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Anemia of prematurity : time for a change in transfusion management?

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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 IVH¹⁷ 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 splanchnic 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 PAGGS-M was used as storage solution. PAGGS-M 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 products⁸⁴, the waste buffy coats were examined on CD34 positive cell content. We found a mean count of residual 2.5×10^6 CD34 positive cells in the premature UCB buffy coats (range 1.2×10^5 – 1.7×10^7 (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), thrombopoietin 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 ($96.7 \pm 0.8\%$) and BM ($98.9 \pm 0.5\%$) was significantly larger compared to UCB ($87.7 \pm 2.7\%$) ($p=0.002$ 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 dexamethason⁹², 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 4.8×10^8 - 4.4×10^{10} expanded erythroblasts. If the whole premature UCB buffy coat would have been cultured without native RBC contamination, this could imply $\pm$ threefold higher number of expanded erythroblasts. As these cells would fully differentiate and mature *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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