Population pharmacokinetic/pharmacodynamic analysis of erythropoiesis kinetics

Mohammad Issa Mahmoud Saleh

University of Iowa

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POPULATION PHARMACOKINETIC / PHARMACODYNAMIC ANALYSIS OF ERYTHROPOIESIS KINETICS

by

Mohammad Issa Mahmoud Saleh

An Abstract

Of a thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Pharmacy in the Graduate College of The University of Iowa

May 2012

Thesis Supervisor: Professor Peter Veng-Pedersen
ABSTRACT

In USA more than 12.5% of all infants are born preterm. Approximately 75% of all perinatal deaths occur among these preterm infants. Preterm infants are frequently very low in birth weight (VLBW) and receive multiple red blood cell (RBC) transfusions. These transfusions pose increased risk of infections and other complications. Since erythropoietin (EPO) stimulates RBC production, EPO treatment of VLBW infants has received attention as a modality for reducing transfusions in this group.

The overall hypothesis of this work is that treatment optimization of EPO of anemia in preterm infants requires a comprehensive knowledge of the behavior of RBC and the pharmacokinetic/pharmacodynamics (PK/PD) relationship between EPO and erythropoiesis. Under that overall hypothesis, the specific aims were: 1) To describe erythropoiesis dynamics in preterm infants, 2) To determine and explain the variability in the response to EPO in preterm infants, 3) To evaluate newborn sheep as an experimental model for erythropoiesis in preterm infants, 4) To test the hypothesis that RBC lifespan is shortened under acute hypoxic stress conditions, 5) To test the hypothesis that EPO receptor (EPOR) pool size increases under hypoxic stress conditions and the change in EPOR pool size can be predicted using EPO clearance measurements, 6) To describe the effect of EPOR pool size changes on erythropoiesis kinetics.

A model that describes erythropoiesis dynamics in preterm infants as a function of the plasma EPO concentration is presented in Chapter 2. In Chapter 3, several covariates are tested for their ability to identify infants with good EPO responsiveness. The lamb is also tested as an animal model for the erythropoiesis in preterm infants (Chapter 4). In Chapters 5-7, the effect of hypoxic stress conditions on RBC survival was explored defining the relation between the efficacy of EPO and survival of RBC produced as a result of EPO administration. RBC lifespan measurement methods are reviewed in Chapter 5. In Chapter 6, a new methodology for the measurement of RBC lifespan under
stress conditions is developed. This new methodology is applied in Chapter 7 to explore the effect of hypoxic stress conditions on the survival of RBC. The study presented in Chapter 8 is undertaken to investigate changes in both EPOR pool size and EPO clearance under hypoxic conditions. An erythropoiesis model that accounts for change in the EPOR pool size under stress conditions is presented in Chapter 9.

Analysis of erythropoiesis dynamics in preterm infants demonstrated that a three fold increase in the amount of RBC produced by preterm infants is possible by EPO administration. This emphasizes the potential of using EPO for the management of anemia in preterm infants. Covariate screening identified gestational age as a potential marker for the responsiveness to EPO treatment. PD analysis results in lambs demonstrated similarities between lambs and preterm infants in different erythropoietic characteristics such as sensitivity to EPO in producing RBC, Hb production rate before birth and blood volume. Survival analysis demonstrated that RBC lifespan is not shortened under acute hypoxic conditions Analysis of EPOR mRNA level demonstrated an up regulation of EPOR level under stress conditions accompanied by a parallel increase in EPO clearance. EPOR up regulation under stress conditions level was incorporated in a PD model presented in Chapter 9. The developed model provides a framework for optimizing EPO dosing. Accordingly, an optimal dosing strategy should in general maximize the interaction between EPO and EPOR. Specifically, EPO should be administered when the number of EPOR are close to maximally up-regulated.

Abstract Approved: ______________________________________
Thesis Supervisor

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Title and Department

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Date
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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Pharmacy in the Graduate College of The University of Iowa

May 2012

Thesis Supervisor: Professor Peter Veng-Pedersen
CERTIFICATE OF APPROVAL

PH.D. THESIS

This is to certify that the Ph.D. thesis of

Mohammad Issa Mahmoud Saleh

has been approved by the Examining Committee for the thesis requirement for the Doctor of Philosophy degree in Pharmacy at the May 2012 graduation.

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To my beloved parents, Issa and Jenia, for their continuous guidance and patience, to my wife, Ahd, for her love, to my daughter Ritan, for being in my life, and to my brothers and sister, Abd Al-Salam, Mahmoud, Ahmad, and Heba for their support.
When an archer misses the mark, he turns and looks for the fault within himself. Failure to hit the bulls-eye is never the fault of the target. To improve your aim - improve yourself.

Glibert Arland, *A Father's Book of Wisdom*
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aspect of my life. Likewise, I thank my brothers, Abd-Alsalam, Mahmoud and, Ahmad, and my little sister Heba for believing in me and encouraging me to pursue my PhD degree. And last, but not least, I must thank my wife Ahd and my daughter Ritan who kept me sane with endless but not always adequately acknowledged love.
ABSTRACT

In USA more than 12.5% of all infants are born preterm. Approximately 75% of all perinatal deaths occur among these preterm infants. Preterm infants are frequently very low in birth weight (VLBW) and receive multiple red blood cell (RBC) transfusions. These transfusions pose increased risk of infections and other complications. Since erythropoietin (EPO) stimulates RBC production, EPO treatment of VLBW infants has received attention as a modality for reducing transfusions in this group.

The overall hypothesis of this work is that treatment optimization of EPO of anemia in preterm infants requires a comprehensive knowledge of the behavior of RBC and the pharmacokinetic/pharmacodynamics (PK/PD) relationship between EPO and erythropoiesis. Under that overall hypothesis, the specific aims were: 1) To describe erythropoiesis dynamics in preterm infants, 2) To determine and explain the variability in the response to EPO in preterm infants, 3) To evaluate newborn sheep as an experimental model for erythropoiesis in preterm infants, 4) To test the hypothesis that RBC lifespan is shortened under acute hypoxic stress conditions, 5) To test the hypothesis that EPO receptor (EPOR) pool size increases under hypoxic stress conditions and the change in EPOR pool size can be predicted using EPO clearance measurements, 6) To describe the effect of EPOR pool size changes on erythropoiesis kinetics.

A model that describes erythropoiesis dynamics in preterm infants as a function of the plasma EPO concentration is presented in Chapter 2. In Chapter 3, several covariates are tested for their ability to identify infants with good EPO responsiveness. The lamb is also tested as an animal model for the erythropoiesis in preterm infants (Chapter 4). In Chapters 5-7, the effect of hypoxic stress conditions on RBC survival was explored defining the relation between the efficacy of EPO and survival of RBC produced as a result of EPO administration. RBC lifespan measurement methods are reviewed in Chapter 5. In Chapter 6, a new methodology for the measurement of RBC lifespan under
stress conditions is developed. This new methodology is applied in Chapter 7 to explore the effect of hypoxic stress conditions on the survival of RBC. The study presented in Chapter 8 is undertaken to investigate changes in both EPOR pool size and EPO clearance under hypoxic conditions. An erythropoiesis model that accounts for change in the EPOR pool size under stress conditions is presented in Chapter 9.

Analysis of erythropoiesis dynamics in preterm infants demonstrated that a three fold increase in the amount of RBC produced by preterm infants is possible by EPO administration. This emphasizes the potential of using EPO for the management of anemia in preterm infants. Covariate screening identified gestational age as a potential marker for the responsiveness to EPO treatment. PD analysis results in lambs demonstrated similarities between lambs and preterm infants in different erythropoietic characteristics such as sensitivity to EPO in producing RBC, Hb production rate before birth and blood volume. Survival analysis demonstrated that RBC lifespan is not shortened under acute hypoxic conditions. Analysis of EPOR mRNA level demonstrated an up regulation of EPOR level under stress conditions accompanied by a parallel increase in EPO clearance. EPOR up regulation under stress conditions level was incorporated in a PD model presented in Chapter 9. The developed model provides a framework for optimizing EPO dosing. Accordingly, an optimal dosing strategy should in general maximize the interaction between EPO and EPOR. Specifically, EPO should be administered when the number of EPOR are close to maximally up-regulated.
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<tr>
<td>%RSE</td>
<td>Percentage standard error</td>
</tr>
<tr>
<td>$^{125}$I-rhEPO</td>
<td>rhEPO labeled with $^{125}$I isotope</td>
</tr>
<tr>
<td>-2LL</td>
<td>Minus two times the log-likelihood value</td>
</tr>
<tr>
<td>AABB</td>
<td>American Association of Blood Banks</td>
</tr>
<tr>
<td>AIC</td>
<td>Akaike’s information criterion</td>
</tr>
<tr>
<td>$a$</td>
<td>Time between the erythroid progenitor cell stimulation by EPO and the first appearance of Hb in the circulation</td>
</tr>
<tr>
<td>ANN</td>
<td>Artificial neural networks</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>$b$</td>
<td>Time between the erythroid progenitor cell stimulation by EPO and their removal from circulation by senescence</td>
</tr>
<tr>
<td>BFU–E</td>
<td>Burst-forming unit–erythroid</td>
</tr>
<tr>
<td>BIC</td>
<td>Bayesian information criterion</td>
</tr>
<tr>
<td>BioRBC</td>
<td>Biotinylated RBCs</td>
</tr>
<tr>
<td>BVN</td>
<td>Bivariate normal distribution</td>
</tr>
<tr>
<td>$C_a(t)$</td>
<td>the concentration of the of $^{125}$I-rhEPO tracer in the blood</td>
</tr>
<tr>
<td>$C_b(t)$</td>
<td>the concentration of endogenous native EPO in the blood</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>$C_{EPO}$</td>
<td>Plasma EPO concentration</td>
</tr>
<tr>
<td>CERA</td>
<td>Continuous erythropoietin receptor activator</td>
</tr>
<tr>
<td>CFU</td>
<td>Committed colony-forming unit</td>
</tr>
<tr>
<td>CFU–E</td>
<td>Colony-forming unit–erythroid</td>
</tr>
<tr>
<td>CFU-GEMM</td>
<td>Colony-forming unit granulocyte erythroid macrophage megakaryocyte</td>
</tr>
<tr>
<td>$\chi^2$</td>
<td>Chi square distribution</td>
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</table>
$Cl$ EPO clearance
$Cl_{NR}$ Non-receptor mediated EPO clearance
$Cl_{R}$ Receptor mediated EPO clearance
CRP C-reactive protein
$\Delta \Delta CT$ Comparative CT method
$EC_{50}$ EPO concentration that results in Hb production rate that is 50% of the scaled $E_{\text{max}}$
$E_{\text{max}}$ Maximum Hb production rate which is scaled to the bodyweight by a $\frac{3}{4}$ power function
EPO Erythropoietin
EPOR Erythropoietin receptors
ESAs Erythropoiesis stimulating agents
$\eta_i$ Individual random effect
$f_{\text{prod}}(t)$ Hb production function
$f_{\text{stim}}(t)$ Hb production stimulation function
$F_T$ Fraction of transfused Hb surviving immediately beyond the transfusion
$GA$ Gestational age
$GA_{\text{mean}}$ Gestational age geometric mean of all subjects included in the study
Hb Hemoglobin
HbA1C Glycated hemoglobin
$Hb_0$ Baseline hemoglobin concentration at birth
$Hb_{\text{endo}}(t)$ Hb produced endogenously
$Hb_{\text{tran}}(t)$ Hb transfused
IL-2 Interleukin 2
IL-6 Interleukin 6
IPRED Predictions using individual parameters
IV Intravenous
IWRES Weighted residuals using predictions using individual parameter estimates

\( k_{\text{prod}}^{\text{in utero}} \) Hb production stimulation function prior to birth

\( MCH_{\text{endo}} \) Mean corpuscular Hb of RBC produced endogenously

\( MCH_{\text{trans}} \) Mean corpuscular Hb of RBC transfused

\( L \) Lifespan

MCV Mean corpuscular volume

MPL Mean potential lifespan

NICU Neonatal Intensive Care Unit

\( \omega^2 \) Variance of individual random effect

\( P \) Typical value of the parameter

PCR Polymerase chain reaction

PD Pharmacodynamics

PEG Polyethylene glycol

\( P_i \) i\(^{th}\) individual’s parameter value

PK Pharmacokinetics

pO\(_2\) oxygen partial pressure

PRED Predictions using population parameters

RBC Red blood cells

rhEPO Recombinant human erythropoietin

RIA Radioimmunoassay

SAEM Stochastic approximation expectation maximization algorithm

SEM Standard error of the mean

SQ Subcutaneous

s-TfR Soluble Transferrin Receptor

S(t) RBC survival function
<table>
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<th>Acronym</th>
<th>Description</th>
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<tr>
<td>SVPC</td>
<td>Standardized visual predictive check</td>
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<tr>
<td>$\tau_{\text{tran}}$</td>
<td>Lifespan of transfused RBC</td>
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<tr>
<td>TIM</td>
<td>Tracer interaction method</td>
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<td>UIR</td>
<td>Unit impulse response</td>
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<td>$V$</td>
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CHAPTER 1. INTRODUCTION

1.1 Background

About 100 years ago, Carnot and Deflandre proposed a humoral factor in the regulation of erythropoiesis. They postulated that there is a hematopoietic factor (called ‘hemopoietine’) in plasma that regulates the production of red blood cells (RBC). This factor is known now as Erythropoietin (EPO). In 1949, Oliva et al demonstrated the erythropoietic activity of exogenous EPO in humans by transfusing plasma from patients with pernicious anemia into normal subjects. Thirty years later, Miyake et al were able to purify a few milligrams of erythropoietin from over 2,500 liters of urine from patients with aplastic anemia. By the year 1985, EPO was cloned successfully with the possibility of large scale production. In 1987, the first clinical trial of EPO demonstrated that EPO treatment was associated with the reduction of RBC transfusions in adults with end-stage renal disease. This finding motivated the research toward using EPO to limit RBC transfusions in preterm infants.

1.2 Erythropoiesis

1.2.1 Erythrocyte differentiation

Erythropoiesis or production of red blood cells is dynamic and highly regulated process by which hematopoietic stem cells give rise to committed erythroid progenitors and then mature into RBC. Erythropoiesis occurs in three defined stages: generation of erythroid committed blast cells from multipotent hematopoietic progenitors; division and differentiation of these morphologically identifiable erythroid progenitor cells; and terminal cellular morphologic changes, including enucleation, to produce reticulocytes.
and ultimately mature red cells. Transformation between different cell types is presented in Figure 1.1.

In the first stage, pluripotential stem cells first form committed myeloid stem cells and committed lymphoid stem cells. The differentiation of stem cells may be a stochastic (random) or deterministic event, or a combination of both. The stem cell pool maintains itself, with little if any depletion, by asymmetric division into a committed colony-forming unit (CFU) and another stem cell. As stem cells differentiate, they lose their ability for self-renewal. Myeloid stem cells then differentiate into a multipotent progenitor cell identified in vitro as colony-forming unit granulocyte erythroid macrophage megakaryocyte (CFU-GEMM). CFU-GEMM in turn gives rise to bipotential progenitors restricted to either the granulocyte/macrophage or the erythroid/megakaryocyte pathways.

In the second stage of erythropoiesis, progenitors committed to the erythroid/megakaryocyte pathway express erythropoietin receptor (EPOR) and are responsive to erythropoietin, whereas cells committed to the granulocyte/macrophage pathway do not express EPOR. The most immature erythroid-restricted progenitor is the burst-forming unit–erythroid (BFU–E) which gives rise to colony-forming unit–erythroid (CFU–E). Early BFU–E are blast-like cells, highly proliferative, and give rise to clustered burst colonies of up to 20,000 cells. BFU–E express low amounts of EPOR but daughter cells derived from them express high amounts of EPOR, become EPO responsive, transferrin receptor positive, and begin to express hemoglobin. CFU–E are highly responsive to EPO, generate smaller colonies, and express many of the gene products required for definitive erythroid development. EPO has been found to stimulate
division and prevent apoptosis of late-stage erythroid cells generated from CFU–E.\textsuperscript{15, 17, 18} Moreover, in tissue culture systems at this stage of development cells begin hemoglobin synthesis and acquire cytoskeletal proteins and cellular adhesion molecules, steps that help define them as nucleated erythroblasts.\textsuperscript{8, 17, 18}

The third stage of erythropoiesis involves morphologically identifiable nucleated precursors that progress from the proerythroblast to basophilic, polychromatophilic, and orthochromatic cell forms leading to the reticulocytes.\textsuperscript{15, 16} During these distinct stages of erythroid differentiation, four major cellular processes proceed: the accumulation of hemoglobin that participates in driving the basophilic to acidophilic cytoplasmic changes seen during maturation, limited erythroblast expansion, a continued decrease in cell size, and nuclear condensation and finally enucleation.\textsuperscript{16, 19} Erythroblasts mature in a microenvironmental niche in bone marrow termed ‘erythroblast islands’ where they are closely associated with macrophages that serve as stromal or nurse cells.\textsuperscript{20-22} In the final stages of erythroid development, reticulocytes exit the marrow and enter the circulation where they mature into erythrocytes. This stage of development is characterized by disassembly of ribosomes, golgi bodies, and other cellular machinery, removal of organelles, enucleation, changes in the cytoskeleton leading to the classic biconcave discoid shape, and then release into the circulation.\textsuperscript{16} The last steps of differentiation again take place in the erythroblast islands, where macrophages participate in terminal maturation of erythrocytes.\textsuperscript{20-22} The mature erythrocyte persists in the circulation for up to 120 days until ‘senescent’, when changes in surface antigen expression or physical characteristics of the cell trigger removal from the blood by macrophages of the reticuloendothelial system.\textsuperscript{23}
1.2.2 Developmental biology of erythropoiesis

During human development, hematopoiesis occurs in three distinct sites: the yolk sac; the fetal liver; and the bone marrow. Red blood cells arise from the yolk sac are also known as “Primitive” red cells. Primitive red cells constitute a distinct transient erythroid lineage that differs from "definitive" red cells that are produced in the fetal liver and bone marrow. In the human, primitive blood islands appear in the yolk-sac membrane at about 16 days of gestation. Primitive erythroblasts differentiate within the bloodstream, remain predominantly nucleated at the end of their maturation and have a very large size (accordingly, they are called “megaloblasts”). The distinctive feature of primitive erythropoiesis at this stage corresponds to the erythropoietin (EPO)-independency. Yolk sac erythropoiesis begins regression by tenth week of gestation and ceases function by sixteenth week.

The liver serves the dominant site of red blood cells production from the 9th to the 24th weeks of gestation. These fetal-liver-derived definitive "macrocyes" are smaller than yolk sac–derived primitive megaloblasts and contain one-third the amount of hemoglobin. Erythroid progenitors derived from hepatic erythropoiesis will differentiate in vitro with erythropoietin alone, in contrast to bone marrow derived BFU-E which requires erythropoietin plus interleukin (IL)-3.

Bone marrow erythropoiesis begins around the thirteen week of gestation to become the predominant erythropoietic organ after the 24th week of gestation and remains so throughout the remainder of fetal life.
1.2.3 Erythropoiesis stimulating agents

There are several different forms of approved erythropoiesis stimulating agents (ESAs), each which contains nearly all of the 165 amino acids of the endogenous EPO molecule but which differ in their glycosylation patterns and/or other side chains, resulting in different half-lives of the compounds.\(^{26, 27}\) Epoetin alpha and beta (rhEPO) contain all 165 amino acids of EPO, only differing slightly in their glycocytlation pattern and have an intravenous (IV) terminal half-life of 4 to 8 hours.\(^{26, 28, 29}\) Darbapoetin alfa has 4 amino acids of the EPO molecule mutated such that 2 additional N-glycan side chains are added, increasing the molecular weight to 38 kD (from 30 kD of endogenous EPO) and the circulating half-life to approximately 24 hours following IV administration.\(^{26, 28}\) Subcutaneous (SQ) administration of epoetin alfa and epoetin beta increases the terminal half-life to 24 hours through “flip-flop” kinetics.\(^{26}\) Similarly, the terminal half-life of SQ administered darbapoetin is increased to 48 hours. Presumably, the “flip-flop” kinetics following SQ administration is due to a fraction of the epoetins being slowly absorbed through the lymphatic system from the administration site.\(^{30, 31}\) A third generation ESA is continuous erythropoietin receptor activator (CERA), which is an EPO molecule attached to methoxy-polyethylene glycol (PEG) polymer chain(s) via amid bonds that doubles the molecular weight to approximately 60 kD.\(^{32}\) The addition of the PEG molecule(s) to EPO results in increasing the terminal elimination half-life of CERA to over 134 hours in humans.\(^{27}\) Apparently due to the long elimination half-life, the terminal kinetics are unaffected by IV or SQ administration.

The pharmacodynamics of ESAs at the EPOR level in erythroid progenitor cells is poorly understood.\(^{33}\) Only 20% to 30% EPOR occupancy by EPO is necessary to
stimulate erythropoiesis. Studies of rhEPO have used once daily, twice weekly, and once weekly administration; however, no clear indication of the optimal dosing strategy for erythropoiesis is evident. Additionally, the weekly IV doses of rhEPO are 30% higher than that needed to achieve the same efficacy with SQ administration, suggesting that the high peaks and low troughs from IV administration are suboptimal for maximizing erythropoiesis. Darbepoetin, with its longer elimination half-life, is generally efficacious at once weekly or once every other week. Furthermore, CERA, with its much longer elimination half-life, is efficacious at administration frequencies of both twice and once a month. The importance of ESA half-life on the pharmacodynamics of erythropoiesis is evident with CERA, which has a lower EPOR binding affinity than epoetins. However, in an in vivo study of rat bone marrow, CERA had more biological activity than an equivalent amount of epoetin, presumably due to its much longer half-life.

1.2.4 Sheep as an animal model for human erythropoiesis

Sheep have often been used as an experimental model because they have similar reticulocytes and red blood cells lifespan to that of humans. Similar to human RBCs, the lifespan of sheep RBCs is estimated at 114 days which is comparable to the value reported in adult human of 120 days. Erythrocyte lifespan in newborn infants is shorter than the lifespan in adult. This observation has been reported in both human and sheep. It can be attributed to the preferential removal of fetal RBCs from the newborn circulation. Fetal RBCs are larger than adult RBCs and express different surface antigens that might explain their short lifespan.
Similar to human, the mechanism of RBC removal from the sheep circulation is primarily due to cellular senescence. Conversely, other animals, such as mice, Red blood cells can be removed from circulation in two ways: random destruction and senescence. Random removal represents random destruction of erythrocytes irrespective of age at any time during their potential lifespan. Those cells that escape the hazard of random destruction eventually disappear, after an approximately constant lifespan, as a result of the process of senescence.\textsuperscript{41}

Additionally, the sites of erythropoiesis, and the types of hemoglobin produced at different developmental stages, most closely resemble the situation in humans.\textsuperscript{42} In sheep three distinct stages of hemoglobin production have been identified in the time span from the 20-day old embryo up to the adult animal. This ontogeny, including the two transition events, is almost completely analogous to the ontogeny of hemoglobin occurring in human. The first transition event involves the switch from embryonic hemoglobin to fetal hemoglobin. This transition is accompanied by the termination of hematopoiesis in the yolk sac blood island and the initiation of hematopoiesis at the embryonic liver.\textsuperscript{43} The second transition event involves the disappearance of fetal hemoglobin and appearance of the adult hemoglobin. In sheep, this transition starts at birth over a time space of about two months starting at birth.\textsuperscript{43, 44}

Erythropoiesis occurs almost exclusively in the bone marrow of adult humans.\textsuperscript{45} In contrast, substantial erythropoiesis in adult mice and other rodents occurs in both the bone marrow and extramedullary in the spleen,\textsuperscript{42, 46} which is an important difference between humans and mice and a limitation of rodents as a model of human
erythropoiesis. In adult sheep, like humans, erythropoiesis occurs primarily in the bone marrow. 42

These similarities justify performing experiments in sheep that cannot be done in human. Those experiments are intended to describe erythropoiesis under normal as well as hypoxic conditions. In other words, sheep can be used to explore erythropoiesis process when the hemoglobin level is about 40% the baseline value. The erythropoietic effect of erythropoietin can be elucidated over a wider range of EPO concentrations compared to the range observed in preterm infants. Based on the similarity in erythropoiesis process between human and sheep, results of these experiments may be extrapolated to the preterm infants.

1.3 Red blood cells

The survival characteristics of erythrocytes depend on the post-conceptional age. Lifespan of erythrocytes determined in term infants (60-70 days) is shorter than the lifespan in adults (120 days). Similarly, the survival of erythrocytes obtained from preterm infants (35-50 days) is shorter than the lifespan in term infants. The lower the post-conceptional age, the greater the degree of reduction in erythrocyte lifespan is. 47 It has been hypothesized that the reduction in lifespan of fetal erythrocytes is a caused by alteration of membrane function and increase susceptibility to mechanical damage. 48 The shortened lifespan of fetal red blood cells is associated with reduced cell rigidity. 49

RBCs, including both reticulocytes and mature erythrocytes, carry oxygen from the lungs to tissues and transport carbon dioxide from the tissues back to the lungs for removal through the Hb contained within them. A single normal human RBC contains approximately 32 pg of Hb and the circulation in an adult contains $3 \times 10^{11}$ RBC. 50 Red
blood cells slowly age following release from the bone marrow and under non-disease state conditions the mechanism of RBC death is primarily due to cellular senescence (i.e. age-related destruction or death).\textsuperscript{51, 52} In healthy human subjects a RBC typically survives approximately 120 days from the time of release as a reticulocyte into the circulation until removal by the reticuloendothelial cells of the liver and spleen.\textsuperscript{51-53} The signal by which a RBC is marked for removal is unknown, but it is thought to be through an accumulation of oxidative stress and damage to the cell surface and intracellular enzymes. There is conflicting evidence on the effect of EPO, if any, on RBC survival. Studies in mice and rats have demonstrated that RBCs produced under stress erythropoiesis (i.e. elevated EPO concentration) have a shortened lifespan.\textsuperscript{54, 55} However, studies conducted in humans suggests that the survival of RBCs produced under stress erythropoiesis is prolonged due to action of EPO on the erythroid progenitor cells that results in improved viability of the resulting RBCs.\textsuperscript{56}

\textbf{1.4 Receptor mediated elimination erythropoietin}

In 1959, Stohlman and Brecher hypothesized that the major site for endogenous EPO degradation is the bone marrow by an EPO receptor-mediated mechanism. This hypothesis was based on the observation that in patients with aplastic anemia who lack erythroid progenitor cells, EPO levels are much higher than in patients with thalassemia intermedia at the same hemoglobin concentration.\textsuperscript{57}

This hypothesis is in line with experimental results indicating that the alteration of bone marrow function results in a significant change in rhEPO pharmacokinetic behavior.\textsuperscript{58, 59} Additionally, higher organ specific uptake of \textsuperscript{125}I-rhEPO in the bone marrow that was observed in rats also supports the hypothesis that the bone marrow is the
major site of EPO degradation. Different studies also provided a substantial indirect evidence of receptor-mediated elimination of EPO. Sawyer et al. demonstrated receptor-mediated elimination of EPO as a result of lysosomal degradation in erythroid cells. Gross and Lodish also reported that 40% of EPO internalized in cells carrying EPO receptor is intracellularly degraded. Recently, Demet et al. demonstrated a highly significant positive linear correlation between EPO receptor mRNA level in the bone marrow and EPO clearance in newborn lambs.

Most studies have reported the nonlinear disposition of rhEPO, primarily indicated by a decrease in clearance with increasing doses. This nonlinear behavior of recombinant EPO suggests that its elimination involves a saturable mechanism. EPO receptor mediated elimination hypothesis explains the nonlinear disposition of EPO.

1.5 Review of Pharmacokinetics of erythropoietin

The pharmacokinetics of rhEPO when administered by intravenous, subcutaneous routes has been extensively investigated. A summary of pharmacokinetic parameters in healthy adults and renal failure patients on hemodialysis is presented in Table 1-1. The volume of distribution ranges from 0.04 to 0.09 L/kg body weight. As a result, peak serum concentration (U/L) immediately after intravenous rhEPO administration is equivalent to the dose (U/kg) multiplied by a factor of 20. Intravenous rhEPO has a terminal plasma elimination half-life of about 4 to 11 hours. A 20% reduction in the half-life of the IV administered rhEPO in renal failure patients compared to normal subjects. However, an earlier study by Kindler et al. reported that the degree of renal failure has a minor effect on the half-life of rhEPO.
The bioavailability a subcutaneous rhEPO dose is about 30%. Peak plasma levels are achieved 12 to 18 hours after a subcutaneous rhEPO administration. The absorption rate is not affected by the total dose administered. However, the clearance was dose dependent that it increases with decreasing the dose.66

In this section, a summary of the models that described that pharmacokinetics of EPO is present:

1.5.1 Two compartment model with linear elimination

Early studies that described the pharmacokinetic behavior of erythropoietin used a biexponential curve (i.e. two compartments).65, 67 Eq.1.1 describes rhEPO plasma concentration after an IV dose:

\[
\frac{dC_{\text{rhEPO}}(t)}{dt} = -\left(K + \frac{G}{\gamma}\right) \cdot C_{\text{rhEPO}} + G \cdot e^{-\gamma t} \ast C_{\text{rhEPO}}
\]

where \( C_{\text{rhEPO}} \) is the plasma rhEPO concentration, \( K \) is the linear elimination pathway 1st order elimination rate constant, \( G \cdot e^{-\gamma t} \) is the distribution function according to disposition decomposition analysis principles and \( \ast \) denotes the convolution linear operator.

The limitation of this model is that it does not account for the nonlinear behavior of rhEPO that is indicated by the increase in the clearance with increasing the rhEPO dose.66

1.5.2 Two compartment with dual elimination from the central compartment (linear and nonlinear)

In 1997, Kato et al. proposed a mechanism based pharmacokinetic model for rhEPO elimination in rats. The model assumes that the drug rapidly distributes after an
IV administration. The elimination occurs from the central compartment through two parallel pathways: linear elimination pathway and nonlinear elimination pathway.\textsuperscript{68} Subsequently, Veng-Pedersen et al. provided an experimental evidence for the dual elimination pathways hypothesis. This was done by altering the rhEPO pharmacokinetics by bone marrow ablation. This experiment demonstrated that the bone marrow is responsible for the nonlinearity observed with rhEPO pharmacokinetics.\textsuperscript{58} The model is presented using the following differential equation:

\[
\frac{dC_{rhEPO}(t)}{dt} = -\left( K + \frac{V_m}{K_m + C_{rhEPO}} + \frac{G}{\gamma} \right) \cdot C_{rhEPO} + G \cdot e^{-\gamma t} \ast C_{rhEPO} \tag{1.2}
\]

where \( C_{rhEPO} \) is the plasma rhEPO concentration, \( K \) is the linear elimination pathway 1\textsuperscript{st} order elimination rate constant, \( G \cdot e^{-\gamma t} \) is the distribution function according to disposition decomposition analysis principles, \( \ast \) denotes the convolution linear operator, and \( V_m \) and \( K_m \) are the Michaelis-Menten parameters corresponding to the nonlinear elimination route.\textsuperscript{69} The advantage of this model is that it is physiologically relevant and in line with experimental results.

### 1.5.3 Two compartment with nonlinear elimination

A model that is based on the previous model is commonly used to describe rhEPO pharmacokinetics.\textsuperscript{70, 71} The model assumes that there is only one elimination pathway from the central compartment that is a nonlinear pathway. The model is described using the following equation:

\[
\frac{dC_{rhEPO}(t)}{dt} = \left( \frac{V_m}{K_m + C_{rhEPO}} + \frac{G}{\gamma} \right) \cdot C_{rhEPO} + G \cdot e^{-\gamma t} \ast C_{rhEPO} \tag{1.3}
\]
This model is beneficial in terms of parameter identifiably with commonly used study designs (e.g. dose escalation studies). Additionally, it is physiologically relevant in terms of accounting for nonlinear behavior of rhEPO. However, this model does not recognize the linear elimination route. This limitation is important if concentration at high dose of rhEPO to be simulated. This might result in a saturated elimination of rhEPO which might overestimate the actual concentration of plasma rhEPO.

1.6 Review of pharmacodynamic (PD) models of erythropoietin

1.6.1 Loeffler et al erythropoiesis kinetics model

One of the earliest models that described the pharmacodynamics of EPO was developed by Loeffler and colleagues. Loeffler et al developed a comprehensive mathematical model that described the erythropoiesis process under normal as well as steady state conditions. As depicted by Figure 1.2, the model can be divided into three subsystems:

1.6.1.1 Renal tissue oxygenation subsystem

The primary laboratory parameter that regulates erythropoietin production in the model was assumed to be tissue oxygen pressure ($PO_2$). Several factors influence $PO_2$, such as: red blood cell mass, oxyhemoglobin dissociation curve, the arterial oxygen pressure and local blood flow.

A decrease in $PO_2$ will stimulate EPO production. Similarly, an increase in $PO_2$ will inhibit the production of EPO. Cases that decrease $PO_2$, include: anemia and hypoxia. In anemia, the red blood cell mass is reduced which decreases the capacity to
transport oxygen from the lung to the tissue. As a result, a lower $PO_2$ is needed to meet the oxygen demand by the tissue. During hypoxia the red blood cell mass is normal but the arterial oxygen pressure is lowered. This will also lead to a reduction in $PO_2$, which in turn will decrease the production of EPO.

### 1.6.1.2 Erythropoietin subsystem

As discussed in section 1.6.1.1, EPO production rate was assumed to depend on tissue oxygen pressure. The equation that describes the change in plasma EPO concentration is given by Eq.1.4.

$$\frac{dC_{EPO}}{dt} = f_{in} - K_{EPO} \cdot C_{EPO}$$  \hspace{1cm} (1.4)

where $C_{EPO}$ is the plasma EPO concentration, $f_{in}$ is EPO production rate normalized by the EPO volume of distribution and $K_{EPO}$ is EPO 1st order elimination rate constant.

The dependence of EPO production rate on tissue oxygen pressure is given by Eq.1.5.

$$f_{in} = f_{in}^{MAX} \cdot \exp \left( - \frac{E \cdot PO_2}{PO_2^{norm}} \right)$$  \hspace{1cm} (1.5)

where $f_{in}^{MAX}$ is the maximum EPO production rate, $E$ is the sensitivity of EPO production to changes in $PO_2$, and $PO_2^{norm}$ is the baseline tissue oxygen pressure under normal conditions.

### 1.6.1.3 Erythropoietic differentiation subsystem

This subsystem considers five compartments each representing one cell type:

a) **Late erythropoietic progenitors (CFU-E)**
In this model, pluripotent hematopoietic stem cells and early erythroid progenitors (BFU-E) were neglected because their status did not change during the study. This assumption is consistent with what is currently known about the responsiveness of hematopoietic cells to EPO. The pluripotent hematopoietic stem cells do not express EPO receptors. BFU-E is to express low amounts of EPO receptors as explained in section 1.2.1. EPO was assumed to affect the production of CFU-E cells as described in the following equations:

\[
\frac{dCFU-E}{dt} = f_{CFU-E} - K_{CFU-E} \cdot (CFU-E)
\]  

(1.6)

where \( CFU-E \) is the late erythroid progenitors, \( f_{CFU-E} \) is the production rate of \( CFU-E \) and \( K_{CFU-E} \) is the 1\(^{st} \) order elimination rate constant of \( CFU-E \). The explicit form of \( f_{CFU-E} \) was described as displayed in Eq.1.7.

\[
f_{CFU-E} = A - B \cdot \exp\left(-C \cdot \left(\frac{C_{EPO}}{C_{norm}^{EPO}}\right)^{0.7}\right)
\]  

(1.7)

where \( A, B, \) and \( C \) are constants, \( C_{norm}^{EPO} \) is the baseline EPO concentration under normal conditions.

b) Proliferative erythroid precursors (PEP)

This compartment represents erythroblasts, basophilic and proliferative polychromatic erythroblasts. The first two compartments (CFU-E and PEP) were assumed to be able to proliferate (i.e. has the ability to undergo mitosis). The change in proliferative erythroid precursors was given as follows:

\[
\frac{dPEP}{dt} = f_{PEP} \cdot K_{CFU-E} \cdot (CFU-E) - K_{PEP} \cdot (PEP)
\]  

(1.8)
where $PEP$ is the proliferative erythroid precursors, $f_{PEP}$ is the production rate of $PEP$ and $K_{PEP}$ is the 1st order elimination rate variable of $PEP$. The explicit for of $f_{PEP}$ was described by the following equation:

$$f_{PEP} = D - F \cdot \exp \left( - G \cdot \left[ \frac{C_{EPO}}{C_{norm_{EPO}}} \right]^{0.7} \right) \quad (1.9)$$

where $D, F$ and $G$ are constants.

The dependence of $K_{PEP}$ on EPO was given by:

$$K_{PEP} = \frac{1}{H - I \cdot \exp \left( - J \cdot \left[ \frac{C_{EPO}}{C_{norm_{EPO}}} \right]^{0.7} \right)} \quad (1.10)$$

where $H, I$, and $J$ are constants.

c) Non-proliferative erythroid precursors (NPEP)

These cells represent non-proliferative polychromatic, orthochromatic erythroblasts and bone marrow reticulocytes. Starting from this stage, cells do not have the ability to proliferate. Another assumption was that EPO do not affect the production of NPEP. However, EPO affect the survival of this population of cells. The equations that describe the behavior of NPEP were:

$$\frac{dNPEP}{dt} = K_{PEP} \cdot (PEP) - K_{NPEP} \cdot (NPEP) \quad (1.11)$$

where $NPEP$ is the non-proliferative erythroid precursor and $K_{NPEP}$ is the 1st order elimination rate variable of $NPEP$.

The dependence of $K_{NPEP}$ on EPO was given by:
\[ K_{NPEP} = \frac{1}{K - L \cdot \exp\left(-M \cdot \left[ \frac{C_{EPO}}{C_{norm, EPO}} \right]^{0.7} \right)} \]  \hspace{1cm} (1.12)

where \( K, L, \) and \( M \) are constants.

d) **Reticulocytes in the circulation (RETI)**

The reticulocytes in the circulation were assumed to independent of EPO. Eq.1.13 describes the change in RETI:

\[ \frac{dRETI}{dt} = K_{NPEP} \cdot (NPEP) - K_{RETI} \cdot RETI \]  \hspace{1cm} (1.13)

where \( RETI \) is the reticulocytes in the circulation and \( K_{RETI} \) is the 1st order elimination rate constant of \( RETI \).

e) **Erythrocytes (ERY)**

The erythrocyte compartment was assumed to be composed of two erythrocytes populations: erythrocytes to be removed by age dependent process (ERYA) and erythrocytes to be removed randomly irrespective to the age structure (ERYR). The change in ERY, ERYA and ERYR was given by the following differential equations:

\[ \frac{dERY}{dt} = \frac{dERYA}{dt} + \frac{dERYR}{dt} \]  \hspace{1cm} (1.14)

with

\[ \frac{dERYA}{dt} = a(h(t)) \cdot h(t) - a(h(t - \tau_{ERY})) \cdot h(t - \tau_{ERY}) \]  \hspace{1cm} (1.15)

and

\[ \frac{dERYR}{dt} = \left(1 - a(h(t))\right) \cdot h(t) - ERYR / \tau_{ERY} \]  \hspace{1cm} (1.16)

where
\[ h(t) = K_{RETI} \cdot RETI \]  

(1.17)

and

\[ \alpha(h(t)) = \exp\left(-0.1 \cdot \left(h(t)/h_{\text{norm}}\right)^2\right) \]  

(1.18)

\( h_{\text{norm}} \) denotes the normal efflux from the RETI compartment and \( \alpha(h(t - \tau_{\text{ERYA}})) \cdot h(t - \tau_{\text{ERYA}}) \) is the influx into subcompartment ERYA delayed by \( \tau_{\text{ERYA}} \).

The model accounted for the effect of tissue oxygenation and EPO on erythropoiesis process. The model was able to describe the general trend for the erythropoietic behavior in mice and rats under different erythropoietic conditions. However, the model was complex and over-parameterized (23-30 parameters). It also implicitly assumed that cells were removed by a first order process which implies that cell removal was a random process and ignores the age structure of cells within a compartment.72-74

1.6.2 Uehlinger et al. erythropoiesis model\textsuperscript{53}

In 1992 Uehlinger et al proposed a clinically oriented model that described the change in hematocrit during rhEPO therapy in uremic anemic patients.\textsuperscript{53} The model assumed that hematocrit (Hct) level is affected by rhEPO through a linear transduction function as explained below. The hematocrit level change as a result of a constant rhEPO dose was described by the following equation:

\[ Hct(t) = Hct(0) + \beta \cdot \int_{0}^{t} S(s) \, ds \]  

(1.19)

where \( Hct(t) \) is the Hct at time \( t \), \( Hct(0) \) is the baseline Hct, \( \beta \) is a constant and \( S(s) \) is the survivor function.
The effect of EPO on Hct level was introduced by affecting the value of $\beta$ as follows:

$$\beta = s \cdot D$$

(1.20)

where $s$ is the dose sensitivity constant and $D$ is the rhEPO dose.

The survivor function under three different scenarios was considered:

a) **All erythrocytes have the same lifespan**

The survivor function was presented as follows:

$$S(t) = \begin{cases} 
1 & \text{for } 0 < t < \tau \\
0 & \text{otherwise}
\end{cases}$$

(1.21)

where $\tau$ is the RBC lifespan.

In this case Eq.1.16 can be simplified into:

$$Hct(t) = Hct(0) + \beta \cdot t - U(t - \tau) \cdot \beta(t - \tau)$$

(1.22)

where ‘$U$’ is a unit step function

$$U(t) = \begin{cases} 
0 & \text{for } t < 0 \\
1 & \text{otherwise}
\end{cases}$$

(1.23)

b) **RBC lifespan distributed around a mean lifespan**

In this case the survivor function was:

$$S(t) = \frac{1}{1 + \exp(\alpha(t - \tau))}$$

(1.24)

where $\alpha$ is a constant (fixed to 0.3 day$^{-1}$).
The limitation of this equation is that at \( t=0 \), the survival function is not equal to 1 which violates the definition of the survival function (Chapter 5, section 5.3).

c) Erythrocytes are removed by two processes (aging and random destruction)

In this case the survivor function was presented as displayed by Eq. 1.25.

\[
S(t) = \frac{\exp(-k \cdot t)}{1 + \exp(\alpha(t - \tau))}
\]  

(1.25)

where \( k \) is a proportionality constant for random destruction and \( \alpha \) is a constant (fixed to 0.3 day\(^{-1}\)).

This formula is not consistent with physiological principles of RBC survival. Similar to Eq. 1.24, using the present formula the value of the survival function does not equal 1 at \( t=0 \). Moreover, the survival function continues indefinitely which implies that some cells are not removed due to senescence.

The Uehlinger et al. erythropoiesis model offers several advantages from a clinical point of view. It provides a formula to calculate the required dose of rhEPO to manage anemia. It also includes a methodology to account for the Hct change as a result of blood transfusion. Perhaps the main limitation from this model is that it was empirical and did not account for important physiological phenomena such as the inhibition of endogenous EPO production by exogenous rhEPO and the lag between rhEPO administration and the appearance of reticulocytes in the circulation. Furthermore, this model cannot be used to extrapolate the effect of dose outside the studied range.
1.6.3 Target-mediated PK/PD model of rhEPO

The concept of indirect response model in combination with lifespan concept was discussed by Kryzanaski et al.\textsuperscript{75} The model described the erythropoiesis process as a series of transduction compartments with different maturation times ("catenary lifespan model").\textsuperscript{76} The model accounted for different feedback processes that affect the regulation of both EPO and hemoglobin production.

As depicted by Figure 1.3, the model can be divided into two subsystems:

a) Erythropoietin subsystem

The disposition of EPO was described by a target mediated drug disposition (TMDD) pharmacokinetic model.\textsuperscript{77} TMDD uses saturable, high affinity receptor binding mechanism as a primary mechanism of nonlinear pharmacokinetics of EPO. Another key assumption of the model is that receptor-mediate endocytosis contributes to EPO elimination.

As displayed in Figure 1.3, EPO was assumed to be produced with a constant production rate \( k_{EPO} \). Free EPO \( (C) \) in the circulation can either distribute into peripheral tissue, bind to free EPO receptors (equilibrium dissociation constant was denoted as \( K_D \)) to form EPO-receptor complex \( (RC) \), or directly eliminated (elimination rate constant was denoted as \( k_{el} \)). EPO-receptor complex can be internalized and degraded, or dissociates to free drug and free receptor \( (R) \). EPO receptors were assumed to be produced with a constant production rate of \( k_{syn} \). The following equations describe the TMDD model of EPO disposition.

\[
\frac{dC_{tot}}{dt} = k_{EPO} - k_{int} \cdot C_{tot} - (k_{el} + k_{pt} - k_{int}) \cdot C + \frac{k_p \cdot A_T}{V_p} \quad (1.26)
\]
\[ \frac{dA_T}{dt} = k_{pt} \cdot C \cdot V_p - k_{tp} \cdot A_T \] (1.27)

\[ \frac{dR_{tot}}{dt} = k_{syn} - (k_{int} - k_{deg}) \cdot (C_{tot} - C) - k_{deg} \cdot R_{tot} \] (1.28)

\[ C = \frac{1}{2} \left( (C_{tot} - R_{tot} - K_D) + \sqrt{(C_{tot} - R_{tot} - K_D)^2 + 4 \cdot K_D \cdot C_{tot}} \right) \] (1.29)

with the following initial conditions:

\[ C_{tot}(0) = \frac{D_{IV} + k_{EPO} \cdot (k_{int} - k_{deg}) \cdot C_0}{k_{int}} \] (1.30)

\[ A_T(0) = \frac{k_{pt} \cdot V_p \cdot C_0}{k_{tp}} \] (1.31)

\[ R_{tot}(0) = \frac{k_{syn} \cdot (K_D + C_0)}{k_{int} \cdot C_0 + k_{deg} \cdot K_D} \] (1.32)

where \( C_{tot} \) is the sum of free drug (\( C \)) and drug-receptor complex (\( RC \)), \( k_{int} \) is the first order rate constant for the internalization and degradation of \( RC \), \( A_T \) is the amount of EPO that distributes to and from tissue, \( k_{pt} \) and \( k_{tp} \) are first order rate constants for the EPO distribution between plasma and tissue, and \( R_{tot} \) is the sum of free receptor (\( R \)) and drug-receptor complex.

b) Pharmacodynamic model

The key feature of the model is that EPO-receptor complex is responsible to it’s the pharmacologic activity of EPO. The PD model consists of four catenary compartments: early erythroid progenitor cells (\( P1 \)), erythroblasts (\( P2 \)), reticulocytes (\( RET \)) and mature red blood cells (\( RBC_{3r} \)). The production of early erythroid progenitors was assumed to be dependent on EPO. In other words, EPO stimulates the production of \( P1 \) cells through a stimulation function \( S(t) \). The Hb level was also
assumed to affect the production of \( P1 \) through an inhibitory function \( (I(t)) \) with parameter \( I_{\text{max}} \) and \( IC_{50} \). The time that \( P1 \) spends before transforming to erythroblasts is defined as their lifespan \( (T_{P1}) \). Similarly, the conversion of early erythroid progenitors to erythroblasts is stimulated by EPO. Erythroblasts will then transform to reticulocytes with a lag time equivalent to erythroblasts lifespan \( (T_{P2}) \). From this stage and on, the erythropoiesis process is not affected by EPO. As a result, the input rate to the RET compartment will be equivalent to the output rate from the P2 compartment. Reticulocytes transforms to \( RBC_M \) with a rate that is equal to the production rate of reticulocytes with a lag time equal to the lifespan of RET \( (T_{RET}) \). At the end, \( RBC_M \) stays in the circulation until the end of their lifespan \( (T_{RBC}) \). Mathematically, the changes in RET and \( RBC_M \) was described according to the following equations:

\[
\frac{d\text{RET}}{dt} = k_{\text{in}} \cdot S(t - T_{P1} - T_{P2}) \cdot S(t - T_{P2}) \cdot I(t - T_{P1} - T_{P2}) - k_{\text{in}} \cdot S(t - T_{P1} - T_{P2} - T_{RET}) \cdot S(t - T_{P2} - T_{RET}) \cdot I(t - T_{P1} - T_{P2} - T_{RET}) 
\]

(1.33)

\[
\frac{dRBC}{dt} = k_{\text{in}} \cdot S(t - T_{P1} - T_{P2} - T_{RET}) \cdot S(t - T_{P2} - T_{RET}) \cdot I(t - T_{P1} - T_{P2} - T_{RET}) - k_{\text{in}} \cdot S(t - T_{P1} - T_{P2} - T_{RET} - T_{RBC}) \cdot S(t - T_{P2} - T_{RET} - T_{RBC}) \cdot I(t - T_{P1} - T_{P2} - T_{RET} - T_{RBC}) 
\]

(1.34)

where \( S(t) = \left(1 + \frac{S_{\text{max}} \cdot RC(t)}{SC_{50} + RC(t)}\right) \), \( I(t) = \left(1 - \frac{I_{\text{max}} \cdot \Delta Hb(t)}{IC_{50} + \Delta Hb(t)}\right) \) \( RET(0) = RET_0 \), \( RBC(0) = RBC_0 \), \( S_{\text{max}} \) is the maximum stimulation of responses by EPO-receptor complex \( (RC) \) and \( SC_{50} \) is the \( RC \) producing half maximal stimulation.
The model was developed based on physiological principles. It also describes the control mechanism associated with the regulation of EPO plasma level and RBC kinetics. It also introduces the concept of lifespan which simplify the model for the cases of reticulocytes and RBCs to be: reticulocytes and RBCs are produced at a specific rate, survive for a specific duration ($T_{\text{RET}}$ and $T_{\text{RBC}}$ respectively) and then are lost. The rate of cell loss must equal to the production rate but it is delayed by the cell lifespan.

Perhaps the main limitation of this model is that it does not include a formula that describes EPO production. Instead, EPO production rate was assumed to be constant. This assumption is not inline with the fact that hypoxia stimulates EPO production. The effect in the hypoxia was included in the model though the use of the change in Hb ($\Delta Hb$) as a marker of hypoxia. It was assumed that the increase in Hb level inhibits the production of progenitor cells directly. However, the increase of Hb level will increase the oxygen carrying capacity of the blood which will inhibit EPO production as a result the decrease in EPO level inhibits progenitor cells production.

One of the assumptions of this model is that the production of EPO receptor is constant. This assumption is violated when dealing with erythropoiesis under stress conditions. As explained in Chapter 8, under stress conditions EPO receptors are up-regulated. This up-regulation can be only explained by the increased production of EPO receptors.

1.6.4 EPO PD modeling using artificial neural networks

Several research groups used artificial neural networks (ANN) to describe erythropoiesis kinetics. Gaweda and colleagues published several articles that applied artificial neural network (ANN) approach to describe the erythropoiesis kinetics in
patients with renal failure.\textsuperscript{82} They developed an approach to predict population response to anemia.\textsuperscript{82} This approach used average monthly hematocrit levels over past 2 months, together with their standard deviations, as well as average doses of erythropoietin over past 2 months as input variables to the ANN model. Gabutti et al implemented ANN successfully to individualize rhEPO therapy in anemic dialysis patients.\textsuperscript{80} The author concluded that the monthly dose adjustments using ANN are superior to physician opinion. The ANN model that was built to predict the monthly adaptations in rhEPO dose was able to detect 48\% of the patients treated with an insufficient rhEPO dose. On the other hand, nephrologists’ opinion allowed detecting 25\% of the patient with insufficient rhEPO dose.\textsuperscript{80}

ANNs are generally able to tolerate missing data and errors in individual measurements.\textsuperscript{83} ANNs also have the advantage of being applicable to translate multivariate non-linear relationships into continuous functions without the need of understanding precisely the underlying relationships between variables.\textsuperscript{80, 84}

The disadvantage of ANN is that they are not physiologically relevant and there is no quantitative understanding of the mechanism behind ANN model.

\textbf{1.6.5 Veng-Pedersen et al erythropoiesis model}

Veng-Pedersen et al has introduced the idea of UIR in the context of cell kinetics. They have published several publications that deal with different aspects of the erythropoiesis process including erythropoietin regulation such as: EPO nonlinearity pharmacokinetic analysis,\textsuperscript{85-87} a proposed mechanism for EPO production in response to acute phlebotomy,\textsuperscript{88, 89} modeling the change of EPO receptor density under stress conditions.\textsuperscript{90} They also proposed new models that provide an in-depth understanding of
the survival or reticulocytes and red blood cells such: time variant models for reticulocytes kinetics, the survival of red blood cells under different environmental conditions, and the effect of EPO receptor change on the erythropoiesis process.

Since it is beyond the scope of this thesis to cover all the aspects of different models introduced by Veng-Pedersen research group, an explanation to the theory behind the model used in this thesis (Chapter 2-3 and 9) was provided in this section.

The change in the Hb level can be simplified in a stimulation function \( f_{stim}(t) \) that is convolved with a unit response function \( UR(t) \) as displayed by Eq.1.35.

\[
Hb(t) = f_{stim}(t) \ast UR(t) = \int_{-\infty}^{t} f_{stim}(u) \cdot UR(t-u) \cdot du
\] (1.35)

Red blood cells were assumed to have a point distribution with a fixed lifespan. In other words, \( UR(t) \) takes one of two possible values 0 and 1. \( UR(t) \) takes a value of 0 when cells are not present in the circulation and 1 when cells are present in the circulation. For a cell that was stimulated at time 0, it will appear in the circulation after a lag time denoted as “a”. The \( UR(t) \) value after stimulation and before appearance in the circulation \( (0 < t < a) \) is 0. After the appearance of the cell in the circulation at time \( a \), it will stay in the circulation for a period that is defined as the lifespan \( (b-a) \). The \( UR(t) \) value after appearance in the circulation and before removal from the circulation \( (a < t < b) \) is 1. After the cell is removed from the circulation the \( UR(t) \) value becomes 0.

\[
UR(t) = \begin{cases} 
1 & \text{for } a < t < b \\
0 & \text{otherwise}
\end{cases}
\] (1.36)

Since \( UR(t-u) \) is 1 for \( a < t-u < b \), Eq.1.35 can be reduced to:
\[ H\text{b}(t) = \int_{t-a}^{t} f_{\text{stim}}(u) \cdot du \]  

(1.37)

The change in Hb level can be described using:

\[ \frac{dH\text{b}(t)}{dt} = \frac{d}{dt} \int_{t-b}^{t-a} f_{\text{stim}}(u) \cdot du \]  

(1.38)

Using Leibniz integration rule:

\[ \frac{dH\text{b}(t)}{dt} = f_{\text{stim}}(t-a) - f_{\text{stim}}(t-b) \]  

(1.39)

The mechanistic interpretation is as follows, the amount of Hb in the circulation is influenced by two processes: input \( (f_{\text{stim}}(t-a)) \) and output \( (f_{\text{stim}}(t-b)) \). The input is the rate of Hb appearance in the circulation which is equivalent to the stimulation rate with a lag time equal to “a”. Similarly, the rate of Hb disappearance (i.e. output) is equivalent to the stimulation rate with a lag time of “b”. The details of the stimulation function and its dependence on EPO is discussed later in this thesis.

This model has been extended to deal with dynamic changes encountered in preterm infant population.\(^{95, 96}\) It also accounts for important variables including: transfusions, phlebotomies, shortened RBC lifespan and blood volume expansion. The model is described in more details in Chapters 2-4.

Another important aspect of erythropoiesis is the dynamic changes in EPO receptors (EPOR) pool size. As explained in Chapter 8, several observations support the hypothesis that EPOR pool size undergoes dynamic changes during phlebotomy-induced anemia. In Chapter 9 a new model that accounts for change in EPOR pool size in modeling erythropoiesis under stress conditions is proposed.
1.7 Anemia of prematurity

During the first two months after birth, the concentration of hemoglobin declines as a result of decreased erythropoiesis rate. This phenomenon is observed in both term and preterm infants. The exposure of the infant to the extra-uterine environment after birth increases tissue oxygenation. This reduces erythropoietin production. As a result, erythropoiesis rate decreases which causes the decline in hemoglobin concentration. The decrease in hemoglobin level in preterm infants is usually not clinically significant. However, in preterm infants the decrease in hemoglobin level is more profound and it is usually referred to as anemia of prematurity.97

Other factors that causes anemia of prematurity include: insufficient EPO production by the liver, blood loss resulting from frequent laboratory blood sampling,98 rapid infant growth, shortened RBC lifespan, and expansion of blood volume.99, 100

1.7.1 Therapy for anemia of prematurity

1.7.1.1 Minimization blood loss

Previous studies have shown that during the first 4 weeks of life in preterm infants with a gestational age of less than 28 weeks, 33.8 mL/kg of blood is removed and 27 mL/kg of blood is transfused.101 Thus, the effect of blood sampling is substantial and plays an important role in the management of anemia of prematurity. Blood loss for clinical samples can be minimized by using inline and microsampling point-of-care testing.102

1.7.1.2 Erythrocyte transfusions

RBC transfusion provides an immediate increase in oxygen delivery to tissues and is an effective and rapid intervention to treat anemia of prematurity. RBC transfusions
can reduce the morbidity associated with anemia, especially anemia of prematurity, and may be life saving in neonates with severe blood loss.\textsuperscript{24}

Although transfusion may be lifesaving, like all medical interventions, it is associated with certain risks. Preterm infants are at increased risk of passive immune hemolysis as a result of receiving an ABO-incompatible RBC transfusion. Fluid overload represents another complication associated with RBC transfusion. Fluid overload occurs when excess fluid is transfused or the transfusion is too rapid. RBC transfusions also pose the risk for transfusion-associated graft versus host disease (TAGVHD). TAGVHD results from the proliferation of donor-derived lymphocytes in response to histocompatibility antigens. Another risk associated with transfusions is transfusion-transmitted infection.\textsuperscript{103}

1.7.1.3 **Recombinant human erythropoietin (rhEPO)**

Low plasma levels of erythropoietin in preterm infants provide a rationale for the use of EPO to prevent or treat anemia of prematurity.\textsuperscript{97, 104} Furthermore, premature infants respond to rhEPO and supplemental iron with a rapid reticulocytosis. Multiple investigations have demonstrated that rhEPO treatment resulted in a significant reduction in RBC transfusions.\textsuperscript{105-108} Other studies reported that EPO treatment does not have a clinically significant effect in the treatment of anemia of prematurity.\textsuperscript{109-111} These differences are likely due to the variable transfusions practices, inter-patient variability, and inconsistencies in the treatment protocol.\textsuperscript{112}

Trials of rhEPO treatment vary in terms of rhEPO dose, timing of initiation, and route of administration. A higher per-kilogram dosage is needed in preterm infants compared to adults. This can be attributed to fact that EPO has a greater volume of
distribution, and greater clearance in preterm infants compared to adults.\textsuperscript{113, 114} Data support the idea that rhEPO might be more efficacious if initiated earlier (before four days postnatal age).\textsuperscript{109, 115} Subcutaneous administration of EPO may be more efficacious as intravenous administration has increased urinary losses.\textsuperscript{116}

Side effects of rhEPO administration reported in adults include hypertension, thrombosis, bone pain, rash, and seizures. These side effects are generally not seen in preterm infants. Pure red-cell aplasia, which has been reported in adults on chronic therapy, has not been reported in infants.\textsuperscript{117} Several studies show decreased absolute neutrophil counts but no increase in infections.\textsuperscript{110, 118-120} Studies also show increased platelet numbers.\textsuperscript{110, 120, 121} Poor weight gain has been cited.\textsuperscript{117} A major concern of rhEPO in prematurity is the extension of functional iron deficiency into tissue iron deficiency. A recent Cochrane review of early rhEPO administration (starting before the 8th day of life) reported a significant increase in the frequency of severe (stages III-V) retinopathy of prematurity.\textsuperscript{122}

1.8 Population pharmacokinetics/Pharmacodynamics (PK/PD)

Population pharmacokinetics/Pharmacodynamics (PK/PD) is defined as the study of PK/PD in the population of interest. In population PK/PD data from all individuals are modeled simultaneously instead of modeling data from each individual separately. Population PK/PD accounts for the different levels of variability (between-subject, within-subject, interoccasion, residual, etc.) using nonlinear mixed effects modeling approach.\textsuperscript{123} Population PK/PD also explore potential covariates such as age, weight, or renal clearance that helps explain the variability observed in PK/PD outcomes.
Population PK/PD approach has several advantages over the individual fitting approach. Population PK/PD improves the power to identify nonlinearity in the structural PK and/or PD model and improves the parameter estimation. Additionally, data obtained from each individual is weighted differently based on the amount of information they supply in estimating the final population parameters. Also missing measurements are handled naturally in the process of population PK/PD modeling. Moreover, population PK/PD provides an advantage in dealing with sparse sampling designs. Furthermore, important covariates which explain subject variability can be identified using population PK/PD.

The application of population PK/PD in exploring the PD of erythropoietin enhances the ability to identify nonlinearity in the erythropoiesis process. Using individual fit approach, might not allow characterizing nonlinearity in erythropoiesis process in preterm infants. This can be explained by the observation that the range of erythropoietin levels measured in some of the infants do not permit characterization of the erythropoietic response at high erythropoietin level. As a result, simpler linear models will be chosen to describe erythropoietic response to erythropoietin. The problem arises if these simpler models are used to predict the response to erythropoietin levels beyond the range of those used to characterize the model. These predictions may result in the false belief that increasing erythropoietin level will result in a greater erythropoietic response when, in actual fact, increasing the erythropoietin level will result in a smaller than expected increase in erythropoietic effect.
1.9 Objectives and specific aims

The primary objective is to identify the PK/PD and physiologic factors important in predicting and maximizing recombinant human erythropoietin’s (rhEPO, “EPO”) erythropoietic effect, based on PK/PD studies of anemic infants and neonatal sheep. Under that overall objective, the specific aims were:

1. To describe erythropoiesis dynamics in preterm infants using population PK/PD modeling approach.
2. To determine and explain the variability in the response to EPO in preterm infants.
3. To evaluate newborn sheep as an experimental model for erythropoiesis in preterm infants.
4. To test the hypothesis that RBC lifespan is shortened under acute hypoxic stress conditions.
5. To test the hypothesis that EPO receptor (EPOR) pool size increases under hypoxic stress conditions and the change in EPOR pool size can be predicted using EPO clearance measurements.
6. To describe the effect of EPOR pool size changes on erythropoiesis kinetics.

1.10 Hypothesis

The overall hypothesis of this work is that treatment optimization of erythropoietin, particularly under dynamic hematological conditions such as stress erythropoiesis and conditions experienced in premature infants, requires a comprehensive knowledge of the behavior of erythrocytes and the complex PK/PD relationship between EPO and erythropoiesis.
Hypothesis 1: EPO plasma level vs. time profile relates in a predictable manner to changes in hemoglobin vs. time profile and can be described by applying population PK/PD model.

Hypothesis 2: The inter-subject variability in EPO’s PD is predictable by several covariates that are identifiable by population PK/PD modeling approach.

Hypothesis 3: The newborn lamb can be used as an animal model for erythropoiesis in preterm infants.

Hypothesis 4: Stress erythropoiesis results in the reduction of the lifespan of RBC produced under stress anemic conditions compared to RBC produced under non anemic conditions.

Hypothesis 5: Stress erythropoiesis results in the up-regulation of EPOR pool size which is accompanied by a parallel increase of EPO clearance as a result of receptor mediated elimination.

Hypothesis 6: EPOR pool size up-regulation directly affects the capacity to produce RBC and its effect can be identified using PK/PD analysis.

1.11 Outline of the thesis

To describe erythropoiesis dynamics in preterm infants as a function of the plasma EPO concentration, a population pharmacokinetics/pharmacodynamics (PK/PD) model was formulated and presented in Chapter 2. The PK/PD model is formulated to account for important variables including transfusions, phlebotomy, shortened RBC lifespan, and blood volume expansion. The model helps in the understanding of the regulation of EPO and hemoglobin (Hb) in preterm infants. In Chapter 3, the analysis performed in the previous Chapter was extended to explain the variability in the response
to rhEPO in preterm infants. This was done by screening for possible covariates to help predict the responsiveness to rhEPO. The information obtained from the population kinetic analysis done with the model is useful in the design of clinical trials with optimum EPO treatment regimen to improve the efficacy and utility of EPO in this patient group.

In Chapter 4, the PD model used in preterm infants (Chapters 2 and 3) is applied to describe erythropoiesis in newborn lambs. The erythropoietic effect of EPO in lambs is studied over a wider range of EPO concentrations compared to the range observed in preterm infants. The estimates of population PK/PD parameters in lambs are compared to the estimates obtained in preterm infants.

One of the key assumptions is that the lifespan of RBC does not change under stress conditions. Previous survival studies on rodents reported a decrease in the lifespan of RBCs under hypoxic conditions. In Chapter 5, a summary of currently used direct methods of RBC lifespan is presented. The quantitative aspects of each method in addition to its advantages or disadvantages are considered. In Chapter 6, a new methodology for the measurement of RBC lifespan under stress conditions is proposed using two biotin densities RBC labeling technique in combination with physiology-based mathematical modeling. In Chapter 7 the hypothesis that the RBC lifespan is shortened under acute hypoxic stress conditions is tested using the tool developed in Chapter 6.

A new aspect of erythropoiesis related to EPO receptor (EPOR) pool size change under stress hypoxic conditions. The study presented in Chapter 8 was undertaken to provide additional direct evidence of the proposed EPOR-based elimination mechanisms
by analysis investigating the relationship between the EPOR mRNA levels and EPO clearance.

A new formulation of the model that accounts for change in the EPOR pool size under stress conditions was presented in Chapter 9. Although the number of EPOR in bone marrow was not measured directly, changes in EPO clearance was assumed to provide an indirect measure of the relative changes in the EPOR pool size. This assumption was based on the finding from previous Chapter.
Table 1-1. Summary of pharmacokinetics parameters of single doses of rhEPO after intravenous (IV) or subcutaneous (SQ) administration in normal adult persons and renal failure patients on hemodialysis

<table>
<thead>
<tr>
<th>Condition</th>
<th>Bioavailability</th>
<th>$t_{\text{max}}$</th>
<th>$t_{1/2} \beta$</th>
<th>$V_d$ (ml/kg)</th>
<th>$\text{Cl}$ (ml/hr/kg)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV Healthy subject</td>
<td>100</td>
<td>0</td>
<td>4-11</td>
<td>40-90</td>
<td>4-15</td>
<td>64, 126-129</td>
</tr>
<tr>
<td>Renal failure patient</td>
<td>100</td>
<td>0</td>
<td>5-10</td>
<td>66-75</td>
<td>11</td>
<td>64, 130-134</td>
</tr>
<tr>
<td>SQ Healthy subject</td>
<td>36-39</td>
<td>9-29</td>
<td>24-79</td>
<td>-</td>
<td>-</td>
<td>64, 66, 127-129</td>
</tr>
<tr>
<td>Renal failure patient</td>
<td>23-49</td>
<td>10-18</td>
<td>9-22</td>
<td>-</td>
<td>-</td>
<td>130, 133-136</td>
</tr>
</tbody>
</table>

$t_{\text{max}}$: time to peak plasma concentration, $t_{1/2} \beta$: elimination half-life in circulation, $V_d$: volume of distribution; $\text{Cl}$: total clearance. The table is reproduced from “Recombinant Human Erythropoietin (rhEPO) in Clinical Oncology Scientific and Clinical Aspects of Anemia in Cancer”. 63
Figure 1.1 Stages of erythropoiesis. Steps of erythrocyte differentiation are shown with defining cellular characteristics based on relative proliferation, cytokine responsiveness, cell surface markers, and/or presence of RNA. CFU-GEMM = colony-forming unit–granulocyte, erythroid, macrophage, megakaryocyte; BFU–E = burst-forming unit–erythroid; EPOR = erythropoietin receptor; CFU–E = colony-forming unit–erythroid; C-Kit R = stem cell factor receptor, CD = CD117; CD71 = transferrin receptor; HGB = hemoglobin synthesis; CD36 = gpIIb, thrombospondin receptor; GPA = glycoporphin A present on human cells only.¹³⁷
Figure 1.2. The scheme of Loeffler et al erythropoiesis kinetics model\textsuperscript{74}
Figure 1.3. The scheme of target-mediated PK/PD model of rhEPO\textsuperscript{76}
CHAPTER 2. POPULATION PHARMACODYNAMICS OF ERYTHROPOIETIN IN PRETERM INFANTS

2.1 Introduction

Erythropoietin (EPO) is a 34 kD glycoprotein hormone that stimulates erythropoiesis.\textsuperscript{138-140} EPO is synthesized primarily in the kidney in adults and in the liver in preterm infants in response to hypoxia.\textsuperscript{141} This dependence on hepatic erythropoietin production in preterm infants may be an important contributor to neonatal anemia because the liver is less sensitive than the kidney to tissue hypoxia in stimulating EPO production.\textsuperscript{98, 142, 143}

Anemia is a frequent complication in very low birth weight (VLBW) premature infants (birth weight < 1500 g) that is usually referred to as anemia of prematurity.\textsuperscript{97} There are two main causes of anemia of prematurity: insufficient EPO production and excessive blood loss resulting from frequent laboratory blood sampling.\textsuperscript{98} Previous studies have shown that during the first 4 weeks of life in preterm infants with a gestational age of less than 28 weeks or birth weights less than 1 kg approximately twice as much blood is transfused as that removed for laboratory blood testing.\textsuperscript{101, 144} Thus, the effect of blood sampling is substantial and need to be considered in the management of anemia and in the assessment of erythopoiesis in preterm infants. Anemia of prematurity is also exacerbated by other factors such as rapid infant growth, shortened red blood cells (RBC) lifespan, and expansion of blood volume with growth.\textsuperscript{99, 100}

For many years, RBC transfusion was the only effective treatment for severe anemia of prematurity. In 1987, the first clinical trial of EPO demonstrated that EPO treatment was demonstrated to reduce the need for RBC transfusions in adults with end-
stage renal disease. This finding motivated the research toward using EPO to limit RBC transfusions in preterm infants. Subsequently, there has been a series of reports demonstrating variable results regarding the benefit of EPO in the treatment of anemia of prematurity. Several trials demonstrated that EPO treatment resulted in a significant reduction in RBC transfusions. However, others reported that the effect of EPO treatment was insufficient in reducing RBC transfusions to be of clinical significance in the treatment of anemia of prematurity. These differences may be due to inconsistent transfusion guidelines, treatment protocols and/or inter-patient variability in EPO responsiveness. Also, since none of the study designs considered the complex pharmacokinetics/pharmacodynamic (PK/PD) of EPO, this likely resulted in empirical EPO doses and dosing schedules that were sub-optimal.

Thus, the goal in this study was to describe erythropoiesis dynamics as a function of plasma EPO concentration using a mechanistic population PK/PD model. The mechanistic PK/PD model developed in this Chapter was formulated to account for important clinical variables affecting erythropoiesis. These included: transfusions, phlebotomies, shortened RBC lifespan and blood volume expansion in their growth. The development of such a model would improve the understanding of EPO’s pharmacodynamics in VLBW preterm infants and, through mechanism and model-driven clinical trials, could lead to improved EPO treatment outcome with a more definite conclusion about the true potential of EPO in treating neonatal anemia.

2.2 Specific aim and hypothesis

The specific aim of this chapter is to describe erythropoiesis dynamics in preterm infants using population PK/PD modeling approach.
The specific hypothesis is that EPO plasma level vs. time profile relates in a predictable manner to changes in hemoglobin vs. time profile and can be described by applying population PK/PD model.

2.3 Materials and Methods

2.3.1 Methods

This study was conducted at the Neonatal Intensive Care Unit (NICU) at the University of Iowa Children’s Hospital between February 2007 and November 2009. The study protocol and data collection were approved by the University of Iowa Human Subject Internal Review Board and all procedures were carried out in accordance with the Declaration of Helsinki. All subjects’ parents or legal guardians signed informed consent.

2.3.2 Subjects

The study enrolment was 27 preterm infants with gestational age <29 weeks and birth weight ≤1500 g. Additional inclusion requirements were treatment with expectation of survival and moderate to severe respiratory distress requiring ventilation. Exclusion criteria were hematological disease (except for anemia of prematurity) and receiving erythropoiesis stimulating agents.

2.3.3 Study Procedures

The details of study procedures and laboratory analysis were not altered from those previously described in the study of 14 infants who are included in current study. Concentrations of Hb and RBCs and plasma EPO concentrations were measured as previously described. All blood samples, i.e., for both research sampling and physician-ordered clinical testing, were included as phlebotomy loss and were
meticulously recorded. The amount of Hb transfused and the time of RBC transfusion were also recorded.

2.3.4 Hb mass balance model

The PD model of EPO is depicted in Figure 2.1. The amount of Hb present in the circulation represents a combination of the Hb produced endogenously \((Hb_{endo}(t))\) and that transfused \((Hb_{tran}(t))\).

\[
Hb(t) = Hb_{endo}(t) + Hb_{tran}(t)  \tag{2.1}
\]

The total number of RBCs present in the systemic circulation, \(RBC(t)\), was delivered by dividing \(Hb_{endo}(t)\) and \(Hb_{trans}(t)\) by their corresponding mean corpuscular Hb, that is \(MCH_{endo}\) and \(MCH_{trans}\), respectively, as follows:

\[
RBC_{total}(t) = \frac{Hb_{endo}(t)}{MCH_{endo}} + \frac{Hb_{trans}(t)}{MCH_{trans}}  \tag{2.2}
\]

The Hb transfused was assumed to have equal representation of RBCs of all ages up to the lifespan of transfused RBCs \(\tau_{tran}\), i.e., having a uniform age distribution. This assumption implies that blood transfused was taken from a donor whose Hb level was at steady state with a time invariant point distribution of RBCs lifespan.\textsuperscript{146} The change in the amount of transfused Hb for the \(i^{th}\) transfusion, resulting from an amount of \(Hb_{\tau}^{i}\) transfused at time \(t_{\tau}^{i}\) was described by a zero order process as displayed in Eq.2.3:

\[
dHb_{\tau}^{i}(t) / dt = \begin{cases} 
    - \frac{F_{\tau} \cdot Hb_{\tau}^{i}}{\tau_{\tau}} & \text{for } 0 < t - t_{\tau}^{i} < \tau_{\tau} \\
    0 & \text{otherwise}
\end{cases}  \tag{2.3}
\]
where $F_r$ denotes the fraction of transfused Hb surviving immediately beyond the transfusion (e.g., if a portion of the RBCs was damaged in storage and removed by the reticuloendothelial system shortly after transfusion). $F_r$ parameter was fixed to 0.875, a midpoint value between 100% recovery and the American Association of Blood Banks requirements that transfused RBCs exhibit 75% or greater recovery after storage.\(^{147}\)

The change in the amount of transfused Hb for multiple RBC transfusions was modeled as the integration of the rate of change in all transfusions:

$$dHb_{\text{tran}}(t)/dt = \sum_{i=1}^{m} dHb_{\text{tran}}^i(t)/dt$$

(2.4)

where $m$ is the number of transfusions.

The model used to describe Hb production assumed that Hb production was stimulated by EPO through a stimulation function $f_{\text{stim}}(t)$ (Eq. 2.5). To account for infant growth, stimulation function was scaled to the bodyweight to the power 3/4.\(^{148}\) Prior to birth ($t<0$), the stimulation function was assumed to be constant, $k_{\text{prod}}^{in\text{ utero}}$, scaled to the bodyweight. Stimulation function after birth ($t\geq0$) was related to plasma EPO concentrations by an $E_{\text{max}}$ model, which resulted in the following Hb production stimulation function:

$$f_{\text{stim}}(t) = \begin{cases} k_{\text{prod}}^{in\text{ utero}} \cdot (W(t))^{3/4} & \text{if } t \leq 0 \\ \frac{E_{\text{max}} \cdot C_{\text{EPO}}}{E_{C50} + C_{\text{EPO}}} \cdot (W(t))^{3/4} & \text{if } t > 0 \end{cases}$$

(2.5)

where $k_{\text{prod}}^{in\text{ utero}}$ is the Hb production rate constant before birth, $W(t)$ is the bodyweight, $E_{\text{max}}$ is the maximum Hb production rate which is scaled to the bodyweight by a $3/4$
power function, $EC_{50}$ is the EPO concentration that results in Hb production rate that is 50% of the scaled $E_{max}$, and $C_{EPO}$ is plasma EPO concentration.

The Hb production model assumes that in the absence of phlebotomies RBCs are removed only by senescence according to the invariant lifespan of the cells.\textsuperscript{51, 149, 150} Accordingly, the change in the amount of Hb produced endogenously is described by:

$$
\frac{dHb_{endo}(t)}{dt} = f_{stim}(t - a) - f_{stim}(t - b) \tag{2.6}
$$

with the initial condition $Hb_{endo}(0) = Hb_0 \cdot V_{tot}(0)$, where $a$ is the time between the erythroid progenitor cell stimulation by EPO and the first appearance of Hb in the circulation, and $b$ is the time between the erythroid progenitor cell stimulation by EPO and their removal from circulation by senescence. The RBCs lifespan is the difference $b-a$. The $Hb_0$ parameter represents baseline Hb concentration at birth and $V_{tot}(0)$ is the blood volume at birth.

The $k_{prod}^{in utero}$ parameter was estimated by solving Eq.2.7:

$$
Hb_0 \cdot V_{tot}(0) = \int_{0-a}^{0-b} k_{prod}^{in utero} \cdot W(t)^{3/4} \cdot dt \tag{2.7}
$$

where the only unknown is $k_{prod}^{in utero}$ and the values of other parameters are the empirical Bayesian estimates. According to mass balance principles, the change in the total amount of Hb in the circulation is the summation of the change in Hb transfused and the change in Hb produced endogenously:

$$
\frac{dHb(t)}{dt} = \frac{dHb_{endo}(t)}{dt} + \frac{dHb_{trans}(t)}{dt} \tag{2.8}
$$
2.3.5 *Correction for Hb phlebotomy loss*

Phlebotomy correction approach was proposed by Freise et al.\textsuperscript{145} The above derivation does not take into account Hb removed from circulation by phlebotomy. The RBCs may undergo a physical removal by the phlebotomy irrespective of their age. Those RBCs that escape removal by phlebotomy will eventually disappear as a result of the senescence process. Accounting for phlebotomy loss is very important when studying erythropoiesis in a neonatal population due to the extent and frequency of the\textsuperscript{101,144} The seemingly small amounts of blood removed for clinical testing has a large influence on the Hb mass balance due to the neonate’s small blood volume and therefore must be rigorously accounted for in the analysis.

The phlebotomy correction term, $PC(t_{\text{start}}, t_{\text{end}})$, involves a start time, $t_{\text{start}}$, and an end time, $t_{\text{end}}$. The time interval over which phlebotomy loss affects the Hb mass balance depends on the lifespan of RBCs, whether the RBCs were transfused or produced endogenously. Assuming $m$ phlebotomies that occurred between $t_{\text{start}}$ and $t_{\text{end}}$, the fraction of Hb that remains in circulation after the $i^{th}$ phlebotomy, denoted $F_p(i)$, that occurred at time $t_p(i)$ is given by Eq.2.9:

$$F_p(i) = 1 - \frac{A_p(t_p(i))}{Hb(t_p(i))} \quad (2.9)$$

where $A_p(t_p(i))$ is the amount phlebotomized at $t_p(i)$

$$PC(t_{\text{start}}, t_{\text{end}}) = \prod_{i=1}^{m} F_p(i) \quad (2.10)$$

where $t_p(1) \geq t_{\text{start}}$ and $t_p(m) < t_{\text{end}}$
\( F_p(i) \) is the fraction of Hb remaining immediately after the \( i^{th} \) phlebotomy relative to the amount present immediately before the \( i^{th} \) phlebotomy. The phlebotomy correction term over the time interval \( t_{start} \) to \( t_{end} \) (i.e., \( PC(t_{start},t_{end}) \)) is the multiplication of fractions remained for all phlebotomies that occurred between \( t_{start} \) and \( t_{end} \) (Eq.2.10). To correct for the volume of transfused Hb removed during phlebotomy, the correction term is the multiplication of the fraction remaining for all phlebotomies that occurred between the time of the previous transfusion \( t^i_{tran} \) and the current time, \( t \). Transfused RBCs that are already removed due to senescence are clearly not affected by phlebotomy. Similar to the amount of Hb transfused, phlebotomy correction for Hb produced is the multiplication of fraction of Hb remaining for all phlebotomies that currently have occurred that equal to the lifespan of produced RBCs. Since Hb produced is modeled as a separate component from Hb transfused, phlebotomy correction for Hb produced is not affected by transfusion. Accordingly, the correction for phlebotomies is given by the following equations:

\[
dHb_{\text{tran}}(t)/dt = \sum_{i=1}^{m} dHb^i_{\text{tran}}(t)/dt
\]  

(2.11)

where \( dHb^i_{\text{tran}}(t)/dt \) is given by:

\[
dHb^i_{\text{tran}}(t)/dt = \begin{cases} 
-PC(t^i_{\text{tran}},t) \cdot \frac{Hb^i_{\text{tran}}}{\tau_{\text{tran}}} & \text{for } 0 < t - t^i_{\text{tran}} < \tau_{\text{tran}} \\
0 & \text{otherwise}
\end{cases}
\]

(2.12)
where $PC(t_{\text{tran}}^i, t)$ is phlebotomy correction between the time of the $i^{th}$ transfusion ($t_{\text{tran}}^i$) and current time ($t$), and $PC(t - \tau_{\text{RBC}}, t)$ is phlebotomy correction between $t - \tau_{\text{RBC}}$ and $t$.

Finally, the estimated amounts of Hb in the circulation were converted into the observed concentrations by dividing by the estimated total blood volume ($V_{\text{total}}(t)$). In order to account for blood volume expansion as a result of growth, total blood volume was adjusted in a direct linear fashion with body weight:

$$V_{\text{total}}(t) = W(t) \cdot V$$

(2.13)

where $V$ is the bodyweight normalized blood volume.

### 2.3.6 Interindividual and Residual Error Model

A log-normal distribution was used to describe parameters distribution:

$$\ln(P_i) = \ln(P) + \eta_i$$

$$\eta_i \sim N(0, \omega^2)$$

(2.14)

where $P_i$ denotes the $i^{th}$ individual’s parameter value and is a function of $P$, the population value of the parameter in preterm infants, and $\eta_i$ is the individual random effect (a zero-mean random variable with the variance $\omega^2$), which accounts for the difference between the population parameter value and the individual value. Parameters were assumed to have a log-normal distribution because they are limited by physiologic constraint that they are supposed to have a positive value.

A proportional error model was used to describe the residual error of the data. The residual error was estimated for Hb and RBC, separately. Correlations between parameters were tested based on graphical inspection of correlation in the empirical Bayesian estimates.
2.3.7 Data Analysis and Model Evaluation

Model fitting and estimation of the population model parameters were performed using the stochastic approximation expectation maximization algorithm (SAEM) for nonlinear mixed-effects models. The SAEM algorithm is implemented in the MATLAB language in the software MONOLIX (version 3.1R2 for windows) with MATLAB version 7.8.0. In order to improve speed of the algorithm, a numerical solver for ordinary differential equation written in C was interfaced to MATLAB was employed. The interface was implemented in Sundial’s TB Matlab toolbox (Sundials version 2.4.0). Model selection was based on standard errors, Bayesian information criterion (BIC), and graphic assessment from MONOLIX output. Standard error estimates were obtained by linearization of the Fisher information matrix of the nonlinear mixed effects model. Models compared include: PD model with nonlinear transduction function (Eq.2.5) and linear transduction function (Eq.2.16), a model in which $k_{\text{prod}}^{\text{in utero}}$ was estimated directly from the structural model as presented by Freise et al, and the present model in which $k_{\text{prod}}^{\text{in utero}}$ was estimated as described by Eq.2.7.

The EPO plasma concentrations were nonparametrically represented using a linear spline function. The infant post-birth bodyweight derived from population-based in utero growth data was represented by a 4th order polynomial fit to the observed bodyweight data to interpolate between bodyweight observations and provide a smooth function of total blood volume. An empirical power function that describes the dynamics of in utero growth was used to calculate in utero body weight which is needed to calculate $f_{\text{stim}}$ before birth.
Finally, a standardized visual predictive check (SVPC) was performed to evaluate the predictive properties of the model described by Wang.\textsuperscript{154} Using the original dataset, one hundred data sets were simulated based on the final model (using final parameter estimates for typical parameter value, inter subject variability and residual variability). The percentile of each observation was plotted vs. time. The percentile ($P_j$) of $j^{th}$ observation for the $i^{th}$ subject is calculated by:

$$P_j = \frac{1}{101} \left( 1 + \sum_{k=1}^{100} \delta_{y,j,k} \right)$$

(2.15)

where $\delta_{y,j,k} = 1$ if $y_j > y_{j,k}'$, otherwise, $\delta_{y,j,k} = 0$, $y_j$ is the actual $j^{th}$ observation for the $i^{th}$ individual, and $y_{j,k}'$ is the $k^{th}$ simulated observation corresponding to $y_j$.

The objective of SVPC was to evaluate the predictability of the model. In other words, SVPC answers the question whether it is possible to reproduce the observed data using simulations. SVPC compare the distribution of the observations and the distribution of the simulated data.

### 2.4 Results

A rich data collection design was employed providing a database composed of 27 patients contributing a total of 2554 Hb concentrations, 568 RBC counts and 1510 EPO concentrations. The subject clinical and laboratory demographic characteristics are summarized in Table 2-1.

The Hb mass balance model fit along with plasma EPO concentration and bodyweight for three representative subjects are shown in Figure 2.2. The model adequately captures the general behavior of the Hb and RBC concentration data. The pharmacodynamic model parameters are summarized in Table 2-2. The lifespan of
transfused RBCs was fixed to 70.8 days, the midpoint of the estimated lifespans of 56.4 and 85.2 days of transfused adult RBCs in preterm infants.\textsuperscript{155, 156} The lifespan of produced RBC was also fixed to 42.5 days based on previous estimates using \textsuperscript{51}Cr.\textsuperscript{47} Additionally, the time between Hb production stimulation by EPO and appearance of produced Hb in the circulation was set equal to 0.448 days based on previous estimates.\textsuperscript{70}

Model evaluation plots for the final model are given in Figures 2.3-2.5. As displayed in Figure 2.3, the plots of observed versus predicted concentrations using population parameters (PRED) as well as predicted concentrations using individual parameters (IPRED) showed good agreement between predicted and observed data. The weighted residuals using population predicted concentrations (WRES) and weighted residuals using individual predicted concentrations (IWRES) weighted residuals versus age (Figure 2.4) were evenly scattered across 0, and there was no apparent trend with the possible exception of the few points at the beginning of the study. The standardized visual predictive check (Figure 2.5) plot demonstrates that observations percentiles are uniformly distributed between 0 and 1. Thus, all the model evaluation plots demonstrate the adequacy of the model and population parameter estimates.

2.5 Discussion

In this study, erythropoiesis dynamics in preterm infants was described using a mechanistic population PD model consistent with EPO’s physiologic role as the primary factor stimulating erythropoiesis. EPO was incorporated in the model to be the main predictor of Hb production rate. Hb production stimulation rate and estimated blood volume were scaled to body weight as it changed in our preterm infant study population. Allometric scaling in the present study was based on standard approaches described in
One of the main findings of this study is that the amount of Hb removed for clinical sampling is 48% of the total amount of Hb transfused. This finding is in agreement with other reports from the literature. In this context, the present PD model is highly relevant in that it encompasses phlebotomy loss as a key model component. Thus, the present PD model permits the exploration of therapeutic approaches in which future decreases in phlebotomy loss (e.g., as a result of advances in non-invasive and nano-technology laboratory testing) can be included in simulation studies focused on reducing the severity of anemia of prematurity.

Fourteen infants who are included in current study were included a previous study. The current study expands this number to 27 and in contrast to the previous study applies a mixed effect modeling approach. The nonlinear mixed effects modeling approach makes use of data across subjects while simultaneously including all relevant data in the analysis. An advantage of the nonlinear mixed effects modeling is that it improves the power to identify an appropriate structural model to improve parameter estimates. In the previous study, it was not possible to identify nonlinearity in the Hb production rate function (“PD transduction function”) in some subjects using an individual fitting approach. Accordingly, a linear Hb production rate function (Eq.2. 16) was previously applied in such infants based on the Akaike’s information criterion. As a result, the individual (non-population-based) approach to model the data may result in simplistic models that exclude important mechanistic components. This is because a single subject’s data, in contrast to all the data considered in a combined way in the
population analysis approach, may not contain sufficient information to identify important structure components of the true model. For example, the range of EPO levels observed in some of the subjects was in the linear range of the PD transduction function (the $E_{\text{max}}$ model) and thus this did not allow a nonlinearity in the PD to be properly determined. This problem is avoided in the present mixed effects modeling approach, thereby improving the derived parameter estimates. Based on Akaike’s information criterion, the nonlinear Hb production rate function was generally preferred over a linear approximation:

$$f_{\text{stim}}(t) = \begin{cases} 
 k_{\text{prod}}^{\text{in utero}} \cdot (W(t))^{3/4} & \text{if } t \leq 0 \\
 \alpha \cdot C_{\text{EPO}} \cdot (W(t))^{3/4} & \text{if } t \leq 0 
\end{cases}$$

(2.16)

The biology related parameters are expected to be similar to the values reported in the literature. Accordingly, previous blood volume estimates determined using $^{131}$-albumin over the first two weeks of life in premature infants was found to be 95 mL/kg compared to 93 mL/kg obtained in the present study. The $MCH_{\text{endo}}$ parameter (Table 2-2) estimated by this study were also comparable to previous literature values of 27-41 pg. As expected, the $MCH_{\text{trans}}$ estimates were lower than $MCH_{\text{endo}}$ because of the larger size of erythrocytes produced by preterm infants. The estimate of $MCH_{\text{trans}}$ (Table 2-2) was also within the reference range of 26-32 pg.

The degree of RBCs removal from the circulation immediately after birth due to RBCs damage as a result of storage lesions was estimated previously to be 2-3% per day of the Hb amount present in the circulation. This corresponds to a Hb destruction rate of 0.240-0.360 g/day at the time of birth using our population estimates of blood volume
and Hb baseline values and assuming a VLBW infant average birth weight of 0.891 kg. Scaling this birth weight to the power $\frac{3}{4}$ transforms the published values to 0.262-0.393 g/day/kg$^{3/4}$, which agrees with the value for $k_{\text{prod}}^{\text{in utero}}$ of 0.421 g/day/ kg$^{3/4}$ determined by this population model-based analysis.

Population model performance evaluation can be categorized based on the objective of the evaluation into two categories: evaluation of the model ability to describe the data and evaluation of the model predictive ability. Model ability to describe the data is usually evaluated using goodness of fit plots (e.g. Figure 2.3 and Figure 2.4) and uncertainty in population parameter estimates (e.g. RSE%). As a result of the growing interest in using population PK/PD modeling to select an appropriate dosing regimen, optimize clinical trial design and make informed go/no-go decisions, advanced model predictive ability evaluation approaches have been developed in the past several years. These approaches include data splitting, resampling techniques and monte carlo simulation.$^{161, 162}$

Different factors contributed to the selection of appropriate predictive ability evaluation approach. Data splitting techniques which require partitioning of the data into two portions; usually for cross validation purposes. One portion of the data is used for model development. The second portion is used to evaluate the model predictive performance. Data splitting techniques require a large dataset in terms of number of subjects. As a result, data splitting technique was not suitable for the present analysis. Resampling techniques (e.g. bootstrapping) require the generation of random samples from the observed data set. Subsequently, the modeling can be applied to each random sample. As a result it would be impractical to test the present model because of the long
run time of the model (each run ~2 days). As a result, monte carlo simulation based technique was selected to evaluate the predictive performance of the presented model.

One of the most commonly used simulation approaches is visual predictive check (VPC). VPC approach include: simulating a large number of datasets that are based on the original dataset, calculating a predictive interval based on the simulated datasets (e.g. 95% predictive interval), and plotting the observed data and the predictive interval on the same plot. Models with valid predictability are the models with variability of the observed data can be reproduced by the simulated data. The main disadvantage of VPC is that it cannot be applied in situation where each individual received an individualized dose (in our case an individualized EPO profile).

Instead of VPC, standardized visual predictive check (SVPC) was performed to evaluate the predictive properties of the model. SVPC provide similar information as a VPC but displays observations and predictions on a standardized scale (a percentile between zero to one). SVPC can be applied to cases with individualized doses.

Several technical difficulties were encountered in the process of implementing the current model in a population framework using the currently “state-of-the-art” specialized software MONOLIX. Firstly, it was challenging to interface user written software to permit implementation of the complex phlebotomy correction, and the use of linear splines and polynomials for nonparametric function representations. To resolve this challenge, the model was implemented in MONOLIX which allows a MATLAB scripting interface. Doing so, however, resulted in a very long run time (i.e., 7-8 days). This problem was overcome by using an ordinary differential equation solver written in C interfaced to MONOLIX via MATLAB. This decreased the run time by 75% (~2 days).
The second difficulty was that, because of the long run time (~2 days), it was impractical to do a bootstrap analysis to evaluate the stability of the final model and estimate the standard error of the parameters. To solve this problem, parameters standard errors were obtained by linearization of the Fisher information matrix of the nonlinear mixed effects model. Because each subject has a unique plasma EPO and weight profile over the first month of life,

In the proposed model the EPO concentration responses are represented as forcing functions in the PD transductions through the use of a fixed linear spline function for each subject. Accordingly, the model indirectly accounts for a possible negative feedback control of production of EPO by the circulating Hb mass, a mechanism that has been suggested previously.89, 164.

2.5.1 Clinical implications of the analysis results on treatment of anemia

Results of the present analysis indicates that preterm infants produced RBCs at a rate that was 33.5% (range: 23.2 – 51.8%) of the maximum production rate during the first month of life. This percent was calculated using the average EPO concentration over the first month of life (mean of 22.3 mU/mL with a range of 13.4 - 47.54 mU/mL) and the population parameter value of $EC_{50}$ of 44.3 mU/mL (Table 2-2). This indicates that preterm infants have the potential to produce additional RBC that could decrease the need for RBC transfusion.

Another finding of this study was that the amount of Hb removed for clinical sampling was only 48% of the total amount of Hb transfused. This finding is in agreement with other reports from the literature.144 In this context, the present PD model
is highly relevant in that it encompasses phlebotomy loss as a key model component. Thus, the present PD model permits the exploration of therapeutic approaches in which future decreases in phlebotomy loss (e.g., as a result of advances in non-invasive and nano-technology laboratory testing) can be included in simulation studies focused on preventative measures to reduce the severity of anemia of prematurity.

2.5.2 Uses of the model in translational medicine

The model presented here was developed for use in the design and analysis of future clinical studies. Firstly, the programming tools developed in the present project can be used as a modeling and simulation framework to investigate the erythropoietic response of various erythropoiesis stimulating agents such as rhEPO. This would increase the understanding of the effects of different types of drugs on erythropoiesis. Secondly, the present model can be used for simulating the Hb profile as a result of various study designs with different dosing strategies. This can facilitate selection of the most appropriate study design for answering the question whether rhEPO is effective in reducing and possibly eliminating the need for RBC transfusions. Thirdly, this model could be applied in the optimization of clinical trial designs. The design of clinical trials is typically both time consuming and requires intensive blood sampling. With the use of population techniques it would be possible to reduce the number of sampling points needed. This could result in less expensive clinical trials and will reduce need for painful blood sampling.

2.6 Conclusion

In summary, a population PK/PD model accounting for the dynamic Hb changes experienced by VLBW infants was simultaneously fitted to plasma EPO, Hb, and RBC
concentrations. The relationship between Hb production rate and EPO concentration was well described by an $E_{\text{max}}$ model. Analysis results indicated that preterm infants have the capacity to produce additional RBCs so that a decrease in RBC transfusions is possible. Additionally, the contribution of blood loss as a result of frequent sampling was found to be a significant contributor to the anemia of prematurity. The model may be applied to a clinical trial simulation to investigate if optimized, individualized EPO dosing applied to subsets of VLBW infants can result in the elimination of the need for RBC transfusions.
Table 2-1. Clinical and Laboratory Characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female</td>
<td>12/15</td>
</tr>
<tr>
<td>Gestational age (week)</td>
<td>26.7 ± 1.29&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Birth weight (kg)</td>
<td>0.89 ± 0.25&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Average EPO concentration over the first month of life (mU/mL)</td>
<td>22.3 ± 8.59&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>First Hb following birth, i.e., baseline Hb (g/dL)</td>
<td>15.6 ± 2.31&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Amount of Hb transfused over the first month of life (g)</td>
<td>12.3 ± 6.24&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Amount of Hb removed over the first month of life (g)</td>
<td>5.90 ± 2.18&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>- Mean ± standard deviation
Table 2-2. Final Parameter Estimates for the population erythropoiesis pharmacodynamic model presented in Eq.2.1-2.14

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Typical Value</th>
<th>RSE%</th>
<th>ω²</th>
<th>RSE%</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V$, mL/kg</td>
<td>Bodyweight normalized blood volume</td>
<td>93.2</td>
<td>6</td>
<td>0.311</td>
<td>15</td>
</tr>
<tr>
<td>$MCH_{endo}$, pg/cell</td>
<td>Mean corpuscular Hb of RBC produced endogenously</td>
<td>35.8</td>
<td>2</td>
<td>0.069</td>
<td>18</td>
</tr>
<tr>
<td>$MCH_{tran}$, pg/cell</td>
<td>Mean corpuscular Hb of RBC transfused</td>
<td>26.9</td>
<td>2</td>
<td>0.073</td>
<td>27</td>
</tr>
<tr>
<td>$Hb_0$, g/dL</td>
<td>Baseline hemoglobin concentration at birth</td>
<td>13.5</td>
<td>3</td>
<td>0.135</td>
<td>14</td>
</tr>
<tr>
<td>$E_{max}$, g/day-$kg^{\frac{3}{4}}$</td>
<td>Maximum Hb production rate which is scaled to the bodyweight by a $\frac{3}{4}$</td>
<td>0.527</td>
<td>21</td>
<td>0.862</td>
<td>15</td>
</tr>
<tr>
<td>$EC_{50}$, mU/mL</td>
<td>EPO concentration that results in Hb production rate that is 50% of the scaled $E_{max}$</td>
<td>44.3</td>
<td>15</td>
<td>NE</td>
<td>NE</td>
</tr>
</tbody>
</table>

Residual error, Hb | 0.093 | 1 | NE | NE |
Residual error, RBC | 0.094 | 3 | NE | NE |

Secondary parameter | Description | Typical value | SD |
<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{in utero}^{pro}$, g/day-$kg^{\frac{3}{4}}$</td>
<td>Hb production stimulation function prior to birth</td>
<td>0.421</td>
<td>0.14</td>
</tr>
</tbody>
</table>

a. NE indicates that the value for $\omega^2$ and its corresponding RSE% were not estimated
Figure 2.1. Diagram of the pharmacodynamic model for the effect of EPO on Hb production and disposition

- $E_{\text{max}}$: Maximum Hb production rate which is scaled to the body weight by a $\frac{3}{4}$ power function
- $EC_{50}$: EPO concentration that results in Hb production rate that is 50% of the scaled $E_{\text{max}}$
- $a$: Time between the erythroid progenitor cell stimulation by EPO and the appearance of RBC in the circulation

- $b$: Time between the erythroid progenitor cell stimulation by EPO and the removal of RBC in the circulation
- $f_{\text{stim}}$: Hb production stimulation function
- Blood transfusion
- Phlebotomy
Figure 2.2. Pharmacodynamic Hb mass balance model fit to three representative infant study subjects. Age represents postnatal age. The first two rows represent plots of observed, predicted concentrations using population parameters (PRED) and predicted concentrations using individual parameters (IPRED) of plasma concentrations of Hb (first row) and RBC (second row) versus time profiles. The last row represents the observed values of EPO concentration (left axis) and bodyweight (right axis).
Figure 2.3. Observed Hb and RBC concentrations (upper and lower rows respectively) versus predicted concentrations using population parameters (PRED) and predicted concentrations using individual parameters (IPRED) of Hb and RBC (left and right columns respectively)
Figure 2.4. Weighted residuals using population predictions (WRES) and weighted residuals using individual predictions (IWRES) versus age. Top panel represent WRES and bottom panel represents IWRES. Left column represents Hb and Right column represents RBC.
Figure 2.5. Standardized visual predictive check (SVPC) plot for erythropoiesis pharmacodynamic model
CHAPTER 3. GESTATIONAL AGE IS A PREDICTOR OF RESPONSE TO ERYTHROPOIETIN IN ANEMIC PREMATURE INFANTS

3.1 Introduction

Erythropoietin (EPO) is a 34 kD glycoprotein hormone that controls fetal, neonatal and adult erythropoiesis. EPO is synthesized by the kidney in adults and by the liver in preterm infants in response to hypoxia. This dependence on hepatic erythropoietin production in preterm infants is important because the liver is less sensitive than the kidney to tissue hypoxia as a stimulus for EPO production. Therefore, there is a poor EPO production response to the anemia frequently reported in preterm infants.

Anemia is a frequent complication in very low birth weight (VLBW) premature infants (birth weight < 1500 g) that is usually referred to as anemia of prematurity. There are two main causes of anemia of prematurity: insufficient EPO production by the liver and excessive blood loss resulting from frequent laboratory blood sampling. Anemia of prematurity is also exacerbated by other factors such as rapid infant growth, shortened red blood cells (RBC) lifespan, and expansion of blood volume.

Besides anemia of prematurity, several types of anemia are associated with a high variability in the response to recombinant human EPO (rhEPO) including: anemia due to chronic kidney disease and anemia associated with cancer treatment. The high variability in response to rhEPO contributes to the controversy in the efficacy of rhEPO in the management of anemia of prematurity. Several trials demonstrated that rhEPO treatment resulted in a significant reduction in RBC transfusions. Other studies reported that rhEPO treatment did not have a clinically significant effect in the treatment of anemia of prematurity.
The pharmacodynamics of erythropoiesis process was investigated in preterm infants. The pharmacodynamics (PD) parameters, namely blood volume and Hb production rate, were scaled to the body weight to account for growth in preterm infants. However, a wide inter-individual variability in the PD parameters was described in preterm infants.

The objective of the present analysis is to explain the variability in the response to rhEPO in preterm infants. This is accomplished by identifying factors influencing the responsiveness of preterm infant to rhEPO. Thereby, it would become more appealing to utilize the information obtained from the present analysis in the identification of separate group of rhEPO responders for whom optimized rhEPO treatment can eliminate the need for all RBC transfusions.

3.1.1 Summary of covariate screening approaches

Procedure used to identify important covariates that affect the value of individual estimated parameter and eventually incorporate these covariates in the population PK/PD model can be described as the combination of the following three steps:

1.) Fitting the population structural model without the incorporation of the covariate effects. This step is performed using specialized population PK/PD software such as NONMEM, MONOLIX, or S-ADAPT.

An example will be fitting a concentration data \( C_{ij} \) to the base model \( f(P_j, t_{ij}) \) according to the following equation:

\[
C_{ij} = f(P_j, t_{ij}) \cdot (1 + \varepsilon_{ij})
\]  

(3.1)
where $C_{ij}$ is the $i^{th}$ observed concentration for the $j^{th}$ individual, $t_{ij}$ is the $i^{th}$ time for the $j^{th}$ individual and $P_j$ is the set of PK/PD parameters for the $j^{th}$ individual, and $\varepsilon_{ij}$ is the residual error.

To describe the parameter inter-individual variability, the following equation is assumed (log-normal):

$$p_{kj} = p_k \cdot \exp(\eta_{kj})$$  \hspace{1cm} (3.2)

where $p_{kj}$ is the estimated parameter value for the $k^{th}$ parameter to the $j^{th}$ subject, $p_k$ is the population estimate for the $k^{th}$ parameter and $\eta_{kj}$ is the random effect that describe inter-individual variability for the $k^{th}$ parameter. $\eta_{kj}$ has a normal distribution with a mean of zero and a variance of $\omega_k^2$:

$$\eta_{ki} \sim N(0, \omega_k^2)$$  \hspace{1cm} (3.3)

The output from this stage include: a set of population parameter estimates, sets of individual parameters estimates, and the inter-individual variability measures. From the values of $\omega_k^2$, parameters with high inter-individual variability are identified. The estimated individual parameters for parameters with high inter-individual variability are used in the next step.

2.) In this step, the relationship between the individual parameter estimates and covariates is explored. This step does not use the population approach. It uses regression techniques for the individual parameter estimates vs. different covariates to identify candidate covariates to be tested later for significance in population PK/PD framework. Procedures used to identify potential covariates are categorized into four categories:

a) Graphics
Graphic can be a very powerful tool for identifying potential covariates if they are constructed appropriately. An example of such plots is the individual parameters estimates vs. each covariate plot. Using this type of plots, the importance of each covariate is determined based on its influence on reducing the variability of the individual parameter estimates.\textsuperscript{167, 168}

The main limitation for this approach is that it is too subjective and is likely to detect only the most obvious covariates. Covariates of marginal significance might not be detected nor might marginal covariates be consistently chosen across analyses. For these reasons, other alternatives have been used as described below.\textsuperscript{167}

b) Stepwise generalized additive modeling

This procedure was employed by Mandema et al.\textsuperscript{169} This procedure uses generalized additive models to describe parameter-covariate relationship. The relation between individual parameter estimates and covariates is described using:

$$p_{ki} = \alpha + f_1(x_{i1}) + f_2(x_{i2}) + \ldots + f_n(x_{in})$$

\textit{(3.4)}

where $\alpha$ is an intercept, the function $f_n()$ represents a spline smoothing function, and $x_{jk}$ is the value for the $j^{th}$ covariate for the $k^{th}$ individual.

This procedure is an iterative procedure. At the first iteration individual parameter estimates are fitted using each covariate separately. Covariates are then compared based on the value of Akaike information criterion (AIC). The covariate with the lowest AIC value will be kept as a potential covariate. At the second iteration the same procedure will be repeated by adding the rest of the covariates individually to the model containing previously identified covariate. Similarly, the covariate with the lowest AIC value will be
kept in the covariate model. This procedure will be repeated until no further decrease in
the AIC value can be obtained.\textsuperscript{169}

The main disadvantage for using generalized additive models is that it does not
consider any possible correlation between parameters or correlation between parameter
and covariate. It also does not account for time varying covariates.\textsuperscript{167}

c) Stepwise multiple linear regression

In this procedure multiple linear regression is used to identify potential covariates
according to the following equation:

\[ p_{ki} = \alpha + \beta_1 \cdot x_{1k} + \beta_2 \cdot x_{2k} + \ldots + \beta_n \cdot x_{nk} \] (3.5)

where \( \alpha \) is an intercept, \( \beta_1 - \beta_n \) are the regression coefficients, and \( x_{jk} \) is the value for
the \( j^{\text{th}} \) covariate for the \( k^{\text{th}} \) individual.

The difference between this approach and generalized additive modeling is that
using this approach linear parameter-covariates relation is constrained to linear
relationship.

Similar to generalized additive models, multiple linear regression approach does
not consider time varying covariates or parameters. It also does not account for
correlation between parameters or between parameters and covariates. Additionally,
using this approach might not detect curvilinear parameter-covariate relationship.

d) Neural networks

Neural networks are computational systems which are developed to simulate the
neurological processing abilities of biological systems and are known for their adaptive
learning and self-organizational capabilities. This technique has been applied to different
perform different tasks in PK/PD. Veng-Pedersen and Modi discussed artificial neural
networks with respect to PD modeling in which neural networks were employed for handling the input rate and effect relationship as measured from the EEG signals.\textsuperscript{170} Moreover, Brier et al have examined the use of neural networks for population pharmacokinetic data analysis and concluded that neural networks and NONMEM provided comparable predictions of plasma drug concentrations.\textsuperscript{171}

Artificial neural networks has been also applied to identify potential covariates in population PK/PD. Haider et al developed a predictive population PK/PD model for repaglinide, an oral hypoglycemic agent, using artificial neural networks. They identified several covariates that affect glucose change response to repaglinide using artificial neural networks.\textsuperscript{172}

The strength of neural networks is that they do not assume a specific model. Instead, they learn to establish the input and output relationships from the data provided to them. This greatly simplifies the modeling work involved untraditional population pharmacokinetic data analysis.\textsuperscript{173}

3.) The third step is fitting the data to a population mixed effects model describing the relationship between covariates and PK parameters. The covariate-parameter relationships found in the second step serve as an initial guess for the final population model. Covariates can be added in stepwise fashion similar to the stepwise addition in the second step. The covariates that will be a part of the final model are the ones that resulted in a significant reduction in the likelihood value.

3.1.1.1 \textbf{Formulating the covariate model with categorical covariates}

Categorical covariates are qualitative measures of certain characteristics of the individual. Categorical covariates can be binary such as the sex (either male or female) or
have multiple categories such as race. To formulate a formula that describes the relation between a categorical covariate (of n categories) and an estimated individual parameter, the following formula can be applied:

\[
p_{ki} = \begin{cases} 
  a_1 & \text{if } \text{covariate} = 1 \\
  a_2 & \text{if } \text{covariate} = 2 \\
  \vdots \\
  a_n & \text{if } \text{covariate} = n 
\end{cases}
\]

(3.6)

where \( \alpha \) is the parameter value corresponding to each covariate category.

3.2 Specific aim and hypothesis

The specific aim of this Chapter is to determine and explain the variability in the response to EPO in preterm infants.

The specific hypothesis is that the inter-subject variability in EPO’s PD is predictable by several covariates that are identifiable by population PK/PD modeling approach.

3.3 Methods

This study was conducted at the Neonatal Intensive Care Unit (NICU) at the University of Iowa Children’s Hospital between February 2007 and November 2009. The study protocol and data collection were approved by the University of Iowa Human Subject Internal Review Board and all procedures were carried out in accordance with the Declaration of Helsinki. All subjects’ parents or legal guardians signed informed consent.

3.3.1 Subjects

27 preterm infants with gestational age \( \leq 29 \) weeks and birth weight \( \leq 1500 \) gm were included in the study. Additional inclusion requirements were treatment with
expectation of survival and moderate to severe respiratory distress requiring ventilation. Exclusion criteria were hematological disease (except for anemia of prematurity), transfusion requirements that were emergent which did not allow controlled sampling, or receiving erythropoiesis stimulating agents.

3.3.2 Study Procedures

The details of study procedures and laboratory analysis were not altered from that discussed previously.\textsuperscript{145} Samples were collected from birth through 30 to 38 days of life. Concentrations of Hb and RBCs were measured using three different instruments namely Radiometer ABL 625 blood gas analyzer (Radiometer America, Inc, Westlake, OH), which measures Hb only; Advia 120 hematology system (Bayer, Tarrytown, NY), and Sysmex XE-2100 automatic hematology analyzer (Sysmex Corporation, Kobe, Japan). Plasma EPO concentrations were measured using a double antibody radioimmunoassay procedure as previously described (lower limit of quantification 1mU/mL).\textsuperscript{174} In this study, a blood sample was considered a phlebotomy hence the amount of Hb removed and the time of phlebotomy were collected. The amount of Hb transfused and the time of transfusion were also recorded.

3.3.3 Covariate data collection

Data were used as covariate are summarized in Table 3-1.

3.3.4 Basic Hb mass balance model

The basic Hb mass balance model used to describe Hb and RBC concentrations are described in Chapter, section 2.3.4.
3.3.5 **Correction for Hb phlebotomy loss**

The present model accounted for the Hb removed from circulation by phlebotomy. The details of the phlebotomy correction are discussed in Chapter 2.

3.3.6 **Interindividual and Residual Error Model**

A log-normal distribution was used to describe interindividual variability. To describe the residual error of the data, a proportional error model was used. The details of interindividual and residual error model are discussed in section 2.3.6.

3.3.7 **Covariate analysis**

The details of covariates screening process is described in section 3.1.1. To summarize, the base model without covariates was used to estimate individual parameters. Using stepwise multiple linear regression approach, potential covariates were identified and included as initial guess for the final model. Multiple linear regression was performed for the natural log transformation of the individual parameter estimates and covariates. Finally, potential covariates were included in a stepwise fashion in the final model. Only covariates that resulted in a significant reduction in the value of in minus two times the log-likelihood value (-2LL) were included in the final model. The -2LL is approximately $\chi^2$-distributed. A difference in -2LL $>3.84$ is significant at the 5% level (one degree of freedom) if nonlinearity and heteroscedasticity in the model is accounted for.

3.3.8 **Data Analysis and Model Evaluation**

Model fitting and estimation of the population model parameters were performed using the stochastic approximation expectation maximization algorithm (SAEM) for nonlinear mixed-effects models. The SAEM algorithm is implemented in the MATLAB
language in the software MONOLIX (version 3.1R2 for windows) with MATLAB version 7.8.0. In order to improve speed of the algorithm, a numerical solver for ordinary differential equation written in C was interfaced to MATLAB was employed. The interface was implemented in Sundial’s TB Matlab toolbox (Sundials version 2.4.0). Model selection was based on standard errors, Bayesian information criterion (BIC), and graphic assessment from MONOLIX output. Standard error estimates were obtained by linearization of the Fisher information matrix of the nonlinear mixed effects model. Models compared include: PD model with nonlinear transduction function (Eq.2.5) and linear transduction function (Eq.2.16), a model in which Hb production stimulation function prior to birth \( k_{\text{prod}}^{\text{in utero}} \) was estimated directly from the structural model as presented by Freise et al, and the present model in which \( k_{\text{prod}}^{\text{in utero}} \) was estimated as described by Eq.2.7.

The EPO plasma concentrations were nonparametrically represented using a linear spline function. The infant bodyweight post-birth was represented by a 4th order polynomial fit to the observed bodyweight data to interpolate between bodyweight observations and provide a smooth function of total blood volume. To calculate in utero body weight which is needed to calculate Hb production stimulation function \( f_{\text{stim}} \) before birth, an empirical power function that describes the dynamics of in utero growth was used.

Finally, a standardized visual predictive check (SVPC) was performed to evaluate the predictive properties of the model described by Wang. Using the original dataset, one hundred data sets were simulated based on the final model (using final parameter estimates for typical parameter value, inter subject variability and residual variability).
The percentile of each observation was plotted vs. time. The percentile \( P_{ij} \) of \( j^{\text{th}} \) observation for the \( i^{\text{th}} \) subject is calculated by:

\[
P_{ij} = \frac{1}{101} \left( 1 + \sum_{k=1}^{100} \delta_{y_{ij,k}} \right)
\]

(3.8)

where \( \delta_{y_{ij,k}} = 1 \) if \( y_{ij} > y'_{ij,k} \), otherwise, \( \delta_{y_{ij,k}} = 0 \). \( y_{ij} \) is the actual \( j^{\text{th}} \) observation for the \( i^{\text{th}} \) individual, and \( y'_{ij,k} \) is the \( k^{\text{th}} \) simulated observation corresponding to \( y_{ij} \).

The objective of SVPC is to test evaluate the predictability of the model. In other words, SVPC answers the question whether it is possible to reproduce the variability observed in the data using simulations. SVPC compare the distribution of the observations and the distribution of the simulated data.

### 3.4 Results

The Hb mass balance model fit along with plasma EPO concentration and bodyweight for three representative subjects are shown in Figure 3.1. The model captures the general behavior of the Hb and RBC concentration data. The pharmacodynamic model parameters are summarized in Table 3-2.

The lifespan of transfused RBCs was set equal to 70.8 days, the midpoint of the estimated lifespans of 56.4 and 85.2 days of transfused adult RBCs in preterm infants.\(^{155,156}\) The lifespan of produced RBC was also fixed to 42.5 days based on previous estimates using \(^{51}\)Cr.\(^{47}\) Additionally, the time between Hb production stimulation by EPO and appearance of produced Hb in the circulation was set equal to 0.448 days based on previous estimates.\(^{70}\)

During the process of covariate screening, stepwise multiple linear regression identified four covariates as potential covariates that contribute to the value of maximum
Hb production rate which is scaled to the bodyweight by a $\frac{3}{4}$ power function ($E_{\text{max}}$). These covariates were: (1) gestational age, (2) Z score of the birth weight, (3) heart rate average over the first two days of life, and (4) nucleated RBC count average over the first two days of life. Multiple linear regression equation that describes the relation between these covariates and the individual estimates of $E_{\text{max}}$, estimated using the base model without covariates, is:

$$\ln(E_{\text{max}}) = \alpha + \beta_1 \ln(GA) + \beta_2 \cdot (Z\text{score}) + \beta_3 \cdot \ln(HR) + \beta_4 \cdot \ln(NRBC)$$

where $\alpha$ is the intercept, $\beta_1, \beta_2, \beta_3, \beta_4$ are the regression coefficients for the gestational age ($GA$), Z score of the birth weight ($Z\text{score}$), heart rate average over the first two days of life ($HR$), and nucleated RBC count average over the first two days of life ($NRBC$) respectively. The values of $GA$, $Z\text{score}$, $HR$, and $NRBC$ were normalized by their corresponding average value. The results of stepwise multiple linear regression are displayed in Table 3-3. Plots for each potential covariate vs. $E_{\text{max}}$ are displayed in Figures 3.2-3.5.

Covariate screening process identified gestational age (GA) to be an important covariate the affect the value of $E_{\text{max}}$. The relation between GA and $E_{\text{max}}$ is described by Eq.3.10. The values of gestational age were normalized by geometric mean, namely $GA_{\text{mean}}$, for infants enrolled in the present study. $E_{\text{max}}$ is the population parameter infants with a gestational age equal to GA, $E_{\text{max}}^a$ is the population parameter value for infants with a gestational age of $GA_{\text{mean}}$. The value of $\beta$ of 10.2 with a standard error of 3.3 indicates a positive significant relationship between $E_{\text{max}}$ and GA ($p<0.01$).
\[ E_{\text{max}} = E_{\text{max}}^0 \cdot \left( \frac{GA}{GA_{\text{mean}}} \right)^\beta \] (3.10)

To test the influence of gestational age on the clinical outcomes of anemia of prematurity, the value of gestational age was plotted against the amount of Hb transfused over the first month of life. Negative correlation between gestational age and amount of Hb transfused can be noticed from Figure 3.2. A mechanistic explanation for this observation is presented in the discussion section.

The goodness of fit plots from the final model are given in Figures 3.7-3.8. As displayed in Figure 3.7, the plots of observed versus predicted concentrations using population parameters (PRED) as well as predicted concentrations using individual parameters (IPRED) showed a good visual agreement between predicted and observed data. The weighted residuals using population predicted concentrations (WRES) and weighted residuals using individual predicted concentrations (IWRES) versus age (Figure 3.8) were evenly scattered across 0, and there was no apparent trend with the possible exception of the few points at the beginning of the study. The SVPC (Figure 3.9) plot demonstrates that observations percentiles were uniformly distributed between 0 and 1. The goodness of fit plots demonstrate the adequacy of the model and the population parameter estimates.

3.5 Discussion

In this study, a part of the variability in the response to rhEPO in preterm infants is explained. The statistical covariate analysis suggested that the erythropoietic efficacy of EPO is increased for preterm infants with larger gestational age.
Stepwise addition detected gestational age, Z score of the birth weight, and average values of nucleated RBC and heart rate over the first two days as potential covariates. However, upon including each potential covariate separately in the population PK/PD model, gestational age resulted in the highest decline in the -2LL (a difference of 19.8 which is equivalent to a p-value of $8.60 \times 10^{-6}$). The addition of the rest of the potential covariates to the base model with gestational age as a covariate did not result in a significant drop on the value of -2LL ($P > 0.05$).

Two observations that demonstrate the importance of considering the gestational age on the treatment of anemia of prematurity were reported in this study. First, the erythropoietic efficacy of EPO is proportional to the gestational age. Second, the amount of Hb transfused over the first 30 days of life is inversely related to the gestational age. Those observations can be attributed to the fact that in this patient population RBCs are produced from two organs, namely the liver and bone marrow. The liver serves the dominant site of red blood cells production from the 9th to the 24th weeks of gestation. Bone marrow erythropoiesis begins around the thirteen week of gestation to become the predominant erythropoietic organ after the 24th week of gestation and remains so throughout the remainder of fetal life.\textsuperscript{16} Taken together, bone marrow may have more potential than the liver for producing RBCs. Compared to preterm infants with small gestational age, infants with large gestational age has a larger fraction of RBCs produced by the bone marrow which may result in more total potential for producing RBCs.

In addition to gestational age other markers are important predictors of response to rhEPO. Since this analysis was done retrospectively, it was not possible to examine all plausible predictors of rhEPO responsiveness. There are many conditions reported as
associated with rhEPO resistance, namely, inflammation, iron deficiency and oxidative stress.\textsuperscript{176-180}

Increased plasma level of inflammatory cytokines such as C-reactive protein (CRP), IL-6, IL-2, IL-1, interferon-\(\gamma\) (IFN-\(\gamma\)), and tumor necrosis factor (TNF) had been described as inflammatory markers for rhEPO resistance in hemodialysis patients.\textsuperscript{181-184} In vitro studies demonstrated that these markers induce erythroid precursor cells apoptosis. This has been proposed as a possible mechanism for rhEPO resistance.\textsuperscript{185} Additionally, TNF, IL-6 and IL-1 can cause rhEPO resistance by promoting iron storage inside macrophages and decreasing plasma level of iron.\textsuperscript{185} Inflammatory cytokines, mainly IL-6, affect iron disposition by stimulating hepcidin production by the liver.

Elevated hepcidin, a type II acute-phase protein produced in the liver,\textsuperscript{186} has been recently proposed as a predictor for rhEPO responsiveness in stable chronic heart failure anemic patients.\textsuperscript{187} Hepcidin is a regulator of iron metabolism.\textsuperscript{186} Hepcidin inhibits iron absorption in the small intestine and sequestering of iron in macrophages. As a result, iron concentration in the circulation will decrease.\textsuperscript{185} Additionally, hepcidin contributes to rhEPO resistance by inhibiting erythroid-progenitor proliferation and survival.\textsuperscript{188}

Iron deficiency markers include mean cell hemoglobin, red cell distribution width, and s-TfR serum levels.\textsuperscript{189} Similar to inflammatory markers, differences in the level iron deficiency markers between rhEPO responders and non-responders has been observed in hemodialysis patients. The investigation of these markers may help identifying additional factors contribute to the erythropoiesis process in preterm infants.

Malondialdehyde and 8-Hydroxy-2-deoxyguanosine are oxidative stress markers that might be used as rhEPO responsiveness markers. Elevated RBC Malondialdehyde
membrane content was reported in hemodialysis patients with rhEPO resistance.\textsuperscript{190} Similarly, 8-hydroxy-2-deoxyguanosine serum levels were positively correlated with rhEPO dose in hemodialysis patients.

3.5.1 Clinical implications of the analysis results on treatment of anemia

The difference in the gestational age (24–29 weeks) showed an approximately seven-fold difference in the value of $E_{\text{max}}$. This conclusion was based on transforming the range of gestational age to a range of $E_{\text{max}}$ based on the mathematical relationship described in Eq.3.12. Using the estimated value of $E_{\text{max}}^0$ of $0.43$ g/day/kg$^{3/4}$, based on the results from Eq.3.12, the population value $E_{\text{max}}$ for preterm infants with a gestational age of 24 weeks was $0.147$ g/day/kg$^{3/4}$. Similarly, the population value $E_{\text{max}}$ for preterm infants with a gestational age of 29 weeks was $1.01$ g/day/kg$^{3/4}$. This result indicates that gestational age needs to be considered when determining the dose of recombinant human EPO (rhEPO) to be given.

3.5.2 Uses of the model in translational medicine

The model presented here was developed for use in the design and analysis of future clinical studies. Firstly, the programming tools developed in the present project can be used as a modeling and simulation framework to investigate the erythropoietic response of various erythropoiesis stimulating agents such as rhEPO. Secondly, the present model can be used for simulating the Hb profile as a result of various study designs with different dosing strategies. This can facilitate selection of the most appropriate study design for answering the question whether rhEPO is effective in reducing and possibly eliminating the need for RBC transfusions. The design of clinical
trials is typically both time consuming and requires intensive blood sampling. With the use of population techniques it would be possible to reduce the number of sampling points needed which might result in the reduction in the total volume of collected blood samples. This could result in less expensive clinical trials and will reduce need for painful blood sampling. Finally, this modeling framework could be used to explore additional covariates, such as CRP, IL-6, soluble IL-2 and s-TfR, that influence the responsiveness to rhEPO.

3.5.3 Limitations

Perhaps the main limitation of the present study is the lack of important covariates that might affect rhEPO responsiveness in preterm infants. An appropriate extension to the present analysis is to include more covariates including: CRP, IL-6, IL-2, IL-1, IFN-γ, TNF, s-TfR, hepcidin, Malondialdehyde and 8-Hydroxy-2-deoxyguanosine. The rationale for recommending these covariates was discussed in section 3.5.

The present study dataset (for a total of 27 subjects) is considered a small dataset for the purpose of covariate screening. Larger datasets have more power to identify potential covariates compared to small datasets. Additionally, smaller datasets tends to falsely identify low-powered covariates to be statistically significant covariates.

3.6 Conclusion

A population PK/PD model accounting for the dynamic Hb changes experienced by these infants was simultaneously fitted to plasma EPO, Hb, and RBC concentrations. The relationship between Hb production rate and EPO concentration was well described by an $E_{max}$ model. Furthermore, covariate analysis suggested that the erythropoietic efficacy of EPO is increased for preterm infants at later gestational ages. This resulted in
an approximately seven-fold difference in the maximum Hb production rate. Additionally, the analysis indicated that preterm infants have the capacity to produce additional RBCs so that a decrease in RBC transfusions is possible. The present model has utility in clinical trial simulations investigating the benefit of rhEPO in the treatment of anemia of prematurity.
Table 3-1. Summary of covariates that were tested for their impact on the pharmacodynamics parameter of interest

<table>
<thead>
<tr>
<th>Neonatal information</th>
<th>Neonatal information (continued)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPAP&lt;sup&gt;a&lt;/sup&gt;</td>
<td>First temperature taken ≤60 minutes</td>
</tr>
<tr>
<td>Intubation</td>
<td>Auxiliary temperature&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Chest compressions</td>
<td>Respiratory rate&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Race (Hispanic vs. nonhispanic)</td>
<td>Blood pressure&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ethnic (Black vs. white)</td>
<td>Heart rate&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Steroids use</td>
<td>Complete blood count results&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Birth weight</td>
<td></td>
</tr>
<tr>
<td>Zscore of the birth weight</td>
<td></td>
</tr>
<tr>
<td>Apgar score (1 min)</td>
<td></td>
</tr>
<tr>
<td>Apgar score (5 min)</td>
<td></td>
</tr>
<tr>
<td>Gestational age</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin level at birth</td>
<td></td>
</tr>
<tr>
<td>Length at birth</td>
<td></td>
</tr>
<tr>
<td>Head size at birth</td>
<td></td>
</tr>
<tr>
<td>Cord pH</td>
<td></td>
</tr>
<tr>
<td>Maternal information</td>
<td></td>
</tr>
<tr>
<td>Mother age</td>
<td></td>
</tr>
<tr>
<td>Insulin dependent diabetes</td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td></td>
</tr>
<tr>
<td>Chorioamnionitis</td>
<td></td>
</tr>
<tr>
<td>Rupture of Membranes</td>
<td></td>
</tr>
<tr>
<td>Steroids prior delivery</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> CPAP: Continuous positive airway pressure

<sup>b</sup> The value used is the average for all measurements over the first two days of life
Table 3-2. Final parameter estimates of the erythropoiesis pharmacodynamics model in preterm infants

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Parameter description</th>
<th>Population parameter</th>
<th>RSE %</th>
<th>$\omega^2$</th>
<th>RSE</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V$, mL/kg</td>
<td>Bodyweight normalized blood volume</td>
<td>91.0</td>
<td>6</td>
<td>0.30</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>$MCH_{endo}$, pg/cell</td>
<td>Mean corpuscular Hb of RBC produced endogenously</td>
<td>35.7</td>
<td>2</td>
<td>0.07</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>$MCH_{tran}$, pg/cell</td>
<td>Mean corpuscular Hb of RBC transfused</td>
<td>27.0</td>
<td>2</td>
<td>0.08</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>$Hb_0$, g/dL</td>
<td>Baseline hemoglobin concentration at birth</td>
<td>13.6</td>
<td>3</td>
<td>0.14</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>$E_{max}^0$, g/day-kg$^{3/4}$</td>
<td>Maximum Hb production rate which is scaled to the bodyweight by a $3/4$</td>
<td>0.43</td>
<td>21</td>
<td>0.73</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>$\beta$</td>
<td>A parameter that relates $E_{max}^0$ to gestational age</td>
<td>10.2</td>
<td>32</td>
<td>NE$^a$</td>
<td>NE$^a$</td>
<td>0.0018</td>
</tr>
<tr>
<td>$EC_{50}$, mU/mL</td>
<td>EPO concentration that results in Hb production rate that is 50% of the scaled $E_{max}$</td>
<td>35.9</td>
<td>24</td>
<td>NE$^a$</td>
<td>NE$^a$</td>
<td></td>
</tr>
<tr>
<td>Residual error, Hb</td>
<td></td>
<td>0.09</td>
<td>1</td>
<td>NE$^a$</td>
<td>NE$^a$</td>
<td></td>
</tr>
<tr>
<td>Residual error, RBC</td>
<td></td>
<td>0.09</td>
<td>3</td>
<td>NE$^a$</td>
<td>NE$^a$</td>
<td></td>
</tr>
<tr>
<td><strong>Secondary parameters</strong></td>
<td>Parameter description</td>
<td><strong>Mean value</strong></td>
<td><strong>SD</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{in utero}$, g/day-kg$^{3/4}$</td>
<td>Hb production stimulation function prior to birth</td>
<td>0.40</td>
<td>0.12</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\) NE indicates that the value for $\omega^2$ and its corresponding RSE% were not estimated
Table 3-3. Stepwise multiple linear regression results summary

<table>
<thead>
<tr>
<th>Estimated value$^{a,b,c}$</th>
<th>Estimated value</th>
<th>Standard error</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$</td>
<td>-0.625</td>
<td>0.098</td>
<td>$2.13 \times 10^{-6}$</td>
</tr>
<tr>
<td>$\beta_1$</td>
<td>9.90</td>
<td>2.07</td>
<td>$8.91 \times 10^{-5}$</td>
</tr>
<tr>
<td>$\beta_2$</td>
<td>-0.426</td>
<td>0.098</td>
<td>$2.75 \times 10^{-4}$</td>
</tr>
<tr>
<td>$\beta_3$</td>
<td>8.67</td>
<td>2.45</td>
<td>0.002</td>
</tr>
<tr>
<td>$\beta_4$</td>
<td>-0.510</td>
<td>0.184</td>
<td>0.011</td>
</tr>
</tbody>
</table>

$^a$ - $\alpha$ is the intercept, $\beta_1$, $\beta_2$, $\beta_3$, and $\beta_4$ are the regression coefficients for the gestational age ($GA$), Z score of the birth weight ($Zscore$), heart rate average over the first two days of life ($HR$), and nucleated RBC count average over the first two days of life ($NRBC$) respectively.

$^b$ - The values of the $E_{max}$ and covariates were log-transformed.

$^c$ - Covariates were normalized by their corresponding average value.
Figure 3.1 Pharmacodynamic Hb mass balance model fit to three representative infant study subjects. The first two rows represent plots of observed, population predicted (PRED), and individual predicted (IPRED) values of plasma concentrations of Hb (first row) and RBC (second row) versus time profiles. The last row represents the observed values of EPO concentration (left axis) and bodyweight (right axis).
Figure 3.2. Maximum Hb production rate which is scaled to the bodyweight by a $\frac{3}{4}$ power function ($E_{\text{max}}$) estimated individual parameters vs. gestational age. The values of $E_{\text{max}}$ are the estimated individual parameters for the base model without covariates.
Figure 3.3. Maximum Hb production rate which is scaled to the bodyweight by a \( \frac{3}{4} \) power function \( (E_{\text{max}}) \) estimated individual parameters vs. Z score for the birth weight. The values of \( E_{\text{max}} \) are the estimated individual parameters for the base model without covariates.
Figure 3.4. Maximum Hb production rate which is scaled to the bodyweight by a $\frac{3}{4}$ power function ($E_{max}$) estimated individual parameters vs. heart rate average over the first two days of life. The values of $E_{max}$ are the estimated individual parameters for the base model without covariates.
Figure 3.5. Maximum Hb production rate which is scaled to the bodyweight by a $\frac{3}{4}$ power function ($E_{\text{max}}$) estimated individual parameters vs. Nucleated RBC count average over the first two days of life. The values of $E_{\text{max}}$ are the estimated individual parameters for the base model without covariates.
Figure 3.6. Amount of hemoglobin transfused over the first month of life versus gestational age
Figure 3.7. Observed Hb and RBC concentrations (upper and lower rows respectively) versus predicted concentrations using population parameters and predicted concentrations using individual parameters (left and right columns respectively)
Figure 3.8. Weighted residuals using population predicted concentrations (WRES) and weighted residuals using individual predicted concentrations (IWRES) versus age. Top panel represent WRES and bottom panel represents IWRES. Left column represents Hb and Right column represents RBC.
Figure 3.9. Standardized visual predictive check (SVPC) Plot for erythropoiesis pharmacodynamic model
CHAPTER 4. THE ASSESSMENT OF NEWBORN LAMB AS AN
ANIMAL MODEL FOR ERYTHROPOIESIS IN PRETERM INFANTS

4.1 Introduction

Anemia is a frequent complication in very low birth weight premature infants (birth weight < 1500 g) that is usually referred to as anemia of prematurity. One of the major causes of anemia of prematurity is blood loss resulting from frequent laboratory sampling. Another key cause of anemia of prematurity is insufficient erythropoietin (EPO) production by the liver. Anemia of prematurity is also exacerbated by endogenous factors such as rapid infant growth, shortened red blood cells (RBC) lifespan, and expansion of blood volume.

For many years, RBC transfusion was the only effective treatment for severe anemia of prematurity. Despite the benefits of transfusions in the treatment of anemia of prematurity, transfusions are associated with the risk of disease transmission. Other risks include fluid overload, hemolytic transfusion reactions, immune-mediated transfusion reactions, extravasation injury, and retinopathy of prematurity.

An important controversy that is still unresolved is the use of EPO in the treatment of anemia of prematurity. Several trials demonstrated that EPO treatment resulted in a significant reduction in RBC transfusions. Other studies reported that EPO treatment does not have a clinically significant effect in the treatment of anemia of prematurity. These differences are likely due to the variable transfusions practices, inter-patient variability, and inconsistencies in the treatment protocol.

Sheep have often been used as an experimental model because they have similar reticulocytes and RBCs lifespan to that of humans. Similar to human, the mechanism of
RBC removal from the sheep circulation is primarily due to cellular senescence. Additionally, the sites of erythropoiesis, and the types of Hb produced at different developmental stages, most closely resemble the situation in humans. These similarities justify performing experiments in sheep that cannot be done in human. These experiments are intended to describe erythropoiesis under normal as well as severely hypoxic conditions. Thus, the sheep model can be used to explore the erythropoietic process when the Hb level is as low as 40% of the baseline value (4-6.8 g/dL). Moreover, the erythropoietic effect of EPO can be elucidated over a wider range of EPO concentrations compared to the range observed in preterm infants. Based on the similarity in erythropoiesis process between human and sheep, results of such experiments may be extrapolated to preterm infants.

The objective of the present study is to compare the erythropoietic characteristics of newborn lambs to preterm infants. This is achieved by describing the kinetics of erythropoiesis in newborn lambs as a function of the plasma EPO concentration using a population pharmacokinetics/pharmacodynamics (PK/PD) model and comparing the parameter estimates to erythropoietic parameters estimated in preterm infants (Chapter 2). The information obtained from the population kinetic analysis is useful in the design of clinical trials with optimum EPO treatment regimen to improve the efficacy and utility of EPO in the treatment of anemia of prematurity.

4.2 Specific aim and hypothesis

The specific aim of this Chapter is to evaluate newborn sheep as an experimental model for erythropoiesis in preterm infants.
The specific hypothesis is that the newborn sheep can be used as an animal model for erythropoiesis in preterm infants

4.3 Materials and Methods

4.3.1 Animals and study outline

All animal experimental procedures were approved by the University of Iowa Institutional Animal Care and Use Committee prior to the study. Thirty-six 1-2 days old lambs were studied. The lambs were housed in an indoor, light- and temperature-controlled environment with the ewes. Jugular venous catheters were inserted under pentobarbital anesthesia. Ampicilin (1g/day) was administered for the first 3 days following the catheter insertion. Iron supplements were given to the mother during pregnancy. Lambs also received iron supplements during the study period.

As illustrated in Figure 4.1, the study was composed of three phases: a tracking phase (First two weeks of life), a stabilization phase and a stress erythropoiesis phases. In the tracking phase, each three newborn lambs were assigned to have a Hb level that matches the Hb level of a preterm infant with anemia of prematurity from a previous study presented in Chapters 2 and 3. In other words, the 36 lambs were assigned to follow the anemic condition of 12 preterm infants with anemia of prematurity. This was done by repeatedly removing and/or transfusing blood from/to the lamb. The RBC transfusions given to the lambs were obtained from the lamb’s mother.

In the stabilization phase the Hb level was brought back to the non-anemic basal level by transfusion to allow stabilization of normal Hb levels for a period of approximately 5-7 days.
The stress erythropoiesis phase started at day 21 after birth by performing a major phlebotomy in which 60% of the lamb’s total body red blood cells were removed. This was accomplished by an exchange transfusion in which the blood removed was centrifuged and the plasma was transfused back. The volume of plasma transfused back was brought to the volume of blood removed by the addition of saline. This procedure decreased the lamb’s Hb level to 4.0-6.8 g/dL. The Hb mass removed with each phlebotomy was calculated by multiplying the volume of blood removed by the Hb concentration measured in the blood sample drawn closest to the time of blood sampling.

Whole blood samples were analyzed with XT 2000, an automated hematology system (Sysmex America Inc. Mundelien, IL). Plasma EPO concentrations were measured using a double antibody radioimmunoassay (RIA) procedure as previously described with a lower limit of quantification of 1mU/mL.193

4.3.2 Hb mass balance model

The basic Hb mass balance model used to describe Hb concentration is described in Chapter, section 2.3.4.

4.3.3 Correction for Hb phlebotomy loss

The present model accounted for the Hb removed from circulation by phlebotomy. The details of the phlebotomy correction are discussed in Chapter 2.

4.3.4 Interindividual and Residual Error Model

A log-normal distribution was used to describe interindividual variability. To describe the residual error of the data, a proportional error model was used. The details of interindividual and residual error model are discussed in section 2.3.6.
4.3.5 Data Analysis and Model Evaluation

Model fitting and estimation of the population model parameters were performed using the stochastic approximation expectation maximization algorithm (SAEM) for nonlinear mixed-effects models. The SAEM algorithm is implemented in the MATLAB language in the software MONOLIX (version 3.1R2 for windows) with MATLAB version 7.8.0. In order to improve speed of the algorithm, Matlab interface solver for ordinary differential equation systems written in C was employed. This interface was implemented in CVODE function from sundialsTB Matlab toolbox (Sundials version 2.4.0).

The EPO plasma concentrations were nonparametrically represented using a linear spline function. The infant bodyweight post-birth was represented by a 4th order polynomial fit to the observed bodyweight data to interpolate between bodyweight observations in order to provide a smooth function of total body blood volume. To account for pre-birth body weight which is needed to calculate \( f_{\text{prod}} \) for \( t < 0 \), an exponential function that describes the dynamics of fetal growth as displayed in Eq. 4.2 was used.\(^{194}\)

\[
W(t) = W(0) \cdot e^{(0.1281 - 0.00038 \cdot (G_{\text{AGE}} + t)) \cdot (G_{\text{AGE}} + t)} \quad \text{for } t < 0
\]

where \( G_{\text{AGE}} \) is the gestational age of the term lamb which was assumed to be 150 days.\(^{195, 196}\)

Finally, a standardized visual predictive check (SVPC) was performed to evaluate the predictive properties of the model described by Wang.\(^{154}\) Using the original dataset, one hundred data sets were simulated based on the final model (using final parameter estimates for typical parameter value, inter subject variability and residual variability).
The percentile of each observation was plotted vs. time. The percentile \((P_{ij})\) of \(j^{th}\) observation for the \(i^{th}\) subject is calculated by:

\[
P_{ij} = \frac{1}{101} \left( 1 + \sum_{k=1}^{100} \delta_{ij,k} \right)
\]

where \(\delta_{ij,k} = 1\) if \(y_{ij} > y'_{ij,k}\), otherwise, \(\delta_{ij,k} = 0\), \(y_{ij}\) is the actual \(j^{th}\) observation for the \(i^{th}\) individual, and \(y'_{ij,k}\) is the \(k^{th}\) simulated observation corresponding to \(y_{ij}\).

The objective of SVPC is to test evaluate the predictability of the model. In other words, SVPC answers the question whether it is possible to reproduce the variability observed in the data using simulations. SVPC compare the distribution of the observations and the distribution of the simulated data.

### 4.4 Results

A rich data collection design was employed with a population providing a database composed of 36 lambs. Individual lamb’s profile contained on average 89 hemoglobin values (range 61 to 111), 91 plasma EPO concentration (range 67 to 113) and 16 bodyweight (range 12 to 27) time points. The bodyweight measurements were well represented by a 4th order polynomial. At the beginning of the study, the baseline hemoglobin was 10.8 ± 1.50 g/dL. At day 21, the hemoglobin level was decreased from 9.87 ± 1.20 g/dL to 4.94 ± 0.64 g/dL as a result of major phlebotomy. The hemoglobin level increased to 8.87 ± 1.12 g/dL at the end of the study. At the beginning of the study, the EPO concentration average was 68.8 ± 63.1 mU/ml, and the average weight was 5.11 ± 1.13 kg. At the end of the study, EPO concentration was 20.96 ± 14.56 mU/mL, and bodyweight was 18.15 ± 3.71 kg.
The Hb profile for three lambs that were matched to a preterm infant Hb profile over the first two weeks of life is presented in Figure 4-3. To account for differences in initial Hb level, the Hb profile was normalized by the initial Hb concentration. The Hb profile for the three lambs showed a good agreement with the Hb profile of the preterm infant.

The Hb mass balance model fit along with plasma EPO concentration and bodyweight for three representative lambs are shown in Figure 4.4. The model captures the general behavior of the Hb concentration data. The pharmacodynamic model parameters are summarized in Table 4-1. The lifespan of both transfused RBCs and produced RBCs were fixed to 120 days based on previous estimates.

The diagnostic plots from the final model are given in Figures 4.5-4.7. As displayed in Figure 4.5, the plots of observed versus predicted concentrations using population parameters (PRED) as well as predicted concentrations using individual parameters (IPRED) showed a good visual agreement between predicted and observed data. The weighted residuals using individual predictions (IWRES) versus age (Figure 4.6) were evenly scattered across 0, and there was no apparent trend. The SVPC (Figure 4.7) plot demonstrates that observations percentiles are uniformly distributed between 0 and 1. The diagnostic plots demonstrate the adequacy of the model and population parameter estimates.

4.5 Discussion

This study provides a quantitative estimation of the rate of Hb synthesis in lambs and considers its functional and mechanistic relationship to the EPO plasma concentration, while accounting for important confounding variables (e.g. phlebotomy,
growth of animal and transfusions). Significant increase in the hemoglobin production rate was generally observed as a result of exposure to high endogenous EPO concentrations in lambs as a result of anemia brought on by phlebotomy. The proposed hemoglobin mass balance model accounted for continuous growth and blood volume expansion as a function of body weight. It also corrected for blood removed due to either regular blood sampling or experimentally controlled phlebotomies. It also accounted for the hemoglobin that was transfused to mimic clinical transfusions used to treat anemia in premature infants.

The production rate of Hb at birth was estimated previously to be 2-3% of the total Hb amount present in the circulation per day. This value was estimated based on ferro-kinetic studies in infants. RBC production rate was estimated at different time points between day 1 and 10 after birth. RBC production at birth was estimated by extrapolation to day 0 after birth. Using the population estimates of blood volume and Hb baseline values and the average birth weight of lambs studied in this thesis, Hb destruction rate can be estimated to be 1.10-1.64 g/day at the time of birth. Scaling this value to the birth weight to the power $3/4$ gives a value that ranges between 0.322-0.483 g/day/kg$^{3/4}$. Hb production stimulation function prior to birth ($k_{prod}^{in\ atro}$) of 0.437 g/day/kg$^{3/4}$ estimated by our model is consistent with previous estimates.

Despite the fact that number of subjects in the current study is small, this data is dense in terms of the number of Hb and EPO measurements per subject that is equal to an average of 89 Hb measurements (range 61 to 111), and 91 plasma EPO concentration determination (range 67 to 113). An alternative approach to the current one is using sparse sampling with larger number of subjects. However, sparse sampling main
limitation applicable in this population is missing important information regarding phlebotomy loss which would introduce substantial error in the estimation. Because Hb removed by phlebotomy represent a significant amount relative to blood Hb, it was necessary to record all information regarding Hb removed by phlebotomy including amount and time which contradicts sparse sampling.

Because of the long computer run time, it was impractical to do bootstrap analysis to evaluate the stability of the final model and estimate the standard error of the parameters. Alternatively, parameters standard errors were obtained by linearization of Fisher information matrix of the nonlinear mixed effects model. Because each subject has a unique profile of EPO and weight data over the first month of life, Visual Predictive Check (VPC) was not applicable in this analysis. Instead of VPC, SVPC was performed to evaluate the predictive properties of the model.\textsuperscript{154}

Perhaps the main limitation of the present model it does not account for the negative feedback control of production of EPO by the circulating Hb mass, an item that has described in previous papers.\textsuperscript{89, 164} Nevertheless, representing EPO levels, by the linear spline function, as a forcing function for the effect on Hb production allow the estimation of different PD parameters without the need to implement the feedback between Hb and EPO. However, it is recognized that in order to best implement the current model in a future clinical trial simulation the feedback between Hb and EPO needs to be incorporated in the model.

The estimated mean value for the time between progenitor cells stimulation by EPO and the appearance of newly produced RBC in the circulation, $\alpha$, was 0.994 days. This is higher than the previously reported values in sheep of 0.72 and 0.797 days.\textsuperscript{91, 197}
This difference can be attributed to the fact that reticulocytes indices were not available in our study. The lack of reticulocyte indices restricted the ability to estimate the value of $\alpha$. Although during stress erythropoiesis the value of $\alpha$ changes, previous results suggest only a minor effect on the observed hemoglobin production. As a result, changes in the value of $\alpha$ were not taken into account in this study.

4.5.1 Comparison of PD parameters between newborn sheep and preterm infants

A summary of PD parameters estimated in preterm infants (Chapter 2) vs. pharmacodynamic parameters estimated newborn lambs is presented in Table 4-2. The value of maximum Hb production rate which is scaled to the bodyweight ($E_{\text{max}}$) in preterm infants (0.527 g/day·kg$^{3/4}$) is lower than the value in lambs (1.62 g/day·kg$^{3/4}$). This indicates that sheep have more capacity in producing Hb. However, the value of EPO concentration that results in Hb production rate that is 50% of the scaled $E_{\text{max}}$, denoted by $EC_{50}$, (44.3 mU/mL in preterm infants vs. 47.1 mU/mL in newborn lambs) indicates that newborn lambs have similar sensitivity to EPO in producing Hb compared to preterm infants. Thus, a similar EPO plasma level is needed in preterm infants compared to newborn lambs to give the same Hb production rate relative to the value of $E_{\text{max}}$. The value of the Hb production before birth ($h_{\text{prod}^{\text{in utero}}}$) in newborn lambs (0.437 g/day·kg$^{3/4}$) was found to be similar to the value estimated in preterm infants (0.421 g/day·kg$^{3/4}$) as presented in Chapters 2. The value of blood volume estimated in our model was comparable to previous estimates of blood volume in preterm infants (98.1 mL/kg in
newborn lambs vs. 95.0 mL/kg in preterm infants). Blood volume in preterm infants was measured using $^{131}$I labeled human albumin.

4.5.2 EPO efficacy for the treatment of prematurity anemia

The benefit of EPO therapy in prematurity anemia is still controversial. While some studies have shown that EPO therapy resulted in a significant reduction in the number and/or volume of RBC transfusions, others have seen little to no significant difference between control and EPO treatment groups. As a part of the experimental design, the lambs were kept under anemic conditions (based on the Hb level) similar to anemic conditions in preterm infants during the first two weeks of life (in the first phase). This has been achieved by transfusing or removing blood from the lamb to obtain a Hb profile that matches the Hb profile of a specified preterm infant. An example of matching lambs to preterm infant is presented in Figure 4-3. The Hb profile for the three lambs matches the Hb profile of an anemic preterm infant (Figure 4-3). However, this Hb level resulted in modest EPO plasma concentrations. The pooled EPO average during the first two weeks was 63.4 mU/mL. A plasma level of 63.4 mU/mL is expected to result in a Hb production rate of 0.93 g/day/kg$^{3/4}$. This was calculated using the population estimates of $E_{\text{max}}$ (1.62 g/day·kg$^{3/4}$) and $EC_{50}$ (47.1 mU/mL) according to Eq.2.5. According to the population estimate of $E_{\text{max}}$, the maximum rate of Hb production rate is predicted to be 1.62 g/day·kg$^{3/4}$, that is equivalent to the population estimate of $E_{\text{max}}$. The maximum possible rate of Hb production is nearly two times (1.62/0.93) the endogenous production rate typically seen without administration of EPO under these anemic conditions. This substantial increase in hemoglobin production rate
expected by proper EPO dosing if extrapolated to anemic preterm infants may potentially decrease the number and quantity of blood transfusion required to treat the anemia.

Based on these results and the hypotheses that these pharmacodynamic relationships extrapolates to human premature infants it is possible to increase the endogenous hemoglobin production rate by a factor of 2 by administering EPO which may result in a significant reduction of the number and quantity of blood transfusion required to treat the anemia.

4.6 Conclusion

Newborn lamb showed significant similarities to preterm infants regarding their erythropoietic characteristics. Results indicate that newborn lambs have more capacity in producing RBC (an $E_{\text{max}}$ value of 1.62 g/day·kg$^{3/4}$ in lambs compared to 0.527 g/day·kg$^{3/4}$ in preterm infants). However, the PD analysis indicates that newborn lambs have similar sensitivity to EPO in producing RBC compared to preterm infants (an $EC_{50}$ of 47.1 mU/mL in lambs compared to 44.3 mU/mL in preterm infants). The value of the Hb production rate before birth in newborn lambs was found to be similar to the value estimated in preterm infants as presented in Chapters 2 (a $k_{\text{prod}}^{\text{in utero}}$ value of 0.437 g/day·kg$^{3/4}$ in lambs compared to 0.421 g/day·kg$^{3/4}$ in preterm infants). The value of blood volume estimated in newborn lambs was comparable to previous estimates of blood volume in preterm infants (98.1 mL/kg in newborn lambs vs. 93.2 mL/kg in preterm infants). Based on the similarity between preterm infants and newborn lambs and the hypotheses that these PD relationships extrapolates to human premature infants it is possible to increase the hemoglobin production rate by a factor of 2 by administering
EPO which may result in a significant reduction of the number and quantity of blood transfusion required to treat the anemia.
Table 4-1. Final parameter estimates of the erythropoiesis pharmacodynamics model in lambs

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Parameter description</th>
<th>Population parameter</th>
<th>RSE%</th>
<th>$\omega^2$</th>
<th>RSE%</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V$, mL/kg</td>
<td>Bodyweight normalized blood volume</td>
<td>94.0</td>
<td>3</td>
<td>0.030</td>
<td>27</td>
</tr>
<tr>
<td>$\alpha$, days</td>
<td>Lag time between Hb production stimulation by EPO and appearance in circulation</td>
<td>0.994</td>
<td>0</td>
<td>NE&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NE&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>$F_T$</td>
<td>Fraction of transfused Hb surviving immediately beyond the transfusion</td>
<td>0.817</td>
<td>1</td>
<td>NE&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NE&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>$Hb_0$, g/dL</td>
<td>Baseline hemoglobin concentration at birth</td>
<td>11.4</td>
<td>4</td>
<td>0.044</td>
<td>24</td>
</tr>
<tr>
<td>$E_{max}$, g/day·kg&lt;sup&gt;3/4&lt;/sup&gt;</td>
<td>Maximum Hb production rate which is scaled to the bodyweight by a 3/4</td>
<td>1.62</td>
<td>1</td>
<td>NE&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NE&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>$EC_{50}$, mU/mL</td>
<td>EPO concentration that results in Hb production rate that is 50% of the scaled $E_{max}$</td>
<td>47.1</td>
<td>13</td>
<td>0.558</td>
<td>24</td>
</tr>
<tr>
<td>Residual error, Hb</td>
<td></td>
<td>0.115</td>
<td>1</td>
<td>NE&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NE&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Secondary parameter</td>
<td>Parameter description</td>
<td>Typical value</td>
<td></td>
<td>SD</td>
<td></td>
</tr>
<tr>
<td>$k_{prod}$, g/day·kg&lt;sup&gt;3/4&lt;/sup&gt;</td>
<td>Hb production stimulation rate prior to birth</td>
<td>0.437</td>
<td></td>
<td>0.114</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> NE: not estimated
Table 4-2. Comparison of the values estimated parameters in newborn lambs to the values estimated in preterm infants (Chapter 2)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Typical Value in newborn lamb (RSE%)</th>
<th>Typical Value in preterm infants (RSE%)</th>
<th>Percent difference&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V$, mL/kg</td>
<td>94.0 (3)</td>
<td>93.2 (6)</td>
<td>0.86%</td>
</tr>
<tr>
<td>α, days</td>
<td>0.994 (0)</td>
<td>0.448 (Fixed)</td>
<td>121%</td>
</tr>
<tr>
<td>$F_t$</td>
<td>0.817 (1)</td>
<td>0.875 (Fixed)</td>
<td>6.63%</td>
</tr>
<tr>
<td>$Hb_0$, g/dL</td>
<td>11.4 (4)</td>
<td>13.5 (3)</td>
<td>15.6%</td>
</tr>
<tr>
<td>$E_{max}$, g/day·kg&lt;sup&gt;0.75&lt;/sup&gt;</td>
<td>1.62 (1)</td>
<td>0.527 (21)</td>
<td>207%</td>
</tr>
<tr>
<td>$EC_{50}$, mU/mL</td>
<td>47.1 (13)</td>
<td>44.3 (15)</td>
<td>6.32%</td>
</tr>
<tr>
<td>$k_{prod}^{in utero}$, g/day·kg&lt;sup&gt;0.75&lt;/sup&gt;</td>
<td>0.437 (NA&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>0.421 (NA&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>3.80%</td>
</tr>
</tbody>
</table>

<sup>a</sup>- percent difference was estimated by dividing the difference in parameter estimates between lamb and preterm infant by the parameter estimate in preterm infant

<sup>b</sup>- NA: not applicable
Figure 4.1. Summary of experimental design. Lambs in groups of 3 were matched to an individual preterm infants investigated in Chapters 2 and 3. In the first phase, the Hb profile of the individual preterm infant was matched in the three lambs. In the second phase, Hb profile in lambs was kept under normal level for about 1 week. In the third phase, a major phlebotomy was performed on lambs to induce stress erythropoiesis.
Figure 4.2. Diagram of the pharmacodynamic model for the effect of EPO on Hb production and disposition

- $E_{\text{max}}$: Maximum Hb production rate which is scaled to the body weight by a $\frac{3}{4}$ power function.
- $EC_{50}$: EPO concentration that results in Hb production rate that is 50% of the scaled $E_{\text{max}}$.
- $a$: Time between the erythroid progenitor cell stimulation by EPO and the appearance of RBC in the circulation.
- $b$: Time between the erythroid progenitor cell stimulation by EPO and the removal of RBC in the circulation.
- $f_{\text{stim}}$: Hb production stimulation function.
- Blood transfusion
- Phlebotomy
Figure 4.3. Tracking phase for three lambs matching the Hb profile for a preterm infant. The Hb profile of the individual preterm infant was matched in the three lambs.
Figure 4.4. Pharmacodynamic Hb mass balance model fit to representative lambs. The first row represent plots of observed, population (i.e. using population parameter estimates) predicted (PRED), and individual (i.e. using individual parameter estimates) predicted (IPRED) values of plasma concentrations of Hb versus time profiles. The second row represents the observed values of EPO concentration (left axis) and bodyweight (right axis).
Figure 4.5. Observed Hb concentrations versus population (i.e. using population parameter estimates) predicted (PRED) and individual (i.e. using individual parameter estimates) predicted (IPRED) concentrations of Hb (left and right columns respectively)
Figure 4.6. Weighted residuals using individual predictions (IWRES) versus age.
Figure 4.7. Standardized visual predictive check (SVPC) Plot for erythropoiesis pharmacodynamic model
CHAPTER 5. RED BLOOD CELL LIFESPAN MEASUREMENT

METHODS

5.1 Introduction

Red blood cell (RBC) lifespan measurement is an important area of research with several clinical applications. In transfusion medicine, RBC lifespan measurement can be used to evaluate the quality of RBC being transfused and their efficacy in correcting for anemia. RBC survival study is also valuable in exploring certain physiological and pathological conditions with altered production or elimination of RBC. These conditions include stress erythropoiesis, sickle cell anemia and immune mediated hemolytic anemia. RBC survival studies are also important in the evaluation of drugs pharmacodynamics (PD). The investigation of RBC and reticulocytes survival has been used to elucidate erythropoietin PD effects on RBC kinetics.\textsuperscript{91, 92, 202} RBC lifespan estimation can be also applied to describe the behavior of certain disease progression factors. Perhaps the most prominent example for this case is glycated hemoglobin (HbA1C). HbA1C is a form of hemoglobin that is measured primarily to identify the average plasma glucose concentration over prolonged period of time in diabetic patients. HbA1C depends on two factors: plasma glucose level and RBC lifespan. Hence, it is important to account for RBC lifespan when describing changes in HbA1C overtime.\textsuperscript{203}

RBC lifespan defined as the time a RBC spends in the circulation starting from its appearance in the circulation until its disappearance. RBC lifespan estimation was first proposed by Ashby in 1919. Ashby devised the differential agglutination technique to estimate RBC lifespan.\textsuperscript{204} Since then, RBC lifespan in different species has been
estimated. In humans, RBC lifespan was estimated to be approximately 120 days. In other mammals, values range from 40 days in mice to 225 days in the llama.\textsuperscript{146}

5.2 Objective

The objective of this Chapter is to review the two methods of direct measurement of red cell lifespan, cohort and random labeling. The mathematical equations that describe each method are elucidated. The advantages and disadvantages of each method are also discussed.

5.3 Red blood survival function \((S(t))\)

Red blood cell survival function is defined as the probability that the survival time of an individual RBC is longer than time \(t\). The survival function is also known as the reliability function or cumulative survival rate.\textsuperscript{205} Mathematical properties of survival function include:\textsuperscript{205}

1. Has a value of one at time zero, 
   \[ S(0) = 1. \]
2. Has a value of zero at infinity, 
   \[ S(\infty) = 0. \]
3. A non increasing function, 
   \[ \frac{dS}{dt} \leq 0. \]

A typical RBC survival function is displayed in Figure 5.1. The value of the survival function starts from 1. If no elimination occurs to a newly produced RBC, then the survival function value stay constant for a certain period after appearance in the circulation. As RBC ages, the probability of remaining in the circulation decrease below 1 until it gets to zero at the end of the survival curve.\textsuperscript{205}

RBC lifespan \((L)\) is obtained from the survival function using Eq.5.1:

\[
L = \int_{0}^{\infty} S(u) du \tag{5.1}
\]
5.4 Modeling RBC kinetics

Assuming that RBC follow a linear disposition kinetics, RBC amount is given by the following equation:

\[ RBC(t) = f_{prod}(t) \ast S(t) \]  

(5.2)

where \( f_{prod}(t) \) is the production rate of RBC, \( \ast \) is the convolution linear operator, \( S(t) \) is the survival function. This equation is equivalent to the following integration form:

\[ RBC(t) = \int_{-\infty}^{t} f_{prod}(u) \cdot S(t-u).du \]  

(5.3)

Under steady state condition, which implies a constant RBC production rate that is equivalent to the RBC removal rate, Eq.5.3 can be represented as:

\[ RBC(t) = \int_{-\infty}^{t} f_{prod}^{ss} \cdot S(t-u).du \]  

(5.4)

At time zero, Eq.5.4 can be rearranged into:

\[ RBC(t = 0) = f_{prod}^{ss} \cdot \int_{0}^{\infty} S(x).dx = f_{prod}^{ss} \cdot L \]  

(5.5)

where \( f_{prod}^{ss} \) is the production rate of RBC under steady state conditions and \( RBC^{ss} \) is the amount of RBC in the circulation under steady state. Since the value of \( \int_{0}^{\infty} S(x).dx \) is equivalent to the lifespan (\( L \)), the amount of RBC under steady state conditions can be described as the result of multiplication of the production rate constant and the lifespan.

5.5 Red blood cell lifespan measurement methods

There are two classes of direct methods for measurement of red cell lifespan: cohort and random labeling. The cohort methods require the incorporation of an
isotopically labeled substance into a group of newly formed cells. If exposure to the label is brief and there is no reuse of label, the labeled cells will be of nearly the same age. Cohort labels result in a pattern characterized by a plateau, the length of which is a measure of erythrocyte lifespan.\textsuperscript{206} In contrast, random-labeling methods use labels that bind with all cells in the circulation regardless of age. Random labels begin to disappear from the circulation immediately, and erythrocyte lifespan is related to the time when all label has vanished. The survival curve of randomly labeled erythrocytes gives a straight line with tailing at the end.\textsuperscript{206}

### 5.5.1 Cohort labeling methods

The idea behind cohort labeling methods is to label a group of RBC at the time of production. This allows tracking this cohort of cells throughout their presence in the circulation starting from their appearance until their disappearance from circulation. An ideal cohort survival curve is displayed in Figure 5.2. The curve is composed of three phases: production phase, plateau phase, and decline phase. The production phase represents the appearance of newly produced labeled RBC in the circulation. No significant elimination or production occurs in the plateau phase. In the third phase, RBC are removed from the circulation.

In this section a summary of equations that describe the behavior of labeled RBC in cohort survival studies is presented. Shemin and Rittenberg described the behavior of labeled RBC in cohort studies in 1946.\textsuperscript{207} They used glycine labeled with $^{15}$N to tag a group of newly produced RBC. The proposed model was based on the following assumptions:

- RBC lifespan distribution is symmetric.
- RBC production rate is constant.

In this study, a human subject ingested $^{15}$N labeled glycine for three days. $^{15}$N concentration in hemin was then followed for 6 months to construct a cohort survival curve (Figure 5.3).

An equation describing the percent of $^{15}$N atoms of the total number of nitrogen atoms in the circulation ($C(t)$) is derived below.

Let

$$f(t) \equiv \text{the } ^{15}\text{N concentration in the hemin (hemin is an iron containing moiety in Hb) synthesized at the time } t$$

$$\alpha \equiv \text{rate of formation of hemin nitrogen in atoms per day}$$

$$N \equiv \text{total hemin nitrogen (atoms) in the blood}$$

$$G(t) \equiv \text{atoms of } ^{15}\text{N in circulating hemin nitrogen at time } t$$

$$S(t) \equiv \text{RBC survival function}$$

Then

$$G(t) = \int_{0}^{t} \alpha \cdot f(u) \cdot S(t-u) \, du$$

(5.6)

Since

$$C(t) = 100 \frac{G(t)}{N}$$

(5.7)

$$C(t) = 100 \frac{\alpha}{N} \int_{0}^{t} f(u) \cdot S(t-u) \, du$$

(5.8)

Since under constant production rate (i.e. steady state conditions), the lifespan is described as
\[
L = \frac{N}{\alpha} \quad \text{(5.9)}
\]

then

\[
C(t) = \frac{100}{L} \int_{0}^{t} f(u) \cdot S(t-u) \, du \quad \text{(5.10)}
\]

In Eq.5.10, let

\[
x = t - u \\
dx = -du
\]

Then

\[
C(t) = -\frac{100}{L} \int_{t}^{0} f(t-x) \cdot S(x) \, dx = \frac{100}{L} \int_{0}^{t} f(t-x) \cdot S(x) \, dx \quad \text{(5.11)}
\]

Despite the assumption of constant production rate of RBC, the incorporation rate of \(^{15}\)N atoms into RBC is not constant. The incorporation of \(^{15}\)N atoms depends on the total number of hemin nitrogen atoms in the circulation, which was assumed constant, and the amount of \(^{15}\)N atoms in the blood, which decrease with time as a result of incorporation in newly produced RBC. Assuming that \(f(t)\) is given by the following equation:

\[
f(t) = \frac{L}{100} C_0 \lambda \cdot e^{-\lambda t} \quad \text{(5.12)}
\]

Eq.5.11 can be represented as:

\[
C(t) = C_0 \lambda \cdot e^{-\lambda t} \int_{0}^{t} e^{\lambda x} \cdot S(x) \, dx \quad \text{(5.13)}
\]

Differentiating with respect to \(t\) yields

\[
\frac{dC}{dt} = -\lambda \cdot C(t) + C_0 \cdot \lambda \cdot S(t) \quad \text{(5.14)}
\]

\[
C_0 \cdot S(t) = \frac{1}{\lambda} \frac{dC}{dt} + C(t) \quad \text{(5.15)}
\]
Differentiation of the previous equations yields

\[
C_0 \cdot \frac{dS}{dt} = \frac{1}{\lambda} \frac{d^2 C}{dt^2} + \frac{dC}{dt}
\]  

(5.16)

The change rate in the survival function \( \frac{dS}{dt} \) can be estimated numerically from the concentration of \(^{15}\text{N}\) in hemin curve. The time at which the maximum value for \( \frac{dS}{dt} \) is obtained is defined as the lifespan \( (L) \).\(^{207}\) This is based on the assumptions that the lifespan distribution curve is unimodal and symmetric around the mean lifespan. As a result of these assumptions, the mean is equal to the mode.

The main advantage of using cohort studies is that they do not require ex vivo manipulation of the cells. On the other hand, cohort labeling methods have several disadvantages that limit their use for RBC survival. Firstly, the labeled can be reused to label another population of RBC after the breakdown of initially labeled RBC or from other sources where the label is incorporated. The reuse of the label was evident in the study conducted by Shemin et al where a significant amount of the label (about 20% of the total labeled RBC) was in the circulation after 200 days. The cohort survival curve also does not go to zero at the end of the study as presented in Figure 5.3. Secondly, it requires taking measurements for a long period to estimate RBC lifespan. For example, to estimate the lifespan of human RBC (about 120 days), assays must be performed for over 120 days. Thirdly, labeled hemoglobin can be removed from the circulating RBC in a process known as hemoglobin vesiculation.\(^{208}\) The loss of label due to vesiculation results in underestimating the actual lifespan because the label is removed from the circulation while the labeled RBC is still circulating. Fourthly, the incorporation of the label into
RBC occurs over long period of time. As a result, the labeled RBC will not have the same age.

5.5.2 Random labeling methods

Random labeling is the labeling of RBC of all ages present in a blood sample at the sampling time point. Commonly used random labeling methods include: differential agglutination method, radioactive chromium ($^{51}$Cr), Diisopropylfluorophosphate labeled with $^{32}$P (DF$^{32}$P), and biotin. An ideal survival curve using these methods is displayed in Figure 5.4. Under steady state erythropoietic conditions, the survival curve is characterized by a linear decline with a tail at the end.

Assuming linear disposition of RBC, the amount of a randomly labeled RBC ($RBC_L$) is given by Eq. 5.17. The labeling time was assumed to be 0.

$$RBC_L(t) = \int_{-\infty}^{0} f_{\text{prod}}^{\text{lab}}(u) \cdot S(t-u) \cdot du \quad , \quad 0 < t$$

(5.17)

where $f_{\text{prod}}^{\text{lab}}(t)$ is the production rate of RBC that were labeled, $S(t)$ is the survival function. Assuming that $f_{\text{prod}}^{\text{lab}}(t)$ is constant, previous equation can be represented as:

$$RBC_L(t) = \int_{-\infty}^{0} f_{\text{prod}}^{ss} \cdot S(t-u) \cdot du \quad , \quad 0 < t$$

(5.18)

where $f_{\text{prod}}^{ss}$ is the production rate of labeled RBC under steady state conditions. The initial amount of labeled RBC ($t=0$) is given by the Eq. 5.19:

$$RBC_L(0) = \int_{-\infty}^{0} f_{\text{prod}}^{ss} \cdot S(0-u) \cdot du$$

(5.19)

Eq. 5.19 can be rearranged into:
\[ RBC_L(0) = f_{\text{prod}}^{\infty} \int_{0}^{\infty} S(u) du \]  \hspace{1cm} (5.20)

Since the value of \( \int_{0}^{\infty} S(u) du \) is equivalent to the lifespan \( L \), the initial amount of labeled RBC can be described as the result of multiplication of the production rate constant and the lifespan. The lifespan is described as presented in Eq.5.22.

\[ RBC_L(0) = f_{\text{prod}}^{\infty} \cdot L \]  \hspace{1cm} (5.21)

\[ L = \frac{RBC_L(0)}{f_{\text{prod}}^{\infty}} \]  \hspace{1cm} (5.22)

Changing the integration variable in Eq.5.19 from \( u \) to \( x=t-u \), results in the following equation:

\[ RBC_L(t) = \int_{t}^{\infty} f_{\text{prod}}^{\infty} \cdot S(x) \cdot dx \hspace{1cm} 0 < t \]  \hspace{1cm} (5.23)

Differentiation of Eq.5.23 using Leibniz rule, the following equation was obtained:

\[ \frac{d[RBC_L(t)]}{dt} = -f_{\text{prod}}^{\infty} \cdot S(t) = -\frac{RBC_L(0)}{L} \cdot S(t) \]  \hspace{1cm} (5.24)

At the time of labeling \( (t=0) \), the value of the survival function \( S(t) \) is 1. As a result, the lifespan can be obtained from the slope of the survival curve at time equals to 0. This result is based on the assumption of a constant rate of RBC production regardless to the shape of the survival function. If \( S(t) \) is characterized by an initial plateau \( (S(t)=1) \) as presented in Figure 5.1, a linear phase from the survival curve will be obtained. From the slope of the linear part of the survival curve the value of the lifespan can be obtained as presented in Figure 5.4. The linear phase has been reported in the survival curve.
observed in healthy adult human and sheep up to 20% of the initial amount of labeled RBC.\textsuperscript{209, 210}

Compared to cohort methods, random labeling methods provide an accurate estimation of the lifespan in a relatively shorter time. Additionally, because labeling usually is performed ex vivo, cross-transfusion studies also are possible. Thus, survival of a patient’s cells in a normal recipient or of normal cells in a patient can be evaluated by this method.

The most prominent disadvantage of random labeling methods is that it assumes a steady state conditions. As a result, this it cannot be used for situation where the steady state assumption is violated. Pathological and physiological states where the steady state assumption is not valid include: acute anemic conditions, erythropoiesis in growing subject such as premature infants, and immune mediated hemolytic anemia. Similar to cohort labeling methods, random methods are affected by hemoglobin vesiculation.\textsuperscript{208} The loss of label due to vesiculation will confound the ability to estimate the actual lifespan because the label is removed from the circulation while the labeled RBC is still circulating.
Figure 5.1: Red blood cell survival function
Figure 5.2: An ideal cohort survival curve. It is composed of three phases: production phase, plateau phase, and decline phase.
Figure 5.3: Survival curve and lifespan calculation reported by Shemin and Rittenberg\textsuperscript{207}
Figure 5.4: An ideal survival curve using random labeling method. The equation ($y = \text{slope} \cdot x + \text{intercept}$) was obtained from the linear part of the curve.
CHAPTER 6. RED BLOOD CELL LIFESPAN MEASUREMENT UNDER NON-STEADY STATE CONDITIONS

6.1 Introduction

There are two classes of direct methods for measurement of red cell lifespan: cohort methods and random labeling methods. The cohort methods require the incorporation of an isotopically labeled substance into a group (“cohort”) of newly formed cells. If exposure to the label is brief and there is no reuse of label, the labeled cells will be of nearly the same age. Cohort labels result in a pattern characterized by a plateau, the length of which is a measure of erythrocyte lifespan (Figure 6.1.A). In contrast, random-labeling methods use labels that bind with all cells in the circulation regardless of age. Random labels begin to disappear from the circulation immediately, and erythrocyte lifespan is related to the time when all label has vanished. The survival curve of randomly labeled erythrocytes gives a straight line with tailing at the end (Figure 6.1.B).

Constant red blood cells (RBC) production rate is commonly assumed for the measurement of RBC lifespan using random labeling techniques. However, measuring the lifespan of RBC produced in response to acute hypoxic stress conditions requires information about the production rate of RBC. Despite the fact that the survival curve of RBC without assuming constant production rate has already been described by Bergner, the author concluded that it is not possible to determine RBC lifespan.

6.2 Objective and related specific aim and hypothesis

The objective of the present analysis is to develop a methodology for the measurement of RBC lifespan under stress conditions. This is done using two biotin
densities RBC labeling technique in combination with mathematical modeling based on physiological principles.

The work presented in this Chapter is an important step toward addressing the specific aim that is to test the hypothesis that RBC lifespan is shortened under acute hypoxic stress conditions.

6.3 Materials and Methods

6.3.1 Animal characteristics.

All sheep studies were approved by the Institutional Animal Care and Use Committees at both the University of Iowa and the University of Arkansas for Medical Sciences. All study animals were female and all were approximately 3 years of age at study entry. All animals had been previously exposed to biotinylated RBCs for a different study protocol. No traces of biotinylated RBCs were measured at the beginning of the present study.

6.3.2 Labeling of RBCs

On the day that RBCs labeled with biotin were to be transfused, a venous blood sample was drawn via the jugular catheter into either syringes containing heparin or a collection bag containing CPD (Baxter Healthcare Corporation, Fenwal Division, Deerfield, IL). These whole blood samples were centrifuged at room temperature; the plasma removed and frozen at -70°C. RBCs were labeled as described below.

6.3.3 Biotin labeling

The method for biotin labeling of RBCs at discrete densities has been previously described. Briefly, autologous RBCs were washed twice in a glucose containing pH 7.4 phosphate wash buffer to remove residual plasma. The washed RBCs were then
biotinylated using sulfo-succinimido-biotin (Pierce Chemical Co., Rockford, IL). In doing so, freshly prepared wash buffer was adjusted to pH 5 to stabilize the biotinylation reagent, and dilutions of this mixture were chosen to yield a pre-determined biotinylation reagent concentration per mL RBC. The biotinylation reaction is conducted at pH 7.4 for 30 min and then was stopped by removing the remaining biotinylating reagent in two additional pH 7.4 washes.

### 6.3.4 Study protocol

The outline of study protocol is presented in Figure 6.2. After a 2-3 weeks of baseline period during which blood was sampled twice weekly for RBC and hemoglobin (Hb) measurements, adult sheep underwent a phlebotomy, i.e. an exchange transfusion using plasma/0.9% normal saline, in which Hb concentration was dropped to 3-4 g/dL. A sample of heparinized blood (● in Figure 6.2) was biotinylated immediately at a biotin density suitable for long-term RBC survival studies. The phlebotomy procedure was continued for 3-4 hr until the desired Hb concentration was reached, and during the time when the autologous “baseline,” steady-state RBC sample (● in Figure 6.2) is being biotinylated. Following biotinylation this blood sample was re-infused on the phlebotomy day.

Phlebotomized sheep was allowed to recover for the next 10-15 days during which blood was sampled once or twice daily for RBC and Hb measurements. Biotinylated RBC enrichment was measured twice per week. Oral iron was administered during this period of maximal stress erythropoiesis in order to ensure sheep had adequate iron for hemoglobin production. Based on previous phlebotomy studies in adult sheep, it is anticipated that after 10-15 days Hb levels will have increased by ~50% relative to
pre-phlebotomy baseline levels. After 10-15 days a second blood sample (② in Figure 6.2) was drawn and biotinylated in a similar fashion to the baseline sample using the other biotin density suitable for long-term RBC survival studies. This “stress erythropoiesis” sample (② in Figure 6.2) which was reinfused on the same day consisted of a mixture of recently produced “stressed” RBCs and pre-existing “baseline” steady-state RBCs. Subsequent blood sampling was continued on a twice a week basis until Biotinylated RBCs was no longer be detected. To account for changing numbers of RBCs after the phlebotomy, measured Biotinylated RBC enrichments was corrected based on the ratio of RBC concentration at the time of sampling over the RBC concentration at the time of labeling.

6.3.5 Quantitation of individual Biotinylated RBC peaks

Mixture modeling approach was done to account for the overlap between the adjacent biotinylated RBC peaks and the unlabeled fluorescent peak as depicted in Figure 6.3. Each Biotinylated RBC peak was assumed to have a lognormal distribution. The tail of the unlabeled RBC was assumed to follow a Weibull distribution.

The choice of lognormal distribution to model labeled RBC and Weibull distribution to model the tail of unlabeled RBC was based on internal data that has known ratios of unlabeled RBC and labeled RBC of different densities. Additionally, this method of peak separation has been used and compared against other method in the current sheep in the first phase. The results for this comparison are recently published by Mock et al. 210
6.3.6 **RBC survival model**

In random labeling studies a sample of red cells of different ages is labeled. The labeled RBC sample typically contains cells of all ages. Assuming that the labeled RBC sample is a representative sample of the RBC in the circulation, the disposition of RBC at the time of sampling can be followed over time.

As displayed by Figure 6.2, at the beginning of the study a sample of the blood was biotinylated and re-administered to the circulation. Let \( N_1(t) \) represents the number of RBC that was produced before the beginning of the study \((t<0)\). As displayed in Figure 6.2, the disposition of \( N_1(t) \) can be followed by following the first biotinylated RBC sample. Similarly, \( N_2(t) \) represents the number of RBC that was produced before \( t=T \), where \( T \) is the time between the first and the second label. The total number of RBC is defined as \( N(t) \).

Assuming that RBC was not removed from the circulation at age less than \( T \), the number of RBC produced between \( t=0 \) (time of major phlebotomy that is the same as the time of the first label) and \( t=T \) (10-15 days after the major phlebotomy that is the same as the time of the second label), denoted by \( N_{\text{NEW}}(t) \), is estimated using mass balance principles. After the major phlebotomy and before the time of the second label, \( N_{\text{NEW}}(t) \) represent the difference between the total number of RBC \( (N(t)) \) and RBC produced before the time of the phlebotomy \( (N_1(t)) \). After the time of the second label, \( N_{\text{NEW}}(t) \) is estimated by taking the difference between RBC produced before the time of the second label \( (N_2(t)) \) and RBC produced before the time of the phlebotomy \( (N_1(t)) \).
To describe the production rate of \( N_{NEW}(t) \), denoted by \( f_{in}(t) \), RBC were assumed to stay in circulation without removal for a minimum of \( T \) (the time between phlebotomy and the second label). In other words, RBCs that were produced after the first biotin label and before the second biotin label were not removed from circulation before the time of the second label. Accordingly, the \( f_{in}(t) \) can be described using:

\[
f_{in}(t) = \begin{cases} 
    \frac{d(N_{NEW}(t))}{dt} & \text{for } 0 < t < T \\
    0 & \text{otherwise}
\end{cases}
\]  

Under the assumption of linear disposition of RBC, the number of \( N_{NEW}(t) \) present in the circulation at time \( t \) was described according to:

\[
N_{NEW}(t) = \int_{-\infty}^{t} f_{in}(u) \cdot S(t-u) \cdot du
\]  

Since \( N_{NEW}(t) \) represent RBC produced between the time of the phlebotomy and the time of the second label, \( f_{in}(t) \) is only defined for \( 0 < t < T \), Eq.6.3 is represented in the following form:

\[
N_{NEW}(t) = \begin{cases} 
    \int_{0}^{t} f_{in}(u) \cdot S(t-u) \cdot du & \text{for } 0 < t < T \\
    \int_{0}^{T} f_{in}(u) \cdot S(t-u) \cdot du & \text{for } T < t \\
    0 & \text{otherwise}
\end{cases}
\]
Assuming a constant blood volume, RBC concentration was used instead of the total number of RBC in the circulation. The assumption that RBC volume is constant is based on the fact that this group represents adult non growing sheep. Additionally, there was no significant weight gain over the study period (p>0.05) as explained in section 6.4.

Since total number of RBC in the circulation The survival function, $S(t)$, was modeled using a Weibull distribution due to the flexibility of the distribution and its support on the non-negative real line. \[ S(t) = \exp \left( - \left( \frac{t - \theta}{\lambda} \right)^k \right) \]  \hspace{1cm} (6.5)

where $\lambda$, $k$, and $\theta$ are the scale, shape, and location parameters, respectively.

The mean potential lifespan was calculated using the following equation:

\[ MPL = \int_0^\infty S(t) \cdot dt \] \hspace{1cm} (6.6)

6.4 Results

The model was applied successfully for 5 out of 6 subjects. One subject was excluded because it passed away at day 85 after the second label. The survival model fit for all subjects are shown in Figure 6.4. The model captures the general behavior of the biotin/RBC data. The survival model parameters are summarized in Table 6-1. The correlation coefficient ($r$) for the fitted values vs. the observed ranged between 0.64-0.95.

The baseline RBC count before at the beginning of the study was $9.14\pm1.16 \times 10^6$ / $\mu l$ (mean ± standard deviation). As a result of phlebotomy, RBC count was reduced to $3.71\pm0.56 \times 10^6$ / $\mu l$. At the end of the study, RBC count increased to $10.65\pm0.82 \times 10^6$ / $\mu l$ that was not significantly different from RBC count at the beginning of the study.
The body weight at the beginning of the study was 91.5±9.77 Kg. The body weight at the end of the study was 90.36±5.00 Kg. Using paired t test, there was no significant difference between the body weight at the beginning of the study compared to the end of the study (p>0.05). Hence, blood volume was assumed to be constant over the study period.

6.5 Discussion

In the present analysis a methodology for the measurement of RBC lifespan under stress conditions was developed. This was done using two biotin densities RBC labeling technique in combination with mathematical modeling based on physiological principles.

Although biotinylation is a random labeling technique, it was implemented in a way to serve the same as cohort methods. Using the proposed methodology enables tracking the behavior of a group of erythrocytes that were produced under stress conditions over time. The use of biotinylated autologous RBCs to measure RBC survival offers additional advantages compared to cohort labeling methods. Biotinylated RBCs does not expose the subject to radioactivity. Biotin does not have toxic effects even when administered intravenously and orally in amounts several orders of magnitude higher than the amount used here. The biotin method with flow cytometric detection has high sensitivity and large linear dynamic range of the cell counter. This method will enhance the ability to accurately follow the labels over long intervals. Unlike cohort labels, biotin label is not reused after the breakdown of initially biotinylated erythrocytes. Additionally, biotin labeling is not affected by hemoglobin vesiculation. As explained in Chapter 5, random labeling (except biotin labeling) and cohort labeling survival methods are affected by hemoglobin vesiculation. The loss of label due to vesiculation
will confound the ability to estimate the actual lifespan because the label is removed from the circulation while the labeled RBC is still circulating.

An important advantage of biotin labeling is its ability to study multiple populations of RBCs simultaneously in the same individual by labeling with biotin at different densities.\textsuperscript{216} With the ability to label multiple populations of RBCs, the present analysis would not have been possible. It allows tracking RBC present in the circulation at the time of phlebotomy (RBC produced before the time of phlebotomy) and RBC present in the circulation 10-15 days after major phlebotomy (RBC produced before 10-15 days after major phlebotomy).

The choice of the two biotin densities was chosen to balance two opposing objectives: 1) to minimize the overlap between the two biotinylated RBC densities for studying RBC survival; and 2) to avoid excessive overlap between the adjacent Biotinylated RBC peaks and the unlabeled fluorescent peak that could confound the determination of Biotinylated RBC enrichment under each peak.\textsuperscript{216}

Perhaps the main limitation of the present methodology and cohort methods is inability to detect the disappearance of young RBCs. As mentioned above, it was assumed that RBCs that are produced in the period between the two biotin labels are not removed from the circulation. Since RBCs are produced under non-steady state environment, it is not possible to describe their removal from the circulation without having a measure of their production rate. In other words, it is not possible using the current methodology to differentiate the production of RBCs and their removal when both processes occur at the same time. This justify the need to assume that newly
produced RBC stays for at least 10-15 days without being removed from the circulation (section 6.3.6).

Other researchers concluded that it is not possible to estimate the lifespan of RBCs that are produced under non-steady state conditions using random labeling technique. This is attributed to the lack of information about the prior history of RBCs that were produced under non-steady state conditions. On the other hand, the proposed methodology makes use of two random labels of biotin with different densities in addition to total RBC count. The first biotin label serve as direct measure of prior history of RBCs. Hence, when the second labeled RBCs were modeled the value of the background (i.e. first label) were subtracted. The value of measuring total RBC count is two fold: to account for the change in RBC count over time and to estimate the production rate.

High variability of the observed points around the fitted line was observed for sheep 1 and sheep 4. This was caused by the high observed overlap between the peak of labeled RBC (second label) and the unlabeled RBC. This confounded the ability to differentiate between the two cell groups. This might have a significant impact on the value of the estimated fraction of labeled RBC.

6.6 Conclusion

In summary, this work adds to previous studies that explored the use of biotin-labeled RBCs to assess RBC survival. Biotin labeling method has been applied in this methodology. The ability of tracking multiple RBC populations simultaneously in the same individual using biotin labeling made the identification of stressed RBC and steady-state RBC possible. The present methodology can be applied in pathological states
involving altered RBC production or removal (e.g. stress erythropoiesis, sickle cell anemia, and immune-mediated hemolytic anemia).
Table 6-1. Survival analysis summary

<table>
<thead>
<tr>
<th>Subject</th>
<th>Lifespan (days)</th>
<th>θ</th>
<th>k</th>
<th>λ</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>87.19</td>
<td>64.6</td>
<td>0.54</td>
<td>12.9</td>
<td>0.64</td>
</tr>
<tr>
<td>2</td>
<td>137.1</td>
<td>30.7</td>
<td>5.59</td>
<td>115</td>
<td>0.90</td>
</tr>
<tr>
<td>3</td>
<td>123.4</td>
<td>10.2</td>
<td>7.47</td>
<td>121</td>
<td>0.92</td>
</tr>
<tr>
<td>4</td>
<td>129.9</td>
<td>11.5</td>
<td>8.89</td>
<td>125</td>
<td>0.72</td>
</tr>
<tr>
<td>5</td>
<td>123.4</td>
<td>67.3</td>
<td>0.96</td>
<td>55.0</td>
<td>0.95</td>
</tr>
<tr>
<td>Mean</td>
<td>120</td>
<td>36.9</td>
<td>4.69</td>
<td>85.8</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>19.3</td>
<td>27.8</td>
<td>3.79</td>
<td>49.7</td>
<td></td>
</tr>
</tbody>
</table>
Figure 6.1. Patterns produced by cohort and random methods of labeling circulating erythrocytes. Cohort survival curves are characterized by an initial increase in label (production phase) followed by a plateau phase where no significant change in the label amount followed by a decline phase in which labeled RBC are removed from the circulation. Random labeling methods survival curve is characterized by an initial linear decline followed by a tail at the end.
Figure 6.2. Diagram for study protocol and model outline. Two biotin labeling were performed at day 0 (phlebotomy day) and 10-15 days later.

- $N$: Total number of RBC
- $N_1$: RBC produced before the time of the first label (before major phlebotomy)
- $N_2$: RBC produced before the time of the second label
- $N_{NEW}$: RBC produced between the time of the first label and the second label
- $T$: Time between first label and second label
Figure 6.3. Mixture modeling to account for the overlap between the adjacent biotinylated RBC peaks and the unlabeled fluorescent peak.
Figure 6.4. Survival plots for the RBCs produced during 10-15 days after the major phlebotomy.
CHAPTER 7. THE EFFECT OF ACUTE HYPOXIC STRESS CONDITIONS ON THE SURVIVAL OF RED BLOOD CELLS

7.1 Introduction

Several researchers reported a decrease in the lifespan of red blood cells (RBCs) under hypoxic conditions. This observation is supported by studies that demonstrate a shortened RBC lifespan in response to a variety of stimuli, including phenylhydrazine anemia, blood loss, altitude exposure and erythropoietin injection. Those studies clearly point to the importance of environment around RBCs in controlling their survival.

The aim of the present investigation was to test the hypothesis that the RBC lifespan is shortened under acute hypoxic stress conditions. It was also intended to test whether the change in RBC survival happens to both blood cells produced and present during the hypoxic environment in the circulation or only to one of them. For these purposes, the lifespan was determined for three RBC populations: RBC produced and resided in the circulation under normal environment, RBC produced under normal environment and resided in the circulation under hypoxic environment, and RBC produced and resided under hypoxic environment.

7.2 Specific aim and hypothesis

The specific aim of this Chapter is to test the hypothesis that RBC lifespan is shortened under acute hypoxic stress conditions.

The specific hypothesis is that stress erythropoiesis results in the reduction of the lifespan of RBC produced under stress anemic conditions compared to RBC produced under non anemic conditions.
7.3 Materials and Methods

7.3.1 Animal characteristics

All sheep studies were approved by the Institutional Animal Care and Use Committees at both the University of Iowa and the University of Arkansas for Medical Sciences. All study animals were adult female and all were approximately 2 years of age at study entry. None had been previously exposed to biotinylated RBCs (BioRBC) or other biotinlyated proteins.

7.3.2 Labeling of RBCs

On the day that RBCs labeled with biotin were to be transfused, venous blood was drawn via the jugular catheter into either syringes containing heparin or a collection bag containing CPD (Baxter Healthcare Corporation, Fenwal Division, Deerfield, IL). These whole blood samples were centrifuged at room temperature; the plasma removed and frozen at -70°C. RBCs were labeled as described below.

7.3.3 Biotin labeling

The method for biotin labeling of RBCs at discrete densities has been previously described.213 Briefly, autologous RBCs were washed twice in a glucose containing pH 7.4 phosphate wash buffer to remove residual plasma. The washed RBCs were then biotinylated using sulfo-succinimido-biotin (Pierce Chemical Co., Rockford, IL). In doing so, freshly prepared wash buffer was adjusted to pH 5 to stabilize the biotinylation reagent, and dilutions of this mixture were chosen to yield a pre-determined biotinylation reagent concentration per mL RBC. The biotinylation reaction is conducted at pH 7.4 for 30 min and then was stopped by removing the remaining biotinylating reagent in two additional pH 7.4 washes.
7.3.4 Study protocol

Throughout this Chapter, the RBC produced and residing in the circulation under normal environment will be referred to as “steady-state RBC1”. Furthermore, the RBC produced under normal environment and resided in the circulation under hypoxic environment is denoted as “steady-state RBC2”. Additionally, “stressed RBC” will be used to refer to the RBC produced and resided under hypoxic environment.

Approximately 1 week prior to the start of the study, a catheter used for infusion and withdrawal was placed percutaneously in a jugular vein (Intracath 16 G x 8 in, Becton Dickinson Infusion Therapy Systems, Inc., Sandy, Utah, USA). Prior to the transfusion of labeled RBCs, daily blood samples were collected for determination of baseline hematologic status, i.e., hemoglobin concentration, RBC counts, and RBC indices.

The study was composed of two phases: steady state and stress erythropoiesis. Study design is outlined in Figure 7.1. The steady state phase starts by biotinylating a sample of RBCs from a non anemic sheep that is under steady state erythropoietic conditions (i.e. the RBC production rate is constant and equivalent to RBC removal rate). After transfusing the first BioRBC sample (steady-state RBC1) into the same sheep that it was obtained from, BioRBC enrichment measurement was performed at weekly intervals throughout the 23-week first phase period (in Figure 7.1). Hemoglobin (Hb), RBC, and hematocrit measurements were also performed at a weekly interval to assess whether erythropoiesis was perturbed during the study period. Hemoglobin, hematocrit, and RBC counts were determined using a hematology analyzer (Sysmex XT 2000i, Sysmex Corp, Kobe, JP).
After the end of the first phase, adult sheep underwent a phlebotomy, ie, an exchange transfusion using plasma/0.9% normal saline, in which Hb concentration was dropped to 3-4 g/dL. A sample of phlebotomized blood was biotinylated immediately at a biotin density suitable for long-term RBC survival studies. The phlebotomy procedure was continued for 3-4 hr until the desired Hb concentration was reached, and during the time when the autologous “baseline,” steady-state RBC2 sample (2 in Figure 7.1) is being biotinylated. Following biotinylation this blood sample was re-infused the same day.

Phlebotomized sheep were allowed to recover for the next 10 - 15 days during which time blood was sampled once or twice daily for Hb, RBC and hematocrit measurements. Biotinylated RBC enrichment was determined twice per week. Oral iron was administered during this period of maximal stress erythropoiesis in order to ensure sheep had adequate iron for hemoglobin production. After 10-15 days a second blood sample (3 in Figure 7.1) was drawn and biotinylated using a biotin density suitable for long-term RBC survival studies. This biotin density was selected to be different from the biotin density used in the previous biotinylation step (i.e. at the time of major phlebotomy). This sample which was is reinfused on the same day consisted of a mixture of recently produced “stressed RBC” and pre-existing “steady-state RBC2”. Subsequent blood sampling was continued on a twice a week basis until Biotinylated RBCs was no longer be detected. To account for changing numbers of RBCs after the phlebotomy, measured biotinylated RBC enrichments was corrected based on the ratio of RBC concentration at the time of sampling over the RBC concentration at the time of labeling.
7.3.5 Quantitation of individual Biotinylated RBC peaks

Mixture modeling approach was done to account for the overlap between the adjacent biotinylated RBC peaks and the unlabeled fluorescent peak as depicted in Figure 7.2. Each biotinylated RBC peak was assumed to have a lognormal distribution. The tail of the unlabeled RBC was assumed to follow a Weibull distribution.

The choice lognormal to model labeled RBC and Weibull to model the tail of unlabeled RBC was based on internal data that has known ratios of unlabeled RBC and labeled RBC of different densities. Additionally, this method of peak separation has been used and compared against other methods in the current sheep in the first phase. The results for this comparison are recently published by Mock et al.210

7.3.6 RBC lifespan estimation for steady state cells

The mean in vivo potential RBC lifespan (L) was estimated for blood cells that were produced under steady state environment (steady-state RBC1 and RBC2) by plotting RBC label concentration (RBC label concentration was obtained by multiplying the fraction labeled by the concentration of RBC) against time post-transfusion and then performing a least-squares linear curve fit of the RBC survival plot (Figure 7.3). MPL was defined by the number of days in the x axis intercept. The survival curves for the BioRBC densities encompassed all data points between 24 h and the time when 20% of each of biotin-labeled RBC population remained. The 20% value was selected because, at this point, several of the RCS curves became nonlinear (Figure 7.3). The details of RBC lifespan measurement using random labeling methods under steady state were discussed in details previously in section 5.5.2.
7.3.7  **RBC lifespan estimation for non-steady state cells**

The lifespan of RBC produced under non-steady state conditions (between the time of the phlebotomy and 10-15 days later), the methodology developed in Chapter 6 was used.

7.3.8  **Statistical analysis**

The statistical analysis was conducted using SAS version 9.0 (SAS Institute Inc., Cary, North Carolina). Repeated measure ANOVA and Tukey’s test for multiple comparisons were conducted to compare the hematologic parameters values over time as depicted in Table 7-1. Repeated measure ANOVA was also used to compare the means of MPL estimates for different RBC populations.

7.4  **Results**

During the study, mean (± SEM) body weight increased from 75.5± 2.0 to 89.9 ± 3.85 kg (P < 0.05). As displayed in Table 7-1, there was no statistical difference in the level of hemoglobin, Hematocrit , mean corpuscular volume (MCV), and RBC at the beginning of phase 1 to the values before the major phlebotomy (before phase 2). On the other hand, there was statistical difference (p<0.05) in the level of hemoglobin, Hematocrit, and RBC at the beginning of phase 2 (right before the major phlebotomy) to the levels at the end of the study. This provides evidence that the animals did not return to the exact steady state they started from during phase 2 of the study. This finding does not affect our results because phase 2 was analyzed without assuming steady state erythropoietic conditions. The MCV for RBCs that were in the circulation during stress environment (i.e. during the first 15 days after major phlebotomy) was significantly larger than steady state RBCs.
The survival results are summarized in Table 7-2. Examination of the survival curves for the 6 study sheep (Figures 7.4-7.7) revealed similarity in the survival characteristics of RBCs regardless to the hematopoietic environment. The MPL for steady-state RBC1 was estimated to be 129 ± 4.94 days (Figure 7.4). The MPL for steady-state RBC2 (123±6.86 days) was not significantly different from steady-state RBC1 (P>0.05) (Figure 7.5). Similarly, the MPL for stressed RBC (120±8.63 days) was not significantly different from steady state cells (P>0.05) (Figures 7.6 and 7.7).

7.5 Discussion

The result of this study does not support the hypothesis that the RBC lifespan is shortened under acute hypoxic stress conditions. The present study extends previous studies in rodents that demonstrated the shortening in the lifespan of red blood cells under stress conditions. In the present study, there is no change in RBC survival duration in either blood cells produced under hypoxic conditions or present during the hypoxic environment in the circulation.

In contrary to our results, several studies reported that the RBC lifespan is shortened under hypoxic stress conditions. A possible explanation for this observation is that all of these studies were done in rodents. The mechanism of RBC removal from the sheep circulation is primarily due to cellular senescence. Conversely, in mice red blood cells can be removed from circulation in two ways: random destruction and senescence. Random removal represents random destruction of erythrocytes irrespective of age at any time during their potential lifespan. Those cells that escape the hazard of random destruction eventually disappear, after an approximately constant lifespan, as a result of the process of senescence. Taken together, it is possible that
rodents RBC are more fragile than sheep RBC because sheep RBC are more resistant to stress conditions. As a result hypoxic stress conditions will affect the survival of rodents RBC with no significant effect on the survival of sheep RBC.

Hypoxic conditions have been reported to affect the size of the produced RBC. As presented in Table 7-1, MCV has been increased as a result of major phlebotomy which returned to baseline values at the end of the study. Larger RBC are less deformable and had shorter lifespan compared to normal RBC which can attribute to the shortened RBC survival observed in rodents. This observation has been reported to take place as a result of variety of stimuli including: hemorrhage, phenylhydrazin induced anemia, and erythropoietin treatment. The fact that these studies were done only in rodents makes the extrapolation of the results to sheep and possibly human questionable.

The relation between RBC volume and survival properties has been explored by Cart et al. They reported the production of macrocytic RBC (RBC with large volume) in response to acute hemorrhage in rabbits. Macrocytic cells disappeared at two rates, with mean survival half lives of 3.1 and 28 days, respectively. Under normal conditions, the survival of RBC followed a single rate with a mean half life of 66 days. This indicates that under acute hypoxic conditions, there is a group of rapidly disappearing macrocytic RBC (with a half life of 3.1 days). This group might have a different characteristics that the macrocytes with longer survival times. Another possible explanation, is that their might be an environmental conditions following acute hypoxic conditions that cause the rapid removal of macrocytic RBC. The survival of macrocytes produced under induced hemolytic anemia in cats was explored by Weiser et al. They also reported that there
are two subpopulations of macrocytes: one with a very short survival and a second subpopulation with normal survival.

Extrapolation of the present findings in sheep to human is supported by the similarity of the erythropoietic process between them. Sheep have often been used as an experimental model because they have similar reticulocytes and red blood cells lifespan to that of humans. The similar to sheep RBCs, the mechanism of RBC removal from human circulation is primarily due to cellular senescence. Additionally, the sites of erythropoiesis, and the types of hemoglobin produced at different developmental stages, most closely resemble the situation in humans. Moreover, erythropoiesis occurs almost exclusively in the bone marrow in both adult humans and sheep.

7.6 Limitation of the present methodology

The main limitation of the present methodology is that it cannot detect the survival properties of RBC populations that survive for less than 10-15 days. Based on previous reports there might be a group of RBC that survives for a very short time that cannot be detected using the present methodology.

7.7 Conclusion

In summary, the result of this study does not support the hypothesis that the RBC lifespan is shortened under acute hypoxic stress conditions. This finding is not inline with previous studies in rodents where a decrease in RBC lifespan was reported. However, if a group of stressed RBC is removed from the circulation in less than 10-15 days the present methodology will not be able to detect such group. Additionally, an increase in RBC volume as a result of phlebotomy is observed that might be related to the production of a new RBC population with a larger size. The present study extends previous studies in
rodents that demonstrated the shortening in the lifespan of red blood cells under stress conditions. In the present study, there is no change in RBC survival duration in either blood cells produced under hypoxic conditions or present during the hypoxic conditions.
Table 7-1. Sheep mean (± SEM) hematological parameters measured

<table>
<thead>
<tr>
<th></th>
<th>Hemoglobin (g/dL)</th>
<th>Hematocrit</th>
<th>MCV (FL)</th>
<th>RBC ($\times 10^6$/μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td>Tukey Grouping$^1$</td>
<td>Mean ± SEM</td>
<td>Tukey Grouping$^1$</td>
</tr>
<tr>
<td>At the beginning of the first phase (day 0)</td>
<td>9.53 ± 0.51</td>
<td>B</td>
<td>0.27 ± 0.01</td>
<td>B</td>
</tr>
<tr>
<td>Three weeks before major phlebotomy (day 180)</td>
<td>10.7 ± 0.65</td>
<td>B</td>
<td>0.31 ± 0.02</td>
<td>B</td>
</tr>
<tr>
<td>Immediately after major phlebotomy (day 200)</td>
<td>4.23 ± 0.33</td>
<td>C</td>
<td>0.13 ± 0.01</td>
<td>D</td>
</tr>
<tr>
<td>10-15 days after major phlebotomy (day 210-215)</td>
<td>5.95 ± 0.38</td>
<td>C</td>
<td>0.19 ± 0.01</td>
<td>C</td>
</tr>
<tr>
<td>At the end of the study (day 380)</td>
<td>12.9 ± 0.74</td>
<td>A</td>
<td>0.36 ± 0.02</td>
<td>A</td>
</tr>
</tbody>
</table>

1- Using Tukey's test, means with the same letter are not significantly different (P > 0.05) and means with different letter are significantly different (P < 0.05).
### Table 7-2. Summary of the survival analysis results

<table>
<thead>
<tr>
<th>Subject</th>
<th>Lifespan for steady-state RBC&lt;sub&gt;1&lt;/sub&gt; (days)</th>
<th>Lifespan for steady-state RBC&lt;sub&gt;2&lt;/sub&gt; (days)</th>
<th>Lifespan for stressed RBC&lt;sub&gt;3&lt;/sub&gt; (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>124</td>
<td>117</td>
<td>87.2</td>
</tr>
<tr>
<td>2</td>
<td>149</td>
<td>115</td>
<td>137</td>
</tr>
<tr>
<td>3</td>
<td>138</td>
<td>112</td>
<td>123</td>
</tr>
<tr>
<td>4</td>
<td>123</td>
<td>140</td>
<td>130</td>
</tr>
<tr>
<td>5</td>
<td>126</td>
<td>146</td>
<td>123</td>
</tr>
<tr>
<td>6</td>
<td>116</td>
<td>104</td>
<td>NA</td>
</tr>
<tr>
<td>Mean</td>
<td>129</td>
<td>123</td>
<td>120&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>SEM</td>
<td>4.94</td>
<td>6.86</td>
<td>8.63&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

1- steady-state RBC<sub>1</sub> are RBC produced and resided in the circulation under normal

2- steady-state RBC<sub>2</sub> are RBC produced under normal environment and resided in the circulation under hypoxic environment

3- stressed RBC are RBC produced and resided under hypoxic environment

4- The mean and SEM are for subjects 1-5. For subject 6, it passed away at day 85 after major phlebotomy so it was not possible to estimate lifespan
Figure 7.1. Outline of study design.
Figure 7.2. Flow cytometry histogram of a representative blood sample after the 3rd biotinylation indicating number of RBCs enumerated relative to log of fluorescent intensity for the 2 populations of biotinylated sheep RBCs. The insert illustrates the mixed model.
Figure 7.3. RBC lifespan estimation for steady state cells. The x axis intercept of the linear regression of the linear part represents the RBC lifespan.

\[ y = \text{slope} \cdot x + \text{intercept} \]

\[ L = -\frac{\text{intercept}}{\text{slope}} \]
Figure 7.4. Survival plots for the first phase in the 6 individual sheep studied. RBC survival was determined independently using 3 populations of autologous BioRBC densities.
Figure 7.5. Survival plots for the biotinylated RBCs transfused immediately after major phlebotomy.
Figure 7.6. Survival plots for the RBCs produced during 10-15 days after the major phlebotomy.
Figure 7.7. Lifespan for the 3 Biotinylated RBC populations. Life: steady-state RBC1 (RBC produced and resided in the circulation under normal environment), steady-state RBC2 (RBC produced under normal environment and resided in the circulation under hypoxic environment), and stressed RBC (RBC produced and resided under hypoxic environment). Error bars represent standard error of the mean (SEM).
CHAPTER 8. EVIDENCE OF RECEPTOR-MEDIATED ELIMINATION OF ERYTHROPOIETIN BY ANALYSIS OF EPO RECEPTOR MRNA EXPRESSION IN BONE MARROW AND ERYTHROPOIETIN CLEARANCE DURING ANEMIA

8.1 Introduction

Erythropoietin (EPO) is a glycoprotein hormone that stimulates the erythroid proliferation and differentiation. EPO exerts its erythropoietic effects through a cell surface receptor (EPOR) on the erythroid progenitor cells (BFU-Es and CFU-Es). Although EPOR expression has been reported in other cell types and tissues, the highest density of EPORs are detected on erythroid progenitor cells in bone marrow.

Binding of EPO to the cell surface induces dimerization of two EPOR molecules, which in turn initiates intracellular signal transduction required for the production of mature red blood cells (RBC). Human EPOR gene has been cloned from a placenta genomic library and characterized intensively. Different EPOR isoforms derived by alternative splicing have been reported for the human, mouse, rat and ovine EPOR genes.

Although the mechanisms involved in EPO elimination and site of degradation is still not completely understood, several studies suggest that receptor-mediated elimination of EPO plays an important central role in its clearance.

The hypothesis of an EPO elimination mechanism via non-recycled EPOR receptors is supported by several observations. Firstly, EPO’s clearance shows transient perturbations in conjunction with large transient perturbations in endogenous EPO levels. Secondly, the EPO clearance is significantly reduced following busulfan-
induced bone marrow ablation. Although these observations are compelling, they only provide indirect evidence for EPO elimination being primarily EPOR-mediated. Thus, the current study was undertaken to provide additional direct evidence of the proposed EporR-based elimination mechanisms by analysis investigating the relationship between the EPOR mRNA levels and EPO clearance. Experimentally this was done by investigating the changes in EPO clearance in association with serial, simultaneously measured EPOR mRNA levels in the bone marrow following a phlebotomy-induced anemia in lambs.

8.2 Specific aim and hypothesis

The specific aim of this Chapter is to test the hypothesis that EPO receptor (EPOR) pool size increases under hypoxic stress conditions and the change in EPOR pool size can be predicted using EPO clearance measurements.

The specific hypothesis is that stress erythropoiesis results in the up-regulation of EPOR pool size which is accompanied by a parallel increase of EPO clearance as a result of receptor mediated elimination.

8.3 Materials and Methods

8.3.1 Animals and study outline

All animal experimental procedures were approved by the University of Iowa Institutional Animal Care and Use Committee prior to the study. Eleven lambs, 3-4 weeks old, were studied. The lambs were housed in an indoor, light- and temperature-controlled environment alongside of their mothers. Jugular venous catheters used for blood sampling were inserted under pentobarbital anesthesia. Ampicilin (1g/day) was administered for the first 3 days following the catheter insertion. Phlebotomy-induced
anemia was achieved by removing 60% of the estimated total body RBC volume from the lambs with initial Hb levels (mean±standard deviation) 9.7±1.1 g/dL. This was accomplished by exchange phlebotomy where equal volumes of autologous plasma (or saline when the plasma volume was insufficient) were transfused for each volume of blood removed. Doing so decreased the lamb’s Hb level to 3.7-4.2 g/dL. For all 11 lambs a baseline EPO PK study and bone marrow aspiration procedure were performed the day before the major phlebotomy and repeated again at approximately 9 days following phlebotomy. In 6 of the 11 lambs an additional EPO PK study and bone marrow aspiration were performed approximately 28 days after the major phlebotomy (Figure 8.1) with Hb levels 9.5±1.5 g/dL. Throughout the study, whole blood samples were analyzed using a XT 2000 automated hematology system analyzer (Sysmex America Inc. Mundelien, IL).

8.3.2 PK studies

Following intravenous bolus dose of a tracer amount of \(^{125}\text{I-rhEPO}\) (less than 0.1 U rhEPO/kg), 14 blood samples were taken over the subsequent 7-hour period. Plasma concentration of \(^{125}\text{I-rhEPO}\) were analyzed by a double antibody immunoprecipitation assay as previously described with a lower level of detection of 0.004 mU/ml.  

8.3.3 Bone marrow aspirate and peripheral blood

collection and RNA isolation

Bone marrow aspirates (2 ml) were collected from the iliac crest into a 5 ml syringe containing heparin (1000 units/ml) using a sterile 18 gauge bone marrow aspiration needle. Bone marrow aspirates were transferred into 2 ml centrifuge tubes and spun at 1000g for 5 minutes after which the plasma was removed and the cell pellet was
mixed with RNAlater Solution (RiboPure™-Blood Kit, Ambion) and stored at -20°C. Total RNA was isolated from stored bone marrow samples using the RiboPure™-Blood Kit from Ambion, Inc. Samples were treated with amplification grade DNaseI (Invitrogen) to ensure the DNA free RNA. Peripheral blood samples (2 ml) were obtained simultaneously as marrow samples and RNA was isolated in a similar matter.

8.3.4 Reverse Transcription and Real-time PCR:

Complementary DNA (cDNA) was generated from 0.5 μg of total RNA using Superscript III Reverse transcriptase (Invitrogen) according to manufacturer’s protocol. Real-time PCR reactions were performed in triplicate in 384-well plates with an ABI Prism 7700 Sequence Detector (Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied BioSystems). Minus RT reactions were also run each time to test for genomic DNA contamination. Real-time PCR primers for sheep EPOR and GAPDH (house keeping gene) genes were designed using partial Ovis Aries cDNA sequences (AY029231 and U94889, respectively). All primers were designed either to span exon-exon junctions or to anneal to different exons thereby preventing genomic DNA amplification. The comparative CT method (ΔΔCT) was used to calculate relative expression of EPOR. The amplification efficiencies of EPOR primer sets (EPORI, EPORII and EPORIII) and GAPDH primer set were confirmed to be approximately equal before the study samples were analyzed.

8.3.5 Pharmacokinetic analysis

The EPO plasma concentration profile from a single intravenous bolus of 125I-rhEPO was best described by a bi-exponential distribution function. Curve fitting was performed using WINFUNFIT, a Windows (Microsoft) version evolved from the general
non-linear regression program FUNFIT.\textsuperscript{239} To account for weight change, EPO clearance values throughout this article are clearance values normalized to the bodyweight.

\subsection*{8.3.6 Statistical analysis}

The statistical analysis was conducted using the R software (Version 2.6.1; \url{http://cran.r-project.org/}). Repeated measure ANOVA was conducted to assess the effect of phlebotomy on mRNA levels and clearance values. Since our data include both fixed and random effects, repeated measure ANOVA was applied using LME (linear mixed effect) function in R (NLME library). Tukey’s test for multiple comparisons was applied using GLHT (general linear hypothesis testing) function in R (multcomp library). The following linear mixed model was used to examine the relationship between EPO clearance values and EPOR mRNA levels:

\begin{equation}
Cl_{i,j} = \alpha_i + \beta_i \cdot mRNA_{i,j} + \varepsilon_{i,j} \tag{8.1}
\end{equation}

\begin{equation}
\varepsilon_{i,j} \sim N(0, \sigma^2) \tag{8.2}
\end{equation}

\begin{equation}
\begin{pmatrix}
\alpha_i \\
\beta_i 
\end{pmatrix} \sim BVN\begin{pmatrix}
\alpha \\
\beta 
\end{pmatrix}, \begin{pmatrix}
\sigma^2_{\alpha} & 0 \\
0 & \sigma^2_{\beta} 
\end{pmatrix} \tag{8.3}
\end{equation}

where, $Cl_{i,j}$ is the $j^{th}$ EPO clearance for the $i^{th}$ subject. The $mRNA_{i,j}$ term denotes the $j^{th}$ EPOR mRNA relative value for the $i^{th}$ subject; $\alpha_i$ and $\beta_i$ are the slope and the intercept for the $i^{th}$ subject; $\varepsilon_{i,j}$ is residual error, and $\sigma^2$ is the variance of the bivariate normal distribution, BVN.

Normal distribution was used to express interindividual variability with diagonal variance covariance matrix (Eqs.8.1-8.3). An additive model was used to describe the residual error. Since absolute number of EPOR mRNA molecules cannot be ascertained by real time PCR, the relative number of EPOR mRNA molecules was derived from
cycle number of gene of interest, EPOR, and house keeping gene, GAPDH, as shown below. The relative number of EPOR mRNA molecules does not indicate the exact number of genes, instead it gives a value proportional to the actual number. While this proportionality constant cannot be determined with the current analysis, it was the same for all samples analyzed. This assumes that the efficiency of the target gene is 2.

\[ \text{mRNA}_{i,j} = 2^{-\Delta CT(Sample)} \]  

and

\[ \Delta CT = CT_{EPOR} - CT_{GAPDH} \]  

where, \( CT_{EPOR} \) is the cycle number of EPOR mRNA, and \( CT_{GAPDH} \) is the cycle number of GAPDH.

8.4 Results

Real time-PCR analyses revealed that 9 days after the major phlebotomy EPOR mRNA levels were higher than pre-phlebotomy baseline levels in all study lambs. The increases for the EPOR mRNA levels ranged between 1.2 and 12.6-fold (Table 8-1). The six lambs that had an additional bone marrow aspirate collected at day 28 post-phlebotomy had EPO mRNA levels that were all significantly lower than those measured at day 9.

As shown in Table 8-2, repeated measure ANOVA analysis of EPOR mRNA levels revealed that compared to baseline day -1, there was a significant (\( p < 0.05 \)) increase in EPOR mRNA levels by 9 days post-phlebotomy, which subsequently decreased in all 6 lambs by 28 days post-phlebotomy. There was no significant difference between EPOR mRNA levels before phlebotomy and 28 days post-phlebotomy.
These sequential changes observed in EPOR mRNA over the four-week study period paralleled those observed for EPO clearance. Ten out of 11 subjects showed an increase in EPO clearance on day 9 post-phlebotomy, decreasing toward baseline levels by day 28. The repeated measure ANOVA analysis conducted to examine the effect of phlebotomy on EPO’s clearance demonstrated that there is a significant increase in EPO’s clearance 9 days post-phlebotomy compared to both the EPO clearance before phlebotomy and 28 days post-phlebotomy (p<0.05). Similar to what was observed for EPOR mRNA levels, EPO’s clearance before the phlebotomy was not significantly different from that at 28 days post-phlebotomy (Table 8-2).

The linear mixed effect modeling of EPO’s clearance using mRNA as a covariate showed that the clearance is positively and linearly related to mRNA by a population slope of 76.8 and a population intercept of 63.1. The 95% confidence interval of the population estimate for the slope is 13.7-139 and for the intercept is 51.1-75.1. Regarding interindividual variability, the coefficient of variation as percent for the individual estimates for the slope is 125.8 and for the intercept is 51.3. The clearance values estimated by the linear mixed effect model plotted against the actual measured clearance are shown in Figure 8.2.

8.5 Discussion

The present study provides compelling new additional evidence that EPOR mRNA is up-regulated as a result of phlebotomy-induced anemia. In further support of this hypothesis is the report that the incubation of murine cell lines with EPO for 6 days increases the number of EPOR per cell without changing EPOR binding affinity. EPOR up-regulation was also reported in rat brain endothelial cell line and cortical
astrocyte cell cultures as a result of hypoxic conditions. Our observation that EPOR mRNA levels decreased towards baseline 28 days after phlebotomy relative to the 9-day sample supports our hypothesis that EPOR up-regulation is due to increased endogenous EPO levels following phlebotomy and is not a developmental growth phenomena.

Since anti-ovine EPOR antibody is not commercially available it was not possible to show the changes in EPOR protein levels. Spandou et al 2004 reported that after hypoxia-ischemia up regulation of EPOR mRNA and protein levels show very similar temporal pattern in rat brain. Therefore, EPOR protein levels are expected to show a similar pattern to EPOR mRNA levels.

Previous studies provided significant evidence that bone marrow plays a major role in EPO elimination and that EPOR is upregulated as a result of phlebotomy-induced anemia in sheep. Similarly, the present study showed an increase in EPO clearance 9 days after phlebotomy. As was observed with EPOR mRNA, the increase observed in EPO clearance is not likely a natural developmental event resulting from growth. This speculation is supported by the observation that EPO clearance decreased toward baseline by 28 days post-phlebotomy. Taken together, these findings strongly support the hypothesis that EPO elimination is largely the result of a receptor-mediated mechanism.

There was a close similarity observed in the behavior of changes in EPOR mRNA and EPO clearance throughout the study during the transition from baseline conditions followed by anemic stress conditions as a result of phlebotomy with a subsequent return towards baseline. This observation supports previously published receptor-mediated model of EPO elimination. Furthermore, results from the present linear mixed effect
model show a linear relationship between EPOR mRNA and EPO clearance. The positive slope observed is significantly different from zero. This indicates that EPO’s clearance increases linearly in parallel with the amount of EPOR mRNA in erythroid progenitor cells. The positive intercept can be explained by a simultaneous EPO elimination through a non-hematopoietic elimination pathway. Studies by other research groups also provide a substantial indirect evidence of receptor-mediated elimination of EPO. Sawyer et al demonstrated receptor-mediated elimination of EPO as a result of lysosomal degradation in erythroid cells. In rats, higher organ specific uptake of $^{125}$I-rhEPO in the bone marrow was also observed. Gross and Lodish also reported that 40% of EPO internalized in cells carrying EPOR is intracellularly degraded.

The effect of phlebotomy induced anemia on the expression of EPOR mRNA isoforms was investigated in the present project. The two EPOR mRNA isoforms were expressed significantly higher following phlebotomy, with no significant change in the ratio of the two isoform transcripts. Further studies are required to investigate whether the longer EPOR isoform is translated into a functional protein and has different characteristics than wild type EPOR.

While it is difficult to predict whether the current findings extrapolate to EPOR expressing non-hematopoietic cells, EPO has been shown to exert cardioprotective and neuroprotective effects. As mentioned above, EPOR up-regulation was also reported in rat brain endothelial cell line and cortical astrocyte cell cultures as a result of hypoxic conditions. Thus one would expect a similar regulatory effect on EPO’s neuroprotective properties.
Linear mixed model was used because it is suitable for dealing with sparse sampling and unbalanced design as in the current study. The data analyzed by the mixed model appeared challenging and several programs were tried including NONMEM and SAS both of which did not converge successfully. Fortunately, successful convergence was obtained using the LME function available in R, but the relative standard error for interindividual estimates could not be determined using R.

8.6 Conclusion

In summary, based on the analysis of data from this study and previous findings in the literature hematopoietic EPOR are expected to be up-regulated as a part of a feedback regulation under conditions of severe tissue hypoxemia, such as occurs with anemia severe enough to result in a high degree of EPO stimulation. The feedback regulation is triggered by a decrease in tissue oxygen (pO$_2$). The pO$_2$ in turn depends on different physiological variables including hemoglobin concentration, arterial pO$_2$, hemoglobin affinity to oxygen, and rate of blood flow. The decrease in pO$_2$ induces an increase in the endogenous EPO production. Increases in EPO stimulate erythropoiesis by several mechanisms that includes preventing the apoptosis of colony forming unit erythroid (CFU-E). Each CFU-E will produce 8 to 60 erythroblasts. The results of the present analysis also suggest that EPO stimulates erythropoiesis by up-regulating EPOR number per cell. Increasing EPOR number will result in an increase in the efficacy of EPO in stimulating erythropoiesis.
Table 8-1. Fold Changes in EPOR mRNA levels and EPO Clearance Pre- and Post-Phlebotomy

<table>
<thead>
<tr>
<th>Lamb ID</th>
<th>Fold change in EPOR mRNA levels relative to pre-phlebotomy</th>
<th>Fold change in EPO Clearance relative to pre-phlebotomy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-phlebotomy</td>
<td>9 days post-phlebotomy</td>
</tr>
<tr>
<td>1</td>
<td>1.0</td>
<td>12.6</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>5.0</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>9.5</td>
</tr>
<tr>
<td>4</td>
<td>1.0</td>
<td>2.8</td>
</tr>
<tr>
<td>5</td>
<td>1.0</td>
<td>3.7</td>
</tr>
<tr>
<td>6</td>
<td>1.0</td>
<td>3.5</td>
</tr>
<tr>
<td>7</td>
<td>1.0</td>
<td>1.2</td>
</tr>
<tr>
<td>8</td>
<td>1.0</td>
<td>2.4</td>
</tr>
<tr>
<td>9</td>
<td>1.0</td>
<td>2.2</td>
</tr>
<tr>
<td>10</td>
<td>1.0</td>
<td>10.2</td>
</tr>
<tr>
<td>11</td>
<td>1.0</td>
<td>1.6</td>
</tr>
</tbody>
</table>

N.D.: Not Done
Table 8-2. Repeated measure ANOVA results for EPOR mRNA levels and EPO linear clearance.*

<table>
<thead>
<tr>
<th></th>
<th>Estimated difference</th>
<th>Standard error</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{Cl}_L^2$ - $\text{Cl}_L^1$</td>
<td>32.7</td>
<td>6.16</td>
<td>&lt;0.0001**</td>
</tr>
<tr>
<td>$\text{Cl}_L^3$ - $\text{Cl}_L^1$</td>
<td>-9.3</td>
<td>7.41</td>
<td>0.42</td>
</tr>
<tr>
<td>$\text{Cl}_L^3$ - $\text{Cl}_L^2$</td>
<td>-42.0</td>
<td>7.41</td>
<td>0.0001**</td>
</tr>
<tr>
<td>$\text{mRNA}^2$ - $\text{mRNA}^1$</td>
<td>0.16</td>
<td>0.03</td>
<td>0.0001**</td>
</tr>
<tr>
<td>$\text{mRNA}^3$ - $\text{mRNA}^1$</td>
<td>0.04</td>
<td>0.04</td>
<td>0.58</td>
</tr>
<tr>
<td>$\text{mRNA}^3$ - $\text{mRNA}^2$</td>
<td>-0.12</td>
<td>0.04</td>
<td>0.01**</td>
</tr>
</tbody>
</table>

* $\text{Cl}_L^1$, $\text{Cl}_L^2$, and $\text{Cl}_L^3$ are clearance (ml/hr/kg) pre-phlebotomy, 9 days post-phlebotomy and 28 days post-phlebotomy respectively. $\text{mRNA}^1$, $\text{mRNA}^2$, and $\text{mRNA}^3$ are mRNA values pre-phlebotomy, 9 days post-phlebotomy, and 28 days post-phlebotomy.

** Indicate significant difference from zero at level of significance < 0.05
Figure 8.1. Graphical representation of the study outline
BM1: Bone marrow aspirate collection before major phlebotomy; BM2: Bone marrow aspirate collection 9 days after major phlebotomy; BM2: Bone marrow aspirate collection 28 days after major phlebotomy; PK1: Pharmacokinetic assay before major phlebotomy; PK1: Pharmacokinetic assay 9 days after major phlebotomy; PK1: Pharmacokinetic assay 28 days after major phlebotomy; n: sample numbers.
Figure 8.2. Estimated EPO clearance values using linear mixed effect model vs. actual clearance values (ml/hr/kg).
CHAPTER 9. PHARMACODYNAMIC ANALYSIS OF STRESS
ERYTHROPOIESIS: CHANGE IN ERYTHROPOIETIN RECEPTOR
POOL SIZE FOLLOWING DOUBLE PHLEBOTOMIES IN SHEEP

9.1 Introduction

Erythropoietin (EPO) is a 30.4 kD glycoprotein hormone, the primary function of which is to regulate erythrocyte production. It does so by binding to specific cell-surface receptor (EPOR) on erythroid progenitor cells located primarily in the bone marrow. This results in the differentiation and proliferation of erythroid progenitors.

Although the mechanisms involved in EPO elimination and site of degradation is still not completely understood, several studies suggest that receptor-mediated elimination of EPO plays an important role in its elimination. Previous studies in sheep provided evidence that bone marrow plays a major role in EPO elimination and that EPOR are up-regulated as a result of phlebotomy-induced anemia.

The objective of the present study in sheep is twofold: Primarily, the study is aimed at analyzing the pharmacodynamics of EPO while considering the complex, feedback-controlled dynamic changes taking place in the EPOR pool during phlebotomy-induced anemia. These events influence hemoglobin (Hb) production and are important determinants for optimizing EPO dosing. Secondarily, the study is aimed at quantifying the relative changes in the size of the EPOR pool. This is done to assess the degree of change in the erythropoietic efficacy of EPO encountered during anemia as a result of regulatory changes in the EPO receptor pool.

Although this study does not directly measure the number of EPOR in bone marrow, changes in EPO clearance provide an indirect measure of the relative changes in
the EPOR pool size. The changes in EPOR pool size are assumed to be linked to the pharmacodynamics of Hb production. To do so, a receptor-mediated disposition model for EPO was linked to the changes in the EPOR pool size.

9.2 Specific aim and hypothesis

The specific aim of this Chapter is to describe the effect of EPOR pool size changes on erythropoiesis kinetics.

The specific hypothesis is that EPOR pool size up-regulation directly affects the capacity to produce RBC and its effect can be identified using PK/PD analysis.

9.3 Materials and Methods

9.3.1 Subjects.

Surgical and experimental procedures were approved by the local institutional animal care review committee prior to the study. Five healthy 2-month-old sheep weighing 21.3±3.63 kg (mean ± SD) were studied. The animals were housed in an indoor, light- and temperature-controlled environment. Surgery was performed under pentobarbital for insertion of jugular venous catheters. Ampicillin (1 g twice a day) was administered daily for the first three postoperative days.

9.3.2 Study protocol

EPO clearance was determined following the intravenous administration of tracer doses of biologically active $^{125}$I-rhEPO ($14 \times 10^4 \pm 2.5 \times 10^4$ cpm/kg) over less than 30 seconds. This is equivalent to 0.1 U/kg EPO or less than 0.01 percent of maximum endogenous EPO concentration. Ten to 15 plasma samples were drawn over the 7–8 hour period following $^{125}$I-rhEPO dosing. To minimize erythrocyte loss, plasma was removed by centrifugation and the red blood cells (RBC) reinfused. $^{125}$I-rhEPO concentrations
were analyzed by an immunoprecipitation assay method as previously described. To establish baseline EPO clearance, two or three $^{125}$I-rhEPO PK studies were done prior to phlebotomy. A hemoglobin level of 3-4 g/dL was established by removing about 60% of the sheep’s RBCs. This was accomplished by an isovolemic exchange phlebotomy where an equal volume of saline was transfused for each volume of blood removed. Each animal underwent two separate phlebotomies 4–6 weeks apart. To determine the pattern of change in EPO clearance following phlebotomy, 7–14 $^{125}$I-rhEPO PK studies were performed following each phlebotomy. Endogenous EPO concentration was measured in triplicate using a double antibody radioimmunoassay (RIA) procedure as previously described. Linear assay values for EPO concentrations were obtained between 10 and 450mU/ml in the sheep RIA.

9.3.3 $^{125}$I-rhEPO disposition model

A mathematical model was used to describe changes in plasma $^{125}$I-rhEPO level. The model applied is based on a tracer interaction method, TIM, combined with disposition decomposition analysis, (Eq.9.1). $C_a(t)$ is the concentration of the $^{125}$I-rhEPO tracer in the blood plasma being sampled, and $C_b(t)$ is the concentration of endogenous EPO.

$$\frac{dC_a(t)}{dt} = -(K + \frac{V_m(t)}{K_m + C_b(t) + C_a(t)}) \cdot C_a(t) + G \cdot e^{-\gamma t} \ast C_a(t)$$

(9.1)

where K is the first order constant of the linear elimination pathway, $V_m$ and $K_m$ are the Michaelis-Menten parameters of the nonlinear elimination pathway, and $G$ and $\gamma$ are distribution function parameters, while $\ast$ denotes convolution.

Since $C_a \ll C_b$ due to the use of tracer doses, Eq.9.1 can be simplified into:
\[
\frac{dC_a(t)}{dt} = -(K + \frac{V_m(t)}{K_m + C_b(t)} + \frac{G}{\gamma}) \cdot C_a(t) + G \cdot e^{-\gamma t} \cdot C_a(t)
\]  (9.2)

Thus, the model proposes that the rate of irreversible elimination of EPO from the sampling compartment occurs via two parallel routes, namely a nonlinear elimination route demonstrating Michaelis-Menten kinetic behavior (represented by parameters \(V_m\) and \(K_m\)), and a linear elimination route demonstrating first order elimination kinetic behavior (rate parameter \(K\)). This elimination kinetics model is consistent with our previous finding that EPO is eliminated nonlinearly via a receptor mediated erythropoietic mechanism by the bone marrow and linearly via a non-erythropoietic mechanism.\(^{89,248}\)

The EPO plasma concentration profile from a single intravenous \(^{125}\)I-rhEPO tracer bolus dose was well described by a biexponential disposition function. The \(^{125}\)I-rhEPO clearance \((Cl(t))\) was calculated using Eq.9.3.

\[
Cl(t) = \frac{D}{AUC}
\]  (9.3)

where \(D\) is the \(^{125}\)I-rhEPO dose and the AUC is the area under the curve for the concentration of \(^{125}\)I-rhEPO time calculated from the biexponential disposition function.

Despite the fact that the clearance function, \(Cl(t)\), presented throughout this work is determined from the biexponential disposition function, a mechanistic interpretation of the \(Cl(t)\) value can still be made based on the model presented in Eq.9.2. Assuming that the concentration of unlabeled EPO \((C_b(t))\) was constant over the relatively short duration of the \(^{125}\)I-rhEPO PK study (7-8 hrs), it follows from Eq.9.2 that the clearance of EPO at the time of \(^{125}\)I-rhEPO PK study can be represented parametrically as shown in Eq.9.4.
EPO's volume of distribution is denoted by $V_{EPO}$. The time in Eqs. 9.1 and 9.2 is relative to the administration of $^{125}$I-rhEPO in the tracer PK study, while the time for the remaining equations is measured relative to the beginning of the study.

\[ Cl(t) = Cl_{NR} + Cl_R(t) \]  \hspace{1cm} (9.5)

\[ Cl_{NR} = K \cdot V_{EPO} \]  \hspace{1cm} (9.6)

\[ Cl_R(t) = \frac{V_m(t)}{K_m + C_b(t)} \cdot V_{EPO} \]  \hspace{1cm} (9.7)

Since previous studies demonstrated that phlebotomy did not have a significant effect on non-receptor mediated clearance,\textsuperscript{58} $Cl_{NR}$ that was assumed to be constant (scaled to the bodyweight) during all PK studies. Receptor mediated clearance on the other hand was assumed to change over study period as a result of changes in number of EPO receptors reflected in the changing value of $V_m(t)$. Based on an assumed proportional relationship between the EPOR pool size and $V_m(t)$, the relative value of EPOR can be described as shown in Eq.9.8.

\[ \frac{EPOR(t)}{EPOR(0)} = \frac{V_m(t)}{V_m(0)} = \frac{Cl_R(t) \cdot (K_m + C_b(t))}{Cl_R(0) \cdot (K_m + C_b(0))} \]  \hspace{1cm} (9.8)

$Cl_{NR}$ was fixed to 6.58 mL/kg/hr as previously determined.\textsuperscript{58} Likewise, the value of $K_m$ was fixed to 523 mU/mL based on our previous analysis.\textsuperscript{248}
9.3.4 Pharmacodynamic Hb mass balance model

Hb production is stimulated by EPO through a stimulation function \( f_{\text{stim}}(t) \):

\[
f_{\text{stim}}(t) = \begin{cases} 
  k_{\text{stim}} \cdot (Wt(t))^{3/4} & \text{if } t \leq 0 \\
  \frac{EPOR(t) \cdot E_{\text{max}} \cdot C_b(t)}{EPOR(0) \cdot EC_{50} + C_b(t)} \cdot (Wt(t))^{3/4} & \text{if } t > 0 
\end{cases}
\]

where \( k_{\text{stim}} \) is the Hb production stimulation rate constant at the start of the study, \( Wt(t) \) is the body weight, \( E_{\text{max}} \) is the maximum Hb production rate scaled to the bodyweight, \( EC_{50} \) is the EPO concentration that results in Hb production rate that is 50% of the scaled \( E_{\text{max}} \).

To account for body weight change, Hb production was assumed to be proportional to the body weight to the power \( \gamma_4 \). \( \text{Hb} \) production before the beginning of the study \((t < 0)\) was assumed to be constant scaled to body weight to the \( \gamma_4 \) power. Hb production rate after the beginning of the study \((t > 0)\) was related to plasma EPO concentrations by a Michaelis-Menten \( E_{\text{max}} \) model. To account for change in number of EPO receptors, Hb production rate was scaled to the relative change in number of receptors by multiplying with the ratio \( \frac{EPOR(t)}{EPOR(0)} \) as shown in Eq. 9.9.

The Hb production model assumed that RBCs have a time invariant lifespan \( (\tau_{RBC}) \) with no variability (i.e. point distribution). \( \text{Hb} \) produced endogenously is then given by:

\[
\frac{dHb(t)}{dt} = f_{\text{stim}}(t-a) - f_{\text{stim}}(t-b)
\]

with initial conditions:
\[ Hb(0) = \int_{t-a}^{t-b} f_{stim}(u) \, du \bigg|_{t=0} = \int_{0-a}^{0-b} f_{stim}(u) \, du \]

where \( a \) is the time between erythroid progenitor cells stimulation by EPO and the appearance of newly produced RBCs in the circulation, and \( b \) is the time between erythroid progenitor cells stimulation by EPO and the removal of the resulting RBCs from circulation by senescence. The RBCs lifespan \( \tau_{RBC} \) is the difference \( b - a \).

In the above model, RBCs were assumed to be removed only by aging when they reach the lifespan. Phlebotomy on the other hand, represents a removal of cells of all ages up to \( \tau_{RBC} \). The time interval over which the phlebotomy affects Hb mass balance depends on the lifespan of RBCs. Assuming \( m \) phlebotomies that occurred over a time period equal to the lifespan of RBCs, the fraction of Hb that remains in circulation after the \( i^{th} \) phlebotomy, denoted \( F_p(i) \), that occurred at time \( t_p(i) \) is given by Eq. 9.11.

Thus, \( F_p(i) \) is the fraction of Hb remaining immediately after the \( i^{th} \) phlebotomy relative to the amount present immediately before the \( i^{th} \) phlebotomy. The phlebotomy correction term at time \( \text{“PC}(t)\)" is the multiplication of fractions remaining for all phlebotomies that occurred between \( t - \tau_{RBC} \) and \( t \).

\[ F_p(i) = 1 - \frac{A_p(t_p(i))}{Hb(t_p(i))} \quad (9.11) \]

where \( A_p(t_p(i)) \) is the amount phlebotomized at \( t_p(i) \)

\[ PC(t) = \prod_{1}^{m} F_p(i) \quad (9.12) \]

where \( t_p(1) \geq t - \tau_{RBC} \) and \( t_p(m) < t \)
\[
\frac{dHb(t)}{dt} = f_{stim}(t-a) - PC(t) \cdot f_{stim}(t-b)
\]  \hspace{1cm} (9.13)

with initial conditions:

\[
Hb(0) = \int_{t-b}^{t-a} f_{stim}(u) \, du \bigg|_{t=0} = \int_{0-b}^{0-a} f_{stim}(u) \, du
\]

The estimated amounts of Hb in the circulation were converted into observed concentrations by dividing by the total blood volume \((V_{Hb}^{total})\). In order to account for blood volume expansion as a result of growth, total blood volume was adjusted according to the body weight:

\[
V_{Hb}^{total}(t) = W(t) \cdot V_{Hb}
\]  \hspace{1cm} (9.14)

### 9.3.5 Sheep body weight

Sheep body weight during the study was estimated using a 4th order polynomial. To estimate the body weight before the beginning of the study and after birth \((-60 < t \leq 0 \text{ days})\), which is needed to calculate \(f_{stim}(t)\), the growth rate (denoted by \(k_{growth}\)) over the first two months of life \((t<60 \text{ days})\) was estimated separately from five lambs. In these lambs, body weight values were collected over the first 50-60 days of life. The average value of \(k_{growth}\) estimated in these lambs was used to estimate body weight for the current sheep subjects before the beginning of the study and after birth \((-60 < t \leq 0 \text{ days})\). To account for pre-birth body weight which is needed to calculate \(f_{stim}(t)\) for \(t<60 \text{ days}\), an exponential function that describes the dynamics of in utero growth was used. \(^{194}\) The explicit equation for body weight is displayed in Eq.9.15.
\[
W_t(t) = \begin{cases} 
  a_0 + a_1 t + a_2 t^2 + a_3 t^3 + a_4 t^4 & \text{for } t > 0 \\
  a_0 + k_{\text{growth}} t & \text{for } -60 < t \leq 0 \\
  wt(t = -60) \cdot \frac{e^{(0.1281 - 0.00038(G_{\text{AGE}} + t + 60))}}{e^{(0.1281 - 0.00038(G_{\text{AGE}} + 60))}} & \text{for } t \leq -60 
\end{cases}
\]

where \(a_0, a_1, a_2, a_3,\) and \(a_4\) are 4th order polynomial parameters and \(G_{\text{AGE}}\) is the gestational age of the term lamb which was fixed to be 150 days.\(^{195, 196}\)

### 9.3.6 Data analysis

All modeling was conducted using WINFUNFIT, a Windows (Microsoft) version evolved from the general nonlinear regression program FUNFIT,\(^{250}\) using ordinary least squares fit to each individual subject’s Hb concentration-time profile. The amount of Hb removed and administered by each phlebotomy, at the time of removal was accounted for by WINFUNFIT using a generalized events processing module. The events processing module integrates the differential equation exactly up to the time of the event and adding or removing the appropriate amount and then continuing integrating the differential equation with new initial conditions set at the appropriate event times. The EPO plasma concentrations were nonparametrically represented using a linear spline function.

To summarize the uncertainty in the individual subject parameter estimates, the relative standard error (RSE\%) of the estimate was calculated for each parameter as:

\[
\text{RSE\%} = \frac{\text{SE}}{\text{P}} \cdot 100
\]

where SE and P are the standard error of the parameter and the estimate of the parameter, respectively.
9.4 Results

The Hb mass balance model fit along with plasma EPO concentration for a representative sheep are shown in Figure 9.1. The model accurately captures the general behavior of the Hb concentration data \( r = 0.95 \pm 0.035 \). The pharmacodynamic model parameters are summarized in Table 9-1.

The average value of \( k_{\text{growth}} \) that describes growth over the first two months of life (t<60 days) was estimated to be 0.285±0.027 kg/day. As displayed in Figure 9.2, the body weight function described accurately the body weight data over the first two months of life (average \( R^2 = 0.996 \), range 0.989-0.998). The body weight data presented in Figure 9.2 represent data over the first two months of life for lambs from different study protocol. This data was included in the analysis to describe the growth over the first two months of life. This information was used to describe Hb production before the beginning of the study (Eq.9.9 and Eq.9.15).

Individual sheep’s Hb concentration-time profiles contained on average 76 Hb values (range 71 to 83), 92 plasma EPO concentrations (range 82 to 107) and 23 bodyweight data time points (range 19 to 32). The body weight measurements are well represented by a 4th order polynomial (Figure 9.3). At the beginning of the study, the baseline Hb was 10.4±2.05 g/dL, the EPO concentration average was 22.7±11.8 mU/ml, the clearance average value was 48.2±10.9 ml/kg/hr, and the average weight was 21.3±3.63 kg. At the end of the study, the Hb average was 9.8±1.55 g/dl, EPO concentration was 18.4±4.72 mU/ml, the clearance average value was 39.2±6.03 ml/kg/hr, and body weight was 32.2±4.58 kg. Paired t-test results indicate that there is no significant difference in the value of Hb, EPO concentration, and clearance between the
values reported at the beginning of the study and at the end of the study (p>0.05). The body weight of the sheep was significantly higher at the end of the study compared to the beginning of the study (p<0.05). Thus, there is a need to account for animal growth in the model.

The newly produced Hb appears in the circulation 2.14±0.95 days after stimulation by erythropoietin. The lifespan of RBCs ($\tau_{RBC} = b - a$, Eq.9.12) was estimated to be 92.7±21.3 days and the bodyweight scaled Hb production constant, $k_{stim}$, was determined to be 0.48±0.12 g/day.kg$^{3/4}$. The Michaelis-Menten $E_{max}$ model parameters were successfully evaluated for all sheep. The predicted maximum achievable Hb production rate, $E_{max}$, was found to be 1.34±0.20 g/day.kg$^{3/4}$, and $EC_{50}$ was found to be 60.5±26.7 mU/mL.

9.5 Discussion

This study provides a quantitative estimation of the rate of Hb synthesis in sheep and considers its functional and mechanistic relationship to the EPO plasma concentration, while also accounting for other important confounding variables, including change in number of EPOR, phlebotomy, and growth. Because of its importance for EPO efficacy and dosing, special attention is focused on the regulatory kinetic mechanism of the EPOR pool size. In contrast to other previous models that describe erythropoiesis,$^{145,251}$ the current model explicitly accounts for changes in the number of EPOR as a result of up-regulation while simultaneously accounting for continuous growth and the expression of blood volume and the volume of distribution expansion as a function of the increase in body weight.
Chapel et al proposed a model that describes the change in the density of EPOR in the form of change in EPO clearance values.\textsuperscript{90} Moreover, a positive linear relation between EPO clearance and EPOR mRNA level has been demonstrated as presented in the previous Chapter.\textsuperscript{247} In this Chapter, information obtained from previous work about EPOR and clearance changes were utilized to describe erythropoiesis under stress conditions. Although other models implicitly accounted for EPOR changes in the form of negative feedback inhibition of progenitor cells production, the previous work did not provide any measure of the change in EPOR density.\textsuperscript{252}

9.5.1 EPO clearance as a measure of EPOR pool size

The clearance parameters obtained by the analysis provides an indirect quantification of the EPOR populations under the well supported assumption that EPO’s elimination is largely via EPORs. The recent study by Nalbant et al (Chapter 8) has shown that the change in EPOR pool size can be estimated as a linear function of the EPO clearance.\textsuperscript{247} From this finding it follows that the quantity of EPOR is proportional to $V_m$ (Eq. 9.8). The pattern of change in EPOR shown in Figure 9.1 was consistently observed for all five study animals after both phlebotomies. The decrease in EPOR that coincides with the increase in the EPO concentration can be explained by a temporary consumption with regulatory feedback correction and the findings from molecular biology investigations that some EPOR are not recycled after interaction with EPO.\textsuperscript{62, 253} Since $V_m(t)$ is proportional to the quantity of EPOR the subsequent increase in $Cl_R(t)$ and $V_m(t)$ indicates an up-regulation of EPOR.

Several studies have demonstrated EPOR up-regulation as a result of exposure to high EPO levels and/or under hypoxic conditions. It was reported that the incubation of
murine cell lines with EPO for 6 days increases the number of EPOR per cell without changing EPOR binding affinity. Bernaudin et al also reported EPOR up-regulation in rat brain endothelial cell line and cortical astrocyte cell cultures as a result of hypoxic conditions.

**9.5.2 RBC Pharmacodynamic Parameters**

The estimated mean value for the lag time, $\alpha$, between erythroid progenitor cells stimulation by EPO and the appearance of newly produced RBCs in the circulation was 2.14 days. This is a higher estimate than the previously reported value in sheep of 0.797 days. This may be explained by the facts that the previous analysis included a peripheral effect site compartment, while the current analysis does not. Also, the lag time was calculated under steady-state conditions while recognizing that the lag time increases under stress conditions, but it was not correct for in this project. The average of individual RBC lifespans ($\tau_{RBC} = b - a$) was found to be 92.7 days compared to the reported value of 120 days in adult sheep.

**9.5.3 EPOR regulatory changes, EPO efficacy and EPO dosing strategy for treatment of anemia.**

It is evident from examining the relative change in the size of the EPOR pool, $\frac{\text{EPOR}(t)}{\text{EPOR}(0)}$ (Figure 9.1) that an up-regulation due to anemia by a factor close to 2 is possible relative to the basal EPOR level and that the lowest and highest EPOR pool sizes can differ by a factor close to 4 ($=2/0.5$). Since according to mass balance principles the rate of progenitor activation is expected to be proportional to the quantity of EPOR, these changes in the EPOR pool size will significantly influence the efficacy of EPO. Such changes need to be considered when formulating an EPO dosing optimization
algorithm. Due to the great similarity of the erythropoietic physiology between mammalian species it is anticipated that a similar EPOR regulatory mechanism model exists in humans. Thus, the kinetic model and mechanism proposed in this pre-clinical study may serve as a starting point for the development of an optimal EPO dosing algorithm to that of neonatal anemia. Because of the mathematical complexity of the present regulatory model, a complete dosing optimization will require extensive computational analysis. However, the simple core principles of the model provide several simple guiding principles: Accordingly, an optimal dosing strategy should in general maximize the interaction between EPO and EPOR. Specifically, EPO should be administered when the number of EPOR are close to maximally up-regulated. Similarly, in recognizing a temporary loss of EPOR following an initial dosing, the subsequent dosing should, to achieve the highest efficacy, be postponed until the EPOR again have increased by the regulatory feed-back control. Several studies have demonstrated that subcutaneous administration of EPO is more efficacious than intravenous dosing,\textsuperscript{113, 254, 255} which according to our model can be explained by the fact that the duration of high plasma EPO concentration is longer in subcutaneous than intravenous administration, thus allowing the exogenous EPO to still be present after the EPORs are up-regulated.

9.6 Conclusion

The present study and previous findings in the literature demonstrates that under conditions of severe tissue hypoxemia hematopoietic EPOR are up-regulated as a part of a feedback regulation. The EPOR pool size, in turn, determines the magnitude of response to EPO in stimulating erythrocyte production. Frequent estimation of EPO clearance as a measure of EPOR pool size and the use of mass balance principles allowed
for the mathematical determination of the in vivo erythropoiesis rate and its relationship to endogenous plasma EPO concentrations under severe hypoxemic conditions. The proposed mechanistically oriented EPO’s PK/PD model could be used as a valuable tool for a better elucidation of EPO’s complex erythropoietic effect and for designing an optimal dosing for EPO to maximize its erythropoietic effect.
Table 9-1. A summary of parameter estimates from the hemoglobin mass balance model

<table>
<thead>
<tr>
<th>Sheep No.</th>
<th>$\alpha$ (day)</th>
<th>$\tau_{\text{RBC}}$ (day)</th>
<th>$E_{\text{max}}$ (g/day.kg$^{0.75}$)</th>
<th>EC$_{50}$ (mU/mL)</th>
<th>$k_{\text{stim}}$ (g/day.kg$^{0.75}$)</th>
<th>$r^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.95</td>
<td>16.5</td>
<td>104 (RSE%$^a$)</td>
<td>1.37 (RSE%$^a$)</td>
<td>97.8 (RSE%$^a$)</td>
<td>0.41 (RSE%$^a$)</td>
</tr>
<tr>
<td>2</td>
<td>2.26</td>
<td>13.3</td>
<td>84.5 (RSE%$^a$)</td>
<td>1.61 (RSE%$^a$)</td>
<td>70.4 (RSE%$^a$)</td>
<td>0.63 (RSE%$^a$)</td>
</tr>
<tr>
<td>3</td>
<td>3.55</td>
<td>8.84</td>
<td>68.8 (RSE%$^a$)</td>
<td>1.36 (RSE%$^a$)</td>
<td>57.1 (RSE%$^a$)</td>
<td>0.33 (RSE%$^a$)</td>
</tr>
<tr>
<td>4</td>
<td>1.71</td>
<td>17.9</td>
<td>124 (RSE%$^a$)</td>
<td>1.28 (RSE%$^a$)</td>
<td>24.6 (RSE%$^a$)</td>
<td>0.49 (RSE%$^a$)</td>
</tr>
<tr>
<td>5</td>
<td>2.22</td>
<td>13.3</td>
<td>82.7 (RSE%$^a$)</td>
<td>1.05 (RSE%$^a$)</td>
<td>52.4 (RSE%$^a$)</td>
<td>0.56 (RSE%$^a$)</td>
</tr>
<tr>
<td>Mean</td>
<td>2.14</td>
<td>14.0</td>
<td>92.7 (RSE%$^a$)</td>
<td>1.34 (RSE%$^a$)</td>
<td>60.5 (RSE%$^a$)</td>
<td>0.48 (RSE%$^a$)</td>
</tr>
</tbody>
</table>

| CV (%)    | 44.4           | 25.1           | 23.1 (RSE%$^a$)               | 15.1 (RSE%$^a$) | 44.2 (RSE%$^a$)               | 24.5 (RSE%$^a$) |

$^a$ RSE%, relative standard error; $^b$ r, correlation coefficient.
Figure 9.1 Representative individual subject fits of model equations to observed plasma hemoglobin, total clearance, erythropoietin, and EPOR fraction
Figure 9.2. Bodyweight values over the first two months of life estimated using linear function \( wt = \alpha_0 + k_{\text{growth}} \cdot t \) vs. actual bodyweight values for all lambs.
Figure 9.3. Bodyweight over the study period in a representative lamb. The curve fitted to the lamb bodyweight is a $4^{th}$ order polynomial.
CHAPTER 10. SUMMARY AND CONCLUSIONS

The overall hypothesis of this work is that treatment optimization of erythropoietin, particularly under dynamic hematological conditions such as stress erythropoiesis and conditions experienced in premature infants, requires a comprehensive knowledge of the behavior of erythrocytes and the complex pharmacokinetic/pharmacodynamics (PK/PD) relationship between erythropoietin (EPO) and erythropoiesis.

The research presented in this dissertation explored three main aspects of erythropoiesis in preterm infants and neonatal sheep under stress hypoxic conditions: the effect of EPO on red blood cell (RBC) production (Chapters 2-4), the effect of hypoxic stress conditions on RBC survival (Chapter 5-7), and the change in EPO receptor (EPOR) under stress conditions (Chapters 8-9).

In Chapter 2, population PK/PD modeling was applied to describe erythropoiesis in preterm infants. The relationship between RBC production rate and EPO concentration was well described by an $E_{\text{max}}$ model. Analysis results indicated that using recombinant human EPO (rhEPO) preterm infants can produce an additional amount of RBC that is on average twice what they produce without the use of rhEPO. Additionally, the cumulative amount of RBC loss as a result of frequent sampling was found to be equivalent to 50% of the amount of RBC transfused.

In Chapter 3, several clinical covariates were tested for their ability to identify preterm infants with good EPO responsiveness. Covariate analysis suggested that the erythropoietic efficacy of EPO is increased for preterm infants at later gestational ages.
This resulted in an approximately seven-fold difference in the maximum Hb production rate for the gestational age range of 24-29 weeks.

Newborn lamb showed significant similarities to preterm infants regarding their erythropoietic characteristics. This was addressed by applying the model developed in Chapter 2 to newborn lambs. Results indicate that newborn lambs have more potential in producing RBC. However, the PD analysis indicates that newborn lambs have similar sensitivity to EPO in producing RBC compared to preterm infants. The value of the Hb production before birth in newborn lambs was found to be similar to the value estimated in preterm infants as presented in Chapters 2 and 4. The value of blood volume estimated in newborn lambs was comparable to previous estimates of blood volume in preterm infants (98.1 mL/kg in newborn lambs vs. 93.2 mL/kg in preterm infants).

The effect of stress conditions on RBC survival was addressed in Chapters 5-7. In Chapter 5, a summary of direct methods for RBC survival measurement was presented. The limitation of these methods that they can be used only under steady state conditions was also addressed. To solve this limitation a new methodology was demonstrated in Chapter 6 by which the survival of RBC under non steady state conditions (such as under stress conditions) was measured. The analysis presented in Chapter 7 tested the hypothesis that stress conditions affect the survival of RBC. It was found that stress conditions did not affect the survival of RBC under stress conditions in sheep.

In Chapter 8, additional evidence that EPOR mRNA is up-regulated as a result of phlebotomy-induced anemia was presented. This increase of EPOR mRNA level was accompanied by a parallel increase in EPO clearance. This finding indicates that EPO is
eliminated by receptor mediated elimination. Additionally, EPO clearance value can be used to predict the degree of EPOR mRNA upregulation.

Based on the finding that EPOR are upregulated under stress conditions (Chapter 8) as a part of a feedback regulation. The EPOR pool size, in turn, determines the magnitude of response to EPO in stimulating erythrocyte production. A new formulation of the model proposed in Chapter 2 was presented in Chapter 9. The new formulation extended the model to account for EPOR upregulation under stress conditions. Frequent estimation of EPO clearance as a measure of EPOR pool size and the use of mass balance principles allowed for the mathematical determination of the in vivo erythropoiesis rate and its relationship to endogenous plasma EPO concentrations under severe hypoxemic conditions. The proposed EPO’s PK/PD model could be used as a valuable tool for a better elucidation of EPO’s complex erythropoietic effect and for designing an optimal dosing for EPO to maximize its erythropoietic effect.
APPENDIX A. MATLAB CODES FOR CHAPTERS 2 AND 3

****Begin prologue

This Matlab code was compiled to Monolix to model the erythropoiesis kinetics as described in Chapters 2 and 3. The instruction of running this script with Monolix are summarized in “Instructions.doc” file located in each of the folders “Chapter 2” and “Chapter 3” in the accompanying CD.

The analysis involves the following files in CD in the folder with the corresponding Chapter number (i.e. Chapter 2 and Chapter 3):

1. Monolix script: \Model\Basic_MODEL.m
2. Hb and RBC data file: \data\HB_RBC_27_COVARIATE.csv
3. EPO data file: \data\INFANT_EPO_27.txt
4. Body weight data file: \data\INFANT_WT_27.txt
5. Gestational age data file: \data\INFANT_GEST_AGE.txt
6. Output folder: \Results

****End prologue

function f = Basic_Model( phi, Id, X )
%Structural MULTIPLE model Basic_Model
%Baseline model no covariates
%
%Model information:
% # outputs = 2
% # regression variables = 1
% # parameters = 13
% # differential equations = 2
%
%f = Basic_Model(phi,Id,X) returns a cell array of size 2
% phi (N x 13) individual parameters.
%
% X{1}, X{2} structures with regression data
% obs (n x 1) = regression variables: x
% newtime = union of all the observation times
%
% Id{1}, Id{2} structures with individual index informations
% obs (n x 1) = individual index for each observation
% Nbs (N x 2) = number of doses (0 if none) and observations per subject
% id_t (n x 1) = indexes of observation times into the union (X.t_obs_u)
%
% where
% N = number of subjects
% n = number of observations
%
% if no arguments are given then we return the informations of the model
persistent iternum tti cputold tempwt tempepo tempgage TRAN PHLEB u_EPO u_WT
GEST_AGE N;
NSUB=27;
N = size(phi,1);
try
    phi;
catch
    f=getdata;
    return
end
XX.dose=X{1}.dose;
if isempty(iternum)
    iternum=1;
    tti=0;
    cputold=cputime;
    tempwt=uiimport('-file');
    tempepo=uiimport('-file');
    tempgage=uiimport('-file');

%SET EPO, WT AND GESTATIONAL AGE VECTORS
%SET EPO VECTOR
u_EPO.ID=zeros(numel(tempepo.ID));
u_EPO.X=zeros(numel(tempepo.ID));
u_EPO.Y=zeros(numel(tempepo.ID));
u_EPO.ID=tempepo.ID;
u_EPO.X=tempepo.TIME;
u_EPO.Y=tempepo.EPO;
%SET WT VECTOR
u_WT.ID=zeros(numel(tempwt.ID));
u_WT.X=zeros(numel(tempwt.ID));
u_WT.Y=zeros(numel(tempwt.ID));
u_WT.ID=tempwt.ID;
u_WT.X=tempwt.TIME;
u_WT.Y=tempwt.WT;
%    for d=1:size(phi,1),
for d=1:NSUB,
    aa=(u_WT.ID==d);
    u_WT.P(d,:)=polyfit(u_WT.X(aa),u_WT.Y(aa),4);
end
%SET GESTATIONAL AGE
GEST_AGE=tempgage.INFANT_GEST_AGE;
%TO SET TRANSFUSION and PHLEBOTOMY
jj=1;
ff=1;
%TRANSFUSIONS
TRAN.ID=zeros(sum((XX.dose(:,2))>0),1);
TRAN.T=zeros(sum((XX.dose(:,2))>0),1);
TRAN.A=zeros(sum((XX.dose(:,2))>0),1);
PHLEB.ID=zeros(sum((XX.dose(:,2))<0),1);
PHLEB.T=zeros(sum((XX.dose(:,2))<0),1);
PHLEB.A=zeros(sum((XX.dose(:,2))<0),1);

TRAN.ID(:)=Id{1}.dose((XX.dose(:,2))>0);
TRAN.T(:)=XX.dose((XX.dose(:,2))>0,1);
TRAN.A(:)=XX.dose((XX.dose(:,2))>0,2);

%PHLEBOTOMY
PHLEB.ID=zeros(sum((XX.dose(:,2))<0),1);
PHLEB.T=zeros(sum((XX.dose(:,2))<0),1);
PHLEB.A=zeros(sum((XX.dose(:,2))<0),1);
PHLEB.F=ones(sum((XX.dose(:,2))<0),1);
PHLEB.ID(:)=Id{1}.dose((XX.dose(:,2))<0);
PHLEB.T(:)=XX.dose((XX.dose(:,2))<0,1);
PHLEB.A(:)=XX.dose((XX.dose(:,2))<0,2);
PHLEB.F(:)=1;
else
% TO SET TRANSFUSION and PHLEBOTOMY
% TRANSFUSIONS
TRAN.ID=zeros(sum((XX.dose(:,2))>0),1);
TRAN.T=zeros(sum((XX.dose(:,2))>0),1);
TRAN.A=zeros(sum((XX.dose(:,2))>0),1);
TRAN.ID(:)=Id{1}.dose((XX.dose(:,2))>0);
TRAN.T(:)=XX.dose((XX.dose(:,2))>0,1);
TRAN.A(:)=XX.dose((XX.dose(:,2))>0,2);

%PHLEBOTOMY
PHLEB.ID=zeros(sum((XX.dose(:,2))<0),1);
PHLEB.T=zeros(sum((XX.dose(:,2))<0),1);
PHLEB.A=zeros(sum((XX.dose(:,2))<0),1);
PHLEB.F=ones(sum((XX.dose(:,2))<0),1);
PHLEB.ID(:)=Id{1}.dose((XX.dose(:,2))<0);
PHLEB.T(:)=XX.dose((XX.dose(:,2))<0,1);
PHLEB.A(:)=XX.dose((XX.dose(:,2))<0,2);
PHLEB.F(:)=1;
end
i_time = X{1}.i_fullobs;
ids = Id{1}.fullobs;
u_time = X{1}.newtime;
AA = phi(:,1);
BB = phi(:,2);
LIFE_SPAN_TRAN = phi(:,3);
FR_UNDAMAGED = phi(:,4);
BLOOD_VOL = phi(:,5);
MRS = phi(:,6);
VMAX = phi(:,7);
KM = phi(:,8);
WTP1 = phi(:,9);
WTP2 = phi(:,10);
BASELINE = phi(:,11);
MCHB_INFANT = phi(:,12);
MCHB_TRAN = phi(:,13);

N = size(phi,1);
DOSE=X{1}.dose;% Dose
tD = DOSE(:,1);
D = DOSE(:,2);
nb_outputs = 2;
nb_ode = 2;
% Write here your initial conditions.
A0=zeros(2,N);
A=zeros(numel(ids),nb_ode);
KIU2=zeros(1,N);
b=0;
for i=1:N,
global CBV CEPO CWTP CWTP1 CWTP2 G_AGE CMRS K_IU EMAX C50
LIFESPAN CWTX CWTY CMCHBI CMCHBT CAA CBB CFR_UNDAMAGED
CMCHBI CMCHBT CBASELINE
if (rem(i,NSUB)>0)
  si=rem(i,NSUB);
else
  si=NSUB;
end
%EPO
ee=(u_EPO.ID==si);

CEPO.X=u_EPO.X(ee);
CEPO.Y=u_EPO.Y(ee);
%WT

aa=(u_WT.ID==si);
CWT.X=u_WT.X(aa);
CWT.Y=u_WT.Y(aa);
CWT.P=polyfit(CWT.X,CWT.Y,4);
CWTX=u_WT.X(aa);
CWTY=u_WT.Y(aa);
CWTP = CWT.P;

% PARAMETERS
CFR_UNDAMAGED = FR_UNDAMAGED(i);
LIFESPAN = LIFE_SPAN_TRAN(i);
CAA = AA(i);
CBB = BB(i);
CWTP1 = WTP1(i);
CWTP2 = WTP2(i);
G_AGE = GEST_AGE(si);
CMRS = MRS(i);
EMAX = (VMAX(i));
C50 = (KM(i));
CMCHBI = MCHB_INFANT(i);
CMCHBT = MCHB_TRAN(i);
CBASELINE = BASELINE(i);
CBV = (BLOOD_VOL(i));

% INITIAL CONDITION
A0(1,i) = CBASELINE * (BLOOD_VOL(i) * WEIGHT(0));
Options = optimset('MaxFunEvals', 100000);
K_IU = fminbnd(@(myfun0, 0, 1);
KIU2(1,i) = K_IU;
A0(2,i) = A0(1,i)/CMCHBI;
global CTRANS CPHLEB;
% set transfusion
jj = (TRAN.ID == i);
CTRANS.T = TRAN.T(jj);
CTRANS.A = TRAN.A(jj);
hh = (PHLEB.ID == i);
CPHLEB.T = PHLEB.T(hh,:);
CPHLEB.A = PHLEB.A(hh,:);
CPHLEB.F = PHLEB.F(hh,:);
ii = (Id{1}.dose == i);
tDi = tD(ii);
nb_dosei = numel(tDi);
DOSEi = DOSE(ii,:);
timek = u_time(i_time(ids == i));
nobs = sum(timek < tDi(1));
% A(nb_ + (1:nobs),:) = A0(i*ones(nobs,1),:);
for we = 1:nobs
    A(nb_ + we,1) = A0(1,i)/(BLOOD_VOL(i) * WEIGHT(timek(we)));
    A(nb_ + we,2) = A0(2,i)/(BLOOD_VOL(i) * WEIGHT(timek(we)));
end
A0(1,i) = A0(1,i) + DOSEi(1,2);
if (DOSEi(1,2) < 0)
A0(2,i)=A0(2,i)+DOSEx(1,2)/CMCHBI;
else
A0(2,i)=A0(2,i)+DOSEx(1,2)/CMCHBT;
end

nb_ = nb_+nobs;
tspan(2) = tDi(1);
end_time = timek(end);
AMOUNT30(1,i)=quad(@IN_PROD_RATE,0-CAA,30-CAA,1.0e-4);
for d = 1 : nb_dosei
if tDi(d)>end_time,
% next individual
break;
end

tspan(1) = tspan(2);
if d==nb_dosei,
tspan(2) = end_time*1.00001; %time intervals
else

tspan(2) = tDi(d+1);
end
if tspan(1)<tspan(2)
curTime=[timek((timek>=tspan(1)) & (timek<tspan(2))); tspan(2)] ;
nb_curTime=numel(curTime);
S= ones(2,nb_curTime);

options = CVodeSetOptions('UserData', phi(i,:),
'RelTol',1.e-4,
'AbsTol',[1.e-8; 1.e-6],
'MaxNumSteps',500,
'LinearSolver','Dense');

CVodeInit(@rhsfn, 'Adams', 'Functional', tspan(1), A0(:,i), options); %non stiff
try
[status,tempT,S(:,1)] = CVode((curTime(1)),'Normal');
catch
S(:,1)=(A0(:,i));
end
for eg=2:nb_curTime
[status,tempT,S(:,eg)] = CVode((curTime(eg)),'Normal');
end

for eg=1:(numel(curTime)-1)
A(nb_+(eg),1)=S(1,eg)/(BLOOD_VOL(i)*WEIGHT(curTime(eg)));
A(nb_+(eg),2)=S(2,eg)/(BLOOD_VOL(i)*WEIGHT(curTime(eg)));
end

nb_ =nb_+nb_curTime-1;
if d==nb_dosei
  if (curTime(end)==DOSEi(d+1,1))
    if (DOSEi(d+1,2)>0)
      A0(1,i)=S(1,end)+FR_UNDAMAGED(i)*DOSEi(d+1,2);
      A0(2,i)=S(2,end)+FR_UNDAMAGED(i)*DOSEi(d+1,2)/CMCHBT;
    else
      CPHLEB.F(CPHLEB.T==curTime(end))=1+(CPHLEB.A(CPHLEB.T==curTime(end))/S(1,end));
      A0(1,i)=S(1,end)+DOSEi(d+1,2);
      A0(2,i)=S(2,end)+DOSEi(d+1,2)*S(2,end)/S(1,end);
    end
  else
    A0(:,i)=S(:,end);
  end
end
end

end

%SET PHLEBOTOMY
hh=(PHLEB.ID==i);
PHLEB.F(hh,:)=CPHLEB.F;
end

if rem(iternum,50)==0
  iternum
  tti
end
A1 = A(:,1);
A2 = A(:,2);

% Define your outputs
f1 = A1 ;
f2 = A2 ;

% Now, you need to retrieve your solution vectors
for k=1:nb_outputs
  Itk=X{k}.Ik;
  eval(['f{',num2str(k),'} (Itk,:);']);
  f{k} = fk;
end

%INITIAL PRODUCTION RATE FUNCTION
function pr=IN_PROD_RATE(T)
global CMRS EMAX C50 TEMP_K_IU
prr=zeros(numel(T),1);
for i=1:numel(T),
    T_WT=WEIGHT(T(i));
    T_EPO=EPO_CONC(T(i));
    if T(i)<0
        prr(i)=TEMP_K_IU*(T_WT^CMRS);
    else
        prr(i)=(EMAX*T_EPO/(C50+T_EPO))*(T_WT^CMRS);
    end
end
pr=prr;

%PRODUCTION RATE FUNCTION
function pr=PROD_RATE(T)
global CMRS K_IU EMAX C50
T_EPO=EPO_CONC(T);
T_WT=WEIGHT(T);
if T<0
    pr=K_IU*(T_WT^CMRS);
else
    pr=(EMAX*T_EPO/(C50+T_EPO))*(T_WT^CMRS);
end

%WEIGHT FUNCTION
function ww=WEIGHT(T)
global CWTP CWTP1 CWTP2 G_AGE CWTX
if T<CWTX(1)
    INTERCEPT=polyval(CWTP,CWTX(1));
    temp=numel(CWTP);
    DERIVATIVE=0;
    for z=1:(temp-1),
        DERIVATIVE=DERIVATIVE+(temp-z)*CWTP(z)*((CWTX(1))^((temp-z-1)));
    end
    wt0=INTERCEPT+DERIVATIVE*(0-CWTX(1));
    if T<0
        ww=(CWTP1*((T+G_AGE)^CWTP2))*(wt0/((CWTP1*(G_AGE^CWTP2))));
    else
        ww=INTERCEPT+DERIVATIVE*(T-CWTX(1));
    end
else
    ww=polyval(CWTP,T);
end
function epo=EPO_CONC(T)
global CEPO
%T<X(0)
if (T<CEPO.X(1)||T>CEPO.X(end))
    epo=(min(CEPO.Y(1),CEPO.Y(end)));
else
    epao=(interp1(CEPO.X,CEPO.Y,T));
    %epao=csapi(CEPO.X,CEPO.Y,T);
    if epao<0
        epao
    end
    epo=epao;
end

%CORRECTION TERM
function cor=COR_TERM(TSTART,TEND)
global CPHLEB
temp=CPHLEB.F(CPHLEB.T>=TSTART & CPHLEB.T<TEND);
if temp==0
    cor=1;
else
    ctemp=1;
    N=numel(temp);
    for i=1:N,
        ctemp=ctemp*temp(i);
    end
    cor=ctemp;
end

function tr=TRAN_TERM(TSTART)
global CTRANS LIFESPAN
if CTRANS.T(1)>TSTART
    tr=0;
else
    temp.T=CTRANS.T(CTRANS.T>=TSTART-LIFESPAN)& CTRANS.T<TSTART);
    temp.A=CTRANS.A(CTRANS.T>=TSTART-LIFESPAN)& CTRANS.T<TSTART);
    tt=0;
    for i=1:numel(temp.T),
        tt=tt+(COR_TERM(temp.T(i),TSTART)*temp.A(i)/LIFESPAN);
    end
    tr=tt;
end

function f = myfun(x)
global CBV CBASELINE CBB CAA TEMP_K_IU
TEMP_K_IU=x;
TEMP=quad(@(IN_PROD_RATE,0-CBB,0-CAA,1.0e-4);
f = ((CBASELINE*(CBV*WEIGHT(0))-TEMP))^2;

function [yd, flag, new_data] = rhsfn(t, y, phi)
global CAA CBB CFR_UNDAMAGED CMCHBI CMCHBT
prod_a=PROD_RATE(t-CAA);
prod_b=COR_TERM(t-(CBB-CAA),t)*PROD_RATE(t-CBB);
tf_term=CFR_UNDAMAGED*TRAN_TERM(t);

%DIFFERENTIAL EQUATION
yd(1)= prod_a-prod_b-tf_term;
yd(2)= ((prod_a-prod_b)/CMCHBI)-(tf_term/CMCHBT);

flag = 0;
new_data = [];
return

% function model_info = getdata
function model_info = getdata
%returns the information about this model
model_info=struct;
model_info.nb_param=13;% number of parameters "phi"
model_info.phi_names={...
    'AA',...
    'BB',...
    'LIFE_SPAN_TRAN',...
    'FR_UNDAMAGED',...
    'BLOOD_VOL',...
    'MRS',...
    'VMAX',...
    'KM',...
    'WTP1',...
    'WTP2',...
    'BASELINE',...
    'MCHB_INFANT',...
    'MCHB_TRAN'};% names of the parameters
model_info.phi_tex={...
    '',...
    '',...
    ''...
model_info.logstruct={...
'L',...
'L',...
'L',...
'L',...
'L',...
'L',...
'L',...
'L',...
'L',...
'L',...
'L',...
'L',...
'L',...
'L',...
'L',...
'L',...
'L',...
'L'};% default distribution of the parameters "phi"

model_info.nb_varex=1;% number of regression variables
model_info.x_names={'x'};% name of the regression variable
model_info.x_tex={''};% tex name of the regression variable for the plots

model_info.nb_outputs=2;% number of outputs
model_info.y_names={...
'y1',...
'y2'};% names of the outputs
model_info.y_tex={...
''};% names of the outputs

model_info.nb_ode=2;% number of differential equations
model_info.ode=[1 1];% outputs that depend on the differential equations

model_info.iop_ode=2;% type of solver

model_info.dose=0;% 1 if the model needs dose information

model_info.cat_model='other';% type of model
model_info.desc='Baseline model no covariates';%description of the model
model_info.outtype={""};%type of each output (" for continuous models)
model_info.hmm=[0,0]; %1 or 0 for each output using a markov chain (every one will use the same
model_info.hmm_memory=-1; %Memory of the markov chain model_info.hmm_states=
0; %Number of states of the markov chain
APPENDIX B. MATLAB CODES FOR CHAPTER 4

****Begin prologue
This Matlab code was compiled to Monolix to model the erythropoiesis kinetics in newborn lambs as described in Chapter 4. The instructions of running this script with Monolix are summarized in “Instructions.doc” file located in each of the folder “Chapter 4” in the accompanying CD.
The analysis involves the following files in CD:
1. Monolix script: 'Chapter 4\Model\Hb_PD_MODEL.m'
2. Hb data file: 'Chapter 4\data\Monoilx_Lamb_DATA.csv'
3. EPO data file: 'Chapter 4\data\EPO.txt'
4. Body weight data file: 'Chapter 4\data\WT.txt'
5. Output folder: 'Chapter 4\Results'

****End prologue

function f = Hb_PD_MODEL( phi, Id, X )
%Structural model Hb_PD_MODEL
%
%Model information:
% # outputs = 1
% # regression variables = 1
% # parameters = 9
% # differential equations = 1
%
%f = Hb_PD_MODEL(phi,Id,X) returns a vector of length n
% phi (N x 9) individual parameters.
%
% X structure with regression data
%  dose (nD x ?) = doses informations (t_dose, dose, ...).
%  obs (n x 1) = regression variables: x
%  newtime = union of all the observation times
%
% Id structure with individual index informations
%  obs (n x 1) = individual index for each observation
%  dose (nD x 1) = individual index for each dose
%  Nbs (N x 2) = number of doses and observations per subject
%  id_t (n x 1) = indexes of observation times into the union (X.t_obs_u)
%
% where
% N = number of subjects
% n = number of observations
%

% if no arguments are given then we return the informations of the model
persistent iternum tti cputold tempwt tempepo tempgage TRAN PHLEB u_EPO u_WT GEST_AGE N;
NSUB=36;
N = size(phi,1);
try
    phi;
catch
    f=getdata;
    return
end
XX.dose=X.dose;
if isempty(iternum)
    iternum=1;
    tti=0;
    cputold=cputime;
    tempwt=uiimport('-file');
    tempepo=uiimport('-file');

  %SET EPO , WT AND GESTATIONAL AGE VECTORS
  %SET EPO VECTOR
  u_EPO.ID=zeros(numel(tempepo.ID));
  u_EPO.X=zeros(numel(tempepo.ID));
  u_EPO.Y=zeros(numel(tempepo.ID));
  u_EPO.ID=tempepo.ID;
  u_EPO.X=tempepo.TIME;
  u_EPO.Y=tempepo.EPO;
  %SET WT VECTOR
  u_WT.ID=zeros(numel(tempwt.ID));
  u_WT.X=zeros(numel(tempwt.ID));
  u_WT.Y=zeros(numel(tempwt.ID));
  u_WT.ID=tempwt.ID;
  u_WT.X=tempwt.TIME;
  u_WT.Y=tempwt.WT;
  % for d=1:size(phi,1),
  for d=1:NSUB,
      aa=(u_WT.ID==d);
      u_WT.P(d,:)=polyfit(u_WT.X(aa),u_WT.Y(aa),4);
  end
  %SET GESTATIONAL AGE
  GEST_AGE=150*ones(NSUB,1);%tempgage.INFANT_GEST_AGE;

  %TO SET TRANSFUSION and PHLEBOTOMY
  jj=1;
  ff=1;
  %TRANSFUSIONS
  TRAN.ID=zeros(sum((XX.dose(:,2))>0),1);
  TRAN.T=zeros(sum((XX.dose(:,2))>0),1);
  TRAN.A=zeros(sum((XX.dose(:,2))>0),1);
PHLEB.ID=zeros(sum((XX.dose(:,2))<0),1);
PHLEB.T=zeros(sum((XX.dose(:,2))<0),1);
PHLEB.A=zeros(sum((XX.dose(:,2))<0),1);

TRAN.ID(:)=ld.dose((XX.dose(:,2))>0);
TRAN.T(:)=XX.dose((XX.dose(:,2))>0,1);
TRAN.A(:)=XX.dose((XX.dose(:,2))>0,2);

%HOLEBOTOMY
PHLEB.ID=zeros(sum((XX.dose(:,2))<0),1);
PHLEB.T=zeros(sum((XX.dose(:,2))<0),1);
PHLEB.A=zeros(sum((XX.dose(:,2))<0),1);
PHLEB.F=ones(sum((XX.dose(:,2))<0),1);
PHLEB.ID(:)=ld.dose((XX.dose(:,2))<0);
PHLEB.T(:)=XX.dose((XX.dose(:,2))<0,1);
PHLEB.A(:)=XX.dose((XX.dose(:,2))<0,2);
PHLEB.F(:)=1;

else

%TO SET TRANSFUSION and PHLEBOTOMY
%TRANSFUSIONS
TRAN.ID=zeros(sum((XX.dose(:,2))>0),1);
TRAN.T=zeros(sum((XX.dose(:,2))>0),1);
TRAN.A=zeros(sum((XX.dose(:,2))>0),1);
TRAN.ID(:)=ld.dose((XX.dose(:,2))>0);
TRAN.T(:)=XX.dose((XX.dose(:,2))>0,1);
TRAN.A(:)=XX.dose((XX.dose(:,2))>0,2);

%PHLEBOTOMY
PHLEB.ID=zeros(sum((XX.dose(:,2))<0),1);
PHLEB.T=zeros(sum((XX.dose(:,2))<0),1);
PHLEB.A=zeros(sum((XX.dose(:,2))<0),1);
PHLEB.F=ones(sum((XX.dose(:,2))<0),1);
PHLEB.ID(:)=ld.dose((XX.dose(:,2))<0);
PHLEB.T(:)=XX.dose((XX.dose(:,2))<0,1);
PHLEB.A(:)=XX.dose((XX.dose(:,2))<0,2);
PHLEB.F(:)=1;

end

iternum=iternum+1;
tti=cputime-cputold;
cputold=cputime;

end

i_time = X.i_fullobs;
ids = ld.fullobs;
u_time = X.newtime;

AA = phi(:,1);
BB = phi(:,2) + AA;
LIFE_SPAN_TRAN = phi(:,3);
FR_UNDAMAGED = phi(:,4);
BLOOD_VOL = phi(:,5);
MRS = phi(:,6);
VMAX = phi(:,7);
KM = phi(:,8);
%WTP1 = phi(:,9);
%WTP2 = phi(:,10);
%SLOPE = phi(:,11);
BASELINE = phi(:,9);

N = size(phi,1);
DOSE = X.dose;% Dose
tD = DOSE(:,1);
D = DOSE(:,2);
%nb_outputs = 1;
nb_ode = 1;

% Write here your initial conditions.
A0 = zeros(1,N);
A = zeros(numel(ids),nb_ode);
KIU2 = zeros(1,N);
nb_ = 0;
for i=1:N,
  %SET EPO AND WT
  global CBV CEPO CWTP CWTP1 CWTP2 G_AGE CMRS K_IU EMAX C50
  LIFESPAN CWTX CWTY CAA CBB CFR_UNDAMAGED CBASELINE
  if (rem(i,NSUB)>0)
    si = rem(i,NSUB);
  else
    si = NSUB;
  end

  %EPO
  ee = (u_EPO.ID == si);
  CEPO.X = u_EPO.X(ee);
  CEPO.Y = u_EPO.Y(ee);
  si = length(CEPO.X);
  %WT
  aa = (u_WT.ID == si);
  CWT.X = u_WT.X(aa);
  CWT.Y = u_WT.Y(aa);
  CWT.P = polyfit(CWT.X, CWT.Y, 4);
  CWTX = u_WT.X(aa);
CWTY = u_WT.Y(aa);
CWTP = CWT.P;

%PARAMETERS
CFR_UNDAMAGED = FR_UNDAMAGED(i);
LIFESPAN = LIFE_SPAN_TRAN(i);
CAA = AA(i);
CBB = BB(i);
CWTP1 = 0.1281; %WTP1(i);
CWTP2 = 0.00038; %WTP2(i);
G_AGE = GEST_AGE(si);
CMRS = MRS(i);
EMAX = VMAX(i);
C50 = KM(i);
CBASELINE = BASELINE(i);
CBV = BLOOD_VOL(i);

%INITIAL CONDITION
A0(1,i) = CBASELINE*(BLOOD_VOL(i)*WEIGHT(0));
K_IU = fminbnd(@(myfun,0,1);
KIU2(1,i) = K_IU;
global CTRANS CPHLEB;
% set transfusion
jj = (TRAN.ID == i);
CTRANS.T = TRAN.T(jj);
CTRANS.A = TRAN.A(jj);
% SET PHLEBOTOMY
hh = (PHLEB.ID == i);
CPHLEB.T = PHLEB.T(hh,:);
CPHLEB.A = PHLEB.A(hh,:);
CPHLEB.F = PHLEB.F(hh,:);
ii = (Id.dose == i);
tDi = tD(ii);
A0(1,i) = A0(1,i) + DOSEi(1,2);
for we = 1:nobs
    A(nb_ + we,1) = A0(1,i)/(BLOOD_VOL(i)*WEIGHT(timek(we)));
end
A0(1,i) = A0(1,i) + DOSEi(1,2);
for d = 1 : nb_dosei
    if tDi(d)>end_time,
        % next individual
        break;
    end
    tspan(1) = tspan(2);
    if d==nb_dosei,
        tspan(2) = end_time*1.00001; % time intervals
    else
        tspan(2) = tDi(d+1);
    end

% Update your initial conditions with the doses
%A0(i,:)=A0(i,:);

if tspan(1)<tspan(2)
    curTime=[timek( (timek>=tspan(1)) & (timek<tspan(2)) ); tspan(2)] ;
    nb_curTime=numel(curTime);
    S= ones(1,nb_curTime);
    options = CVodeSetOptions('UserData', phi(i,:),
        'RelTol',1.e-4,...
        'AbsTol',1.e-8,...
        'MaxNumSteps',500,...
        'LinearSolver','Dense');

    CVodeInit(@rhsfn, 'Adams', 'Functional', tspan(1), A0(:,i), options); % non stiff

    try
        [status,tempT,S(:,1)] = CVode((curTime(1)),'Normal');
    catch
        S(:,1)=(A0(:,i));
    end
    S(:,1);

    for eg=2:nb_curTime
        curTime(eg);
        EPO_CONC(curTime(eg));
        % length(curTime)
        [status,tempT,S(:,eg)] = CVode((curTime(eg)),'Normal');
        %[status,tempT,S(:,eg)]
    end

    for eg=1:(numel(curTime)-1)
        A(nb_+(eg),1)=S(1,eg)/(BLOOD_VOL(i)*WEIGHT(curTime(eg)));
nb_ = nb_ + nb_curTime-1;

if d==nb_dosei
    if (curTime(end)==DOSEi(d+1,1))
        if (DOSEi(d+1,2)>0)
            A0(1,i)=S(1,end)+FR_UNDAMAGED(i)*DOSEi(d+1,2);
        else
            CPHLEB.F(CPHLEB.T==curTime(end))=1+(CPHLEB.A(CPHLEB.T==curTime(end))/S(1,end));
            A0(1,i)=S(1,end)+DOSEi(d+1,2);
        end
    else
        end
    else
        A0(:,i)=S(:,end);
    end
end

%SET PHLEBOTOMY
hh=(PHLEB.ID==i);
PHLEB.F(hh,:)=CPHLEB.F;
end

if rem(iternum,50)==0
    iternum
    tti
    KIU2
end
A1 = A(:,1);
A1;
% Define your outputs
f = A1 ;

% Now, you need to retrieve your solution vectors
Itk = X.Ik;

f = f(Itk,:);

%INITIAL PRODUCTION RATE FUNCTION
function pr=IN_PROD_RATE(T)
global CMRS EMAX C50 TEMP_K_IU
prr=zeros(numel(T),1);
for i=1:numel(T),
    T_WT=WEIGHT(T(i));
end
T_EPO=EPO_CONC(T(i));

if T(i)<0
    pr(i)=TEMP_K_IU*(T_WT^CMRS);
else
    pr(i)=(EMAX*T_EPO/(C50+T_EPO))*(T_WT^CMRS);
end

end

pr=prr;

%PRODUCTION RATE FUNCTION
function pr=PROD_RATE(T)
    global CMRS K_IU EMAX C50
    T_EPO=EPO_CONC(T);
    T_WT=WEIGHT(T);
    if T<0
        pr=K_IU*(T_WT^CMRS);
    else
        pr=(EMAX*T_EPO/(C50+T_EPO))*(T_WT^CMRS);
    end
end

function ww=WEIGHT(T)
    global CWTP CWTP1 CWTP2 G_AGE CWTX
    if T<CWTX(1)
        INTERCEPT=polyval(CWTP,CWTX(1));
        temp=numel(CWTP);
        DERIVATIVE=0;
        for z=1:(temp-1),
            DERIVATIVE=DERIVATIVE+(temp-z)*CWTP(z)*((CWTX(1))^(temp-z-1));
        end
        wt0=INTERCEPT+DERIVATIVE*(0-CWTX(1));
        if T<0
            ww=(CWTP1*((T+G_AGE)^CWTP2))*(wt0/((CWTP1*(G_AGE^CWTP2))));
            ww=wt0*exp((CWTP1-CWTP2*(T+G_AGE))*(T+G_AGE))/exp((CWTP1-
                CWTP2*(G_AGE))*(G_AGE));
        else
            ww=INTERCEPT+DERIVATIVE*(T-CWTX(1));
        end
    else
        ww=polyval(CWTP,T);
    end
end

function epo=EPO_CONC(T)
global CEPO
%T<X(0)
if (T<CEPO.X(1)||T>CEPO.X(end))
    epo=(min(CEPO.Y(1),CEPO.Y(end)));
else
    epao=(interp1(CEPO.X,CEPO.Y,T));
%epao=csapi(CEPO.X,CEPO.Y,T);
if epao<0
    epao
    1
end
epo=epao;
end

%CORRECTION TERM
function cor=COR_TERM(TSTART,TEND)
global CPHLEB
temp=CPHLEB.F(CPHLEB.T>=TSTART & CPHLEB.T<TEND);
if temp==0
    cor=1;
else
    ctemp=1;
    N=numel(temp);
    for i=1:N,
        ctemp=ctemp*temp(i);
    end
    cor=ctemp;
end

function tr=TRAN_TERM(TSTART)
global CTRANS LIFESPAN
if CTRANS.T(1)>TSTART
    tr=0;
else
    temp.T=CTRANS.T(CTRANS.T>=(TSTART-LIFESPAN)& CTRANS.T<TSTART);
    temp.A=CTRANS.A(CTRANS.T>=(TSTART-LIFESPAN)& CTRANS.T<TSTART);
    tt=0;
    for i=1:numel(temp.T),
        tt=tt+(COR_TERM(temp.T(i),TSTART)*temp.A(i)/LIFESPAN);
    end
    tr=tt;
end

function f = myfun(x)
global CBV CBASELINE CBB CAA TEMP_K_IU
TEMP_K_IU = x;
TEMP = quad(@(IN_PROD_RATE, 0 - CBB, 0 - CAA, 1.0e-4);
f = ((CBASELINE * (CBV * WEIGHT(0)) - TEMP))^2;

function [yd, flag, new_data] = rhsfn(t, y, phi)
global CAA CBB CFR_UNDAMAGED

prod_a = PROD_RATE(t - CAA);
prod_b = COR_TERM(t - (CBB - CAA), t) * PROD_RATE(t - CBB);
tf_term = CFR_UNDAMAGED * TRAN_TERM(t);

% DIFFERENTIAL EQUATION
yd(1) = prod_a - prod_b - tf_term;
flag = 0;
new_data = [];
return

function model_info = getdata
% returns the information about this model

model_info = struct;

model_info.nb_param = 9; % number of parameters "phi"
model_info.phi_names = {...
    'AA', ...
    'TauProd', ...
    'LIFE_SPAN_TRAN', ...
    'FR_UNDAMAGED', ...
    'BLOOD_VOL', ...
    'MRS', ...
    'VMAX', ...
    'KM', ...
    'BASELINE'}; % names of the parameters
model_info.phi_tex = {...
    '...', ...
    '...', ...
    '...', ...
    '...', ...
    '...', ...
    '...', ...
    '...', ...
    '...', ...
    '...', ...
}; % tex names of the parameters for the plots
model_info.logstruct = {...
    'L', ...
    'L', ...
model_info.nb_varex=1;% number of regression variables
model_info.x_names={'x'};% name of the regression variable
model_info.x_tex={''};% tex name of the regression variable for the plots

model_info.nb_outputs=1;% number of outputs
model_info.y_names={'y'};% name of the output
model_info.y_tex={''};% tex name of the output for the plots

model_info.nb_ode=1;% number of differential equations
model_info.ode=1;% name of the output

model_info.iop_ode=2;% type of solver

model_info.dose=1;% 1 if the model needs dose information

model_info.cat_model='other';% type of model

model_info.desc='';% description of the model

model_info.outtype={''};% type of each output (" for continuous models)

model_info.hmm=0;  % 1 or 0 for each output using a markov chain (every one will use the same
model_info.hmm_memory=-1; % Memory of the markov chain
model_info.hmm_states= 0; % Number of states of the markov chain
APPENDIX C. WINFUNFIT CODES FOR CHAPTER 6 AND CHAPTER 7

Appendix C.1 WINFUNFIT code for the peak separation algorithm

****Begin prologue
This FORTRAN program performs peak separation for biotinylated RBC data with multiple biotin densities as explained in sections 6.3.5 and 7.3.5. Mixture modeling approach was done to account for the overlap between the adjacent biotinylated RBC peaks and the unlabeled fluorescent peak as depicted in Figure 5.3. Each Biotinylated RBC peak was assumed to have a lognormal distribution. The tail of the unlabeled RBC was assumed to follow a Weibull distribution.
The analysis involves the following files in CD in the folder “Chapter 6 and 7\Biotinylated Cell Peak Algorithm” with the corresponding Chapter number (i.e. Chapter 6 and Chapter 7):
1) Main program file FORTRAN code: \Biotinylated Cell Peak Algorithm\Parametric\PARAMETRIC_CELL_ALGORITHM_V01.5.F90
2) WINFUNFIT files located in the folder “Biotinylated Cell Peak Algorithm\WINFUNFIT”: ALLWINFUNFIT.lib, FUNFIT_RESOURCES.res and DUMMY_USERMODEL_ODE_JACOBIAN.F90
3) Data files and results: \Biotinylated Cell Peak Algorithm\Parametric\Sheep Files

****End prologue

! FILENAME = PARAMETRIC_CELL_ALGORITHM_V01.5.F90
!
! PURPOSE: To identify and quantify the different biotin density labeled RBC populations.
!
! ALGORITHM: 1.) Determines the proportions (P1-P5) of each biotin labeled cell peaks using a
! an Exponential distribution for the unlabeled peak and Normal distributions
! for the labeled peaks.
!
! NOTES: The data set read in directly by WINFUNFIT is assumed to be the FITC-
avidin histogram.
!
! CHANGES/REVISIONS:
! VERSION 1.0 OCT 16, 2007 Original code (modified from
CELL_ALGORITHM_V02.F90).
! VERSION 1.1 OCT 19, 2007 Replaced the exponential distribution with a
 normalized and
! constained quadratic spline function.
! VERSION 1.2 NOV 20, 2007 Reparameterized the unlabeled peak proportion to
 prevent
! the total proportion from exceeding 1.0
VERSION 1.3 NOV 20, 2007 Adjusted model so that the channel 1 (x=0) data is no longer removed as noise and instead is considered to be unlabeled RBCs.

VERSION 1.4 JAN 08, 2008 Modified to only model the trailing right edge of the unlabeled cells in order to improve the model fitting (i.e. the objective function will only be influenced by the overlapping region of the unlabeled peak and the labeled peaks).

VERSION 1.5 JAN 08, 2008 Modified model to provide user control of when to start modeling unlabeled cells.

! ============= DUMMY CALL TO SUCCESSFULLY LINK WITH WINFUNFIT LIBRARIES =============
SUBROUTINE USERMODEL_ODE(X,Y,YPRIME,P,NP,IFUN)
END SUBROUTINE USERMODEL_ODE

! ============= MODEL START =============
SUBROUTINE USERMODEL(X,Y,P,NP,IFUN)
IMPLICIT NONE

! PARAMETER DECLARATIONS
INTEGER :: NP, IFUN, NUM
REAL*8 :: X, Y, P(*)
INTEGER, PARAMETER :: NEQN = 1, LENSTRING = 180, NPS = 500,
MAXOBS = 1024, MAXPEAKS = 5
REAL*8, PARAMETER :: EPSILON = 0.001, MINX=1D0,
MAXX=10000D0, RELERR = 0.01, PI=3.141592654
INTEGER :: J, LUN, NOBS, XPOS_UM, JPOS
REAL*8 :: NPEAKS, P1, P2, P3, P4, P5, PE, RATE, DELTAN2,
THETAE, X_UM, &
DELTAN3, DELTAN4, DELTAN5, SIGMAN1, SIGMAN2,
SIGMAN3, SIGMAN4, SIGMAN5, &
THETAN1, THETAN2, THETAN3, THETAN4, THETAN5,
SUMP, EXP_DENS, &
NORM1_DENS, NORM2_DENS, NORM3_DENS,
NORM4_DENS, NORM5_DENS, &
SCALAR, XT(MAXOBS), YT(MAXOBS), YSUM, PYUM,
UM_YSUM, YMAX, CONTROL

LOGICAL, SAVE :: SHOWIT, PLOTSAVED, NEWFIT = .TRUE.
CHARACTER (LEN=256) :: ID, DATAFILENAME
NPEAKS = P(1) ! NUMBER OF BIOTIN LABELED PEAKS (FIXED VALUE BETWEEN 0 AND MAXPEAKS)
P1 = P(2) ! BIOTIN LABELED PEAK 1 PROPORTION (FIX TO 0.0 IF NPEAKS = 0)
P2 = P(3) ! BIOTIN LABELED PEAK 2 PROPORTION (FIX TO 0.0 IF NPEAKS < 2)
P3 = P(4) ! BIOTIN LABELED PEAK 3 PROPORTION (FIX TO 0.0 IF NPEAKS < 3)
P4 = P(5) ! BIOTIN LABELED PEAK 4 PROPORTION (FIX TO 0.0 IF NPEAKS < 4)
P5 = P(6) ! BIOTIN LABELED PEAK 5 PROPORTION (FIX TO 0.0 IF NPEAKS < 5)
RATE = P(7) ! RATE OF DECLINE OF THE EXPONENTIAL DISTRIBUTION
THETAN1 = P(8) ! THETA (LOCATION) PARAMETER OF BIOTIN LABELED PEAK 1 NORMAL DISTRIBUTION (FIX IF NPEAKS = 0)
SIGMAN1 = P(9) ! SIGMA (SCALE) PARAMETER OF BIOTIN LABELED PEAK 1 NORMAL DISTRIBUTION (FIX IF NPEAKS = 0)
DELTAN2 = P(10) ! THETA (LOCATION) PARAMETER OF BIOTIN LABELED PEAK 2 NORMAL DISTRIBUTION (FIX IF NPEAKS < 2)
SIGMAN2 = P(11) ! SIGMA (SCALE) PARAMETER OF BIOTIN LABELED PEAK 2 NORMAL DISTRIBUTION (FIX IF NPEAKS < 2)
DELTAN3 = P(12) ! THETA (LOCATION) PARAMETER OF BIOTIN LABELED PEAK 3 NORMAL DISTRIBUTION (FIX IF NPEAKS < 3)
SIGMAN3 = P(13) ! SIGMA (SCALE) PARAMETER OF BIOTIN LABELED PEAK 3 NORMAL DISTRIBUTION (FIX IF NPEAKS < 3)
DELTAN4 = P(14) ! THETA (LOCATION) PARAMETER OF BIOTIN LABELED PEAK 4 NORMAL DISTRIBUTION (FIX IF NPEAKS < 4)
SIGMAN4 = P(15) ! SIGMA (SCALE) PARAMETER OF BIOTIN LABELED PEAK 4 NORMAL DISTRIBUTION (FIX IF NPEAKS < 4)
DELTAN5 = P(16) ! THETA (LOCATION) PARAMETER OF BIOTIN LABELED PEAK 5 NORMAL DISTRIBUTION (FIX IF NPEAKS < 5)
SIGMAN5 = P(17) ! SIGMA (SCALE) PARAMETER OF BIOTIN LABELED PEAK 5 NORMAL DISTRIBUTION (FIX IF NPEAKS < 5)
SCALAR = P(18) ! LINEAR SCALAR OF MIXTURE DISTRIBUTION
CONTROL = P(19) ! CONTROL PARAMETER: IF \geq 0.0 USER SETS X VALUE TO BEGIN MODELING WITH CONTROL VALUE,
            ! OTHERWISE, ITS BASED ON NEGATIVE CONTROL VALUE AS THE NUMBER OF STANDARD DEVIATIONS
            ! FROM THE LEFT OF THE 1ST LABELED PEAK MEAN.
! ============= START OF PRELIMINARY PREPARATION SECTION

IF (IFUN == -1000) THEN

! SET PARAMETER NAMES
IF(NP /= 19) STOP '***** ERROR: INCONSISTENT NUMBER OF PARAMETERS IN PARAMETER FILE.*****'
CALL SETFUNFITPARAMETERNAME(1, " NO. PEAKS")
CALL SETFUNFITPARAMETERNAME(2, " P1")
CALL SETFUNFITPARAMETERNAME(3, " P2")
CALL SETFUNFITPARAMETERNAME(4, " P3")
CALL SETFUNFITPARAMETERNAME(5, " P4")
CALL SETFUNFITPARAMETERNAME(6, " P5")
CALL SETFUNFITPARAMETERNAME(7, " RATE")
CALL SETFUNFITPARAMETERNAME(8, " THETA PEAK 1")
CALL SETFUNFITPARAMETERNAME(9, " SIGMA PEAK 1")
CALL SETFUNFITPARAMETERNAME(10, " DELTA PEAK 2")
CALL SETFUNFITPARAMETERNAME(11, " SIGMA PEAK 2")
CALL SETFUNFITPARAMETERNAME(12, " DELTA PEAK 3")
CALL SETFUNFITPARAMETERNAME(13, " SIGMA PEAK 3")
CALL SETFUNFITPARAMETERNAME(14, " DELTA PEAK 4")
CALL SETFUNFITPARAMETERNAME(15, " SIGMA PEAK 4")
CALL SETFUNFITPARAMETERNAME(16, " DELTA PEAK 5")
CALL SETFUNFITPARAMETERNAME(17, " SIGMA PEAK 5")
CALL SETFUNFITPARAMETERNAME(18, " SCALAR")
CALL SETFUNFITPARAMETERNAME(19, " CONTROL")

! PRINT BASIC FORMATING/ASSUMPTIONS OF FITTING
PRINT*
PRINT*, 'IT IS ASSumed THAT:
PRINT*, ' 1. X-AXIS VALUES ARE LOG-TRANSFORMED (BASE 10).
PRINT*, ' 2. CHANNEL 1 (X =0) DATA ARE UNLABELED RBCS.
PRINT*
READ*
END IF

! ============= END OF PRELIMINARY PREPARATION SECTION

! ENSURE THE NUMBER OF PEAKS IS WITHIN THE BOUNDS
IF ( ( INT(NPEAKS) > MAXPEAKS ) .OR. ( INT(NPEAKS) < 0 ) ) STOP '***** ERROR: NO. OF PEAKS OUTSIDE MODEL BOUNDS.*****'

! GET VALUES OF CHANNELS
CALL GET_XYOB1S(1,XT,YT,NOBS)
IF ( NOBS > MAXOBS ) STOP '***** ERROR: NO. OBSERVATIONS GREATER THAN MODEL MAXIMUM.*****'
! DETERMINE X POSITION OF LAST UMODELED CHANNEL
IF ( CONTROL < 0D0 ) THEN
   THETAE = THETAN1 + CONTROL*SIGMAN1
ELSE
   THETAE = CONTROL
ENDIF
XPOS_UM = 1
DO J=2, NOBS
   IF ( XT(J) > THETAE ) EXIT
   XPOS_UM = J
END DO
X_UM = XT(XPOS_UM)

! DETERMINE PROPORTION OF TOTAL NO. OF CELL THAT ARE NOT
MODELED
UM_YSUM = 0D0
DO J = 1, XPOS_UM
   UM_YSUM = UM_YSUM + YT(J)
ENDDO
YSUM = SUM(YT)
PYUM = UM_YSUM/YSUM

! SET THE 'P' VALUES OF PEAKS THAT EXCEED NPEAKS TO 0.0
IF ( INT(NPEAKS) < MAXPEAKS ) THEN
   DO J= NPEAKS, MAXPEAKS - 1
      P(2+J) = 0D0
   END DO
END IF
P1 = P(2)
P2 = P(3)
P3 = P(4)
P4 = P(5)
P5 = P(6)

! DEFINE BIONTIN LABELED PEAK LOCATION PARAMETERS (ORDERED
SUCH THAT THETAN1 <...< THETAN5)
THETAN2 = THETAN1 + DELTAN2
THETAN3 = THETAN2 + DELTAN3
THETAN4 = THETAN3 + DELTAN4
THETAN5 = THETAN4 + DELTAN5

! DETERMINE VALUE OF EXPONENTIAL DISTRIBUTION
IF ( X <= X_UM ) THEN
   EXP_DENS = 0D0
ELSE
    EXP_DENS = RATE*EXP(-RATE*(X - X_UM))
END IF

! DETERMINE VALUES OF OTHER DENSITIES
NORM1_DENS = (1/(SIGMAN1*SQRT(2D0*PI)))*DEXP(-(((X-
THETAN1)**2D0)/(2D0*(SIGMAN1**2D0))))
NORM2_DENS = (1/(SIGMAN2*SQRT(2D0*PI)))*DEXP(-(((X-
THETAN2)**2D0)/(2D0*(SIGMAN2**2D0))))
NORM3_DENS = (1/(SIGMAN3*SQRT(2D0*PI)))*DEXP(-(((X-
THETAN3)**2D0)/(2D0*(SIGMAN3**2D0))))
NORM4_DENS = (1/(SIGMAN4*SQRT(2D0*PI)))*DEXP(-(((X-
THETAN4)**2D0)/(2D0*(SIGMAN4**2D0))))
NORM5_DENS = (1/(SIGMAN5*SQRT(2D0*PI)))*DEXP(-(((X-
THETAN5)**2D0)/(2D0*(SIGMAN5**2D0))))

! SET PE
SUMP = P1 + P2 + P3 + P4 + P5
PE = MAX(0D0,1D0 - SUMP)

! MODEL FITTING
IF (IFUN == 1) THEN
    IF ( X > X_UM ) THEN
        Y = SCALAR*(PE*EXP_DENS + P1*NORM1_DENS + P2*NORM2_DENS +
P3*NORM3_DENS + P4*NORM4_DENS + P5*NORM5_DENS)
    ELSE
        ! FIND POSITION OF Y
        DO J=1,NOBS
            JPOS = J
            IF ( X == XT(J) ) EXIT
        END DO
        Y = YT(JPOS)
    ENDIF
END IF

! ============= USER OUTPUT =============
IF (IFUN == 0) THEN
    ! WRITE OUT USER CONTROLS AND OTHER RESULTING
    ! CALCULATIONS
    DO J = 1,NP
        CALL GETFUNFITPARAMETERNAME (J,PNAME)
        WRITE(*,"(1X,I2,1X,A,G14.4)") J, PNAME, P(J)
    ENDDO
    WRITE(*,"(4X,'PE', 18X, G14.4)") PE
WRITE(*,"
(4X,'PYUM', 18X, G14.4)") PYUM
WRITE(*,"
(4X,'UNLABELED PEAK PERCENTAGE: ', F7.4,'%)")
(PYUM+PE*(1D0-PYUM))*100D0
IF ( INT(NPEAKS) > 0D0 ) THEN
  WRITE(*,"
(/,1X,'LABELED PEAK PERCENTAGES')")
  DO J= 0, NPEAKS - 1
     WRITE(*,"
('PEAK ',I1,': ', 8X, F14.4,'%')") J+1, P(2+J)*(1D0-
  PYUM)*100D0
  END DO
END IF

! ============= START OF PLOTS =============
! DETERMINE IF PLOTS WANTED
CALL PROMT(SHOWIT)
IF (SHOWIT) THEN

! CREATE PLOT OF FITTED VS. OBSERVED FREQUENCY
CALL GETDATAFILENAME(DATAFILENAME)
!            CALL ADDMARGINTEXT(DATAFILENAME)
CALL LEFTLABEL ('FREQUENCY')
YMAX = 0D0
DO J=XPOS_UM, NOBS
   YMAX = MAX(YMAX,YT(J))
END DO
CALL BEGINXAT_D(X_UM)
CALL ENDELFTAT_D(YMAX)
CALL ADDFITTEDCURVELEFT(1)
CALL INCLUDECURVELEFT_D(XT,YT,NOBS,0)
CALL XLABEL('LOG(FITC)')
CALL DISPLAYPLOT
CALL RECORDPLOTIFSAVED(3)
CALL RECORDPLOTIFSAVED(LUN)
END IF

! ============= END OF PLOTS =============

! SAVE PARAMETERS WITH NAMES TO OUTPUT FILE IF REQUESTED
PRINT*
PRINT*, 'WOULD YOU LIKE PARAMETERS WITH NAMES SAVED TO A
FILE?'
!        READ (*,**) RESPONSE
RESPONSE="Y"
PRINT*
IF ( RESPONSE == 'Y' .OR. RESPONSE == 'y' ) THEN
WRITE(3,"(//)"
DO J = 1, NP
    CALL GETFUNFITPARAMETERNAME(J,PNAME)
    WRITE(3,"(1X,A,G14.6)") PNAME, P(J)
ENDDO
WRITE(3,"(1X, 'PE', 16X, G14.4)") PE
WRITE(3,"(1X,'PYUM', 16X, G14.4)"") PYUM
WRITE(3,"(/,'*************************************************')"
WRITE(3,"(/,1X,'UNLABELED PEAK PERCENTAGE: ', F7.4,'%')") (PYUM + PE*(1D0-PYUM))*100D0
    IF ( INT(NPEAKS) > 0D0 ) THEN
        WRITE(3,"(/,1X,'LABELED PEAK PERCENTAGES')")
        DO J= 0, NPEAKS - 1
            WRITE(3,"(4X,'PEAK ',I1, ': ', 8X, F14.4,'%')") J+1, P(2+J)*(1D0-PYUM)*100D0
        END DO
    END IF
END IF
END IF
! ============= END OF ALGORITHM =============
RETURN
ENTRY MODELID(ID)  !*********  N O N  O P T I O N A L  D E F I N I T I O N
SECTION *************
    ID = 'PARAMETRIC_CELL_ALGORITHM_V01.5.F90' ! RECORD THE ID
FOR THE MODEL USED IN THE FITTING :
    RETURN
END SUBROUTINE USERMODEL

! ============= END OF FILE =============
Appendix C.2 WINFUNFIT code for survival analysis for stressed RBC

****Begin prologue

This FORTRAN program performs survival analysis for the RBC produced under non steady state conditions \( N_{\text{NEW}}(t) \) as explained in Chapters 6 and 7. The behavior of \( N_{\text{NEW}}(t) \) is given by the following equations:

\[
N_{\text{NEW}}(t) = \begin{cases} 
N(t) - N_1(t) & \text{for } 0 < t < T \\
N_2(t) - N_1(t) & \text{for } T < t \\
0 & \text{o.w}
\end{cases}
\]  

(6.1)

\[
f_{\text{in}}(t) = \begin{cases} 
\frac{d(N_{\text{NEW}}(t))}{dt} & \text{for } 0 < t < T \\
0 & \text{o.w}
\end{cases}
\]  

(6.2)

The number of \( N_{\text{NEW}}(t) \) present in the circulation at time \( t \) was described according to:

\[
N_{\text{NEW}}(t) = \int_{-\infty}^{t} f_{\text{in}}(u) \cdot S(t-u) \cdot du
\]  

(6.3)

Since \( f_{\text{in}}(t) \) is only defined for \( 0 < t < T \), Eq.5.3 is represented in the following form:

\[
N_{\text{NEW}}(t) = \begin{cases} 
\int_{0}^{t} f_{\text{in}}(u) \cdot S(t-u) \cdot du & \text{for } 0 < t < T \\
\int_{0}^{T} f_{\text{in}}(u) \cdot S(t-u) \cdot du & \text{for } T < t \\
0 & \text{o.w}
\end{cases}
\]  

(6.4)

The survival function, \( S(t) \), was modeled using a Weibull distribution was modeled as a Weibull distribution due to the flexibility of the distribution and its support on the non-negative real line:

\[
S(t) = \exp \left( -\left[ \frac{t - \theta}{\lambda} \right]^k \right)
\]  

(6.5)

where \( \lambda, k, \) and \( \theta \) are the scale, shape, and location parameters, respectively.

The mean potential lifespan was calculated using the following equation:

\[
MPL = \int_{0}^{\infty} S(t) \cdot dt
\]  

(6.6)

The analysis involves the following files in CD in the folder “\Chapter 6 and 7\Stress Erythropoiesis MPL”:
1) Main program file FORTRAN code: \MAIN.F90
2) UIR subroutine: UIR.F90
3) RBC production subroutine: RBC PRODUCTION.F90
4) WINFUNFIT files located in the folder “\WINFUNFIT”: ALLWINFUNFIT.lib, FUNFIT_RESOURCES.res and DUMMY_USERMODEL_ODE_JACOBIAN.F90
5) Data files: \Stress Survival studies
6) Results folder: \Results March 2011

****End prologue
Appendix C.2.1 Main.F90

SUBROUTINE USERMODEL_ODE(T,Y,YPRIME,P,NP,IFUN) ! REQUIRED NAME: USERMODEL_ODE (DO NOT CHANGE)
  USE RBC_PROD_MODULE
  USE UNIT_IMPULSE_RESPONSE
  IMPLICIT REAL*8 (A-H,O-Z) ! REQUIRED TO USE REAL*8 FOR NON-INTEGERS
  DIMENSION Y(*), YPRIME(*), P(*) ! NOTE USE REAL*8 FOR ALL CONSTANTS
  LOGICAL EVENT_IS_ACTIVE ! * <= REMEMBER TO INCLUDE THIS!

  !***********************************************8

  IF(IFUN > 0 )THEN ! ------------------------------------
    WRITE(*,*)RBC_PRODUCTION_RATE(T)
    YPRIME(1)=UIR(T)*RBC_PRODUCTION_RATE(T)
    YPRIME(2)=RBC_PRODUCTION_RATE(T)
  END IF

RETURN
END

!===============================================================

! THE FOLLOWING SUBROUTINE (USERMODEL):
! (1) DEFINES THE EQUATIONS TO BE FITTED
! (2) ASSIGN 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 RBC_PROD_MODULE
USE UNIT_IMPULSE_RESPONSE
IMPLICIT REAL*8 (A-H,O-Z)

REAL*8, PARAMETER :: ABSERR = 0D0, RELERR = 0.001
REAL*8::TSTART,TEND
REAL*8,EXTERNAL::TOTAL_RBC_PROD_RATE, UIR2
INTEGER,PARAMETER::NEQN = 2, NPAR=4
DIMENSION YZERO (NEQN),TZERO(NEQN)
REAL*8::N0
REAL*8::TIMEZERO=32.0639D0
DIMENSION P(*)
LOGICAL SHOWIT
CHARACTER*256 ID, DATAFILENAME
INTEGER::IERR,LUN=3
CHARACTER*2::CH_IFUN

TSTART=1.04D0
TEND=14.96D0

! TSTART=1
! TEND=9.54D0
! TEND=15.85D0

!----
! 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,"THETA") ! DISCONTINUED INPUT
RATE FOR YPRIME(3) PERTUBATION (EVENT PARAMETER P(1))
CALL SetFunfitParameterName(2,"K") ! A BOLUS INPUT PERTURBING
Y(2) (EVENT PARAMETER P(2))
CALL SetFunfitParameterName(3,"LAMBDA") ! INITIAL (AND SS VALUE)
FOR Y1
CALL SetFunfitParameterName(4,"N0") ! INITIAL (AND SS VALUE) FOR Y2

CALL GET_RBC_PROD_DATA
THETA=P(1)
K=P(2)
LAMBDA=P(3)
N0=P(4)
CURRENT_TIME=TEND
CALL DQDAGS(TOTAL_RBC_PROD_RATE, TSTART, TEND, ABSERR, RELERR, YY, ESTERR)
WRITE(*,*)YY
JFUN = 1
CALL INTEGRATE_USERMODEL_ODE(TEND,Y,P,NP,JFUN,TSTART,0D0,NEQN,JFUN)
WRITE(*,*)Y
ENDIF

IF(IFUN > 0 ) THEN
  THETA=P(1)
  K=P(2)
  LAMBDA=P(3)
  N0=P(4)
  CURRENT_TIME=T
  JFUN = IFUN
  !CALL INTEGRATE_USERMODEL_ODE(T,Y,P,NP,IFUN,TZERO,YZERO,NEQN,JFUN)
  CALL INTEGRATE_USERMODEL_ODE(TEND,YY,P,NP,IFUN,TSTART,0D0,NEQN,JFUN)
  Y=P(4)*YY
  ! WRITE(*,*)Y
  ! CALL INTEGRATE_USERMODEL_ODE(TEND,YY,P,NP,2,TSTART,0D0,NEQN,2)
  ! WRITE(*,*)T,YY
  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
!CURRENT_TIME=TEND
CALL DQDAGS(UIR2, 0D0,12500D0, ABSERR, RELERR, YY, ESTERR)
WRITE(*,*)YY
WRITE(LUN,"(/, ' Mean Potential lifespan (D):    ',G12.4)")YY
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 LEFTLABEL("RBC COUNT")         ! LABEL FOR LEFT Y-AXIS
CALL TITLE("LABELED RBC SURVIVAL")  ! TITLE OF PLOT
CALL XLABEL("TIME (DAY)")           ! LABEL FOR X-AXIS
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 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

!-----------------------------------------------------------------------------------------------------------
- ! IMPORTANT ! IMPORTANT ! IMPORTANT ! IMPORTANT ! IMPORTANT !
!-----------------------------------------------------------------------------------------------------------
-

! ***** NON 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 = 'STRESS ERYTHROPOIESIS SURVIVAL (MIS, V.1)'!* <= CHANGE THIS STRING EVERY TIME YOU

RETURN
REAL*8 FUNCTION TOTAL_RBC_PROD_RATE(X)
  USE RBC_PROD_MODULE
  USE UNIT_IMPULSE_RESPONSE
  IMPLICIT NONE
  REAL*8 :: X
  TOTAL_RBC_PROD_RATE=UIR(X)*RBC_PRODUCTION_RATE(X)
END FUNCTION TOTAL_RBC_PROD_RATE

REAL*8 FUNCTION UIR2(X)
  USE RBC_PROD_MODULE
  USE UNIT_IMPULSE_RESPONSE
  REAL*8 :: X
  IF (X<0D0) THEN
    UIR2=0.0D0
  ELSE
    UIR2=DEXP(-1D0*(((MAX(0D0,X-THETA))/LAMBDA)**K))
  ENDIF
END FUNCTION UIR2
Appendix C.2.2 RBC PRODUCTION.F90

MODULE RBC_PROD_MODULE
USE NUMERICAL_LIBRARIES
IMPLICIT NONE
DOUBLE PRECISION,ALLOCATABLE,DIMENSION(:)::TPROD,APROD
INTEGER::NPROD
INTEGER,PARAMETER::DEGREE=4
REAL*8, DIMENSION(1:DEGREE+1) :: BWCOEF, SSPOLY
REAL*8, DIMENSION(1:10):: STAT
INTEGER::I,J,IOERROR,STATUS
SAVE
CONTAINS

SUBROUTINE GET_RBC_PROD_DATA
IMPLICIT NONE
REAL*8::TEMP1,TEMP2
CHARACTER*256 DATAFILENAME2

!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
PRINT*, "ENTER THE FILE FOR CUMULATIVE AMOUNT PRODUCED OF 
RBCs"
    CALL FILESELECT("TXT", DATAFILENAME2, J)
    WRITE(*,"(' SELECTED FILENAME: ', A256)"") DATAFILENAME2
    OPEN(UNIT=13,FILE=DATAFILENAME2,STATUS='OLD',ACTION='READ'
\ ,IOSTAT=IOERROR)
    NPROD=0
    IF (IOERROR == 0) THEN
    DO     !
            READ(13,*, IOSTAT=IOERROR)TEMP1,TEMP2
            IF (IOERROR/=0) EXIT
            I=I+1
            TPROD(I)=TEMP1
            APROD(I)=TEMP2
    END DO
    ALLOCATE(TPROD(NPROD),STAT=STATUS)
    ALLOCATE(APROD(NPROD),STAT=STATUS)
    REWIND (UNIT=13)
    I=0
    DO     !
            READ(13,*, IOSTAT=IOERROR)TEMP1,TEMP2
            IF (IOERROR/=0) EXIT
            I=I+1
            TPROD(I)=TEMP1
            APROD(I)=TEMP2
    END DO
    CLOSE (UNIT=13)
ELSE
WRITE(*,*)'FILE OPEN FAILED STATUS =',IOERROR,DATAFILENAME2
ENDIF
!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
CALL DRCURV(NPROD,TPROD,APROD, DEGREE, BWCOEF, SSPOLY, STAT)
END SUBROUTINE GET_RBC_PROD_DATA

REAL*8 FUNCTION RBC_PRODUCTION_RATE(TPR)
  IMPLICIT NONE
  REAL*8::TPR
  REAL*8::SLOPE
  SLOPE=0D0
!WRITE(*,*)BWCOEF(1:DEGREE+1)
  DO I=2,DEGREE+1
    SLOPE=SLOPE+BWCOEF(I)*DBLE(I-1)*TPR**(DBLE(I-2))
  END DO
  RBC_PRODUCTION_RATE=SLOPE
END FUNCTION RBC_PRODUCTION_RATE

END MODULE RBC_PROD_MODULE
Appendix C.2.3 UIR.F90

MODULE UNIT_IMPULSE_RESPONSE
IMPLICIT NONE
REAL*8::THETA,K,LAMBDA,CURRENT_TIME

CONTAINS

REAL*8 FUNCTION UIR(X)
  REAL*8::X
  IF (X<0D0) THEN
    UIR=0.0D0
  ELSE
    IF (CURRENT_TIME>100D0) WRITE(*,*) DEXP(-1D0*(((MAX(0D0,CURRENT_TIME-X-THETA))/LAMBDA)**K))
    UIR=DEXP(-1D0*(((MAX(0D0,CURRENT_TIME-X-THETA))/LAMBDA)**K))
  ENDIF
END FUNCTION UIR
END MODULE UNIT_IMPULSE_RESPONSE
APPENDIX D. SOFTWARE CODES FOR CHAPTERS 8

Appendix D.1 WINFUNFIT code for EPO clearance calculation used in Chapters 8 and 9

****Begin prologue

The EPO plasma concentration profile from a single intravenous $^{125}$I-rhEPO tracer bolus dose was well described by a biexponential disposition function. The $^{125}$I-rhEPO clearance ($Cl(t)$) was calculated using:

$$Cl(t) = \frac{D}{AUC}$$

where D is the $^{125}$I-rhEPO dose and the AUC is the area under the curve for the concentration of $^{125}$I-rhEPO time calculated from the biexponential disposition function. The analysis involves the following files in CD in the folder “\Chapter 8\Clearance calculation\Two comp model PK study”:

1) Main program file FORTRAN code: ‘MAIN.F90’
2) WINFUNFIT files located in the folder “\WINFUNFIT”: ALLWINFUNFIT.lib, FUNFIT_RESOURCES.res and DUMMY_USERMODEL_ODE_JACOBIAN.F90
3) Data files: ‘\DATA’

****End prologue

! FILENAME = Main.F90
!
! PURPOSE: To calculate the clearance in EPO PK studies
!
! Instructions to calculate the clearance based on the PK studies
! 1-The data files are contained in the folder titled "DATA".
! 2-Run "Two comp model PK study.dsw"
! 3-Change the dose and the weight in the fortran main file

! ALGORITHM: 1.) Fit a two exponential for radio labeled EPO and calculate the Clearance based on the dose and the AUC
!
!
! CHANGES/REVISIONS:
! VERSION 1.0 FEB 13, 2008 Original code

!--------------------------------------------------------------------------------------------
SUBROUTINE USERMODEL_ODE(T,Y,YPRIME,P,NP,IFUN)  ! REQUIRED
NAME: USERMODEL_ODE (DO NOT CHANGE)
IMPLICIT REAL*8 (A-H,O-Z) ! REQUIRED TO USE REAL*8 FOR NON-INTEGERS
DIMENSION Y(*), YPRIME(*), P(*) ! NOTE USE REAL*8 FOR ALL CONSTANTS
!LOGICAL EVENT_IS_ACTIVE                      !* <= REMEMBER TO INCLUDE THIS!

IF(IFUN == 1 )THEN !------------------------------------

END IF

RETURN
END

!===============================================================

SUBROUTINE USERMODEL(T,C,P,NP,IFUN) ! USERMODEL IS A REQUIRED NAME (DO NOT CHANGE)
! USE EVENTSTRUCTURE                   !*  <= MUST DECLARE EVENT DATA TYPE WHEN USING EVENT PROCESSING
IMPLICIT REAL*8 (A-H,O-Z)            !   REQUIRED  TO USE REAL*8 FOR ALL NON INTEGERS
PARAMETER  (NEQN = 1)                !   REQUIRED, WE HAVE 3 DIFF EQUATIONS IN THIS CASE
DIMENSION YZERO (NEQN)               !   REQUIRED
REAL*8::A,B,alpha,Beta,DUMMY,DOSE = 1742868,WT= 10.1
DIMENSION P(*)                     !   REQUIRED
LOGICAL SHOWIT
CHARACTER*256 ID, DATAFILENAME
! TYPE (EVENTTYPE) EVENT(1)            !* <= MUST DECLARE EVENT DATA TYPE WHEN USING EVENT PROCESSING

!----
! 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,"A")
CALL SetFunfitParameterName(2,"alpha")
CALL SetFunfitParameterName(3,"B")
CALL SetFunfitParameterName(4,"Beta_Alpha")
CALL SetFunfitParameterName(5,"Clearance")
CALL SetFunfitParameterName(6,"Beta")
CALL SetFunfitParameterName(7,"AUC")
CALL SetFunfitParameterName(8,"NORM_CLEARANCE")

A=P(1)
alpha=P(2)
B=P(3)
Beta_Alpha=P(4)
P(6)=P(2)+P(4)
Beta =P(6)
P(7) = (A/alpha)+(B/Beta)
AUC =P(7)
P(5)= DOSE/P(7)
Clearance = P(5)
P(8)=DOSE/(P(7)*WT)
NORM_CLEARANCE=P(8)

IF(IFUN == 1 ) THEN
   JY = IFUN
   C=(P(1)*DEXP(-T*P(2)))+(P(3)*DEXP(-T*(P(6))))
RETURN
ENDIF
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("PPT'D CPM/ML") ! LABEL FOR LEFT Y-AXIS
   CALL TITLE("PPTD CPMS VS TIME") ! TITLE OF PLOT
   CALL XLABEL("TIME") ! 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
!

!----------------------------------------------------------------------------------------------------------------------------
!
! IMPORTANT! IMPORTANT! IMPORTANT! IMPORTANT!
!----------------------------------------------------------------------------------------------------------------------------
!
!
!

******** NON OPTIONAL DEFINITION SECTION ******
!

**** THIS IS FOR THE RECORDING OF THE MODEL USED IN THE FITTING

ENTRY MODELID(ID)
   ID = 'TWO COMPARTMENT MODEL FOR PK DATA (MIS, V.1)'!* <= CAHNGE THIS STRING EVERY TIME YOU
   ! !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
   ! MAKE CHANGES IN THE ABOVE

SUBRROUTINE(S)

RETURN

END
!

-- -------------------------------------------------- E N D --------------------------------------------------
--
Appendix D.2 R code for Statistical analysis of clearance and mRNA conducted in Chapters 8

****Begin prologue

The statistical analysis was conducted using R software (Version 2.6.1; http://cran.r-project.org/). Repeated measure ANOVA was conducted to assess the effect of phlebotomy on mRNA levels and clearance values. Since our data include both fixed and random effects, repeated measure ANOVA was applied using LME (linear mixed effect) function in R (NLME library). The following linear mixed model was used to examine the relationship between EPO clearance values and EPOR mRNA levels:

\[
CL_{i,j} = \alpha_i + \beta_i \cdot mRNA_{i,j} + \epsilon_{i,j}
\]

\[
\epsilon_{i,j} \sim N(0, \sigma^2)
\]

\[
\begin{pmatrix}
\alpha_i \\
\beta_i
\end{pmatrix} \sim BVN
\begin{pmatrix}
\alpha^2 \\
\beta^2
\end{pmatrix}
\]

****End prologue

```r
require(nlme)
SUB<-c(1,1,2,2,3,3,4,4,5,5,6,6,6,6,6,7,7,7,7,8,8,8,8,8,9,9,9,10,10,10,11,11,11)
OBS<-c("OB1","OB2","OB1","OB2","OB1","OB2","OB1","OB2","OB1","OB2","OB1","OB2","OB3","OB1","OB2","OB3","OB1","OB2","OB3","OB1","OB2","OB3","OB1","OB2","OB3","OB1","OB2","OB3")
CL<-c(58.65,132.55,51.23,68.14,65.89,77.09,73.33,99.73,54.92,98.31,51.64,115.16,70.75,87.23,43.33,65.89,92.9,54.69,80.48,103.82,46.68,69.06,64.23,43.51,59.7,108.65,61.49)
MR<-c(0.010871288,0.136786713,0.047366143,0.235424004,0.041234622,0.38958229,0.140957616,0.400534939,0.130609644,0.488579984,0.113702299,0.393199484,0.26061644,0.171545914,0.208290171,0.1093212463,0.041713745,0.10153155,0.046606231,0.010289802,0.022600296,0.016280804,0.008167534,0.082875645,0.024373524,0.049485043,0.079084531,0.0038955504)
#MR<-1*MR1
d <- data.frame(SUB,CL,MR,OBS)
CLANOVA<-lme(CL ~ OBS-1, data = d,random=~1|SUB)
intervals(CLANOVA)
summary(CLANOVA)
require(graphics)
boxplot(CL ~ OBS, data = d)
MRANOVA<-lme(MR ~ OBS-1, data = d,random=~1|SUB)
intervals(MRANOVA)
summary(MRANOVA)
boxplot(MR ~ OBS, data = d)
mix <- lme(CL ~ MR, data = d,random=~1+MR|SUB)
```
mix
summary(mix)
intervals(mix, which="fixed")
anova(mix)
fmlOrthF.lis <- lmList(CL ~ MR|SUB, data = d)
coef( fmlOrthF.lis )
intervals( fmlOrthF.lis )
f<-predict( fmlOrthF.lis )
lme( fmlOrthF.lis )
dd <- data.frame(f,CL)
plot(dd)
o<-c(min(f),max(f))
plot(o,o, type = "l",xlab="PREDICTED",ylab="ACTUAL")
points(dd, cex = 1.5, col = "blue",pch=19)
APPENDIX E. WINFUNFIT CODES FOR CHAPTER 9

****Begin prologue
This FORTRAN program performs modeling of the erythropoiesis kinetics model discussed in Chapter 9.
The analysis involves the following files in CD in the folder “\Chapter 9”:
1) Main program file FORTRAN code: MAIN.F90
2) Hb production stimulation function: FSTIM_EPO_MODULE.F90
3) Linear spline subroutine: LINEAR SPLINE.F90
4) Phlebotomy and transfusion module: PHLEBOTOMY TRANSFUSION MODULE.F90
5) Body weight subroutine: WEIGHT.F90
6) WINFUNFIT files located in the folder “\WINFUNFIT”: ALLWINFUNFIT.lib, FUNFIT_RESOURCES.res and DUMMY_USERMODEL_ODE_JACOBIAN.F90
7) Data files: \DATA
8) Results folder: \Results

****End prologue

Appendix E.1 Fortran code for MAIN.F90

! FILENAME = LAMB_SHEEP.F90
!
! PURPOSE: TO DETERMINE THE PRODUCTION RATE OF HEMOGLOBIN IN NEONATAL SHEEPS
! WHILE ACCOUNTING FOR EPO RECEPTOR UP-REGULATION ,SHEEP GROWTH, PHLEBOTIMES, AND TRANSFUSIONS
!
! MODEL: CLEARANCE WAS USED AS A MEASURE OF EPO RECEPTOR LEVEL. THE STIMULATION RATE IS A FUNCTION OF EPO CONCENTRATION. A POINT DISTRIBUTION OF
! RBC POTENTIAL LIFESPANS IS ASSUMED FOR BOTH THE ENDOGENOUSLY PRODUCED AND TRANSFUSED
! RBCS. THE LIFESPANS OF RBCS DETERMINES THE RATE OF HEMOGLOBIN LOSS. THE STIMULATION
! RATE AND BLOOD VOLUME IS PROPORTIONAL TO THE BODY WEIGHT. PHLEBOTOMY AND TRANSFUSION
! EVENTS ARE ASSUMED TO BE INSTANTANEOUS EVENTS.
!
! UNITS:
! HEMOGLOBIN (HGB) G/DL
! TIME DAYS
! BODY WEIGHT KG
! BLOOD VOLUME DL/KG
! PHLEBOTOMIES G HGB
! TRANSFUSIONS G HGB
! STIMULATION RATES G HGB/DAY/(KG**MRS)
! ERYTHROPOIETIN MU/ML
! CLEARANCE MU/HOUR
!
!
! REVISIONS:
! VERSION 1.0 APRIL 14, 2009 ORIGINAL CODE-MIS.
!
!
! ============= DIFFERENTIAL EQUATION(S) OF MODEL =============
SUBROUTINE USERMODEL_ODE(T,Y,YPRIME,P,NP,IFUN)
USE PHLEBOTOMY_TRANSFUSION_MODULE
USE FSTIM_EPO_MODULE
USE BODYWEIGHT_MODULE
    USE LINEAR_SPLINE
IMPLICIT NONE

INTEGER, PARAMETER :: MAXCOEFF = 100, MAXEVENTS = 250
INTEGER :: NP, IFUN, NO_PHLEBOTOMIES, NOCOEFF, J
REAL*8 :: T, Y(*), YPRIME(*), P(*), A, B, CORRECTION_TERM, &
TRANSFUSION_TERM, TEMPA, &
TEMPY, TEMPF,Y2LA,Y2LB,KCL0,KCL1,KCL2,KCL3
LOGICAL :: EVENT_IS_ACTIVE
REAL*8, EXTERNAL :: TOTAL_BODY_STIM_RATE,YLAG

IF(IFUN.EQ.1.OR.IFUN.EQ.2) THEN
    ! ============= PARAMETER DECLARATIONS =============
    A = P(1)
    B = P(2)
    LT = P(3)
    FDAMAGED = 1D0 - P(4)
    MRS = P(6)
    GESTATIONAL_AGE_AT_BIRTH = P(7)
    VMKM = P(8)
    IKM = P(9)
        K_INUTERO=P(10)
        WTP1=P(11)
        WTP2=P(12)
        WTP3=P(13)

    ! ============= CALCULATE PHLEBOTOMY CORRECTION AND
TRANSFUSION TERM =============
    CALL GET_NUMBER_PHLEBOTOMIES(T, NO_PHLEBOTOMIES)
    IF ( NO_PHLEBOTOMIES > 0 ) THEN
        IF ( EVENT_IS_ACTIVE(NO_PHLEBOTOMIES) ) THEN
CALL GET_PHLEBOTOMY_AMOUNT_VALUE(NO_PHLEBOTOMIES, TEMPA)
CALL UPDATE_FRACTION_REMAINING_VALUE(NO_PHLEBOTOMIES, 1D0 - TEMPA/Y(1))
END IF
END IF
CALL GET_PHLEBOTOMY_CORRECTION_TERM(T - (B - A), T, CORRECTION_TERM)
TRANSFUSION_TERM=0D0
! ============= DIFFERENTIAL EQUATION(S) =============
YPRIME(1) = TOTAL_BODY_STIM_RATE(T-A) - TOTAL_BODY_STIM_RATE(T-B)*CORRECTION_TERM
YPRIME(2) = TOTAL_BODY_STIM_RATE(T-A)
ENDIF
END SUBROUTINE USERMODEL_ODE

! ============= MODEL (EXCLUDING DIFFERENTIAL EQUATIONS(S)) =============
SUBROUTINE USERMODEL(T,Y,P,NP,IFUN)
USE PHLEBOTOMY_TRANSFUSION_MODULE
USE FSTIM_EPO_MODULE
USE BODYWEIGHT_MODULE
USE NUMERICAL_LIBRARIES
IMPLICIT NONE
INTEGER, PARAMETER :: NEQN = 2, NPAR=21, 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,STATUS,ZZ,NCL,ROR
REAL*8 :: T, Y, P(*), YZERO(NEQN), TZERO(NEQN), TEMPT, TEMPA, TEMPX(MAXN), &
TEMPY(MAXN), BVOL, COEFFICIENTS(MAXCOEFF), ESTERR, HBZERO, A, B, &
XMAX, HBTOTALPROD, HBTOTALPHLEB, HBTOTALTRANS, TEMPSUM, TY, TX, TR, &
MAXHBMAMT, TX1(MAXN), TX2(MAXN), TY1(MAXN), TY2(MAXN), TYMAX, &
MONTHHBTOTALPROD, FUNDAMAGED, XEPO(MAXN), YEPO(MAXN), &
KCL0,KCL1,KCL2,KCL3,FLAG1,FLAG2,MXEPO,XCL(MAXN),YCL(MAXN),CLP(L(MAXN))
! DOUBLE
PRECISION,ALLOCATABLE,DIMENSION(:)::LOGYEPO,LOGXEPO
LOGICAL, SAVE :: SHOWIT, PLOTSaved
CHARACTER (LEN=256) :: ID, DATAFILENAME
CHARACTER (LEN=20)  :: PNAME
CHARACTER (LEN=1)   :: RESPONSE
REAL*8, EXTERNAL :: TOTAL_BODY_STIM_RATE,YLAG

! THEOSE VARIABLES JUST TO MANAGE PHLEBOTOMIES
INTEGER::PHN
REAL*8::TEMP1,TEMP2,TEMP3,PHT(MAXN),PHX(MAXN),PHF(MAXN),VSAMPLE,VCL,VPH11,VPH12,VPH21,VPH22

! ============= PRELIMINARY PREPARATION SECTION ==============
IF (IFUN == -1000) THEN
  IF(NP /= NPAR) STOP ' USERMODEL: INCONSISTENT NUMBER OF PARAMETERS IN PARAMETER FILE'

! ============= PARAMETER NAMES ==============
CALL SETFUNFITPARAMETERNAME(1, " A (DAY)")
CALL SETFUNFITPARAMETERNAME(2, " B (DAY)")
CALL SETFUNFITPARAMETERNAME(3, " DO NOT USE")
CALL SETFUNFITPARAMETERNAME(4, " DO NOT USE")
CALL SETFUNFITPARAMETERNAME(5, " BLOOD VOL (DL/KG)")
CALL SETFUNFITPARAMETERNAME(6, " METAB RATE SCALAR")
CALL SETFUNFITPARAMETERNAME(7, " GA AT BIRTH (DAYS)")
CALL SETFUNFITPARAMETERNAME(8, " VMAX (G/DAY*(KG**MRS))")
CALL SETFUNFITPARAMETERNAME(9, " KM (MU/ML)")
CALL SETFUNFITPARAMETERNAME(10, " K_INUTERO")
CALL SETFUNFITPARAMETERNAME(11, " WTP1")
CALL SETFUNFITPARAMETERNAME(12, " WTP2")
CALL SETFUNFITPARAMETERNAME(13, " WTSLOPE")
CALL SETFUNFITPARAMETERNAME(14, " VSAMPLE")
CALL SETFUNFITPARAMETERNAME(15, " VCL")
CALL SETFUNFITPARAMETERNAME(16, " VPH11")
CALL SETFUNFITPARAMETERNAME(17, " VPH12")
CALL SETFUNFITPARAMETERNAME(18, " LINEAR CLEARANCE")
CALL SETFUNFITPARAMETERNAME(19, " EPO ELIMINATION EC50")
CALL SETFUNFITPARAMETERNAME(20, " VPH21")
CALL SETFUNFITPARAMETERNAME(21, " VPH22")
! ============= 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 *, 'PHLEBOTOMY AMOUNT-TIME DATA:'
CALL FILESELECT("DAT", DATAFILENAME, J)

PHN=0

OPEN (UNIT=10, FILE=DATAFILENAME, STATUS='OLD', ACTION='READ', IOSTAT=status)

IF (STATUS == 0) THEN

DO
   READ(10,*, IOSTAT=status) TEMP1,TEMP2,TEMP3
   IF (STATUS /=0) EXIT
   PHN=PHN+1
   PHT(PHN)=TEMP1
   PHF(PHN)=TEMP2
   PHX(PHN)=TEMP3
END DO

ELSE
   WRITE(*,3002)STATUS
   3002 FORMAT (1X,'PHLEBOTOMY FILE OPEN FAILED status= ',I6)
END IF

NPHLEB=PHN

!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!

TEMPN = MAXN
PRINT *
PRINT *, 'BODY WEIGHT-TIME DATA:'
CALL GET_XY_DATA_FROM_FUNFIT_FILE (TEMPX,TEMPY,TEMPN)
IF ( TEMPN > MAXN ) STOP ' TOO MANY BODY WEIGHTS. ADJUST THE MAXN APPROPRIATELY'
CALL SET_BODYWEIGHT_TIME_VECTOR(TEMPN, TEMPX)
CALL SET_BODYWEIGHT_VALUE_VECTOR(TEMPN, TEMPY)

TEMPN = MAXN
PRINT*
PRINT*, 'EPO CONCENTRATION-TIME DATA:'
CALL GET_XY_DATA_FROM_FUNFIT_FILE (TEMPX,TEMPY,TEMPN)
IF ( TEMPN > MAXN ) STOP ' TOO MANY EPO DATA POINTS. ADJUST THE
MAXN APPROPRIATELY'
CALL
SET_LINEAR_SPLINE_PARAMETERS(TEMPX,TEMPY,TEMPN)
    STATUS=0

    XEPO = TEMPX
    YEPO = TEMPY
    NEPO = TEMPN
    TEMPN = MAXN
PRINT*
PRINT*, 'CLEARANCE-TIME DATA:'
CALL GET_XY_DATA_FROM_FUNFIT_FILE (TEMPX,TEMPY,TEMPN)
IF ( TEMPN > MAXN ) STOP ' TOO MANY CLEARANCE DATA POINTS.
ADJUST THE MAXN APPROPRIATELY'
CALL
SET_LINEAR_SPLINE_PARAMETERS2(TEMPX,TEMPY,TEMPN)
    STATUS=0
    XCL=TEMPX
    YCL=TEMPY
    NCL=TEMPN

DO ROR=1,NCL
    CLPL(ROR)=((CLR(XCL(ROR))-LCL)*(CEPO(XCL(ROR))+KDEPO))/((CLR(0D0)-
LCL)*(CEPO(0D0)+KDEPO))
ENDDO

    FUNDAMAGED = P(4)

    ! ============ SELCECT THE ODE SOLVER AND PLOTTING OPTIONS
--------------
    CALL USE_DELAY_ODE_SOLVER
    CALL DO_NOT_USE_DELAY_ODE_PLOTS
    END IF
NTRANS=0
    ! =========== PARAMETER DECLARATIONS ==========
    A = P(1)
    B = P(2)
    LT = P(3)
    FUNDAMAGED = P(4)
    FDAMAGED = 1D0 - FUNDAMAGED
BVOL = P(5)
MRS = P(6)
GESTATIONAL_AGE_AT_BIRTH = P(7)
VMKM = P(8)
IKM = P(9)
WTP1 = P(11)
WTP2 = P(12)
WTP3 = P(13)
K_INUTERO = P(10)
VSAMPLE = P(14)
VCL = P(15)
VPH11 = P(16)
VPH12 = P(17)
LCL = P(18)
KDEPO = P(19)
VPH21 = P(20)
VPH22 = P(21)
CURRENT_AGE = 60

IF ( NPHLEB > 0 ) THEN
DO J = 1, NPHLEB
  TEMPX(J) = PHT(J)
  IF (PHF(J) < 1.5) THEN
    TEMPY(J) = VSAMPLE * PHX(J)
  ELSEIF (PHF(J) < 2.5) THEN
    TEMPY(J) = VCL * PHX(J)
  ELSEIF (PHF(J) < 3.5) THEN
    TEMPY(J) = VPH11 * PHX(J)
  ELSEIF (PHF(J) < 4.5) THEN
    TEMPY(J) = VPH12 * PHX(J)
  ELSEIF (PHF(J) < 5.5) THEN
    TEMPY(J) = VPH21 * PHX(J)
  ELSEIF (PHF(J) < 6.5) THEN
    TEMPY(J) = VPH22 * PHX(J)
  ENDIF
  TEMPT = TEMPX(J)
  TEMPA = TEMPY(J)
  CALL REGISTER_EVENT(J, TEMPT, TEMPT, -TEMPA, 1)
END DO
END IF
NOEVENTS = NPHLEB
CALL COMPLETE_THE_EVENT_REGISTRATION
TEMPN=NPHLEB
CALL SET_PHLEBOTOMY_TIME_VECTOR(TEMPN,
TEMPX)
CALL SET_PHLEBOTOMY_AMOUNT_VECTOR(TEMPN,
TEMPY)
END IF

! ============= DIFFERENTIAL EQUATION INITIAL CONDITIONS
=============
TZERO(1) = TIMEZERO
CALL DQDAGS(TOTAL_BODY_STIM_RATE, TZERO(1) - B, TZERO(1) - A,
ABSERR, RELERR, YZERO(1), ESTERR)

! ============= MODEL FITTING SECTION ==============
IF(IFUN.EQ.1) THEN
  JFUN = IFUN
  CALL
  INTEGRATE_USERMODEL_ODE(T,Y,P,NP,IFUN,TZERO,YZERO,NEQN,JFUN)
  Y = Y/(BVOL*BODYWEIGHT(T))
END IF

! ============= USER OUTPUT SECTION SECTION ==============
IF ( IFUN == 0 ) THEN

! ============= SECONDARY PARAMETER CALCULATIONS AND
WRITE STATEMENTS ==============
CALL GET_GLOBAL_XMAX(XMAX)
T=TZERO(1)+ XMAX

CALL
INTEGRATE_USERMODEL_ODE(T,HBTOTALPROD,P,NP,2,TZERO,YZERO,NEQN,2)
T=TZERO(1)+ 30D0
CALL
INTEGRATE_USERMODEL_ODE(T,MONTHHBTOTALPROD,P,NP,2,TZERO,YZERO,
NEQN,2)
HBTOTALPHLEB = 0
IF ( NPHLEB > 0 ) THEN
  DO J = 1, NPHLEB
    CALL GET_PHLEBOTOMY_AMOUNT_VALUE(J, TEMPA)
    HBTOTALPHLEB = HBTOTALPHLEB + TEMPA
  END DO
END IF
HBTOTALTRANS = 0
IF ( NTRANS > 0 ) THEN
DO J = 1, NTRANS
   CALL GET_TRANSFUSION_AMOUNT_VALUE(J, TEMPA)
   HBTOTALTRANS = HBTOTALTRANS + TEMPA
END DO
END IF
DO J = 1, NP
   CALL GETFUNFITPARAMETERNAME (J,PNAME)
   WRITE(*,"(1X,I2,1X,A,G14.4)"), J, PNAME, P(J)
ENDDO
IF (IKM > 0D0) THEN
   WRITE(*,"(4X,'VM (G/DAY*(KG**MRS))',1X,G14.4)") VMKM/IKM
   WRITE(*,"(4X,'KM (MU/ML)',11X,G14.4)") 1/IKM
END IF
WRITE(*,",(,' INITIAL AMOUNT OF HEMOGLOBIN PRESENT (G):",G12.4)") YZERO(1)
WRITE(*,",(,' TOTAL AMOUNT OF HEMOGLOBIN REMOVED (G):",G12.4)") HBTOTALPHLEB
WRITE(*,",(,' TOTAL AMOUNT OF HEMOGLOBIN TRANSFUSED (G):",G12.4)") HBTOTALTRANS
WRITE(*,",(,' TOTAL AMOUNT OF HEMOGLOBIN PRODUCED (G):",G12.4)") HBTOTALPROD
WRITE(*,",(,' AMT OF HEMOGLOBIN PRODUCED OVER 30 DAYS (G):",G12.4)") MONTHHBTOTALPROD

! ============= SAVING OF SECONDARY PARAMETERS AND CONSTRUCTION OF USER PLOTS =============
CALL PROMT(SHOWIT)
IF (SHOWIT) THEN
   WRITE(*,",(,' PLEASE ENTER THE SUBJECT NUMBER:')")
   READ(*,*) SUBNO
   WRITE(LUN,"(/,' LAMB NUMBER: ',I6,/)"), SUBNO
   IF (IKM > 0D0) THEN
      WRITE(LUN,"(/,' VM (G/DAY*(KG**MRS)):',10X,G12.4)") VMKM/IKM
      WRITE(LUN,"(' KM (MU/ML):',20X,G12.4)") 1/IKM
   END IF
   WRITE(LUN,"(/, ' INITIAL AMOUNT OF HEMOGLOBIN PRESENT (G):",G12.4)") YZERO(1)
   WRITE(LUN,"(/, ' TOTAL AMOUNT OF HEMOGLOBIN REMOVED (G):",G12.4)") HBTOTALPHLEB
   WRITE(LUN,"(' TOTAL AMOUNT OF HEMOGLOBIN TRANSFUSED (G):",G12.4)") HBTOTALTRANS
   WRITE(LUN,"(' TOTAL AMOUNT OF HEMOGLOBIN PRODUCED (G):",G12.4)") HBTOTALPROD
   WRITE(LUN,"(/,' AMT OF HEMOGLOBIN PRODUCED OVER 30 DAYS (G):",G12.4)") MONTHHBTOTALPROD
WRITE(LUN,"(/' NUMBER OF PHLEBOTOMIES: ',G12.4") NPHLEB
WRITE(LUN,"(/' NUMBER OF TRANSFUSIONS: ',G12.4") NTRANS

! ============= CONSTRUCTION OF PLOTS OF BODY WEIGHT, PRODUCTION RATE, BLOOD VOLUME, AND EPO CONCENTRATION ==============

WRITE(*,"(/,' WOULD YOU LIKE PLOTS OF BODY WEIGHT, PRODUCTION RATE, BLOOD VOLUME, AND EPO CONCENTRATION?')")
READ(*,*) RESPONSE
IF ( RESPONSE == 'Y' .OR. RESPONSE == 'y' ) THEN
CALL ADD_MARGIN_TEXT_WITH_INTEGER_NUMBER_ADDED('LAMB:', SUBNO)
CALL TITLE('BODYWEIGHT')
CALL DEFINE_GRID_FOR_CURVE(TZERO(1), TZERO(1) + XMAX, MAXN)

JOB = 0
DO
  CALL CONSTRUCT_CURVE(TX, TY, JOB)
  IF ( JOB /= 1 ) EXIT
  TY = BODYWEIGHT(TX)
END DO
CALL GET_BODYWEIGHT_TIME_VECTOR(TEMPX, TEMPN)
CALL GET_BODYWEIGHT_VALUE_VECTOR(TEMPY, TEMPN)
CALL LEFT_LABEL('BODYWEIGHT (KG)')
CALL X_LABEL('TIME (DAYS)')
CALL END_X_AT(XMAX)
CALL ADD_POINTS_LEFT(TEMPX, TEMPY, TEMPN)
CALL ADD_CONSTRUCTED_CURVE
CALL PLOT_IN_AREA(1, 4)

CALL TITLE('TOTAL BLOOD VOLUME')
CALL DEFINE_GRID_FOR_CURVE(TZERO(1), TZERO(1) + XMAX, MAXN)

JOB = 0
DO
  CALL CONSTRUCT_CURVE(TX, TY, JOB)
  IF ( JOB /= 1 ) EXIT
  TY = BVOL*BODYWEIGHT(TX)
END DO
CALL LEFT_LABEL('BLOOD VOLUME (DL)')
CALL X_LABEL('TIME (DAYS)')
CALL END_X_AT(XMAX)
CALL ADD_POINTS_LEFT(TEMPX, TEMPY, TEMPN)
CALL ADD_CONSTRUCTED_CURVE
CALL PLOT_IN_AREA(2, 4)
CALL TITLE('TOTAL HB PROD. RATE')
CALL DEFINE_GRID_FOR_CURVE(TZERO(1), TZERO(1) + XMAX, MAXN)

JOB = 0
DO
   CALL CONSTRUCT_CURVE(TX, TY, JOB)
   IF ( JOB /= 1 ) EXIT
   TY = TOTAL_BODY_STIM_RATE(TX - A)
END DO
CALL LEFT_LABEL('PROD. RATE (G/DAY)')
CALL X_LABEL('TIME (DAYS)')
CALL END_X_AT(XMAX)
CALL ADD_CONSTRUCTED_CURVE
CALL PLOT_IN_AREA(3, 4)

CALL TITLE('EPO CONCENTRATION')
CALL DEFINE_GRID_FOR_CURVE(TZERO(1), TZERO(1) + XMAX, MAXN)

JOB = 0
DO
   CALL CONSTRUCT_CURVE(TX, TY, JOB)
   IF ( JOB /= 1 ) EXIT
   TY = CEPO(TX)
END DO
CALL LEFT_LABEL('EPO CONC. (MU/ML)')
CALL X_LABEL('TIME (DAYS)')
CALL END_X_AT(XMAX)
CALL ADD_POINTS_LEFT(XEPO, YEPO, NEPO)
CALL ADD_CONSTRUCTED_CURVE
CALL PLOT_IN_AREA(4, 4)

CALL DISPLAY_PLOT
CALL RECORDPLOTIFSAVED(LUN)

END IF
!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
! ============= CONSTRUCTION OF PLOTS OF BACK-EXTRAPOLATED BODY WEIGHT, STIMULATION RATE, AND EPO CONCENTRATION =============
WRITE(*,./(,' WOULD YOU LIKE PLOTS OF BACK-EXTRAPOLATED BODY WEIGHT, STIMULATION RATE, AND EPO CONCENTRATION?'))
READ(*,*) RESPONSE
IF ( RESPONSE == 'Y' .OR. RESPONSE == 'y' ) THEN
   CALL ADD_MARGIN_TEXT_WITH_INTEGER_NUMBER_ADDED('-LAMB:', SUBNO)
   CALL TITLE('BODYWEIGHT')
CALL DEFINE_GRID_FOR_CURVE(TZERO(1) - B, TZERO(1) + XMAX, MAXN)
JOB = 0
DO
  CALL CONSTRUCT_CURVE(TX, TY, JOB)
  IF ( JOB /= 1 ) EXIT
  TY = BODYWEIGHT(TX)
END DO
CALL GET_BODYWEIGHT_TIME_VECTOR(TEMPX, TEMPN)
CALL GET_BODYWEIGHT_VALUE_VECTOR(TEMPY, TEMPN)
CALL LEFT_LABEL('BODYWEIGHT (KG)')
CALL X_LABEL('TIME (DAYS)')
CALL END_X_AT(XMAX)
CALL ADD_CONSTRUCTED_CURVE
CALL PLOT_IN_AREA(1, 4)

CALL TITLE('EPO CONCENTRATION')
CALL DEFINE_GRID_FOR_CURVE(TZERO(1) - B, TZERO(1) + XMAX, MAXN)
JOB = 0
DO
  CALL CONSTRUCT_CURVE(TX, TY, JOB)
  IF ( JOB /= 1 ) EXIT
  TY = CEPO(TX)
END DO
CALL LEFT_LABEL('EPO CONC. (MU/ML)')
CALL X_LABEL('TIME (DAYS)')
CALL END_X_AT(XMAX)
CALL ADD_CONSTRUCTED_CURVE
CALL PLOT_IN_AREA(2, 4)

CALL TITLE('TOTAL HB STIM. RATE')
CALL DEFINE_GRID_FOR_CURVE(TZERO(1) - B, TZERO(1) + XMAX, MAXN)
JOB = 0
DO
  CALL CONSTRUCT_CURVE(TX, TY, JOB)
  IF ( JOB /= 1 ) EXIT
  TY = TOTAL_BODY_STIM_RATE(TX)
END DO
CALL LEFT_LABEL('STIM. RATE (G/DAY)')
CALL X_LABEL('TIME (DAYS)')
CALL END_X_AT(XMAX)
CALL ADD_CONSTRUCTED_CURVE
CALL PLOT_IN_AREA(3, 4)
CALL DISPLAY_PLOT
CALL RECORDPLOTIFSAVED(LUN)
END IF

! ============= CONSTRUCTION OF PLOTS OF CUMULATIVE
PHLEBOTIMIES, TRANSFUSIONS, PRODUCTION, =============
! AND PREDICTED HEMOGLOBIN AMOUNTS IN THE SUBJECT
WRITE(*,'(/,' WOULD YOU LIKE PLOTS OF CUMULATIVE
PHLEBOTOMIES, TRANSFUSIONS, PRODUCTION,'/, &
' AND PREDICTED HEMOGLOBIN AMOUNTS IN THE
SUBJECT?')")
READ(*,*) RESPONSE
IF ( RESPONSE == 'Y' .OR. RESPONSE == 'y' ) THEN
   TX = TZERO(1)
   MAXHBAMT = YZERO(1)
   DO
       TX = TX + (XMAX - TZERO(1))/REAL(MAXN,8)
       IF ( TX > XMAX ) EXIT
       CALL
       INTEGRATE_USERMODEL_ODE(TX,TY,P,NP,1,TZERO,YZERO,NEQN,1)
       MAXHBAMT = MAX(TY, MAXHBAMT)
   END DO
   CALL ADD_MARGIN_TEXT_WITH_INTEGER_NUMBER_ADDED('LAMB:', SUBNO)
   CALL TITLE_WITH_VALUE_ADDED('TOTAL AMT. REMOVED =',HBTOTALPHLEB)
   IF ( NPHLEB > 0 ) THEN
       TEMPSUM = 0D0
       DO J = 1, NPHLEB
           CALL GET_TIME_OF_PHLEBOTOMY(J, TEMPT)
           CALL GET_PHLEBOTOMY_AMOUNT_VALUE(J, TEMPA)
           TEMPX(J*2-1) = TEMPT
           IF ( J > 1 ) THEN
               TEMPY(J*2-1) = TEMPY(J*2-2)
           ELSE
               TEMPY(J*2-1) = 0D0
           END IF
           TEMPX(J*2) = TEMPT
           IF ( J > 1 ) THEN
               TEMPY(J*2) = TEMPY(J*2-2) + TEMPA
           ELSE
               TEMPY(J*2) = 0D0 + TEMPA
           END IF
       END DO
       TEMPX(NPHLEB*2+1) = XMAX
       TEMPY(NPHLEB*2+1) = TEMPY(NPHLEB*2)
ELSE
   TEMPX(1) = 0D0
   TEMPY(1) = 0D0
END IF
CALL LEFT_LABEL('CUMULATIVE AMT. REMOVED (G)')
CALL X_LABEL('TIME (DAYS)')
CALL BEGIN_LEFT_AT(0D0)
CALL END_LEFT_AT(MAX(HBTOTALPROD, HBTOTALPHLEB,
                      HBTOTALTRANS, MAXHBAMT))
CALL BEGIN_X_AT(TZERO(1))
CALL END_X_AT(XMAX)
CALL ADD_CURVE_LEFT(TEMPX, TEMPY, NPHLEB*2+1)
CALL PLOT_IN_AREA(1,4)
CALL TITLE_WITH_VALUE_ADDED('TOTAL AMT. TRANSFUSED
 = ', HBTOTALTRANS)
IF ( NTRANS > 0 ) THEN
   TEMPSUM = 0D0
   DO J = 1, NTRANS
      CALL GET_TIME_OF_TRANSFUSION(J, TEMPT)
      CALL GET_TRANSFUSION_AMOUNT_VALUE(J, TEMPA)
      TEMPX(J*2-1) = TEMPT
      IF ( J > 1 ) THEN
         TEMPY(J*2-1) = TEMPY(J*2-2)
      ELSE
         TEMPY(J*2-1) = 0D0
      END IF
      TEMPX(J*2) = TEMPT
      IF ( J > 1 ) THEN
         TEMPY(J*2) = TEMPY(J*2-2) + TEMPA
      ELSE
         TEMPY(J*2) = 0D0 + TEMPA
      END IF
   END DO
   TEMPX(NTRANS*2+1) = XMAX
   TEMPY(NTRANS*2+1) = TEMPY(NTRANS*2)
ELSE
   TEMPX(1) = 0D0
   TEMPY(1) = 0D0
END IF
CALL LEFT_LABEL('CUMULATIVE AMT. TRANS. (G)')
CALL X_LABEL('TIME (DAYS)')
CALL BEGIN_LEFT_AT(0D0)
CALL END_LEFT_AT(MAX(HBTOTALPROD, HBTOTALPHLEB,
                      HBTOTALTRANS, MAXHBAMT))
CALL BEGIN_X_AT(TZERO(1))
CALL END_X_AT(XMAX)
CALL ADD_CURVE_LEFT(TEMPIX, TEMPY, NTRANS*2+1)
CALL PLOT_IN_AREA(2,4)

CALL TITLE_WITH_VALUE_ADDED('TOTAL AMT. PRODUCED =', HBOTALPROD)
CALL DEFINE_GRID_FOR_CURVE(TZERO(1), TZERO(1) + XMAX, MAXN)
JOB = 0
DO
   CALL CONSTRUCT_CURVE(TX, TY, JOB)
   IF ( JOB /= 1 ) EXIT
   CALL DQDAGS(TOTAL_BODY_STIM_RATE, TZERO(1) - A, TX - A, ABSERR, RELERR, TY, ESTERR)
END DO
CALL LEFT_LABEL('CUMULATIVE AMT. PRODUCED (G)')
CALL X_LABEL('TIME (DAYS)')
CALL BEGIN_LEFT_AT(0D0)
CALL END_LEFT_AT(MAX(HBTOTALPROD, HBOTALPHLEB, HBOTALTRANS, MAXHBAMT))
CALL BEGIN_X_AT(TZERO(1))
CALL END_X_AT(XMAX)
CALL ADD_CONSTRUCTED_CURVE
CALL PLOT_IN_AREA(3,4)

CALL TITLE_WITH_INTEGER_NUMBER_ADDED('PRED. AMT. HGB IN LAMB', SUBNO)
CALL DEFINE_GRID_FOR_CURVE(TZERO(1), TZERO(1) + XMAX, MAXN)
JOB = 0
DO
   CALL CONSTRUCT_CURVE(TX, TY, JOB)
   IF ( JOB /= 1 ) EXIT
   CALL INTEGRATE_USERMODEL_ODE(TX,TY,P,NP,1,TZERO,YZERO,NEQN,1)
END DO
CALL LEFT_LABEL('HEMOGLOBIN AMT. (G)')
CALL X_LABEL('TIME (DAYS)')
CALL BEGIN_LEFT_AT(0D0)
CALL END_LEFT_AT(MAX(HBTOTALPROD, HBOTALPHLEB, HBOTALTRANS, MAXHBAMT))
CALL BEGIN_X_AT(TZERO(1))
CALL END_X_AT(XMAX)
CALL ADD_CONSTRUCTED_CURVE
CALL PLOT_IN_AREA(4,4)
CALL DISPLAY_PLOT
CALL RECORDPLOTIFSAVED(LUN)
END IF

! ============= CONSTRUCTION OF A SINGLE PLOT OF OBSERVED
AND FITTED HEMOGLOBIN CONCENTRATIONS =============
WRITE(*, "/,' WOULD YOU LIKE A PLOT OF THE OBSERVED AND
FITTED HEMOGLOBIN CONCENTRATIONS??")
READ(*,*) RESPONSE
IF ( RESPONSE == 'Y' .OR. RESPONSE == 'y' ) THEN
    CALL ADD_MARGIN_TEXT_WITH_INTEGER_NUMBER_ADDED('-
    LAMB:', SUBNO)
    CALL TITLE_WITH_INTEGER_NUMBER_ADDED('LAMB:', SUBNO)
    CALL DEFINE_GRID_FOR_CURVE(TZERO(1), TZERO(1) + XMAX,
    MAXN)
    JOB = 0
    DO
        CALL CONSTRUCT_CURVE(TX, TY, JOB)
        IF ( JOB /= 1 ) EXIT
        CALL INTEGRATE_USERMODEL_ODE(TX,TY,P,NP,1,TZERO,YZERO,NEQN,1)
        TY = TY/(BVOL*BODYWEIGHT(TX))
    END DO
    CALL LEFT_LABEL('HEMOGLOBIN (G/DL)')
    CALL X_LABEL('TIME (DAYS)')
    CALL BEGIN_LEFT_AT(0D0)
    CALL BEGIN_X_AT(TZERO(1))
    CALL END_X_AT(XMAX)
    CALL ADDOBSERVATIONSLEFT(1)
    CALL ADD_CONSTRUCTED_CURVE
    CALL DISPLAY_PLOT
    CALL RECORDPLOTIFSAVED(LUN)
END IF
END IF

! ============= PLOT HGB AND EPO ONLY
! ============= CONSTRUCTION OF A SINGLE PLOT OF OBSERVED
AND FITTED HEMOGLOBIN CONCENTRATIONS =============
CALL ADD_MARGIN_TEXT_WITH_INTEGER_NUMBER_ADDED('-
LAMB:', SUBNO)
CALL TITLE_WITH_INTEGER_NUMBER_ADDED('LAMB:', SUBNO)
CALL DEFINE_GRID_FOR_CURVE(TZERO(1), TZERO(1) + XMAX,
MAXN)
    JOB = 0
DO
  CALL CONSTRUCT_CURVE(TX, TY, JOB)
  IF ( JOB /= 1 ) EXIT
  CALL INTEGRATE_USERMODEL_ODE(TX, TY, P, NP, 1, TZERO, YZERO, NEQN, 1)
  TY = TY/(BVOL*BODYWEIGHT(TX))
END DO
CALL LEFT_LABEL('HEMOGLOBIN (G/DL)')
CALL X_LABEL('TIME (DAYS)')
CALL BEGIN_LEFT_AT(0D0)
CALL BEGIN_X_AT(TZERO(1))
CALL END_X_AT(XMAX)
  CALL RIGHT_LABEL('EPO (MU/ML)')
     MAXEPO=0
     DO ZZ=1,NEPO
        IF(YEPO(ZZ)>MAXEPO)THEN
           MAXEPO=YEPO(ZZ)
        ENDIF
     ENDDO
     MAXEPO=1.2D0*MAXEPO
     CALL END_RIGHT_AT(MAXEPO)
  CALL INCLUDE_POINTS_RIGHT(XEPO,YEPO,NEPO,4)
  CALL ADD_CURVE_RIGHT(XEPO,YEPO,NEPO)
  CALL DISPLAY_PLOT
  CALL RECORDPLOTIFSACTIVE(SAVED(LUN))
!
!   ============= PLOT HGB AND CLEARANCE ONLY
CALL ADD_MARGIN_TEXT_WITH_INTEGER_NUMBER_ADDED('-LAMB: ', SUBNO)
CALL TITLE_WITH_INTEGER_NUMBER_ADDED('LAMB: ', SUBNO)
CALL DEFINE_GRID_FOR_CURVE(TZERO(1), TZERO(1) + XMAX, MAXN)
  JOB = 0
DO
  CALL CONSTRUCT_CURVE(TX, TY, JOB)
  IF ( JOB /= 1 ) EXIT
  CALL INTEGRATE_USERMODEL_ODE(TX, TY, P, NP, 1, TZERO, YZERO, NEQN, 1)
  TY = TY/(BVOL*BODYWEIGHT(TX))
END DO
CALL LEFT_LABEL('HEMOGLOBIN (G/DL)')
CALL X_LABEL('TIME (DAYS)')
CALL BEGIN_LEFT_AT(0D0)
CALL BEGIN_X_AT(TZERO(1))
CALL END_X_AT(XMAX)
CALL ADDOBSERVATIONSLEFT(1)
CALL ADD_CONSTRUCTED_CURVE
    CALL RIGHT_LABEL('CLEARANCE (ML/HR/KG)')
    MAXEPO=0
    DO ZZ=1,NCL
        IF(YCL(ZZ)>MAXEPO) THEN
            MAXEPO=YCL(ZZ)
        ENDIF
    ENDDO
    MAXEPO=2.60D0*MAXEPO
    CALL END_RIGHT_AT(MAXEPO)
    CALL INCLUDE_POINTS_RIGHT(XCL,YCL,NCL,1)
    CALL ADD_CURVE_RIGHT(XCL,YCL,NCL)
    CALL DISPLAY_PLOT
    CALL RECORDPLOTIFSAVED(LUN)

! ============= CONSTRUCTION OF A SINGLE PLOT OF
OBSERVED AND FITTED HEMOGLOBIN CONCENTRATIONS =============
WRITE(*,*)W
READ(*,*)R
IF ( R == 'Y' .OR. R == 'y' ) THEN
    CALL ADD_MARGIN_TEXT_WITH_INTEGER_NUMBER_ADDED('-LAMB:', SUBNO)
    CALL TITLE_WITH_INTEGER_NUMBER_ADDED('LAMB:', SUBNO)
    CALL DEFINE_GRID_FOR_CURVE(TZERO(1), TZERO(1) + XMAX, MAXN)
    JOB = 0
    DO
        CALL CONSTRUCT_CURVE(TX, TY, JOB)
        IF ( JOB /= 1 ) EXIT
    CALL INTEGRATE_USERMODEL_ODE(TX,TY,P,NP,1,TZERO,YZERO,NEQN,1)
    TY = TY/(BVOL*BODYWEIGHT(TX))
    END DO
    CALL LEFT_LABEL('HEMOGLOBIN (G/DL)')
    CALL X_LABEL('TIME (DAYS)')
    CALL BEGIN_LEFT_AT(0D0)
    CALL BEGIN_X_AT(TZERO(1))
    CALL END_X_AT(XMAX)
    CALL ADDOBSERVATIONSLEFT(1)
    CALL ADD_CONSTRUCTED_CURVE
    CALL RIGHT_LABEL('CLEARANCE (ML/HR/KG)')
    MAXEPO=0
    DO ZZ=1,NCL
IF(YCL(ZZ) > MAXEPO) THEN
  MAXEPO = YCL(ZZ)
ENDIF
ENDDO
MAXEPO = 2.60D0 * MAXEPO
CALL END_RIGHT_AT(MAXEPO)
CALL INCLUDE_POINTS_RIGHT(XCL, YCL, NCL, 1)
CALL ADD_CURVE_RIGHT(XCL, YCL, NCL)
CALL PLOT_IN_AREA(1, 2)
CALL DEFINE_GRID_FOR_CURVE(TZERO(1), TZERO(1) + XMAX, MAXN)
JOB = 0
DO ZZ = 1, NCL
  CLPL(ZZ) = ((YCL(ZZ) - LCL) * (CEPO(XCL(ZZ)) + KDEPO)) / ((CLR(0D0) - LCL) * (CEPO(0D0) + KDEPO))
ENDDO
CALL INCLUDE_POINTS_LEFT(XCL, CLPL, NCL, 2)
CALL ADD_CURVE_LEFT(XCL, CLPL, NCL)
DO
  CALL CONSTRUCT_CURVE(TX, TY, JOB)
  IF (JOB /= 1) EXIT
  TY = ((CLR(TX) - LCL) * (CEPO(TX) + KDEPO)) / ((CLR(0D0) - LCL) * (CEPO(0D0) + KDEPO))
END DO
CALL LEFT_LABEL('CHANGE IN NUMBER OF RECEPTORS')
CALL X_LABEL('TIME (DAYS)')
CALL BEGIN_LEFT_AT(0D0)
CALL BEGIN_X_AT(TZERO(1))
CALL RIGHT_LABEL('EPO (MU/ML)')
MAXEPO = 0
DO ZZ = 1, NEPO
  IF(YEPO(ZZ) > MAXEPO) THEN
    MAXEPO = YEPO(ZZ)
  ENDIF
ENDDO
MAXEPO = 1.2D0 * MAXEPO
CALL END_RIGHT_AT(MAXEPO)
CALL INCLUDE_POINTS_RIGHT(XEPO, YEPO, NEPO, 4)
CALL ADD_CURVE_RIGHT(XEPO, YEPO, NEPO)
CALL PLOT_IN_AREA(2, 2)
CALL DISPLAY_PLOT
CALL RECORD_PLOT_IF_SAVED(LUN)
END IF
END IF
RETURN
! =============== RECORD THE ID FOR THE MODEL USED IN THE FITTING
==============
ENTRY MODELID(ID)
    ID = 'VLBW_V2.0.F90'
RETURN
END SUBROUTINE USERMODEL

! ============= EXTERNAL FUNCTIONS =============
REAL*8 FUNCTION TOTAL_BODY_STIM_RATE(X)
    ! PURPOSE: TO CALCULATE THE TOTAL BODY STIMULATION RATE OF
    ! HEMOGLOBIN AT TIME X
    USE FSTIM_EPO_MODULE
    USE BODYWEIGHT_MODULE
    IMPLICIT NONE
    REAL*8 :: X
    IF (X.LE.0D0) THEN
        TOTAL_BODY_STIM_RATE = ((BODYWEIGHT(X))**MRS)*K_INUTERO
    ELSE
        TOTAL_BODY_STIM_RATE = ((BODYWEIGHT(X))**MRS)*FSTIM(X)
    ENDIF
END FUNCTION TOTAL_BODY_STIM_RATE
! ================ END OF FILE ===============
Appendix E.2 Fortran code for FSTIM_EPO_MODULE.F90

! FILENAME = FSTIM_EPO_MODULE_V2.0.F90
!
! PURPOSE: TO STORE PARAMETERS AND ROUTINES TO CALCULATE
! THE STIMULATION RATE AND EPO CONCENTRATION
!
! REVISIONS:
! VERSION 2.0 DEC 15, 2008 ORIGINAL CODE-.
!
! FSTIM: STIMULATION RATE
! VMKM: FSTIM PARAMETER EQUAL TO VM/KM IN A MICHAELIS-MENTEN
EQUATION
! IKM: FSTIM PARAMETER EQUAL TO 1/KM (I.E. INVERSE KM) IN A
MICHAELIS-MENTEN EQUATION
! MRS: METABOLIC RATE SCALAR
! CEPO: EPO CONCENTRATION
!

MODULE FSTIM_EPO_MODULE
USE LINEAR_SPLINE
USE LINEAR_SPLINE2
USE BODYWEIGHT_MODULE
IMPLICIT NONE
SAVE

REAL*8 :: VMKM, IKM, MRS, K_INUTERO, KDEPO, LCL
REAL*8, PARAMETER :: T_ZERO = 0D0
REAL*8, EXTERNAL :: LINEAR_SPLINE_FITTED_VALUE
CONTAINS
REAL*8 FUNCTION CEPO(T)
! PURPOSE: TO CACLUATE THE VALUE OF THE EPO CONCENTRATION
!
! NOTE: LINEAR FORWARD EXTRAPOLATION USED AND LINEAR
BACKWARD
! EXTRAPOLATION USED BACK TO TIME 0 (I.E. BIRTH)
THROUGH
! CUBIC GCV. PRIOR TO BIRTH CONSTANT EXTRAPOLATION
USED
! FROM THE LINEAR EXTRAPOLATED CONCENTRATION AT
BIRTH.
IMPLICIT NONE
REAL*8 :: T
IF ( T >= T_ZERO ) THEN
CALL CUBIC_GCV(T, CEPO)
CEPO = (DEXP(DEXP(CEPO)))
CALL EVALUATE_LINEAR_SPLINE(T,CEPO)

ELSE
   CALL CUBIC_GCV(T_ZERO,CEPO)
   !CEPO=(DEXP(DEXP(CEPO)))
   CALL EVALUATE_LINEAR_SPLINE(T_ZERO,CEPO)
END IF

END FUNCTION CEPO

REAL*8 FUNCTION CLR(T)
! PURPOSE: TO CALCULATE THE VALUE OF THE EPO CONCENTRATION
! NOTE: LINEAR FORWARD EXTRAPOLATION USED AND LINEAR BACKWARD
! EXTRAPOLATION USED BACK TO TIME 0 (I.E. BIRTH)
 THROUGH
! CUBIC GCV. PRIOR TO BIRTH CONSTANT EXTRAPOLATION USED
! FROM THE LINEAR EXTRAPOLATED CONCENTRATION AT BIRTH.
IMPLICIT NONE
REAL*8 :: T
IF ( T >= T_ZERO ) THEN
   CALL CUBIC_GCV(T,CEPO)
   ! CEPO=(DEXP(DEXP(CEPO)))
   CALL EVALUATE_LINEAR_SPLINE2(T,CLR)
ELSE
   CALL CUBIC_GCV(T_ZERO,CEPO)
   !CEPO=(DEXP(DEXP(CEPO)))
   CALL EVALUATE_LINEAR_SPLINE2(T_ZERO,CLR)
END IF

END FUNCTION CLR

REAL*8 FUNCTION FSTIM(T)
! PURPOSE: TO CALCULATE THE VALUE OF THE STIMULATION RATE FUNCTION
! WHERE T IS THE TIME
IMPLICIT NONE
REAL*8 :: T,CLNR,CLNR0
CLNR0=(CLR(0D0)-LCL)*(CEPO(0D0)+KDEPO)
CLNR=(CLR(T)-LCL)*(CEPO(T)+KDEPO)
!FSTIM =
(VMKM/10000)*CEPO(T)*CLNR*((BODYWEIGHT(T))**0.25)/(CEPO(T) + IKM)
  FSTIM = (VMKM)*CEPO(T)*(CLNR0)/(CEPO(T) + IKM)
  !FSTIM = (VMKM)*CEPO(T)*(1D0)/(CEPO(T) + IKM)
END FUNCTION FSTIM

REAL*8 FUNCTION QSTIM(T)
  ! PURPOSE: TO CALCULATE THE VALUE OF THE STIMULATION RATE
  FUNCTION
    ! WHERE T IS THE CONCENTRATION
  IMPLICIT NONE
  REAL*8 :: T
  QSTIM = VMKM*T/(IKM*1D0 + T)
END FUNCTION QSTIM

END MODULE FSTIM_EPO_MODULE
Module LINEAR_SPLINE
IMPLICIT NONE
PRIVATE
PUBLIC SET_LINEAR_SPLINE_PARAMETERS,
     ,EVALUATE_LINEAR_SPLINE
INTEGER,SAVE::N,LJ=1
INTEGER,PARAMETER::NMAX=500
REAL*8,DIMENSION(NMAX),SAVE::SLOPE,INTERCEPT,X,Y
CONTAINS
SUBROUTINE SET_LINEAR_SPLINE_PARAMETERS(XX,YY,NN)
! PURPOSE: TO SET LINEAR SPLINE PARAMETERS (I.E. SLOPE AND INTERCEPT)
IMPLICIT NONE
INTEGER,INTENT(IN)::NN
REAL*8,INTENT(IN),DIMENSION(XX,YY)
INTEGER::J
REAL*8::DX,DY
N=NN
DO J=1,N
   X(J)=XX(J)
   Y(J)=YY(J)
END DO
DO J=2,N
   SLOPE(J)=SLOPE(J-1)+(X(J)-X(J-1))/DX
   INTERCEPT(J)=INTERCEPT(J-1)+(Y(J)-Y(J-1))/DY
END DO
DX=X(J)-X(J-1)
IF ( DX == 0D0 ) THEN
WRITE(*,'(" CHECK THE DATA FITTED BY LINEAR SPLINE ', G12.4")') J
STOP ' ERROR! TWO OBSERVATIONS CANNOT OCCUR AT THE SAME TIME'
END IF
DY=Y(J)-Y(J-1)
SLOPE(J-1)=DY/DX
INTERCEPT(J-1)=Y(J-1)
END DO
END SUBROUTINE SET_LINEAR_SPLINE_PARAMETERS

SUBROUTINE EVALUATE_LINEAR_SPLINE(T,CALCY)
! PURPOSE: TO EVALUATE THE FITTED VALUE AT GIVEN T VALUE
IMPLICIT NONE
REAL*8,INTENT(IN)::T
REAL*8,INTENT(OUT)::CALCY
INTEGER::J
IF ( T >= X(LJ) .AND. T < X(LJ+1) ) THEN
  CALCY=SLOPE(LJ)*(T-X(LJ))+INTERCEPT(LJ)
RETURN
ELSEIF ( T >= X(LJ+1) .AND. T < X(LJ+2) ) THEN
  CALCY=SLOPE(LJ+1)*(T-X(LJ+1))+INTERCEPT(LJ+1)
  LJ=LJ+1
RETURN
ELSEIF ( T < X(1) ) THEN
  CALCY=Y(1)
RETURN
ELSEIF ( T > X(N) ) THEN
  CALCY=Y(N)
RETURN
ELSE
  DO J=1,N
    IF ( T >= X(J) .AND. T < X(J+1) ) THEN
      CALCY=SLOPE(J)*(T-X(J))+INTERCEPT(J)
      LJ=J
    END IF
  END DO
END IF
END SUBROUTINE EVALUATE_LINEAR_SPLINE
END MODULE LINEAR_SPLINE
Appendix E.4 Fortran code for PHLEBOTOMY TRANSFUSION MODULE.F90

! FILENAME = PHLEBOTOMY_TRANSFUSION_MODULE_V1.2.F90
!
! PURPOSE: TO STORE DATA AND Routines TO ACCOUNT FOR THE
! EFFECT OF
! THE PHLEBOTOMIES AND TRANSFUSIONS ON THE
HEMOGLOBIN/RBC COUNT
!
!
! REVISIONS:
! VERSION 1.0 JUN 6, 2008 ORIGINAL CODE EXTENDED FROM
PHLEBOTOMY_AND_TRANSFUSION_MODULE_V1.1-.
! 1.1 JUL 18, 2008 SIMPLIFIED THE CALCULATION OF THE
PHLEBOTOMY CORRECTIONS BY MULTIPLYING ALL THE
! FRACTION REMAININGS BETWEEN ENTRY OF THE CELL
OF INTEREST INTO THE SAMPLING
! COMPARTMENT AND THE CURRENT TIME-.
! 1.2 DEC 17, 2008 MODIFIED TO REMOVE REMAINING LIFESPAN
DISTRIBUTION SUBROUTINES
!
! MAXPHLEB: MAXIMUM NUMBER OF PHLEBOTOMIES ALLOWED
! MAXTRANS: MAXIMUM NUMBER OF TRANSFUSIONS ALLOWED
! NOPHLEB: NUMBER OF PHLEBOTOMIES
! NOTRANS: NUMBER OF TRANSFUSIONS
! LT: LIFESPAN OF TRANSFUSED RBCS
! FDAMAGED: FRACTION OF TRANSFUSED CELLS THAT ARE DAMAGED
AND IMMEDIATELY REMOVED
! UPON TRANSFUSION
! TP: VECTOR OF PHLEBOTOMY TIMES ORDERED FROM FIRST TO LAST
! AP: VECTOR OF PHLEBOTOMY AMOUNTS ORDERED FROM FIRST TO LAST
! FP: VECTOR OF PHLEBOTOMY FRACTIONS REMAINING ORDERED FROM
FIRST TO LAST
! TT: VECTOR OF TRANSFUSION TIMES ORDERED FROM FIRST TO LAST
! AP: VECTOR OF TRANSFUSION AMOUNTS ORDERED FROM FIRST TO LAST

MODULE PHLEBOTOMY_TRANSFUSION_MODULE
IMPLICIT NONE
SAVE

INTEGER, PARAMETER :: MAXPHLEB = 250, MAXTRANS = 25
INTEGER, PRIVATE :: NOPHLEB, NOTRANS
REAL*8 :: LT, FDAMAGED
REAL*8, DIMENSION(MAXPHLEB+1), PRIVATE :: TP
REAL*8, DIMENSION(MAXPHLEB), PRIVATE :: AP, FP
REAL*8, DIMENSION(MAXTRANS), PRIVATE :: TT, AT

CONTAINS
SUBROUTINE SET_PHLEBOTOMY_TIME_VECTOR(N, T)
! PURPOSE: TO SET THE VALUES OF THE PHLEBOTOMY TIME VECTOR
IMPLICIT NONE
INTEGER, INTENT(IN) :: N
REAL*8, DIMENSION(N), INTENT(IN) :: T
INTEGER :: J
IF ( N > MAXPHLEB ) STOP ’ ERROR! DIMENSION OF PHLEBOTOMY TIME VECTOR LARGER THAN MAXIMUM SIZE’
NOPHLEB = N
DO J = 1, N
TP(J) = T(J)
IF ( J > 1 ) THEN
IF ( TP(J) == TP(J-1) ) THEN
WRITE(*,”(‘ TWO PHLEBOTOMIES AT TIME ’, G12.4)”) TP(J)
STOP ’ ERROR! TWO PHLEBOTOMIES CANNOT OCCUR AT THE SAME TIME’
END IF
END IF
END DO
DO J = N+1, MAXPHLEB+1
TP(J) = TP(1) - 1D0
END DO
END SUBROUTINE SET_PHLEBOTOMY_TIME_VECTOR

SUBROUTINE GET_NUMBER_PHLEBOTOMIES(T, N)
! PURPOSE: TO DETERMINE THE NUMBER OF PHLEBOTOMIES AT AND PRECEEDING TIME T
IMPLICIT NONE
REAL*8, INTENT(IN) :: T
INTEGER, INTENT(OUT) :: N
INTEGER :: J
DO J = 1, NOPHLEB
N = J - 1
IF ( TP(J) > T ) EXIT
END DO
IF ( TP(NOPHLEB) <= T ) THEN
N = NOPHLEB
END IF
END SUBROUTINE GET_NUMBER_PHLEBOTOMIES
SUBROUTINE GET_NUMBER_PHLEBOTOMIES_PRECEEDING(T, N)
! PURPOSE: TO DETERMINE THE NUMBER OF PHLEBOTOMIES
PRECEEDING TIME T
IMPLICIT NONE
REAL*8, INTENT(IN) :: T
INTEGER, INTENT(OUT) :: N
INTEGER :: J
DO J = 1, NOPHLEB
  N = J - 1
  IF ( TP(J) >= T ) EXIT
END DO
IF( TP( NOPHLEB ) < T ) THEN
  N = NOPHLEB
END IF
END SUBROUTINE GET_NUMBER_PHLEBOTOMIES_PRECEEDING

SUBROUTINE GET_TIME_OF_PHLEBOTOMY(N, T)
! PURPOSE: TO DETERMINE THE TIME OF PHLEBOTOMY N
IMPLICIT NONE
INTEGER, INTENT(IN) :: N
REAL*8, INTENT(OUT) :: T
T = TP(N)
END SUBROUTINE GET_TIME_OF_PHLEBOTOMY

SUBROUTINE GET_TOTAL_NUMBER_PHLEBOTOMIES(N)
! PURPOSE: TO DETERMINE THE TOTAL NUMBER OF PHLEBOTOMIES
IMPLICIT NONE
INTEGER, INTENT(OUT) :: N
N = NOPHLEB
END SUBROUTINE GET_TOTAL_NUMBER_PHLEBOTOMIES

SUBROUTINE SET_PHLEBOTOMY_AMOUNT VECTOR(N, A)
! PURPOSE: TO SET THE VALUES OF THE AMOUNTS OF BLOOD REMOVED
BY PHLEBOTOMY VECTOR
IMPLICIT NONE
INTEGER, INTENT(IN) :: N
REAL*8, DIMENSION(N), INTENT(IN) :: A
INTEGER :: J
IF( N > MAXPHLEB ) STOP ' ERROR! DIMENSION OF PHLEBOTOMY
AMOUNT VECTOR LARGER THAN MAXIMUM SIZE'
IF( N /= NOPHLEB ) STOP ' ERROR! INCONSITENT NUMBER OF
PHLEBOTOMY AMOUNTS AND TIMES'
DO J = 1, N
SUBROUTINE SET_PHLEBOTOMY_AMOUNT_VECTOR(N, AP)
    ! PURPOSE: TO SET THE AMOUNT OF BLOOD REMOVED BY PHLEBOTOMY 'N'
    INTEGER, INTENT(IN) :: N
    REAL*8, INTENT(INOUT) :: AP(N)
    IF ( N > NOPHLEB ) STOP ' ERROR! REQUESTED VALUES EXCEEDS THE NUMBER OF PHLEBOTOMIES'
    IF ( N < 1 ) STOP ' ERROR! THE REQUESTED PHLEBOTOMY NUMBER MUST BE POSITIVE'
    AP(N) = A(N)
END SUBROUTINE SET_PHLEBOTOMY_AMOUNT_VECTOR

SUBROUTINE GET_PHLEBOTOMY_AMOUNT_VALUE(N, AVALUE)
    ! PURPOSE: TO GET THE VALUE OF THE AMOUNT OF BLOOD REMOVED BY PHLEBOTOMY 'N'
    ! IMPLICIT NONE
    INTEGER, INTENT(IN) :: N
    REAL*8, INTENT(OUT) :: AVALUE
    IF ( N > NOPHLEB ) STOP ' ERROR! REQUESTED VALUES EXCEEDS THE NUMBER OF PHLEBOTOMIES'
    IF ( N < 1 ) STOP ' ERROR! THE REQUESTED PHLEBOTOMY NUMBER MUST BE POSITIVE'
    AVALUE = AP(N)
END SUBROUTINE GET_PHLEBOTOMY_AMOUNT_VALUE

SUBROUTINE SET_FRACTION_REMAINING_VECTOR(N, F)
    ! PURPOSE: TO SET THE VALUES OF THE PHLEBOTOMY FRACTIONS REMAINING VECTOR
    ! IMPLICIT NONE
    INTEGER, INTENT(IN) :: N
    REAL*8, DIMENSION(N), INTENT(IN) :: F
    INTEGER :: J
    IF ( N > MAXPHLEB ) STOP ' ERROR! DIMENSION OF FRACTION REMAINING VECTOR LARGER THAN MAXIMUM SIZE'
    IF ( N /= NOPHLEB ) STOP ' ERROR! INCONSITENT NUMBER OF FRACTION REMAINING VALUES AND TIMES'
    DO J = 1, N
        FP(J) = F(J)
    END DO
    IF ( N < MAXPHLEB ) THEN
        DO J = N+1, MAXPHLEB
            FP(J) = 0D0
        END DO
    END IF
END SUBROUTINE SET_FRACTION_REMAINING_VECTOR
SUBROUTINE UPDATE_FRACTION_REMAINING_VALUE(N, FVALUE)
! PURPOSE: TO UPDATE A SINGLE VALUE AT POSITION J OF THE
PHLEBOTOMY FRACTIONS REMAINING VECTOR
IMPLICIT NONE
INTEGER, INTENT(IN) :: N
REAL*8, INTENT(IN) :: FVALUE
IF ( N > NOPHLEB ) STOP ' ERROR! REQUESTED VALUES EXCEEDS THE
NUMBER OF FRACTIONS REMOVED'
IF ( N < 1 ) STOP ' ERROR! THE REQUESTED FRACTION REMAINING
NUMBER MUST BE POSITIVE'
FP(N) = FVALUE
END SUBROUTINE UPDATE_FRACTION_REMAINING_VALUE

SUBROUTINE GET_FRACTION_REMAINING_VALUE(N, FVALUE)
! PURPOSE: TO GET A SINGLE VALUE AT POSITION J OF THE
PHLEBOTOMY FRACTIONS REMOVED VECTOR
IMPLICIT NONE
INTEGER, INTENT(IN) :: N
REAL*8, INTENT(OUT) :: FVALUE
IF ( N > NOPHLEB ) STOP ' ERROR! REQUESTED VALUES EXCEEDS THE
TOTAL NUMBER OF FRACTIONS REMAINING'
IF ( N < 1 ) STOP ' ERROR! THE REQUESTED FRACTION REMAINING
NUMBER MUST BE POSITIVE'
FVALUE = FP(N)
END SUBROUTINE GET_FRACTION_REMAINING_VALUE

SUBROUTINE GET_PHLEBOTOMY_CORRECTION_TERM(TSTART, TEND, CT)
! PURPOSE: TO CALCULATE THE TOTAL CORRECTION TERM (CT) FOR
! THE PHLEBOTOMIES CONDUCTED BETWEEN TIME TSTART AND TEND
IMPLICIT NONE
REAL*8, INTENT(IN) :: TSTART, TEND
REAL*8, INTENT(OUT) :: CT
INTEGER :: NSTART, NEND, J
IF ( TSTART > TEND) STOP ' ERROR! THE START TIME MUST BE LESS
THAN OR EQUAL TO THE STOP TIME'
CALL GET_NUMBER_PHLEBOTOMIES_PRECEEDING(TSTART, NSTART)
CALL GET_NUMBER_PHLEBOTOMIES_PRECEEDING(TEND, NEND)
CT = 1D0
IF ( (TSTART == TEND) .OR. (NSTART == NEND) ) THEN
RETURN
ELSE
DO J = NSTART+1, NEND

```
CT = FP(J)*CT
END DO
END IF
END SUBROUTINE GET_PHLEBOTOMY_CORRECTION_TERM

SUBROUTINE SET_TRANSFUSION_TIME_VECTOR(N, T)
! PURPOSE: TO SET THE VALUES OF THE TRANSFUSION TIME VECTOR
IMPLICIT NONE
INTEGER, INTENT(IN) :: N
REAL*8, DIMENSION(N), INTENT(IN) :: T
INTEGER :: J
IF (N > MAXTRANS) STOP 'ERROR! DIMENSION OF TRANSFUSION TIME VECTOR LARGER THAN MAXIMUM SIZE'
DO J = 1, N
   TT(J) = T(J)
END DO
IF (N < MAXTRANS) THEN
   DO J = N+1, MAXTRANS
      TT(J) = 10000D0
   END DO
END IF
NOTRANS = N
END SUBROUTINE SET_TRANSFUSION_TIME_VECTOR

SUBROUTINE SET_TRANSFUSION_AMOUNT_VECTOR(N, A)
! PURPOSE: TO SET THE VALUES OF THE AMOUNTS OF BLOOD ADDED BY TRANSFUSION VECTOR
IMPLICIT NONE
INTEGER, INTENT(IN) :: N
REAL*8, DIMENSION(N), INTENT(IN) :: A
INTEGER :: J
IF (N > MAXTRANS) STOP 'ERROR! DIMENSION OF TRANSFUSION AMOUNT VECTOR LARGER THAN MAXIMUM SIZE'
IF (N /= NOTRANS) STOP 'ERROR! INCONSISTENT NUMBER OF TRANSFUSION AMOUNTS AND TIMES'
DO J = 1, N
   AT(J) = A(J)
END DO
IF (N < MAXTRANS) THEN
   DO J = N+1, MAXTRANS
      AT(J) = 0D0
   END DO
END IF
END SUBROUTINE SET_TRANSFUSION_AMOUNT_VECTOR
SUBROUTINE GET_TOTAL_NUMBER_TRANSFUSIONS(N)
! PURPOSE: TO DETERMINE THE TOTAL NUMBER OF TRANSFUSIONS
IMPLICIT NONE
INTEGER, INTENT(OUT) :: N
N = NOTRANS
END SUBROUTINE GET_TOTAL_NUMBER_TRANSFUSIONS

SUBROUTINE GET_TIME_OF_TRANSFUSION(N, T)
! PURPOSE: TO DETERMINE THE TIME OF TRANSFUSION N
IMPLICIT NONE
INTEGER, INTENT(IN) :: N
REAL*8, INTENT(OUT) :: T
IF ( N > NOTRANS ) STOP 'ERROR! REQUESTED VALUES EXCEEDS THE NUMBER OF TRANSFUSIONS'
IF ( N < 1 ) STOP 'ERROR! THE REQUESTED TRANSFUSION NUMBER MUST BE POSITIVE'
T = TT(N)
END SUBROUTINE GET_TIME_OF_TRANSFUSION

SUBROUTINE GET_TRANSFUSION_AMOUNT_VALUE(N, AVALUE)
! PURPOSE: TO GET THE VALUE OF THE AMOUNT OF BLOOD GIVEN BY TRANSFUSION 'N'
IMPLICIT NONE
INTEGER, INTENT(IN) :: N
REAL*8, INTENT(OUT) :: AVALUE
IF ( N > NOTRANS ) STOP 'ERROR! REQUESTED VALUES EXCEEDS THE NUMBER OF TRANSFUSIONS'
IF ( N < 1 ) STOP 'ERROR! THE REQUESTED TRANSFUSION NUMBER MUST BE POSITIVE'
AVALUE = AT(N)
END SUBROUTINE GET_TRANSFUSION_AMOUNT_VALUE

SUBROUTINE GET_TRANSFUSION_TERM(T, VALUE)
! PURPOSE: TO CALCULATE THE TOTAL TRANSFUSION TERM AT TIME 'T' WITH CORRECTION FOR PHLEBOTOMIES
IMPLICIT NONE
REAL*8, INTENT(IN) :: T
REAL*8, INTENT(OUT) :: VALUE
INTEGER :: NOPHLEB, J, K
REAL*8 :: CT

REAL*8 :: USTEP, HDIST
VALUE = 0D0
IF ( T < TT(1) ) THEN
  RETURN
ELSE
  DO J = 1, NOTRANS
    CT = 1D0
    IF ( TT(J) <= T ) THEN
      CALL GET_PHLEBOTOMY_CORRECTION_TERM(TT(J), T, CT)
    END IF
    VALUE = USTEP(T-TT(J))*CT*HDIST(T - TT(J))*AT(J)*(1D0 - FDAMAGED) + VALUE
  END DO
END IF
END SUBROUTINE GET_TRANSFUSION_TERM

REAL*8 FUNCTION USTEP(X)
  ! PURPOSE: TO CALCULATE THE UNIT STEP FUNCTION
  IMPLICIT NONE
  REAL*8 :: X

  IF ( X >= 0D0 ) THEN
    USTEP = 1D0
  ELSE
    USTEP = 0D0
  END IF
END FUNCTION USTEP

REAL*8 FUNCTION HDIST(X)
  ! PURPOSE: TO CALCULATE THE REMAINING LIFESPAN DISTRIBUTION OF TRANSFUSED RBCS. NOTE: A 'POINT' DISTRIBUTION OF LT WITH STEADY-STATE PRODUCTION RATE IS ASSUMED.
  IMPLICIT NONE
  REAL*8 :: X

  IF ( X < 0 ) THEN
    HDIST = 0
  ELSE IF ( X <= LT ) THEN
    HDIST = 1/LT
  ELSE
    HDIST = 0D0
  END IF
END FUNCTION HDIST
END MODULE PHLEBOTOMY_TRANSFUSION_MODULE
Appendix E.5 Fortran code for WEIGHT.F90

! FILENAME = BODYWEIGHT_MODULE_V1.5.F90
!
! PURPOSE: TO STORE PARAMETERS AND ROUTINES TO CALCULATE
! THE BODY WEIGHT, WHICH IS DONE BY A SMOOTHED CUBIC SPLINE
! OF THE OBSERVED BODY WEIGHTS VS. TIME
!
!
! MAXBWPOINTS: MAXIMUM NUMBER OF BODY WEIGHT MEASUREMENTS
ALLOWED
! DEGREE: DEGREE OF THE FITTED POLYNOMIAL
! BWT: VECTOR OF MEASURED BODY WEIGHT TIMES
! BWV: VECTOR OF MEASURED BODY WEIGHTS
! BWCOEF: VECTOR OF FITTED POLYNOMIAL COEFFICIENTS
! SSPOLY: VECTOR CONTAINING THE SEQUENTIAL SUM OF SQUARES
!

MODULE BODYWEIGHT_MODULE
  USE NUMERICAL_LIBRARIES
  IMPLICIT NONE
  SAVE

  INTEGER, PARAMETER :: MAXBWPOINTS = 50, DEGREE = 4
  REAL*8 ::
  GESTATIONAL_AGE_AT_BIRTH,WTP1,WTP2,WTP3,CURRENT_AGE
  INTEGER, PRIVATE :: BWPOINTS
  REAL*8, DIMENSION(1:MAXBWPOINTS), PRIVATE :: BWT, BWV
  REAL*8, DIMENSION(1:DEGREE+1), PRIVATE :: BWCOEF, SSPOLY
  REAL*8, DIMENSION(1:10), PRIVATE :: STAT

  CONTAINS
    SUBROUTINE SET_BODYWEIGHT_TIME_VECTOR(N, T)
      ! PURPOSE: TO SET THE BODY WEIGHT MEASUREMENT TIME VECTOR
      IMPLICIT NONE
      INTEGER, INTENT(IN) :: N
      REAL*8, DIMENSION(1:N), INTENT(IN) :: T
      INTEGER :: J
      IF ( N > MAXBWPOINTS ) STOP ' ERROR! DIMENSION OF BODY WEIGHT VECTOR LARGER THAN MAXIMUM SIZE'
      BWPOINTS = N
      DO J = 1, N
        BWT(J) = T(J)
      END DO
      IF ( T(1) < 0D0 ) STOP ' ERROR! THE FIRST BODYWEIGHT MEASUREMENT TIME MUST NON-NEGATIVE'

  END SUBROUTINE SET_BODYWEIGHT_TIME_VECTOR

END MODULE BODYWEIGHT_MODULE
IF ( N < MAXBWPOINTS ) THEN
    DO J = N+1, MAXBWPOINTS
        BWT(J) = BWT(1) - 1D0
    END DO
END IF
END SUBROUTINE SET_BODYWEIGHT_TIME_VECTOR

SUBROUTINE SET_BODYWEIGHT_VALUE_VECTOR(N, V)
    ! PURPOSE: TO SET THE BODY WEIGHT VALUE VECTOR AND TO
    ! COMPUTE THE SMOOTHED QUARTIC SPLINE.
    IMPLICIT NONE
    INTEGER, INTENT(IN) :: N
    REAL*8, DIMENSION(1:N), INTENT(IN) :: V
    INTEGER :: J
    IF ( N > MAXBWPOINTS ) STOP ' ERROR! DIMENSION OF BODY WEIGHT VECTOR LARGER THAN MAXIMUM SIZE'
    IF ( N /= BWPOINTS ) STOP ' ERROR! INCONSITENT NUMBER OF BODY WEIGHTS AND TIMES'
    DO J = 1, N
        BWV(J) = V(J)
    END DO
    IF ( N < MAXBWPOINTS ) THEN
        DO J = N+1, MAXBWPOINTS
            BWV(J) = -1D0
        END DO
    END IF
    CALL DRCURV(BWPOINTS, BWT, BWV, DEGREE, BWCOEF, SSPOLY, STAT)
END SUBROUTINE SET_BODYWEIGHT_VALUE_VECTOR

SUBROUTINE GET_FITTED_BODYWEIGHT_R2(VALUE)
    ! PURPOSE: TO GET THE R^2 OF THE POLYNOMIAL FITTED TO THE BODY WEIGHT DATA
    IMPLICIT NONE
    REAL*8, INTENT(OUT) :: VALUE
    VALUE = STAT(5)
END SUBROUTINE GET_FITTED_BODYWEIGHT_R2

SUBROUTINE GET_BODYWEIGHT_TIME_VECTOR(T, N)
    ! PURPOSE: TO GET THE BODY WEIGHT MEASUREMENT TIME VECTOR
    IMPLICIT NONE
    REAL*8, DIMENSION(1:BWPOINTS), INTENT(OUT) :: T
    INTEGER, INTENT(OUT) :: N
    T = BWT
N = BWPOINTS
END SUBROUTINE GET_BODYWEIGHT_TIME_VECTOR

SUBROUTINE GET_BODYWEIGHT_VALUE_VECTOR(V, N)
  ! PURPOSE: TO GET THE BODY WEIGHT VALUE VECTOR
  IMPLICIT NONE
  REAL*8, DIMENSION(1:BWPOINTS), INTENT(OUT) :: V
  INTEGER, INTENT(OUT) :: N
  V = BWV
  N = BWPOINTS
END SUBROUTINE GET_BODYWEIGHT_VALUE_VECTOR

REAL*8 FUNCTION BODYWEIGHT(T)
  ! PURPOSE: TO CALCULATE THE BODY WEIGHT VALUE AT TIME T USING THE
  ! POLYNOMIAL REPRESENTATION, LINEAR FORWARD EXTRAPOLATION
  ! AND LINEAR BACKWARD EXTRAPOLATION UP TO TIME 0, IF NECESSARY.
  !
  IMPLICIT NONE
  REAL*8 :: C1 = 1.4446E-08 ! C1: INTERCEPT OF BODY WEIGHT CURVE FROM 22 TO 32 WEEKS OF GESTATIONAL AGE
  REAL*8 :: C2 = 0.4483 ! C2: EXPONENT OF BODY WEIGHT CURVE FROM 22 TO 32 WEEKS OF GESTATIONAL AGE
  REAL*8 :: T, X, INTERCEPT, DERIVATIVE, BIRTHBW, SLOPE
  SLOPE=WTP3
  INTERCEPT=BWCOEF(1)
  BIRTHBW=SLOPE*-1D0*CURRENT_AGE+INTERCEPT

  IF ((T >= BWT(1)) .AND. (T <= BWT(BWPOINTS))) THEN
    BODYWEIGHT = BWCOEF(1) + BWCOEF(2)*T + BWCOEF(3)*(T**2) + BWCOEF(4)*(T**3) + BWCOEF(5)*(T**4)
  ELSE IF ( T < (0D0-CURRENT_AGE) ) THEN
    X = GESTATIONAL_AGE_AT_BIRTH + T+CURRENT_AGE
    BODYWEIGHT = BIRTHBW*DEXP(WTP1*X)*DEXP(-WTP2*(X**2))/(DEXP(WTP1*GESTATIONAL_AGE_AT_BIRTH)*DEXP(-WTP2*(GESTATIONAL_AGE_AT_BIRTH**2)))
  ELSE IF ( T < BWT(1) ) THEN
    
    
  END FUNCTION BODYWEIGHT
BODYWEIGHT = INTERCEPT + SLOPE*T

ELSE

INTERCEPT = BWCOEF(1) + BWCOEF(2)*BWT(BWPOINTS) + BWCOEF(3)*(BWT(BWPOINTS)**2) + BWCOEF(4)*(BWT(BWPOINTS)**3) + BWCOEF(5)*(BWT(BWPOINTS)**4)
DERIVATIVE = BWCOEF(2) + 2D0*BWCOEF(3)*BWT(BWPOINTS) + 3D0*BWCOEF(4)*(BWT(BWPOINTS)**2) + 4D0*BWCOEF(5)*(BWT(BWPOINTS)**3)
BODYWEIGHT = INTERCEPT + DERIVATIVE*(T - BWT(BWPOINTS))

END IF
END FUNCTION BODYWEIGHT
END MODULE BODYWEIGHT_MODULE
APPENDIX F. PUBLICATIONS AND SUBMITTED MANUSCRIPTS


REFERENCES


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