Title: Erythropoietic response to endogenous erythropoietin in premature very low birth weight infants†

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d) **Nonstandard abbreviations:**

\(a\)  time between production of progenitor cells to release of subsequently produced RBC into the circulation

AIC  Akaike’s information criterion

\(b\)  time between production of progenitor cells and age-related death of subsequently produced RBC

CBC  complete blood count

\(C_{EPO}\)  plasma EPO concentration

\(E_{max}\)  maximum bodyweight scaled Hb production rate

EPO  erythropoietin
\( f_{\text{prod}}^{\text{ex utero}}(t) \) post-birth \((ex \ utero)\) bodyweight scaled Hb production rate

\( f_{\text{prod}}^{\text{total}}(t) \) total body Hb production rate

\( f_{\text{trans}}(t) \) function proportional to rate of Hb loss of transfused adult Hb

\( F_{Pj} \) fraction of Hb remaining immediately after the \( j^{th} \) phlebotomy

\( F_T \) fraction of transfused Hb surviving immediately beyond the time of transfusion

\( Hb_{\text{in vivo}}(t) \) amount of Hb present in the circulation that was produced \( in \ vivo \)

\( Hb_{\text{total}}(t) \) total amount of Hb present in the circulation

\( Hb_{\text{trans}}(t) \) amount of Hb present in the circulation that was transfused

\( Hb_{Pj} \) amount of Hb removed by the \( j^{th} \) phlebotomy

\( Hb_{Ti} \) amount of Hb administered at the \( i^{th} \) transfusion

Hct hematocrit

\( k_{\text{prod}}^{\text{in utero}} \) pre-birth \((in \ utero)\) bodyweight scaled Hb production rate constant

\( L_{\text{in vivo}} \) RBC lifespan of infant cells produced \( in \ vivo \)

\( L_{\text{trans}} \) RBC lifespan of transfused cells

\( MCH_{\text{in vivo}} \) mean corpuscular Hb of infant cells produced \( in \ vivo \)

\( MCH_{\text{trans}} \) mean corpuscular Hb of transfused cells

\( m(t) \) bodyweight mass over time

MSE\% mean percent standard error of the estimate

NICU neonatal intensive care unit

PD pharmacodynamic
RBC  red blood cell
rHuEPO recombinant human erythropoietin
t  time relative to birth
$T_{Pj}$ time of the $j^{th}$ phlebotomy
$T_{Ti}$ time of the $i^{th}$ transfusion
$V_N$ bodyweight mass normalized blood volume
$V_{total}(t)$ total blood volume
VLBW very low birth weight

e) **Recommended section assignment:** Other
Abstract

Despite the common occurrence of anemia in very low birth weight (VLBW) infants, the erythropoiesis and Hb production rates and their relationship to plasma erythropoietin (EPO) concentrations remain unknown in these subjects. To determine these quantities, all blood removed by phlebotomy and administered by red blood cell (RBC) transfusion over the first 30 days of life was recorded in 14 ventilated VLBW infants born at 24-28 weeks of gestation. Discarded blood from frequent clinically ordered laboratory blood samples was used to construct plasma EPO, Hb, and RBC concentration-time profiles for each infant. A pharmacodynamic Hb mass balance model that accounted for the dynamic hematological conditions experienced by these infants was simultaneously fitted to the plasma EPO, Hb, and RBC concentrations from each individual subject, while accounting for subject growth. Based on the model estimates an average of 4.69 g of Hb were produced over the first 30 days of life, compared to 5.97 g removed by phlebotomies and 12.3 g administered by transfusions. These high transfusion amounts were consistent with a relatively short RBC lifespan and rapidly expanding blood volume with infant growth. The estimated mean bodyweight-scaled Hb production rate dropped nearly 3-fold following birth to 0.144 g/day·(kg)³⁴. Though only estimated in a subset of the subjects, the mean plasma EPO $EC_{50}$ of 28.5 mU/mL and maximum Hb production rate ($E_{max}$) indicated that a several-fold increase in Hb production rate could be achieved with only a modest increase in plasma EPO concentrations.
Introduction

The anemia of prematurity occurs in all very low birth weight (VLBW) premature infants (birth weight < 1500 g) and is exacerbated by iatrogenic blood loss resulting from frequent laboratory blood sampling for managing clinical illness (Strauss, 1995; Lin et al., 2000; Madan et al., 2005; Widness et al., 2005). The resulting development of clinically significant anemia is managed by the administration of red blood cell (RBC) transfusions, creating dynamic changes in the hemoglobin (Hb) mass in these infants due to both the physical removal and administration of erythrocytes. In infants, as well as adults, RBCs are produced from erythroid progenitor cells located primarily in the bone marrow, though some residual erythropoiesis may still occur in the liver and spleen of preterm infants (Brugnara and Platt, 2003; Hoffman et al., 2005). The development and expansion of erythroid progenitor cells into mature RBCs is primarily controlled by erythropoietin (EPO), a 30.4 kD glycoprotein hormone produced in response to oxygen need by the pertibular cells of the kidney in the adult and possibly also the liver in preterm infants (Brown, 1988; Moritz et al., 1997; Brugnara and Platt, 2003; Hoffman et al., 2005). Additionally, under non-disease state conditions the mechanism of RBC death or removal from the circulation is primarily to due to cellular senescence (i.e. age related cell death) (Landaw, 1988).

Despite the common occurrence of anemia in VLBW infants, the erythropoiesis rate in these subjects remains unknown. This is largely due to the complications in determining the erythropoiesis rate caused by frequent phlebotomies and RBC transfusions altering the RBC/Hb mass, and the effect each phlebotomy on the RBC/Hb removal rate due to the lifespan based disposition of RBCs (Landaw, 1988; Freise et al., 2007; Freise et al., 2008b). Previous studies have demonstrated that on average 33.8 mL/kg of blood are removed and 27.0 mL/kg transfused
during the first 4 weeks of life in infants born at a gestational age of less than 28 weeks (Madsen et al., 2000) and other studies have reported even higher phlebotomy blood loss volumes in the first 2 weeks of life alone (Lin et al., 2000; Widness et al., 2005). Thus the effects of the physical removal and administration of RBCs are substantial and cannot be ignored. Other complications in determining the erythropoiesis rate in preterm VLBW infants include increases in total blood volume as it expands with infant growth and the mixture of endogenously produced RBCs and exogenously administered adult donor RBCs following transfusion, the former which generally have shorter lifespans (Brugnara and Platt, 2003; Strauss et al., 2004).

Knowledge of the in vivo erythropoiesis and Hb production rates and their relationship to plasma EPO concentrations in these infants would provide an understanding of these subject’s ability to compensate for phlebotomy blood loss, and thereby providing a reference for evaluating the potential improvement of erythropoiesis through administration of recombinant human EPO (rHuEPO) and other erythropoiesis stimulating agents. Since the administration of RBC transfusions carries infectious and non-infectious risks (Galel and Fontaine, 2006; Ohlsson and Aher, 2006), knowledge of the in vivo erythropoiesis rate is also important for assessing the potential therapeutic strategies to reduce or eliminate RBC transfusion risks. Thus the objective of the current study was to estimate the in vivo erythropoiesis and Hb production rates and their relationship to endogenous plasma EPO concentrations in preterm VLBW infants. To achieve this aim, the amount of Hb removed by phlebotomy and administered by RBC transfusion to 14 preterm VLBW infants were recorded and a pharmacodynamic (PD) Hb mass balance model formulated that accounts for the dynamic hematological conditions experienced by these infants. The model was subsequently fitted simultaneously to each infant’s observed endogenous plasma
EPO, Hb, RBC count, and bodyweight profiles over time to estimate the erythropoiesis rate and its relationship to endogenous plasma EPO concentrations.

Methods

Subjects. A consecutive sample of 14 inborn infants between 24 and 28 weeks of gestation being cared for in the Neonatal Intensive Care Unit (NICU) at the University of Iowa Children’s Hospital were enrolled between February 2007 and February 2008. Additional inclusion requirements were treatment with expectation of survival and moderate to severe respiratory distress requiring ventilation. Infants were excluded with: hematological disease (except for anemia associated with phlebotomy blood loss and prematurity), alloimmune hemolytic anemia, diffuse intravascular coagulation, transfusion requirements that were emergent which did not allow controlled sampling, or who received erythropoiesis stimulating agents. The study was approved by the University of Iowa Human Subject Internal Review Board and all procedures carried out in accordance with the Declaration of Helsinki. All subjects’ parents or legal guardians signed informed consent.

Study Procedures. Phlebotomy blood samples from study subjects were weighed (AE50 Mettler Balance Scale, Mettler-Toledo Inc, Columbus, OH) and recorded immediately after collection from birth through 30 to 37 days of life. The collection tube weights were subtracted from the samples and converted to a blood volume based on the specific gravity of blood of 1.05 g/mL (Trudnowski and Rico, 1974). If a phlebotomy blood sample was mistakenly not weighed (~4% of all samples), then the mean sample weight for the type of clinical test drawn was substituted. An additional 38 μL of blood was added to each phlebotomy collected by capillary heel stick to account for blood wiped away prior to collection and bleeding after collection. The
38 μL of blood was determined by weighing the volume of blood estimated to be equivalent (as determined by visual assessment) to the amount routinely removed from the skin prior to collection and loss after sample collection by capillary heel stick. Additionally, the same amount of blood was added to samples collected from indwelling arterial or venous lines due to loss by clearing of the catheter prior to collection. Analysis of catheter fluid used to clear the lines from a sample of infants indicated that from 10 to 114 μL (median of 38 μL) of blood was loss with each blood sample drawn from an indwelling catheter.

The Hb mass removed with each phlebotomy was calculated by multiplying the volume of blood removed by the Hb concentration measured in the blood sample drawn closest to the time of blood sampling. Concentrations of Hb and RBCs were measured from several sources including: clinically ordered blood gases and electrolytes (Hb only) using a Radiometer ABL 625 blood gas analyzer (Radiometer America, Inc, Westlake, OH), clinically ordered and study protocol driven complete blood counts (CBC) using an Advia 120 hematology system (Bayer, Tarrytown, NY), and excess blood recovered from other clinical tests using a Sysmex XE-2100 automatic hematology analyzer (Sysmex Corporation, Kobe, Japan) operated in normal (if sufficient blood volume (> 200 μL) was available) or capillary mode. If blood collected at the same time was measured by multiple instruments, the average Hb or RBC concentration from all instruments was used. We have previously demonstrated that Hb and RBC concentrations measured on recovered blood using the Sysmex XE-2100 is stable for up to 72 hours at 4°C or room temperature and unaffected by the use of EDTA or heparin as an anticoagulant or operation in capillary mode (Freise et al., 2008a). In addition to the Hb and RBC concentrations from clinically ordered tests, research blood samples were collected weekly and prior to and approximately daily after the first RBC transfusion for 10 days to ensure adequate sampling
density. However, if a CBC was ordered by the attending physician(s) on the corresponding
days, then research blood samples were not collected to avoid duplicate sample collection. The
total amount of blood removed by the research blood samples was limited to less than 1.6
mL/week/kg.

Plasma samples for EPO concentration determination were also collected by
centrifugation from excess blood recovered from the clinical tests and research samples
described above. If the plasma volume from the sample was insufficient to conduct a plasma
EPO determination, then the plasma was pooled with other samples within an approximately 8
hour time window. The “collection time” for these pooled plasma samples used for the data
analysis was the weighted average (based on the relative plasma volume contribution to the total
sample) of the collection times of the individual samples that made up the pooled samples.
Plasma EPO concentrations were measured using a double antibody radioimmunoassay (RIA)
procedure as previously described (lower limit of quantification 1mU/mL) (Widness et al.,

The volumes of packed RBCs (85% hematocrit (Hct)) administered by syringe (30 of 60
cc BD™ Syringe, Becton, Dickinson and Company, Franklin Lakes, NH) and infusion pump, as
well as the start and stop times of all RBC transfusions were recorded. The Hb mass
administered to individual infants was calculated based on the measured Hb concentrations of
the transfusate, or if not directly measured then based on a typical Hb concentration of 28.3
g/dL. The decision to treat an individual subject’s anemia by administration of RBC transfusions
was made by the physician responsible for the subject’s patient care according to NICU
guidelines (Strauss, 2008). From the known amounts of Hb removed by phlebotomy and
administered by transfusion to each infant and the observed Hb, RBC, plasma EPO
concentration, and bodyweight profiles over time, the erythropoiesis and Hb production rates and their relationship to endogenous plasma EPO concentrations were estimated using the PD Hb mass balance model described below.

**Pharmacodynamic Hb mass balance model.** The PD Hb mass balance model assumed that the behavior or disposition of the Hb and RBCs in the absence of phlebotomies was lifespan based (i.e. based on removal of RBCs from the circulation through cellular aging/senescence) (Landaw, 1988; Krzyzanski et al., 2006; Freise et al., 2008b). A time invariant “point distribution” (i.e. no variability) of RBC lifespans was assumed (Krzyzanski et al., 2006; Freise et al., 2007). The Hb mass or amount in the infants was modeled as the summation of two separate components, (1) Hb contained in RBCs produced by the infant *in vivo* (\(Hb_{\text{in vivo}}\)) and (2) Hb contained in transfused RBCs (\(Hb_{\text{trans}}\)).

The Hb production rate of the first Hb component, \(Hb_{\text{in vivo}}\), was assumed to be proportional to bodyweight or mass scaled to the ¾ power, as many metabolic processes are in physiology and pharmacokinetics (Savage et al., 2004; Meibohm et al., 2005; Anderson and Holford, 2009), to account for the changing body mass of the fetus/infant as the subject matured. Prior to birth, the bodyweight scaled Hb production rate was assumed to be constant and was denoted by \(k_{\text{prod}}^{\text{in utero}}\). While post-birth the body mass scaled Hb production rate, denoted by \(f_{\text{prod}}^{\text{ex utero}}(t)\), was assumed to be a function of time \(t\) through changes in the plasma EPO concentration \(C_{\text{EPO}}(t)\). Thus the total body Hb production rate \(f_{\text{prod}}^{\text{total}}(t)\) is a function of time through changes in both the body mass and the plasma EPO concentration over time as given by:

\[
f_{\text{prod}}^{\text{total}}(t) = f_{\text{prod}}(t) \cdot m(t)^{\frac{3}{4}} \tag{1}
\]

where \(m(t)\) is the body mass and:
\[ f_{\text{prod}}(t) = \begin{cases} L_{\text{prod}}^{\text{in utero}} & \text{if } t \leq 0 \\ f_{\text{prod}}^{\text{ex utero}}(C_{\text{EPO}}(t)) & \text{if } t > 0 \end{cases} \]

and \( t = 0 \) denotes the time of birth. A Michaelis-Menten or hyperbolic \( E_{\text{max}} \) model of plasma EPO concentration changes over time was used for \( f_{\text{prod}}^{\text{ex utero}}(t) \); however, due to the limited plasma EPO concentration range observed for many subjects, the model was parameterized as:

\[ f_{\text{prod}}^{\text{ex utero}}(t) = \frac{p_1 \cdot C_{\text{EPO}}(t)}{1 + p_2 \cdot C_{\text{EPO}}(t)} \]

such that \( p_1 = E_{\text{max}} / EC_{50} \) and \( p_2 = (EC_{50})^{-1} \), where \( E_{\text{max}} \) is the maximum bodyweight scaled Hb production rate and \( EC_{50} \) is the plasma EPO concentration that results in 50% of \( E_{\text{max}} \). This parameterization allows for the nonlinear production rate function given by Eq. 3 to reduce to a linear function by setting \( p_2 = 0 \) when only operating in the approximately linear range of the \( E_{\text{max}} \) model (i.e. when \( C_{\text{EPO}} << EC_{50} \), see Data analysis section below).

Let the time between production of progenitor cells outside the systemic circulation to release of the subsequently produced RBC into the circulation be denoted by \( a \) and the RBC lifespan of cells produced \( \text{in vivo} \) be denoted by \( L_{\text{in vivo}} \). Hence the time between production of progenitor cells and age-related death of RBCs, denoted by \( b \), is given by \( b = a + L_{\text{in vivo}} \). Then in the absence of any phlebotomies the rate of change in the Hb amount that was produced \( \text{in vivo} \) is given by (Freise et al., 2007):

\[ \frac{dHb_{\text{in vivo}}}{dt} \equiv Hb_{\text{in vivo}}'(t) = f_{\text{prod}}^{\text{total}}(t-a) - f_{\text{prod}}^{\text{total}}(t-b) \]

with initial conditions given by:
\[ Hb_{in\,vivo}(0) = \int_{t=a}^{t=b} f_{prod}^{total}(u) \, du \bigg|_{u=0}^{u=t} = \int_{0}^{a} f_{prod}^{total}(u) \, du \]

Thus the input rate \((f_{prod}^{total}(t-a))\) and the output rate \((f_{prod}^{total}(t-b))\) of the \textit{in vivo} Hb amounts are simply time shifted total body Hb production rates.

The second Hb mass component, the Hb from the RBC transfusions \((Hb_{trans})\), was accounted for through superposition by adding the Hb mass transfused at each transfusion and then accounting for a linear rate of decline of the transfused cells. The linear rate of decline arises from assuming a constant Hb production rate and a constant RBC lifespan in the RBC donor subjects. Thus in the absence of any phlebotomies:

\[
Hb_{trans}(t) = \begin{cases} 
Hb_{trans}(T_{i}) & \text{for } t = T_{i} \\
Hb_{trans}(t) + F_{T} \cdot Hb_{trans} & \text{for } t = T_{i} + \varepsilon
\end{cases} \quad i = 1 \text{ to } m \tag{5}
\]

where \(T_{i}\) is the time of the \(i^{th}\) transfusion, \(Hb_{trans}\) is the amount Hb administered at the \(i^{th}\) transfusion, \(\varepsilon\) denotes an infinitesimally small time increment, \(0 \leq F_{T} \leq 1\) is the fraction of transfused RBCs surviving immediately beyond the transfusion (e.g.
if a portion of the RBCs were damaged in storage and removed by the reticuloendothelial system shortly after transfusion), and \(m\) is the number of transfusions. Though all RBC transfusions were administered over a 3 to 4 hour time period, the effect of the transfusion on the Hb mass was approximated assuming that the cells were administered as a bolus. The linear rate of decline of the amount transfused Hb is given by:

\[
Hb_{trans}'(t) = -F_{T} \cdot \sum_{i=1}^{m} Hb_{trans} \cdot f_{trans}(t - T_{i}), \quad Hb_{trans}(0) = 0 \tag{6}
\]

with:
\[
f_{\text{trans}}(t) = \begin{cases} 
\frac{1}{L_{\text{trans}}} & \text{for } 0 \leq t < L_{\text{trans}} \\
0 & \text{otherwise}
\end{cases}
\]

where \( L_{\text{trans}} \) is the RBC lifespan of the transfused cells from the donor subject. The storage age of the donor blood was not accounted for in the model since long-term RBC survival is unaffected and the short-term recovery is still within AABB requirements (Luten et al., 2008; Roback et al., 2008). By superposition, summation of \( Hb_{\text{in vivo}}(t) \) and \( Hb_{\text{trans}}(t) \) gives the total amount of Hb present in the systemic circulation:

\[
Hb_{\text{total}}(t) = Hb_{\text{in vivo}}(t) + Hb_{\text{trans}}(t)
\] (7)

The total number of RBCs present in the systemic circulation, \( RBC_{\text{total}}(t) \), was given by dividing the \( Hb_{\text{in vivo}}(t) \) and the \( Hb_{\text{trans}}(t) \) by the corresponding mean corpuscular Hb, that is \( MCH_{\text{in vivo}} \) and \( MCH_{\text{trans}} \), respectively, as given by:

\[
RBC_{\text{total}}(t) = \frac{Hb_{\text{in vivo}}(t)}{MCH_{\text{in vivo}}} + \frac{Hb_{\text{trans}}(t)}{MCH_{\text{trans}}}
\] (8)

A separate \( MCH \) was used for the infant \textit{in vivo} and adult transfused RBCs since infant cells are generally larger and contain more Hb than adult cells (Hoffman et al., 2005).

The above presented equations are only applicable in the absence of any phlebotomies. Since the studied infants were subjected to numerous and frequent phlebotomies for clinical testing purposes, corrections to the above equations were needed. To do so, at the time of each phlebotomy \( Hb_{\text{in vivo}}(t) \) was corrected as:

\[
Hb_{\text{in vivo}}(t) = \begin{cases} 
Hb_{\text{in vivo}}(t) & \text{for } t = T_{pj} \\
Hb_{\text{in vivo}}(t) - Hb_{pj} \cdot \frac{Hb_{\text{in vivo}}(T_{pj})}{Hb_{\text{total}}(T_{pj})} & \text{for } t = T_{pj} + \epsilon, \quad j = 1 \text{ to } n
\end{cases}
\] (9)

and \( Hb_{\text{trans}}(t) \) was corrected as:
\[ H_b^{\text{trans}}(t) = \begin{cases} H_b^{\text{trans}}(t) & \text{for } t = T_{P_j} \\ H_b^{\text{trans}}(t) - H_b^{\text{prod}}(t) & \text{for } t = T_{P_j} + e, \quad j = 1 \text{ to } n \end{cases} \]

where \( T_{P_j} \) is the time of the \( j^{th} \) phlebotomy, \( H_b^{\text{prod}} \) is the amount of Hb removed by the \( j^{th} \) phlebotomy, and \( n \) is the total number of phlebotomies.

Additionally, the negative terms in the differential Eq. 4 and Eq. 6 presented above (i.e. \( f_{\text{prod}}^\text{total}(t-b) \) and \( H_b^{\text{trans}}(t-T_{P_j}) \cdot f_{\text{prod}}^\text{total}(t-T_{P_j}) \), respectively) must be corrected for Hb removed by the phlebotomies. These negative terms represent the output of Hb from the system due to cell age-related death of RBCs, and thus if not corrected the Hb will be “removed” twice, once due to the correction presented above in Eq. 9 and Eq. 10 at the time of each phlebotomy and a second time when the Hb would have been “removed” from the systemic circulation in the absence of the phlebotomies due to age-related death of RBCs. Since the cells physically removed by the phlebotomy cannot be removed again through the differential equation terms that represent removal by cell death (Krzyzanski and Jusko, 2002), these terms must be modified so that the equations remain correct. Let \( F_{P_j} \) be the fraction of Hb remaining immediately after the \( j^{th} \) phlebotomy relative to the amount present immediately before the \( j^{th} \) phlebotomy, thus:

\[ F_{P_j} = \frac{H_b^{\text{total}}(T_{P_j}) - H_b^{\text{prod}}(T_{P_j})}{H_b^{\text{total}}(T_{P_j})} \]

Additionally, if \( F_{P_j} \) are ordered from the first to the last phlebotomy such that \( T_{P_{j+1}} > T_{P_j} \), then the exact phlebotomy correction factors to multiply these negative terms with in the differential equation is given by:

\[ \text{phlebotomy correction factor} = \begin{cases} \prod_{j=k}^{l} F_{P_j} & \text{if } l \geq k \text{ and } T_{P_k} < t \\ 1 & \text{otherwise} \end{cases} \]
where \( k \) is the first phlebotomy after entry of the cells of interest into the systemic circulation and \( l \) is the last phlebotomy prior to the current time \( t \) (see Appendix I for derivation).

Therefore, as long as \( T_{pk} < t \), \( k \) is the first phlebotomy after time \( t - L_{in\,vivo} \) for the \( f^{\text{total}}(t-b) \) term and the first phlebotomy after time \( t - T_{Ti} \) for each \( Hb_{Ti} \cdot f^{\text{trans}}(t - T_{Ti}) \) term. The presented phlebotomy correction factor following \( l - k + 1 \) phlebotomies is consistent with formulas previously derived following only 1 or 2 phlebotomies (Freise et al., 2007; Freise et al., 2008b).

Finally, the amounts estimated from \( Hb_{\text{total}}(t) \) and \( RBC_{\text{total}}(t) \) were converted into the observed concentrations by the model estimated total blood volume. The total blood volume, \( V_{\text{total}} \), was assumed to be proportional to the infant body mass (Sisson et al., 1959; Meibohm et al., 2005), and was given by:

\[
V_{\text{total}}(t) = m(t) \cdot V_N
\]  

(13)

where \( V_N \) is the bodyweight or mass normalized blood volume.

**Data analysis.** All modeling and simulations were conducted using WINFUNFIT, a Windows (Microsoft) version evolved from the general nonlinear regression program FUNFIT (Veng-Pedersen, 1977), using ordinary unweighted least squares fit to each individual subject’s Hb and RBC concentration-time profile. The amount of Hb removed and administered by each phlebotomy and transfusion, respectively, at the time of removal or administration was accounted for by WINFUNFIT using a generalized events processing module. The events processing module integrates the differential equation exactly up to the time of the event before adding or removing the appropriate amount and then continuing on integrating the differential equation from the new initial conditions set immediately after each successive event.
The EPO plasma concentrations were nonparametrically represented using a generalized cross validated cubic spline function (Hutchinson and deHoog, 1985). The infant bodyweight post-birth was represented by a 4th order polynomial fit to the observed bodyweight data to interpolate between bodyweight observations and provide a smooth function of total blood volume. To account for the in utero body masses, which are needed to calculate $f_{\text{prod}}(t)$ when $t \leq 0$, a power function was fitted to the mean body weights of over 10,000 live singleton births 22 to 32 weeks of gestational age (Arbuckle et al., 1993). Then for each infant, the in utero body masses were calculated based on their gestational age and linearly scaled such that function predicted birth body mass was continuous with the bodyweight at birth. Thus both plasma EPO concentrations and bodyweights acted as forcing functions in the model.

To account for the fact that 24-hour post-transfusion recoveries ($\text{PTR}_{24}$) are generally less than 100% (Hess et al., 2003), $F_T$ was fixed to 0.875, the midpoint between 100% recovery and the AABB requirements that transfused RBCs must exhibit 75% or greater recovery after storage (Roback et al., 2008). The lifespans of the adult transfused ($L_{\text{trans}}$) was fixed to 70.8 days, the midpoint of the estimated lifespans of 56.4 and 85.2 days of transfused adult RBCs in preterm infants (Bard and Widness, 1997; Strauss et al., 2004). Additionally, the time between production of progenitor cells outside the circulation to release of the subsequently produced RBC into the circulation ($a$) was set equal to 3 days based on previous estimates (Izak, 1977; McKenzie, 1988; Hoffman et al., 2005; Krzyzanski et al., 2005). The $MCH_{\text{trans}}$ parameter was set equal to the measured $MCH$ for each unit of transfused blood, or if not measured for a particular unit of blood then the mean value of all the measured units (27.5 pg/cell). For each subject, the decision to use a nonlinear ($p_2$ estimated) or linear ($p_2 = 0$) bodyweight scaled Hb production rate function (Eq. 3) was made using the Akaike’s Information Criterion (AIC). All
remaining parameters of the model (i.e. $L_{\text{in} \text{vivo}}$, $V_N$, $MCH_{\text{in} \text{vivo}}$, $k_{\text{prod}}^{\text{in } \text{utero}}$, and $E_{max}/EC_{50}$) were estimated in all subjects.

To summarize the uncertainty in the individual subject parameter estimates, the mean percent standard error (MSE%) of the estimate was calculated for each parameter as:

$$\text{MSE}\% = \frac{1}{n} \sum_{i=1}^{n} \left( \frac{SE_i}{P_i} \right) \times 100$$

where $SE_i$ and $P_i$ are the standard error of the parameter and the estimate of the parameter for the $i^{th}$ subject, respectively, and $n$ is the number of subjects for which the parameter was estimated.

Results

Subject characteristics. The mean gestational age of the 14 infant study subjects was 27 weeks (range, 25.0 to 28.6) and mean birth weight was 0.840 kg (range, 0.548 to 1.49). Five males and 9 females were studied. A summary of the number of phlebotomies and RBC transfusions and the corresponding amounts of Hb removed and administered per study subject, respectively, are displayed in Table 1. All subjects who received transfusions were administered more Hb than was removed by phlebotomy. Approximately twice as much Hb was administered by transfusion as removed by phlebotomy. In 54 of the 57 transfusions administered, the volume of packed RBCs (85% Hct) administered was 15 mL/kg. The mean storage age of all RBC transfusions was 11.0 (range 2 to 42) days. Due to the severity of cardiorespiratory disease encountered in the first few weeks of life, the majority of RBC transfusions administered to these infants occurred at a Hct of approximately 35%. During the entire approximately 1 month study period, the average number of phlebotomies performed per subject per day was 4.5 (range, 1.6 to
7.1. The mean rate of blood removal by phlebotomy began very high at 10.1 mL/day/kg of bodyweight on the first day of life and subsequently decreased over the next few days (Figure 1). On average 48.3% of the blood removed in the first 30 days of life was removed during the first 7 days of life. The mean daily rate of phlebotomy blood removal over the study period was 1.72 mL/day/kg of bodyweight. The plasma EPO concentrations were variable and declined approximately 3-fold from 45 mU/mL immediately after birth to an average of 15.5 ± 6.55 (mean ± SD) mU/mL from 5 days of age onward (Figure 2).

**Pharmacodynamic Hb mass balance model.** The Hb mass balance model fit to the Hb and RBC concentration-time profiles, along with the plasma EPO concentration and bodyweight data (with fitted curves) for two representative subjects are displayed in Figure 3 (individual subject data fittings are given in supplemental material, Supplemental Figures 1-14). General agreement between the model fit and the Hb and RBC concentrations was observed (R² range 0.868 to 0.978). The administrations of the RBC transfusions are indicated by the vertical lines in the model predicted Hb and RBC concentration-time profiles. The rapid decline in the Hb and RBC concentrations after each transfusion is due to a combination of phlebotomy blood loss, blood volume expansion with growth (minor component), and RBC age-related death of both endogenously produced and transfused RBCs. Individual subject’s Hb concentration-time profiles contained on average 91 Hb (range, 28 to 172), 27 RBC (range, 13 to 44), 50 plasma EPO (range, 10 to 96) concentration and 32 bodyweight (range, 30 to 37) measurements. The observed plasma EPO concentrations and bodyweights were also well represented by the fitted cross-validated cubic spline and fourth order polynomial, respectively (Figure 3). The estimates of the parameters are displayed in Table 2 (individual subject parameters are given in supplemental material, in Supplemental Table 1). The parameters were well estimated with a
MSE% of < 15% for all but the Hb production rate function parameters. The relatively high MSE% for $E_{max}/E_{C_{50}}$ is due to a single subject having a high standard error of 1300%. Calculation of the MSE% for $E_{max}/E_{C_{50}}$ without this subject results in a MSE% of only 24.6%.

The $E_{C_{50}}^{-1}$ parameter, and thus $E_{max}$ and $E_{C_{50}}$ secondary parameters, were only determined in 6 subjects where the nonlinear Hb production rate function (Eq. 3) was preferred over a linear function based on AIC. The estimated amounts of Hb present at birth and produced (and released into the circulation) over the first 30 days of life, as well as the observed amounts of Hb removed by phlebotomy and administered by RBC transfusion, are displayed in Table 3. By 30 days of age, the estimated cumulative amount of Hb released into the circulation in the 14 VLBW study subjects ranged from 0.64 to 14.2 g/kg of birth weight.

Discussion

The in vivo erythropoiesis rate and its relationship to endogenous plasma EPO concentrations in VLBW infants over the first 30 days of life was successfully estimated utilizing a PD-based Hb mass balance model with: (1) detailed accounting of all blood removed and transfused, (2) frequently sampled Hb, RBC, and plasma EPO concentration-time profiles created through recovery of excess blood collected, and (3) recording of serial bodyweights to account for growth and blood volume expansion. The estimated mean post-birth bodyweight scaled Hb production rate over the first 30 days of life (average $\overline{f_{prod}^{exu}}$ of 0.144 g/day·kg$^{3/4}$ of bodyweight can serve as a quantitative reference for the erythropoietic ability of VLBW infants, under conditions of mild anemia due to receiving clinically ordered RBC transfusions, to compensate for Hb removed by phlebotomy and Hb dilution due to blood volume expansion as a result of growth. Furthermore, this reference and the post-birth Hb production rate parameters
estimates ($E_{max}/EC_{50}$ and $EC_{50}^{-1}$) are both important for assessing the potential to reduce or eliminate RBC transfusion risks through administration of exogenous rHuEPO or other erythropoiesis stimulating agent.

In healthy term infants the Hb production rates have been shown to decrease approximately 8-fold at day 10 of life relative to day 1 of life (Garby et al., 1963). A decrease was also observed in the current study as the mean production rates dropped 3-fold from 0.414 g/day· kg$^{3/4}$ at birth to an average of 0.144 g/day· kg$^{3/4}$ post-birth (Table 2). The mean post-birth Hb production rate estimate of 0.144 g/day· kg$^{3/4}$ (corresponding to an erythropoiesis rate of 3.84 × 10$^9$ RBCs/day· kg$^{3/4}$) is approximately half the production rate in healthy adults of 0.260 g/day· kg$^{3/4}$ (6.3 g/day in a 70 kg adult) (Hoffman et al., 2005). This modest Hb production rate in these infants suggests that the production rate is suppressed. Specifically, the administration of RBC transfusions to these infants, which prevents more severe anemia, may be limiting Hb production particularly considering that adults under severe chronic anemic conditions are capable of increasing their Hb production rate 3- to 5-fold above normal (Hillman and Finch, 1967). The possible limiting effect of RBC transfusion on erythropoiesis is illustrated in Panel A of Figure 3, where the plasma EPO concentrations (top) drop following each RBC transfusion. However, a similar pattern in the relationship between transfusions and plasma EPO concentrations was not observed in all subjects. For example, the representative subject displayed in Panel B of Figure 3 does not show any obvious relationship between RBC transfusions and plasma EPO concentrations. Either way, any effect of RBC transfusion on EPO is accounted for in the model since the cubic spline curve fitted to the plasma EPO concentrations acts as a forcing function. However, it is acknowledged that the model would be more physiologically complete if there was a negative feedback between Hb concentrations and EPO production.
The estimates of $E_{\text{max}}$ and $EC_{50}$ in Table 2 may be biased since they could only be determined in a subset of the infants ($n = 6$) where the production rate was operating in the nonlinear (i.e. higher) plasma EPO concentration range. That is, in this subset of 6 infants the Hb production rate was approaching saturation while in the other 8 infants no production rate saturation was present in the observed plasma EPO concentration range. However, the mean estimates of $E_{\text{max}}$ and $EC_{50}$ suggest that the erythropoiesis or Hb production rate could be increased several-fold from of 0.144 g/day·kg$^{34}$ of bodyweight up to 0.566 g/day·kg$^{34}$ with only a relatively modest increases in the plasma EPO concentration, since the mean estimated $EC_{50}$ was only 28.5 mU/mL. The increased plasma EPO concentrations and erythropoiesis rate could be achieved with exogenous administration of rHuEPO due to its identical amino acid sequence and biological equivalence to the natural hormone (Egrie et al., 1986). However, previous research has demonstrated limited success in the administration of rHuEPO to sufficiently increase erythropoiesis such that a clinically relevant reduction in RBC transfusions is achieved in preterm VLBW infants (Ohlsson and Aher, 2006). The exact reasons for the apparent limited efficacy of rHuEPO in infants are unknown, but they may be related to excessive phlebotomy blood loss, suboptimal dosing regimens and insufficient understanding of the complex PDs of the response of erythrocytes to EPO. Alternatively, increased plasma EPO concentrations and erythropoiesis may also be achieved by allowing the Hct to fall lower before administration of a RBC transfusion(s). The increased hypoxia due to a lower Hct would likely increase endogenous EPO production and subsequently increase erythropoiesis.

The estimated 4-fold maximal increase in Hb production rate is consistent with the estimates in adults under severe chronic anemic conditions where 3- to 5-fold increase in Hb production rate occurs (Hillman and Finch, 1967). Additionally, the mean maximal Hb
production rate estimate of 0.566 g/day \cdot kg^{3/4} was also similar to the estimated mean in utero Hb production rate from the current study of 0.414 g/day \cdot kg^{3/4}, which is a more hypoxic environment than that experienced post-birth and thus may also represent a near maximal Hb production rate. Similar to the postnatal fall in erythropoiesis experienced by these infants, the plasma EPO concentrations (Figure 2) also fell following birth.

Previous RBC lifespan estimates based on $^{51}$Cr labeled RBCs from premature infants of 35 to 50 days (Brugnara and Platt, 2003) are similar to the results obtained in the current study of 65.8 days (Table 2). As expected, the estimated infant lifespans were shorter than the typically referenced adult RBC lifespan of 120 days (Landaw, 1988; Brugnara and Platt, 2003). The estimated mean blood volume of 97.7 mL/kg in the first weeks of life (Table 2) is consistent with other measurements of blood volume in term and premature infants ranging from 89 to 110 mL/kg during the first 2 weeks of life (Sisson et al., 1959; Usher and Lind, 1965; Simon et al., 1998).

The number (Table 1) and rate of phlebotomy blood loss (Figure 1) in these VLBW infants was extensive and contributes substantially to their anemia and RBC transfusion need. It was estimated that on average over 50% of the Hb present at birth was removed during the first 30 days of life (Table 3). Additionally, the mean amount of Hb removed was greater than the estimated total amount of Hb produced and inputted into the circulation by these infants in the first 30 days of life. The transfused Hb amount was approximately twice the phlebotomy blood loss. Furthermore, as suggested by some subject’s plasma EPO concentration-time profiles and discussed above, the administration of RBC transfusions may contribute to continued transfusion need by suppressing endogenous EPO production and the subsequent erythropoiesis. The effect of the estimated amount of blood loss (38 µL) added at each phlebotomy due to blood wiped
away prior to capillary heel stick blood collection and clearing of blood from catheter lines at each indwelling catheter blood collection was minimal, as halving or doubling this estimated amount only changed the average blood loss by -6.07% or 12.1%, respectively.

The common goal with rHuEPO and other erythropoiesis stimulating agent therapy in preterm VLBW infants is not just to reduce the number of RBC transfusions, but to eliminate them altogether and thus the disease transmission and other risks associated with them (Ohlsson and Aher, 2006). With the common use of stored RBCs from a single donor, multiple RBC transfusions carry little additional risk of disease transmission than a single transfusion. Based on the estimated amount of Hb produced and the measured amount of Hb transfused (Table 3), an erythropoiesis stimulating agent would need to increase the Hb production rate 2- to 3-fold to maintain Hb concentrations high enough to avoid the need for a RBC transfusion in the first 30 days of life under the current clinical practice guidelines utilized in the study. If the phlebotomy blood volume removed due to clinical testing could be substantially reduced and/or if the Hct percentage used in determining whether a transfusion is needed was decreased, then the Hb production rate needed to avoid a transfusion in these subjects would be less (Ohlsson and Aher, 2006).

**Conclusion.** In summary, a PD model that accounts comprehensively for phlebotomy losses and RBC transfusions was fitted to endogenous plasma EPO, Hb, RBC, and bodyweight profiles over time from 14 VLBW infants. Detailed recording of all blood removed from and administered to these infants and use of a Hb mass balance model allowed for a mathematically rigorous determination of the *in vivo* erythropoiesis and Hb production rates and their relationship to endogenous plasma EPO concentrations under the dynamic and complex hematological conditions routinely experienced by VLBW infants in first 30 days of life. The
estimated parameters of the PD Hb mass balance model were consistent with other reports in the literature using direct measurement techniques, further supporting the utility of the proposed model. Future work with this model and parameter estimates, including direct measurements of blood volume and RBC lifespan in these subjects, will allow for an assessment of the potential to eliminate RBC transfusions in VLBW infants through administration of erythropoiesis stimulating agents and/or changes in other clinical practices.

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References


Footnotes

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Legends for Figures

Figure 1. Mean rate of phlebotomy blood loss versus subject age

Figure 2. Plasma EPO concentrations versus subject age.

The solid line represents a non-parametric smoothing spline fit to the observed data (circles)

Figure 3. Pharmacodynamic Hb mass balance model fit to representative subjects.

The symbols represent the observed data and the lines represent the model fit. Panels A (left-hand side) and B (right-hand side) are different subjects. (Individual subject data fittings are given in supplemental material, Supplemental Figures 1-14)
### Table 1. Individual subject Hb, phlebotomy, and transfusion characteristics during study period \((n = 14)\)

<table>
<thead>
<tr>
<th>First Hb concentration following birth (g/dL)</th>
<th>Study period (days)</th>
<th>No. of phlebotomies</th>
<th>No. of transfusions</th>
<th>Total amount of Hb removed by phlebotomies (g)</th>
<th>Total amount of Hb transfused (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>16.3</td>
<td>32.1</td>
<td>143</td>
<td>4.07</td>
<td>6.27</td>
</tr>
<tr>
<td>S.D.</td>
<td>2.3</td>
<td>2.46</td>
<td>56.1</td>
<td>2.50</td>
<td>2.58</td>
</tr>
<tr>
<td>Minimum</td>
<td>11.6</td>
<td>29.7</td>
<td>48</td>
<td>0</td>
<td>2.12</td>
</tr>
<tr>
<td>Maximum</td>
<td>19.3</td>
<td>37.3</td>
<td>239</td>
<td>8</td>
<td>10.6</td>
</tr>
</tbody>
</table>
Table 2. Estimated parameter summary from the pharmacodynamic Hb mass balance model ($n = 14$)

(individual subject parameters are given in supplemental material, in Supplemental Table 1)

<table>
<thead>
<tr>
<th>$L_{\text{in vivo}}$</th>
<th>$V_N$</th>
<th>$MCH_{\text{in vivo}}$</th>
<th>$k_{\text{in utero prod}}$</th>
<th>$E_{\text{max}}/EC_{50}$</th>
<th>$1/EC_{50}$</th>
<th>Avg.</th>
<th>$E_{\text{max}}$</th>
<th>$EC_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(day)</td>
<td>(mL/kg)</td>
<td>(pg/cell)</td>
<td>(g/day·kg$^{0.75}$)</td>
<td>(g·mL/day·mU·kg$^{0.75}$)</td>
<td>(mL/mU)</td>
<td>(g/day·kg$^{0.75}$)</td>
<td>(mU/mL)</td>
<td>(g/day·kg$^{0.75}$)</td>
</tr>
<tr>
<td>Mean</td>
<td>65.8</td>
<td>97.7</td>
<td>37.5</td>
<td>0.414</td>
<td>0.0140</td>
<td>0.062</td>
<td>0.144</td>
<td>0.566</td>
</tr>
<tr>
<td>S.D.</td>
<td>42.7</td>
<td>31.5</td>
<td>3.1</td>
<td>0.156</td>
<td>0.0127</td>
<td>0.037</td>
<td>0.109</td>
<td>0.372</td>
</tr>
<tr>
<td>MSE% $^c$</td>
<td>11.6%</td>
<td>6.2%</td>
<td>9.2%</td>
<td>7.4%</td>
<td>123%</td>
<td>33.6%</td>
<td>NA $^d$</td>
<td>NA $^d$</td>
</tr>
</tbody>
</table>

$^a$ Only determined in 6 out of 14 subjects based on AIC; $^b$ Secondary parameter; $^c$ MSE% indicates mean percent standard error of parameter estimate; $^d$ NA indicates not applicable.
Table 3. Observed and estimated Hb amounts over the first 30 days of life from the pharmacodynamic Hb mass balance model ($n = 14$)

<table>
<thead>
<tr>
<th></th>
<th>Estimated amount</th>
<th>Estimated amount</th>
<th>Observed amount</th>
<th>Observed amount</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>present at birth</td>
<td>produced and released into the circulation</td>
<td>removed</td>
<td>transfused</td>
</tr>
<tr>
<td></td>
<td>(g)</td>
<td>(g)</td>
<td>(g)</td>
<td>(g)</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td>10.3</td>
<td>4.69</td>
<td>5.97</td>
<td>12.3</td>
</tr>
<tr>
<td><strong>S.D.</strong></td>
<td>2.70</td>
<td>3.32</td>
<td>2.53</td>
<td>7.01</td>
</tr>
</tbody>
</table>
Figure 3

A

B

Hemoglobin (g/dL)

EPO (mU/mL)

RBC ($10^6/μL$)

Bodyweight (kg)

Age (days)
Appendix I: Derivation of the phlebotomy correction factor for the differential equation output terms

Let event $A_j$ be the removal of RBCs (cells) or Hb by the $j^{th}$ phlebotomy and thus the complement, $A_j^C$, is the event that cells or Hb are not removed by the $j^{th}$ phlebotomy. Obviously cells that entered the systemic circulation after the $k-1$ phlebotomy cannot be affected by the phlebotomies conducted prior to the $k^{th}$ phlebotomy and thus probability of removal by these phlebotomies is zero. Similarly, cells that exit the circulation due to the age-related cellular death prior to the $l+1$ phlebotomy cannot be affected by phlebotomies after the $l^{th}$ phlebotomy and the probability of removal by these phlebotomies is also zero. Therefore, only the effect of phlebotomies $k$ through $l$ on the differential equations need to be accounted, given that at least one phlebotomy was conducted between entry and exit (due to age related cell death) of the cells from the circulation. The quantity of interest then is the probability that a cell is not removed by phlebotomies $k$ through $l$, as this is the fraction of remaining cells which will exit via the age-related death through the appropriate differential equation term. This quantity can also be written as the probability of the intersection of events $A_k^C$ through $A_l^C$ (i.e. $P(A_k^C \cap A_{k+1}^C \cap \ldots \cap A_l^C)$). The conditional probability of not being removed by the $j^{th}$ phlebotomy is given by:

$$P(A_k^C) = F_{p_k}$$

$$P(A_{k+1}^C | A_k^C) = F_{p_{k+1}}$$

$$P(A_{k+2}^C | A_k^C \cap A_{k+1}^C) = F_{p_{k+2}}$$

$$\vdots$$

$$\vdots$$
\[ P(A^C_k \mid A^C_k \cap A^C_{k+1} \cap \ldots \cap A^C_{l-1}) = F_{pj} \]

where \( P(\cdot | \cdot) \) denotes the conditional probability. By rearrangement of Bayes theorem and substitution from above:

\[ P(A^C_k \cap A^C_{k+1}) = P(A^C_{k+1} \mid A^C_k) \cdot P(A^C_k) = F_{pk+1} \cdot F_{pk} \]

\[ P(A^C_k \cap A^C_{k+1} \cap A^C_{k+2}) = P(A^C_{k+2} \mid A^C_k \cap A^C_{k+1}) \cdot P(A^C_k \cap A^C_{k+1}) = F_{pk+2} \cdot F_{pk+1} \cdot F_{pk} \]

which generalizes to:

\[ P(A^C_k \cap A^C_{k+1} \cap \ldots \cap A^C_j) = P(A^C_j \mid A^C_k \cap A^C_{k+1} \cap \ldots \cap A^C_{j-1}) \cdot P(A^C_k \cap A^C_{k+1} \cap \ldots \cap A^C_{j-1}) \]

\[ = F_{pj} \cdot \prod_{j=k}^{l-1} F_{pj} \]

\[ = \prod_{j=k}^{l} F_{pj} \]

If no phlebotomies were performed between entry and exit (due to age related cellular death) of the cells from the circulation, then no correction factor is needed, or equivalently the correction factor is equal to one. This completes the derivation of the phlebotomy correction factor for the differential equation output terms.