Statistical analysis plan

Randomized controlled trial on the use of EPO to reduce postnatal transfusions in neonates with red blood cell alloimmunization treated with intrauterine transfusions

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1. Introduction

The EPO-4-Rh study is a single center randomized controlled trial to evaluate the role of darbepoetin alfa (a long acting agent of erythropoietin or EPO) in reducing the incidence of anemia in infants with hemolytic disease of the fetus and newborn (HDFN), treated with one or more intrauterine transfusion (IUT), compared to a control group of infants with HDFN without darbepoetin alfa treatment. The trial is registered with ClinicalTrials.gov, identifier NCT03104426.
The primary hypothesis is that treatment with EPO will reduce the number of postnatal transfusions per infant in a population of severe HDFN, which is defined by the need for intrauterine transfusion. Recruitment of patients started in October 2017.

2. Background

The mainstay of antenatal treatment of fetal anemia due to red cell alloimmunization is (serial) IUT.[1,2] The mainstay of postnatal treatment in HDFN is (1) intensive phototherapy and, if necessary, exchange transfusion to treat hyperbilirubinemia and prevent kernicterus, and (2) postnatal transfusions to treat anemia.[3] Up to 80% of infants with HDFN treated with IUT require at least one postnatal transfusion for anemia during the first 3 months of life, with a median of two postnatal transfusions involving two hospital admissions per infant.[3-5] Postnatal anemia can be divided in early (up to 7 days of life) due to ongoing hemolysis and late anemia (7 days - 3 months of age).
Two mechanisms are attributed late anemia: “hypo-regenerative anemia” characterized by depressed erythropoiesis[6] and “late anemia of hemolytic disease” caused by persisting antibodies, destroying young erythrocytes.[4,7] “Hypo-regenerative anemia” occurs in particular in neonates treated with several IUTs as a result of bone marrow suppression.[4,6] Other contributing factors for late anemia have been reported such as severity of HDFN, reduced use of exchange transfusions during the neonatal period and reduced survival of transfused red blood cells.[3] Finally, erythropoietin deficiency is also considered as a possible contributing factor to late anemia.[8-13]
Erythropoietin (EPO) has been increasingly used in full-term and particular in preterm neonates to prevent or reduce neonatal anemia without short or long-term adverse effects.[12,14-16] Several small studies and casuistic reports suggest that neonates with HDFN may benefit from treatment with EPO to reduce the risk of delayed anemia and subsequent transfusions.[8-13] However, other authors found that EPO may be less effective than expected.[17] Due to the lack of evidence, routine use of EPO is currently not recommended.[3] To determine a role for EPO in this group of patients, a well-designed randomized controlled clinical trial of sufficient sample size is required.[13] Potentially, EPO stabilizes the hemoglobin levels of these infants and thus prevents anemia, hospital admissions for

transfusions and potential transfusion reactions, creating a more stable and natural postnatal course for patients with HDFN. In this scenario, the current management of weekly out-patient visits and weekly blood draws for hemoglobin level measurements, may be reduced, further contributing to reduction of the burden for these infants.

3. Objective

The primary objective of this study is to investigate whether darbepoetin alfa is effective in reducing the incidence of late anemia in infants with HDFN treated with IUT and therefore in decreasing the number of postnatal transfusions per infant, compared to a control group of infants receiving standard care without darbepoetin alfa treatment.

4. Methods

4.1 Study population

All (near) term neonates (gestational age ≥ 35 weeks) with HDFN (due to D, C, c, E, Kell or other red blood cell alloimmunization) treated with IUT and admitted to the Leiden University Medical Center (LUMC) from May 2017 are eligible for the study. The LUMC is the single national referral center in the Netherlands for pregnancies complicated by maternal red blood cell alloimmunization. A prenatal national screening program in the Netherlands indicates referral to the LUMC in case of elevated antibody titers tested in maternal serum ≥1/2 in Kell immunization and ≥1/16 in D or other types of alloimmunization or in case of an elevated ADCC (antibody-dependent cell-mediated cytotoxicity) assay ≥ 50% in case of D immunization ≥ 30% in case of other blood group antigens. These pregnancies are monitored by serial Doppler measurements to assess the velocity of the middle cerebral artery (MCA), which is considered the most accurate non-invasive predictor of fetal anemia. If MCA Doppler exceeds 1.5 multiples of the median (MoM) or if signs of hydrops are present, treatment with IUT is indicated. Intrauterine transfusion is usually continued until 34-35 weeks gestation to progress these pregnancies to term.[18] In general, labour and admission of the neonate to the Neonatology department of the LUMC is highly recommended in these pregnancies. Approximately 15 eligible patients are treated in the LUMC annually.

Postnatal anemia in this study is defined as a need for one or more erythrocyte transfusions. The LUMC transfusion guideline recommends a postnatal transfusion of 15 ml/kg irradiated erythrocytes in full-term neonates with HDFN when hemoglobin levels fall below 10.5 g/dL (6.5 mmol/L, day 0-6), below 8.9 g/dL (5.5 mmol/L, day 7-13) and below 7.2 g/dL (4.5 mmol/L, from day 14). These cut-off

values are communicated with referral hospitals after discharge from the LUMC and the research team is in contact with these hospitals to ensure transfusions are given according to these cut-off values.

4.1.1 Inclusion and exclusion criteria

Inclusion criteria:

- Gestational age ≥ 35 weeks;
- Treatment with IUT only due red blood cell alloimmunization;
- Birth at the LUMC.

Exclusion criteria:

- Early onset proven neonatal sepsis.

4.2 Study design

Randomized controlled trial without placebo. Included neonates will be randomized at birth to treatment with EPO (intervention group) or "standard care", with 1:1 allocation, to be randomized in varying blocks of 4 and 6, no stratification is applied. In the treatment group, EPO (darbepoetin alfa) is administered subcutaneously at a dosage of 10 µg/kg once a week, starting at approximately day 7, for a period of 8 weeks. Treatment is administered during weekly home visits in the treatment arm after discharge from the LUMC. Concomitant therapy with folate (0.25 mg/day) is given in both groups (standard practice). Concomitant iron therapy is given if ferritin level drops below 75 microg/l (standard practice). Weekly routine measurements of complete blood counts (including hemoglobin level, hematocrit and reticulocyte count) will be performed in both groups (standard practice). EPO is discontinued if hemoglobin level is ≥ 13 g/dL after at least 4 weeks of treatment with EPO. In EPO treated infants, blood pressure will be measured for safety reasons at onset of treatment, after four weeks and eight weeks. Monthly measurements of liver enzymes (aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), gamma-glutamyl transferase (γGT) and lactate dehydrogenase (LDH)) will also be performed in all groups (standard practice). In addition, the neonatal EPO-level will be determined at birth in both groups. The number of postnatal transfusions received during the first 3 months of life and hemoglobin levels prior to the postnatal transfusion are recorded. After initial discharge from the LUMC, postnatal transfusions are recommended to be performed when hemoglobin levels fall below aforementioned cut-off values of hemoglobin.

Because of these clearly defined guideline and hemoglobin measures, blinding of caregivers to treatment allocation and use of a placebo was not deemed necessary.
Collected data, including history of pregnancy, neonatal clinical records from the initial hospitalization at the LUMC and records from additional admissions and outpatient clinic visits in other hospitals than the LUMC, were collected for each included patient with written consent of parents or caregivers. Data was upon collection blinded and entered into an online secured database (CASTOR) by a member of the research team with in this database blinding of treatment allocation. The datamanager and statistician involved are blinded as well. After all data have been collected and entered in the database, and after data cleaning and finalization of the statistical analysis plan, the database will be locked and the data will be unblinded to all parties involved.

4.2.1 Data safety monitoring board

A data safety monitoring board (DSMB) is installed to review the trial's progress including data quality and safety data. No interim review regarding efficacy will be performed and therefore the efficacy data can remain blinded throughout the course of the study. Safety data, consisting of serious adverse events, adverse events and suspected unexpected serious adverse events are reported directly from the researchers to the DSMB and will be unblinded. Recruitment data (e.g. data return rates, treatment compliance) will be provided to the DSMB by the research team.
The DSMB will meet at least once a year. An extra meeting may be scheduled on request of the principal investigators, study coordinator or of the members of the DSMB.

4.3 End points

Primary end point:

- Number of postnatal transfusions per infant from birth up to 3 months of life.

Secondary end points:

- Number of postnatal transfusions per infant ≥7 days up to 3 months of life.
- The percentage of infants requiring a postnatal transfusion up to 3 months of life;
- Time from birth to first postnatal transfusion (days);
- Hemoglobin level at first postnatal transfusion (g/dl);
- Number of days of hospitalization and readmission(s) associated with erythrocyte transfusion(s);
- Course of hemoglobin up to 3 months of life.

Safety end points:

- The percentage of infants with a systolic blood pressure ≥ 2 SD above age adjusted mean systolic blood pressure during treatment;
- The percentage of infants with ferritin levels >200 µg/L during treatment.

4.4 Sample size calculation

Based on the (scarce) results in the literature, we expect a 50% reduction in the median number of total postnatal transfusions per patient with EPO treatment, from a median of 2 to 1. For sample size calculation we hypothesized a shift in the distribution of number of transfusions per infant as depicted in Figure 1. The distribution in the 'care as usual' group are based on data from 2000-2014. Based on these expected frequencies, group sample sizes of 21 achieve 81% power to detect a difference of 1.1 between the null hypothesis that both group means are 1.9 and the alternative hypothesis that the mean of the EPO group is 0.8 with estimated group standard deviations of 1.5 and 0.9 and with a significance level (alpha) of 0.05 using a two-sided Mann-Whitney test. The drop-out percentage is estimated at 5%, adding (42/0.95 = 44) 1 infant to each group's sample size.

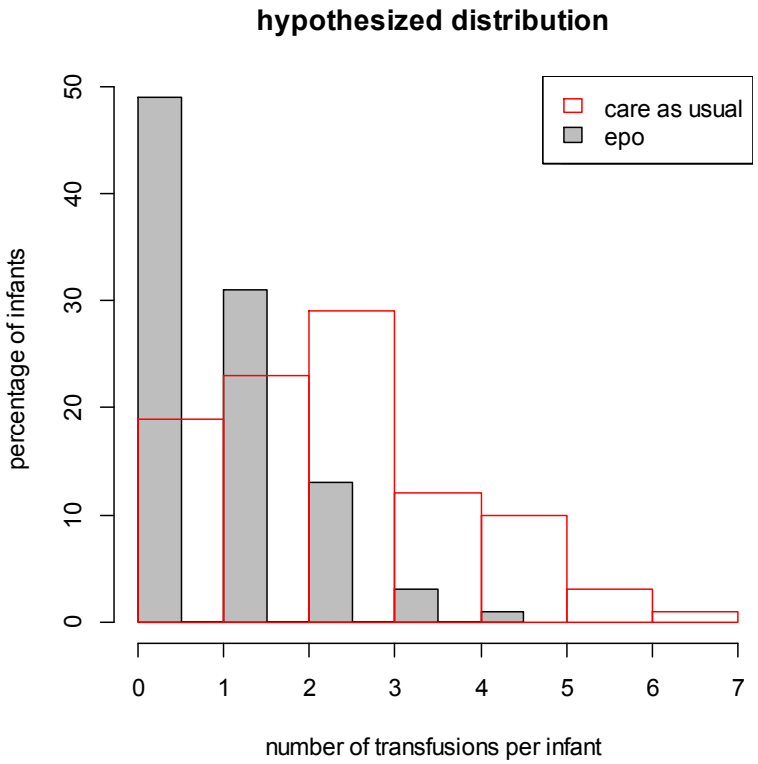

Figure 1. Hypothesized distribution of number of postnatal transfusions

5. Statistical analyses

5.1 Analysis sets

If after randomization parents or caregivers choose to withdraw the infant from participation before day 7, the patient will be replaced and its data excluded from all analyses.
Both intention-to-treat (ITT) and per protocol (PP) analysis will be carried out to study the differences in all outcome measures between the intervention and control group and will be presented. Intention to treat is our primary choice of analysis. The ITT analysis set consists of all randomized patients who did not withdraw before day 7, classified according to allocated treatment regardless of whether they received the assigned treatment and or any protocol deviations. Patients with major protocol deviations will be excluded from the PP analysis set. For patients assigned to the treatment arm, receiving less than 4 out of 8 darbepoetin injections is considered a major protocol violation. Furthermore, in the PP analysis patients will be classified according to treatment assignment.

If the difference between the ITT and PP analysis set is <10%, no per protocol analysis will be performed.

5.2 Analysis of primary and secondary outcomes

The total number of postnatal transfusions per infant from birth to the end of the follow-up period, the number of postnatal transfusions per infant ≥7 days (which is at the start of intervention in the treatment group) and the number of days of additional admissions for postnatal erythrocyte transfusions will be compared between both groups using a Mann-Whitney U test. The time from birth to first postnatal transfusion will be compared between both groups using a log rank test and will be displayed using Kaplan-Meier survival curves (in this case transfusion-free survival) by treatment group. The period of follow-up in this study is 90 days and as such the end point for included infants that do not receive transfusions during this period, is 90 days in the survival analysis. The percentage of infants requiring a postnatal transfusion up to 3 months of life will be compared between both groups using a $\chi 2$ test. The average or median number, depending on shape of distribution, of EPO injections in the treatment arm will be reported, as well as a summary of reasons to discontinue EPO treatment.

A multiple linear regression analysis will be performed to correct the association between treatment assignment and the primary outcome (number of postnatal transfusions). The following potential

confounders identified from previous research [unpublished data] are to be corrected for if, despite randomization, there is a baseline difference between the groups: type of alloimmunization, EPO-levels at birth, number of IUTs and treatment with exchange transfusion. In case of a non-normal distribution of the number of postnatal transfusions per neonate, log transformation will be applied. If the assumptions underlying linear regression are violated, or if the value zero occurs often, a Poisson regression will be fitted. If the number of postnatal transfusions per infant <7 days of life shows a difference between the two groups, a sensitivity analysis will be performed by performing the multiple linear regression analysis (or Poisson regression) with and without the transfusions administered <7 days. This will differentiate between the clinical course of early and late anemia and will evaluate more specifically the effect of EPO treatment, which is started at 7 days of age.

A linear mixed model will be fitted to assess the course of hemoglobin in the treatment and control group which will be graphically depicted. Each infant has repeated weekly hemoglobin measurements as advised in the protocol, but as the frequency of measurements is not mandatory, the data may show missing values at different time points. These missing values are expected to be at random and not completely at random, as it can be anticipated that a measurement is omitted after a previous positive (high) hemoglobin measure which justifies a longer interval between measurements due to the low risk of sudden anemia warranting transfusion. The course of hemoglobin in both groups is expected to have a negative slope after birth due to ongoing hemolysis and a physiological decline of fetal hemoglobin, which will stabilize over the course of several weeks and then show a positive slope as normal physiology recovers or is artificially raised with postnatal transfusions.

To account for the correlation between repeated measurements on the same subject, a random intercept will be included and, if model fit improves, a random slope in the model. The fixed effects will be treatment, time (with a non-linear functional form that will later be determined to match the shape of the expected de- and increase described above) and the interaction treatment*time. The primary assessment of whether the two groups differ in their hemoglobin course over time will be based on a test on the interaction treatment*time (a test which will correspond with the most suitable functional form).

Throughout the planned analyses, a p-value below 0.05 is regarded as statistically significant. Statistical analysis will be performed using IBM SPSS Statistics 23.0 (Chicago, Illinois, USA).

5.1 Figures and tables

Figure 1. Flowchart of study participants

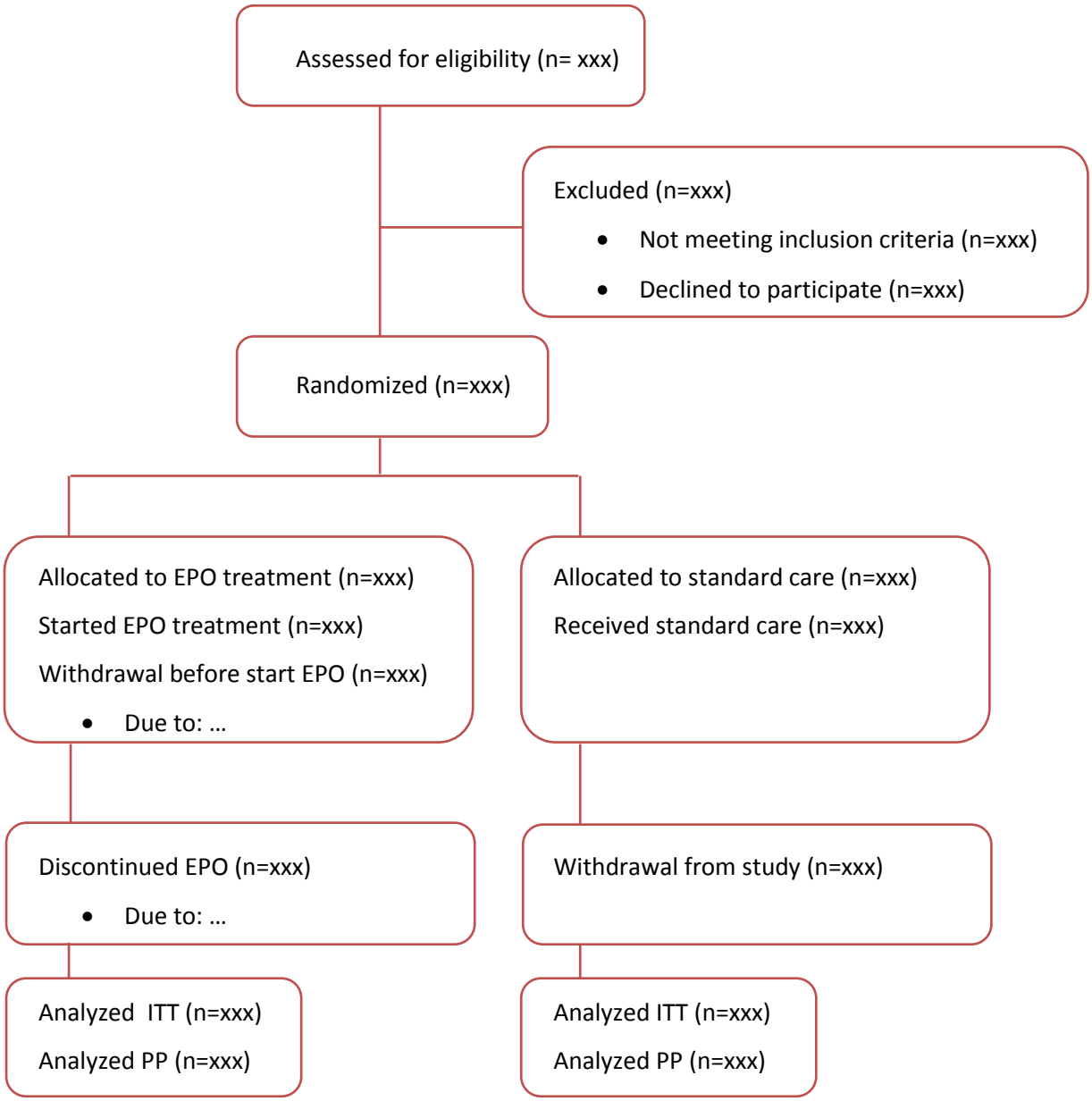

Figure 2. Kaplan Meier survival curves for time to first postnatal transfusion by treatment group

Figure 3. Hemoglobin course over time by treatment group

Table 1. Baseline demographic and clinical characteristics by treatment group

	EPO treatment (n=22)	**Standard care** (n=22)
Male - n (%)		
Cesarean delivery - n (%)		
Gestational age at birth - weeks		
Number of IUTs per neonate		
Gestational age at first IUT - weeks		
Hemoglobin level at first IUT - g/dl		
Birth weight - gram		
Hemoglobin level at birth - g/dl		
Reticulocyte count at birth - ‰		
Endogenous erythropoietin at birth - U/L		
Apgar score at 5 minutes		
RhD alloimmunization - n (%)		
Rhc alloimmunization - n (%)		
Kell alloimmunization - n (%)		
Phototherapy - days		
Treatment with exchange transfusions - n (%)		
Maximum bilirubin - mg/dl		

[a] Value given as mean ± SD

[b] Value given as median (IQR)

Table 2. Postnatal transfusions and safety measures in EPO treated infants and infants treated according to standard care

	EPO treatment (n=22)	**Standard care** (n=22)	**p-value**
Total number of RBC transfusions per neonate			
Number of RBC transfusions per neonate ≥7 days			
Treatment with postnatal RBC transfusion - n (%)			
Number of days before first transfusion			[c]
Hemoglobin level at first transfusion - g/dl		-	
Initial hospitalization - days			
Additional hospitalization - days			
Infants with systolic blood pressure ≥ 2 SD above age adjusted mean during treatment - n (%)		-	
Infants with ferritin levels >200 µg/L during treatment - n (%)		-	

[a] Value given as mean ± SD

[b] Value given as median (IQR)

[c] Log rank test

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