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Evaluation of erythropoiesis in anemic low birth weight preterm infants

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EVALUATION OF ERYTHROPOIESIS IN ANEMIC LOW BIRTH WEIGHT PRETERM INFANTS

by

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CERTIFICATE OF APPROVAL

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PH.D. THESIS

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To my parents, my siblings and my beloved wife

Numbers have an important story to tell. They rely on you to give them a voice.

Stephen Few

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ABSTRACT

Anemia of prematurity is characterized by a progressive decline in hemoglobin level during the first month of life. Unlike term newborns, preterm infants become anemic and often require red blood cell transfusions. Various factors contribute to the development of this anemia. These include short infant red blood cell (RBC) lifespan, decline in erythropoiesis rate after birth, and blood losses caused by repeated phlebotomies.

The objectives of this work were to develop novel models to evaluate fetal and neonatal erythropoiesis, and to study in vivo adult and neonatal RBC survival in low birth weight preterm anemic infants. The model developed to evaluate fetal erythropoiesis was based on the in utero growth of the fetus over time. Neonatal erythropoiesis rate was estimated using a hemoglobin (Hb) mass-balance based method that has the advantage of not relying on specific structural pharmacodynamic model assumptions to describe the Hb production, but instead utilizes simple mass balance principles and nonparametric regression analysis to quantify the amount of Hb produced and the Hb production rate during the first month of life. To study RBC survival, two separate models, one describing the elimination of neonatal RBCs produced under non-steady state conditions, and the second describing the elimination of adult RBCs produced under steady state conditions were developed and applied to the RBC survival data obtained from low birth weight anemic preterm infants. The proposed mathematical models and its implementation provides a flexible framework to study both *in utero* non-steady state (non-SS) fetal erythropoiesis and neonatal erythropoiesis in newborn infants.

PUBLIC ABSTRACT

Anemia of prematurity (AOP) is an exaggerated, pathologic response of the preterm infant to the transition from a relatively hypoxic state before birth to a relatively hyperoxic state with increased tissue oxygenation after birth that leads to a decline in erythropoietin (EPO) concentration. Three basic mechanisms are responsible for the development of AOP, (1) inadequate RBC production, (2) shortened RBC life span, and (3) blood loss.

The objectives of this work were to develop and evaluate novel mathematical models to evaluate fetal and neonatal erythropoiesis, and to study *in vivo* adult and neonatal RBC survival in low birth weight preterm anemic infants. The proposed models and its implementation provide a flexible framework to study both non-steady state (non-SS) fetal erythropoiesis and neonatal erythropoiesis in anemic newborn infants.

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LIST OF SYMBOLS AND ABBREVIATIONS

RBC	Red blood cell
Hb	Hemoglobin
VLBW	Very low birth weight infant
ELBW	Extremely low birth weight infant
GA	Gestational age
Epo	Erythropoietin
Non-SS	Non-steady state
NICU	Neonatal intensive care unit
$Hb_T(t)$	Total amount of hemoglobin present in circulation at any time t
$Hb_B(t)$	Hb amount present at birth which still are present at time t
$Hb_P(t)$	Hb amount produced after birth still present in circulation at time t
$Hb_{TR}(t)$	Hb amount transfused after birth still present in circulation at time t
$\hat{H}b_P(t)$	Predicted amount of Hb produced at time t
$BW(t)$	*In utero* body weight at time t
MCH	Mean corpuscular hemoglobin
Hct	Hematocrit
L_{TRj}	Lifespan of transfused RBCs from the j^{th} transfusion

F_T	Fraction of transfused RBCs surviving immediately after the transfusion
R(t)	*In utero* erythropoiesis rate
α	Rate of change in *in utero* fetal RBC lifespan
k	Scaling factor that relates *in utero* growth to fetal erythropoiesis rate
L(0)	Lifespan of RBCs present at birth ($t = 0$)
NTR	Number of donor RBC transfusions
F_{RMi}	Fraction of total Hb remaining after i^{th} phlebotomy
PD	Pharmacodynamic
t	Time
BioRBC	Biotinylated RBC
Hct	Hematocrit
F_L	Fraction of labeled RBCs
n(0)	Number of RBCs produced *in utero* that are present in circulation at birth
$n_E(t)$	Number of RBCs produced *in utero* that are eliminated from circulation at time t
n(t)	Number of RBCs produced *in utero* that remain in circulation at time t
PCF	Phlebotomy correction factor
PTR_{24}	Initial post transfusion recovery
MPL	Mean potential lifespan

MA	Mean age
TRCS	Transfused RBC survival
MRL	Mean remaining lifespan
MRT	Mean residence time
F(t)	Fraction of the transfused RBCs remaining in circulation at time *t*

CHAPTER 1. INTRODUCTION

1.1 BACKGROUND

Red blood cells are the most common cell type found in blood. These cells are primarily responsible for providing oxygenation to tissues and are crucial for the healthy existence of all vertebrate organisms. Human blood contains approximately 5×10^6 erythrocytes per microliter. The normal range is 4.7×10^6 to 6.1×10^6 erythrocytes per microliter for males, and 4.2×10^6 to 5.4×10^6 erythrocytes per microliter for females. These cells are produced in the bone marrow and released continuously into circulation.

The determination of RBC production rates and RBC survival has been an interest to researchers for many years. In healthy adults, where the RBCs are produced under steady-state conditions, $\sim 2.4 \times 10^6$ new erythrocytes are produced in the bone marrow and released into circulation each second. In addition, ~1% of the erythrocytes are cleared from the circulation every day and replaced by new RBCs.

Much of the earlier work on erythropoiesis and RBC survival assumed constant RBC production rates. While this assumption may be valid for healthy adults, it cannot be applied to more complex erythropoiesis conditions such as stress erythropoiesis, fetal/neonatal erythropoiesis, or erythropoiesis in patients with chronic renal disease. In these conditions, the RBC production rates are not constant but vary significantly with time. In addition, the RBC lifespan in circulation may also vary with time.

1.2 ANEMIA OF PREMATURITY

All newborn infants experience a decline in circulating RBCs during the first weeks of life (1). In healthy term infants, this postnatal drop in hemoglobin levels is well tolerated, does not require therapy, and is referred to as the "physiological anemia of infancy" (2). In premature very low birth weight (VLBW, <1500 g) and extremely low birth weight (ELBW, <1000 g) infants, the postnatal drop in Hb concentration is associated with abnormal clinical signs, require therapeutic intervention, and is referred to as "anemia of prematurity" (AOP) (1).

AOP is not a physiological condition and several factors are reported to play a role in its pathogenesis. A large number of low birth weight preterm infants are born before the last trimester of gestation and thus, they are deprived of most of the iron transport from the mother (1). These infants are also deprived of a major share of the fetal erythropoiesis that takes place during the last trimester before birth (1). Preterm infants also have diminished plasma Epo levels compared to term infants (3, 4).

AOP is also exaggerated by non-physiological factors such as frequent clinical blood sampling (phlebotomy) for serial laboratory tests. These tests include blood gases, electrolytes, blood cultures and counts (1). Low birth weight preterm neonates have the smallest circulating RBC volume but require the most frequent blood sampling. As a result, these infants suffer the greatest proportional loss of RBCs from their circulation compared to healthier neonates. In preterm infants requiring intensive care, the mean volume of blood removed for clinical sampling is reported in the range of 0.8 to 3.1 mL/kg per day (5).

1.2.1 Donor RBC transfusions to treat anemia of prematurity

Until the early 1990s, the only available treatment option for AOP was RBC transfusion from healthy adult donors. Most low birth weight preterm infants born before 30 weeks gestation required at least one RBC transfusion during their initial hospitalization. Early RBC transfusions (first 2 weeks of life) were given to compensate for acute blood loss due to multiple blood sampling during critical illness. Following the first 2 weeks of life, critically ill anemic preterm neonates are transfused clinically manage their symptomatic AOP.

Although there are no universal guidelines for transfusing RBCs to preterm neonates, most RBC transfusions administered to infants consist of 15 ± 5 mL/kg RBCs transfused over 2-4 hours. These transfusions are given to maintain a level of blood hemoglobin or hematocrit that is optimal for each infant's clinical condition (1). The most commonly reported guidelines used by neonatologists to treat AOP are listed in Table 1.1 (1).

1.2.2 Recombinant erythropoietin to limit RBC transfusions

The introduction of recombinant human erythropoietin (rHuEpo) has revolutionized the treatment of patients with HIV infection, and other hematological and oncological disorders. Recombinant human Epo was initially introduced for the treatment of anemia associated with chronic kidney disease (6). The first commercialized rHuEpo, Epoetin alfa, is a 165 amino acid glycoprotein with an average molecular weight of 30,400 Da (7). Epoetin beta, also contains 165 amino acids but differ slightly in the glycosylation pattern (7, 8). Both epoetin alpha and beta have an intravenous (IV) half-life of 4 to 8

hours (7, 8). Darbapoietin alfa has increased molecular weight of approximately 38,000 Da and has an IV half-life of 24 hours (7, 8). A third generation erythropoiesis stimulating agent (ESA) is continuous erythropoietin receptor activator (CERA), which has an approximate molecular weight of 60,000 Da and has a reported terminal elimination half-life of 134 hours in humans (9, 10).

Previous studies on Epo responsiveness of erythroid progenitor cells of preterm neonatal have identified inadequate Epo production as a major cause of AOP (11). The well-established low plasma Epo levels and the reported responsive RBC progenitor cells in neonates provide a rational basis to consider rHuEpo as a potential treatment for AOP (1, 11). By the end of 1999, over 20 controlled clinical trials were conducted to assess the efficacy of rHuEpo to eliminate RBC transfusions in anemic preterm infants (12). Although the trials reduced the number of RBC transfusions administered to infants compared to controls, a meta-analysis of the rHuEpo clinical trials concluded that the magnitude of the drug's effect was relatively modest and of questionable clinical importance (12).

1.3 ERYTHROPOIESIS

Erythropoiesis is regulated via a well-established oxygen-dependent negative feedback loop mediated by the hormone erythropoietin (Epo). One of the earliest reports of this feedback control mechanism was proposed by Paul Bert in 1878. He proposed that the observed increase in the red blood cell number at high altitudes was to compensate for the lower oxygen tension (13). In 1906, DeFlandre and Carnot hypothesized that the erythropoietic feedback control mechanism was mediated via a humoral factor. This hypothesis was later confirmed by Erslev in 1953 (14). This humoral factor, Epo, was

purified in 1971 (15), and was later cloned in 1985 (16, 17).

Erythropoiesis (RBC production) is the result of a proliferation and differentiation pathway that becomes progressively restricted to the erythroid lineage. Hematopoietic progenitor cells residing in the bone marrow differentiate into burst-forming unit erythroid (BFUe) and then into colony-forming unit (CFUe) cells (18, 19). CFUe cells further differentiate into proerythrobalsts, and finally into erythroblasts (6). The late-stage erythrobalsts begin to take up iron, undergo enucleation, and form reticulocytes that are released into circulation. After several days, the reticulocytes mature into circulating RBCs that provide oxygenation to tissues (6).

1.3.1 Fetal erythropoiesis

In neonates, erythropoiesis is divided into two phases, the fetal erythropoiesis before birth and the neonatal erythropoiesis after birth. The former takes place at three main sites during fetal life, namely the yolk sac, the liver, and the bone marrow (20). During the first trimester, fetal erythropoiesis occurs at the yolk sac and is predominantly megaloblastic (21). In the second trimester, the hepatic erythropoiesis takes over and continues until the beginning of the third trimester (21). During the third trimester, fetal erythropoiesis shifts to the bone marrow and continues to become the primary site of erythropoiesis after birth (21).

1.3.2 Neonatal erythropoiesis

Erythropoiesis decreases rapidly during the first week after birth. This drop in erythropoiesis has been supported by studies of bone marrow (22), iron kinetics (23) and reticulocytes (24). During this time, the endogenous Epo levels also decline

drastically (21). It is well substantiated that the decrease in neonatal erythropoiesis during the first week of life is primarily due to improved tissue oxygenation and cessation of Epo production (21). After the first week of life, and during the next several weeks, erythropoiesis continues at a low rate and the total red cell volume (RCV) decreases (21). The reported presence of erythropoiesis inhibitors in newborn infant plasma suggest that inhibitory mechanism also play a role in the suppression of erythropoiesis postnatally (25).

1.4 RBC SURVIVAL

Human RBCs survive in a non-random manner, and is removed from the circulation based on their lifespan (26). The measurement of human red blood cell (RBC) survival (RCS) is an old but still challenging area of research, and a wide variety of methods have been utilized for this purpose. The first accurate method to determine RBC lifespan using the differential agglutination technique was proposed by Winifred Ashby in 1919 (27). This technique was accurate, and remained the standard for determining allogeneic RBC lifespan for almost 40 years. Over the next several years, research has focused on developing RBC labels that are able to track the RBCs in circulation for longer time with better accuracy. These labels can be classified into two general types: cohort labeling, in which RBCs of a certain age are labeled, and population labeling, in which all RBCs present at a moment in time are labeled irrespective of their age (28).

1.4.1 RBC labels

Chromium, ^{51}Cr

^{51}Cr is currently the most commonly used radioactive RBC population label and has in the past been considered as the "gold standard" in RBC labeling studies. It binds non-covalently to hemoglobin in RBCs, and over 90% of the label is incorporated into the RBCs. When the labeled RBCs are reinfused back into circulation, Cr elutes from the labeled RBCs at a rate of about 1% per day (29). The advantages of this label are that this labeling method is more convenient than earlier methods and is standardized. Since the half-life is 27.8 days, this method is suitable for short 30 day RBC survival studies (26). There are several disadvantages of using the ^{51}Cr labeling method. Since this is a radioactive label, it cannot be used in children and pregnant women (26). The elution of the label from the RBCs has to be mathematically corrected to estimate RBC lifespan accurately. The combination of radioactive decay and elution makes it difficult to follow the labeled RBCs accurately for the entire RBC lifespan.

Biotin

The use of biotin labeled RBCs to evaluate RBC survival is now considered as the new gold standard for such evaluations (30). Biotin is a nonradioactive RBC population label that covalently binds to RBCs. RBCs are reacted with sulfo- N-hydroxysuccinimide(NHS)-biotin or NHS-biotin to covalently label membrane proteins with biotin (31). These RBCs are then reacted with fluorescently conjugated streptavidin and then quantified by flow cytometry. This method has several advantages as compared to ^{51}Cr labeling method. There is no loss of label from the biotin labeled

RBCs, and thus it is possible to accurately determine track the labeled RBCs for their entire lifespan (30). The ability to place different levels of biotin on RBCs and to distinguish them in the flow cytometer makes it possible to track multiple RBC populations concurrently in the same study subject (30). Finally, since biotin is nonradioactive, it can be used to study RBC survival is vulnerable populations including newborns, children and pregnant women (30). The disadvantage of the biotin label is the potential development of antibodies against biotinylated RBCs (32). Although transient antibodies were detected in few subjects in a reported biotin-labeled RBC study, none of these antibodies had an effect on the survival of the labeled RBCs (32).

1.5 HYPOTHESIS

The central hypothesis of this work is that erythropoiesis and RBC survival in anemic low birth weight neonates can be described and characterized by novel mathematical models that accurately accounts for clinical transfusions, phlebotomies and neonatal growth. Hypothesis 1: The non-SS *in utero* RBC production can be accurately described using a model that relates the *in utero* growth of the fetus to fetal erythropoiesis. Hypothesis 2: The dynamic changes in post-natal erythropoiesis in low birth neonates can be accurately quantified using a Hb mass balance-based semiparametric method. Hypothesis 3: Allogeneic RBCs from healthy adults would survive about twice as long as autologous RBCs in low birth weight infants.

1.6 OBJECTIVES

The overall objective of this work was to evaluate fetal and neonatal erythropoiesis, and to study RBC survival in low birth weight preterm infants. The specific aims of this work were:

1) to present a method that utilizes cord blood or infant blood RBCs collected within the first days after birth to study both the non-SS *in utero* RBC production and the changes in *in utero* RBC lifespan over time; and to apply this method to *in vivo* RBC disappearance curves of umbilical cord RBCs from critically ill very low birth weight preterm infants tracked via a biotin label.

2) to present a Hb mass balance-based semiparametric method that utilizes infant Hb data from the first 30 post-natal days ("month of life" hereafter) to evaluate the dynamic changes in post-natal erythropoiesis rate in newborn infants; and to apply this method to Hb data from 79 critically ill low birth weight preterm anemic infants to estimate the cumulative amount of Hb produced, to study the changes in neonatal erythropoiesis rate during the first month of life, and to determine the inter-subject variability in post-natal Hb production.

3) to develop a quantitative method to describe *in vivo* RBC survival of neonatal and adult RBCs that were transfused concurrently into a newborn infant while also accounting for confounding factors including multiple phlebotomies, clinical transfusions and growth; and to apply this method to estimate the RBC lifespan of neonatal and adult RBCs from the *in vivo* BioRBC disappearance curves from critically ill low birth weight preterm infants.

4) to introduce the *MRL* parameter to quantify transfused red cell survival (TRCS), and present a simple algorithm for its evaluation; to discuss the merits of *MRL* relative to mean potential lifespan and other parameters for quantifying TRCS; and to demonstrate the evaluation of MRL in various clinical scenarios with the purpose of providing examples of evaluations for discussing conceptual differences relative to other parameters for TRCS.

1.7 OUTLINE OF THESIS

A novel method that accounts for the non-SS in-utero erythropoiesis is developed and presented in Chapter 2. The mathematical model considers both changes in rate of in-utero erythropoiesis and fetal RBC lifespan, and also accurately accounts for the confounding effects of multiple phlebotomies, clinical transfusions and fetal growth.

In Chapter 3, a mass-balance based semi-parametric method is introduced and applied to evaluate neonatal erythropoiesis. Non-parametric techniques including cubic splines were utilized to estimate the amount of Hb produced and the body-weight normalized post-natal Hb production rate during the first 30 days after birth in low birth weight infants.

Chapter 4 introduces a method to describe *in vivo* RBC survival of neonatal and adult RBCs that were transfused concurrently into a newborn infant. Two separate models, one describing the elimination of neonatal RBCs produced under non-steady state conditions, and the second describing the elimination of adult RBCs produced under steady state conditions, were applied to biotinylated RBC data from VLBW preterm anemic infants to estimate the adult and neonatal RBC lifespan.

Chapter 5 introduces a new clinically relevant parameter to assess the quality of transfused RBCs. The new parameter, the mean remaining lifespan was used to quantify transfused RBC survival in two RBC survival data sets.

Table 1.1 Most commonly reported guidelines used by neonatologists to treat anemia of prematurity (1).

Transfuse to maintain the blood hematocrit per each clinical transfusion: >40% for severe cardiopulmonary disease >30% for moderate cardiopulmonary disease >30% for major surgery >25% for symptomatic anemia > 20% for asymptomatic anemia

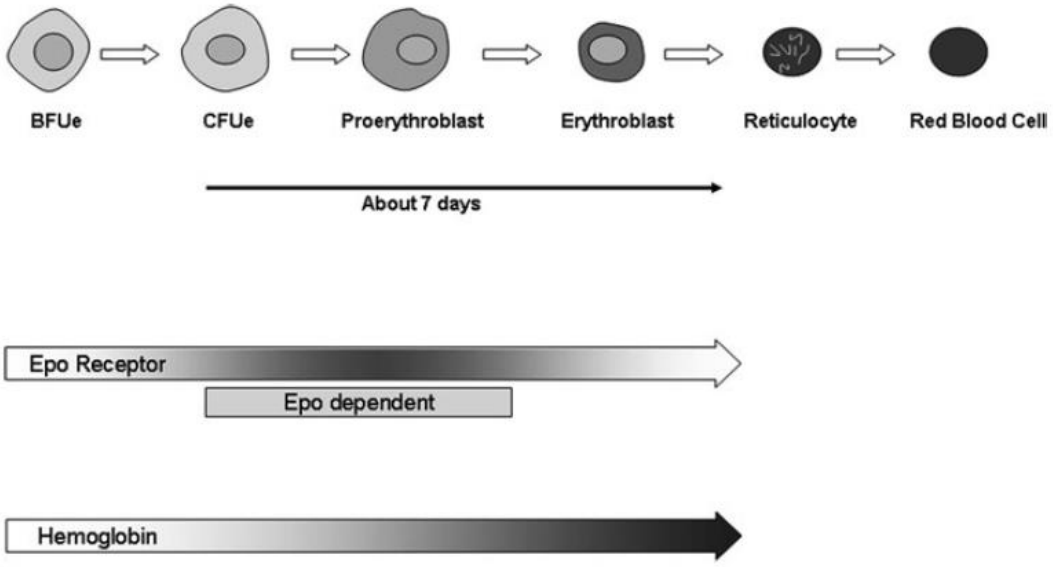

Figure 1.1. Differentiation of erythroid progenitor cells. Burst-forming unit erythroids differentiate in the bone marrow into colony-forming unit erythroid and ultimately into red blood cells that are released into circulation (6).

CHAPTER 2. A METHOD TO EVALUATE FETAL ERYTHROPOIESIS FROM POSTNATAL SURVIVAL OF FETAL RBCs

2.1 ABSTRACT

Fetal RBCs are produced during a period of very rapid growth and stimulated erythropoiesis under hypoxic intrauterine conditions. Fetal RBC lifespan varies with gestational age (GA) and is shorter than that in healthy adults. Due to the special kinetic properties of lifespan-based survival of human RBCs, a mathematical model-based kinetic analysis of the survival of fetal RBCs shortly after birth provides a unique opportunity to "look backward in time" to evaluate fetal erythropoiesis. This work introduces a novel method that utilizes postnatal *in vivo* RBC survival data collected within 2 days after birth to study both non-steady state (non-SS) *in utero* RBC production and changing fetal RBC lifespan over time. The effect of changes in erythropoiesis rate and RBC lifespan and the effect of multiple postnatal phlebotomies on the RBC survival curves were investigated using model-based simulations. This mathematical model, which considers both changes in rate of erythropoiesis and RBC lifespan and which accurately accounts for the confounding effect of multiple phlebotomies, was applied to survival curves for biotin labeled RBCs from ten anemic very low birth weight preterm infants. The estimated mean fetal RBC production rate scaled by body weight was 1.07×10^7 RBCs/day/g, and the mean RBC lifespan at birth was 52.1 days; these values are consistent with reported values. The *in utero* RBC lifespan increased at a rate of 0.51 days per day of gestation. We conclude that the proposed mathematical model and its implementation provides a flexible framework to study *in utero* non-SS fetal erythropoiesis in newborn infants.

2.2 INTRODUCTION

Erythropoietic status in newborn infants is determined by two phases, the fetal erythropoiesis before birth and neonatal erythropoiesis after birth. The former is important in understanding the mechanisms involved in red blood cell (RBC) production in the hypoxic intrauterine environment, while the latter provides a better understanding of the newborn infant's ability to compensate for the expected decline in post-natal erythropoietic activity that predictably results from the increased oxygen availability and resulting down regulation of erythropoietin synthesis and release (33-35). In anemic preterm infants, the latter also provides information about the critically ill infant's ability to compensate for blood loss due to the multiple clinical phlebotomies that commonly results from required neonatal care. Determining the postnatal erythropoiesis rate would also help in assessing and improving efficacy of strategies such as erythropoietin therapy that are aimed at reducing or eliminating RBC transfusions.

The RBCs present at birth have been formed during the latter part of fetal life (36). During this period, the fetus experiences rapid growth and as a result, the rate of RBC production is high (37). The progressive increase in hemoglobin (Hb) concentration and erythrocyte content in whole blood that is observed during the course of intrauterine development provides ample evidence that the RBC production increases with time *in utero*, leading to high Hb values at birth (21, 38, 39).

Past studies indicate that the RBC lifespan of a term newborn infant is only about two thirds (60 to 80 days) that of a healthy adult (40). Further, RBC lifespan is even shorter in preterm infants than in full term infants and appears to decrease with

birth weight (34). Likewise, in ovine fetuses, the fetal RBC lifespan increases with GA and is less than that in the adult (41). Since the Hb level at any time is dependent on both the production and the survival of RBCs, a better understanding of the intrauterine changes in fetal RBC lifespan is crucial in evaluating fetal erythropoiesis in newborn infants.

Several studies have utilized human fetal RBCs isolated from either umbilical cord or placentas at delivery to study *in utero* erythropoiesis (34, 36, 42, 43). These studies primarily focused on estimating the fetal RBC lifespan and typically comparing fetal RBC lifespan to adult RBC lifespan. However, these studies did not evaluate the non-steady state (non-SS) conditions under which the cord blood RBCs were produced. This simplification may have confounded previous conclusions. Because cord blood RBCs were formed during the latter part of fetal life and their survival is lifespan-based, the mathematical analysis of the survival curves of these RBCs provide a unique opportunity to "look backward in time" for evaluating the phase of fetal erythropoiesis in newborn infants.

The objectives of the present study are the following: 1) to present a novel method that utilizes cord blood or infant blood RBCs collected within the first days after birth to study both the non-SS *in utero* RBC production and the changes in *in utero* RBC lifespan over time; and 2) to apply this method to *in vivo* RBC disappearance curves of umbilical cord RBCs from critically ill very low birth weight (VLBW) preterm infants tracked via a biotin label.

2.3 MATERIALS AND METHODS

2.3.1 The mathematical model

Intrauterine growth: Intrauterine growth was estimated using the birth weight as a function of GA data of Arbuckle et al (44). These data from over one million live births represents one of the largest live birth data sets available that includes GA (44). The 50th birth weight percentile-GA data was digitally extracted for male singleton, female singleton, male twin and female twin live births. A fourth order polynomial function, which provided the best fit to each dataset, was used to model the change in birth weight with GA (Equation 2.1 and Figure 2.1). It was assumed that the data generated from the birth weights of preterm infants was representative of intrauterine growth of fetuses remaining *in utero* up to the time of birth. For GA less than that included in this data set (i.e., less than 154 days), an exponential function (Equation 2.1 and Figure 2.1) was used for estimating intrauterine growth. The *in utero* body weight, *BW(GA)*, can be expressed as (Figure 2.1):

$$BW(GA) = \begin{cases} A \cdot GA^4 + B \cdot GA^3 + C \cdot GA^2 + D \cdot GA + E & GA > 154 \\ M \cdot (e^{\gamma \cdot GA} - 1) & 0 < GA \leq 154 \end{cases} \quad (2.1)$$

where *GA* is the gestational age of the infant measured in days and *A, B, C, D, E, M, γ* are parameters listed in Table 2.1.

In utero erythropoiesis rate**:** The *in utero* erythropoiesis rate, *R(t)*, is considered to be proportional to the body weight and accordingly is expressed as:

$$R(t) = k \cdot BW(t + GA) \qquad t \leq 0 \qquad (2.2)$$

where *BW(t)* is the *in utero* body weight of the infant at time *t*, *k* is a scaling/proportionality factor, and *t* is the time relative to the time of birth (*t=0*). At the time of birth, i.e., when *t=0*, *R(0)* will be proportional to *BW(GA)*, and for any time *t* thereafter, *R(t)* will be proportional to the infant body weight at time *t+GA*. For the specific case that assumes a lifespan-based disposition with a fixed *in utero* RBC lifespan (i.e., no change in *in utero* RBC lifespan with time), the number of RBCs produced *in utero* present at the time of birth is given by:

$$n(0) = \int_{-L(0)}^{0} R(t)\, dt \qquad (2.3)$$

In equation 2.3, the *in utero* RBC production rate is integrated from *–L(0)* to zero, where *L(0)* represents the fixed fetal RBC lifespan that is also equal to the RBC lifespan at the time of birth (*t*=0). To consider the more complex case of variable *in utero* RBC lifespan, the lower integration limit in equation 2.3 has to be modified. Previously published data in ovine fetuses indicate that the fetal RBC lifespan increases approximately linearly with GA (41). Considering a similar case in humans, the linear change in *in utero* fetal RBC lifespan with time, can be expressed as:

$$L(t) = L(0) + \alpha t \qquad t \leq 0 \qquad (2.4)$$

where α is the slope parameter that describes the rate of change in fetal RBC lifespan with time. Let *x* be defined as an intermediate variable defining the integration limits for the number of RBCs produced *in utero*, which are eliminated after birth, obtained as follows:

$$x = t - L(x) \qquad x \leq 0 \qquad (2.5)$$

From equations 4 and 5, we get:

$$x = t - L(0) - \alpha x \qquad (2.6)$$

$$x = \frac{t - L(0)}{1 + \alpha} \qquad (2.7)$$

Equation 2.7 defines the integration limits in the case of a linear change in *in utero* RBC lifespan. Equation 3 then becomes (Figure 2.2):

$$n(0) = \int_{\frac{-L(0)}{1+\alpha}}^{0} R(t)\, dt \qquad (2.8)$$

Furthermore, the number of RBCs produced *in utero* that are removed from circulation up until time *t* after birth, is then given by (Figure 2.2):

$$n_E(t) = \int_{\frac{-L(0)}{1+\alpha}}^{\frac{t-L(0)}{1+\alpha}} R(u)\, du \qquad (2.9)$$

The number of RBCs that were produced *in utero* that remain in circulation after birth at time *t*, can then be calculated as:

$$n(t) = n(0) - n_E(t) \qquad (2.10)$$

where *n(0)* is the number of RBCs produced *in utero* that are present at the time of birth. If a small fraction of these RBCs are removed, labeled and reinfused back into the same infant, then:

$$n_L(t) = F_L \cdot n(t) \qquad (2.11)$$

where $n_L(t)$ is the number of labeled RBCs produced *in utero* that remain in circulation after birth at time *t*, and F_L is the fraction of labeled RBCs relative to the total number of RBCs present. The amount of Hb present in the labeled RBCs produced *in utero* that remain in circulation after birth at time *t* can be given as:

$$Hb_L(t) = MCH \cdot n_L(t) \qquad (2.12)$$

where *MCH* is the mean corpuscular hemoglobin of the RBCs. Substituting equations 2.1, 2.2, 2.8-2.11 in equation 2.12, and integrating, the final model to calculate the amount of Hb present in labeled RBCs produced *in utero* and remaining in circulation after birth at time *t* can be given as:

$$Hb_L(t) = \begin{cases} F_L \cdot MCH \cdot k \cdot \left[\frac{M}{\gamma} \cdot S_1(t) + M \cdot S_2(t) + S_3\right] & t \le (p - GA)(1+\alpha) + L(0) \\ F_L \cdot MCH \cdot k \cdot S_4(t) & L(0) \ge t > (p - GA)(1+\alpha) + L(0) \end{cases} \qquad (2.13)$$

where p=154 days (Equation 2.1) and,

$$S_1(t) = e^{\gamma \cdot p} - e^{\gamma \cdot \left(GA + \frac{t - L(0)}{1+\alpha}\right)} \qquad (2.14)$$

$$S_2(t) = GA - p + \frac{t - L(0)}{1+\alpha} \qquad (2.15)$$

$$S_3 = \begin{cases} \frac{A}{5} \cdot (GA^5 - p^5) + \frac{B}{4} \cdot (GA^4 - p^4) + \frac{C}{3} \cdot (GA^3 - p^3) \\ \quad + \frac{D}{2} \cdot (GA^2 - p^2) + E \cdot (GA - p) & GA \ge p \\ 0 & GA < p \end{cases} \qquad (2.16)$$

$$S_4(t) = \frac{A}{5} \cdot \left(GA^5 - \left(GA + \frac{t - L(0)}{1 + \alpha} \right)^5 \right) + \frac{B}{4} \cdot \left(GA^4 - \left(GA + \frac{t - L(0)}{1 + \alpha} \right)^4 \right) + \frac{C}{3} \cdot \left(GA^3 - \left(GA + \frac{t - L(0)}{1 + \alpha} \right)^3 \right) + \frac{D}{2} \cdot \left(GA^2 - \left(GA + \frac{t - L(0)}{1 + \alpha} \right)^2 \right) + E \cdot \left(\frac{L(0) - t}{1 + \alpha} \right) \quad (2.17)$$

It is assumed that the disposition of Hb/RBCs was lifespan based (i.e., RBCs were removed from circulation through cellular aging/senescence) (45-48). The model also assumed a single point distribution of RBC lifespans, i.e., RBCs produced at a given time *in utero*, have the same RBC lifespan.

Accurately accounting for phlebotomies in the analysis

Newborn infants are subjected to multiple phlebotomies for clinical testing purposes; accordingly, equation 2.13 must be modified to accurately account for the perturbations in the Hb level caused by the phlebotomies. We accounted for the loss of labeled RBCs from circulation as previously described (49-51). Details of the phlebotomy correction are described in Appendix A.

2.3.2 Subjects

Ten VLBW preterm infants between 24 and 28 weeks gestation being cared for in the Neonatal Intensive Care Unit (NICU) at the University of Iowa Children's Hospital were enrolled in this study. The study was approved by the University of Iowa Human Subject Internal Review Board. All subject's parents or legal guardians provided written informed consent as part of an ongoing consent process. Inclusion

criteria included treatment with expectation of survival and moderate to severe respiratory distress requiring mechanical ventilation. Exclusion criteria included hematological diseases (except for anemia associated with phlebotomy loss and prematurity), alloimmune hemolytic anemia, diffuse intravascular coagulation, thrombosis, and transfusion requirements that were emergent and did not allow controlled sampling.

2.3.3 Biotinylation of RBCs and FACs analysis

The measurement of red cell survival using RBCs labeled with biotin (BioRBCs) is practical, reliable, accurate and safe (31, 52, 53). RBCs were labeled with biotin as previously described (31, 52). Briefly, RBCs from study subjects were washed twice and prepared at 25% hematocrit (Hct). The biotinylation reagent sulfo NHS-biotin was dissolved, and used to label RBCs at a discrete biotin density. After a 30 minute reaction, the BioRBCs were washed twice, filtered and transfused. The percent of BioRBCs in post-transfusion blood samples was determined by flow cytometric enumeration after staining with Avidin conjugated with Alexa Fluor 488as previously described (31, 52). Cord blood/infant autologous RBCs were biotinylated as described earlier and reinfused back into the same infant. Each infant received only a single BioRBC transfusion during the study period. All BioRBC transfusions were administered within the first two days of life.

2.3.4 BioRBC survival

Cord blood RBCs collected at the time of birth are comprised entirely of fetal RBCs. Autologous infant RBCs collected very close to time of birth also are comprised

almost entirely of fetal RBCs. RBC production falls several fold after birth and thus, the number of RBC produced after birth within the first 2 days is only a very small fraction compared to the total number of fetal RBCs that are present in the infant at the time of birth. Because of this and due to the limited number of infants that receive cord blood RBC transfusions, this analysis also included infants that receive RBC transfusions from autologous RBCs taken within first 2 days of birth.

Phlebotomy blood samples from birth through the end of the BioRBC study period were weighed and recorded immediately after collection. The blood collection tube weights were subtracted from the total weights and converted to the volume of blood removed based on a specific gravity of blood of 1.05 (54). The Hb mass removed with each phlebotomy was calculated by multiplying the volume of blood removed times the Hb concentration measured at the time of blood sampling. In addition to the BioRBC transfusion, the infants also received additional unlabeled RBC transfusions at various times based on severity of anemia. The decision to treat with RBC transfusions was made by the physician in accordance with NICU guidelines (55). The volume of packed RBCs administered (85% Hct) and the time of RBC transfusions were recorded for use in the analysis. The *MCH* parameter was set equal to 37.5 pg/cell based on previous estimates (49, 50).

2.3.5 Data analysis

The amount of Hb present in BioRBCs over time, $Hb_L(t)$ (Equation 2.13), was modeled instead of the total number of BioRBCs over time, $n_L(t)$ (Equation 2.11). Flow cytometric analysis of BioRBCs is an enumeration technique. At each BioRBC sampling time, the fraction of BioRBCs relative to the total number of RBCs in the

sample can be accurately estimated. To calculate the total absolute number of BioRBCs in the infant circulation at a particular sampling time, $n_L(t)$ (Equation 2.11), this fraction has to be multiplied by the total number of infant RBCs in circulation at that sampling time. In this study, the total number of RBCs in circulation could not be measured at the time of each BioRBC sample. Instead, the Hb concentration measurements available for each BioRBC sampling times was used to estimate the absolute amount of Hb present in the BioRBCs in infant circulation over time (Equation 2.13).

All modeling and simulations were conducted using WINFUNFIT, a Windows (Microsoft) version evolved from the general nonlinear regression program FUNFIT (56), using ordinary least squares fit to each individual subject's Hb amount-time profile. To characterize the uncertainty in the estimates of the individual subject parameters, the standard deviation (SD) and the percent coefficient of variation (CV %) of the estimates were calculated for each parameter.

2.4 RESULTS

2.4.1 Subject characteristics

The mean GA of the 10 newborn subjects was 180.7 days (range, 162 to 194). The mean birth weight was 0.815 kg (range, 0.564 to 1.250 kg). Three males (all singletons) and seven females (six singletons and one twin) were studied. Of the 10 infants, three received cord blood BioRBCs and seven received biotin labeled autologous RBCs drawn within the first two days of birth. The infants underwent an average of 142 phlebotomies (range, 50 to 271). The average number of RBC transfusions was 4.7 (range, 1 to 12). For all transfusions administered, the volume of packed RBCs (85% Hct) administered was 15 mL/kg.

2.4.2 Model simulations

Figure 2.3 shows the effect of varying individual parameters (α, k and $L(0)$) and the effect of multiple clinical phlebotomies on the model predicted BioRBC survival curves. Figure 2.3A shows the effect of varying α, the slope associated with the rate of change in fetal RBC lifespan (Equation 2.4), on the simulated BioRBC survival curve with all other model parameters fixed at specified values: $L(0)$=80 days; k=0.60x10^8 RBCs/day/g; no clinical phlebotomies. Similarly, Figure 2.3B shows the effect of varying $L(0)$, the RBC lifespan at the time of birth, on the simulated BioRBC survival curve with fixed parameters of α=0, k=0.60x10^8 RBCs/day/g, and no clinical phlebotomies. Figure 2.3C shows the effect of multiple clinical phlebotomies on the simulated BioRBC survival curve with fixed parameters of $L(0)$=80 days, k=0.60x10^8 RBCs/day/g and α=0. Finally, the effect of varying k, the scaling factor associated with

the fetal RBC production rate, on the model predicted BioRBC survival curve is depicted in Figure 2.3D with fixed parameters of α=0, $L(0)$=80 days, and no clinical phlebotomies.

2.4.3 Model fit to infant data

The model (Equation 2.13) fit to the Hb amount-time profiles, along with the cumulative amount of Hb removed for four representative subjects are displayed in Figure 2.4. General agreement between the model fit and the Hb amount data was observed. The estimates of the parameters are summarized in Table 2.2.

2.5 DISCUSSION

This study introduces a novel method for utilizing the *in vivo* disappearance of cord blood RBCs of newborn infants labeled *ex vivo* to evaluate fetal erythropoiesis. Although RBC survival curves have been used previously for estimating fetal RBC lifespan, the use of these data for evaluating fetal erythropoiesis has not been previously described.

2.5.1 Model simulations

The effect of individual parameters (α, k and $L(0)$) and the effect of multiple clinical phlebotomies on the model predicted BioRBC survival curves are displayed in Figure 2.3. Parameter α describes the rate of change (slope) in fetal RBC lifespan with time (Equation 2.4). When α is zero (Figure 2.3A), the fetal RBC lifespan is fixed *in utero*, i.e., the fetal RBC lifespan does not change with development during this time. When α is negative, the model assumes that the fetal RBC lifespan decreases with fetal development and approaches $L(0)$, the lifespan at time of birth (t=0). Finally, when α is positive, the model assumes that the fetal RBC lifespan increases with fetal development and approaches the RBC lifespan at the time of birth. The last case is likely the most physiologically relevant because previous studies are consistent with the inference that infant RBCs have shorter lifespans than those of healthy adults and that RBC lifespan is shorter in preterm infants than in term infants (34, 40, 41).

$L(0)$ describes the RBC lifespan at the time of infant birth (t=0). Since the model describes the survival of cord blood RBCs, the RBCs produced at the time of birth are the youngest RBCs in the collected cord blood sample. When this sample is labeled and

reinfused back into the same infant, the youngest RBCs will remain in the circulation the longest. Thus, *L(0)* represents the time the youngest RBCs in the population will survive. For example, if *L(0)* was 80 days, then the youngest labeled cord blood RBCs will be removed from circulation 80 days after these cells where introduced into the circulation. Beyond 80 days, none of the labeled RBCs will be present in infant circulation. Thus, *L(0)* determines the end point of the RBC survival curve (Figure 2.3B).

Newborn infants, especially critically ill preterm anemic low birth infants, are subjected to multiple clinical blood sampling as part of their routine care and management. Any phlebotomy following the transfusion of labeled RBCs will perturb the RBC survival curve. This perturbation becomes increasingly significant as infants are subjected to more and more phlebotomies. Each phlebotomy removes a certain fraction of the labeled RBCs from the circulation resulting in a decline in RBC survival curve. As shown in Figure 2.3C, the effect of 109 clinical phlebotomies on labeled RBCs significantly affected the shape of the model predicted RBC survival curve.

Finally, *k* is the scaling parameter that relates the *in utero* growth of the infant to the rate of fetal erythropoiesis (Equation 2.2). When all the other factors in the model are kept the same (i.e., same GA, gender, singleton/twin), a larger value of *k* indicates that the infant has a higher fetal erythropoiesis rate as compared to an infant with lower *k* value (Figure 2.3D).

2.5.2 Model fit to infant data

The applicability of the model has been described using an example data set of cord blood/autologous RBCs from ten VLBW preterm infants that were labeled with biotin. The model successfully described the elimination of cord blood/autologous BioRBCs in these infants (Figure 2.4) and accounted for: 1) all blood removed and transfused; and 2) an increase in body weight due to infant growth and blood volume expansion.

A positive value for α indicates that the fetal RBC lifespan increases with fetal development. In the present study, the mean model estimate for α of 0.5076 (Table 2.2) suggests that fetal RBC lifespan increased at a rate of ~0.51 day/day gestation in the 10 subjects, and is similar to what has been previously reported in ovine fetuses (41).

The mean RBC lifespan at the time of birth, *L(0)*, was 52.06 days (Table 2.2). This is similar to the previous range of RBC lifespan estimates of 35 to 50 days based on ^{51}Cr labeled RBCs (57). As expected, the estimated infant RBC lifespans were shorter than reported adult RBC lifespan of 120 days (58). The reduced fetal RBC lifespan as compared to adult RBC lifespan may be due the differences in the conditions under which these RBCs were produced. RBCs in healthy adults are produced under steady state conditions. In contrast, fetal RBCs are produced under hypoxic intrauterine conditions during a period of rapid increase in the number of circulating RBCs. This results in a forced accelerated maturation of fetal RBCs and thus resembles "stress erythropoiesis" of later life.

The mean body weight scaled fetal RBC production rate, *k*, of $1.07x10^7$ RBCs/day/g (which corresponds to an erythropoiesis rate of $1.07x10^{10}$ RBCs/day in a 1000 g infant) was similar to previously reported *in utero* Hb stimulation rate of 0.414 $g/day.kg^{3/4}$ (which corresponds to $1.104x10^{10}$ RBCs/day in a 1000 g infant) (49). The estimated *in utero* erythropoiesis rate was also approximately three fold higher than the RBC production rate after birth (49). Previous studies of bone marrow, reticulocytes and iron kinetics has unequivocally substantiated this drop in erythropoiesis after birth (21).

Clinical Significance

Due to practical and ethical concerns with fetal blood sampling, there is very limited information available on the dynamic changes associated with *in utero* RBC production. This study introduces a novel method to utilize cord blood RBCs collected at the time of birth to look "backward in time" to evaluate fetal erythropoiesis. The proposed model can be used to study fetal erythropoiesis under non-steady state conditions while also accounting for *in utero* changes in fetal RBC lifespan. Given the gestational age of a newborn infant, this model can be used to estimate the RBC production rate at the time of birth and also to estimate how soon the RBCs present at the time of birth are removed from infant circulation after birth. The data derived from this study are clinically relevant in that they enhance understanding fetal and neonatal anemia, and can help to guide the evaluation of therapeutic interventions in the future.

Limitations of the model

The proposed model assumes that the fetal RBC lifespan varies linearly with GA. Although this assumption is based on previously published studies in ovine fetuses (41), it has yet to be verified in humans. Due to obvious ethical and regulatory concerns with fetal sampling, there is limited information regarding the intrauterine changes in human fetal RBC lifespan. The aim of the proposed model was to introduce a novel approach of utilizing cord blood RBC survival data to better understand fetal erythropoiesis. A similar model to that which we proposed here can be derived for non-linear intrauterine changes in RBC lifespan with gestation. The model also assumed an exponential function to describe intrauterine growth of the fetus before 154 days gestation (Equation 2.1 and Figure 2.1). Further experimental evidence is needed to validate this assumption.

The model also assumes that the fetal erythropoiesis rate is a function of the *in utero* growth of the fetus. During the latter part of fetal life, the fetus grows rapidly and there is a rapid increase in fetal weight. This increases the demand for oxygenation of the fetal tissues and must be met by a proportional increase in *in utero* RBC production rate. Further experimental evidence is needed to validate this assumption.

2.6 CONCLUSION

The present study for the first time demonstrates a novel and versatile method for utilizing labeled cord blood RBCs of newborn infants to study non-SS fetal erythropoiesis. This method also accounts for changes in fetal RBC lifespan with GA. The model was successfully applied to cord blood/autologous BioRBC survival data from 10 VLBW preterm anemic infants. The estimated parameters of the model were consistent with previously reported literature values, further supporting the utility of this model. Future investigations that study a greater number of infants encompassing a greater GA spectrum are needed to allow for identification of associations between rate of fetal erythropoiesis and important factors influencing fetal erythropoiesis and fetal RBC lifespan.

Table 2.1. Parameter estimates obtained by fitting Equation 2.2 to birth weight-GA data extracted from Arbuckle et al (44).

	Male, Singleton	**Female, Singleton**	**Male, Twin**	**Female, Twin**
A ($g \cdot day^{-4}$)	-1.21×10^{-5}	-1.60×10^{-5}	-9.09×10^{-6}	-1.28×10^{-5}
B ($g \cdot day^{-3}$)	9.37×10^{-3}	1.28×10^{-2}	6.72×10^{-3}	1.01×10^{-2}
C ($g \cdot day^{-2}$)	-2.53	-3.66	-1.73	-2.87
D ($g \cdot day^{-1}$)	3.0×10^{2}	4.61×10^{2}	1.98×10^{2}	3.67×10^{2}
E (g)	-1.30×10^{4}	-2.15×10^{4}	-8.40×10^{3}	-1.78×10^{4}
M (g)	46.8	30.7	25.6	13.6
γ (day^{-1})	1.65×10^{-2}	1.86×10^{-2}	1.97×10^{-2}	2.27×10^{-2}

Table 2.2. Estimated parameter summary from the Hb mass model (n = 10).

	α (days/day)	$L(0)$ (day)	k (x10^7 RBCs/day/g)
Mean	0.508	52.1	1.07
SD	0.065	10.8	0.51
CV (%)	12.8	20.7	47.7

SD: Standard Deviation

CV: Coefficient of Variation

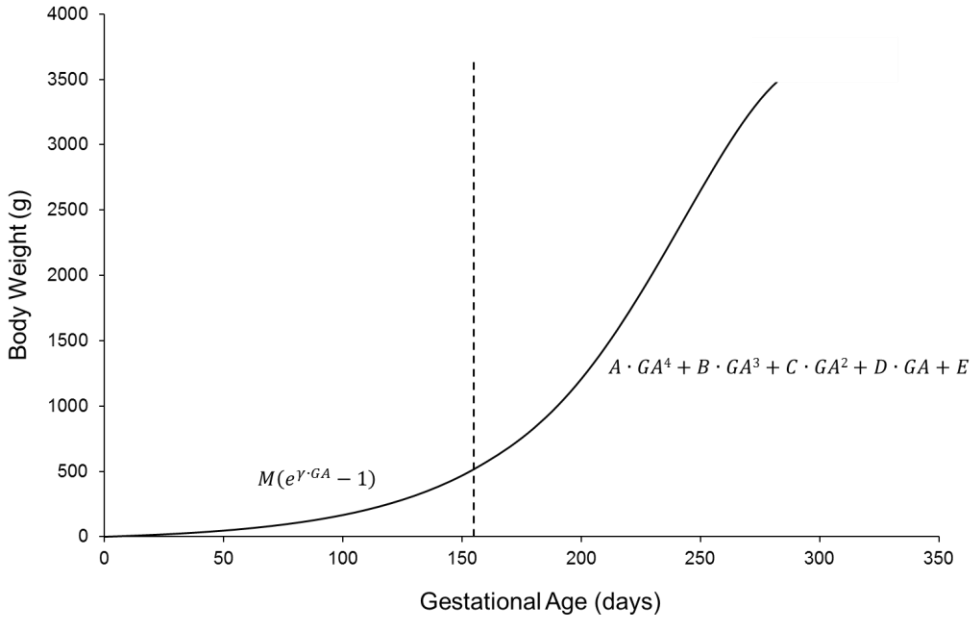

Figure 2.1. Intrauterine growth of newborn infants. The birth weight vs. GA data (50th birth weight percentile) extracted from Arbuckle et al (44) was used to approximate the intrauterine growth for male singleton, female singleton, male twin and female twin infants. For GA greater than 154 days (dashed line), each data set was separately fitted with a fourth order polynomial function (Equation 2.1). For GA less than that included in this birth cohort, i.e., less than 154 days, an exponential function (Equation 2.1) was used for extrapolation of intrauterine growth. The values of parameters *A, B, C, D, E, M, γ* are listed in Table I.

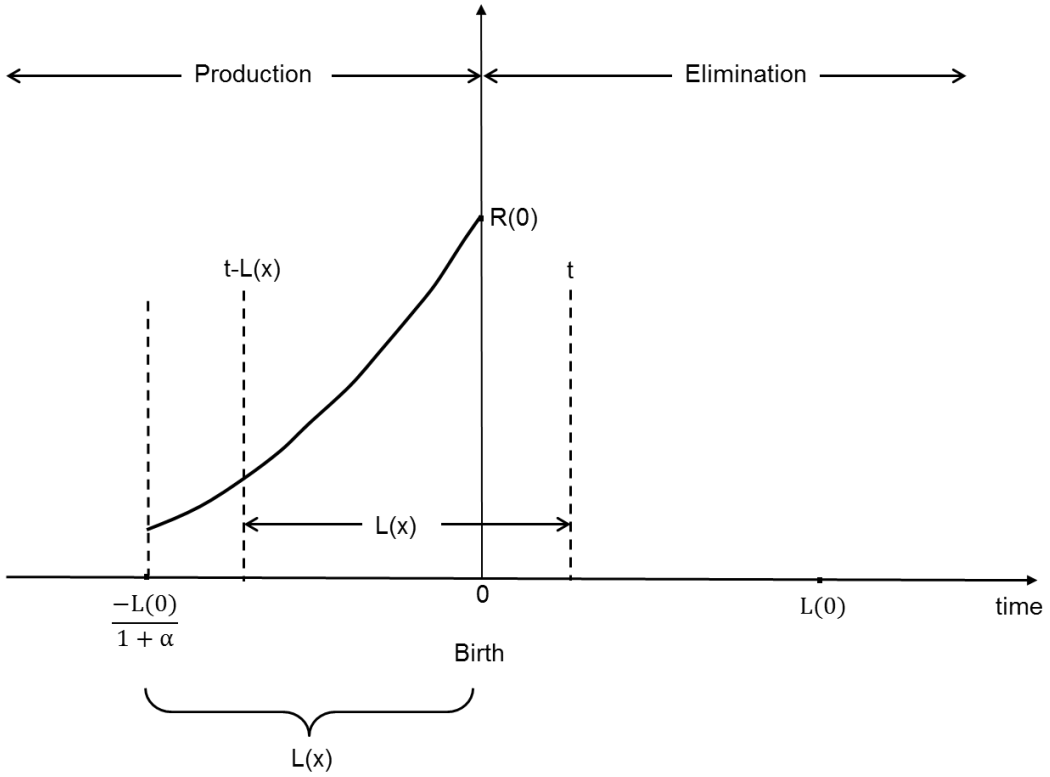

Figure 2.2. Non-SS fetal erythropoiesis in newborn infants. The solid line represents the changes in fetal erythropoiesis rate up to the time of birth. *R(0)* represents the RBC production rate at time of birth (*t*=0). The fetal erythropoiesis rate is proportional to the *in utero* infant body weight (Equation 2.2). Fetal RBC lifespan, *L(x)*, varies linearly with time (Equation 2.3).

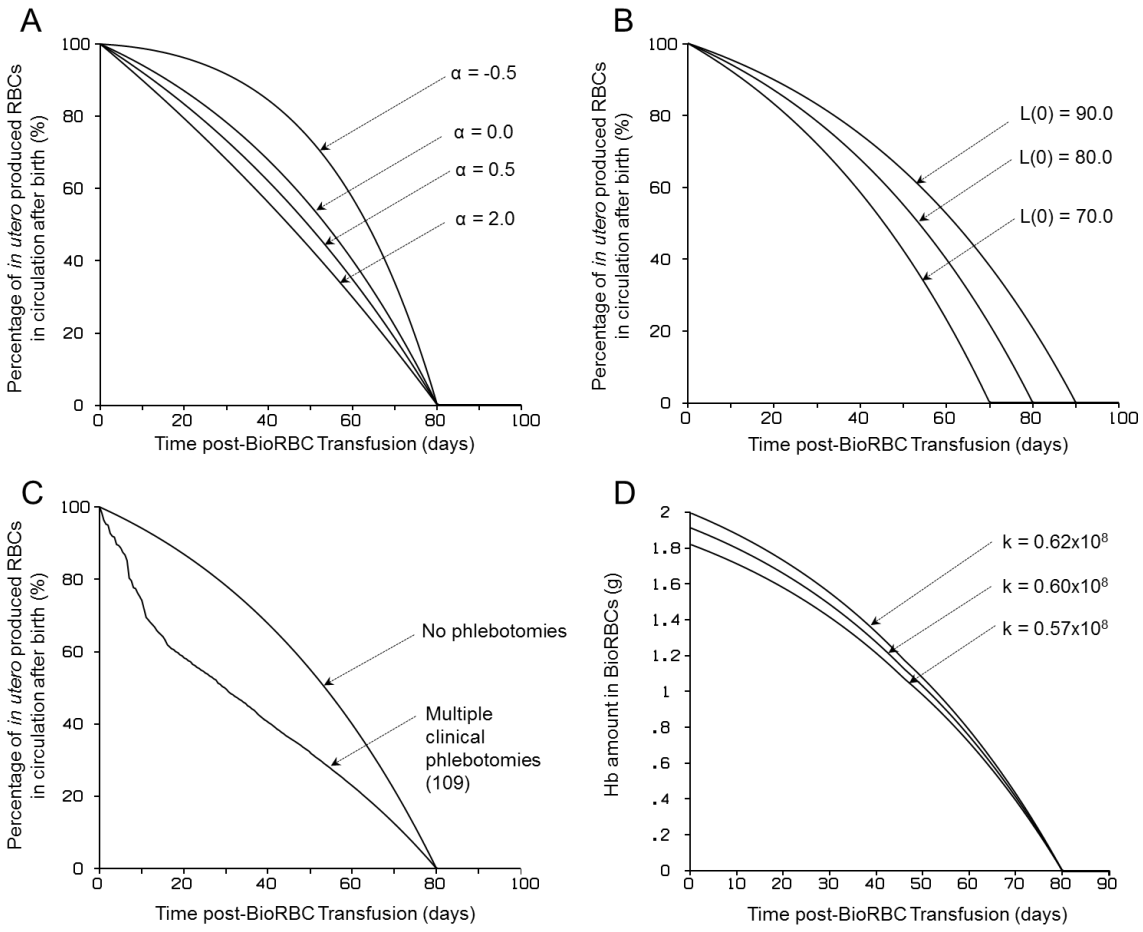

Figure 2.3. Model simulated cord blood RBC survival curves. Figure 2.3A shows the effect of varying α (Equation 2.5), on the simulated RBC survival curve. All other model parameters were fixed at specified values ($L(0)$=80 days, k=0.60x10^8 RBCs/day/g with no clinical phlebotomies). Figure 2.3B shows the effect of varying $L(0)$ on the simulated RBC survival curve (α=0.0, k=0.60x10^8 RBCs/day/g with no clinical phlebotomies). Figure 2.3C shows the effect of multiple clinical phlebotomies on the simulated RBC survival curve ($L(0)$=80 days, k=0.60x10^8 RBCs/day/g and α=0.0). The effect of varying k on the model predicted RBC survival curve can be observed in Figure 2.3D (α=0.0, $L(0)$=80 days with no clinical phlebotomies).

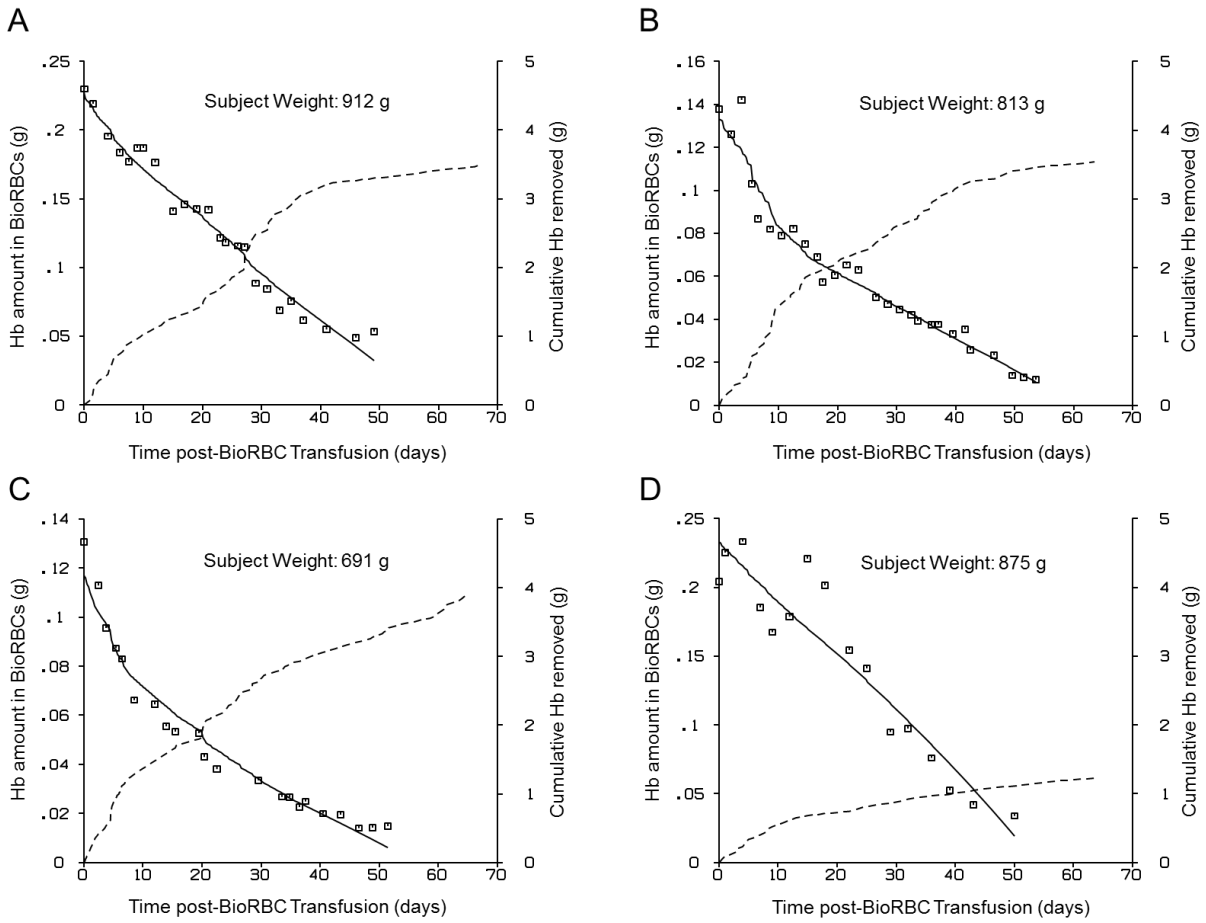

Figure 2.4. Model fit to Hb amount-time data for four subjects. The open squares represent Hb amount data points and the solid line shows the model fit (Equation 2.10). The dashed line represents the cumulative amount of Hb removed from the infant during the same time interval.

CHAPTER 3. A MASS BALANCE-BASED SEMIPARAMETRIC APPROACH TO EVALUATE NEONATAL ERYTHROPOIESIS

3.1 ABSTRACT

Post-natal hemoglobin (Hb) production in anemic preterm infants is determined by several factors including the endogenous erythropoietin levels, allogeneic RBC transfusions administered to treat anemia and developmental age. As a result, their post-natal Hb production rate can vary considerably. This work introduces a novel Hb mass balance-based semiparametric approach that utilizes infant blood concentrations of Hb from the first 30 post-natal days to estimate the amount of Hb produced and the erythropoiesis rate in newborn infants. The proposed method has the advantage of not relying on specific structural pharmacodynamic model assumptions to describe the Hb production, but instead utilizes simple mass balance principles and nonparametric regression analysis. The developed method was applied to the Hb data from 79 critically ill anemic very low birth weight preterm infants to evaluate the dynamic changes in erythropoiesis during the first month of life and to determine the inter-subject variability in Hb production. The estimated mean (± SD) cumulative amount of Hb produced by the infants over the first month of life was 6.6 ± 3.4 g (mean body weight: 0.768 kg), and the mean estimated body weight-scaled Hb production rate over the same period was 0.23 ± 0.12 g/d/kg. A significant positive correlation was observed between infant gestational age and the mean body weight-scaled Hb production rate of the infant over the first month of life ($P<0.05$). We conclude that the proposed mathematical approach and its implementation provides a flexible framework to evaluate post-natal erythropoiesis in newborn infants.

3.2 INTRODUCTION

All newborn infants experience a decline in blood concentrations of hemoglobin ("Hb levels" hereafter) during the first weeks of life. In healthy term infants, this post-natal drop in Hb levels is well tolerated, does not require therapy, and is commonly referred to as "physiological anemia of infancy" (1, 2, 59). In critically ill very low birth weight (VLBW, birth weight < 1500 g) and extremely low birth weight (ELBW, birth weight < 1000 g) preterm infants, the Hb levels falls to significantly lower levels than term infants, and often require one or more RBC transfusions as treatment for clinically significant "anemia of prematurity" (1, 2).

A major factor responsible for the post-natal decline in Hb levels is a substantial decline in the rate of erythropoiesis, especially during the first weeks of extra-uterine life (21). Previous studies of bone marrow histology, iron kinetics and peripheral blood reticulocyte concentrations are consistent with the observed post-natal decrease in Hb production (60-62). This decrease results from increased oxygen availability and resultant down regulation of erythropoietin (Epo) synthesis and release (33-35). After the first week of life, erythropoiesis continues at a low rate and the rate of Hb elimination exceeds its production (21).

In anemic VLBW and ELBW preterm infants, a better understanding of post-natal erythropoiesis is crucial in evaluating their ability to compensate for blood loss due to the multiple clinical phlebotomies that those who are critically ill require. Knowledge of their post-natal erythropoiesis rate would also help in evaluating the potential for enhancing erythropoiesis using strategies such as Epo administration with a goal of substantially reducing or eliminating RBC transfusions.

Previous studies of neonatal erythropoiesis assumed specific structural pharmacodynamic (PD) models to describe the regulation of post-natal Hb production rate over time (49, 50). In these models, the post-natal Hb production was assumed to be stimulated by Epo through a stimulation function. The stimulation function was related to plasma Epo concentrations by an E_{max} model (49, 50). While useful, this approach is highly dependent on defining the correct relationship between the plasma Epo levels and the Hb stimulation rate.

In this work, we introduce a new method that utilizes simple mass balance principles to calculate the amount of Hb produced after birth and subsequently utilize nonparametric cubic spline functions to estimate the post-natal Hb production rate. This novel approach does not assume any structural PD model to describe the Hb simulation rate or its functional relationship to the plasma Epo concentration. This results in a more robust and rational approach for evaluating post-natal erythropoiesis in newborn infants when compared to earlier methods.

The two specific objectives of the present study are: 1) to present a novel Hb mass balance-based semiparametric method that utilizes infant Hb data from the first 30 post-natal days ("month of life" hereafter) to evaluate the dynamic changes in post-natal erythropoiesis rate in newborn infants; and 2) to apply this method to Hb data from 79 critically ill VLBW and ELBW preterm anemic infants to estimate the cumulative amount of Hb produced, to study the changes in neonatal erythropoiesis rate during the first month of life, and to determine the inter-subject variability in post-natal Hb production.

3.3 METHODS

3.3.1 Subjects

Seventy-nine low birth weight preterm anemic infants (including 27 infants from our previous study (50)), less than 29 wk GA being cared for in the Neonatal Intensive Care Unit (NICU) at the University of Iowa Children's Hospital were enrolled in this study. The study was approved by the University of Iowa Human Subject Internal Review Board. For each subject, at least one parent or legal guardian provided written informed consent. Inclusion criteria included treatment with expectation of survival and respiratory distress requiring mechanical ventilation. Exclusion criteria included hematological diseases (other than anemia associated with phlebotomy loss and prematurity), diffuse intravascular coagulation, thrombosis, and transfusion requirements that were emergent and did not allow controlled sampling.

Clinically ordered laboratory phlebotomy blood samples from birth through the end of the first 30 post-natal days were weighed and recorded immediately after collection. The weight of the blood collection tube was subtracted from the total weight of tube and blood sample, and this blood sample weight was converted to the volume of blood removed based on the estimated specific gravity of whole blood of 1.05 (54). The Hb mass removed with each phlebotomy was calculated by multiplying the volume of blood removed by the Hb concentration measured at that time. The decision to treat with RBC transfusions was made by the physician in accordance with NICU guidelines (55). For all transfusions, the volume of packed RBCs administered (85% Hct) was 15 mL/kg.

3.3.2 Mass balance-based approach

The total Hb present at any time *t* after birth, $Hb_T(t)$, can be calculated as:

$$Hb_T(t) = Hb_B(t) + Hb_P(t) + Hb_{TR}(t) \qquad (3.1)$$

where $Hb_T(t)$ ≡ total amount of hemoglobin present in circulation at any time *t*; $Hb_B(t)$ ≡ amount of hemoglobin present at time of birth ($t = 0$) that are still present at time *t*; $Hb_P(t)$ ≡ amount of hemoglobin produced after birth that are still present in circulation at time *t*; $Hb_{TR}(t)$ ≡ amount of hemoglobin transfused after birth that are still present in circulation at time *t*. The disposition of Hb was assumed to be lifespan-based (i.e., based on removal of RBCs from the circulation through cellular aging/senescence) (45).

Hb present at time of birth that are still present at time t ($Hb_B(t)$)

Disposition of Hb present at birth in the absence of phlebotomies: The Hb present at the time of birth comes from RBCs produced *in utero* up until the time of birth (t=0). These RBCs are produced during a period of rapid fetal growth and stimulated erythropoiesis under hypoxic intrauterine conditions. In addition, RBC lifespan varies with gestational age (GA) at birth (i.e., the time between last menstrual period and day of delivery of an infant), and is less than that in healthy adults (40, 41). The mathematical model that accounts for both the non-steady state (non-SS) *in utero* RBC production and changing fetal RBC lifespan over time has been detailed in our previous work (63). In brief, the *in utero* body weight, $BW(GA)$, which increases rapidly over time, can be expressed as:

$$BW(GA) = \begin{cases} A \cdot GA^4 + B \cdot GA^3 + C \cdot GA^2 + D \cdot GA + E & GA > 154 \\ M \cdot (e^{\gamma \cdot GA} - 1) & 0 < GA \leq 154 \end{cases} \quad (3.2)$$

where *GA* is the gestational age at birth of the infant measured in days and *A, B, C, D, E, M, γ* are fixed parameters that were set equal to previously reported values (Table 2.1) (63). The *in utero* erythropoiesis rate, *R(t)*, is considered to be proportional to the *in utero* body weight and is expressed as:

$$R(t) = k \cdot BW(t + GA) \qquad t \leq 0 \quad (3.3)$$

where *BW(t)* is the *in utero* body weight at time *t*, *k* is the scaling/proportionality factor that relates the *in utero* growth to fetal erythropoiesis rate, and *t* is the time relative to birth (*t=0*). To account for *in utero* changes in RBC lifespan with advancing GA, the fetal RBC lifespan, *L(t)*, was assumed to vary linearly with time, and can be expressed as:

$$L(t) = L(0) + \alpha t \qquad t \leq 0 \quad (3.4)$$

where *α* is the slope parameter describing the rate of change in fetal RBC lifespan with time and *L(0)* represents the RBC lifespan at the time of birth (*t*=0).

The final model (derivation detailed in our previous work (63)) used in calculating the amount of Hb present in RBCs produced *in utero* and remaining in circulation after birth at time *t* can be given as:

$$Hb_B(t) = \begin{cases} MCH \cdot k \cdot \left[\frac{M}{\gamma} \cdot S_1(t) + M \cdot S_2(t) + S_3\right] & t \leq (p - GA)(1 + \alpha) + L(0) \\ MCH \cdot k \cdot S_4(t) & L(0) \geq t > (p - GA)(1 + \alpha) + L(0) \end{cases} \quad (3.5)$$

where *p*=154 days (Equation 3.2) and,

$$S_1(t) = e^{\gamma \cdot p} - e^{\gamma \cdot \left(GA + \frac{t - L(0)}{1+\alpha}\right)} \quad (3.6)$$

$$S_2(t) = GA - p + \frac{t - L(0)}{1 + \alpha} \quad (3.7)$$

$$S_3 = \begin{cases} \frac{A}{5} \cdot (GA^5 - p^5) + \frac{B}{4} \cdot (GA^4 - p^4) + \frac{C}{3} \cdot (GA^3 - p^3) + \frac{D}{2} \cdot (GA^2 - p^2) + E \cdot (GA - p) & GA \geq p \\ 0 & GA < p \end{cases} \quad (3.8)$$

$$S_4(t) = \frac{A}{5} \cdot \left(GA^5 - \left(GA + \frac{t - L(0)}{1 + \alpha}\right)^5\right) + \frac{B}{4} \cdot \left(GA^4 - \left(GA + \frac{t - L(0)}{1 + \alpha}\right)^4\right) + \frac{C}{3} \cdot \left(GA^3 - \left(GA + \frac{t - L(0)}{1 + \alpha}\right)^3\right) + \frac{D}{2} \cdot \left(GA^2 - \left(GA + \frac{t - L(0)}{1 + \alpha}\right)^2\right) + E \cdot \left(\frac{L(0) - t}{1 + \alpha}\right) \quad (3.9)$$

where *MCH* is the mean corpuscular Hb for the RBCs present at birth and was set as equal to 37.5 pg/cell (49, 50).

Disposition of Hb present at birth in the presence of phlebotomies: Newborn VLBW and ELBW preterm infants are subjected to substantial number of phlebotomies for clinical testing purposes; accordingly, Equation 3.5 must be corrected to accurately account for the perturbations in Hb levels caused by the phlebotomies. The loss of fetal RBCs from infant's circulation was accounted for by introducing a phlebotomy correction factor as previously described (49, 50, 63). Details of the phlebotomy correction are described in the Appendix.

Hb transfused after birth that are still present at time t ($Hb_{TR}(t)$)

Disposition of transfused Hb in the absence of phlebotomies: The Hb from multiple RBC transfusions (Hb_{TR}), were accounted for through superposition by adding the Hb mass transfused with transfusion and then accounting for the linear rate of decline of the transfused RBCs. This linear rate of decline arises from assuming normal hematologic steady-state conditions and a constant RBC life span in the adult RBC donor subjects (30). Thus, the behavior of the transfused RBCs is given by:

$$Hb_{TR}(t) = \sum_{j=1}^{NTR} Hb_{TRj}(t) \qquad Hb_{TR}(0) = 0 \qquad (3.10)$$

where *NTR* is the number of RBC transfusions and $Hb_{TRj}(t)$ represents the hemoglobin amount remaining at time *t* from the j^{th} transfusion and is given by:

$$Hb_{TRj}(t) = \begin{cases} \dfrac{F_T \cdot Hb_{TR}(t_j) \cdot (L_{TRj} + t_j - t)}{L_{TRj}} & t_j \le t \le t_j + L_{TRj} \\ 0 & otherwise \end{cases} \qquad (3.11)$$

where t_j is the time of the j^{th} transfusion, L_{TRj} is the lifespan of transfused RBCs from the j^{th} transfusion, and F_T is the fraction of transfused RBCs surviving immediately after the transfusion and was set equal to 0.875 (49, 50).

Disposition of transfused Hb in the presence of phlebotomies: The fraction of transfused Hb remaining after each phlebotomy and the phlebotomy correction factor are calculated and applied as described earlier. Although all RBC transfusions were

administered over a 3 to 4-h time period, the effect of the transfusion on the Hb mass was approximated assuming that the cells were administered as a bolus.

Hb produced after birth that are still present at time t ($Hb_P(t)$)

The hemoglobin produced after birth, $Hb_P(t)$, can then be calculated as:

$$Hb_P(t) = Hb_T(t) - Hb_B(t) - Hb_{TR}(t) \qquad (3.12)$$

where the $Hb_T(t)$ is the total hemoglobin amount present in circulation at time *t*, while the $Hb_B(t)$ and $Hb_{TR}(t)$ are calculated as described earlier.

In the absence of phlebotomies, Equation 3.12 can be used to calculate the absolute amount of Hb produced after birth. But in the presence of multiple phlebotomies, Equation 3.12 can only be used to calculate the absolute amount of Hb produced after birth that is remaining after phlebotomies. Hence, the net amount of Hb produced after birth (including the amount removed by phlebotomies), would be greater than that calculated from Equation 3.12. To account for the Hb removed due to phlebotomies, Equation 3.12 can be modified by incorporating the phlebotomy correction factor (Appendix A):

$$Hb_P(t) = \frac{Hb_T(t) - Hb_B(t) - Hb_{TR}(t)}{Phlebotomy\ Correction\ Factor} \qquad (3.13)$$

Equation 3.13 can be used to calculate the total amount of Hb produced from birth to the end of the study period. In this study, we assumed that the RBC lifespan of the produced RBCs was longer than the 30-day study period, (i.e., none of the RBCs produced after birth are removed from the circulation during the study period). Equation

3.13 can be then be used to calculate the cumulative amount of Hb produced after birth throughout the study period.

3.3.3 Iman conover regression fit

The Iman Conover regression is a well-established nonparametric method that makes use of the rank transform approach in regression (64). This method is particularly advantageous when the dependent variable is a monotonic function of the independent variable, even when the monotonic relationship is non-linear in nature (64). Since the cumulative Hb produced after birth is monotonically increasing with time, the Iman Conover regression fit to the estimated Hb-time data (Equation 3.13) was used for predicting the cumulative amount of Hb produced after birth over time.

3.3.4 Nelder-Mead Objective Function Minimization and Cubic Spline Fit

The cumulative amount of Hb produced after birth (Equation 3.13) is dependent on several parameters used to describe $Hb_B(t)$ and $Hb_{TR}(t)$. As described earlier, two separate models were used to describe the disposition of $Hb_B(t)$ and $Hb_{TR}(t)$. The disposition of Hb produced before birth that remains in circulation at any time *t* after birth (Eqs. 5-9), $Hb_B(t)$, is dependent on the following model parameters: $L(0)$, k and α. Similarly, for the transfused Hb (Eqs. 10-11), the disposition of the Hb administered for each transfusion, $Hb_{TR}(t)$, is dependent on the lifespan of the transfused RBCs, L_{TRj}. These parameters were optimized by minimizing the sum of the absolute value of the residuals as defined by the objective function:

$$Objective\ Function = \sum_{i=1}^{N} \left| Hb_P(t_i) - \widehat{Hb}_P(t_i) \right| \qquad (3.14)$$

where $Hb_P(t_i)$ is calculated as described in Equation 3.13, and $\hat{Hb}_P(t_i)$ is the predicted amount of Hb produced at time t_i from the regression fit to the data. The value of the objective function (Equation 3.14) was minimized by the Nelder-Mead simplex method (65).

The final predicted cumulative amount of Hb produced were then represented by a nonparametric cubic smoothing spline function (66). The Hb production rate (i.e., neonatal erythropoiesis rate) at any time during the first month of life was then evaluated as the first derivative of this cubic spline. Finally, the body weight-scaled Hb production rate was calculated during the first month of life by normalizing the estimated Hb production rate by the body weight at that time.

3.3.5 Data analysis

Data analyses were performed in R version 3.0.3 using the RStudio integrated development environment (67, 68). The Nelder-Mead objective function minimization, Iman Conover nonparametric regression and cubic spline fits were all conducted using WINFUNFIT, a Windows (Microsoft) version evolved from the general nonlinear regression program FUNFIT (56). A regression slope *t*-test (slope $\neq 0$) was used to evaluate the linear correlation observed between infant gestational age and the mean body weight-scaled Hb production rate over the first week and month of life. Statistical differences were considered to be significant for values of $P<0.05$.

The total blood volume was assumed to be proportional to the infant body mass. The total Hb amount present in infant circulation at any time *t*, $Hb_T(t)$, was estimated by multiplying the measured Hb concentration times the total blood volume at time *t*.

3.4 RESULTS

3.4.1 Subject characteristics

The mean GA of the 79 newborn subjects was 25.6 wk (range, 22.4 to 28.6 wk). Thirty-five males (27 singletons and 8 twins) and 44 females (35 singletons and 9 twins) were studied. The infants underwent an average of 104 phlebotomies (range, 36 to 215). The mean birth weight was 0.768 kg (range, 0.412 to 1.487 kg). The average number of RBC transfusions administered during the study period was 3.7 (range, 0 to 10).

3.4.2 Mass balance-based semiparametric approach

Figure 3.1 shows an overlay of $Hb_T(t)$, i.e., the total amount of Hb present in infant circulation at any time t after birth, and Hb_{TR} (t)+ $Hb_B(t)$, i.e., the sum of the transfused Hb (Eqs. 10-11) and the Hb produced in utero up to time of birth remaining in circulation at any time t after birth (Eqs. 5-9) for a representative infant study subject. The sum of $Hb_B(t)$ and $Hb_{TR}(t)$ was corrected to account for the loss of Hb due to multiple phlebotomies. The difference between the model predicted solid line and the individual data points provides an estimate of the cumulative amount of Hb produced by the infant during the first month of life, before accounting for the loss of Hb due to phlebotomies.

The Iman Conover regression fit to the calculated cumulative amount of Hb produced after accounting for the loss due to phlebotomies (Equation 3.13) is shown in Figure 3.2. Figure 3.3 shows the nonparametric cubic smoothing spline fit to $\hat{Hb}_P(t)$, the predicted cumulative amount of Hb produced at time *t* from the Iman Conover

regression fit to the data. The mean (± SD) parameter estimates obtained from the Nelder-Mead minimization of the objective function (Equation 3.14) were: $L(0)$ = 35.5 ± 12.8 d, $k = 1.70\times10^{7} \pm 0.65\times10^{7}$ RBCs/d/g, α = 0.83 ± 0.59 and L_{TR} = 51.6 ± 26.3 d.

The cumulative Hb produced during the first month of life, and the dynamic changes in body weight-scaled Hb production rate during the same period are displayed for four representative subjects in Figure 3.4. The results of the PD analysis utilizing the mass balance-based semiparametric method for the 79 low birth weight infants are summarized in Table 3.1. Finally, to test the influence of GA at birth on the post-natal Hb production, the mean body weight-scaled Hb production rate over the first week of life for all study subjects was plotted against their GA (Figure 3.5). A significant positive correlation was found between GA and the mean body weight-scaled Hb production rate over the first week of life ($P<0.05$). A similar significant positive correlation was also found between GA and the mean body weight-scaled Hb production rate over the first month of infant life ($P<0.05$).

3.5 DISCUSSION

In premature newborns, the relationship between Epo and Hb levels is complex. In addition to the impact of Epo on stimulating Hb production, there are several others factors including P_{50}, 2, 3- diphosphoglycerate, cardiac output, and mixed venous oxygen that also play a role in the Epo response (69-71). Attempts to develop a structured model to describe neonatal erythropoiesis should factor all these variables into the model to successfully evaluate the post-natal Hb production in these infants. Previous attempts to evaluate the PD of Epo focused solely on structured parametric PD models that related Hb production to the plasma Epo concentrations (45, 49, 50, 72, 73). Due to the inherent complexities associated with evaluating neonatal erythropoiesis, a nonparametric or semiparametric approach would be more suitable for this purpose.

This study introduces a mass balance-based semiparametric approach to evaluate the PD of Epo in newborn infants. The advantage of this approach over previously developed methods for describing neonatal erythropoiesis is that this approach does not *a priori* assume any specific structured PD model for describing post-natal Hb production. Instead, the cumulative amount of Hb produced over the first month of life is calculated by mass balance principles, and nonparametric methods (cubic spline) are utilized in evaluating the dynamic changes in Hb production rate over the first month of life.

As illustrated in Figures 3.1 and 3.2, the mass balance-based method permits successful estimation of the cumulative amount of Hb produced over the first month of life. The solid line in Figure 3.1 represents a prediction and is not a model fit. The estimated Hb data were fitted with the Iman Conover nonparametric regression to

determine the cumulative amount of Hb produced post-natally at any time during the first month of life (Figure 3.2). The Iman Conover regression ensures that cumulative amount of Hb produced is monotonically increasing with time. This is important because, by definition, the cumulative amount Hb produced cannot decrease with time. Furthermore, since the first derivative of the cumulative amount of Hb produced yields the post-natal Hb production rate, a hypothetical decrease in the cumulative amount of Hb produced would indicate a negative post-natal Hb production rate in the infant. This would be physiologically meaningless.

The predicted cumulative amount of Hb produced as obtained from the Iman Conover regression were fitted with a nonparametric cubic smoothing spline (Figure 3.3). In contrast to lower order polynomial functions, cubic smoothing splines have the advantage of being more flexible, and thus are better able to describe the local behavior of a curve. When studying neonatal erythropoiesis, especially in anemic VLBW and ELBW preterm infants, the rate of post-natal Hb production can vary considerably based on several factors including allogeneic RBC transfusions, endogenous Epo levels, and disease conditions. These dynamic changes in erythropoiesis are best captured by fitting the flexible cubic smoothing spline function as displayed in Figure 3.2b.

Using this approach, we were able to successfully estimate the post-natal Hb production rate in the newborn infants (Figure 3.4). We observed that the post-natal Hb production rate was high at birth and then dropped to lower levels within the first few days of life. After the first week, the Hb production rate remained at lower levels until the end of the one-month study period. This drop in post-natal Hb production after birth

is in agreement with previous reports and is consistent with the observed increase in oxygen availability and resultant down-regulation of Epo synthesis and release (33-35).

The estimated mean cumulative amount of Hb produced for the 79 infants (mean body weight: 0.768 kg) over the first month of life was 6.6 g (SD, 3.4) (Table 3.1) and is similar to previously reported value of 4.7 g (SD, 3.3) in our previously reported study of 14 preterm infants (49). The mean estimated body weight-scaled Hb production rate over the first month of life was 0.226 g/d/kg (SD, 0.119) and was below our previously reported maximum post-natal Hb production rate of 0.43 g/d/kg$^{3/4}$ (50).

Finally, the importance of GA of the newborn infant on the post-natal Hb production was investigated (Figure 3.5). It was observed that the mean body weight-scaled Hb production rate during the first week of life increased with GA. This is in agreement with our previous population PD study that identified GA as the most important covariate affecting the PD of endogenous Epo in anemic VLBW preterm infants (50).

Limitations of the study

The mass balance-based approach detailed in this work is a "semiparametric" approach that does not *a priori* assume any specific structured model to evaluate the amount of Hb produced after birth ($Hb_P(t)$). It does however, define two separate models to describe the disposition of Hb from the other two populations of RBCs present in the infant circulation (Equation 3.1). These two include the Hb produced before birth that remain in circulation after birth at time *t*, $Hb_B(t)$, and the Hb transfused to infants at any time during the study that remain in circulation at time *t*, $Hb_{TR}(t)$. The

disposition of Hb from these two RBC populations was modeled as described earlier using Eqs. 5-9 and Eqs. 10-11.

In this study, the measured Hb data demonstrated moderate variability of about 20%. Since the proposed method is based on mass balance principles, the Hb data measured in the infants should be as accurate and precise as possible. In future, with improved infant Hb measurements, this proposed method could be applied to even more accurately quantify post-natal Hb production in newborn infants.

Clinical Significance

Compared to healthy adults, erythropoiesis in newborns, especially VLBW and ELBW preterm anemic infants, has not been well studied. It is during the first month of life, when their severity of illness is usually at its peak, that anemic preterm infants experience a large number clinical phlebotomies, require multiple allogeneic RBC transfusions, and exhibit a rapid increase in body weight, all of which affects their ability to produce Hb. In this study, mass balance principles and nonparametric regression techniques were used to evaluate the dynamic changes in post-natal Hb production during the first month of life. Results from the present study of 79 anemic low birth weight preterm infants indicate that the GA of the infant is an important factor in determining the post-natal erythropoiesis in the infant. Infants with higher GA were able to produce greater amounts of post-natal Hb normalized for body weight, making the more mature infants less susceptible to developing anemia. Finally, the knowledge gained from this study on the dynamic changes in post-natal Hb production will be helpful in evaluating the potential treatment strategies for improvement of erythropoiesis, such as Epo therapy, aimed at reducing or eliminating RBC transfusions.

3.6 CONCLUSION

In summary, this work introduces a mass balance-based semiparametric approach that does not assume any structural PD model for describing the dynamic changes associated with neonatal erythropoiesis during the first month of life. Due to the inherent complexities associated with neonatal erythropoiesis in VLBW and ELBW preterm anemic infants, this more flexible approach that is based on fewer assumptions, offers a more direct and suitable way for evaluating the PD effect of Epo and other erythropoiesis stimulating agents compared to earlier methods. Future work with this approach includes studying the relationship between the Hb production rates and the plasma Epo concentrations and identifying covariates that in addition to GA have a significant effect on the post-natal Hb production in VLBW and ELBW infants.

Table 3.1 Summary of infant pharmacodynamic estimates from the Hb mass balance-based semiparametric method (n = 79).

	Cumulative amount of Hb produced during the first week of life (g)	**Cumulative amount of Hb produced during the first month of life** (g)	**Mean Hb production rate during the first week of life** (g/d/kg)	**Mean Hb production rate during the first month of life** (g/d/kg)
Mean	1.50	6.60	0.24	0.23
SD	0.72	3.44	0.12	0.12

SD: Standard Deviation

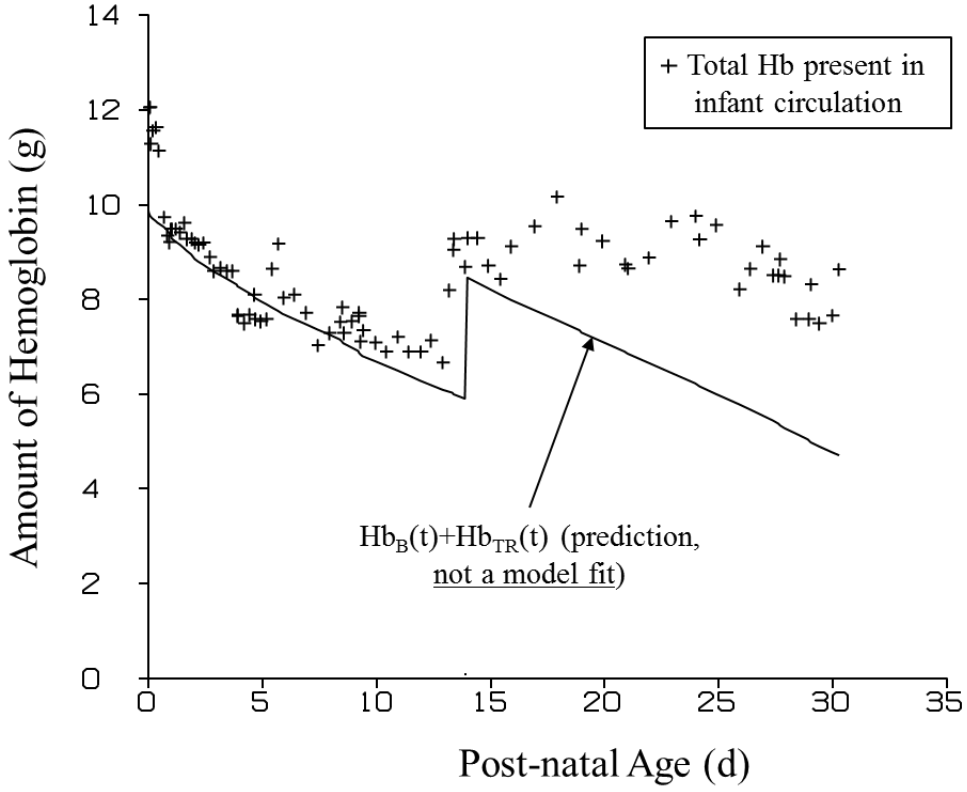

Figure 3.1. Hb amount present in the circulation of a representative infant during the first month of life. The individual data shown (+) represent $Hb_T(t)$, the total amount of Hb present in infant circulation at any time during the first month of life (Eq.1). The solid line represents $Hb_B(t)+Hb_{TR}(t)$, the sum of transfused Hb and the Hb produced *in utero* prior to birth and remaining in the infant's circulation following birth after accounting for phlebotomy loss. The solid line represents a prediction and is not a model fit. The difference between the solid line and the data points (+) represent $Hb_P(t)$, the amount of Hb produced by the infant at that time before accounting for phlebotomy loss.

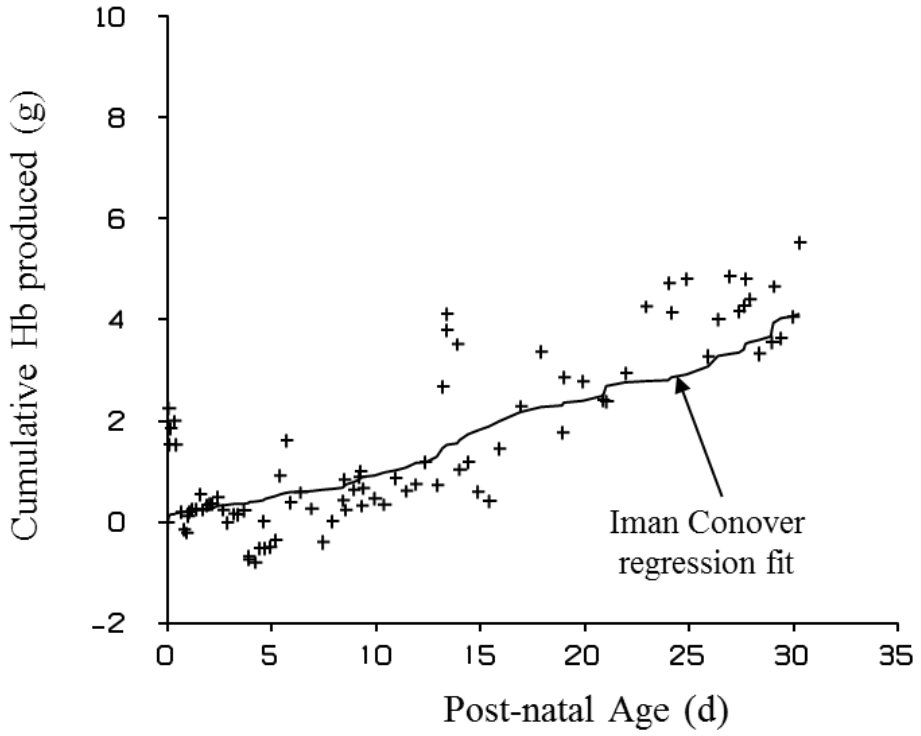

Figure 3.2. Iman Conover nonparametric regression fit to infant Hb data. The amount of Hb produced during the first month of life ($Hb_P(t)$) was estimated using Eq. 13. These data were then fit with the Iman Conover nonparametric regression. The solid line represents the regression fit to the calculated $Hb_P(t)$ data (+).

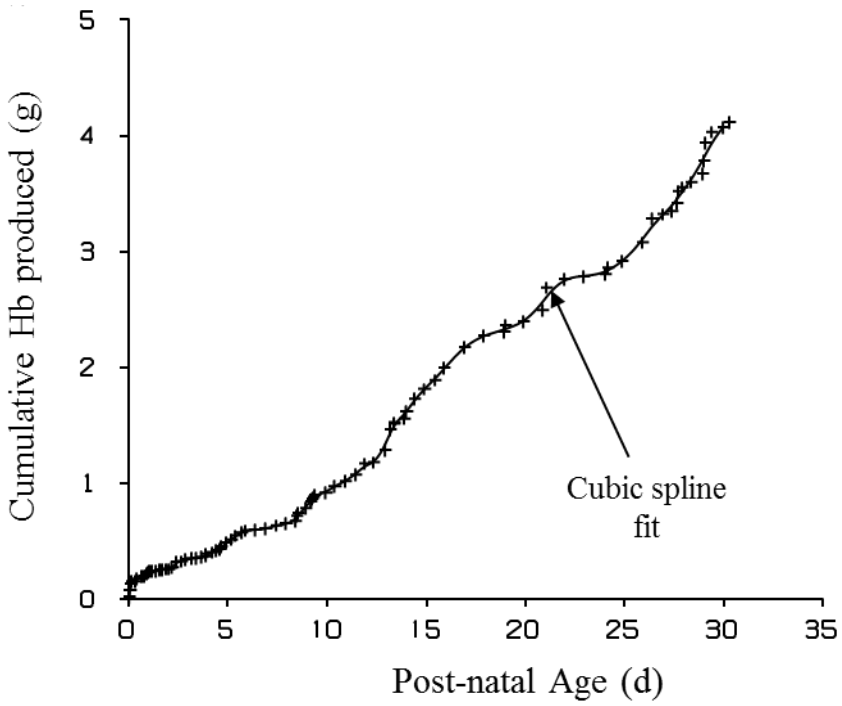

Figure 3.3. Cubic spline fit to infant Hb data. The final predicted cumulative Hb amounts from the Iman conover regression fit (+) were fitted with a nonparametric cubic smoothing spline function (solid line).

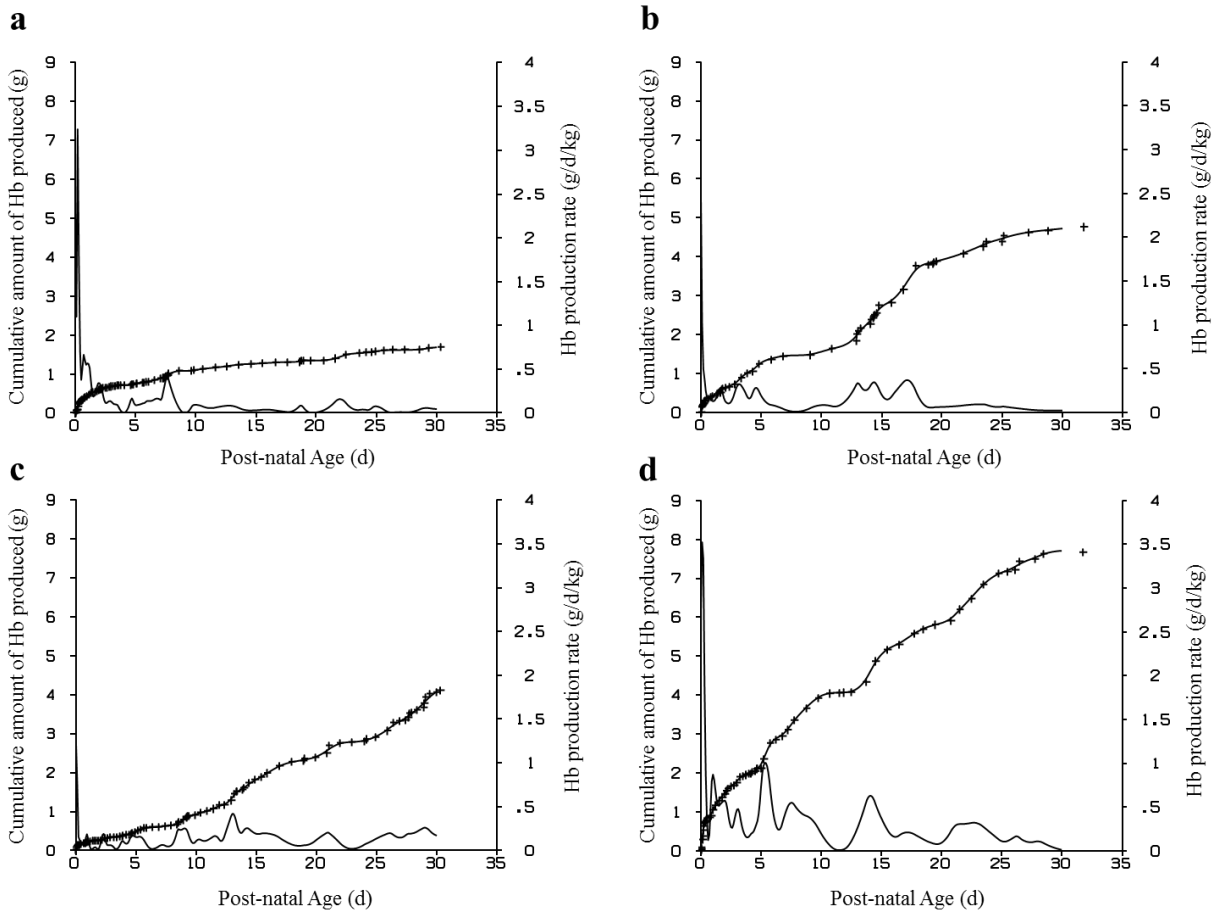

Figure 3.4. a-d Dynamic change in post-natal Hb production during the first month of life for four representative subjects. The solid line represents the cubic smoothing spline fit to the estimated cumulative Hb produced (+). The other solid line represents the dynamic changes in body weight-scaled Hb production rate during the first month of life.

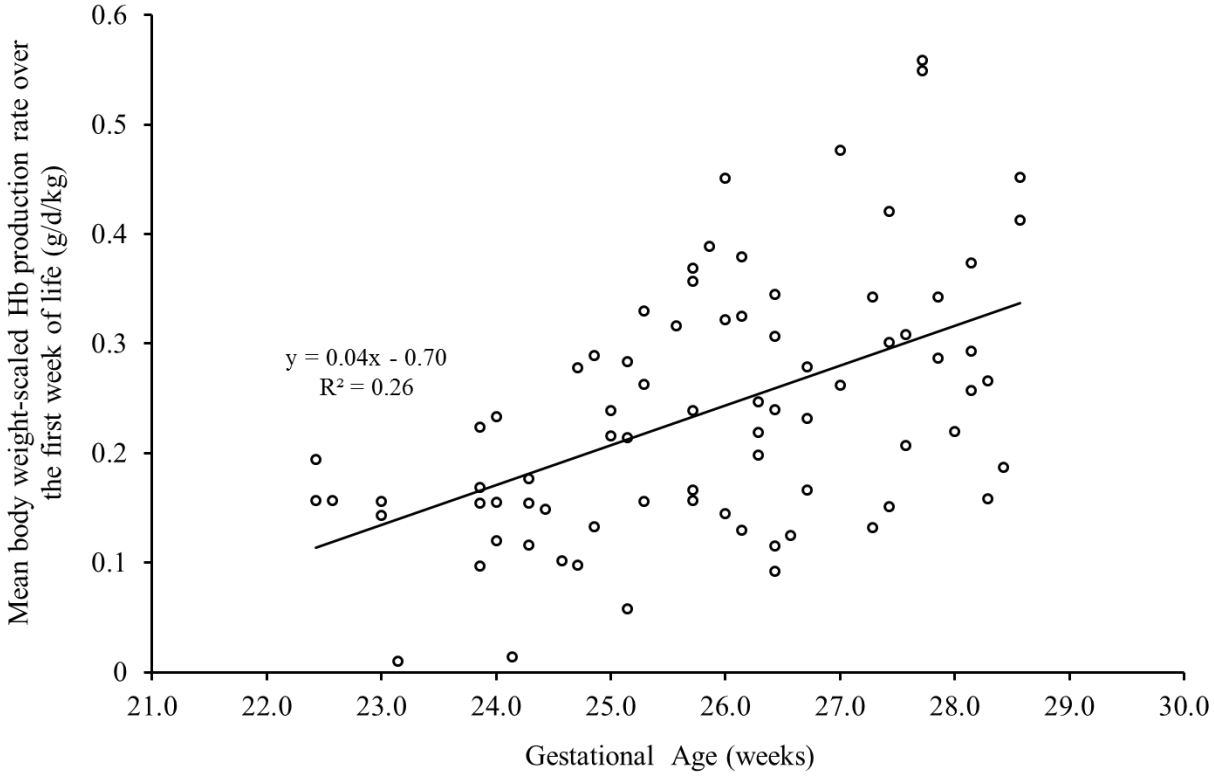

Figure 3.5. Influence of GA on the post-natal Hb production. The individual data points represent the estimated body weight-scaled post-natal Hb production rate over the first week of life vs. the GA for the 79 VLBW and ELBW anemic preterm study infants.

CHAPTER 4. ESTIMATION OF ADULT AND NEONATAL RBC LIFESPANS IN VERY LOW BIRTH WEIGHT ANEMIC NEONATES USING RBCS LABELED AT MULTIPLE BIOTIN DENSITIES

4.1 ABSTRACT

Concurrent post-transfusion tracking of labeled autologous neonatal and allogeneic adult red blood cells (RBCs) in neonates provides a unique opportunity to evaluate the *in vivo* survival of adult and neonatal RBCs simultaneously in the same study subject. In this study, RBCs from the first allogeneic adult RBC transfusion and from autologous infant blood were labeled at two discretely different biotin densities (BioRBCs) and simultaneously transfused to very low birth weight (VLBW) ventilated neonates. Two separate models, one describing the elimination of neonatal RBCs produced under non-steady state conditions, and the second describing the elimination of adult RBCs produced under steady state conditions, were applied to BioRBC data from 15 VLBW preterm anemic infants to estimate the adult and neonatal RBC lifespan. The mean (± *SD*) RBC lifespan of neonatal RBCs was estimated as 54.2 ± 11.3 d, and was significantly shorter than the mean adult RBC lifespan of 70.1 ± 19.1 d ($P<0.05$). A significant positive correlation was observed between allogeneic adult RBC lifespan and the infant body weight ($P<0.05$). We conclude that both the extrinsic environmental factors and intrinsic infant-adult RBC differences play a role in ultimately determining RBC survival *in vivo*.

4.2 INTRODUCTION

Red blood cells (RBCs) from neonates differ in many ways from those in healthy adults. Neonatal RBCs are produced during a period of rapid erythropoiesis with expanding blood volume (55). In contrast, RBCs from healthy adults with a relatively constant body weight and blood volume, are produced under normal hematologic steady state conditions. Neonatal RBCs are also poorly deformable, more fragile, and larger in size (74, 75). These inherent differences between adult and neonatal RBCs can significantly affect their *in vivo* long-term survival (lifespan).

The environment in which the RBCs are circulating may also play a role in determining their long-term survival. The mean *in vivo* RBC lifespan of adult RBCs in healthy adults is approximately 120 d (47). The same cells when transfused to a severely anemic infant may survive for a much shorter time. Conversely, if neonatal RBCs are transfused to healthy adults, the cells may survive for longer time due to the favorable environmental conditions.

The determination of red blood cell (RBC) lifespan is an active area of research, and provides important information about the factors that affect RBC lifespan *in vivo*. A wide variety of RBC labeling methods have been developed for this purpose. These methods can be classified into two general types: cohort labeling and random labeling. Cohort labeling involves labeling RBCs of a certain age, while random or population labeling method labels all RBCs present at a moment in time irrespective of their age.

Most commonly used RBC population labels, including ^{51}Cr and ^{32}P, allow only one population of RBCs to be labeled and studied at a time. This limits the applicability of these methods, especially if the researcher is interested in investigating the survival

of multiple separate RBC populations concurrently in the individual study subject. For example, when anemic infant is transfused with adult donor RBCs, after transfusion, there will be two separate RBC populations present in the infant's circulation. The two RBC populations may have different *in vivo* survival properties which require them to be studied using methods that are able to uniquely identify each RBC population post-transfusion.

Biotin is a non-radioactive, covalent population label that is used to label membrane proteins on the surface of RBCs (26). The measurement of red cell survival using RBCs labeled with biotin (BioRBCs) is practical, reliable, accurate and safe (31, 52). A unique advantage of biotin label is that RBCs can be labeled at discretely different biotin densities which make them ideal for studying the survival of multiple separate RBC populations concurrently in the same subject.

The specific objectives of the present study were: 1) to develop a quantitative method to describe *in vivo* RBC survival of neonatal and adult RBCs that were transfused concurrently into a newborn infant while also accounting for confounding factors including multiple phlebotomies, clinical transfusions and growth; and 2) to apply this method to estimate the RBC lifespan of neonatal and adult RBCs from the *in vivo* BioRBC disappearance curves from critically ill very low birth weight (VLBW) preterm infants.

4.3 METHODS

4.3.1 Subjects

Fifteen VLBW preterm anemic infants, less than 29 wk gestation age (GA) being cared for in the Neonatal Intensive Care Unit (NICU) at the University of Iowa Children's Hospital were enrolled in this study. The study was approved by the University of Iowa Human Subject Internal Review Board. Inclusion criteria included treatment with expectation of survival and respiratory distress requiring mechanical ventilation. Exclusion criteria included diffuse intravascular coagulation, thrombosis, hematological diseases (except for anemia associated with phlebotomy loss and prematurity) and transfusion requirements that were emergent and did not allow controlled sampling. For each subject, at least one parent or legal guardian provided written informed consent.

4.3.2 Biotinylation of RBCs and flow cytometric RBC analysis

Neonatal and adult RBCs were labeled with different biotin densities as previously described (31, 52). Briefly, RBCs were washed twice and prepared at 25% hematocrit (Hct). The biotinylation reagent sulfo NHS-biotin was dissolved, and used to label RBCs at a discrete biotin density. After a 30 minute reaction, the BioRBCs were washed twice, filtered and transfused (Figure 4.1). The percent of BioRBCs in post-transfusion blood samples was determined by flow cytometric enumeration after staining with Avidin conjugated with Alexa Fluor 488 as previously described (31, 52).

Clinically ordered laboratory phlebotomy blood samples from birth through the end of the BioRBC study period were weighed and recorded immediately after

collection. The weight of the blood collection tube was subtracted from the total weight of tube and blood sample, and this blood sample weight was converted to the volume of blood removed based on the estimated specific gravity of whole blood of 1.05 (54). The Hb mass removed with each phlebotomy was calculated by multiplying the volume of blood removed times the Hb concentration measured at the time of blood sampling. In addition to the BioRBC transfusion, the infants also received additional unlabeled RBC transfusions at various times based on severity of anemia. The decision to treat with RBC transfusions was made by the physician in accordance with NICU guidelines (55). For all transfusions administered, the volume of packed RBCs (85% Hct) administered was 15 mL/kg. The time of RBC transfusions were recorded for use in the analysis.

4.3.3 The model

Model for adult donor RBC survival

RBCs from healthy adult donors are produced under normal hematologic steady-state conditions. If these cells were isolated, labeled and transfused to a subject, then a certain fraction of the original pool of labeled RBCs will be removed from the circulation each day, leading to a linear survival curve. This linear survival curve can then be extrapolated to the time axis to estimate the adult donor RBC lifespan. The model to describe this linear decline of labeled RBCs/Hb after transfusion at time *t* is given as:

$$Hb_L(t) = \begin{cases} Hb_L(0) \cdot \left[1 - \frac{t}{L(0)}\right] & 0 \le t \le L(0) \\ 0 & otherwise \end{cases} \qquad (4.1)$$

where $Hb_L(t)$ represents the amount of Hb present in labeled RBCs at time *t*, $L(0)$ is the lifespan of the labeled adult donor RBCs.

Model for neonatal RBC survival

Unlike adult RBCs, neonatal RBCs are produced under non steady-state (non-SS) conditions. To describe the survival of neonatal RBCs, a mathematical model that accounts for non-SS RBC production has been detailed in our previous work (63). In brief, *in utero* growth was estimated using the birth weight as a function of GA data of Arbuckle et al (44), and can be expressed as:

$$BW(GA) = \begin{cases} A \cdot GA^4 + B \cdot GA^3 + C \cdot GA^2 + D \cdot GA + E & GA > 154 \\ M \cdot (e^{\gamma \cdot GA} - 1) & 0 < GA \leq 154 \end{cases} \quad (4.2)$$

where *GA* is the gestational age at birth measured in days and $A, B, C, D, E, F, M, \gamma$ are fixed parameters that were set equal to previously reported values (Table 2.1) (63). The erythropoiesis rate, $R(t)$, is considered to be proportional to the body weight and accordingly is expressed as:

$$R(t) = k \cdot BW(t + GA) \qquad t \leq 0 \quad (4.3)$$

where $BW(t)$ is the body weight at time *t*, *k* is a scaling factor that relates the *in utero* growth to fetal erythropoiesis rate. The final model to calculate the amount of Hb present in neonatal labeled RBCs remaining in circulation can be given as:

$$Hb_L(t) = \begin{cases} F_L \cdot MCH \cdot k \cdot \left[\frac{M}{\gamma} \cdot S_1(t) + M \cdot S_2(t) + S_3\right] & t \leq (p - GA)(1 + \alpha) + L(0) \\ F_L \cdot MCH \cdot k \cdot S_4(t) & L(0) \geq t > (p - GA)(1 + \alpha) + L(0) \end{cases} \quad (4.4)$$

where p=154 d (Equation 4.2) and,

$$S_1(t) = e^{\gamma \cdot p} - e^{\gamma \cdot \left(GA + \frac{t - L(0)}{1+\alpha}\right)} \quad (4.5)$$

$$S_2(t) = GA - p + \frac{t - L(0)}{1 + \alpha} \quad (4.6)$$

S_3

$$= \begin{cases} \frac{A}{5} \cdot (GA^5 - p^5) + \frac{B}{4} \cdot (GA^4 - p^4) + \frac{C}{3} \cdot (GA^3 - p^3) + \frac{D}{2} \cdot (GA^2 - p^2) + E \cdot (GA - p) & GA \geq p \\ 0 & GA < p \end{cases} \quad (4.7)$$

$$S_4(t) = \frac{A}{5} \cdot \left(GA^5 - \left(GA + \frac{t - L(0)}{1 + \alpha}\right)^5\right) + \frac{B}{4} \cdot \left(GA^4 - \left(GA + \frac{t - L(0)}{1 + \alpha}\right)^4\right) + \frac{C}{3} \cdot \left(GA^3 - \left(GA + \frac{t - L(0)}{1 + \alpha}\right)^3\right) + \frac{D}{2} \cdot \left(GA^2 - \left(GA + \frac{t - L(0)}{1 + \alpha}\right)^2\right) + E \cdot \left(\frac{L(0) - t}{1 + \alpha}\right) \quad (4.8)$$

where *MCH* is the mean corpuscular Hb for neonatal RBCs and was set equal to previously reported value of 37.5 pg/cell (49). In both models, the disposition of Hb/RBCs was assumed to be lifespan based (i.e., RBCs were removed from circulation through cellular aging/senescence) (45-48).

Accurately accounting for phlebotomies in the analysis

Newborn infants are subjected to substantial number of phlebotomies for clinical testing purposes; accordingly, Equations 4.1 and 4.4 must be corrected to accurately account for the loss of BioRBCs due to phlebotomies. We accounted for the loss of labeled RBCs from circulation as previously described (49-51). Details of the phlebotomy correction are described in the Appendix A.

4.3.4 Data analysis

Data analyses were performed in R version 3.0.3 using the RStudio integrated development environment (67, 68). All modeling were conducted using WINFUNFIT, a Windows (Microsoft) version evolved from the general nonlinear regression program FUNFIT (56), using ordinary least squares fit to each individual subjects Hb amount-time profile. To characterize the uncertainty in the estimates of the individual subject parameters, the standard deviation (SD) of the estimates were calculated for each parameter. Neonatal and adult RBC lifespans were compared using a two tailed paired *t*-test. Statistical differences were considered to be significant for values of $P<0.05$.

4.4 RESULTS

Subject characteristics

The mean body weight of the 15 anemic VLBW infants at the time of BioRBC transfusion was 0.742 kg (range, 0.494 to 1.042 kg). The mean GA of the infants was 178 d (range, 162 to 190). Five males (all singletons) and 10 females (7 singletons and 3 twins) were studied. During the BioRBC study period, study infants received a total of 1 to 9 clinically ordered RBC transfusions.

Biotinylation of RBCs and survival

Both adult donor and neonatal RBCs were successfully biotinylated with different discrete biotin densities and transfused concurrently to the infants. Almost all the transfused adult BioRBCs were recovered 24 h post-transfusion indicating the absence of storage effects on RBC survival. *Ex vivo* labeling of RBCs with low density biotin did not seem to have any effect on the removal rate of adult or neonatal BioRBCs.

Model fit to BioRBC data

The non-SS neonatal RBC survival model (Equation 4.4) fit to the Hb amount-time profiles for the same 2 representative subjects are displayed in Figures 4.2A and 4.2B. General agreement between the model fit and the infant Hb amount data was observed. The mean model estimated RBC lifespan of neonatal RBCs in neonatal infants was 54.2 ± 11.3 d. The mean value of *k*, the scaling parameter (Equation 4.3), was estimated as 0.92×10^7 RBCs/d/g.

The steady-state adult RBC survival model (Equation 4.1) fit to the adult Hb amount-time profiles for 2 representative subjects are displayed in Figures 4.2C and 4.2D. The mean (± *SD*) model estimated RBC lifespan of adult donor RBCs in neonatal infants was 70.1 ± 19.1 d. The long-term RBC survival of adult RBCs in neonatal infants was shorter than that in healthy adults (~120 d). A two-tailed paired *t*-test was used to compare the model estimated lifespans of adult and neonatal RBCs. The *in vivo* survival of adult RBCs was significantly greater than neonatal RBCs ($P<0.05$, Figure 4.3).

Finally, to test the influence of infant body weight on RBC lifespan, the mean estimated adult and infant RBC lifespans were plotted against infant body weight. A significant positive correlation was observed between infant body weight and *in vivo* allogeneic adult RBC lifespan ($P<0.05$, Figure 4.4).

4.5 DISCUSSION

The use of BioRBCs to measure long-term *in vivo* RBC survival offers several important advantages as compared to previous methods such as radiolabeling with ^{51}Cr or ^{32}P. BioRBCs are more sensitive and can be accurately tracked over longer periods of time.(76) Biotin binds securely to the surface of RBCs, and thus enable highly reliable determinations when analyzed by flow cytometry. BioRBCs are also non-radioactive, which makes them suitable for directly evaluating RBC survival in vulnerable study populations including fetuses, infants, children and pregnant woman.(77)

In this study, autologous infant and allogeneic adult RBCs labeled at two discretely different biotin densities were transfused to anemic VLBW preterm infants to study the concurrent post-transfusion *in vivo* survival. The advantage of this approach over previous methods is that this method enables to study the survival of both adult and infant RBC populations concurrently in the same study subject.

As mentioned earlier, neonatal RBCs and adult RBCs are produced under different conditions (i.e., SS vs. non-SS conditions), and thus, a direct comparison of the two BioRBC survival curves would be misleading and incorrect. Instead, two separate models, one describing the elimination of infant RBCs produced under non-SS conditions, and the second describing the elimination of adult RBCs produced under SS conditions are needed to accurately estimate and compare the lifespan of these two RBC populations. Furthermore, the infants underwent additional clinical transfusions, multiple phlebotomies and increase in blood volume, all of which can affect the BioRBC survival curves. In this study, all these confounders were accounted for to accurately estimate the RBC lifespan of both these RBC populations.

The estimated mean (± *SD*) lifespan of adult RBCs transfused to anemic VLBW infants was 70.1 ± 19.1 d, and was shorter than the 110-120 d lifespan previously reported for adult RBCs transfused to healthy adults.(78) This provides strong evidence that there is an environmental effect that shortens the survival of adult RBCs in infant circulation.

The effect of environment on RBC survival has been previously demonstrated in uremic patients through cross-transfusion experiments. When healthy recipients were transfused with RBCs from uremic patients, the RBC survival improved, whereas healthy donor RBCs had impaired survival when transfused to uremic patients.(79, 80) This indicates that the uremic environment is responsible for the decreased RBC lifespan than an intrinsic cell defect.

The shortened RBC lifespan of allogeneic adult RBCs in anemic VLBW infant circulation is due to a similar environmental effect. Although the exact mechanism for this is not clearly understood, one possible explanation could be the decreased deformability of adult RBCs in infant circulation. A previous study in which adult donor RBCs were transfused to severely anemic D+ fetuses found that adult RBCs had decreased deformability after being introduced into the fetal environment.(81) The impaired deformability would shorten the adult RBC survival due to the increased mechanical stress during the passage through the capillaries. A significant increase of the cholesterol-to-phospholipid (C/P) ratio in adult RBCs was considered as the primary cause for the decreased deformability of the donor RBCs.(81)

Infants also have a higher body weight normalized cardiac output than adults. This result in the donor RBCs undergoing more number of capillary passages per unit

time in the infant circulation compared to that in healthy adults. This combination of decreased RBC deformability and increased number of trips of adult RBCs in anemic VLBW infant circulation would lead to increased mechanical damage, and a faster removal of adult RBCs from infant circulation as compared to that in healthy adults. The proposed mechanism also provides an explanation for the observed increased lifespan of fetal RBCs after a severe fetomaternal hemorrhage in D- and ABO matched mothers.(82)

The estimated mean (± *SD*) lifespan of neonatal RBCs was 54.2 ± 11.3 d, and was similar to the previous range of RBC lifespan estimates of 35 to 50 d based on ^{51}Cr labeled RBCs.(57) The lifespan of neonatal RBCs was significantly lower than the estimated lifespan of adult transfused RBCs ($P<0.05$, two tailed paired *t*-test). This indicates that in addition to the environmental effect, the difference in the physical properties between adult and infant RBCs, such as those related to surface charge, aggregation, filterability and fragility(83) may also play a role in determining the *in vivo* RBC survival.

Finally, the mean adult RBC lifespan in infant circulation significantly increased with infant body weight (Figure 4.4). This indicates that allogeneic donor RBCs are able to survive longer in larger infants as compared to infants with lower body weights. This reduced survival of allogeneic donor RBCs in lower body weight infants provides an explanation for the greater number of RBC transfusions required to clinically manage anemia in VLBW and extremely low birth weight (< 1000 g) infants.(84-86)

Study limitations

In this study, the total RBC count could not be measured at the time of each BioRBC sample. Due to this limitation, the Hb concentration measurements available for each BioRBC sampling times was used to estimate the absolute amount of Hb present in the BioRBCs in the infant circulation over time. This estimated Hb present in the BioRBCs was then subsequently modeled instead of the number of BioRBCs present in the infant circulation.

This study included a relatively small sample of 15 study subjects that may not be representative of all VLBW preterm infants. In future, larger number of study subjects encompassing a greater GA and body weight spectrum need to be studied.

Clinical significance

The results from this study provide strong evidence of an environmental effect that decreased the survival of transfused RBCs in anemic VLBW infants. Since transfused donor RBCs survived for shorter time in the infant circulation, the anemic infants would require multiple donor RBC transfusions at regular intervals as treatment for clinically significant anemia of prematurity. The estimated allogeneic adult RBC lifespan was significantly longer than the neonatal RBC lifespan. This suggests that previously reported clinical strategies including delayed cord clamping and umbilical cord milking may have limited ability to replace the need for allogeneic RBC transfusion in anemic VLBW preterm infant.

In summary, the present study introduces a quantitative method to describe *in vivo* RBC survival of neonatal RBCs produced under non-SS conditions, and adult RBCs produced under SS conditions while also accounting for confounding factors

including multiple phlebotomies, clinical transfusions and growth. The method was successfully applied to estimate the *in vivo* RBC lifespan of allogeneic adult RBCs and autologous infant RBCs in anemic VLBW preterm infants. Future work involves identifying possible extrinsic environmental factors that are responsible for decreasing the transfused RBC survival in these anemic VLBW preterm infants.

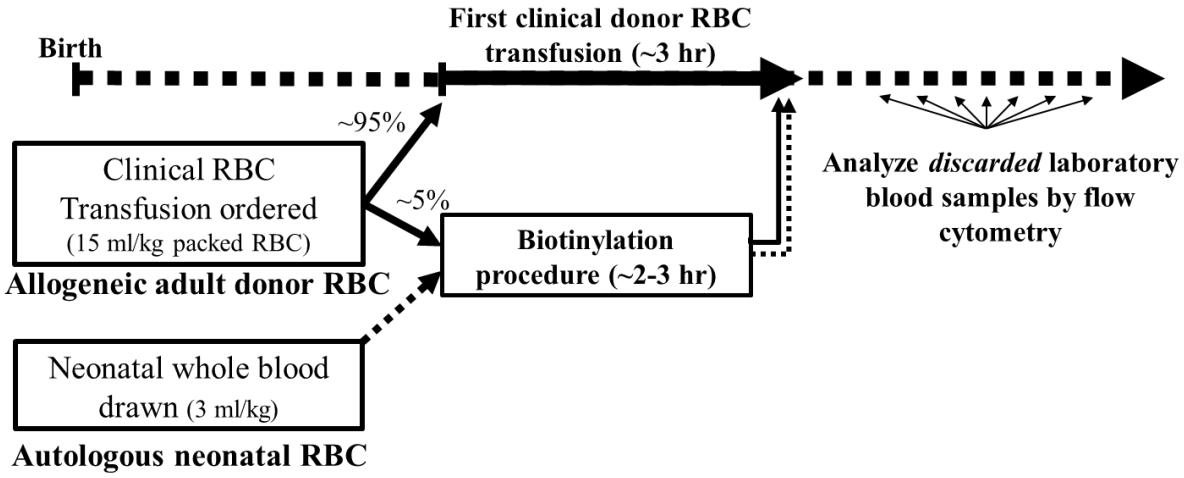

Figure 4.1. RBC biotinylation and quantitative flow cytometric analysis. Allogeneic adult donor RBCs and autologous neonatal RBCs were labeled at two discreetly different biotin densities and transfused to VLBW anemic infants at the time of the first clinical RBC transfusion. The discarded laboratory samples post-transfusion were analyzed by flow cytometric enumeration to determine the fraction of biotin-labeled adult and neonatal RBCs that were remaining in infant circulation.

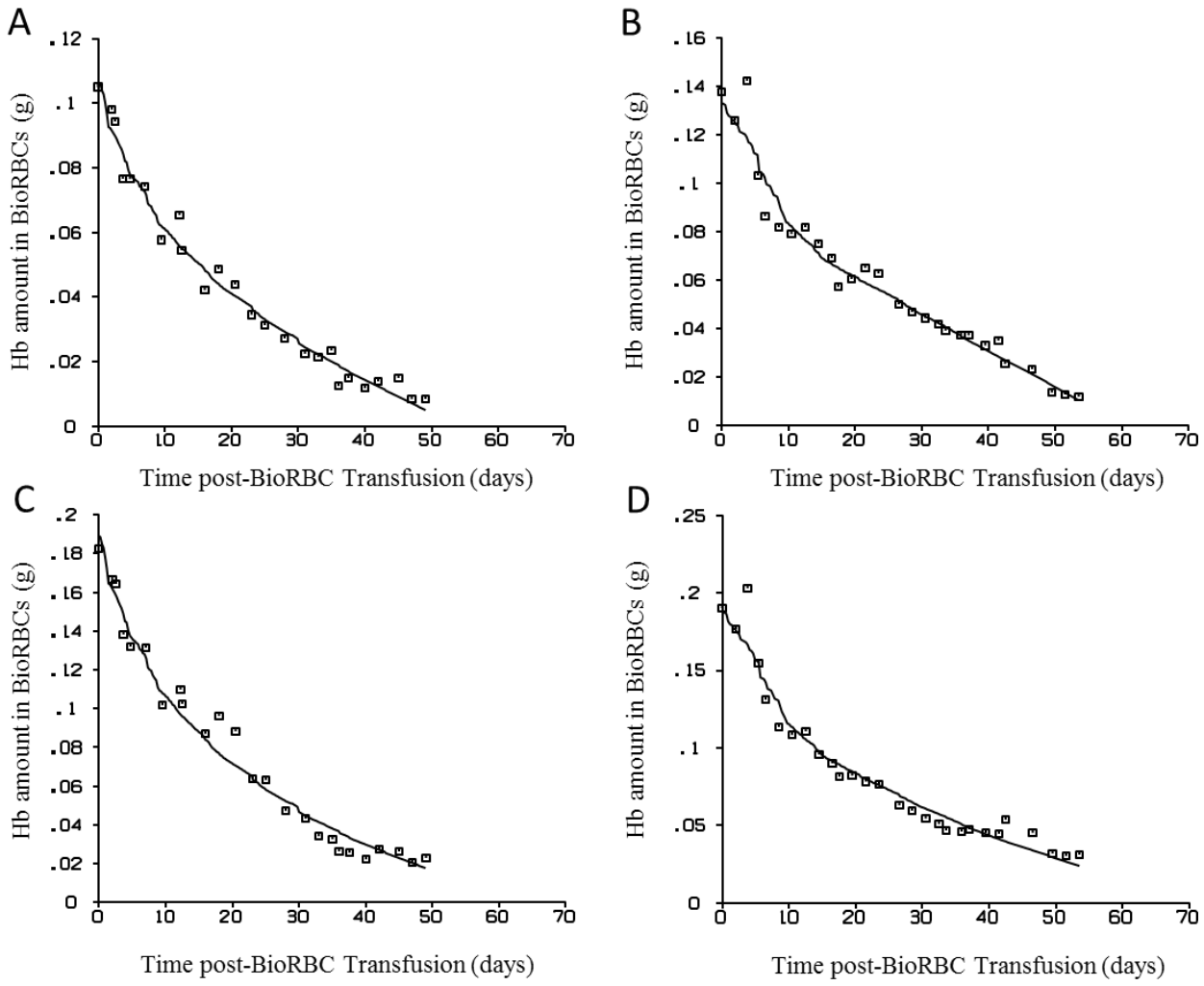

Figure 4.2. Model fit to Hb amount-time data. (**A, B**) The non-SS neonatal RBC survival model (Equation 4.4) fit (*solid line*) to the Hb amount in autologous neonatal BioRBCs (*open squares*) for two representative study subjects are displayed. (**C, D**) The steady-state adult RBC survival model (Equation 4.1) fit (*solid line*) to the Hb amount in allogeneic adult donor BioRBCs (*open squares*) for the same two representative study subjects are displayed. General agreement between both the model fits and the infant Hb amount data was observed. The non-smooth nature of the curves is due to the curve accounting for multiple clinical phlebotomies.

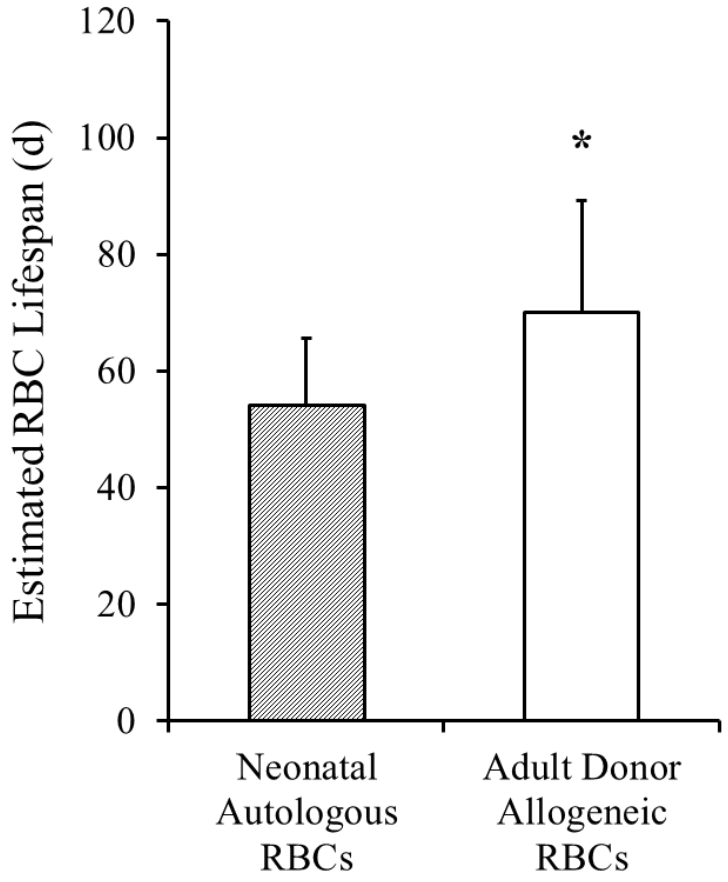

Figure 4.3. The mean (± *SD*) RBC lifespan of neonatal autologous and adult allogeneic RBCs in VLBW anemic infants. The lifespan of neonatal RBCs was significantly lower than the estimated lifespan of adult transfused RBCs ($P<0.05$, two tailed paired *t*-test).

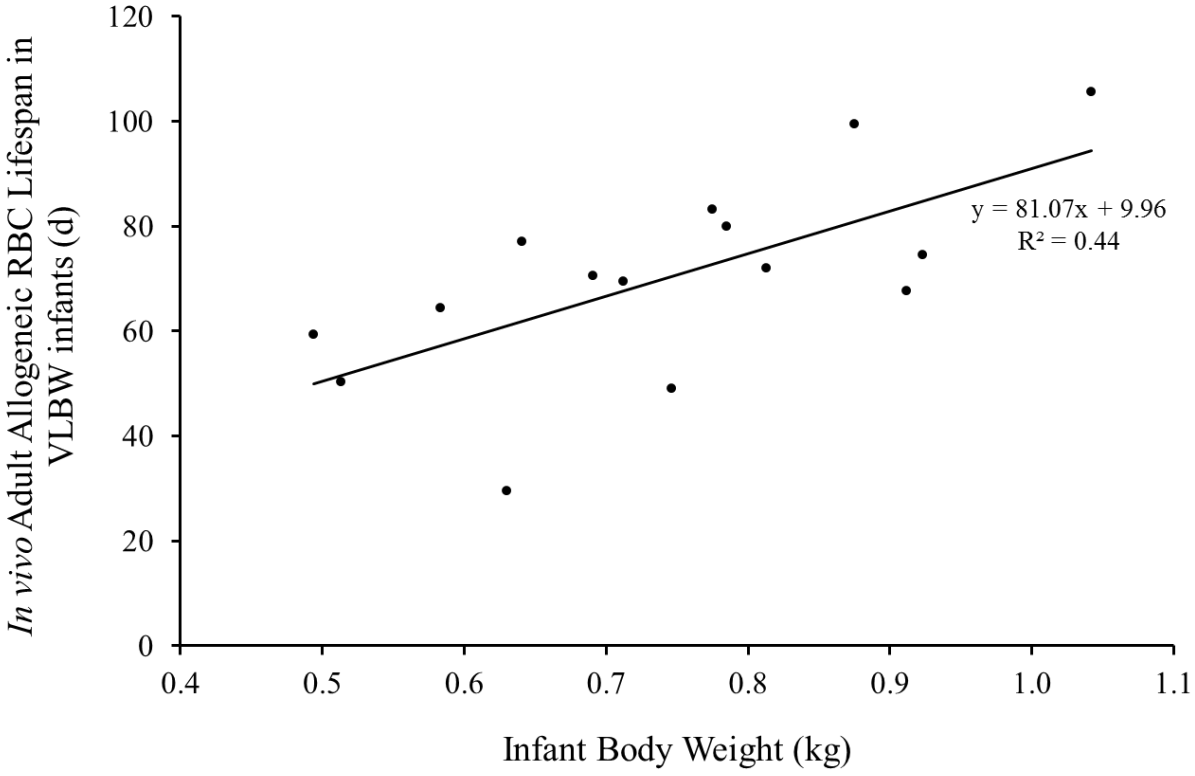

Figure 4.4. Influence of infant body weight on allogeneic adult RBC lifespan. The individual data points represent the estimated adult RBC lifespan plotted against the infant body weight for the 15 VLBW anemic preterm infants. The mean adult RBC lifespan in infant circulation was found to significantly increase with infant body weight ($P<0.05$).

CHAPTER 5. MEAN REMAINING LIFE SPAN: A NEW CLINICALLY RELEVANT PARAMETER TO ASSESS THE QUALITY OF TRANSFUSED RED BLOOD CELLS

5.1 ABSTRACT

Quality of transfused red blood cells (RBC) to treat anemia depends on its potential for oxygen delivery, governed by two properties: 1) initial post transfusion recovery (PTR_{24}); and 2) lifespan of initially surviving RBCs. The latter property is poorly evaluated by the traditional mean potential lifespan (*MPL*) or mean cell age (*MA*), because these parameters do not evaluate how long transfused RBCs remain in circulation. Furthermore, evaluation of *MPL* is based on two problematic assumptions regarding transfused RBCs: 1) they were produced at a constant steady state rate; 2) they have similar storage lifespans. This work introduces a new parameter, the mean remaining lifespan (*MRL*) to quantify transfused RBC survival (TRCS) and presents a simple algorithm for its evaluation. The *MRL* was calculated for four adult subjects with sickle cell disease and four adult diabetic and non-diabetic subjects using RBC survival data sets with existing TRCS parameters. The RBC survival curves in the sickle cell subjects were non-linear with rapid decline in survival within the first 5 d. The *MRL* was approximately 4.6 d. Thus, the *MRL* was indicative of the survival of all transfused RBCs. For the diabetic and non-diabetic subjects, the RBC disappearance curves did not deviate substantially from a linear decline. Thus, the estimates for *MRL* ranging from 39-51 d are similar to the *MA* previously computed. *MRL* overcomes limitations of previously proposed TRCS parameters, is simpler to calculate, and is physiologically and clinically more appropriate.

5.2 INTRODUCTION

The goal of administering RBC transfusions is to increase the circulating blood oxygen content in anemic individuals to improve tissue oxygenation. Regulatory licensing dealing with the short-term quality of transfused RBCs has focused on the proportion of viable post-transfusion RBCs recovered at 24 h (PTR_{24}) (87-89). Labeling is used to evaluate red cell survival (RCS) of transfused RBCs. FDA regulatory standards for RBC products specify that: "Recovery of greater than 75 percent of radiolabeled RBC 24 h after infusion into autologous donors."(90) (91).

In addition to PTR_{24}, other long-term RBC kinetic parameters most commonly used to characterize RCS include half-life (T_{50}), mean potential lifespan (*MPL*) and mean cell age (*MA*). The T_{50} is defined as the time post transfusion when 50 percent of the transfused RBCs remain in the circulation. The RBC mean age (MA) represents the mean age of RBCs at time of transfusion and is derived from the mathematical relationship between the age distribution of the RBCs and their disappearance rate (92). If all the cells had the same age, then the death rate (i.e., same as survival function) takes the form of a straight line (93). The mean red-cell lifespan as defined by the International Committee for Standardization in Hematology is the mean survival time of all circulating RBCs irrespective of their destruction mechanism, i.e., random destructions vs. senescence (94). If the transfused donor RBCs were produced under steady-state conditions of erythropoiesis and have the same survival properties, i.e., the same intrinsic lifespan, then the RCS curve exhibits a linear decline typically quantified by linear least square regression. The *MPL* is then obtained by simple linear extrapolation to intersection with the time axis (95).

This linear extrapolation time point used to evaluate *MPL* represents the time when the "youngest" of the RBCs transfused is removed from the circulation and thus is a poor overall representation of red cell survival. A *MPL* value of 120 d evaluated by the extrapolation method does not indicate that transfused RBCs remain, on average, in circulation in the recipient for 120 d. Instead, *MPL* indicates the time when the youngest, most viable RBCs at the time of RBC labeling were removed from the circulation. Such cells normally represent only a small fraction of the transfused RBCs. Thus, *MPL* is not an adequate representation of the overall survival of the transfused RBCs. Normally transfused RBCs have ages ranging from zero to the maximum lifespan. This is consistent with the fact that the quantity of transfused RBCs immediately declines after transfusion and continues to do so until those cells that were the youngest at the time of labeling are removed from the circulation, i.e., at the *MPL* time point.

Clearly, the quantity of transfused RBCs in terms of oxygen delivery capacity is poorly quantified by the *MPL* parameter. Logically, a parameter quantifying the duration that donor RBCs remain in the recipient's circulation would be a better choice. In this communication the mean remaining lifespan (*MRL*) parameter is proposed as such a parameter.

The objectives of this analysis are to: 1) introduce the *MRL* parameter to quantify TRCS and present a simple algorithm for its evaluation; 2) discuss the merits of *MRL* relative to *MPL* and other parameters for quantifying TRCS; and 3) demonstrate the evaluation of MRL in various clinical scenarios with the purpose of

providing examples of evaluations for discussing conceptual differences relative to other parameters for TRCS.

5.3 METHODS

5.3.1 Age, remaining lifespan and total lifespan of RBCs

To illustrate the rationale for proposing MRL as a parameter for quantifying TRCS, it is useful to consider a hypothetical example of three individual RBCs that are transfused at arbitrary time, t_0 (Figure 5.1). The ages of the three RBCs at the time (t_0) of the transfusion are denoted a_1, a_2 and a_3. After transfusion, the three RBCs exhibit a remaining lifespan of r_1, r_2 and r_3. The total lifespan of each RBC is the sum of the age at time of transfusion plus the remaining lifespan, e.g. $L_1 = a_1 + r_1$. By summing the total RBC lifespan and averaging these, it becomes clear that: mean RBC age at time of transfusion + MRL = mean total lifespan.

Of the three mean parameters, the MRL is the only parameter that quantifies how long, overall, the transfused RBCs remain in circulation in the recipient and is the most suitable parameter to quantify TRCS.

MRL calculation

The MRL parameter, which is analogous to the mean residence time (MRT) that has been extensively discussed in a pharmacokinetic context (96, 97), is calculated as the area under the curve of the fraction (F(t)) of the transfused RBCs remaining in circulation versus time:

$$MRL = \int_0^\infty F(t)\ dt \qquad (5.1)$$

For practicality reasons, it may be more suitable to deal with the MRL representing 95 percent of the transfused RBCs. The reason for this modification is the fact that it is practically impossible to follow the disappearance until all the transfused RBCs have been taken out of circulation. Accordingly, a more practical MRT parameter is evaluated by the following expression

$$MRL_{0.95} = \int_0^{t_{0.95}} F(t)\ dt \qquad (5.2)$$

where $t_{0.95}$ is the time when 95 percent of the transfused cells have disappeared from the circulation. This parameter is derived by interpolation of the fraction of RBCs remaining versus time curve.

Consideration of all transfused RBCs instead of those RBCs recovered at 24 h (PTR_{24})

The fraction remaining, *F(t)*, may be defined in either of two ways depending on whether the interest is in evaluating all transfused RBCs or just those initially surviving, e.g., cells still present 24 h post-transfusion.

F(t) defined for all transfused RBCs is the ratio of quantity of cells remaining at time *t* and the quantity transfused, while *F(t)* for RBCs initially surviving after 24 h is the ratio between the quantity at time *t* and the quantity initially surviving.

If F_{PTR24} defines the fraction of transfused RBCs initially surviving post-transfusion, then the relationship between the total MRL for all transfused cells (MRL_{ALL}) and MRL for the cells initially surviving 24 h is:

$$\mathrm{MRL_{ALL}} = \mathrm{FPTR_{24}} \cdot \mathrm{MRL_{24}} \qquad (5.3)$$

MRL_{ALL} is a valuable parameter because it is more comprehensive than MRL_{24} in the way that it includes all transfused RBCs in the calculation of the mean remaining lifespan parameter. Equation 5.4 is derived considering the RBCs initially removed to have a MRL of zero. Thus, the total mean remaining lifespan considering all cells is the weighted (according to proportions) average of the two averages, i.e.,

$$MRL_{ALL} = (1 - FPTR_{24}) \cdot 0 + FPTR_{24} \cdot MRL_{24} = FPTR_{24} \cdot MRL_{24}. \quad (5.4)$$

Importantly, MRL_{ALL} incorporates both the property of initially survival and the survival property (MRL) of the surviving cells.

Relationship of MRL to T_{max} and T_{50}

In addition to MPL, two parameters T_{max} and T_{50} have been recommended to quantify the survival of transfused RBCs (98). T_{max} is defined as the longest time a transfused RBC will remain in circulation after transfusion, while T_{50} denotes the time when 50 percent of transfused RBCs remain in circulation.

In a rare, hypothetical case, MRL relates in a simple way to these two parameters:

$$MRL = \frac{T_{\max}}{2} = T_{50} \quad (5.5)$$

This case requires two assumptions: 1) all transfused RBCs have identical survival in the donor, during storage and while circulating in recipient; 2) transfused RBCs were produced at a constant, steady state rate for as long as their lifespan in the donor.

5.3.2 Example illustrations with subject data

Two RBC survival data sets were selected from previous publications to illustrate the calculation of MRL (92, 99):

Example 1 (Figure 5.2) illustrates biotin labeled RBC non-fetal cell (F cell) survival curves in four adult study subjects with sickle cell disease (99). In this study, 10 ml of autologous sickle cells were labeled with biotin and reinfused (99). The labeled RBCs were identified by flow cytometry. The percentage of F-cells was determined as a function of time after reinfusion using a 2 color flow cytometric analysis (99).

Example 2 (Figure 5.3) illustrates the RBC lifespan using a biotin label in diabetic and non-diabetic adult subjects (92). Both Type 1 and Type 2 diabetic subjects were selected based on age greater than or equal to 14 y and stable diabetic control (92). Non-diabetic subjects who were hematologically normal were recruited from the general population (92). *Ex vivo* biotinylation with flow cytometric analysis was used to determine the survival of autologous RBCs. Up to 10 ml of RBCs were labeled with biotin under sterile conditions and re-infused. Initial post-infusion blood samples were obtained after 10 min, 20 min, 2 h, and 24 h (92). Additional blood samples, were taken after 2 d, one wk, 2 wk, and at subsequent 2-wk intervals until the fraction of biotinylated cells fell below 5 percent of the initial percentage of biotin labeled RBCs (92).

5.4 RESULTS

Example 1. Survival of non-F cells in sickle cell disease subjects (Figure 5.2)

The $MRL_{0.95}$ calculated for the four study subjects in Example 1 are shown in Table 5.1. Based on the survival curves for four study subjects with sickle cell disease, it is clear that the RBCs from individuals with sickle cell disease undergo rapid clearance during the first few days post-transfusion.

As illustrated in Figure 5.2, the RBCs are removed rapidly from circulation under disease conditions leading to a largely convex elimination versus time profile. Under these circumstances, it is difficult to estimate the *MPL*. Even if *MPL* was estimated using non-linear extrapolation, it would not provide a meaningful estimate for the survival of transfused RBCs. To illustrate this point, in Figure 5.2A, it can be observed that the percent survival of labeled RBCs drops down close to zero percent at ~ 20 d. This indicates that the youngest labeled RBCs are cleared from the circulation at ~ 20 d. The $MRL_{0.95}$ estimate for the same subject provides a value of only 4.6 d. If Figure 5.2A is used as a reference, one observes that less than 20 percent of the labeled RBCs remain in the circulation 8 d post-transfusion. Therefore, a lifespan estimate of 20 d is misleading as an estimate for the overall survival of the transfused RBCs and thus not useful as a clinical evaluation of the potential oxygen carrying capacity of the transfused cells. A similar analysis can be done with Figures 5.2B, 5.2C and 5.2D. In every case, it is observed that the survival curves are non-linear with rapid decline in survival (to less than 50 percent of labeled cells) within the first 5 d. Thus, any parameter used to describe RCS should be able to capture this rapid decline. The mean

$MRL_{0.95}$ for the four subjects is ~ 4.6 d. Clearly, the $MRL_{0.95}$ provides a more meaningful evaluation of the survival of the transfused RBCs.

Example 2. Survival of RBCs in diabetic and non-diabetic study subjects (Figure 5.3)

The RBC disappearance curves (Figure 5.3) in diabetic and non-diabetic subjects further illustrate the advantage of *MRL* (92). Again, because of the curvature of the four RBC disappearance curves in these examples, it is not meaningful to use linear extrapolation of all the data points to determine the RBC lifespan. Linear extrapolation of the first few points of the disappearance curve would underestimate of the true mean RBC lifespan while a linear extrapolation of the final few points would overestimate the true mean RBC lifespan of transfused RBCs. Moreover, it does not provide information regarding how the older RBCs behave in the circulation. The $MRL_{0.95}$ for each of the four subject's RBC disappearance curves were computed and compared to the mean cell age computed previously (Table 5.2).

The *MA* estimates used for comparison to the MRL estimates were calculated by the method described previously by Cohen et al (92). Briefly, the maximum survival time was computed by extrapolation of the final two points of the curve to the time axis (92). This was followed by fitting cubic equations to the survival data (92). The first derivative of the survival curve generates the death rate as a function of time, which was used to determine the initial cell age distribution (92). The mean age at any postinfusion time *t* was then computed from the recalculated death rate function and age distribution at each time t (92). Additional details on the derivation of the *MA* parameter are provided by Lindsell et al (93).

5.5 DISCUSSION

The present study demonstrates that applying mean remaining lifespan is simpler to calculate as compared to previously proposed parameters used to characterize the survival of transfused RBCs and that doing so also overcomes their limitations. In addition, MRL is a physiologically and clinically relevant parameter for quantifying the survival of transfused RBCs. The relative merits of the *MRL* parameter are illustrated in the two clinical examples provided: 1) among individuals with markedly shortened red cell survival as a result of sickle cell disease; and 2) among individuals with diabetes mellitus who demonstrated subject to subject variability in RBC survival.

Ambiguity in the Lifespan Notation

Intrinsically the total lifespan of an individual RBC encompasses the time from when the RBC enters the donor bloodstream from the marrow until the time it is cleared from the circulation in the donor or the transfused recipient. The notation "total lifespan" above is consistent with this intrinsic definition of a lifespan. Accordingly, "lifespan in recipient" or "lifespan of transfused RBC" are confusing terms that should be avoided. This is particularly relevant if the rate of RBC aging truly is different in the donor and recipient, a situation quite likely to be present when donors and recipients are fundamentally different, e.g., an adult donor and a newborn infant recipient.

The term "remaining lifespan" avoids such confusion by being more clearly defined. It also provides a clinically more meaningful parameter for the oxygenation potential of transfused RBCs. Furthermore the evaluation of the MRL does not depend on a steady state assumption for the production of the transfused RBCs. In contrast, the *MPL* parameter, unfortunately, has the above ambiguity associated with lifespan. For

the casual reader, *MPL* may incorrectly be interpreted in several different ways: e.g., as the mean lifespan of donor RBCs in the donor, as the mean lifespan of fresh new donor cells in the recipient, or as the mean remaining lifespan of transfused cells. None of these interpretations would be correct.

MRL as an alternative to MA

The estimates for the RBC *MA* and *MRL* were similar in Table 5.2 because the RBC disappearance curves for the four study subjects do not deviate substantially from a linear decline (Figure 5.3). For RBCs produced under steady state conditions with similar lifespan, the MA will be equal to the MRL. However, this equality will not be true under non-steady state production of RBCs. Also, the MA relates to the mean age of the cells at the time the cells enter the recipient circulation, i.e., the time spent in the donor prior to transfusion to the recipient (Figure 5.1), which is far from as relevant as the time spend in the recipient, and thus can be misleading. Also, the *MA* cannot be directly calculated from the RCS curve post-transfusion in the recipient. In contrast, the *MRL* is directly calculated from the RBC post-transfusion disappearance curve and relates directly to the time the transfused cells spend in the recipient, not the donor.

MRL as an alternative to MPL

The mean remaining lifespan (*MRL*) indicates the mean *in vivo* survival of all the RBCs that are transfused to the recipient. If all transfused RBCs have same fixed lifespan, then the *MRL* will be equal to the T_{50} if produced under steady state conditions. Thus, T_{50} is not as useful a parameter because this parameter is only an accurate estimate of *MRL* for situations in which the steady state production of the transfused RBCs also exhibits a fixed lifespan. Since the older RBCs are cleared earlier from the

circulation than the younger RBCs, *MRL* is less than *MPL*. In contrast, *MRL* accurately accounts for the reduced lifespan in the recipient of the older transfused cells. The MRL represents a true mean survival of the cells in the recipient even when the transfused RBCs have been produced under non-steady state conditions.

While it may be tempting to assume that *MPL* represents the survival of RBCs in the donor as well as in the recipient, this assumption is not valid when "environmental effects" altering RBC survival are considered. The RBC membrane is composed of 39.5 percent proteins and 35.1 percent lipids, both of which are susceptible to oxidative modifications (100). RBCs have high content of oxygen and hemoglobin, making them susceptible to oxidative damage (101). Mature RBCs are more susceptible to oxidative damage than other cells due to their inability to synthesize new proteins or overcome damage to cellular components (102). These factors indicate that mature RBCs are more prone to damage than newly produced cells. In contrast to *MPL*, the *MRL* does not have this limitation to its interpretation since the MRL parameter, as its name clearly indicates, only pertains to survival of the transfused RBCs in the recipients, and thus inherently considers the environmental effects.

The *MRL* as an accurate estimate of the potential oxygen delivery assumes that independent of their age and their remaining lifespan, transfused RBCs deliver the same amount of oxygen to tissues irrespective of their age. This assumption may not be valid if the affinity of oxygen to hemoglobin changes with the age of the transfused RBCs. With prolonged *ex- vivo* storage of RBCs, there is a reduction in 2,3-diphosphoglycerate (2,3-DPG), a major allosteric modifier of the hemoglobin (Hb) oxygen affinity (103).

Further work needs to be done to account for the changes in oxygen delivery potential of transfused RBCs that occur with age.

In conclusion, the mean remaining lifespan has been proposed as a more clinically relevant parameter for predicting the quality of stored RBCs used for transfusion. Relative to mean potential lifespan and mean age, estimation of *MRL* requires fewer assumptions and is more meaningful in the evaluation of red cell survival in the recipient and the potential oxygen delivery of the transfused RBCs. In contrast to *MPL, MRL* is easily estimated under non-steady state conditions. This makes it a more universally applicable than *MPL* or *MA* for quantifying the quality of transfused RBCs.

Table 5.1. Mean remaining lifespan ($MRL_{0.95}$) for each study subject in Example 1

Subject ID	**Figure**	**$MRL_{0.95}$, days**
A	Figure 5.2 A	4.62
B	Figure 5.2 B	3.92
C	Figure 5.2 C	4.08
D	Figure 5.2 D	5.78

The RBC survival data from the published paper by Franco et al (99).

Table 5.2 Mean remaining lifespan ($MRL_{0.95}$) for each study subject in Example 2

Subject ID	**Figure**	**RBC MA*, (d)**	**$MRL_{0.95}$, (d)**
A (DM 3)	Figure 5.3 A	45.4	44.3
B (NDM 3)	Figure 5.3 B	38.4	39.4
C (DM 5)	Figure 5.3 C	49.3	49.1
D (NDM 4)	Figure 5.3 D	51.6	50.9

* The RBC mean cell age data from the published paper by Cohen et al (92). The method used for mean cell age calculation was adapted from previous published literature (93).

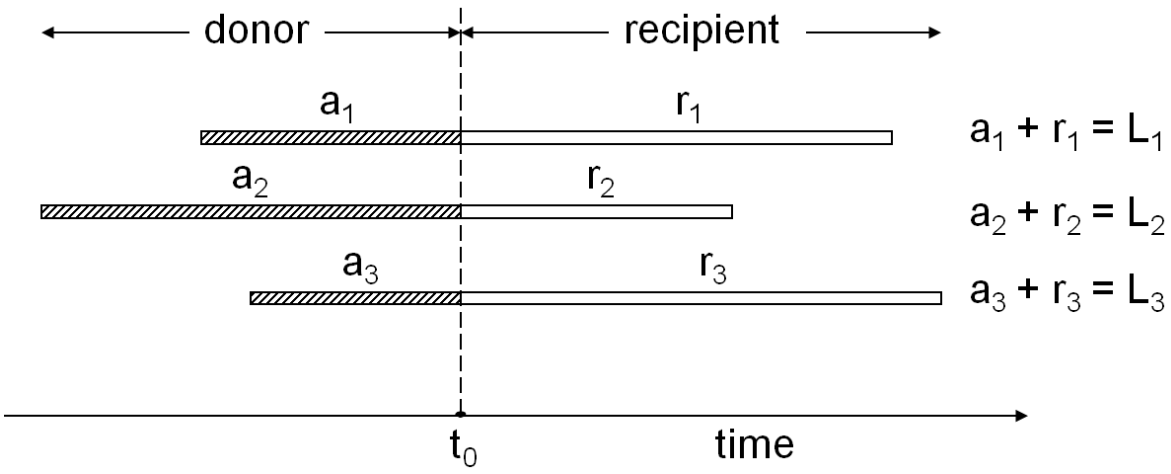

Figure 5.1. Relationship between MPL, MRL and MA. Three RBCs are transfused to a recipient at time t_0. The mean of a_1, a_2 and a_3 represents the MA of the transfused RBCs. Mean of L_1, L_2 and L_3 represents the MPL of the transfused RBCs. The mean of r_1, r_2 and r_3 represents the MRL, i.e., the mean time the cells will remain in the circulation relative to time t_0, the time of transfusion.

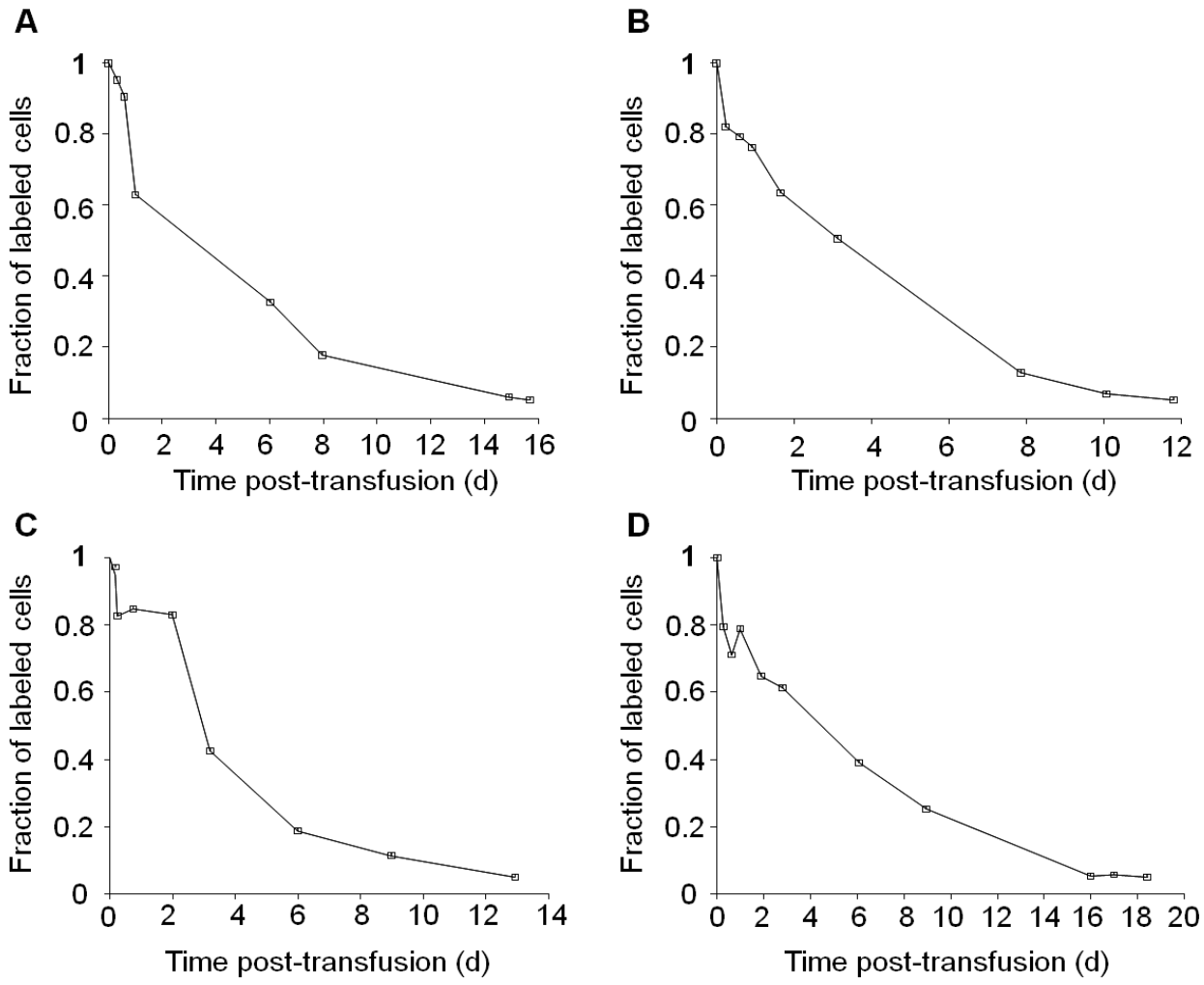

Figure 5.2. Survival of biotin labeled non-F cells in patients with sickle cell disease. Panels A, B, C and D illustrate the survival of biotin labeled non-F cells for four study subjects with sickle cell disease (99). The last data point in the graphs is calculated by linear interpolation and represents the time at which only 5 percent of the labeled cells remain in circulation. The mean remaining lifespan ($MRL_{0.95}$) is calculated from the RCS curve by computing the area under the curve until 95 percent of the labeled cells are removed from circulation. All survival data points below 5 percent are omitted, as they are not used in $MRL_{0.95}$ calculations.

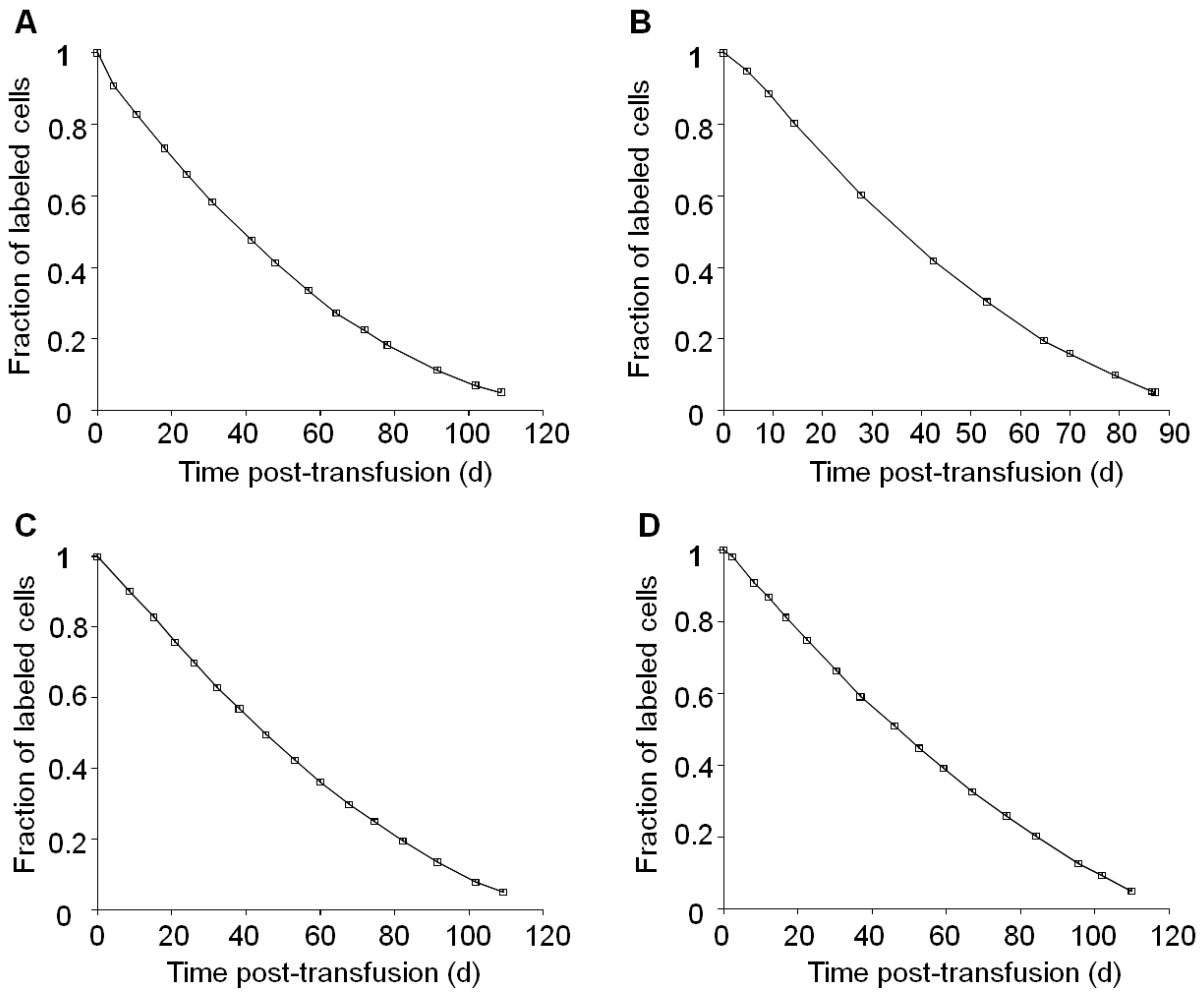

Figure 5.3. The survival of RBCs in diabetic and non-diabetic study subjects. Panels A - D illustrate the survival of RBCs in diabetic (A, C) and non-diabetic (B, D) subjects. The mean remaining lifespan ($MRL_{0.95}$) is calculated from the RBC survival curve by computing the area under the curve until 95 percent of the labeled cells are removed from circulation.

APPENDIX A. PHLEBOTOMY CORRECTION FACTOR

Correction for phlebotomies

Let us consider the ith phlebotomy was performed at time t_{pi} that removed a certain fraction of Hb from circulation. The fraction remaining after the ith phlebotomy, $F_{RMi.}$is given by equation A1:

$$F_{RMi} = \frac{Hb_T(t_{Pi}) - Hb_{RMi}}{Hb_T(t_{Pi})} \qquad (A1)$$

, where Hb_{RMi} is the hemoglobin removed due to the ith phlebotomy at time t_{pi}. For multiple phlebotomies, the phlebotomy correction factor (PCF) can be calculated as shown in equation A2,

$$PCF = \begin{cases} \prod_{i=j}^{q} F_{RMi} & if\ q \geq j\ and\ t_{Pi} < t \\ 1 & otherwise \end{cases} \qquad (A2)$$

, where the fraction remaining after each phlebotomy are ordered from the first to the last phlebotomy, j is the first phlebotomy after entry of the RBCs into the systemic circulation and q is the last phlebotomy prior to the current time t. In this case, j represents the first phlebotomy after the BioRBCs are introduced into the circulation, and thus the equation A2 now can be written as equation A3. The derived PCF is then used to account for loss of RBCs due to multiple clinical phlebotomies.

$$PCF = \begin{cases} \prod_{i=1}^{q} F_{RMi} & if\ q \geq 1\ and\ t_{Pi} < t \\ 1 & otherwise \end{cases} \qquad (A3)$$

APPENDIX B. FORTRAN AND WINFUNFIT SUBROUTINES

B.1 FORTRAN subroutines for chapter 2

```
! FILENAME = BIORBC_SURVIVAL_MODEL_DIFF_V1.1.F90
!
! PURPOSE:  TO MODEL THE BIORBC SURVIVAL CURVE FOR IN-UTERO RBC
PRODUCTION
!
!=================================================================
==========================
!
! THE FOLLOWING SUBROUTINE (USERMODEL):
! (1) DEFINES THE EQUATIONS TO BE FITTED
! (2) ASSIGNS NAMES TO THE PARAMETERS (IFUN=-1000 CALL)
! (3) ALLOWS THE USER TO DEFINE AND REGISTER EVENT (IFUN =-1000
CALL)
! (4) INTERACTIVELY ALLOWS THE USER TO SELECT THE ALGORITHM TO BE
!     USED BY WINFUNFIT FOR THE INTEGRATION OF THE DIFFERENTIAL
EQUATIONS
!     SPECIFIED IN THE SUBROUTINE "USERMODEL_ODE" GIVEN ABOVE.
! (5) PROVIDES THE USER THE OPPORTUNITY TO MAKE SPECIAL
CALCULATIONS
!     AND PLOTS AFTER WINFUNFIT HAS COMPLETED A FITTING TO A DATA
SET (IFUN=0 CALL)
!-----------------------------------------------------------------
---------------------------

SUBROUTINE USERMODEL(T,Y,P,NP,IFUN)  !   USERMODEL IS A REQUIRED
NAME (DO NOT CHANGE)
    USE PHLEBOTOMY_TRANSFUSION_MODULE
    USE NUMERICAL_LIBRARIES

    INTEGER, PARAMETER  :: NEQN = 1, NPAR=13, MAXN = 500, MAXCOEFF
= 100, LUN = 3
    REAL*8, PARAMETER   :: FACTOR = 2D0, TOLERANCE = 1.0D-7,
TIMEZERO = 0D0, ABSERR = 0D0, &
                           RELERR = 0.001
    INTEGER             :: NP, IFUN, JFUN, NSIGDIGITS, NUM,
NOEVENTS, NPHLEB, NTRANS, &
                           TEMPN, NOCOEFF, J, SUBNO, JOB, TN, K,
KLAST, NEPO, TEMN, Q
    REAL*8              :: T, Y(*), TZER, CTT, AMT(NEQN), P(*),
YZERO(NEQN), TZERO(NEQN), TEMPT, TEMPA, TEMPX(MAXN), &
                           TEMPY(MAXN), BVOL,
COEFFICIENTS(MAXCOEFF), ESTERR, HBZERO, A1, &
                           XMAX, HBTOTALPROD, HBTOTALPHLEB,
HBTOTALTRANS, TEMPSUM, TY, TX, TR, &
```

```
                          MAXHBAMT, TX1(MAXN), TX2(MAXN),
TY1(MAXN), TY2(MAXN), TYMAX, BRET, LTRBC, LTRET, &
                          MONTHHBTOTALPROD, FUNDAMAGED,
XEPO(MAXN), YEPO(MAXN), MCHE, MCHT, CV, FRETT, &
                          TRANS_FRAC, RET_TRANS_FRAC, BRBC, A2,
INPUT1, INPUT2, AVGSTIMRATE, A3, T3, INPUT3, TEMX(MAXN),TEMY(MAXN),
CUM(MAXN), TAT=0D0
    LOGICAL, SAVE       :: SHOWIT, PLOTSAVED
    CHARACTER (LEN=256) :: ID, DATAFILENAME
    CHARACTER (LEN=20)  :: PNAME
    CHARACTER (LEN=1)   :: RESPONSE

!----
! BEFORE FITTING WE WOULD LIKE TO GIVE NAMES TO THE PARAMETERS AND
! SELECT THE ALGORITHM FOR THE INTEGRATION OF THE DIFF EQUATIONS
!----
IF(IFUN == -1000)THEN
! THIS SECTION (IFUN.EQ.-1000) ALLOW YOU TO SET OPTIONS BEFORE THE
! START OF THE FITTING TO THE DATA (WHICH OCCURS WHEN IFUN = -1000)
!-------------------------------------------------------------------
-----
!ASSIGN NAMES (HIGHLY RECOMMENDED FOR READABILITY OF OUTPUT)

        CALL SetFunfitParameterName(1,"ALPHA")
!! SLOPE OF LINEAR CHANGE IN LIFESPAN
        CALL SetFunfitParameterName(2,"F")
!! COEFFICIENT FOR SINGLE EXPONENTIAL FUNCTION
        CALL SetFunfitParameterName(3,"k")
             !! SCALAR PARAMETER RELATING BW TO INUTERO RBC
PRODUCTION RATE
        CALL SetFunfitParameterName(4,"LB")
             !! LIFESPAN OF THE RED BLOOD CELLS AT BIRTH
        CALL SetFunfitParameterName(5,"GA")
             !! GESTATIONAL AGE OF THE INFANT IN DAYS: FIXED
PARAMETER
        CALL SetFunfitParameterName(6,"A")                        !!
COEFFICIENT OF X^4 FOR THE BW FOURTH ORDER POLYNOMIAL: FIXED
PARAMETER
        CALL SetFunfitParameterName(7,"B")
!! COEFFICIENT OF X^3 FOR THE BW FOURTH ORDER POLYNOMIAL: FIXED
PARAMETER
        CALL SetFunfitParameterName(8,"C")
             !! COEFFICIENT OF X^2 FOR THE BW FOURTH ORDER
POLYNOMIAL: FIXED PARAMETER
        CALL SetFunfitParameterName(9,"D")
             !! COEFFICIENT OF X^1 FOR THE BW FOURTH ORDER
POLYNOMIAL: FIXED PARAMETER
        CALL SetFunfitParameterName(10,"E")
             !! COEFFICIENT OF X^0 FOR THE BW FOURTH ORDER
POLYNOMIAL: FIXED PARAMETER
        CALL SetFunfitParameterName(11,"GAMMA")
             !! EXPONENTIAL TERM COEFFICIENT FOR SINGLE EXPONENTIAL
FUNCTION: FIXED PARAMETER
```

```
        CALL SetFunfitParameterName(12,"FR")
             !! FRACTION OF RBCS RELATIVE TO THE TOTAL NUMBER OF
RBCS PRODUCED THAT ARE LABELLED AND
        CALL SetFunfitParameterName(13,"MCH")
             !! MCH VALUE IN G/CELL

                                !! REINFUSED FOR TRACKING STUDY
(BIORBC): FIXED PARAMETER
!-------------------------------------------------------------------
-----

! ============= SET PHLEBOTOMY, TRANSFUSION, STIMULATION RATE
KNOTS, AND BODYWEIGHT VECTORS =============
!                   NOTE: THE TIME/KNOT VECTORS MUST BE SET BEFORE
THE AMOUNT/FVALUE VECTORS
!
        TEMPN = MAXN
        PRINT*
        PRINT*,' PHLEBOTOMY FRACTION REMAINING-TIME DATA:'
        CALL GET_XY_DATA_FROM_FUNFIT_FILE (TEMPX,TEMPY,TEMPN)
        IF ( TEMPN > MAXN ) STOP ' TOO MANY PHLEBOTOMY DATA POINTS.
ADJUST THE MAXN APPROPRIATELY'
        CALL SET_PHLEBOTOMY_TIME_VECTOR(TEMPN, TEMPX)
        CALL SET_FRACTION_REMAINING_VECTOR(TEMPN, TEMPY)
!
!-------------------------------------------------------------------
-----
        TEMN = MAXN
        PRINT*
        PRINT*,' CUMULATIVE_HB_REMOVED_GRAMS-TIME DATA:'
        CALL GET_XY_DATA_FROM_FUNFIT_FILE (TEMX,TEMY,TEMN)
        IF ( TEMN > MAXN ) STOP ' TOO MANY PHLEBOTOMY DATA POINTS.
ADJUST THE MAXN APPROPRIATELY'

DO Q=1, TEMN

      IF(Q==1) THEN
            TAT = 0D0
      ELSE
            TAT = CUM(Q-1)
      ENDIF

      CUM(Q)= TAT + TEMY(Q)
      TAT = 0D0

END DO

!-------------------------------------------------------------------
-----
  RETURN
ENDIF

!===================================================================
================================================
```

```
! THIS SECTION DOES THE INTEGRATION OF THE ODEs AND PROVIDES THE
PREDICTED VALUES TO BE FITTED TO THE DATA
! FOR THE 3 VARIABLES Y(1), Y(2) AND Y(3) (IFUN = 1,2 AND 3)
IF(IFUN == 1) THEN


      BOUNDARY: IF(T <= ((154.-P(5))*(1D0+P(1))+P(4))) THEN
                                                                    !!
CASE 1

                  TZER = 0D0
                  CTT = 0D0
                  CALL GET_PHLEBOTOMY_CORRECTION_TERM(TZER, T, CTT)

                  Y(1) =
CTT*P(13)*(P(12))*((((P(6)*(1D0/5D0)*(((P(5))**5.)-((154.)**5.)))
     + &                                !! NUMBER OF RBCS REMAINING
AT ANY TIME T AFTER BIRTH

      (P(7)*(1D0/4D0)*(((P(5))**4.)-((154.)**4.)))             + &
                                  !! MULTIPLY BY THE FRACTION OF
TOTAL RBCS THAT ARE BIOTIN LABELLED AND REINFUSED

      (P(8)*(1D0/3D0)*(((P(5))**3.)-((154.)**3.)))             + &

      (P(9)*(1D0/2D0)*(((P(5))**2.)-((154.)**2.)))             + &
                                       (P(10)*((P(5))-
(154.))))*P(3))                              + &
                                       (((P(3)*P(2))/(P(11)))
                                       * &
                                       ((EXP(154D0*P(11)))-
(EXP(P(11)*P(5)+                    &
                                       (P(11)*(MIN(0D0, (T-
P(4))))/(1D0+P(1)))))))                + &
                                       ((P(3)*P(2))
                                             * &
                                       (((MIN(0D0, (T-
P(4))))/(1D0+P(1)))-154D0+P(5))))


!                 Y(1) =
CTT*(P(12))*(((((P(6)*(1D0/5D0)*(((P(5))**5.)-((154.)**5.)))   + &
                                  !! PERCENTAGE OF RBCS REMAINING
AT ANY TIME T AFTER BIRTH
!
      (P(7)*(1D0/4D0)*(((P(5))**4.)-((154.)**4.)))             + &
                                  !! MULTIPLY BY THE FRACTION OF
TOTAL RBCS THAT ARE BIOTIN LABELLED AND REINFUSED
!
      (P(8)*(1D0/3D0)*(((P(5))**3.)-((154.)**3.)))             + &
!
      (P(9)*(1D0/2D0)*(((P(5))**2.)-((154.)**2.)))             + &
!                                      (P(10)*((P(5))-
(154.))))*P(3))                              + &
```

```
!                                          (((P(3)*P(2))/(P(11)))
                                           * &
!                                          ((EXP(154D0*P(11)))-
(EXP(P(11)*P(5)+                      &
!                                          (P(11)*(MIN(0D0, (T-
P(4))))/(1D0+P(1)))))))                    + &
!                                          ((P(3)*P(2))
                                                * &
!                                          (((MIN(0D0, (T-
P(4))))/(1D0+P(1)))-154D0+P(5)))) / &
!                                          ((((P(3)*P(2))/(P(11)))
                                                * &
!                                          (((EXP(154D0*P(11)))-
(EXP((P(11)*P(5))-                           &
!
      (P(11)*((P(4))/(1D0+P(1)))))))
      - &
!                                          (P(11)*(154D0-
P(5)+((P(4))/(1D0+P(1)))))))               + &
!                                          (P(3)

           * &         !! TOTAL NUMBER OF INUTERO RBCS PRESENT AT
BIRTH (T=0)
!                                          ((P(6)*(1D0/5D0)

      * &
!                                          (((P(5))**5.)-
((154D0)**5.)))
           + &
!                                          (P(7)*(1D0/4D0)

           * &
!                                          (((P(5))**4.)-
((154D0)**4.)))
           + &
!                                          (P(8)*(1D0/3D0)

           * &
!                                          (((P(5))**3.)-
((154D0)**3.)))
           + &
!                                          (P(9)*(1D0/2D0)

           * &
!                                          (((P(5))**2.)-
((154D0)**2.)))
           + &
!                                          (P(10)*(1D0/1D0)

      * &
!                                          (((P(5))**1.)-
((154D0)**1.)))))))*100D0
```

```
!                N(0) =      (((P(3)*P(2))/(P(11)))
                                          * &
           !! TOTAL NUMBER OF INUTERO RBCS PRESENT AT BIRTH (T=0)
!                                         (((EXP(154D0*P(11)))-
(EXP(P(11)*P(5)-                     &
!
      (P(11)*((P(4))/(1D0+P(1)))))))
      - &
!                                         (P(11)*(154D0-
P(5)+((P(4))/(1D0+P(1)))))))              + &
!                                         (P(3)

           * &         !! TOTAL NUMBER OF INUTERO RBCS PRESENT AT
BIRTH (T=0)
!                                         ((P(6)*(1D0/5D0)

      * &
!                                         (((P(5))**5.)-
((154D0)**5.)))
           + &
!                                         (P(7)*(1D0/4D0)

           * &
!                                         (((P(5))**4.)-
((154D0)**4.)))
           + &
!                                         (P(8)*(1D0/3D0)

           * &
!                                         (((P(5))**3.)-
((154D0)**3.)))
           + &
!                                         (P(9)*(1D0/2D0)

           * &
!                                         (((P(5))**2.)-
((154D0)**2.)))
           + &
!                                         (P(10)*(1D0/1D0)

      * &
!                                         (((P(5))**1.)-
((154D0)**1.))))))

      ELSE

                TZER = 0D0
                CTT = 0D0
                CALL GET_PHLEBOTOMY_CORRECTION_TERM(TZER, T, CTT)

                Y(1) = CTT*P(13)*(P(12))*(P(3)

      * &         !! NUMBER OF RBCS REMAINING AT ANY TIME T AFTER
BIRTH
```

```
                                    ((P(6)*(1D0/5D0)
                                                                    * &
                                    (((P(5))**5.)-(((((MIN(0D0, (T-
P(4))))/(1D0+P(1)))+P(5)))**5.)))       + &
                                    (P(7)*(1D0/4D0)

      * &
                                    (((P(5))**4.)-(((((MIN(0D0, (T-
P(4))))/(1D0+P(1)))+P(5)))**4.)))       + &
                                    (P(8)*(1D0/3D0)

      * &
                                    (((P(5))**3.)-(((((MIN(0D0, (T-
P(4))))/(1D0+P(1)))+P(5)))**3.)))       + &
                                    (P(9)*(1D0/2D0)

      * &
                                    (((P(5))**2.)-(((((MIN(0D0, (T-
P(4))))/(1D0+P(1)))+P(5)))**2.)))       + &
                                    (P(10)*(1D0/1D0)
                                                                    * &
                                    (((P(5))**1.)-(((((MIN(0D0, (T-
P(4))))/(1D0+P(1)))+P(5)))**1.))))))



!                Y(1) = CTT*(P(12))*((P(3)
                                                                    * &
           !! PERCENTAGE OF RBCS REMAINING AT ANY TIME T AFTER
BIRTH
!                                   ((P(6)*(1D0/5D0)
                                                                    * &
!                                   (((P(5))**5.)-(((((MIN(0D0, (T-
P(4))))/(1D0+P(1)))+P(5)))**5.)))       + &
!                                   (P(7)*(1D0/4D0)

      * &
!                                   (((P(5))**4.)-(((((MIN(0D0, (T-
P(4))))/(1D0+P(1)))+P(5)))**4.)))       + &
!                                   (P(8)*(1D0/3D0)

      * &
!                                   (((P(5))**3.)-(((((MIN(0D0, (T-
P(4))))/(1D0+P(1)))+P(5)))**3.)))       + &
!                                   (P(9)*(1D0/2D0)

      * &
!                                   (((P(5))**2.)-(((((MIN(0D0, (T-
P(4))))/(1D0+P(1)))+P(5)))**2.)))       + &
!                                   (P(10)*(1D0/1D0)
                                                                    * &
!                                   (((P(5))**1.)-(((((MIN(0D0, (T-
P(4))))/(1D0+P(1)))+P(5)))**1.))))))          / &
```

```
!                                    ((((P(3)*P(2))/(P(11)))

      * &
!                                    (((EXP(154D0*P(11)))-
(EXP((P(11)*P(5))-
        &
!                                    (P(11)*((P(4))/(1D0+P(1)))))))
                                                                  - &

!                                    (P(11)*(154D0-
P(5)+((P(4))/(1D0+P(1)))))))
      + &
!                                    (P(3)

      * &         !! TOTAL NUMBER OF INUTERO RBCS PRESENT AT BIRTH
(T=0)
!                                    ((P(6)*(1D0/5D0)
                                                                  * &
!                                    (((P(5))**5.)-((154D0)**5.)))
                                                                  + &
!                                    (P(7)*(1D0/4D0)

      * &
!                                    (((P(5))**4.)-((154D0)**4.)))
                                                                  + &
!                                    (P(8)*(1D0/3D0)

      * &
!                                    (((P(5))**3.)-((154D0)**3.)))
                                                                  + &
!                                    (P(9)*(1D0/2D0)

      * &
!                                    (((P(5))**2.)-((154D0)**2.)))
                                                                  + &
!                                    (P(10)*(1D0/1D0)
                                                                  * &
!                                    (((P(5))**1.)-
((154D0)**1.)))))))*100D0



!                N(0) =      (P(3)

      * &         !! TOTAL NUMBER OF INUTERO RBCS PRESENT AT BIRTH
(T=0)
!                                    ((P(6)*(1D0/5D0)
                                                                  * &
!                                    (((P(5))**5.)-((154D0)**5.)))
                                                                  + &
!                                    (P(7)*(1D0/4D0)

      * &
```

```
!                                  (((P(5))**4.)-((154D0)**4.)))
                                                                + &
!                                  (P(8)*(1D0/3D0)

      * &
!                                  (((P(5))**3.)-((154D0)**3.)))
                                                                + &
!                                  (P(9)*(1D0/2D0)

      * &
!                                  (((P(5))**2.)-((154D0)**2.)))
                                                                + &
!                                  (P(10)*(1D0/1D0)
                                                                * &
!                                  (((P(5))**1.)-((154D0)**1.)))))


      ENDIF BOUNDARY

  RETURN
END IF

! THIS SECTION IS THE SPECIAL OPTIONAL USER OUTPUT SECTION THAT
WILL BE EXECUTED
! WHEN WINFUNFIT IS DONE WITH THE FITTING TO THE CURRENT DATA SET
! (INDICATED BY WINFUNFIT CALLING USERMODEL WITH IFUN=0)
IF(IFUN.EQ.0)THEN
    CALL PROMT(SHOWIT) ! DO WE NEED TO SHOW THE USER PLOT? THIS
CALL STARTS A DIALOG WITH THE USER
    IF(SHOWIT) THEN    ! THE USER WANTED TO SHOW USER PLOT(S)
!!----
!!   USER DESIGNED 'SPECIAL' PLOTS :
!!-----
    CALL GETDATAFILENAME(DATAFILENAME)
    CALL ADDMARGINTEXT(DATAFILENAME)             ! PUT THE DATA
FILE NAME IN THE RIGHT MARGIN OF PLOT
    CALL ADDOBSERVATIONSLEFT(1)                  ! ADDS
OBSERVATIONS (FUNCTION 1) WITH A LEFT Y-AXIS
    CALL ADDFITTEDCURVELEFT(1)                   ! ADDS FITTED
CURVE (FUNCTION 1) WITH A LEFT Y-AXIS
    CALL LEFTLABEL('HB AMOUNT IN BIORBCS (G)')               !
LABEL FOR LEFT Y-AXIS

      CALL INCLUDE_CURVE_RIGHT(TEMX, CUM, TEMN, 2)
      CALL RIGHT_LABEL("CUMULATIVE HB REMOVED (G)")
      CALL END_RIGHT_AT(9D0)
      CALL END_X_AT(70D0)
      CALL ADD_ZERO_LEFT
    CALL TITLE('FETAL ERYTHROPOIESIS MODEL')       ! TITLE OF PLOT
    CALL XLABEL('TIME POST-BIORBC TRANSFUSION(DAYS)')
! LABEL FOR X-AXIS
    CALL DISPLAYPLOT                             ! THIS WILL
CONSTRUCT AND DISPLAY THE PLOT
```

```
!! THIS WILL RECORD THE UNIQUE PLOT ID (PLOT SN) IF PLOT IS SAVED
    CALL GETLUNOUTPUT(LUN)                          ! GET LOGICAL
UNIT NUMBER USED FOR STANDARD OUTPUT
    CALL RECORDPLOTIFSAVED(LUN) ! IF USER SAVES THE PLOT ITS SN
WILL BE RECORDED
    CALL RECORDPLOTIFSAVED(3)   ! IF USER SAVES THE PLOT ITS SN
WILL BE RECORDED ON UNIT 3 (USER OUTPUT SECTION)
    ENDIF
  ENDIF
  RETURN
!-------------------------------------------------------------------
-----------------------------------------
!                      ******* N O N  OPTIONAL DEFINITION SECTION
*******
!
! **** THIS IS FOR THE RECORDING OF THE MODEL USED IN THE FITTING
! **** ALWAYS, ALWAYS, ALWAYS! USE A DIFFERENT NAME OR VERSION
NUMBER WHEN YOU MAKE CHANGES IN THE MODEL

ENTRY MODELID(ID)                                       !!
  ID = 'INUTERORBC (V.1.0)' !* <= CHANGE THIS STRING EVERY TIME YOU
                                                                  !
MAKE CHANGES IN THE ABOVE SUBRROUTINE(S)
  RETURN
END SUBROUTINE USERMODEL
```

B.2 FORTRAN subroutines for chapter 3

```
PROGRAM WINWIN
!IMPLICIT NONE

CHARACTER (LEN=3)           :: SUBNUM = '028'                   !!
CHANGE THIS THREE DIGIT NUMBER FOR EVERY INFANT
INTEGER, PARAMETER                :: A=100, B=105, C=4, E=100, NN=7
!! VALUES HAVE TO BE CHANGED BASED ON NO. OF ROWS IN DATA
DOUBLE PRECISION            :: TIME= 30D0, VALUE, VALUEE= 31.50
REAL*8                                  :: TEMP=0.0, HBUO = 0.0,
TUTR = 45.0, HBL = 0.0, AAAA=0.0, AAB=0.0, BBB = 0.0, YYY=0.0, &
                                           FRA =0.85, ADDD=0.0,
AGGG=0.0, AAAB=0.0,AAAC=0.0,AAAD=0.0,AAAE=0.0, MNPRODRATE=0.0,
MNPRODRATESVN=0.0 !HBT = 0.0
                                                        !! TUTR:
LIFESPAN OF RBCS PRESENT AT TIME OF BIRTH.
                                                        !! TTRAN:
LIFESPAN OF TRANSFUSED RBCS
                                                        !! FRA:
FRACTION OF TRANSFUSED RBCS SURVIVING IMMEDIATELY BEYOND THE
TRANSFUSION.
                                                        !! HBUO:
INTIAL VALUE FOR HB PRESENT AT BIRTH
```

```
                                                        !! AAA,AAB,
BBB, YYY: TEMPORARY VARIABLES

REAL*8                                    :: ALPHA = 0.5076, MCOEFF =
30.70, KCOEFF = 1.0573E7 , GA = 170.0, ACOEFF = -1.60E-5, &
                                                  BCOEFF = 1.28E-2,
CCOEFF = -3.66, DCOEFF = 4.61E2, ECOEFF = -2.15E4, GAMMA =1.86E-2,
MCH = 37.50E-12

      CHARACTER (LEN=13) :: TOTHBNAME = '_TOTAL_HB.DAT'        !!
THESE ARE CHARACTER VARIABLES
CHARACTER (LEN=7)  :: INFANT = 'INFANT_'                       !!
THAT ARE USED LATER TO READ
CHARACTER (LEN=17) :: TRANS = '_TRANSFUSIONS.DAT'        !! THE
FILES TO INPUT THE DATA.
CHARACTER (LEN=15) :: PHLEBO = '_PHLEBOTOMY.DAT'         !!
CHARACTER (LEN=11) :: OUTPT = '_OUTPUT.DAT'         !!
!CHARACTER (LEN=8)  :: EPOM = '_EPO.DAT'
CHARACTER (LEN=15) :: BWT = '_BODYWEIGHT.DAT'       !!
                           !!
CHARACTER (LEN=30) :: FILE1, FILE2, FILE3, FILE4, FILE5, FILE6
!! THE NAME OF THE FOUR FILES THAT ARE TO BE READ. NEEDS TO HAVE
ENOUGH CHARACTER LENGTH TO ACCOMODATE THE ENTIRE FILE NAME !!

INTEGER                                   :: MODEN=0, MAXEVALN =
17000, NEVALN=0, STATUS=0, STATAT=0, STATU=0, &
                                                 STAT=0, STA=0, ST=0,
I=0, J=0, K=0, L=0, M=0, NVALS=0, NUMBER=0          !! STATUS,
STATU, STAT,STA ARE


                           !! READ STATUS VARIABLES. I-L ARE LOOP
VARIABLES.
REAL*8, DIMENSION(A)         :: HBUDAT, THBDAT, HBMDAT, AGEDAT,
HBPRD, HBPRDCM, HBTTT, HBPPRD, HBT, HBI, HH, ABDIFFHBP, ABDIFFH,
SS, SSE
!REAL*8, DIMENSION(D)        :: AG, EPOO
REAL*8, DIMENSION(A, 2)            :: THB, HBU, HBM, HBTT,
HBTTA=0.0, HBTTB=0.0, HBTTC=0.0, HBTTD=0.0, HBTTE=0.0, HBTTF=0.0
!! TWO DIMENSIONAL ARRAY TO STORE TOTAL HB DATA, CALCULATED HB AT
BIRTH, HB TRANSFUSED + BIRTH, HB TRANSFUSED
REAL*8, DIMENSION(B, 2)            :: PHL                        !!
TWO DIMENSIONAL ARRAY TO STORE PHLEBOTOMY DATA
REAL*8, DIMENSION(C, 2)            :: TRA                        !!
TWO DIMENSIONAL ARRAY TO STORE TRANSFUSION DATA
!REAL*8, DIMENSION(D, 2)           :: EP                         !!
TWO DIMENSIONAL ARRAY TO STORE ERYTHROPOIETIN DATA
REAL*8, DIMENSION(E, 2)            :: BWTT                        !!
TWO DIMENSIONAL ARRAY TO STORE BODYWEIGHT DATA

REAL*8, DIMENSION(301, 5)          :: SPLINDAT

REAL*8, DIMENSION(NN)        :: XX = (/40.00, 1.89103E7, 1.11076,
35.06, 10.06, 35.06, 35.06/), &
```

```
                                                XXA = (/30.00,
1.90E7, 1.11, 30.06, 5.05, 23.06, 5.06/), &
                                                XXB = (/85.06,
1.90E7, 1.1124, 65.06, 65.06, 90.06, 120.06/)

!REAL*8, DIMENSION(5)        :: AD = (/1., 2., 3., 4., 5./)
!1.777903E7
FILE1 = INFANT // SUBNUM // TOTHBNAME                    !! CHANGE
THE 'SUBNUM' ABOVE BASED ON WHICH
FILE2 = INFANT // SUBNUM // TRANS                        !! SUBJECT
NUMBER FILES ARE BEING ANALYZED.
FILE3 = INFANT // SUBNUM // PHLEBO                       !! ALWAYS
SAVE ALL THE INPUT FILENAMES IN
!FILE4 = INFANT // SUBNUM // EPOM                         !! THE
SAME FILENAME FORMAT.
FILE5 = INFANT // SUBNUM // BWT                       !!
FILE6 = INFANT // SUBNUM // OUTPT

OPEN (UNIT = 24, FILE = FILE1, STATUS='OLD', ACTION='READ',
IOSTAT=STATUS)            !! READ DATA FROM USB (TOTAL HB FILE)

                                                           !! MAKE
SURE NONE OF DATA FILES HAVE ANY HEADER INFORMATION.

OPEN (UNIT = 26, FILE = FILE2, STATUS='OLD', ACTION='READ',
IOSTAT=STATU)             !! READ DATA FROM USB (RBC TRANSFUSIONS
FILE)

OPEN (UNIT = 28, FILE = FILE3, STATUS='OLD', ACTION='READ',
IOSTAT=STAT)              !! READ DATA FROM USB (PHLEBOTOMIES FILE)

!OPEN (UNIT = 30, FILE = FILE4, STATUS='OLD', ACTION='READ',
IOSTAT=STA)               !! READ DATA FROM USB (EPO DATA FILE)

OPEN (UNIT = 32, FILE = FILE5, STATUS='OLD', ACTION='READ',
IOSTAT=ST)              !! READ DATA FROM USB (BODYWEIGHT FILE)

OPEN (UNIT = 34, FILE = FILE6, STATUS='REPLACE', ACTION='WRITE',
IOSTAT=STATAT)              !! WRITE FINAL OUTPUTS

!! THE FIRST STEP IS TO CHECK IF ALL THE FILES ARE READ CORRECTLY
BY THE PROGRAM. USE WRITE STATEMENTS TO VERIFY DATA READ IN
CORRECTLY.
!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
FILEOPEN: IF(STATUS == 0 .AND. STATU == 0 .AND. STATU == 0 .AND.
STA == 0 .AND. STATAT == 0) THEN               !!ALL FOUR FILES
ARE READ CORRECTLY

    WRITEDO:DO I = 1, A
                !! NAME ALL DO LOOPS. IT BECOMES EASIER TO DEBUG
THE CODE.

        READ(24,*, IOSTAT=STATUS) THB(I, 1), THB(I, 2)       !!
READ & STORE THE TOTAL HB VS AGE DATA
```

```
!        WRITE(*,*) THB(I, 1), THB(I, 2)

    END DO WRITEDO

    WRITEDOO:DO J = 1, C

        READ(26,*, IOSTAT=STATUS) TRA(J, 1), TRA(J, 2)       !!
READ & STORE THE TRANSFUSED HB VS AGE DATA
!        WRITE(*,*) TRA(J, 1), TRA(J, 2)

    END DO WRITEDOO

    WRITEDON:DO K = 1, B

        READ(28,*, IOSTAT=STATUS) PHL(K, 1), PHL(K, 2)       !!
READ & STORE THE FRACTION REMAINING PHLEBOTOMY VS AGE DATA
!        WRITE(*,*) PHL(K, 1), PHL(K, 2)

    END DO WRITEDON
!
!    WRITEDONE:DO L = 1, D
!
!        READ(30,*, IOSTAT=STATUS) EP(L, 1), EP(L, 2)           !!
READ & STORE THE EPO DAT VS AGE
!        WRITE(*,*) PHL(K, 1), PHL(K, 2)

!    END DO WRITEDONE

    WRITEDONN:DO M = 1, E

        READ(32,*, IOSTAT=STATUS) BWTT(M, 1), BWTT(M, 2)       !!
READ & STORE THE BODYWEIGHT VS AGE DATA
!        WRITE(*,*) BWTT(M, 1), BWTT(M, 2)

    END DO WRITEDONN


ELSE FILEOPEN                                          !! ERROR IN
READING FILES. PRINT ERROR CODE.


    WRITE(*, 1040) STATUS
    1040 FORMAT (1X, 'FILE OPEN FAILED--STATUS = ', I6) !! PRINT
ERROR

END IF FILEOPEN

CLOSE (UNIT = 24)
CLOSE (UNIT = 26)
CLOSE (UNIT = 28)
!CLOSE (UNIT = 30)
CLOSE (UNIT = 32)
```

```
!! ALL FILES READ AND STORED CORRECTLY. NEXT STEP: MODEL HB PRESENT
AT TIME OF BIRTH (HBU)


NELDER: DO

MODELDO: DO I= 1, A

    HBU(I, 1) = THB(I, 1)                                    !! COPY
THE AGE DATA TO HBU


      BOUNDARY: IF(HBU(I, 1) <= ((154.-GA)*(1D0+XX(3))+XX(1))) THEN

      !! CASE 1


                  HBU(I, 2) = MCH*((((ACOEFF*(1D0/5D0)*(((GA)**5.)-
((154.)**5.)))         + &                                !! NUMBER
OF RBCS REMAINING AT ANY TIME T AFTER BIRTH

      (BCOEFF*(1D0/4D0)*(((GA)**4.)-((154.)**4.)))             + &
                                  !! MULTIPLY BY THE FRACTION OF
TOTAL RBCS THAT ARE BIOTIN LABELLED AND REINFUSED

      (CCOEFF*(1D0/3D0)*(((GA)**3.)-((154.)**3.)))             + &

      (DCOEFF*(1D0/2D0)*(((GA)**2.)-((154.)**2.)))             + &
                                        (ECOEFF*((GA)-
(154.))))*XX(2))                                    + &
                                        (((XX(2)*MCOEFF)/(GAMMA))
                                              * &
                                        ((EXP(154D0*GAMMA))-
(EXP(GAMMA*GA+                          &
                                        (GAMMA*(MIN(0D0, (HBU(I,
1)-XX(1))))/(1D0+XX(3)))))))      + &
                                        ((XX(2)*MCOEFF)
                                                  * &
                                        (((MIN(0D0, (HBU(I, 1)-
XX(1))))/(1D0+XX(3)))-154D0+GA)))


      ELSE


                  HBU(I, 2) = MCH*(XX(2)
                                                              * &
            !! NUMBER OF RBCS REMAINING AT ANY TIME T AFTER BIRTH
                                  ((ACOEFF*(1D0/5D0)
                                                              * &
                                  (((GA)**5.)-(((((MIN(0D0, (HBU(I,
1)-XX(1))))/(1D0+XX(3)))+GA))**5.)))          + &
                                  (BCOEFF*(1D0/4D0)
                                                        * &
```

```
                                    (((GA)**4.)-(((((MIN(0D0, (HBU(I,
1)-XX(1))))/(1D0+XX(3)))+GA))**4.)))          + &
                                    (CCOEFF*(1D0/3D0)
                                                                  * &
                                    (((GA)**3.)-(((((MIN(0D0, (HBU(I,
1)-XX(1))))/(1D0+XX(3)))+GA))**3.)))          + &
                                    (DCOEFF*(1D0/2D0)
                                                                  * &
                                    (((GA)**2.)-(((((MIN(0D0, (HBU(I,
1)-XX(1))))/(1D0+XX(3)))+GA))**2.)))          + &
                                    (ECOEFF*(1D0/1D0)
                                                                  * &
                                    (((GA)**1.)-(((((MIN(0D0, (HBU(I,
1)-XX(1))))/(1D0+XX(3)))+GA))**1.)))))

      ENDIF BOUNDARY

    !WRITE(*,*) HBU(I, 1), HBU(I, 2)

END DO MODELDO

HBM = HBU                                                      !! HBM
IS THE SAME AS HBU, BUT WE USE HBM TO ADD TRANSFUSION AND PHLB
EFFECTS LATER

!! NEXT STEP: UPDATE THE MODEL WITH TRANSFUSIONS GIVEN AFTER BIRTH
(TRA)
!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
HBTTA(:,1) = THB(:,1)
HBTTB(:,1) = THB(:,1)
HBTTC(:,1) = THB(:,1)
HBTTD(:,1) = THB(:,1)
                                       !! AGE
HBTTE(:,1) = THB(:,1)

DO I= 1, A                                                  !! FIRST
TRANSFUSION

      IF(((TRA(1, 1)+XX(4)-HBTTA(I,1))/XX(4)) .LE. 1.0) THEN
!! MAXIMUM VALUE OF PARANTHESIS SHOULD BE 1.

            HBTTA(I,2) = FRA*(TRA(1, 2)*((MAX(0.,(TRA(1, 1)+XX(4)-
HBTTA(I,1))))/XX(4)))

!           HBT(T,1) = T
!           WRITE(*,*) HBT(T), T

      ELSE

            HBTTA(I, 2) = 0.0
!           WRITE(*,*) HBT(T)
!

      ENDIF
```

```
END DO

!WRITE(*,*) HBTTA

DO I= 1, A                                                    !! SECOND
TRANSFUSION

      IF(((TRA(2, 1)+XX(5)-HBTTB(I,1))/XX(5)) .LE. 1.0) THEN
!! MAXIMUM VALUE OF PARANTHESIS SHOULD BE 1.

!           TEMP = HBTTB(I, 2)
            HBTTB(I,2) = FRA*(TRA(2, 2)*((MAX(0.,(TRA(2, 1)+XX(5)-
HBTTB(I,1))))/XX(5)))

            TEMP = 0.0

      ENDIF

!           HBT(T,1) = T                                          !!
AGE

END DO


DO I= 1, A                                                    !! THIRD
TRANSFUSION

      IF(((TRA(3, 1)+XX(6)-HBTTC(I,1))/XX(6)) .LE. 1.0) THEN
!! MAXIMUM VALUE OF PARANTHESIS SHOULD BE 1.

!           TEMP = HBTTC(I, 2)
            HBTTC(I,2) = FRA*(TRA(3, 2)*((MAX(0.,(TRA(3, 1)+XX(6)-
HBTTC(I,1))))/XX(6)))

            TEMP = 0.0

      ENDIF

!           HBT(T,1) = T                                          !!
AGE

END DO

!
DO I= 1, A                                                    !! FOURTH
TRANSFUSION

      IF(((TRA(4, 1)+XX(7)-HBTTD(I,1))/XX(7)) .LE. 1.0) THEN
!! MAXIMUM VALUE OF PARANTHESIS SHOULD BE 1.

!           TEMP = HBTTD(I, 2)
            HBTTD(I,2) = FRA*(TRA(4, 2)*((MAX(0.,(TRA(4, 1)+XX(7)-
HBTTD(I,1))))/XX(7)))
```

```
            TEMP = 0.0

      ENDIF

!           HBT(T,1) = T                                                !!
AGE

END DO

!!
!!DO I= 1, A                                                  !! FIFTH
TRANSFUSION
!!
!!    IF(((TRA(5, 1)+XX(8)-HBTTD(I,1))/XX(8)) .LE. 1.0) THEN
!! MAXIMUM VALUE OF PARANTHESIS SHOULD BE 1.
!!
!!!         TEMP = HBTTD(I, 2)
!!          HBTTF(I,2) = FRA*(TRA(5, 2)*((MAX(0.,(TRA(5, 1)+XX(8)-
HBTTD(I,1))))/XX(8)))
!!
!!          TEMP = 0.0
!!
!!    ENDIF
!
!!          HBT(T,1) = T                                                !!
AGE
!
!!END DO


HBTTE(:,2) = HBTTA(:,2) + HBTTB(:,2) + HBTTC(:,2) + HBTTD(:,2)+
HBTTF(:,2)

SS = HBTTE(:,2)                                          !! TOTAL
TRANSFUSED HB BEFORE PHLB CORRECTION

!WRITE(*,*) TRA

! HBTTE HAS ALL THE TRANSFUSED RBC DATA BEFORE PHLEBOTOMY
CORRECTION


!HBTT(:,1) = HBM(:,1)
!HBTT(:,2) = HBM(:,2) - HBU(:,2)                          !! ONLY ALL
OF THE TRANSFUSED RBCS BEFORE PHLEBOTOMY

!WRITE(*,*) HBTTA

!! NEXT STEP: UPDATE THE MODEL WITH PHLEBOTOMIES CONDUCTED AFTER
BIRTH (PHL)

MDL: DO J = 1, B                                                    !!
TO ACCOUNT FOR ALL PHLEBOTOMIES
```

```
    MODELDS: DO I = 1, A


        IF(HBM(I,1) .GE. PHL(J,1)) THEN

            AAAA = HBTTA(I,2)
!! HB MODELED 1ST TRANSFUSION ONLY

            AAAB = HBTTB(I,2)
!! HB MODELED 2ND TRANSFUSION ONLY

            AAAC = HBTTC(I,2)
!! HB MODELED 3RD TRANSFUSION ONLY

            AAAD = HBTTD(I,2)
!! HB MODELED 4RTH TRANSFUSION ONLY

            AAAE = HBTTF(I,2)
!! HB MODELED 5TH TRANSFUSION ONLY

            BBB = HBU(I,2)                                          !!
HB PRESENT AT TIME OF BIRTH

            HBU(I, 2) = PHL(J,2)*BBB

                IF(PHL(J,1) .GT. TRA(1,1)) THEN
!! IMPORTANT: SINCE HBTT(:,2) IS THE SUM OF ALL TRANSFUSED RBCS,
THIS CORRECTION APPLIES FOR ALL

                 HBTTA(I, 2) = PHL(J,2)*AAAA
           !! FUTURE TRANSFUSIONS AFTER TIME TRA(1,1).

                ELSE IF(PHL(J,1) .GT. TRA(2,1)) THEN

                 HBTTB(I, 2) = PHL(J,2)*AAAB

                ELSE IF(PHL(J,1) .GT. TRA(3,1)) THEN

                 HBTTC(I, 2) = PHL(J,2)*AAAC

                ELSE IF(PHL(J,1) .GT. TRA(4,1)) THEN

                 HBTTD(I, 2) = PHL(J,2)*AAAD

!               ELSE IF(PHL(J,1) .GT. TRA(5,1)) THEN
!
!                HBTTF(I, 2) = PHL(J,2)*AAAE

                END IF

!           WRITE(*,*) HBM(I,1), BBB, HBU(I, 2), AAAA, HBTT(I,2)

        END IF
```

```
    END DO MODELDS

END DO MDL

HBTTE(:,2) = HBTTA(:,2) + HBTTB(:,2) + HBTTC(:,2) + HBTTD(:,2)+
HBTTF(:,2)

SSE = HBTTE(:,2)                                          !! TOTAL
TRANSFUSED HB AFTER PHLB CORRECTION

HBM(:,2) = HBU(:,2) + HBTTE(:,2)

!! NEXT STEP: CALCULATE THE QUANTITY PRODUCED AND PRINT THE RESULTS

MODELDDD: DO I= 1, A

    THBDAT(I) = THB(I,2)

    HBMDAT(I) = HBM(I,2)

    HBPRD(I)  = THBDAT(I)-HBMDAT(I)
                        !! HB PRODUCED AFTER BIRTH,BUT HAVE TO ADD
THE FRACTION REMOVED DUE TO PHLEBOTOMIES

!    IF(HBPRD(I) .LT. 0.0) THEN
!
!        HBPRD(I)  = 0.0
                              !! HB PRODUCED AFTER BIRTH CANT BE
NEGATIVE. SO SET THE LOWER LIMIT TO ZERO.
!
!     END IF

!    WRITE(*,*) THB(I,1), THB(I, 2), HBU(I, 2), HBM(I,2)

END DO MODELDDD

MDLM: DO J = 1, B
                        !! TO ACCOUNT FOR ALL PHLEBOTOMIES

    MODELDSM: DO I = 1, A

        IF(HBM(I,1) .GE. PHL(J,1)) THEN

            AAB = HBPRD(I)
                 !! HB PRODUCED

                 HBPRD(I) = AAB/PHL(J,2)
                       !! ADD FRACTION REMOVED DUE TO PHLEBOTOMY

        END IF

    END DO MODELDSM
```

```
END DO MDLM

!*****************************************************************
*****************
HBPRD(1) = 0.0
!HBPRD(2) = 0.0
!HBPRD(9) = 0.0
!HBPRD(8) = 0.0
!*****************************************************************
*****************
                                                    !! NO HB PRODUCED
AT TIME 0
!WRITE(*,*) HBPRD
!WRITE(*,*)
'*************************************************************'

CALL IMAN_CONOVER_REGRESSION(HBPRD, A, HBPPRD)
!WRITE(*,*) HBPPRD

AGGG = SUM(ABS(HBPRD-HBPPRD))                          !! SUM OF
ABSOLUTE DIFFERENCE. MINIMISE THIS FOR BEST ESTIMATE.
!WRITE(*,*) AGGG


! NELDER MEAD MINIMIZATION. MINIMISE THE SUM OF THE ABSOLUTE
DIFFERENCE.

!      CALL NELMIN_BC(X,XA,XB,N,FX,MODE,MAXEVAL,NEVAL)
      CALL NELMIN_BC(XX,XXA,XXB,NN,AGGG,MODEN,MAXEVALN,NEVALN)

      IF(MODEN.GT.1)EXIT
!      FX = FUNCTION OF X TO BE MINIMIZED. (DEFINED BY THE USER)

END DO NELDER
!      IF(MODE.NE.2) SOME USER ERROR HAS OCCURED
!-----------------------------------------------------------------
-------------------------------------------------------------

WRITE(*,*)
'*********************************************************'
WRITE(*,*) 'MODE VALUE OF 2 MEANS MINIMUM FOUND. FINAL MODE VALUE
=', MODEN
WRITE(*,*)
'*********************************************************'

!*****************************************************************
**************
 !PRINT FINAL EVALS
!WRITE(*,*)
'*********************************************************'
!
DO I=1,NN

      WRITE(*,*) XX(I)
```

```
END DO

! FIT CUBIC SPLINE TO REGRESSION POINTS HBPPRD
!WRITE(*,*) HBPPRD
!! FINAL YHATS FROM THE REGRESSION
AGEDAT = THB(:,1)
!! THE AGE DATA IS SAVED IN AGEDAT

! AGEDAT ARE THE X VALUES AND HBPPRD ARE THE Y VALUES FOR THE CUBIC
SPLINE

!! NEXT STEP: FIT CUBIC SPLINE TO THE AGEDAT,HBPPRD DATA AND PLOT
THE FIRST DERIVATIVE OF THE CUBIC SPLINE.

CALL GENERATE_OBJECTS_FROM_XY_DATA(BWTT(:,1), BWTT(:,2), E,
'BODYWT') !! BW DATA
CALL GENERATE_FITTED_CURVE('GCV_CUBIC_SPLINE', 'BODYWT', 'GC')


CALL SET_CV_VALUE_FOR_SPLINE_FIT(VALUEE)             !! THE CV
VALUE CONTROLS THE SMOOTHNESS OF THE CUBIC SPLINE.

CALL CUBIC_GCV_FIT (AGEDAT,HBPPRD,A)                   !! GCV FIT
TO THE DATA

INTIALIZEARRAY: DO I= 1,301                            !! GENERATE
301 POINTS FROM 0 TO 30 DAYS

      SPLINDAT(I,1) = 0.10*(REAL(I)-1D0)                   !! TIME
POINTS FOR WHICH THE CUBIC SPLINE HAS TO BE EVALUATED

      SPLINDAT(I,2) = 0D0

      SPLINDAT(I,3) = 0D0

END DO INTIALIZEARRAY

!WRITE(*,*) SPLINDAT(:,1)                              !! THE
GENERATED POINTS ARE CORRECT AND WORKS CORRECTLY

CALCUSPL: DO I= 1,301

      CALL CUBIC_GCV(SPLINDAT(I,1),SPLINDAT(I,2))           !!
CALCULATE CUBIC SPLINE

      CALL CUBIC_GCV_DERIVATIVE(SPLINDAT(I,1),SPLINDAT(I,3)) !!
CALCULATE CUBIC SPLINE FIRST DERIVATIVE

      CALL GET_VALUE_OF_CUBIC_SPLINE('GC', SPLINDAT(I,1),
SPLINDAT(I,4)) !! CALCULATE BW AT EACH TIME PT

      SPLINDAT(I,5) = SPLINDAT(I,3)/SPLINDAT(I,4)
```

```
      IF(I .EQ. 71) THEN

            WRITE(*,*) 'CUMULATIVE HB PRODUCED AFTER BIRTH OVER 7
DAYS OF LIFE:', SPLINDAT(I,2)

      ELSE IF(I .EQ. 301) THEN

            WRITE(*,*) 'CUMULATIVE HB PRODUCED AFTER BIRTH OVER 30
DAYS OF LIFE:', SPLINDAT(I,2)
!           WRITE(*,*) 'HB PRODUCTION RATE NORMALISED TO BW AT 30
DAYS OF LIFE:', SPLINDAT(I,3)

      END IF

END DO CALCUSPL

MNPRODRATE = SUM(SPLINDAT(:,3))/301D0                              !!
MEAN RBC PRODUCTION RATE OVER 30 DAYS OF LIFE

MNPRODRATESVN = SUM(SPLINDAT(1:71,3))/71D0
!! MEAN RBC PRODUCTION RATE OVER 7 DAYS OF LIFE

!WRITE(*,*) SPLINDAT(:,1)

!WRITE(*,*) SPLINDAT(:,3)     !SPLINDAT(:,4), SPLINDAT(:,5)

WRITE(*,*) 'MEAN HB PRODUCTION RATE OVER 30 DAYS OF LIFE:',
MNPRODRATE

WRITE(*,*) 'MEAN HB PRODUCTION RATE OVER FIRST 7 DAYS OF LIFE:',
MNPRODRATESVN


WRITE(34, 1060) SPLINDAT(71,2), SPLINDAT(301,2), MNPRODRATE,
MNPRODRATESVN
1060 FORMAT (1X, 'CUMULATIVE HB PRODUCED AFTER BIRTH OVER 7 DAYS OF
LIFE = ', F10.3, /,&
                 'CUMULATIVE HB PRODUCED AFTER BIRTH OVER 30 DAYS
OF LIFE:', F10.3, /,&
                 'MEAN HB PRODUCTION RATE OVER 30 DAYS OF LIFE:',
F10.3, /,&
                 'MEAN HB PRODUCTION RATE OVER FIRST 7 DAYS OF
LIFE:', F10.3) !! OUTPUT


CLOSE (UNIT = 34)

HBUDAT = HBU(:,2)
!AG = EP(:,1)
!EPOO = EP(:,2)
HBTTT = HBTT(:,2)

!! NEXT STEP: PLOTTING THE OUTPUT USING PVPPLOT.
```

```
CALL TITLE(FILE1(1:10))                                    !! LABEL
PLOT WITH CONCATENATED FILE NAME

CALL X_LABEL('DAY OF LIFE')
CALL LEFT_LABEL('CUMULATIVE HB PRODUCED (G)')
CALL RIGHT_LABEL('HB PROD RATE (G/DAY/KG)')
CALL BEGIN_LEFT_AT(0D0)
CALL END_LEFT_AT(9D0)
CALL BEGIN_X_AT(0D0)
CALL END_X_AT(35D0)


CALL BEGIN_RIGHT_AT(0D0)
CALL END_RIGHT_AT(3D0)

CALL INCLUDE_POINTS(AGEDAT, HBPPRD, A, 3)

CALL ADD_CURVE(SPLINDAT(:,1),SPLINDAT(:,2),301)

!CALL ADD_CURVE_RIGHT(SPLINDAT(:,1),SPLINDAT(:,3),301)

CALL ADD_CURVE_RIGHT(SPLINDAT(:,1),SPLINDAT(:,5),301)  !!
BODYWEIGHT NORMALISED RBC PRODUCTION RATE

!CALL GENERATE_OBJECTS_FROM_XY_DATA(AGEDAT, HBPPRD, A, 'CUMHB')
!
!CALL GET_VALUE_OF_CUBIC_SPLINE('FITTED_CURVE', TIME, VALUE)
!WRITE(*,*) 'CUMULATIVE HB PRODUCED AFTER BIRTH FOR THE FIRST 30
DAYS AFTER BIRTH = ', VALUE
!CALL GENERATE_FITTED_CURVE('CUBIC_POLYNOMIAL', 'CUMHB', 'CUBIC')
!! CUBIC FIT
!CALL ADD_TO_PLOT('CUBIC', 'ADD_LINEAR_SPLINE_LEFT')
!CALL ADD_TO_PLOT('CUBIC', 'ADD_XY_DATA_LEFT')

!CALL ADD_TO_PLOT('CUBIC', 'ADD_CUBIC_SPLINE_LEFT')
!
!CALL ADD_TO_PLOT('CUBIC', 'ADD_CUBIC_SPLINE_DERIVATIVE_RIGHT')

CALL PLOT_IN_AREA(4,4)

CALL X_LABEL('DAY OF LIFE')
CALL LEFT_LABEL('HEMOGLOBIN (G)')
CALL BEGIN_LEFT_AT(0D0)
CALL INCLUDE_POINTS(AGEDAT, THBDAT, A, 3)
CALL INCLUDE_CURVE_LEFT(AGEDAT, HBMDAT, A, 0)
CALL INCLUDE_CURVE_LEFT(AGEDAT, HBTTE(:,2), A, 1)
CALL INCLUDE_CURVE_LEFT(AGEDAT, SS, A, 2)              !! SS
REPRESENTS SUM OF ALL TRANSFUSIONS BEFORE PHLEBOTOMY
CALL INCLUDE_CURVE_LEFT(AGEDAT, SSE, A, 4)
CALL PLOT_IN_AREA(1,4)
!!
!CALL TITLE('SUBJECT 302')
CALL X_LABEL('DAY OF LIFE')
CALL LEFT_LABEL('HEMOGLOBIN (G)')
```

```
!CALL RIGHT_LABEL('EPO (MU/ML)')
!CALL INCLUDE_CURVE_LEFT(AGEDAT, HBPRD, A, 4)
CALL INCLUDE_POINTS(AGEDAT, HBPRD, A, 3)
CALL INCLUDE_CURVE_LEFT(AGEDAT, HBPPRD, A, 0)
!CALL INCLUDE_CURVE_LEFT(AGEDAT, HBPRDCM, A, 1)
!CALL INCLUDE_POINTS_RIGHT(AG, EPOO, D, 2)
!CALL INCLUDE_CURVE_RIGHT(AG, EPOO, D, 2)
CALL PLOT_IN_AREA(2,4)

CALL DISPLAY_PLOT
CALL GET_SERIAL_NUMBER_IF_PLOT_IS_SAVED(NUMBER)
IF(NUMBER/=0)PRINT*, 'THE PLOT SERIAL NUMBER FOR THE SAVED PLOT IS:
', NUMBER

STOP

END PROGRAM WINWIN
```

B.3 FORTRAN subroutines for chapter 4

```
! FILENAME = BIORBC_SURVIVAL_MODEL_DIFF_V1.1.F90 (SS ADULT RBCS)
!
! PURPOSE:  TO MODEL THE BIORBC SURVIVAL CURVE WHILE ACCOUNTING FOR
MULTIPLE CLINICAL PHLEBOTOMIES,
!            TRANSFUSIONS AND INCREASE IN BODY WEIGHT
!
! REVISIONS:
!   VERSION  1.0    JUL 02, 2012
!   VERSION  1.1    MAR 06, 2015    !
!=================================================================
==========================
!
! THE FOLLOWING SUBROUTINE (USERMODEL):
! (1) DEFINES THE EQUATIONS TO BE FITTED
! (2) ASSIGNS NAMES TO THE PARAMETERS (IFUN=-1000 CALL)
! (3) ALLOWS THE USER TO DEFINE AND REGISTER EVENT (IFUN =-1000
CALL)
! (4) INTERACTIVELY ALLOWS THE USER TO SELECT THE ALGORITHM TO BE
!     USED BY WINFUNFIT FOR THE INTEGRATION OF THE DIFFERENTIAL
EQUATIONS
!     SPECIFIED IN THE SUBROUTINE "USERMODEL_ODE" GIVEN ABOVE.
! (5) PROVIDES THE USER THE OPPORTUNITY TO MAKE SPECIAL
CALCULATIONS
!     AND PLOTS AFTER WINFUNFIT HAS COMPLETED A FITTING TO A DATA
SET (IFUN=0 CALL)
!-----------------------------------------------------------------
--------------------------

SUBROUTINE USERMODEL(T,Y,P,NP,IFUN)  !   USERMODEL IS A REQUIRED
NAME (DO NOT CHANGE)
```

```
    USE PHLEBOTOMY_TRANSFUSION_MODULE
    USE NUMERICAL_LIBRARIES

    INTEGER, PARAMETER  :: NEQN = 1, NPAR=3, MAXN = 500, MAXCOEFF =
100, LUN = 3
    REAL*8, PARAMETER   :: FACTOR = 2D0, TOLERANCE = 1.0D-7,
TIMEZERO = 0D0, ABSERR = 0D0, &
                           RELERR = 0.001
    INTEGER             :: NP, IFUN, JFUN, NSIGDIGITS, NUM,
NOEVENTS, NPHLEB, NTRANS, &
                           TEMPN, NOCOEFF, J, SUBNO, JOB, TN, K,
KLAST, NEPO
    REAL*8              :: T, Y, TZER, CTT, AMT(NEQN), P(*),
YZERO(NEQN), TZERO(NEQN), TEMPT, TEMPA, TEMPX(MAXN), &
                           TEMPY(MAXN), BVOL,
COEFFICIENTS(MAXCOEFF), ESTERR, HBZERO, A1, &
                           XMAX, HBTOTALPROD, HBTOTALPHLEB,
HBTOTALTRANS, TEMPSUM, TY, TX, TR, &
                           MAXHBAMT, TX1(MAXN), TX2(MAXN),
TY1(MAXN), TY2(MAXN), TYMAX, BRET, LTRBC, LTRET, &
                           MONTHHBTOTALPROD, FUNDAMAGED,
XEPO(MAXN), YEPO(MAXN), MCHE, MCHT, CV, FRETT, &
                           TRANS_FRAC, RET_TRANS_FRAC, BRBC, A2,
INPUT1, INPUT2, AVGSTIMRATE, A3, T3, INPUT3
    LOGICAL, SAVE       :: SHOWIT, PLOTSAVED
    CHARACTER (LEN=256) :: ID, DATAFILENAME
    CHARACTER (LEN=20)  :: PNAME
    CHARACTER (LEN=1)   :: RESPONSE

!----
! BEFORE FITTING WE WOULD LIKE TO GIVE NAMES TO THE PARAMETERS AND
! SELECT THE ALGORITM FOR THE INTEGRATION OF THE DIFF EQUATIONS
!----
IF(IFUN == -1000)THEN
! THIS SECTION (IFUN.EQ.-1000) ALLOW YOU TO SET OPTIONS BEFORE THE
! START OF THE FITTING TO THE DATA (WHICH OCCURS WHEN IFUN = -1000)
!-------------------------------------------------------------------
-----
!ASSIGN NAMES (HIGHLY RECOMMENDED FOR READABILITY OF OUTPUT)

       CALL SetFunfitParameterName(1,"YZERO")
!! HB AMOUNT (GM) AT TIME T=0, THE TIME OF BIORBC TRANSFUSION
(NORMALISED)
       CALL SetFunfitParameterName(2,"TAU")
!! LIFESPAN OF THE RED BLOOD CELLS
!      CALL SetFunfitParameterName(3,"FRACRMVD")
!! FRACTION OF BIORBC REMOVED WITH ZERO SURVIVAL
!-------------------------------------------------------------------
-----

! ============= SET PHLEBOTOMY, TRANSFUSION, STIMULATION RATE
KNOTS, AND BODYWEIGHT VECTORS =============
!                   NOTE: THE TIME/KNOT VECTORS MUST BE SET BEFORE
THE AMOUNT/FVALUE VECTORS
```

```
        TEMPN = MAXN
        PRINT*
        PRINT*,' PHLEBOTOMY FRACTION REMAINING-TIME DATA:'
        CALL GET_XY_DATA_FROM_FUNFIT_FILE (TEMPX,TEMPY,TEMPN)
        IF ( TEMPN > MAXN ) STOP ' TOO MANY PHLEBOTOMY DATA POINTS.
ADJUST THE MAXN APPROPRIATELY'
        CALL SET_PHLEBOTOMY_TIME_VECTOR(TEMPN, TEMPX)
        CALL SET_FRACTION_REMAINING_VECTOR(TEMPN, TEMPY)

!-----------------------------------------------------------------
-----

  RETURN
ENDIF

!=================================================================
================================================
! THIS SECTION DOES THE INTEGRATION OF THE ODEs AND PROVIDES THE
PREDICTED VALUES TO BE FITTED TO THE DATA
! FOR THE 3 VARIABLES Y(1), Y(2) AND Y(3) (IFUN = 1,2 AND 3)
IF(IFUN == 1) THEN

      TZER = 0D0
                  !!
      CALL GET_PHLEBOTOMY_CORRECTION_TERM(TZER, T, CTT)       !!
TZERO IS A CONSTANT TSTART TIME OF TRANSFUSION & T IS THE TEND
WHERE WE NEED TO CALCULATE THE CORRECTION TERM CTT
!     WRITE(*,*) T, CTT
!     Y = P(4) * P(3) * MAX(0D0, 1D0 - ((T-P(1))/P(2))) * CTT
!! F IS THE PRODUCT OF THE FRACTION REMAINING AFTER PHLEBOTOMIES
UNTIL TIME T
      Y = P(1) * (MAX(0D0, 1D0 - (T/P(2)))) * CTT        !! F IS
THE PRODUCT OF THE FRACTION REMAINING AFTER PHLEBOTOMIES UNTIL TIME
T
!     Y = P(5) * P(3) * MAX(0D0, 1D0 - ((T-P(1))/P(2))) * CTT +
(1D0 - P(5)) * P(3) * MAX(0D0, 1D0 - ((T-P(1))/P(4))) * CTT
!! F IS THE PRODUCT OF THE FRACTION REMAINING AFTER PHLEBOTOMIES
UNTIL TIME T

  RETURN
ENDIF

! THIS SECTION IS THE SPECIAL OPTIONAL USER OUTPUT SECTION THAT
WILL BE EXECUTED
! WHEN WINFUNFIT IS DONE WITH THE FITTING TO THE CURRENT DATA SET
! (INDICATED BY WINFUNFIT CALLING USERMODEL WITH IFUN=0)
IF(IFUN.EQ.0)THEN
    CALL PROMT(SHOWIT) ! DO WE NEED TO SHOW THE USER PLOT? THIS
CALL STARTS A DIALOG WITH THE USER
    IF(SHOWIT) THEN    ! THE USER WANTED TO SHOW USER PLOT(S)
!----
!   USER DESIGNED 'SPECIAL' PLOTS :
!-----
```

```
    CALL GETDATAFILENAME(DATAFILENAME)
!    CALL ADDMARGINTEXT(DATAFILENAME)              ! PUT THE DATA
FILE NAME IN THE RIGHT MARGIN OF PLOT
    CALL ADDOBSERVATIONSLEFT(1)                   ! ADDS
OBSERVATIONS (FUNCTION 1) WITH A LEFT Y-AXIS
    CALL ADDFITTEDCURVELEFT(1)                    ! ADDS FITTED
CURVE (FUNCTION 1) WITH A LEFT Y-AXIS
    CALL LEFTLABEL('HB IN BIORBC G/ML')               ! LABEL FOR
LEFT Y-AXIS

      CALL END_X_AT(70D0)
      CALL ADD_ZERO_LEFT
    CALL TITLE('BIORBC STUDY')       ! TITLE OF PLOT
    CALL XLABEL('TIME POST-BIORBC TRANSFUSION (DAYS)')
! LABEL FOR X-AXIS
    CALL DISPLAYPLOT                              ! THIS WILL
CONSTRUCT AND DISPLAY THE PLOT
! THIS WILL RECORD THE UNIQUE PLOT ID (PLOT SN) IF PLOT IS SAVED
    CALL GETLUNOUTPUT(LUN)                        ! GET LOGICAL
UNIT NUMBER USED FOR STANDARD OUTPUT
    CALL RECORDPLOTIFSAVED(LUN) ! IF USER SAVES THE PLOT ITS SN
WILL BE RECORDED
    CALL RECORDPLOTIFSAVED(3)   ! IF USER SAVES THE PLOT ITS SN
WILL BE RECORDED ON UNIT 3 (USER OUTPUT SECTION)
    ENDIF
  ENDIF
  RETURN

!                     ******* N O N  OPTIONAL DEFINITION SECTION
*******
!
! **** THIS IS FOR THE RECORDING OF THE MODEL USED IN THE FITTING
! **** ALWAYS, ALWAYS, ALWAYS! USE A DIFFERENT NAME OR VERSION
NUMBER WHEN YOU MAKE CHANGES IN THE MODEL

  ENTRY MODELID(ID)
  ID = 'BIORBC (V.1.1)' !* <= CHANGE THIS STRING EVERY TIME YOU
                                                                  !
MAKE CHANGES IN THE ABOVE SUBRROUTINE(S)
  RETURN
  END
!-- ------------------------------------------------ E N D ---------
----------------------------------------


! FILENAME = BIORBC_SURVIVAL_MODEL_DIFF_V1.1.F90 (NON-SS INFANT
RBC)
!
! PURPOSE:  TO MODEL THE BIORBC SURVIVAL CURVE FOR IN-UTERO RBC
PRODUCTION
!
! REVISIONS:
!   VERSION  1.0
```

```
!     VERSION  1.3    MAR 03, 2015    ADDDED CUMULATIVE HB REMOVED
CALCULATION AND PLOT.
!=================================================================
=========================
!/////////////////////////////////////////////////////////////////
/////////////////////////
!=================================================================
=========================
!
! THE FOLLOWING SUBROUTINE (USERMODEL):
! (1) DEFINES THE EQUATIONS TO BE FITTED
! (2) ASSIGNS NAMES TO THE PARAMETERS (IFUN=-1000 CALL)
! (3) ALLOWS THE USER TO DEFINE AND REGISTER EVENT (IFUN =-1000
CALL)
! (4) INTERACTIVELY ALLOWS THE USER TO SELECT THE ALGORITHM TO BE
!     USED BY WINFUNFIT FOR THE INTEGRATION OF THE DIFFERENTIAL
EQUATIONS
!     SPECIFIED IN THE SUBROUTINE "USERMODEL_ODE" GIVEN ABOVE.
! (5) PROVIDES THE USER THE OPPORTUNITY TO MAKE SPECIAL
CALCULATIONS
!     AND PLOTS AFTER WINFUNFIT HAS COMPLETED A FITTING TO A DATA
SET (IFUN=0 CALL)
!-----------------------------------------------------------------
-------------------------

SUBROUTINE USERMODEL(T,Y,P,NP,IFUN)  !   USERMODEL IS A REQUIRED
NAME (DO NOT CHANGE)
    USE PHLEBOTOMY_TRANSFUSION_MODULE
    USE NUMERICAL_LIBRARIES

    INTEGER, PARAMETER  :: NEQN = 1, NPAR=13, MAXN = 500, MAXCOEFF
= 100, LUN = 3
    REAL*8, PARAMETER   :: FACTOR = 2D0, TOLERANCE = 1.0D-7,
TIMEZERO = 0D0, ABSERR = 0D0, &
                           RELERR = 0.001
    INTEGER             :: NP, IFUN, JFUN, NSIGDIGITS, NUM,
NOEVENTS, NPHLEB, NTRANS, &
                           TEMPN, NOCOEFF, J, SUBNO, JOB, TN, K,
KLAST, NEPO, TEMN, Q
    REAL*8              :: T, Y(*), TZER, CTT, AMT(NEQN), P(*),
YZERO(NEQN), TZERO(NEQN), TEMPT, TEMPA, TEMPX(MAXN), &
                           TEMPY(MAXN), BVOL,
COEFFICIENTS(MAXCOEFF), ESTERR, HBZERO, A1, &
                           XMAX, HBTOTALPROD, HBTOTALPHLEB,
HBTOTALTRANS, TEMPSUM, TY, TX, TR, &
                           MAXHBAMT, TX1(MAXN), TX2(MAXN),
TY1(MAXN), TY2(MAXN), TYMAX, BRET, LTRBC, LTRET, &
                           MONTHHBTOTALPROD, FUNDAMAGED,
XEPO(MAXN), YEPO(MAXN), MCHE, MCHT, CV, FRETT, &
                           TRANS_FRAC, RET_TRANS_FRAC, BRBC, A2,
INPUT1, INPUT2, AVGSTIMRATE, A3, T3, INPUT3, TEMX(MAXN),TEMY(MAXN),
CUM(MAXN), TAT=0D0
    LOGICAL, SAVE       :: SHOWIT, PLOTSAVED
    CHARACTER (LEN=256) :: ID, DATAFILENAME
```

```
    CHARACTER (LEN=20)  :: PNAME
    CHARACTER (LEN=1)   :: RESPONSE

!----
! BEFORE FITTING WE WOULD LIKE TO GIVE NAMES TO THE PARAMETERS AND
! SELECT THE ALGORITHM FOR THE INTEGRATION OF THE DIFF EQUATIONS
!----
IF(IFUN == -1000)THEN
! THIS SECTION (IFUN.EQ.-1000) ALLOW YOU TO SET OPTIONS BEFORE THE
! START OF THE FITTING TO THE DATA (WHICH OCCURS WHEN IFUN = -1000)
!-----------------------------------------------------------------
-----
!ASSIGN NAMES (HIGHLY RECOMMENDED FOR READABILITY OF OUTPUT)

        CALL SetFunfitParameterName(1,"ALPHA")
!! SLOPE OF LINEAR CHANGE IN LIFESPAN
        CALL SetFunfitParameterName(2,"F")
!! COEFFICIENT FOR SINGLE EXPONENTIAL FUNCTION
        CALL SetFunfitParameterName(3,"k")
             !! SCALAR PARAMETER RELATING BW TO INUTERO RBC
PRODUCTION RATE
        CALL SetFunfitParameterName(4,"LB")
             !! LIFESPAN OF THE RED BLOOD CELLS AT BIRTH
        CALL SetFunfitParameterName(5,"GA")
             !! GESTATIONAL AGE OF THE INFANT IN DAYS: FIXED
PARAMETER
        CALL SetFunfitParameterName(6,"A")                      !!
COEFFICIENT OF X^4 FOR THE BW FOURTH ORDER POLYNOMIAL: FIXED
PARAMETER
        CALL SetFunfitParameterName(7,"B")
!! COEFFICIENT OF X^3 FOR THE BW FOURTH ORDER POLYNOMIAL: FIXED
PARAMETER
        CALL SetFunfitParameterName(8,"C")
             !! COEFFICIENT OF X^2 FOR THE BW FOURTH ORDER
POLYNOMIAL: FIXED PARAMETER
        CALL SetFunfitParameterName(9,"D")
             !! COEFFICIENT OF X^1 FOR THE BW FOURTH ORDER
POLYNOMIAL: FIXED PARAMETER
        CALL SetFunfitParameterName(10,"E")
             !! COEFFICIENT OF X^0 FOR THE BW FOURTH ORDER
POLYNOMIAL: FIXED PARAMETER
        CALL SetFunfitParameterName(11,"GAMMA")
             !! EXPONENTIAL TERM COEFFICIENT FOR SINGLE EXPONENTIAL
FUNCTION: FIXED PARAMETER
        CALL SetFunfitParameterName(12,"FR")
             !! FRACTION OF RBCS RELATIVE TO THE TOTAL NUMBER OF
RBCS PRODUCED THAT ARE LABELLED AND
        CALL SetFunfitParameterName(13,"MCH")
             !! MCH VALUE IN G/CELL

                              !! REINFUSED FOR TRACKING STUDY
(BIORBC): FIXED PARAMETER
!-----------------------------------------------------------------
-----
```

```
! ============= SET PHLEBOTOMY, TRANSFUSION, STIMULATION RATE
KNOTS, AND BODYWEIGHT VECTORS =============
!                   NOTE: THE TIME/KNOT VECTORS MUST BE SET BEFORE
THE AMOUNT/FVALUE VECTORS
!
        TEMPN = MAXN
        PRINT*
        PRINT*,' PHLEBOTOMY FRACTION REMAINING-TIME DATA:'
        CALL GET_XY_DATA_FROM_FUNFIT_FILE (TEMPX,TEMPY,TEMPN)
        IF ( TEMPN > MAXN ) STOP ' TOO MANY PHLEBOTOMY DATA POINTS.
ADJUST THE MAXN APPROPRIATELY'
        CALL SET_PHLEBOTOMY_TIME_VECTOR(TEMPN, TEMPX)
        CALL SET_FRACTION_REMAINING_VECTOR(TEMPN, TEMPY)
!
!-----------------------------------------------------------------
-----
!        TEMN = MAXN
!        PRINT*
!        PRINT*,' CUMULATIVE_HB_REMOVED_GRAMS-TIME DATA:'
!        CALL GET_XY_DATA_FROM_FUNFIT_FILE (TEMX,TEMY,TEMN)
!        IF ( TEMN > MAXN ) STOP ' TOO MANY PHLEBOTOMY DATA POINTS.
ADJUST THE MAXN APPROPRIATELY'
!
!DO Q=1, TEMN
!
!     IF(Q==1) THEN
!          TAT = 0D0
!     ELSE
!          TAT = CUM(Q-1)
!     ENDIF
!
!     CUM(Q)= TAT + TEMY(Q)
!     TAT = 0D0
!
!END DO
!

!
!-----------------------------------------------------------------
-----
  RETURN
ENDIF

!=================================================================
==================================================
! THIS SECTION DOES THE INTEGRATION OF THE ODEs AND PROVIDES THE
PREDICTED VALUES TO BE FITTED TO THE DATA
! FOR THE 3 VARIABLES Y(1), Y(2) AND Y(3) (IFUN = 1,2 AND 3)
IF(IFUN == 1) THEN
```

```
      BOUNDARY: IF(T <= ((154.-P(5))*(1D0+P(1))+P(4))) THEN
                                                                    !!
CASE 1

                  TZER = 0D0
                  CTT = 0D0
                  CALL GET_PHLEBOTOMY_CORRECTION_TERM(TZER, T, CTT)

                  Y(1) =
CTT*P(13)*(P(12))*((((P(6)*(1D0/5D0)*(((P(5))**5.)-((154.)**5.)))
      + &                                  !! NUMBER OF RBCS REMAINING
AT ANY TIME T AFTER BIRTH

      (P(7)*(1D0/4D0)*(((P(5))**4.)-((154.)**4.)))             + &
                                     !! MULTIPLY BY THE FRACTION OF
TOTAL RBCS THAT ARE BIOTIN LABELLED AND REINFUSED

      (P(8)*(1D0/3D0)*(((P(5))**3.)-((154.)**3.)))             + &

      (P(9)*(1D0/2D0)*(((P(5))**2.)-((154.)**2.)))             + &
                                         (P(10)*((P(5))-
(154.))))*P(3))                                 + &
                                         (((P(3)*P(2))/(P(11)))
                                         * &
                                         ((EXP(154D0*P(11)))-
(EXP(P(11)*P(5)+                      &
                                         (P(11)*(MIN(0D0, (T-
P(4))))/(1D0+P(1)))))))                  + &
                                         ((P(3)*P(2))
                                               * &
                                         (((MIN(0D0, (T-
P(4))))/(1D0+P(1)))-154D0+P(5))))


!                 Y(1) =
CTT*(P(12))*(((((P(6)*(1D0/5D0)*(((P(5))**5.)-((154.)**5.)))   + &
                                     !! PERCENTAGE OF RBCS REMAINING
AT ANY TIME T AFTER BIRTH
!
      (P(7)*(1D0/4D0)*(((P(5))**4.)-((154.)**4.)))             + &
                                     !! MULTIPLY BY THE FRACTION OF
TOTAL RBCS THAT ARE BIOTIN LABELLED AND REINFUSED
!
      (P(8)*(1D0/3D0)*(((P(5))**3.)-((154.)**3.)))             + &
!
      (P(9)*(1D0/2D0)*(((P(5))**2.)-((154.)**2.)))             + &
!                                        (P(10)*((P(5))-
(154.))))*P(3))                                 + &
!                                        (((P(3)*P(2))/(P(11)))
                                         * &
!                                        ((EXP(154D0*P(11)))-
(EXP(P(11)*P(5)+                      &
!                                        (P(11)*(MIN(0D0, (T-
P(4))))/(1D0+P(1)))))))                  + &
```

```
!                                          ((P(3)*P(2))
                                                 * &
!                                          (((MIN(0D0, (T-
P(4))))/(1D0+P(1)))-154D0+P(5)))) / &
!                                          ((((P(3)*P(2))/(P(11)))
                                                 * &
!                                          (((EXP(154D0*P(11)))-
(EXP((P(11)*P(5))-                          &
!
      (P(11)*((P(4))/(1D0+P(1)))))))
      - &
!                                          (P(11)*(154D0-
P(5)+((P(4))/(1D0+P(1)))))))               + &
!                                          (P(3)

            * &         !! TOTAL NUMBER OF INUTERO RBCS PRESENT AT
BIRTH (T=0)
!                                          ((P(6)*(1D0/5D0)

      * &
!                                          (((P(5))**5.)-
((154D0)**5.)))
           + &
!                                          (P(7)*(1D0/4D0)

           * &
!                                          (((P(5))**4.)-
((154D0)**4.)))
           + &
!                                          (P(8)*(1D0/3D0)

           * &
!                                          (((P(5))**3.)-
((154D0)**3.)))
           + &
!                                          (P(9)*(1D0/2D0)

           * &
!                                          (((P(5))**2.)-
((154D0)**2.)))
           + &
!                                          (P(10)*(1D0/1D0)

      * &
!                                          (((P(5))**1.)-
((154D0)**1.)))))))*100D0



!                N(0) =      (((P(3)*P(2))/(P(11)))
                                          * &

      ELSE
```

```
                TZER = 0D0
                CTT = 0D0
                CALL GET_PHLEBOTOMY_CORRECTION_TERM(TZER, T, CTT)

                Y(1) = CTT*P(13)*(P(12))*(P(3)

     * &        !! NUMBER OF RBCS REMAINING AT ANY TIME T AFTER
BIRTH
                                    ((P(6)*(1D0/5D0)
                                                                * &
                                    (((P(5))**5.)-(((((MIN(0D0, (T-
P(4))))/(1D0+P(1)))+P(5)))**5.)))       + &
                                    (P(7)*(1D0/4D0)

     * &
                                    (((P(5))**4.)-(((((MIN(0D0, (T-
P(4))))/(1D0+P(1)))+P(5)))**4.)))       + &
                                    (P(8)*(1D0/3D0)

     * &
                                    (((P(5))**3.)-(((((MIN(0D0, (T-
P(4))))/(1D0+P(1)))+P(5)))**3.)))       + &
                                    (P(9)*(1D0/2D0)

     * &
                                    (((P(5))**2.)-(((((MIN(0D0, (T-
P(4))))/(1D0+P(1)))+P(5)))**2.)))       + &
                                    (P(10)*(1D0/1D0)
                                                                * &
                                    (((P(5))**1.)-(((((MIN(0D0, (T-
P(4))))/(1D0+P(1)))+P(5)))**1.))))))



!               Y(1) = CTT*(P(12))*((P(3)
                                                                * &
           !! PERCENTAGE OF RBCS REMAINING AT ANY TIME T AFTER
BIRTH
!                                   ((P(6)*(1D0/5D0)
                                                                * &
!                                   (((P(5))**5.)-(((((MIN(0D0, (T-
P(4))))/(1D0+P(1)))+P(5)))**5.)))       + &
!                                   (P(7)*(1D0/4D0)

     * &
!                                   (((P(5))**4.)-(((((MIN(0D0, (T-
P(4))))/(1D0+P(1)))+P(5)))**4.)))       + &
!                                   (P(8)*(1D0/3D0)

     * &
!                                   (((P(5))**3.)-(((((MIN(0D0, (T-
P(4))))/(1D0+P(1)))+P(5)))**3.)))       + &
```

```
!                                    (P(9)*(1D0/2D0)

      * &
!                                    (((P(5))**2.)-(((((MIN(0D0, (T-
P(4))))/(1D0+P(1)))+P(5)))**2.)))       + &
!                                    (P(10)*(1D0/1D0)
                                                                    * &
!                                    (((P(5))**1.)-(((((MIN(0D0, (T-
P(4))))/(1D0+P(1)))+P(5)))**1.)))))          / &
!                                    ((((P(3)*P(2))/(P(11)))

      * &
!                                    (((EXP(154D0*P(11)))-
(EXP((P(11)*P(5))-
        &
!                                    (P(11)*((P(4))/(1D0+P(1)))))))
                                                                    - &

!                                    (P(11)*(154D0-
P(5)+((P(4))/(1D0+P(1)))))))
      + &
!                                    (P(3)

      * &         !! TOTAL NUMBER OF INUTERO RBCS PRESENT AT BIRTH
(T=0)
!                                    ((P(6)*(1D0/5D0)
                                                                    * &
!                                    (((P(5))**5.)-((154D0)**5.)))
                                                                    + &
!                                    (P(7)*(1D0/4D0)

      * &
!                                    (((P(5))**4.)-((154D0)**4.)))
                                                                    + &
!                                    (P(8)*(1D0/3D0)

      * &
!                                    (((P(5))**3.)-((154D0)**3.)))
                                                                    + &
!                                    (P(9)*(1D0/2D0)

      * &
!                                    (((P(5))**2.)-((154D0)**2.)))
                                                                    + &
!                                    (P(10)*(1D0/1D0)
                                                                    * &
!                                    (((P(5))**1.)-
((154D0)**1.)))))))*100D0



!                 N(0) =      (P(3)
```

```
      * &         !! TOTAL NUMBER OF INUTERO RBCS PRESENT AT BIRTH
(T=0)
!                                    ((P(6)*(1D0/5D0)
                                                                  * &
!                                    (((P(5))**5.)-((154D0)**5.)))
                                                                  + &
!                                    (P(7)*(1D0/4D0)

      * &
!                                    (((P(5))**4.)-((154D0)**4.)))
                                                                  + &
!                                    (P(8)*(1D0/3D0)

      * &
!                                    (((P(5))**3.)-((154D0)**3.)))
                                                                  + &
!                                    (P(9)*(1D0/2D0)

      * &
!                                    (((P(5))**2.)-((154D0)**2.)))
                                                                  + &
!                                    (P(10)*(1D0/1D0)
                                                                  * &
!                                    (((P(5))**1.)-((154D0)**1.)))))



      ENDIF BOUNDARY

  RETURN
END IF

! THIS SECTION IS THE SPECIAL OPTIONAL USER OUTPUT SECTION THAT
WILL BE EXECUTED
! WHEN WINFUNFIT IS DONE WITH THE FITTING TO THE CURRENT DATA SET
! (INDICATED BY WINFUNFIT CALLING USERMODEL WITH IFUN=0)
IF(IFUN.EQ.0)THEN
    CALL PROMT(SHOWIT) ! DO WE NEED TO SHOW THE USER PLOT? THIS
CALL STARTS A DIALOG WITH THE USER
    IF(SHOWIT) THEN    ! THE USER WANTED TO SHOW USER PLOT(S)
!!----
!!   USER DESIGNED 'SPECIAL' PLOTS :
!!-----
    CALL GETDATAFILENAME(DATAFILENAME)
!    CALL ADDMARGINTEXT(DATAFILENAME)              ! PUT THE DATA
FILE NAME IN THE RIGHT MARGIN OF PLOT
    CALL ADDOBSERVATIONSLEFT(1)                  ! ADDS
OBSERVATIONS (FUNCTION 1) WITH A LEFT Y-AXIS
    CALL ADDFITTEDCURVELEFT(1)                   ! ADDS FITTED
CURVE (FUNCTION 1) WITH A LEFT Y-AXIS
    CALL LEFTLABEL('HB AMOUNT IN BIORBCS (G)')               !
LABEL FOR LEFT Y-AXIS
!     CALL INCLUDE_CURVE_RIGHT(TEMX, CUM, TEMN, 2)
!     CALL RIGHT_LABEL("CUMULATIVE HB REMOVED (G)")
```

```
!     CALL END_RIGHT_AT(5D0)
      CALL END_X_AT(70D0)
      CALL ADD_ZERO_LEFT
! ADDITION OF LINES END HERE
    CALL TITLE('FETAL ERYTHROPOIESIS MODEL')       ! TITLE OF PLOT
    CALL XLABEL('TIME POST-BIORBC TRANSFUSION(DAYS)')
! LABEL FOR X-AXIS
    CALL DISPLAYPLOT                               ! THIS WILL
CONSTRUCT AND DISPLAY THE PLOT
!! THIS WILL RECORD THE UNIQUE PLOT ID (PLOT SN) IF PLOT IS SAVED
    CALL GETLUNOUTPUT(LUN)                         ! GET LOGICAL
UNIT NUMBER USED FOR STANDARD OUTPUT
    CALL RECORDPLOTIFSAVED(LUN) ! IF USER SAVES THE PLOT ITS SN
WILL BE RECORDED
    CALL RECORDPLOTIFSAVED(3)   ! IF USER SAVES THE PLOT ITS SN
WILL BE RECORDED ON UNIT 3 (USER OUTPUT SECTION)
    ENDIF
  ENDIF
  RETURN
!-------------------------------------------------------------------
----------------------------------------
!                     ******* N O N  OPTIONAL DEFINITION SECTION
*******
!
! **** THIS IS FOR THE RECORDING OF THE MODEL USED IN THE FITTING
! **** ALWAYS, ALWAYS, ALWAYS! USE A DIFFERENT NAME OR VERSION
NUMBER WHEN YOU MAKE CHANGES IN THE MODEL

ENTRY MODELID(ID)                                    !!
  ID = 'INUTERORBC (V.1.3)' !* <= CHANGE THIS STRING EVERY TIME YOU
                                                                  !
MAKE CHANGES IN THE ABOVE SUBRROUTINE(S)
  RETURN
END SUBROUTINE USERMODEL
!-- ------------------------------------------------ E N D ---------
----------------------------------------
```

B.4 FORTRAN subroutines for chapter 5

```
!Purpose:
!   TO CALCULATE THE AREA UNDER THE CURVE (95%) FOR A GIVEN SET OF
DATA POINTS (FINITE DATA SET)
!
IMPLICIT NONE

INTEGER:: NVALS, IERROR, I
REAL:: XVALUE, YVALUE, AUC1=0, SLOPE=0.
REAL*8, DIMENSION(1000):: XVAL
REAL*8, DIMENSION(1000):: YVAL
REAL*8, DIMENSION(1000):: YGVAL
```

```
! make sure the xvals and yvals are sperated by a space and are not
tab spaced
!OPEN(UNIT=8, FILE='C:\\patA.TXT', STATUS='OLD', ACTION='READ',
IOSTAT=IERROR)

OPEN(UNIT=8, FILE='H:\\data1.TXT', STATUS='OLD', ACTION='READ',
IOSTAT=IERROR)


WRITE(*,*) ' ',IERROR

openif:IF(IERROR == 0) THEN

      DO
            READ(8,*, IOSTAT=IERROR) XVALUE, YVALUE
            IF(IERROR /= 0) EXIT
            NVALS = NVALS +1

            IF(YVALUE >= 5.) THEN
                  XVAL(NVALS) = XVALUE
                  YVAL(NVALS) = YVALUE

            ELSE

                  SLOPE = (YVAL(NVALS-1)-YVALUE)/(XVAL(NVALS-1)-
XVALUE)  !NOTE THAT THIS IS DONE TO CALCULATE THE AUC ONLY UPTO 95%
AND NOT THE TOTAL
                  YVAL(NVALS) = 5.
                  XVAL(NVALS) = (5. - YVAL(NVALS - 1))/SLOPE +
XVAL(NVALS - 1)

            EXIT

            END IF

      END DO

      YVAL = YVAL/100.     ! TO NORMALISE FRACTION TO 1
      YGVAL = YVAL
      !next step is the calculation of AUC (we need to get close to
the 0.95 level so do linear interpolation to get the exact value

      DO I=1, NVALS-1

            AUC1 = AUC1 + ((0.5)*(YVAL(I) + YVAL(I+1))*(XVAL(I+1)-
XVAL(I)))  !THIS IS TO CALCULATE THE TOTAL AREA UNDER THE CURVE
UNTIL THE LAST DATA POINT....
            WRITE(*,*) AUC1
      END DO

      WRITE(*, 1010) AUC1   ! TO CHECK IF IT READS CORRECTLY
      1010 FORMAT('FINAL AUC:', F10.4)

!     CALL ADD_POINTS (XVAL,YGVAL,NVALS)
```

```
!     CALL ADD_CURVE(XVAL,YGVAL,NVALS)
!     CALL X_LABEL('DAYS POST BIORBC TRANSFUSION')
!     CALL LEFT_LABEL ('FRACTION OF LABELLED CELLS')
!     CALL DISPLAY_PLOT

      readif:IF(IERROR > 0) THEN

                  WRITE(*,*) 'AN ERROR OCCURRED WHILE READING'

            ELSE

                  WRITE(*,*) 'END OF FILE REACHED'

      END IF readif

ELSE openif

    WRITE(*,*) 'ERROR OPENING THE FILE IOSTAT=', IERROR

END IF openif

CLOSE (UNIT = 8)

END PROGRAM AUC
!-- ------------------------------------------------ E N D ---------
-----------------------------------------
```

APPENDIX C. PUBLICATIONS AND SUBMITTED MANUSCRIPTS

Kuruvilla DJ, Widness JA, Nalbant D, Schmidt R, Mock D, Veng-Pedersen P. A mass balance-based semiparametric approach to evaluate neonatal erythropoiesis. ***The AAPS Journal.*** 2015 (Accepted, In press)

Kuruvilla DJ, Widness JA, Nalbant D, Schmidt R, Mock D, Veng-Pedersen P. A method to evaluate fetal erythropoiesis from postnatal survival of fetal RBCs. ***The AAPS Journal.*** 2015; *17* (5), 1246-54

Widness JA, **Kuruvilla DJ**, Mock D, Matthews N, Nalbant D, Cress G, Schmidt R, Strauss R, Zimmerman MB, Veng-Pedersen P. Post-Transfusion Red Cell Survival of Neonatal Autologous and Adult Donor Biotin Labeled RBC Measured Concurrently in Very Low Birth Weight Neonates Are Not Different. ***The Journal of Pediatrics.*** 2015 (Accepted, In press)

Kuruvilla DJ, Nalbant D, Widness JA, Veng-Pedersen P. Mean remaining life span: a new clinically relevant parameter to assess the quality of transfused red blood cells. ***Transfusion.*** 2014; *54* (10pt2), 2724-9

Yoo J, **Kuruvilla DJ**, D'Mello SR, Salem AK, Bowden NB. New class of biodegradable polymers formed from reactions of an inorganic functional group. ***Macromolecules.*** 2012; *45* (5), 2292-2300

Kuruvilla DJ, Salem AK. Nanotechnology and Nanomedicine in Diabetes: An Overview. Science Publishers. 2012

REFERENCES

1. Strauss RG. Anaemia of prematurity: Pathophysiology and treatment. Blood Rev. 2010;24(6):221-5.
2. Dallman PR. Anemia of prematurity. Annu Rev Med. 1981;32(1):143-60.
3. Stockman JA, 3rd, Graeber JE, Clark DA, McClellan K, Garcia JF, Kavey RE. Anemia of prematurity: determinants of the erythropoietin response. J Pediatr. 1984;105(5):786-92.
4. Brown MS, Garcia JF, Phibbs RH, Dallman PR. Decreased response of plasma immunoreactive erythropoietin to "available oxygen" in anemia of prematurity. J Pediatr. 1984;105(5):793-8.
5. Obladen M, Sachsenweger M, Stahnke M. Blood sampling in very low birth weight infants receiving different levels of intensive care. Eur J Pediatr. 1988;147(4):399-404.
6. Elliott S. Erythropoiesis-stimulating agents. Cancer Treat Res. 2011;157:55-74.
7. Jurado Garcia JM, Torres Sanchez E, Olmos Hidalgo D, Alba Conejo E. Erythropoietin pharmacology. Clin Transl Oncol. 2007;9(11):715-22.
8. Jelkmann W. The enigma of the metabolic fate of circulating erythropoietin (Epo) in view of the pharmacokinetics of the recombinant drugs rhEpo and NESP. Eur J Haematol. 2002;69(5-6):265-74.
9. Macdougall IC. CERA (Continuous Erythropoietin Receptor Activator): a new erythropoiesis-stimulating agent for the treatment of anemia. Curr Hematol Rep. 2005;4(6):436-40.
10. Topf JM. CERA: third-generation erythropoiesis-stimulating agent. Expert Opin Pharmacother. 2008;9(5):839-49.
11. Rhondeau SM, Christensen RD, Ross MP, Rothstein G, Simmons MA. Responsiveness to recombinant human erythropoietin of marrow erythroid progenitors from infants with the "anemia of prematurity". J Pediatr. 1988;112(6):935-40.
12. Vamvakas EC, Strauss RG. Meta-analysis of controlled clinical trials studying the efficacy of rHuEPO in reducing blood transfusions in the anemia of prematurity. Transfusion (Paris). 2001;41(3):406-15.
13. Haase VH. Hypoxic regulation of erythropoiesis and iron metabolism. Am J Physiol Renal Physiol. 2010;299(1):F1-13.
14. Erslev A. Humoral regulation of red cell production. Blood. 1953;8(4):349-57.
15. Goldwasser E, Kung CK. Purification of erythropoietin. Proc Natl Acad Sci U S A. 1971;68(4):697-8.
16. Lin FK, Suggs S, Lin CH, Browne JK, Smalling R, Egrie JC, et al. Cloning and expression of the human erythropoietin gene. Proc Natl Acad Sci U S A. 1985;82(22):7580-4.
17. Jacobs K, Shoemaker C, Rudersdorf R, Neill SD, Kaufman RJ, Mufson A, et al. Isolation and characterization of genomic and cDNA clones of human erythropoietin. Nature. 1985;313(6005):806-10.
18. Uoshima N, Ozawa M, Kimura S, Tanaka K, Wada K, Kobayashi Y, et al. Changes in c-Kit expression and effects of SCF during differentiation of human erythroid progenitor cells. Br J Haematol. 1995;91(1):30-6.

19. Broudy VC, Lin N, Zsebo KM, Birkett NC, Smith KA, Bernstein ID, et al. Isolation and characterization of a monoclonal antibody that recognizes the human c-kit receptor. Blood. 1992;79(2):338-46.
20. Yoffey JM. The stem cell problem in the fetus. Isr J Med Sci. 1971;7(7):825-33.
21. Finne P, Halvorsen S. Regulation of erythropoiesis in the fetus and newborn. Arch Dis Child. 1972;47(255):683.
22. Kalpaktsoglou PK, Emery JL. The effect of birth on the haemopoietic tissue of the human bone marrow: a histological study. Br J Haematol. 1965;11:453-60.
23. Garby L, Sjolin S, Vuille JC. Studies on erythro-kinetics in infancy. II. The relative rate of synthesis of haemoglobin F and haemoglobin A during the first months of life. Acta Paediatr. 1962;51:245-54.
24. Seip M. The reticulocyte level, and the erythrocyte production judged from reticulocyte studies, in newborn infants during the first week of life. Acta Paediatr. 1955;44(4):355-69.
25. Skjaelaaen P, Halvorsen S. Inhibition of erythropoiesis by plasma from newborn infants. Acta Paediatr Scand. 1971;60(3):301-8.
26. Franco RS. Measurement of Red Cell Lifespan and Aging. Transfusion Medicine and Hemotherapy. 2012;39(5):302-7.
27. Ashby W. The determination of the length of life of transfused blood corpuscles in man. J Exp Med. 1919;29(3):267-81.
28. Krzyzanski W, Brier ME, Creed TM, Gaweda AE. Reticulocyte-based estimation of red blood cell lifespan. Exp Hematol. 2013;41(9):817-22.
29. Recommended methods for radioisotope red-cell survival studies. A report by the ICSH panel on diagnostic applications of radioisotopes in haematology. Br J Haematol. 1971;21(2):241-50.
30. Mock DM, Widness JA, Veng-Pedersen P, Strauss RG, Cancelas JA, Cohen RM, et al. Measurement of posttransfusion red cell survival with the biotin label. Transfus Med Rev. 2014;28(3):114-25.
31. Mock DM, Matthews NI, Zhu S, Strauss RG, Schmidt RL, Nalbant D, et al. Red blood cell (RBC) survival determined in humans using RBCs labeled at multiple biotin densities. Transfusion (Paris). 2011;51(5):1047-57.
32. Cordle DG, Strauss RG, Lankford G, Mock DM. Antibodies provoked by the transfusion of biotin-labeled red cells. Transfusion (Paris). 1999;39(10):1065-9.
33. Kett JC. Anemia in infancy. Pediatr Rev. 2012;33(4):186-7.
34. Kaplan E, Hsu KS. Determination of erythrocyte survival in newborn infants by means of Cr51-labelled erythrocytes. Pediatrics. 1961;27(3):354-61.
35. Sturgeon P. Volumetric and microscopic pattern of bone marrow in normal infants and children: II. Cytologic pattern. Pediatrics. 1951;7(5):642-50.
36. Bratteby L-E, Garby L, Wadman B. Studies on erythro-kinetics in infancy:XII. Survival in adult recipients of cord blood red cells labelled in vitro with di-isopropyl fluorophosphonate (DF32P). Acta Pædiatrica. 1968;57(4):305-10.
37. Palis J, Segel G. Developmental biology of erythropoiesis. Blood Rev. 1998;12(2):106-14.
38. Thomas DB, Yoffey J. Human foetal haemopoiesis I. The cellular composition of foetal blood. Br J Haematol. 1962;8(3):290-5.

39. Walker J, Turnbull EN. Hæmoglobin and red cells in the human fœtus: and their relation to the oxygen content of the blood in the vessels of the umbilical cord. The Lancet. 1953;262(6781):312-8.
40. Pearson HA. Life-span of the fetal red blood cell. The Journal of pediatrics. 1967;70(2):166-71.
41. Brace RA, Langendörfer C, Song T-B, Mock DM. Red blood cell life span in the ovine fetus. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 2000;279(4):R1196-R204.
42. Hollingsworth JW. Lifespan of fetal erythrocytes. The Journal of Laboratory and Clinical Medicine.45(3):469-73.
43. Bratteby L-E, Garby L, Groth T, Schneider W, Wadman B. Studies on erythro-kinetics in infancy:XIII. The mean life span and the life span frequency function of red blood cells formed during foetal life. Acta Pædiatrica. 1968;57(4):311-20.
44. Arbuckle TE, Wilkins R, Sherman GJ. Birth weight percentiles by gestational age in Canada. Obstet Gynecol. 1993;81(1):39-48.
45. Krzyzanski W, Ramakrishnan R, Jusko W. Basic pharmacodynamic models for agents that alter production of natural cells. J Pharmacokinet Biopharm. 1999;27(5):467-89.
46. Krzyzanski W, Woo S, Jusko W. Pharmacodynamic Models for Agents that Alter Production of Natural Cells with Various Distributions of Lifespans. J Pharmacokinet Pharmacodyn. 2006;33(2):125-66.
47. Landaw SA. Factors that accelerate or retard red blood cell senescence. Blood Cells. 1988;14(1):47-67.
48. Freise K, Widness J, Schmidt R, Veng-Pedersen P. Modeling time variant distributions of cellular lifespans: increases in circulating reticulocyte lifespans following double phlebotomies in sheep. J Pharmacokinet Pharmacodyn. 2008;35(3):285-323.
49. Freise KJ, Widness JA, Veng-Pedersen P. Erythropoietic response to endogenous erythropoietin in premature very low birth weight infants. J Pharmacol Exp Ther. 2010;332(1):229-37.
50. Saleh MI, Nalbant D, Widness JA, Veng-Pedersen P. Population pharmacodynamic analysis of erythropoiesis in preterm infants for determining the anemia treatment potential of erythropoietin. 2013;304(9):R772-R81.
51. Saleh MI, Widness JA, Veng-Pedersen P. Pharmacodynamic analysis of stress erythropoiesis: change in erythropoietin receptor pool size following double phlebotomies in sheep. Biopharm Drug Dispos. 2011;32(3):10.1002/bdd.743.
52. Mock DM, Widness JA, Strauss RG, Franco RS. Posttransfusion red blood cell (RBC) survival determined using biotin-labeled RBCs has distinct advantages over labeling with 51Cr. Transfusion (Paris). 2012;52(7):1596.
53. Kuruvilla DJ, Nalbant D, Widness JA, Veng-Pedersen P. Mean remaining life span: a new clinically relevant parameter to assess the quality of transfused red blood cells. Transfusion (Paris). 2014;54(10pt2):2724-9.
54. Trudnowski RJ, Rico RC. Specific gravity of blood and plasma at 4 and 37 °C. Clin Chem. 1974;20(5):615-6.

55. Strauss RG. How I transfuse red blood cells and platelets to infants with the anemia and thrombocytopenia of prematurity. Transfusion (Paris). 2008;48(2):209-17.
56. Veng-Pedersen P. Curve fitting and modeling in pharmacokinetics and some practical experiences with NONLIN and a new program FUNFIT. J Pharmacokinet Biopharm. 1977;5(5):513-31.
57. Brugnara C, Platt OS. The neonatal erythrocyte and its disorders. Nathan and Oski's hematology of infancy and childhood. 2003;1:36-66.
58. Kruse A, Uehlinger DE, Gotch F, Kotanko P, Levin NW. Red blood cell lifespan, erythropoiesis and hemoglobin control. Contrib Nephrol. 2008;161:247-54.
59. Kett JC. Anemia in infancy. Blood. 2012;1599:1678.
60. Kalpaktsoglou PK, Emery JL. The effect of birth on the haemopoietic tissue of the human bone marrow. A histological study. Br J Haematol. 1965;11(4):453-60.
61. Garby L, SjÖLin S, Vuille J-C. Studies on erythro-kinetics in infancy. Acta Pædiatrica. 1962;51(2):245-54.
62. Seip M. The reticulocyte level, and the erythrocyte production judged from reticulocyte studies, in newborn infants during the first week of life. Acta Paediatr. 1955;44(4):355-69.
63. Kuruvilla D, Widness J, Nalbant D, Schmidt R, Mock D, Veng-Pedersen P. A method to evaluate fetal erythropoiesis from postnatal survival of fetal RBCs. AAPS J. 2015:1-9.
64. Iman RL, Conover WJ. The use of the rank transform in regression. Technometrics. 1979;21(4):499-509.
65. Nelder JA, Mead R. A simplex method for function minimization. The Computer Journal. 1965;7(4):308-13.
66. Reinsch C. Smoothing by spline functions. Numer Math. 1967;10(3):177-83.
67. R Core Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2014.
68. Racine JS. RStudio: A Platform-Independent IDE for R and Sweave. Journal of Applied Econometrics. 2012;27(1):167-72.
69. Doyle JJ. The role of erythropoietin in the anemia of prematurity. Semin Perinatol. 1997;21(1):20-7.
70. Stockman III JA, Garcia JF, Oski FA. The anemia of prematurity: factors governing the erythropoietin response. N Engl J Med. 1977;296(12):647-50.
71. Hågå P, Meberg A, Halvorsen S. Plasma erythropoietin concentrations during the early anemia of prematurity. Acta Pædiatrica. 1983;72(6):827-31.
72. Krzyzanski W, Wyska E. Pharmacokinetics and pharmacodynamics of erythropoietin receptor in healthy volunteers. Naunyn-Schmied Arch Pharmacol. 2008;377(4-6):637-45.
73. Woo S, Krzyzanski W, Jusko W. Target-mediated pharmacokinetic and pharmacodynamic model of recombinant human erythropoietin (rHuEPO). J Pharmacokinet Pharmacodyn. 2007;34(6):849-68.
74. Linderkamp O, Wu PYK, Meiselman HJ. Geometry of Neonatal and Adult Red Blood Cells. Pediatr Res. 1983;17(4):250-3.

75. Linderkamp O, Friederichs E, Meiselman HJ. Mechanical and Geometrical Properties of Density-Separated Neonatal and Adult Erythrocytes. Pediatr Res. 1993;34(5):688-93.
76. Mock DM, Lankford GL, Widness JA, Burmeister LF, Kahn D, Strauss RG. Measurement of red cell survival using biotin-labeled red cells: validation against 51Cr-labeled red cells. Transfusion (Paris). 1999;39(2):156-62.
77. Diekema DS. Conducting ethical research in pediatrics: A brief historical overview and review of pediatric regulations. The Journal of Pediatrics. 2006;149(1, Supplement):S3-S11.
78. Klein HG, Anstee DJ. Mollison's blood transfusion in clinical medicine: John Wiley & Sons; 2008.
79. Desforges JF, Dawson JP. The anemia of renal failure. AMA Arch Intern Med. 1958;101(2):326-32.
80. Loge JP, Lange RD, Moore CV. Characterization of the anemia associated with chronic renal insufficiency. The American Journal of Medicine. 1958;24(1):4-18.
81. Egberts J, Hardeman MR, Luykx LM. Decreased deformability of donor red blood cells after intrauterine transfusion in the human fetus: possible reason for their reduced life span? Transfusion (Paris). 2004;44(8):1231-7.
82. Sebring ES, Polesky HF. Fetomaternal hemorrhage: incidence, risk factors, time of occurrence, and clinical effects. Transfusion (Paris). 1990;30(4):344-57.
83. Stockman J. Physical properties of the neonatal red blood cell. Developmental and Neonatal Haematology Raven Press, New York. 1988:297-323.
84. Strauss RG. Red blood cell transfusion practices in the neonate. Clin Perinatol. 1995;22(3):641-55.
85. Strauss RG. TRansfusion therapy in neonates. Am J Dis Child. 1991;145(8):904-11.
86. Widness JA, Seward VJ, Kromer IJ, Burmeister LF, Bell EF, Strauss RG. Changing patterns of red blood cell transfusion in very low birth weight infants. The Journal of Pediatrics. 1996;129(5):680-7.
87. Europe. Co. Guide to the preparation, use and quality assurance of blood components. 11th ed ed: Council of Europe Publishing; 2005.
88. Hess JR, Hill HR, Oliver CK, Lippert LE, Rugg N, Joines AD, et al. Twelve-week RBC storage. Transfusion. 2003;43(7):867-72.
89. Högman CF, Meryman HT. Red blood cells intended for transfusion: quality criteria revisited. Transfusion. 2006;46(1):137-42.
90. Allene C-G. Dating of Irradiated Blood Products 2004.
91. Dumont LJ, AuBuchon JP, Biomedical Excellence for Safer Transfusion C. Evaluation of proposed FDA criteria for the evaluation of radiolabeled red cell recovery trials. Transfusion. 2008;48(6):1053-60.
92. Cohen RM, Franco RS, Khera PK, Smith EP, Lindsell CJ, Ciraolo PJ, et al. Red cell life span heterogeneity in hematologically normal people is sufficient to alter HbA1c. Blood. 2008;112(10):4284-91.
93. Lindsell CJ, Franco RS, Smith EP, Joiner CH, Cohen RM. A method for the continuous calculation of the age of labeled red blood cells. American Journal of Hematology. 2008;83(6):454-7.

94. Recommended Method for Radioisotope Red-Cell Survival Studies. International Committee for Standardization in Haematology*. British Journal of Haematology. 1980;45(4):659-66.
95. Eadie GS, Brown IW. Analytical Review: Red Blood Cell Survival Studies. Blood. 1953;8(12):1110-36.
96. Veng-Pedersen P. Mean time parameters in pharmacokinetics. Definition, computation and clinical implications (Part II). Clin Pharmacokinet. 1989;17(6):424-40.
97. Veng-Pedersen P. Mean time parameters in pharmacokinetics. Definition, computation and clinical implications (Part I). Clin Pharmacokinet. 1989;17(5):345-66.
98. Brown Iw JHHF. Recovery and in vivo survival of human red cells: Studies of red cells after storage up to six and one-fourth months at subzero temperatures. Archives of Surgery. 1953;66(3):267-82.
99. Franco RS, Yasin Z, Palascak MB, Ciraolo P, Joiner CH, Rucknagel DL. The effect of fetal hemoglobin on the survival characteristics of sickle cells. Blood. 2006;108(3):1073-6.
100. Pasini EM, Kirkegaard M, Mortensen P, Lutz HU, Thomas AW, Mann M. In-depth analysis of the membrane and cytosolic proteome of red blood cells. Blood. 2006;108(3):791-801.
101. Bryszewska M, Zavodnik IB, Niekurzak A, Szosland K. Oxidative processes in red blood cells from normal and diabetic individuals. Biochem Mol Biol Int. 1995;37(2):345-54.
102. Sivilotti ML. Oxidant stress and haemolysis of the human erythrocyte. Toxicol Rev. 2004;23(3):169-88.
103. D'Almeida Mark S, Gray D, Martin C, Ellis Christopher G, Chin-Yee Ian H. Effect of prophylactic transfusion of stored RBCs on oxygen reserve in response to acute isovolemic hemorrhage in a rodent model. Transfusion. 2001;41(7):950-6.