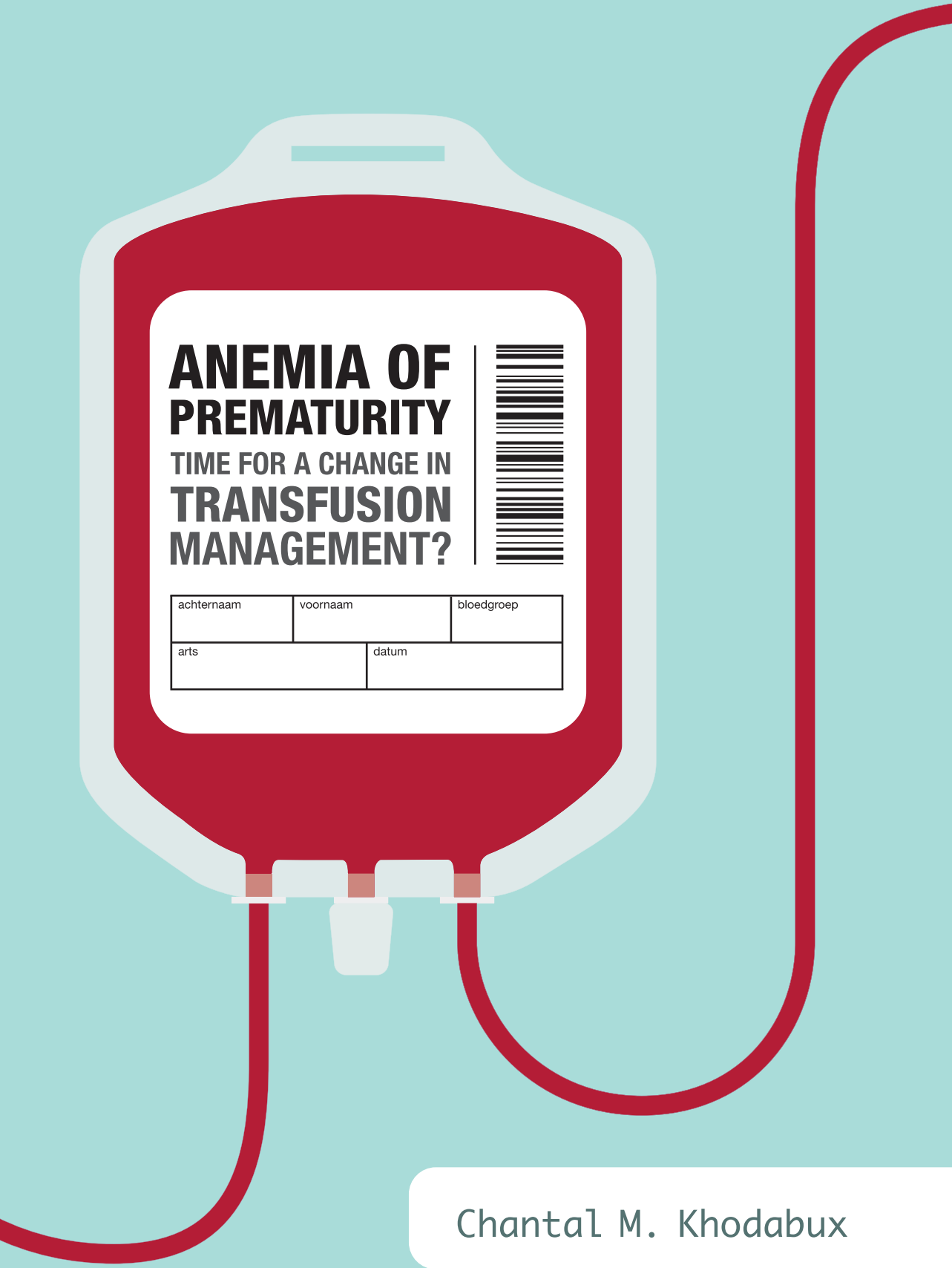

Chantal M. Khodabux

Anemia of prematurity

Time for a change in transfusion management?

Chantal Khodabux

The research described in this thesis was conducted at the Sanquin Blood Bank South-West Region, the Leiden University Medical Center (Department of Obstetrics and Division of Neonatology, Department of Pediatrics) and the University Medical Center Utrecht (Department of Obstetrics and Department of Neonatology). The studies were financed by ZonMW, grant number 945-04-609.

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Time for a change in transfusion management?

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Voor mijn ouders

Contents

1

Introduction

Each year approximately 2200 very premature infants, with a gestational age < 32 weeks, are born in the Netherlands.[1] 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.[2] More than 50% of the infants born before 32 gestational weeks receive RBC transfusions early in life because of this anemia.[3-5] 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.[6] 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.[7-8] Clinical symptoms of AOP are rather non-specific and include poor weight gain, tachycardia, bradycardia, apnea, and an increased need for supplemental oxygen.[9] 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.[10-11] 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.[12] 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.[13-15] Of these laboratory parameters, a high initial Hb is associated with reduced transfusion needs.[14] VEGF levels > 140pg/ml indicate tissue hypoxia and probably can serve as a marker for transfusion.[15] 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.[16]

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.[17]

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.[18] 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.[19]

In some countries, single donor programs have been evaluated, in which one adult RBC donation is dedicated to one or two premature infants.[20-23] Such strategies to reduce the donor exposure have been shown to be feasible[22] and cost-effective [23], but are not standard practice in every neonatal center around the world.

Several randomized studies compared a restrictive transfusion strategy with a more liberal threshold.[24-26] Use of restrictive transfusion triggers resulted in either a lower number of transfusions per infant,[24,26] or fewer infants receiving transfusions.[25] 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.[24] This was not observed by Kirpalani et al and Chen et al.[25-26] 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.[26] 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.[27] 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.[28] 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.[29] 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.[30] 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.[24-25] Bell et al transfused products with a hematocrit between 0.80 and 0.85 L/L.[24] The products transfused in the study by Kirpalani et al were washed before transfusion and the actual hematocrit of these products was not reported.[25]

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.[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.[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.[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.[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.[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).[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.[43-46] In addition, several studies have shown that blood transfusion may suppress neonatal hematopoiesis at 6-7 weeks after birth.[30-33]

Blood transfusions also have beneficial effects. Several studies demonstrated that RBC transfusions improve cerebral, splanchic tissue and renal oxygenation in anemic premature infants.[47-49] A hemoglobin level of 6 mmol/l has been proposed as a critical threshold for cerebral oxygenation to be at risk.[49] Next to this, possible beneficial neuro-developmental effects cannot be ruled out.[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.[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.[8]

Alternatives for RBC transfusion in premature infants

Recombinant Human Erythropoietin

Several studies found low plasma levels of endogenous erythropoietin (EPO) in premature infants.[30-33] 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.[52] 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).[53-55] This potential adverse effect of rH-EPO was supported by experimental studies.[56-57]

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.[53-55]

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.[58-60] 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.[59-60] In other studies the amount of harvested UCB was only correlated to gestational age.[12,58] 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.[12] However, practical feasibility of the clinical application of autologous UCB remained to be investigated.

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]

Altogether, there is a need for a robust methodology in which the delay in cord clamping and position of the infant after birth is well defined, although a delay of about 30 seconds as routine practice in premature infants without need for resuscitation offers a clear clinical benefit.

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.

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A comparative cohort study on transfusion practice and outcome in two Dutch tertiary neonatal centers

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Abstract

Objective: To investigate how a red blood cell transfusion volume of 15 or 20 mL/kg bodyweight affects total number of administered transfusions and neonatal complications in premature infants born before 32 gestational weeks.

Methods: In this observational study we analysed clinical data from two cohorts of 218 and 241 premature infants admitted to two neonatal centers which used the same transfusion guideline and product, but different transfusion volumes. Outcome parameters were number of administered transfusions and the composite outcome of bronchopulmonary dysplasia, retinopathy of prematurity, intra-ventricular haemorrhage and mortality.

Results: The proportion transfused infants was significantly lower (59% vs 77%) in the center using a lower transfusion volume of 15 mL/kg. In infants born between 24^{+0}-27^{+6} gestational weeks a similar proportion received transfusions in both centers, with an equal number of transfusions per infant. In infants born between 28^{+0}-31^{+6} gestational weeks the proportion of transfused infants (49% vs 74%) was significantly higher in the center using a larger transfusion volume. In these infants transfusion with 20 mL/kg resulted however in a mean reduction of 1 transfusion episode per infant. The higher proportion of transfused infants was associated with a higher pre-transfusion hematocrit in less ill infants, suggesting the use of different triggers based on clinical grounds. Composite clinical complications were similar in both cohorts.

Conclusion: Clinical neonatal outcome was similar disregard of a higher proportion of transfused patients and a higher total amount of RBC transfused in one of the centers. A larger transfusion volume of 20 mL/kg prolonged the interval until next transfusion and can reduce donor exposure in infants born between 28^{+0}-31^{+6} weeks of gestation.

Introduction

The number of red blood cell (RBC) transfusions in premature infants has successfully declined the past decade due to the use of specific transfusion guidelines.[1-3] These comprise recommendations for transfusion triggers based on the post-natal age of the infant and the need for respiratory support, as well as a recommended transfusion volume range from 10 to 20 mL/kg body weight, an equivalent of 2-4 units of RBC in adults. These guidelines are however not evidence based. In addition, strategies to limit donor exposure by dedicating a blood product to one or two premature infants have been evaluated.[4-5] Single-donor programs have been shown to be feasible[6] and cost-effective[7,] but are not standard practice in every neonatal center. In spite of these guidelines, premature infants are still frequently transfused.

Recently the use of restrictive transfusion triggers has been addressed in several randomized controlled trials.[8-9] Two randomized studies, in which a restrictive and a more liberal transfusion strategy were compared, showed that the use of restrictive transfusion triggers resulted in a lower number of transfusions per infant or fewer infants receiving transfusions.[8-9] The results of the first study by Bell et al, suggested that infants under a more restrictive transfusion policy may experience a higher rate of neurologic sequelae.[8] These adverse effects were not observed in the larger so called Premature Infants in Need for Transfusion (PINT) study by Kirpalani.[9] Lowering the transfusion triggers may be feasible; however; this should still be conducted in well designed clinical trials. Studies on the impact of a higher transfusion volume per kg body weight on the total number of transfusions per infant are scarce. Paul and colleagues compared transfusion with 10 and 20 mL/kg in 13 very low birth weight infants and found higher post-transfusion hematocrits when transfused with 20 mL/kg. Whether the higher volume resulted in a lower number of transfusions per infant could not be confirmed in this study.[10] Wong et al compared 15 mL with 20 mL/kg and reported significant higher post-transfusion hematocrits, but a higher transfusion volume did not result in a lower number of transfusions per infant.[11]

It is difficult to compare transfusion volume reliably as the RBC volume is dependent on the product used. For instance, although the transfusion volume per kg in the studies by Bell and Kirpalani was similar, the transfused products were different. Bell et al transfused products that had a hematocrit between 80 and 85%.[8] The products transfused in the PINT study were washed before transfusion and the actual hematocrit of these products was not mentioned.[9] How differences in RBC volume in transfusion products affect total transfusion needs and outcome is unknown.

In this study we compared clinical data from two of the ten Dutch tertiary neonatal centers, using the same transfusion thresholds[12] and the same transfusion product, but a different transfusion volume per kg bodyweight. Our aim was to investigate whether a different transfusion volume affected the total number of received transfusions until discharge to home and clinical relevant morbidity in premature infants born before 32 weeks of gestation.

Material and Methods

This study was approved by the medical ethical committees of the Leiden University Medical Center and the University Medical Center Utrecht. Informed consent was obtained from the parents of all included infants.

Subjects Studied

Between December 2004 and October 2006, clinical data from premature infants born before 32 weeks of gestation were registered in Unit A. In Unit B, data collection was performed from March 2005 until October 2006. Infants suffering from allo-immune haemolytic disease, congenital infections or those in need of major surgery (ie gastro-intestinal or cardio-thoracic surgery) were excluded from analysis. All other infants were included. Data on gestational age, birth weight, gender, Apgar score, mortality, endotracheal ventilation, transfusion parameters (number of transfusions, increase in hematocrit, time interval until next transfusion and volume transfused) and the CRIB (Clinical Risk Index for Babies) II score were collected on clinical request forms from birth until discharge.[13] The CRIB II score is a risk adjustment tool that comprises 5 items (gestational age, gender, birth weight, temperature and base excess at admission) and a score ranging from 0 to 27, with higher scores indicating a higher risk of mortality and morbidity. Data on neonatal complications, i.e. retinopathy of prematurity (ROP)[14], bronchopulmonary dysplasia (BPD)[15] and intra-ventricular haemorrhage (IVH)[16], and length of stay until discharge home, were obtained from our hospitals or the referral hospitals.

Transfusion guideline

All infants were transfused according to the Dutch consensus for blood transfusion.[12] The recommended transfusion triggers vary with postnatal age and need for respiratory support.

- In the first 24 hours after birth:<Hb 8 mmol/L (13g/dL) (hematocrit range 0.38-0.40 L/L) capillary (or < 7 mmol/L arterial (11g/dL)) (hematocrit range 0.32-0.35 L/L)
- Stable infants with cardio-respiratory problems and/or mechanical ventilation: Hb 7 mmol/L (11g/dL) (hematocrit range 0.32-0.35 L/L) capillary
- Infants with a postnatal age < 4 weeks: Hb 6 mmol/L (10g/dL) capillary (hematocrit range 0.27-0.3 L/L); and infants with a postnatal age > 4 weeks: Hb 4.5 mmol/L (7g/dL) (hematocrit range 0.2-0.23 L/L) capillary. In case of symptomatic anemia, it is recommended that transfusion should take place at higher triggers.

Transfusion volume per kg body weight was different between the hospitals; 15 mL/kg in Unit A and 20 mL/kg in Unit B. These transfusion volumes were not specially chosen for this study, but were part of the standard practice in the neonatal intensive care units. The same transfusion product was used in both hospitals. All products consisted of pre-storage filtered RBC stored in additive solution SAG-M, with a hematocrit between 0.55 and 0.65 L/L. The products were irradiated with 25 Gy less than 24 hours before transfusion.

Outcome parameters

Primary outcome included mortality and a composite of clinical relevant morbidities (ROP, BPD and IVH). The total number of transfusions, transfused volume, transfusion triggers, post-transfusion hematocrit measured 24 hrs after transfusion and length of stay were specified as secondary outcome parameters.

Statistical analysis

All variables were analysed by univariate analysis for continuous variables and Chi-Square or Fishers exact probability test for nominal variables (SPSS 12). Backward stepwise logistic regression analysis was used for the independent effect of the Apgar score, CRIB II score, mechanical ventilation and hospital of admittance, on the transfusion needs. A *p*-value of less than 0.05 was considered significant.

Results

Study population

In the, respectively, 22 and 19 month study period, a total of 221 infants in Unit A and 248 infants in Unit B were born before 32 weeks of gestation. After admittance three and seven infants respectively were in need of major surgery, and were excluded from analysis. A total of 459 infants were included in this study, 218 infants in Unit A and 241 infants in Unit B. Both groups were comparable with regard to birth weight, gestational age, gender, median Apgar scores, median CRIB II scores and need for endotracheal ventilation (Table 1). The CRIB II score was incomplete in 27 infants from the Unit B cohort and these were indicated as missing in further analysis. All data on other variables were complete.

Administered transfusions and transfusion parameters

In Unit A 128 out of 218 (59%) infants born before 32 weeks of gestation received red blood cell transfusions, compared to 186 out of 241 (77%) infants in Unit B (p <0.001). In the infants born between 24^{+0}-27^{+6} weeks of gestation respectively 95 % and 91% of the infants received transfusions. In the group born between 28^{+0} and 31^{+6} weeks the proportion of infants receiving blood transfusions was respectively 49% (n=86) in Unit A and 74% (n=147) in Unit B (p <0.001). (Table 1) Because of this difference, we also analysed the cohorts according to gestational age. In the following two paragraphs the results from the infants born between 24^{+0}-27^{+6} weeks and from the infants born between 28^{+0}-31^{+6} weeks of gestation are shown separately.

Table 1: Clinical characteristics of the study population

	Unit A (n=218)	Unit B (n=241)
Birth weight, g (mean ± SD)	1256 ± 358	1261 ± 343
Gestational age weeks (mean, range)	29^{+3} (25^{+0}-31^{+6})	29^{+3} (25^{+0}-31^{+6})
- 24^{+0}-27^{+6} weeks, % (n)	20% (44)	18% (43)
- 28^{+0}-31^{+6} weeks, % (n)	80% (174)	82% (198)
Born from singleton pregnancy, % (n)	62% (134)	66% (159)
Born from multiple pregnancy, % (n)	38% (84)	34% (82)
Male gender, % (n)	57% (125)	56% (135)
CRIB II Score (median, range)	7 (1-16)	6 (1-14)*
AS 5' <7, % (n)	12% (27)	11% (26)
Cord clamping time, s (median, range)	5 (0-30)	10 (0-120)
Endotracheal ventilation % (n)	48% (105)	42% (100)
Ventilation days, median (range)	4 (1-89)	6 (1-95)
Infants requiring transfusion, % (n)	59% (128)#	77% (186)#
-24-28 weeks, % (n)	95% (42)	91% (39)
-28-32 weeks, % (n)	49% (86)#	74% (147)#

Abbreviations: CRIB: clinical risk index for babies; AS: Apgar score
*Median CRIB II score of 214 infants, 27 scores in Unit B were incomplete
#*p-value* <0.01

In the group infants born between 24^{+0}-27^{+6} weeks, the number of infants receiving transfusions was comparable, respectively 42 out of 44 (95%) in Unit A and 39 out of 43 (91%) in Unit B (p= 0.43). Also the mean number of transfusions per infant was similar, 5.1 ± 2.9 in Unit A and 5.2 ± 2.3 in Unit B (p= 0.88, mean difference 0.1, 95% CI (-1.2 – 1.1)). Consequently this resulted in a significant difference in total transfusion volume of 77 versus 104 mL per kg body weight. The median first day of transfusion was day 5 (range 1-33) in Unit A and day 3 (range 1-49) in Unit B (p <0.01). The median interval until the next transfusion was 7 days (range 0-44) in Unit A and 9 (range 0-59) in Unit B (p= 0.02). The pre-transfusion hematocrit and hematocrit increment after transfusion (±24 hours) were not statistically different. (Table 2)

The number of infants born between 28^{+0}-31^{+6} weeks that received transfusions was significantly higher in Unit B. The mean number of transfusions per transfused infant was respectively 3 ± 2.7 in Unit A and 2 ± 1.5 in Unit B (p < 0.01, mean difference 1.0, 95% CI (0.3 – 2.0)). The mean volumes transfused per kg body weight were comparable (45 mL per kg in Unit A and 40 mL per kg in Unit B). The median first day of transfusion was comparable in Unit A and B, respectively day 8 (range 1-77) and day 8 (range 1-84), but the interval until the next transfusion was 9 days (range 0-97) in Unit A and 14 days (range 0-65) (p <0.01) in Unit B. Transfusion trigger and hematocrit increment after transfusion showed no differences between both groups. (Table 2)

Table 2: Transfusion parameters in the transfused infants according to gestational age

	24^{+0}-27^{+6} weeks			28^{+0}-31^{+6} weeks		
	Unit A (n=42)	Unit B (n=39)	*p-value*	Unit A (n=86)	Unit B (n=147)	*p-value*
Total number Tx, mean ± SD	5.1 ± 2.9*	5.2 ± 2.3*	0.88	3.0 ±2.7*	2.0 ± 1.5*	<0.01
Total volume transfused, mL/kg, mean ± SD	77 ± 44	104 ± 46	<0.01	45 ± 41	40 ± 30	0.20
1st Tx day, median (range)	5 (1-33)	3 (1-49)	<0.01	8 (1-77)	8 (1-84)	0.99
Interval until next Tx, days median (range)	7 (0-44)	9 (0-59)	0.02	9 (0-97)	14 (0-65)	<0.01
Hematocrit trigger, %	34.9 ± 4.7	35.4 ± 2.9	0.18	34.6 ± 4.6	34.6 ± 3.6	0.99
Hematocrit increment#, %	10.1 ± 5.0	9.7 ± 4.3	0.38	10.1 ± 5.9	10.7 ± 4.2	0.33

*transfusion volume per kg: unit A 15ml/kg and unit B 20ml/kg; # calculated ± 24hours after transfusion. Abbreviation: Tx: transfusion

Transfusion triggers in practice

Within the first 24 hours after birth, a Hb of lower than 8 mmol/L (13g/dL) is recommended as transfusion trigger. Respectively 17 (8%) and 32 (13%) infants were transfused in Unit A and B within 24 hours after birth. The mean hematocrits before transfusion were respectively 34.3 ± 8.1 and 33.6 ± 5.8 (p= 0.71). After 24 hours, the recommended trigger depends on whether an infant suffers from cardio-respiratory problems. We compared the proportion of transfused infants in both units that also had endotracheal ventilation. These proportions were similar in both cohorts, 85 infants out of 218 in Unit A (39%) and 90 infants out of 241 in Unit B (37%). The mean hematocrit before transfusion was respectively 35.1 ± 4.6 and 35.2 ± 3.1 in both cohorts.

The proportion of transfused infants without endotracheal ventilation was 43 out of 218 in Unit A (19%) and 96 out of 241 in Unit B (40%). The mean hematocrit before transfusion was respectively 32.1 ± 4.3 in Unit A and 34.4 ± 3.7 in Unit B (p= 0.001 mean difference 2.3; 95%CI (-3.7; -0.78)).

Clinical outcome

Composite mortality and neonatal complications in both total cohorts as well when analysed according to gestational age were comparable. (Table 3A and 3B).

Table 3A: Neonatal mortality and morbidity in the study cohorts

	Unit A (n=218)	Unit B (n=241)	*p-value*
Composite mortality, BPD, ROP and IVH, % (n)	47% (102/218)	41% (100/241)	p= 0.26 OR 1.2 (0.9-1.8)
Mortality, n (%)	5% (11/218)	5% (13/241)	p= 1.00 OR 1.1 (0.5-2.4)
Survived with BPD (total), n (%)	26% (53/207)	22% (51/228)	p= 0.43 OR 0.8 (0.5-1.3)
≥ grade 2, n (%)	10% (20/207)	12% (28/228)	p= 0.44 OR 1.3 (0.7-2.4)
Survived with ROP (total), n (%)	9% (18/207)	5% (11/228)	p= 0.12 OR 0.5 (0.3-1.2)
≥ grade 3, n (%)	4% (7/207)	2% (5/228)	p= 0.56 OR 0.6 (0.2-2.1)
Survived with IVH (total), n (%)	23% (47/207)	21% (47/228)	p= 0.64 OR 1.1 (0.7-1.8)
≥grade 3, n (%)	3% (7/207)	3% (7/228)	p= 1.00 OR 0.9 (0.3-2.6)
Length of stay, median (IQR)	58 days (45-76)	56 days (47-69)	p=0.41

Abbreviations: BPD: bronchopulmonary dysplasia, ROP: retinopathy of prematurity, IVH: intraventricular hemorrhage, IQR: interquartile range

Table 3B: Neonatal morbidity and mortality according to gestational age in the study cohorts

	24^{+0}-27^{+6} weeks			28^{+0}-31^{+6} weeks		
	Unit A (n=44)	Unit B (n=43)	*p-value*	Unit A (n=174)	Unit B (n=198)	*p-value*
Composite mortality, BPD, ROP and IVH, % (n)	77% (34/44)	81% (35/43)	p= 0.79 OR 0.8 (0.3-2.2)	39% (68/174)	33% (65/198)	p= 0.23 OR 1.3 (0.9-2.0)
Mortality, n (%)	20% (9/44)	16% (7/43)	p=0.78 OR 1.3 (0.4-3.9)	1% (2/174)	3% (6/198)	p= 0.29 OR 0.4 (0.1-1.9)
Survived with BPD (total), n (%)	57% (20/35)	69% (25/36)	p=0.33 OR 0.6 (0.2-1.6)	19% (33/172)	14% (26/192)	p= 0.16 OR 1.5 (0.9-2.7)
≥ grade 2, n (%)	31% (11/35)	39% (14/36)	p=0.62 OR 0.7 (0.3-1.9)	5% (9/172)	7% (14/192)	p= 0.52 OR 0.7 (0.3-1.7)
Survived with ROP (total), n (%)	23% (8/35)	17% (6/36)	p=0.56 OR 1.5 (0.5-4.8)	6% (10/172)	3% (5/192)	p= 0.19 OR 2.3 (0.8-6.9)
≥ grade 3, n (%)	9% (3/35)	11% (4/36)	p=1.00 OR 0.8 (0.2-3.6)	2% (4/172)	1% (1/192)	p= 0.19 OR 4.5 (0.5-41)
Survived with IVH (total), n (%)	26% (9/35)	33% (12/36)	p=0.60 OR 0.7 (0.2-1.9)	22% (38/172)	18% (35/192)	p= 0.36 OR 1.3 (0.8-2.1)
≥grade 3, n (%)	11% (4/35)	6% (2/36)	p=0.67 OR 2.1 (0.4-12)	2% (3/172)	3% (5/192)	p=0.73 OR 0.7 (0.2-2.8)
Length of stay, median (IQR)	59 (46-71)	60 (48-72)	p=0.58	58 (45-77)	54 (47-68)	p=0.26

Abbreviations: BPD: bronchopulmonary dysplasia, ROP: retinopathy of prematurity, IVH: intraventricular hemorrhage, IQR: interquartile range range

Predictors of receiving a transfusion

The variables Apgar score, mechanical ventilation, CRIB II score and hospital of admittance were included in a multivariate analysis. The hospital of admittance (OR 4.7 (95% CI 2.7-8.0)), mechanical ventilation (OR 3.6 (95% CI 1.9-6.4)) and the CRIB II score (OR 1.5 (95% CI 1.4-1.7)) were independent predictors of transfusion needs (p <0.001). (Table 4) Because the median CRIB II scores, the number of infants with mechanical ventilation and duration of ventilation were comparable between both cohorts (Table 1), differences in proportion of transfused infants and transfusion outcome could only be explained by differences in hospital transfusion practice.

Table 4: Predictors of receiving transfusions after premature birth < 32 gestational weeks

	*p-value**	OR	95% CI of OR
Hospital of admittance	<0.001	4.7	2.7-8.0
CRIB II score	<0.001	1.5	1.4-1.7
Mechanical Ventilation	<0.001	3.6	1.9-6.4

*included variables: CRIB II score, Apgar score, mechanical ventilation, hospital of admittance

Discussion

More than half of the infants born before 32 weeks of gestation received blood transfusions early in life. This is in accordance with published practice since the introduction of specified transfusion guidelines for premature infants.[1,17] Postulated transfusion triggers and transfusion volumes per kg body weight in the current guidelines remain topics of discussion. In the Netherlands, all ten perinatal centers use transfusion triggers that are recommended in the Dutch transfusion guideline. The transfusion volume per kg body weight however varies within a range of 10-20 mL/kg. In this observational study we aimed to investigate whether a different standard transfusion volume per kg body weight had impact on the total number of administered transfusions and clinical outcome in two cohorts of premature infants born before 32 gestational weeks.

Although the demographic parameters were similar between both cohorts, we surprisingly observed a significant difference in the number of infants receiving RBC transfusions, 59% in Unit A and 77 % in Unit B (Table 1). When we analysed the cohorts according to their gestational age at birth, almost all infants born between 24^{+0}-27^{+6} weeks of gestation received RBC transfusions in both units. Transfusion with 20 mL/kg did not reduce the total number of transfusions and increased the transfused RBC volume by 35%. As we observed no differences in hematocrit before and after transfusions between Unit A and B, we can only speculate about possible explanations for this difference. These may be due to larger phlebotomy losses in Unit B, or differences in fluid intake or less use of diuretics. The greater volume transfused in infants in Unit B and thereby a greater volume of hemoglobin A, was not associated with a deleterious effects on the health status of these infants. In both units these extreme low gestational age infants suffered from

considerable mortality and such extensive morbidity that a possible deleterious effect of a larger transfused volume remains undetected (Table 3B).

Analysis of the two cohorts born between 28^{+0}-31^{+6} weeks of gestation showed a significant difference in the proportion of transfused infants (49% versus 74 %) between both units. Although the number of administered transfusions per infant in Unit A was higher; when adjusted for the smaller transfusion volume per kg bodyweight; the transfused infants received comparable volumes of donor blood (Table 2). If a center does not apply a donor reduction policy by reservation of a RBC unit for the same patient, transfusion with a lower volume can lead to a higher donor exposure. The first transfusion day was similar between both cohorts. The interval between transfusions however was significantly shorter in Unit A, which used a lower transfusion volume. Comparing the clinical outcome of both 28^{+0}-31^{+6} week cohorts, we observed no statistical differences. Although in Unit A, fewer infants received transfusions, this was not associated with a poorer clinical outcome, compared to Unit B.

Surprisingly, we did not observe a higher hematocrit increase after transfusion with a larger amount of red cells. In the study performed by Paul et al, the higher hematocrit increment after a larger transfusion volume was measured 8 hours after transfusion instead of after 24 hours as in our study. The use of furosemide or other diuretics given as a single dose post-transfusion is not standard practice in our centers. Nevertheless we saw benefit of the larger volume with respect to a longer interval to next transfusion.
In view of the difference in number of transfused infants that were born after 28^{+0}-31^{+6} gestational weeks, we looked at the transfusion triggers that had been used in infants in relation to the recommended transfusion triggers, i.e. infants less than 24 hours after birth and infants with and without endotracheal ventilation. Only in infants older than one day and without endotracheal ventilation, we observed a higher pre-transfusion hematocrit in Unit B. This higher trigger could explain the larger number of transfused infants. The decision to transfuse could be influenced by a difference in clinical symptoms that were not recorded, e.g. apnea or arrhythmia or to a different perception of clinical symptoms by the physicians. In view of these possible differences, a scenario-based survey between the neonatal intensive care units could help to identify under which circumstances the decision to transfuse differs most.

Logistic regression analysis confirmed the hospital of admittance as an important independent predictor of the administration of transfusions. Since the median CRIB II scores and number of infants with mechanical ventilation in both units were comparable, differences in the proportion of transfused infants and outcome could only be explained by different transfusion and/or other policies (e.g. phlebotomy, fluid management, clinical opinion) in the hospitals.

Observational studies like this have many limitations. All data were collected and analysed in retrospect. Because we expected that the indication for transfusion would be similar, we anticipated on investigating the effect of a different transfusion volume using the same transfusion product. This clinical research question was initiated by observations in critically ill adult patients after cardiac surgery revealing that postoperative complications were associated with transfusions of 4 units or more, representing more than 15 mL/kg bodyweight.[18] Overall we observed no differences in clinical composite complications and cannot conclude that a smaller or larger transfusion dose is to be preferred. Transfusing with 20 mL/kg may reduce donor exposure in infants born between 28^{+0}-31^{+6} weeks of gestation if a single donor program is not used. Despite the use of the same national guideline, the clinical motivation for the decision to transfuse appeared to differ between both hospitals, in our study especially in case of more stable infants. Audits among neonatal intensive care units, could be used to better understand why such differences exist when the same guidelines are supposedly being used.

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3

Long term outcome in relationship to neonatal transfusion volume in extremely premature infants: a comparative cohort study

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Abstract

Background: In premature born infants, red blood cell (RBC) transfusions have been associated with both beneficial and detrimental sequels. Upon RBC transfusion, improvement in cerebral blood flow and oxygenation have been observed, while a more liberal transfusion policy may be associated with a better developmental outcome. The effect of the transfusion volume on long-term outcome is not known.

Methods: We performed an observational follow-up study of a cohort of extremely premature born infants, treated in two neonatal intensive care units using a different transfusion volume (15 mL/kg in Unit A and 20 mL/kg in Unit B). The primary outcome was a composite of post discharge mortality, neuromotor developmental delay, blindness or deafness, evaluated at a mean corrected age (CA) of 24 months related to the transfusion volume/kg bodyweight administered during the postnatal hospital stay.

Results: Despite the difference in transfusion volume in two groups of clinically comparable infants, they received a similar mean number of transfusions (5.5 ± 3.2 versus 5.5 ± 2.3 respectively in Unit A and B). The total transfused volume in unit A was 79 ± 47 mL/kg and 108 ± 47 mL/kg in unit B (p = 0.02). The total transfused RBC volume per kg bodyweight was not an independent predictor of the composite outcome (p = 0.96, OR 1.0 (CI 0.9-1.1).

Conclusion: There was no relationship between the composite outcome at 24 months CA and transfusion volume received during the postnatal hospital stay. As there was no clinical advantage of the higher transfusion volume, a more restrictive volume will reduce total transfusion volume and donor exposure. Future research on the optimal transfusion volume per event to extreme preterm infants should include larger, prospective studies with a longer follow-up period through to childhood or even adolescence.

Background

There is ongoing uncertainty whether transfusion of red blood cells (RBC) in the neonatal period influences the clinical outcome and development of premature infants. In the international literature there is discussion about the optimal transfusion volume and trigger.[1] We previously published a study comparing two transfusion dosages of the same RBC product in two Dutch tertiary care neonatal units (NICUs) using the same transfusion trigger protocols.[2] No difference in short term outcome and mortality was observed in extremely and very preterm infants when a dose of 15 mL/kg had been administered compared to a volume of 20 mL/kg bodyweight. Little is known about the long term follow-up of extremely premature infants after RBC transfusion. Only a few randomized trials have been published on long term outcome in premature infants with difference in transfusion practice.[3-4] However, these were studies (the Premature Infants in Need of Transfusion (PINT) trial and the Iowa trial) comparing liberal and restrictive transfusion triggers, exposing the infants in the restrictive group to the possible risks of a low hemoglobin level.[5-6] In sequel to our study on short term outcome we also wanted to compare long term outcome in the same group of extremely premature infants treated with different transfusion volumes but with otherwise comparable transfusion triggers and transfusion products. In our follow-up study we evaluated post discharge mortality, neuromotor developmental outcome and disabilities in a cohort of extremely premature born infants born before 28 gestational weeks in two Dutch tertiary NICUs.

Methods

Ethics

In the Netherlands no ethical approval is required for this type of research as no new intervention or treatment is studied. All collected data were anonymous.

Study population

This is a follow-up study of a cohort of extremely premature infants born before 28 gestational weeks that participated in an earlier study on transfusion practice and short term outcome in two Dutch NICUs. All infants were transfused according to the same transfusion triggers and with a similar RBC blood product with only a different transfusion volume per event, i.e. 15 and 20 mL/kg bodyweight. The study design was previously described.[2] The primary outcome measure was a composite of post discharge mortality, neuromotor developmental outcome and disabilities.

Transfusion guideline and product

All infants were transfused according to the Dutch consensus for blood transfusion 2004.[7] The recommended transfusion triggers vary with postnatal age, degree of illness and need for respiratory support:

- Hb < 8 mmol/L (13 g/dL) (hematocrit (Hct) range 0.38-0.40 L/L) capillary (or < 7 mmol/L arterial (11 g/dL) (Hct 0.32-0.35 L/L)): the first 24 hours after birth in all infants with clinical symptoms of anemia (tachycardia, supplemental oxygen need, apnoea, bradycardia); in all infants on mechanical ventilation or severely ill.
- Hb < 7 mmol/L (12 g/dL) (Hct 0.32-0.35 L/L) capillary: reasonably stable infants with cardio-respiratory problems (patent ductus arteriosus, apnoea, bronchopulmonary dysplasia, need for supplemental oxygen).
- Hb < 6 mmol/L (10 g/dL) (Hct 0.27-0.30 L/L) capillary: stable premature infants with a postnatal age < 4 weeks.
- Hb < 4.5 mmol/L (7 g/dL) (Hct 0.2-0.23 L/L) capillary: stable infants with a postnatal age > 4 weeks if there are no signs of anemia (apnoea, tachycardia, poor weight gain, poor feeding).

In case of symptomatic anemia, transfusion is recommended at a higher threshold. Transfusion volume per kg bodyweight was different between the two hospitals; 15 mL/kg in Unit A and 20 mL/kg in Unit B. This transfusion volume was part of the standard practice in the hospitals and was not chosen for study purposes. The same transfusion product was used in both hospitals. All products consisted of pre-storage filtered RBC stored in additive solution Saline Adenine Glucose Mannitol (SAG-M) (maximum storage time 35 days), with a Hct of 0.58 ± 0.05 L/L. The products were irradiated with 25 Gy less than 24 hours before transfusion. Preventative measures for anemia of prematurity were not standardized. Erythropoietin was not used in the study units. Iron supplementation was started 6 to 8 weeks after birth if there had been no previous RBC transfusion. After RBC transfusion iron supplementation was postponed for four weeks because of the assumed iron load given with the transfusion.

Data collection and outcome parameters

Infants with a syndrome or congenital/hereditary anomaly known to cause a neuromotor developmental delay were excluded from analysis. Data on neuromotor developmental outcome, major disabilities (deafness, blindness) and survival at a mean (± SD) corrected age (CA) of 24 ± 3.4 months were obtained from each child's outpatient follow-up physician, child-psychologist and/or pediatric physiotherapist, who were all trained in neonatal follow-up. Developmental examination was done in different hospitals. The children were assessed with various instruments depending on the hospital of follow-up. Instruments used were the Dutch 2nd version of the Bayley Scales of Infant Development-II (BSID-II-NL)[8], the Griffiths Mental Development Scales[9], Alberta Infant Motor Scale (AIMS)[10], and the Hempel[11], Touwen[12], and van Wiechen[13] assessments of neuromotor development. The children were classified as normal, mildly delayed or severely

delayed, using the cut-off values of each test, classifying severe neuromotor developmental delay as a score of more than 2 SD below average and mild neuromotor developmental delay as a score 1 to 2 SD below the mean. Non-cooperative children received a general assessment by the physician, pediatric physiotherapist and/or child-psychologist. A parental questionnaire was sent to the parents of the children if follow-up at age 2 years was unknown by any of the previously mentioned professionals. Our primary outcome was the composite of post discharge mortality, severe hearing or visual impairment, or neuromotor developmental delay at 24 months CA.
Visual impairment was defined as <20/200 of best eye, hearing impairment was defined as the need for a hearing aid or cochlear implant. Neuromotor developmental delay was defined as a score more than 1 SD below the mean. The definition for bronchopulmonary dysplasia (National Institutes of health Consensus, USA) was used as described by Ehrenkranz et al.[14] For retinopathy of prematurity the revised international classification was used.[15] Intraventricular hemorrhage was graded according to Volpe.[16]

Statistical Analysis

All variables were analyzed by univariate analysis for continuous variables and Chi-Square or Fishers exact probability test for nominal variables. Backward step wise logistic regression analysis was used for the independent effect of the following factors: hospital (representing transfusion dose and unknown factors), number of RBC transfusions, total transfused RBC volume per kg bodyweight, gender, gestational age, birth weight, CRIB II score and Apgar- score at 5 minutes. (SPSS 17 Chicago, United States of America). A *p*-value of less than 0.05 was considered significant.

Results

Eighty-seven extremely premature infants were included in our earlier cohort study (44 in Unit A, 43 in Unit B). Four infants died the first day of life and were excluded from this follow-up study (1 lung hypoplasia, 1 massive pulmonary hemorrhage, 1 severe perinatal asphyxia and 1 cause unknown). None of these children had received a RBC transfusion. Twelve other patients died in the neonatal period for various reasons; they had all received RBC transfusions. They were also excluded from follow-up. Seventy-one of the 87 infants (82%) left the hospital alive (in total 9 in unit A and 7 in unit B died ($p = 0.78$). There were no deaths after discharge from the units. One other patient was excluded from follow-up analysis because of a severe myopathy. Three children were lost to follow-up in the first year of life; one due to emigration, of the other two (twins) the reason is unknown, leaving 67/70 (96%) of the eligible infants surviving the postnatal period for neuromotor developmental follow-up.

There were no statistically significant differences when comparing the baseline characteristics of the patients from both units (Table 1). The number of transfusions administered was similar, 5.5 ± 3.2 (Unit A) versus 5.5 ± 2.3 (Unit B) respectively. As the volume per transfusion event per center differed, the mean total volume transfused in unit A and B was significantly different (79 ± 47 mL/kg versus 108 ± 47 mL/ kg (p = 0.02) respectively). Neuromotor development was evaluated at a mean 24 ± 3.4 months CA. One infant had a severe visual impairment and one child had severe hearing loss. Forty-seven of 67 (70.1%) infants showed normal neuromotor development for corrected age. Seventeen infants (25.4%) had a mild developmental delay and three infants were severely impaired (4.5%). There was no statistically significant difference in the transfused volume for the primary outcome (composite of post discharge mortality, neuromotor developmental delay, blindness or deafness) compared to children with a normal outcome (105 ± 52 mL versus 90 ± 47 mL respectively) (p = 0.96, OR 1.0 (0.9-1.1). In our multivariate analysis none of the tested variables reached statistical significance for an independent association with the composite outcome (Table 2).

Table 1: Characteristics of patients per unit included in follow-up study*

Variable	Total (n=67)	Unit A (n=31)	Unit B (n=36)	*p-value*
Male	41 (61)	23 (74)	18 (50)	0.05
Gestational age, weeks	26 6/7 ± 4/7	26 6/7 ± 4/7	26 6/7 ± 4/7	0.65
Birth weight, g	900 ± 187	906 ± 178	895 ± 197	0.59
Apgar Score at 5 minutes, median (IQR)	8 (7-9)	8 (7-9)	8 (7-9)	0.88
Intraventricular hemorrhage ≥ grade 3	6 (9)	4 (13)	2 (6)	0.40
Bronchopulmonary dysplasia ≥ grade 2	23 (34)	9 (29)	14 (39)	0.35
Retinopathy of prematurity ≥ grade 3	8 (12)	4 (13)	4 (11)	1.00
Total number of transfusions	5.5 ± 2.7	5.5 ± 3.2	5.5 ± 2.3	0.98
mL per kg RBC administered	95 ± 49	79 ± 47	108 ± 47	0.02
Composite outcome	21 (31)	9 (29)	12 (33)	0.71

*Data are reported as number (%) or mean ± SD, or as indicated otherwise
Abbreviations: IQR: interquartile range

Table 2: Predictors of the composite outcome*

Variable	*p-value*	OR	CI of OR (95%)
Hospital	0.8	1.6	0.1-24.7
Number of RBC tranfusions	1.0	1.0	0.2-4.8
mL per kg RBC transfused	1.0	1.0	0.9-1.1
Gender	0.2	0.4	0.0-1.6
Gestational age	0.8	1.0	0.9-1.1
Birth Weight	0.4	1.0	1.0-1.0
CRIB II score	0.6	1.0	1.0-1.0
Apgar Score	0.3	1.2	0.8-1.9

* Composed of neuromotor developmental delay, post discharge mortality, blindness or deafness

Discussion

Transfusion of RBC has been associated with negative and positive effects on clinical outcome. Our study showed no significant differences between a smaller and a larger transfusion volume regarding a composite outcome of post discharge mortality, neuromotor developmental outcome and disabilities at 2 years CA. This would imply that a smaller transfusion volume rather has advantages (generally limiting donor exposure and costs), than negative effects on neuromotor developmental outcome. It is possible that no effects were seen because the difference in transfusion volume per transfusion event was not large enough. On the other hand, the total transfusion volume/kg during the post natal hospital stay was significantly different in the two NICUs. Several studies associated RBC transfusion with the development of retinopathy of prematurity[17-20] and chronic lung disease.[21-23] It is hypothesized that iron overload, caused by multiple RBC transfusions, increases oxidative stress leading to free radical-induced injury to the premature retina and developing lungs.[24-25] However, anemia has been associated with negative effects as well and in particular the brain may be susceptible to low hemoglobin levels. Bell et al, in a randomized study, found a statistically significant higher incidence in the number of infants with intracranial hemorrhage grade IV or periventricular leukomalacia when a restrictive transfusion threshold was applied as compared to a more liberal threshold.[5] On the other hand, neither Chen[24] nor Kirpalani (PINT trial)[6] found a statistically significant difference in the occurrence of (severe) intracranial pathology. However, the follow-up study by Whyte et al, suggested that preterm infants treated with a more liberal transfusion regime may have a better developmental outcome.[4] The premature infants included in this multicenter PINT trial were analyzed at 18-21 months CA with regard to mortality, cerebral palsy, severe visual impairment, hearing-loss, a Bayley-Mental Developmental Index (MDI) score < 70, and a composite of these variables. Although there was no statistically significant difference in composite outcome (45% in the restrictive and 38% in the liberal group), the difference in cognitive delay (MDI score < 70) approached statistical significance in favor of the liberal group. A post-hoc analysis with cognitive delay redefined (MDI score more than 1SD below the age-standardized mean) showed a significant difference favoring the liberal threshold group. This suggests that premature infants may benefit from a higher hemoglobin threshold for transfusion.[4] In the study by Nopoulos et al on the brain structure of 12 year old children previously enrolled in the Iowa trial by Bell and colleagues, only 44 of the 100 children participated.[3] The children who were exposed to the liberal transfusion threshold appeared to have a substantially smaller intracranial volume compared to the children transfused according to the restrictive guideline. Nopoulos mentions unpublished data on long-term cognitive follow-up showing (non-significant but) overall poorer outcome in the group of liberal transfused children. Another recent study has shown that RBC transfusions increase cerebral oxygenation, thereby decreasing the risk of tissue hypoxia.[26] Mercer et al showed that premature male infants after delayed cord clamping, had a better developmental outcome at seven months CA compared

to infants after immediate cord clamping.[27] It is conceivable that upon transfusion of a larger volume, an increase in cerebral flow and better oxygenation can be obtained with a similar effect as described by Mercer. In transfusion guidelines, the range of recommended RBC volume per kilogram bodyweight is rather wide, 10-20 mL/kg. Given the variation in Hct between used RBC products, the guidelines result in a huge variation in actually transfused RBC. We observed no influence of transfused volume RBC per kg bodyweight on a composite of impaired neuromotor development, post discharge mortality and deafness or blindness in extremely preterm born infants evaluated at 24 months CA. This finding remained unchanged after logistic regression analysis correcting for other factors relevant for outcome. The two Dutch NICUs participating in this study used the same transfusion guideline, transfusion trigger and transfusion product, which virtually excludes important differences in degree of anemia between infants. Our study has its limits being a retrospective, non-randomized trial. The neuromotor development was assessed using various different tests. In Unit B all children were assessed by the same special educator who is also a pediatric physiotherapist (ICvH) using the same test (BSID-II-NL) in 95% of the children. The children from Unit A were assessed using various validated tests, performed in different hospitals by different professionals. It may be an incentive to perform a larger, prospective randomized controlled trial, focusing not only on the transfusion trigger but also on the total transfused RBC volume to recommend an optimal transfusion trigger and dose.

Conclusion

A high total transfused volume of RBC per kg was not correlated with the composite outcome of impaired neuromotor developmental outcome, post discharge mortality, blindness or deafness when analyzed at 24 ± 3.4 months CA, hereby questioning the previously presumed possible detrimental effects of RBC transfusions.

Recommendations

Due to differences in neonatal transfusion practice, conclusions on the effect of maintaining a higher Hb value by the use of liberal transfusion triggers or a larger volume of RBC per transfusion are not based on strong evidence. A larger transfusion volume may not be associated with deleterious effects, whereas a smaller transfusion volume may limit donor exposure. Through randomized clinical studies, the effect of more liberal or restrictive triggers on long-term outcome can be studied, as well as the combination with a low (10 mL/kg) or high (20 mL/kg) transfusion volume. A longitudinal study to school age or even adolescence looking for instance at academic competencies might give further insight in the effects of different transfusion volumes.

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4

Erythropoietin levels in premature neonates in relation to red blood cell transfusions

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Submitted

Abstract

Objective: The role of erythropoietin (EPO) in physiological anemia of the premature newborn (AOP) and its relation with red blood cell (RBC) transfusions is still unclear and may depend on neonatal complications, frequent phlebotomies and transfusion policy.

Study design: Using waste material we frequently measured endogenous EPO levels in 46 premature neonates born < 36 gestational weeks, in their first month of life. These levels were correlated to hematological parameters when concomitantly determined, clinical parameters and administered transfusions.

Results: Thirty-six of 46 neonates received ≥1 transfusions starting at median day 4. EPO levels were not correlated with individual (pre-transfusion) hemoglobin levels, although overall higher EPO levels were present in the transfused cohort in the first 4 days after birth (8mU/mL; 5-95 percentile range <1.4–112.5 mU/mL) compared to the period thereafter (3mU/mL; 5-95 percentile range <1.4–23.9 mU/mL) (p <0.001). In neonates with a lower Apgar score and respiratory failure higher EPO levels were observed. EPO levels declined after every administered RBC transfusion; albeit this was only statistically significant after the first transfusion. Incidental high EPO levels > 500mU/mL were associated with life-threatening conditions.

Conclusion: Newborns with AOP and requiring transfusions had higher initial EPO levels, which declined after starting transfusion treatment. We did not find a statistically significant suppressive effect of cumulative RBC transfusions in the first month after premature birth.

Introduction

Transfusion of red blood cells (RBC) is an important element in the treatment of premature neonates. These transfusions have been associated with potential negative clinical outcomes, although causality has not been proven.[1-2] Introduction of transfusion guidelines has led to fewer administered transfusions. Nowadays, most transfusions are administered in the first month of life after premature birth.[3] The transfusion needs in these first weeks result from a combination of physiological and iatrogenic factors. These include the short life span of fetal RBCs, an increasing blood volume due to relative rapid growth, an inadequate erythropoietin (EPO) response to the level of anemia and frequent phlebotomies for diagnostics.[4-5] In premature neonates EPO is mainly produced by the liver, which is less prompt responsive to hypoxia. This results in a lower EPO level than would be expected for the degree of anemia.[6] Per transfusion, premature neonates receive 10 to 20 mL packed RBCs (with an hematocrit (Ht) between 0.60 and 0.80 L/L) per kg bodyweight, depending on local transfusion practice and guideline.[7] This results in a wide range of RBC volume administered. These differences in transfusion practice probably suppress EPO production in variable degrees. Few studies investigated the effect of RBC transfusion on EPO levels and they reported transfusion related suppression of EPO within 48 hours to 7 days after RBC transfusion.[9-10] This suppression normalized 14 days after transfusion.[10] However, most studies reported on premature neonates with a postnatal age of > 4 weeks who already had received multiple transfusions before the EPO levels were measured.[9-10] A small recent study which used a RBC product with a Ht of 0.80-0.85 L/L, transfused approximately twofold of the hemoglobin (Hb) mass that was lost due to blood withdrawal and in this study the EPO production was found suppressed by every transfusion.[8]

The effects of a liberal transfusion policy, aiming to maintain a higher hematocrit, on the neuro-development of premature neonates have been studied. These studies however showed conflicting results. Maintaining a higher hematocrit was associated with a better cognitive development at the age of 18-21 months corrected age[11], but also associated with a worse neuro-cognitive profile and a reduced brain volume.[12-13] A postulated explanation was the suppression of endogenous EPO production due to liberal transfusion management. Lower EPO levels could result in a decreased anti-inflammatory function and impaired cell recovery after brain injury.[12-14] However, in a recent report on acute physiological effects of RBC transfusions, the mean EPO levels before RBC transfusion were similar between the patients in the group transfused at a higher hematocrit compared to the lower hematocrit group.[15] Unfortunately, in this study the effects of multiple RBC transfusions on EPO levels were not reported, so a stronger reduction of EPO after a liberal transfusion strategy cannot be precluded.

Recently it was proposed that administration of EPO after severe brain injury resulting from asphyxia or intra-ventricular hemorrhage could be neuro-protective, but the most optimal EPO dosage regimen is unclear. Besides a presumed deleterious effect of EPO treatment on retinopathy

of prematurity, also neuro-toxic effects of high dose EPO are possible.[14] More subtle brain damage in newborns could however be negatively affected by early RBC transfusions by lowering the endogenous EPO level that may intercede with a potential physiological neuro-protective EPO effect.[12-13, 16] Evaluation of intrinsic EPO levels after premature birth in presence or absence of RBC transfusions can attend studies investigating optimal EPO dosages, especially those focusing on early treatment. Few studies are available that report endogenous EPO levels and the effect of RBC transfusions through the first weeks of life after premature birth. Non-transfused premature neonates were reported to have a mean EPO level of 9.7 mU/mL (range ± 1 *SE* 7.4-10.3 mU/mL) during the first month of life.[17] Anemic premature neonates were reported to have higher (anemic 20 ± 1.08 mU/mL vs non-anemic 14 ± 1.06 mU/mL) EPO levels.[18] Yamashita studied both non-transfused and transfused very low birth weight premature neonates and reported EPO levels in the first week of life ranging from < 5-307 mU/mL, while beyond this first week EPO levels were <20 mU/mL (day 7-50 after birth).[19]

In this report we frequently measured EPO levels in the first weeks of life using waste material in relation to pre-transfusion Hb and administered RBC transfusions using a restrictive transfusion trigger in a group of 46 premature neonates born before 36 gestational weeks.

Material and Methods

Study population and material

In six consecutive months, 46 premature neonates born before 36 gestational weeks admitted at our neonatal intensive care unit were included in this study. Exclusion criteria were congenital abnormalities, hemolytic disease of the newborn and neonates in need of surgery. None of the neonates received recombinant human EPO. The follow-up period was up to 1 month after delivery or until discharge to another hospital or home. Clinical data of the study patients were collected retrospectively. Data on birth weight, gender, Apgar scores, number of RBC transfusions, type of respiratory support, sepsis (clinically suspect and/or positive blood culture), severe intra-ventricular hemorrhage (≥ grade 3) and mortality were registered.

Transfusion policy and RBC product

All patients were transfused according to the operative Dutch guideline for blood transfusion. The triggers in short; within the first 24 hours after birth < Hb 8 mmol/L (13g/dL); stable neonates with cardio-respiratory problems and/or mechanical ventilation < Hb 7 mmol/L (11g/dL); postnatal age < 4 weeks: < Hb 6 mmol/L (10g/dL).[20] The standard transfusion product consisted of pre-storage filtered adult RBC stored in saline adenine glucose-mannitol with a hematocrit of 0.60 ± 0.05 L/L, irradiated if transfused to an neonate weighing <1500 g or gestational age < 32 weeks. The transfusion volume was 15 mL per kg body weight.

EPO measurements

Waste material from these neonates was collected from our clinical chemical laboratory. Before collection, all material was kept between 2-6˚C up to 72 hours after blood withdrawal, thereafter all material was frozen and kept at -40˚C. The EPO Enzyme Linked Immuno Sorbent Assay (ELISA) (IBL, Hamburg, Germany) was used for all measurements. The lower limit of detection of the EPO ELISA was 1.4 mU/mL. Per single measurement, at least 50 µL material was necessary. To validate the use of waste material refrigerated for up to 72 hours, we aliquoted plasma from premature neonates, cord blood and adult blood bank donors which was kept at varying time intervals at 2-6˚C; <24 hours, between 24-48 hours and up to 72 hours until freezing at -40˚C. Additional spiking experiments were performed with respectively 10 and 20 mU EPO alpha per sample. All tests were performed in duplicate. EPO levels remained stable at 2-6˚C during the time series in all samples (R^2: 0.998), indicating that the waste material could be used for testing.

Outcome parameters and statistical analysis

EPO levels were correlated to hematological parameters (Hb, hematocrit, number of nucleated RBC) if the waste material was drawn at the same time as the complete blood count. The first EPO value after birth was correlated to clinical parameters at birth (gestational age, birth weight, Apgar score ≤ 6 at 5 minutes after birth). We also examined EPO levels according to the method of respiratory support and to clinical and/or proven sepsis. Subsequent EPO levels were correlated to the number of administered RBC transfusions prior to EPO measurement. All EPO measurements that fell below the detection level of 1.4 mU/mL, were counted as 0 mU/mL. To test the correlation between continuous variables the coefficient of determination R^2 was calculated. We used a linear mixed model analysis for repeated measures to test changes in EPO levels before and after a RBC transfusion. The fixed effect was the number of administered RBC transfusions up to that moment. The number of measurements per patient differed as well as the number of administered transfusions per patient. Therefore both the patient variable and the number of administered transfusions at the time of EPO level measurement, were included as covariates to correct for inter-patient and intra-patient variability. The distribution of EPO levels was highly skewed, with the most data having low values and a some (extreme) high values. To normalize the data distribution, logarithmic transformation was applied to the values. To include the measurements of '0' mU/mL, a constant was added to all measurements The log-transformed data were used in the analysis. The mean estimates were computed by back-transformation of the log-means. A p-value of <0.05 was considered statistically significant.

Ethics

In the Netherlands no ethical approval is required for this type of research with the use of waste material, as no new intervention or treatment is studied. All collected data were anonymous.

Results

Study population

The 46 neonates are described in Table 1. Thirty-six neonates (78%) received RBC transfusions, with a mean of 2.6 ± 1.5 transfusions per neonate in the first month after birth. The first RBC transfusion was administered at median day 4 after birth (95% range 1-9 days). The mean pre-transfusion hematocrit was 0.34 ± 0.04 L/L and the mean post-transfusion hematocrit was 0.40 ± 0.04 L/L. The mean Ht of non-transfused neonates was 0.46 ± 0.03 L/L in the first week after birth, and 0.36 ± 0.05 L/L in the weeks thereafter. The transfused neonates were more premature compared to the non-transfused neonates, had a lower birth weight and suffered from more complications. More material had been collected from transfused neonates compared to non-transfused premature neonates; reflecting that these children were sicker and exposed to more phlebotomies for diagnostic purposes and consequently more anemic. Transfused and non transfused neonates differed significantly (Table 1); both patient groups were analyzed separately as the non-transfused neonates could not serve as controls for the transfused neonates.

Table 1: Patient characteristics and measured erythropoietin levels

	Transfused (n=36)*	Non-transfused (n=10)*
Gestational weeks, mean (range)	27 4/7 (25 4/7-35 0/7)*	31 0/7 (29 1/7-32 2/7)*
Birth weight, g mean ± SD	1052 ± 256*	1359 ± 258*
Gender - Boys: Girls, n	26:10*	4:6*
Apgar score at 1 minute, median (range)	5 (1-9)	7 (2-10)
Apgar score at 5 minutes, median (range)	8 (6-9)	9 (5-10)
Respiratory support, % neonates (n)	100% (36)	70% (7)
Mechanical ventilation‡, days median (range)	9 (1-22)*	0 (0-3)*
Clinical Complications		
Sepsis§, % neonates (n)	64% (23)	40% (4)
Severe IVH (≥ grade 3), % (n)	8% (3)	0% (0)
Mortality, % (n)	6% (2)	0% (0)
Mean Hb level, mmol/l, week 1	8.3 ± 1.0	10.1 ± 1.4
Mean Hb level, mmol/l, after week 1	8.3 ± 0.9	8.4 ± 1.5
Samples collected after premature birth, n	336	69
-Week 1, n samples	151	37
-After week 1, n samples	185	32
Median Erythropoietin levels mU/mL - *first 4 days* (5-95 percentile range)	8.0 (<1.4 – 112.5)†	<1.4 (<1.4 – 60.6)
Median Erythropoietin levels mU/mL - *after day 4* (5-95 percentile range)	3.0 (<1.4 – 23.9)†	3.0 (<1.4 – 45.6)

Abbreviations: IVH: intra-ventricular hemorrhage
* *p-value* <0.05, † *p-value* <0.001
‡ Synchronized Intermittent Mandatory Ventilation or High Frequency Oscillation, §defined by clinically suspect and/or positive blood culture

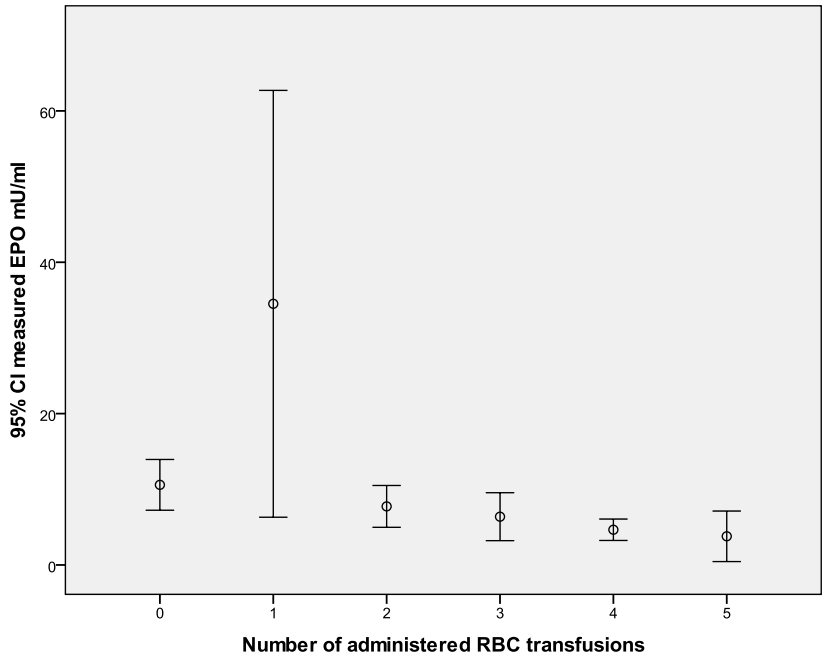

Figure 1: EPO levels measured in the group of transfused neonates (n=36).
The mean and 95% Confidence Interval of the EPO levels (mU/mL) were plotted against the number of administered RBC transfusions at the time of EPO measurement.
0 = before RBC transfusion

Endogenous EPO levels

Seventy percent (n=405; n=336 from transfused neonates / n=69 from non-transfused neonates) of the collected waste samples could be used for EPO measurements in duplicate. Most samples were collected in the first two weeks of life. In the group of neonates that required RBC transfusion (n=36), the first EPO levels measured within 24 hours after birth were not correlated with the first measured Hb concentration (R^2: 0.102), birth weight (R^2: 0.204), gestational age (R^2: 0.102). EPO levels from neonates with an Apgar score of ≤ 6 at 5 minutes after birth were 25 ± 34.4 mU/mL (n=11) compared to the neonates with a better start after birth 8.6 ± 11.9 mU/mL (n=25) (p-value = 0.17).

During the first month in general, EPO levels were not significantly correlated to Hb concentration (R^2: 0.003) and nucleated RBC count (R^2: 0.153) when examined concurrently.

EPO levels during the first week after premature birth were highly variable and after the first week of life we observed overall lower EPO concentrations. As the first RBC transfusion was administered at median day 4, we examined the EPO levels in the first 4 days and the period thereafter. Median EPO level in the first 4 days was 8 mU/mL (5-95 percentile range <1.4 – 112.5 mU/mL). In the period thereafter the median EPO level was significantly lower, 3 mU/mL (5-95

percentile range <1.4 – 23.9 mU/mL) (*p*-value <0.001). EPO levels measured in neonates requiring mechanical ventilation (22.4 ± 97.2 mU/mL) was higher compared to neonates requiring CPAP (8.7 ± 52.8 mU/mL) or requiring no respiratory support (7.2 ± 13 mU/mL). This however was not statistically significant (*p*-value 0.147, one way ANOVA for differences between groups). Neonates in the transfused group who suffered from sepsis had similar EPO levels compared to the other neonates in the same group. (data not shown)

The non-transfused neonates (n=10) were analyzed in a similar manner. EPO levels after birth were not correlated with the first Hb level (R^2:0.044), birth weight (R^2: 0.511), gestational age (R^2:0.011). Only 1 out of 10 neonates had an Apgar score of ≤ 6 at 5 minutes after birth. Median EPO level in the first 4 days after birth was <1.4mU/mL (5-95 percentile range (<1.4 – 60.6 mU/mL) and in the period thereafter 3 mU/mL (5-95 percentile range <1.4 -45.6 mU/mL).

A significant proportion of the samples had EPO levels below the detection limit of 1.4 mU/mL. This was the case in respectively 35% of the samples from the transfused neonates and 48% of the non-transfused neonates.

EPO levels were correlated to the number of RBC transfusions administered prior to EPO measurement with the use of a linear mixed model to correct for repeated measurements within the patients. Both the patient variable and the number of administered transfusions were included to correct for random effects. Per transfusion administered, the decline in EPO level was between -1.3 and -1.9 mU/mL. The decline in EPO after every administered RBC transfusion is reported in Table 2. This decline in EPO was only statistically significant after the first RBC transfusion, a drop of -1.3 mU/mL (*p*-value 0.018). The decline in EPO values after subsequent RBC transfusions were not significantly correlated.

In the first week after birth, incidental high EPO levels were observed, exceeding adult reference values, ranging from over 100 mU/mL to 1200 mU/mL. Two out of three extremely high EPO levels (>500 mU/mL) were observed in neonates who died early after birth. One neonate born at a gestational age of 26 weeks suffered from severe intra-ventricular hemorrhage (≥ grade 3); the other neonate was born after 32 gestational weeks but had severe intra-uterine growth retardation and died because of cardio-respiratory failure. The third surviving neonate was born after 30 gestational weeks and suffered from severe intra-ventricular hemorrhage (≥ grade 3) and sepsis.

Table 2: EPO in relation to administered RBC transfusions.

	Mean estimate	95% confidence interval	*p-value*
After 1 RBC transfusion	-1.3 mU/ml	-1.9 ; -0.45	0.018
After 2 RBC transfusions	-1.5 mU/ml	-2.0 ; -0.76	0.066
After 3 RBC transfusions	-1.7 mU/ml	-2.1 ; -0.95	0.166
After 4 RBC transfusions	-1.6 mU/ml	-2.1 ; -0.95	0.128
After 5 RBC transfusions	-1.9 mU/ml	-2.3 ; -1.34	0.606

Mean estimates (back transformation of the log means) were calculated after every increase in number of administered RBC transfusions

Discussion

In this observational study we measured endogenous EPO levels in premature neonates born before 36 gestational weeks during their first month of life. To avoid extra bloodletting we validated EPO measurement in waste material from blood withdrawn for other clinically indicated diagnostic tests. This enabled us to determine EPO levels approximately 4 times during the first week of life and 1-3 times per week in the period thereafter.

In 37% of all samples, EPO levels were below the detection limit of our assay of 1.4 mU/mL (35% in the transfused group and 48% of the samples in the non-transfused group). Other studies reported higher[17-18] or similar[19] ranges of endogenous EPO levels during the first month of life. These differences could be secondary to the use of different techniques as in most studies a radio-immuno assay (RIA) was used. Comparison of EPO measurement kits showed that the performance of RIA methods was best across the range of 40-150 mU/mL, but more poorly at the lower end of the detection range. The ELISA method we used showed good precision across lower EPO levels.[21]

Thirty-six neonates received RBC transfusions during their first month of life. Ten neonates did not receive transfusions. Both groups differed significantly in basic demographics and were analyzed separately. Transfused neonates suffered from more respiratory and infectious complications and more blood samples had been withdrawn for diagnostic tests, which probably contributed to the transfusion needs. During the first 4 days after premature birth, EPO levels were higher in the anemic neonates who needed transfusions, albeit with a wide range. We observed no relationship between individual EPO concentrations and the Hb levels. Although the differences were not statistically significant; the EPO levels of both neonates with an Apgar score of ≤ 6 at 5 minutes after birth and neonates requiring mechanical ventilation, were clearly higher. This rather suggests that the higher EPO concentrations in the first week of life may be explained by a higher clinical stress level and/or more other sources of hypoxic stimuli. Higher EPO levels in cord blood have previously been found associated with neonatal stress and severe intra-ventricular hemorrhage.[22-23] Also in septicemia higher EPO levels have been recorded.[24] In our cohort EPO levels were however not higher in neonates suffering from sepsis. Extremely high EPO levels in three of our patients were probably indicative for life-threatening events and two of these three neonates died. Because in extremely stressful situations EPO levels can be extraordinary high, it may be advised to measure EPO prior to administration of recombinant human EPO for neuro-protective indications to avoid extreme high EPO levels in severely ill premature neonates.

In the transfused neonates the EPO concentrations declined significantly after the first week of life. To unravel cause or coincidence, we performed a linear mixed model analysis to examine the relation between measured EPO levels and the administered transfusions. Per administered RBC transfusion the EPO levels declined. After the first RBC transfusion this decrease was statistically significant. The continuing decline in EPO after following transfusion was however

not significantly related to the number of administered RBC transfusions. Although statistically not significant the cumulated decrease in EPO could still be clinically important; i.e. after the mean number of administered transfusions (approximately 3), the cumulative decline in EPO level could be more than 4 mU/ml. It is possible that the decline in EPO in our cohort is less distinct after transfusion due to the relative low RBC volume administered per transfusion compared to a RBC product with a higher hematocrit or transfused at a higher volume per kg bodyweight, which also could worsen the suppression of nucleated red blood cells.[8, 25]

In the non-transfused premature neonates EPO levels slightly increased and after the first week of life the EPO levels in both transfused and non-transfused neonates were similar, albeit that after day 18 no samples were available of non-transfused patients.

In conclusion, in a large group of prematurely born infants, we showed by frequent determination of EPO levels in waste material, an initial higher EPO value in the first week of life in the premature newborns that needed RBC transfusions, without a relationship with the individual Hb levels. EPO levels tended to be higher with other causes of hypoxic stress, although we did not find a significant relationship with a low Apgar score at 5 minutes after birth, need for respiratory support or sepsis. In agreement with other studies, EPO levels declined after every RBC transfusion.[8-10] This decline could only be significantly correlated to the first RBC transfusion. It is however possible that differences in transfusion policy (amount of RBC transfused per kg body weight) result in variable degrees of EPO suppression.

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5

The use of cord blood for transfusion purposes:

current status

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Abstract

Although the use of umbilical cord blood (UCB) for transfusion purposes has been proposed decades ago, the employ is still limited. In this article we review studies evaluating UCB collection efficiency and sterility, examine processing and storage of UCB derived red blood cells (RBC) and discuss clinical studies in which UCB was used for transfusion purposes.
Efforts to provide preterm newborns with autologous RBC derived from UCB have not been very successful. UCB collected after full term deliveries can however easily be processed into RBC products and could be used autologous in case surgery of the neonate is indicated early after birth, or for allogeneic small volume paediatric transfusions. To harvest enough UCB volume, immediate clamping of the umbilical cord is commonly used as standard practice. Although delayed cord clamping has shown to improve the iron status in full term infants; for small for gestational age infants this has not been demonstrated. In addition, an increased need for phototherapy after delayed clamping exists. Altogether, we could find no disencouraging evidence to collect UCB, which could be processed into an easy available RBC product for paediatric transfusion in resource-restricted countries.

Introduction

The use of umbilical cord blood (UCB) has become widely established for haematopoietic stem cell transplantation. Although most collections of UCB are merely for its haematopoietic progenitor cell content; the remainder of the red blood cells (RBCs) could serve transfusion purposes. Since Halbrecht in 1939 published his experience on using placental blood for transfusions,[1] many efforts have been undertaken to investigate its use in clinical practice.[2-5] Many concerns existed however, mainly about the bacterial contamination rate and the risk of blood clotting.[6-7] The improved methods to collect UCB in a sterile manner and the development of RBC storage media, stimulated investigations on the use of UCB derived RBCs for clinical purposes.[2,4,8] Although the number of cord blood banks worldwide has increased significantly in the past years, there is still no international consensus on the procedure of UCB collection. As low volume is one of the most common reasons to discard a UCB unit[9], this is of course also a challenge when one aims to use cord blood RBCs for transfusion purposes.

To evaluate its state of the art, we have reviewed the current literature on 4 major topics that contribute to the feasibility of the use of UCB derived RBCs for transfusion purposes, namely; UCB collection efficiency and sterility, UCB processing into a RBC product, storage of UCB derived red cells and the clinical use of UCB transfusion.

Material & Methods

Literature searches were performed in Pubmed and Medline (period until December 2008) using the following keywords; 'umbilical cord blood'; 'blood volume'; 'collection method' and 'umbilical cord blood or placental blood transfusion'. After excluding all duplicates, 696 papers were screened on title and topic. After the first screening, 154 papers remained and were read thoroughly by both authors. The reference lists of all included papers were also screened for additional relevant titles. Papers were included if they met at least one of the following outcome parameters: 1. UCB collection method, harvested UCB volume and sterility; 2. The influence of cord clamping time and harvested UCB volume; 3. UCB blood component processing also reporting on the RBC volume after processing; 4. storage parameters of UCB derived RBC; or 5. the use of UCB for autologous or allogeneic RBC transfusion.

Results

Comparison of UCB collection methods

The most common methods to collect UCB is by puncture of umbilical cord vessels either before (*in utero*) or after (*ex utero*) placental delivery.

Randomised studies

Four randomised studies, conducted by 2 groups of investigators, comparing *in* and *ex utero* UCB collection after full term deliveries, were found. Surbek and colleagues have performed two studies in which they compared both methods, using random allocation before delivery. In their first study they investigated whether *in* or *ex utero* collection was superior after vaginal deliveries.[10] The mean harvested UCB volumes were respectively 83.26 ± 7.9 mL and 48.42 ± 4.07 mL, respectively using the *in* and *ex utero* collection method (p=0.0007). In their second study they used the same design after caesarean deliveries.[11] In this study the mean UCB volumes were respectively 93 ± 7.5 mL and 66 ± 6.6 mL using the *in* and *ex utero* collection method (p=0.004). Although the studies are rather small, the results suggest that in both vaginal and caesarean deliveries, *in utero* UCB collection resulted in significant higher UCB volumes. Two other randomised studies performed by Pafumi and colleagues, compared *in* and *ex utero* collection after caesarean deliveries and supported the results from Surbek et al.[12-13] In the first smaller study *in utero* collection resulted in a mean UCB volume of 90.7 ± 6 mL compared to 60.9 ± 13.7 mL after *ex utero* collection. Although the mean volumes collected in their second larger study were lower than in their first study (74.9 ± 7 mL vs 35.8 ± 3.6 mL), *in utero* collection in both studies resulted in higher harvested UCB volumes compared to *ex utero* collection.[13] (Table 1)

Table 1: Randomised studies comparing UCB collection methods after full term deliveries

	Authors (ref nr)	Mode of delivery	N	cord clamping time	*in utero* harvested (ml)*	*ex utero* harvested (ml)*	*p-value*
1	Surbek et al (10)	V	42	<30 sec	83.26 ± 7.9 (n=23)	48.42 ± 4.07 (n=19)	0.0007
2	Surbek et al (11)	CS	40	<10 sec	93 ± 7.5 (n=21)	66 ± 6.6 (n=19)	0.004
3	Pafumi et al (12)	CS	47	nm	90.7 ± 6.0 (n=21)	60.9 ± 13.7 (n=26)	<0.001
4	Pafumi et al (13)	CS	149	nm	74.93 ± 7.1 (n=73)	35.78 ± 3.6 (n=76)	0.013

Data are indicated as mean ± SD
Abbreviations: ref nr: reference number; UCB: umbilical cord blood; V: vaginal delivery; CS: Caesarean Section; nm: not mentioned, decided by the operator; Symbols: * excluding anti-coagulant

Observational studies

Most of these studies are retrospective reports of the cord blood bank experience using one or two collection methods. To be able to evaluate the efficiency by method, we included only the studies that reported at least on both collection method and harvested volume. In 33 observational studies, the efficacy of the collection method could be evaluated. In Table 2, nine studies are shown which reported on both *in* and *ex utero* collection, and if possible, divided per mode of delivery. Most of the studies report only data of processed and banked products. In addition, however, the discard rate after collection was reported in some studies, which we could also use to compare the collection method efficiency within one center, but not between the centers as the discard cut-off values differed significantly. The most common reasons to discard UCB products mentioned in the studies were a low UCB volume and low TNC count.

In seven studies, comprising a range of 151 to 1722 deliveries per study, the comparison of an *in* and ex *utero* collection method showed no statistically significant differences in harvested UCB volumes.[14,15,18,20-23] In three of these studies, the collection method depended on the mode of delivery[18,20, 23] and in two other studies the collection method was evaluated solely after caesarean deliveries.[21-22] In the studies by Reboredo et al, Sparrow et al and Kögler et al, in case of a caesarean section only an *ex utero* collection method was used.[18,20, 23] Sparrow et al found in a rather small cohort of 157 deliveries no differences between *in* and *ex utero* harvested volume after vaginal deliveries, but *ex utero* collection after caesarean sections resulted in a higher median UCB volume compared to the volumes collected after vaginal deliveries.[18] Tamburini et al and Solves et al compared an *in* and *ex utero* collection method after caesarean sections.[21-22] Their results, showing no differences between both methods, are in contrast to the results reported by Surbek et al and Pafumi et al in controlled randomised studies, which reported higher harvested volumes when an *in utero* method was used after caesarean section.[11-13]

Solves and colleagues reported a significant higher harvested UCB volume when an *in utero* collection method was used after vaginal deliveries in two studies.[16-17] Because the discard rates in both studies were similar we included only the largest study in Table 2.[16] When an *in utero* collection method was used the discard rate was lower compared to ex *utero* harvesting, respectively 25% vs 33%, thereby supporting the results found by Surbek et al.[11, 16-17]

Merely one smaller observational study reported a significant higher harvested UCB volume when an extra uterine collection method was used. In this study by Yamada et al, the *ex utero* collection method after caesarean section resulted in a significant higher mean UCB volume compared to vaginal deliveries after which an *in utero* method was used.[19] This is however in contrast to the study by Reboredo et al, which found similar harvested UCB volumes after *in utero* collection after vaginal deliveries and *ex utero* collection after caesarean section.[20] An explanation for these different observations may be small differences in timing of cord clamping, although reported within 30 seconds after birth, and the position of the newborn after birth, between the two types of delivery which could have influenced the harvested volumes significantly.[19,24] (Table 2)

Table 2: Observational studies comparing *in* and *ex utero* UCB collection

#	Authors (ref nr)	mode of delivery	N	collection method	cord clamping time	*in utero* harvested (ml)	*ex utero* harvested (ml)	*p-value*	Discard % (cutoff value)	bacterial contamination (%)
1	Kögler et al (23)	V+CS	574	In utero (n=450 V) Ex utero (n=124 CS)	nm	74 ± 25¥ (25-191)	65 ± 27¥ (12-160)	ns	total 7% (≥40 ml UCB)	<1%
2	Wall et al (14)	V+CS	334	In utero (n=293 V) Ex utero (n=41 CS)	nm	81 (40-170)	75 (41-140)	ns	total 50% (≥7.10^8 WBC)	2-5%
3	Yamada et al (19)	V+CS	155	In utero (n=126 V) Ex utero (n=29 CS)	<30 sec	84.2 ± 25.3* 27.4 ± 8.4 ml/kg BW	103.9 ± 33.6* 36.5 ± 9.7 ml/kg BW	p=0.0005	na	nm
4	Reboredo et al (20)	V+CS	576	In utero (n=472 V) Ex utero (n=104 CS)	<30 sec	93.8 ± 25.2*	92.6 ± 20.4*	ns	total 40% (≥4.10^8 WBC)	5%
5	Lasky et al (15) (multicenter trial in 5 centers)	V	1722	In utero (n=245) Ex utero respectively (n=395), (n=469), (n=216), (n=397) per center	nm	76.8 ± 26.4 (range 40-162)*	85.9 ± 27.5 93 ± 30.5 72.5 ± 23.2 85.8 ± 28.7 (range 32-247)*	ns	In utero :53 % Ex utero: 34-52% (≥6.10^8TNC)	0-3%
6	Sparrow et al (18)	V	157	In utero (n=58) Ex utero (n=99)	nm	67 (36-153)*	63 (30-148)*	ns	nm (≥5.10^8 WBC)	nm
		V+CS	119	In utero (n=58 V) Ex utero (n=61 CS)	nm	67 (36-153)*	76 (40-162)*	p= 0.0001	nm (≥5.10^8 WBC)	nm
7	Solves et al (16)	V	848	In utero (n=484) Ex utero (n=364)	nm	107 ± 51	99 ± 28	p<0.05	25% vs 33% (≥5.10^8 TNC)	1-5%
8	Tamburini et al (21)	CS	151	In utero (n=69) Ex utero (n=82)	nm	110 (52-190)	102 (54-192)	ns	62% vs 60% (≥1.10^9 TNC)	0%
9	Solves et al (22)	CS	253	In utero (n=113) Ex utero (n=140)	nm	88.6 ± 32.5	92.5 ± 27.6	ns	nm (≥5.10^8 TNC)	0%

Data are indicated as mean ± SD or as median (range), or as indicated otherwise
Abbreviations: ref nr: reference number; UCB: umbilical cord blood; V: vaginal; CS: caesarean section; nm: not mentioned; na: not applicable; WBC: white blood cells; TNC: total nucleated count.
Symbols: ¥: mean ± SEM; *excluding anti-coagulant

Twenty-five centers reported on either *in utero* or *ex utero* UCB collection. (Table 3-4) Like the earlier studies mentioned, these studies reported characteristics of processed or banked products. In some studies the discard rate was reported or could be calculated. The nine studies in which only *in utero* collection was used, comprising over 13.000 deliveries (range 79-8623 deliveries per study) reported a broad range in harvested UCB volume, 20-256 mL in individual cases but also an approximately twofold difference, from 56 mL to over 130 mL (the last including 25 mL anti-coagulant) in mean harvested UCB volumes, between centers. (Table 3) [25-33] In four studies the discard rate was reported. In the study by Reed and colleagues, the proportion unbanked units was rather low compared to the other studies.[29] This could be explained by the different setting of this study, which had a specific focus on sibling cord blood banking for children with malignant and non-malignant disease. In the other studies by Wada et al, Nakagawa et al and Wu et al, a discard rate range between 40-48% was reported.[28-30]

The nine studies reporting on over 22.000 deliveries (range 130 – 9205 per center) and *ex utero* collections of UCB are mentioned in Table 4, if possible, divided per mode of delivery.[17-18, 34-40] A discard rate range between 18% and 46% was reported in 5 out of 9 studies. Exceptional is the 18% discard rate of one study by Donaldson et al, which is likely due to the pre-collection assessment of the placenta by the mid-wife collectors attempting only collection when they were anticipated to be successful, and thereby lowering the total discard rate. The studies by Sparrow et al, Kurtzberg et al, and Donaldson et al showed higher harvested UCB volumes after caesarean section when an *ex utero* collection method was used.[18, 37, 39] (Table 4)

Altogether; although the data from the randomised studies suggest that after caesarean deliveries the *in utero* collection method is superior,[11-13] this is not supported by large observational studies. The superiority of *in utero* collection after vaginal deliveries in harvesting larger UCB volumes was shown in one randomised study[10], supported by two large observational studies.[16-17] Analysis of all studies show that efficacy of the UCB collection used is closely related to the mode of delivery. Differences in cord clamping time and the position of the newborn before clamping could not be evaluated from these studies as either early clamping or discretion of the obstetrician regarding cord clamping and position of the newborn was reported.

Table 3: Studies reporting on *in utero* UCB collection

#	Authors (ref nr)	mode of delivery	N	cord clamping time	*in* utero harvested UCB (ml)	Bacterial contamination %	Discard rate %
1	Brossard et al (25)	V	158	<15 sec	92 ± 36 (30-200)	0%	nm
2	Bertolini et al (26)	V (N=393) CS (n=62)	455	10-120 sec	V: 71 ± 31 CS: 61 ± 17	3-5%	nm
3	Ademokun et al (27)	V	201	nm	56.6 ± 2 (20-187)*	12%	nm
4	Pafumi et al (28)	V (n=429) CS (n=434)	863	Early	V: 69 ± 23 CS: 79 ± 27	nm	nm
5	Reed et al (29)	V+CS	540	nm	Total 103 ± 32.7 (banked units n=506)^ 42.3 ± 8.2 (unbanked units n=36) total range (32-256)	nm	7% (≥3.10^{8}TNC)
6	Wada et al (30)	V	897	nm	79.4 ± 24.8 (40.8-191.5)*	3%	45% (≥6.10^{8}TNC)
7	Nakagawa et al (31)	V	572	nm	60.8 ± 0.9 (20-182)*	nm	40% (≥3.5.10^{8}TNC)
8	Wu et al (32)	V	8623	nm	95.5 (60-227)*	1%	48% (≥60ml)
9	Manegold et al (33)	CS	79	nm	130 (128.3-142.4) (n=45 primary CS) 135 (118.4-148.1) (n=13 failure to progress) 131 (119.5-142.6) (n=21 fetal distress)	nm	nm

Data are indicated as mean ± SD, median (range) or as indicated otherwise
Abbreviations: ref nr: reference number; UCB: umbilical cord blood; V: vaginal; CS: caesarean section; nm: not mentioned or decided by obstetrician or midwife.
Symbols: * excluding anti-coagulant, ^:including anti-coagulant, ¥: mean ± SEM;

Table 4: Studies reporting on *ex utero* UCB collection

#	Authors (ref nr)	mode of delivery	N	cord clamping time	*ex utero* harvested UCB (ml)	bacterial contamination %	Discard % (cutoff)
1	Armitage et al (34)	V+CS	1000	nm	Total: 78 (41-179) (n=666)	4%	33% ($\leq 4.10^8$ TNC)
2	Donaldson et al (35)	V (n=464) CS (n=285)	749	nm	V: 92 ± 32 CS: 107 ± 36[a]	2.3%	18% (60 ml)
3	Brune et al (36)	V+CS	130	nm	Approx. 20ml/kg independent of BW	0%	nm
4	Sparrow et al (18)	V (n=99) CS (n=61)	160[b]	nm	V: 62 (30-148)* CS: 76 (40-162)*[a]	nm	nm ($\leq 5.10^8$ WBC)
5	Aroviita et al (37)	V (n=326) CS (n=262)	588	nm	Total: 69 (28-116)	<1%	34% ($\leq 8.10^8$ WBC)
6	Jones et al (38)	V+CS	9205	nm	Total: 72 ± 0.27¥	nm	nm (40 ml)
7	Solves et al (17)	V (n=305) CS (n=70)	375	nm	V: 98 ± 28.4 CS: 102.5 ± 21.65	1-5%	33% vs 46% ($\leq 5.10^8$ TNC)
8	Kurtzberg et al (39)	V (n=6472) CS (n=2200)	8730	nm	V: 76.6 CS: 89.2[a]	2%	36% ($\leq 6.10^8$ TNC)
9	Askari et al (40)	V+CS	1628	nm	Total: 85.2 (30-226)	nm	nm

Data are indicated as mean ± SD, median (range) or as indicated otherwise.
Abbreviations: ref nr: reference number; UCB: umbilical cord blood; V: vaginal; CS: Caesarean Section; nm: not mentioned or decided by obstetrician or midwife, WBC: white blood cells, TNC: total nucleated count
Symbols:*excluding anti-coagulant; [a]CS higher than V *p-value* <0.001. [b]: only ex utero collections included, ¥: mean ± SEM

Other UCB collection methods

Besides the comparison of *in* or *ex utero* collection, seven studies applied a modified UCB collection technique.[19,41-46] In five of these studies it was investigated whether additional *in* or *ex utero* perfusion using heparinized NaCl or another anti-coagulant resulted in a higher yield of UCB. In one study an additional pressure device was tested to collect the remaining UCB from the placenta *ex utero*. One other study compared the influence of position of the infant after birth on the in utero collected UCB volume. When the newborn was kept above the level of the placenta before cord clamping, the harvested volume was significantly higher, compared to a lower position below the level of the placenta, 87.7 ± 14.5 mL vs 41.7 ± 8.9 mL.[19] With each of the adaptations tested, the researchers were able to enlarge the harvested UCB volume. (Table 5)[19, 41-46] As most of these modified techniques make use of additional punctures and or a combination of a collection bag-system and syringe, it is not unconceivable that bacterial contamination could occur at a higher rate. Next to this it is important to work out whether such modifications could be easily and ethically implemented in a standard procedure in the delivery room.

Microbial Contamination

In the majority of the observational studies the bacterial contamination rate was mentioned. (Tables 2-5) Some studies mentioned that due to extensive training the contamination rates could be lowered significantly.[20,23] Many studies did however not mention the cultured volume, shown to be of relevance for the chance of finding bacterial contamination.[47] Overall, the percentage of contaminated UCB collections was below 5%, suggesting that there is no difference in contamination risk when an *in* or *ex utero* collection method is used. Only the study by Ademokun et al reported a higher contamination rate of 12%.[27]

In regard to the other collection methods mentioned in Table 5, the study by Elchalal et al showed a rather high contamination rate because of the open systems that were used.[42] The proportion contaminated UCB products used in the clinical studies on autologous UCB transfusion was between 0% and 9%, a similar range compared to the other rates reported.[48-51]

Collection of UCB after preterm birth

In several studies UCB was also collected after preterm deliveries. Brune et al and Garritsen et al used both *in* and *ex utero* UCB collection methods and showed that it was possible to harvest approximately 20 mL per kg bodyweight independent of the birth weight.[52-53] In the study by Eichler and colleagues, the harvested UCB volume per kg birth weight decreased with the infants' maturity. A maximum of 43 mL per kg body weight was harvested in the most immature infants. The absolute amount of harvested UCB did however increase with the birth weight.[48] Surbek et al collected a median volume of 21 mL (range 8-38) after deliveries between 22 and 32 gestational weeks and a median volume of 49 mL (range 21-103) after deliveries between 33 and 36 weeks of gestation (p<0.001), this however could not correlated to the birth weight of the infants.[54-55]

Also in the studies by Jansen et al and Khodabux et al, the collected UCB volume could be correlated to gestational age but this however could not be correlated to the birth weight.[51,56]

UCB collection, cord clamping practice in relation to the effects on newborns

Bertolini and colleagues showed that when the cord was clamped within 30 seconds after birth, a significant larger volume UCB could be harvested, but that this resulted in a lower Hb level of the infants during the first four days of life. When UCB was collected after early clamping, within 30 second, compared to late clamping within 35-180 seconds, respectively a volume of 76 ± 22 mL vs 39 ± 17 mL was collected. The mean neonatal haemoglobin levels were respectively 16 ± 1.1 g/dL in the early clamping group and 17.4 ± 1.3 dL in the late clamping group.[26] This indicated that early clamping resulted in a small decrease of haemoglobin, raising ethical questions of whether this small deprivation of the newborns own cells should be allowed.[57] The WHO has recommended in 2007 that 'the cord should not be clamped as early as necessary', this was however graded as a low evidence recommendation.[58] This means that cord clamping practice is still at the discretion of the obstetrician or midwife. A recent Cochrane review investigated the effects of cord clamping time on the neonatal outcome of full term newborns. Early cord clamping was associated with a significant decrease in haemoglobin level during the first 48 hours after birth (WMD -1.34 g/dL, 95% CI -1.88 to -0.88) compared to delayed clamping, which in addition also approved the iron status at the age of 6 months. One important disadvantage of delayed clamping was however a 2% (95% CI 0.04 to 0) higher risk of jaundice requiring phototherapy.[59] In taking the decision to clamp the cord early or late, on should considerate the potential risk of either deprivation of iron or the risk of jaundice requiring phototherapy.

In the case of preterm infants born before 37 weeks, a recent systematic review showed that a brief delay in cord clamping of at least 30 seconds was safe to use and this was also associated with lower transfusion needs and a significant lower incidence of intra-ventricular haemorrhage. There is however still need for a standardized delayed clamping method regarding, timing of the delay, position of the child after birth and consensus about possible resuscitation, to test the robustness of this method.[60]

Table 5: Studies reporting on a modified UCB collection method

#	Authors (ref nr)	mode of delivery	N	collection method	cord clamping time	harvested UCB (ml)	bacterial contamination %
1	Donaldson et al (41)	V+CS	114	In utero additional saline flushing	0-4 min	in utero 55 (10-140) additional 33.5 (10-115) total: 95 (50-195)	nm
2	Elchalal et al (42)	V	75	In utero (n=25) vs Flush drain open system (n=25) vs Flush and drain in standard bag (n=25)	nm	76 ± 32 174 ± 43 174 ± 41	28% 48% 16%
3	Belvedère et al (43)	V+CS	48	Ex utero Additional (pressure) device	nm	Ex utero: 46.7 ± 25.6 Additional:14.3 ± 7.8 total 60.9 ± 29.2 (17-141.5)	nm
4	Pafumi et al (24)	V	51	In utero - upper position (n=28) vs In utero - lower position (n=23)	<30 sec	87.67 ± 14.54 41.68 ± 8.91	nm
5	Bornstein et al (44)	V	44	In utero Additional saline flushing	nm	81.7 ± 35 40.9 ± 28.7*	3%
6	Skoric et al (45)	V	99	In utero: gravity (n=50) vs In utero: saline flushing (n=49)	nm	86 ± 29 103 ± 35	nm
7	Tsagias et al (46)	V	15	In utero Additional saline flushing	nm	60.6 ± 9 64.7 ± 18.8	7%

Data are indicated as mean ± SD, median (range) or as indicated otherwise.
Abbreviations: ref nr: reference number; UCB: umbilical cord blood; V: vaginal; CS: caesarean section, nm: not mentioned or decided by obstetrician or midwife
Symbols: *excluding anti-coagulant

Cord blood RBC products and storage parameters

Studies reporting on UCB processing into a RBC product are scarce. Armitage et al and Godinho et al used centrifugation to deplete the buffy-coat from the RBC.[61-62] The mean RBC loss in these studies were respectively 53% and 34% (range 5-77) in UCB units smaller than 80 mL, compared to 56% (range 32-75) in UCB units that were larger than 80 mL. Two studies, investigating the use of autologous UCB transfusion in premature infants, used a similar processing method, by centrifugation and extraction using the Optipress, to prepare UCB derived RBC. In the study performed by Eichler and colleagues, 81 % of the premature UCB collections (34 out of 42) could be processed in this manner. A minimal net volume of 30 mL UCB appeared necessary for this procedure. The successfully processed UCB units (n=34) had a mean hematocrit of 62.7 ± 12.4 L/L and a RBC component volume of 28.6 ± 18.8 mL.[48] Garritsen and colleagues used a similar semi-automated processing method, but did not report the UCB derived RBC recovery after processing. The final autologous UCB products in this study had a mean volume of 23.4 ± 9.1 mL and a mean hematocrit of 52.6 ± 10 L/L.[53]

Another method to process UCB into a RBC product is by filtration. Brune and colleagues showed that hollow fibre in-line filtration by gravity could be used for RBC separation from UCB. The filtrated RBC units had a mean volume of 63.3 ± 13.5 mL, a mean hematocrit of 56 ± 6 L/L and there was no white blood cell contamination. The recovery of RBC in the filtered unit was unfortunately not mentioned.[63] The advantage of this method is that it is less laborious compared to the semi-automated centrifugation method. Filtration was however only possible with a minimal volume of 60 mL UCB or larger, whereas centrifugational separation was also possible with smaller volumes; although the RBC loss was proportionally higher when the initial UCB volume was lower.

To investigate whether harvested cord blood could be banked for transfusion purposes, several studies were performed which investigated red cell lesions in stored cord blood units (Table 6). Brandes et al stored whole blood UCB during 8 days in CPD at 2-6°C degrees. During this storage period, there was a modest loss in ATP, but 2.3 DPG declined very rapidly. Hemolysis remained below 1% and potassium rose to a mean value of 16.7mmol/L.[2] Horn stored whole blood UCB during 21 days in either CPD or CPDA and showed that UCB red cells were more affected by storage compared to adult red cells. Storage in CPDA was better than in CPD resulting in higher ATP levels, 47 µmol/gHb vs 1.2 µmol/gHb, after 21 days compared to CPD. The decline in 2.3 DPG and pH were similar between both storage media.[4] Bifano et al stored placental whole blood in CPDA for 28 days.[8] Hemolysis rate was minimal, from 0.29 ± 0.07 % at day 0 to 0.39 ± 0.05 % at day 28 and the increase in potassium mmol/L was comparable to stored adult red blood cells during a similar period. The studies performed by Garritsen et al, Brune et al and Widing et al, showed however different results.[52, 63-64] In these last three studies, cord blood was processed into RBC units and stored in SAG-M or PAGGS-M for 35 days. In contrast to the results from Bifano and colleagues, the haemolysis rate in these three studies increased significantly in

time. After 35 days of storage a mean haemolysis rate of respectively 1.1 ± 0.8 % was seen in the study by Garritsen et al and 1.0 ± 0.7% in the study by Brune et al.[53, 63] The use of storage medium PAGGS-M was however superior to SAG-M in the study performed by Widing et al. The haemolysis rates were respectively 0.3 % (range 0.2-0.4) compared to 0.9 % (range 0.6-1.1).[64] Altogether these results show that at a considerable part of the stored UCB derived RBC units had surpassed the European haemolysis limit of 0.8% and the American haemolysis limit of 1%. The pH levels in the UCB products after 35 days storage were also significant lower, compared to adult RBC products.[63] The decline in pH level in the filtered RBC products was lower compared to the products processed by centrifugation. At day 35 the pH levels were respectively 6.4 ± 0.1 and 6.1 ± 0.1. It is possible that small differences in the ratio of SAG-M to the amount of RBC could explain this observation.[53,63] (Table 6) All studies show that UCB red cells deteriorate faster during storage compared to adult red cells, estimated from haemolysis increase and pH decrease. The results suggest that whole blood storage causes less deterioration than RBC enrichment by centrifugation or filtration and storage in preservation solutes. From the better results obtained with the storage solution PAGGSM, which contains additional phosphate and guanosine compared to SAG-M, it is suggested that not the manipulation of cord blood, but rather the preservative solution may contribute to the storage damage of the RCB. When adapted to a shorter storage period, it is likely that the UCB products would have a similar metabolic profile suiting all product quality parameters as for adult red cells, making them acceptable for transfusion.

Table 6: Studies on cord blood derived red cell storage

Authors (ref nr)	N	harvested CB ml	bacterial contami-nation	storage period	material	storage medium	Red cell parameters after storage period					
							hct (l/l)	hemolysis %	ATP (µg/gHb)	2.3 DPG (µg/gHb)	pH	potassium mmol/L
1. Brandes et al (2)	8	nm	0%	8 days	WB	CPD	nm	nm	3.9 (range 3.1-4.9)	3.93 ± 0.24	nm	16.7 ± 4.7
2. Horn et al (4)	11	nm	Nm	21 days	WB	CPD	nm	nm	1.2 ± 0.14	1.75 ± 0.05	6.65 ±0.08	nm
					WB	CPDA	nm	nm	2.47 ±0.19	0.60 ± 0.025	6.60 ±0.16	nm
3. Bifano et al (8)	25	65 (30-119)	8%	28 days	WB	CPDA	41 ± 2	0.39 ± 0.05	3.46 ± 0.56	1.31 ± 0.28	6.51 ± 0.12	32.0 ± 5.9
4. Garritsen et al (53)	12	24.5-88.3 20ml/kg	1.85%	35 days	RBC	SAG-M	55.2 ± 11.1	1.1 ± 0.8	1.2 ± 0.5	nm	6.1 ± 0.1	nm
5. Brune et al (52)	12	62.5 ± 13.5	Nm	35 days	RBC	SAG-M	57.1 ± 4.8	1.0 ± 0.7	nm	nm	6.4 ± 0.1	nm
6. Widing et al (64)	37	89.6 (35.5-172)	3%	35 days	RBC	SAG-M	±80	0.9 (0.6-1.1)	nm	nm	nm	range 60-70
		(8.5-39.3 ml/kg)		35 days	RBC	PAGGS-M	±80	0.5 (0.4-0.8)	nm	nm	nm	range 60-70

Data are indicated as mean ± SD, median (range) or as indicated otherwise.
Abbreviations: ref nr: reference number; CB: cord blood; nm: not mentioned; WB: whole blood; RBC: red blood cells; CPD: citrate phosphate dextrose; CDPA: SAG-M: saline adenine glucose mannitol; PAGGS-M: phosphate adenine glucose, guanosine, mannitol.

Clinical use of UCB derived red cells for autologous transfusion purposes

The total volume of UCB that can be harvested for transfusion is of course limited. Therefore, as a single transfusion product, it could only be used for small volume RBC transfusions for paediatric patients. In particular premature infants receive frequently RBC transfusions early in life. In a study by Ballin and colleagues, several aspects of UCB collection for neonatal autologous transfusion were investigated. They evaluated the thrombin activation by quantification of prothrombin fragments, one and 10 minutes after cord clamping. All cultures were negative and similar amounts of prothrombin fragments 1 and 2 were seen at one and ten minutes after cord clamping. These results encouraged the authors to administer autologous UCB to a premature infant. The collected UCB served two packed cell transfusions in this case. No untoward transfusion reactions were seen.[65] In the study performed by Eichler et al mentioned above, 4 products were used for autologous transfusion without any untoward adverse effects.[48] Until present, one randomised study has been performed that investigated the clinical use of autologous UCB for the treatment of anemia of prematurity. In this trial, autologous UCB derived RBC products were available for 27% of the transfused infants and could cover 58% (range 25-100%) of the administered transfusions. This study also showed that harvesting of enough UCB from preterm placentas was more problematic when the infants were born before 28 weeks of gestation.[51] Next to premature infants, autologous UCB transfusion could also serve transfusion requirements of newborns in need of surgery after birth, for instance due to congenital malformations. In a case report by Hosono et al, an infant with a huge saccrococcygal teratoma underwent autologous UCB transfusion safely and no additional allogeneic transfusions were needed.[66] In two series of newborns in need of surgery shortly after birth, allogeneic transfusions could be avoided in respectively 36% (9 out of 25 patients) and 70% (7 out of 10) of the cases.[49,67] In all reported cases, no adverse reactions were observed. Brune et al examined the efficacy and safety of autologous UCB transfusions in a series of 52 newborns consisting of 30 premature infants and 22 newborns in need of surgery. The mean increase in Hb after transfusion and the Hb decline the days after autologous UCB transfusion were compared with allogeneic transfusion. They found a similar Hb increase after autologous or allogeneic transfusion, respectively 3.1 and 3.0 g/dL. However, the Hb decline was significantly accelerated after autologous transfusion, 0.32 compared to 0.24 g/dL. Based on their findings, they estimated that homologue transfusion could be replaced by autologous UCB in 40% of the premature infants with a birth weight of 1000-2500 g and newborns in need of surgery to correct congenital malformations.[67] Their first finding could however not be supported by the only randomised study available, which reported that in case of infants born after 30 weeks of gestation, autologous blood was only available in 19% of the infants with a transfusion indication.[51] In the case of extreme low birth weight infants or newborns in need of more complex abdominal or heart surgery, additional allogeneic transfusions would be unavoidable (Table 7).[67]

Regarding the collection of premature UCB for autologous RBC transfusion there are two important limitations which should be outlined. First, although mothers with an increased risk of infection, i.e. maternal fever or prolonged rupture of the membranes, were excluded from UCB collection, there was still an increased likelihood of microbial contamination compared to standard allogeneic RBC products.[51] This increased risk of non-sterility resulted in products that were discarded and should be accounted for in the production costs. Secondly, as already mentioned, collection of UCB from premature placenta's resulted in less blood volume compared to UCB collection after term deliveries. Considering the lower volumes that can be collected, the volume anticoagulant in the collection system could be adjusted to avoid low blood to CPDA ratios. Weisbach et al showed that too much CPDA with respect to the blood volume, could result in an increased hemolysis rate and lower pH.[68] Although such data are however not available yet for UCB, on should consider this.

Clinical use of UCB derived red cells for allogeneic transfusion purposes

Transfusion of UCB in the allogeneic setting has been extensively studied by a research group in India. From 1999 onwards, this group has administered fresh whole blood cord blood transfusions and studied is safety and efficacy.[69-78] In their research setting, UCB was only collected after caesarean section. After collection, all units were typed for ABO/Rhesus and screened for HBV, HCV, HIV and malaria. The collected UCB was stored in CPDA, kept at 1-4°C degrees and transfused within 72 hours after collection. The largest reported series consisted of 129 patients, mostly adults, suffering from anemia in consequence of advanced cancer, aplastic anemia, thallasaemia, ankylosing spondilitis, rheumatoid arthritis or lupus erythromatosis. In this study a total of 413 UCB units were harvested with a mean volume of 86 ± 7.6 mL. These units had a mean Hb of 16.2 ± 1.8 g/dL. Before transfusion, the UCB units were bedside filtered. The number of UCB transfusions that were administered per patient ranged from 1-33 units. No adverse clinical or immunological transfusion reactions were seen.[70] Although not studied extensively, and reported only in the thallasaemia patients, no pulmonary problems were observed. In view of its higher affinity for oxygen, on should consider this in the decision whether or not to use HbF bearing RBC in acute hypoxic patients, even when no alternatives are present.

The use of whole blood UCB transfusions as a treatment for paediatric and adult patients suffering from malaria has also been studied. In this series, 94 UCB units were harvested and transfused to 39 patients. The UCB units had a mean volume of 81 ± 6.6 mL and a mean Hb of 16.4 ± 1.6 g/dL. Each patient received two pooled fresh whole blood UCB units at a time (range 2-6 units per patient) depending on availability and compatibility. The age of the patients ranged from 8-72 years. The post transfusion increase in Hb ranged from 0.5-1.6 g/dL. All transfusions were well tolerated and no transfusion related side effects were observed.[71]

Altogether Bhattcharya reported on over 500 fresh whole blood filtered cord blood transfusions administered to anaemic paediatric and adult patients suffering from a variety of underlying conditions. During follow up of these patients no transfusion related hazards were observed. These data suggest that in case of resource restricted areas, the use of allogeneic whole blood cord blood may be a good alternative when adult donor RBC products are scarce.

Conclusion and recommendations

The clinical use of UCB for RBC transfusion purposes is still limited. In neonatal transfusion practice, efforts have been made to provide premature infants, especially those born before 32 gestational weeks, with autologous RBC.[48,50-53] For another group of neonates, those with antenatal confirmed anomalies which can be corrected short after birth, UCB collection for autologous transfusion has been shown more efficient in avoiding allogeneic transfusions compared to infants suffering from anemia of prematurity.[49,67] Experience with UCB in the allogeneic setting is still limited. In India no adverse transfusion effects were seen in a wide variety of patients that received (pooled) allogeneic fresh whole blood UCB transfusions.[69-78] Altogether, the clinical feasibility of using UCB for transfusion purposes depends on the intended patient groups. A better evaluation on the collection method efficiency pointed at harvesting the largest possible UCB volume would be of benefit for the transplantation setting but would also enlarge the possibilities for transfusion purposes. Besides whole blood transfusion and bedside filtration; hollow fiber in-line filtration seems to be a possibly attractive option to process UCB into an easy and ready available RBC product, although due to the availability of filters suiting this purpose, this method is currently only effective for total volumes greater than or equal to 60 mL.[62]

The use of UCB for small volume allogeneic transfusions in anaemic children in Africa or in malaria endemic areas has also been proposed.[79] A preclinical study showed that donation and transfusion of UCB would be acceptable to women living in Mombasa, Kenya.[80] Planning of cord blood donation programs as suggested by Hassall et al could be of benefit of many children in sub-Saharan Africa. Immediately cord clamping after birth seems however mandatory for efficient UCB collection. The possible advantages and disadvantages of immediate cord clamping should be weighed and discussed with the mother before donation. An important point of concern is that in case of small for gestational age infants, who are more likely to have a increased morbidity, the beneficial effects of delayed clamping have not been demonstrated yet. For autologous transfusion in neonates, it is probably only worth the effort to collect and store UCB in the neonatal surgery setting. In view of the small volumes RBC per unit that can be collected, it is most likely that anaemic children in need of small volume transfusions in resource-restricted countries would benefit most from this easy available transfusion product.

Table 7: Studies investigating autologous cord blood transfusion

Authors (ref nr)	Study design	N	Patient characteristics	Harvested UCB	bacterial contami-nation %	N transfused patients	Could receive autologous UCB transfusion	autologous UCB coverage on total transfusions
1. Ballin et al (65)	Case report	1	Premature born	35 ml (28 ml/kg)	nm	1 (100%)	1 (100%)	100%
2. Eichler et al (48)	Observational	47	Premature born	56.4 ± 33 (34 ± 16 ml/kg)	8.6%	21 (45%)	4 (19%)	6% of the transfusions
3. Imura et al (49)	Observational	50	newborns in need of surgery	72 ± 54 (27 ± 18 ml/kg)	6%	26 (52%)	25 (96%)	100% in 9 out of 25 patients
4.Taguchi et al (67)	Observational	12	newborns in need of surgery	64 ± 36 ml	nm	11 (92%)	10 (91%)	100% in 7 out of 10 patients
5. Brune et al (50)	Observational	52	Premature newborns (n=30) and newborns in need of surgery (n=22)	nm	nm	52 (100%)	52 (100%)	100% in 9 out of 22 premature newborns with BW 1000-2500g 100% in 3 out of 7 surgical newborns with congenital anomalies
6. Hosono et al (66)	Case report	1	newborn in need of surgery	100 ml (26 ml/kg)	nm	1	1 (100%)	100%
7. Khodabux et al (51)	RCT	215	Premature newborns born < 32 gestational weeks	BW <1000g 16 ± 15 ml/kg BW1000-1250g 18± 18 ml/kg BW>1250g 20± 23 ml/kg	5%	94 (44%)	25 (27%)	58% (range 25-100) of the transfusions

Data are indicated as mean ± SD or as median (range).
Abbreviations: ref nr: reference number; BW: birth weight; UCB: umbilical cord blood; nm; not mentioned; RCT: randomized clinical trial

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6

Processing cord blood from premature infants into autologous red blood cell products for transfusion

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Abstract

Background & Objective: The use of umbilical cord blood (UCB) for transfusion purposes has gained interest the past years. UCB transfusion could serve premature infants, who often need red blood cell (RBC) transfusions early in life.

Material & Methods: We investigated the suitability of different storage media. UCB was collected after 25 0/7 – 35 6/7 gestational weeks and centrifuged to concentrate RBC subsequently stored in saline-adenine-glucose-mannitol (SAG-M) or in additive-solution-3 (AS-3), or stored as whole blood in citrate-phosphate-dextrose-adenine-1. Quality parameters were measured at 7 day intervals during 35 days, and compared to the standard RBC product.

Results: White blood cell- and platelet counts were higher in the UCB products. In the fractionated units, hemolysis remained below 1.0% in 64% after 14 days, and in 30% after 21 days. Storage in SAG-M or AS-3 showed similar quality. Whole blood UCB showed better pH and hemolysis rates after 21 days.

Conclusion: UCB can be processed into autologous products for premature infants. Shelf-life is limited to 14-21 days and compares unfavourably to stored whole blood. Considering the early transfusion needs in these infants, a short shelf-life would not be a practical objection.

Introduction

In the past years, the use of umbilical cord blood (UCB) for transfusion purposes has gained the interest of clinicians.[1-4] UCB could be used for either autologous or allogeneic transfusion purposes. For allogeneic use, whole blood UCB from term newborns has been used for paediatric and adult patients suffering from malaria.[5] In the autologous setting, the use of UCB for transfusion has shown to be effective in avoiding allogeneic transfusions in neonates in need of surgery after birth.[6-7] Next to these infants, autologous UCB could serve transfusion requirements in premature infants.[1, 8-10] Despite the use of transfusion guidelines with restrictive triggers, premature infants are still frequently transfused.[11-14] Allogeneic red blood cell (RBC) transfusions have been incriminated to have adverse effects on the neonatal outcome, in particular on 'oxidative' diseases like bronchopulmonary dysplasia and retinopathy of prematurity.[15-17] Whether the use of autologous RBC may overcome these possible side effects is still unknown. Several studies showed that it is feasible to collect UCB after premature birth for autologous RBC transfusion.[2,9-10] In a randomized study we reported earlier on the logistics and the costs to incorporate autologous UCB transfusion for anemia of prematurity in standard blood bank practice.[9]
In this paper we present storage and quality parameters of UCB derived RBC, collected after premature birth, and stored in different extended storage media.

Material and Methods

UCB collection from premature infants

UCB was collected after deliveries between 25 0/7 - 35 6/7 gestational weeks. Exclusion criteria were active blood group incompatibility, hemoglobinopathies, maternal infections (HIV, HBV, HCV, HTLV, and syphilis), premature rupture of the membranes with fever treated with antibiotics, and congenital infections (CMV, Parvovirus B19, Toxoplasmosis). Informed consent for study participation was obtained from (one of) the parents before delivery. After a vaginal delivery, premature UCB (P-UCB) was collected from the undelivered placenta. In case of a caesarean section, collection was performed after the placenta had been delivered. The collection system consisted of a 150 mL bag (Fresenius, Bad Homburg, Germany) containing 5 ml of citrate-phosphate-dextrose-adenine-1 (CPDA-1) (Baxter, IL), a collection tubing system with 2 attached needles (Medisize, Hillegom, the Netherlands) and a syringe (Medisize, Hillegom, the Netherlands) containing (additional) 5 ml CPDA-1. The umbilical cord was sterilized with iodine, the cord vein was punctured and P-UCB was collected by gravity. After collection, the additional 5 mL CPDA-1 in the syringe was added to the collected P-UCB. The collection bag was then stored at 2-6 ° C, prior to transport to the blood bank for further processing.

Fractionation of the P-UCB

A half mL whole blood was used to perform complete blood counts (Beckman Coulter Onyx, Brea, USA) and blood group typing (ABO-RhD, Diamed, Cressier, Switzerland). The remaining P-UCB was transferred to a closed centrifuge circuit (15 minutes at 1010x g, Biosafe Sepax, Eysins, Switzerland). This procedure was started if the collected total blood volume was at least 15 mL (excluding anticoagulant). RBCs were separated from buffy-coat and plasma and adjusted with either saline-adenine-glucose-mannitol (SAG-M) (Fresenius, Bad Homburg, Germany) or additive solution 3 (AS-3) (Braintree, MA) to a red blood cell concentrate (RBCC) with a hematocrit between 0.55 and 0.65 L/L. At least 10 mL of the removed CPDA-1-plasma was used for aerobic and anaerobic bacterial culture (BacTalert, Durham, USA).[18] P-UCB was not tested on contamination of maternal cells. All processing was done within 24 hours after collection. Subsequently, P-UCB products were stored during 35 days at 2-6°C. At 7 day intervals 0.5 mL was removed for quality parameters.

Whole blood storage

UCB collections with a CPDA-1 : blood ratio of 1: < 4 were discarded. Twelve P-UCB collections with an anticoagulant : blood ratio of 1: ≥ 4, were stored as whole blood during 21 days at 2-6°C. Prior to storage, a volume of 10 mL whole blood UCB was used for aerobic and anaerobic bacterial culture (BacTalert, Durham). At 7 day intervals 0.5 mL was removed for quality parameters.

Storage stability

For the quality control we performed an automatic blood cell count (Beckman Coulter Onyx). Undiluted RBCC or whole blood P-UCB was used for sodium, potassium, free Hb and pH measurements. Hemolysis, lactate, glucose and osmotic resistance measurements were done using diluted RBCC or whole blood UCB (5 times diluted in NaCl 0.9%). Samples were centrifuged at 10.000 rpm (9615 x g) for 3 minutes. After centrifugation, the supernatant was kept at minus 80° C until analysis. Sodium and potassium supernatant levels were determined using a standard flame photometry method. The pH was measured on a blood gas analyzer (Bayer rapidlab, Siemens Healthcare Diagnostics, Deerfield, IL). Glucose, lactate, free Hb, hemolysis and osmotic resistance were measured by optical density on a Versamax® plate reader. (Molecular Devices, Silicon Valley, CA) For osmotic resistance measurement, a series of 0.2% - 0.8% NaCl was used to determine the NaCl concentration resulting in 50 % hemolysis.

Storage parameters were compared with pre-expiration values from 10 standard pedi-packs stored for 35 days under the same conditions. The standard pedi-pack consisted of 65 mL adult donor pre-storage leukocyte-depleted, RBC stored in SAG-M. Pedi-pack products have a hematocrit between 0.55 and 0.65 L/L and a white blood cell (WBC) count < 1x10e6/ unit (EC guideline 2004/33/EC).[19]

Statistical and data analysis

Data are presented as mean ± SD unless indicated otherwise. SPSS (version 16, Chicago, IL) for Windows was used for data analysis. Continuous variables were analyzed using independent t-tests. In view of the number of parameters included in our analysis, a *p*-value <0.01 was considered significant.

Results

P-UCB products

Fifty-nine collections were used for the validation study. Forty-seven of these were processed using a closed centrifuge circuit (Biosafe Sepax, Eysins, Switzerland), of which 34 were stored in SAG-M, with a mean volume of 34.4 ± 13.4 mL (including anticoagulant); and 13 in AS-3 with a mean total volume of 32.2 ± 20.5 mL (including anticoagulant), during 35 days at 2-6° C. Twelve P-UCB collections were stored as whole blood during 21 days, mean volume 52.1 ± 20.9 mL (median anticoagulant : blood ratio 1:6, range 1:4 – 1:8). Seven percent of all products were microbially contaminated (4 of 59). The time interval in which we detected positive blood cultures was 13.2 hours – 74.4 hours after processing. The pathogens were identified as Coagulase negative staphylococcus species, Escherichia Coli species and Streptococcus agalactiae B species (2x).

Storage and quality characteristics - fractionated P-UCB products

The fractionated P-UCB products had a mean hematocrit of 0.605 ± 0.04 L/L. At the start, the RBCC products differed in lactate, sodium and potassium levels depending on the storage media (p<0.01). (Figure 1A-B) Remarkably, also the hemolysis rate at the start was higher in RBCC stored in AS-3 compared to SAGM (p<0.001). (Figure 1C)

During storage, we observed similar increases in lactate, potassium and hemolysis, as well as decreases in glucose, sodium and pH, when either stored in SAG-M or AS-3. (Figure 1A-C) After 14 days of storage, 47% (22 of 47) of the fractionated units had a hemolysis rate below 0.8%, and 64% (30 of 47) of the units below a rate of 1.0 %. After 21 days of storage, these hemolysis rates were respectively 21% (10 of 47) below 0.8% and 30 % (14 of 47) below 1.0% hemolysis. After 35 days of storage the mean corpuscular volume (MCV) of RBC stored in SAG-M was higher, compared to the RBC stored in AS-3 (p=0.018) (Table 1).

The P-UCB product storage data at 21 and 35 days were compared with data at pre-expiration day 35 from standard pedi-packs. WBC and platelet counts were higher in all P-UCB products. (Table 1). During storage the hematocrit of the P-UCB products gradually increased as did the MCV. (Table 1) Compared to the standard pedi-pack, the SAG-M products displayed significant differences in pH, hemolysis, and free Hb after 21 storage days (p<0.01). In addition, after 35 days lactate and potassium were also significantly higher in the P-UCB products stored in SAG-M

compared to the standard pedi-pack (p<0.01). Storage of the P-UCB products in AS-3 showed significantly higher free Hb and sodium levels after 21 days of storage (p<0.01) compared to the standard pedipack. At day 35, pH, hemolysis rate and potassium were also significantly different (p<0.01). (Table 2) Although the higher hemolysis rates indicated that the P-UCB derived RBC degraded significantly during storage, this was not seen in the osmotic resistance measurements. Storage in SAG-M or AS-3 showed no significant differences in osmotic resistance compared to the standard pedi-pack. Gestational age at the time of P-UCB collection had no influence on the storage parameters (data not shown).

Figure 1: Quality parameters of fractionated P-UCB products (stored in SAG-M or AS-3) and whole blood P-UCB products (stored in CPDA-1) during 35 days of storage

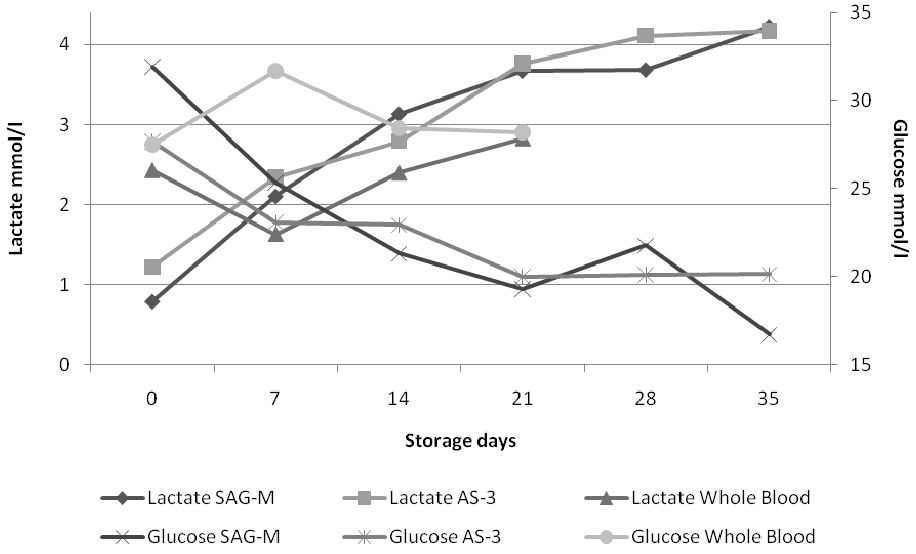

Figure 1A: Lactate and glucose levels during storage
Whole blood lactate at day 21 was significantly lower than SAG-M and AS-3 (p<0.001)
Whole blood glucose at day 21 was significantly higher than AS-3 (p<0.001)
Abbreviations:P-UCB: premature umbilical cord blood; SAG-M: Saline Adenin Glucose Mannitol; AS-3: additive solution 3; CPDA-1: citrate phosphate dextrose adenine 1

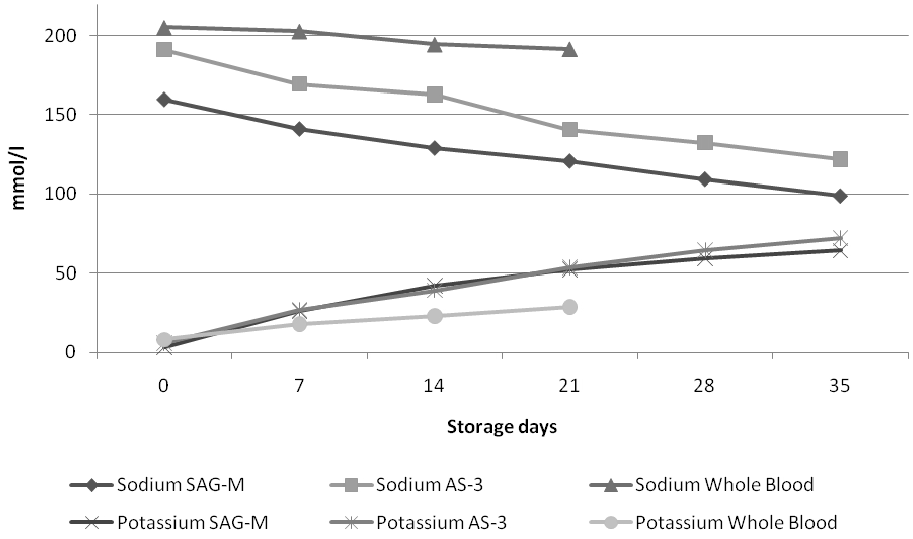

Figure 1B: Sodium and potassium levels during storage
Sodium levels in SAG-M was significantly lower compared to AS-3 and whole blood (p<0.01)
Potassium levels in whole blood was significantly lower compared to SAG-M and AS-3 (p<0.01)
Abbreviations:P-UCB: premature umbilical cord blood; SAG-M: Saline Adenin Glucose Mannitol; AS-3: additive solution 3; CPDA-1: citrate phosphate dextrose adenine 1

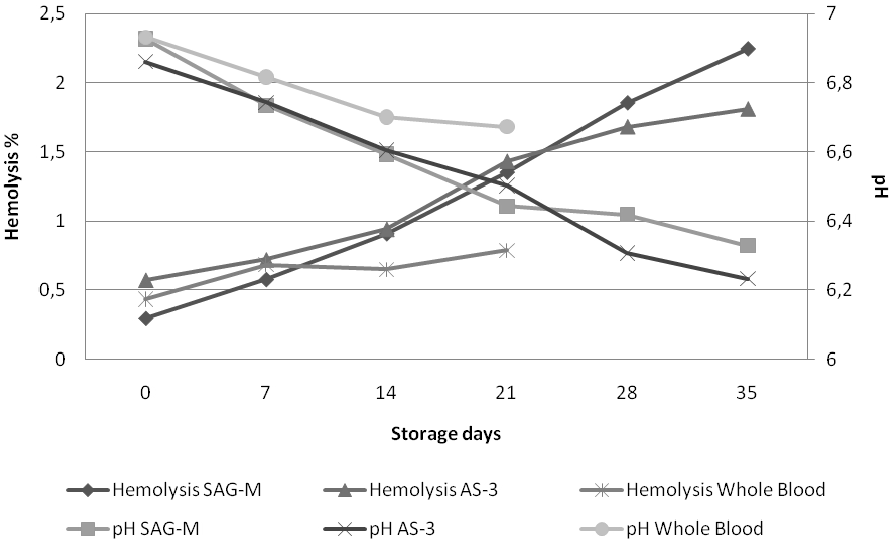

Figure 1C: Hemolysis rate and pH during storage
AS-3 hemolysis rate at day 0 was significantly lower than SAG-M (p<0.001)
Whole blood hemolysis rate at day 21 was significantly lower than SAG-M and AS-3 (p<0.001)
Whole blood pH at day 21 was significantly higher than SAG-M and AS-3 (p<0.001)
Abbreviations:P-UCB: premature umbilical cord blood; SAG-M: Saline Adenin Glucose Mannitol; AS-3: additive solution 3; CPDA-1: citrate phosphate dextrose adenine 1

Table 1: Product cell content after storage 21-35 days at 2-6° C

	SAG-M (n=34)^	AS-3 (n=13)^	Whole blood (n=12)^	Standard Pedi-pack (n=10)*
After 21 days of storage				
RBC concentration, x10e12/l	5.4 ± 0.48	5.6 ± 0.49	3.7 ± 0.88#	
MCV, fl	117.9 ± 6.67	113.8 ± 6.39	110.8 ± 5.98	
Hematocrit, l/l	0.64 ± 0.05	0.64 ± 0.06	0.41 ± 0.08 #	
WBC concentration, x10e9/l	4.5 ± 2.27	4.5 ± 1.74	7.1 ± 4.48	
Thrombocyte concentration, x10e9/l	94 ± 40.7	96 ± 44.6	175 ± 102.4	
After 35 days of storage				
RBC concentration, x10e12/l	5.5 ± 0.50	5.6 ± 0.45	Na	6.5 ± 0.51
MCV, fl	122.1 ± 7.6	116.1 ± 6.9	Na	90.5 ± 3.87
Hematocrit, l/l	0.67 ± 0.05	0.65 ± 0.06	Na	0.58 ± 0.03
WBC concentration, x10e9/l	3.9 ± 2.16	3.7 ± 1.51	Na	<0.001
Thrombocyte concentration, x10e9/l	83.3 ± 37.7	96 ± 43.9	Na	<0.015

Data are indicated as mean ± standard deviation, or as indicated otherwise
RBC: red blood cells; MCV: mean corpuscular volume; WBC: white blood cell; SAG-M: saline adenine glucose mannitol; AS-3: additive solution 3; Na: not applicable
*: only pre-expiration measurement at day 35; ^: $p<0.01$ compared to Standard pedipack; #: $p<0.0001$ compared to SAG-M/AS-3/ standard pedi-pack

P-UCB whole blood storage

After 21 days of storage, the increase in hemolysis and MCV and the decrease in pH, were less pronounced in P-UCB stored as unprocessed whole blood, compared to the P-UCB RBCC in storage solutions. (Figure 1-C) In addition, the whole blood P-UCB products had significant lower lactate-, maintained higher glucose- and had lower potassium levels (Figure 1 A-B). The pH and hemolysis rate in the whole blood products after 21 days were not statistically different when compared to the pre-expiration data of the standard product. The osmotic resistance of the whole blood UCB derived RBC was also similar. (Table 2)

Table 2: Red cell biochemical parameters after storage 21-35 days at 2-6° C

	SAG-M (n=34)	AS-3 (n=13)	Whole blood (n=12)	Standard Pedipack (n=10)*
After 21 days of storage				
pH	6.44 ± 0.13†^ (6.09-6.72)	6.5 ± 0.96~ (6.34-6.63)	6.67 ± 0.19 (6.36-7.02)	
Hemolysis rate, %	1.35 ± 0.64‡~ (0.42-3.47)	1.43 ± 0.91 (0.56-3.36)	0.79 ± 0.42 (0.30-1.59)	
Free Hemoglobin, g/dL	0.63 ± 0.31‡^ (0.18-1.16)	0.82 ± 0.69‡~ (0.23-2.38)	0.13 ± 0.06 (0.05-0.23)	
Osmotic resistance (% NaCl), median (IQR)[1]	0.51 (0.49-0.54)	0.49 (0.46-0.51)	0.48 (0.47-0.51)	
Sodium, mmol/l	120.7 ±12.9#^ (94-143)	140.6 ± 11.9#^ (115-155)	191.5 ± 29.8‡ (163-262)	
Potassium, mmol/l	52.4 ± 10.1^ (36.0-77.0)	53.7 ± 10.8^ (35.9-77.5)	28.8 ± 7.9‡ (16.9-45.3)	
Glucose, mmol/l	19.3 ± 6.3 (10.6-25.1)	19.9 ± 10.5 (7.31-44.5)	28.2 ± 14.9 (10.4-57.3)	
Lactate, mmol/l	3.67 ± 0.71^ (2.36-5.1)	3.76 ± 0.77~ (2.79-5.3)	2.8 ± 0.62† (1.39-3.51)	
After 35 days of storage				
pH	6.23 ± 0.16‡ (6.00-6.78)	6.32 ± 0.06‡ (6.25-6.74)	Na	6.56 ± 0.14 (6.38-6.74)
Hemolysis rate, %	2.2 ± 0.67‡ (1.1-3.5)	1.8 ± 1† (0.76-3.67)	Na	0.7 ± 0.14 (0.32-0.84)
Free Hemoglobin, g/dL	1.05 ± 0.4‡ (0.48-1.88)	1.32 ± 1.2† (0.29-3.38)	Na	0.13 ± 0.03 (0.05-0.18)
Osmotic resistance (% NaCl), median (IQR)[1]	0.53 (0.50-0.61)	0.49 (0.47-0.59)	Na	0.52 (0.51-0.57)
Sodium, mmol/l	106.6 ± 12.8# (82-129)	122.1 ± 12.1# (98-140)	Na	113.8 ± 1.8 (111-117)
Potassium, mmol/l	69.3 ± 11.3‡ (50-91)	72.2 ± 12.4‡ (51-95)	Na	45.2 ± 3.0 (41-50)
Glucose, mmol/l	16.7 ± 10.7 (2-28)	20.1 ± 10.9 (7-40)	Na	22.1 ± 6.3 (9.7-29.7)
Lactate, mmol/l	4.2 ± 0.8† (2.6-5.9)	4.2 ± 1.1 (1.1-5.3)	Na	3.45 ± 0.31(1.1-5.25)

Data are indicated as mean ± standard deviation (min-max range), or as indicated otherwise
SAG-M: saline adenine glucose mannitol; AS-3: additive solution 3; Na: not applicable
[1]:% NaCl causing 50% lysis of RBC
*: only pre-expiration measurement at day 35
†: Compared to day 35 standard pedi-pack p <0.01; ‡: Compared to day 35 standard pedi-pack p<0.001
#: SAG-M compared to AS-3 p <0.001; ~: Compared to Whole blood p<0.01
^: Compared to Whole blood p<0.001

Discussion

In this study we collected P-UCB which was either fractionated using a closed centrifugation circuit and stored in extended storage medium SAG-M or AS-3 or stored as whole blood in CPDA-1. Quality parameters were compared to the standard pedi-pack, consisting of leukocyte-depleted filtered adult donor RBC after 35 days of storage, which are validated for neonatal transfusions.

Comparison of the P-UCB products stored in either SAG-M or AS-3 showed no differences in biochemical parameters. Fractionated P-UCB stored in SAG-M or AS-3 showed a higher hemolysis rate and increase in MCV compared to the standard pedi-pack. The mean hematocrit of the fractionated P-UCB products fulfilled product release requirements of standard pedi-packs. However, residual WBC and platelet counts in the P-UCB products were significantly higher as compared to pre-storage filtered pedi-packs[19] (Tables 1 and 2), indicating that the centrifugation method aiming to remove the buffy-coat with a low loss of RBC, in fact removed few WBCs and platelets. Hemolysis rates after 14 days of storage maintained below the European limit of 0.8% in 47% of the fractionated P-UCB units and below the American limit of 1.0% in 64% of the fractionated P-UCB units. After 21 storage days, 21% of the fractionated P-UCB product remained below 0.8% hemolysis and 30 % below 1.0% hemolysis. This indicates that these P-UCB products have a significant shorter shelf life, and frequent quality control is necessary if these products are used in clinical practice. The osmotic resistance, also an indicator of RBC fragility, did however not differ compared to the standard pedi-pack. This could suggest that mechanical stress, due to collection and/or processing, contributes more to the vulnerability of the cord blood red cells, rather than the fragility caused by swelling of the cells during storage.

The storage parameters from the whole blood P-UCB stored in CPDA-1 showed a better pH, lower hemolysis rate and lower lactate and potassium levels up to 21 days, when compared to the fractionated P-UCB. This may be the result of a lower RBC concentration, resulting in a more diluted cell product including nutrients, or that fetal RBC are less resistant to the mechanical stress of centrifugation. An advantage of whole blood storage is furthermore that it is a less laborious product and there is no RBC loss due to processing, while immature hematopoietic precursor cells, probably of benefit for the infant are not removed along with the leukocytes. The drawback is that stored whole blood showed variability in cell count and in the ratio CPDA-1: collected blood. Despite higher as well as lower ratio's (median 1:6; range 1:4 to 1:8) compared to standard RBCC products (anticoagulant: blood ratio - 1:7), the biochemical parameters maintained rather well up to 14-21 days of storage. However, in view of the lower and variable hematocrit and the high WBC content, it is more complicated to formulate quality parameters under blood bank conditions to use whole blood as an alternative for allogeneic RBC transfusion in premature infants. In comparison to leuko-reduced UCB products, the higher platelet and WBC count could increase the risk on the formation of platelet-leukocyte aggregates upon cold storage

and exert immunomodulation.[20] The WBC in cord blood are more naïve and immature. As such it might be that these cells would have less potential to induce an immunosuppressive effect in a recipient as compared to allogeneic adult WBC. However, studies in adults showed that the clinical immunomodulatory effects on post transfusion infections and mortality of allogeneic and autologous leukocyte-(aggregate)-containing RBC products are controversial.[21]

Another drawback of using whole blood is the volume needed for appropriate microbial testing, in contrast to the waste plasma that was used after P-UCB fractionation. Although a volume of 10 mL (even 20 mL) is optimal for bacterial culture[18]; the use of a smaller volume for microbial testing, for instance 1-2 mL blood, as is used in neonatal practice[22]; is acceptable in cord blood banking. In this study we observed a contamination rate of 7%, which is not at variance with collections for cord blood bank purposes.[23] The pathogens that were identified are known to cause neonatal sepsis. In clinical practice an incubation period of 72 hours is kept before a blood culture is released, as this interval is sufficient to detect all clinically important pathogens.[22] To prevent transfusion of a contaminated autologous product, a similar quarantine period could be held for product release, as most pathogens were found within this time interval.

A few studies on storage of cord blood RBC have been reported. Our P-UCB products stored in SAG-M had significant higher hemolysis rates after 35 days of storage compared to the studies by Garritsen et al, Brune et al and Widing et al, who also stored fractionated cord blood RBC in SAG-M. We found after 35 storage days, a mean hemolysis rate of 2.2 ± 0.67%, compared to their observations, respectively 1.1 ± 0.8%, 1.0 ± 0.7% and 0.9% (range 0.6-1.1). [3, 20,24] The pH in our SAG-M stored P-UCB was comparable to the centrifuged UCB products in the study by Garritsen et al and the filtrated UCB products in the study by Brune et al; 6.23 ± 0.16 versus respectively 6.1 ± 0.1 and 6.4 ± 0.1.[3, 24] It may be that the premature RBC we collected may be more fragile after processing compared to RBC derived from full term cord blood, which generally contains a mean proportion of 30% HbA.[25] Also the more fragile vessels in the preterm placenta may enhance RBC damage during UCB collection.

Similarly, in whole blood P-UCB, hemolysis rate after 21 days of storage (0.79 ± 0.42%) exceeded the rate reported by Bifano et al after 28 days of storage of whole blood UCB collected at term (0.39 ± 0.05%), despite similar mean hematocrit and pH of our whole blood products compared to Bifano et al (Ht, 0.41 ± 0.08 L/L versus 0.41 ± 0.02 L/L and pH 6.67 ± 0.19 versus 6.51 ± 0.12, respectively).[26]

Brune et al have shown that filtration of UCB is also feasible, but RBC loss was only acceptable when at least 60 mL UCB was available.[3] Premature placentas are smaller and several studies showed that the volume of UCB that can be collected is either related to gestational age or birth weight.[1-2, 10, 27-28] Subsequently, the collected volumes are often less than 60 mL and would need adjusted filters to prevent significant RBC loss.

P-UCB products stored in either SAG-M or AS-3 showed a significant increase in hemolysis and decrease in pH compared to the standard pedi-pack. This underscores that macrocytic fetal RBC

may be more vulnerable during storage. In particular, in SAG-M, this swelling was evident. (Table 1) Osmotic fragility was, however, not different when compared to the standard pedi-pack. This vulnerability was not related to the degree of prematurity of the cord blood RBC.

In our clinical study we used the autologous P-UCB products stored in SAG-M for transfusion needs in the first 21 days after birth, under the condition that in case of product release between 14 and 21 days of storage, hemolysis rate was < 0.8%. We showed that premature infants, in particular born before 30 weeks, could be treated with autologous UCB. The first transfusion needs in these infants were at median day 6 after birth (interquartile range 2-13, total range 0-33).[9] Considering the early transfusion needs of these premature infants, the shorter shelf life of autologous P-UCB would not be a major obstacle.

In conclusion, P-UCB can be collected and RBC can be stored for approximately 14 – 21 days for autologous transfusion to premature infants. SAG-M or AS-3 as extended storage media for packed P-UCB cells had no advantage over whole blood storage on the quality of the RBC. P-UCB derived RBC seem more vulnerable for mechanical stress and/or storage at higher hematocrit in extended storage media. Although we showed that the use of autologous P-UCB red cells under blood bank qualifications, including viral testing, is cost-increasing[9], the use of an autologous UCB product for premature infants could potentially be further developed as an alternative for allogeneic transfusion.

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7

A clinical study on the feasibility of autologous cord blood transfusion for anemia of prematurity

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This trial is registered as PUCB-trial ISRCTN01566504.

Abstract

Background: To investigate the use of autologous red blood cells (RBC) derived from umbilical cord blood (UCB), as an alternative for allogeneic transfusions in premature infants admitted to a tertiary neonatal center.

Study design and Methods: UCB collection was performed at deliveries <32 weeks of gestation and processed into autologous RBC products. Premature infants requiring a RBC transfusion were randomized to an autologous or allogeneic product. Primary endpoint was a ≥50% reduction in allogeneic transfusion needs.

Results: 57% of the collections harvested enough volume (≥15mL) for processing. After processing, autologous products (≥10mL/kg) were available for 36% of the total study population and for 27% of the transfused infants, and could cover 58% (range 25-100%) of the transfusion needs within the 21 day product shelf life.
Availability of autologous products depended most on the gestational age. Infants born between 24 and 28 weeks had the lowest availability (17%). All products however would be useful in view of their high transfusion needs. Availability was highest (48%) for the total group of infants born between 28 and 30 weeks. For 42% of the infants with transfusion needs in this group, autologous products were available. For the infants born between 30 and 32 weeks, autologous products were available for 36% of the infants. Transfusion needs in this group were however much lower compared to the other gestational groups.

Conclusion: Autologous RBC derived from UCB could not replace 50% of allogeneic transfusions due to the low UCB volumes collected and subsequent low product availability.

Introduction

The combination of an impaired erythropoietin response to anemia[1-2], a shorter life span of hemoglobin F bearing erythrocytes and high phlebotomy losses[2-4] results in a rapid decline in hemoglobin concentration in premature infants. More than 50% of these infants receive allogeneic blood transfusions early in life[5-8], placing them at risk to presumed negative transfusion effects, such as transmittance of infectious agents and immune suppression.[9] Umbilical cord blood (UCB) has often been suggested as a source for autologous transfusions. [10-12] Due to increasing use of cord blood for transplantation purposes, progress has been made on aseptic collection of UCB and processing of small bloodvolumes.[13-15]

Approximately 20 mL of UCB per kg of body weight can be harvested irrespective of birth weight[13] and several infants have been transfused with autologous UCB safely.[16-20] We previously investigated the potential coverage of autologous UCB in transfusion needs in a preclinical pilot study. This study suggested that UCB collection could be efficient for infants born between 29 and 31 weeks of gestation.[21] However, feasibility of the clinical application of autologous UCB remained to be investigated. To answer this question we performed a randomized clinical trial on the use of autologous cord blood red cell transfusions in premature infants. Our objective was to assess the practical and economical feasibility of autologous red blood cell transfusion and to investigate whether this application could result in a meaningful reduction in the use of allogeneic blood products.

Materials and Methods

This double-blind randomized controlled trial was conducted between January 2005 and October 2006 at the Leiden University Medical Center. The Medical Ethical Committee of the Leiden University Medical Centre approved the study protocol. This trial is registered as PUCB-trial ISRCTN01566504 (www.trialregister.nl).

Study population

Informed consent for study participation was obtained from (one of) the parents before delivery. Infants included were expected to be born before 32 weeks of gestation. According to Sanquin Blood bank guidelines for release of blood products[22], maternal blood was tested for TPHA (treponema pallidum agglutination assay), anti-HBV, anti-HTLV, anti-HIV and anti-HCV antibodies and nucleic acid testing on HIV and HCV. In the weekends STAT testing was performed. Exclusion criteria were maternal antibodies against minor blood group antigens, hemoglobinopathies, maternal infections (HIV, HBV, HCV, HTLV, and syphilis), premature rupture of the membranes and fever that were treated with antibiotics, and congenital infections (CMV, Parvovirus B19, and Toxoplasmosis).

Outcomes

Primary outcome parameter was a reduction of ≥50% in the use of allogeneic RBC products in the first 21 days after birth. Secondary outcome parameters were composite neonatal complications (bronchopulmonary dysplasia, retinopathy of prematurity, intraventricular hemorrhage), length of hospitalization, mortality and cost efficacy.

UCB collection

A specially trained student team collected UCB. After a vaginal delivery, UCB was collected from the undelivered placenta. In case of a cesarean section, collection was performed after the placenta had been delivered. The collection system consisted of a 150 ml bag (Fresenius, Bad Homburg, Germany), a collection tubing system (Medisize, Hillegom, the Netherlands) with 5 mL CPDA-1 (Baxter, Illinois, USA) and a syringe (Medisize, Hillegom, the Netherlands) with 5 mL CPDA-1. The umbilical cord was sterilized with iodine, the cord vein was punctured and UCB was collected by gravity.

In monochorionic deliveries, UCB of both infants was collected in one bag for potential use for both infants. In dichorionic deliveries, two separate collection systems were used.

Blood processing by the Biosafe Sepax

A half mL whole blood was used to perform complete blood counts (Beckman Coulter Onyx, Miami, USA) and blood group typing (ABO-RhD, Diamed, Cressier, Switzerland). The remaining UCB was processed using a closed centrifuge circuit (Biosafe Sepax, Eysins, Switzerland). The procedure was started if total blood volume was at least 15 mL (excluding anti-coagulant). RBCs were separated from buffy-coat and plasma and adjusted with SAG-M to a red blood cell concentrate (RBCC) with a hematocrit between 55% and 65%. At least 10 mL of CPDA-1-plasma was used for aerobic and anaerobic bacterial culturing (BacTalert, Durham, USA).[23] UCB was not tested on contamination of maternal cells.

All processing was done within 24 hours after collection. Autologous RBC in SAG-M with a final volume of ≥ 10mL per kg of body weight of the child were stored for 21 days at 2-6°. Upon request, products were released with a negative bacterial screening or if request was within 7 days, with a negative culture up to date. The requested volume was transferred by sterile docking to a syringe (Medisize, Hillegom, the Netherlands). Autologous RBC products were not irradiated before transfusion, in contrast to the allogeneic donor products. All processes were performed under responsibility of the Sanquin Blood bank. Data regarding the product parameters (ATP, 2.3 DPG, hemolysis and pH) will be published separately, but were within allowed limits for standard packed cells stored for 35 days.

Randomization

All patients with parental consent remained in the study, even if delivery occurred beyond 32 weeks of gestation, to calculate total costs, including the extra costs for maternal virology. All live born infants were stratified according to gestational age, respectively 24-28, 28-30 and 30-32 weeks and were registered as "premature experimental product" (PEP) patients, each with a unique study number. Upon request for a transfusion, the request form was marked with a PEP label and sent to the hospital blood transfusion service. An employee of the blood transfusion department drew a sealed envelope in the correct gestational age group. The infants were randomly assigned to a standard or an autologous product. If an autologous product was not available, a standard product (irradiated leukodepleted packed RBC in SAG-M with a hematocrit between 55% and 65%) was delivered. Both types of transfusion products were delivered in a syringe (Medisize, Hillegom, the Netherlands) to guarantee blinding of the clinical staff of the neonatal intensive care unit.

Transfusion protocol

All infants were transfused according to the Dutch consensus guidelines for blood transfusion.[24] This is the same protocol that was used before start of the study. Transfusion volume according to the guideline was 10-15 mL per kg body weight. Allogeneic blood products were cross-matched with maternal serum and irradiated with 25 Gy. All transfusions were monitored and untoward reactions recorded, according to the hospital hemovigilance protocol.

Clinical data collection

Information on gestational age, gender, Apgar scores, birth weight, umbilical arterial pH, method of delivery, singleton or multiple birth, CRIB II score (Clinical Risk Index for Babies)[25], length of stay, mechanical ventilation and supplemental O_2, were systematically registered in clinical request forms. Data on RBC transfusions ≤ 21 days and > 21 days after birth and neonatal complications (infections, use of antibiotics, retinopathy of prematurity[26], bronchopulmonary dysplasia[27], intraventricular hemorrhage) were obtained from the clinical charts and computerized medical records. Six months after birth, standardized information on medical complications was collected from the parents and from the secondary hospital after transfer from our center.

Sample size

We anticipated a reduction of ≥ 50% in the use of allogeneic RBC products to be worth the effort. To detect this reduction, with a power of 90% and α <0.05, two groups of 85 eligible transfused premature infants had to be included. Taking into account that ± 40% of the infants will not require any RBC transfusion and an expected mortality of 10-15%, a total of 325 infants would need to be included.

Statistical and data analysis

Data are presented as mean ± SD unless indicated otherwise. SPSS (version 12) for Windows was used for data analysis. Data were analyzed on an intention to treat basis and a secondary analysis was performed comparing autologous and allogeneic transfused neonates (according to treatment (ATT) analysis). Continuous variables were analyzed using independent t-tests. Other variables were analyzed using the Fisher's exact test or non-parametric tests. A *p*-value <0.05 was considered significant. Interim analysis was planned 1.5 year after the start of the study to confirm feasibility.

Results

UCB collection and processing

From January 2005 until September 2006, 427 women were admitted with imminent preterm delivery. One hundred and eighty women delivered in our hospital before 32 weeks of gestation. Three women were excluded directly post partum because of sepsis and one infant was excluded because of a congenital CMV infection. A total of 176 women (and 215 infants) met the study criteria for UCB collection (Figure 1). Within this group, 195 collections (from 132 singleton, 24 mono- and 20 dichorionic deliveries, in one dichorionic delivery was only one collection due to intra-uterine mortality) were possible. In 176 cases an attempt was made to collect UCB (Table 1). In 19 cases, there was no collection attempt due to several factors: student team not called in (n=12), umbilical cord torn off (n=3), placenta not intact (n=2) or reason not recorded (n=2). In 58 cases UCB was collected from an intra-uterine placenta and 98 times from an extra-uterine placenta (of which 76 after cesarean sections). In 19 cases an intra-uterine attempt was followed by an additional extra-uterine collection. In one case the record on the placenta was missing.

Of the 176 attempts, 101 (57.4%) harvested a UCB volume of ≥ 15 mL, suitable for Sepax processing. Total volumes, including CPDA-1, from the processed UCB in the three groups according to gestational age were respectively, 32 ± 7.7 mL (16 collections for 18 infants born between 24 and 28 weeks), 44 ± 27.4 mL (32 collections for 36 infants born between 28 and 30 weeks) and 33 ± 13.3 mL (53 collections for 58 infants born between 30 and 32 weeks).

Harvested UCB (excluding anti-coagulant) was respectively 16 ± 15 mL/kg for infants with a birth weight <1000g; 18 ± 18 mL/kg for infants weighing between 1000-1250g and 20 ± 23 mL/kg for infants with a birth weight >1250g. Collected UCB volume was not well correlated with birth weight (r^2=0.24).

Thirty-four blood processing procedures did not result in an approved autologous product (technical failure n=4; final volume too small n=27; other reasons n=3). In total 67 autologous products remained (66.3%, of 101) of which three were withdrawn due to a positive bacterial culture (Coagulase-negative staphylococci n=2, Gram-negative rods n=1). All three cultures were positive within 24 hours. Thus after processing and quality control, 64 suitable autologous products were available for transfusion (36.4% of all 176 collections) (Table 1).

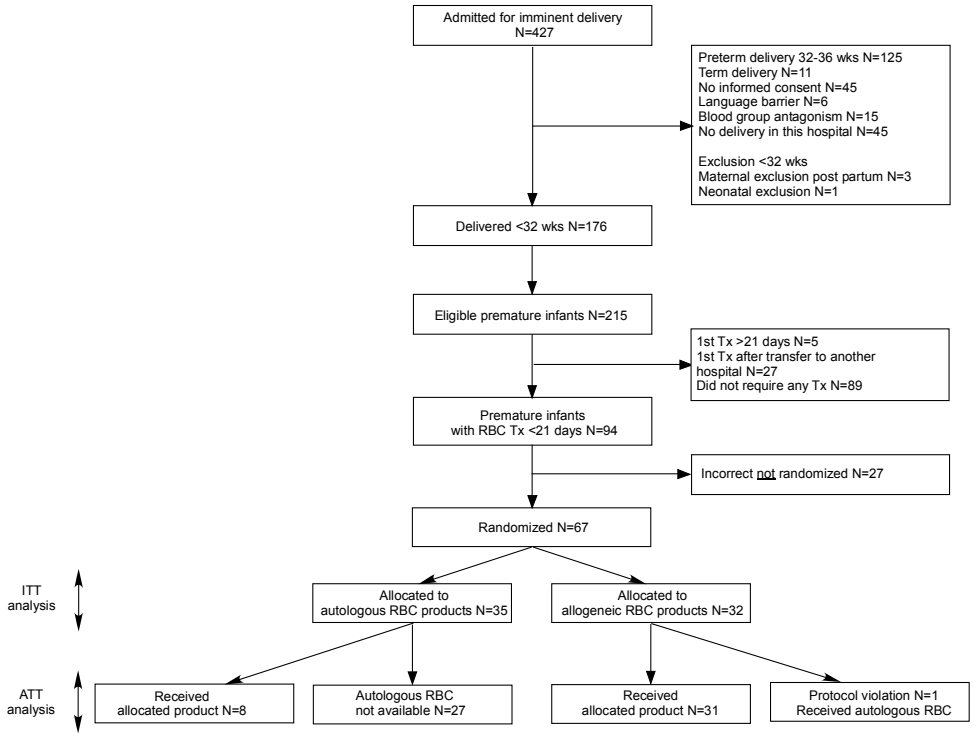

Figure 1: Flow chart – Patient Selection and Randomization scheme
Abbreviations: Tx: transfusion; ITT: intention to treat analysis; ATT: analysis according to transfusion; RBC: red blood cells

Table 1: Collected UCB and autologous RBCC processing

	Total	24-28 weeks	28-30 weeks	30-32 weeks
Eligible infants born <32 weeks, n	215	45	72	98
Possible collections, n	195	41	64	90
No collection attempt, n	19	6	8	5
Collection attempts, n	176	35	56	85
UCB volume < 15 ml	75 (43%)	19 (54%)	24 (43%)	32 (38%)
UCB volume ≥ 15 ml & processed	101(57%)	16 (46%)	32 (57%)	53 (62%)
Autologous RBCC after processing	67	6	27	34
Products with a positive bacterial culture	3	0	0	3
Available autologous RBCC for the total study population	64 (36%)	6 (17%)	27 (48%)	31 (36%)

Abbreviations: UCB: umbilical cord blood; RBCC: red blood cell concentrate

Study population

In the study period, 215 infants were born before 32 weeks of gestation. Of these, five infants required RBC transfusions after 21 days, 27 received their first RBC transfusion after transfer to another hospital, and 89 infants required no transfusions at all. Ninety-four infants required transfusions within 21 days after birth and should have been randomized upon transfusion request. However 27 infants that met the entry criteria for randomization were missed (Figure 1). For these infants, the standard RBC product, a pedi-pack of 65 ml irradiated leukodepleted packed RBC, was requested instead of a study product. In 9 of these cases the urgent transfusion indication within 24 hours after birth could have been the reason for these violations. For the other 18 protocol violations no specific cause was found. The 27 eligible, but non-randomized infants were compared with the two randomized groups to exclude possible confounding factors. All transfused patients remained included in the follow-up analysis, except one infant in the non-randomized group who was lost to follow-up. Baseline demographic and clinical characteristics of all transfused patients were comparable (Table 2). Sixty-seven randomized infants remained, 35 in the autologous arm and 32 in allogeneic arm (Figure 1).

Table 2: Demographic and clinical variables of infants with transfusion indication ≤21days (n=94)

	Autologous group n=35	Allogeneic group n=32	Non-randomized N=27
Birth weight, g, mean ± SD	1050 ± 357	1080 ± 317	1045 ± 358
Gestational age, weeks (range)	28^{+2} (25^{+0}-31^{+5})	28^{+1} (25^{+2}-31^{+6})	28^{+4} (25^{+3}-31^{+5})
-24-28 weeks, n	13	15	11
-28-30 weeks, n	16	12	8
-30-32 weeks, n	6	5	8
Male n, %	26 (74.3%)*	20 (62.5%)*	13 (48.1%)*
Live born infants from twin pregnancy, n	15	12	11
- Monochorionic	8	8	6
- Dichorionic	7	4	5
Vaginal delivery/Cesarean section, n	18:17	21:11	14:13
AS 1′ median (IQR)	6 (4-8)	6 (4-7)	7 (3-8)
AS 5′ median (IQR)	8 (7-9)	8 (7-8)	8 (6-9)
Arterial pH, mean ± SD	7.27 ± 0.1 (n=21)	7.27 ± 0.1 (n=24)	7.28 ± 0.1 (n=18)
Hematocrit after birth %, mean ± SD	44 ± 7	41 ± 10	42 ± 6
Cord clamping time, sec, mean ± SD	7 ± 4 (n=28)	8 ± 4 (n=27)	8 ± 6 (n=22)
CRIB II, median (IQR)	9 (8-11)	10 (7-12)	10 (7-12)
Infants with supplemental O_2, n	33	31	24

Abbreviations: CRIB II: clinical risk index for babies, AS: apgar Score; IQR: interquartile range *most unequally distributed variable p=0.11

Primary outcome

Transfusion triggers in the groups were comparable (Table 3). In the autologous group 2.5 ± 1.4 RBC transfusions were given, 2.7 ± 2.2 transfusions in the allogeneic group (p=0.65) and the non-randomized group received 2.3 ± 1.6 standard pedi-packs. Intention to treat analysis showed that the total number of allogeneic transfusions given was 2.3 ± 1.4 in the autologous group and 2.7 ± 2.2 transfusions in the allogeneic group. This mean difference of 0.4 allogeneic units (15%) in the autologous group was not significant (p=0.38). A reduction of allogenic RBC of 25% was present in infants born between 28 and 32 weeks of gestation (Table 3).

Table 3: Transfused RBC units and transfusion parameters

	Autologous group N=35	Allogeneic group N=32	*p**	Non-randomized n=27
Total transfusions ≤21days Mean ± SD	2.5 ± 1.4	2.7 ± 2.2	0.65	2.3 ± 1.6
-24-28 weeks	3.1 ± 1.1	3.1 ± 2.4	0.98	2.6 ± 1.0
-28-30 weeks	2.5 ± 1.7	2.8 ± 2.2	0.74	2.0 ± 2.1
-30-32 weeks	1.3 ± 0.5	1.6 ± 0.9	0.58	2.4 ± 1.9
Pre hematocrit, % mean ± SD	35.5 ± 4.2	34.4 ± 6.4	0.99	36.1 ± 4.0
Post hematocrit, % mean ± SD	45.7 ± 6.1	44.5 ± 6.5	0.49	44.5 ± 5.2
1st transfusion, day, median (IQR)	4 (2-9)	3 (2-9)	0.25	4 (2-10)
Interval till next transfusion, days, median (IQR)	3 (2-6)	4 (2-8)	0.93	4 (2-6)
Allogeneic RBC				
Allogeneic transfusions ≤21days mean ± SD	2.3 ± 1.4	2.7 ± 2.2	0.38	
-24-28 weeks	3.0 ± 1.1	3.0 ± 2.4	1.00	
-28-30 weeks	2.1 ± 1.6	2.8 ± 2.2	0.41	
-30-32 weeks	1.2 ± 0.4	1.6 ± 0.9	0.36	

Abbreviations: RBC: red blood cells; IQR: interquartile range
* autologous versus allogeneic group

Analysis according to autologous or allogeneic transfusion

Eight infants (23%) in the autologous group received the allocated product. For 27 (77%) infants an autologous product was not available. One infant in the allogeneic group received erroneously an autologous product. After transfusion of the autologous RBC products (n=9) the average hematocrit increase was 9.8% ± 5.5 %, comparable with the increase after an allogeneic transfusion of 10% ± 5.2 %. Median interval until the next transfusion in these infants was 3 days, comparable to the infants who only received allogeneic products. For two infants the available autologous RBC volume only covered 1 of the 4 transfusions they required in the first 21 days. In three infants available autologous RBC covered half of the required transfusions. Three infants received autologous RBC products only (Table 4). No adverse effects were observed.

Because the study was randomized, 11 autologous products were available for infants assigned to allogeneic RBC and 6 products for non-randomized infants. The potential transfusion coverage of these products was estimated. If this had not been a randomized study, the available products would have covered 58% (range 25-100%) of the transfusion needs ≤ 21 days per infant (Table 5).

Table 4: Transfused autologous RBC products and reduction in allogeneic transfusion

Patient # (stratum)	Total Tx ≤21 days (n)	Received Autol Tx (n)	Received Allog Tx (n)	Reduction in Allog Tx (n)
1 (24-28 weeks)	4	1	3	25%
2 (24-28 weeks)	2	1	1	50%
3 (28-30 weeks)	4	1	3	25%
4 (28-30 weeks)	2	1	1	50%
5 (28-30 weeks)	2	1	1	50%
6 (28-30 weeks)	2	1	1	50%
7 (28-30 weeks)	1	1	0	100%
8 (28-30 weeks)	1	1	0	100%
9 (30-32 weeks)	1	1	0	100%
Pre hematocrit, % mean ± SD	37.5 ± 5.2	36.0 ± 6.0	39.0 ± 4.0	
Post hematocrit, % mean ± SD	46.5 ± 7.3	45.8 ± 8.3	47.2 ± 6.5	

Abbreviations: Tx: transfusion; Autol: autologous; Allog: allogeneic, IQR: interquartile range

Table 5: Premature infants with available autologous RBC product in relation to the actually received transfusions

Stratum	Tx needs ≤21 days, n	Infants, n	Autologous RBC ml/kg, mean ± SD	Coverage in Tx needs (%)
24-28 weeks	No tx	0	-	-
	1 tx	1	14 ± 0	100%
	2 tx	2	13 ± 3	50%
	>2 tx	3	11 ± 3	25-33%[1]
28-30 weeks	No tx	12	16 ± 4	-
	1 tx	7	27 ± 12	100%
	2 tx	5	18 ± 8	50-100%[2]
	>2 tx	3	30 ± 11	25-71%[3]
30-32 weeks	No tx	27	17 ± 8	-
	1 tx	3	19 ± 9	100%
	2 tx	1	11 ± 0	50%
	>2 tx	0	-	-

Abbreviation: Tx: transfusion
[1]: 2 infants required 4 transfusions and 1 required 3 transfusions; coverage was respectively 25% and 33%
[2]: 4 infants with 50% coverage, 1 with 100%
[3]: Infants required respectively 3, 4 and 7 transfusions with coverages of respectively 33%, 25% and 71%

Secondary outcomes

Clinical outcome variables are shown in Table 6. Length of stay was registered in our center. Neonatal complications were registered during the complete hospital stay and the six-month follow-up period. The most unequally distributed variable in intention to treat analysis was length of stay (p=0.19), in favor of the allogeneic group and the composite mortality and morbidity (p=0.20) in analysis according to transfusion, in favor of the autologous transfused infants. Analysis of the outcome variables showed no significant differences between the groups.

Table 6: Neonatal Mortality and Morbidity

	ITT		ATT		Non-randomized (N=27)
	Autol (N=35)	Allog (N=32)	Autol (N=9)	Allog (N=58)	
Composite morbidity and mortality, n, %	27 (77%)	24 (75%)	5 (56%)§	46 (79%)§	19 (70%)
Mortality n	3 (9%)	4 (13%)	0 (0%)	7 (12%)	3 (11%)
IVH n	10 (29%)	14 (44%)	2 (22%)	22 (38%)	9 (33%)
≥ grade 3	4 (11%)	4 (13%)	0 (0%)	8 (14%)	1 (4%)
ROP n	8 (23%)	5 (16%)	1 (11%)	12 (21%)	2 (7%)
≥ grade 3	1(3%)	1 (3%)	0 (0%)	2 (3%)	1 (4%)
BPD n	20 (57%)	10 (31%)	4 (44%)	26 (45%)	14 (52%)
≥ grade 2	11 (31%)	5 (16%)	1 (11%)	15 (26%)	6 (22%)
Length of stay, Median (IQR) days	30 (15-48)*	23 (13-39)*	30 (14-37)	28 (15-44)	21 (9-48)
in NICU	19 (14-30)	20 (10-30)	18 (11-31)	20 (11-29)	16 (8-30)

ITT: intention to treat analysis; ATT: according to treatment; Autol: autologous; Allog: allogeneic. *: most unequally distributed parameter in ITT analysis, p=0.19
§: most unequally distributed parameter in ATT analysis, p=0.20

Collection and Production efficacy

Overall successful UCB collection rate (≥ 15ml blood excluding anti-coagulance) was low (57%). Most of the collections were performed from an extra-uterine placenta. Analysis of harvested volume per collection method showed that collections from an intrauterine placenta resulted in higher UCB volumes (37.7 ± 20.2 ml) than collections from an extra-uterine placenta (30.6 ± 22.1 ml). Hence, the proportion processed UCBs after an intra-uterine collection was larger. We also analyzed production efficacy of the Sepax device. Of the 101 successful UCB collections, 64 autologous products remained after processing, mainly because of loss of RBC during the procedure. Efficacy per group was lowest in the 24 and 28 wks group, 16 productions resulted in only 6 RBCC with the required volume and hematocrit. However, each of these infants required transfusions. Processing of the UCBs from the 30 and 32 week group resulted in the highest number of products, but as the infants in this group had much lower transfusion needs, most of these products remained unused. UCB collection as well as processing was optimal for the infants

born between 28-30 weeks. In particular, 69% of the intrauterine collections in this group were large enough for processing to red cell concentrates and resulted all in autologous products. More than half of the available products in this group were intended for infants with transfusion needs and could cover approximately 75% of the transfusion needs in this group (Table 5).

Estimation of costs

Maternal virology testing was performed for 202 cases. For 26 cases testing was performed, but the delivery occurred beyond 32 weeks. In 18 cases UCB was processed in the weekend for which STAT testing was performed, resulting in a huge increase of the total costs. The costs for UCB collection disposables were made for 176 cases, the costs for Sepax processing for 101 cases and the costs for quality control for 67 products. Ultimately the direct cost for 25 indicated RBC products were almost 15 times higher than to the costs of a standard pedi-pack (Table 7). Without STAT testing, the costs would be 6.5 times higher.

Table 7: Autologous RBCC cost estimation

	N	€ per unit	Total costs
Maternal virology (regular)	202	€ 15.50	€ 3.131
STAT maternal virology (weekends)	18	€ 1.330	€ 23.940
UCB collection disposables	176	€ 14.17	€ 2.494
Sepax kit for processing	101	€ 117.09	€ 11.826
Quality control (ABO-RhD, BacTalert)	67	€ 7.31	€ 490
Available autologous products	64	€ 654	
Cost per indicated product	25	€ 1.675	
Cost per indicated product (excluded STAT)	25	€ 729	
Standard pedi-pack, irradiated		€ 114	

RBCC: red blood cell concentrate

Interim analysis

Interim analysis revealed low availability of autologous products due to a low number of successful UCB collections. This availability of < 40 % autologous products for all premature infants with collected UCB would preclude that the primary endpoint of a reduction of at least 50% in use of allogeneic RBC products would be attainable. Therefore we decided to stop enrollment in October 2006 after inclusion of 215 infants and 94 transfused infants.

Discussion

In this clinical trial we collected UCB after premature deliveries < 32 weeks and randomized premature infants who required a RBC transfusion to either an autologous or allogeneic product. From the start of the study we considered a reduction of ≥ 50% in use of allogeneic blood

worth the effort. Interim analysis revealed that for 27% of the transfused premature infants an autologous RBC product was available for at least one transfusion. Therefore this trial was stopped prematurely.

Eight out of 35 patients received autologous RBC in the group assigned to autologous products. Possible clinical benefits of these autologous transfusions could therefore not be evaluated, although post-transfusion hematocrit increment, interval until next transfusion, incidence of adverse events and composite neonatal complications were not at variance. This was in accordance with the results observed by other groups.[17-20] Unfortunately, 27 infants were not randomized. This high number of protocol violations can not be fully explained. However, analysis of the non-randomized infants showed comparable demographics and outcome, suggesting no selection bias.

Our collection methods resulted in 101 UCBs that could be processed, of which only 25 available products were intended for transfused infants. This implies that for each effective product three extra products were made unnecessary. Considering all products would have been used for patients with a transfusion indication (in case of a non-randomized study), 21 of the 25 products would have been large enough to cover at least 50 % of the transfusion needs per infant in the first 21 days after birth.

Despite the lower number of successful UCB collections for the infants born between 24 and 28 weeks, almost every available product would be useful, in view of the large number of infants (87%) with transfusion needs (39 out of 45). For the infants born between 28 and 30 weeks, UCB collection volumes were more often sufficient for processing. In this group of infants, 50% (36 out of 72) had transfusion needs. UCB collections for the infants born between 30 and 32 weeks were most successful according to our definition of ≥ 15ml whole blood. After processing however, a smaller number of products remained available. In addition, transfusion needs in this group were much lower compared to the other gestational groups (Tables 1 and 2). Taken this all together, UCB collection and autologous RBCC production was most efficient for the infants born between 28 and 30 weeks compared to the other gestational groups. In this group autologous RBC was available for 42% of the infants that required transfusions. Harvested UCB was not enough to cover the transfusion needs of the smallest infants and the autologous products made for infants born between 30 and 32 weeks remained unused due to lower transfusion needs.

Some possible improvements of efficacy need to be considered. Only 57% of all collection attempts resulted in a UCB volume large enough for Sepax processing. Most of the successful collections were harvested from an intrauterine placenta. Restriction of UCB collection to only intrauterine harvesting[28] would increase production efficacy by 25%. In addition, this resulted for infants born between 24 and 28 weeks, in a 10% lower RBC recovery compared to the other processed UCBs, because of relative higher cell losses in these smaller collections (data not shown). RBC separation by filtration has been described as an alternative.[29-30] However; the intrinsic volume of the filters could also result in absolute red cell volume loss. Furthermore, doing

regular maternal virology testing instead of expensive STAT testing can reduce the costs. In this study, the release of 18% of the UCB collections suitable for Sepax processing would be delayed for a maximum of 72 hours.

For future exploration of the possible clinical effects of autologous cord blood, UCB harvesting must be more efficient. Additional placental perfusion with heparinized saline after UCB collection has been described as an effective method for enlarging the total collected volume.[31] Whether this method is also useful in the smaller and more fragile placentas after premature deliveries remains to be investigated. The restriction to infants born before 30 weeks of gestation, the use of intrauterine collections only (also after cesarean sections) and a processing procedure with higher red cell recovery could result in higher volumes and available products expecting to cover > 50% of the transfusion needs. Another approach of autologous transfusion is placental transfusion by delayed cord clamping.[32-36] Recent studies show that delayed cord clamping is associated with reduced transfusion needs and a lower risk of IVH in premature infants.[32, 35] However, the described time range of delayed clamping was broad and varied from 30 till 120 seconds.[32] Although the results seem promising, it is desirable that they are confirmed by larger studies with a uniform definition and method of delayed cord clamping, in particular in critically ill children with a low Apgar score and a high risk of transfusion probability.[21]

In conclusion, autologous RBC derived from UCB could replace allogeneic transfusions for 27% of the infants in need of transfusions. Whether an autologous product was available for an infant with transfusion needs depended most on the gestational age. UCB collection and processing for infants born between 28 and 30 weeks was most efficient. In this trial a small number of autologous RBCC were actually transfused, therefore no clinical benefits could be determined. Whether premature infants may benefit clinically from autologous blood remains a question unanswered at this moment.

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8

Exploring the use of expanded erythroid cells for autologous transfusion for anemia of prematurity

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Abstract

Background: Autologous cord blood (CB) red blood cells (RBCs) can partly substitute transfusion needs in premature infants suffering from anemia. To explore whether expanded CB cells could provide additional autologous cells suitable for transfusion, we set up a simple one-step protocol to expand premature CB cells.

Study design and methods: CB buffy coat cells and isolated CD34 positive ($CD34^{pos}$) cells from premature and full-term CB and adult blood were tested with several combinations of growth factors while omitting xenogeneic proteins from the culture medium. Cell differentiation was analyzed serially during 21 days using flow cytometry, progenitor assays, and high performance liquid chromatography.

Results: Expanded CB buffy coat cells resulted in a threefold higher number of erythroblasts than the isolated $CD34^{pos}$cells. However, the RBCs contaminating the buffy coat remained present during the culture with uncertain quality. Premature and full-term CB $CD34^{pos}$cells had similar fold expansion capacity and erythroid differentiation. With the use of interleukin-3, stem cell factor, and erythropoietin, the fold increases of all $CD34^{pos}$cell sources were similar: CB 3942 ± 1554, adult peripheral mobilized blood 4702 ± 1826, and bone marrow (BM) 4143 ± 1908. The proportion of CD235a expression indicating erythroblast presence on Day 21 was slightly higher in the adult $CD34^{pos}$cell sources: peripheral blood stem cells (96.7 ± 0.8%) and BM (98.9 ± 0.5%) compared to CB (87.7 ± 2.7%; $p = 0.002$). We were not able to induce further erythroid maturation *in vitro*.

Conclusion: This explorative study showed that fairly pure autologous erythroid expanded cell populations could be obtained by a simple culture method, which should be optimized. Future challenges comprise obtaining *ex vivo* enucleation of RBCs with the use of a minimal manipulating approach, which can add up to autologous RBCs derived from CB in the treatment of anemia of prematurity.

Introduction

Potentially deleterious effects of allogeneic red blood cell (RBC) transfusions have led to the development of alternatives for RBC transfusion. Explored alternatives are the use of hemoglobin (Hb)-based oxygen carriers, recombinant human erythropoietin (EPO), and intra- or postoperative RBC salvage. For the treatment of anemia of prematurity these options are however no suitable alternatives.[1-5] Currently, *ex vivo* expansion of CD34positive ($CD34^{pos}$) hematopoietic stem cells derived from adult blood or cord blood (CB) embodies the center of research on transfusion alternatives. Clinical-grade RBCs have been generated *ex vivo*, and also the use of expanded erythroblasts as an alternative transfusion product has been suggested.[6-12] Clinical application of these expanded cells is, however, still held back due to the high costs, the complex production process, and the presence of xenogeneic material in the culture medium.[12] Alongside the potential use of *ex vivo* expanded RBCs for allogeneic transfusion purposes, these cells could also serve for autologous transfusions for patients with a high probability of transfusion needs, that is, premature infants suffering from anemia of prematurity.[13,14] A therapeutic allogeneic RBC transfusion for adults contains approximately 2 ± 10^{12} RBCs preserved in 350 mL of storage medium. Premature infants receive, depending on the hospital transfusion practice, 10 to 20 mL of RBC product per kg of body weight per transfusion. Extremely low birth weight infants weighing less than 1000 g are most often in need of transfusions. For these infants approximately 5x10^{10} to 10x10^{10} RBCs would be enough for a transfusion. We previously showed that autologous RBCs could be harvested from CB collected after premature birth and, if successful, could cover up to 58% of the transfusion needs.[15] These premature CB-derived RBC transfusion products had a shorter shelf life (maximally 21 days) compared to adult RBCs, which can be stored for 35 days.[16] To obtain RBCs, the premature CB was buffy coat depleted by centrifugation and the removed buffy coats contained approximately 40% of the white blood cells (WBCs). We evaluated whether this WBC fraction or the isolated $CD34^{pos}$ cells could provide additional expanded autologous RBCs. We aimed to set up a simple one-step culture protocol using only human proteins and recombinant human cytokines in the culture medium. In view of the practical transfusion needs of premature infants, we focused on the cultured cells that could be obtained within 21 days of expansion, which could theoretically supplement autologous CB RBCs, which have a shelf life up to 21 days.[16] To test the efficiency and efficacy of this culture protocol, we compared both premature and full-term CB and cells derived from adult blood and bone marrow (BM).

Material and methods

Cell sources

Premature CB units were obtained after parental consent as part of our prospective clinical study.[15] CB units from full-term deliveries were obtained from the Umbilical Cord Blood Bank Leiden from donors who consented for experimental use of the CB. Mobilized peripheral blood stem cell (PBSC) samples were obtained from healthy adult donors. Before donation, patient consent was obtained for experimental use of a small part of the collection for research (approx. 2%). Adult BM samples were obtained from Stem Cell Technologies, Grenoble, France.

Cell isolation and culture medium

Buffy coat preparation

To produce a transfusion product from premature CB, the autologous RBCs and buffy coat were separated by centrifugation.[16] The recovery of autologous CB RBCs was 75 ± 8%. Consequently, the premature CB buffy coats contained a relatively large RBC fraction. Further RBC depletion from the buffy coat by hydroxyethyl starch (HES) sedimentation (ratio 1:4, HES:blood) and centrifugation at 50 ± *g* for 10 minutes at room temperature resulted in a further RBC reduction of 45 ± 18%. The remaining buffy coat fraction after HES sedimentation was washed with phosphate-buffered saline (PBS; Hospital Pharmacy LUMC, Leiden, the Netherlands) and 500,000 total nucleated cells (TNCs) per well were seeded on 24-well culture plates.

$CD34^{pos}$ cell isolation

Mononuclear cells (MNCs) were separated by a Ficoll-Isopaque gradient (1.079 g/cm3; Hospital Pharmacy LUMC) centrifuged and enriched in $CD34^{pos}$ cells by using columns (MiniMACS,Miltenyi Biotec, Bergisch Gladbach, Germany). Purity of the enriched cells was 77 to at least 95%; 50,000 $CD34^{pos}$ cells were seeded per well.

Culture medium and growth factors

The HES buffy coat fraction and the isolated $CD34^{pos}$ cells were incubated at 37°C and 5% CO2 humidified atmosphere for 21 days. The culture medium consisted of Iscove's modified Dulbecco's medium (Invitrogen, Carlsbad, CA), supplemented with 20% human AB-citrated plasma (Sanquin Blood Bank South West, Rotterdam, the Netherlands), 0.165 mg/mL CaCl2 (Sigma, Steinheim, Germany), 1% penicillin-streptomycin (Invitrogen), 0.34% human serum albumin (Sanquin Blood Bank, Amsterdam, the Netherlands), 10 U/mL heparin (Hospital Pharmacy LUMC), 0.5 mg/mL human transferring (Sigma), 0.05 mmol/L 2b-mercaptoethanol (Sigma), and 0.001 mol/L dexamethasone (Hospital Pharmacy LUMC). Cells were cultured in presence of several combinations of recombinant human 3 ng/mL thrombopoietin (TPO; KIRIN Brewery Co. Ltd, Yokohama, Japan), 50 ng/mL recombinant human stem cell factor (SCF; Endogen, Rockford,

IL), or/and 10 ng/mL recombinant human interleukin-3 (IL-3; Peprotech, London, UK), as early acting growth factors, in combination with 4 IU/mL human recombinant EPO (Eprex, Janssen-Cilag, Tilburg, the Netherlands). Half of the medium was refreshed at least once weekly or more often if cell proliferation increased. Both TPO and IL-3 were removed by washing after 6 days of culture. SCF and EPO were present during the entire 21-day culture period. The cultured cells were harvested on Days 4, 7, 10, 14, and 21. TNCs were counted on an automated blood cell counter (AcT 10 cell analyzer, Beckman Coulter, Woerden, the Netherlands). Expansion rate was calculated as follows: harvested TNCs, divided by the original number of seeded cells per well and corrected for the dilution factor for well transfer.

Induction of enucleation

To induce further differentiation and maturation of the expanded erythroblasts, we added thyroid hormone and insulin in the last week of culture.[17-19] $CD34^{pos}$ CB cells were cultured during 10 days in the standard medium with IL-3, SCF, and EPO to induce erythroid expansion. On Day 10 the expanded cells were washed with PBS and reseeded in the standard medium without dexamethasone and additionally supplemented with 10 mg/mL insulin (Sigma-Aldrich, Zwijndrecht, the Netherlands) and 1 mmol/L thyroid hormone (T3; Sigma-Aldrich). The culture media of these cells were refreshed every other day. On Day 17 all wells were harvested for counting, phenotypic analysis, and cell smear preparation.

Phenotypic analysis

After being harvested and counted, the expanded cells were both washed and resuspended in PBS. The cells were incubated with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated antibodies for 20 minutes at room temperature and resuspended in PBS before measurement. Anti-CD45-FITC, anti-CD34-PE, anti-CD14-FITC, anti-CD15-PE, anti-CD62p-FITC, anti-CD41-PE, anti-CD34-FITC, anti-CD71-PE, anti-CD36-FITC, 7-aminoactinomycin D (Beckman Coulter), and CD235a (glycophorin A)-PE (Dako, Glostrup, Denmark) were used for analysis. Controls were isotype FITC and PE antibodies.

Nucleated RBCs (NRBCs) were enumerated by flow cytometry using propidium iodide and CD45-FITC identified cells with a low forward and side scatter.[20] All analyses were performed on a flow cytometer (FC 500, Beckman Coulter).

Colony-forming assay

Colony-forming unit (CFU) and erythroid burst-forming unit (BFU-E) progenitors were assayed in methylcellulose cultures (Methocult GF H4434, Stem Cell Technologies) on Day 0 and after 7, 10, 14, and 21 days of *ex vivo* expansion. Cell concentrations of $0.32x10^4$/mL on Day 0; $0.32x10^5$/mL on Day 7; and $0.32\ x10^6$/mL on Days 10, 14, and 21 were seeded. The cultures were incubated at 37°C and 5% CO2 in humidified atmosphere. Colonies were counted after 14 days.

Unless otherwise specified, all erythroid colonies (BFU-E; CFU-granulocyte, erythrocyte; CFU-megakaryocyte, erythrocyte; and CFU-granulocyte, erythrocyte, monocyte, megakaryocyte) were combined and counted as total erythroid colony formation expressed per 10^3 plated (expanded) cells.

Cell morphology

Cell morphology was analyzed on Days 17 and 21 on smears of the expanded cells. The smears were stained with May-Grunwald-Giemsa. Photographs were made with a digital camera (AxioCam MRc5, Zeiss, Gottingen, Germany), enlargement 100 ± /1.30 oil, ∞/0.17.

Hb analysis

Harvested cells were washed with NaCl 0.9% and kept at minus 80°C until processing. The separation by reversed phase high-performance liquid chromatography (HPLC) of the globin chains was performed with HPLC (AKTA Purifier 100, GE Healthcare Europe, Diegem, Belgium) with a sample injector and a variable UV detector operating at 215 nm. A LiChrospher 100 RP-8 column (VWR, Amsterdam, the Netherlands) was used and elution was obtained in a linear gradient of 25 column volumes of acetonitrile, methanol, NaCl in ultrapure water (milliQ, Millipore Corp., Billerica, MA) at a flow rate of 0.8 mL/ min. The gradient started with 30% Solvent B (acetonitrile, methanol, 0.155 mol/L NaCl 68:4:28) and 70% Solvent A (acetonitrile, methanol, 0.077 mol/L NaCl 26:33:41) and ended with 55% Solvent B.

Statistical analysis

Data are presented as mean ± SD from generally three separate experiments unless indicated otherwise. Computer software (SPSS for Windows, Version 18, Chicago, IL) was used for data analysis. Continuous variables were analyzed using independent t tests or one-way analysis of variance (ANOVA) for differences between more than two groups. Bonferroni correction was used to correct for multiple testing if applicable. A *p*-value of less than 0.05 was considered significant.

Results

Premature CB cells after fractionation

After processing of the autologous CB products as described in our earlier clinical study, approximately 40% of the WBCs were removed by buffy coat depletion. The premature CB RBCs had a mean cell volume of 117 ± 5 fL. The products had hematocrit levels of 0.60 ± 0.05 L/L and contained a median of 5.1 ± 10^9 CB RBCs/mL (range, $3.6x10^9$- $6.6x10^9$/mL).[16] The absolute RBC number per product was (median) $10.8x10^{10}$ (range, $2.8x10^{10}$- $3.5x10^{11}$). The residual buffy coats (n = 10) contained a median of $1.9x10^8$ TNCs (range, $2.4x10^7$-$6.8x10^8$) and a median of $2.5x10^6$ $CD34^{pos}$ cells (range, $1.2x10^5$-$1.1x10^7$).

Expansion of premature CB buffy coats

Expansion of the premature CB buffy coat HES fraction was performed with the use of SCF and EPO (n = 3). Cells expressing both CD36 and CD235a could be distinguished from the native RBCs that were present at the start of the culture. After 21 days, the mean fold TNC increase was 48.3 ± 16.8. Total CD235a^{high} expression on Day 21 was 91.4 ± 9.6%, of which a minor proportion of 5.4 ± 7% coexpressed CD36^{high} (indicative of basophilic or polychromatic erythroblasts). The major proportion of these cells were CD36^{neg}CD235a^{high} (indicative of polychromatic or orthochromatic erythroblasts). CD71 (transferrin receptor) expression was 80.7 ± 16%. The fold increase of CD34^{pos} fractions from the premature CB buffy coats with SCF and EPO was 1324 ± 1295, with total CD235a^{high} expression of 92.7 ± 5.6% on Day 21 of which 13 ± 2.1% coexpressed CD36^{high}. Total CD71 expression was 96.7 ± 1.5%. Thus the buffy coat HES fractions yielded approximately three times more erythroblasts than the CD34^{pos} fractions without obvious differences in purity and erythroblast maturation.

Premature and full-term CD34^{pos} CB cells

We then compared CD34^{pos} fractions from premature and full-term CB. Addition of SCF and EPO to the culture medium resulted in a higher expansion rate compared to TPO and EPO ($p < 0.001$). Premature and full-term CB had a similar fold increase. Also the appearance of erythroid cell markers was similar (Fig. 1). SCF-EPO cultures had a higher fold increase with similar proportions of CD36^{high}CD235a^{high} cells and CD36$^{low/neg}$CD235a^{high} cells, respectively, 19 ± 5.6% in full-term CB and 13 ± 2.0% in premature CB, and 75.7 ± 7.2% in full-term CB and 80 ± 6% in premature CB, respectively (n = 6 full-term CB vs. n = 3 premature CB). In the premature CB cultures expanded with SCF and EPO, we counted a similar amount of NRBCs per harvested well, respectively, $1.5x10^6$ ± $0.9x10^6$ NRBCs in premature CB and $1.3x10^6$ ± $1.2x10^6$ NRBCs in full-term CB. Given the similar fold expansion rate and generation of erythroid cells in both premature and full-term CB cultures, full-term CB was used for subsequent experiments.

Growth factor combinations: erythroid cell expansion and differentiation

Addition of TPO to SCF and EPO did not result in a substantial synergistic effect, in contrast to supplementation of IL-3, which resulted in a higher expansion rate of full-term CB compared to the other combinations (one-way ANOVA for differences between the groups $p = 0.04$; Fig. 1). Erythroid marker expression in the tested growth factor combinations were almost similar. The TPO-EPO combination resulted in a higher percentage of CD41 positive cells (10.2 ± 2.6%) as well as in residual CD34^{pos} cells after 21 days of culture compared to the other combinations. There was no difference in lineage negative cells (defined as CD235a/CD45-negative cells; Table 1). Although statistically not different, the proportion of CD36^{high}CD235a^{high} cells in the IL-3-SCF-EPO combination was higher, suggestive for less advanced erythroblast maturation. Expanded NRBCs on Day 21 were similar in SCF-EPO ($1.3x10^6$ ± $1.2x10^6$/well), TPO-SCF-EPO ($2.1x10^6$

± 3.2x10^6/well), and IL-3-SCF-EPO (5.9 x10^5 ± 4.5x10^5/well; one-way ANOVA for differences between the groups, p = 0.779; after correction for fold expansion, p = 0.545). Because of the optimal expansion rate of the TNCs, the combination IL-3-SCF-EPO was used for subsequent experiments to compare CB and adult CD34^{pos} cell sources. Removal of dexamethasone and addition of insulin and thyroid hormone to the culture medium was compared to the standard medium. Fold expansion of the TNCs was similar, respectively, 192 ± 210 for standard and 188 ± 257 for the two-step method. NRBCs were respectively 7x10^5 ± 2.4x10^5/well for the standard medium and 6.6x10^5 ± 2.2x10^5/well for the two-step method. After correction for well passaging the NRBC yield was also similar. We observed small differences in erythroid surface marker expression. Total CD235a expression was, respectively, 97.7 ± 1.2% for standard and 99.4 ± 0.3% for two-step method (p = 0.067). CD36^{high} coexpression was, respectively, 11.7 ± 6% and 3.8 ± 2.4% (p = 0.052), indicating more mature erythroid cells in the two-step method. Total CD71 expression was similar; 98.2 ± 0.6% in standard and 97.3 ± 1.7% in the two-step method. However, cell smears showed similar erythroid morphology without reticulocytes or RBCs in either of the cultures (not shown).

Table 1: Cell surface marker expression of expanded full term cord blood CD34^{pos} at day 21

	TPO-EPO (n=6)	SCF-EPO (n=6)	TPO-SCF-EPO (n=3)	IL3-SCF-EPO (n=3)
Fold expansion	47 ± 41	988 ± 842	802 ± 364	3942 ± 1554
CD36^{pos}-CD235a^{neg}	11.4 ± 3.5 %*	4.4 ± 2 %	6.0 ± 1.7 %	9.4 ± 1.2 %
CD36^{pos}-CD235a^{pos}	8.4 ± 6.3 %	17.6 ± 7.8 %	17 ± 10.4 %	37.2 ± 2.4 %
CD36^{neg}-CD235a^{pos}	75.2 ± 8 %	76.2 ± 9.2 %	75.7 ± 11.5 %	50.6 ± 0.2 %
CD36^{neg}-CD235a^{neg}	5.1 ± 5.9 %	1.8 ± 1.3 %	1.3 ± 1.2 %	2.8 ± 1.5 %
CD235a^{neg} –CD45^{neg}	5 ± 3.9 %	2.9 ± 1.1 %	2.7 ± 0.6 %	5.1 ± 0.8 %
CD71^{pos}	86.7 ± 4.8 %	92 ± 5.3 %	92.3 ± 4.0 %	93 ± 0.9 %
CD41^{pos}	10.2 ± 2.6 %†	0%	0.7 ± 1.1 %	0.2 ± 0.2 %
CD34^{pos}	2.8 ± 1.6 %	0.3 ± 0.4 %	0.3 ± 0.6 %	0.6 ± 0.3 %

*: TPO-EPO compared SCF-EPO and SCF-TPO-EPO p=0.01 after correction for multiple testing. †: TPO-EPO compared to all other combinations p<0.001 after correction for multiple testing.
Abbreviations: TPO: Thrombopoietin, EPO: erythropoietin, SCF: Stem Cell Factor; IL-3: interleukin 3; CB: cord blood

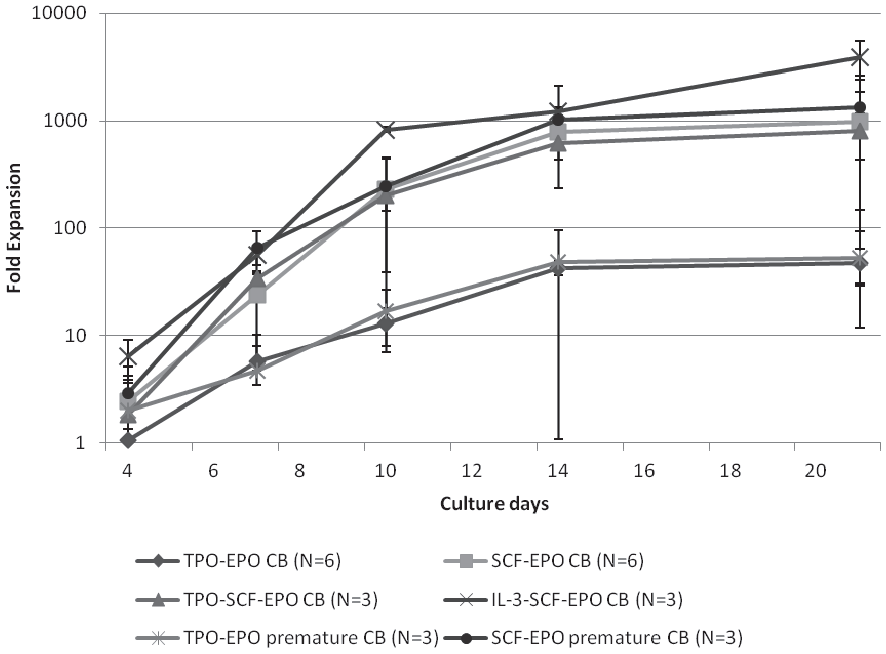

Figure 1. Fold expansion of premature and term cord blood
Abbreviations: TPO: Thrombopoietin, EPO: erythropoietin, SCF: Stem Cell Factor; CB: cord blood, IL3: interleukin 3

Erythroblast formation from adult PBSC-, BM-, and CB-derived CD34^{pos} cells

After 21 days of expansion with IL-3-SCF-EPO we observed similar fold expansion rates between the three CD34^{pos} cell sources: 3942 ± 1554 fold for full-term CB, 4702 ± 1826 fold for mobilized PBSCs, and 4143 ± 1908 for BM (one-way ANOVA for differences between the groups, $p = 0.82$; Fig. 2). Erythroid total CD235a expression on Day 21 is depicted in Table 2. Adult CD34^{pos} cell cultures were more purely erythroid compared to CB; CD235a expression was, respectively, 87.7 ± 2.7% in CB, 96.7 ± 0.8% in mobilized PBSCs, and 98.9 ± 0.5% in BM ($p = 0.002$ after correction for multiple testing). In the CB cultures there were more lineage-negative cells, depicted as both CD235a/CD45 negative, instead of remaining CD34^{pos} cells. CD71 expression, indicative of proliferating cells, was higher in the adult cell cultures compared to the CB cultures ($p = 0.032$ after correction for multiple testing; Table 2). The development of CD235a^{pos} expression was plotted against CD36^{pos} cell expression. In adult BM and PBSC cultures we observed earlier presence of CD36^{high}CD235a^{high} cells, indicative of basophilic and/or polychromatic erythroblast formation (Fig. 3). NRBC counts on Day 21 were $5.9x10^5 \pm 4.5x10^5$/well in CB, $9.4x10^5 \pm 0.6x10^5$/well in mobilized PBSCs, and $2.6x10^6 \pm 1.3x10^6$/well in BM. After correction for the number of cell passages and fold expansion, the absolute NRBC numbers in the BM cultures were statistically higher compared to CB ($p = 0.022$) and PB ($p = 0.027$). Myeloid cell presence at 21 days estimated

by CD14 and CD15 expression was similar: 2 ± 0.9% in CB, 0.7 ± 0.5% in mobilized PBSCs, and 1.4 ± 1.7% in BM (one-way ANOVA difference between the groups, $p = 0.43$).

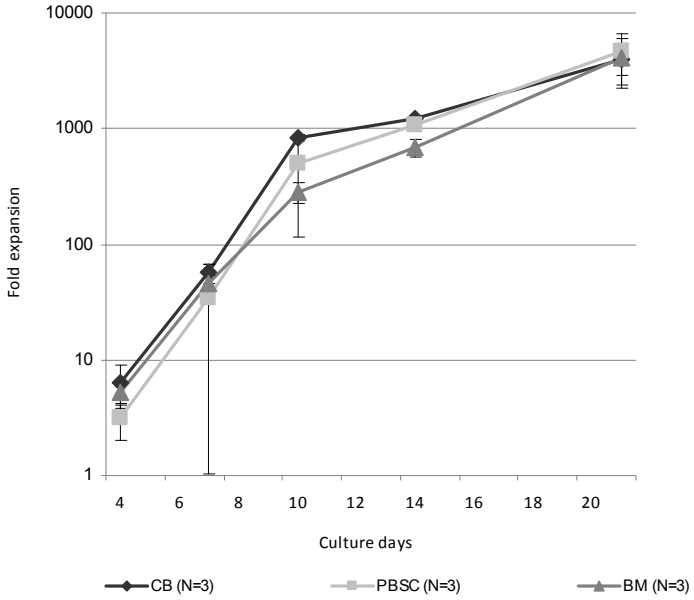

Figure 2. Comparison of fold expansion of cord blood and adult blood
Abbreviations: EPO: erythropoietin, IL3: interleukin 3, SCF: Stem Cell Factor; CB: cord blood, PBSC: mobilized peripheral blood; BM: bone marrow

Table 2: Cell surface marker expression after 21 days of expansion with IL3, SCF and EPO, in relation to adult (PBSC/BM) or CB derived $CD34^{pos}$ cell source

	CB	PBSC	BM	*p-value*
Fold expansion	3942 ± 1554	4702 ± 1826	4143 ± 1908	ns
$CD36^{pos}$-$CD235a^{neg}$	9.4 ± 1.2 %*	2.7 ± 0.7 %	0.9 ± 0.5 %	<0.001
$CD36^{pos}$-$CD235a^{pos}$	37.2 ± 2.4 %	54.4 ± 11.2 %	48.2 ± 9.3 %	ns
$CD36^{neg}$-$CD235a^{pos}$	50.6 ± 0.2 %	42.4 ± 11.9 %	50.4 ± 9.4%	ns
$CD36^{neg}$-$CD235a^{neg}$	2.8 ± 1.5 %†	0.6 ± 0.1 %	0.3 ± 0.1 %	0.04
$CD235a^{neg}$–$CD45^{neg}$	5.1 ± 0.8 %‡	1.0 ± 0.2 %	0.4 ± 0.1 %	0.002
$CD71^{pos}$	93 ± 0.9 %§	97.9 ± 1.5 %	96.8 ± 1.5 %	0.032
$CD41^{pos}$	0.2 ± 0.2 %	0.1 ± 0.1 %	0.4 ± 0.2 %	ns
$CD34^{pos}$	0.6 ± 0.3 %	5.1 ± 4.2 %	3.3 ± 2.5 %	ns

*: CB statistically significant different compared to both PBSC and BM after correction for multiple testing; †: CB statistically significant compared to BM after correction for multiple testing. ‡: CB statistically significant different compared to both PBSC and BM after correction for multiple testing; §: CB statistically significant different compared to both PBSC and BM after correction for multiple testing
Abbreviations: CB: cord blood, PBSC: mobilized peripheral blood; BM: bone marrow; EPO: erythropoietin, SCF: Stem Cell Factor; IL3: interleukin 3

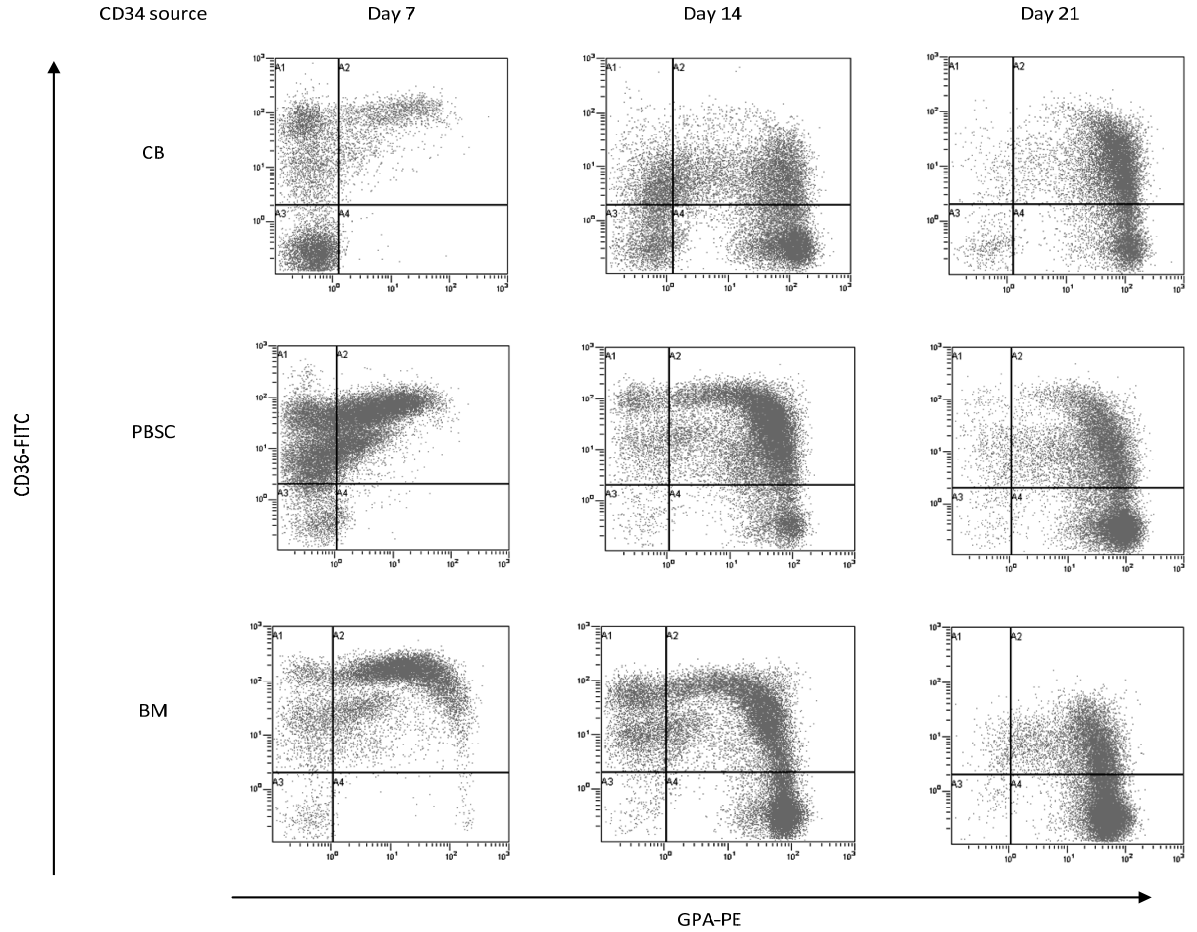

Figure 3. CD235a (GPA) cell expression during expansion plotted against CD36 expression. (All pictures are representative of three separate experiments.)

Colony-forming capacity of expanded cells

The colony-forming capacity of the expanded adult and CB cells were measured on Days 7, 10, 14, and 21 of culture. After 10 days of expansion only few $CD34^{pos}$ cells could be detected in the CB cultures (0.4 ± 0.4%), compared to PBSCs (8.2 ± 2%) and BM (21.5 ± 3%). Nevertheless, after 7 and 10 days of expansion, both the expanded adult and the CB cells were still able to form BFU-E, although between Day 7 and Day 10 the colony-forming capacity sharply declined. After Day 10 there were almost no colonies formed. The expanded BM cells were less able to form BFU-E colonies after 7 days of expansion compared to CB and PBSCs (Table 3).

Table 3: Colony forming capacity of expanded adult and cord blood cells

Material	Colonies per 1000 seeded cells	Days of culture		
		Day 0	Day 7	Day 10
CB	Total HPC colonies	438 ± 13*	152 ± 4	13 ± 3*
	Total Erythroid colonies	169 ± 18*	104 ± 31	4 ± 4
	BFU-E colonies	59 ± 22	91 ± 39	4 ± 3
PBSC	Total HPC colonies	118 ± 119	120 ± 53	4 ± 3
	Total Erythroid colonies	27 ± 33	79 ± 25	2 ± 2
	BFU-E colonies	25 ± 30	79 ± 25	2 ± 2
BM	Total HPC colonies	138 ± 77	17 ± 4†	3 ± 4
	Total Erythroid colonies	72 ± 46	11 ± 2†	3 ± 3
	BFU-E colonies	62 ± 38	11 ± 1‡	3 ± 3

*CB significantly different compared to PBSC and BM ($p<0.04$) after correction for multiple testing; † BM significantly different compared to PBSC and CB ($p<0.03$) after correction for multiple testing; ‡BM significantly different compared to CB ($p<0.03$) after correction for multiple testing.
Abbreviations and symbols: CB: cord blood; PBSC: mobilized peripheral blood; BM: bone marrow; HPC: hematopoietic progenitor cell colonies; BFU-E: erythroid burst forming unit; Total erythroid: BFU-E, CFU-GE, CFU-ME and CFU-GEMM

Hb analysis

On Days 11, 14, and 21, the globin chain expression was examined by reversed-phase HPLC. The HbA:HbF expression rates in the CB cultures were, respectively, 49:51 on Day 11, 37:63 on Day 14, and 24:76 on Day 21. In the adult $CD34^{pos}$ cell cultures the HbA:HbF expression rates on Day 21 were 82:18 for BM and 84:16 for PB.

Cell morphology

We observed several stages of erythropoiesis. The majority of the cells were polychromatic erythroblasts, but also basophilic and orthochromatic erythroblasts were present. We also detected a few proerythroblasts. Reticulocytes and mature RBCs were not detected. Besides the expected stages of erythropoiesis we also detected a trinuclear cell in one of the smears, which can be a sign of either dyserythropoiesis or stress erythropoiesis. The cell membrane of this last cell was not intact and was possibly in degradation (Fig. 4).

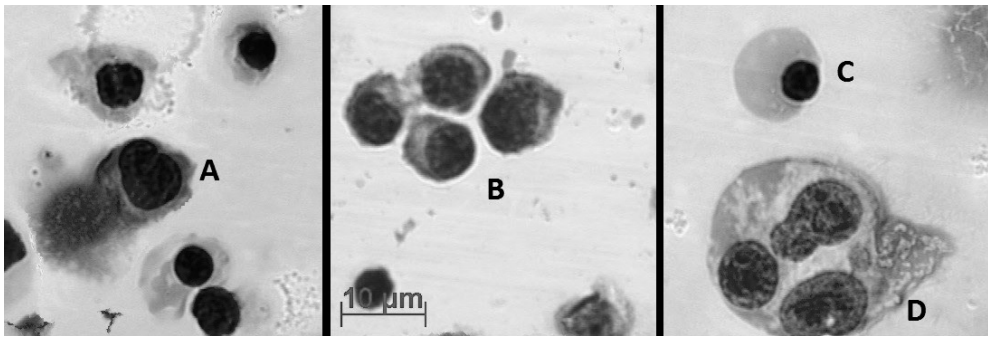

Figure 4. Erythroid progenitor cells after 21 days of expansion
A: pro-erythroblast
B: several basophilic erythroblasts
C: orthochromatic erythroblast
D: trinuclear cell

Potential quantitative erythroblast formation after ex vivo expansion

The buffy coats contained an absolute number of 2.4 ± 10^7 to 6.8 ± 10^8 nucleated cells and contained an absolute range of 1.2 ± 10^5 to 1.1 ± 10^7 $CD34^{pos}$ cells (n = 10). With the use of the optimal combination of IL-3, SCF, and EPO, this would lead to a range of 4.8 ± 10^8 to 4.4 ± 10^{10} expanded erythroblasts when isolated $CD34^{pos}$ cells from the premature buffy coats are cultured. Based on the threefold higher yield of erythroblasts when the buffy coat was used instead of $CD34^{pos}$ cells, this could imply approximately 5 ± 10^{10} cells if the premature buffy coats could have been cultured without RBC contamination.

Discussion

Current research on *in vitro* production of RBCs provides methods to expand adult or CB MNCs and $CD34^{pos}$ cells into mature RBCs suitable for transfusion.[6-12] The use of expanded erythroblasts has also been mentioned as an alternative transfusion product, as these cells seem to mature *in vivo* when a suitable mouse model was used.[21,22] In an earlier study we showed that autologous RBCs derived from premature CB could be used for transfusion purposes. This amount of harvested autologous RBCs is limited. The premature CB buffy coat that was removed during autologous RBC production contained $CD34^{pos}$ and other progenitor cells. We aimed for a simple one-step culture intended to obtain additional RBCs. Ideally these expanded autologous cells would be available within 14 to 21 days when the shelf life of CB RBCs had expired.[16] Expansion of the premature CB buffy coat yielded three times more erythroblasts than $CD34^{pos}$ cells, confirming the results of van den Akker and colleagues.[17] However, the CB buffy coats contained a relatively large amount of CB RBCs. During 21 days of culture these RBCs may have acquired "storage" lesions posing a risk factor for transfusion. We further explored the erythroid differentiation characteristics in

our one-step protocol with $CD34^{pos}$ cells. In line with previous studies, the combination of IL-3, SCF, and EPO was most effective.[11,23] Premature and full-term CB had similar expansion potential and erythroid marker appearance. The premature CB buffy coats contained a median of 2.5 ± 10^6 $CD34^{pos}$ cells (range, $1.2x10^5$-$1.1x10^7$). With the observed mean fold expansion rate of 3942 ± 1554, a range of $4.8x10^8$ to $4.4x10^{10}$ (median, $1.2x10^{10}$) erythroblasts could be generated in theory. Only the maximum of this range would provide just enough RBCs for one neonatal transfusion for very low birth weight neonates. We were unable to induce further differentiation and enucleation of the $CD36^{neg}CD71^{high}CD235a^{high}$ polychromatic erythroblasts by adjustment of the culture medium with insulin and thyroid hormones and removal of dexamethasone. It is possible either that a density purification step is essential for further differentiation of the erythroblasts or that our supplemented growth factor combination was still too simple.[17,18] Adult PBSC-, BM-, and CB-derived $CD34^{pos}$ cells all had a similar fold increase. The adult cells had a more accelerated erythroid differentiation compared to CB (Fig. 3). Moreover, a higher residual lineage negative fraction remained in the CB cultures, whereas more $CD34^{pos}$ cells remained in adult cultures. Loss of CD34 expression, with maintenance of expansion depending on growth factors toward hematopoietic particular lineages, has been previously observed to be typical for CB.[24] Myeloid cell presence was low in all cultures. All cultures finally consisted of greater than 90% erythroid cells. The colony forming capacity was not diminished at least until up to 10 culture days, indicating that *in vivo* erythroid proliferation and differentiation could be possible after 10 days of expansion. The increased HbF expression during culture was expected in view of the erythropoietic stress conditions present during culture and was also shown in the adult cultures.[25] The cell smears at the end of the culture showed a few proerythroblasts and all stages of erythroblasts through normoblast. We did not observe reticulocytes or RBCs. We also observed signs of dyserythropoiesis or stress erythropoiesis, which may be explained due to the continuous drive to proliferate in the cultures.

Recently the literature on *ex vivo* expansion of $CD34^{pos}$ and MNCs has been summarized, showing that MNCs from both adult and CB could generate on average 10 to 100 times more erythroblasts than the corresponding $CD34^{pos}$ cells.[18] Compared to other studies, we generated a lower number of erythroblasts aiming for a single-step culture method suitable for standard blood bank conditions in closed systems. Only a minority of the studies, reviewed by Migliaccio and colleagues[18] used xenofree medium. The fold increase varied between factors of 50 and 1.10^6 depending on the cell source or growth factor combination used; enucleation rate was either not given or ranged between 30 and 100%.[10,11,18,26] Baek and coworkers[9] also used non xenogeneic proteins but these cells were cultured upon human mesenchymal stem cells. They reached approximately 10^4-fold increase with an enucleation rate of greater than 60%. The combination IL-3, SCF, and EPO was used in all of these studies and seems to be fundamental for ex vivo expansion.[9-11,26] Furthermore, an extensive set of steroids and cytokines, addition of lipids or other components like d-mannitol, trace elements, glucocorticoid antagonists, or multiple

culture phases resulted in complex procedures.[10,11,18,26] Of these, the highest fold expansion was reached by Miharada and coworkers[10] who used vascular endothelial growth factor and insulin growth factor-2. With our approach, we did not reach a 100% CD235a positive cell population, in view of the remaining lineage negative cells. For autologous use, however, this is not an obstacle. Expanded erythroblasts have been suggested as an alternative for transfusion.[12,27] Most *in vivo* mice models, however, are not all suitable for studying expanded human erythroblast maturation and enucleation, as human erythroid cells are removed by the mouse spleen.[21,22,28] Although the splenectomized NOD/SCID/ IL2Rgnull mice model seems more suitable for *in vivo* human erythroblast tracking, in this model human RBC counts in peripheral mouse blood also remained low. Alongside improving our culture method, our production process of autologous CB products should be adapted. During processing approximately 60% of the total WBCs remained in the RBC product[16] and, vice versa a relatively large amount of autologous RBCs remained in the buffy coats. A better separation method, preferentially by filtration and recovery of WBCs from the filter[29] would lead to a larger product for autologous transfusion and an increased proportion of nucleated cells suitable for ex vivo expansion.

Hence, next to salvage of CB RBCs to prepare an autologous transfusion product, we provide preliminary results indicating the potential use of the remaining progenitor cells to obtain an additional RBCs in the early neonatal period during which most transfusions are required. Despite apparently suboptimal harvest and culture conditions, our results suggest that it may be worthwhile to put effort in further optimization and generation of clinical-grade culture conditions and *in vivo* differentiation and functionality testing. It should be emphasized, however, that the expansion experiments we performed merely indicate the possible feasibility of this approach. Obviously, major hurdles must be overcome before *ex vivo* expanded CB cells can actually supplement autologous CB RBCs in the treatment of anemia of prematurity. The use of premature CB for autologous RBC transfusion purposes has been critically summarized.[30] Nevertheless both our previous and current feasibility study suggest that complete avoidance of allogeneic RBC transfusions for anemia of prematurity may be in reach in the future when combining a more efficient RBC separation with a larger availability of residual $CD34^{pos}$ and progenitor cells that can be used for both transfusion and *ex vivo* expansion. Where there is a will, there will be a way. In our opinion, the full potential of premature CB for autologous use has not been fully explored yet and offers ample room for progress.

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General discussion

Premature infants are among the most frequently transfused patients. The number of administered red blood cell (RBC) transfusions is inversely related to gestational age; resulting in the most immature infants being exposed to the most RBC transfusions.[1] Allogeneic RBC transfusions have been associated with a negative clinical outcome. A correlation between RBC transfusion and accompanying morbidity due to prematurity, such as intraventricular hemorrhage (IVH), retinopathy of prematurity, chronic lung disease and necrotizing enterocolitis (NEC), has been reported.[2-7] In addition, RBC transfusion has been associated with increased respiratory support needs in neonates.[8] An association between RBC transfusion and neonatal mortality has also been brought up. Although transfusion was stated to be an independent predictive risk factor, causality remains to be proven.[9] RBC transfusion however as a co-factor which can worsen clinical outcome is not completely unlikely.[10] During the last decades, transfusion guidelines have been implemented with more restrictive transfusion triggers, adjusted for postnatal age and cardio-respiratory condition. Use of these guidelines resulted in a significantly lower number of RBC transfusions administered to premature infants; without an increase in length of stay or morbidity.[11-13] Although these guidelines have positive effects on donor exposure and risk for blood transmissible infectious diseases, these guidelines are more empirically based than evidence based. Consequently, uniformity in neonatal transfusion practice is still not within reach.[14-16] Recent clinical trials have provided some evidence that the use of restrictive transfusion triggers are feasible.[17-19] Restrictive transfusion practice, according to the lower thresholds reported in these trials, did not have a significant impact on neonatal morbidity and mortality.[20]

However, neonatal and in particular long-term outcome yielded controversial results depending on the postnatal age of evaluation. Restrictive triggers were associated with short term negative neurological sequelae including grade 3-4 IVH[17] and liberal triggers were associated with a possible better neurodevelopment at the age of 18-21 months corrected age.[21] In contrast, the infants in the liberal group showed a reduced brain volume at an average age of 12 years and the infants included in the restrictive group had better cognitive functions.[22-23] These results should be interpreted with caution. For instance, the Bayley Scale for Infant Development Mental Development Index used 18-21 month after birth has a poor prognostic value for long term outcome.[24] In addition, in an earlier study Cooke et al showed that there was no relation between cognitive function at the age of 12-13 years and perinatal brain damage.[25] Consequently, the relation between neonatal transfusion practice and neuro-cognitive outcome still needs other well designed clinical trials with long follow-up.

Alternative strategies in the treatment and prevention of anemia of prematurity, are the use of recombinant Human Erythropoietin (EPO), the use of micro-techniques to minimize phlebotomy losses and delayed cord clamping.[26-32] Use of recombinant Human EPO for treatment of anemia of prematurity has shown to be ineffective with respect to blood sparing. [26-28] In contrast, minimizing blood losses for diagnostics and late cord clamping resulted in fewer administered RBC transfusions, but cannot completely prevent the transfusion needs in premature infants.[29-32]

Late cord clamping was associated with more clinical benefits, including an improved circulatory stability, less IVH and a lower risk for NEC.[30] The use of near infra red spectroscopy (NIRS) or doppler sonography to identify which infants are in need of transfusion showed promising results.[33-34]

In summary, the administration of allogeneic RBC transfusions to premature infants has been reduced during the past decade. Whereas the survival rate of premature infants has continued to improve, the clinical effects of allogeneic RBC transfusions on neonatal outcome are still under scrutiny over its possible impact on long term outcome.

In this thesis, we analyzed RBC transfusion practice in two Dutch tertiary centers and its effects on neonatal outcome of premature infants born before 32 gestational weeks. Furthermore, we investigated the use of autologous umbilical cord blood (UCB) as an alternative for allogeneic RBC transfusions.

Neonatal transfusion practice in the Netherlands

In **Chapter 2** we describe the implementation of the transfusion guideline, set up by the Dutch Institute for Health Care Improvement in 2004, in two neonatal intensive care units.[35-36] Besides recommended transfusion triggers, a transfusion volume range of 10 to 15 ml/kg bodyweight was advised. In other guidelines a wider range of 10 to 20 ml/kg bodyweight is recommended.[37] The two tertiary neonatal centers in our study used the same transfusion triggers and the same RBC products but a different transfusion volume per kg bodyweight.

The proportion of transfused infants was significantly different, 59% vs. 77%, surprisingly with the lowest percentage in the center using a smaller transfusion volume of 15 ml per kg bodyweight. In the younger infants born between 25 0/7 and 27 6/7 gestational weeks, we observed no differences, despite the difference in RBC transfusion volume, in the percentage of transfused patients and in transfusion events. The percentage of transfused infants born between 28 0/7 and 31 6/7 gestational weeks was significantly higher in the center using a larger transfusion volume (74% vs.49%). Transfusion with 20 ml per kg resulted in a mean reduction of one transfusion episode per infant. The higher percentage of transfused infants was associated with a higher pre-transfusion hematocrit in less premature infants, which suggests the use of different triggers based on interpretation of clinical indicators. A larger transfusion volume of 20 ml per kg prolonged the interval until the next transfusion and could reduce donor exposure in infants born between a gestational age of 28 0/7 weeks and 31 6/7 weeks.[36]

On the other hand reduction of donor exposure is also obtainable by implementation of a pedipack reservation approach using the same donor for all transfusions until expiration date of 35 days. These differences in neonatal transfusion practice are not peculiar.[15,38] To evaluate short and long term clinical effects of RBC transfusion; standardization of transfusion practice with regard to transfusion trigger, RBC product hematocrit and transfusion volume is crucial. Proposed adjustments to induce a more uniform transfusion practice include on one hand

implementation of the care-givers perception in the transfusion trigger and on the other hand use of a computerized transfusion product ordering and monitoring.[39-40] But also other parameters like heart rate, regional tissue oxygenation or blood flow velocity could be included in the decision to transfuse.[33,41-42] Further studies are necessary to identify whether these advices can contribute to better compliance to the transfusion guideline.

Recently, the national Dutch transfusion guideline has been revised. Strategies attending a decrease in neonatal anemia such as late cord clamping and the use of micro-techniques for diagnostics to reduce the phlebotomy volumes, have been included in the transfusion guideline. Recommendations on transfusion triggers and transfusion volume have also been adjusted. The earlier recommendation to maintain an hemoglobin (Hb) level ≥ 8 mmol/l during the first 24 hours has been abandoned. A low Hb level direct post-partum is caused by either acute blood loss or acute hemolysis due to i.e. rhesus antagonism. It is now recommended that the decision to transfuse RBCs within 24 hours after birth should be based on clinical grounds, rather than the Hb level at that moment. Transfusion of a higher volume per kg bodyweight (15ml/kg vs 20ml/kg) did not lead to significant better outcome or reduction in transfusion episodes for premature neonates with the highest transfusion needs. Aiming to a more distinct guideline, the earlier recommended range (10-15ml/kg) in transfusion volume has been adjusted to 15 ml/kg bodyweight.[43]

Transfusion effects on neonatal clinical outcome

The relation between RBC transfusion and clinical outcome has been studied in different ways. Earlier mentioned "trigger studies" investigated the safety of the transfusion threshold and related this to clinical outcome.[17-19] Target studies, in which the efficacy and effects of the total administered RBC volume are investigated, are available in only a few studies with a small number of patients.[44-46]

Short term clinical outcome

In our observational comparative study (**Chapter 2**) we evaluated the effect of the total administered RBC transfusion volume on short-term neonatal outcome, consisting of a composite of mortality, IVH, retinopathy of prematurity and chronic lung disease. Clinical neonatal outcome was similar, regardless of a higher proportion of transfused patients and a higher total amount of RBCs transfused in one of the centers and despite the difference in transfusion volume. This suggests that an absolute larger transfused RBC volume was not associated with a worse short-term clinical outcome in the two cohorts studied.[36]

To establish appropriate transfusion triggers and to estimate when to use which trigger in premature infants is extremely complex. Recent studies using NIRS to monitor tissue oxygen saturation showed that after RBC transfusion oxygen saturation increased in cerebral, renal

and splanchic tissues.[47-49] This technique could theoretically assist in evaluating the timing of a RBC transfusion to prevent hypoxia and to avoid possible transfusion related oxidative stress in premature infants. Early transfusions have been associated with the development of IVH.[50-51] RBC transfusion in the first week of life doubled the risk of worsening of grade 1 IVH.[51] Late RBC transfusions have been related to the incidence of NEC.[6,52] More studies investigating the causal relation between RBC transfusions; including timing of transfusion and transfusion volume; and these neonatal complications are essential for establishment of future transfusion guidelines.

Long-term clinical outcome

There are few studies available which investigate the relation between RBC transfusion and long-term outcome in premature infants. Whyte et al compared neurodevelopmental outcome at a corrected age of 18-21 months among premature infants transfused to maintain high or low hemoglobin levels during early neonatal care. They observed a non-statistically significant better outcome when a more liberal transfusion strategy was used.[21] The long-term evaluation of infants in the study by Bell et al showed opposite results, but these children were also evaluated at a different age.[17,22-23] The infants in the Iowa study included in the liberal transfusion group had a smaller brain volume and a worse neurocognitive profile compared to the restricted transfusion group.[22-23] These studies investigated the effect of a lower transfusion Hb threshold and did not correlate the actual total administered RBC volume and the Hb target with long-term clinical outcome.

We performed an observational follow-up study (**Chapter 3**) of a group of extremely premature infants at a corrected age of 24 months in two tertiary neonatal centers using a RBC transfusion volume of either 15 or 20 ml/kg bodyweight. One cohort was assessed using the Bayley Scale of Infant Development II, all performed by the same psychologist. The other cohort was assessed using various validated tests, performed in different hospitals. In the Netherlands it is not yet common practice to use the same assessment tool for developmental follow-up. We observed no differences in neuromotor development. Our study had several limitations, including a small study population, different tools to evaluate neuromotor development and the retrospective disposition of the study.[53] In view of the earlier mentioned studies, it cannot be ruled out that the total volume of administered donor RBC has an effect on neuromotor development in extremely premature infants. Future well designed randomized trials, preferentially using the same method for neurodevelopmental outcome, are pivotal to evaluate whether there is an optimal Hb target and thus transfusion volume in relation to long-term neonatal outcome.[54]

Erythropoietin response to RBC transfusion

RBC transfusions suppress endogenous erythropoietin (EPO) production.[55] Most (older) studies report a transfusion associated decrease in EPO levels, however this has been measured late in the hospital course, after the patients have received frequent RBC transfusions.[55-60] Neonatal

transfusion practice has changed since these studies were performed.[11] The transfusion triggers have become more restrictive for stable infants after the postnatal age of 4 weeks and most RBC transfusions are now administered in the first month after premature birth.[1] Animal studies have shown that EPO has neuro-protective properties.[61] More restrictive RBC transfusion practices could in theory attend endogenous EPO production and consequently help in preventing or overcoming brain injury.[61] To study these effects we should know more about EPO levels in premature infants. In adults with a normal hematocrit, EPO values vary between 2.6 and 18.5 mU/ml.[63] In healthy children (one month – 16 years old) reference EPO values are slightly higher with a mean value of 15.8 mU/ml and 95% range of 9.1-27.6 mU/ml.[64] For premature infants, in particular during the first month of life, no exact data are available.

We measured EPO levels in 46 premature infants, born in 6 consecutive months (**Chapter 4**). We used waste material, so no extra blood loss was necessary for our measurements. Consequently, we were only able to measure EPO levels during the first month of life. Thereafter, most infants had been transferred to peripheral centers. None of the infants received recombinant human-EPO. Thirty six out of 46 received at least one transfusion. EPO levels were not correlated to Hb or hematocrit values. EPO is also a marker for stress.[65-66] EPO levels >500 mU/ml were associated with life threatening conditions. Although we did not find a significant relationship between EPO levels and a worse Apgar score, the need for respiratory support or sepsis. EPO levels declined after every administered RBC transfusion. We did not find a significant suppressive effect of cumulative RBC transfusions in the first month of life. It is however possible that differences in transfusion practice (used triggers, transfusion volume or hematocrit of the transfusion product) can influence this decline in EPO in variable degrees.

Umbilical Cord Blood for transfusion purposes

Collection of umbilical cord blood

Umbilical cord blood (UCB) can be collected by puncture of the umbilical cord vessels either before (*in utero*) or after (*ex utero*) placental delivery. Few randomized studies exist, mainly performed in a caesarean section setting (**Chapter 5**).[67-70] These studies showed that *in utero* collection results in significantly higher UCB volumes. This was supported by a large observational study by Solves et al.[71] Of note, immediate clamping of the cord is an important factor contributing to a larger harvested UCB volume. Collection of UCB after caesarean section resulted in less contamination.[72] Reported microbial contamination of collected UCB in several clinical studies was between 0% and 9%.[73-77]

Processing and storage of UCB for transfusion purposes

There are few studies reporting on UCB processing into RBC products (**Chapter 5**). Eichler and Garritsen used a centrifugational separation technique.[74,78] A minimal net volume of 30 ml UCB appeared necessary to obtain approved UCB derived RBC products.[74] Hollow fibre in-line filtration

by gravity could be an elegant method to be used for RBC separation from UCB, because this method is less laborious and may exert less mechanical stress compared to the centrifugation method. This technique requires a minimum of 60 ml UCB and is therefore not a suitable alternative for processing premature UCB because the collected volumes after premature delivery are much smaller.[74,79-80]

Several studies have been performed to explore red cell lesions in stored UCB units. After storage up to 28 days in citrate-phosphate-dextrose-adenine (CPDA), whole blood UCB using autologous plasma has an acceptable mean pH of 6.51 and haemolysis rate of 0.39 ± 0.05 %.[81-82] Storage of UCB previously processed into RBC products gives different results. UCB derived packed RBC units, supplemented and stored in saline-adenine-glucose-mannitol (SAG-M) or phosphate-adenine-glucose-guanosine-saline-mannitol (PAGGS-M) for 35 days [78-79, 83] had significantly higher mean haemolysis rates.[78-79] Storage in PAGGS-M resulted in a lower haemolysis rate compared to SAG-M.[83] In view of the increase in haemolysis and decrease in pH, it can be concluded that UCB red cells deteriorate faster during storage than adult red cells. The whole blood storage (in autologous CPDA- plasma) parameters suggest that UCB derived RBCs deteriorate faster after processing by centrifugation or filtration and storage in preservation medium as compared to adult RBCs. The study by Widing et al showed better results when PAGGSM was used as storage solution. PAGGSM contains additional phosphate and guanosine compared to SAG-M.[83] This finding suggests that not (only) the manipulation of cord blood, but rather the preservative solution may contribute to the storage damage of UCB derived RBCs.

There is limited data on storage of premature RBCs. For our clinical study on the use of premature UCB for autologous transfusion purposes, we first performed a validation study (**Chapter 6**). We stored premature UCB as whole blood in CPDA or as a fractionated RBC component in SAG-M or additional solution 3 (AS-3). Fractionation was performed using a closed centrifugation circuit (15 min at 1010 g; Biosafe Sepax, Eysins,

Switzerland) suited to use different blood volumes. Stored as whole blood the red cell parameters maintained rather well up to 14 days of storage.[84] The lower and variable hematocrit and the high white blood cell content, is a disadvantage to use whole blood stored RBC as an alternative for allogeneic RBC transfusion in premature infants. Moreover as will be later discussed, harvesting the white blood cell fraction, which contains the cord blood stem cells, could be of future benefit. Storage parameters of UCB derived RBC components in SAG-M or AS-3 were similar, but both less optimal than whole blood storage. We observed a higher haemolysis rate compared to the studies by Garritsen et al, Brune et al, and Widing et al.[78-79,83] The pH during storage in SAG-M was similar compared to the studies by Brune and Garritsen.[78-79] It is possible that premature UCB derived RBCs are more fragile and less resistant to mechanical stress during fractionation.

Altogether, we can conclude that premature UCB derived RBCs can be collected for transfusion purposes. In view of the red cell lesion parameters, shelf life of these products would be maximally 21 days and therefore much shorter than the standard donor pedi-pack RBCs. In current transfusion practice, most premature infants receive RBC transfusions in the first weeks of life.[85]

Clinical implementation - autologous UCB transfusion

We performed a randomized clinical study to investigate the feasibility of autologous premature UCB transfusions for the treatment of anemia of prematurity (**Chapter 7**). Premature UCB was collected after deliveries between 25 0/7 – 31 6/7 gestational weeks. In 57% of all collections, the harvested UCB volume was adequate for processing into a RBC product. After processing and quality control there were autologous products approved for release for transfusion available for 36% of the total study population. In the context of a blinded RCT, the proportion of transfused infants with an available autologous RBC product was 27%. These products could cover a mean of 58% of the transfusion needs of these infants (range 25-100%). The transfusion needs from the infants born after 30 gestational weeks was low, in our study 19% of these infants received RBC transfusions.[1] As a result of these findings, we concluded that the collection of premature UCB for infants born after 30 gestational weeks was less efficient, resulting in 80% of available autologous UCB products which were not used. The collection of UCB for younger infants appeared to be more efficient. For infants born before 28 gestational weeks, UCB collection was however not often sufficient for successful processing, but in view of the high transfusion needs, every available product would be used. For slightly older infants, born between 28 0/7 and 30 6/7 gestational weeks, the availability of autologous product was most efficient.[77]

Expansion of UCB derived CD34 positive cells into erythroid cells

UCB contains a large content of CD34 positive hematopoietic stem cells and other progenitor cells. These multi-potent cells can be expanded into clinical grade RBCs using specific combinations of cytokines and growth factors.[86-90] In addition, expanded erythroblasts have been proposed as an alternative for the standard transfusion product. Clinical implementation of these expanded cells for transfusion is however held back due to the use of xenogeneic proteins in the culture medium and expensive complex multistep protocols.[91]

After processing of the premature UCB into RBC products[84], the waste buffy coats were examined on CD34 positive cell content. We found a mean count of residual $2.5x10^6$ CD34 positive cells in the premature UCB buffy coats (range $1.2x10^5$ – $1.7x10^7$ (n=10)). We evaluated whether expansion of these waste buffy coats could provide additional red cells for transfusion to supplement our autologous premature UCB cell products that have a shelf life of 14-21 days.[84]

We have set up a simple one-step liquid culture protocol in which we tested several combinations of recombinant human Stem Cell Factor (SCF), Interleukin 3 (IL-3), trombopoietin and EPO, while omitting xenogeneic cytokines and proteins from the culture medium (**Chapter 8**). We tested the whole waste buffy coat after premature UCB processing and isolated CD34 positive cells from premature and full term cord blood and adult mobilized peripheral blood (PBSC) and bone marrow (BM). Expansion of the whole premature UCB buffy coat was more effective in gaining erythroblasts and resulted in a ± 3 fold higher number of erythroblasts compared to isolated CD34 positive cells from premature UCB. Due to our processing method there was however

a significant proportion of native RBCs present in the buffy coat. During culture it cannot be precluded that these native cells are subject to red cell storage lesion. Therefore the subsequent experiments were performed with isolated CD34 positive cells. Premature and full term CD34 positive cells isolated from UCB had a similar fold increase and a similar erythroid differentiation pattern. The CD34 positive cells from full term UCB and the adult cell sources had similar fold expansion rates (between 4000 and 4700 fold) after 21 days of culture with SCF, IL3 and EPO. On day 21, the expanded cell cultures were pure erythroid. The proportion of CD235a (Glycophorin-A) expressing cells, reflecting erythroblast differentiation and maturation, in adult PBSC (96.7±0.8%) and BM (98.9±0.5%) was significantly larger compared to UCB (87.7±2.7%) ($p=0.002$ after correction for multiple testing). Residual cells in the UCB cultures existed of CD71 positive and CD235a-CD45 negative cells, reflecting proliferating cells that had not differentiated towards a specific cell lineage. Based on the cell surface marker expression of the majority of the expanded cells; CD235a positive/CD71 positive/CD36 negative; we concluded that we obtained mostly polychromatic erythroblasts in our culture system. Our attempt to induce *ex vivo* enucleation by adding an extra culture phase which included supplementation of insulin and thyroid hormone to the culture medium, combined with removal of dexamethason[92], was unsuccessful.

With the optimal growth factor combination of IL3, SCF and EPO we calculated that expansion of CD34 positive cells would lead to a range of $4.8x10^8$- $4.4x10^{10}$ expanded erythroblasts. If the whole premature UCB buffy coat would have been cultured without native RBC contamination, this could imply ± threefold higher number of expanded erythroblasts. As these cells would fully differentiate and maturate *in vivo*, theoretically enough cells could be expanded to obtain for extremely low birth infant infants at least one additional red cell transfusion.

There is however much room for optimization of techniques to increase the yield. Future studies should first focus on the *in vivo* functionality and safety of expanded red cells have to prove suitability of expanded cell transfusion. Although our harvesting method and culture conditions were suboptimal, the results suggest that it is worthwhile to put effort in further optimization and generation of clinical grade culture conditions, and *in vivo* differentiation and functionality testing. Combined with the use of autologous UCB derived RBCs, expansion of autologous cells could assist in minimizing neonatal exposure to allogeneic RBC products.

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Summary

The association between allogeneic RBC transfusions and a negative clinical outcome in premature neonates has contributed to the introduction of blood transfusion guidelines with recommended transfusion triggers. Despite the use of a national blood transfusion guideline significant differences exist concerning the total amount of allogeneic RBC transfusions administered to premature infants. However, as shown by comparison of two Dutch tertiary neonatal centers, a more liberal transfusion strategy and a higher transfusion volume was not associated with obvious poor short-term outcome, nor delayed neuromotor development at 2 years of age. A methodological bias due to the use of different assessment tools for neuromotor development or a too small study population however cannot be ruled out and long-term follow-up evaluation is lacking.

Due to use of the current transfusion triggers, most transfusions are administered early in life. Therefore less suppression of endogenous EPO could be expected. Future studies focusing on transfusion triggers and transfused RBC volume can elucidate whether differences in transfusion practices result in variable degrees of EPO supression.

Umbilical cord blood may be an easy accessible alternative for allogeneic RBC transfusions but can also be used for autologous purposes. Premature infants born < 30 gestational weeks suffering from anemia of prematurity can be treated with autologous RBCs derived from UCB. *Ex vivo* expansion of UCB derived CD34 positive stem cells and progenitor cells provides a potential source of *ex vivo* expanded RBC for autologous use, and as such could supplement autologous UCB red cells in minimizing allogeneic RBC transfusions. In the future, the culturing methods should be optimized for clinical use.

Current neonatal transfusion practice and clinical studies have focused on more restrictive transfusion triggers and optimizing RBC product volume and hematocrit to minimize allogeneic RBC transfusions. Use of autologous UCB cells and expanded UCB red cells can assist in reducing allogeneic blood. However, much remains to be done before this can be achieved. Until then, the presumed disadvantages of allogeneic RBCs on neonatal outcome should be further clarified, because avoidance of allogeneic transfusions is unattainable in current clinical practice.

Recommendations

- Future studies on RBC transfusion triggers and/or effects should mention transfusion volume as well as product hematocrit. As such international data on actual transfused RBC effects can be compared in a more precise manner.
- Follow up studies on transfusion related outcome should use a uniform method for neuro-cognitive testing and also a longer follow up period (at least up to school age) is recommended.
- To widen the use of autologous cord blood transfusion; there exist ample possibilities for improvement of collection and fractionating techniques of umbilical RBC products and of erythrocyte expansion protocols from UCB leukocytes.

Samenvatting

Te vroeg geboren baby's worden vaak behandeld met rode bloedcel (RBC) transfusies vanwege bloedarmoede.[1] Deze RBCs zijn afkomstig van gezonde volwassen donoren. In de literatuur wordt een relatie gelegd tussen deze transfusies en nadelige neonatale klinische uitkomsten.[2-6] Een causaal verband is echter tot nu toe niet bewezen.[7]

De invoering van nationale bloedtransfusierichtlijnen heeft er toe geleid dat het aantal toegediende RBC transfusies aan te vroeg geboren baby's significant is verminderd.[8-10] De achtergrond van deze transfusierichtlijnen is echter empirisch; wetenschappelijke ondersteuning voor het gebruik van de geadviseerde transfusiedrempels en transfusievolume ontbreekt. Resultaten van studies naar het gebruik van meer restrictieve en liberale transfusiedrempels toonden dat een restrictiever transfusiebeleid leidde tot minder toegediende transfusies.[11-13] De klinische uitkomsten van een restrictief of meer liberaal transfusie beleid waren echter minder eenduidig. Eén studie toonde dat een restrictief transfusiebeleid leidde tot meer neurologische problemen op de korte termijn.[11] Alhoewel een liberaal transfusiebeleid geassocieerd was met een mogelijke betere neuro-motore ontwikkeling op de gecorrigeerde leeftijd van 18-21 maanden, bleek daarentegen dat dit beleid ook geassocieerd is met een kleiner hersenvolume en een minder goed cognitief profiel op de schoolgaande leeftijd.[14-16] Interpretatie van deze resultaten is niet eenvoudig. De methoden om de ontwikkeling op de jonge kinderleeftijd te testen hebben een lage voorspellende waarde voor het cognitief functioneren op de schoolgaande leeftijd, waarbij geen relatie is gevonden tussen perinatale hersenschade en cognitief functioneren op 12 jarige leeftijd.[17-18] Toekomstige studies zullen moeten laten zien wat de invloed is van RBC transfusie op cognitieve uitkomsten.

Andere onderzochte methoden in de strijd tegen bloedarmoede bij te vroeg geborenen zijn het gebruik van recombinant humaan Erytropoëtine (EPO), vermindering van het bloedverlies door afname voor diagnostiek en later afnavelen na een vroeggeboorte. Het gebruik van recombinant humaan EPO bij te vroeg geboren baby's is niet doelmatig en effectief gebleken.[19-21] Daarentegen leidden zowel minder bloedafnamen voor diagnostiek als later afnavelen wel degelijk tot minder toegediende transfusies, alhoewel RBC transfusie niet volledig kon worden voorkomen.[22-23]

Kortom, het gebruik van allogene RBC transfusies aan te vroeg geboren baby's is significant verminderd. Aangezien te vroeg geborenen een steeds betere overleving hebben, is het belangrijk om zowel de korte als de lange termijn effecten van deze transfusies in kaart te brengen.

In dit proefschrift hebben we in twee Nederlandse neonatale intensive care units (NICU's) transfusie effecten en uitkomsten in kaart gebracht. Daarnaast hebben we onderzocht of RBCs uit eigen navelstrengbloed als alternatief gebruikt kunnen worden voor het standaard donor RBC product.

Neonataal transfusie beleid in Nederland

De Nederlandse bloedtransfusierichtlijn is opgesteld door het CBO Kwaliteitsinstituut voor de Gezondheidszorg.[24] In deze richtlijn worden aanbevelingen gedaan voor de transfusiedrempel, maar geen eenduidig advies gegeven voor de transfusietarget, te bereiken door een lager of

hoger transfusievolume per kg lichaamsgewicht. Daarom hebben we in twee Nederlandse NICU's onderzoek gedaan naar de effecten van een verschillend transfusie volume (15ml/kg vs 20 ml/kg) op de neonatale uitkomsten. Beide centra hanteerden de Nederlandse transfusierichtlijn en gebruikten hetzelfde transfusieproduct. Desondanks zagen we grote verschillen. Het percentage te vroeg geboren baby's dat werd behandeld met transfusies was duidelijk hoger in het centrum dat een volume van 20 ml/kg hanteerde (77% van de baby's) in vergelijking met het andere centrum (59% van de baby's). Dit werd vooral bepaald door een groot verschil in getransfundeerde baby's geboren na 28 tot 32 weken zwangerschapsduur (74% versus 49 %). Bij de jongere premature geboren vóór 28 weken zwangerschapsduur werd een vergelijkbaar aantal baby's behandeld met RBC transfusies. Ondanks het gebruik van de nationale richtlijn waren er sterke aanwijzingen dat frequent transfusies werden toegediend op klinische gronden ondanks dat de aanbevolen transfusiedrempel niet was bereikt.
Het effect van een verschillend transfusievolume was niet eenduidig. Bij de jongere baby's geboren vóór 28 weken zwangerschapsduur, resulteerde het hogere volume niet in een geringer aantal transfusies. Bij de baby's geboren tussen 28-32 weken zwangerschapsduur zagen we bij transfusie met 20 ml per kg een vermindering van één transfusie episode per kind.[25]
In 2011 werd de CBO richtlijn voor bloedtransfusie toediening herzien. De belangrijkste wijzigingen in de richtlijn zijn als volgt; de aanbeveling om laat af te navelen (30 tot max. 180 sec) indien klinisch mogelijk is opgenomen in de richtlijn; de transfusie drempel van 8 mmol/l voor de eerste 24 uur na geboorte is verlaten, en het aanbevolen transfusievolume per kg lichaamsgewicht is veranderd naar 15 mL/kg lichaamsgewicht in plaats van de eerder aanbevolen range van 10 tot 15 mL/kg lichaamsgewicht.[26]

Deze verschillen in transfusiebeleid staan niet op zichzelf. Ook in andere landen is gebleken dat er ondanks de invoering van een nationale transfusierichtlijn belangrijke verschillen bestaan in de praktijk.[27-29] Voorstellen om de compliantie van de zorgverlener te vergroten omvatten onder andere het gebruik van een elektronisch transfusieproduct bestel- en monitorsysteem en het implementeren van de klinische beoordeling van de zorgverlener binnen de richtlijn.[30-31] Met het oog op de aangetoonde verschillen met betrekking tot het stellen van de transfusie indicatie en het effect van het gebruikte transfusie volume op de blootstelling aan donorbloed, is het belangrijk om uit te zoeken welke aanpassing bijdraagt aan de beste compliantie.

Transfusie effecten op neonatale klinische uitkomsten

Korte en lange termijn klinische uitkomsten

In bovengenoemde neonatale cohorten hebben we gekeken naar de invloed van het aantal RBC transfusies en het toegediende transfusievolume op zowel korte als lange termijn neonatale uitkomsten. Op de korte termijn zagen we geen verschillen tussen beide groepen met betrekking tot frequentie van overlijden, hersenbloedingen, prematuren retinopathie en bronchopulmonaire

dysplasie, ondanks dat er in één centrum een significant grotere groep kinderen werd behandeld met RBC transfusies en een groter transfusievolume.[25]
Er is weinig bekend over de relatie tussen RBC transfusie en neonatale uitkomsten op de langere termijn. Zoals eerder genoemd is de literatuur niet eenduidig.[14-16]
Wij hebben de relatie tussen transfusie volume en neuro-motore ontwikkeling geëvalueerd in twee groepen extreem te vroeg geboren baby's, geboren vóór 28 weken zwangerschapsduur. Het aantal toegediende transfusies was vergelijkbaar tussen beide groepen. We vonden geen verschil in neuro-motore ontwikkeling op de gecorrigeerde leeftijd van (mediaan) 24 maanden bij een groter of kleiner toegediend transfusievolume. Echter, onze studie had een aantal beperkingen waaronder een relatief kleine studiepopulatie, verschillen in evaluatie methoden en het observationele karakter van de studie.[32] Het kan dus niet worden uitgesloten dat het totale volume van een RBC transfusie invloed heeft op de neuro-motore ontwikkeling van extreem te vroeg geboren kinderen. Goed opgezette gerandomiseerde klinische studies zijn essentieel om dit verder te kunnen onderzoeken.

Het effect van RBC transfusie op EPO
Meerdere (oudere) studies hebben laten zien dat RBC transfusies bij premature pasgeborenen de endogene EPO productie kunnen onderdrukken. Dit EPO onderdrukkend effect werd echter pas na een aantal weken postnataal gemeten, wanneer de kinderen al een groot aantal transfusies hadden gekregen.[33-36]
Het huidige neonatale transfusie beleid is aanzienlijk anders dan in deze oudere studies. Dankzij de invoering van nationale transfusierichtlijnen, met aanbevolen transfusiedrempels, is het aantal toegediende RBC transfusies significant gedaald.[8-10] Dit heeft ertoe geleid dat de meeste transfusies worden toegediend in de eerste maand na geboorte.
Meerdere dierexperimentele studies hebben laten zien dat EPO neuro-protectieve eigenschappen heeft.[37-38] Een restrictiever transfusiebeleid zou in theorie de endogene EPO productie minder onderdrukken en kunnen bijdragen aan betere neuro-protectie en het voorkomen van hersenschade op de langere termijn. Om deze theorie te ondersteunen zouden we meer moeten weten van EPO waarden bij te vroeg geboren baby's. Volwassenen zonder bloedarmoede hebben EPO waarden tussen de 2.6 – 18.5 mU/mL.[39] Kinderen (tussen 1 maand -16 jaar) hebben iets hogere EPO waarden, tussen de 9.1-27.6 mU/mL.[40-42] Van te vroeg geboren baby's geboren hebben we beperkt beschikbare gegevens.
We hebben daarom bij 46 prematuur geboren baby's EPO concentraties gemeten in plasma. We hebben alleen spijtmateriaal gebruikt, dat wil zeggen, bloed wat over was van bloedafnamen voor reguliere diagnostiek. Hierdoor waren we alleen in staat om vooral in de eerste maand na geboorte onze metingen te verrichten, omdat enerzijds veel kinderen daarna werden overgeplaatst naar ziekenhuizen elders, en anderzijds omdat in de eerste maand de meeste bloedtesten worden verricht. De kinderen kregen in deze periode geen rh-EPO toegediend. De baby's die

wel transfusies kregen toegediend hadden hogere EPO waarden dan de niet-getransfundeerde te vroeg geboren zuigelingen. Dit reflecteert de diepere bloedarmoede bij deze kinderen en mogelijk een compensatoire EPO respons op deze anemie. Anderzijds is ook bekend dat EPO een marker is voor stress. Ook klinisch zieker zijn zou dus in theorie kunnen bijdragen aan een hogere EPO productie. De waarden laten zien dat vroege transfusies in de eerste levensmaand de EPO productie niet duidelijk onderdrukken.

Navelstrengbloed voor transfusiedoeleinden

Het afnemen van navelstrengbloed

Navelstrengbloed kan afgenomen worden vóór (*in utero*) of na (*ex utero*) de geboorte van de placenta. *In utero* afname van navelstrengbloed leidt tot aanzienlijk hogere bloedvolumes, zowel na een vaginale bevalling als na een keizersnede.[43-46] Daarnaast is het vroeg afklemmen van de navelstreng ook een belangrijke factor die bijdraagt aan een groter volume van de bloedafname.[47] Bacteriële contaminatie komt voor in 0 tot 9% van de navelstrengbloedafnames.[48-52]

Het verwerken en bewaren van navelstrengbloed voor transfusiedoeleinden

Navelstrengbloed kan met behulp van centrifugatie bewerkt worden tot een RBC concentraat.[49,53] Ook zonder centrifugeren is scheiding van RBCs uit navelstrengbloed mogelijk. Deze techniek heet 'hollow-fibre in-line' filtratie en maakt alleen gebruik van zwaartekracht. Deze methode veroorzaakt mogelijk minder schade aan rode bloedcellen, maar is alleen doelmatig met een minimaal afgenomen volume van 60 mL navelstrengbloed, aangezien anders relatief teveel RBCs achterblijven in het filtersysteem.[54] Aangezien de afgenomen volumina na een premature geboorte kleiner zijn, lijkt deze methode vooralsnog niet geschikt voor het opwerken van prematuur navelstrengbloed.[52]

Voor het bewaren van navelstrengbloed RBC zijn verschillende bewaarvloeistoffen getest. Het bewaren van volbloed uit de navelstreng in citraat-fosfaat-dextrose-adenine en autoloog plasma toonde goede parameters na een bewaarduur van 21-28 dagen; een gemiddelde pH van 6.6 na 21 dagen, en een gemiddelde pH van 6.51 en een hemolyse van 0.39 ± 0.05% na 28 dagen.[55-56] Aangezien in Nederland geldt dat de hemolyse <0.8% moet zijn ten tijde van een transfusie, hebben deze studies laten zien dat het bewaren van volbloed navelstrengbloed voldoet aan deze strenge eis.

Het bewaren van door centrifugatie opgewerkte navelstrengbloed RBC in bewaarvloeistoffen, zoals gebruikt voor RBCs van bloeddonoren, leidde tot minder goede resultaten. Bij het bewaren van navelstrengbloed RBCs in saline-adenine-glucose-mannitol (SAG-M) of phosphate-adenine-glucose-guanosine-saline-mannitol (PAGGS-M) werd gezien dat de hemolyse aanzienlijk toenam in de tijd.[53,57] PAGGS-M bleek een betere bewaarvloeistof dan SAG-M, met lagere hemolyse waarden.[57] Gezien de toename in hemolyse en de pH daling kunnen we concluderen dat navelstrengbloed RBCs sneller in kwaliteit achteruitgaan indien deze cellen bewerkt en bewaard

worden onder omstandigheden die zijn geoptimaliseerd voor volwassen RBCs. Aangezien PAGGS-M de beste resultaten gaf, concluderen we dat niet alleen de opwerkprocedure middels centrifugatie, maar ook het bewaarmedium kan bijdragen aan schade aan foetale RBCs tijdens het bewaren.

Er is weinig bekend over de optimale bewaarcondities van afgenomen navelstrengbloed van prematuur geborenen. Voor onze klinische studie over het gebruik van prematuur navelstrengbloed voor autologe transfusie, hebben we eerst een navelstrengbloedproduct validatie studie opgezet.[58] We hebben prematuur navelstrengbloed opgeslagen als volbloed in CPDA of als, door centrifugatie gefractioneerd, RBC component in SAG-M of Additional Solution 3 (AS-3). De volbloed RBC kwaliteitparameters na 21 dagen waren beter dan van de RBC componenten. Echter, gezien de lagere en variabele hematocriet en de hoge witte bloedcel contaminatie, die na centrifugatie verlaagd werd met gemiddeld 37%, is volbloed minder aantrekkelijk voor autologe transfusie. SAG-M of AS-3 waren even goed als bewaarvloeistof. De hemolyse in onze studie was hoger in vergelijking met andere studies[53-54, 57], maar de pH was wel vergelijkbaar.[53-54] Het is mogelijk dat navelstrengbloed RBC van prematuren kwetsbaarder zijn en minder bestand tegen mechanische stress tijdens centrifugering. Al met al kunnen we concluderen dat navelstrengbloed RBC van prematuur geborenen geschikt is voor autologe transfusie. Met het oog op de RBC bewaarparameters, is de houdbaarheid van deze producten aanzienlijk korter (tot maximaal 21 dagen) dan van standaard volwassen RBC bewaard als 'pedi-pack'. Maar aangezien premature baby's vrij snel na geboorte transfusie behoefte hebben[1], is dit geen praktisch bezwaar.

Klinische toepasbaarheid – Autologe navelstrengbloedtransfusie

In een gerandomiseerde studie hebben we de haalbaarheid van autologe navelstrengbloed transfusies onderzocht voor de behandeling van anemie van de prematuriteit. Prematuur navelstrengbloed werd afgenomen na bevallingen bij een termijn van 25 tot 32 weken zwangerschap. In 57% van de gevallen was er voldoende (> 15 ml) navelstrengbloed geoogst voor verdere bewerking tot een autoloog RBC product. Na het opwerken, het uitvoeren van dezelfde kwaliteitstesten als voor volwassen donor RBCs en bacteriële testen van alle navelstrengbloed RBC producten, waren er goedgekeurde autologe producten beschikbaar voor 36% van de totale studie populatie. Voor 27% van de prematuren met een daadwerkelijke transfusiebehoefte was er een autoloog RBC product beschikbaar, dat gemiddeld 58% van de transfusiebehoefte van deze pasgeborenen kon dekken (range van 25-100%).[52]

Het kweken van navelstrengbloed CD34 positieve cellen naar rode cellen

Navelstrengbloed is een belangrijke bron voor CD34 positieve hematopoïetische stamcellen en van andere voorlopercellen. Deze multipotente cellen kunnen ondermeer worden opgekweekt tot RBC geschikt voor transfusie.[59-63] Het gebruik van gekweekte rode voorlopercellen uit navelstrengbloed is overigens momenteel een ontwikkeling die met veelbelovende resultaten

wordt geëxploreerd als een toekomstig alternatief voor het standaard transfusie product. De daadwerkelijke toepasbaarheid van deze gekweekte cellen voor transfusie is nog niet bekend. Met name het gebruik van groeimedia die dierlijke eiwitten bevatten en dure complexe kweekprotocollen zijn debet aan het feit dat de toepasbaarheid nog niet uitgebreid is onderzocht in de kliniek.[63]

Na het bewerken van het prematuur navelstrengbloed tot een autoloog RBC product hebben we in de afgescheiden buffy coat, die een groot deel van de witte bloedcelfractie bevat, het aantal CD34 positieve stamcellen bepaald. We vonden gemiddeld $2.5x10^6$ CD34 positieve cellen per buffy coat (range $1.2x10^5$-$1.7x10^7$; n = 10). Onze hypothese was dat het kweken van deze stamcel bevattende buffy coats zou kunnen leiden tot extra rode (voorloper) cellen, geschikt voor transfusie na 21 dagen kweek, wanneer de bewaarduur van het autologe RBC navelstrengbloed product verlopen zou zijn.

We hebben hiertoe een eenvoudige 1-staps kweekmethode opgezet met gebruik van recombinant-humaan Stem Cell Factor (SCF), Interleukine-3 (IL-3) en EPO, zonder het gebruik van dierlijke eiwitten. De efficiëntie van onze methode hebben we getest met zowel CD34 positieve cellen uit (prematuur en a terme) navelstrengbloed, en uit CD34 positieve celbronnen van volwassenen (gemobiliseerd perifeer bloed (PBSC) en beenmerg (BM)). Na 21 kweekdagen hadden we een celvermenigvuldiging van gemiddeld 4000 tot 4700 keer in zowel de navelstrengbloed kweken als de PBSC en BM kweken. Na 21 dagen kweken bestonden alle kweken uit rode voorlopercellen. We berekenden dat met onze gemiddelde vermenigvuldigingssnelheid, we $4.7x10^8$ tot $4.4x10^{10}$ rode voorlopercellen kunnen genereren. Deze voorlopercellen kunnen *in vivo* verder uitrijpen tot rode cellen. In theorie is het aantal CD34 positieve stam en voorlopercellen in de buffy coat van navelstrengbloed van prematuren buffy coat met de behaalde celvermenigvuldigingsfactor, voldoende voor tenminste één extra neonatale transfusie. Verdere optimalisatie van de kweekmedia en het *in vivo* testen van het patroon van uitrijping en functionaliteit van deze rode voorlopercellen, zal ons dichter bij een gekweekt autoloog rode bloedcelproduct brengen. In combinatie met ons autologe RBC navelstrengproduct, kan dit leiden tot verder verminderen, of eventueel elimineren van de blootstelling aan allogene bloedtransfusies aan premature neonaten.

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Curriculum Vitae

Chantal Muriël Khodabux werd geboren op 6 augustus 1978 te Amsterdam. In 1996 behaalde zij haar VWO diploma aan het Pieter Nieuwland College te Amsterdam. Hierna studeerde zij eerst medische biologie aan de Vrije Universiteit te Amsterdam, waarna zij in 1998 een overstap maakte naar de studie Geneeskunde aan de Universiteit van Amsterdam. Na het behalen van het artsendiploma in 2005 heeft ze gewerkt als arts-assistent interne geneeskunde. In de loop van 2005 wisselde ze de kliniek in voor een promotietraject. Bij de Sanquin Bloedbank en het Leids Universitair Medisch Centrum deed ze onderzoek naar het gebruik van autoloog navelstrengbloed voor transfusies aan te vroeg geboren neonaten. De resultaten hiervan zijn in dit proefschrift beschreven. In de afrondende fase van haar proefschrift heeft ze gewerkt als arts-assistent kindergeneeskunde. Momenteel werkt ze als jeugdarts.