Neonatal anemia and red blood cell transfusions: finding the optimal balance
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Chapter 11
General discussion and future perspectives
Chapter 11

Anemia is a common comorbidity in (preterm) infants admitted to the neonatal intensive care unit (NICU). Most very preterm infants develop anemia, and red blood cell (RBC) transfusions are, therefore, a frequently used neonatal treatment. Differences in transfusion practices may account for at least some of the large variety in long-term outcomes of the high-risk NICU population. As a result, research is required on both the short-term and long-term impact of the RBC transfusion strategy in medically vulnerable anemic infants, including preterm infants and term born infants born after perinatal asphyxia. Despite improvements in survival, the incidence of disability in the preterm population has not diminished accordingly. Since therapeutic hypothermia has been introduced, still up to 50% of infants with moderate to severe perinatal asphyxia die or has severe long-term neurological sequelae. A major long-term adverse outcome for these high-risk infants is neurodevelopmental and behavioral problems. Understanding the risk factors for abnormal neurodevelopmental outcome (NDO) is critical for implementing intervention strategies to improve outcome. Some of the key risk factors for adverse outcome are biological factors that are not modifiable following preterm birth: gestational age, birth weight, male sex, and being part of multiple birth. However, there are also factors with potential impact on developmental outcome that can be targeted for improvement. One of them is management of anemia, particularly RBC transfusion strategies. The most optimal strategy is, however, not clear as yet.

The primary aim of this thesis was to clarify controversial issues regarding neonatal anemia and RBC transfusions, specifically regarding the effects of anemic hypoxia on the one hand, and adverse effects of RBC transfusions on the other. This thesis provides insight into the short-term effects of both neonatal anemia and RBC transfusions on the brain and the intestine of preterm infants. Furthermore, this thesis provides evidence for an improved neurological outcome when using a lower limit of cerebral oxygen saturation to guide RBC transfusions in anemic preterm infants, rather than a threshold solely based on hemoglobin (Hb) level. Finally, this thesis addressed the influence of perinatal anemia in neonatal encephalopathy. The results of this thesis may therefore contribute to individualizing and improving NICU treatment for these vulnerable infants regarding anemia and RBC transfusions. The main findings are summarized in Table 1.
Table 1. Main findings of the thesis. Associations between anemia and red blood cell transfusions, and cerebral and intestinal outcome measures.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Brain (Ch. 3 and 5)</th>
<th>Outcome measure</th>
<th>Low hemoglobin level</th>
<th>Red blood cell transfusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preterm infants (GA &lt; 32wks)</td>
<td>$r_{SO_2}$</td>
<td>↓</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Preterm GMs quality</td>
<td>↓</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GMOS</td>
<td>↓</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>Intestine (Ch. 4, 6 and 7)</td>
<td>$r_{SO_2}$</td>
<td>↓</td>
<td>$&lt;24$ h before RBCT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$r_{SO_2}$ variability</td>
<td>↓</td>
<td>$≤3$ days before RBCT</td>
<td>$\approx$</td>
</tr>
<tr>
<td></td>
<td>8-isoprostane</td>
<td>NI</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td></td>
<td>I-FABP</td>
<td>↑</td>
<td>$≤6$ days before RBCT</td>
<td></td>
</tr>
<tr>
<td>Term infants who underwent TH because of NE (Ch. 10)</td>
<td>Brain (Ch. 10)</td>
<td>$r_{SO_2}$</td>
<td>↓</td>
<td>NI</td>
</tr>
<tr>
<td></td>
<td>Abnormal aEEG background pattern</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>aEEG epileptic activity</td>
<td>↓</td>
<td>NI</td>
<td></td>
</tr>
<tr>
<td></td>
<td>aEEG SWC</td>
<td>↓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outcome (Ch. 9 and 10)</td>
<td>Mortality</td>
<td>↑</td>
<td>NI</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NDO at 2-3y</td>
<td>↑</td>
<td>NI</td>
<td></td>
</tr>
</tbody>
</table>

Red arrows indicate negative associations, green arrows indicate positive associations, and arrow direction indicates the direction of the association.

Abbreviations: aEEG, amplitude-integrated electroencephalography; GA, gestational age; GMs, general movements; GMOS, general movement optimality score; I-FABP, intestinal fatty acid-binding protein; NDO, neurodevelopmental outcome; NE, neonatal encephalopathy; NI, not investigated; RBCT, red blood cell transfusion; $r_{SO_2}$, cerebral tissue oxygen saturation; $r_{SO_2}$, splanchnic tissue oxygen saturation; SWC, sleep-wake cycling; TH, therapeutic hypothermia.
Main findings

Part I – Literature overview
In the first part of this thesis (Chapter 2), we present a systematic overview of the literature on the impact of anemia and RBC transfusions during NICU admission on cerebral tissue oxygen saturation ($r_cSO_2$), measured using near-infrared spectroscopy (NIRS), brain injury and structural brain development, and NDO in very preterm infants. From the literature to date, we conclude that anemia of varying severity indeed appears to reduce cerebral oxygen supply. RBC transfusions, conversely, improve cerebral oxygen supply. These results indicate that anemia and RBC transfusions contribute significantly to brain development and NDO, possibly by its association with cerebral oxygen saturation: severe anemia during NICU admission seems to be associated with disturbances of structural brain development, even though findings on long-term outcome suggest potential neuroprotective benefits from a restrictive transfusion strategy. Based on the results of this systematic review, individualized care regarding RBC transfusions, with attention to cerebral tissue oxygen saturation, seems reasonable and even desirable in preterm infants.

Part II – Anemia and RBC transfusions in preterm infants during their NICU stay
This part of the thesis focuses on anemia and RBC transfusions in a cohort of preterm infants during their NICU stay. In these preterm infants, Hb level was strongly related to $r_cSO_2$. Hemoglobin level on Day 1 was associated with impaired neurological functioning on Day 8, as measured in terms of quality of general movements (GMs) (Chapter 3). Early neurological functioning, expressed by the quality of GMs and its detailed score, improved instantly in preterm infants after their first RBC transfusion, as did $r_cSO_2$ (Chapter 5). Regarding the intestine, Hb level before RBC transfusion was associated with signs of intestinal cell injury in anemic preterm infants during the first weeks after birth (Chapter 4). The magnitude of markers of intestinal injury were associated with lower Hb levels and with diminished variability of splanchnic oxygenation (Chapter 4). RBC transfusions were associated with biomarkers of oxidative stress and intestinal injury in preterm infants. Moreover, both biomarkers were also strongly interrelated (Chapter 6). Reduced splanchnic oxygen saturation variability, more pronounced than actual level of splanchnic tissue oxygen saturation ($r_cSO_2$), may represent hypoxic and/or hyperoxic intestinal injury (Chapter 4, 6 and 7). Finally, an individualized strategy using a lower $r_cSO_2$ limit to guide RBC transfusions in preterm infants improves the neurological outcome at 3 months corrected age (CA), compared with a transfusion threshold based only on Hb level (Chapter 8).

Part III – Perinatal anemia in term infants with neonatal encephalopathy
In this part we present the association between perinatal anemia and mortality and NDO in term infants with neonatal encephalopathy (NE). Perinatal anemia was associated with higher mortality. Anemic survivors, however, had a favourable NDO during infancy (Chapter 9). Infants with perinatal anemia showed lower $r_cSO_2$ and more frequently suppressed amplitude-integrated electroencephalography (aEEG) background patterns, but fewer seizures during therapeutic hypothermia (TH) (Chapter 10).
General discussion
Balance between neonatal anemia and red blood cell transfusions

Preterm infants often develop anemia and frequently require RBC transfusions during neonatal intensive care. Although it is widely known that anemia can cause hypoxic injury, consensus is lacking on the optimal RBC transfusion practice during NICU stay. Results of studies on liberal versus restrictive transfusion thresholds with regard to short-term effects on apnea, tachycardia, and bradycardia, and long-term effects, especially the neurological outcome, were contradictory.3-10 Recently published trials, however, demonstrate evidence on the safety of a more restrictive RBC threshold.11,12 Concerns regarding the association between RBC transfusions and development of necrotizing enterocolitis (NEC) may further restrict transfusion policies in preterm infants. Nevertheless, severe anemia may cause harm in preterm infants13, emphasizing the need to reconsider too restricted transfusion guidelines. Transfusing preterm infants to improve oxygen-carrying capacity or restricting RBC transfusion strategies to avoid transfusion-associated risks may both potentially impair cerebral and intestinal short-term and long-term development.

The preterm brain
Anemia and the preterm brain

We found that anemia, by its effect on the oxygen-carrying capacity, has a major impact on cerebral tissue oxygen saturation (Chapter 2 and 3). It was already known that the preterm brain is particularly vulnerable to hypoxia.14 We now confirmed this by demonstrating in preterm infants that lower Hb levels on Day 1 were independently associated with poorer neurological functioning on Day 8, as reflected by the quality of GMs including its detailed scoring (general movement optimality score, GMOS) (Chapter 3). We measured the neurological condition already after the first week, which supports, although not confirms, the notion of causality between Hb level on Day 1 and impaired neurological functioning. Even so, general movement assessment (GMA) on Day 8 is the closest moment to the risk factor of interest, that is, Hb level at Day 1. The association between impaired neurological functioning on Day 8 and low Hb on Day 1 possibly reflects cerebral injury, appearing to be, at least partly, mediated by low $r_2SO_2$ on Day 1 (Chapter 3). Hemoglobin levels on the day of GMA did not remain associated with neurological functioning on Day 8. This suggests that the cerebral injury following low Hb levels is related to the timing of anemia, of which the first day after birth appeared to be the most sensitive period (Chapter 3). Apart from its prognostic value15-19, GMA is also the most reliable method at this young age to assess the integrity of the young nervous system.20,21 We speculate that a low Hb level and its concomitant low $r_2SO_2$ particularly on Day 1 have an impact on optimal brain functioning and development of injury. The first day after birth can be especially characterized by a state of low cerebral blood flow and high oxygen demand.14 Previously, low $r_2SO_2$ on the first day after birth has also been shown to be predictive for poorer NDO during infancy.22
Potential pathological effects of severe or prolonged anemia may first be, (acute) hypoperfusion and hypoxia result in end-organ ischemic injury, such as hypoxic-ischemic white matter injury, intestinal ischemia, and renal failure. Second, progressive anemia may increase the frequency of apnea, tachypnea, tachycardia, and hypoxia. Third, reperfusion injury may also account for part of the end-organ injury.

The association between low Hb levels and low cerebral oxygen saturation may reflect an underlying mechanism of cerebral anemic hypoxic injury or dysfunction. The associations we found between pre-transfusion Hb levels and pre-transfusion \( r_{SO_2} \), and between pre-transfusion Hb levels and lower GMOS before RBC transfusion also support this hypothesis (Chapter 5). The presence of low \( r_{SO_2} \) suggests the presence of cerebral hypoxia. Hypoxia implies an imbalance between oxygen supply and demand, in other words limited oxygen availability to meet the tissue’s metabolic demand. The development of white matter injury, which is associated with neurodevelopmental delay, may, in part, result in poorer outcomes through the exposure to either early severe or prolonged anemia. We speculate about possible mechanisms. First, hypoxia in itself triggers a cascade of events, both compensated (physiological) and non-compensated (pathological). The pathological scenario leads to cellular changes involving enzyme activities, mitochondrial function, membrane transport, and antioxidant defenses, resulting in a failure of ATP production and an altered cell membrane potential, favoring the influx of water into the cell. Pathological (anemic) hypoxia results, in this way, in the production of cytotoxic edema, which in itself is a cause for neuronal injury. Second, pre-oligodendrocytes are particularly susceptible to hypoxic-ischemic related injury, because of their immature, relatively low antioxidant capacity. These progenitors of oligodendrocytes dominate the white matter of preterm infants. Third, preterm infants likely compensate for decreased \( r_{SO_2} \) by increasing oxygen extraction. High levels of cerebral tissue oxygen extraction (cFTOE) have been associated with an increased risk of developing intraventricular hemorrhage (IVH). Since Hb level is an important determinant of \( r_{SO_2} \) and cFTOE, cerebral anemic hypoxia might in this way be involved in brain injury. This speculation is supported by the higher incidence of severe IVH and periventricular leukomalacia in preterm infants in infants treated following the restrictive RBC transfusion strategy of the IOWA trial (Chapter 2). Conversely, Kirpalani et al. reported a slightly lower incidence of IVH in the restrictive threshold group of the PINT trial. Both recently published large randomized controlled trials, however, did not report an adverse effect of a restrictive RBC transfusion strategy on the incidence of severe IVH. Fourth, oxidative stress on account of hypoxia-ischemia, measured by high levels of cord blood isoprostane, has also been associated with white matter injury in preterm infants.

Red blood cell transfusions and the preterm brain

The RBC transfusion helps preterm infants by increasing circulatory Hb, improving tissue oxygen saturation, and reducing the need for compensatory mechanisms (Chapter 2 and 5). We now add that RBC transfusions seem to improve the neurological condition instantly in preterm infants, as expressed by general and detailed GMA (Chapter 5). Following RBC transfusion, both neurological functioning and cerebral oxygen saturation quickly improved, suggesting the improved neurological condition to be mediated by adequate oxygen supply to the brain.
As improved early neurological functioning is associated with improved oxygen-carrying capacity, that is, Hb level, and its concomitant improved $r_c SO_2$, the RBC transfusion may also benefit long-term neurological development by preventing anemic hypoxic injury. In line with our results, Andersen et al. also already speculated that an RBC transfusion may be neuroprotective.25

The improved oxygen-carrying capacity from the RBC transfusion potentially contributed to the improved infants’ neurological condition. Intermittent episodes of hypoxemia were previously associated with adverse long-term outcomes.16 However, the two recently published large randomized controlled trials did not confirm significant benefits of an increased number of RBC transfusions on long-term outcome, instead, they found that a liberal RBC transfusion strategy did not improve survival or neurodevelopmental impairments.11,12 A possible explanation might be the suggested side effects of RBC transfusions through oxidative stress inducing negative effects on the central nervous system.37,38 Another explanation might be an inflammatory response to RBC transfusions during NICU stay. This may be sex-dependent, because girls seem to benefit more from a restrictive transfusion strategy than boys, as girls show poorer cognitive outcomes with increased number of transfusions, while boys show poorer outcomes in the presence of more severe anemia.9,10,39,40 In our results, we also found a more pronounced beneficial effect of number of transfusions in boys, as compared to girls. The fact that delayed cord clamping, which gives the newborn infant a higher circulating blood volume and higher Hb/Ht levels, was previously also associated with more pronounced protective effects for boys, supports the theory that additional blood volume may have sex-specific neuroprotective effects.41

**Cerebral hypoxia and cerebral hyperoxia, measured using near-infrared spectroscopy**

Previously, the underlying role of cerebral hypoxia and hyperoxia regarding poorer outcomes of preterm infants was investigated by several researchers. They reported both low $r_c SO_2$, or an increased burden of cerebral hypoxia, and also high $r_c SO_2$, or an increased burden of cerebral hyperoxia, to be associated with poorer NDO.22,42,43

This thesis provides evidence that cerebral oxygen saturation is a promising tool for predicting neurological outcome in preterm infants (Chapter 2, 3, and 5). Anemia and the resultant decrease in oxygen-carrying capacity presumably increase the burden of cerebral hypoxia in preterm infants (Chapter 2 and 3). Correcting anemia by RBC transfusions is presumed to enhance oxygen supply to vital organs, including the brain (Chapter 2 and 5). Lower $r_c SO_2$ is associated with more desaturations and hypoxic episodes in preterm infants.44-47 Early low $r_c SO_2$ may partly explain the poorer neurological condition that we found in preterm infants on Day 8, whereas increased $r_c SO_2$ after the first RBC transfusion in preterm infants had a moderate association with the improvement in neurological functioning (Chapter 3 and 5). Conversely, exposure to hyperoxia has also been associated with cell injury in the developing brain.25,37,48,49 Possibly, the actual brain injury happens after re-oxygenation. Hyperoxia causes oxidative stress because of a cascade of free radical production and toxic reactive oxygen species. Prolonged cerebral hyperoxia has, for example, been associated with higher risks of retinopathy of prematurity.50

Thus, in our opinion, our results demonstrate the added value of NIRS-derived $r_c SO_2$ measurements. However, we have to address the limitations of NIRS as a technique, the most
critical concerning validity and precision, and the generalizability of \( r_{SO_2} \) values between devices and sensors. Nevertheless, strong relations between \( r_{SO_2} \) measurements and outcome up until 2 to 3 years of age have been published, and we found important associations in relation to the brain and its development, as reflected by GMA.

**Individualized treatment for red blood cell transfusions in preterm infants**

Both evidence for the benefits and risks of anemia (Chapter 3 and 4) and of RBC transfusions (Chapter 5, 6, and 7) has raised potential concern for the safety of very strict RBC transfusion thresholds tolerating severe anemia, particularly since transfusion practices solely based on Hb level may not adequately incorporate an individual patient’s clinical status and illness severity. Hemoglobin level on its own may also not fully reflect tissue oxygenation in vital organs, such as the brain.

This thesis provides evidence on the added value of an individualized RBC transfusion strategy using NIRS-based \( r_{SO_2} \) measurements in preterm infants (Chapter 8). Preterm infants randomized to an RBC transfusion strategy using a lower \( r_{SO_2} \) limit as transfusion threshold, had a favorable neurological outcome at the CA of 3 months, as measured by their early motor repertoire, compared to control infants who were treated in accordance with routine clinical care using a fixed Hb threshold for RBC transfusion (Chapter 8). Currently, the early motor repertoire at three months CA, consisting of evaluation of the qualitative and quantitative movement and postural patterns, is considered the best available method to assess the integrity of the young nervous system and the short-term neurological outcome with excellent diagnostic and prognostic values for both motor impairments and neurological dysfunction at school age. The main findings of Chapter 8 are presented in Table 2.
Table 2. Main findings of the POCKET study (Chapter 8)

<table>
<thead>
<tr>
<th>RBCT threshold</th>
<th>Number of RBCT</th>
<th>Mean Hb level throughout trial</th>
<th>Mean rSO₂ throughout trial</th>
<th>IVH &gt; Grade II at 40wks PMA</th>
<th>NEC ≥ Bell’s stage II at 40wks PMA</th>
<th>In-hospital mortality</th>
<th>Early motor repertoire at 3 months’ corrected age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Based on rSO₂ limit</td>
<td>55 RBCTs in 21 infants</td>
<td>8.7 mmol/L *</td>
<td>75% *</td>
<td>n = 3</td>
<td>n = 8</td>
<td>n = 3</td>
<td>22 infants (50%) had an optimal early motor repertoire * Median MOS-R 25 (IQR 24 – 26) * 44 infants (98%) had normal FM</td>
</tr>
<tr>
<td>Based on fixed Hb threshold</td>
<td>25 RBCTs in 12 infants</td>
<td>9.4 mmol/L</td>
<td>72%</td>
<td>n = 5</td>
<td>n = 6</td>
<td>n = 1</td>
<td>13 infants (27%) had an optimal early motor repertoire Median MOS-R 24 (IQR 22 – 26) 50 infants (100%) had normal FM</td>
</tr>
</tbody>
</table>

Abbreviations: FM, fidgety movements; MOS-R, motor optimality score-revised; Hb, hemoglobin; IQR, interquartile range; IVH, intraventricular hemorrhage; NEC, necrotizing enterocolitis; PMA, postmenstrual age; POCKET, Preterm Oxygenation of the Cerebrum: Key for Erythrocyte-transfusion Threshold; RBCT, red blood cell transfusion; rSO₂, cerebral tissue oxygen saturation; * p<0.05 between randomisation groups.
Cerebral oxygenation guiding red blood cell transfusions

Over the past years, several other authors also suggested that tissue oxygenation itself may play an important role in identifying the trigger for RBC transfusion. During anemia, both a decrease in the oxygen-carrying capacity of blood and a decrease in blood viscosity occurs, possibly resulting in increased cerebral blood flow. Compensatory increased cerebral blood flow during anemia, however, seems insufficient to normalize cerebral oxygenation. Understanding the factors related with tissue oxygen extraction may guide clinicians in the decision making for RBC transfusions. High cFTOE under baseline conditions leaves little reserve to meet brain tissue oxygen demands, particularly during oxygen desaturations. Therefore, cerebral oxygen supply may become compromised more easily in those infants with frequent desaturations and hypoxemic episodes, potentially threatening the integrity of the preterm brain. It may be clinically important to identify the vulnerable group with low cerebral oxygenation to administer RBC transfusions at the right time, improving their short-term and long-term outcomes. Our findings of Chapter 8 support this notion, on which we will elaborate in the next paragraph.

Preventing cerebral hypoxia

Despite of administering more RBC transfusions, mean Hb level during the study period was slightly lower in infants in the intervention group compared to infants in the control group (Chapter 8). Probably due to the RBC transfusions, mean rSO₂ was slightly higher in infants in the intervention group than in control infants. This suggests that moderate anemia might be well-tolerated in preterm infants as long as the cerebral oxygen saturation is sufficient. In this trial we investigated the effect of preventing prolonged cerebral hypoxia in anemic preterm infants. Although the optimum timing of intervention continues to be a matter of debate in the neonatal literature, there is accumulating evidence showing the beneficial effects of early intervention on preventing cerebral hypoxia.

We found no differences in the presence of both in-hospital mortality, and neonatal morbidities such as IVH, NEC, bronchopulmonary dysplasia, and retinopathy of prematurity (Chapter 8). These morbidities are associated with hypoxia and ischemia-reperfusion damage in varying degrees. Our trial, however, was underpowered to detect differences in secondary outcomes. Despite of the similar incidence in NEC in both groups, the impact of this RBC transfusion strategy on splanchnic oxygenation and intestinal injury is an aspect that needs further attention.

The preterm intestine

Anemia and the preterm intestine

It has been proposed that anemia may lead to a decreased intestinal oxygen delivery. A limited intestinal oxygen supply may result in intestinal hypoxia, possibly leading to impaired mucosal integrity and intestinal injury. We are the first to present biochemical evidence for intestinal injury in the presence of anemia in preterm infants prior to RBC transfusion, as lower Hb levels were associated with higher urinary levels of intestinal fatty acid-binding protein (I-FABP), a
specific biomarker for intestinal cell injury.\textsuperscript{58} Low $r_S O_2$ shortly before RBC transfusion was also associated with I-FABP level (Chapter 4). These results suggest that anemia and intestinal tissue hypoxia are associated with (subclinical) intestinal injury.

Anemia may be related to intestinal hypoxic injury through several potential mechanisms. First, preterm infants are able to compensate for a decreasing Hb level by circulatory adjustments, such as increased cardiac output and increased tissue oxygen extraction.\textsuperscript{13} Profound anemia, however, results in tissue hypoxia when oxygen supply does not meet its demand. Particularly the preterm intestine may have a smaller reserve to respond to disturbed intestinal oxygenation.\textsuperscript{59} Second, we speculate that preterm infants in the presence of severe anemia may have a preferential flow to the brain, leading to a reduced intestinal blood flow, aggravating intestinal hypoxia. In contrast to the relatively well controlled blood flow to and consistent metabolism of the brain, the $r_S O_2$ is highly dependent on the balance between oxygen supply and varying demand. Decreased $r_S O_2$ may, thus, be secondary to either reduced splanchnic oxygen delivery as a result of anemia, or to an increased oxygen consumption. Third, anemic infants may have less ability to meet the relatively high metabolic demand of feeding and digestion, potentially making them more vulnerable to an imbalanced oxygen supply and demand.\textsuperscript{60} In this thesis, however, we did not investigate the effect of feeding. Ongoing trials comparing transfusion thresholds and the effect of feeding during anemia and RBC transfusions will hopefully provide important information.

**Red blood cell transfusions and the preterm intestine**

Red blood cell transfusions have the ability to improve splanchnic tissue oxygen saturation.\textsuperscript{45,61} We confirmed this in Chapter 6, in which we demonstrated increased $r_S O_2$ after RBC transfusion, even up to 24-hours later. Lack of baseline $r_S O_2$ before transfusion may have precluded us from finding differences in $r_S O_2$ after RBC transfusion in Chapter 7. Limited success of the transfusion, reflected by a relatively small increase in Hb and $r_S O_2$, was associated with intestinal injury and NEC development (Chapter 6 and 7).

Over the past years, the relation between splanchnic oxygen saturation and intestinal injury has received increasing attention.\textsuperscript{62-70} In line with our results described in Chapter 6 and 7, Marin et al. reported greater $r_S O_2$ fluctuations and also decreased $r_S O_2$ in infants who developed NEC after RBC transfusion compared with infants who did not develop NEC after transfusion.\textsuperscript{64} Furthermore, relatively high $r_S O_2$ values following relatively low values were previously seen in infants who developed NEC after transfusion, supporting the role of ischemia-reperfusion injury.\textsuperscript{63,71} Nevertheless, the fact that we found that a relatively small increase in $r_S O_2$ after RBC transfusion was associated with intestinal injury (Chapter 6), seems to contradict this theory of re-oxygenation injury. Based on our results described in Chapter 4, 6, and 7, and the conflicting reports on high or low actual $r_S O_2$ values in infants who developed NEC, we speculate that the role of splanchnic perfusion on intestinal injury during and after RBC transfusion is predominantly reflected in an altered $r_S O_2$ variability, rather than in the absolute $r_S O_2$ value.

Following RBC transfusions we also found an increase in urinary 8-isoprostanate and I-FABP, biomarkers for oxidative stress and intestinal injury, respectively (Chapter 6). Our result on the strong relation between the magnitude of change in 8-isoprostanate and I-FABP levels from before to after RBC transfusion offers a potential explanation for the association between RBC
transfusions and intestinal injury (Chapter 6). It suggests an important role of oxidative stress in
the pathogenesis of intestinal cell injury following RBC transfusion, that could predispose infants
to NEC. A similar association we found in Chapter 6, between oxidative stress and intestinal injury,
has also been observed in cord blood of late preterm infants. A possible explanation through
which the RBC transfusion may influence injury of intestinal wall integrity, is the imbalance
between oxygen supply and demand. The anemic hypoxic state followed by the hyperoxic state
after RBC transfusion may lead to intervals of recurrent hypoxia and re-oxygenation. These
intermittent changes in the tissue oxygenation may lead to an increase in oxidative stress, which
may consequently cause intestinal injury. This, however, remains speculative as we were not
able to confirm the association between oxidative stress and splanchnic oxygen saturation. A
possible explanation for the transfusion-associated oxidative stress to be less reflected by actual
rSO2 values, would be that rSO2 does not properly reflect oxygen availability to the tissue, as it
measures predominantly oxygen saturation of venous blood. Another speculative explanation for
transfusion-related injury is a potential transfusion-related immune response, characterized by an
increased pro-inflammatory cytokine production and epithelial activation. Several bioactive
substances, such as circulating iron, might have contribute to such a transfusion-related immune
response. An uncontrolled inflammatory response has previously been associated with continuing
intestinal injury, and a reduced capacity for repair of the intestinal mucosa.

**Development of necrotizing enterocolitis**

The current concerns that a common neonatal physiological state (anemia) and intervention
(RBC transfusion) may predispose preterm infants to NEC development, make that
understanding the potential causal effects of anemia and RBC transfusions together with the
underlying pathophysiology is important to both NICU clinicians and researchers.

A causal link between severe anemia, RBC transfusions, and NEC has been proposed, but
not proven. Because RBC transfusions are used to treat anemia, and more severely anemic infants
are more likely to be transfused, anemia and transfusion are difficult to separate in observational
studies. Several possible pathophysiological mechanisms regarding RBC transfusion strategies
have been addressed that may explain the pathogenesis of transfusion-associated NEC. In
our studies, we offered biochemical evidence that both anemia and the RBC transfusions are
associated with intestinal injury (Chapter 4 and 6).

The results described in Chapter 4 and 6 suggest that both anemia and the RBC transfusion
increase the susceptibility for NEC development, and provide support for the hypothesis that
the development of NEC after RBC transfusion is probably not related to a single factor but
more or less dependent on a combination of factors. Infants in our sample, who developed NEC
subsequently after RBC transfusion tended to have a lower pre-transfusion Hb level than infants
who did not develop NEC after transfusion, which supports the aforementioned hypothesis. The
combination of the exposure to anemia and RBC transfusions contributing to NEC development
may be explained by a phenomenon similar to Knudson’s two-hit hypothesis. The incidence
of consecutive manifestations, in our case anemia and the subsequent RBC transfusion, may
be determining repetitive factors in developing transfusion-related intestinal injury. Potentially,
anemia and RBC transfusions may even be the fourth and fifth hit, on top of preterm birth with
an immature intestine, a potential aberrant intestinal microbiome\textsuperscript{82}, and an underdeveloped innate immune system with Toll-like receptor 4 activation.\textsuperscript{83}

A study in mice investigated the interaction between anemia and RBC transfusions and tried to find the underlying pathophysiological mechanism that could explain transfusion-related intestinal injury.\textsuperscript{84} Increased intestinal permeability and increased macrophage activation was found in both anemic and anemic transfused mice. An increase in biomarkers for intestinal injury, however, was only found in the anemic transfused mice, as compared with their anemic controls. Besides, an RBC transfusion did not cause harm when administered to mice in the absence of preceding anemia. This might indicate a progressive susceptibility for intestinal injury following RBC transfusion after the presence of severe anemia. Furthermore, this may support the hypothesis of re-oxygenation injury rather than a transfusion-related immune response. We speculate that anemia might be the priming step, whereas the RBC transfusion might be the activation step inducing intestinal injury, in some infants leading to NEC.

**Splanchnic oxygen saturation and its variability, measured using near-infrared spectroscopy**

Near-infrared spectroscopy monitoring shows real-time organ oxygen saturation that correlates with changes in systemic or regional perfusion.\textsuperscript{85} The validity of splanchnic oxygen saturation measurements, however, is still under debate, as a result of its inter- and intravariability.\textsuperscript{86-88} It remains challenging to identify variability due to changes in metabolism from other factors, such as gas-fluid surfaces, intestinal peristalsis, and gut movements.\textsuperscript{87,88} Even so, previously strong correlations have been reported between r$_{SO_2}$ measurements and Doppler flow measurements of the superior mesenteric artery,\textsuperscript{86,89} between r$_{SO_2}$ and (progression of) NEC development\textsuperscript{66-68}, and between r$_{SO_2}$ and intestinal recovery after NEC\textsuperscript{90}, supporting that r$_{SO_2}$ measurements are feasible as indicator for splanchnic perfusion. The use of r$_{SO_2}$ measurements may yet be useful in monitoring anemic infants for early detection of altered splanchnic tissue oxygenation, suggesting inadequate oxygenation, and onset of intestinal injury (Chapter 4).

Since infants before and after NEC onset showed different splanchnic oxygen saturation patterns, partly reflected in the r$_{SO_2}$ variability, variability measurements might be of added value to gather information about the relation between intestinal perfusion, intestinal injury, and the potential development of NEC.\textsuperscript{62,63,67,91} We feel that adding variability measurements may improve identifying intestinal injury, as supported by our results described in Chapter 4, 6, and 7. Baseline r$_{SO_2}$ is in itself much more variable than r$_{SO_2}$. The stable r$_{SO_2}$ probably demonstrates the autoregulatory capacity of the cerebral circulatory system, and a more constant balance between oxygen supply and consumption in the brain compared to the intestine.\textsuperscript{85} In other words, the larger r$_{SO_2}$ variability may reflect real-time changes in blood flow to the splanchnic tissue versus momentary alterations in oxygen consumption.

Lower r$_{SO_2}$ variability, as reflected by the coefficient of variation (CoVar), was associated with higher I-FABP levels in anemic infants, already from three days prior to RBC transfusion (Chapter 4). Furthermore, a reduced r$_{SO_2}$ variability after RBC transfusion was associated with higher 8-isoprostane and I-FABP levels, biomarkers for oxidative stress and intestinal injury (Chapter 6). Finally, the smaller ranges of r$_{SO_2}$ we found after RBC transfusion in infants who
subsequently developed NEC also implicate a diminished variability (Chapter 7). Our findings of a diminished rSO₂ variability are reminiscent of fetal and neonatal heart rate. Minimal variability in fetal heart rate is thought to result from cerebral hypoxemia, acidosis and failure of fetal compensatory mechanisms to maintain adequate cerebral oxygenation. Decreased neonatal heart rate variability may reflect a reduced ability to adapt to changes in autonomic nervous system activity. Aforementioned explanations may also account for rSO₂. The preceding hypoxia and subsequent re-oxygenation after RBC transfusion may result in a reduced rSO₂ variability, that seems to be associated with intestinal cell injury. We speculate therefore, that a diminished variability either reflects (post-)hypoxic intestinal injury and/or shows a reduced capacity of the splanchnic vasculature to adapt to changes in the balance between oxygen supply and demand. A reduced rSO₂ variability may, thus, be indicative of intestinal distress (Chapter 4, 6, and 7). Furthermore, diminished splanchnic vascular variability after RBC transfusion might be an early marker for NEC development, representing part of the early process of transfusion-associated NEC (Chapter 6 and 7).

In line with our results, Cortez et al. observed a loss of rSO₂ variability in two infants who developed NEC, already 24-48h before clinical diagnosis. In another large cohort, infants who developed NEC also showed lower rSO₂ variability at time of clinical suspicion, supporting the diagnosis of NEC, as well as during the 24h after suspicion has arisen. In contrast, Bailey et al. suggested that the higher rSO₂ variability they found could be mainly explained by poor vascular regulation capabilities, leading to such variability in perfusion and oxygen delivery after RBC transfusion. However, no patients monitored in their study actually developed NEC in the post-RBC transfusion period. Therefore, this strengthens our hypothesis that a reduced rSO₂ variability plays a role in transfusion-associated NEC development.

The term brain after anemic neonatal encephalopathy

Neonatal encephalopathy (NE) is clinically defined as acute or subacute brain injury due to perinatal asphyxia, and occurs in 1-6 per 1000 term births. Perinatal asphyxia results in cerebral hypoxia and ischemia, due to inadequate perfusion and an impaired oxygen exchange caused by either fetal factors, maternal factors or neonatal cardiorespiratory failure. Severe prenatal or perinatal blood loss may account for perinatal asphyxia, as a result of fetomaternal transfusion or fetal blood loss through the placenta or umbilical cord.

Neonatal and neurodevelopmental outcome

Severe perinatal anemia is a rare but serious pregnancy complication. In line with our results described in Chapter 9 and 10, perinatal death varies in literature between 31% to 50%. Strikingly, survivors of anemic NE had a favorable NDO at two-to-three years of age, compared with survivors of non-anemic NE (Chapter 9 and 10). Anemia co-occurring with NE may be characterized as a short and acute hypoxic-ischemic incident together with hypovolemia. Conversely, NE due to other causes may predominantly be characterized by a more prolonged and repetitive state of hypoxia and subsequent ischemia, leading to another pattern of brain injury. Another possible explanation may be that perinatal anemia itself did not affect the long-
term outcome, as supported by the results of another study in which they reported no signs of injury on MRI of the thalamus and basal ganglia in surviving term-born infants born with severe perinatal anemia. In our study, however, we were unable to show differences on cerebral MRI between infants with anemic and non-anemic NE (Chapter 9).

**Cerebral monitoring in infants with anemic NE during therapeutic hypothermia**

Longitudinal recordings of \( r_{\text{SO}_2} \) and electrocortical activity might elucidate possible pathophysiological mechanisms for the opposite associations between anemic NE and mortality, and anemic NE and NDO. Anemic infants had lower \( r_{\text{SO}_2} \) and more frequently abnormal aEEG background patterns during TH than infants with non-anemic NE (Chapter 10). Less oxygenated blood may have resulted in impaired cerebral oxygen delivery, inadequate to fulfill the metabolic demand of the brain, which is highly dependent on adequate oxygen and nutrient supply. Severe anemia may have resulted in an absolute deficit of the oxygen-carrying capacity of blood leading to severe brain injury, as reflected by the suppressed and flat aEEG background patterns in those infants (Chapter 10). Although mean \( r_{\text{SO}_2} \) during the first 6-hours did not seem extremely low in anemic infants, \( r_{\text{SO}_2} \) values may have been below previously established hypoxic-ischemic thresholds in piglets. As we calculated mean \( r_{\text{SO}_2} \) over the first 6-hours after birth, earliest postnatal \( r_{\text{SO}_2} \) values and those before birth may have been lower. Furthermore, most anemic infants received their first RBC transfusion already within a few hours after birth which also accounted for increased mean \( r_{\text{SO}_2} \) values. These factors may have resulted in an overestimation of the \( r_{\text{SO}_2} \) during the first hours after birth.

Conversely, the relatively low \( r_{\text{SO}_2} \) in anemic infants with NE during 6 to 48-hours after birth may have accounted for less cerebral hyperoxia and less oxidative stress, potentially explaining part of the favourable long-term outcome. Hyperoxia may reflect a diminished cerebral consumption as a result of severe brain injury, but might also account for (further) injury itself. The biological defenses against hyperoxia may not be as robust as against hypoxia in newborn infants. An alternative explanation might be that the lower absolute oxygen-carrying capacity during anemia has resulted in less free oxygen radical production and therefore lower oxidative stress. This, however, remains speculative as we did not measure biomarkers for oxidative stress in our study. The seizure activity, seen less frequent in anemic infants, might also provide an explanation for the favourable long-term outcome, as seizure burden may cause additional brain injury. We hypothesize that seizure activity might even be a better expression of brain injury, as aEEG background patterns are more affected by sedatives, known to cause a time-related depression of electrocortical activity after administration.

**Future perspectives**

This thesis provides insight in the balance between neonatal anemia and red blood cell transfusions, particularly in its potential benefits and risks in both preterm infants born before 32 weeks of gestation and term infants with NE born after perinatal asphyxia. An individualized RBC transfusion strategy, based on cerebral oxygen saturation measurements, seems promising
in preterm infants. Many uncertainties, however, still remain, regarding the effects of neonatal anemia and RBC transfusions. Preventing cerebral hypoxia as part of an RBC transfusion strategy in preterm infants needs to be further explored. Furthermore, based on the association between both anemia and RBC transfusions, and intestinal injury, additional research is needed to assess potential anemia treatment approaches to protect the preterm intestine from injury. Finally, the suggested advantage of perinatal anemia in NE for long-term outcome needs to be addressed in larger studies and may give clues towards therapeutic measures regarding preventing cerebral hyperoxia.

An important finding of this thesis is that preterm infants who were treated following an rSO\textsubscript{2}-guided RBC transfusion strategy had a favourable neurological outcome at 3 months CA. It is important to assess whether this finding can be confirmed by multicenter studies with longer follow-up. Results of this thesis support the notion of preventing cerebral hypoxia in very preterm anemic infants. Exact limits, however, should be further explored. A decreasing r\textsubscript{SO\textsubscript{2}} limit may be feasible with increasing postnatal age. The results of the SafeBooC II trial may provide some answers.\textsuperscript{111}

Future studies regarding individualized RBC transfusion treatment should also focus on how to protect the preterm intestine, and not only the brain, from injury. The preterm brain takes advantage from sufficient r\textsubscript{SO\textsubscript{2}}, partly maintained by RBC transfusions and partly through redistribution of blood to the brain during hypoxic circumstances. The intestine, however, is vulnerable to both anemic hypoxia and hyperoxic re-oxygenation and/or oxidative stress following RBC transfusion. Ongoing trials investigating feeding practices during RBC transfusion may shed light on clinical decisions regarding RBC transfusions and enteral feedings during the transfusion, possibly reducing intestinal injury.\textsuperscript{112}

Identification of an appropriate and effective biomarker for RBC transfusion may contribute to individualized RBC transfusion treatment. Near-infrared spectroscopy measurements may provide the key in finding the optimal balance between anemia and RBC transfusions. Additional NIRS-based measurements may help better understand how anemia and RBC transfusions, with its varying levels of organ tissue oxygenation, interact with other mediators to influence intestinal injury in preterm infants. First, observing variability measures needed to validly measure splanchnic oxygenation requires further elucidation since r\textsubscript{SO\textsubscript{2}} variability may be a preferable and more suitable indicator for (healthy) splanchnic perfusion. Second, splanchnic-cerebral oxygenation ratios may also improve the identification of intestinal distress.

Apart from RBC transfusion strategies, clinicians and researchers should focus on prevention of neonatal anemia, by minimizing (unnecessary) phlebotomy-related blood loss and provision of delayed cord clamping. Furthermore, the role of erythropoietin needs further attention in this context, which may provide neuroprotection and might also influence growth and development of the gastrointestinal tract.

Finally, the benefits and risks of perinatal anemia and RBC transfusions in NE should be further explored. Primarily, further studies may identify possible interventions targeting at avoiding high cerebral oxygen saturation, by either lowering the Hb transfusion threshold, or for example using a r\textsubscript{SO\textsubscript{2}} limit for RBC transfusion in anemic infants with NE. Secondarily, pharmacological neuroprotection is considered as a potential target of hypoxic-ischemic brain
injury. Anti-oxidative and anti-inflammatory medications, such as allopurinol or 2-iminobiotin might have the potential to prevent further brain injury and to improve neonatal and neurodevelopmental outcomes.\textsuperscript{113,114} Whether these approaches are also beneficial in infants in whom the NE is caused by severe anemia has to be subject to further study.

Conclusions

In conclusion, neonatal anemia is associated with both poorer neurological functioning and intestinal cell injury in preterm born infants. Red blood cell transfusions immediately benefit the preterm brain, possibly mediated by the increase in cerebral oxygen saturation, but may also be harmful to the preterm intestine.

Regarding the brain and neurological development, this thesis provides evidence that RBC transfusions outweigh the disadvantages of moderate to severe anemia. Moderate anemia may be well-tolerated by preterm infants, provided that sufficient cerebral oxygen saturation values are met to secure optimal brain functioning and brain development. Preventing cerebral hypoxia may underlie these findings. With regard to the intestine, the balance between anemia and RBC transfusions remains largely unclear and the contributions of both anemic hypoxia and the hyperoxic transfusion hit to intestinal injury need further elucidation. Further studies should focus on individualized RBC transfusion treatment including approaches to protect the preterm intestine. The presence of variability in splanchnic oxygen saturation seems to be important for adequate splanchnic perfusion. Diminished variability in splanchnic oxygen saturation reflects intestinal distress. There are some explanations. First, the splanchnic vasculature is insufficiently able to adapt to changes in oxygen supply and demand. Second, it may reflect intestinal hypoxic injury that occurred following the preceding hypoxia and subsequent re-oxygenation. Based on the results of this thesis, we suggest that anemia and RBC transfusions may contribute to NEC development as part of a multiple hit process. This thesis further demonstrates that near-infrared spectroscopy measurements seems promising for finding the optimal moment and threshold for RBC transfusions in this vulnerable preterm population.

Perinatal anemia causing term neonatal encephalopathy is associated with high risks of mortality in term infants. Neurodevelopmental outcome after anemic NE, however, is favourable. The less frequently occurring high cerebral oxygenation and seizure activity, and a less pronounced re-oxygenation phase with potentially less oxidative stress after birth, might, at least partly, explain the favourable outcome in the infants with perinatal anemia who survive.
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