Official Title: **Red Blood Cell Survival Following Transfusion in Infants**

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This large variability between infants is also observed in other important metrics of EPO responsiveness. For example, we calculated the ratio (Hb/AUC) of the amount of Hb produced by each infant over 30 days (a cumulative erythropoiesis indicator) to the area under curve of endogenous Epo plasma concentration (a metric of cumulative EPO stimulation). **Fig 14** depicts a plot of that ratio against infant subject number (assigned by order of entry into the study) and confirms that there is considerable variation in Epo responsiveness among individual infants.

C.4.i. Robustness of Kinetic Estimations

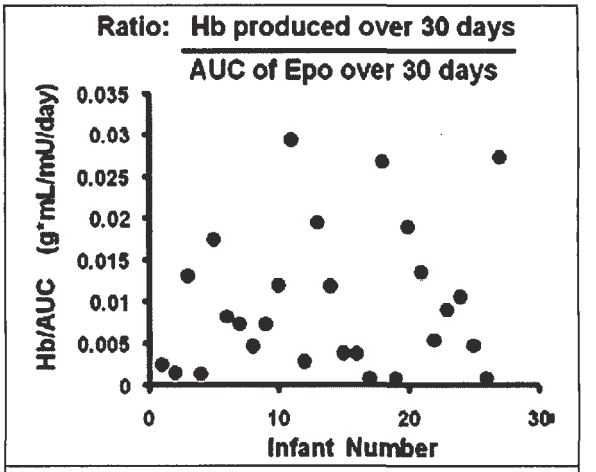

Fig 14. Large intersubject variability was observed in the ratio of the amount of Hb produced over the first 30 d of life (a cumulative indicator of erythropoiesis) divided by the integrated area under the curve of the endogenous Epo plasma concentration (a metric of cumulative EPO stimulation) ("Hb/AUC") for the 27 VLBW infants Project 3□

Robustness of Pharmacodynamic Parameter Estimations. Reviewers expressed concern about the robustness of the essential parameters of model, particularly of the transduction functions. This is an understandable concern because accuracy of prediction of Epo responsiveness depends on the robustness of the model. The details and derivation of the PD model used to predict Epo responsiveness have been previously described[3]. To determine the robustness of the individual subject parameter estimates, the mean percent standard error (MSE%) of the estimate was calculated for each parameter. The parameters were well estimated with a MSE% of <15% for all except the two PD transduction function parameters: E_{MAX} (the maximum Hb production rate) and EC_{50} (the plasma EPO concentration that results in 50% of E_{MAX}). Large variations in MSE% for EC_{50} and E_{MAX} are commonly seen in PD analysis, but this degree of variability is not detrimental to the analysis because it is their ratio (E_{MAX}/EC_{50}) which best summarizes the PD transduction curve with respect to predictions. Although the MSE% for the ratio E_{MAX}/EC_{50} was somewhat high (MSE%=123%), this was due to a single outlier subject who showed very little nonlinearity in the PD transduction function (MSE%=1300%). Calculation of the MSE% for E_{MAX}/EC_{50} without this subject results in a MSE% of only 24.6%, indicating an excellent robustness in the computer estimations. A similarly, excellent estimation of robustness was found for $1/EC_{50}$ (MSE% = 33.6%).

Robustness of Hb production Estimation. To demonstrate the robustness of our PD model's estimates of the amount of Hb produced, we compared the model results to those obtained by a nonparametric estimation of the amount of Hb produced. The nonparametric estimate of Hb produced calculated the difference between the amount of Hb transfused and the amount of Hb removed (by phlebotomy and due to RBC aging) but made no assumptions about the mechanism of the Hb production other than that the RBC in the VLBW infants had a fixed lifespan of 42.5 days [11]. A paired *t* test comparing both estimates found no significant difference ($p>0.05$), supporting the robustness of the Hb production estimation determined by our PD model.

Additional Support Our Assertion re. Robustness in Kinetic Estimations. Besides mathematical modeling, another indication of the utility of the model is the ability to identify covariates that predict Epo responsiveness that seem reasonable in the context of existing studies. We have thus far identified 5 clinical covariates indicating a statistically significant difference between the predicted 14 good and 13 poor Epo responders (**Table 3**). Each makes sense in the context of existing clinical studies. ***The detection of these statistically significant covariates gives further indirect evidence for the robustness of our estimation of the PD parameters and the Hb production. More importantly, it supports our assertion that computer estimations and simulations demonstrate the feasibility of our proposed study and points to a successful outcome.***

C.4.j. Application of Alternative Approaches

To realize the benefits of a personalized approach to eliminate RBCTX in VLBW, a practical, alternative modeling approach for identifying good Epo responders will also be applied [7]. This approach makes use of RBC and reticulocyte indices readily available in all NICUs and considers the physiological and pathological population dynamics of circulating human RBCs and provides an exciting opportunity for predictions that we will explore further. This new approach was derived from the theory of statistical physics and applied to hospital laboratory data. It provides a master equation model for RBC maturation and clearance. This new methodology showed a striking utility of identifying many pre-anemic patients weeks before anemia became clinically

detectable. Predictions were based on data from standard clinical CBC parameters—RBC volume and Hb content—and were done from a joint probability distribution function. The equation derived dealing with the non-equilibrium statistical dynamics of the joint probability distribution function of the RBCs provides a "snapshot" evaluation of the RBC pathophysiology with demonstrated predictive value. The study is not powered to detect significance for any of the secondary outcomes, eg, genetic markers and individual cytokines and biomarkers.

C.5. Specific Aim 1 (Infant Study 1)

Aim: Determine clinical and laboratory covariates (ie, patient-specific characteristics) controlling the large inter-subject variability in Epo's PD using data from Epo dosing of VLBW infants

Hypothesis: The inter-subject variability in Epo's PD is predictable by several covariates that are identifiable using our modeling approach

C.5.a. Feasibility

In our preliminary study we identified 5 covariates that exhibited a statistically significant difference between the 14 infants predicted to be good Epo responders and the 13 infants predicted to be poor Epo responders (**Table 3**). The identification of these covariates supports the robustness of our estimation of the PD parameters (transduction function parameters) and other parameters used in predicting Epo responsiveness.

C.5.b. Rationale

In Specific Aim 1 we propose that a large part of the observed PD variability is not random but explainable. We further propose that, among the many laboratory (see **Table 1**) and clinical factors that will be systematically quantitated in Infant Study 1, we will identify covariates that are associated with much of this variability.

C.5.c. Research Design

We will address Specific Aim 1 in three steps. Step 1: Individual transduction functions will be constructed and the related PD parameters determined in all infants based on the Epo levels that result from the optimized dosing regimen and the Hb produced (determined by mass balance). The parameter estimation will be done using the modeling approach described previously [3]. Step 2. Infants will be separated into two groups of about equal size based on high and low PD efficacy (PD efficacy is the area under the PD transduction curve, **Fig 12**). Creating two groups of equal size is supported by our computer modeling of data from the first 27 infants indicating that about 50 percent of the infants will be good Epo responders. Step 3. A statistical comparison (*t* test) of covariate candidates for the two groups of infants will be conducted. Covariates showing a significant difference ($p<0.05$) will be included in the final group of covariates incorporated into the population PK/PD model developed at the end of Infant Study 1 (see Specific Aim 3).

C.5.d. Sample size

The sample size needed for identifying covariates of Epo responsiveness was determined using the sample size calculation for logistic regression by Hsieh et al [13]. In a 2-step process: 1) sample size was calculated for simple bi-variate tests (*t*-test for continuous variables; Pearson chi-square for proportions); and 2) an upward adjustment by a variance inflation factor (VIF) based on covariate correlations was then applied. Assuming 50% poor and 50% good Epo responders and a multiple correlation $r^2= 0.3$ among the covariates (or VIF=1.43) based on the data from our pilot study of 27 babies, a total sample size of n=100 infants will be needed to detect an effect size of 0.85 SD in the difference of the continuous covariate mean between good and poor Epo responders at the 0.01 significance level with 0.80 power. Using the SD estimates from our pilot study of 27 babies, the detectable mean differences between predicted good and poor Epo responders for covariates already identified (**Table 3**) are close to or smaller than the difference seen in our pilot study, providing evidence of the feasibility of our experimental design and sample size estimate.

Table 3: Covariate predictors of good & poor Epo responders	**SD**	**Detectable difference**	**Observed difference**
Birth Wt *	0.25	0.21	0.30
Gestational age *	1.29	1.08	1.50
Mean corpuscular Hb (MCH) *	32.6	27.4	30.0
First 24 hr phlebotomy loss *	4.70	3.95	5.40
Mean arterial blood pressure *	6.17	5.18	7.30
* These statistically significant covariates were obtained by testing for group differences while considering several covariate candidates in the predicted 14 good and 13 poor Epo responders (see **Fig 7**).			

C.5.e. Expected outcome

Among the covariates considered in Specific Aim 1 (eg, clinical neonatal/maternal factors, blood cell parameters, cytokines linked to erythropoiesis, inflammation biomarkers, oxidative stress, iron status, and genetic factors), we will identify several that correlate significantly with Epo PD.

C.5.f. Potential problems

Because we have already demonstrated that we can recruit and perform all the procedures planned (eg, meticulously control the data acquisition procedures for Hb mass balance, complete infant demographics, etc.), we do not expect any problems at the UI site. However, the reviewers correctly point out that the University of Minnesota (UM) group has less experience with these demanding studies. We were remiss in not clarifying previously that our recruiting plans incorporated a 6-month period of cross training with the UM team prior to enrolling the first University of Minnesota infant in Infant Study 1.

C.6. Specific Aim 2 (Infant Study 1)

Aim: Determine the lifespan of fetal RBC in the gestational age spectrum of the study infants

Hypothesis: There exists a significant inter-subject variability in the lifespan of fetal RBCs in VLBW infants that is predictable based on gestational age

C.6.a. Justification

Net Epo responsiveness as reflected in Hb level depends on two components: Epo PD and RBC lifespan (**Fig 15**). By determining RBC lifespan, we will explain inter-subject variability of Epo responsiveness resulting from one of these components. Studies of ovine fetal RBC lifespan have shown a linear correlation to gestational age [14].

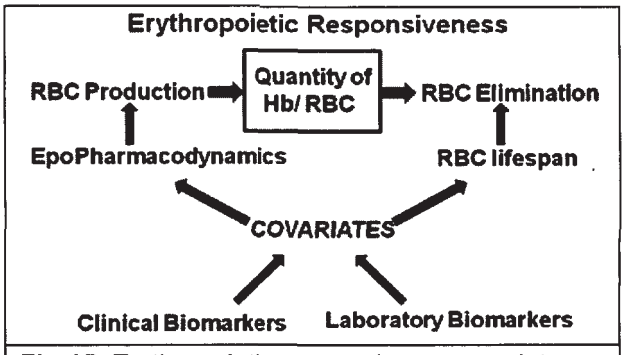

Fig 15. Erythropoietic responsiveness as determine by determination of Epo PD and RBC lifespan

C.6.b. Feasibility

RBC lifespan will be determined using the BioRBC method already developed in current PPG.

C.6.c. Research Design

We will address Specific Aim 2 in four steps. Step 1: Individual fetal RBC lifespans will be determined in 30 VLBW infants enrolled at UI for the RBC lifespan study. Step 2: The fetal lifespan data will be examined for its correlation with gestational age. If the correlation is statistically significant, gestational age will be included in the final selection of covariates for the population PK/PD model to be developed at the end of Infant Study 1 as described under Specific Aim 3. Step 3. Infants will be separated into two groups based on the median fetal RBC lifespan (use of the median to separate the two groups is based on our computer predictions of Epo responsiveness indicating that about 50 percent of the infants will be good Epo responders). Step 4. A statistical comparison (t-test) of covariate candidates for the two groups of infants will be conducted. Covariates showing a significant group difference ($p<0.05$) will be included in the final selection of covariates as described under Specific Aim 3. Steps 3 and 4 are included to look for any additional covariates in addition to gestational age that correlate with fetal RBC lifespan.

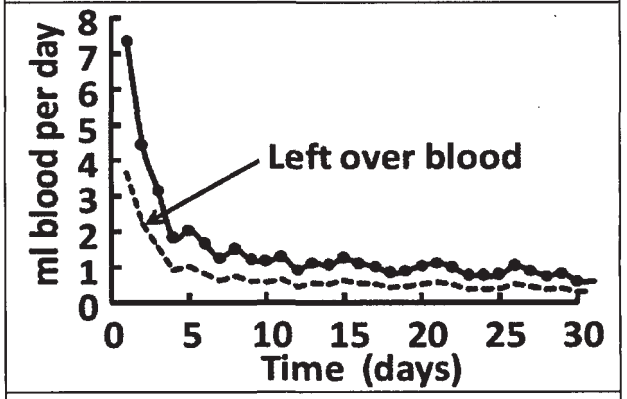

Fig 16. Blood loss for clinical testing averaged over first 30 d of life for 27 VLBW Project 3 infants. With help from the NICU clinical lab staff >95% of blood samples were retrieved, weighed and available for research analysis (see "Human Subjects" p 210).

C.6.d. BioRBC procedure

The description and reference to publications giving details of the biotinylated RBC (BioRBC) method for determining the RBC lifespan are presented in Core B, "C.1.a. Biotin-Labeled RBCs for RCV and RCS," p 403.

C.6.e. Blood required for assays

In our current Project 3 study, an average of 740 µL of discarded waste blood was left over per day (**Fig 8** and **Fig 16**). Since <20 µL of blood is needed for the each determination of BioRBC survival and only 250-350 µL is needed for the biomarkers (**Table 1**), there is sufficient blood for determination of autologous BioRBCs, enabling accurate lifespan determinations.

C.6.f. Expected Outcome

The relationship between lifespan of fetal RBCs and gestational age will be similar to that which we observed in the ovine fetus.

C.6.g. Sample size

Considering the ovine fetus to be a good model for human fetal RBC lifespan, we used results from our ovine fetal study [14] (RBC life span in days, lifespan SD=5.8 d and correlation r=0.83 between lifespan and gestational age) in the human infant sample size calculation. From our pilot study of 27 babies, we assumed gestational age SD was 1.3 d. At the 0.05 significance level with 0.80 power, a sample size of n=30 babies will be needed to detect a slope of at least 1.32 in the linear regression of RBC lifespan versus gestational age. This is similar to the RBC lifespan versus gestational age regression slope of 1.37 in the ovine fetus.

C.6.h. Potential Problems

Departure from prior steady-state (SS) condition. Lifespan determination assessed by labeling a population of RBCs and tracking the label disappearance requires an assumption about the rate of RBC production *prior* to the labeling. Most commonly a prior steady-state (SS) condition is assumed. However, this may not be a good approximation for fetal RBCs. **Alternative strategies to solve problem:** The prior production rate can be estimated, assuming each RBC has the same fixed lifespan ("point distribution" assumption).

C.7. Specific Aim 3 (Infant Study 1)

Aim: Derive an individualized, optimal Epo dosing algorithm and an individualized prediction model for the Epo responsiveness in VLBW infants from the results in Specific Aims 1 and 2

Hypothesis: Cross validation-type computer simulations based on individualized and optimized Epo dosing in a subgroup of VLBW infants with good Epo responsiveness, identified by the prediction model, will indicate that RBCTX can be avoided in a select group of VLBW infants

C.7.a. Justification

Due to the large inter-individual variability in Epo responsiveness it is critical to develop an individualized Epo responsiveness prediction model and optimization algorithm for Epo dosing to achieve the objective of eliminating the need for RBCTX in a select group of VLBW infants.

C.7.b. Feasibility

Our preliminary computer analysis has shown a statistically significant correlation between predicted Epo responsiveness in our 27 VLBW infants and several covariates (**Table 3**).

C.7.c. Research Design

Epo Responsiveness Prediction Mode Development. The research design for Specific Aims 1 and 2 described above will identify covariates for Epo PD and fetal RBC lifespan through correlation analysis. These are the major determinates of Epo responsiveness and, as such, are the starting point for the population PK/PD model to be developed under SA 3. To produce the expanded prediction model presented schematically in **Fig 10,** relevant covariate candidates will be identified and included in the model. In this final covariate selection and model building, we will apply advanced, state-of-the-art analysis procedures developed and used successfully in population kinetic modeling [15-20]. The basis of this approach is described in "General Program Introduction" section B.6.c. on p 130. Incorporating the most promising covariate candidates, we will apply a multivariate regression analysis using a generalized additive model (GAM [20, 21]). In addition, we will apply a full set, step-down variable reduction technique in the GAM analysis together with bootstrap tree-based modeling using Xpose which is a S-PLUS based covariate model building aid for the powerful PK/PD software program, NONMEM [21].

Epo Dosing Algorithm Development. The development of the dosing optimization algorithm will be analogous to that described under "Epo dosing algorithm (Infant Study 1)" except that the PK/PD model will incorporate covariates selected in Specific Aim 3 to provide *individualized* dosing for improved dosing efficacy.

C.7.d. *Expected Outcome.* The model will accurately predict individual Epo responsiveness to optimized Epo dosing and will provide strong support for our hypothesis that RBCTX can be completely eliminated in a select group of VLBW infants.

C.7.e. Sample size

Since in Aim 3 only computer simulations based on the fitted model will be applied (no new data will be utilized), there is no rationale for performing a sample size estimate.