Hb was 70 g/L in the neonatal period and only 30 g/L at 4 months.

For children from 6 months onwards, pediatric RIs for the RBC biomarkers have also been reported by Aldrimer et al. (138). Children in these studies were prospectively recruited in healthcare centers in the region of Falun, Sweden. For Hb, the study defined 6 months to 7 years as one age group based on the criteria of Lahti et al. (139). Very few children below the age of 12 months were included. As reported in Paper III, results for 12-month-old infants in our study were mainly in agreement with these data.

In a recent initiative, Mohammadi et al. defined RIs by direct methodology in Iranian presumably healthy infants (140). Their age groups were defined as from birth to 4 months and from 4 months to 30 months. The RBC dynamics during the first year of life in their study deviates from the findings of our study (Paper III).

For example, their lower reference limit for Hb first days in life is defined as 92 g/L. At the postnatal age 48 to 118 hours this is considerably lower than the lower reference limit defined Paper III (147 g/L) for a presumably healthy infant. This can be explained by the wide age groups defined by Mohammadi et al. and the lower reference limit in their study might be heavily influenced by data collected at postnatal age 2 months (141).

In the last few decades, most modern established infant RIs have been based on patient populations (16-20, 142, 143). With these RI methodologies, exclusions are usually based on data cleaning and the application of different mathematical criteria. RIs established with these approaches therefore heavily depend on the prevalence and the severity of disease in the patient population (3). Results are also influenced by the particular mathematical method used and on its underlying assumptions. In particular, this could be an issue with RIs based on data containing only hospital inpatients, such as the neonatal intensive care units.

Several indirect methodology reference intervals are based on the Hoffman approach (144). This method of defining RIs assumes that the Gaussian distribution within the database of patient results corresponds to a healthy population. Thereby, this range determines the reference intervals. Worthy of note, calculating RIs according to methodologies based on this assumption could have limitations (13, 113, 145). According to Shaw et al., the approach even resulted in negative numbers for the lower reference limit of iron concentration in a pediatric population (146).

In the data-mining initiatives such as the study reported by Zierk et al., the statistical assumption is instead based on the fact that RIs can be defined within the subpopulation of data that allows Box-Cox transformation to a Gaussian distribution (20). Besides the hypothetical relationship between the mathematical assumption and health, this could also be conflicting with a methodological observation in our study. Transforming the RBC biomarker data for the healthy infants by Box-Cox did not produce a satisfactory Gaussian distribution (unpublished data). Therefore, a non-parametric approach was chosen when defining RIs in Paper III.

The findings in Paper III, demonstrating deviations between indirectly derived and directly derived RIs at well-specified time points, show that further research with the aim of deepened understanding of data cleaning processes and indirect technique mathematical modelling are needed. Such initiatives, for example based on clustering analyses, are ongoing for older children (147) but are to the best of my knowledge not yet explored for infants the first year of life.

Dynamics

Hemoglobin and red blood cells in infants after term gestations

The current knowledge about the RBC dynamics in healthy infants was mainly established in the 1950s and -60s. In light of the technical advancements and harmonization activities between measurement systems, the numerical findings presented in these early studies risk being outdated. Nevertheless, the results of our study can be expected to follow the same dynamics after birth as these older studies.

In utero, the environment is relatively hypoxic. This hypoxia holds the pulmonary vasculature constricted through the action of hypoxia-inducible factors (148) thereby shunting oxygenated blood through the fetal circulation. At birth, with the oxygenation of the lungs, reversal of the pulmonary vasoconstriction allows the entire cardiac output to participate in gas exchange (149). The transition after birth also results in a fluid shift where an excess of extracellular fluid is redistributed.

With this fluid shift follows an increase in Hb concentrations. We observed an increase of 27% at the lower limit and 15% at the upper limit between umbilical cord blood and 48 to 118 hours (Paper III). These results might indicate slightly higher values compared with the observation by Gairdner et al. in the 1950s reporting a mean increase of 12% (51). A possible explanation for a smaller percentual increase observed in the older study, could be the differences in umbilical cord clamping practices. The older study meticulously practiced immediate clamping of the umbilical cord while the current studies are based on infants with delayed clamping.

The lower oxygen levels in utero also trigger an increased erythropoiesis, through hypoxia-inducible-factor activation of the Epo transcription gene (150). After birth, following higher oxygen saturation, the concentrations of Epo decrease. By this, fewer erythroid progenitors continue the erythropoietic lineage. Subsequently, the peripheral RBC count, as well as Hb, will gradually decrease. This occurs until a nadir is reached at 2 months (51). In the studies upon which this dissertation is based, no blood samples of the healthy infants were taken at 2 months. However, in a study published earlier this year Nielsen et al. reported a lower reference limit of 92 g/L at 2 months of age in Danish infants (141). This Hb concentration is substantially lower than the nadir 110 g/L suggested by Gairdner et al. in the 1950s (51). Importantly, the methodology to measure Hb in the older study differs from the methodology used today (117). The older study measured oxyHb and applied a correction factor of 0.87 to adjust venous blood to arterial. The basis of modern techniques is usually a conversion of Hb before measurement (60, 151, 152). Alternatively, a measurement across multiple wavelengths that reports the sum of the total Hb derivatives is used (153).

After 2 months, if sufficient iron is available to support erythropoiesis, Hb will gradually start to increase (154). But, since Gairdner et al. was limited to the age

interval between birth and 3 months they hypothesized that 110 g/L was the lower concentration where the bone marrow would respond (51). This presumed limit of 110 g/L has persisted until modern days (155, 156). Moe et al., however reported values already as early as the 1960s, indicating increases by approximately 4% between 4 to 12 months. This is coherent with the increase of approximately 5% observed in Paper III. The expected dynamics for Hb are shown in **Figure 7**. Contrary to our observations, a study using direct techniques published in 2021 suggested a single RI for Hb for infants 2 weeks to 36 months of age (157). This study however based this suggestion on a sample of approximately 20 infants differing in age from 2 weeks to 4 months, the median infant age in the cohort was 15 months.

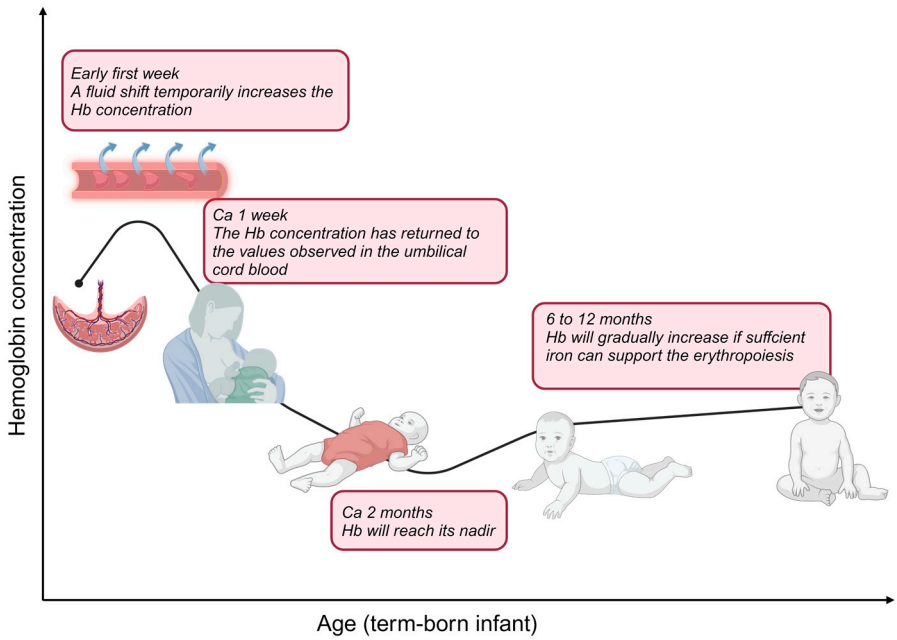


Figure 7. A schematic drawing of the dynamics in hemoglobin concentrations during the first year of life in a healthy term-born infant. The high hemoglobin concentrations early after birth increase further due to a shift of fluid from the vascular space. A gradual decrease is thereafter observed until 2 months of age. After 6 months Hb will continue to increase if the erythropoiesis is supported by the presence of sufficient iron.

Hemoglobin and red blood cells after extremely preterm birth

With preterm birth, the postnatal decrease of Hb concentration tends to be more rapid and severe (53). This has so far mainly been explained by the decreasing Epo stimulation following extra-uterine oxygen levels at birth, aggravated by losses due

to repeated blood sampling (158). The results of Paper IV could add further aspects to this model. Some extremely preterm born infants early after birth had a large proportion of peripheral RBCs with a particularly high Hb content (Hyper-He). As this proportion rapidly decreased after birth, infants with Hb distributed in high-content RBCs could be more at risk of developing severe anemia.

The rapid decrease in the proportion of Hyper-He cells is intriguing. It could be due to sampling-related blood loss and/or a dilution effect from pRBC transfusions. A further possibility is differing RBC properties, making them more susceptible to the loss of RBC membrane integrity. This hypothesis would be coherent with an earlier finding of an osmotically fragile Hb dense population in the umbilical cord blood of newborns (159). RBC survival has been estimated at only 35 to 60 days for preterm neonates compared with 60 to 80 days for the neonate born term (160, 161).

Additionally, the results of our study suggest that the peripheral RBC population show gestational age and growth dependent heterogeneity. This would be in line with the recent questioning of the classical model of HSC derived erythropoiesis. The precast hierarchy could hypothetically point to gestational age transitions where erythropoiesis in different lineages, with different niches as well as signaling could result in a differing number of cell divisions.

As for the initial hematopoietic wave RBCs, an extended RBC survival has been reported by Kingsley et al. (30). In their pre-clinical model, cells from this early origin were found in the peripheral blood even five days after birth. To the best of my knowledge, little is known about how the extent of erythropoietic transitions vary between individuals at the timing of extremely preterm birth.

Iron stores in infants after term gestations

The ferritin reference limits increased between the umbilical cord blood samples and 48-72 hours (Paper I). This increase (>130% at the lower limit and >20% at the upper limit) was more pronounced than the corresponding increase observed for Hb and the RBC counts (ca 25% at the lower limit and 15% at the upper limit). Thus, this could suggest influence on ferritin concentrations from other variables than the fluid shift.

Hypothetically, acute phase response triggered ferritin increases from labor might not have been sufficiently excluded by the stringent CRP criteria. Ferritin concentrations can remain elevated for several weeks after the acute phase response thereby persisting in the postnatal period (162, 163). The results therefore imply that ferritin, as a biomarker of iron stores first weeks in life could be unreliable.

The ferritin RIs defined in Paper I were compared with other studies using the Roche Cobas assay. Parkin et al. (2017) reported discrete RIs in Canadian infants defining 10 days to 12 months as one age group (164). Thus, a comparison of the longitudinal dynamics in our study with their results was not feasible. Worthy of note however was the lower reference limit reported in their study. Already from 10 days of age the lower limits were only 9 and 10 µg/L for boys and girls, respectively.

Their low reference limits might point towards an inclusion of iron deficient infants as they are even lower numerical findings than for the 12 months infants in our study.

The RIs from Paper I were also compared with the results reported by Bohn et al. in the CALIPER project in 2019 (135). They defined RIs for infants <6 months based on a total of 47 infants from outpatient clinics and maternity wards. The low ferritin limit suggested by their study, only 8 µg/L at 4 months, could also suggest an inclusion of infants with iron deficiency. In addition, their study points towards increasing iron stores between 1 and 12 months. This is unexpected for a healthy term-born infant where iron stores, on the contrary, are expected to decrease in the first year of life (165). The results from Paper I are depicted in **Figure 8**.

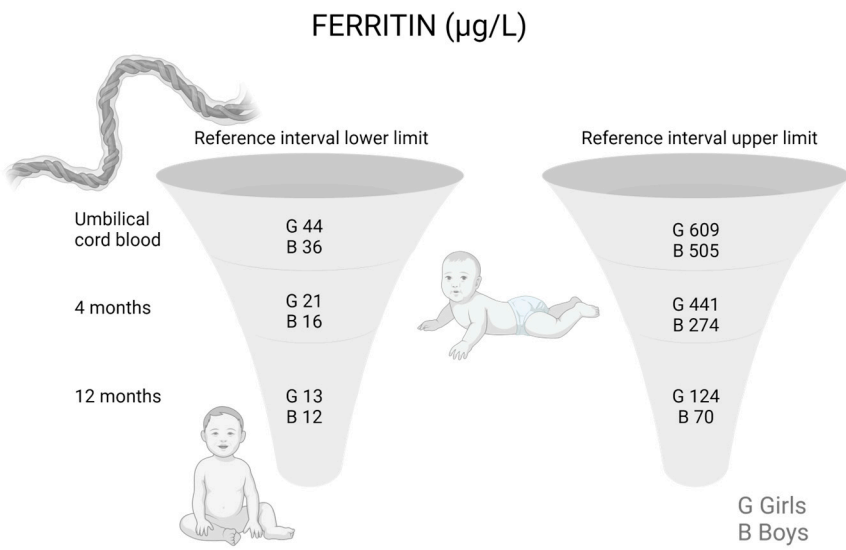


Figure 8. In healthy infants, the concentration of ferritin decrease the first year of life as the iron stores are used. The reference limits depicted in this figure are reproduced from Paper I (166).

Iron uptake in infants after term gestations

For sTfR, the RI upper limit during the first year of life reached its highest value in the umbilical cord blood (9.5 mg/L) and its lowest value at 4 months (5.7 mg/L) (Paper II). The infant upper reference limits were thereby considerably higher than both adult men (5.0 mg/L) and adult women (4.4 mg/L) (167).

The finding of high concentrations soon after birth is coherent with two previous reports where sTfR has been suggested to be a biomarker of erythropoietic activity (90, 168). However, we found a substantial variation in our population where the upper and lower reference limits spanned from 9.5 mg/L to as low as 2.4 mg/L.

Thus, for some infants sTfR concentration at birth was lower than at no other measured time point in the first year of life. As all presumably healthy term-born infants after uneventful pregnancies could be assumed to have increased erythropoietic activity at birth, an alternative hypothesis is needed.

The unexpected finding of an association between CRP and sTfR (Paper II), could offer an alternative explanation. Contractions during normal labor could, in addition to cytokine release, also due to the variations in oxygenation induce hypoxia inducible factor signaling. By that, increasing transferrin receptor expression in RBC precursors (169, 170).

Regardless, as for the other time points, it can not be excluded that the possibility of detecting a statistically significant effect was limited by the few number of individuals with increased CRP concentrations.

The sTfR concentration has previously been questioned as regards reflecting iron status in the newborn (90). Adding to this evidence is the rapid clearance of sTfR observed in our study (>12% at the upper limit) between umbilical cord and 48-72 hours (Paper II). The postnatal age when sTfR concentrations could reflect an upregulation in membrane receptors due to iron deficiency remains to be determined.

Further comparisons with other studies of sTfR dynamics during infancy were not possible with the currently available literature data. There were large inconsistencies in the results found due to the lack of assay standardization, arbitrarily chosen age groups, small study cohorts and issues with statistical methodology (Paper II).

Differences between the sexes

In Paper I, ferritin RIs were partitioned into subgroups by sex at all four time points from birth to 12 months. At 12 months, the upper reference limit differed by 54 µg/L between girls and boys, corresponding to a 44% difference.

These observed differences between the sexes are in conflict with the results published in 2019 by the CALIPER project. They reported equal numerical findings in girls and boys (135). This inconsistency is likely due to the differing sizes of the study cohorts. The study in Paper I was based on 456 infants, while the CALIPER cohort defined RIs on the results from 47 infants.

Differences between the sexes for ferritin are supported by the findings from both animal studies and studies of human fetuses and infants (67, 72-74). Although mean differences were not investigated in this work, the estimated difference of 44% at the upper reference limit for an infant at 12-months of age is coherent to the earlier studies by Domellöf et al. as they reported a statistically significant mean difference between girls and boys of 30% at 9 months of age (73). Similarly, the reported mean

difference reported by Emond et al. in the study of British 8-months old infants was 26% (74).

The differences between the sexes in Hb and MCV found in the study by Domellöf et al., could not be demonstrated in the RI studies within this project. This could be explained by differing methodological approaches as the current studies have investigated the lower and upper reference limits. A statistical testing of the mean differences was not performed in our study. Other plausible explanations are different interventions in the original studies (cord clamping vs iron supplementation) as well as differing imprecision in instrumentation and assays (Sysmex only vs Sysmex and Cell-Dyn, Roche vs Ramco). Furthermore, it could be due to the differing ages at which testing was performed.

Conclusions and future perspectives

This work has studied aspects of iron status dynamics and RBC dynamics in term-born infants following uncomplicated pregnancies. Thereafter, RBC dynamics in infants born extremely preterm have been further investigated.

The data presented in the first three RI studies can support interpretation of patient's results. Comparative data are also required for epidemiological studies and further studies are required regarding the observed disagreements with the WHO classifications for Hb and ferritin.

The dynamics observed in this and other studies call for the development of other comparative tools than the reference intervals in their classical discrete form. A suggestion for future work could be the development of continuous percentile charts or reference charts similar to the tools currently in use to assess infant growth. When age groups are defined in direct RI studies, care has to be taken that the age intervals reflect the physiological changes and the expected dynamics.

Gestational age is a variable generally not imported to the laboratory information systems. Improvements in this aspect is of particular importance. Studies with a cross-disciplinary approach aimed at developing technical solutions for enhanced presentation and reporting of lab results are warranted.

The indirect RI methodologies rely on data cleaning in combination with mathematical algorithms. Comparing our results with published RIs based on mathematically trimmed hospital data show that the development of indirect techniques needs further work for providing reproducible results. Such research initiatives are ongoing, but for older children. As comparative tools in the development of techniques, the data on the reference interval width reported in Papers I, II and III could be particularly useful. These are less affected by potential biases between measurement systems. In addition, studies that qualitatively investigate the clinical background of why and when an infant blood test is ordered, and the different clinical contexts, could provide insights on more reproducible data-cleaning strategies. A further way forward, could be the development of new mathematical approaches combining data from indirect and direct RI studies.

For ferritin, the results in this dissertation confirm differences between girls and boys during infancy. It needs to be further elucidated as to whether boys have an increased risk of infant iron deficiency.

The ferritin dynamics observed in this work might point to potential interference from the acute phase response in the assessment of iron stores in the neonatal period despite the stringent exclusions based on CRP. As regards sTfR: the high variation

within the population, the association with CRP at 48 to 96 hours, the rapid clearance observed at the upper limit in the newborn period as well as the high concentrations at the upper limit compared with adults, add to the existing evidence that points towards sTfR not being a biomarker for iron deficiency during the initial time period after birth. Thereby, neither ferritin nor sTfR may reflect iron status in the neonatal period. It remains to be determined at what age these biomarkers are reliable for the estimation of infant iron status and further studies of Ret-He as an alternative biomarker might offer a way forward.

With regard to most biomarkers studied, there is a large variation within the healthy population soon after birth compared with later on during infancy. This implies a substantial variation in fetal life iron acquisition and RBC properties. Further studies are needed to elucidate which maternal or fetal factors that are most important for fetal iron acquisition.

The results of Paper IV further underline the unique properties of the RBC population at the gestational age of extremely preterm births. The Hyper-He RBCs seemed to be mainly lost, or diluted by RBCs of adult origin, in the first 14 postnatal days. Frequent blood sampling could deprive the infant of large densely packed RBCs. The cells could hypothetically be important oxygen carriers in the transition after birth and their role for infant development during this important time frame needs to be further investigated. Moreover, technical developments reducing the need for frequent blood sampling as well as the actual blood volumes, are required.

The proportion of Hyper-He RBCs was associated with birth weight and gestational age. Furthermore, infants born after fetal growth restriction had seemingly higher proportions. Possibly, these increased proportions could be due to the degree of stress erythropoiesis. It remains to be determined if Hyper-He could be a potential biomarker to describe an individually increased risk of severe Hb nadirs in the first postnatal weeks.

Further research to better understand the transitional erythropoiesis, the iron interplay and the development of postnatal anemia is needed, in the aim of developing future clinical treatment options for the optimized support of the infants' endogenous erythropoiesis.

Acknowledgements

I have during these years learned how the deep commitment and dedication, huge amounts of work and the impressive collaboration of many, are foundations upon which clinical research is based. I would by this like to express my heartfelt gratitude to everyone who has contributed to this work and to my PhD studies.

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I would also like to thank the funders who made this work possible.

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Figures: Dissertation at a glance, 1-3, 7, 8 and the Graphical summaries were created using Biorender.com

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