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Introduction
Each year approximately 2200 very premature infants, with a gestational age < 32 weeks, are born in the Netherlands. Anemia of prematurity (AOP) is a condition which is often seen in these infants. This AOP is an exaggerated form of the normal physiologic anemia seen in full term infants and is inversely related to gestational age at birth. More than 50% of the infants born before 32 gestational weeks receive RBC transfusions early in life because of this anemia. The aetiology of AOP is multi-factorial. The shorter life span of hemoglobin (Hb) F bearing red blood cells (RBC) and the relatively rapid growth, contribute to the fall in neonatal Hb. Another important factor contributing to the decrease in neonatal Hb is blood loss due to frequent phlebotomies for diagnostics during the first weeks of life. Several studies have shown that replacement of iatrogenic blood loss is one of the major reasons for administering RBC transfusions. Clinical symptoms of AOP are rather non-specific and include poor weight gain, tachycardia, bradycardia, apnea, and an increased need for supplemental oxygen. Studies that investigated which clinical parameters could predict the transfusion needs in premature infants, showed that gestational age and birth weight were the only independent variables. In one other study, the combination of gestational age and Apgar score contributed to the prediction of transfusion needs in infants born after 32 weeks of gestation. Cardiac output, heart rate and respiratory rate did not help in predicting blood transfusion needs. Laboratory parameters, such as erythropoietin, initial Hb, vascular endothelial growth factor (VEGF) and lactate, have also been studied as predictive markers for transfusion needs. Of these laboratory parameters, a high initial Hb is associated with reduced transfusion needs. VEGF levels > 140pg/ml indicate tissue hypoxia and probably can serve as a marker for transfusion. Because there is no single factor predictive for transfusion needs in these infants, in practice the decision to transfuse is frequently based on a combination of postnatal age, hematocrit value and clinical manifestations.

**Neonatal transfusion practice**

In the Netherlands, the Dutch Institute for Health Care Improvement has set up a transfusion guideline in which specific triggers are recommended for use in neonatal intensive care. These thresholds vary depending on postnatal age and the presence of cardio-respiratory problems. Infants suffering from such problems are transfused at a higher threshold. In absence of these problems, the triggers become more restrictive with increasing postnatal age. The standard RBC transfusion product, a pedi-pack (one-fifth of an adult unit), consists of pre-storage filtered red blood cells (RBC) derived from voluntary healthy adult donors, stored in extended storage medium consisting of saline, adenine, glucose and mannitol (SAG-M), and has a hematocrit between 0.55-0.65 L/L. The shelf life of this product is 35 days. Transfusion volume per kg recipient bodyweight differs per neonatal center. In international literature, a transfusion volume range of 10-20 mL per kg body weight is reported. For infants with a birth weight of <1500 grams and/or a gestational age < 32 weeks, pedi-packs are irradiated with 25 Gray before administration.
In some countries, single donor programs have been evaluated, in which one adult RBC donation is dedicated to one or two premature infants. Use of restrictive transfusion triggers resulted in either a lower number of transfusions per infant or fewer infants receiving transfusions. However, with respect to outcome these studies reported different results. Bell and colleagues suggested that infants under a more restrictive transfusion policy were more at risk for neurologic sequelae. This was not observed by Kirpalani et al and Chen et al. However, the latter study reported that the infants who received a total transfusion volume of more than 30 mL over 30 days were more prone to develop chronic lung disease. This risk could decline by the use of more restrictive triggers. The infants enrolled in the multi-center study by Kirpalani were evaluated at 18 to 21 months of corrected age. The primary composite outcome in this study was death or survival with any of either severe visual or the presence of cerebral palsy, or cognitive delay, or hearing impairment. They observed no statistically significant difference in such worse primary outcome, but the difference in cognitive delay (Mental Development Index score < 70) approached statistical significance. A post-hoc analysis with redefined cognitive delay (Mental Development Index score < 85) showed a significant difference favoring the liberal threshold group. This study provided some evidence of benefit from a higher hemoglobin threshold for transfusion primarily through this secondary analysis of cognitive delay. In contrast to this finding, Nopoulos et al reported that premature infants receiving transfusions according to the liberal guideline, earlier included in the study by Bell et al, had a reduced brain volume at a mean age of 12 years. These findings indicate that further research is required regarding the transfusion effect on neuro-developmental outcome in premature infants. Altogether, lowering the transfusion triggers may be feasible but not without potential clinical risks. As such, additional controlled clinical trials are desirable.

The broad transfusion volume range from 10 to 20 mL per kg body weight used for infants, an equivalent of 2 to 4 units of RBC in adults, has hardly been investigated. Paul et al studied the effect of RBC transfusions with 10 and 20 mL per kg and found higher hematocrit and hemoglobin values after transfusion with 20 mL per kg in accordance with the double dose. However, whether the higher volume resulted in a lower total number of transfusions per infant could not be confirmed. Wong et al examined transfusion volumes of 15 mL and 20 mL per kg and also reported higher post-transfusion hematocrits. A higher transfusion volume did not result in a lower number of transfusions per infant. Comparison of transfusion volume relies on the hematocrit of the used product. For instance, although the transfusion volume per kg was similar in the randomised studies by Bell and Kirpalani, the transfused products were different. Bell et al transfused products with a hematocrit between 0.80 and 0.85 L/L. The products transfused in the study by Kirpalani et al were washed before transfusion and the actual hematocrit of these products was not reported.
Furthermore, there is evidence that allogeneic RBC transfusion suppresses premature hematopoiesis. Several clinical series demonstrated a decrease in endogenous EPO concentration in premature infants after RBC transfusion.\textsuperscript{30-33} These studies, however, used a more liberal transfusion strategy than the current transfusion guidelines. How differences in total RBC volume, transfused according to the current transfusion practice or RBC product volume, affect total transfusion needs and outcome is unknown.

Compliance to neonatal transfusion guidelines is still a subject of research.\textsuperscript{34-35} Implementation of an electronic transfusion ordering and monitoring system and the inclusion of care-givers perception of patients transfusion needs in the guideline have been suggested as options to provide better adherence.\textsuperscript{35-36} With more uniform neonatal transfusion practice, we could gain more and better evidence for transfusion related effects on neonatal outcome.

**Clinical sequelae associated with RBC transfusion**

Allogeneic RBC transfusions have often been incriminated to have clinical disadvantages.\textsuperscript{37} Despite the stringent quality control of the Blood Banks regarding donor recruiting and product preparation, a very small risk of blood transmitted infectious diseases still exists.\textsuperscript{38} Furthermore, RBC transfusion has shown to be associated with higher risk for severe neonatal conditions like bronchopulmonary dysplasia (BPD), retinopathy of prematurity (ROP) and necrotizing enterocolitis (NEC).\textsuperscript{37, 39-42} Despite many efforts to relate these free radical associated disorders with the administration of a RBC transfusion, causality has not been proven.\textsuperscript{43-46} In addition, several studies have shown that blood transfusion may suppress neonatal hematopoiesis at 6-7 weeks after birth.\textsuperscript{30-33}

Blood transfusions also have beneficial effects. Several studies demonstrated that RBC transfusions improve cerebral, splanic tissue and renal oxygenation in anemic premature infants.\textsuperscript{47-49} A hemoglobin level of 6 mmol/l has been proposed as a critical threshold for cerebral oxygenation to be at risk.\textsuperscript{49} Next to this, possible beneficial neuro-developmental effects cannot be ruled out.\textsuperscript{27} To investigate whether a causal relation exists between allogeneic RBC transfusions and the occurrence of neonatal complications, one has to overcome several difficulties. Transfused premature infants are more often born at a younger gestational age and suffer from multiple organ insufficiencies because of their immaturity.\textsuperscript{50-51} To evaluate a transfusion effect in these infants, a well controlled non-transfused group would be necessary. However, with decreasing gestational age, more infants receive transfusions, making formation of a control group not possible most of the time. Another hurdle in investigating transfusion effects is to equalize the amount of blood needed for investigation, as phlebotomies for diagnostic purposes are a major cause for RBC transfusions.\textsuperscript{8}
Alternatives for RBC transfusion in premature infants

Recombinant Human Erythropoietin

Several studies found low plasma levels of endogenous erythropoietin (EPO) in premature infants. These measured levels were below expected levels for the degree of anemia in adults. Comparison with anemic adults with similar degrees of anemia, showed 10-100 times lower EPO values in infants. This finding provided the rationale to investigate the use of recombinant human EPO (rH-EPO) in premature infants. Over 40 randomized trials investigating the effects of rH-EPO have been performed. Aher and Ohlsson published three meta-analyses on behalf of the Cochrane Collaboration, which investigated respectively: the use of early administration of rH-EPO (within 8 days after birth), late administration of rH-EPO (between day 8-28 after birth) and also compared the effect of early versus late administration of rH-EPO. Primary objective was to assess safety and efficacy of the use of rH-EPO in reducing the number of administered RBC transfusions and the volume of transfused blood in mL per kg body weight. Use of both early and late rH-EPO resulted in a small reduction of RBC transfusions, but this decrease was of limited clinical importance because many infants already had received blood transfusions before study entry. The reduction in donor-exposure was < 1 donor per transfused infant and a reduction of 6 mL transfused blood per kg. However, early rH-EPO administration was associated with a higher incidence of severe ROP (grade 3). This potential adverse effect of rH-EPO was supported by experimental studies.

Altogether, administration of rH-EPO was not associated with a clinical significant reduction in RBC transfusions, neither in the volume of RBCs transfused per kg nor in a significant reduction in donor exposure, and therefore cannot be recommended as an alternative in the treatment of AOP. In addition, treatment of premature infants with rH-EPO should not be done without considering the higher risk of ROP.

Autologous umbilical cord blood transfusion

Autologous umbilical cord blood (UCB) has often been suggested as an alternative for allogeneic RBC transfusions in the treatment of AOP. Due to increasing use of UCB for transplantation purposes, progress has been made on aseptic collection of UCB and processing of small blood volumes. Some studies reported that approximately 20 mL of UCB per kg of body weight could be harvested irrespective of birth weight. In other studies the amount of harvested UCB was only correlated to gestational age. The potential coverage of transfusion needs with autologous UCB has been investigated in a preclinical study. This study suggested that collection of UCB could be efficient for premature infants born between 29 and 31 gestational weeks. However, practical feasibility of the clinical application of autologous UCB remained to be investigated.
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Autologous transfusion by delayed clamping
Transfusion of autologous cord blood can also be carried out by delaying the moment of cord clamping after birth or by repeated milking of the cord towards the infant.61-62 Rabe et al performed a systematic review of ten randomized studies comprising 454 premature infants. A delay in cord clamping of at least 30 seconds was associated with lower transfusion needs, and more importantly, a significant lower rate of intra-ventricular haemorrhage.61 Delayed cord clamping is also associated with better cerebral oxygenation and may protect very low birth weight male infants against motor disability at 7 months corrected age.63-64 Strauss et al performed a randomized trial in which premature infants born between 30 and 36 gestational weeks without the need for resuscitation were allocated to either early or delayed clamping. A 1 minute delay in clamping resulted in an increased Hct and RBC volume, but more infants in the delayed group were in need of phototherapy and the bilirubin levels and duration of phototherapy were similar between both groups.65 Although this simple manoeuvre has benefits, there may be potential risks like polycythemia and increased jaundice needing phototherapy. Besides these clinical aspects there are also methodological considerations. Definitions regarding the time interval for delayed clamping differ among studies, but a delay of 30 seconds has shown to be safe. Furthermore, the body position of the newborn after birth has shown to affect the amount of auto-transfusion.66 Premature infants are often in need of direct resuscitation after birth and this should be taken into account when one intends to apply delayed cord clamping practice. Lastly, the amount of blood transfused during delayed clamping cannot be measured by a reliable method.67

Ex vivo expansion of UCB hematopoietic stem cells
UCB contains a high amount of hematopoietic stem and progenitor cells. In the past few years, progress has been made in culturing these precursor cells ex vivo towards the erythroid lineage.68-70 Using either a murine or human stroma layer or a specific combination of growth factors and cytokines, it is possible to obtain in vitro expanded mature RBC.69 The protocols reported in the medical literature have several disadvantages. Most protocols consist of multiple stages, each with a specific growth factor and cytokine combination, which makes them rather laborious and expensive. In addition, some protocols use culture medium with animal protein. Altogether, this makes ex vivo expansion of (autologous) hematopoietic UCB stem and progenitor cells not easy to translate into a clinical grade process. To provide a clinical grade transfusion product, the expanded product should be well defined and the development of these products need a process that should be usable in a cost-effective manner.71 These challenges are currently under investigation.72
Objective of this thesis
In this thesis, we investigated to following objectives:

1. Short term and long term clinical effects of allogeneic RBC transfusions in premature infants.
2. The effect of different RBC transfusion volumes on neonatal outcome in premature infants.
3. The use of autologous cord blood as an alternative for allogeneic RBC transfusions.

Outline of this thesis
Dutch neonatal transfusion practice was evaluated in two tertiary neonatal centers. Main points of study were transfusion outcome and short-term clinical follow-up (Chapter 2). Long-term follow-up of developmental disabilities of extreme low gestational age premature infants, born before 28 gestational weeks, in relation to RBC transfusion were evaluated at a corrected age of 24 months (Chapter 3). The effects of RBC transfusions in premature infants were evaluated by erythropoietin (EPO) measurement in serum using an ELISA assay. EPO levels were measured in waste material from blood drawn for diagnostics in the first month of life, because most premature infants receive the most RBC transfusions early after birth (Chapter 4).

In Chapter 5, the current literature on the use of UCB for autologous and allogeneic transfusion purposes is reviewed. Topics regarding UCB collection methods, UCB processing into a transfusion product and clinical experience with allogeneic and autologous UCB transfusions are discussed.

In Chapter 6, the red cell lesion parameters of stored whole blood premature UCB products and premature UCB derived RBC products stored in SAG-M or Additive Solution 3 (AS-3) are described. The use of autologous cord blood RBC transfusion for the treatment of AOP was investigated in a randomized clinical trial (Chapter 7).

In Chapter 8 we discuss whether ex vivo expansion of UCB derived hematopoietic stem and progenitor cells could help in harvesting additional autologous red cells for transfusion purposes in the future.
Reference list


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