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Cellular lifespan based pharmacodynamic analysis of erythropoiesis

Kevin Jay Freise
University of Iowa

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**CELLULAR LIFESPAN BASED PHARMACODYNAMIC ANALYSIS OF
ERYTHROPOIESIS**

by

Kevin Jay Freise

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

July 2009

Thesis Supervisor: Professor Peter Veng-Pedersen

ABSTRACT

The disposition of cells whose mechanism of death is related to the age of the cell cannot be appropriately represented by pharmacodynamic (PD) models where the elimination rate is related to the number of cells. In cells with age-related death their disposition is determined by their lifespan. Thus in these cells PD models of cellular response must incorporate a lifespan component. Previous cellular lifespan PD models assumed that the lifespan of cells is predetermined and does not vary over time. However, in many instances these assumptions are inappropriate and thus extensions to the existing models are needed. An important application of these time variant PD models is determining the erythropoiesis rate, since the lifespan of reticulocytes and mature erythrocytes are known to change over time under specific conditions.

The objectives of this work were to develop a general time variant lifespan-based PD model of cellular response and to use the model to determine the dynamic changes over time in both the erythrocyte lifespan and erythropoiesis rate under a variety of complex conditions. An initial time variant cellular lifespan model was formulated assuming no variability in lifespans and used to determine the dynamic changes in both the reticulocyte lifespan and erythropoiesis rate in sheep. Subsequently, the time variant model was extended to account for a distribution of cellular lifespans, which resulted in better capturing the physiology of sheep erythrocyte maturation. The model was then further extended to account for the effect of changes in the environment on cell lifespans and used to determine the effect of chemotherapy administration on sheep erythrocytes. In order to conduct studies on erythropoiesis in premature very low birth weight (VLBW) infants the ability to accurately measure erythrocytes and hemoglobin from clinically

collected excess blood was validated. Then an in depth analysis of the relationship between erythropoietin, erythrocytes, and hemoglobin was conducted in a clinical study of premature VLBW infants that accounted for the dynamic hematological conditions experienced by these subjects. This analysis indicated that a nearly 4-fold increase in erythropoiesis could be achieved with only a modest increase in plasma erythropoietin concentrations.

Abstract Approved: _____
Thesis Supervisor

Title and Department

Date

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Graduate College
The University of Iowa
Iowa City, Iowa

CERTIFICATE OF APPROVAL

PH.D. THESIS

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

Kevin Jay Freise

has been approved by the Examining Committee
for the thesis requirement for the Doctor of Philosophy
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To my beloved wife, Hilary, and our daughter, Isabel

The meaning of this degree is that the recipient of instruction is examined for the last time in his life, and is pronounced completely full. After this, no new ideas can be imparted to him.

Stephen Leacock, *Sunshine Sketches of a Little Town*

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LIST OF SYMBOLS AND ABBREVIATIONS

$*$	convolution operator
$1\{\cdot\}$	indicator function
$a, a(s)$	time invariant and time variant “point” cellular release time delay, respectively
AIC	Akaike’s Information Criterion
b	time from stimulation of a precursor cell to transformation into a new cell type or senescence/destruction of the subsequently released cell(s)
BV	blood volume
BFU-E	burst forming unit-erythroid
CBC	complete blood count
$C(t)$	concentration
$C_{bio}(t)$	biophase EPO concentration
$C_p(t)$	plasma EPO concentration
CERA	continuous erythropoietin receptor activator
CFU-E	colony forming unit-erythroid
DDE	delay differential equation
Δ	small time increment
$e(t, z)$	cumulative measure of the effect of the environment
e_{avg}	average value of the environmental effect function

$E\{\cdot\}, E\{\cdot \cdot\}$	mathematical expectation and conditional expectation of a random variable, respectively
EC_{50}	$C_p(t)$ or $C_{bio}(t)$ that results in 50% of maximal erythrocyte/hemoglobin stimulation rate
E_{max}	maximal erythrocyte/hemoglobin stimulation rate
EDTA	ethylenediaminetetraacetic acid
EPO	erythropoietin
EPO-R	erythropoietin receptor
ε	infinitesimally small time increment
F, F_1, F_2, F_{pj}	fraction of cells remaining following a phlebotomy (subscript number or j denotes phlebotomy number)
F_T	fraction of transfused Hb surviving immediately beyond the time of transfusion
$f_{prod}(t)$	production rate (i.e. input rate into the sampling compartment)
$f_{stim}(t)$	stimulation rate
$f_{stim}^{ex\ utero}(t)$	post-birth (<i>ex utero</i>) bodyweight scaled Hb stimulation rate
$f_{stim}^{total}(t)$	total body Hb stimulation rate
$f_{trans}(t)$	function proportional to rate of Hb loss of transfused adult Hb
f_{prod}^{SS}	steady-state production rate
f_{stim}^{SS}	steady-state stimulation rate
$g(t), h(t)$	environmental effect functions

Hb	hemoglobin
$Hb_{in\ vivo}(t)$	amount of Hb present in the in the circulation that was produced <i>in vivo</i>
$Hb_{total}(t)$	total amount of Hb present in the circulation
$Hb_{trans}(t)$	amount of Hb present in the circulation that was transfused
Hb_{p_j}	amount of Hb removed by the j^{th} phlebotomy
Hb_{T_i}	amount of Hb administered at the i^{th} transfusion
Hct	hematocrit
HIF-1	hypoxia-inducible factor-1
HPC	hematopoietic progenitor cells
IG	immature granulocytes
IPF	immature platelet fraction
IRF	immature reticulocyte fraction
k_{bio}	biophase conduction function parameter
k_{out}	first-order output rate constant
$k_{stim}^{in\ utero}$	pre-birth (<i>in utero</i>) bodyweight scaled Hb stimulation rate constant
K_{RET}	hemoglobin to reticulocyte count conversion constant
$l, k, \text{ and } \theta(t)$	scale, shape, and time variant location parameters of the Weibull distribution, respectively
$L_{in\ vivo}$	RBC lifespan of infant cells produced <i>in vivo</i>
L_{trans}	RBC lifespan of transfused cells
$\ell(\tau, \cdot)$	time variant p.d.f. of cellular lifespans

$\ell_b(\tau, z)$	baseline p.d.f. of cellular lifespans
$\ell_e(\tau, z)$	observed p.d.f. of cellular lifespans with an environmental effect
$\lambda_b(t, z)$	baseline hazard function
$\lambda_e(t, z)$	observed hazard function with an environmental effect
$m(t)$	subject mass (bodyweight)
MSE%	mean percent standard error
MCH	mean corpuscular hemoglobin
$MCH_{in\ vivo}$	mean corpuscular Hb of infant cells produced <i>in vivo</i>
MCH_{trans}	mean corpuscular Hb of transfused cells
MCV	mean corpuscular volume
MPV	mean platelet volume
M	positive multiple of the baseline environmental effect function value
NICU	neonatal intensive care unit
NRBC	nucleated red blood cell
μ_b	mean of the baseline cellular lifespan
μ_{ss}	steady-state mean cellular lifespan
$\mu_{RET}(t)$	time variant circulating reticulocyte lifespan
$\mu_{RET,0}$	initial (t_0) or baseline (mean) circulating reticulocyte lifespan
$\mu_{RET,END}$	end circulating reticulocyte lifespan
$\mu_{RET,MAX}$	maximal (mean) circulating reticulocyte lifespan

$N(t)$	number of cells in the sampling compartment
N_p, N_{p1}, N_{p2}	number of cells removed by phlebotomy (subscript denotes phlebotomy number)
ODE	ordinary differential equation
ω, Ω	cellular release time delay, capital omega refers to random release time delay variable
PD	pharmacodynamic
PK	pharmacokinetic
$P(\cdot)$	probability
p.d.f.	probability density function
$r(\omega, s)$	time variant p.d.f. of cellular release time delays
RBC	red blood cell (may be sub-scripted with other terms)
RDW	red blood cell distribution width
RET	reticulocyte (may be sub-scripted with other terms)
RET He	reticulocyte mean corpuscular hemoglobin
rHuEPO	recombinant human erythropoietin
RMI	reticulocyte maturity index
RNA	ribonucleic acid
σ	standard deviation of the baseline cellular lifespan
s	time of stimulation
SD	standard deviation
SE	standard error
$S_b(t, z)$	baseline survival function

$S_e(t, z)$	observed survival function with an environmental effect
SS	steady-state (super- or sub-scripted with other terms)
t	time
t_0	initial time
$T_{decline}$	time period over which the production rate declines from f_{prod}^{SS} to zero
$T_P, T_{P1}, T_{P2}, T_{Pj}$	time of phlebotomy (subscript number or j denotes phlebotomy number)
T_{Ti}	time of the i^{th} transfusion
τ, T	cellular lifespan, i.e. the time from input into the sampling space to the time of output from the sampling space (capital tau refers to random cellular lifespan variable)
T_1	time post-phlebotomy that steady-state τ values are deviated from
T_2	time post-phlebotomy that steady-state τ values are returned to ($T_2 > T_1$)
θ	time invariant location parameter of the Weibull distribution
θ_0	initial (t_0) location parameter value of the Weibull distribution
u, v	arbitrary integration variables
$U(\cdot)$	unit step function
UR	unit response
$UR_\ell, UR_\ell(t, z)$	time variant unit response defined by $\ell(\tau, z)$
$UR_r, UR_r(t, s)$	time variant unit response defined by $r(\omega, s)$

V_n, V_N	bodyweight mass normalized blood volume
$V(t), V_{total}(t)$	total sampling space or blood volume
VLBW	very low birth weight
WBC	white blood cell
$x(t)$	time of stimulation of cells currently entering the sampling compartment using a “point distribution” cellular lifespan model
z	time of production
$z(t)$	logarithmically transformed $a(x(t))$

CHAPTER 1. INTRODUCTION

1.1 Background

Determination of the lifespans and production rates of cells has been an interest to researchers for many years. Some of the first work determined the lifespans of red blood cells (RBCs) from cell survival curves (1-3). However, much of the early work assumed constant production rates and distributions of cellular lifespans and thus the field remains an active area of research (4-6). With the development of many new drugs that affect important cell populations, such as cancerous, erythrocyte, platelet, leukocyte, and bacterial cell populations, the study of the effect of these new drugs on both the production and destruction of cells is an important consideration for optimal dosing and understanding of cellular behavior. For cell death mechanisms that are related to the age of the cell, i.e. time since production/stimulation, the lifespan distributions and age structure of the population are important for understanding the effect of the therapeutic agent and/or endogenous hormones. In particular, an accurate quantification of the pharmacokinetic/pharmacodynamic (PK/PD) relationship between a therapeutic agent or endogenous hormone(s) and cellular production (or destruction for a chemotherapeutic agent) cannot be determined without accounting for the age of the cells affected by the agent. Anemia and erythropoietin (EPO) stimulated erythropoiesis is one large area of research involving cellular response to an endogenous hormone/therapeutic agent (7-11).

1.2 Erythropoiesis, erythrocytes, and anemia

1.2.1 Erythropoiesis

Erythropoiesis is primarily controlled by EPO, a 30.4 kD glycoprotein hormone produced by the peritubular cells of the kidney in response to oxygen need (12-14).

Oxygen need is sensed through hypoxia-inducible factor-1 (HIF-1), a transcription factor for the EPO gene (13). In the absence of oxygen HIF-1 levels increase due to decreased proteasome degradation leading to an increase in EPO production. Since EPO increases erythropoiesis and thus oxygen carrying capacity of the blood, it is negatively regulated by its functional end product, hemoglobin (Hb), contained in circulating RBCs. EPO binds to 78 kD specific cell surface erythropoietin receptors (EPO-Rs) of the cytokine receptor superfamily (12, 15, 16). EPO-Rs are located primarily on erythroid progenitor cells; however, they have also been identified in many other tissues but at a much lower density than that observed on erythroid progenitor cells (17). EPO induces homodimerization of 2 EPO-Rs on the cell surface and following binding it is internalized and a substantial fraction of EPO is degraded in the lysosomes (15, 16, 18, 19). Thus EPO disposition is controlled in part through receptor- or target-mediated elimination (20-22). Additional EPO metabolism is thought to occur in the kidney and liver, with less than 10% excreted in the urine (13, 23). The amino acid sequence homology of EPO between humans and other mammals is high, at 80-82% amino acid identity with the sequence in pig, sheep, mouse, and rat (14).

Several nutrients are important for effective erythropoiesis including adequate supplies of folate, vitamin B₁₂, and iron, among others (24). Among these nutrients, iron is a particularly important nutrient for erythropoiesis and a maximal response to EPO since 4 iron molecules are contained in a single Hb molecule (1 in each heme group). Iron deficiency results in reduced erythropoiesis and still remains the most common cause of anemia in the United States of America and worldwide (25). Erythropoiesis also requires the action of interleukin-3 (IL-3), though EPO is the principal hormonal

regulator of erythropoiesis (14). Other cytokines thought to be involved in erythropoiesis include granulocyte colony-stimulating factor (G-CSF), stem cell factor (SCF), and insulin growth factor-1 (IGF-1) (26), though these primarily effect only the most immature progenitor cells (e.g. pluripotent stem cells, burst forming units-erythroid, etc.) (27).

Erythropoiesis occurs almost exclusively in the bone marrow of adult humans (12). In contrast, substantial erythropoiesis in adult mice and other rodents occurs in both the bone marrow and extramedullary in the spleen (14, 28), 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 (14). In fact, sheep most closely follow erythropoiesis in humans in terms of sites of erythropoiesis and the types hemoglobins produced, making them an important and common animal model of human erythropoiesis (14, 29). The large body size of sheep also makes them attractive research subjects since repeated blood sampling can be conducted with minimal impact on hematology homeostasis. In humans and animals, the site of erythropoiesis changes with the stage of development, beginning first in the yolk sac as 3- to 4- week old embryo in humans (12, 29). Erythropoiesis then switches to the liver and the spleen by about 8 weeks of age, and begins in the bone marrow by about 12 to 16 weeks. By birth in full-term infants all erythropoiesis has switched to the bone marrow.

The prevailing model of erythropoiesis is that erythroid progenitor cells develop from pluripotent stem cells in a stochastic fashion (26, 30). These erythroid progenitor cells are committed to the erythroid lineage, and hence cannot develop into other non-

erythroid blood cells (e.g. white blood cells). The erythroid progenitor cells are classified as either burst forming units-erythroid (BFU-E) or colony forming units-erythroid (CFU-E) by *in vitro* culture and are the first progenitor cells that are responsive to EPO (12). Erythropoietin acts on these erythroid progenitor cells by preventing apoptosis and by accelerating their development toward Hb producing cells (12). BFU-Es are less mature than CFU-Es and develop into the CFU-Es via action of EPO and other cytokines. The CFU-Es develop further into proerythroblasts, with the greatest number of EPO-Rs generally identified at this cell type transition. The number of EPO-Rs on an erythroid progenitor cell is generally considered to be 1000 per cell (16, 19, 23, 26). *In vivo*, erythropoiesis occurs in erythroblastic islands, which are specialized niches within the bone marrow where erythroblasts proliferate, differentiate, and enucleate (31). The erythroblastic islands are made up of a central macrophage surrounded by erythroblasts at various stages of development. During maturation of the erythroblasts the Hb content of the cells increases and the cell size decreases (12). Additionally, the number of EPO-Rs steadily decreases from the proerythroblast stage onwards. By the time a reticulocyte forms through nucleus extrusion of erythroblasts there is no detectable EPO binding, indicating the loss of all EPO-Rs by this stage of erythroid cell development. The maturation and proliferation of erythroblasts in the bone marrow is thought to occur over a 3-7 day period (32-34). However, this processes may be shorter *in vivo* and seems to be accelerated or shortened under conditions of high EPO concentrations (12, 35).

1.2.2 Erythrocyte maturation and senescence

Reticulocytes are the immature erythrocytes containing residual ribosomal ribonucleic acid (RNA) that initially reside in the bone marrow (12). They further mature

in the bone marrow for 1 or more days following enucleation (in humans) before being released into the systemic circulation where maturation into a mature erythrocyte (i.e. RBC) occurs (32). Maturation of reticulocytes primarily involves the removal of ribosomal RNA, remodeling of the plasma membrane, and a progressive decrease in cell size (12, 36, 37). Remodeling of the plasma membrane during reticulocyte maturation involves changes in the proteins that form the cytoskeletal network through removal of spectrin free regions of the membrane (38), though the exact processes involved are poorly understood. Reticulocytes are commonly used to monitor the “real-time” functional state of the erythroid bone marrow and play an important role in diagnoses and management of anemia (36, 37, 39-42).

In humans, the majority of the erythrocytes released from the bone marrow into the systemic circulation are reticulocytes (36, 37, 43). Under basal erythropoietic conditions (i.e. non-anemic or non-erythropoietically stimulated) in humans, reticulocytes have a lifespan in the systemic circulation of approximately 24 hours before developing into mature RBCs. However, during stress erythropoiesis (i.e. stimulated erythropoietic conditions), the reticulocyte lifespan in the circulation increases to an estimated 2-3 days (39). The reticulocytes produced under stress erythropoiesis also contain more residual RNA, are larger, and have less flexible plasma membranes than those produced under normal basal conditions, and therefore are thought to be immature reticulocytes that under “normal” physiological conditions reside in the bone marrow until being released as more mature reticulocytes (37, 39, 44, 45). In animal species such as ruminants and horses, however, the majority of the erythrocytes are released as mature RBC's under basal erythropoietic conditions (36, 37, 43). Similar to humans though, in ruminants the

percentage of reticulocytes increases dramatically during stress erythropoiesis (43, 46). Therefore like humans, younger erythrocytes are also released following stress erythropoiesis in these species. Accordingly, the reticulocyte counts increase under stress erythropoiesis not only due to increased reticulocyte production in response to EPO stimulation, but also due to a longer lifespan in the systemic circulation.

In addition to stimulating erythropoiesis, EPO also seems to indirectly affect the release of the immature reticulocytes from the bone marrow. Within 12 to 24 hours of plasma EPO increases the immature reticulocyte fraction increases and bone marrow reticulocytes decrease (47, 48). Since marrow reticulocytes have no EPO-Rs, the release of bone marrow reticulocytes is thought to be controlled by bone marrow sinuses through cell deformability/size exclusion (49, 50). It has been demonstrated that increased concentrations of plasma EPO increases the number and size of the apertures of the sinus wall (50). As reticulocytes mature they become smaller and more flexible, thus the size of the marrow sinuses may be a key determinant to their release. This process could be related, in part, to the increased circulating lifespan of reticulocytes during stress erythropoiesis. Peripheral oxygen concentration may also play a role in the behavior of sinus wall apertures independent of the effects of EPO.

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 3×10^{11} RBC (51). Red blood cells slowly age following release from the bone marrow and under non-disease state conditions the mechanism of RBC death is primarily to due to cellular

senescence (i.e. age-related destruction or death) (32, 52). In healthy human subjects a RBC typically survives 120 days from the time of release as a reticulocyte into the circulation until removal by the reticuloendothelial (RE) cells of the liver and spleen (32, 52, 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. Similar to human RBCs, the lifespan of sheep RBCs is estimated at 114 days and RBC removal from the circulation is related to cell age (54). In contrast to humans and sheep, in mice the lifespan of RBCs is much shorter, at only 20-30 days, and the mechanism of cell removal is considered to primarily be an age-independent or random process (4, 43). 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 (55, 56). 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 (57).

1.2.3 Types of anemia

Anemia remains a common disease with estimates of 1.5 to 1.7 billion people (prevalence of 22.9-26.7%) affected worldwide (58). It is a disease with a diverse etiology ranging from absence of erythroid cells in the bone marrow, to kidney failure, to nutritional deficiencies, to genetic defects in Hb synthesis, to blood loss, among others. Non-blood loss anemia may be broadly classified into two types, hypoproliferative and hemolytic (24). In hypoproliferative anemia the body cannot produce an adequate number of erythrocytes to an appropriate stimulus (i.e. reduced oxygen carrying capacity of the

blood). Depending on the exact etiology, administration of recombinant human EPO (rHuEPO) may treat the underlying cause of the anemia. Examples include the treatment of anemia with rHuEPO during chronic renal failure or during chronic inflammatory diseases (27, 59), both which reduce the production of endogenous EPO by the kidney. However, administration of rHuEPO would obviously have limited ability to correct hypoproliferative anemia caused by marrow damage through either drugs, radiation, infection, malignancy or other causes without first reversing the marrow damage (37). In contrast, with hemolytic anemia, which is caused by the premature destruction of erythrocytes, rHuEPO remains only a palliative treatment and does not address the underlying disease process.

Lately, much attention has focused on the treatment of very low birth weight (VLBW, < 1000 g) premature infant neonatal anemia with rHuEPO in order to reduce the number of RBC transfusions (7, 60-62). Reducing the number of RBC transfusions in these infants reduces the risk viral infection, retinopathy of prematurity, and bronchopulmonary dysplasia, and may reduce costs (62). Administration of rHuEPO represents one approach to reducing the number of RBC transfusions; however, previous research has demonstrated limited success of exogenous rHuEPO administration in neonatal infants despite a documented high capacity for erythropoiesis in infants and proven success of the therapy in anemic adults (9, 60, 62, 63). The exact reasons for the apparent limited efficacy of rHuEPO in infants are unknown, but it may be related to suboptimal dosing and insufficient understanding of the complex pharmacodynamics of the response of erythrocytes to rHuEPO.

1.3 Erythropoiesis stimulating agents

There are several different forms of approved erythropoiesis stimulating agents (ESAs), each which contains nearly all or 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 (64, 65). Epoetin alpha and beta (rHuEPO) contain all 165 amino acids of EPO, only differing slightly in their glycosylation pattern and have an intravenous (IV) terminal half-life of 4 to 8 hours (21, 64, 66). 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 (21, 64). Subcutaneous (SQ) administration of epoetin alfa and epoetin beta increases the terminal half-life to 24 hours through “flip-flop” kinetics (64). 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 (67, 68). 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 (69). 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 (65). Apparently due to the long elimination half-life, the terminal kinetics are unaffected by IV or SQ administration.

The pharmacodynamics of ESAs at the EPO-R level in erythroid progenitor cells is poorly understood (23). Only 20% to 30% EPO-R occupancy by EPO is necessary to stimulate erythropoiesis. Studies of rHuEPO have used once daily, thrice weekly, 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 rHuEPO are 30% higher than that needed to achieve the same efficacy with SQ elimination (23), 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 (65). The importance of ESA half-life on the PDs of erythropoiesis is evident with CERA, which has a lower EPO-R 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.

1.4 Pharmacodynamic models of cellular response

1.4.1 Random cell removal models

One of the most common techniques for modeling the PK/PD relationship between therapeutic agents and the cellular populations is the compartmental or cellular “pool” model, in which cells are transferred between one or more compartments by first-order processes (70, 71), as given by:

$$\begin{aligned}
\frac{dN_1}{dt} &= f_{prod}(t) - k_{out} \cdot N_1(t) \\
\frac{dN_2}{dt} &= k_{out} \cdot N_1(t) - k_{out} \cdot N_2(t) \\
&\vdots \\
\frac{dN_n}{dt} &= k_{out} \cdot N_{n-1}(t) - k_{out} \cdot N_n(t)
\end{aligned} \tag{1.1}$$

where $N_i(t)$ is the number of cells in the i^{th} compartment at time t , $f_{prod}(t)$ is the cellular production rate, k_{out} is the first-order output rate constant, and n is the number of compartments. This model creates a delay between cellular production or input rate into the first cell compartment and the output rate from the last cell compartment; however, a major limitation of this model is that it ignores the age structure of cells within a compartment, treating all cells within the compartment as equally likely to be transferred out of the compartment. Thus cells in these models are removed from each compartment by random, age-independent, first order processes. While these cellular compartmental models are easy to implement numerically using a system of ordinary differential equations (ODEs) as given in Eq. 1.1, they often ignore the underlying physiology of the system. However, for some cell populations or disease conditions where cells are removed by random age-independent processes, such as the penicillin induced immune hemolytic anemia (37), these models may be appropriate.

1.4.2 Cellular lifespan models

For cells like reticulocytes and mature erythrocytes in sheep and humans whose “removal” from the sampling compartment is primarily determined by developmental processes (i.e. transformation into a mature RBC) and cellular senescence, respectively, more physiologically realistic models are needed that incorporate a cellular lifespan component. These cellular lifespan PD models fall into the broad class of indirect

response models. In a lifespan model, the disposition kinetics of the cells in the body are determined by the lifespan of individual cells and the distributions of such lifespans in the cellular population. In their simplest form, all cells have the same lifespan (i.e. a “point distribution” of lifespans), resulting in:

$$\frac{dN}{dt} = f_{prod}(t) - f_{prod}(t - \tau) \quad (1.2)$$

where τ is the cellular lifespan. Thus in a cellular lifespan model the output rate (i.e. $f_{prod}(t - \tau)$) is given by the negative lifespan shifted cellular production rate (i.e. input rate). The cellular lifespan models have diverse potential applications to many cell types (e.g. erythrocytes, bacteria, cancerous cells) (5, 6); however, most publications to date in PD have focused populations of blood cells (5, 11, 35, 72-75).

1.5 Time variant cellular lifespan PD models

Early cellular lifespan PD models assumed a single “point distribution” of cellular lifespans that does not vary over time (i.e. time invariant) (Eq. 1.2). More recently, models have been introduced that account for a time invariant distribution of cell lifespans (6), that is the variability in cell lifespans between individual cells is incorporated into the model through a constant lifespan distribution. The time invariant lifespan assumption has been partially addressed by allowing for two different point reticulocyte maturation times or lifespans, a baseline and a stress-erythropoiesis maturation (11, 76). However, models that determine dynamic changes over time in cellular lifespan of erythrocytes or other cell populations have not been presented nor have the dynamic changes in erythrocyte lifespans been previously quantified.

1.6 Outline of the thesis

To account for changes in lifespans of reticulocytes and other cells, a time variant cellular lifespan model was formulated assuming a simple “point distribution” of cell lifespans and is presented in Chapter 2. The model was integrated into a PK/PD analysis of EPO, reticulocytes, and Hb response in phlebotomized sheep, demonstrating the dynamic changes in both the reticulocyte lifespan and Hb production rate during stress erythropoiesis. In Chapter 3 the time variant cellular lifespan model was extended to account for a distribution of cellular lifespans and applied to EPO, reticulocyte, and RBC response in double phlebotomized sheep. The formulated model better captured the physiology of sheep erythrocyte maturation and is a more generally applicable model of time variant cellular lifespans. However, the model still suffered the limitation that all time variance in lifespans occurs at the time of stimulation/production and that no changes in the environment (e.g. the systemic circulation for RBCs) following production of the cells could affect their lifespan. In Chapter 4 these limitations were overcome by further extending the cellular lifespan model in Chapter 3 to account for the effect of changes in the environment on cellular lifespans by incorporating time variant models of survival analysis from statistical and engineering fields. The formulated environmental effect cellular lifespan model was applied to sheep RBC and Hb response following high-dose chemotherapy administration of busulfan to sheep.

Chapter 5 is a validation of the stability of infant cord blood collected into either sodium heparin or ethylenediaminetetraacetic acid (EDTA) anticoagulant containing tubes and the ability to dilute small volumes of blood for analysis on the Sysmex XE-2100 automated hematology analyzer. The stability of the blood in both anticoagulants

and the ability to dilute small blood volumes allowed for in depth study of the relationship between endogenous EPO, RBCs, and Hb in a clinical study of premature VLBW infants presented in Chapter 6. In that study, detailed accounting of all blood removed by phlebotomy and administered by transfusion was conducted and incorporated into a cellular lifespan model of erythrocyte disposition with exact corrections for these frequent exogenous perturbations of the system. This analysis represents the first quantitative estimates of the erythropoiesis and Hb production rates and their relationship to EPO in these VLBW infants.

1.7 Objectives

The overall hypothesis of this work is that treatment optimization of erythropoietin, particularly under dynamic hematological conditions such as stress erythropoiesis and that experienced in premature infants, requires a mechanistic pharmacodynamic understanding of erythrocyte disposition. Under that overall hypothesis, the specific objectives were: 1.) to develop a time variant cellular lifespan model to dynamically determine the changes in the circulating reticulocyte lifespan under stress erythropoiesis, 2.) to present a more general PD model that incorporates a time variant distribution of cellular lifespans and successfully fit the presented model to erythrocyte data following stress erythropoiesis to estimate the changes in the circulating reticulocyte lifespan, 3.) to further extend the time variant cellular lifespan PD models to allow for the incorporation of environmental effects on the cellular lifespan, 4.) to validate the use of selected hematological parameters when determined from infant blood that uses sodium heparin as the anticoagulant, diluted, and/or stored for up to 72 hours,

and 5.) to estimate the *in vivo* erythropoiesis and Hb production rates and their relationship to endogenous plasma EPO concentrations in VLBW preterm infants.

CHAPTER 2. PHARMACODYNAMIC ANALYSIS OF TIME-VARIANT CELLULAR DISPOSITION: RETICULOCYTE DISPOSITION CHANGES IN PHLEBOTOMIZED SHEEP

2.1 Introduction

Reticulocytes are immature red blood cells (RBCs) that initially reside in the bone marrow and subsequently are released into the systemic circulation where they develop into mature RBCs (32). Reticulocytes are produced from erythroid progenitor cells (BFU-E and CFU-E) located primarily in the bone marrow. They are commonly used to monitor the “real-time” functional state of the erythroid bone marrow and play an important role in diagnoses and management of anemia (36, 37, 39-42). Measurement of the reticulocyte count is valuable for determining if a patient has a functionally normal response to either endogenously produced or exogenously administered erythropoietin (EPO) (40, 77). Reticulocyte counts are also used to monitor bone marrow suppression by chemotherapy (78) and bone marrow engraftment following bone marrow transplantation (79-82). The maturation of erythroid progenitor cells into reticulocytes and ultimately mature RBCs is primarily controlled by EPO, a 30.4 kD glycoprotein hormone produced by the peritubular cells of the kidney in response to oxygen need (12, 14). During the development from erythroid progenitor cells their hemoglobin (Hb) content increases until it develops into a reticulocyte upon nucleus extrusion, where further maturation primarily involves the removal of ribosomal RNA, remodeling of the plasma membrane, and a progressive decrease in cell size, increasing the concentration of Hb on a mass/volume basis, but not increasing the overall amount of Hb within the cell

(36, 37, 51). Therefore, a reticulocyte cell has approximately the same amount of Hb as a RBC (45).

Generally, under normal erythropoietic conditions in humans (i.e. non-erythropoietically stimulated), the reticulocyte lifespan in the systemic circulation is approximately 24 hours before maturation into RBCs (36, 37). However, during stress erythropoiesis (i.e. stimulated erythropoietic conditions), the reticulocyte circulating lifespan or residence time in the circulation increases to an estimated 2-3 days (39). Reticulocytes produced under stress erythropoiesis also contain more residual ribosomal RNA, are larger, and have less flexible plasma membranes than those produced under normal conditions, and therefore are thought to be immature reticulocytes that under “normal” physiological conditions reside in the bone marrow until being released as more mature reticulocytes (37, 39, 44, 45). Accordingly, the reticulocyte counts increase under stress erythropoiesis not only due to increased reticulocyte production in response to EPO stimulation, but also due to a longer lifespan in the systemic circulation. To account for the effect of this increased circulating reticulocyte lifespan on the reticulocyte count in clinical practice, one “rule of thumb” approach has been to divide the observed reticulocyte count or percentage by two to obtain a “more accurate” index of reticulocyte production when they are produced under stress erythropoiesis (37). More sophisticated quantitative approaches utilize the maturity of the produced reticulocytes, referred to as the reticulocyte maturity index (RMI). The RMI is based on of individual reticulocyte RNA content commonly determined by fluorescence intensity measurements. However, the problem with existing methods utilizing RMI is that there is no standard method that corrects for the observed reticulocyte counts based on the RMI. Furthermore, these

methods suffer from the difficulty in standardizing and cross-correlating across different instruments and laboratories (36).

Although different terminology is often used to describe reticulocyte or cellular lifespan, in this work it is defined as the time a cell resides in the sampling compartment (e.g. systemic blood circulation) as the cell type of interest (e.g. reticulocyte). Analogous terminology used by other authors includes “residence time” and “maturation time” (72, 73, 76). Previous EPO-reticulocyte pharmacokinetic/pharmacodynamic (PK/PD) models have commonly assumed that all cells have a time invariant and the same common lifespan or maturation time (i.e. “point distribution”) (5, 35, 72-75). Recently, EPO-reticulocyte PK/PD models have been presented that allow for a fixed (time invariant) distribution of cell lifespans (6). The time invariant lifespan assumption has been partially addressed by allowing for two different reticulocyte maturation times, a baseline and a stress-erythropoiesis maturation (76). Other models that partially address the stress erythropoiesis effects on increasing reticulocyte lifespan incorporate an erythropoietin dose dependent lifespan (6). However, the dynamic changes in the circulating reticulocyte lifespan throughout the time course of stress erythropoiesis were not determined in any of the above studies. Given the known change in reticulocyte lifespan in the systemic circulation following stress erythropoiesis, the clinical importance of the reticulocyte count/lifespan change, and the limited attempts to quantify these changes; the primary objective of the present analysis was to dynamically determine the changes in the circulating reticulocyte lifespan due to phlebotomy induced stress erythropoiesis. To achieve this aim, a time variant cellular lifespan or disposition

model was developed and integrated into a comprehensive pharmacodynamic analysis of EPO, reticulocyte, and Hb response in phlebotomized sheep.

2.2 Theoretical

2.2.1 Unit response function

The unit response (UR) function of a linear cellular disposition, Figure 2.1 (panel A), for hematopoietic cells may be described as:

$$UR(t) = U(t-a) - U(t-b) \quad b > a \geq 0 \quad (2.1)$$

where a is the time delay it takes for an individual cell to appear in the sampling compartment (i.e. systemic circulation) following precursor stimulation, b is the time the subsequent cell exits the sampling compartment, and ' t ' is measured, as a and b , relative to the time of stimulation (time s in Figure 2.1, panel A). U is the unit step function defined by:

$$U(t) = \begin{cases} 1 & \text{if } t \geq 0 \\ 0 & \text{otherwise} \end{cases} \quad (2.2)$$

Therefore, $UR(t)$ takes on the value of 1 when the cell is present, and a value of 0 when the cell is not present in the sampling compartment.

2.2.2 Time invariant cellular disposition

Consider hematopoietic progenitor cells, which in the following will be referred to simply as "cells", that are to be stimulated over time by a stimulation function, $f_{stim}(t)$, which typically depends on time through endogenous growth factor(s) and/or exogenous drug. The dependence of $f_{stim}(t)$ on these factors will be considered subsequently. The units for $f_{stim}(t)$ are numbers of cells stimulated per unit time. Let it be assumed that these cells act fairly independently of each other, then one can apply the superposition

principle. In the case where all cells have the same *time invariant* disposition parameters (a and b , Figure 2.1, panel A) it follows that the number of cells present in the systemic circulation, $N(t)$, is given by:

$$N(t) = f_{stim}(t) * UR(t) \equiv \int_{-\infty}^t f_{stim}(u) \cdot UR(t-u) du \quad (2.3)$$

where $*$ denotes the convolution operator. The integration limit of $-\infty$ in Eq. 2.3 is to be interpreted to consider “all prior history” of the system. The above equation assumes a point distribution of cell lifespans (i.e. zero variance). In order to readily fit Eq. 2.3 to observed data, it is convenient to transform it into a differential equation form. Equation 2.3 can be separated into two components as:

$$N(t) = \int_{-\infty}^{t_0} f_{stim}(u) \cdot UR(t-u) du + \int_{t_0}^t f_{stim}(u) \cdot UR(t-u) du, \quad t > t_0 \quad (2.4)$$

where t_0 is the initial observation time. If we define:

$$I_g(t) \equiv \int_{-\infty}^{t_0} f_{stim}(u) \cdot UR(t-u) du \quad (2.5)$$

$$I_h(t) \equiv \int_{t_0}^t f_{stim}(u) \cdot UR(t-u) du \quad (2.6)$$

Then Eq. 2.4 becomes:

$$N(t) = I_g(t) + I_h(t) \quad (2.7)$$

If steady-state (SS) conditions are assumed to exist prior to t_0 so that:

$$f_{stim}(t) = f_{stim}(t_0) = f_{stim}^{SS}, \quad t \leq t_0 \quad (2.8)$$

where f_{stim}^{SS} is the steady-state stimulation rate, then:

$$I_g(t) = f_{stim}^{SS} \int_{-\infty}^{t_0} UR(t-u) du = f_{stim}^{SS} \int_{t-t_0}^{\infty} UR(u) du \quad (2.9)$$

Differentiating Eq. 2.9 with respect to time gives:

$$\frac{dI_g}{dt} \equiv I_g'(t) = -f_{stim}^{SS} \cdot UR(t-t_0) = -f_{stim}^{SS} [U(t-t_0-a) - U(t-t_0-b)] \quad (2.10)$$

Evaluation and differentiation of Eq. 2.6, Appendix A.1, results in:

$$I_h'(t) = f_{stim}(t-a) \cdot U(t-t_0-a) - f_{stim}(t-b) \cdot U(t-t_0-b) \quad (2.11)$$

Differentiation of Eq. 2.7 and substitution of Eq. 2.10 and Eq. 2.11 results in:

$$N'(t) = [f_{stim}(t-a) - f_{stim}^{SS}] \cdot U(t-t_0-a) - [f_{stim}(t-b) - f_{stim}^{SS}] \cdot U(t-t_0-b), \quad t > t_0 \quad (2.12)$$

with:

$$N(t) = N(t_0) = f_{stim}^{SS} \cdot (b-a), \quad t \leq t_0 \quad (2.13)$$

2.2.3 Extension to time variant disposition parameters

Next consider the case where the cellular disposition parameters are *time variant*.

That is, the lifespan in the sampling compartment, $b-a$, is a function of time. For simplicity we consider the time variance of the lifespan to be due only to the a parameter (Figure 2.1, panel B). In this case the UR function becomes:

$$UR(t, s) = U(t-a(s)) - U(t-b), \quad b > a(s) \geq 0, \quad t \geq s \quad (2.14)$$

Here s is the time that the cell is stimulated (Figure 2.1, panel B). The value of a is considered variable and determined at the time the cell is stimulated and remains fixed at that value thereafter. Again, a point distribution of cell lifespans, at a given stimulation time, is assumed.

For this time variant case, Eq. 2.4 becomes:

$$N(t) = \int_{-\infty}^{t_0} f_{stim}(u) \cdot UR(t-u, u) du + \int_{t_0}^t f_{stim}(u) \cdot UR(t-u, u) du, \quad t > t_0 \quad (2.15)$$

If similar to before we assume steady-state conditions for $t \leq t_0$, then:

$$a(t) = a(t_0) = a_{SS}, \quad t \leq t_0 \quad (2.16)$$

where a_{SS} is the steady-state 'a' value. Due to the steady-state assumption the first term, $I_g(t)$, becomes nearly identical to Eq. 2.5, and similar to before we get:

$$I_g'(t) = -f_{stim}^{SS} \cdot [U(t - t_0 - a_{SS}) - U(t - t_0 - b)] \quad (2.17)$$

However, this time we write:

$$I_h(t) = \int_{t_0}^t f_{stim}(u) \cdot UR(t - u, u) du = \int_{t_0}^t f_{stim}(u) \cdot U(t - u - a(u)) du - \int_{t_0}^t f_{stim}(u) \cdot U(t - u - b) du \quad (2.18)$$

For convenience in derivation we define:

$$I_h(t) \equiv I_{h1}(t) - I_{h2}(t) \quad (2.19)$$

$$I_{h1}(t) \equiv \int_{t_0}^t f_{stim}(u) \cdot U(t - u - a(u)) du \quad (2.20)$$

$$I_{h2}(t) \equiv \int_{t_0}^t f_{stim}(u) \cdot U(t - u - b) du \quad (2.21)$$

Due to the unit step function, $U(t)$, the integrand of Eq. 2.20 will be zero in the integration region $u = t_0$ to $u = t$ for u values for which $t - u - a(u) < 0$. Thus, the switch between zero and non-zero integrand values will occur at $u = x$ value(s) where x , that is a function of time, is the solution(s) to the equation:

$$x(t) = t - a(x(t)) \quad (2.22)$$

Potentially Eq. 2.22 may have multiple solutions for x at the current time t . However, as evident from the graphical representation of the solution(s) to Eq. 2.22 given in Figure 2.2, if the $a(t)$ never has a slope, $a'(t)$, less than or equal to -1 then Eq. 2.22 only has

one solution (Figure 2.2, time t_1). For a more detailed proof, see Appendix A.2.

Accordingly, Eq. 2.20 becomes:

$$I_{hl}(t) = 0, \quad t - t_0 < a(t_0) = a_{SS}, \quad a'(t) > -1 \quad (2.23)$$

$$I_{hl}(t) = \int_{t_0}^{x(t)} f_{stim}(u) du = \int_{t_0}^{t-a(x(t))} f_{stim}(u) du, \quad t - t_0 \geq a(t_0) = a_{SS}, \quad a'(t) > -1 \quad (2.24)$$

It is recognized from Eq. 2.22 that x is simply the time for the progenitor cell stimulation of those “new” cells that are currently entering the systemic circulation at time t .

Differentiation of Eq. 2.23 and Eq. 2.24 gives:

$$I_{hl}'(t) = 0, \quad t - t_0 < a_{SS} \quad (2.25)$$

$$I_{hl}'(t) = f_{stim}(x(t)) \cdot x'(t), \quad t - t_0 \geq a_{SS} \quad (2.26)$$

Eq. 2.25 and Eq. 2.26 generalize to:

$$I_{hl}'(t) = x'(t) \cdot f_{stim}(x(t)) \cdot U(t - t_0 - a_{SS}) \quad (2.27)$$

Similar to Eq. 2.6, evaluation and differentiation of Eq. 2.21 generalizes to:

$$I_{h2}'(t) = f_{stim}(t - b) \cdot U(t - t_0 - b) \quad (2.28)$$

Differentiation of Eq. 2.19 and substitution of Eq. 2.27 and Eq. 2.28, followed by subsequent substitution of the resulting equation along with Eq. 2.17 into the time variant equivalent of differentiated Eq. 2.7 results in:

$$N'(t) = \left[x'(t) \cdot f_{stim}(x(t)) - f_{stim}^{SS} \right] \cdot U(t - t_0 - a_{SS}) - \left[f_{stim}(t - b) - f_{stim}^{SS} \right] \cdot U(t - t_0 - b), \quad t > t_0 \quad (2.29)$$

with:

$$x'(t) = \frac{I}{1 + a'(x(t))}, \quad t > t_0, \quad a'(t) > -1 \quad (2.30)$$

$$N(t) = N(t_0) = f_{stim}^{SS} \cdot (b - a_{SS}), \quad t \leq t_0 \quad (2.31)$$

$$x(t) = t - a_{SS}, \quad t \leq t_0 \quad (2.32)$$

$$a(t) = a(t_0) = a_{SS}, \quad t \leq t_0 \quad (2.33)$$

2.2.4 Correction for cells removed by phlebotomy

Let F denote the fraction of cells remaining momentarily after the time of the phlebotomy, denoted T_p , then the equation to be integrated changes to a new “initial” condition at time T_p according to:

$$N(t) = \begin{cases} N(T_p) & \text{for } t = T_p \\ F \cdot N(T_p) & \text{for } t = T_p + \varepsilon \end{cases} \quad (2.34)$$

where ε denotes an infinitesimally small time increment. However, just a change in a boundary condition according to Eq. 2.34 is insufficient to correct for the cells mechanically removed. The differential equation, Eq. 2.29, being integrated across the phlebotomy boundary point ($t = T_p$) must also be changed. If the original equation (Eq. 2.29) is not changed then this equation will for some time period beginning immediately after the phlebotomy underestimate the number of cells because it contains an elimination component (i.e. $I_{h2}'(t)$) that includes those cells mechanically removed at time T_p , which should not be included in the elimination. To correct for this, one needs to add a term that accounts for the elimination rate of those cells removed at time T_p identical to their elimination had they not been removed by phlebotomy. Accordingly, the correction term (Appendix A.3) to be *added* is:

$$[1-F] \cdot [U(t-T_p) - U(t-(T_p + b - a(x(T_p))))] \cdot f_{stim}(t-b) \quad (2.35)$$

In deriving Eq. 2.35 it is recognized that the oldest cells in the removed cell population would be eliminated *in vivo* at $t = T_p$ with a (projected) rate of $[1-F] \cdot f_{stim}(T_p - b)$, while the youngest cells would be eliminated at $t = T_p + b - a(x(T_p))$ with a rate of $[1-F] \cdot f_{stim}(T_p - a(x(T_p)))$. Thus, the correction term is active from the time period $t = T_p$ to $t = T_p + b - a(x(T_p))$, consistent with the above considerations.

2.2.5 Summary of general key equation

For optimal readability the *general key equation*, which follows from Eq. 2.29 and Eq. 2.35, is produced below together with support equations, although the latter equations are reproduced from above:

$$N'(t) = \left[x'(t) \cdot f_{stim}(x(t)) - f_{stim}^{SS} \right] \cdot U(t-t_0 - a_{SS}) - \left[f_{stim}(t-b) - f_{stim}^{SS} \right] \cdot U(t-t_0 - b) + [1-F] \cdot [U(t-T_p) - U(t-(T_p + b - a(x(T_p))))] \cdot f_{stim}(t-b), \quad t > t_0 \quad (2.36)$$

with:

$$x(t) = t - a(x(t))$$

$$x'(t) = \frac{1}{1 + a'(x(t))}, \quad t > t_0 \text{ and } a'(t) > -1$$

$$N(t) = N(t_0) = f_{stim}^{SS} \cdot (b - a_{SS}), \quad t \leq t_0$$

$$x(t) = t - a_{SS}, \quad t \leq t_0$$

$$a(t) = a(t_0) = a_{SS}, \quad t \leq t_0$$

$$f_{stim}(t) = f_{stim}(t_0) = f_{stim}^{SS}, \quad t \leq t_0$$

$$b > a(t) \geq 0$$

$$N(t) = \begin{cases} N(T_p) & \text{for } t = T_p \\ F \cdot N(T_p) & \text{for } t = T_p + \varepsilon \end{cases}$$

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

2.2.6 Specific modeling of time variant disposition

The above Eq. 2.36 describes the general case considering *any* arbitrary variation over time in the $a(t)$ “parameter”. The following section is aimed at specifically modeling the $a(t)$ variation over time.

The time variant cellular disposition parameters are not readily determined by modeling cellular count data from the cells of interest alone. The observed response is a function of both the stimulation rate and the disposition “parameters”, which are both changing as functions of time, causing difficulty in the estimation procedure. One possible solution is to utilize shared disposition parameters or functions from “coupled” cell data (i.e. cells that transform into each other) to assist in the simultaneous determination of the stimulation rate and the disposition “parameters”.

To determine the time variant reticulocyte circulating lifespan, the present analysis utilizes the shared input and disposition functions of reticulocytes and Hb (i.e. RBCs) to overcome the above problem, since a reticulocyte has approximately the same amount of Hb as a mature RBC (51). Accordingly, both the reticulocyte count and Hb response share the same time delay, $a(t)$, for appearance in the systemic circulation. The release of the Hb into the systemic circulation is assumed to enter the circulation within a reticulocyte before it matures into an RBC. The b parameter of the reticulocyte disposition, denoted by b_{RET} , was assumed to be time invariant. Furthermore, both the

reticulocytes and Hb share the stimulation function, f_{stim} , which is multiplied by a conversion constant, K_{RET} , to change from units of Hb to number of reticulocytes (i.e. has units of 10^3 cells/g of Hb). Additionally, since the same fraction of cells remaining will occur for RBCs (i.e. Hb) and reticulocytes, both share the F parameter as well. Thus, reticulocytes and Hb are “coupled” through several shared parameters and functions which generally assist in the analysis.

2.2.7 The stimulation function

Measurement of hematopoietic cells *in vivo* is done in terms of concentrations, such as number of erythroid cells or grams of Hb per volume of blood, therefore the number of cells (or grams of Hb), N , is divided by the volume, V . However, in the analysis, due to the measurements units, the V fuses with the parameters of the stimulation function, f_{stim} , so that it is not estimated directly in the regression analysis. Erythropoietin was considered the stimulator for the stimulation of the progenitor cells. Accordingly, $C_{bio}(t)$ is the concentration of EPO at the biophase (effect site). The analysis indicated that $C_{bio}(t)$ is kinetically distinct from the EPO plasma concentration, $C_p(t)$, and the following convolution relationship:

$$C_{bio}(t) = k_{bio} \cdot e^{-k_{bio} \cdot t} * C_p(t) \quad (2.37)$$

was used to model the plasma/biophase relationship resulting in the following differential equation:

$$C_{bio}'(t) = k_{bio} \cdot C_p(t) - k_{bio} \cdot C_{bio}(t), \quad t > t_0 \quad (2.38)$$

with:

$$C_{bio}(t) = C_{bio}(t_0) = C_{bio}^{SS} = C_p^{SS}, \quad t \leq t_0 \quad (2.39)$$

where C_{bio}^{SS} and C_p^{SS} denote the steady-state effect site and plasma EPO concentrations, respectively, and C_p^{SS} was set equal to the initial (first observation) fitted plasma EPO concentration. The stimulation of the erythroid progenitor cells (quantified in terms of Hb) was related to C_e by the Hill equation:

$$f_{stim}(t) = \frac{E_{max} \cdot C_{bio}(t)}{EC_{50} + C_{bio}(t)} \quad (2.40)$$

with:

$$f_{stim}^{SS} = f_{stim}(t) = \frac{E_{max} \cdot C_{bio}(t)}{EC_{50} + C_{bio}(t)} = \frac{E_{max} \cdot C_{bio}^{SS}}{EC_{50} + C_{bio}^{SS}} = \frac{E_{max} \cdot C_p^{SS}}{EC_{50} + C_p^{SS}}, \quad t \leq t_0 \quad (2.41)$$

where f_{stim}^{SS} is the steady-state stimulation rate prior to time t_0 , E_{max} is the maximal Hb stimulation rate in g/dL/day, and EC_{50} is the biophase EPO concentration that results in 50% of maximal Hb stimulation rate (E_{max}).

2.2.8 Specific PD model

The analysis involved simultaneously fitting to both the Hb and reticulocyte count concentration vs. time profiles, denoted $Hb(t)$ and $RET(t)$, respectively. Let b_{RBC} denote the time the RBCs are removed from the systemic circulation relative to progenitor cell stimulation (i.e. the RBC equivalent ‘ b ’ parameter from Eq. 2.14 and Figure 2.1) and b_{RET} denote the time the reticulocytes mature into RBCs relative to the stimulation. Both the b_{RET} and b_{RBC} parameters are considered time invariant. As before, let $a(t)$ denote the time that reticulocytes enter into the systemic circulation relative to progenitor stimulation. The time variance of $a(t)$ is shared by the Hb kinetics because the

release of Hb into the systemic circulation is assumed to initially enter within a reticulocyte. To ensure that $b_{RET} > a(t) \geq 0$ for all t and for computational convenience, it is helpful to parameterize $a(t)$ as:

$$a(t) = b_{RET} - \mu_{RET}(t), \quad b_{RET} \geq \mu_{RET}(t) > 0 \quad (2.42)$$

Therefore, $\mu_{RET}(t)$ is the (time variant) circulating reticulocyte lifespan. The parameterization of Eq. 2.36 using Eq. 2.42 (Appendix A.4) with the appropriate substitutions (i.e. Eq. 2.40 and Eq. 2.41) from above results in the following *specific key equations*:

$$\begin{aligned} Hb'(t) = & \left[\left[\left[1 - z'(t) \cdot e^{z(t)} \right] \cdot f_{stim}(t - e^{z(t)}) - f_{stim}^{SS} \right] \cdot U(t - t_0 - (b_{RET} - \mu_{RET,SS})) \right. \\ & - \left. \left[f_{stim}(t - b_{RBC}) - f_{stim}^{SS} \right] \cdot U(t - t_0 - b_{RBC}) \right. \\ & \left. + [1 - F] \cdot \left[U(t - T_P) - U(t - (T_P + b_{RBC} - (b_{RET} - \mu_{RET}(T_P - e^{z(T_P)})))) \right] \right] \cdot f_{stim}(t - b_{RBC}) \end{aligned} \quad ,$$

$$t > t_0 \quad (2.43)$$

$$RET'(t) = K_{RET} \cdot \left\{ \begin{aligned} & \left[\left[1 - z'(t) \cdot e^{z(t)} \right] \cdot f_{stim}(t - e^{z(t)}) - f_{stim}^{SS} \right] \cdot U(t - t_0 - (b_{RET} - \mu_{RET,SS})) \\ & - \left[f_{stim}(t - b_{RET}) - f_{stim}^{SS} \right] \cdot U(t - t_0 - b_{RET}) \\ & + [1 - F] \cdot \left[U(t - T_P) - U(t - (T_P + \mu_{RET}(T_P - e^{z(T_P)})))) \right] \cdot f_{stim}(t - b_{RET}) \end{aligned} \right\},$$

$$t > t_0 \quad (2.44)$$

with:

$$z'(t) = \frac{-\mu_{RET}'(t - e^{z(t)})}{1 - \mu_{RET}'(t - e^{z(t)})} \cdot e^{-z(t)}, \quad t > t_0 \text{ and } \mu_{RET}'(t) < 1 \quad (2.45)$$

$$Hb(t) = Hb(t_0) = f_{stim}^{SS} \cdot (b_{RBC} - (b_{RET} - \mu_{RET,SS})), \quad t \leq t_0 \quad (2.46)$$

$$RET(t) = RET(t_0) = K_{RET} \cdot f_{stim}^{SS} \cdot \mu_{RET,SS}, \quad t \leq t_0 \quad (2.47)$$

$$z(t) = z(t_0) = \ln(b_{RET} - \mu_{RET,SS}), \quad t \leq t_0 \quad (2.48)$$

$$Hb(t) = \begin{cases} Hb(T_p) & \text{for } t = T_p \\ F \cdot Hb(T_p) & \text{for } t = T_p + \varepsilon \end{cases} \quad (2.49)$$

$$RET(t) = \begin{cases} RET(T_p) & \text{for } t = T_p \\ F \cdot RET(T_p) & \text{for } t = T_p + \varepsilon \end{cases} \quad (2.50)$$

The final model is schematically detailed in Figure 2.3.

2.3 Materials and methods

2.3.1 Animals

All animal care and experimental procedures were approved by the University of Iowa Institutional Animal Care and Use Committee. Five healthy young adult sheep ranging in age from 2-4 months old and weighing 21.2 ± 3.60 kg (mean \pm SD) were selected. Animals were housed in an indoor, light- and temperature-controlled environment, with *ad lib* access to feed and water. Prior to study initiation, jugular venous catheters were aseptically placed under pentobarbital anesthesia. Intravenous ampicillin (1 g) was administered daily for 3 days following catheter placement.

2.3.2 Study protocol

Blood samples (~0.5 mL/sample) for plasma EPO, reticulocyte counts, and Hb were collected for 5-7 days to determine baseline values prior to conducting a single controlled phlebotomy over several hours to induce acute anemia. Animals were phlebotomized to Hb concentration of approximately 4 g/dL. To maintain a constant blood volume during the procedure, equal volumes 0.9% NaCl solution were infused for each volume of blood removed. Blood samples were subsequently collected at least daily for 25-30 days post-phlebotomy. No iron supplementation other than that in the animal's feed was given. Plasma iron and total iron binding capacity (TIBC) were monitored to

ensure animals did not become iron deficient. To minimize Hb and red cell loss due to frequent blood sampling, blood was centrifuged, the plasma removed, and the red cells re-infused.

2.3.3 Sample analysis

Plasma EPO concentrations were measured in triplicate using a double antibody radioimmunoassay (RIA) procedure as previously described (lower limit of quantification 1 mU/mL) (83). Only the mean of concentration of the triplicate plasma EPO concentration determinations was utilized for modeling fitting. All samples from the same animal were measured in the same assay to reduce variability. The reticulocyte count was determined by flow cytometry (FACScan, Becton-Dickinson, San Jose, CA, USA) as previously described (84). Hemoglobin concentrations were measured spectrophotometrically using an IL482 CO-oximeter (Instrumentation Laboratories, Watham, MA). In total, 30-45 samples were analyzed per variable per subject.

2.3.4 PD modeling details

The proposed PD model is schematically detailed in Figure 2.3. The pharmacokinetics of EPO (i.e. EPO plasma concentrations) were nonparametrically represented using a generalized cross validated cubic spline function, with nodes at every data point (85). The Hb and reticulocyte-plasma EPO concentration relationship was modeled by simultaneously fitting Eq. 2.38 through Eq. 2.40 and Eq. 2.43 through Eq. 2.50 to the modeled plasma EPO, observed blood Hb, and observed reticulocyte count concentration-time data of each subject. The lag time parameter, b_{RBC} , between progenitor cell stimulation and RBC removal from the systemic circulation was fixed at ' $a_{SS} + red\ cell\ (RBC +\ reticulocyte)\ lifespan$ '. The normal red cell lifespan has

previously been determined in sheep using [^{14}C] cyanate label and found to be 114 days (54). A fifth order constrained spline was used to semiparametrically estimate the circulating reticulocyte lifespan, $\mu_{RET}(t)$, as a function of time. The spline function was constrained to remain at the pre-phlebotomy steady-state value ($\mu_{RET,0} = \mu_{RET,SS}$) until some time (T_1) after the phlebotomy ($\mu_{RET}'(t) = 0$ for $t \leq T_1 < T_2$) and then return to a steady-state value ($\mu_{RET,END}$) at some later point (T_2 , $\mu_{RET}'(t) = 0$ for $t \geq T_2$), as detailed in Appendix A.5.

2.3.5 Computational details

All modeling was conducted using WINFUNFIT, a Windows (Microsoft) version evolved from the general nonlinear regression program FUNFIT (86), using weighted least squares. Data points were weighted by y_{obs}^{-2} , where y_{obs} is the observed Hb or reticulocyte concentration. The analysis required the numerical solution to delay differential equations (DDE) because of the lag times involved in the mathematical model. This was done by WINFUNFIT using a DDE solver module that is based on an adaptation of the code "RETARD" (87). The fraction of Hb and reticulocytes remaining, F , was estimated by WINFUNFIT using a generalized events processing module that allows parameterized events to change during the iterative parameter estimation procedure. Accordingly, the magnitude and time for the events and delays can be estimated simultaneously with other unknown parameters of the mathematical model. Akaike's Information Criterion (AIC) was used to compare the *time variant* model with 11 estimated parameters (4 cellular production parameters (E_{max} , EC_{50} , k_{bio} , and F) and 7 residence time parameters/coefficients (b_{RET} and the spline parameters/coefficients)) to

the identical *time invariant* model with only 6 estimated parameters (4 cellular production and 2 residence time parameters (a and b_{RET})) (88).

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

$$\text{MSE\%} = \frac{1}{n} \cdot \sum_{i=1}^n \frac{SE_i}{|P_i|} \cdot 100 \quad (2.51)$$

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

2.3.6 Statistical analysis

Mean reticulocyte lifespans in the systemic circulation were compared using paired two tailed Student’s t-test at selected points in time using Microsoft® Excel 2002 SP3 (Microsoft Corporation, Redmond, WA, USA). Statistically significant differences were determined at the $\alpha = 0.05$ type I error rate.

2.4 Results

The simultaneous fit to the EPO, reticulocyte, and Hb data for two representative subjects is shown in Figure 2.4. Good agreement between the observed and predicted values is evident from the fits (overall $R = 0.978 \pm 0.0084$ (mean \pm SD), Hb $R = 0.981 \pm 0.0203$, reticulocyte $R = 0.966 \pm 0.0114$). The PD model parameters are summarized in Table 2.1. The mean determined maximal rate of Hb production, E_{max} , was estimated to be 1.29 ± 0.471 g/dL/day. The mean lag time between progenitor stimulation and reticulocyte disappearance from the systemic circulation, b_{RET} , was estimated at 1.24 ± 0.167 days and the mean pre-phlebotomy steady-state circulating reticulocyte lifespan,

$\mu_{RET,0}$, was estimated to be 0.447 ± 0.100 days. Accordingly, the mean pre-phlebotomy steady-state delay between progenitor stimulation and reticulocyte (Hb) appearance in the systemic circulation, a_{SS} , was estimated at 0.797 ± 0.193 days.

The reticulocyte systemic circulation lifespan determined for each subject rapidly increased shortly after the phlebotomy, reaching a maximal value of 0.761-1.24 days at 5.95 ± 0.899 days post-phlebotomy (Figure 2.5). Figure 2.5 displays the circulating lifespan for cells *currently being stimulated*, not the lifespan of reticulocytes currently entering the bloodstream or circulating in the systemic blood. The time post-phlebotomy that the circulating lifespan began to deviate from steady-state conditions was estimated at 0.730 ± 0.834 days. The maximal circulating lifespan was 2.01-2.64 fold higher than the initial steady-state circulating lifespan ($p < 0.01$). Following establishment of the maximal circulating lifespan, the circulating lifespan rapidly dropped in all subjects below its pre-phlebotomy value, to a minimum value of 0.471-0.906 fold of the initial steady-state circulating lifespan ($p < 0.05$). It then remained at that value or increased to a new steady-state value. On average, the circulating lifespan returned to steady-state conditions 15.4 ± 2.36 days following the phlebotomy. In all but one subject, the ending steady-state reticulocyte circulating lifespan, $\mu_{RET,END}$, was near the initial value ($p > 0.25$, including and excluding the single subject with the ending circulating lifespan well above the initial value). The reason for this different behavior in the single subject is not clear, but it is evident from the subject's data (Figure 2.4, panel B) that the reticulocyte count was substantially elevated compared to the baseline count well past the phlebotomy, supporting that the circulating reticulocyte lifespan likely remained elevated.

The MSE% for the primary parameter estimates are also displayed in Table 2.1. The other circulating lifespan parameters (see Appendix A.5), T_1 and T_2 , had MSE% of 1.2% and 1.4%, respectively. The two unconstrained coefficients of the circulating lifespan fifth order spline function, α_4 and α_5 , had MSE% of 0.7% and $> 1000\%$, respectively. The high MSE% for α_5 was due to two subjects, as the other three subjects had a MSE% of 0.6%. Modeling the data instead with a fourth order circulating lifespan spline function still resulted in a good model fit to the data for the two subjects with the high SE for the α_5 coefficient, however, it resulted in an unacceptably poor fit to the data from the other three subjects. Therefore, a fifth order spline function was used to model the time variant circulating lifespan as the minimal model that resulted in an acceptable fit to the all the subject data.

In all subjects, the time *variant* circulating reticulocyte lifespan model was superior to the identical time *invariant* circulating reticulocyte lifespan model, where a single circulating lifespan was estimated, based on AIC. The difference in the fits between these two models for a representative subject is displayed in Figure 2.6.

2.5 Discussion

2.5.1 Cellular production

The determined steady-state delay between effect-site EPO stimulation and reticulocyte or Hb appearance in the systemic circulation, the a_{SS} value, of 0.797 ± 0.193 days is similar to the corresponding parameter we have determined in previous sheep phlebotomy studies of 0.72 days (76). The current model includes an effect site compartment, while the previous analysis did not. In contrast to the previous analysis (76), this analysis also includes Hb data in the estimation procedure. The determined a_{SS}

value (0.797 days or 19.1 hours) is approximately the same as a similar physiological parameter determined in cynomolgus monkeys of 14.95 hours and in humans of 10.76 hours (74, 75).

The determined C_p^{SS} value and $f_{stim}(t)$ function for each subject estimates a steady-state Hb production of 0.0929 ± 0.0472 g/kg/day, assuming a blood volume of 0.75 dL per kg bodyweight. Furthermore, the maximum production rate of Hb following the observed EPO stimulation (i.e. maximum C_{bio}) was estimated at 0.504 ± 0.0422 g/kg/day, indicating that endogenous EPO released under maximally stimulated acute anemic conditions can increase Hb (RBC) production approximately 5-fold. The determined 5-fold increase in Hb production following acute anemia in sheep is similar to the 3 to 5-fold increase reported in human patients (89).

2.5.2 Reticulocyte lifespan

As would be predicted from the current understanding in the scientific community of reticulocyte maturation under stress erythropoiesis, the circulating reticulocyte lifespan increased post-phlebotomy (Figure 2.5 and Figure 2.6, panel B). The mean pre-phlebotomy steady-state reticulocyte lifespan, $\mu_{RET,0}$, was estimated at 0.447 ± 0.100 days (10.7 hours) (Table 2.1). The estimated $\mu_{RET,0}$ is similar to other estimates in sheep of 0.61 days and the commonly reported value of 1 day in humans (36, 76, 90). From the steady-state value, the reticulocyte lifespan increased 2.01 to 2.64-fold during stress erythropoiesis, before dropping below pre-phlebotomy values. The determined maximally increased reticulocyte lifespan following a phlebotomy is approximately the same as that previously estimated *in vivo* in sheep and humans of 4.5 fold and 2-3 fold, respectively (76, 90) . It is also very close to the commonly recognized increase in

circulating reticulocyte lifespan in humans due to stress erythropoiesis of 2-3 days (39). The drop of the mean reticulocyte lifespan below the pre-phlebotomy steady-state value is not generally noted in the literature, and may be due to an overshoot/rebound phenomenon often observed in biological systems.

It is important to note that the determined circulating reticulocyte lifespan is not the true reticulocyte lifespan. The latter is not possible to determine accurately *in vivo* by only sampling in the systemic circulation. A reticulocyte initially normally exists in the bone marrow of sheep and humans (32, 36, 90), and therefore cannot be observed until it is released from the marrow into the systemic circulation. Accordingly, the determined drop below baseline circulating reticulocyte lifespan post-phlebotomy most likely represents the release of more mature reticulocytes from the bone marrow into the circulation through some rebound/overshoot phenomenon.

2.5.3 Model details

Utilizing a time variant reticulocyte lifespan model was superior to a time invariant reticulocyte lifespan model for all subjects based on the AIC (Figure 2.6). As can also be seen in Figure 2.6, an adequate simultaneous fit to both the reticulocyte and Hb data is not possible with a time invariant reticulocyte lifespan model. The current model is also an improvement over previous EPO, reticulocyte, and Hb integrated PK/PD models published by our laboratory in accounting for Hb production (72), since previous models did not include the Hb in reticulocytes as part of the total measured Hb. The Hb contained in the reticulocyte, as previously modeled, only added to the measured Hb upon maturation of the reticulocyte into a RBC. Furthermore, previous models used the percent reticulocytes instead of the absolute reticulocyte count, the former which is not as

easily interpreted physiologically. The current analysis also provides an exact correction for the effect of removing erythroid cells during the phlebotomy process.

Because the current model shared functions through the improved Hb accounting, a time variant circulating reticulocyte lifespan could be modeled, which is widely known to occur physiologically, but apparently has not been previously modeled. It may be physiologically relevant to include time variance in the b_{RET} parameter in addition to the a parameter. However, the time variant residence could not be modeled as such because a variable b_{RET} value in addition to a variable a value would not be possible to determine in a reliable way. Since reticulocytes are released from the bone marrow at a younger age following stress erythropoiesis, modeling the time variance due to changes in the a value accounts for the primary physiological features of reticulocytes produced under stress erythropoiesis. The ability to estimate a time variant a value is primarily due to the simultaneous fitting to the “coupled” Hb and reticulocyte responses. Two key features of the “coupled” responses are the longer residence time or lifespan in the systemic circulation of Hb compared to reticulocytes and the larger pool of Hb relative to reticulocytes. Accordingly, a substantial change in the a value, and thus the Hb and reticulocyte input, has a relatively large and rapid effect on the observed reticulocyte response, but only a relatively minor effect on the observed Hb response. Alternatively, a model with a time *invariant* a parameter but with a time *variant* b parameter could be formulated and fitted to the data to estimate the reticulocyte lifespan in the systemic circulation. However, a model formulated as such would not acknowledge the primary features of erythropoiesis physiology. The model also determined a time variant Hb lifespan in the systemic circulation. However, we only report and discuss the results of

the model in terms of the change in the circulating reticulocyte lifespan, μ_{RET} , and the observed reticulocyte response due to the small magnitude of the percent change in the Hb lifespan (<1%), the fixing of the b_{RBC} parameter, and the limited observation time relative to the lifespan of Hb.

Another important model assumption is the fixed b_{RBC} value of ‘ $a_{SS} + red\ cell\ lifespan$ ’ for all cells, since it has been experimentally demonstrated in mice and rats that red cells produced under stress erythropoietic conditions have a shortened lifespan or residence time in the systemic circulation (55, 56). However, the lifespan of the red cells would need to be reduced by greater than 70% (i.e. to less than 30 days) to have an effect, since this was the longest period of time of observation post-phlebotomy. A final simplifying assumption is that a single, calculated Hb to reticulocyte count conversion constant, K_{RET} , was used for all subjects. The K_{RET} parameter acts only as a scalar though, so any changes in the magnitude of the reticulocyte lifespan would still be observed even if the value for K_{RET} varied from subject-to-subject. Additionally, a constant K_{RET} would be inappropriate if the amount of Hb per erythroid cell varied with time, as may occur if subjects became iron deficient. However, monitoring of iron status did not indicate that any subjects became iron deficient throughout the time course of the experiments.

The model, as currently described, does not account for a distribution(s) of cell lifespans, since it was assumed that all cells stimulated at a given time have the same “pre-determined” lifespan. However, previous work has demonstrated little advantage in moving to a more complicated lifespan distribution model compared to a point distribution model due to problems estimating the standard deviation of the cell lifespans

(6). The simultaneous determination of a cellular distribution(s) and changes in the individual a and/or b values would be mutually confounding and could create tremendous estimation problems.

The present analysis did not indicate that $a'(t)$ at any time fell at or below a value of -1 (nor did $\mu_{RET}'(t)$ fall at or above a corresponding value of $+1$). Thus, the problem associated with accounting for multiple solutions, possible according to Eq. 2.22, fortunately did not arise in this analysis (see Figure 2.2, showing multiple solutions when $a'(t)$ becomes < -1). Physiologically, what occurs when multiple solutions to Eq. 2.22 arise is that cells that were stimulated at a later point in time are entering the sampling compartment prior to cells that were stimulated earlier (i.e. “younger” cells are entering prior to “older” cells), resulting in multiple groups (solutions) of cells that were stimulated at different times entering the systemic circulation at exactly the same time. Additionally, mathematically Eq. 2.30 becomes undefined when $a'(t) = -1$, because there is an infinite number of solutions along the finite range where $a'(t)$ continuously equals -1 . If the analysis had indicated that $a'(t)$ at any time fell at or below -1 , we would have had to resort to much more complex numerical solutions to the already complex delay-type differential equations describing the proposed model.

The time variant cellular disposition model presented in the theoretical section is applicable to modeling any cellular response where changes in cellular lifespan is expected and may be extended to incorporate unique features of the specific physiological system being modeled. Furthermore, the specific EPO, reticulocyte, and Hb PK/PD model that was presented could readily be applied to humans, both adult and

neonate, and other species. Future modeling work and more “complete” data may also allow for the relaxation of certain model assumptions (i.e. a point distribution of cell lifespans), developing an even more general PD model of cellular responses.

2.6 Conclusion

In summary, by sharing parameters and functions between the measured Hb and reticulocyte count response, the reticulocyte lifespan in the systemic circulation was accurately determined as it changed with time. The utilized methodology allows for the dynamic determination of time variant cellular disposition or lifespan without directly measuring the cellular lifespan. The determined increase in circulating reticulocyte lifespan from reticulocytes produced under stress erythropoiesis and the determined increase in Hb production is consistent with other experimental data and with the scientific community’s current understanding of erythropoiesis physiology. Furthermore, the determined reduction below baseline lifespan time post-phlebotomy may serve to generate future experiments for determining why or how physiologically the reduction in circulating reticulocyte lifespans occurs *in vivo* in sheep, and ultimately in humans.

Table 2.1. Parameter estimates from the time-variant reticulocyte lifespan model ($n = 5$).

Cellular Production Parameters						
	E_{max} (g/dL/day)	EC_{50} (mU/mL)	k_{bio} (1/day)	$I-F$		
Mean	1.29	198	0.367	0.610		
SD	0.471	83.0	0.112	0.0860		
MSE%	5.2%	5.3%	12.5%	12.5%		
Lifespan Parameters						
	a_{SS}^* (day)	b_{RET} (day)	$\mu_{RET,0}$ (day)	$\mu_{RET,END}$ (day)	$\mu_{RET,MAX}^{**}$ (day)	$\mu_{RET,MIN}^{**}$ (day)
Mean	0.797	1.24	0.447	0.551†	0.996‡	0.306‡
SD	0.193	0.167	0.100	0.319	0.181	0.0990
MSE%	NA	2.8%	4.1%	4.5%	NA	NA

SD: Standard deviation

MSE%: Mean percent standard error

*Secondary parameter

**Calculated value: the maximum or minimum determined $\mu_{RET}(t)$ from time T_1 to T_2 .

† Not significantly different from $\mu_{RET,0}$ ($p > 0.05$)

‡ Significantly different from $\mu_{RET,0}$ ($p < 0.05$)

NA: Not applicable

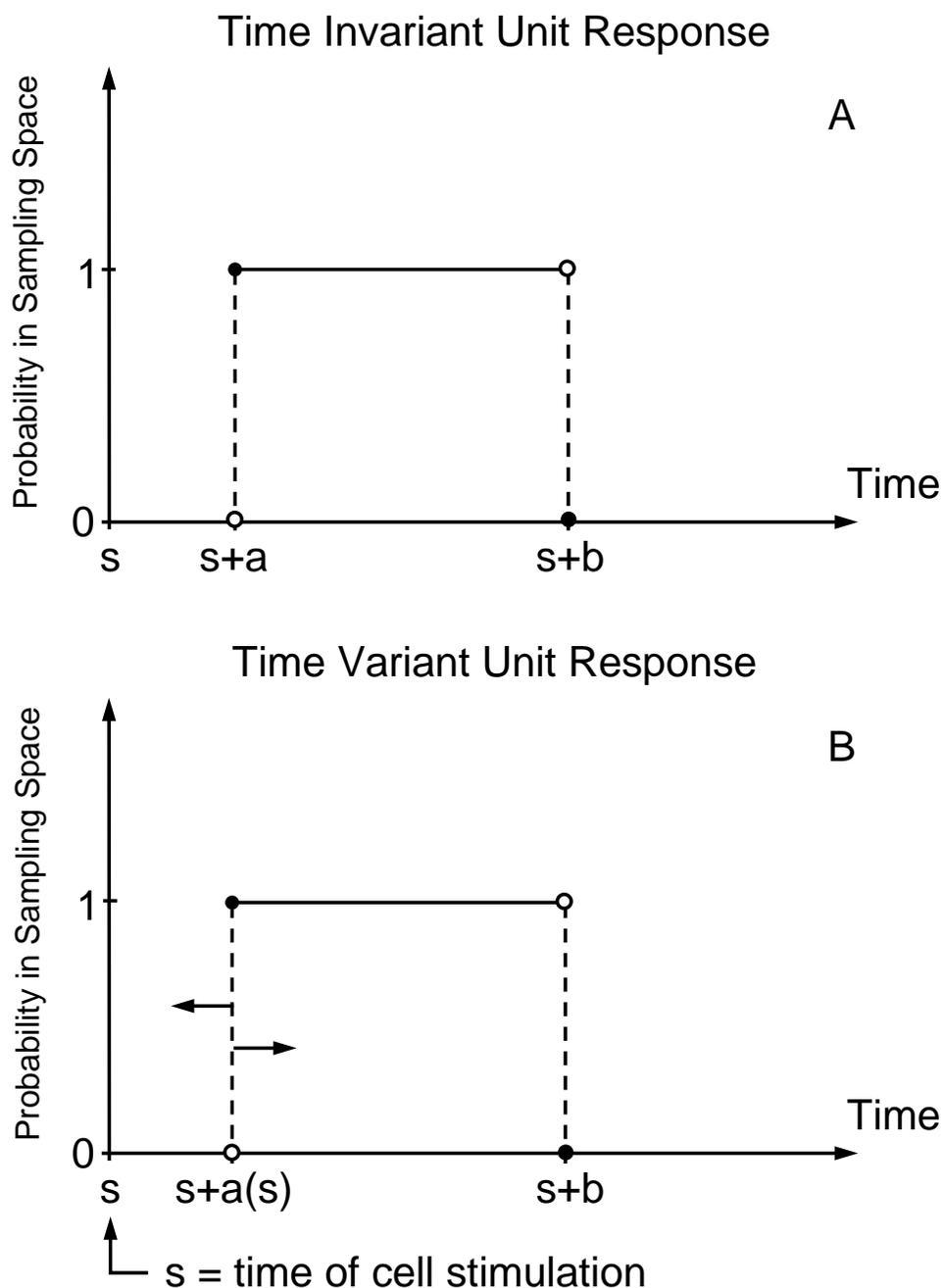


Figure 2.1. Unit response characterizing the disposition function of a single cell.

The cell is present in the systemic circulation when $UR(t)=1$. Thus, a and b represent the disposition function parameters for the appearance and disappearance of the cell from the sampling compartment, respectively, and s is the time of precursor cell stimulation. Time variance of the UR is considered (lower Panel B) by assuming the a value to be variable and determined at the time ($t = s$) of the precursor cell stimulation.

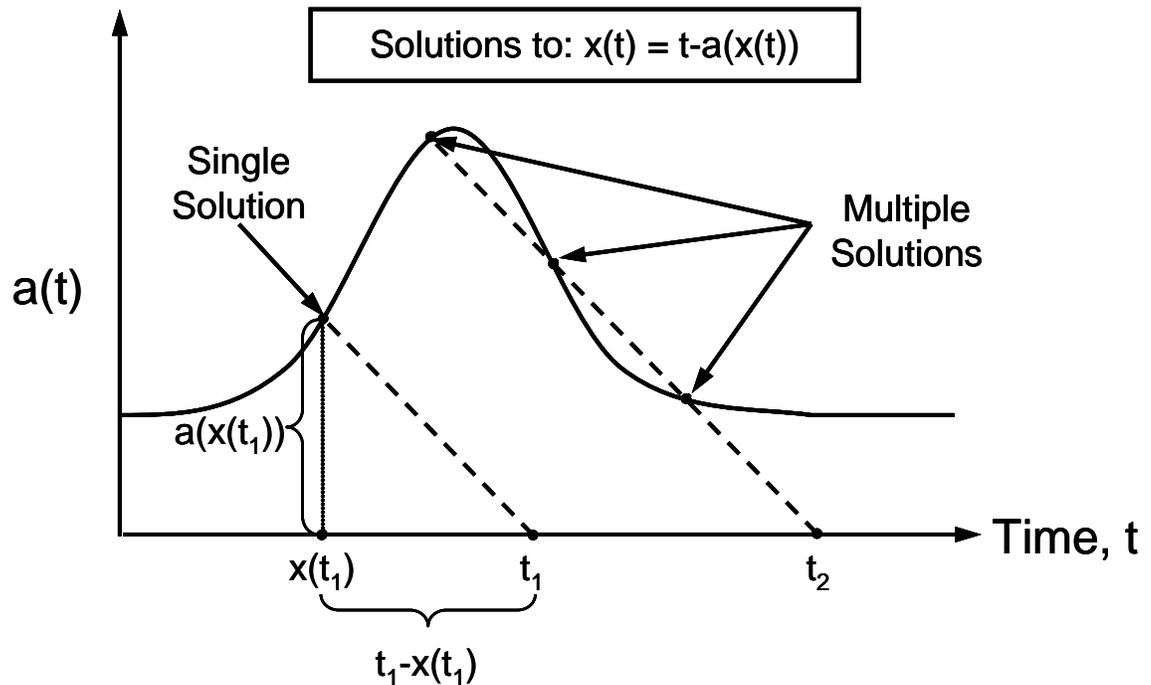


Figure 2.2. Illustration of the relationship between ' t ', ' $x(t)$ ', ' $a(t)$ ' and the condition for multiple solutions to Eq. 2.22.

The scale on the ' $a(t)$ ' and the time axes are the same. The slope of the dashed lines are -1 . At time ' t_1 ', the ' a ' value of cells currently entering the observed compartment is ' $a(x(t_1))$ ', which is equal to ' $t_1 - x(t_1)$ '. Therefore, ' $x(t)$ ' is the time currently entering cells were stimulated at, from which the appropriate ' a ' value for these entering cells can be determined. At time ' t_2 ', three ' $a(x(t))$ ' and ' $x(t)$ ' exactly satisfy the boxed equation (Eq. 2.22), resulting in multiple solutions. The multiple solutions occur when $a'(t)$ becomes ≤ -1 . Physiologically, at time ' t_2 ' cells stimulated at three different points in time are entering the sampling compartment at exactly the same time.

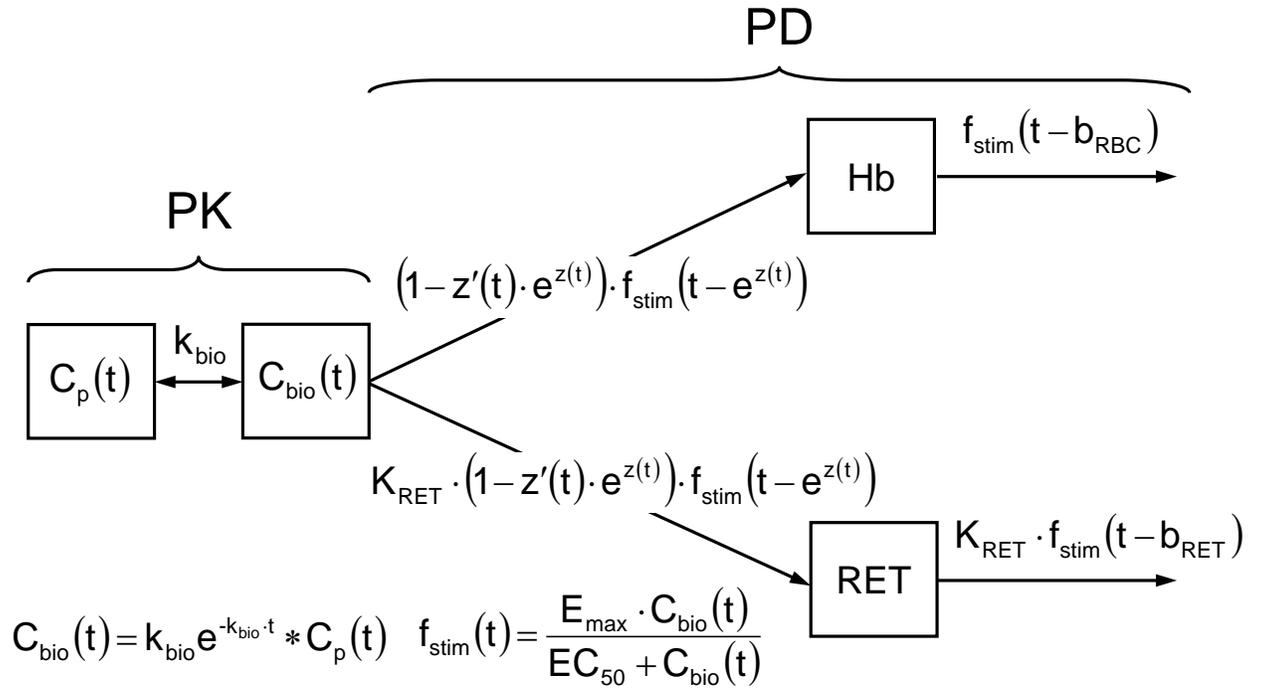


Figure 2.3. PD model schematic of the time-variant reticulocyte lifespan model.
Symbols are defined in the Glossary.

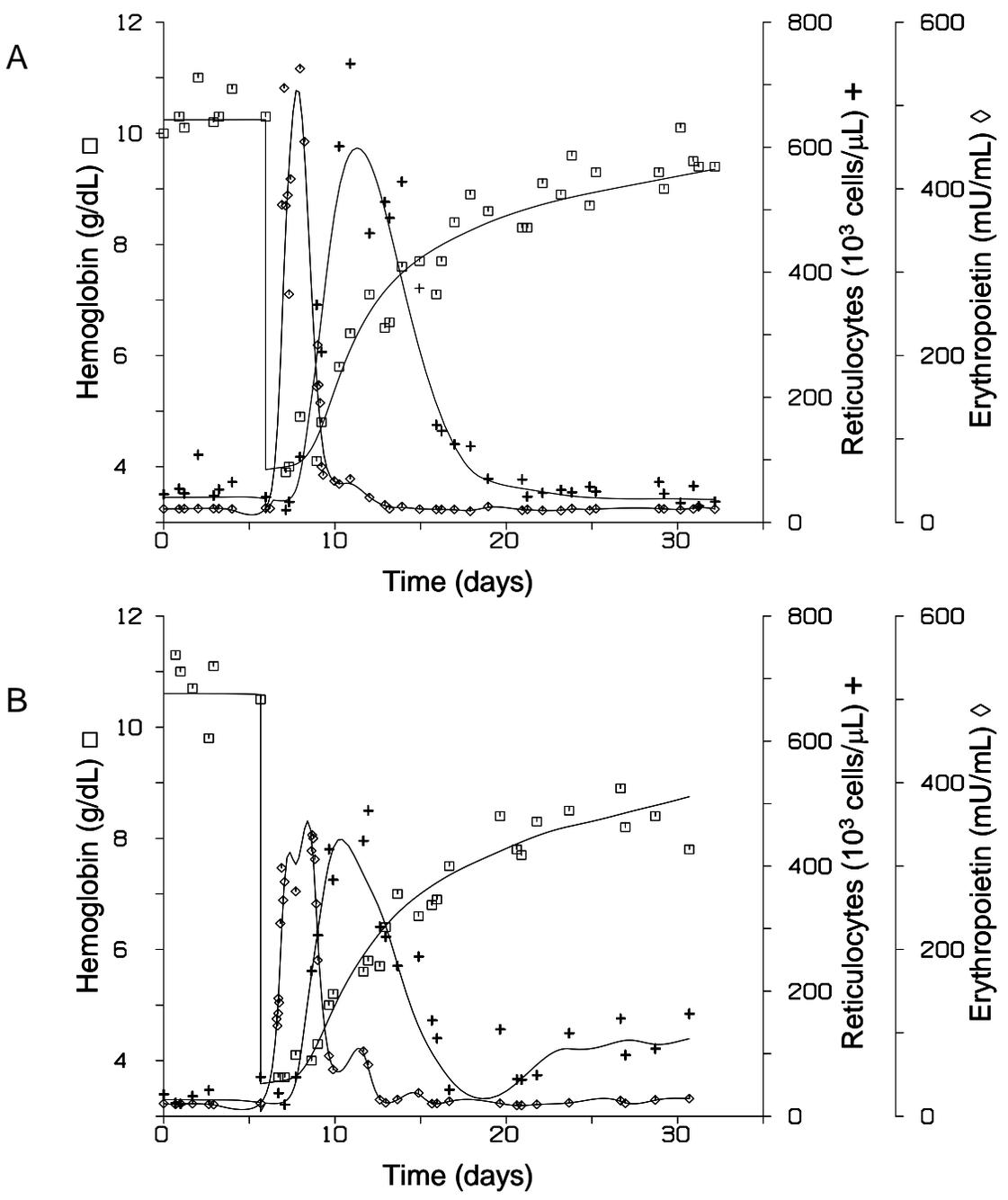


Figure 2.4. Representative model fits (lines) to individual subject data (symbols). Observed plasma erythropoietin (\diamond), hemoglobin (\square), and reticulocyte (+) count concentrations are displayed. Panels A and B are different subjects.

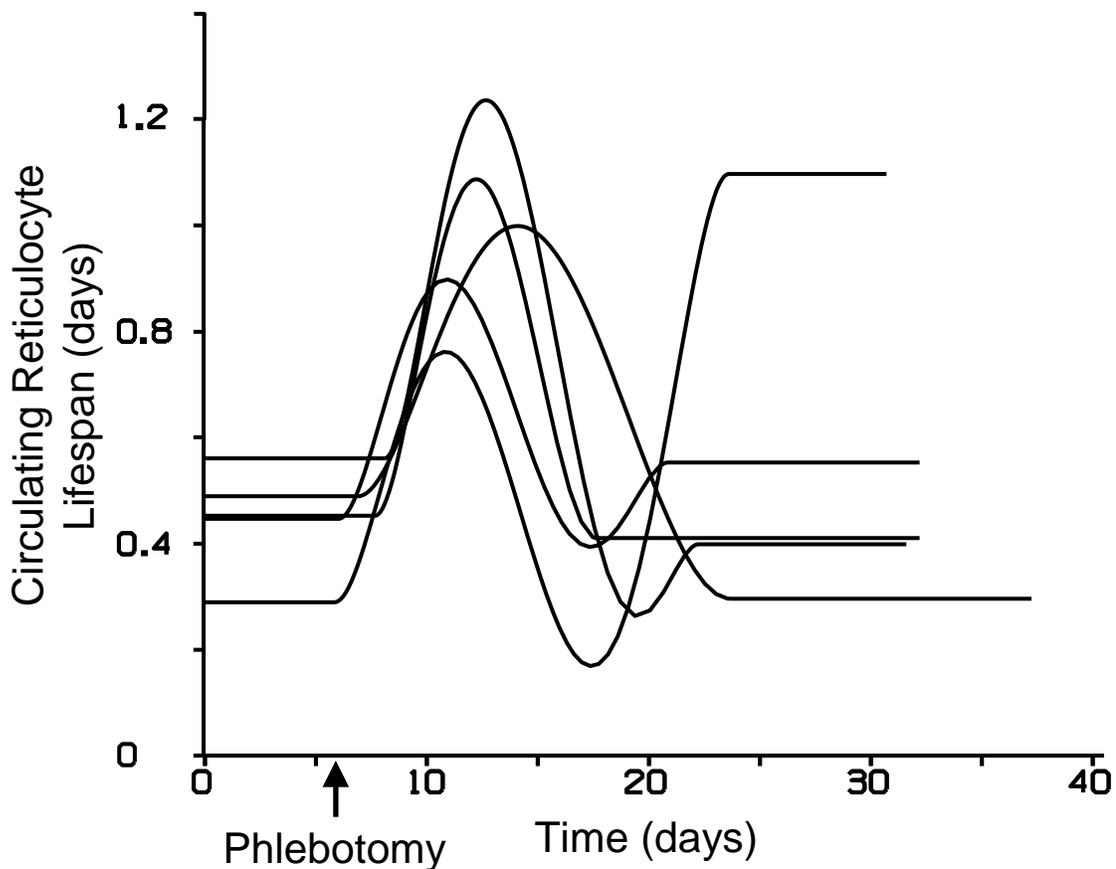


Figure 2.5. Determined circulating reticulocyte lifespans for the individual subject model fits ($n = 5$).

The circulating reticulocyte lifespans were represented using a semiparametric spline function and are displayed as the lifespans, $\mu_{RET}(t)$, of cells currently being stimulated.

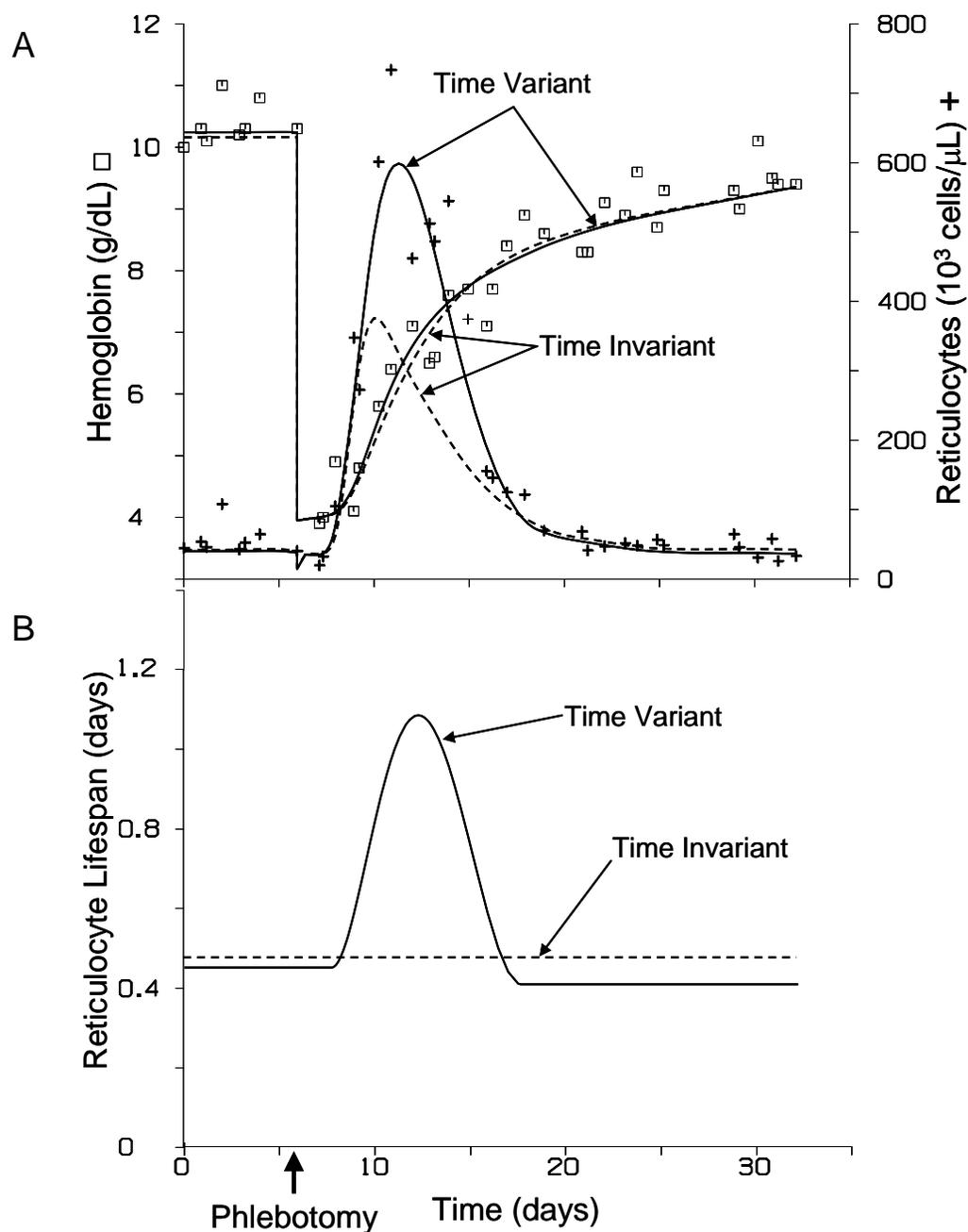


Figure 2.6. Representative model fit to subject data fit using a time *variant* or time *invariant* circulating reticulocyte lifespan model. The observed hemoglobin (\square) and reticulocyte (+) count concentrations along with the model fits (lines) are displayed in Panel A. The solid lines (—) are the predicted values with the time *variant* model and the dashed lines (- -) are the predicted values with the time *invariant* model. Panel B displays the determined reticulocyte lifespans, $\mu_{RET}(t)$, of cells currently being stimulated for the representative subject using the time *variant* (—) or time *invariant* (- -) model.

**CHAPTER 3. MODELING TIME VARIANT DISTRIBUTIONS OF CELLULAR
LIFESPANS: INCREASES IN CIRCULATING RETICULOCYTE LIFESPANS
FOLLOWING DOUBLE PHLEBOTOMIES IN SHEEP**

3.1 Introduction

Determination of the lifespan distributions of cells has been an interest to researchers for many years. Some of the first work determined the lifespans of red blood cells from cell survival curves (1-3). However, much of the early work assumes constant production rates and distributions of cellular lifespans. With the development of many new drugs that affect important cell populations, such as cancerous, erythrocyte, leukocyte, platelet, and bacterial cell populations, the study of the effect of these new drugs on both the production and destruction is an important consideration for optimal dosing. For cell death mechanisms that are related to the age of the cell, i.e. time since production, the lifespan distributions and age structure of the population are vitally important for understanding the effect of the therapeutic agent.

With respect to red blood cells (RBC), under non-disease state conditions the mechanism of cell death is primarily to due to cellular senescence (i.e. the expiration of the cellular lifespan) (52). The two primary RBC types in the systemic circulation are reticulocytes and mature erythrocytes, the former which is just an immature RBC. Reticulocytes are produced from erythroid progenitor cells located primarily in the bone marrow, where they initially reside and subsequently are released from into the systemic circulation (32). The maturation of erythroid progenitor cells into reticulocytes and ultimately RBCs is primarily controlled by erythropoietin (EPO), a 30.4 kD glycoprotein hormone produced by the peritubular cells of the kidney in response to oxygen need (12,

14). During the development from erythroid progenitor cells their hemoglobin content increases until it develops into a reticulocyte upon nucleus extrusion, where further maturation primarily involves the removal of ribosomal ribonucleic acid (RNA), remodeling of the plasma membrane, and a progressive decrease in cell size (12, 36, 37).

In humans the majority of the erythrocytes released from the bone marrow into the systemic circulation are reticulocytes, while in other species such as ruminants and horses, under basal erythropoietic conditions (i.e. non-anemic or non-erythropoietically stimulated) the majority of the erythrocytes are released as mature RBCs (36, 37, 43). In general, under basal erythropoietic conditions in humans, reticulocytes have a lifespan in the systemic circulation of approximately 24 hours before developing into mature RBCs. However, during stress erythropoiesis (i.e. stimulated erythropoietic conditions), the reticulocyte lifespan in the circulation increases to an estimated 2-3 days (39). In humans, the reticulocytes produced under stress erythropoiesis also contain more residual ribosomal RNA, are larger, and have less flexible plasma membranes than those produced under normal basal conditions, and therefore are thought to be immature reticulocytes that under “normal” physiological conditions reside in the bone marrow until being released as more mature reticulocytes (37, 39, 44, 45). Similarly in animals such as ruminants with a low basal percentage of erythrocytes released as reticulocytes, the percentage of reticulocytes increases dramatically during stress erythropoiesis (43, 46). Therefore like humans, younger erythrocytes are also released following stress erythropoiesis in these species. Accordingly, the reticulocyte counts increase under stress erythropoiesis not only due to increased reticulocyte production in response to EPO stimulation, but also due to a longer lifespan in the systemic circulation.

One of the most common techniques for modeling the pharmacokinetic/pharmacodynamic (PK/PD) relationship between therapeutic agents, such as EPO, and the cellular populations is the compartmental or cellular “pool” model, in which cells are transferred between compartments by first-order processes (71). A major limitation of this model is that it completely ignores the age structure of cells within a compartment, treating all cells within the compartment as equally likely to be transferred out of the compartment. For cells like reticulocytes and mature erythrocytes whose “removal” from the sampling compartment is primarily determined by a developmental processes (i.e. transformation into a mature RBC) and cellular senescence, respectively, more physiologically realistic models incorporate a cellular lifespan component (5, 11, 35, 53, 72-75). However, nearly all of these PK/PD models assumed a single “point distribution” of cellular lifespans shared by all cells that does not vary over time (i.e. time invariant). More recently, models have been introduced that account for a *time invariant* distribution of cell lifespans (6) and *time variant* “point distribution” of cellular lifespans (91).

To date, a PD cellular response model that incorporates a *time variant* distribution of cellular lifespans has not been presented and successfully fitted to data. Additionally, a time variant distribution of reticulocyte lifespans has not been described following induction of stress erythropoiesis conditions, nor have estimates of changes in the proportion of erythrocytes released as reticulocytes and mature RBCs been previously obtained. Therefore, the objectives of the current work were: 1.) to present a general PD model that incorporates a time variant distribution of cellular lifespans, 2.) to successfully fit the presented model to erythrocyte data following stress erythropoiesis to estimate the changes in the circulating reticulocyte lifespan and proportion of erythrocytes released

into the systemic circulation as reticulocytes, and 3.) to compare the presented model to other cellular lifespan models.

3.2 Theoretical

3.2.1 *Time variant cellular disposition*

Let $\ell(\tau, z)$ denote the *time variant* probability density function (p.d.f.) of cellular lifespans, where τ is the cellular lifespan and z is an arbitrary time of production (Figure 3.1, Panel A). More specifically, the cellular lifespan is defined for a particular cell type of interest as the time from input into the sampling space to the time of output from the sampling space, which may be due to: cellular death/senescence, transformation into a different cell type, and/or irreversible removal from the sampling space. Therefore, the lifespan of a cell is determined by the definition of both the cell type and the sampling space. Under the above definition of cellular lifespan it could also be described as the residence time in the sampling space as the cell type of interest. Additionally, cellular production is defined as the physical input of cells into the sampling space. Let it be assumed that at the time of production each cell is assigned a unique lifespan which is not further affected by subsequent environmental conditions following production. Therefore, the z variable references the lifespan distribution to the particular time of production. Since it is assumed that after production the lifespan of the cells is not affected by the environment, the cells act independent of each other following entry into the sampling space and therefore have a linear cellular disposition (91). Due to the above properties, each cell is assigned an individual probability of survival after production that may vary with the time of production. The probability of cellular survival to a particular time t

after production is given by the time variant unit response (UR_ℓ) function (91), which can also be viewed as a survival function from failure time data analysis (92). Accordingly:

$$UR_\ell(t, z) = P(T > t - z, z) = \int_{t-z}^{\infty} \ell(\tau, z) d\tau = 1 - \int_0^{t-z} \ell(\tau, z) d\tau, \quad t \geq z \quad (3.1)$$

where t is the current time, T denotes a random cellular lifespan variable, and $P(T > t - z, z)$ denotes the probability that $T > t - z$ for cells produced at time z . Therefore the UR_ℓ is the cellular disposition, as illustrated in Figure 3.1 (Panel B). Let $f_{prod}(t)$ denote the production (i.e. input) rate of cells into the sampling space, which is typically a function of time through endogenous growth factors and/or exogenous drug, and let Δz denote a small time increment. Then the number of cells currently present in the sampling space at time t that were produced at a previous time z is given by the product of the number of cells produced at time z and the probability that these cells have survived to time t (i.e. $UR_\ell(t, z)$):

$$f_{prod}(z) \cdot \Delta z \cdot UR_\ell(t, z) \quad (3.2)$$

Summation of Eq. 3.2 by integration across all time prior to t followed by substitution of Eq. 3.1 into the resulting equation gives the *general key equation* for the total number of cells in the current sampling space population when modeling a time variant cellular lifespan distribution:

$$\begin{aligned} N(t) &= \lim_{\Delta z \rightarrow 0} \sum_{Z: z \leq t} f_{prod}(z) \cdot \Delta z \cdot UR_\ell(t, z) = \int_{-\infty}^t f_{prod}(u) \cdot UR_\ell(t, u) du \\ &= \int_{-\infty}^t f_{prod}(u) \cdot \left[1 - \left[\int_0^{t-u} \ell(\tau, u) d\tau \right] \right] du \end{aligned} \quad (3.3)$$

As can be observed from Eq. 3.3, the number of cells in the sampling space is given by an integral of the product of the number of cells produced at a previous time and the

probability that the cells produced at that previous time are present in the sampling space at the current time. The lower integration limit of $-\infty$ in Eq. 3.3 is to be interpreted to consider “all prior history” of the system that affects $N(t)$. Due to the finite lifespan of cells, in reality the lower limit may be explicitly stated as t minus the maximal cellular lifespan, if known. Differentiation of Eq. 3.3 results in:

$$\frac{dN}{dt} = f_{prod}(t) - \int_{-\infty}^t f_{prod}(u) \cdot \ell(t-u, u) du \quad (3.4)$$

as previously presented (11). Thus the input rate into sampling space at time t is given by $f_{prod}(t)$ and the output rate from the sampling space is given by $\int_{-\infty}^t f_{prod}(u) \cdot \ell(t-u, u) du$.

3.2.2 Accounting for subject growth

Measurement of cells *in vivo* is often done in terms of concentrations, $C(t)$, such as number of reticulocytes per volume of blood, therefore, Eq. 3.3 is divided by the total sampling space volume, $V(t)$, resulting in:

$$C(t) = \frac{N(t)}{V(t)} \quad (3.5)$$

If the subject is mature or the observation time window/cell lifespans are short relative to the rate of change in the sampling space volume, then $V(t)$ can reasonably be assumed to be constant, simplifying Eq. 3.5. However, if the subject is growing, and therefore the sampling space volume is changing with time, and the time window and/or cell lifespan is relatively long, the dilution of the cellular concentration due to volume expansion must be considered.

3.2.3 Corrections for cell removal

To improve the analysis of the cell population it is necessary to correct for the effect of external removal of cells, such as a phlebotomy. Let F denote the fraction of cells remaining immediately following a phlebotomy conducted at time T_p , then to correct for a phlebotomy when $t \geq T_p$ Eq. 3.3 simply becomes (see Appendix B.1):

$$N(t) = F \cdot \int_{-\infty}^{T_p} f_{prod}(u) \cdot \left[1 - \left[\int_0^{t-u} \ell(\tau, u) d\tau \right] \right] du + \int_{T_p}^t f_{prod}(u) \cdot \left[1 - \left[\int_0^{t-u} \ell(\tau, u) d\tau \right] \right] du, \quad t \geq T_p \quad (3.6)$$

3.2.4 Calculation of the sampling space volume

By the external removal from or addition of cells to the sampling space, the volume of the sampling space can be estimated under the assumption that the total sampling space volume remains constant. Following an acute phlebotomy (i.e. removal of blood cells) the original blood volume is reestablished within 24-48 hours if no plasma volume expanders are administered (37, 93), therefore the assumption of a constant blood volume (i.e. the sampling space) is reasonable when dealing with populations of blood cells and the 24-48 hour lag-time for reestablishment of the blood volume is considered. The original blood volume will be reestablished even more rapidly if plasma or other blood volume expander is administered, due to increased osmotic pressure in the vascular system. In case of an acute removal or addition of cells, the sampling space volume is given by:

$$V(T_p) = \frac{N_p}{\Delta C} \quad (3.7)$$

where N_p denotes the number of cells removed or added and ΔC denotes the magnitude of the change in the cell concentration due to the phlebotomy or transfusion, respectively.

In the case of a transfusion, the additional assumption must also be made that a substantial fraction of the transfused cells are not rapidly removed from the sampling space, such as splenic removal of transfused RBCs from the systemic circulation due to damage that occurred during the storage or transfusion process.

3.2.5 Alternative parameterization of a time variance

For many populations of cells there is a time delay between the activation or stimulation of cells and the physical input of the stimulated cells into the sampling space. An example is stimulation of erythroid precursor cells in the bone marrow (i.e. outside the sampling space) and the subsequent release of the stimulated cells into the systemic circulation (i.e. the sampling space) as either reticulocytes or mature RBCs. In this instance a time variant cellular lifespan can be alternatively parameterized as follows. Let the time of cellular stimulation be denoted by s and let ω denote the time delay from cellular stimulation to appearance or release of the subsequently stimulated cell(s). Then a time variant cellular disposition can be accounted for by assuming the time delay of the release of a cell to be a random variable and the time from stimulation of the cell to the time of output from the sampling space to be a fixed period of time, denoted b . As before, the output from the sampling space may be due to cellular death/senescence, transformation into a different cell type, and/or irreversible removal. Let $r(\omega, s)$ denote a time variant p.d.f. of release time delays into the sampling space (Figure 3.1, Panel C). Then the probability that a cell is present in the sampling space as the cell type of interest is given by the intersection of the events that the time since stimulation is less than b and that the cell has been released into the sampling space. In this instance let it also be assumed that the probability of these two events are independent of each other and that at

the time of stimulation each cell is assigned a unique release time delay which is not further affected by subsequent environmental conditions following stimulation; thus the cells act independent of each other following stimulation and have a linear cellular disposition. Therefore, the probability that a cell resulting from progenitor cell stimulation at time s is present in the sampling space at time t can be defined in terms of a unit response of the cell type of interest, denoted UR_r (Appendix B.2), and is given by:

$$UR_r(t, s) = 1\{t - s < b\} \cdot P(\Omega \leq t - s, s) = [1 - U(t - s - b)] \cdot \int_0^{t-s} r(\omega, s) d\omega, \quad t \geq s \quad (3.8)$$

where $1\{X\}$ is the indicator function which is equal to 1 if X is true and 0 otherwise, Ω is a random release time delay variable, and U is the unit step function described by:

$$U(t) = \begin{cases} 1 & \text{if } t \geq 0 \\ 0 & \text{otherwise} \end{cases} \quad (3.9)$$

It can be observed from Eq. 3.8 and Figure 3.1 (Panel D), if $\omega \geq b$ then the UR_r has a value of 0, as logically expected. If not all the cells stimulated at time s have been released yet by time b , then an UR_r value of 0 can be interpreted as a fraction of the cells died prior to release into the sampling space and/or a fraction of the cells transformed into a different cell type prior to release.

Let $f_{stim}(t)$ denote the stimulation rate of cells, then similar to Eq. 3.3 the number of cells in the population is given by integration across all prior time of the product of $f_{stim}(t)$ and Eq. 3.8:

$$\begin{aligned}
N(t) &= \int_{-\infty}^t f_{stim}(u) \cdot \left[[1 - U(t - u - b)] \cdot \int_0^{t-u} r(\omega, u) d\omega \right] du \\
&= \int_{t-b}^t f_{stim}(u) \cdot \left[\int_0^{t-u} r(\omega, u) d\omega \right] du
\end{aligned} \tag{3.10}$$

which is the analogous equation to Eq. 3.3. The unit step function is eliminated in the simplification step of Eq. 3.10 since it is recognized that the integrand will have a value of 0 at all times when $u < t - b$. Similar to Eq. 3.3 and Eq. 3.6, a correction for a phlebotomy is needed for Eq. 3.10 when the time interval from $t - b$ to t contains T_p (i.e. contains a phlebotomy). During this time interval the equation for $N(t)$ (Appendix B.3) then becomes:

$$\begin{aligned}
N(t) &= \int_{t-b}^t f_{stim}(u) \cdot \left[\int_0^{t-u} r(\omega, u) d\omega \right] du - [1 - F] \cdot \int_{t-b}^{T_p} f_{stim}(u) \cdot \left[\int_0^{T_p-u} r(\omega, u) d\omega \right] du, \\
& \qquad \qquad \qquad t - b < T_p \leq t
\end{aligned} \tag{3.11}$$

The p.d.f.'s $\ell(\tau, \cdot)$ and $r(\omega, s)$ are related by the expression:

$$\ell(\tau, s) = \begin{cases} \frac{r(b - \tau, s)}{\int_0^b r(\omega, s) d\omega} & \text{if } 0 \leq \tau < b \\ 0 & \text{otherwise} \end{cases} \tag{3.12}$$

as derived in Appendix B.4. The p.d.f. $\ell(\tau, \cdot)$ is now indexed by s instead of z , as the time of stimulation is when the unit response was defined for a cell. As can be observed from Eq. 3.12, a time variant cellular lifespan is still being modeled by considering the release time delay from stimulation to release to be a random variable with a fixed time period between stimulation and output from the sampling space of the subsequently released cell.

3.3 Materials and methods

3.3.1 Animals

All animal care and experimental procedures were approved by the University of Iowa Institutional Animal Care and Use Committee. Four healthy young adult sheep approximately 4 months old and weighing 23.5 ± 1.14 kg (mean \pm SD) at the beginning of the experiment were utilized. Animals were housed in an indoor, light- and temperature-controlled environment, with *ad lib* access to feed and water. Prior to study initiation, jugular venous catheters were aseptically placed under pentobarbital anesthesia. Intravenous ampicillin (1 g) was administered daily for 3 days following catheter placement.

3.3.2 Study protocol

Blood samples (~0.5 mL/sample) for plasma EPO, reticulocyte counts, and RBC determination were collected for 5–12 days to determine baseline values prior to conducting the first of two controlled phlebotomies over several hours to induce acute anemia. The second phlebotomy was conducted 8 days later. For each phlebotomy, animals were phlebotomized to hemoglobin concentrations of 3-5 g/dL. To maintain a constant blood volume during the procedure, the plasma removed during the phlebotomy was collected and infused back into the animal. Additionally, a volume 0.9% NaCl solution was infused so that a 1-to-1 total volume of fluid exchange was conducted during each phlebotomy. The total number of RBCs removed at each phlebotomy was determined by assaying a sample of the removed volume. Blood samples were collected 1-4 times daily between the phlebotomies and for 15-42 days following the second phlebotomy. Animal weights were also recorded upon study initiation and 1-2 times

weekly throughout the course of the experiments. No iron supplementation other than that in the animal's feed was given. To minimize erythrocyte loss due to frequent blood sampling, blood was centrifuged, the plasma for EPO determination removed, and the unused red cells re-infused.

3.3.3 Sample analysis

Plasma EPO concentrations were measured in triplicate using a double antibody radioimmunoassay (RIA) procedure as previously described (lower limit of quantification 1mU/mL) (83). Only the mean of concentration of the triplicate plasma EPO concentration determinations was utilized for modeling fitting. All samples from the same animal were measured in the same assay to reduce variability. The reticulocyte and RBC counts were determined using the ADIVIA® 120 Hematology System (Bayer Corp., Tarrytown, NY), a flow cytometry instrument which counts RBCs by electrical impedance and determines reticulocyte percentage based on forward light scatter and fluorescence of a nucleic acid stain. In total, for each subject approximately 40–50 samples were analyzed for erythrocyte counts and 50-100 samples were analyzed for plasma EPO determination.

3.3.4 Specific model formulation

Erythropoietin was considered to be the stimulator of the erythroid cell precursors. The stimulation rate was related to the plasma EPO concentration (C_p) with time using a systems analysis approach that focuses on their overall functional relationship (94). A biophase conduction function and a Hill equation transduction function were utilized, specifically, the biophase concentration (C_{bio}) was determined by:

$$C_{bio}(t) = k_{bio} \cdot \exp(-k_{bio} \cdot t) * C_p(t) \quad (3.13)$$

where ‘*’ denotes the convolution operator and k_{bio} is the biophase conduction function parameter. For $t \leq t_0$, C_p was set to the initial (first observation) fitted plasma EPO concentration (i.e. steady-state plasma EPO concentration assumption). The EPO plasma concentrations were nonparametrically represented using a generalized cross validated cubic spline function (85), and the convolution of the fitted cubic spline with the conduction function given in Eq. 3.13 was analytically determined. The stimulation rate was subsequently related to C_{bio} by the transduction function given by:

$$f_{stim}(t) = \frac{E_{max} \cdot C_{bio}(t)}{EC_{50} + C_{bio}(t)} \cdot m(t) \quad (3.14)$$

where f_{stim} is the redefined stimulation function from Eq. 3.10 that depends on $C_{bio}(t)$ and the animal mass, $m(t)$, E_{max} is the maximal erythrocyte (RBC) stimulation rate in cells/kg/day, and EC_{50} is the biophase EPO concentration that results in 50% of maximal erythrocyte stimulation rate (E_{max}). The mass of the animal was incorporated into the model to account for subject growth prior to and during the experiment, as it is likely that the total mass of erythrocytes produced (stimulated) would increase with growth as the erythropoietic progenitor cell mass increases. As with the stimulation rate, proportionality between blood volume and animal mass was assumed to account for growth induced blood volume expansion during the experiment, as given by:

$$V(t) = V_n \cdot m(t) \quad (3.15)$$

where V_n is the mass normalized constant total blood volume. Hence the modeled sampling space is defined as the total blood volume of the systemic circulation. Due to

the relatively young age and rapid growth of lambs, the animal mass, $m(t)$, was represented as a monoexponential fit to the animal weight data as given by:

$$m(t) = A \cdot \exp(\alpha \cdot [t - t_0]) \quad (3.16)$$

where A is the body mass (weight) at time t_0 and α is a first-order growth rate constant.

The release time delay p.d.f., $r(\omega, s)$, from Eq. 3.10 was modeled as a Weibull distribution due to the flexibility of the distribution, its support on the non-negative real line, and the analytical solution to its cumulative distribution function. Specifically:

$$r(\omega, s) = \begin{cases} \frac{k}{l} \cdot \left[\frac{\omega - \theta(s)}{l} \right]^{k-1} \cdot \exp\left(-\left[\frac{\omega - \theta(s)}{l} \right]^k\right) & \text{for } \omega \geq \theta(s) \text{ and } 0 \leq \omega < \infty \\ 0 & \text{otherwise} \end{cases} \quad (3.17)$$

with:

$$l > 0, k > 0, \text{ and } \theta(s) \geq 0 \text{ for all } s$$

where l , k , and $\theta(s)$ are the scale, shape, and location parameters, respectively, with only the location parameter being time variant. From Eq. 3.8 and Eq. 3.17 it follows that:

$$\int_0^{t-s} r(\omega, s) d\omega = \begin{cases} 1 - \exp\left(-\left[\frac{t-s-\theta(s)}{l} \right]^k\right) & \text{for } t-s \geq \theta(s) \text{ and } 0 \leq t-s < \infty \\ 0 & \text{otherwise} \end{cases} \quad (3.18)$$

The time variance of the distribution was assumed to enter through the location parameter due to the simplicity of the interpretation in changes of $\theta(s)$. However, the presented model and the numerical implementation (see below) readily extends time variance in the other distribution parameters.

To account for the double phlebotomies, let T_{p1} and T_{p2} be defined as the time of the first and second phlebotomy, respectively, and let F_1 and F_2 be the corresponding

fraction of the cells remaining after the phlebotomy. Then from extensions of Eq. 3.11 to two phlebotomies (Appendix B.5), the number of cells present at time t is given by:

$$\begin{aligned}
N(t) = & \int_{t-b}^t f_{stim}(u) \cdot \left[\int_0^{t-u} r(\omega, u) d\omega \right] du - U(t - T_{P1}) \cdot [1 - F_1] \cdot \int_{\min(T_{P1}, t-b)}^{T_{P1}} f_{stim}(u) \cdot \left[\int_0^{T_{P1}-u} r(\omega, u) d\omega \right] du \\
& - U(t - T_{P2}) \cdot [1 - F_2] \cdot \int_{\min(T_{P2}, t-b)}^{T_{P2}} f_{stim}(u) \cdot \left[\int_0^{T_{P2}-u} r(\omega, u) d\omega \right] du \\
& + U(t - T_{P2}) \cdot [1 - F_2] \cdot [1 - F_1] \cdot \int_{\min(T_{P1}, t-b)}^{T_{P1}} f_{stim}(u) \cdot \left[\int_0^{T_{P1}-u} r(\omega, u) d\omega \right] du
\end{aligned} \tag{3.19}$$

with:

$$F_i = 1 - \frac{N_{Pi}}{N(T_{Pi} - \varepsilon)}, \quad i = 1, 2 \tag{3.20}$$

where $\min(T_{Pi}, t - b)$ is the minimum of T_{Pi} and $t - b$, $f_{stim}(u)$ is given by Eq. 3.13 and Eq. 3.14, and $\int_0^{t-u} r(\omega, u) d\omega$ is given by Eq. 3.18. Additionally, ε denotes an infinitely small time increment and N_{P1} and N_{P2} the number of cells removed by the first and second phlebotomy, respectively. The fraction of cells remaining after a phlebotomy will be the same for both reticulocytes and RBCs, and therefore only a single F_1 and F_2 is calculated for both populations of cells. For $t \leq t_0$, the release time delay distribution was assumed to remain at the initial (t_0) release time delay distribution (i.e. $r(\omega, s) = r(\omega, t_0)$ for $s \leq t_0$). An end-constrained quadratic spline function was used to nonparametrically estimate the Weibull distribution time variant location parameter (i.e. $\theta(s)$) of the release time delay distribution, as further detailed in Appendix B.6.

Specifically, let the time from stimulation of a erythroid precursor cell to transformation of the subsequently stimulated reticulocyte (either in the marrow or

systemic circulation) into a mature RBC be denoted as b_{RET} and the time from stimulation of a erythroid precursor cell to senescence/destruction of the subsequently stimulated RBC (immature + mature) be denoted as b_{RBC} . Due to the fact that a reticulocyte is an immature RBC, then replacement of b by b_{RET} or b_{RBC} in Eq. 3.19 gives the fitted equation for the number of reticulocytes ($N_{RET}(t)$) or the number of RBCs ($N_{RBC}(t)$), respectively. The modeled relationship between $r(\omega, s)$, b_{RET} , and b_{RBC} is schematically illustrated in Figure 3.2 (Panel A). Thus from Eq. 3.5 and Eq. 3.7 the concentration of reticulocytes ($C_{RET}(t)$) and RBCs ($C_{RBC}(t)$) are given by:

$$C_{RET}(t) = \frac{N_{RET}(t)}{V(t)} \quad (3.21)$$

$$C_{RBC}(t) = \frac{N_{RBC}(t)}{V(t)} \quad (3.22)$$

where $V(t)$ is given by Eq. 3.15. The parameter for the time from stimulation of a erythroid precursor cell to senescence/destruction of the subsequently stimulated RBC, b_{RBC} , was fixed to ‘ $E\{\Omega\} + \text{RBC lifespan}$ ’, where $E\{\}$ denotes the mathematical expectation of a random variable and in this instance is the expectation taken with respect to the initial steady-state release time delay distribution (i.e. $r(\omega, t_0)$). The normal RBC lifespan has previously been determined in sheep using [^{14}C] cyanate label and found to be 114 days (54).

The reticulocyte and RBC-plasma EPO concentration relationship, accounting for subject growth, was modeled by simultaneously fitting Eq. 3.21 and Eq. 3.22 (along with supporting Eq. 3.13 through Eq. 3.15 and Eq. 3.18 through Eq. 3.20) using the fitted

plasma EPO concentration and animal mass, the observed reticulocyte count, and the observed RBC count concentration-time data of each subject.

3.3.5 Comparison to other lifespan models

The formulated time variant cellular lifespan distribution model (Figure 3.2, Panel A) was compared to the identical time invariant cellular lifespan distribution model, as well as the time variant and time invariant “point distribution” cellular lifespan models. Each model was fit to data from each animal. Replacement of $r(\omega, u)$ in Eq. 3.19 with $r(\omega)$ gives the following identical *time invariant* cellular lifespan distribution model:

$$r(\omega) = \begin{cases} \frac{k}{l} \cdot \left[\frac{\omega - \theta}{l} \right]^{k-1} \cdot \exp\left(-\left[\frac{\omega - \theta}{l} \right]^k\right) & \text{for } \omega \geq \theta \text{ and } 0 \leq \omega < \infty \\ 0 & \text{otherwise} \end{cases} \quad (3.23)$$

where θ is the time invariant location parameter of the Weibull distribution (Figure 3.2, Panel B). In the time invariant cellular lifespan distribution model the distribution of release time delays (and lifespans) is constant and independent of the time of stimulation. Models of time invariant distributions of cellular lifespans have previously been described in detail (6).

The *time variant* “point distribution” cellular lifespan model (Figure 3.2, Panel C), is obtained by replacing in Eq. 3.19 the time variant Weibull distribution (i.e. $r(\omega, u)$ given by Eq. 3.17) with a time variant dirac delta function, $\delta(\omega - a(u))$, which upon simplification gives:

$$\begin{aligned}
N(t) = & \int_{t-b}^{x(t)} f_{stim}(u) du - U(t - T_{P1}) \cdot [1 - F_1] \cdot \int_{\min(x(T_{P1}), t-b)}^{x(T_{P1})} f_{stim}(u) du \\
& - U(t - T_{P2}) \cdot [1 - F_2] \cdot \int_{\min(x(T_{P2}), t-b)}^{x(T_{P2})} f_{stim}(u) du \\
& + U(t - T_{P2}) \cdot [1 - F_2] \cdot [1 - F_1] \cdot \int_{\min(x(T_{P1}), t-b)}^{x(T_{P1})} f_{stim}(u) du
\end{aligned} \tag{3.24}$$

with:

$$x(t) = t - a(x(t))$$

$$a'(s) > -1$$

$$b_{RET} > a(s) \geq 0$$

where $x(t)$ is the time of stimulation of cells currently entering the sampling compartment and $a(s)$ is the time variant “point” cellular release time delay given by the end constrained quadratic spline function of the same form as that used for $\theta(s)$ (Appendix B.6). The constraint that $a'(s) > -1$ is needed to ensure a unique solution to $x(t)$ as previously discussed (91). Additionally, $a(s)$ is constrained to be less than b_{RET} because if $a(s) \geq b_{RET}$ then no reticulocytes would be present in the sampling compartment (at least for some period of time), which was never observed. For the time variant “point distribution” model all cells stimulated at a given stimulation time have the same release time delay and lifespan, the latter which is defined by $b - a(s)$. However, cells stimulated at different times may have different release time delays and lifespans. The time variant and time invariant “point distribution” cellular lifespan model and the $x(t)$ function have previously been presented (91).

The *time invariant* “point distribution” cellular lifespan model, is obtained by replacing in Eq. 3.19 the time variant Weibull distribution with a time invariant dirac delta function, $\delta(\omega - a)$, which upon simplification gives:

$$\begin{aligned}
N(t) = & \int_{t-b}^{t-a} f_{stim}(u) du - U(t - T_{P1}) \cdot [1 - F_1] \cdot \int_{\min(T_{P1}-a, t-b)}^{T_{P1}-a} f_{stim}(u) du \\
& - U(t - T_{P2}) \cdot [1 - F_2] \cdot \int_{\min(T_{P2}-a, t-b)}^{T_{P2}-a} f_{stim}(u) du \\
& + U(t - T_{P2}) \cdot [1 - F_2] \cdot [1 - F_1] \cdot \int_{\min(T_{P1}-a, t-b)}^{T_{P1}-a} f_{stim}(u) du
\end{aligned} \tag{3.25}$$

where:

$$b_{RET} > a \geq 0$$

and a is the time invariant ‘‘point’’ cellular release time delay (Figure 3.2, Panel D). In the time invariant ‘‘point distribution’’ cellular lifespan model all cells have the identical release time delay and lifespan (i.e. resulting in a constant value for the lifespan, $b - a$), regardless of the time of stimulation. The relationship between the time variant and time invariant ‘‘point distribution’’ cellular lifespan models can be also be observed by replacement of $a(s)$ in Eq. 3.24 with the constant a , which upon simplification will give Eq. 3.25.

The differences between the four cellular lifespan models are illustrated in Figure 3.2. The objective function value, Akaike’s Information Criterion (AIC) value (88), and the squared correlation coefficient (R^2) of the observed vs. predicted concentrations (across all animals) were used as goodness-of-fit criteria to compare the four different cellular lifespan models. Additionally, the means and standard deviations of the common parameters of the 4 different models were compared.

3.3.6 Computational details

All modeling was conducted using WINFUNFIT, a Windows (Microsoft) version evolved from the general nonlinear regression program FUNFIT (86), using weighted least squares. Motivated by the enumeration of the cellular data (i.e. a Poisson process) and the large differences in scale of the reticulocyte and RBC data, data points were

weighted by y_{obs}^{-1} , where y_{obs} is the observed reticulocyte or RBC concentration. The fitted models required the numerical solution to a one dimensional integral (Eq. 3.19, Eq. 3.24, and Eq. 3.25). This was done by using the FORTRAN 90 subroutine QDAGS from the IMSL® Math Library (Version 3.0, Visual Numerics Inc., Houston, TX). QDAGS is a univariate quadrature adaptive general-purpose integrator that is an implementation of the routine QAGS (95). The relative error for the QDAGS routine was set at 0.1% for all numerical integrations. Additionally, the implicit function $x(t)$ had to be solved to determine the upper integration bound of Eq. 3.24, which was done using the FORTRAN 90 subroutine ZREAL from the IMSL® Math Library. ZREAL is a nonlinear equation solver that finds the zero of a real function using Müller's method. The relative error for the ZREAL routine was set at 0.01%.

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

$$\text{MSE\%} = \frac{1}{n} \cdot \sum_{i=1}^n \frac{\text{SE}_i}{|P_i|} \cdot 100 \quad (3.26)$$

where SE_i and P_i are the standard error of the parameter and the estimate of the parameter for the i^{th} subject, respectively, and n is the number of subjects. The mean lifespan of circulating reticulocytes at the current stimulation time was determined over time for each subject by analytically calculating the mathematical expectation of the circulating lifespan, conditional on s (i.e. $E\{T|s\}$), with the expectation taken with respect to the distribution given by Eq. 3.12, where $r(\cdot, s)$ is the fitted Weibull distribution (Appendix B.7).

3.3.7 Statistical analysis

For the time variant lifespan distribution model, the minimum, maximum, and study end mean circulating reticulocyte lifespans at the current stimulation time were statistically compared to the mean initial (t_0) or baseline lifespan (denoted by $\mu_{RET,0}$) with paired two tailed Student's t-tests using Microsoft® Excel 2002 SP3 (Microsoft Corporation, Redmond,WA). Statistically significant differences were determined at the $\alpha = 0.05$ type I experimentwise error rate. To control the experimentwise error rate inflation due to multiple comparisons, a stepdown Bonferroni method was used to adjust the *p-values* from the paired t-tests (96).

3.4 Results

The profiles and the simultaneous fit to the plasma EPO, reticulocyte, and RBC concentration data for two representative animals (Panel A and B) is displayed in Figure 3.3 for the time variant cellular lifespan distribution model. The dynamic relationship between the phlebotomy induced anemia, plasma EPO, reticulocyte, and RBC concentrations was modeled. The plasma EPO concentrations rapidly rose within hours of both phlebotomies, and then returned to baseline concentrations approximately 5 days post-phlebotomy. The reticulocyte concentrations began to increase 1 to 2 days following the rise in plasma EPO concentrations, peaking several days later, while the RBC concentrations steadily rose following both phlebotomies. The formulated time variant cellular lifespan distribution model also fit the data very well across a wide range of concentrations. The R^2 of observed vs. predicted reticulocyte concentrations was 0.952 and observed vs. predicted RBC concentrations was 0.964 across all animals.

The PD parameters of the time variant lifespan distribution model are summarized in Table 3.1. In general, the parameters of the model were well estimated with MSE% of less than 20% for all parameters, with many less than 5%. Not surprisingly, the scale (λ) and shape (k) parameters of the release time delay p.d.f. were not as well estimated and had higher MSE%. A relatively high amount of subject to subject variability was observed in the E_{max} and EC_{50} parameters of the transduction function, with means of $4.97 \cdot 10^{10}$ cells/kg/day and 66.6 mU/mL, respectively. The weight normalized total blood volume was estimated at 81.0 ± 2.38 (mean \pm SD) mL/kg or 8.10%, consistent with the standard blood volume estimates in mammals of 6% to 11% of body weight (46), and slightly higher than means previously determined in sheep ranging from 57.6 to 74.4 mL/kg (97). The incorporation of blood volume expansion due to animal growth was an important consideration of the model, as animals grew on average 4.1 kg or 17% of their initial body weights over the course of the experiments. The average initial (t_0) body weight and rate constant of growth were estimated at 22.3 ± 2.31 kg and 0.00379 ± 0.00385 1/day, respectively. The monoexponential fit to body weight data from the presented two representative subjects is displayed in Figure 3.3 (inset). While the monoexponential function (Eq. 3.16) does not fit the observed animal weight data exactly, it is used to represent the lean body mass that will be more representative of changes in erythropoietic progenitor cell mass and blood volume, since these will not likely change substantially with transient increases and losses of adipose that may be represented in the observed body weight. Furthermore, an exponential model was utilized for the body weight instead of a linear model to prevent the possibility of negative animal body weight prior to time t_0 . From the number of measured cells removed by each

phlebotomy and the model estimated number of cells in the circulation immediately prior to each phlebotomy the fraction of cells removed following the first and second phlebotomies (F_1 and F_2 , respectively) were estimated at 0.409 ± 0.0721 and 0.571 ± 0.0963 , respectively. The baseline minimum release time delay between stimulation in the bone marrow and the subsequent release of the stimulated erythrocyte(s) into the systemic circulation (θ_0) was estimated at 1.28 ± 0.343 days, while the mean baseline release time delay of an erythrocyte was 2.26 ± 0.729 days with a constant (i.e. time invariant) standard deviation of 0.659 ± 0.289 days. Finally, the time between stimulation in the bone marrow and maturation of the erythrocyte from a reticulocyte to a mature RBC (b_{RET}) was estimated at 1.99 ± 0.518 days.

The average mean circulating reticulocyte lifespan at the current stimulation time is displayed in Figure 3.4. From the baseline value of 0.304 days ($\mu_{RET,0}$, Table 3.1) it rapidly increased over 3-fold following the first phlebotomy to a value of 1.03 days ($p = 0.009$). Following the initial peak, the mean circulating reticulocyte lifespan dropped down to near baseline values before rising again after the second phlebotomy. Following the second reticulocyte lifespan peak, the study end mean lifespan dropped to a value of 0.218 days, similar to the baseline value ($p > 0.05$). The minimum mean circulating reticulocyte lifespan was also not significantly different from the baseline lifespan ($p > 0.05$), nor was the minimum lifespan between the two phlebotomies significantly different from $\mu_{RET,0}$ ($p > 0.05$). The average percentage of erythrocytes at baseline (i.e. at day 0) being released as reticulocytes was estimated at 43.0% (57.0% released as mature RBCs), while the average maximal proportion of stimulated cells to be released as reticulocytes was estimated at 89.5%, with only 10.5% of stimulated cells

released as mature RBCs. The two-fold increase in the percentage of erythrocytes being released as reticulocytes (and nearly six-fold decrease in the percentage released as mature RBCs) illustrates the dramatic changes that occur in both the release time delay distribution and the type of erythrocytes being released under stress erythropoietic conditions in sheep.

The comparison of the time variant distribution, the time invariant distribution, the time variant “point distribution”, and time invariant “point distribution” of cellular lifespan models is summarized in Table 3.2. A schematic of the model differences is displayed in Figure 3.2. In general, all models fit the RBC data equally well, with R^2 values near 0.96, however, the time invariant models fit the reticulocyte data poorly with R^2 values near 0.46. The mean objective function value was the smallest for the time variant lifespan distribution model. However, in 3 of the 4 animals the time variant “point distribution” was preferred over the other models based on the AIC. In the remaining animal the AIC was the lowest for the time variant distribution model. On average, AIC was the smallest for the time variant “point distribution” model. The stimulation function parameters (i.e. E_{max} , EC_{50} , and k_{bio}) and the weight normalized total blood volume were very similar across all models indicating a good model robustness for estimation of these parameters among the models. The mean b_{RET} was similar among most of the models, except for the time invariant “point distribution” cellular lifespan model. The baseline circulating reticulocyte lifespan ($\mu_{RET,0}$) was approximately 3-fold lower in the “point distribution” models than in the distribution models. For the time variant lifespan models the mean maximal circulating reticulocyte lifespans ($\mu_{RET,MAX}$) in each animal

were similar, however, the increase from baseline was nearly 10-fold for the “point distribution” model.

3.5 Discussion

Two basic parameterizations of a time variant cellular lifespan distribution PD model were formulated to account for changes over time in the underlying lifespan p.d.f. of cellular populations. The presented model extends recent cellular lifespan models that assumed a single (i.e. a “point distribution”) *time variant* cellular lifespan (91) and models that assumed a *time invariant* distribution of cellular lifespans (6). Two important assumptions of the proposed PD model are: 1.) the stochastic independence of cells, and 2.) that following production/stimulation subsequent changes in the environment do not alter the cellular disposition. The effects of subject growth on production or stimulation rate and on sampling space volume were also incorporated into the model. Additionally, the time variance of the underlying lifespan distribution can readily be incorporated into any of the parameters of the lifespan p.d.f., as either a nonparametric function of time or if more is known about the biological system, a function of the cellular production environment. By choosing a flexible arbitrary p.d.f. with a corresponding cumulative density function (c.d.f.) that can be analytically or otherwise rapidly evaluated, the proposed PD model can be fit to observed cellular concentration data requiring only a suitable numerical one dimensional integration solver. Furthermore, the production or stimulation rate in the model can be any analytical function that depends on endogenous growth factors and/or exogenous drugs.

A single time variant release time delay p.d.f. was utilized in the fitted equations for both the reticulocytes and RBCs (Eq. 3.19), with a fixed time, b_{RET} , from stimulation

of an erythropoietic cell to transformation from a reticulocytes (an immature RBC) into a mature RBC (Figure 3.2, Panel A). The presented model allows for a fraction of the red cells to be released directly into the systemic circulation as mature RBCs, as cells with release time delays $\geq b_{RET}$ are released into the sampling compartment as mature erythrocytes. The ability to account for the release of mature RBCs is particularly important when dealing with ruminants, such as sheep, that under basal, non-erythropoietically stimulated conditions release the majority of their red cells into the systemic circulation directly from the bone marrow as mature RBCs (43), and thus partially explaining the very low basal reticulocyte percentage in ruminants (0.1-0.2%) (46). While a common release time delay p.d.f. is justified on a physiological basis, a fixed time of development from stimulation of progenitor cells to development into a mature RBC is a simplification of the underlying physiology. However, the utilized time variant cellular lifespan distribution model was chosen based on the knowledge that: less developmentally mature and hence younger reticulocytes (RBCs) are released under stress erythropoiesis (37, 39, 44, 45), and that under basal, non-erythropoietically stimulated conditions sheep release the majority of their red cells directly from the bone marrow as mature RBCs (43). Thus, the model captures the primary kinetic features of the immature RBC physiology, which would not be possible using a direct parameterization of a time variant reticulocyte lifespan (i.e. as given by Eq. 3.3).

The distribution of circulating reticulocyte lifespans at the current stimulation time can be determined from Eq. 3.12, and subsequently the mean circulating reticulocyte lifespan was calculated (Appendix B.7), as displayed in Figure 3.4. Similar to previous results with a single phlebotomy and a “point distribution” of circulating reticulocyte

lifespans (91), the mean circulating reticulocyte lifespan rapidly increased approximately 3-fold shortly after the first phlebotomy ($p = 0.009$). However, unlike previous results, the mean reticulocyte lifespan did not drop significantly below the baseline lifespan following either phlebotomy ($p > 0.05$). The difference may be attributed to the small sample sizes in both studies ($n = 5$ and $n = 4$, respectively), accounting for subject growth in the present analysis, and/or the incorporation of a distribution of lifespans instead of a single lifespan, among other factors. The circulating reticulocyte lifespan in Figure 3.4 begins to increase prior to the 2nd phlebotomy, which may be the same but somewhat muted rebound phenomenon previously observed following a single acute phlebotomy (91). The estimated approximately 3-fold increase in the circulating reticulocyte lifespan in both sheep studies is consistent with estimates in humans of a 2-3 fold increase following stress erythropoiesis (90) and with the exogenous administration of erythropoietin (11).

The time variant cellular lifespan distribution model represents the most general case and the other three models are simplified “special cases” of this model. The comparison of the four cellular lifespan models (Figure 3.2) indicates that the time variant models were preferred to the time invariant models based on the objective function, R^2 , and AIC values (Table 3.2). The two time variant cellular lifespan models resulted in similar fits, with the time variant “point distribution” model resulting in a lower AIC in three of the four animals. Hence, in the majority of cases the more complicated time variant distribution cellular lifespan model was not preferred to the time variant “point distribution” cellular lifespan model. These results comparing the distribution and the “point distribution” cellular lifespan models are consistent with

results obtained by other investigators comparing a time invariant distribution cellular lifespan model to a time invariant “point distribution” cellular lifespan model (6). However, the time variant “point distribution” model resulted in a very short estimate of the baseline circulating reticulocyte lifespan of 0.123 ± 0.0487 days and a maximal increase in the circulating reticulocyte lifespan of nearly 10-fold, which is inconsistent with estimated increases in circulating reticulocyte lifespan in humans of 2-3 fold following stress erythropoiesis (11, 90). The most likely reason for the apparent physiologically unrealistic estimates of these values is that the “point distribution” of cellular release time delays for this model (Figure 3.2, Panel C) requires that all erythrocytes released from the bone marrow into the systemic circulation be released as reticulocytes. While the model could be extended to allow for all erythrocytes to be released from the bone marrow as either reticulocytes or mature RBCs depending on the stimulation time, this would cause the fitted reticulocyte concentration to drop to zero when $a(s) \geq b_{RET}$, which was never observed. The constraint on the type of erythrocyte released into the systemic circulation given by the time variant “point distribution” cellular lifespan model is in contrast to the more general nature of the time variant distribution model (Figure 3.2, Panel A). With the distribution model, erythrocytes with a release time delay (i.e. ω) less than b_{RET} enter the systemic circulation as a reticulocyte while erythrocytes with an $\omega \geq b_{RET}$ enter the circulation as a mature RBC, consistent with the known physiology in sheep where a fraction of the erythrocytes enter the circulation as a mature RBC (43, 46). Thus the underlying physiology must be considered when selecting the most appropriate cellular lifespan model, and not just the goodness-of-fit criteria (e.g. AIC).

The addition of a distribution of release time delays to the time variant lifespan model over a time variant single “point distribution” lifespan (91) offers modeling cellular responses in a more physiologically realistic manner. The described model allowed for the estimation of the proportion of the erythrocytes being released directly into the systemic circulation from the bone marrow as mature RBCs. Apparently, estimates of the proportion of erythrocytes released as reticulocytes or RBCs have not been previously determined in sheep. Other potential applications of the presented model are to account for changes in RBC lifespan that are due to production under stress erythropoiesis conditions, as previously demonstrated in some animal models (52, 55, 56). Even though not accounted for in the model, a reduced RBC lifespan due to stress erythropoiesis stimulation conditions was not a concern because the lifespan would have to be reduced to less than 50 days to have an effect on the modeling, since this was the longest time period of observation following the first phlebotomy.

In addition to the stochastic independence assumption of cells, the other key assumption of the model is that the disposition of cells *following* production or stimulation is not affected by changes in the environmental conditions. Apparently, all PD models of cellular response presented to date either implicitly utilize this assumption (6, 11, 53, 72, 76, 91), or assume that cellular age has no effect on the probability of cellular death/transformation (i.e. a cell “pool” or “random hit lifespan” model) (70, 71, 98). The lack of a “environmental effect” assumption may not be reasonable if the environmental conditions that a cell is exposed to over its lifetime vary substantially over time, particularly for cells with relatively long lifespan (relative to the rate of change in the environmental conditions). For circulating reticulocytes, which have a relatively short

lifespan, there is evidence in rats that the RNA content (and hence age) of cells depends on the conditions under which the reticulocytes developed (99). Similar conclusions have been obtained in humans, that under normal conditions the properties of the erythrocyte lifespan are determined by the conditions under which they are formed (52). Thus for reticulocytes their disposition may well be determined at the time of stimulation. However, if changes in the environmental conditions (e.g. plasma EPO concentrations) *following* production or stimulation of reticulocytes (or mature RBCs) do substantially affect their circulating lifespan this key assumption of the current model would be violated. Hence, extensions of the presented model to incorporate the effects of changing environmental conditions on the disposition of the cells are still needed, particularly under pathological disease conditions. Further work in this area is detailed in the next chapter.

3.6 Conclusion

In summary, a time variant cellular lifespan distribution PD model was formulated to account for changes over time in the underlying lifespan probability density function of cellular populations. The model extends recent cellular lifespan models that assumed a single (i.e. a “point distribution”) *time variant* cellular lifespan and models that assumed a *time invariant* distribution of cellular lifespans. Furthermore, the model developed in the present study was used to determine the time variant circulating reticulocyte lifespan in sheep following stress erythropoiesis conditions. The proportion of erythrocytes released from the bone marrow as reticulocytes was estimated by the model to increase over 2-fold following phlebotomy. The time variant cellular lifespan distribution model was compared to three simpler cellular lifespan models derived as

specific cases of the proposed time variant lifespan model. These comparisons indicated the importance of accounting for a time variant cellular lifespan for reticulocytes stimulated under stress erythropoiesis conditions. Additionally, they indicated that the selection of the most appropriate model should not solely be based on conventional goodness-of-fit metrics but must also consider the underlying cellular physiology. The presented PD model readily allows incorporation of time variant lifespan distributions when considering populations of cells whose production or stimulation depends on endogenous growth factors and/or exogenous drugs.

Table 3.1. Parameter estimates for the reticulocyte and RBC time variant lifespan (release time delay) distribution model ($n = 4$).

	E_{max} (10^{10} cells/kg/day)	EC_{50} (mU/mL)	k_{bio} (1/day)	V_n (mL/kg)	θ_0 (day)	l	k	b_{RET} (day)	$\mu_{RET,0}^*$ (day)
Mean	4.97	66.6	0.126	81.0	1.28	1.09	1.55	1.99	0.304
SD	2.08	35.4	0.0271	3.28	0.343	0.476	0.285	0.518	0.0862
MSE%	0.5%	0.7%	11.2%	1.0%	4.4%	10.7%	17.2%	2.6%	NA

*Secondary parameter

SD: Standard deviation

MSE% : Mean percent standard error

NA: Not applicable

Table 3.2. Comparison of goodness-of-fit criteria and common parameter estimates of four different cellular lifespan models ($n = 4$).*

	Time variant distribution	Time invariant distribution	Time variant “point distribution”	Time invariant “point distribution”
No. of fitted parameters	21	8	19	6
R^2 reticulocytes**	0.952	0.455	0.942	0.470
R^2 RBCs**	0.964	0.966	0.964	0.962
Objective function	62 300 (37 300)	152 000 (73 100)	63 100 (35 100)	157 000 (73 100)
AIC	629 (98.4)	685 (91.9)	627 (93.8)	684 (86.8)
E_{max} (10^7 cells/kg/day)	4.97 (2.08)	5.36 (2.00)	5.43 (2.44)	5.65 (2.67)
EC_{50} (mU/mL)	66.6 (35.4)	71.0 (32.0)	71.7 (41.0)	77.5 (47.1)
k_{bio} (1/day)	0.126 (0.0271)	0.146 (0.0680)	0.119 (0.0276)	0.137 (0.0595)
V_n (mL/kg)	81.0 (3.28)	82.9 (4.85)	83.5 (3.48)	81.9 (4.83)
b_{RET} (day)	1.99 (0.518)	1.76 (0.047)	1.90 (0.289)	1.28 (0.127)
$\mu_{RET,0} \dagger$ (day)	0.304 (0.0862)	0.403 (0.162)	0.123 (0.0487)	0.135 (0.049)
$\mu_{RET,MAX} \dagger$ (day)	1.12 (0.238)	NA	1.02 (0.198)	NA

Table 3.2. Continued.

* Values represented as mean (standard deviation)

** Only a single R^2 value was determined across all animals

† Secondary parameter

NA: Not applicable

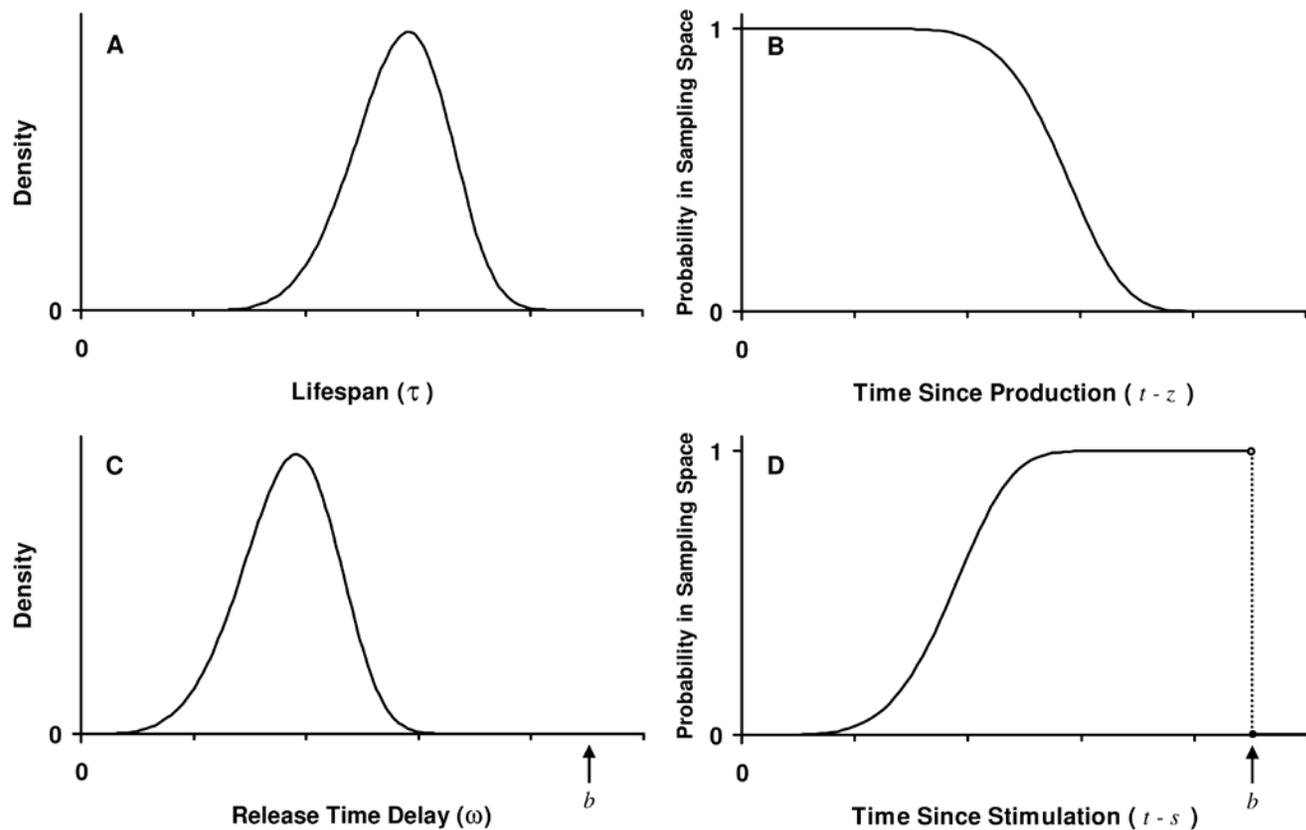


Figure 3.1. Illustration of the relationship between the time variant lifespan or release time delay distributions and the corresponding unit responses.

The time variant lifespan distribution, $\ell(\tau, z)$ is displayed in Panel A, and the corresponding unit response, $UR_l(t, z)$, from Eq. 3.3 is displayed in Panel B. The relationship between the time variant release time delay distribution, $r(\omega, s)$, and the corresponding unit response, $UR_r(t, s)$, from Eq. 3.8 is displayed in Panel C and D, respectively.

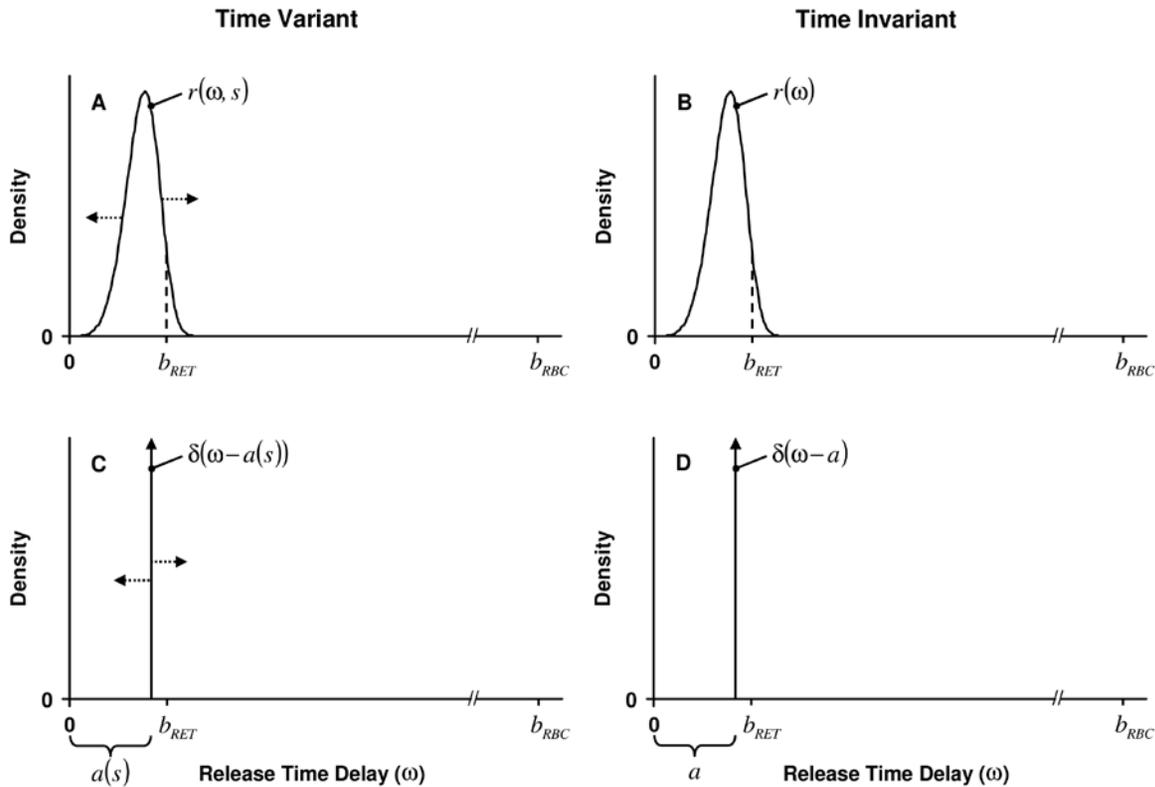


Figure 3.2. Schematic of the differences between the time variant and invariant “point distribution” and distribution cellular lifespan models.

The time variant distribution, time invariant distribution, time variant “point distribution”, and time invariant “point distribution” are displayed in Panels A, B, C, and D, respectively. Time variance is illustrated by horizontal dotted arrows. Also illustrated in each panel is the relationship between each of the release time delay “distributions”, the time from stimulation of an erythroid precursor cell to transformation of the subsequently stimulated reticulocyte into a mature RBC (b_{RET}), and the time from stimulation of a erythroid precursor cell to senescence/destruction of the subsequently stimulated RBC (b_{RBC}). The stimulation time is denoted by s .

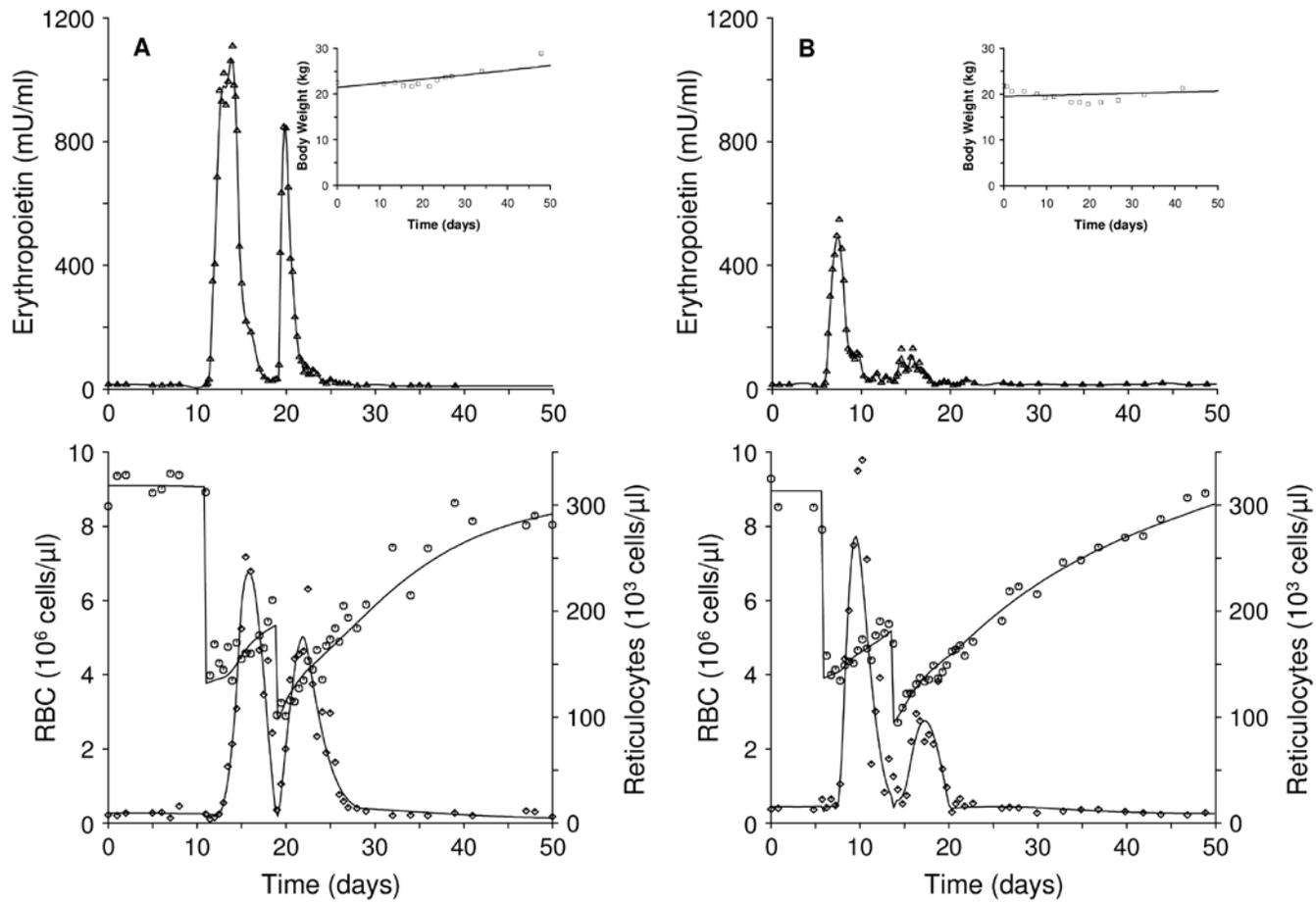


Figure 3.3. Representative individual subject fits (curves) of the time variant lifespan distribution model to observed data (symbols). Plasma EPO (Δ) concentrations, reticulocyte (\diamond) concentrations, RBC (\circ) concentrations, and body weight (\square) (inset) are displayed. Panels A and B are different subjects.

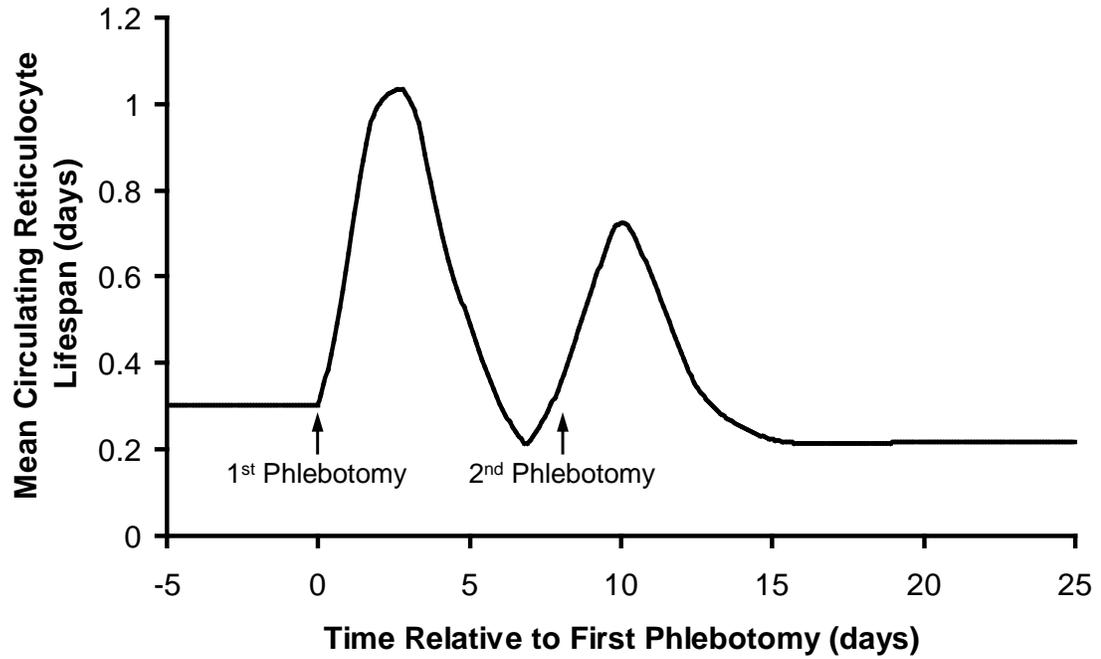


Figure 3.4. Average mean circulating reticulocyte lifespan for the time variant lifespan distribution model. Average lifespan calculated according to Appendix B.7 from the parameter and $\theta(t)$ estimates for each individual subject ($n = 4$).

CHAPTER 4. PHARMACODYNAMIC MODELING OF THE EFFECT OF CHANGES IN THE ENVIRONMENT ON CELLULAR LIFESPAN AND CELLULAR RESPONSE

4.1 Introduction

Many drugs impart their primary pharmacodynamic (PD) effect on the number of cells of a particular cell type. Cell populations commonly targeted include erythrocytes, leukocytes, platelets, cancerous cells, and bacteria. Additionally, some drugs may have side effects on non-targeted cellular populations, e.g. chemotherapeutic agents on non-cancerous cells. In order to optimally dose these drugs to properly balance efficacy and cellular toxicity an understanding of the PD effect on the production and destruction of these cells is needed. Thus, lifespan distributions and age structure of the cellular population are important to accurately quantify pharmacodynamically.

The lifespan based models of cellular response fall into the broad class of indirect response models (5, 6, 53, 70, 91, 100-102). Early lifespan based PD models assumed a single “point distribution” of cellular lifespans that does not vary over time (i.e. time invariant). More recently, models have been introduced that account for a time invariant (6, 70) and time variant distributions of cell lifespans (100). The previous PD models of time variant cellular lifespans (91, 100, 101), as well as the time invariant lifespan models, assumed that the lifespan distribution and thus probability of cellular survival was defined for an individual cell at the time of cellular production. Hence the time variant disposition of the cellular lifespans was assumed to be only due to changes in the conditions cells were produced or formed under; and subsequent changes in the environmental conditions after their production were assumed to have no effect on the

disposition of the cells. This assumption may not be reasonable if the environmental conditions that a cell is exposed to vary substantially over time. The term “environmental effect” in this chapter will refer specifically to effect of the environmental conditions on cellular lifespan that are induced by changes in the environment occurring *after* the cell is produced (e.g. while present in the sampling space), and is to be distinguished from changes in the conditions under which the cell is formed.

The neglect of and need to account for environmental effects on cellular behavior using mathematical models has been recently recognized in areas such as red blood cell (RBC) survival in patients with uremia that are treated with erythropoietin and RBC transfusions (103). Other examples of “environmental effects” on cellular lifespan include: increases in the concentration of reactive oxygen species that RBCs are exposed to due to chemotherapy administration (104, 105), increases in RBC oxidative stress due to hemodialysis (106), decreased platelet activation due to administration of antiplatelet drugs (107), and changes in the availability of nutrients of cancerous cells due to the administration of anti-angiogenic drugs (108). The study of the effect of different storage conditions and time periods on RBC aging and the survival of the transfused RBC cells is also directly related to the environmental effects on cell lifespan, where storage induced acceleration of the aging process is thought to occur (109).

The objectives of the current work are: 1.) to extend the previously formulated time variant cellular lifespan PD models to allow for the incorporation of environmental effects on the cellular lifespan, 2.) to examine by simulation the effect of varying the level of the environmental effects on the observed cellular response curve, 3.) and to

present a data analysis example of the proposed environmental effect models considering the cellular response following hematopoietic busulfan ablation in sheep.

4.2 Theoretical

4.2.1 Effect of changes in the cellular environment

Let $\ell_b(\tau, z)$ denote the “baseline” probability density function (p.d.f.) of cellular lifespans where τ is the cellular lifespan in the sampling space and z is an arbitrary time of production. Cellular production is here defined as the physical input of cells into the sampling space. The p.d.f. $\ell_b(\tau, z)$ is the baseline lifespan distribution that is defined at the time of production for cells produced at time z , which would be observed if the sampling space environment had no effect on the cellular lifespan distribution. Thus, in the context of a constant cellular environment that has no effect on the cell lifespans, a corresponding baseline time variant survival function, S_b , can be defined:

$$S_b(t, z) = P(T > t - z, z) = 1 - \int_0^{t-z} \ell_b(\tau, z) d\tau, \quad t \geq z \quad (4.1)$$

where t is the current time, T denotes a random cellular lifespan variable, and $P(T > t - z, z)$ denotes the probability that $T > t - z$ for cells produced at time z . Accordingly, $S_b(t, z)$ defines the cellular disposition or probability of a cell being present in the sampling space if the environmental conditions cells are exposed to after production does not affect the baseline lifespan p.d.f.. The baseline survival function is analogous to the previously defined unit response function (91, 100). However, a unit response does not apply when dealing with environmental effects because the contribution of prior production to the probability a cell is present is no longer an exclusive function of elapsed time since production (i.e. $t - z$).

The effect of the environment on the cellular disposition can be considered, as previously proposed in survival analysis (92, 110), by utilization of a positive function, $e(t, z)$, that is the effect of the sampling space environment on the cellular disposition:

$$S_e(t, z) = P(T > e(t, z) \cdot [t - z], z) = 1 - \int_0^{e(t, z)[t-z]} \ell_b(\tau, z) d\tau, \quad t \geq z \quad (4.2)$$

where S_e is the observed survival function due to the environmental effects. The function $e(t, z)$ is a *cumulative measure* of the effect of the environment over the time period from time z of production to the current time t (i.e. time period that the cells have been in the environment since production) and acts by accelerating and decelerating the age related death/removal process or life of the cells. Accordingly, $e(t, z) > 1$ and $e(t, z) < 1$ correspond to a reduction and a prolongation of the cellular survival, respectively. The constraint that $e(t, z) > 0$ ensures that all cells have a finite lifespan. Furthermore, to ensure that the probability a cell is present cannot increase with time, i.e. ensure that the survival function is not increasing, the following additional constraint on $e(t, z)$ must be considered:

$$\left[\frac{d}{dt} e(t, z) \right] \cdot [t - z] + e(t, z) > 0 \quad (4.3)$$

Taking the negative derivative of Eq. 4.2 gives:

$$-\frac{d}{dt} S_e(t, z) = \left[\left[\frac{d}{dt} e(t, z) \right] \cdot [t - z] + e(t, z) \right] \cdot \ell_b(e(t, z) \cdot [t - z], z), \quad t \geq z \quad (4.4)$$

Followed by substitution of $z + \tau$ for t in Eq. 4.4 gives the observed cellular lifespan distribution due to the environmental effects of cells produced at time z , denoted $\ell_e(\tau, z)$, since the lifespan (i.e. τ) being evaluated in Eq. 4.4 is given by $t - z$:

$$\ell_e(\tau, z) = \left[\left[\frac{d}{d\tau} e(z + \tau, z) \right] \cdot \tau + e(z + \tau, z) \right] \cdot \ell_b(e(z + \tau, z) \cdot \tau, z), \quad \tau \geq 0 \quad (4.5)$$

The necessity of the constraint imposed by Eq. 4.3 is also evident in Eq. 4.5, as it ensures that the observed lifespan p.d.f. can never take on a negative value.

Let $f_{prod}(t)$ denote the production (i.e. input) rate of cells into the sampling space, which is typically a function of time through endogenous growth factors and/or exogenous drug. Then as previously presented (100), the number of cells present in the sampling space, denoted $N(t)$, is given by the integral of the product of the number of cells produced at a previous times and the probability that the cells produced at the previous times are present in the sampling space at the current time (i.e. $S_e(t, z)$):

$$\begin{aligned} N(t) &= \int_{-\infty}^t f_{prod}(u) \cdot S_e(t, u) du \\ &= \int_{-\infty}^t f_{prod}(u) \cdot \left[1 - \left[\int_0^{e^{(t,u)}[t-u]} \ell_b(\tau, u) d\tau \right] \right] du \end{aligned} \quad (4.6)$$

Eq. 4.6 gives the *key general equation* for modeling time variant cellular lifespans using an accelerated life model that allows for the incorporation of changes in the cellular lifespans due to both time variant effects of the environment following production (i.e. through $e(t, z)$) and due to time variance in the conditions cells are formed under at the time of production (i.e. through $\ell_b(\tau, z)$). The lower integration limit of $-\infty$ in Eq. 4.6 is to be interpreted to consider “all prior history” of the system that affects $N(t)$. Due to the finite lifespan of cells, the lower limit can alternatively be explicitly stated as t minus the maximal cellular lifespan, if known.

4.2.2 Incorporation of the effect of the environment

The constraints imposed on $e(t, z)$, particularly by Eq. 4.3, limit the functional forms that may be considered to incorporate the environmental effect. One particularly attractive formulation of $e(t, z)$ proposed here is given by:

$$e(t, z) = \begin{cases} \frac{\int_z^t g(u) du}{t - z} & \text{if } t > z, \\ g(t) & \text{if } t = z \end{cases}, \quad g(t) > 0 \quad (4.7)$$

where $g(t)$ is the environmental effect function, which is a measure of the effect of the current environmental conditions at time t . Thus, if $g(t) = 1$ then environmental conditions at time t would have no effect on the baseline lifespan distribution. Calculation of Eq. 4.7 when $g(t) = 1$ for all t results in a value of $e(t, z) = 1$ for all t and z such that $t \geq z$, agreeing with the interpretation of this function. Further inspection of Eq. 4.7 reveals that under this formulation $e(t, z)$ is the average value of the environmental effect function over the time period that a cell has been in the sampling space environment following production. Thus this mathematical formulation of $e(t, z)$ agrees with the definition given above as $e(t, z)$ being a *cumulative measure* of the effect of the environment on the cellular disposition. Furthermore, $e(t, z)$ given by Eq. 4.7 satisfies the constraint that $e(t, z) > 0$ and the constraint given by Eq. 4.3 (Appendix C.1). However, any number formulations of $e(t, z)$ that satisfy the above constraints are possible (i.e. formulations where $e(t, z)$ is not the average value).

4.2.3 Alternative formulations of the environmental effect

The above formulated accelerated life time variant lifespan model given by Eq. 4.2 and Eq. 4.6 represents just one formulation for incorporating the environmental effects on cellular disposition. Alternatively, the post-production time variant environmental effects could be incorporated using a time dependent relative risk model for the survival function (92), which represents the second class of the most commonly used models in survival analysis. Accordingly, the environmental effect function, now denoted by $h(t)$ for the relative risk model, directly affects the baseline hazard function, denoted by $\lambda_b(t, z)$, by altering the instantaneous risk of death/removal relative to the risk of death/removal given by the baseline hazard function. By definition (92, 110, 111), the hazard function is the rate of cell removal from the sampling compartment at time t conditional on survival of the cells to time t . Thus:

$$\lambda_b(t, z) = \frac{\ell_b(t-z, z)}{S_b(t, z)}, \quad t \geq z \quad (4.8)$$

If in the model formulation $h(t)$ directly affects $\lambda_b(t, z)$, as proposed above, then the observed hazard function, $\lambda_e(t, z)$, is given by:

$$\lambda_e(t, z) = \lambda_b(t, z) \cdot h(t) = \frac{\ell_b(t-z, z)}{S_b(t, z)} \cdot h(t), \quad h(t) > 0, \quad t \geq z \quad (4.9)$$

Resulting in the following observed survival function (92):

$$\begin{aligned}
S_e(t, z) &= \exp\left[-\int_z^t \lambda_e(v, z) dv\right] = \exp\left[-\int_z^t \lambda_b(v, z) \cdot h(v) dv\right] \\
&= \exp\left[-\int_z^t \frac{\ell_b(v-z, z)}{S_b(v, z)} \cdot h(v) dv\right], \quad h(t) > 0, t \geq z
\end{aligned}
\tag{4.10}$$

where $S_b(t, z)$ is given by Eq. 4.1. Similar to the accelerated life model, when $h(t) > 1$ the survival of the cells is reduced and conversely when $h(t) < 1$ the survival of the cells is prolonged. However, it is observed from the model formulation and the different entry of $g(t)$ and $h(t)$ into Eq. 4.2 (through Eq. 4.7) and Eq. 4.10, respectively, that the exact interpretation and the effect of the environmental effect function is not the same in the accelerated life and relative risk model formulations. Paralleling the derivation of Eq. 4.5, taking the negative derivative of Eq. 4.10 followed by substitution of $z + \tau$ for t gives the observed cellular lifespan distribution due to the environmental effects of cells produced at time z for the relative risk model:

$$\ell_e(\tau, z) = h(z + \tau) \cdot \frac{\ell_b(\tau, z)}{S_b(z + \tau, z)} \cdot \exp\left[-\int_z^{z+\tau} \frac{\ell_b(v-z, z)}{S_b(v, z)} \cdot h(v) dv\right], \quad h(t) > 0
\tag{4.11}$$

Furthermore, substitution of Eq. 4.10 into Eq. 4.6 as the observed survival function due to the environmental effects gives an alternative *general equation* for modeling time variant cellular lifespans that incorporates the effect of the environment using a relative risk model instead of an accelerated life model:

$$\begin{aligned}
N(t) &= \int_{-\infty}^t f_{prod}(u) \cdot S_e(t, u) du \\
&= \int_{-\infty}^t f_{prod}(u) \cdot \left[\exp \left[- \int_u^t \frac{\ell_b(v-u, u)}{S_b(v, u)} \cdot h(v) dv \right] \right] du
\end{aligned} \tag{4.12}$$

However, in contrast to the accelerated lifespan model above there is no direct physical interpretation of the effect of the environmental conditions (i.e. $h(t)$) on the probability of survival of the cells in the sampling compartment (92). The absence of a physical interpretation of $h(t)$ may limit the applicability of the relative risk model formulation from a physiological perspective.

4.3 Materials and methods

4.3.1 Simulations

A series of simulations were conducted to compare the two environmental effect models by varying the magnitude and shape of the environmental effect functions on the observed cellular response curve ($N(t)$). For all simulations the production rate, denoted by f_{prod}^{SS} , remained constant with a value of 3×10^9 cells/kg/day, the normal steady-state RBC production rate in humans (12). The constant baseline cellular lifespan p.d.f. followed a two parameter Weibull distribution with a mean of 120 days and a standard deviation of 15 days. The Weibull distribution was chosen due to the analytical solution to the integral given in Eq. 4.2 and in Eq. 4.10 if $g(t)$ and $h(t)$, respectively, are represented as polynomials. The mean and standard deviation were chosen to match typical RBC lifespan values in humans (11, 51-53). The environmental effect functions, $g(t)$ and $h(t)$ for the accelerated life and relative risk models, respectively, were arbitrarily chosen to result in a 16-fold range in mean steady-state cellular lifespans (μ_{SS}): 30, 60, 120, 240, and 480 days, which would be observed if the extremum from

baseline (i.e. $g(t), h(t)=1$) of the simulated environmental effect function profiles were maintained indefinitely. Three different environmental effect function profiles were considered: 1.) a gradual change in the environmental conditions from baseline followed by an immediate return to baseline, 2.) an immediate change from baseline followed by gradual return to baseline, and 3.) moderate to rapid change from baseline followed by the temporary establishment of a new steady-state environmental conditions before a final moderate to rapid return to baseline environmental conditions (Figure 4.1).

Specifically, profile 1 (Figure 4.1, Panels A and B) was simulated as follows:

$$g(t), h(t) = \begin{cases} 1 & \text{if } t \leq 0 \\ 1 + \left[\frac{M-1}{3 \cdot \mu_b} \right] \cdot t & \text{if } 0 < t \leq 3 \cdot \mu_b \\ 1 & \text{if } t > 3 \cdot \mu_b \end{cases} \quad (4.13)$$

where μ_b is the mean baseline cellular lifespan (i.e. 120 days) and M is a positive multiple of the baseline environmental effect function value that was standardized for each model to give steady-state, i.e. $g(t), h(t) = M$, mean cellular lifespans of 30, 60, 120, 240, and 480 days (i.e. 0.25X, 0.5X, 1X, 2X, and 4X of μ_b , respectively). Profile 2 (Figure 4.1, Panels C and D) was simulated as:

$$g(t), h(t) = \begin{cases} 1 & \text{if } t \leq 0 \\ M - \left[\frac{M-1}{3 \cdot \mu_b} \right] \cdot t & \text{if } 0 < t \leq 3 \cdot \mu_b \\ 1 & \text{if } t > 3 \cdot \mu_b \end{cases} \quad (4.14)$$

Finally, profile 3 (Figure 4.1, Panels E and F) was simulated as:

$$g(t), h(t) = \begin{cases} 1 & \text{if } t \leq 0 \\ 1 + \left[\frac{M-1}{\frac{1}{4} \cdot \mu_b} \right] \cdot t & \text{if } 0 < t \leq \frac{1}{4} \cdot \mu_b \\ M & \text{if } \frac{1}{4} \cdot \mu_b < t \leq \frac{11}{4} \cdot \mu_b \\ M - \left[\frac{M-1}{\frac{1}{4} \cdot \mu_b} \right] \cdot \left[t - \frac{11}{4} \cdot \mu_b \right] & \text{if } \frac{11}{4} \cdot \mu_b < t \leq 3 \cdot \mu_b \\ 1 & \text{if } t > 3 \cdot \mu_b \end{cases} \quad (4.15)$$

The observed number of cells, $N(t)$, were simulated using the accelerated life model given by Eq. 4.6 and Eq. 4.7 and the relative risk model given by Eq. 4.12. For the accelerated life model the mean cellular lifespan under steady-state environmental conditions (i.e. $g(t) = M$) is given by (Appendix C.2):

$$E_{\ell_e} \{T | z, g(t) = M\} = \frac{E_{\ell_e} \{T | z, g(t) = 1\}}{M} = \frac{E_{\ell_b} \{T | z\}}{M} \quad (4.16)$$

where T is a random cellular lifespan variable, $E_{\ell_e} \{\cdot\}$ is the mathematical expectation taken with respect to $\ell_e(\tau, z)$ (i.e. Eq. 4.5), and $E_{\ell_b} \{\cdot\}$ is the mathematical expectation taken with respect to $\ell_b(\tau, z)$. Thus from Eq. 4.16 the standard M value equaled 4, 2, 1, 0.5, and 0.25 for the accelerated lifespan model to achieve the selected steady-state mean cellular lifespans of 30, 60, 120, 240, and 480 days, respectively. For the relative risk model, the standardized value of M for $h(t)$ giving the above selected mean cellular lifespans under steady-state conditions was determined numerically from the mathematical expectation of T taken with respect to the negative first derivative of $S_e(t, z)$ (i.e. Eq. 4.11) for different steady-state values of $h(t)$. The resulting values of M for the relative risk model were 6.05×10^5 , 7.78×10^2 , 1, 1.29×10^{-3} , and 1.65×10^{-6} corresponding to mean steady-state cellular lifespan of 30, 60, 120, 240, and 480 days, respectively. Additionally, to directly observe the effect of $g(t)$ and $h(t)$ on the baseline

cellular lifespan distribution, the observed cellular lifespan distributions due to the environmental effect (i.e. $\ell_e(\tau, z)$) were plotted under steady-state environmental conditions for both models with $g(t), h(t) = M$ from above.

4.3.2 Environmental effect of busulfan administration

The accelerated life and relative risk environmental effect cellular lifespan PD models given by Eq. 4.6 and Eq. 4.12, respectively, were fit to the data following hematopoietic ablation by busulfan administration in sheep to present an example of how the environmental effects may be estimated by the two models.

All animal care and experimental procedures were approved by the University of Iowa Institutional Animal Care and Use Committee. Six healthy young adult sheep approximately 4 months old and weighing 29.9 ± 2.59 kg (mean \pm SD) at the beginning of the experiment were utilized. Animals were housed in an indoor, light- and temperature-controlled environment, with *ad lib* access to feed and water. Prior to study initiation, jugular venous catheters were aseptically placed under pentobarbital anesthesia. Intravenous ampicillin (1 g) was administered daily for 3 days following catheter placement.

The busulfan chemotherapy was chosen to ensure complete ablation of the bone marrow and erythropoietic cells without jeopardizing the animal health (112). Following catheter placement, 5.5 mg/kg busulfan was administered orally twice a day for three consecutive days. Intravenous ampicillin (1 g) was administered daily following chemotherapy initiation to prevent infection due to the ablation of the immune system. Animals were clinically monitored for adverse events of chemotherapy such as weight loss, wool loss, blood in urine or stools, fever, etc. Blood samples (~ 1 mL/sample) were

collected approximately once per day and measured for hemoglobin concentrations using an IL482 CO-oximeter (Instrumentation Laboratories, Watham, MA) and RBC concentrations using a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL). To prevent undue animal distress, studies were terminated and sheep were euthanized using an intravenous over-dosage of pentobarbital when the hemoglobin concentrations reached approximately 3 g/dL.

The production rate was assumed to be constant for $t \leq t_0$ (denoted by f_{prod}^{SS}), where t_0 is the time of initiation of the busulfan administration, and then to linearly drop to a production rate of 0 over 8 days ($T_{decline}$). The production rate was assumed to drop to 0 by 8 days post-initiation of the busulfan therapy because previous experiments indicated that by 8 days no colony-forming units erythroids (CFU-E) are present (112), which are the bone marrow erythroid progenitor cells that directly develop into hemoglobinized cells (12). The RBC lifespan distribution in the absence of environmental effects (i.e. $\ell_b(\tau, z)$) was assumed to be time invariant (i.e. $\ell_b(\tau, z) = \ell_b(\tau)$) and follow a Weibull distribution with a fixed mean (μ) of 114 days as previously determined in sheep (54). The standard deviation (σ) of the baseline lifespan distribution was assumed to be similar to that typically cited for humans and fixed to 15 days (52, 53). A time invariant baseline lifespan distribution was assumed since the focus of this work was the time variance of cellular lifespan due to *environmental effects* and to minimize the complexity of the analysis. Additionally, a time invariant baseline normal distribution of the RBC lifespan with mean μ of 114 days and standard deviation σ of 15 days was fitted to the data; however, the results were not presented in detail due to numerical problems encountered in the evaluation of relative risk environmental effect

model in this case. For the accelerated life model $e(t, z)$ was given by Eq. 4.7. A “bottom-up” approach was used to estimate the value of the environmental effect functions without creating a functional relationship to any measured variables (113), since the exact mechanism that causes the reduced lifespan of the RBCs is unknown. The environmental effect functions, $g(t)$ and $h(t)$, were semi-parametrically represented and assumed to be equal to 1 for $t \leq t_0$, and then follow a positive and continuous linear spline with constant forward extrapolation beyond the last node. The number of nodes utilized, which was four, was empirically chosen to provide sufficient flexibility to adequately fit the data from all subjects. To convert from number of cells per kg to concentration of cells, the estimated number of RBCs from Eq. 4.6 and Eq. 4.12 for the accelerated life and relative risk models, respectively, were divided by a fixed, previously determined, blood volume (BV) of 74.4 mL/kg (114). The hemoglobin concentrations were estimated by multiplying the estimated concentration of RBCs by the model estimated mean corpuscular hemoglobin (MCH) parameter in picograms per cell.

4.3.3 Computational details

All simulations and modeling were conducted using WINFUNFIT, a Windows (Microsoft) version evolved from the general nonlinear regression program FUNFIT (86), using ordinary least squares. The simulated and fitted models required a solution to multiple integrals. The integral in the observed survival functions (i.e. $S_e(t, z)$) given by Eq. 4.2 and Eq. 4.10 were analytically determined, as was the integral in Eq. 4.7 ($e(t, z)$) for the accelerated life model. Finally, the numerical solution to the outer integral from $-\infty$ to t in Eq. 4.6 and Eq. 4.12 was determined using the FORTRAN 90 subroutine QDAGS from the IMSL® Math Library (Version 3.0, Visual Numerics Inc., Houston,

TX). QDAGS is a univariate quadrature adaptive general-purpose integrator that is an implementation of the routine QAGS (95). The relative error for the QDAGS routine was set at 0.1% for all numerical integrations. The model given by Eq. 4.6 for the accelerated life model and by Eq. 4.12 for the relative risk model was simultaneously fit to both the sheep RBC and hemoglobin data by multiplying the estimated RBC concentrations by the estimated *MCH* parameter. The model fits to the sheep RBC and hemoglobin data were compared using the Akaike's Information Criterion (AIC) value, with the number of estimated parameters and spline coefficients included in the calculation (88).

4.4 Results

4.4.1 Simulations

Figure 4.2 displays the effect on the cellular response of a gradual change from baseline environmental effect (i.e. baseline effect given by $g(t)$ or $h(t)=1$) followed by an immediate return to baseline after three mean (μ_b) baseline cellular lifespans (Profile 1, Eq. 4.13), as displayed in Figure 4.1, Panels A and B for the accelerated life and relative risk models, respectively. An increase (decrease) in the value of $g(t)$ or $h(t)$ in the accelerated life and relative risk models caused a decrease (increase) in the cell number. For the accelerated life model, all five magnitudes of simulated environmental effect function values resulted in distinct cellular response curves. However, for the relative risk model the two lowest values of simulated environmental effects, corresponding to the two highest μ_{ss} (i.e. 2X and 4X of μ_b), resulted in cellular response curves that virtually overlaid each other. For the accelerated life environmental effect model, all cell number response curves deviated and returned to baseline cell numbers at the same time regardless of the magnitude of the perturbation from steady-state but for

the relative risk model the time to return to steady-state appears to vary with both the magnitude and direction of the change in the environmental effect functions. For both models, the return to baseline cell numbers begins immediately upon the drop in the environmental effects, as can be observed from the “break” in the curve at 360 days (i.e. $3 \cdot \mu_b$). Additionally, as can be analytically determined from Eq. 4.6 or Eq. 4.12 when the cellular production rate and lifespan distribution are constant (i.e. at steady-state) with no environmental effects (i.e. $g(t)$ or $h(t)=1$) (Appendix C.3), the baseline number of cells is given by $f_{prod}^{SS} \cdot \mu_b = 360 \times 10^9$ cells/kg.

Figure 4.3 displays the effect on the cellular response of an immediate change from baseline environmental effects followed by gradual return to baseline environmental effects (Profile 2, Eq. 4.14), as displayed in Figure 4.1, Panels C and D for the accelerated life and relative risk models, respectively. Similar to before, an increase (decrease) in the environmental effects caused a decrease (increase) in the cell number. Again for the relative risk model the two lowest values of simulated environmental effect function values resulted in cellular response curves that virtually overlaid each other. Unlike the previously simulated cell number response curves (Figure 4.2), the shapes of the curves are “smoother,” as expected.

Figure 4.4 displays the effect on the cellular response of a moderate to rapid change from baseline environmental effects followed by a new temporary steady-state environmental effect function values and a final moderate to rapid return to baseline environmental effects (Profile 3, Eq. 4.15), as displayed in Figure 4.1, Panels E and F for the accelerated life and relative risk models, respectively. Steady-state cell numbers were achieved within the time period of perturbation of the environmental effects for 4 out of

the 5 simulated steady-state environmental effect function values. However, steady-state cell numbers were not achieved for the lowest value of the environmental effects, which corresponds to the longest μ_{ss} (i.e. 4X of μ_b). As designed, the same steady-state number of cells was achieved for both models when steady-state cell numbers were reached. The relative risk model reached the new steady-state values more rapidly than the accelerated life model for the same standardized environmental effect function profiles. Unlike the previous simulated profiles using the relative risk model (Figure 4.2 and Figure 4.3), each level of environmental effects resulted in a distinct cellular response profile. Additionally, the time for the relative risk model to return to baseline cell number following the drop in the environmental effects back to baseline appeared to depend on the direction and the magnitude the environmental effects were changed. For the accelerated life model, the time to return to the baseline cell number was independent of the direction and magnitude the environmental effects were changed.

Figure 4.5 displays the effect of steady-state environmental conditions on the observed cellular lifespan distribution for both the accelerated life and relative risk models. As expected, as μ_{ss} increased the mass of the distribution shifted to longer lifespans. Additionally, the variance in the lifespan also increased with increasing μ_{ss} for both models. Furthermore, under steady-state environmental conditions the observed lifespan distributions were identical for the accelerated life and relative risk models when they were constrained to have the same mean observed cellular lifespan arising from a baseline Weibull distribution. However, this is not generally true for any baseline cellular lifespan distribution. If a normal distribution is instead used as the baseline distribution

then the observed cellular lifespan densities would not have been equal even when constrained to have the same mean observed cellular lifespan.

4.4.2 Environmental effect busulfan administration

The profiles and simultaneous fit to the RBC and hemoglobin concentrations using the accelerated life and relative risk models, as well as the estimated environmental effect function values, for a representative sheep following hematopoietic ablation by busulfan administration at time 0 is displayed in Figure 4.6. In general, the sheep RBC and hemoglobin concentrations gradually declined for the first two weeks following busulfan administration, and then rapidly declined at an increased rate over the next week before study termination. The rapid decline in the RBC and hemoglobin concentrations was paralleled by a rapid increase in the estimated environmental effect function values. Both models fit the data similarly well and gave similar parameter estimates (Table 4.1), except for the parameters related to the environmental effects, consistent with the different magnitude of environmental effects necessary to achieve the same steady-state cell number. The relative risk model gave the lowest mean AIC value; however, in 2 of the 6 subjects the AIC was lowest for the accelerated life model. The steady-state cellular production rate (f_{prod}^{SS}) was estimated at 6.20 ± 0.384 and $6.15 \pm 0.402 \cdot 10^9$ cells/kg/day (mean (C.V.)) for the accelerated life and relative risk models, respectively, and the mean corpuscular hemoglobin (MCH) was estimated at 11.8 pg per cell for both models. The average environmental effect function values (e_{avg}) from the initial time of busulfan administration (t_0) to the last observation (t_{last}) were calculated at 3.49 ± 1.03 and 115 ± 118 for the accelerated life and relative risk models (Note: for the accelerated life model, $e_{avg} = e(t_{last}, t_0)$, where $e(t, z)$ is given by Eq. 4.7). Using a normal p.d.f. instead of a

Weibull p.d.f. to represent the baseline cellular lifespan distribution for the accelerated life environmental effect model resulted in very similar estimates of the model parameters ($6.12 \pm 0.271 \cdot 10^9$ cells/kg/day, 11.8 ± 0.602 pg, and 3.52 ± 1.02 for f_{prod}^{SS} , MCH , and e_{avg} , respectively). The use of a normal distribution for the relative risk environmental effect model could not be successfully implemented due to excessive numerical integration errors associated with the lack of an analytical solution to the Eq. 10 when the environmental effects are represented as a linear spline function.

Assuming that no environmental effect was present but allowing the cellular production rate to drop to 0 immediately following busulfan administration initiation did not result in as good of a fit to the data compared to the environmental effect model, as shown for a representative subject by the dashed and solid lines, respectively, in Figure 4.7. Additionally, assuming the cellular production rate dropped linearly over 8 days, as done above, and no environmental effect but allowing the mean and standard deviation of the baseline cellular lifespan distribution to be estimated also did not result in as good of fit to the data (dotted line, Figure 4.7). The AIC's were 37.0 ± 27.6 , 3.39 ± 16.2 , and -37.3 ± 30.4 for the instantaneous stop in production (no environmental effect), mean and standard deviation of cellular lifespan estimated (no environment effect), and accelerated life environmental effect models, respectively. The AIC was -38.4 ± 30.0 for the relative risk environmental effect model. In fact, both the accelerated life and relative risk environmental effects models had a lower AIC value for all subjects than either of the above sub-models that assumed no environmental effect. Furthermore, without the environmental effect being incorporated the mean baseline cellular lifespan distribution was estimated at 41.9 ± 13.4 days, much lower than previous estimates in sheep of $114 \pm$

12.0 days using a [^{14}C] cyanate label (54) and 75 to 153 days using ^{59}Fe pulse-chase methodology (115).

4.5 Discussion

A PD model for incorporating time variant environmental effects on cellular lifespans and cellular responses was formulated and presented using the two basic classes of models from survival analysis, an accelerated life and a relative risk model. The models extend previous models of time variant cellular lifespans (91, 100), allowing for time variance in the cellular lifespans to be due to both changes in the baseline cellular lifespan distribution (i.e. $\ell_b(\tau, z)$) and changes in the environmental effects (i.e. $g(t)$ or $h(t)$). The model formulation allows for a flexible incorporation of the environmental effect function with minimal constraints on the form of the function representing the environmental effects other than what is dictated from obvious physiological considerations. The environmental effect of drugs on the cellular lifespan can readily be incorporated into the model through the environmental effect functions (i.e. $g(t)$ and $h(t)$), which can be modeled to depend on time through changes in drug concentrations. Additionally, multiple changes in the environment that effect the cellular lifespan can be incorporated into the model through the environmental effect functions if a specific model of these functions of the environmental changes is proposed. The model formulation also allows for the utilization of any arbitrary p.d.f. to represent the baseline cellular lifespan distribution for the accelerated life model. The formulated accelerated life and relative risk models of environmental effects were successfully fitted to the RBC and hemoglobin concentration profiles of 6 sheep following hematopoietic ablation by busulfan administration. Comparisons of the environmental effect models to models

without the environmental effect component indicated that the environment effect needs to be considered to more fully explain the observed change in the cellular response.

Simulations demonstrated a different shape to the cellular responses following standardized changes in the environmental effects using the accelerated life model compared to the relative risk model (Figure 4.2 through Figure 4.1). Another prominent difference in the models demonstrated by the simulations is the time to return to steady-state cellular response following the return of the environmental conditions to baseline. The time to return to baseline depended on the direction and magnitude of the changes in the environmental effects for the relative risk model, but appeared to be independent of the environmental effect change for the accelerated life model. The shape in the cellular responses is in some ways intrinsic to the model formulations, as the environmental effect functions, $g(t)$ and $h(t)$, enter the observed survival functions (Eq. 4.6 through Eq. 4.7 and Eq. 4.12, respectively) in very different mathematical forms for the accelerated life and relative risk models, respectively. This can be illustrated by examining the relationship between the steady-state cell number, denoted by N_{SS} , and the steady-state environmental effects when assuming both the cellular production rate and the baseline cellular lifespan (i.e. $\ell_b(\tau, z) = \ell_b(\tau)$) are constant. From Appendix C.2 and Appendix C.3 it is evident that the relationship between N_{SS} and $g(t)$ for the accelerated life model is given by:

$$N_{SS} = \frac{f_{prod}^{SS} \cdot \mu_b}{g_{SS}} \quad (4.17)$$

where g_{SS} is the steady-state environmental effects. Thus the steady-state cellular response is inversely proportional to the steady-state environmental effects for the

accelerated life environmental effect model. In contrast, for the relative risk model the relationship between N_{SS} and $h(t)$ at steady-state, denoted by h_{SS} , is given by (using results from relative risk regression modeling (92)):

$$N_{SS} = f_{prod}^{SS} \cdot \int_0^{\infty} \left[1 - \int_0^u \ell_b(\tau) d\tau \right]^{h_{SS}} du \quad (4.18)$$

Obviously the relationship between the steady-state cellular response and the environmental effects for the relative risk environmental effect model is more complex than the inversely proportional relationship that exists for the accelerated life model.

Additionally, there are several limitations to the relative risk model that are not present for the accelerated life model. One limitation is the absence of a direct physical interpretation of the environmental effect function on the probability of the survival of cells in the relative risk model (92). This limitation reduces the ability to directly interpret the meaning of the changes in the environmental effects. A second limitation of the relative risk model is in not being able to define the baseline hazard function ($\lambda_b(t, z)$) in Eq. 4.8 when a dirac delta function is used as the baseline lifespan distribution. In that case, $\ell_b(\tau, z) = \delta(\tau - a, z)$, where $\delta(\cdot, z)$ is a dirac delta function defined at time z and a is the single “point” cellular lifespan. Defining $\lambda_b(t, z)$ as zero except for at $t = z + a$ results in a model that cannot account for an environmental effect since the observed hazard function in Eq. 4.9 will be equal to the baseline hazard function regardless of the value of the environmental conditions ($h(t)$). Therefore, the relative risk environmental effect model does not apply to a “point distribution” of cellular lifespans, which is often a practical and sufficient simplification of the cellular lifespan PD model (6). The accelerated life model does not have this limitation.

A clear distinction of the presented environmental effects cellular lifespan model must be made from other cellular response PD models where environmental effect mediated cell removal is a random, i.e. age-independent, process. Examples of these types of models include simplified models that assumed a time invariant “point distribution” of cellular lifespans and a time invariant first-order removal processes in addition to the age-dependent removal processes (116, 117). The removal of cells from the sampling space in the current models is still an age-dependent process; however, the age-dependent removal is modified by the environmental effects. This removal process due to the environmental effects is different than that given by environmental effects that cause cell removal by an age-independent mechanism, as would be the case if some cells are removed by first order processes prior to the predetermined lifespan (116, 117). A discussion of modeling environmental effects that cause an age-independent removal process is beyond the scope of this work.

Hematopoietic ablation of 6 sheep by busulfan administration caused very little initial increase in the estimated environmental effects in the first week, followed by a rapid increase in $g(t)$ and $h(t)$ beginning between 1 and 2 weeks following busulfan administration initiation (Figure 4.6). The exact cause of the environmental effect increase is unknown, but could be do to a variety of factors. One likely candidate for accelerating the removal of the RBCs from the systemic circulation is an increase in the oxidative stress that the RBCs are exposed to in the systemic circulation. Previous research has demonstrated an increase in the oxidative stress status of individuals undergoing high-dose chemotherapy for peripheral blood stem cell transplantation through a decrease in the plasma antioxidant concentrations and a corresponding increase

in oxidative stress markers (105, 118). Furthermore, the peak increase in the oxidative stress markers corresponded to 7 to 9 days following the initiation of high-dose chemotherapy, similar to the 1 to 2 week time frame for the rise in the estimated environmental effects. Other studies have also demonstrated increases in free radical generation and lipid peroxidation *in vivo* following chemotherapy administration (119). Increases in oxidative stress and oxidant damage of RBCs are important determinants in the premature removal of erythrocytes from the systemic circulation (104, 120). Since oxidative damage to RBCs would accumulate over time, it is mechanistically appealing to use the accelerated life environmental effect model compared to the relative risk model, as the accelerated life model is formulated in terms of the cumulative effect of the environment over the lifespan of a cell (i.e. $e(t, z)$ in Eq. 4.6), even though on average AIC was lowest for the relative risk model (Table 4.1). Additionally, other researchers also hypothesize that RBC senescence is primarily due to the cumulative stress a cell is exposed to during its time in the systemic circulation (52), further supporting the use of the accelerated life environmental effect model in this instance. However, since no markers of oxidant stress were measured in the present study other environmental effects of hematopoietic ablation by busulfan administration certainly could have caused the observed increased removal of cells.

The importance of including an environmental effect into the cellular lifespan distribution model under certain situations is illustrated with a representative subject fits given in Figure 4.7 for models that assumed no environmental effects. Neither estimation of the baseline mean and standard deviation of the baseline cellular lifespan distribution nor assuming that busulfan administration immediately dropped the production rate to

zero resulted in models that fit the observed RBC data as well, based on AIC. While the assumption of the functional form of the RBC production rate (i.e. $f_{prod}(t)$) will influence the observed cellular response (Figure 4.6 and Figure 4.7) and the estimated environmental effects (Figure 4.6), alternative assumptions on the production rate, such as it remained positive beyond 8 days, would result in a more gradual decline in the RBC and hemoglobin concentrations for the same given environmental effect.

The proposed environmental effect PD models may have applications in a variety of disciplines involving the PDs and/or kinetics of cellular populations that are exposed to changing environmental conditions over time. One area of potential application is the management of anemia in patients with limited renal function by treatment with erythropoietin and RBC transfusions (103). These patients often have impaired RBC survival due to the uremic environment that alter the RBC membrane and cytoskeleton characteristics and are subjected to increased RBC oxidative stress through hemodialysis (103, 106). The study of the effect of different storage conditions and time periods on RBC aging and the survival of the transfused RBC cells is also closely related to the proposed models, where storage induced acceleration of the aging process is thought to occur (109). As discussed above, the model has further potential applications in understanding the decreases in RBC cell survival due to increases in the concentration of reactive oxygen species that RBCs are exposed to from chemotherapy administration (104, 105). Finally, other areas of application include dynamically accounting for decreased platelet activation and increased survival due to administration of antiplatelet drugs and decreased survival of cancerous cells due to changes in the availability of nutrients from the administration of anti-angiogenic drugs (107, 108).

4.6 Conclusion

In summary, the previously formulated time variant cellular lifespan PD models were extended to incorporate environmental effects on the cell lifespan using the two basic classes of models from survival analysis: accelerated life and relative risk models. Due to the more direct physical interpretation of the environmental effects, a relatively simple steady-state relationship between cellular response and environmental effects, and the ability to reduce the model to a “point” baseline lifespan distribution, the accelerated life environmental effect model may offer some advantages. The analysis of a data example using RBC and hemoglobin concentration data of 6 sheep following hematopoietic ablation by busulfan administration indicated the environmental effects began to increase from baseline 1 to 2 weeks following initiation of ablation and that competing models that did not incorporate an environmental effect could not describe the data as well. The presented cellular lifespan PD model allows for the incorporation of arbitrary changes in the conditions of the cellular environment and the effect of these changes on the probability of age-dependent cellular survival.

Table 4.1. Parameter summary and comparison between the accelerated life and relative risk environmental effect cellular lifespan PD model ($n = 6$).*

Parameter	Accelerated Life Model		Relative Risk Model	
	Mean	SD	Mean	SD
AIC	-37.3	30.4	-38.4	30.0
f_{prod}^{SS} (10^9 cells/kg/day)	6.20	0.403	6.15	0.402
$T_{decline}$ (days)	8.00**	--	8.00**	--
MCH (pg)	11.8	0.595	11.8	0.614
BV (mL/kg)	74.4**	--	74.4**	--
μ (days)	114**	--	114**	--
σ (days)	15.0**	--	15.0**	--
e_{avg} †	3.49	1.03	115	118

* A Weibull distribution of cellular lifespans was assumed and the models were fitted to sheep RBC and hemoglobin concentrations following hematopoietic ablation with busulfan.

** Fixed parameter

† Secondary parameter, average environmental effect function values from the time of initiation of busulfan administration (t_0) to the last observation time (t_{last})

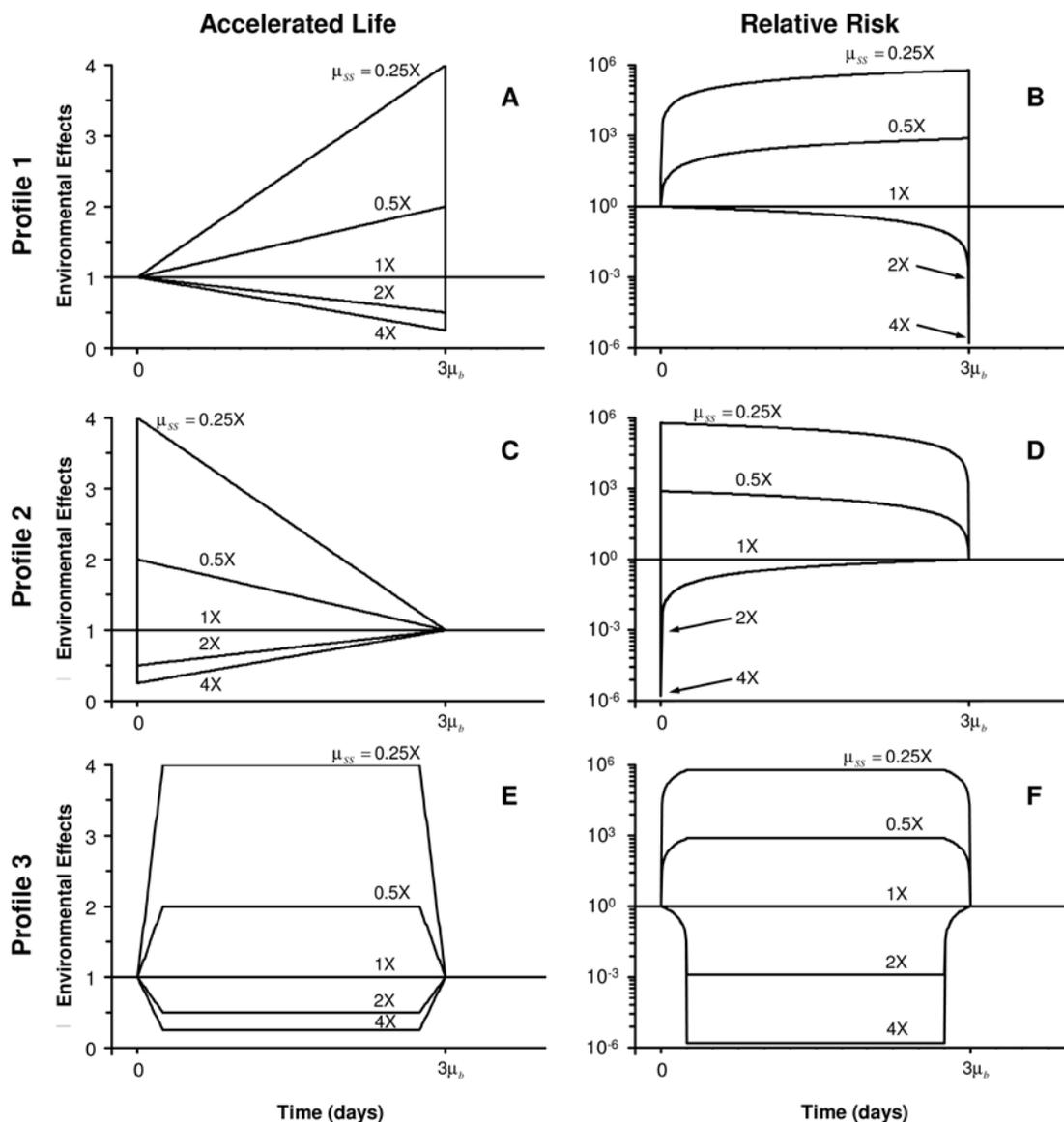


Figure 4.1. The three simulated environmental effect function profiles for the accelerated life and relative risk models.

Profile 1 is given by Panels A and B, Profile 2 is given by Panels C and D, and Profile 3 is given Panels E and F for the accelerated life (Panels A, C, and E) and the relative risk (Panels B, D, and F) models. μ_{ss} is the steady-state mean cellular lifespan, as a multiple of the baseline mean cellular lifespan (i.e. 0.25X, 0.5X, 1X, 2X, and 4X), that would be reached if the extremum from baseline of the simulated environmental effect function profile was maintained indefinitely and μ_b is the mean baseline cellular lifespan. The simulated environmental effect functions for the relative risk model (right Panels) are plotted on a log y-axis scale due to the extreme values needed to achieve the multiples of μ_b .

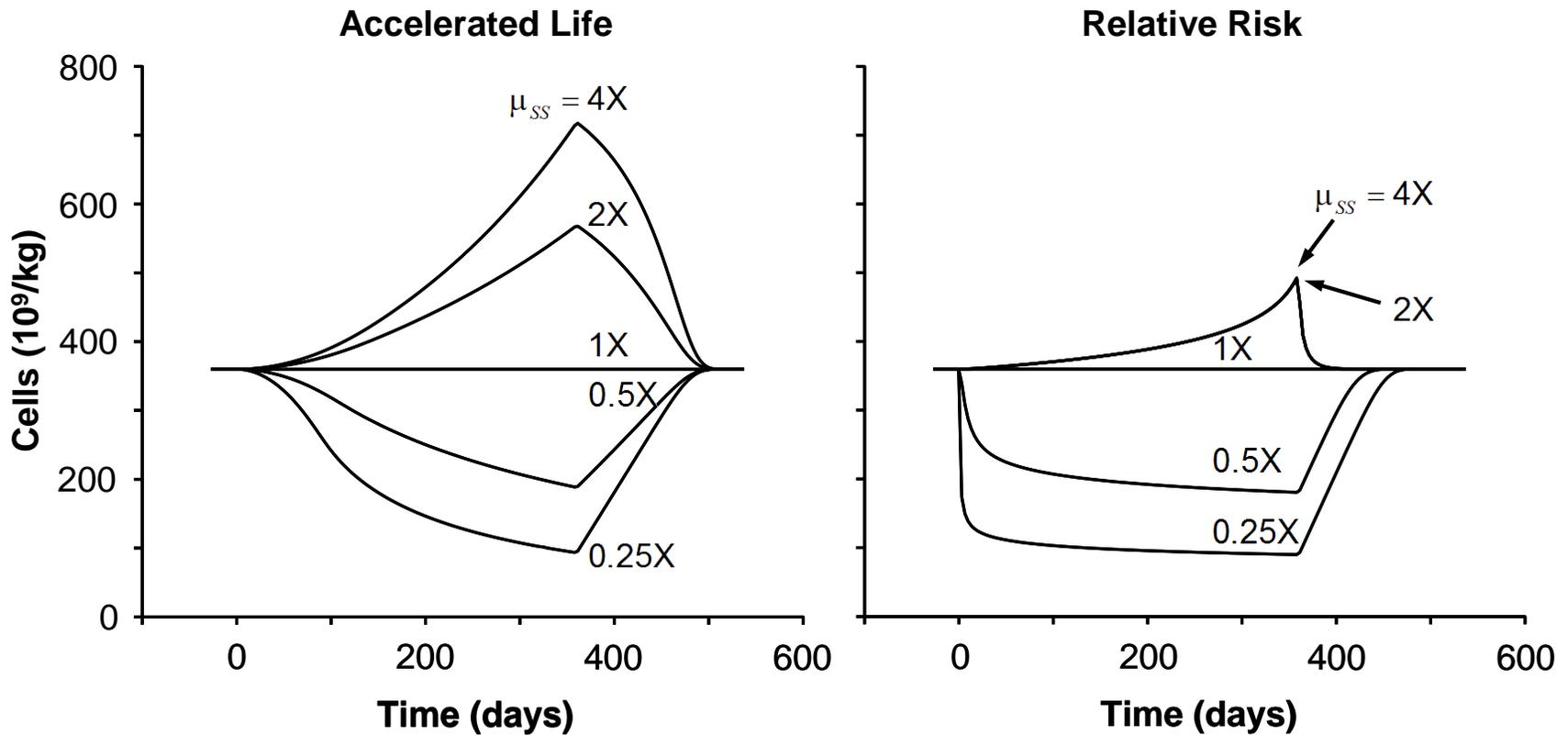


Figure 4.2. The effect on the cellular response of a gradual change from baseline environmental effects followed by an immediate return to baseline environmental effects. The change in the environmental effects is given by Profile 1 (Eq. 4.13 as displayed in Figure 4.1, Panels A and B for an accelerated life and a relative risk environmental effect models, respectively). The cellular production rate and baseline lifespan distribution were constant with values of 3×10^9 cells/day and a mean and standard deviation of 120 and 15 days, respectively.

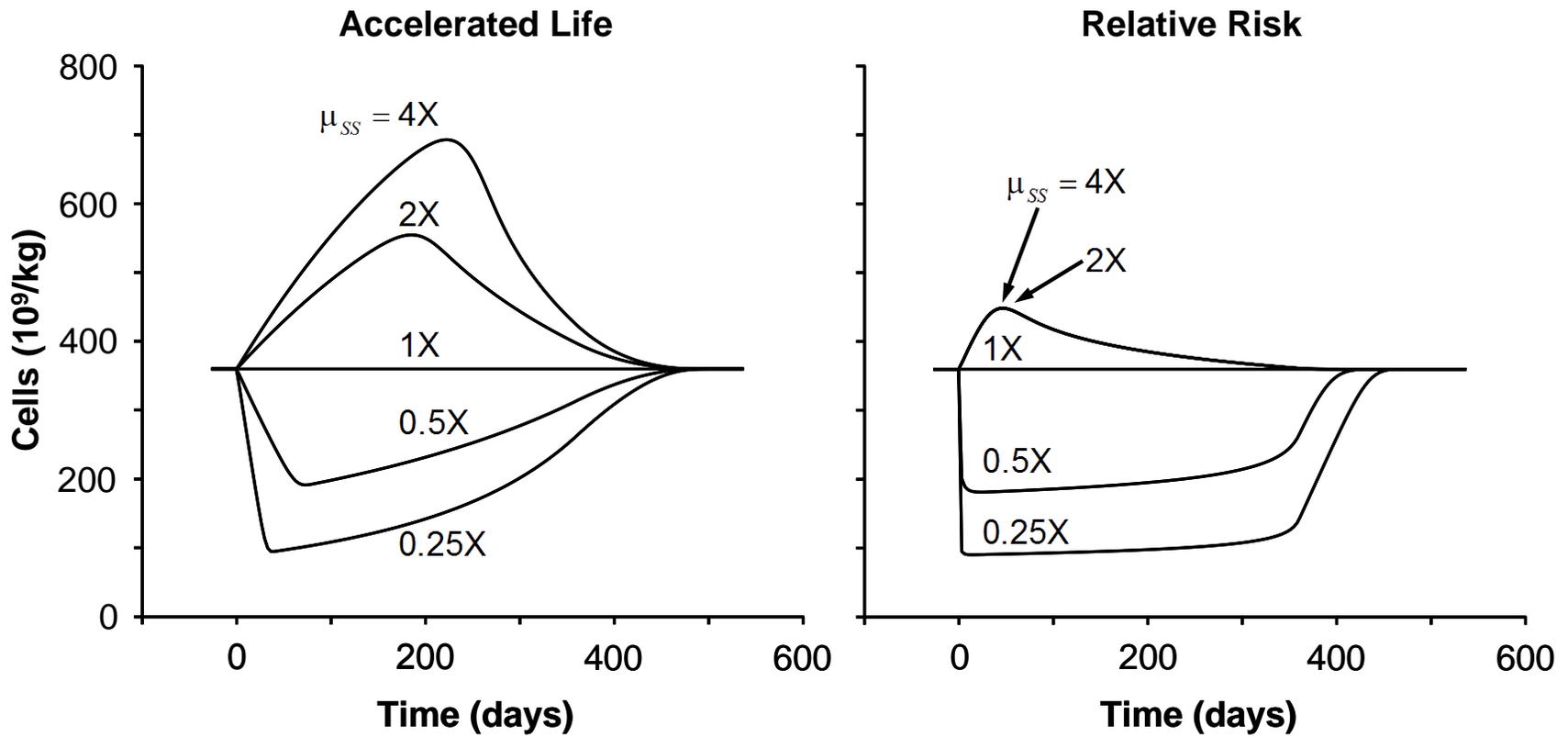


Figure 4.3. The effect on the cellular response of an immediate change from baseline environmental effects followed by gradual return to baseline environmental effects. The change in the environmental effects is given by Profile 2 (Eq. 4.14 as displayed in Figure 4.1, Panels C and D for an accelerated life and a relative risk environmental effect models, respectively). The cellular production rate and baseline lifespan distribution were constant with values of 3×10^9 cells/day and a mean and standard deviation of 120 and 15 days, respectively.

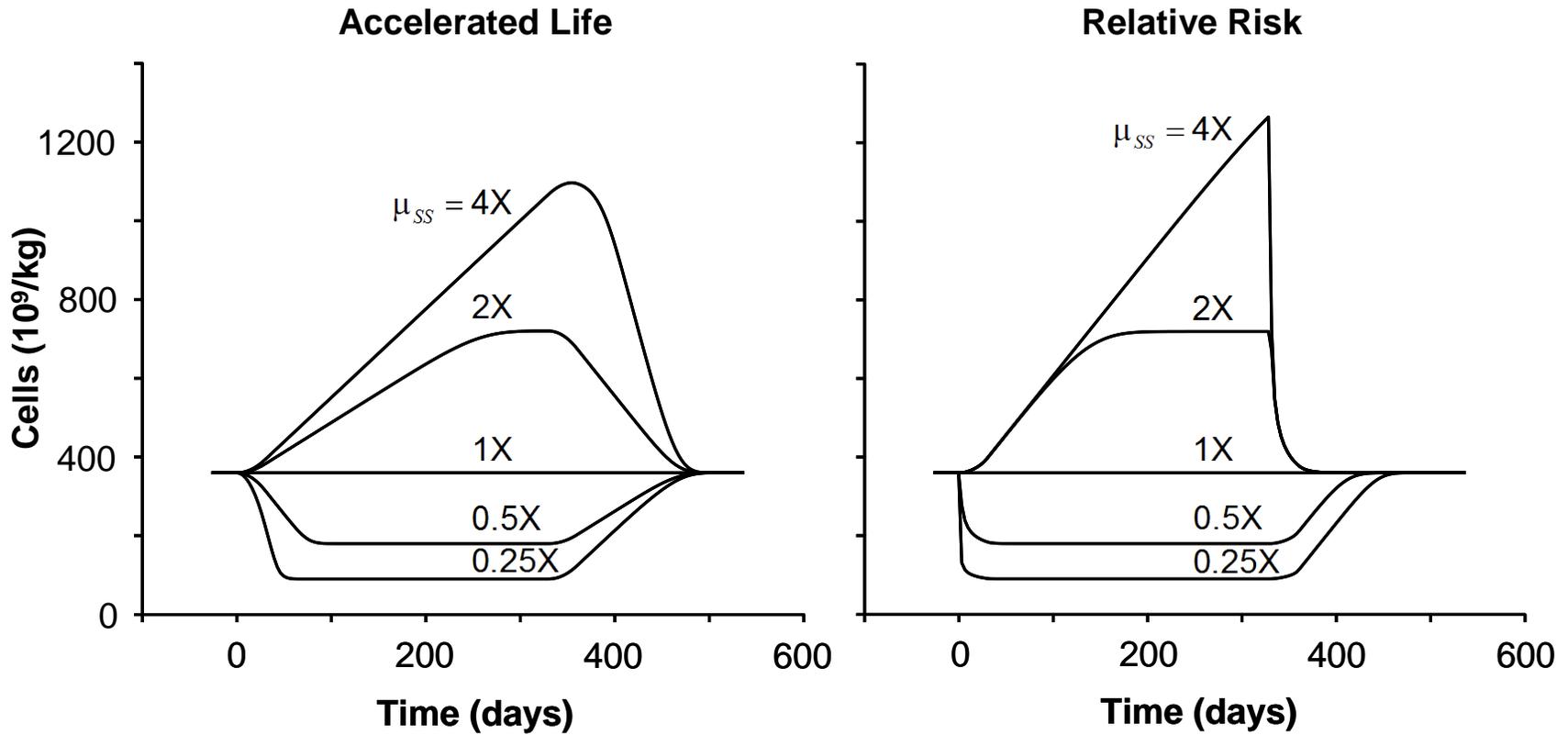


Figure 4.4. The effect on the cellular response of a moderate to rapid change from baseline environmental effects followed by a new steady-state environmental effect and a final moderate to rapid return to baseline environmental effects. The change in the environmental effects is given by Profile 3 (Eq. 4.15 as displayed in Figure 4.1, Panels E and F for an accelerated life and a relative risk environmental effect models, respectively). The cellular production rate and baseline lifespan distribution were constant with values of 3×10^9 cells/day and a mean and standard deviation of 120 and 15 days, respectively.

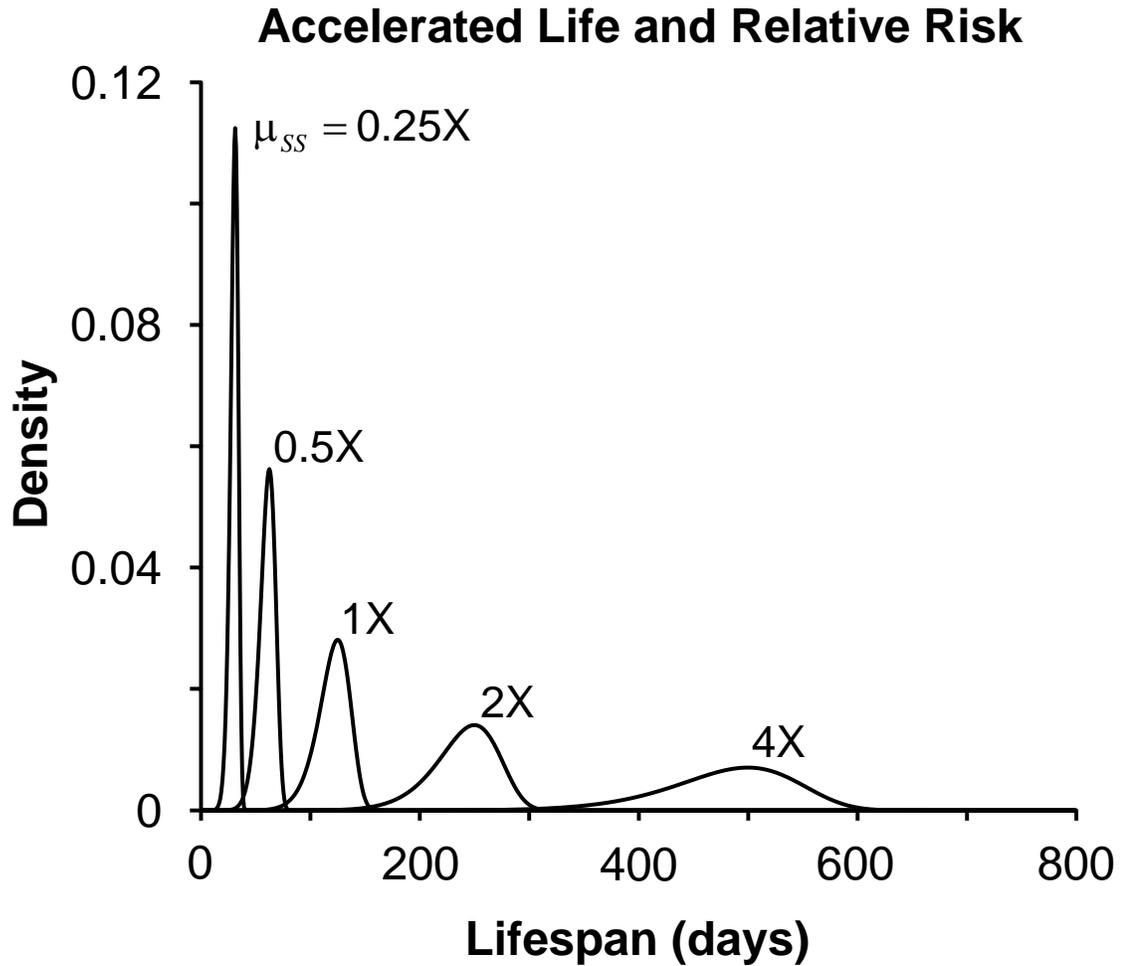


Figure 4.5. The effect of steady-state environmental conditions ($g(t), h(t) = M$) on the observed cellular lifespan distribution. The cellular lifespan distribution ($\ell_e(\tau, z)$) is given by Eq. 4.5 and Eq. 4.11 for the accelerated life and relative risk models, respectively. The baseline lifespan distribution ($\ell_b(\tau, z), 1X$) was a Weibull distribution with a mean and standard deviation of 120 and 15 days, respectively.

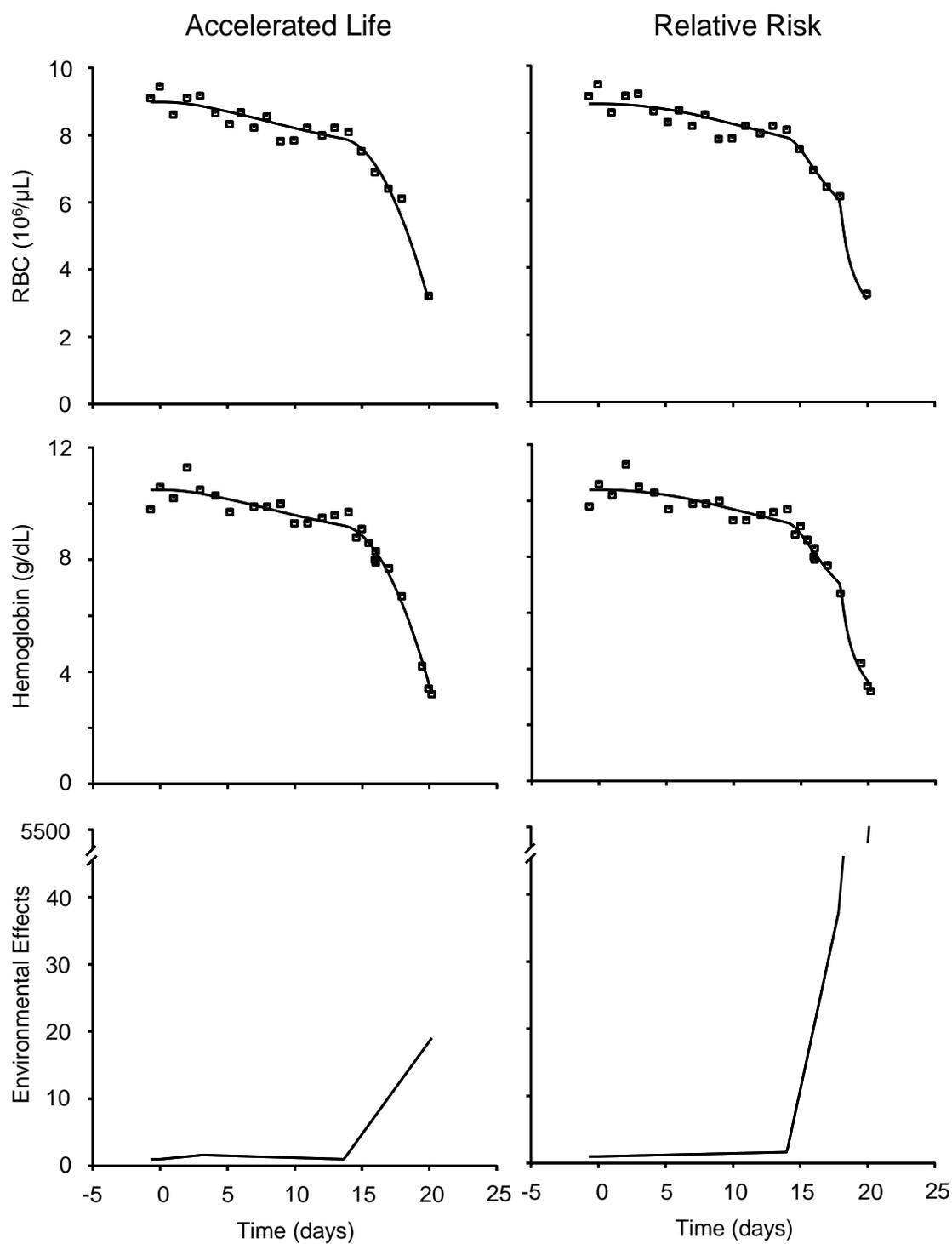


Figure 4.6. Representative animal subject fit (lines) of the environmental effect cellular lifespan PD models to the observed sheep RBC and hemoglobin concentrations (squares) following hematopoietic ablation with busulfan. The estimated environmental effects overtime are displayed in the bottom panels.

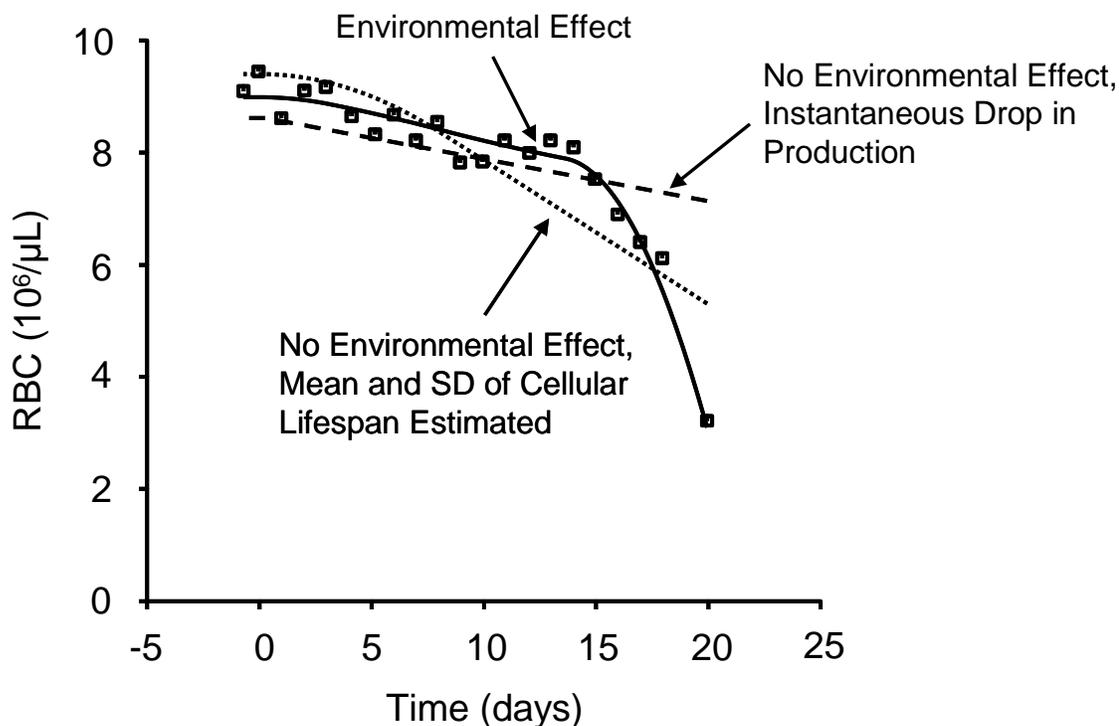


Figure 4.7. Accelerated life environmental effect model fits (lines) to a representative animal subject RBC concentrations (squares) following hematopoietic ablation with busulfan assuming three different model scenarios. 1.) Environmental Effect (solid line) – environmental effects estimated, production rate drops linearly over 8 days to 0, mean and standard deviation of the baseline cellular lifespan distribution fixed to 114 and 15 days, respectively, 2.) No Environmental Effect, Instantaneous Stop of Production (dashed line) – no environmental effects incorporated, production rate drops instantly to 0 at time 0, mean and standard deviation of the baseline cellular lifespan distribution fixed to 114 and 15 days, respectively, and 3.) No Environmental Effect, Mean and SD of Cellular Lifespan Estimated (dotted line) – no environmental effects estimated, production rate drops linearly over 8 days to 0, mean and standard deviation of the baseline cellular lifespan distribution estimated (37.6 and 1.47 days, respectively, for this subject).

**CHAPTER 5. THE EFFECT OF ANTICOAGULANT, STORAGE
TEMPERATURE, AND DILUTION ON CORD BLOOD HEMATOLOGY
PARAMETERS OVER TIME**

5.1 Introduction

Phlebotomy blood loss for clinical laboratory testing in the neonatal intensive care unit (NICU) is a major contributor to anemia and the resultant red blood cell (RBC) transfusions administered to critically ill, very low birth weight (VLBW) infants (121-123). Indeed, reduction in the volume of blood drawn for laboratory testing has been shown to reduce the number of RBC transfusions (122, 123). Therefore, the development of laboratory methodologies that require smaller blood volumes for clinical and research testing is an important objective for improving neonatal care by preventing anemia and thereby avoiding the risks and cost associated with RBC transfusion.

Hematology analyzers utilizing flow cytometry are routinely used to provide rapid, reliable, and accurate determination of hematology parameters in the clinical setting. Generally, blood samples are collected in tubes containing the recommended anticoagulant, ethylenediaminetetraacetic acid (EDTA), and sent to the laboratory for immediate analysis. In the setting of the NICU, 250 to 500 μL samples of whole blood are typically required for hematological testing. In the NICU setting, blood collected for clinical laboratory testing 1.) is often drawn in heparin, or anticoagulants other than EDTA, and 2.) is often drawn in volumes that are in excess of that needed. If properly saved and labeled, the limited volumes of leftover anticoagulated whole blood ordered for clinically indicated purposes could be retrieved, diluted, and analyzed to yield valuable clinical and/or research data without the need for drawing additional blood.

Available samples are commonly collected in sodium heparin containing tubes for analysis of blood gases, electrolytes, and other blood chemistries.

Previous studies in adults have demonstrated the stability of many hematological parameters over time (124-127) and have documented which parameters are affected when sodium heparin is used in place of EDTA (126, 127). None of the studies have investigated these effects on fetal or neonatal blood, nor have they investigated the effect of sample dilution on hematological parameters. Furthermore, these effects have not been evaluated for the most recent hematological variables analyzed with the newer, highly sophisticated hematology analyzers. Examples of these newer measurements include the hematopoietic progenitor cell (HPC) count, nucleated red blood cell (NRBC) count, immature reticulocyte fraction (IRF), and reticulocyte hemoglobin content (RET He).

Therefore, using fetal umbilical cord blood as a surrogate for neonatal blood, the objective of the current study was to determine the effect of EDTA versus sodium heparin on selected hematological parameters kept at different storage temperatures for up to 72 hours. We also sought to examine the effect of a 1:5 dilution of cord blood on hematological parameters using the manufacturer's cellular diluting solution while maintaining samples at room temperature for 4 hours. The findings of these studies have important clinical and research implications for critically ill, VLBW infants relative to reducing the volume of blood required for laboratory testing and the resultant number of RBC transfusions.

5.2 Materials and methods

Eight study subjects born after uncomplicated pregnancy, labor and delivery were enrolled. This included 6 term and 2 preterm (< 37 weeks post-menstrual age) infants. All

procedures were approved by the University of Iowa Human Subject Internal Review Board Committee and parental written consent was obtained for all study subjects.

Immediately after delivery of the placenta, fetal cord blood samples were collected in EDTA and sodium heparin containing Vacutainer® tubes (BD Vacutainer® sodium heparin, 143 USP Units, Reference #367874, and K₂ EDTA, 10.8 mg, Reference #367899, Franklin Lakes, NJ, USA). Only tubes that filled completely were included. Blood samples from individual collection tubes were split into twelve 0.5 mL aliquots and stored at room temperature (~20° C) or 4° C (6 aliquots per storage temperature). Selected hematological parameters were determined serially on individual aliquots over 72 hours, the period of time over which stored, retrieved blood remaining from clinical analyses might commonly be available. Separate aliquots from each anticoagulant tube at the two temperatures were measured in manual mode at 0, 4, 8, 24, 48, and 72 hours using the Sysmex XE-2100 automatic hematology analyzer (Sysmex Corporation, Kobe, Japan). The analyzer was calibrated and quality controlled according to the manufacturer's recommendations. Immediately after collection, aliquots from both the EDTA and heparin samples were diluted 1:5 with the cellular suspension medium CELLPACK (Sysmex Corporation). The diluted EDTA and heparin blood samples were aliquoted in five 0.2 mL volumes and stored at room temperature for up to 4 hours, the estimated period of time before diluted blood samples might commonly be analyzed. Aliquots of the 1:5 diluted blood samples were analyzed in capillary mode after 0, 0.5, 1, 2, and 4 hours using the same hematology analyzer.

The effects of anticoagulant and dilution at time 0 were analyzed for the hematological parameters using a linear mixed effects model, with subject as a random

effect and anticoagulant, dilution, and their interaction as fixed effects. The effects of anticoagulant and storage temperature over time for undiluted cord blood hematological results were analyzed using a linear mixed effects repeated measures model, with subject as a random effect and anticoagulant, temperature, time, and their interactions as fixed effects. The effect of anticoagulant over time on diluted cord blood was analyzed similarly using subject as a random effect, and using anticoagulant, time, and their interactions as fixed effects.

The correlation between repeated measurements on the same subject for both diluted and undiluted blood was modeled as a Toeplitz (diagonal constant) variance-covariance matrix structure, based on the Akaike's Information Criterion (88). The null hypothesis that $|\mu_1 - \mu_0| \leq 5\% \cdot \mu_0$ was tested against the alternative hypothesis that $|\mu_1 - \mu_0| > 5\% \cdot \mu_0$ using a Student's t-test, where μ_1 is the mean value of the treatment combination of interest and μ_0 is the mean baseline value of the control treatment. A difference of larger than 5% from μ_0 was selected for the alternative hypothesis as smaller differences are not generally of clinical and/or biological relevance. For the effect of anticoagulant and dilution at time 0 analysis, the baseline control treatment was the mean value of the EDTA/undiluted/time 0 treatment for both the 4° C and room temperature samples. Similarly, for the effect of anticoagulant and storage temperature over time for undiluted blood analysis and for the effect of anticoagulant over time on diluted blood analysis, the baseline control treatments were the "EDTA/4° C/undiluted/time 0 treatment" and "EDTA/diluted/time 0 treatment," respectively. Statistically significant differences were determined at the $\alpha = 0.05$ probability of type I error rate. Dunnett's post hoc adjustment was performed for multiple comparisons (128).

All statistical analyses were conducted using SAS® for Windows (Version 9.1.3, Service Pack 4, SAS® Institute Inc., Cary, NC).

5.3 Results

Collection of cord blood with sodium heparin as the anticoagulant did not result in any statistically significant effects on any of hematological parameters that were greater than 5% of the EDTA values of undiluted blood samples measured immediately after collection except for platelets, immature platelet fraction (IPF), and HPC (Figure 5.1). For undiluted samples, the platelet count was lower and the IPF was higher when heparin was used as the anticoagulant rather than EDTA. Similarly, dilution of fresh cord blood with CELLPACK did not affect any of the hematological parameters more than 5% of the EDTA/undiluted blood parameters values except for reticulocyte count and RET He (Figure 5.1). With dilution, reticulocyte count dropped from 214 ± 10.8 (mean \pm standard error of the mean) to $190 \pm 8.88 \times 10^3$ cells/ μ L (-11.2%) and RET He dropped from 32.6 ± 0.415 to 26.3 ± 0.510 (-19.6%), irrespective of the anticoagulant. Additionally, there was a statistically significant effect of dilution greater than 5% of the control treatment on white blood cell (WBC) counts when a statistical test was conducted across both diluted EDTA and heparin treatments simultaneously. However, the individual EDTA/diluted and heparin/diluted white blood cell (WBC) count effects were not statistically greater than 5% of the control treatment. Unlike reticulocytes and RET He, however, dilution with CELLPACK increased the WBC count across anticoagulants by 13.3%.

In general, the undiluted cord blood hematological parameters changed little over the 72 hour post-collection period irrespective of the storage temperature or the

anticoagulant (Table 5.1). Exceptions to this were the WBC count, immature granulocyte (IG) count, HPC count, IPF, mean corpuscular volume (MCV) (Figure 5.2, Panel C), platelets (Figure 5.2, Panel D), and RET He. Other than platelets, IG count, IPF, and MCV, none of these changes were statistically greater than 5% of the EDTA/4° C/undiluted/time 0 hour value until after 24 hours after blood collection. While the 5% difference limit seems large relative to quality control variances for hematological parameters, in the NICU even greater variances are observed for capillary blood sampling relative to venous or arterial sampling (129, 130). For all hematological parameters evaluated other than HPC count, whole blood collected into EDTA tubes and stored at 4° C did not change more than 5% of the time 0 hour value through 72 hours of storage. When blood was collected into heparin containing tubes, storage at room temperature was superior to storage at 4° C after 48 to 72 hours for WBC count, IG count, IPF, and RET He. Conversely, for MCV storage at 4° C was superior to storage at room temperature over time irrespective of anticoagulant. The RBC and reticulocyte count did not change statistically over time, irrespective of the anticoagulant or storage temperature (Figure 5.2, Panels A and B, respectively). In contrast, MCV slowly increased over time with either EDTA or heparin as anticoagulants when stored at room temperature.

Following 1:5 dilution in CELLPACK, there were no statistically significant changes in the hematological parameters studied which were greater than 5% of mean EDTA/diluted/time 0 hour value over the 4 hour study period when stored at room temperature (Figure 5.2). As noted above, the only statistically significant effect was that of heparin as the anticoagulant on platelets.

5.4 Discussion

In the present study, we demonstrate that the Sysmex XE-2100 hematology analyzer is capable of reducing physician-ordered laboratory blood loss by nearly an order of magnitude (from the 250 to 500 μL commonly requested by hospital laboratories to 40 μL) while providing hematological results for the same expansive group of analytes. In using umbilical cord blood at delivery as a surrogate for neonatal blood, we also demonstrated that with few exceptions that anticoagulated whole blood results are unaffected by 1.) storage for up to 72 hours at 4° C, 2.) storage for up to 24 hours at room temperature, after which cell swelling occurs as indicated by greater MCV, and 3.) the use heparin over EDTA, the recommended anticoagulant for hematological parameters, except for lower platelet and HPC counts and increases in IPF with heparin. The present findings have potentially important clinical and research implications for reducing iatrogenic phlebotomy loss in the critical care setting without sacrificing either the accuracy or the scope of laboratory test results.

As technology advances, the capability of chemistry and hematology analyzers used in clinical settings continues to expand while the sample volumes required by these instruments continue to decrease. In the intensive care setting, an important consequence of these technological advances is less iatrogenic blood loss. As a result, patients are less prone to develop anemia and require fewer RBC transfusions. Despite these technologic advances, even greater progress is needed, particularly in the NICU setting where iatrogenic blood loss and resultant anemia among VLBW infants remains the primary reason for RBC transfusions (121-123).

For measurement of all hematological parameters, EDTA is the recommended anticoagulant (131). Nonetheless, we found that analysis of cord blood immediately after collection using heparin as the anticoagulant had no effect on the primary hematological parameters other than lower platelet counts (Figure 5.1). Similar findings have been reported for hematological parameters in adults (132, 133). The effect of heparin on lowering progenitor cell counts and increasing IPF has not been previously reported in adults, perhaps because both are relatively recent reported additions of some hematological analyzers.

While dilution of fresh cord blood with CELLPACK initially affected only reticulocyte count, RET He, and WBC count (Figure 5.1), hematology parameters did not vary further over 4 hours at room temperature following dilution (Table 5.2). This indicates that anticoagulated whole blood samples do not need to be analyzed immediately following dilution to obtain accurate results. Despite the effects of dilution on these hematology parameters, the ability to analyze hematological parameters with as little as 40 μ L of whole blood when using a 1:5 dilution and analysis in capillary mode on the Sysmex XE-2100 instrument offers the advantages of markedly decreasing phlebotomy loss and of allowing capillary blood draws as a reliable alternative to venous sampling (134). If similar laboratory findings are demonstrated in adults, these features may also be of importance for the elderly in nursing homes and for critically ill, VLBW premature infants, who by virtue of their extremely small size and need for frequent laboratory monitoring, quickly become clinically anemic to the extent that they require frequent RBC transfusions in the early weeks of life (121-123). Recent studies by our

group in VLBW infants have demonstrated that reducing the volume of blood drawn for laboratory testing may reduce the number of RBC transfusions (122, 123).

In the NICU setting, blood collected for clinical laboratory testing is 1.) often drawn in heparin, or anticoagulants other than EDTA, and 2.) often drawn in volumes in excess of that needed. Hence, the reduced blood volume needed when using dilutions and capillary mode allows for substantially reduced blood volumes to be collected, and combined with the stability of the hematological values over several days (Table 5.1 and Table 5.2) permits “leftover” anticoagulated blood from other routine clinical tests to be retrieved, diluted, and analyzed for clinical and/or research purposes. Since in the critical care setting, a majority of samples are collected in heparin, this provides a rich source of data without incurring additional blood loss.

Though most hematological parameters were stable over 72 hours, the MCV increase indicated cell swelling over time when undiluted blood was stored at room temperature; however, it did not change when stored at 4° C (Figure 5.2, Panel C). The temperature-dependent swelling in RBC for both EDTA and heparin is consistent with previous reports in humans (124-126, 135) and dogs at room temperature (136). Both the undiluted RBC and reticulocyte counts did not substantially change over 72 hours regardless of anticoagulant and storage temperature (Table 5.1 and Figure 5.2, Panels A and B, respectively). The stability of reticulocytes is consistent with other reports using Sysmex instrumentation demonstrating that reticulocytes drawn in EDTA from adults are stable for 48 hours when stored at either room temperature or 4° C (124, 127). Other reports have demonstrated reticulocyte stability between 1 and 3 days when stored at 4° C or 24° C (137). Of note, in our study key clinical hematological parameters including

hemoglobin concentration, mean corpuscular hemoglobin (MCH), RBC distribution width (RDW), mean platelet volume (MPV), IRF, and NRBC also were not affected by anticoagulant, storage temperature, or storage time. Our results of RDW, MPV, IRF, and NRBC are in variance from those of de Baca et al. (124), who have reported significant changes/variability in these parameters upon storage of EDTA-anticoagulated blood at room temperature for over 24 hours. The observed variances may, however, be attributed to the differences in sample size (8 vs. 40) and specimen type (normal vs. normal and abnormal) utilized in the two studies. Recently, Hedberg and Lehto (125) reported changes in RDW at 48 and 72 hr of EDTA samples stored at room temperature (but not in IRF).

Not surprisingly, some of the newer hematological parameters that have inherently low cell numbers were more variable than the more “classical” parameters. In particular, the IG, HPC, and NRBC count were substantially more variable (Figure 5.1). Accordingly, detecting changes in the mean value using heparin over time required larger percent changes (Table 5.1 and Table 5.2). Given the high variability of these parameters, caution is required when interpreting change over time, particularly when heparin is used as the anticoagulant.

5.5 Conclusion

In summary, we have demonstrated that with its capability of utilizing 40 μ L anticoagulated whole blood samples diluted 1:5 with CELLPACK, the Sysmex XE-2100 hematology analyzer is capable of reducing iatrogenic laboratory blood loss by nearly an order of magnitude (from 250-500 μ L to 40 μ L). The results provided include nearly the same expansive panel of hematological parameters as determined on undiluted whole

blood samples. Using umbilical cord blood at delivery as a surrogate for neonatal blood, we also demonstrated that the use of sodium heparin instead of EDTA as anticoagulant has little effect on the measured hematological parameters of cord blood other than lowering platelet and HPC counts and increasing IPF. In general the primary hematological parameters were stable over 72 hours at both room temperature and 4° C storage temperatures, except for swelling of RBCs after 24 hours of room temperature storage. However, dilution of cord blood 1:5 in CELLPACK had a significant initial effect on WBC count, reticulocyte count, and reticulocyte hemoglobin amount values. Of note, after dilution with CELLPACK, no further changes in any measured hematology values were observed through 4 hours of storage at room temperature. The stability of the hematological parameters over time and the ability to reduce the blood volume required for hematological testing has important clinical and research implications for improving patient care by reducing anemia and the need for and the risks associated with RBC transfusions. This is particularly true of VLBW premature infants for whom iatrogenic blood loss remains the primary cause of symptomatic anemia.

Table 5.1. Mean undiluted cord blood hematological parameter results by anticoagulant, storage temperature, and duration of storage ($n = 8$).

Variable	Anticoagulant	Temp.*	Time (hours)					
			0	4	8	24	48	72
WBC (/ μ L)	EDTA	4° C	9418	9464	9604	9894	9943	9725
		RT	9619	9533	9480	9544	9751	10084
	Heparin	4° C	9110	9219	8904	8645	7991	7511**
		RT	9003	9036	9166	9131	8970	9238
IG (/ μ L)	EDTA	4° C	178	188	203	175	156	135
		RT	205	198	180	179	188	129
	Heparin	4° C	179	213	296	370**	416**	431**
		RT	183	168	164	164	176	199
HPC (/ μ L)	EDTA	4° C	9.14	15.1	12.6	14.0	16.0	20.3**
		RT	5.43	10.7	10.0	11.7	12.0	12.0
	Heparin	4° C	2.14	2.57	1.00	2.00	4.29	4.14
		RT	2.43	1.00	0.71	0.43	0.43	0.43
Platelets (10^3 / μ L)	EDTA	4° C	271	244	243	240	250	255
		RT	275	282	282	279	278	277
	Heparin	4° C	198**	49.8**	60.9**	53.4**	72.0**	85.9**
		RT	191**	171**	180**	161**	204**	199**
MPV (fL)	EDTA	4° C	10.2	10.4	10.5	10.4	10.7	10.6
		RT	10.2	10.5	10.4	10.6	10.8	10.9
	Heparin	4° C	10.0	10.4	10.4	10.5	10.8	11.2
		RT	9.7	10.3	10.3	10.4	9.9	10.1
IPF (%)	EDTA	4° C	3.00	3.74	3.65	4.19	4.06	4.30
		RT	3.20	3.26	3.74	3.74	4.45	4.99
	Heparin	4° C	4.63	16.1**	13.8**	14.8**	16.1**	18.0**
		RT	4.51	7.75	7.56	8.39	5.60	6.64

Table 5.1. Continued.

Variable	Anticoagulant	Temp.*	Time (hours)					
			0	4	8	24	48	72
Hb (g/dL)	EDTA	4° C	15.9	15.9	15.8	16.0	15.9	15.9
		RT	15.8	15.8	15.7	16.0	15.9	16.0
	Heparin	4° C	15.4	15.4	15.3	15.5	15.6	15.6
		RT	15.4	15.4	15.4	15.4	15.5	15.0
RBC (10 ⁶ /μL)	EDTA	4° C	4.32	4.32	4.32	4.34	4.35	4.33
		RT	4.31	4.27	4.28	4.28	4.29	4.28
	Heparin	4° C	4.19	4.21	4.20	4.24	4.26	4.26
		RT	4.23	4.22	4.19	4.23	4.21	4.20
MCV (fL)	EDTA	4° C	108	108	108	108	109	110
		RT	108	110	111	116**	121**	123**
	Heparin	4° C	106	106	106	107	108	108
		RT	106	107	108	114	119*	121**
RDW (%)	EDTA	4° C	16.8	16.7	16.6	16.6	16.4	16.3
		RT	16.8	16.8	16.9	17.3	17.4	17.4
	Heparin	4° C	16.6	16.6	16.5	16.3	16.2	16.1
		RT	16.6	16.6	16.6	17.1	17.1	17.0
MCH (pg)	EDTA	4° C	36.9	36.7	36.7	36.7	36.6	36.7
		RT	36.7	36.9	36.7	37.3	37.1	37.3
	Heparin	4° C	36.8	36.6	36.4	36.7	36.5	36.6
		RT	36.4	36.6	36.6	36.5	36.9	35.6
RET. He (pg)	EDTA	4° C	32.3	31.6	31.6	32.6	34.4	35.1
		RT	32.4	33.1	33.2	33.7	34.3	34.7
	Heparin	4° C	32.8	32.5	32.6	33.7	35.3**	35.7**
		RT	33.1	33.7	33.9	34.4	34.4	34.1

Table 5.1. Continued.

Variable	Anticoagulant	Temp.*	Time (hours)					
			0	4	8	24	48	72
RET. (10 ³ /μL)	EDTA	4° C	216	214	214	217	217	210
		RT	215	215	217	219	220	226
	Heparin	4° C	213	215	216	223	233	237
		RT	212	214	212	217	216	241
IRF (%)	EDTA	4° C	37.9	37.9	37.9	38.3	39.5	39.0
		RT	40.1	39.7	41.4	39.0	40.1	39.6
	Heparin	4° C	37.2	37.4	38.4	38.1	36.9	36.6
		RT	38.0	37.3	38.6	38.0	36.6	34.5
NRBC (/μL)	EDTA	4° C	960	956	995	1011	1078	1048
		RT	973	953	998	901	895	879
	Heparin	4° C	868	926	961	943	901	904
		RT	878	886	855	856	864	851

* RT: room temperature

** Absolute value of the difference from the mean EDTA/4° C/undiluted/time 0 hour treatment significantly greater than 5% (p < 0.05)

Table 5.2. Mean diluted cord blood hematological parameter results by anticoagulant and duration of storage ($n = 8$).

Variable	Anticoagulant	Storage Period (hours)				
		0	0.5	1	2	4
WBC (μL)	EDTA	10846	10896	10908	10898	11020
	Heparin	10216	10196	10335	10310	10334
Platelets ($10^3/\mu\text{L}$)	EDTA	304	306	306	300	311
	Heparin	229*	222*	226*	228*	229*
IPF (%)	EDTA	4.48	5.04	4.46	4.39	4.73
	Heparin	5.40	5.09	4.74	5.05	4.95
Hb (g/dL)	EDTA	15.8	15.8	15.8	15.7	15.8
	Heparin	15.6	15.5	15.4	15.5	15.5
RBC ($10^6/\mu\text{L}$)	EDTA	4.33	4.35	4.35	4.32	4.34
	Heparin	4.25	4.24	4.21	4.27	4.24
MCV (fL)	EDTA	110	110	110	110	110
	Heparin	109	109	108	108	109
MCH (pg)	EDTA	36.6	36.4	36.3	36.4	36.3
	Heparin	36.7	36.6	36.6	36.4	36.6
RET He (pg)	EDTA	26.1	26.5	26.6	27.7	28.2
	Heparin	26.2	26.5	26.9	27.3	28.1
Reticulocyte ($10^3/\mu\text{L}$)	EDTA	188	196	196	191	201
	Heparin	190	188	192	199	196
IFR (%)	EDTA	40.8	44.1	41.1	42.3	40.4
	Heparin	41.8	42.4	42.6	40.8	44.0

* Absolute value of the difference from the mean EDTA/diluted/time 0 hour treatment significantly greater than 5% ($p < 0.05$)

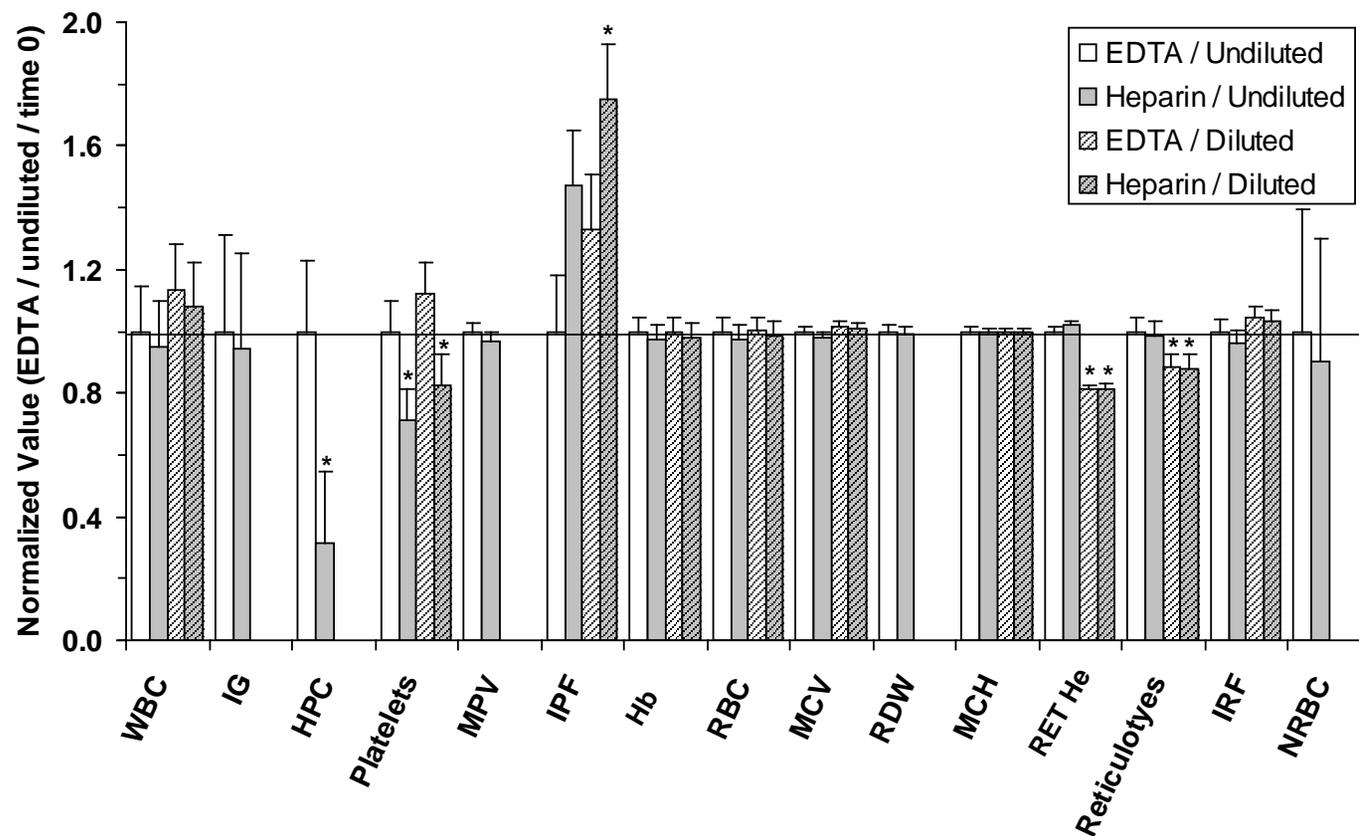


Figure 5.1. Mean hematological values immediately after collection normalized by the mean EDTA/undiluted/time 0 hour sample value.

Horizontal line indicates the normalized value of 1.0 and bars represent the normalized standard error ($n = 8$). Hematological parameters missing for the diluted samples are not reported by the Sysmex XE-2100 automatic hematology analyzer when operated in capillary (i.e., diluted) mode.

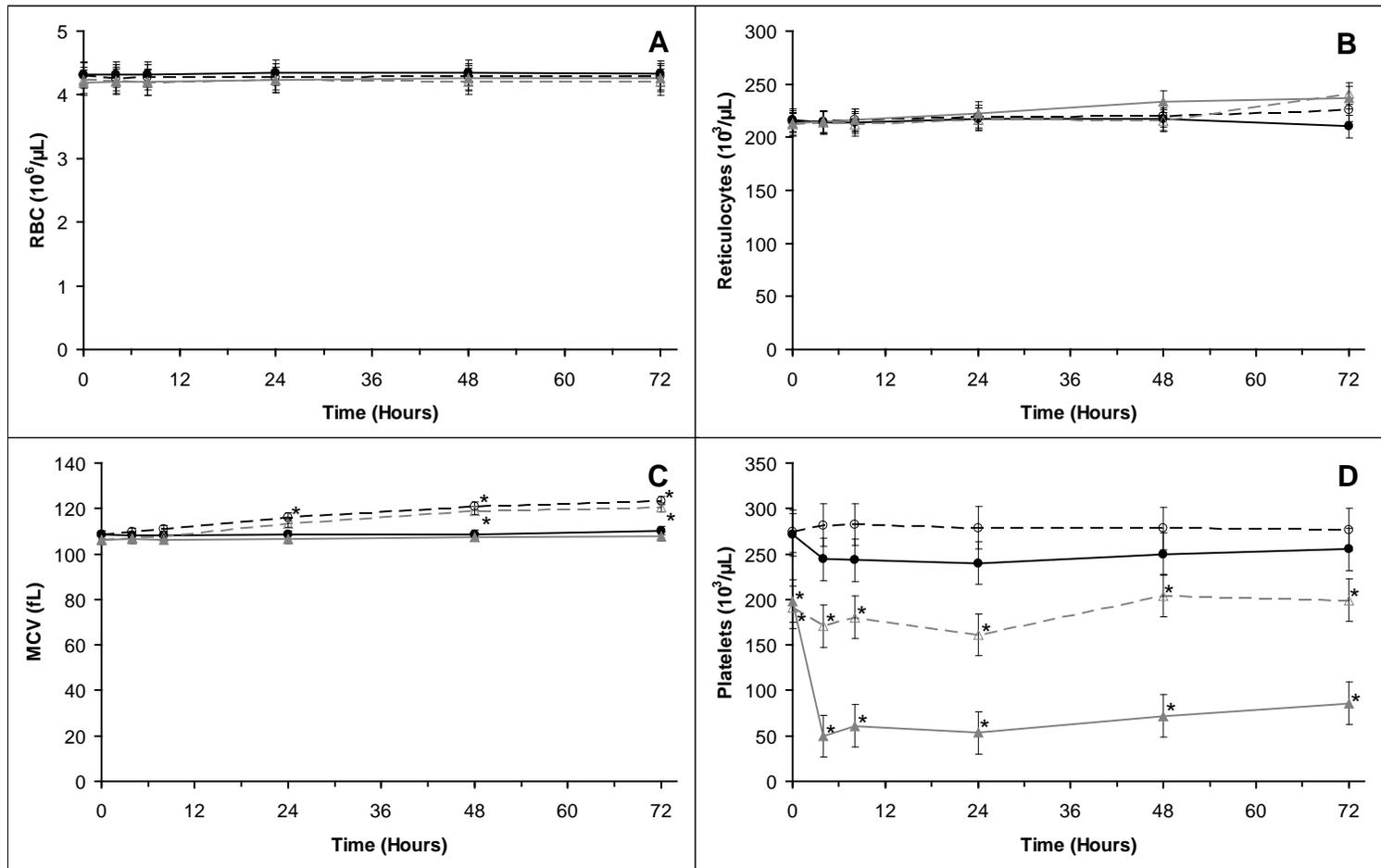


Figure 5.2. Mean RBC (Panel A), reticulocytes (Panel B), MCV (Panel C) and platelets (Panel D) versus time by treatment ($n = 8$). EDTA - 4°C (●), EDTA - room temp. (○), heparin - 4°C (▲), and heparin - room temp. (△) – undiluted treatments. Bars represent the standard error.

CHAPTER 6. ERYTHROPOIETIC RESPONSE TO ENDOGENOUS ERYTHROPOIETIN IN PREMATURE VERY LOW BIRTH WEIGHT INFANTS

6.1 Introduction

The anemia of prematurity occurs in all very low birth weight (VLBW) premature infants (birth weight < 1500 g) and is exacerbated by iatrogenic blood loss resulting from frequent laboratory blood sampling for managing clinical illness (121-123, 138). The resulting development of clinically significant anemia is managed by the administration of red blood cell (RBC) transfusions, creating dynamic changes in the hemoglobin (Hb) mass in these infants due to both the physical removal and administration of erythrocytes. In infants, as well as adults, RBCs are produced from erythroid progenitor cells located primarily in the bone marrow, though some residual erythropoiesis may still occur in the liver and spleen of preterm infants (12, 139). The development and expansion of erythroid progenitor cells into mature RBCs is primarily controlled by erythropoietin (EPO), a 30.4 kD glycoprotein hormone produced in response to oxygen need by the peritubular cells of the kidney in the adult and possibly also the liver in preterm infants (12, 14, 139-141). The most immature form of RBCs present in the circulation are the reticulocytes, which are distinguished from mature RBCs by the presence of residual ribosomal ribonucleic acid (RNA). Reticulocytes normally have a lifespan in the systemic circulation of approximately 1 day before developing into mature RBCs. Under non-disease state conditions the mechanism of RBC death or removal from the circulation is primarily to due to cellular senescence (i.e. age related cell death) (52).

Despite the common occurrence of anemia in VLBW infants, the erythropoiesis rate in these subjects remains unknown. This is largely due to the complications in

determining the erythropoiesis rate caused by frequent phlebotomies and RBC transfusions altering the RBC/Hb mass, and the effect each phlebotomy on the RBC/Hb removal rate due to the lifespan based disposition of RBCs (52, 91, 100). Previous studies have demonstrated that on average 33.8 mL/kg of blood are removed and 27.0 mL/kg are transfused during the first 4 weeks of life in infants born at a gestational age of less than 28 weeks (142) and other studies have reported even higher phlebotomy blood loss volumes in the first 2 weeks of life alone (121, 122). Thus the effects of the physical removal and administration of RBCs are substantial and cannot be ignored. Other complications in determining the erythropoiesis rate in preterm VLBW infants include increases in total blood volume as it expands with infant growth (143) and the mixture of endogenously produced RBCs and exogenously administered adult donor RBCs following transfusion, the former which generally have shorter lifespans (139, 144).

Knowledge of the *in vivo* erythropoiesis and Hb production rates and their relationship to plasma EPO concentrations in these infants would provide an understanding of these subject's ability to compensate for phlebotomy blood loss, and thereby providing a reference for evaluating the potential improvement of erythropoiesis through administration of recombinant human EPO (rHuEPO) and other erythropoiesis stimulating agents. Since the administration of RBC transfusions carries infectious and non-infectious risks (62, 145), knowledge of the *in vivo* erythropoiesis rate is also important for assessing the potential therapeutic strategies to reduce or eliminate RBC transfusion risks. Thus the objective of the current study was to estimate the *in vivo* erythropoiesis and Hb production rates and their relationship to endogenous plasma EPO concentrations in preterm VLBW infants. To achieve this aim, the amount of Hb

removed by phlebotomy and administered by RBC transfusion to 14 preterm VLBW infants were recorded and a pharmacodynamic (PD) Hb mass balance model formulated that accounts for the dynamic hematological conditions experienced by these infants. The model was subsequently fitted simultaneously to each infant's observed endogenous plasma EPO, Hb, RBC count, and bodyweight profiles over time to estimate the erythropoiesis rate and its relationship to endogenous plasma EPO concentrations.

6.2 Materials and methods

6.2.1 Subjects

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

6.2.2 Study procedures

Phlebotomy blood samples from study subjects were weighed and recorded immediately after collection from birth through 30 to 37 days of life. The collection tube

weights were subtracted from the samples and converted to a blood volume based on the specific gravity of blood of 1.05 g/mL (146). If a phlebotomy blood sample was mistakenly not weighed (~ 4% of all samples), then the mean sample weight for the type of clinical test drawn was substituted. An additional 38 μ L of blood was added to each phlebotomy collected by capillary heel stick to account for blood wiped away prior to collection and bleeding after collection. The 38 μ L of blood was determined by weighing the volume of blood estimated to be equivalent (as determined by visual assessment) to the amount routinely removed from the skin prior to collection and loss after sample collection by capillary heel stick. Additionally, the same amount of blood was added to samples collected from indwelling arterial or venous lines due to loss by clearing of the catheter prior to collection. Analysis of catheter fluid used to clear the lines from a sample of infants indicated that from 10 to 114 μ L (median of 38 μ L) of blood was lost with each blood sample drawn from an indwelling catheter.

The Hb mass removed with each phlebotomy was calculated by multiplying the volume of blood removed by the Hb concentration measured in the blood sample drawn closest to the time of blood sampling. Concentrations of Hb, RBC, and reticulocytes were measured from several sources including: clinically ordered blood gases and electrolytes (Hb only) using a Radiometer ABL 625 blood gas analyzer (Radiometer America, Inc, Westlake, OH), clinically ordered and study protocol driven complete blood counts (CBC) using an Advia® 120 hematology system (Bayer, Tarrytown, NY), and excess blood recovered from other clinical tests using a Sysmex XE-2100 automatic hematology analyzer (Sysmex Corporation, Kobe, Japan) operated in normal (if sufficient blood volume (> 200 μ L) was available) or capillary mode. If blood collected at the same time

was measured by multiple instruments, the average Hb, RBC, or reticulocyte concentration from all instruments was used. We have previously demonstrated that Hb, RBC, and reticulocyte concentrations measured on recovered blood using the Sysmex XE-2100 is stable for up to 72 hours at 4° C or room temperature and unaffected by the use of EDTA or heparin as an anticoagulant (147). Hb and RBC concentrations were also found to not be affected by operation using the capillary mode; however, reticulocyte concentrations were on average 11.2% lower when operated in capillary mode. To correct for this, reticulocyte concentrations determined in capillary mode were multiplied by the ratio of the normal to capillary mode concentrations (i.e. multiplied by 1.13) (147), which was externally validated on 23 infant samples to give corrected reticulocyte concentrations within 5% of the concentration determined in normal mode ($p = 0.787$). In addition to the Hb, RBC, and reticulocyte concentrations from clinically ordered tests, research blood samples were collected weekly and prior to and approximately daily after the first RBC transfusion for 10 days to ensure adequate sampling density. However, if a CBC was ordered by the attending physician(s) on the corresponding days, then research blood samples were not collected to avoid duplicate sample collection. The total amount of blood removed by the research blood samples was limited to less than 1.6 mL/week/kg.

Plasma samples for EPO concentration determination were also collected by centrifugation from excess blood recovered from the clinical tests and research samples described above. If the plasma volume from sample was insufficient to conduct a plasma EPO determination, then the plasma was pooled with other samples within an approximately 8 hour time window. The “collection time” for these pooled plasma

samples used for the data analysis was the weighted average (based on the relative plasma volume contribution to the total sample) of the collection times of the individual samples that made up the pooled samples. Plasma EPO concentrations were measured using a double antibody radioimmunoassay (RIA) procedure as previously described (lower limit of quantification 1 mU/mL) (148).

The volumes of packed RBCs (85% hematocrit (Hct)) administered and the start and stop times of all RBC transfusions were recorded. The Hb mass administered to individual infants was calculated based on the measured Hb concentrations of the transfusate, or if not directly measured then based on a typical Hb concentration of 28.3 g/dL. The decision to treat an individual subject's anemia by administration of RBC transfusions was made by the physician responsible for the subject's patient care according to NICU guidelines (149). From the known amounts of Hb removed by phlebotomy and administered by transfusion to each infant and the observed Hb, RBC, plasma EPO concentration, and bodyweight profiles over time, the erythropoiesis and Hb production rates and their relationship to endogenous plasma EPO concentrations were estimated using the PD Hb mass balance model described below.

6.2.3 Pharmacodynamic hemoglobin mass balance model

The PD Hb mass balance model assumed that the behavior or disposition of the Hb and RBCs in the absence of phlebotomies was lifespan based (i.e. based on removal of RBC from the circulation through cellular aging/senescence) (5, 6, 52, 100). A time invariant "point distribution" (i.e. no variability) of RBC lifespans was assumed (6, 91). The Hb mass or amount in the infants was modeled as the summation of two separate

components, 1.) Hb contained in RBC produced by the infant *in vivo* ($Hb_{in\ vivo}$) and 2.) Hb contained in transfused RBC (Hb_{trans}).

The Hb stimulation rate of the first Hb component, $Hb_{in\ vivo}$, was assumed to be proportional to bodyweight or mass scaled to the $3/4$ power, as many metabolic processes are in physiology and pharmacokinetics (150-152), to account for the changing body mass of the fetus/infant as the subject matured. Prior to birth, the bodyweight scaled Hb stimulation rate was assumed to be constant and was denoted by $k_{stim}^{in\ utero}$. While post-birth the body mass scaled Hb stimulation rate, denoted by $f_{stim}^{ex\ utero}(t)$, was assumed to be a function of time (t) through changes in the plasma EPO concentration ($C_p(t)$). Thus the total body Hb stimulation rate ($f_{stim}^{total}(t)$) is a function of time through changes in both the body mass and the plasma EPO concentration over time as given by:

$$f_{stim}^{total}(t) = f_{stim}(t) \cdot m(t)^{3/4} \quad (6.1)$$

where $m(t)$ is the body mass and :

$$f_{stim}(t) = \begin{cases} k_{stim}^{in\ utero} & \text{if } t \leq 0 \\ f_{stim}^{ex\ utero}(C_p(t)) & \text{if } t > 0 \end{cases} \quad (6.2)$$

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

$$f_{stim}^{ex\ utero}(t) = \frac{p_1 \cdot C_p(t)}{1 + p_2 \cdot C_p(t)} \quad (6.3)$$

such that $p_1 = E_{max}/EC_{50}$ and $p_2 = (EC_{50})^{-1}$, where E_{max} is the maximum bodyweight scaled Hb stimulation rate and EC_{50} is the plasma EPO concentration that results in 50% of E_{max} . This parameterization allows for the nonlinear stimulation rate function given by Eq. 6.3 to reduce to a linear function by setting $p_2 = 0$ when only operating in the approximately linear range of the E_{max} model (i.e. when $C_p \ll EC_{50}$).

Let the time delay between stimulation of progenitor cells outside the systemic circulation to release (i.e. production) of the subsequently stimulated RBC into the circulation be denoted by a and the RBC lifespan of cells produced *in vivo* be denoted by $L_{in vivo}$. Hence the time between stimulation of precursor or progenitor cells and age-related/senescent death of RBC, denoted by b , is given by $b = a + L_{in vivo}$. Then in the absence of any phlebotomies the rate of change in the Hb amount that was produced *in vivo* is given by (5, 91):

$$\frac{dHb_{in vivo}}{dt} \equiv Hb_{in vivo}'(t) = f_{stim}^{total}(t-a) - f_{stim}^{total}(t-b) \quad (6.4)$$

with initial conditions given by:

$$Hb_{in vivo}(0) = \int_{t-b}^{t-a} f_{stim}^{total}(u) du \Big|_{t=0} = \int_{0-b}^{0-a} f_{stim}^{total}(u) du$$

Thus the input rate ($f_{stim}^{total}(t-a)$) and the output rate ($f_{stim}^{total}(t-b)$) of the *in vivo* Hb amounts are simply time shifted total body Hb stimulation rates.

The second Hb mass component, the Hb from the RBC transfusions (Hb_{trans}), was accounted for through superposition by adding the Hb mass transfused at each transfusion and then accounting for a linear rate of decline of the transfused cells. The linear rate of

decline arises from assuming a constant Hb production/stimulation rate and a constant RBC lifespan in the RBC donor subjects. Thus in the absence of any phlebotomies:

$$Hb_{trans}(t) = \begin{cases} Hb_{trans}(t) & \text{for } t = T_{Ti} \\ Hb_{trans}(t) + F_T \cdot Hb_{Ti} & \text{for } t = T_{Ti} + \varepsilon \end{cases} \quad i = 1 \text{ to } m \quad (6.5)$$

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

$$Hb_{trans}'(t) = -F_T \cdot \sum_{i=1}^m Hb_{Ti} \cdot f_{trans}(t - T_{Ti}), \quad Hb_{trans}(0) = 0 \quad (6.6)$$

with:

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

where L_{trans} is the RBC lifespan of the transfused cells from the donor subject. By superposition, summation of $Hb_{in\ vivo}(t)$ and $Hb_{trans}(t)$ gives the total amount of Hb present in the systemic circulation:

$$Hb_{total}(t) = Hb_{in\ vivo}(t) + Hb_{trans}(t) \quad (6.7)$$

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

$$RBC_{total}(t) = \frac{Hb_{in\ vivo}(t)}{MCH_{in\ vivo}} + \frac{Hb_{trans}(t)}{MCH_{trans}} \quad (6.8)$$

A separate MCH was used for the infant *in vivo* and adult transfused RBCs since infant cells are generally larger and contain more Hb than adult cells (51).

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

$$Hb_{in\ vivo}(t) = \begin{cases} Hb_{in\ vivo}(t) & \text{for } t = T_{P_j} \\ Hb_{in\ vivo}(t) - Hb_{P_j} \cdot \frac{Hb_{in\ vivo}(T_{P_j})}{Hb_{total}(T_{P_j})} & \text{for } t = T_{P_j} + \varepsilon, \end{cases} \quad j = 1 \text{ to } n \quad (6.9)$$

and $Hb_{trans}(t)$ was corrected as:

$$Hb_{trans}(t) = \begin{cases} Hb_{trans}(t) & \text{for } t = T_{P_j} \\ Hb_{trans}(t) - Hb_{P_j} \cdot \frac{Hb_{trans}(T_{P_j})}{Hb_{total}(T_{P_j})} & \text{for } t = T_{P_j} + \varepsilon, \end{cases} \quad j = 1 \text{ to } n \quad (6.10)$$

where T_{P_j} is the time of the j^{th} phlebotomy, Hb_{P_j} is the amount of Hb removed by the j^{th} phlebotomy, and n is the total number of phlebotomies.

Additionally, the negative terms in the differential Eq. 6.4 and Eq. 6.6 presented above (i.e. $f_{stim}^{total}(t-b)$ and $Hb_{T_i} \cdot f_{trans}(t-T_{T_i})$, respectively) must be corrected for Hb removed by the phlebotomies. These negative terms represent the output of Hb from the system due to cell age-related death of RBCs, and thus if not corrected the Hb will be

“removed” twice, once due to the correction presented above in Eq. 6.9 and Eq. 6.10 at the time of each phlebotomy and a second time when the Hb would have been “removed” from the systemic circulation in the absence of the phlebotomies due to age-related death of RBC. Since the cells physically removed by the phlebotomy cannot be removed again through the differential equation terms that represent removal by cell death, these terms must be modified so that the equations remain correct. Let F_{P_j} be the fraction of Hb remaining immediately after the j^{th} phlebotomy relative to the amount present immediately before the j^{th} phlebotomy, thus:

$$F_{P_j} = \frac{Hb_{total}(T_{P_j}) - Hb_{P_j}}{Hb_{total}(T_{P_j})} \quad (6.11)$$

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

$$\text{phlebotomy correction factor} = \begin{cases} \prod_{j=k}^l F_{P_j} & \text{if } l \geq k \text{ and } T_{P_k} < t \\ 1 & \text{otherwise} \end{cases} \quad (6.12)$$

where k is the first phlebotomy after entry of the cells of interest into the systemic circulation and l is the last phlebotomy prior to the current time t (see Appendix D.1 for derivation). Therefore, as long as $T_{P_k} < t$, k is the first phlebotomy after time $t - L_{in\ vivo}$ for the $f_{stim}^{total}(t - b)$ term and the first phlebotomy after time $t - T_{Ti}$ for each $Hb_{Ti} \cdot f_{trans}(t - T_{Ti})$ term. The presented phlebotomy correction factor following $l - k + 1$ phlebotomies is consistent with formulas previously derived following only 1 or 2 phlebotomies (91, 100).

Finally, the amounts estimated from $Hb_{total}(t)$ and $RBC_{total}(t)$ were converted into the observed concentrations by the model estimated total blood volume. The total blood volume, V_{total} , was assumed to be proportional to the infant body mass (150, 153), and was given by:

$$V_{total}(t) = m(t) \cdot V_N \quad (6.13)$$

where V_N is the bodyweight or mass normalized blood volume.

6.2.4 Data analysis

All modeling and simulations were conducted using WINFUNFIT, a Windows (Microsoft) version evolved from the general nonlinear regression program FUNFIT (86), using ordinary least squares fit to each individual subject's Hb and RBC concentration-time profile. The amount of Hb removed and administered by each phlebotomy and transfusion, respectively, at the time of removal or administration was accounted for by WINFUNFIT using a generalized events processing module. The events processing module integrates the differential equation exactly up to the time of the event before adding or removing the appropriate amount and then continuing on integrating the differential equation from the new initial conditions set immediately after each successive event.

The EPO plasma concentrations were nonparametrically represented using a generalized cross validated cubic spline function (85). The infant bodyweight post-birth was represented by a 4th order polynomial fit to the observed bodyweight data to interpolate between bodyweight observations and provide a smooth function of total blood volume. To account for the *in utero* body masses, which are needed to calculate

$f_{stim}^{total}(t)$ when $t \leq 0$, a power function was fitted to the mean body weights of over

10,000 live singleton births 22 to 32 weeks of gestational age (154). Then for each infant, the *in utero* body masses were calculated based on their gestational age and linearly scaled such that function predicted birth body mass was continuous with the bodyweight at birth. Thus both plasma EPO concentrations and bodyweights acted as forcing functions in the model.

To account for the fact that 24-hour post-transfusion recoveries (PTR_{24}) are generally less than 100% (155), F_T was fixed to 0.875, the midpoint between 100% recovery and the FDA requirement that transfused RBC must exhibit 75% or greater recovery after storage (156). The lifespans of the adult transfused (L_{trans}) was fixed to 70.8 days, the midpoint of the estimated lifespans of 56.4 and 85.2 days of transfused adult RBC in preterm infants (144, 157). Additionally, the time between stimulation of progenitor cells outside the circulation to release of the subsequently stimulated RBC into the circulation (a) was set equal to 3 days based on previous estimates (32-35). The MCH_{trans} parameter was set equal to the measured MCH for each unit of transfused blood, or if not measured for a particular unit of blood then the mean value of all the measured units. For each subject, the decision to use a nonlinear (p_2 estimated) or linear ($p_2 = 0$) bodyweight scaled Hb stimulation rate function (Eq. 6.3) was made using the Akaike's Information Criterion (AIC) (88). All remaining parameters of the model (i.e.

$L_{in\ vivo}$, V_N , $MCH_{in\ vivo}$, $k_{stim}^{in\ utero}$, and E_{max}/EC_{50}) were estimated in all subjects.

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

$$\text{MSE}\% = \frac{1}{n} \cdot \sum_{i=1}^n \frac{\text{SE}_i}{|P_i|} \cdot 100 \quad (6.14)$$

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

6.3 Results

6.3.1 Subject characteristics

The mean gestational age of the 14 infant study subjects was 27.0 weeks (range, 25.0 to 28.6) and mean birth weight was 0.840 kg (range, 0.548 to 1.49 kg). Five males and 9 females were studied. A summary of the number of phlebotomies and RBC transfusions and the corresponding amounts of Hb removed and administered per study subject, respectively, are displayed in Table 6.1. All subjects who received transfusions were administered more Hb than was removed by phlebotomy. Approximately twice as much Hb was administered by transfusion as removed by phlebotomy. In 54 of the 57 transfusions administered, the volume of packed RBCs (85% Hct) administered was 15 mL/kg. Due to the severity of cardiorespiratory disease encountered in the first few weeks of life, the majority of RBC transfusions administered to these infants occurred at a Hct of approximately 35%. During the entire approximately 1 month study period, the average number of phlebotomies performed per subject per day was 4.5 (range, 1.6 to 7.1). The mean rate of blood removal by phlebotomy began very high at 10.1 mL/day/kg of bodyweight on the first day of life and subsequently decreased over the next few days (Figure 6.1). On average 48.3% of the blood removed in the first 30 days of life was removed during the first 7 days of life. The mean daily rate of phlebotomy blood removal over the study period was 1.72 mL/day/kg of bodyweight. The plasma EPO

concentrations were variable and declined approximately 3-fold from 45 mU/mL immediately after birth to an average of 15.5 ± 6.55 (mean \pm SD) mU/mL from 5 days of age onward (Figure 6.2). The reticulocyte counts were also variable and typically dropped from approximately day 2 through day 7 of life from a baseline count of around 250 to 300×10^3 cells/ μ L to approximately 100×10^3 cells/ μ L (Figure 6.3). A small rise in reticulocyte counts following the nadir at around day 7 of life was observed in some subjects.

6.3.2 Pharmacodynamic hemoglobin mass balance model

The Hb mass balance model fit to the Hb and RBC concentration-time profiles, along with the plasma EPO concentration and bodyweight data (with fitted curves) for two representative subjects are displayed in Figure 6.4. General agreement between the model fit and the Hb and RBC concentrations was observed. The administrations of the RBC transfusions are indicated by the vertical lines in the model predicted Hb and RBC concentration-time profiles. The rapid decline in the Hb and RBC concentrations after each transfusion is due to a combination of phlebotomy blood loss, blood volume expansion with growth, and RBC age-related death of both endogenously produced and transfused RBCs. Individual subject's Hb concentration-time profiles contained on average 91 Hb (range, 28 to 172), 27 RBC (range, 13 to 44), 50 plasma EPO (range, 10 to 96) concentration and 32 bodyweight (range, 30 to 37) measurements. The observed plasma EPO concentrations and bodyweights were also well represented by the fitted cross-validated cubic spline and fourth order polynomial, respectively (Figure 6.4). The estimates of the parameters are displayed in Table 6.2. The parameters were well estimated with a MSE% of $< 15\%$ for all but the Hb stimulation rate function parameters.

The relatively high MSE% for E_{max}/EC_{50} is due to a single subject having a high standard error of 1300%. Calculation of the MSE% for E_{max}/EC_{50} without this subject results in a MSE% of only 24.6%. The EC_{50}^{-1} parameter, and thus E_{max} and EC_{50} secondary parameters, were only determined in 6 subjects where the nonlinear Hb stimulation rate function (Eq. 6.3) was preferred over a linear function based on AIC. The estimated amounts of Hb present at birth and produced over the first 30 days of life, as well as the observed amounts of Hb removed by phlebotomy and administered by RBC transfusion, are displayed in Table 6.3. By 30 days of age, the estimated cumulative amount of Hb released into the circulation in the 14 VLBW study subjects ranged from 0.64 to 14.2 g/kg of birth weight.

6.4 Discussion

The *in vivo* erythropoiesis rate and its relationship to endogenous plasma EPO concentrations in VLBW infants over the first 30 days of life was successfully estimated utilizing a PD-based Hb mass balance model with: 1.) detailed accounting of all blood removed and transfused, 2.) frequently sampled Hb, RBC, and plasma EPO concentration-time profiles created through recovery of excess blood collected, and 3.) recording of serial bodyweights to account for growth and blood volume expansion. The estimated mean post-birth bodyweight scaled Hb stimulation rate over the first 30 days of life (average $f_{stim}^{ex\ utero}$) of $0.144 \text{ g/day} \cdot \text{kg}^{3/4}$ of bodyweight can serve as a quantitative reference for the erythropoietic ability of VLBW infants, under conditions of mild anemia due to receiving clinically ordered RBC transfusions, to compensate for Hb removed by phlebotomy and Hb dilution due to blood volume expansion as a result of growth. Furthermore, this reference and the post-birth Hb stimulation rate parameters

estimates (E_{max}/EC_{50} and EC_{50}^{-1}) are both important for assessing the potential to reduce or eliminate RBC transfusion risks through administration of exogenous rHuEPO.

The mean post-birth bodyweight scaled Hb stimulation rate estimate of 0.144 g/day·kg^{3/4} (corresponding to an erythropoiesis rate of 3.84×10^9 RBCs/day·kg^{3/4}) is approximately half the estimated Hb stimulation rate in healthy adults of 0.260 g/day·kg^{3/4} (6.3 g/day in a 70 kg adult) (12). This modest Hb stimulation rate in these infants suggests that the production is suppressed. Specifically, the administration of RBC transfusions to these infants, which prevents more severe anemia, may be limiting Hb stimulation particularly considering that adults under severe chronic anemic conditions are capable of increasing their Hb stimulation rate 3- to 5-fold above normal (89). The possible limiting effect of RBC transfusion on erythropoiesis is illustrated in Panel A of Figure 6.4, where the plasma EPO concentrations (top) drop following each RBC transfusion. However, a similar pattern in the relationship between transfusions and plasma EPO concentrations was not observed in all subjects. For example, the representative subject displayed in Panel B of Figure 6.4 does not show any obvious relationship between RBC transfusions and plasma EPO concentrations. The differences in the subjects could be due to a variety of reasons, such as different severity or type of illness. In healthy term infants the Hb production/stimulation rates have been shown to decrease substantially at day 10 of life relative to day 1 of life (158). This was also observed in the current study as the mean Hb stimulation rates dropped 3-fold from 0.414 g/day·kg^{3/4} at birth to an average of 0.144 g/day·kg^{3/4} post-birth (Table 6.2).

The estimates of E_{max} and EC_{50} in Table 6.2 may be biased since they could only be determined in a subset of the infants ($n = 6$) where the stimulation rate was operating

in the nonlinear (i.e. higher) plasma EPO concentration range. That is, in this subset of 6 infants the Hb stimulation rate was approaching saturation while in the other 8 infants no stimulation rate saturation was present in the observed plasma EPO concentration range. However, the mean estimates of E_{max} and EC_{50} suggest that the erythropoiesis or Hb stimulation rate could be increased approximately 4-fold from of $0.144 \text{ g/day} \cdot \text{kg}^{3/4}$ of bodyweight to $0.566 \text{ g/day} \cdot \text{kg}^{3/4}$ with only a relatively modest increases in the plasma EPO concentration, since the mean estimated EC_{50} was only 28.5 mU/mL. As a reference, the maximum plasma EPO concentration in the 6 subjects that EC_{50} was estimated for was $51.1 \pm 21.7 \text{ mU/mL}$. The estimated 4-fold maximal increase in Hb stimulation rate is consistent with the estimates in adults under severe chronic anemic conditions where 3- to 5-fold increase in Hb stimulation rate occurs (89). Additionally, the mean maximal Hb stimulation rate estimate of $0.566 \text{ g/day} \cdot \text{kg}^{3/4}$ was also similar to the estimated mean *in utero* Hb stimulation rate from the current study of $0.414 \text{ g/day} \cdot \text{kg}^{3/4}$, which is a more hypoxic environment than that experienced post-birth and thus may also represent a near maximal Hb stimulation rate.

Similar to the postnatal fall in erythropoiesis experienced by these infants, both the plasma EPO concentrations (Figure 6.2) and reticulocyte counts (Figure 6.3) also fell following birth. While reticulocytes are often used to monitor erythropoiesis in subjects due to their short circulating lifespan of approximately 1 day (36, 37), the incorporation of the reticulocytes into the PD Hb mass balance model is complicated by the changes in their circulating lifespan under conditions of high erythropoiesis rates (i.e. “stress” erythropoiesis) (11, 39, 91, 100). Since it is known that the *in utero* erythropoiesis stimulation rate is higher than the *ex utero* stimulation rate due to a transition from a

relatively hypoxic environment to a normoxic environment at birth (158), the lifespan of the reticulocytes in the circulation would likely change over the course of the experiment, perhaps as much as 2- to 3-fold. While the complications to the modeling due to changes in reticulocyte lifespan can readily be accounted (11, 91, 100), reticulocytes add little quantitative information to the estimation of the erythropoiesis rate and serve more as a qualitative marker of erythropoiesis.

Previous RBC lifespan estimates based on ^{51}Cr labeled RBCs from premature infants of 35 to 50 days (139) are similar to the results obtained in the current study of 65.8 days (Table 6.2). As expected, the estimated infant lifespans were shorter than the typically referenced adult RBC lifespan of 120 days (32, 52, 139). The estimated mean blood volume of 97.7 mL/kg in the first weeks of life (Table 6.2) is consistent with other measurements of blood volume in term and premature infants ranging from 89 to 110 mL/kg during the first 2 weeks of life (153, 159, 160).

The number (Table 6.1) and rate of phlebotomy blood loss (Figure 6.1) in these VLBW infants was extensive and contributes substantially to their anemia and RBC transfusion need. It was estimated that on average over 50% of the Hb present at birth was removed during the first 30 days of life (Table 6.3). Additionally, the mean amount of Hb removed was greater than the estimated total amount of Hb produced by these infants in the first 30 days of life. The transfused Hb amount was approximately twice the phlebotomy blood loss. Furthermore, as suggested by some subject's plasma EPO concentration-time profiles and discussed above, the administration of RBC transfusions may contribute to continued transfusion need by suppressing endogenous EPO production and the subsequent EPO stimulated erythropoiesis.

The common goal with rHuEPO and other erythropoiesis stimulating agent therapy in preterm VLBW infants is not just to reduce the number of RBC transfusions, but to eliminate them altogether and thus the disease transmission and other risks associated with them (62). With the common use of stored RBCs from a single donor (161), multiple RBC transfusions carry little additional risk of disease transmission than a single transfusion. Based on the estimated amount of Hb produced and the measured amount of Hb transfused (Table 6.3), an erythropoiesis stimulating agent would need to increase the Hb stimulation rate 2- to 3-fold to maintain Hb concentrations high enough to avoid the need for a RBC transfusion in the first 30 days of life under the current clinical practice guidelines utilized in the study. If the phlebotomy blood volume removed due to clinical testing could be substantially reduced and/or if the Hct percentage used in determining whether a transfusion is needed was decreased, then the Hb stimulation rate needed to avoid a transfusion in these subjects would be less (62). The interaction of these variables on RBC transfusion need may be more thoroughly evaluated in future studies.

6.5 Conclusion

In summary, a PD model that accounts comprehensively for phlebotomy losses and RBC transfusions was fitted to endogenous plasma EPO, Hb, RBC, and bodyweight profiles over time from 14 VLBW infants. Detailed recording of all blood removed from and administered to these infants and use of a Hb mass balance model allowed for a mathematically rigorous determination of the *in vivo* erythropoiesis and Hb production rates and their relationship to endogenous plasma EPO concentrations under the dynamic and complex hematological conditions routinely experienced by VLBW infants in first 30

days of life. The estimated parameters of the PD Hb mass balance model were consistent with other reports in the literature using direct measurement techniques, further supporting the utility of the proposed model. Future work with this model and parameter estimates, including direct measurements of blood volume and RBC lifespan in these subjects, will allow for an assessment of the potential to eliminate RBC transfusions in VLBW infants through administration of erythropoiesis stimulating agents and/or changes in other clinical practices.

Table 6.1. Individual subject Hb, phlebotomy, and transfusion characteristics during study period ($n = 14$).

	First Hb concentration following birth (g/dL)	Study period (days)	No. of phlebotomies	No. of transfusions	Total amount of Hb removed by phlebotomies (g)	Total amount of Hb transfused (g)
Mean	16.3	32.1	143	4.07	6.27	14.4
SD	2.3	2.46	56.1	2.50	2.58	6.77
Minimum	11.6	29.7	48	0	2.12	0.00
Maximum	19.3	37.3	239	8	10.6	24.3

SD: Standard deviation

Table 6.2. Estimated parameter summary from pharmacodynamic Hb mass balance model ($n = 14$).

	$L_{in\ vivo}$ (day)	V_N (mL/kg)	$MCH_{in\ vivo}$ (pg/cell)	$k_{stim}^{in\ utero}$ (g/day·kg ^{3/4})	E_{max}/EC_{50} (g·mL/day·mU· kg ^{0.75})	$1/EC_{50}$ * (mL/mU)	Avg. $f_{stim}^{ex\ utero}$ ** (g/day· kg ^{3/4})	$E_{max}^{*,**}$ (g/day· kg ^{0.75})	$EC_{50}^{*,**}$ (mU/mL)
Mean	65.8	97.7	37.5	0.414	0.0140	0.062	0.144	0.566	28.5
SD	42.7	31.5	3.1	0.156	0.0127	0.037	0.109	0.372	28.8
MSE%	11.6%	6.2%	9.2%	7.4%	123%	33.6%	NA	NA	NA

*Only determined in 6 out of 14 subjects based on AIC

**Secondary parameter

SD: Standard deviation

MSE%: Mean percent standard error of parameter estimate

NA: Not applicable

Table 6.3. Observed and estimated Hb amounts over the first 30 days of life from the pharmacodynamic Hb mass balance model ($n = 14$).

	Estimated amount present at birth (g)	Estimated amount produced (g)	Observed amount removed (g)	Observed amount transfused (g)
Mean	10.3	4.69	5.97	12.3
SD	2.70	3.32	2.53	7.01

SD: Standard deviation

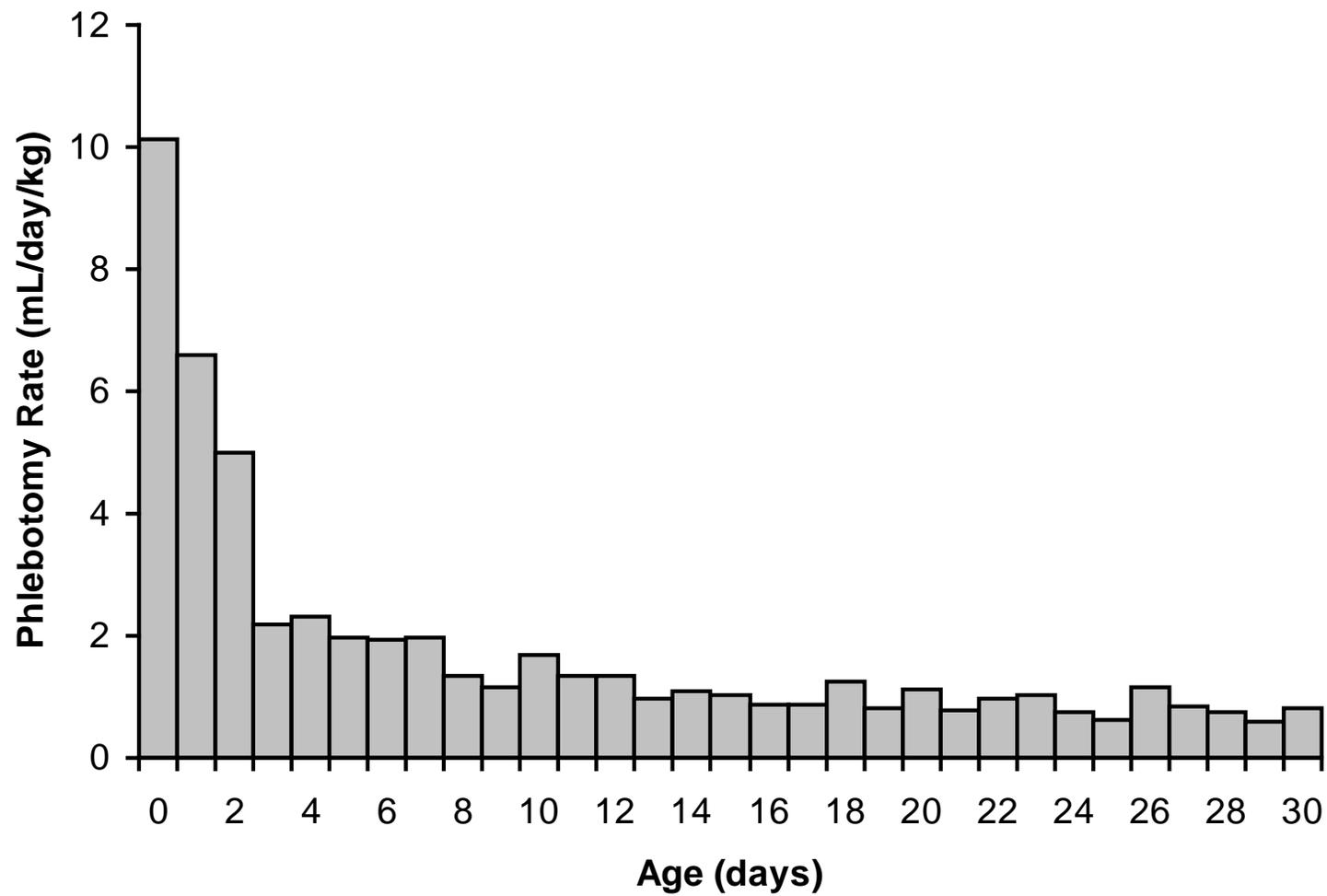


Figure 6.1. Mean rate of phlebotomy blood loss versus subject age.

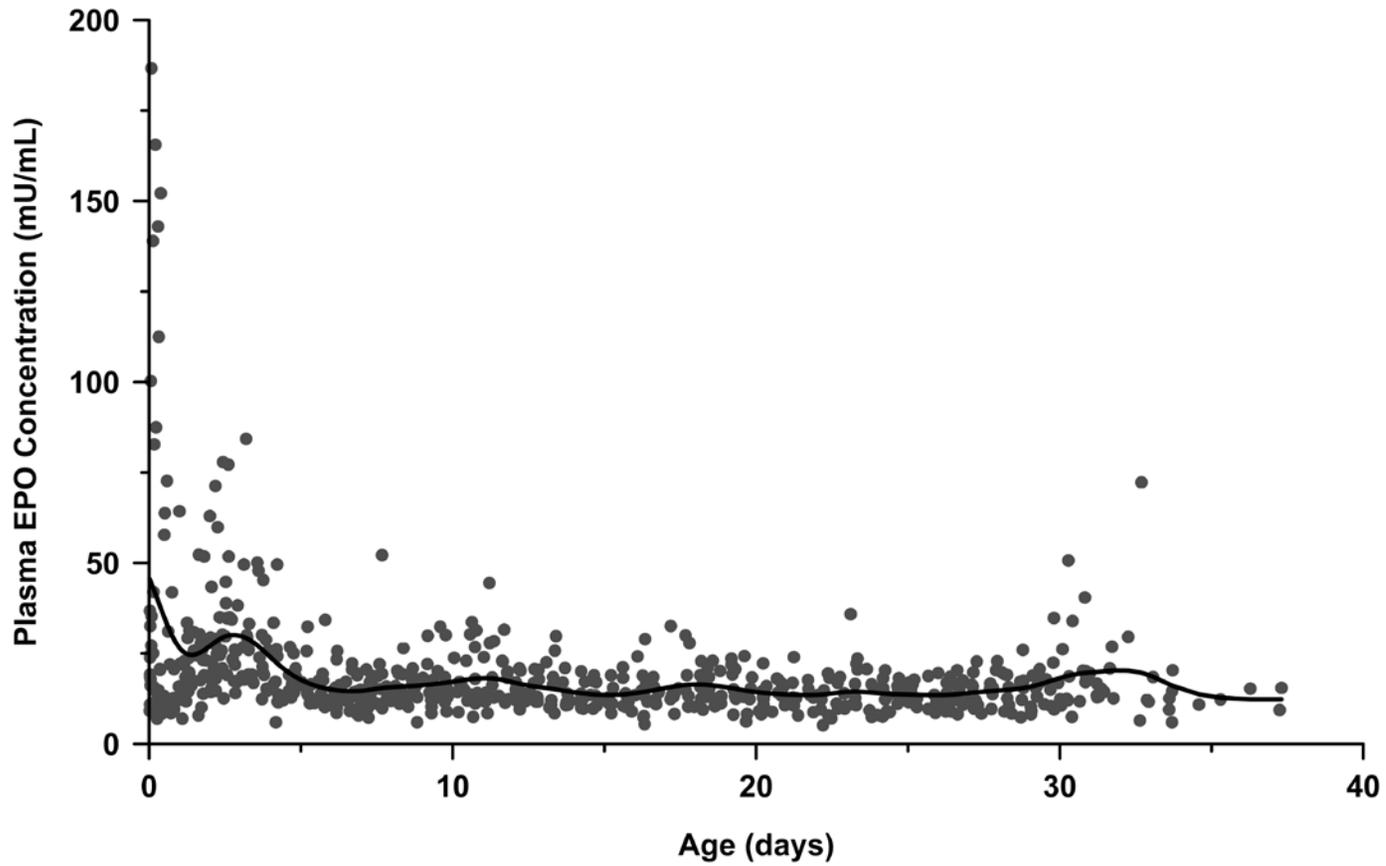


Figure 6.2. Plasma EPO concentrations versus subject age.
The solid line represents a non-parametric smoothing spline fit to the observed data (circles).

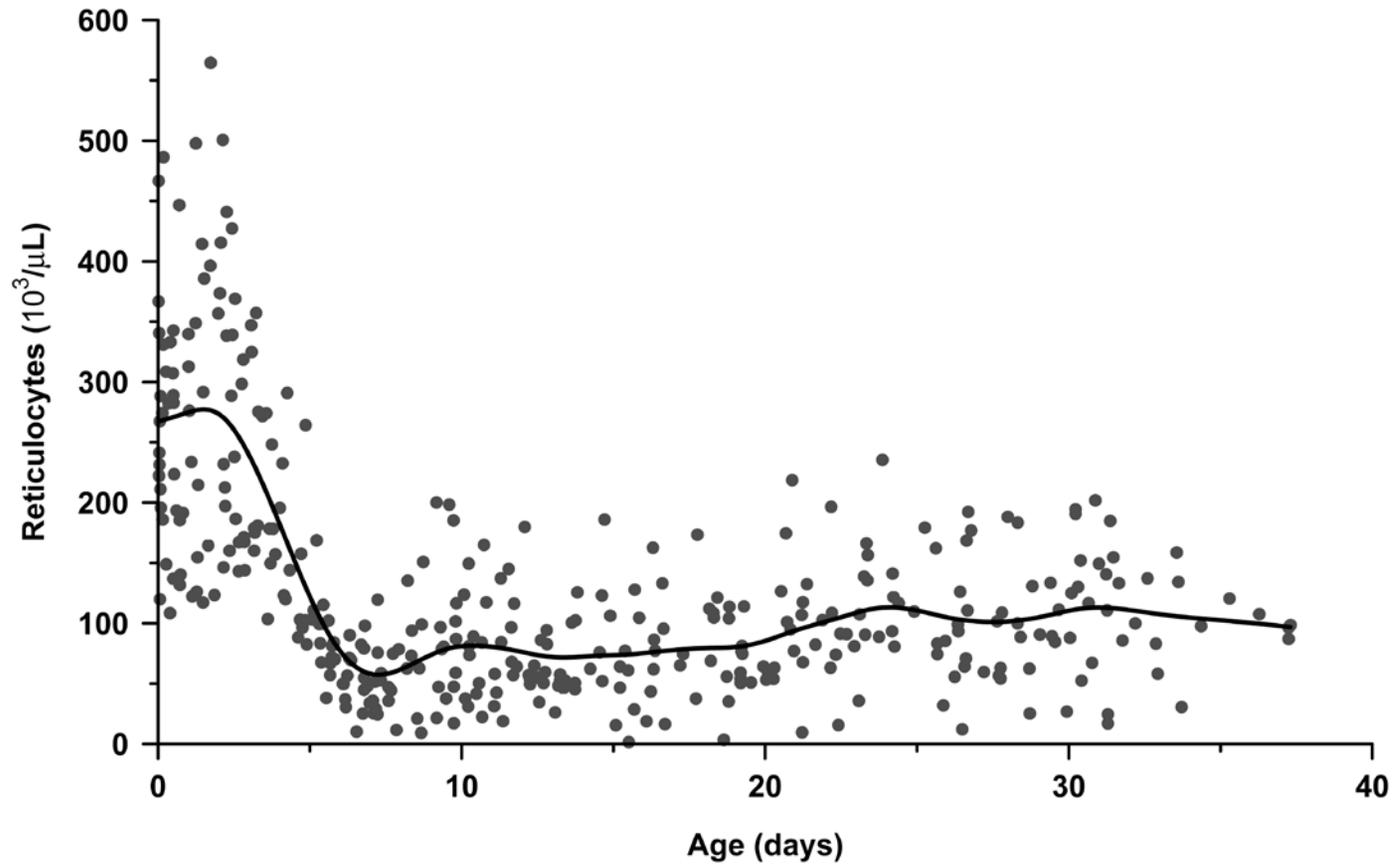


Figure 6.3. Reticulocyte counts versus subject age.
The solid line represents a non-parametric smoothing spline fit to the observed data (circles).

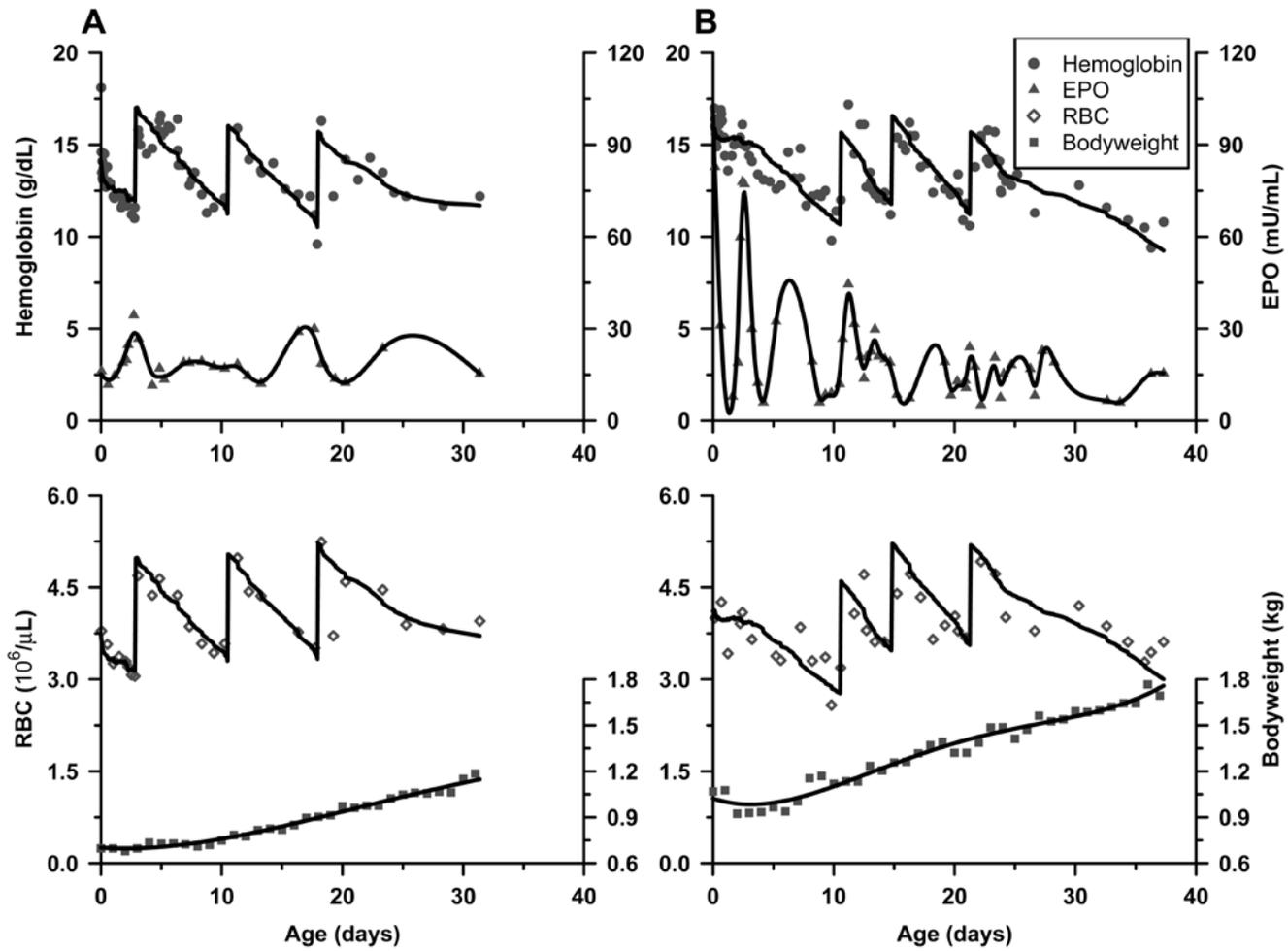


Figure 6.4. Pharmacodynamic Hb mass balance model fit to representative subjects.

The symbols represent the observed data and the lines represent the model fit. Panels A (left-hand side) and B (right-hand side) are different subjects.

APPENDIX A. DERIVATIONS AND PROOFS FOR CHAPTER 2

A.1 Derivation of Eq. 2.11

By evaluation of Eq. 2.6:

$$I_h(t) = 0, \quad \text{for } t - t_0 < a \quad (\text{A.1.1})$$

$$I_h(t) = \int_{t_0}^{t-a} f_{stim}(u) du, \quad \text{for } a \leq t - t_0 < b \quad (\text{A.1.2})$$

$$I_h(t) = \int_{t-b}^{t-a} f_{stim}(u) du, \quad \text{for } t - t_0 \geq b > a \quad (\text{A.1.3})$$

Differentiation of Eq. A.1.1 through A.1.3 gives:

$$I_h'(t) = 0, \quad \text{for } t - t_0 < a \quad (\text{A.1.4})$$

$$I_h'(t) = f_{stim}(t - a), \quad \text{for } a \leq t - t_0 < b \quad (\text{A.1.5})$$

$$I_h'(t) = f_{stim}(t - a) - f_{stim}(t - b), \quad \text{for } t - t_0 \geq b > a \quad (\text{A.1.6})$$

Eq. A.1.4 through A.1.6 generalize to:

$$I_h'(t) = f_{stim}(t - a) \cdot U(t - t_0 - a) - f_{stim}(t - b) \cdot U(t - t_0 - b) \quad (\text{A.1.7})$$

which proves Eq. 2.11.

A.2 Proof of unique solution to Eq. 2.22 when $a'(t) > -1$

Eq. 2.22 may be written as:

$$G(y, a(y)) \equiv y - t + a(y) = 0 \quad (\text{A.2.1})$$

with $y \equiv x(t)$. Treating 't' as fixed the Jacobian determinant, $|J_G|$, of Eq. A.2.1 is:

$$|J_G| = \frac{\partial(y - t + a(y))}{\partial y} = 1 + a'(y) \quad (\text{A.2.2})$$

Thus by the Implicit Function Theorem (162), Eq. 2.22 has a unique solution if $|J_G| \neq 0$, i.e. if $1 + a'(y) \neq 0$, which is satisfied when $a'(y) > -1$ or when $a'(y) < -1$ for all 'y'.

The condition $a'(t) > -1$ for all 't' values ensures that $a'(y) > -1$ for all 'y' values, thus ensuring a unique solution to Eq. 2.22.

A.3 Derivation of Eq. 2.35

It is recognized that the phlebotomy at time $t = T_p$ can only remove cells already present in the circulation. If we separate Eq. 2.15 such as:

$$N(t) = \int_{-\infty}^{T_p - a(x(T_p))} f_{stim}(u) \cdot UR(t - u, u) du + \int_{T_p - a(x(T_p))}^t f_{stim}(u) \cdot UR(t - u, u) du, \quad t = T_p \quad (\text{A.3.1})$$

and define:

$$I_j(t) \equiv \int_{-\infty}^{T_p - a(x(T_p))} f_{stim}(u) \cdot UR(t - u, u) du \quad (\text{A.3.2})$$

$$I_k(t) \equiv \int_{T_p - a(x(T_p))}^t f_{stim}(u) \cdot UR(t - u, u) du \quad (\text{A.3.3})$$

It is apparent that the $I_j(t)$ term gives all the cells that have already entered into the circulation at time, $t = T_p$, and thus all the cells that can be removed by the phlebotomy. By evaluation of Eq. A.3.2:

$$I_j(t) = \int_{t-b}^{t-a(x(t))} f_{stim}(u) du, \quad t - T_p < 0 \quad (\text{A.3.4})$$

$$I_j(t) = \int_{t-b}^{T_p - a(x(T_p))} f_{stim}(u) du, \quad 0 \leq t - T_p < b - a(x(t)) \quad (\text{A.3.5})$$

$$I_j(t) = 0, \quad t - T_p \geq b - a(x(t)) \quad (\text{A.3.6})$$

Since the $I_j(t)$ term represents the cells that can be removed by the phlebotomy, it is multiplied by F of Eq. 2.34 at time $t = T_p + \varepsilon$, resulting in:

$$N(t) = F \cdot I_j(t) + I_k(t) = I_j(t) + I_k(t) - [1 - F] \cdot I_j(t), \quad \text{for } t > T_p \quad (\text{A.3.7})$$

The first term on the right hand side of Eq. A.3.7, $I_j(t) + I_k(t)$, is recognized as $N(t)$ of Eq. 2.15, which if differentiated will result in Eq. 2.29. Therefore, $-[1 - F] \cdot I_j(t)$ represents the correction term. Since $t - T_p > 0$ in Eq. A.3.7, $I_j(t)$ only has non zero values from time $t = T_p$ to $t = T_p + b - a(x(T_p))$ as given by Eq. A.3.5. Differentiation of Eq. A.3.5 results in:

$$I_j'(t) = -f_{stim}(t - b), \quad T_p \leq t < T_p + b - a(x(T_p)) \quad (\text{A.3.8})$$

Thus the correction term generalizes to:

$$+ [1 - F] \cdot [U(t - T_p) - U(t - (T_p + b - a(x(T_p))))] \cdot f_{stim}(t - b) \quad (\text{A.3.9})$$

which proves Eq. A.35.

A.4 Alternative parameterization of Eq. 2.36

A useful alternative parameterization to ensure that the conditions of Eq. 2.42 hold and for computational convenience follows: from equation Eq. 2.22 define:

$$t - x(t) = a(x(t)) \equiv e^{z(t)} \quad (\text{A.4.1})$$

The exponential transformation ensures that $a(x(t)) > 0$ for any $x(t)$ value.

Rearrangement and differentiation give:

$$x(t) = t - e^{z(t)} \quad (\text{A.4.2})$$

$$x'(t) = 1 - z'(t) \cdot e^{z(t)} \quad (\text{A.4.3})$$

From Eq. 2.33 and Eq. 2.42 it follows that:

$$\mu_{RET}(t) = \mu_{RET}(t_0) = \mu_{RET,SS} = b - a_{SS}, \quad t \leq t_0 \quad (\text{A.4.4})$$

$$a(x(t)) = b - \mu_{RET}(x(t)) \quad (\text{A.4.5})$$

Substituting Eq. A.4.2 through Eq. A.4.5 into Eq. 2.36 results in:

$$N'(t) = \begin{cases} \left[1 - z'(t) \cdot e^{z(t)} \right] \cdot f_{stim}(t - e^{z(t)}) - f_{stim}^{SS} \cdot U(t - t_0 - (b - \mu_{RET,SS})) \\ - \left[f_{stim}(t - b) - f_{stim}^{SS} \right] \cdot U(t - t_0 - b) \\ + \left[1 - F \right] \cdot \left[U(t - T_P) - U(t - (T_P + \mu_{RET}(T_P - e^{z(T_P)}))) \right] \cdot f_{stim}(t - b) \end{cases}, \quad t > t_0 \quad (\text{A.4.6})$$

with:

$$N(t) = N(t_0) = f_{stim}^{SS} \cdot \mu_{RET,SS}, \quad t \leq t_0 \quad (\text{A.4.7})$$

$$N(t) = \begin{cases} N(T_P) & \text{for } t = T_P \\ F \cdot N(T_P) & \text{for } t = T_P + \varepsilon \end{cases} \quad (\text{A.4.8})$$

Additionally, substitution of Eq. A.4.2 into the right-hand side of Eq. A.4.1 results in:

$$a(t - e^{z(t)}) = e^{z(t)} \quad (\text{A.4.9})$$

Differentiation of Eq. A.4.5 and A.4.9, with substitution and rearrangement results in the following additional conditions to Eq. A.4.6:

$$z'(t) = \frac{-\mu_{RET}'(t - e^{z(t)}) \cdot e^{-z(t)}}{1 - \mu_{RET}'(t - e^{z(t)})}, \quad t > t_0 \text{ and } \mu_{RET}'(t) < 1 \quad (\text{A.4.10})$$

$$z(t) = z(t_0) = \ln(b - \mu_{RET,SS}), \quad t \leq t_0 \quad (\text{A.4.11})$$

$$\mu_{RET}(t) = \mu_{RET}(t_0) = \mu_{RET,SS}, \quad t \leq t_0 \quad (\text{A.4.12})$$

The above $\mu_{RET}'(t) < 1$ constraint corresponds to $a'(t) > -1$ to ensure that Eq. 2.22 has a single solution (Figure 2.2 and Appendix A.2).

A.5 Circulating reticulocyte lifespan spline function

The reticulocyte systemic circulation lifespan, $\mu_{RET}(t)$, was modeled as follows:

$$\mu_{RET}(t) = \mu_{RET,0}, \quad t < T_1 \quad (\text{A.5.1})$$

$$\mu_{RET}(t) = \alpha_0 + \alpha_1 t + \alpha_2 t^2 + \alpha_3 t^3 + \alpha_4 t^4 + \alpha_5 t^5, \quad T_1 \leq t \leq T_2 \quad (\text{A.5.2})$$

$$\mu_{RET}(t) = \mu_{RET,END}, \quad t > T_2 \quad (\text{A.5.3})$$

Such that:

$$T_P < T_1 < T_2 \quad (\text{A.5.4})$$

$$\mu_{RET}(T_1) = \mu_{RET,0} \quad (\text{A.5.5})$$

$$\mu_{RET}(T_2) = \mu_{RET,END} \quad (\text{A.5.6})$$

$$\mu_{RET}'(T_1) = \mu_{RET}'(T_2) = 0 \quad (\text{A.5.7})$$

Therefore:

$$\mu_{RET,SS} = \mu_{RET,0} \quad (\text{A.5.8})$$

In order to satisfy the above constraints, the fifth order polynomial that represents $\mu_{RET}(t)$ when $T_1 \leq t \leq T_2$ was parameterized in terms of the parametric terms, $\mu_{RET,0}$, $\mu_{RET,END}$, T_1 and T_2 , and the nonparametric coefficients, α_4 and α_5 .

APPENDIX B. DERIVATIONS AND PROOFS FOR CHAPTER 3

B.1 Derivation of Eq. 3.6

To account for the removal by phlebotomy of a certain fraction, $1 - F$, of cells at time $t = T_p$ is equivalent to label this fraction of cells at time T_p and only counting the unlabeled cells.

$$N_{tot}(t) = N_{lab}(t) + N_{unlab}(t) \quad (\text{B.1.1})$$

where:

$$N_{tot}(t) \equiv \text{total number of cells}$$

$$N_{lab}(t) \equiv \text{number of labeled cells}$$

$$N_{unlab}(t) \equiv \text{number of unlabeled cells}$$

The interest is to quantify the number of unlabeled cells (i.e. the cells not removed by the phlebotomy), which from Eq. B.1.1 is given by:

$$N(t) \equiv N_{unlab}(t) = N_{tot}(t) - N_{lab}(t) \quad (\text{B.1.2})$$

If it is assumed that the cells behave independent of each other regardless of being labeled or not (which is a basic assumption of the derivation), then the superposition principle holds. Let the probability that a cell that enters the sampling space at time z is still present at time $z + x$, where x is a non-negative time value, be denoted by $P(x, z)$, then according to the superposition principle that arises from a linear cellular disposition:

$$N_{tot}(t) = \int_{-\infty}^t f_{prod}(u) \cdot P(t - u, u) du \quad (\text{B.1.3})$$

Eq. B.1.3 can be written as:

$$\begin{aligned} N_{tot}(t) &= \int_{-\infty}^t f_{prod}(u) \cdot P(t - u, u) du \\ &= \int_{-\infty}^{\min(t, T_p)} f_{prod}(u) \cdot P(t - u, u) du + \int_{\min(t, T_p)}^t f_{prod}(u) \cdot P(t - u, u) du \end{aligned} \quad (\text{B.1.4})$$

where $\min(t, T_p)$ is the minimum value of t and T_p . If the production of cells ($f_{prod}(t)$), i.e. input of new cells into the sampling space, is stopped at time T_p then the second integral in Eq. B.1.4 is equal to zero for $t > T_p$ since $f_{prod}(t) = 0$. Additionally, if $t \leq T_p$ the second integral is still equal to zero. Hence total number of cells would be:

$$N_{tot}(t) = \int_{-\infty}^{\min(t, T_p)} f_{prod}(u) \cdot P(t - u, u) du \quad (\text{B.1.5})$$

Equation B.1.5 is equivalent to labeling all cells in the sampling space at time T_p and counting the number of labeled cells thereafter. Thus, if only a fraction, $1 - F$, of the cells present at time T_p are labeled (i.e. removed by the phlebotomy) then the number of labeled cells is:

$$N_{lab}(t) = U(t - T_p) \cdot [1 - F] \cdot \int_{-\infty}^{T_p} f_{prod}(u) \cdot P(t - u, u) du \quad (\text{B.1.6})$$

where U is the unit step function described by:

$$U(t) = \begin{cases} 1 & \text{if } t \geq 0 \\ 0 & \text{otherwise} \end{cases} \quad (\text{B.1.7})$$

which has been introduced in Eq. B.1.6 to make the equation valid for any value of t . Equations B.1.2, B.1.3, and B.1.6 give:

$$N(t) = \int_{-\infty}^t f_{prod}(u) \cdot P(t-u, u) du - U(t-T_p) \cdot [1-F] \cdot \int_{-\infty}^{T_p} f_{prod}(u) \cdot P(t-u, u) du \quad (\text{B.1.8})$$

If $\ell(\tau, z)$ denotes the *time variant* p.d.f. of cellular lifespans, where τ is the cellular lifespan and z is an arbitrary time of production, then:

$$P(x, z) = 1 - \int_0^x \ell(\tau, z) d\tau \quad (\text{B.1.9})$$

which can be recognized as the unit response of Eq. 3.1. Inserting Eq. B.1.9 into Eq. B.1.8 gives:

$$N(t) = \int_{-\infty}^t f_{prod}(u) \cdot \left[1 - \left[\int_0^{t-u} \ell(\tau, u) d\tau \right] \right] du - U(t-T_p) \cdot [1-F] \cdot \int_{-\infty}^{T_p} f_{prod}(u) \cdot \left[1 - \left[\int_0^{t-u} \ell(\tau, u) d\tau \right] \right] du \quad (\text{B.1.10})$$

For $t \geq T_p$ $U(t-T_p) \equiv 1$ and Eq. B.1.10 simplifies to the following expression:

$$N(t) = F \cdot \int_{-\infty}^{T_p} f_{prod}(u) \cdot \left[1 - \left[\int_0^{t-u} \ell(\tau, u) d\tau \right] \right] du + \int_{T_p}^t f_{prod}(u) \cdot \left[1 - \left[\int_0^{t-u} \ell(\tau, u) d\tau \right] \right] du, \quad t \geq T_p \quad (\text{B.1.11})$$

Completing the derivation of Eq. 3.6.

B.2 Derivation of Eq. 3.8

The probability that a cell is present in the sampling space is given by the intersection of two events: the time since stimulation is less than b (Event 1) and the cell has been released into the sampling space (Event 2). The probabilities of these two individual events are given by:

$$P(\text{Event 1}) = P(t-s < b) = 1 - \{t-s < b\} = 1 - U(t-s-b) \quad (\text{B.2.1})$$

$$P(\text{Event 2}) = P(\Omega \leq t, s) = \int_0^{t-s} r(\omega, s) d\omega, \quad t \geq s, \quad 0 \leq \omega < \infty \quad (\text{B.2.2})$$

Due to the independence assumption of the two events, the probability of both events occurring (i.e. the intersection of the events) is simply the product of the individual event probabilities, as given by:

$$P(\text{Event 1} \cap \text{Event 2}) = P(\text{Event 1}) \cdot P(\text{Event 2}) = [1 - U(t-s-b)] \cdot \int_0^{t-s} r(\omega, s) d\omega, \quad t \geq s, \quad 0 \leq \omega < \infty \quad (\text{B.2.3})$$

Given the assumed independent disposition of cells following stimulation, the probability that a cell is present in the sampling space is the unit response of the cell, completing the derivation of Eq. 3.8.

B.3 Derivation of Eq. 3.11

Following the derivation of Eq. 3.6 (Appendix B.1), from Eq. B.1.2 the interest is to quantify the number of unlabeled cells (i.e. the cells not removed by the phlebotomy). Let the probability that the time since stimulation, x , for a cell is less than some positive constant b (i.e. probability of Event 1) be denoted by $P_1(x)$. Equivalently, $P_1(x)$ can be thought of as the probability that a cell exists as the cell type of interest (either outside or in the sampling space). Additionally, let the probability that a cell stimulated at time s has been released into the sampling space at time $s + x$, be denoted by $P_2(x, s)$ (i.e. probability of Event 2). If these two events are assumed to be independent (as is assumed in the model formulation, see Appendix B.2), then the probability that a cell is present in the sampling space as the cell type of interest (i.e. the intersection of Event 1 and Event 2) is given by the multiplication of these probabilities. Then according to the superposition principle that arises from a linear cellular disposition:

$$N_{tot}(t) = \int_{-\infty}^t f_{stim}(u) \cdot P_1(t-u) \cdot P_2(t-u, u) du \quad (\text{B.3.1})$$

which can also be written as:

$$\begin{aligned} N_{tot}(t) &= \int_{-\infty}^t f_{stim}(u) \cdot P_1(t-u) \cdot P_2(t-u, u) du \\ &= \int_{-\infty}^{\min(t, T_p)} f_{stim}(u) \cdot P_1(t-u) \cdot P_2(t-u, u) du + \int_{\min(t, T_p)}^t f_{stim}(u) \cdot P_1(t-u) \cdot P_2(t-u, u) du \\ &= \int_{-\infty}^{\min(t, T_p)} f_{stim}(u) \cdot P_1(t-u) \cdot [P_2(\min(t, T_p) - u, u) + P_2(t-u, u) - P_2(\min(t, T_p) - u, u)] du \\ &\quad + \int_{\min(t, T_p)}^t f_{stim}(u) \cdot P_1(t-u) \cdot P_2(t-u, u) du \\ &= \int_{-\infty}^{\min(t, T_p)} f_{stim}(u) \cdot P_1(t-u) \cdot P_2(\min(t, T_p) - u, u) du \\ &\quad + \int_{-\infty}^{\min(t, T_p)} f_{stim}(u) \cdot P_1(t-u) \cdot [P_2(t-u, u) - P_2(\min(t, T_p) - u, u)] du \\ &\quad + \int_{\min(t, T_p)}^t f_{stim}(u) \cdot P_1(t-u) \cdot P_2(t-u, u) du \end{aligned} \quad (\text{B.3.2})$$

where $\min(t, T_p)$ is the minimum value of t and T_p . If the input of new cells into the sampling space were to be stopped at time T_p , then $f_{stim}(t)$ must be equal to zero when $t \geq T_p$ giving:

$$\begin{aligned}
N_{tot}(t) &= \int_{-\infty}^{\min(t, T_p)} f_{stim}(u) \cdot P_1(t-u) \cdot P_2(\min(t, T_p) - u, u) du \\
&\quad + \int_{-\infty}^{\min(t, T_p)} f_{stim}(u) \cdot P_1(t-u) \cdot [P_2(t-u, u) - P_2(\min(t, T_p) - u, u)] du \\
&\quad + \int_{\min(t, T_p)}^t 0 \cdot P_1(t-u) \cdot P_2(t-u, u) du \\
&= \int_{-\infty}^{\min(t, T_p)} f_{stim}(u) \cdot P_1(t-u) \cdot P_2(\min(t, T_p) - u, u) du \\
&\quad + \int_{-\infty}^{\min(t, T_p)} f_{stim}(u) \cdot P_1(t-u) \cdot [P_2(t-u, u) - P_2(\min(t, T_p) - u, u)] du
\end{aligned} \tag{B.3.3}$$

Likewise, if $t < T_p$ then the integral of the third integrand of Eq. B.3.2 would also be equal to zero, hence Eq. B.3.3 is true for all t . Furthermore, when $t > T_p$ it is recognized that $P_2(t-s, s) - P_2(\min(t, T_p) - s, s)$ is the probability that a cell stimulated at time s is released into the sampling space between time T_p and time t (if $t \leq T_p$ it's the probability of release between time t and time t , which is equal to zero). However, since the input of new cells stopped at time T_p , then $P_2(t-s, s) - P_2(\min(t, T_p) - s, s)$ must also be equal to zero giving:

$$\begin{aligned}
N_{tot}(t) &= \int_{-\infty}^{\min(t, T_p)} f_{stim}(u) \cdot P_1(t-u) \cdot P_2(\min(t, T_p) - u, u) du \\
&\quad + \int_{-\infty}^{\min(t, T_p)} f_{stim}(u) \cdot P_1(t-u) \cdot [0] du \\
&= \int_{-\infty}^{\min(t, T_p)} f_{stim}(u) \cdot P_1(t-u) \cdot P_2(\min(t, T_p) - u, u) du
\end{aligned} \tag{B.3.4}$$

Equation B.3.4 is equivalent to labeling all cells in the sampling space at time T_p and counting the number of labeled cells. Thus if only a fraction, $1 - F$, of the cells present at time T_p are labeled (i.e. removed by the phlebotomy) then the number of labeled cells is:

$$N_{lab}(t) = U(t - T_p) \cdot [1 - F] \cdot \int_{-\infty}^{T_p} f_{stim}(u) \cdot P_1(t-u) \cdot P_2(T_p - u, u) du \tag{B.3.5}$$

Equations B.1.2, B.3.1, and B.3.5 give:

$$\begin{aligned}
N(t) &= \int_{-\infty}^t f_{stim}(u) \cdot P_1(t-u) \cdot P_2(t-u, u) du \\
&\quad - U(t - T_p) \cdot [1 - F] \cdot \int_{-\infty}^{T_p} f_{stim}(u) \cdot P_1(t-u) \cdot P_2(T_p - u, u) du
\end{aligned} \tag{B.3.6}$$

The probability that x is less than some positive constant b (i.e. $P_1(x)$) is either 1 or 0, since x either is or is not less than b , respectively. Therefore:

$$P_1(x) = 1 - U(x - b) \tag{B.3.7}$$

which can be recognized as the probability of Event 1 of Eq. B.2.1. If $r(\omega, s)$ is the *time variant* p.d.f of cellular release time delays, where ω is the cellular release time delay and s is an arbitrary time of stimulation, then:

$$P_2(x, s) = \int_0^x r(\omega, s) d\omega \tag{B.3.8}$$

which can be recognized as the probability of Event 2 of Eq. B.2.2. Substitution of Eq. B.3.7 and Eq. B.3.8 in Eq. B.3.6 results in:

$$\begin{aligned}
N(t) &= \int_{-\infty}^t f_{stim}(u) \cdot [1 - U(t - u - b)] \cdot \left[\int_0^{t-u} r(\omega, u) d\omega \right] du \\
&\quad - U(t - T_p) \cdot [1 - F] \cdot \int_{-\infty}^{T_p} f_{stim}(u) \cdot [1 - U(t - u - b)] \cdot \left[\int_0^{T_p - u} r(\omega, u) d\omega \right] du \\
&= \int_{t-b}^t f_{stim}(u) \cdot \left[\int_0^{t-u} r(\omega, u) d\omega \right] du - U(t - T_p) \cdot [1 - F] \cdot \int_{\min(T_p, t-b)}^{T_p} f_{stim}(u) \cdot \left[\int_0^{T_p - u} r(\omega, u) d\omega \right] du
\end{aligned} \tag{B.3.9}$$

The unit step functions of the integrands are eliminated in the simplification step of Eq. B.3.9 since it is recognized that the integrand will have a value of 0 at all times when $u < t - b$. However, by eliminating the units step functions the lower bound of the first integral in the second term of Eq. B.3.9 must then be constrained to be the $\min(T_p, t - b)$ to maintain the integrals evaluation to zero at all values of $u < t - b$. For $t - b < T_p \leq t$ Eq. B.3.9 further simplifies to the following expression:

$$\begin{aligned}
N(t) &= \int_{t-b}^t f_{stim}(u) \cdot \left[\int_0^{t-u} r(\omega, u) d\omega \right] du - [1 - F] \cdot \int_{t-b}^{T_p} f_{stim}(u) \cdot \left[\int_0^{T_p - u} r(\omega, u) d\omega \right] du, \\
&\qquad\qquad\qquad t - b < T_p \leq t \qquad\qquad\qquad \text{(B.3.10)}
\end{aligned}$$

It can also be observed from Eq. B.3.9 that if T_p is not contained in the time interval from $t - b$ to t , only the first term is non-zero, giving the solution identical to Eq. 3.10. This completes the derivation of Eq. 3.11.

B.4 Derivation of Eq. 3.12

The cellular lifespan for a particular cell type of interest, defined as the time period from release of a cell into the sampling space to the time the cell is removed (or transformed) from the sampling space, is given by:

$$\tau = b - \omega, \qquad 0 \leq \tau < b \tag{B.4.1}$$

where ω denotes the time delay from cellular stimulation to appearance or release of the subsequently stimulated cell(s). Therefore it follows that:

$$\ell(\tau, s) \propto r(b - \tau, s), \qquad 0 \leq \tau < b \tag{B.4.2}$$

The normalizing factor for Eq. B.4.2 is the definite integral of the release time delay p.d.f. from 0 to b , giving:

$$\ell(\tau, s) = \frac{r(b - \tau, s)}{\int_0^b r(\omega, s) d\omega}, \qquad 0 \leq \tau < b \tag{B.4.3}$$

Completing the derivation of Eq. 3.12.

B.5 Derivation of Eq. 3.19

Following the labeled cell derivation from Appendix B.1 and Appendix B.3, let the number of cells removed at time T_{p1} be denoted by $N_{lab1}(t)$ and the number of cells removed at time T_{p2} be denoted by $N_{lab2}(t)$, then the total number of cells is given by:

$$N_{tot}(t) = N_{lab1}(t) + N_{lab2}(t) + N_{unlab}(t) \quad (\text{B.5.1})$$

where $N_{unlab}(t)$ is the number of unlabeled cells. Again, the interest is to quantify the number of unlabeled cells (i.e. cells not removed by the phlebotomies), which from Eq. B.5.1 is given by:

$$N(t) \equiv N_{unlab}(t) = N_{tot}(t) - N_{lab1}(t) - N_{lab2}(t) \quad (\text{B.5.2})$$

From Eq. B.3.1:

$$N_{tot}(t) = \int_{-\infty}^t f_{stim}(u) \cdot P_1(t-u) \cdot P_2(t-u, u) du \quad (\text{B.5.3})$$

and from Eq. B.3.5:

$$N_{lab1}(t) = U(t - T_{P1}) \cdot [1 - F_1] \cdot \int_{-\infty}^{T_{P1}} f_{stim}(u) \cdot P_1(t-u) \cdot P_2(T_{P1} - u, u) du \quad (\text{B.5.4})$$

where $P_1(x)$ and $P_2(x, s)$ are defined as before (Appendix B.3). From Eq. B.3.6 the total number of cells excluding cells labeled at time T_{P1} is given by:

$$\begin{aligned} N_{-lab1}(t) \equiv N_{tot}(t) - N_{lab1}(t) &= \int_{-\infty}^t f_{stim}(u) \cdot P_1(t-u) \cdot P_2(t-u, u) du \\ &\quad - U(t - T_{P1}) \cdot [1 - F_1] \cdot \int_{-\infty}^{T_{P1}} f_{stim}(u) \cdot P_1(t-u) \cdot P_2(T_{P1} - u, u) du \end{aligned} \quad (\text{B.5.5})$$

From Eq. B.3.2 through Eq. B.3.4 it is realized that if the input of cells into the sampling space is stopped at time T_{P2} the first integrand in Eq. B.5.5 is equal to:

$$\int_{-\infty}^{\min(t, T_{P2})} f_{stim}(u) \cdot P_1(t-u) \cdot P_2(\min(t, T_{P2}) - u, u) du \quad (\text{B.5.6})$$

giving:

$$\begin{aligned} N_{-lab1}(t) &= \int_{-\infty}^{\min(t, T_{P2})} f_{stim}(u) \cdot P_1(t-u) \cdot P_2(\min(t, T_{P2}) - u, u) du \\ &\quad - U(t - T_{P1}) \cdot [1 - F_1] \cdot \int_{-\infty}^{T_{P1}} f_{stim}(u) \cdot P_1(t-u) \cdot P_2(T_{P1} - u, u) du \end{aligned} \quad (\text{B.5.7})$$

where Eq. B.5.7 is equivalent to labeling all cells in the sampling space at time T_{P2} and counting the number of cells with the only the label given at time T_{P2} . Thus if only a fraction, $1 - F_2$, of the cells present at time T_{P2} are labeled (i.e. removed by the second phlebotomy) then the number of cells labeled at time T_{P2} is:

$$\begin{aligned} N_{lab2}(t) &= U(t - T_{P2}) \cdot [1 - F_2] \cdot \left[\int_{-\infty}^{T_{P2}} f_{stim}(u) \cdot P_1(t-u) \cdot P_2(T_{P2} - u, u) du \right. \\ &\quad \left. - U(t - T_{P1}) \cdot [1 - F_1] \cdot \int_{-\infty}^{T_{P1}} f_{stim}(u) \cdot P_1(t-u) \cdot P_2(T_{P1} - u, u) du \right] \\ &= U(t - T_{P2}) \cdot [1 - F_2] \cdot \int_{-\infty}^{T_{P2}} f_{stim}(u) \cdot P_1(t-u) \cdot P_2(T_{P2} - u, u) du \\ &\quad - U(t - T_{P2}) \cdot [1 - F_2] \cdot [1 - F_1] \cdot \int_{-\infty}^{T_{P1}} f_{stim}(u) \cdot P_1(t-u) \cdot P_2(T_{P1} - u, u) du \end{aligned} \quad (\text{B.5.8})$$

Equations B.5.2, B.5.3, B.5.4, and B.5.8 give:

$$\begin{aligned}
N(t) &= \int_{-\infty}^t f_{stim}(u) \cdot P_1(t-u) \cdot P_2(t-u, u) du \\
&\quad - U(t-T_{P_1}) \cdot [1-F_1] \cdot \int_{-\infty}^{T_{P_1}} f_{stim}(u) \cdot P_1(t-u) \cdot P_2(T_{P_1}-u, u) du \\
&\quad - U(t-T_{P_2}) \cdot [1-F_2] \cdot \int_{-\infty}^{T_{P_2}} f_{stim}(u) \cdot P_1(t-u) \cdot P_2(T_{P_2}-u, u) du \\
&\quad + U(t-T_{P_2}) \cdot [1-F_2] \cdot [1-F_1] \cdot \int_{-\infty}^{T_{P_1}} f_{stim}(u) \cdot P_1(t-u) \cdot P_2(T_{P_1}-u, u) du
\end{aligned} \tag{B.5.9}$$

Substitution of Eq. B.3.7 and Eq. B.3.8 in Eq. B.5.9 results in:

$$\begin{aligned}
N(t) &= \int_{-\infty}^t f_{stim}(u) \cdot [1-U(t-u-b)] \cdot \left[\int_0^{t-u} r(\omega, u) d\omega \right] du \\
&\quad - U(t-T_{P_1}) \cdot [1-F_1] \cdot \int_{-\infty}^{T_{P_1}} f_{stim}(u) \cdot [1-U(t-u-b)] \cdot \left[\int_0^{T_{P_1}-u} r(\omega, u) d\omega \right] du \\
&\quad - U(t-T_{P_2}) \cdot [1-F_2] \cdot \int_{-\infty}^{T_{P_2}} f_{stim}(u) \cdot [1-U(t-u-b)] \cdot \left[\int_0^{T_{P_2}-u} r(\omega, u) d\omega \right] du \\
&\quad + U(t-T_{P_2}) \cdot [1-F_2] \cdot [1-F_1] \cdot \int_{-\infty}^{T_{P_1}} f_{stim}(u) \cdot [1-U(t-u-b)] \cdot \left[\int_0^{T_{P_1}-u} r(\omega, u) d\omega \right] du \\
&= \int_{t-b}^t f_{stim}(u) \cdot \left[\int_0^{t-u} r(\omega, u) d\omega \right] du - U(t-T_{P_1}) \cdot [1-F_1] \cdot \int_{\min(T_{P_1}, t-b)}^{T_{P_1}} f_{stim}(u) \cdot \left[\int_0^{T_{P_1}-u} r(\omega, u) d\omega \right] du \\
&\quad - U(t-T_{P_2}) \cdot [1-F_2] \cdot \int_{\min(T_{P_2}, t-b)}^{T_{P_2}} f_{stim}(u) \cdot \left[\int_0^{T_{P_2}-u} r(\omega, u) d\omega \right] du \\
&\quad + U(t-T_{P_2}) \cdot [1-F_2] \cdot [1-F_1] \cdot \int_{\min(T_{P_1}, t-b)}^{T_{P_1}} f_{stim}(u) \cdot \left[\int_0^{T_{P_1}-u} r(\omega, u) d\omega \right] du
\end{aligned} \tag{B.5.10}$$

The unit step functions of the integrands are eliminated in the simplification step of Eq. B.5.10 since it is recognized that the integrand will have a value of 0 at all times when $u < t-b$. However, by eliminating the units step functions the lower bound of the first integrals of the last three terms must then be constrained to be the $\min(T_{P_i}, t-b)$, $i=1,2$, to maintain the integrals evaluation to zero at all values of $u < t-b$. This completes the derivation of Eq. 3.19.

B.6 Time variant location parameter spline function

If we denote the nodes of the spline function by T_i ($T_{i+1} > T_i$, $i=1,2,\dots,8$), the time variant Weibull distribution location parameter value, $\theta(t)$, was modeled as an end-constrained quadratic spline function as given by:

$$\theta(t) = S(t) \equiv S_i(t) \tag{B.6.1}$$

where,

$$S_i(t) = \sum_{j=0}^2 \alpha_{j,i} \cdot (t-T_i)^j, \quad T_i \leq t < T_{i+1}, \quad i=1,2,\dots,7 \tag{B.6.2}$$

Subject to continuity conditions on the function and its derivative:

$$S(T_i - \varepsilon) = S(T_i) \quad (\text{B.6.3})$$

$$S'(T_i - \varepsilon) = S'(T_i) \quad (\text{B.6.4})$$

and boundary constraints:

$$S(t) = S_1(T_1) \quad \text{for} \quad t < T_1 \equiv T_{p1} \quad (\text{B.6.5})$$

$$S(t) = S_7(T_8 - \varepsilon) \quad \text{for} \quad t \geq T_8 \leq t_{last} \quad (\text{B.6.6})$$

where ε denotes an infinitely small time increment and t_{last} denotes the time of the last observation. Since for the Weibull distribution $\theta \geq 0$, the spline function was constrained to be non-negative by:

$$\theta(t) = \text{maximum}(\theta(t), 0) \quad (\text{B.6.7})$$

In all, 7 nodes and 6 unconstrained spline coefficients were estimated.

B.7 Derivation of $E\{T | s\}$

The mean circulating reticulocyte lifespan is given by the $E\{T | s\}$ taken with respect to the distribution given by Eq. 3.12, with $r(\cdot, s)$ as the fitted Weibull distribution. Therefore from Eq. 3.12 and Eq. 3.17:

$$\begin{aligned} E\{T | s\} &= \frac{\int_0^b \tau \cdot r(b - \tau, s) d\tau}{\int_0^b r(\omega, s) d\omega} \\ &= \frac{\int_0^{b-\theta(s)} \tau \cdot \frac{k}{\lambda} \cdot \left[\frac{b - \tau - \theta(s)}{\lambda} \right]^{k-1} \cdot \exp\left(-\left[\frac{b - \tau - \theta(s)}{\lambda} \right]^k\right) d\tau}{\int_{\theta(s)}^b \frac{k}{\lambda} \cdot \left[\frac{\omega - \theta(s)}{\lambda} \right]^{k-1} \cdot \exp\left(-\left[\frac{\omega - \theta(s)}{\lambda} \right]^k\right) d\omega} \end{aligned} \quad (\text{B.7.1})$$

and by Eq. 3.18 the denominator of Eq. B.7.1 becomes:

$$1 - \exp\left(-\left[\frac{b - \theta(s)}{\lambda} \right]^k\right) \quad \text{for } b \geq \theta(s) \text{ and } 0 \text{ otherwise} \quad (\text{B.7.2})$$

If we define:

$$u = \left[\frac{b - \tau - \theta(s)}{\lambda} \right]^k \quad (\text{B.7.3})$$

Then by substitution of u into the numerator of Eq. B.7.1, it becomes:

$$-\int_{\left[\frac{b-\theta(s)}{\lambda} \right]^k}^0 \left[b - \theta(s) - \lambda \cdot u^{1/k} \right] \cdot \frac{k}{\lambda} \cdot \left[u^{1/k} \right]^{k-1} \cdot \exp(-u) \cdot \frac{\lambda \cdot u^{(1/k)-1}}{k} du \quad (\text{B.7.4})$$

which can be simplified to:

$$\begin{aligned}
&= \int_0^{\left[\frac{b-\theta(s)}{\lambda}\right]^k} \left[b - \theta(s) - \lambda \cdot u^{1/k} \right] \cdot u^{(k-1)/k} \cdot u^{(1-k)/k} \cdot \exp(-u) du \\
&= [b - \theta(s)] \cdot \int_0^{\left[\frac{b-\theta(s)}{\lambda}\right]^k} u^{(k-1+1-k)/k} \cdot \exp(-u) du - \lambda \cdot \int_0^{\left[\frac{b-\theta(s)}{\lambda}\right]^k} u^{(k-1+1-k+1)/k} \cdot \exp(-u) du \\
&= [b - \theta(s)] \cdot \int_0^{\left[\frac{b-\theta(s)}{\lambda}\right]^k} \exp(-u) du - \lambda \cdot \int_0^{\left[\frac{b-\theta(s)}{\lambda}\right]^k} u^{1/k} \cdot \exp(-u) du \\
&= [b - \theta(s)] \cdot \left[1 - \exp\left(-\left[\frac{b-\theta(s)}{\lambda}\right]^k\right) \right] - \lambda \cdot \gamma\left(1 + \frac{1}{k}, \left[\frac{b-\theta(s)}{\lambda}\right]^k\right)
\end{aligned} \tag{B.7.5}$$

where $\gamma(a, x)$ is the lower incomplete gamma function with integrand exponent parameter a and upper limit of the integral x . Dividing the numerator (Eq. B.7.5) by the denominator (Eq. B.7.2) of Eq. B.7.1 results in:

$$E\{T | s\} = [b - \theta(s)] - \frac{\lambda \cdot \gamma\left(1 + \frac{1}{k}, \left[\frac{b-\theta(s)}{\lambda}\right]^k\right)}{\left[1 - \exp\left(-\left[\frac{b-\theta(s)}{\lambda}\right]^k\right)\right]} \quad \text{for } b > \theta(s) \tag{B.7.6}$$

Completing the derivation of $E\{T | s\}$.

APPENDIX C. DERIVATIONS AND PROOFS FOR CHAPTER 4

C.1 Proof that Eq. 4.7 satisfies constraints

Since by definition $g(t)$ is > 0 for all t , then for $t > z$ both the numerator and denominator of Eq. 4.7 are greater than zero. Thus $e(t, z)$ is > 0 when t is $> z$. Furthermore, since $g(t)$ is > 0 then $e(t, z)$ is > 0 if $t = z$, proving that Eq. 4.7 satisfies the positive constraint imposed on $e(t, z)$.

Differentiation of Eq. 4.7 with respect to t when $t > z$ results in:

$$\frac{d}{dt}e(t, z) = \frac{g(t) \cdot [t - z] - \int_z^t g(u) du}{[t - z]^2} \quad (\text{C.1.1})$$

Substitution of Eq. C.1.1 into Eq. 4.3 results in:

$$\begin{aligned} \left[\frac{d}{dt}e(t, z) \right] \cdot [t - z] + e(t, z) &= \frac{g(t) \cdot [t - z] - \int_z^t g(u) du}{[t - z]^2} \cdot [t - z] + e(t, z) \\ &= \frac{g(t) \cdot [t - z] - \int_z^t g(u) du}{[t - z]} + e(t, z) \\ &= g(t) - \frac{\int_z^t g(u) du}{[t - z]} + e(t, z) \\ &= g(t) - e(t, z) + e(t, z) \\ &= g(t) > 0 \end{aligned} \quad (\text{C.1.2})$$

Finally, since at time $t = z$ the difference $t - z$ in the left-hand-side of Eq. C.1.2 equals zero, Eq. 4.3 is also equal to Eq. C.1.2 when $t = z$. Proving that Eq. 4.7 also satisfies the conditions imposed by Eq. 4.3 and completing the proof.

C.2 Derivation of Eq. 4.16

From Eq. 4.7, if $g(t) = M$ for all t then $e(t, z) = M$ for all $t \geq z$. Thus the mathematical expectation of T taken with respect to $\ell_e(\tau, z)$ is given from Eq. 4.5:

$$\begin{aligned} E_{\ell_e} \{T | z, g(t) = M\} &= \int_0^{\infty} \tau \cdot \ell_e(\tau, z) d\tau \\ &= \int_0^{\infty} \tau \cdot \left[\left[\frac{d}{d\tau} e(z + \tau, z) \right] \cdot \tau + e(z + \tau, z) \right] \cdot \ell_b(e(z + \tau, z) \cdot \tau, z) d\tau \\ &= \int_0^{\infty} \tau \cdot [[0] \cdot \tau + M] \cdot \ell_b(M \cdot \tau, z) d\tau \\ &= \int_0^{\infty} M \cdot \tau \cdot \ell_b(M \cdot \tau, z) d\tau \end{aligned}$$

By change of variables:

$$\int_0^\infty M \cdot \tau \cdot \ell_b(M \cdot \tau, z) d\tau = \frac{\int_0^\infty u \cdot \ell_b(u, z) du}{M}$$

$$= \frac{E_{\ell_b} \{T | z\}}{M}$$

Completing the derivation of Eq. 4.16.

C.3 Derivation of the baseline number of cells

From both Eq. 4.6 and Eq. 4.12:

$$N(t) = \int_{-\infty}^t f_{prod}(u) \cdot S_e(t, u) du \quad (C.3.1)$$

If the production rate remains constant, denoted by f_{prod}^{SS} , then:

$$N(t) = \int_{-\infty}^t f_{prod}^{SS} \cdot S_e(t, u) du \quad (C.3.2)$$

$$= f_{prod}^{SS} \cdot \int_{-\infty}^t S_e(t, u) du$$

If additionally the baseline distribution is constant (i.e. $\ell_b(\tau, z) = \ell_b(\tau)$) and there are no environmental effects present (i.e. $g(t) = 1$ or $h(t) = 1$), then:

$$S_e(t, z) = S_b(t, z) = 1 - \int_0^{t-z} \ell_b(\tau) d\tau \quad (C.3.3)$$

for $S_e(t, z)$ given by both Eq. 4.2 (with Eq. 4.7) and Eq. 4.10. Thus Eq. C.3.2 becomes

$$N(t) = f_{prod}^{SS} \cdot \int_{-\infty}^t S_b(t, u) du$$

$$= f_{prod}^{SS} \cdot \int_{-\infty}^t \left[1 - \int_0^{t-u} \ell_b(\tau) d\tau \right] du \quad (C.3.4)$$

$$= -f_{prod}^{SS} \cdot \int_{-\infty}^0 \left[1 - \int_0^u \ell_b(\tau) d\tau \right] du$$

$$= f_{prod}^{SS} \cdot \int_0^\infty \left[1 - \int_0^u \ell_b(\tau) d\tau \right] du$$

Using a well known identity from survival analysis (92, 163), the integral in Eq. C.3.4 is equal to the mathematical expectation of τ taken with respect to the p.d.f. of the survival function being integrated across, giving:

$$N(t) = f_{prod}^{SS} \cdot \mu_b \quad (C.3.5)$$

Completing the derivation of the baseline number of cells.

APPENDIX D. DERIVATIONS AND PROOFS FOR CHAPTER 6

D.1 Derivation of the phlebotomy correction factor

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

$$\begin{aligned} P(A_k^C) &= F_{Pk} \\ P(A_{k+1}^C | A_k^C) &= F_{Pk+1} \\ P(A_{k+2}^C | A_k^C \cap A_{k+1}^C) &= F_{Pk+2} \\ &\vdots = \vdots \\ P(A_l^C | A_k^C \cap A_{k+1}^C \cap \dots \cap A_{l-1}^C) &= F_{Pl} \end{aligned}$$

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

$$\begin{aligned} P(A_k^C \cap A_{k+1}^C) &= P(A_{k+1}^C | A_k^C) \cdot P(A_k^C) = F_{Pk+1} \cdot F_{Pk} \\ P(A_k^C \cap A_{k+1}^C \cap A_{k+2}^C) &= P(A_{k+2}^C | A_k^C \cap A_{k+1}^C) \cdot P(A_k^C \cap A_{k+1}^C) = F_{Pk+2} \cdot F_{Pk+1} \cdot F_{Pk} \end{aligned}$$

which generalizes to:

$$\begin{aligned} P(A_k^C \cap A_{k+1}^C \cap \dots \cap A_l^C) &= P(A_l^C | A_k^C \cap A_{k+1}^C \cap \dots \cap A_{l-1}^C) \cdot P(A_k^C \cap A_{k+1}^C \cap \dots \cap A_{l-1}^C) \\ &= F_{Pl} \cdot \prod_{j=k}^{l-1} F_{Pj} \\ &= \prod_{j=k}^l F_{Pj} \end{aligned}$$

If no phlebotomies were performed between entry and exit (due to age related cellular death) of the cells from the circulation, then no correction factor is needed, or

equivalently the correction factor is equal to one. This completes the derivation of the phlebotomy correction factor for the differential equation output terms.

APPENDIX E. FORTRAN AND WINFUNFIT SUBROUTINES

E.1 Common Fortran subroutines for multiple chapters

E.1.1 GCV.FOR

```

C*****
  SUBROUTINE CUBIC_GCV_FIT (T,F,N) !*** NOTE: CONVERTED TO
REAL*8 VARIABLES
  IMPLICIT REAL*8 (A-H,O-Z)
  * VERSION 2.3 (15 SEPTEMBER 2006) CORRECTED ERROR IN
CUBIC_GCV_COEFFICIENTS ROUTINE (KJF)
  * 2.2 (OCTOBER 2005) CONVERTED TO USE "CONTAINS" TO EMBED THE
SUPPORT ROUTINES
  *
  * COPYRIGHT PV-P
  *
  * INPUT:
  *
  * T,F  ARRAYS OF LENGTH N TO BE FITTED BY CUBIC
  *      GENERALIZED CROSS VALIDATION (F=F(T))
  *
  * R E S T R I C T I O N S : N MUST NOT EXCEED 2000
  *

  SAVE C,Y,TSAVE,NSAVE, USE_DEFAULT_VALUES, CV_VALUE
  PARAMETER (NMAX=2000,NWK=7*(NMAX+2),IC=NMAX-1)
  REAL*8, PARAMETER :: ONE=1D0, ZERO=0D0 , HUNDRED=100D0 !
NEW JAN 2005
  REAL*8, PARAMETER :: DF_DEFAULT=ONE, VAR_DEFAULT=-ONE !
NEW JAN 2005
  REAL*8      :: CV_VALUE, CV_VALUE_SPECIFIED    ! NEW JAN 2005
  REAL*8      :: VAR_MAX, VAR_CALC                ! NEW JAN 2005
  REAL*8      :: YZERO,SLOPE,SSLINEFIT,SSTOTAL   ! NEW JAN 2005
  REAL*8      :: ERROR_VARIANCE_LINE            ! NEW JAN 2005
  LOGICAL     :: USE_DEFAULT_VALUES              ! NEW JAN 2005
  LOGICAL, PARAMETER :: DEBUGMODE = .FALSE.
  DATA USE_DEFAULT_VALUES /.TRUE./
  REAL*8 Y(NMAX),DF(NMAX),
& C(IC,3),SE(NMAX),WK(NWK),VAR,D,Z2,
& SSSE,SSRES
  REAL*8 T(*), F(*), TSAVE(NMAX)
  IF(N.GT.NMAX)STOP '** ERROR (CUBIC_GCV_FIT) : TOO LARGE ARRAY'
  IF(N.LT.2) STOP '** ERROR (CUBIC_GCV_FIT) : TOO SMALL ARRAY'

  IF(USE_DEFAULT_VALUES) THEN

```

```

    VAR = - ONE
    DO J = 1,N
    DF(J) = ONE
    TSAVE(J) = T(J)
    ENDDO
ELSE                                     ! NEW JAN 2005
=====
    CALL GET_ERROR_VARIANCE_STRAIGHT_LINE_FIT
    &                                     (T,F,N,ERROR_VARIANCE_LINE) ! NEW JAN 2005
    IF(DEBUGMODE) PRINT*, ' ERROR_VARIANCE_LINE',
ERROR_VARIANCE_LINE
!     CALL SUBROUTINE STRAIGHT_LINE_FIT
(X,Y,NXY,YZERO,SLOPE,SSFIT,SSTOTAL)
    CALL STRAIGHT_LINE_FIT (T,F,N,YZERO,SLOPE,SSLINEFIT,SSTOTAL)
    IF(DEBUGMODE) THEN
    PRINT*, ' SSLINEFIT, SSLINEFIT/N =',SSLINEFIT, SSLINEFIT/N
    PRINT*, ' SLINEFIT/(N-2) =',SSLINEFIT/(N -2)
    ENDIF
    VAR = ONE
    DO J= 1,N
    DF(J) = ABS(F(J)*CV_VALUE/HUNDRED)
    TSAVE(J) = T(J)
    ENDDO
    ENDIF                                     !
=====

    NSAVE = N
    CALL CUBGCV (T,F,DF,N,Y,C,IC,VAR,1,SE,WK,IER)
    USE_DEFAULT_VALUES = .TRUE.  ! RESET TO DEFAULT VALUE  ! NEW
JAN 2005
! DEBUG -----
!     WK   - WORK VECTOR OF LENGTH 7*(N + 2). ON NORMAL EXIT
THE
!     FIRST 7 VALUES OF WK ARE ASSIGNED AS FOLLOWS:-
!
!     WK(1) = SMOOTHING PARAMETER (= RHO/(RHO + 1))
!     WK(2) = ESTIMATE OF THE NUMBER OF DEGREES OF
!             FREEDOM OF THE RESIDUAL SUM OF SQUARES
!     WK(3) = GENERALIZED CROSS VALIDATION
!     WK(4) = MEAN SQUARE RESIDUAL
!     WK(5) = ESTIMATE OF THE TRUE MEAN SQUARE ERROR
!             AT THE DATA POINTS
!     WK(6) = ESTIMATE OF THE ERROR VARIANCE
!     WK(7) = MEAN SQUARE VALUE OF THE DF(I)
!
!     IF WK(1)=0 (RHO=0) AN INTERPOLATING NATURAL CUBIC

```

```

!           SPLINE HAS BEEN CALCULATED.
!           IF WK(1)=1 (RHO=INFINITE) A LEAST SQUARES
!           REGRESSION LINE HAS BEEN CALCULATED.
!           WK(2) IS AN ESTIMATE OF THE NUMBER OF DEGREES OF
!           FREEDOM OF THE RESIDUAL WHICH REDUCES TO THE
!           USUAL VALUE OF N-2 WHEN A LEAST SQUARES
REGRESSION
!           LINE IS CALCULATED.
!           WK(3),WK(4),WK(5) ARE CALCULATED WITH THE DF(I)
!           SCALED TO HAVE MEAN SQUARE VALUE 1. THE
!           UNSCALED VALUES OF WK(3),WK(4),WK(5) MAY BE
!           CALCULATED BY DIVIDING BY WK(7).
!           WK(6) COINCIDES WITH THE OUTPUT VALUE OF VAR IF
!           VAR IS NEGATIVE ON INPUT. IT IS CALCULATED WITH
!           THE UNSCALED VALUES OF THE DF(I) TO FACILITATE
!           COMPARISONS WITH A PRIORI VARIANCE ESTIMATES.
!
IF(DEBUGMODE) THEN
  PRINT*, 'CUBIC_GCV_FIT:'
  PRINT*, ' 1 SMOOTHING PARAMETER (= RHO/(RHO + 1)):',WK(1) !
  PRINT*, ' 2 ESTIMATED DF OF THE RESIDUAL SUM OF SQUARES:',WK(2)
!
  PRINT*, ' 3 GENERALIZED CROSS VALIDATION:',WK(3) !
  PRINT*, ' 4 MEAN SQUARE RESIDUAL: ',WK(4) !
  PRINT*, ' 5 ESTIMATE OF THE TRUE MEAN SQUARE ERROR:',WK(5) !
  PRINT*, ' 6 ESTIMATE OF THE ERROR VARIANCE:',WK(6) !
  PRINT*, ' 7 MEAN SQUARE VALUE OF THE DF(I):',WK(7) !
  PRINT*, ' 3/7 =',WK(3)/WK(7) !
  PRINT*, ' 4/7 =',WK(4)/WK(7) !
  PRINT*, ' 5/7 =',WK(5)/WK(7) !
ENDIF
! DEBUG -----
IF(IER.NE.0) THEN
  PRINT*, '** ERROR (CUBIC_GCV_FIT): IER =',IER
  STOP
ENDIF
RETURN
!*****
  ENTRY SET_CV_VALUE_FOR_SPLINE_FIT(CV_VALUE_SPECIFIED) !
NEW JAN 2005
  CV_VALUE = CV_VALUE_SPECIFIED
  USE_DEFAULT_VALUES = .FALSE.
  RETURN
!*****
  ENTRY CUBIC_GCV(TCALC,FCALC) ! INPUT: TCALC, OUTPUT: FCALC =
VALUE OF SPLINE AT TCALC

```

```

IF(TCALC.LT.TSAVE(1))THEN ! LINEAR BACKWARD EXTRAPOLATION
FCALC = Y(1) + C(1,1)*(TCALC-TSAVE(1))
RETURN
ENDIF
IF(TCALC.GT.TSAVE(NSAVE))THEN ! LINEAR FORWARD
EXTRAPOLATION
  NSM1 = NSAVE - 1
  DEND = TSAVE(NSAVE) - TSAVE(NSM1)
  YEND = ((C(NSM1,3)*DEND+C(NSM1,2))*DEND+C(NSM1,1))*DEND+Y(J)
  YPEND = ((3D0*C(NSM1,3)*DEND+2D0*C(NSM1,2))*DEND+C(NSM1,1))
FCALC = YEND + YPEND*(TCALC-TSAVE(NSAVE))
RETURN
ENDIF
DO J = 1,NSAVE-1
IF(TCALC.LE.TSAVE(J+1)) THEN
D = TCALC-TSAVE(J)
FCALC = ((C(J,3)*D+C(J,2))*D+C(J,1))*D+Y(J)
EXIT
ENDIF
ENDDO
RETURN
!****
ENTRY CUBIC_GCV_DERIVATIVE(TCALC,FCALC) ! INPUT: TCALC,
OUTPUT: FCALC = VALUE OF SPLINE DERIVATIVE AT TCALC
IF(TCALC.LT.TSAVE(1))THEN ! LINEAR BACKWARD EXTRAPOLATION
FCALC = C(1,1)
RETURN
ENDIF
IF(TCALC.GT.TSAVE(NSAVE))THEN ! LINEAR FORWARD
EXTRAPOLATION
  NSM1 = NSAVE - 1
  DEND = TSAVE(NSAVE) - TSAVE(NSM1)
  FCALC = ((3D0*C(NSM1,3)*DEND+2D0*C(NSM1,2))*DEND+C(NSM1,1))
RETURN
ENDIF
DO J = 1,NSAVE-1
IF(TCALC.LE.TSAVE(J+1)) THEN
D = DBLE(TCALC-TSAVE(J))
FCALC = (3D0*C(J,3)*D+2D0*C(J,2))*D + C(J,1)
EXIT
ENDIF
ENDDO
RETURN
!****
ENTRY CUBIC_GCV_COEFFICIENTS(TOBS,AZ,A1,A2,A3)

```

```

! INPUT= TOBS, OUTPUT= A,B,C,D = SPLINE COEFFICIENTS FOR SEGMENT
STARTING AT T=TOBS
! NOTE THIS ROUTINE MUST BE CALLED MULTIPLE TIMES TO GET
COEFFICIENTS FOR ALL N-1 SEGMENTS.
!
! SPLINE = AZ + A1*X + A2*X*X + A3*X*X*X WITH X=DISTANCE TO START
OF SPLINE SEGMENT
!
! TOBS = START OF SPLINE SEGMENT = ONE OF THE T'S IN THE FITTED
DATA SET, EXCLUDING THE LAST T (T(N))
  DO J = 1,NSAVE-1
    IF(TOBS.LT.TSAVE(J+1)) THEN
      AZ = Y(J)
      A1 = C(J,1)
      A2 = C(J,2)
      A3 = C(J,3)
    EXIT
  ENDIF
ENDDO
  IF(TOBS.EQ.TSAVE(NSAVE)) THEN
    AZ = Y(NSAVE-1)
    A1 = C(NSAVE-1,1)
    A2 = C(NSAVE-1,2)
    A3 = C(NSAVE-1,3)
  ENDIF
  RETURN
!////////////////////////////////////
CONTAINS
*****
! ALGORITHM 642 COLLECTED ALGORITHMS FROM ACM.
! ALGORITHM APPEARED IN ACM-TRANS. MATH. SOFTWARE, VOL.12, NO.
2,
! JUN., 1986, P. 150.
! SUBROUTINE NAME - CUBGCV
!-----
! AUTHOR - M.F.HUTCHINSON
! CSIRO DIVISION OF MATHEMATICS AND STATISTICS
! P.O. BOX 1965
! CANBERRA, ACT 2601
! AUSTRALIA
!
! LATEST REVISION - 15 AUGUST 1985
!
! PURPOSE - CUBIC SPLINE DATA SMOOTHER
!
! USAGE - CALL CUBGCV (X,F,DF,N,Y,C,IC,VAR,JOB,SE,WK,IER)

```

```

!
! ARGUMENTS  X   - VECTOR OF LENGTH N CONTAINING THE
!              ABSCISSAE OF THE N DATA POINTS
!              (X(I),F(I)) I=1..N. (INPUT) X
!              MUST BE ORDERED SO THAT
!              X(I) .LT. X(I+1).
!
!   F   - VECTOR OF LENGTH N CONTAINING THE
!         ORDINATES (OR FUNCTION VALUES)
!         OF THE N DATA POINTS (INPUT).
!
!   DF  - VECTOR OF LENGTH N. (INPUT/OUTPUT)
!         DF(I) IS THE RELATIVE STANDARD DEVIATION
!         OF THE ERROR ASSOCIATED WITH DATA POINT I.
!         EACH DF(I) MUST BE POSITIVE.  THE VALUES IN
!         DF ARE SCALED BY THE SUBROUTINE SO THAT
!         THEIR MEAN SQUARE VALUE IS 1, AND UNSCALED
!         AGAIN ON NORMAL EXIT.
!         THE MEAN SQUARE VALUE OF THE DF(I) IS RETURNED
!         IN WK(7) ON NORMAL EXIT.
!         IF THE ABSOLUTE STANDARD DEVIATIONS ARE KNOWN,
!         THESE SHOULD BE PROVIDED IN DF AND THE ERROR
!         VARIANCE PARAMETER VAR (SEE BELOW) SHOULD THEN
!         BE SET TO 1.
!         IF THE RELATIVE STANDARD DEVIATIONS ARE UNKNOWN,
!         SET EACH DF(I)=1.
!
!   N   - NUMBER OF DATA POINTS (INPUT).
!         N MUST BE .GE. 3.
!
!   Y,C - SPLINE COEFFICIENTS. (OUTPUT) Y
!         IS A VECTOR OF LENGTH N. C IS
!         AN N-1 BY 3 MATRIX. THE VALUE
!         OF THE SPLINE APPROXIMATION AT T IS
!          $S(T) = ((C(I,3)*D + C(I,2))*D + C(I,1))*D + Y(I)$ 
!         WHERE X(I).LE.T.LT.X(I+1) AND
!         D = T-X(I).
!
!   IC  - ROW DIMENSION OF MATRIX C EXACTLY
!         AS SPECIFIED IN THE DIMENSION
!         STATEMENT IN THE CALLING PROGRAM. (INPUT)
!
!   VAR - ERROR VARIANCE. (INPUT/OUTPUT)
!         IF VAR IS NEGATIVE (I.E. UNKNOWN) THEN
!         THE SMOOTHING PARAMETER IS DETERMINED
!         BY MINIMIZING THE GENERALIZED CROSS VALIDATION
!         AND AN ESTIMATE OF THE ERROR VARIANCE IS
!         RETURNED IN VAR.
!         IF VAR IS NON-NEGATIVE (I.E. KNOWN) THEN THE
!         SMOOTHING PARAMETER IS DETERMINED TO MINIMIZE
!         AN ESTIMATE, WHICH DEPENDS ON VAR, OF THE TRUE
!         MEAN SQUARE ERROR, AND VAR IS UNCHANGED.
!

```

! IN PARTICULAR, IF VAR IS ZERO, THEN AN
 ! INTERPOLATING NATURAL CUBIC SPLINE IS CALCULATED.
 ! VAR SHOULD BE SET TO 1 IF ABSOLUTE STANDARD
 ! DEVIATIONS HAVE BEEN PROVIDED IN DF (SEE ABOVE).
 ! JOB - JOB SELECTION PARAMETER. (INPUT)
 ! JOB = 0 SHOULD BE SELECTED IF POINT STANDARD ERROR
 ! ESTIMATES ARE NOT REQUIRED IN SE.
 ! JOB = 1 SHOULD BE SELECTED IF POINT STANDARD ERROR
 ! ESTIMATES ARE REQUIRED IN SE.
 ! SE - VECTOR OF LENGTH N CONTAINING BAYESIAN STANDARD
 ! ERROR ESTIMATES OF THE FITTED SPLINE VALUES IN Y.
 ! SE IS NOT REFERENCED IF JOB=0. (OUTPUT)
 ! WK - WORK VECTOR OF LENGTH $7*(N + 2)$. ON NORMAL EXIT
 THE
 ! FIRST 7 VALUES OF WK ARE ASSIGNED AS FOLLOWS:-
 !
 ! WK(1) = SMOOTHING PARAMETER (= $\text{RHO}/(\text{RHO} + 1)$)
 ! WK(2) = ESTIMATE OF THE NUMBER OF DEGREES OF
 ! FREEDOM OF THE RESIDUAL SUM OF SQUARES
 ! WK(3) = GENERALIZED CROSS VALIDATION
 ! WK(4) = MEAN SQUARE RESIDUAL
 ! WK(5) = ESTIMATE OF THE TRUE MEAN SQUARE ERROR
 ! AT THE DATA POINTS
 ! WK(6) = ESTIMATE OF THE ERROR VARIANCE
 ! WK(7) = MEAN SQUARE VALUE OF THE DF(I)
 !
 ! IF WK(1)=0 (RHO=0) AN INTERPOLATING NATURAL CUBIC
 ! SPLINE HAS BEEN CALCULATED.
 ! IF WK(1)=1 (RHO=INFINITE) A LEAST SQUARES
 ! REGRESSION LINE HAS BEEN CALCULATED.
 ! WK(2) IS AN ESTIMATE OF THE NUMBER OF DEGREES OF
 ! FREEDOM OF THE RESIDUAL WHICH REDUCES TO THE
 ! USUAL VALUE OF N-2 WHEN A LEAST SQUARES
 REGRESSION
 ! LINE IS CALCULATED.
 ! WK(3),WK(4),WK(5) ARE CALCULATED WITH THE DF(I)
 ! SCALED TO HAVE MEAN SQUARE VALUE 1. THE
 ! UNSCALED VALUES OF WK(3),WK(4),WK(5) MAY BE
 ! CALCULATED BY DIVIDING BY WK(7).
 ! WK(6) COINCIDES WITH THE OUTPUT VALUE OF VAR IF
 ! VAR IS NEGATIVE ON INPUT. IT IS CALCULATED WITH
 ! THE UNSCALED VALUES OF THE DF(I) TO FACILITATE
 ! COMPARISONS WITH A PRIORI VARIANCE ESTIMATES.
 !
 ! IER - ERROR PARAMETER. (OUTPUT)
 ! TERMINAL ERROR

```

!           IER = 129, IC IS LESS THAN N-1.
!           IER = 130, N IS LESS THAN 3.
!           IER = 131, INPUT ABSCISSAE ARE NOT
!           ORDERED SO THAT X(I).LT.X(I+1).
!           IER = 132, DF(I) IS NOT POSITIVE FOR SOME I.
!           IER = 133, JOB IS NOT 0 OR 1.
!
! PRECISION/HARDWARE - DOUBLE
!
! REQUIRED ROUTINES - SPINT1,SPFIT1,SPCOF1,SPERR1
!
! REMARKS    THE NUMBER OF ARITHMETIC OPERATIONS REQUIRED BY
THE
!           SUBROUTINE IS PROPORTIONAL TO N. THE SUBROUTINE
!           USES AN ALGORITHM DEVELOPED BY M.F. HUTCHINSON AND
!           F.R. DE HOOG, 'SMOOTHING NOISY DATA WITH SPLINE
!           FUNCTIONS', NUMER. MATH. (IN PRESS)
!
!-----
!
SUBROUTINE CUBGCV(X,F,DF,N,Y,C,IC,VAR,JOB,SE,WK,IER)
  IMPLICIT REAL*8 (A-H,O-Z)
!
!---SPECIFICATIONS FOR ARGUMENTS---
  INTEGER N,IC,JOB,IER
  REAL*8    X(N),F(N),DF(N),Y(N),C(IC,3),SE(N),VAR,
.           WK(0:N+1,7)
!
!---SPECIFICATIONS FOR LOCAL VARIABLES---
  REAL*8    DELTA,ERR,GF1,GF2,GF3,GF4,R1,R2,R3,R4,TAU,RATIO,
.           AVH,AVDF,AVAR,ZERO,ONE,STAT(6),P,Q
!
  DATA RATIO/2.0D0/
  DATA TAU/1.618033989D0/
  DATA ZERO,ONE/0.0D0,1.0D0/
!
!---INITIALIZE---
  IER = 133
  IF (JOB.LT.0 .OR. JOB.GT.1) GO TO 140
  CALL SPINT1(X,AVH,F,DF,AVDF,N,Y,C,IC,WK,WK(0,4),IER)
  IF (IER.NE.0) GO TO 140
  AVAR = VAR
  IF (VAR.GT.ZERO) AVAR = VAR*AVDF*AVDF
!
!---CHECK FOR ZERO VARIANCE---
  IF (VAR.NE.ZERO) GO TO 10

```

```

R1 = ZERO
GO TO 90
!
!---FIND LOCAL MINIMUM OF GCV OR THE EXPECTED MEAN SQUARE
ERROR---
10 R1 = ONE
R2 = RATIO*R1
CALL SPFIT1(X,AVH,DF,N,R2,P,Q,GF2,AVAR,STAT,Y,C,IC,WK,WK(0,4),
. WK(0,6),WK(0,7))
20 CALL SPFIT1(X,AVH,DF,N,R1,P,Q,GF1,AVAR,STAT,Y,C,IC,WK,WK(0,4),
. WK(0,6),WK(0,7))
IF (GF1.GT.GF2) GO TO 30
!
!---EXIT IF P ZERO---
IF (P.LE.ZERO) GO TO 100
R2 = R1
GF2 = GF1
R1 = R1/RATIO
GO TO 20

30 R3 = RATIO*R2
40 CALL SPFIT1(X,AVH,DF,N,R3,P,Q,GF3,AVAR,STAT,Y,C,IC,WK,WK(0,4),
. WK(0,6),WK(0,7))
IF (GF3.GT.GF2) GO TO 50
!
!---EXIT IF Q ZERO---
IF (Q.LE.ZERO) GO TO 100
R2 = R3
GF2 = GF3
R3 = RATIO*R3
GO TO 40

50 R2 = R3
GF2 = GF3
DELTA = (R2-R1)/TAU
R4 = R1 + DELTA
R3 = R2 - DELTA
CALL SPFIT1(X,AVH,DF,N,R3,P,Q,GF3,AVAR,STAT,Y,C,IC,WK,WK(0,4),
. WK(0,6),WK(0,7))
CALL SPFIT1(X,AVH,DF,N,R4,P,Q,GF4,AVAR,STAT,Y,C,IC,WK,WK(0,4),
. WK(0,6),WK(0,7))
!
!---GOLDEN SECTION SEARCH FOR LOCAL MINIMUM---
60 IF (GF3.GT.GF4) GO TO 70
R2 = R4
GF2 = GF4

```

```

R4 = R3
GF4 = GF3
DELTA = DELTA/TAU
R3 = R2 - DELTA
CALL SPFIT1(X,AVH,DF,N,R3,P,Q,GF3,AVAR,STAT,Y,C,IC,WK,WK(0,4),
.      WK(0,6),WK(0,7))
GO TO 80

70 R1 = R3
GF1 = GF3
R3 = R4
GF3 = GF4
DELTA = DELTA/TAU
R4 = R1 + DELTA
CALL SPFIT1(X,AVH,DF,N,R4,P,Q,GF4,AVAR,STAT,Y,C,IC,WK,WK(0,4),
.      WK(0,6),WK(0,7))
80 ERR = (R2-R1)/ (R1+R2)
IF (ERR*ERR+ONE.GT.ONE .AND. ERR.GT.1.0D-6) GO TO 60
R1 = (R1+R2)*0.5D0
!
!---CALCULATE SPLINE COEFFICIENTS---
90 CALL SPFIT1(X,AVH,DF,N,R1,P,Q,GF1,AVAR,STAT,Y,C,IC,WK,WK(0,4),
.      WK(0,6),WK(0,7))
100 CALL SPCOF1(X,AVH,F,DF,N,P,Q,Y,C,IC,WK(0,6),WK(0,7))
!
!---OPTIONALLY CALCULATE STANDARD ERROR ESTIMATES---
IF (VAR.GE.ZERO) GO TO 110
AVAR = STAT(6)
VAR = AVAR/ (AVDF*AVDF)
110 IF (JOB.EQ.1) CALL SPERR1(X,AVH,DF,N,WK,P,AVAR,SE)
!
!---UNSCALE DF---
DO 120 I = 1,N
DF(I) = DF(I)*AVDF
120 CONTINUE
!
!--PUT STATISTICS IN WK---
DO 130 I = 0,5
WK(I,1) = STAT(I+1)
130 CONTINUE
WK(5,1) = STAT(6)/ (AVDF*AVDF)
WK(6,1) = AVDF*AVDF
GO TO 150
!
!---CHECK FOR ERROR CONDITION---
140 CONTINUE

```

```

! IF (IER.NE.0) CONTINUE
150 RETURN
END SUBROUTINE CUBGCV
!*****
SUBROUTINE SPINT1(X,AVH,Y,DY,AVDY,N,A,C,IC,R,T,IER)
IMPLICIT REAL*8 (A-H,O-Z)
!
! INITIALIZES THE ARRAYS C, R AND T FOR ONE DIMENSIONAL CUBIC
! SMOOTHING SPLINE FITTING BY SUBROUTINE SPFIT1. THE VALUES
! DF(I) ARE SCALED SO THAT THE SUM OF THEIR SQUARES IS N
! AND THE AVERAGE OF THE DIFFERENCES X(I+1) - X(I) IS CALCULATED
! IN AVH IN ORDER TO AVOID UNDERFLOW AND OVERFLOW PROBLEMS IN
! SPFIT1.
!
! SUBROUTINE SETS IER IF ELEMENTS OF X ARE NON-INCREASING,
! IF N IS LESS THAN 3, IF IC IS LESS THAN N-1 OR IF DY(I) IS
! NOT POSITIVE FOR SOME I.
!
!---SPECIFICATIONS FOR ARGUMENTS---
INTEGER N,IC,IER
REAL*8 X(N),Y(N),DY(N),A(N),C(IC,3),R(0:N+1,3),
. T(0:N+1,2),AVH,AVDY
!
!---SPECIFICATIONS FOR LOCAL VARIABLES---
INTEGER I
REAL*8 E,F,G,H,ZERO
DATA ZERO/0.0D0/
!
!---INITIALIZATION AND INPUT CHECKING---
IER = 0
IF (N.LT.3) GO TO 60
IF (IC.LT.N-1) GO TO 70
!
!---GET AVERAGE X SPACING IN AVH---
G = ZERO
DO 10 I = 1,N - 1
H = X(I+1) - X(I)
IF (H.LE.ZERO) GO TO 80
G = G + H
10 CONTINUE
AVH = G/ (N-1)
!
!---SCALE RELATIVE WEIGHTS---
G = ZERO
DO 20 I = 1,N
IF (DY(I).LE.ZERO) GO TO 90

```

```

      G = G + DY(I)*DY(I)
20 CONTINUE
      AVDY = DSQRT(G/N)
!
      DO 30 I = 1,N
          DY(I) = DY(I)/AVDY
30 CONTINUE
!
!---INITIALIZE H,F---
      H = (X(2)-X(1))/AVH
      F = (Y(2)-Y(1))/H
!
!---CALCULATE A,T,R---
      DO 40 I = 2,N - 1
          G = H
          H = (X(I+1)-X(I))/AVH
          E = F
          F = (Y(I+1)-Y(I))/H
          A(I) = F - E
          T(I,1) = 2.0D0* (G+H)/3.0D0
          T(I,2) = H/3.0D0
          R(I,3) = DY(I-1)/G
          R(I,1) = DY(I+1)/H
          R(I,2) = -DY(I)/G - DY(I)/H
40 CONTINUE
!
!---CALCULATE C = R'*R---
      R(N,2) = ZERO
      R(N,3) = ZERO
      R(N+1,3) = ZERO
      DO 50 I = 2,N - 1
          C(I,1) = R(I,1)*R(I,1) + R(I,2)*R(I,2) + R(I,3)*R(I,3)
          C(I,2) = R(I,1)*R(I+1,2) + R(I,2)*R(I+1,3)
          C(I,3) = R(I,1)*R(I+2,3)
50 CONTINUE
      RETURN
!
!---ERROR CONDITIONS---
60 IER = 130
      RETURN

70 IER = 129
      RETURN

80 IER = 131
      RETURN

```

```

90 IER = 132
  RETURN
  END SUBROUTINE SPINT1
!*****
*****
  SUBROUTINE
SPFIT1(X,AVH,DY,N,RHO,P,Q,FUN,VAR,STAT,A,C,IC,R,T,U,V)
  IMPLICIT REAL*8 (A-H,O-Z)
!
! FITS A CUBIC SMOOTHING SPLINE TO DATA WITH RELATIVE
! WEIGHTING DY FOR A GIVEN VALUE OF THE SMOOTHING PARAMETER
! RHO USING AN ALGORITHM BASED ON THAT OF C.H. REINSCH (1967),
! NUMER. MATH. 10, 177-183.
!
! THE TRACE OF THE INFLUENCE MATRIX IS CALCULATED USING AN
! ALGORITHM DEVELOPED BY M.F.HUTCHINSON AND F.R.DE HOOG
(NUMER.
! MATH., IN PRESS), ENABLING THE GENERALIZED CROSS VALIDATION
! AND RELATED STATISTICS TO BE CALCULATED IN ORDER N
OPERATIONS.
!
! THE ARRAYS A, C, R AND T ARE ASSUMED TO HAVE BEEN INITIALIZED
! BY THE SUBROUTINE SPINT1. OVERFLOW AND UNDERFLOW PROBLEMS
ARE
! AVOIDED BY USING  $P=RHO/(1 + RHO)$  AND  $Q=1/(1 + RHO)$  INSTEAD OF
! RHO AND BY SCALING THE DIFFERENCES  $X(I+1) - X(I)$  BY AVH.
!
! THE VALUES IN DF ARE ASSUMED TO HAVE BEEN SCALED SO THAT THE
! SUM OF THEIR SQUARED VALUES IS N. THE VALUE IN VAR, WHEN IT IS
! NON-NEGATIVE, IS ASSUMED TO HAVE BEEN SCALED TO COMPENSATE
FOR
! THE SCALING OF THE VALUES IN DF.
!
! THE VALUE RETURNED IN FUN IS AN ESTIMATE OF THE TRUE MEAN
SQUARE
! WHEN VAR IS NON-NEGATIVE, AND IS THE GENERALIZED CROSS
VALIDATION
! WHEN VAR IS NEGATIVE.
!
!---SPECIFICATIONS FOR ARGUMENTS---
  INTEGER IC,N
  REAL*8      X(N),DY(N),RHO,STAT(6),A(N),C(IC,3),R(0:N+1,3),
.            T(0:N+1,2),U(0:N+1),V(0:N+1),FUN,VAR,AVH,P,Q
!
!---LOCAL VARIABLES---
```

```

INTEGER I
REAL*8      E,F,G,H,ZERO,ONE,TWO,RHO1
DATA ZERO,ONE,TWO/0.0D0,1.0D0,2.0D0/
!
!---USE P AND Q INSTEAD OF RHO TO PREVENT OVERFLOW OR
UNDERFLOW---
  RHO1 = ONE + RHO
  P = RHO/RHO1
  Q = ONE/RHO1
  IF (RHO1.EQ.ONE) P = ZERO
  IF (RHO1.EQ.RHO) Q = ZERO
!
!---RATIONAL CHOLESKY DECOMPOSITION OF P*C + Q*T---
  F = ZERO
  G = ZERO
  H = ZERO
  DO 10 I = 0,1
    R(I,1) = ZERO
10 CONTINUE
  DO 20 I = 2,N - 1
    R(I-2,3) = G*R(I-2,1)
    R(I-1,2) = F*R(I-1,1)
    R(I,1) = ONE/ (P*C(I,1)+Q*T(I,1)-F*R(I-1,2)-G*R(I-2,3))
    F = P*C(I,2) + Q*T(I,2) - H*R(I-1,2)
    G = H
    H = P*C(I,3)
20 CONTINUE
!
!---SOLVE FOR U---
  U(0) = ZERO
  U(1) = ZERO
  DO 30 I = 2,N - 1
    U(I) = A(I) - R(I-1,2)*U(I-1) - R(I-2,3)*U(I-2)
30 CONTINUE
  U(N) = ZERO
  U(N+1) = ZERO
  DO 40 I = N - 1,2,-1
    U(I) = R(I,1)*U(I) - R(I,2)*U(I+1) - R(I,3)*U(I+2)
40 CONTINUE
!
!---CALCULATE RESIDUAL VECTOR V---
  E = ZERO
  H = ZERO
  DO 50 I = 1,N - 1
    G = H
    H = (U(I+1)-U(I))/ ((X(I+1)-X(I))/AVH)

```

```

      V(I) = DY(I)* (H-G)
      E = E + V(I)*V(I)
50 CONTINUE
      V(N) = DY(N)* (-H)
      E = E + V(N)*V(N)
!
!---CALCULATE UPPER THREE BANDS OF INVERSE MATRIX---
      R(N,1) = ZERO
      R(N,2) = ZERO
      R(N+1,1) = ZERO
      DO 60 I = N - 1,2,-1
        G = R(I,2)
        H = R(I,3)
        R(I,2) = -G*R(I+1,1) - H*R(I+1,2)
        R(I,3) = -G*R(I+1,2) - H*R(I+2,1)
        R(I,1) = R(I,1) - G*R(I,2) - H*R(I,3)
      60 CONTINUE
!
!---CALCULATE TRACE---
      F = ZERO
      G = ZERO
      H = ZERO
      DO 70 I = 2,N - 1
        F = F + R(I,1)*C(I,1)
        G = G + R(I,2)*C(I,2)
        H = H + R(I,3)*C(I,3)
      70 CONTINUE
      F = F + TWO* (G+H)
!
!---CALCULATE STATISTICS---
      STAT(1) = P
      STAT(2) = F*P
      STAT(3) = N*E/ (F*F)
      STAT(4) = E*P*P/N
      STAT(6) = E*P/F
      IF (VAR.GE.ZERO) GO TO 80
      STAT(5) = STAT(6) - STAT(4)
      FUN = STAT(3)
      GO TO 90

80 STAT(5) = DMAX1(STAT(4)-TWO*VAR*STAT(2)/N+VAR,ZERO)
      FUN = STAT(5)
90 RETURN
      END SUBROUTINE SPFIT1
!*****
****

```

```

SUBROUTINE SPERR1(X,AVH,DY,N,R,P,VAR,SE)
  IMPLICIT REAL*8 (A-H,O-Z)
  !
  ! CALCULATES BAYESIAN ESTIMATES OF THE STANDARD ERRORS OF THE
  ! FITTED
  ! VALUES OF A CUBIC SMOOTHING SPLINE BY CALCULATING THE
  ! DIAGONAL ELEMENTS
  ! OF THE INFLUENCE MATRIX.
  !
  !---SPECIFICATIONS FOR ARGUMENTS---
  INTEGER N
  REAL*8      X(N),DY(N),R(0:N+1,3),SE(N),AVH,P,VAR
  !
  !---SPECIFICATIONS FOR LOCAL VARIABLES---
  INTEGER I
  REAL*8      F,G,H,F1,G1,H1,ZERO,ONE
  DATA ZERO,ONE/0.0D0,1.0D0/
  !
  !---INITIALIZE---
  H = AVH/ (X(2)-X(1))
  SE(1) = ONE - P*DY(1)*DY(1)*H*H*R(2,1)
  R(1,1) = ZERO
  R(1,2) = ZERO
  R(1,3) = ZERO
  !
  !---CALCULATE DIAGONAL ELEMENTS---
  DO 10 I = 2,N - 1
    F = H
    H = AVH/ (X(I+1)-X(I))
    G = -F - H
    F1 = F*R(I-1,1) + G*R(I-1,2) + H*R(I-1,3)
    G1 = F*R(I-1,2) + G*R(I,1) + H*R(I,2)
    H1 = F*R(I-1,3) + G*R(I,2) + H*R(I+1,1)
    SE(I) = ONE - P*DY(I)*DY(I)* (F*F1+G*G1+H*H1)
  10 CONTINUE
  SE(N) = ONE - P*DY(N)*DY(N)*H*H*R(N-1,1)
  !
  !---CALCULATE STANDARD ERROR ESTIMATES---
  DO 20 I = 1,N
    SE(I) = DSQRT(DMAX1(SE(I)*VAR,ZERO))*DY(I)
  20 CONTINUE
  RETURN
  END SUBROUTINE SPERR1
  !*****
  SUBROUTINE SPCOF1(X,AVH,Y,DY,N,P,Q,A,C,IC,U,V)

```

```

      IMPLICIT REAL*8 (A-H,O-Z)
!
! CALCULATES COEFFICIENTS OF A CUBIC SMOOTHING SPLINE FROM
! PARAMETERS CALCULATED BY SUBROUTINE SPFIT1.
!
!--SPECIFICATIONS FOR ARGUMENTS---
      INTEGER IC,N
      REAL*8      X(N),Y(N),DY(N),P,Q,A(N),C(IC,3),U(0:N+1),
      .          V(0:N+1),AVH
!
!--SPECIFICATIONS FOR LOCAL VARIABLES---
      INTEGER I
      REAL*8      H,QH
!
!--CALCULATE A---
      QH = Q/ (AVH*AVH)
      DO 10 I = 1,N
        A(I) = Y(I) - P*DY(I)*V(I)
        U(I) = QH*U(I)
      10 CONTINUE
!
!--CALCULATE C---
      DO 20 I = 1,N - 1
        H = X(I+1) - X(I)
        C(I,3) = (U(I+1)-U(I))/ (3.0D0*H)
        C(I,1) = (A(I+1)-A(I))/H - (H*C(I,3)+U(I))*H
        C(I,2) = U(I)
      20 CONTINUE
      RETURN
      END SUBROUTINE SPCOF1
!*****
      SUBROUTINE STRAIGHT_LINE_FIT
(X,Y,NXY,YZERO,SLOPE,SSFIT,SSTOTAL)
      IMPLICIT NONE
      REAL*8, INTENT (IN)  :: X(*), Y(*)
      REAL*8, INTENT (OUT) :: YZERO ! INTERCEPT
      REAL*8, INTENT (OUT) :: SLOPE ! SLOPE
      REAL*8, INTENT (OUT) :: SSFIT ! SS OF RESIDUALS
      REAL*8, INTENT (OUT) :: SSTOTAL ! SS OF Y AROUND MEAN VALUE OF
Y
      INTEGER, INTENT (IN)  :: NXY ! NUMBER OF POINTS

      REAL*8, PARAMETER    :: ZERO=0D0
      REAL*8               :: SX,SY,SSX,SSY,SXY,N
      INTEGER              :: J
      SX =ZERO

```

```

SY = ZERO
SSY = ZERO
SSX = ZERO
SXY = ZERO
DO J = 1,NXY
SX =SX + X(J)
SY = SY + Y(J)
SSY = SSY + Y(J)*Y(J)
SSX = SSX + X(J)*X(J)
SXY = SXY + X(J)*Y(J)
ENDDO
N = NXY
SLOPE = (SXY-SX*SY/N)/(SSX-SX*SX/N)
YZERO = (SY - SLOPE*SX)/N
SSTOTAL = SSY - SY*SY/N
SSFIT = SSTOTAL - (SXY-SX*SY/N)*SLOPE
RETURN
END SUBROUTINE STRAIGHT_LINE_FIT
!*****
*****
SUBROUTINE GET_ERROR_VARIANCE_STRAIGHT_LINE_FIT
&          (X,Y,NXY,ERROR_VARIANCE) ! NEW JAN 2005
IMPLICIT NONE
REAL*8, INTENT (IN)  :: X(*), Y(*)
REAL*8, INTENT (OUT) :: ERROR_VARIANCE
INTEGER, INTENT (IN)  :: NXY          ! NUMBER OF POINTS

REAL*8, PARAMETER    :: ZERO=0D0, TWO=2D0
REAL*8               :: SX,SY,SSX,SSY,SXY,N,SLOPE,SSTOTAL
INTEGER              :: J
SX =ZERO
SY = ZERO
SSY = ZERO
SSX = ZERO
SXY = ZERO
DO J = 1,NXY
  SX =SX + X(J)
  SY = SY + Y(J)
  SSY = SSY + Y(J)*Y(J)
  SSX = SSX + X(J)*X(J)
  SXY = SXY + X(J)*Y(J)
ENDDO
N = NXY
SLOPE = (SXY-SX*SY/N)/(SSX-SX*SX/N)
SSTOTAL = SSY - SY*SY/N
ERROR_VARIANCE = (SSTOTAL - (SXY-SX*SY/N)*SLOPE)/(N-TWO)

```

```

        RETURN
    END SUBROUTINE GET_ERROR_VARIANCE_STRAIGHT_LINE_FIT
!*****
*****
    END SUBROUTINE CUBIC_GCV_FIT
!.....END OF CUBGCV.FOR

```

E.1.2 SUB...XY_DATA_FROM_FUNFIT_FILE.F90

```

SUBROUTINE GET_XY_DATA_FROM_FUNFIT_FILE (X,Y,N)
! READS THE FIRST X,Y DATA SET IN A STANDARD WINFUNFIT FILE THAT
MAY CONTAIN COMMENT LINES.
! NOTE:
! ON INPUT N MUST BE SET EQUAL TO THE DIMENSION OF THE X, Y
ARRAYS ASSIGNED IN THE CALLING PROGRAM.
! THE RETURNED VALUE OF N IS THE ACTUAL DIMENSION OF THE X, Y
DATA READ FROM THE FILE.
    IMPLICIT NONE
    INTEGER, INTENT (INOUT)          :: N
    REAL*8, INTENT (INOUT)           :: X(*), Y(*)

    INTEGER, PARAMETER                :: MAXLINES = 10000, LENSTRING =
80
    INTEGER                            :: J, JS, K, NN, IERR
    CHARACTER (LEN=256)                :: DATAFILENAME
    CHARACTER (LEN=LENSTRING)         :: STRING

    PRINT*, " SELECT THE FILE CONTAINING THE DATA TO BE READ"
    CALL FILESELECT("DAT", DATAFILENAME, J)
    WRITE(*, "(' SELECTED FILENAME: ', A256)") DATAFILENAME
    OPEN(UNIT=101, FILE=DATAFILENAME, STATUS='OLD', IOSTAT = IERR)
    IF(IERR /= 0) STOP ' GET_XY_DATA_FROM_FUNFIT_FILE: ERROR IN
OPENING DATA FILE'
    NN = 0
    DO J= 1, MAXLINES
        JS = J
        STRING(1:LENSTRING) = "
        READ (101,"(A)", END = 10, IOSTAT = IERR) STRING
        IF(IERR /= 0) STOP ' GET_XY_DATA_FROM_FUNFIT_FILE: ERROR IN
READING DATA FILE'
        K = LEN_TRIM(ADJUSTL(STRING))
        IF(STRING(1:1) == 'C' .OR. STRING(1:1) == 'c' .OR. STRING(1:1) == " .OR. K
<= 2) CYCLE
        NN = NN + 1
        IF(NN > N) STOP ' GET_XY_DATA_FROM_FUNFIT_FILE: ASSIGNED
DIMENSION OF X, Y TOO SMALL'

```

```

      READ(String,*, IOSTAT = IERR) X(NN), Y(NN)
      IF(IERR /= 0) STOP ' GET_XY_DATA_FROM_FUNFIT_FILE: ERROR IN
DATA FILE LIKELY, PLEASE CHECK'
      ENDDO
      10 IF (JS == MAXLINES) STOP ' GET_XY_DATA_FROM_FUNFIT_FILE: DATA
FILE CONTAINS TOO MANY LINES (RECORDS)'
      N = NN
      CLOSE (101,STATUS='SAVE')
END SUBROUTINE GET_XY_DATA_FROM_FUNFIT_FILE

```

E.2 Fortran subroutines for Chapter 2.

E.2.1 HB_PROD_V5.14.F90

```

! FILENAME = HB_PROD_V5.14.F90
!
! PURPOSE: ANALYSIS HEMOGLOBIN PRODUCTION RATE RELATIVE TO
ERYTHROPOIETIN CONCENTRATIONS POST-PHLEBOTOMY
!
! MODEL: EPO CONCENTRATIONS MODELED USING A GCV CUBIC SPLINE
FIT AND STIMULATES HB PRODUCTION USING AN EFFECT COMPARTMENT
!   MODEL. EFFECT COMPARTMENT CONCENTRATION IS LINKED TO HB
STIMULATION USING A SIGMOID EMAX MODEL. HB APPEARS IN THE
!   BLOOD "A" DAYS AFTER STIMULATION AND DISAPPEARS "D" DAYS
AFTER STIMULATION (CONSTANT LIFESPAN DISTRIBUTION MODEL).
!   CAN CHOOSE BETWEEN DIFFERENT COMBINATIONS OF STEADY-
STATE AND NON STEADY-STATE INTITAL CONDITIONS. RETICULOCYTES
!   APPEAR IN THE BLOOD "A" DAYS AFTER STIMULATION AND
DISAPPEAR FROM THE BLOOD "B" DAYS AFTER STIMULATION.
!   NOTE: THIS SOLUTION DOES NOT WORK IF BMAPRIME(X) >= 1 AT
ANY TIME T
!
!   Y(1) = HEMOGLOBIN CONCENTRATION
!   Y(2) = RETICULOCYTE CELL CONCENTRATION
!   Y(3) = EFFECT COMPARTMENT EPO CONCENTRATION
!   Y(4) = BMA(T) VALUE (I.E. B - A(T))
!   Y(5) = Z(T) ( I.E. LN(B - BMA(T - BMA(T))) )
!
! NOTES
!   IT IS ASSUMED THAT:
!     1. EPO CONCENTRATIONS ARE IN MILLIUNITS/MILLILITER
!     2. HEMOBLOBIN CONCENTRATIONS ARE IN GRAMS/DECILITER
!     3. RETICULOCYTE CONCENTRATIONS ARE IN 1000
CELLS/MICROLITER'
!     4. TIME IS GIVEN IN DAYS
!

```

! CHANGES/REVISIONS:

! VERSION 1.0 APR 12, 2006 ORIGINAL CODE (KF).

! VERSION 2.0 APR 18, 2006 ADDED THE ABILITY TO CHOOSE BETWEEN STEADY-STATE AND NON STEADY-STATE INITIAL CONDITIONS. THE ESTIMATED

! CE_INITIAL IS USED TO DETERMINE THE BACKGROUND HB RESPONSE. ALSO REMOVED THE ABILITY TO ALLOW FOR

! PARTITIONING OF DRUG AT STEADY-STATE BETWEEN PLASMA AND THE EFFECT COMPARTMENT (K1E) DUE TO PARAMETER UNIDENTIFIABILITY.

! VERSION 2.1 APR 18, 2006 EXTENDED THE CHOICE BETWEEN STEADY-STATE AND NON STEADY-STATE INITIAL CONDITIONS TO BE LINKED TO THE BACKGROUND

! RESPONSE IN MULTIPLE WAYS, AS WELL AS ESTIMATE THE BACKGROUND HB RESPONSE

! VERSION 3.0 MAY 17, 2006 ADDED FITTING TO RETICULOCYTE DATA BY ESTIMATING THE RETICULOCYTE BLOOD RESIDENCE TIME (B - A PARAMETER)

! NON-PARAMETRICALLY (CUBIC POLYNOMIAL CONSTRAINED TO POSITIVE VALUE)

! VERSION 3.1 MAY 18, 2006 SWITCHED ORDER OF DIFFERENTIAL EQUATIONS 2 AND 3

! VERSION 3.2 MAY 19, 2006 CORRECTED A MISTAKE IN THE CORRECTION OF RETICULOCYTE LOSS DUE TO PHLEBOTOMY (LINE 194 AND 195) AND ADDED

! FOURTH POLYNOMIAL TERM

! VERSION 4.3 MAY 25, 2006 CHANGED TO A PARAMETRIC ESTIMATION OF THE RETICULOCYTED BLOOD RESIDENCE TIME (B - A PARAMETER) BY USING

! A FEEDBACK MODEL (DIFFERENTIAL EQUATIONS 4 AND 5)

! VERSION 4.6 MAY 26, 2006 REDUCED THE COMPLEXITY OF THE FEEDBACK MODEL AND USED ABSOLUTE VALUE IN AN ATTEMPT TO CONSTRAIN DIFFERENTIAL

! EQUATION 4 TO POSITIVE VALUES

! VERSION 4.12 MAY 30, 2006 DROPPED ABSOLUTE VAULE AND INSTEAD MULTIPLIED THE WHOLE DIFFERENTIAL EQUATION 4 (YPRIME(4)) BY ITSELF(Y(4)) TO

! CONSTRAIN IT TO POSITIVE VALUES

! VERSION 4.14 MAY 30, 2006 EXPANDED TO ALLOW FOR FIRST ORDER OR SECOND ORDER OUTPUT OF B - A DIFFERENTIAL EQUATION (EQN. 4)

! NOTE: IF USING FIRST ORDER OUTPUT 'ESSENTIALLY' DROP DIFFERENTIAL EQUATION 5 (SET EQUAL TO ZERO)

! VERSION 5.0 JUN 23, 2006 THE DETERMINATION OF "B" HAS BEEN CORRECTED FROM THE APPROXIMATION USED IN MODEL 3 AND MODEL 4,

! AND IS NOW EXACT. "B" IS MODELED AS A FUNCTION
 OF THE ACTIVATION RATE.
 ! VERSION 5.1 JUN 26, 2006 CORRECTED INITIAL CONDITIONS AND
 MADE BPFUNC A POLYNOMIAL
 ! VERSION 5.2 JUN 27, 2006 CHANGED TO BMA(T) AND Z(T) MODEL TO
 CONSTRAIN THE VALUES OF BMA(T) TO BE POSITIVE USING A
 EXPONENTIAL POLYNOMIAL
 ! FUNCTION
 ! VERSION 5.4 JUL 10, 2006 CORRECTED EQUATIONS USING X(T) AND
 Z(T) TO ALLOW FOR CHANGES IN RESPONSE SOLELY DUE TO CHANGES IN
 THE B(T)
 ! VERSION 5.7 JUL 12, 2006 REFORMULATED IN TERMS OF BMA(T)
 INSTEAD OF B(T) TO CONSTRAIN EQUATION PROPERLY (I.E. B > A)
 ! VERSION 5.10 JUL 13, 2006 CHANGED FORM OF BMA(T) POLYNOMIAL
 ! VERSION 5.11 JUL 14, 2006 CHANGED THE 5TH ORDER POLYNOMIAL
 FROM VERSION 5.10 SO THAT ITS CONSTRAINED TO HAVE START
 CHANGING WITH A
 ! A DERIVATIVE OF 0.0 AT T1 AND A VALUE OF BMA0
 AND TO STOP CHANING WITH A DERIVATIVE OF 0.0 AT T2 WITH A VALUE
 ! OF BMA_END
 ! VERSION 5.12 AUG 9, 2006 REFORMULATED TO HAVE A TIME
 VARIANT 'A' VALUE INSTEAD OF A TIME VARIANT 'B' VALUE
 ! VERSION 5.13 AUG 10, 2006 REFORMULATED WITH A TIME VARIANT
 'A' AGAIN, BUT DOING SO BY MODELING A TIME VARIANT BMA INSTEAD
 OF 'A' DIRECTLY
 ! VERSION 5.14 FEB 26, 2007 DEBUGGING CODE ADDED TO GET
 STATISTICAL OUTPUTS WORKING
 !
 !
 !
 SUBROUTINE USERMODEL_ODE(T,Y,YPRIME,P,NP,IFUN)
 IMPLICIT NONE
 INTEGER :: NP, IFUN, K = 1, i
 REAL*8 :: T, Y(*), P(*)

 REAL*8 :: YPRIME(*)
 REAL*8 :: D, HBSTIM, VM, KM, N, SS, KE0, HBDROP, CESS,
 HB_INITIAL, CE_INITIAL, TWO = 2D0, &
 T_FIRST, EPO, EPO_FIRST, EPO0, ZERO = 0D0, ONE = 1D0,
 START_VALUE, STOP_VALUE, TPHLEB, CE_MAX = 0.0, &
 T_FIRST_EPO, EPO_HAT_T_FIRST_EPO, T_LAST_EPO,
 EPO_HAT_T_LAST_EPO, TMD, Y3TMEZ, Y3TMD, YLAG, EPO_POST, &
 HBSTIMEZ, HBSTIMD, CF, TMB, B, Z, EZ, TMEZ,
 Y4TMEZ_TPHLEB, Y4TMEZ, &
 Y3TMB, HBSTIMB, TIME_VAR, X, MAX_VAL = 500D0,
 BMA0, BMA_END, T1, T2, P1, P2, DMA0

```

REAL*8, DIMENSION(0:5)      :: COEFFICIENTS = 0.0

LOGICAL                      :: EVENT_IS_ACTIVE, DEBUG
CHARACTER(LEN=12)           :: INDICATOR

COMMON /EPO_INFO/ T_FIRST_EPO, EPO_HAT_T_FIRST_EPO,
T_LAST_EPO, EPO_HAT_T_LAST_EPO, CE_MAX, DEBUG
EXTERNAL HBSTIM, EVENT_IS_ACTIVE

IF(IFUN == 1 .OR. IFUN == 2) THEN

  !HEMOGLOBIN PRODUCTION/DESTRUCTION PRIMARY PARAMETERS
  B      = P(1) ! LAG TIME (B) BETWEEN EPO ACTIVATION AND
MATURATION OF RETICULOCYTES INTO RBCS
  DMA0   = P(2) ! BASELINE HB BLOOD RESIDENCE TIME (D - A0)
  KM     = P(3) ! PARAMETER USED TO DEFINE THE ACTIVATION RATE
OF HB BY EPO (SIGMOID EMAX MODEL)
  VM     = P(4) ! MAXIMUM HB ACTIVATION RATE BY EPO (SIGMOID
EMAX MODEL)
  N      = P(5) ! SIGMOIDICITY PARAMETER TO DEFINE THE
ACTIVATION RATE OF HB BY EPO (SIGMOID EMAX MODEL)
  SS     = P(6) ! INDICATOR VARIABLE:
          ! SS=1.0 INDICATES STEADY-STATE INITIAL CONDITIONS
ASSUMED FOR HB AND CE.
          !   BACKGROUND HB A FUNCTION OF CESS (REDUCED
MODEL)
          ! SS=2.0 INDICATES STEADY-STATE INITIAL CONDITIONS
ASSUMED FOR CE BUT INITIAL CONDITIONS FOR HB ESTIMATED.
          !   BACKGROUND HB A FUNCTION OF CESS
          ! OTHERWISE:
          !   INDICATES STEADY-STATE INITIAL CONDITIONS
ASSUMED FOR HB BUT INITIAL CONDITIONS FOR CE ESTIMATED.
          !   BACKGROUND HB A FUNCTION OF CE_INITIAL
  KE0    = P(7) ! RATE CONSTANT FROM EFFECT COMPARTMENT
  HBDROP = P(8) ! PARAMETER THAT DETERMINES (IN
TRANSFORMED FORM) THE FRACTIONAL DROP IN HB DUE TO
PHLEBOTOMY
  TPHLEB = P(9) ! TIME FOR PHLEBOTOMY (MEASURED ON SAME
TIME SCALE AS TFIRST = 1ST OBSERVATION TIME)
  HB_INITIAL = P(10) ! ESTIMATED INITIAL HB CONCENTRATION
  CE_INITIAL = P(11) ! ESTIMATED INITIAL EFFECT COMPARTMENT
CONC.
  TIME_VAR = P(12) ! INDICATOR VARIABLE:
          ! TIME_VAR=1.0 INDICATES TIME VARIANT A
          ! OTHERWISE, TIME INVARIANCE OF A IS ASSUMED

```

```

! RETICULOCYTE/HEMOGLOBIN LIFESPAN PARAMETERS
  CF      = P(13) ! CONVERSION FACTOR FROM HEMOGLOBIN TO
RETICULOCYTES
  BMA0    = P(14) ! INITIAL OR BASELINE (SS) BMA VALUE
  BMA_END = P(15) ! FINAL (SS) BMA VALUE
  T1      = P(16) ! TIME OF THE START OF DEVIATION FROM SS BMA
VALUES (BMA0)
  T2      = P(17) ! TIME OF THE RETURN TO SS BMA VALUE (BMA_END)
  P1      = P(18) ! ALPHA4 COEFFICIENT
  P2      = P(19) ! ALPHA5 COEFFICIENT

! SECONDARY PARAMETERS
  TMB = T - B      ! TIME AT T-B
  D = B - BMA0 + DMA0 ! LAG TIME (D) BETWEEN EPO ACTIVATION AND
AND DESTRUCTION OF PRODUCED HEMOGLOBIN (SENESCENCE OF RBCS)
  TMD = T - D      ! TIME AT T-D
  EPO = ZERO      ! EPO CONCENTRATION FROM CUBIC SPLINE FIT
(INITIALIZED TO 0) AT TIME T
  EPO0 = EPO_HAT_T_FIRST_EPO      ! SET EPO INITIAL = VALUE OF
FITTED CUBIC SPLINE AT FIRST EPO OBSERVATION
  EPO_POST = EPO_HAT_T_LAST_EPO    ! SET EPO POST = VALUE OF
FITTED CUBIC SPLINE AT LAST EPO OBSERVATION

! DETERMINE IF TIME VARIANT A OR NOT
  IF ( (TIME_VAR < ONE - 0.000001) .OR. (TIME_VAR > ONE + 0.000001) )
THEN
  BMA_END = BMA0
  P1 = ZERO
  P2 = ZERO
ENDIF

!CALCULATE COEFFICIENTS OF A(T) FUNCTION
  CALL CALC_POLY_TERMS(BMA0, BMA_END, T1, T2, P1, P2,
COEFFICIENTS)

! STEADY-STATE EFFECT COMPARTMENT CONCENTRATION (CESS)
  IF ( SS == ONE .OR. SS == TWO) THEN
    CESS = EPO0
  ELSE
    CESS = CE_INITIAL
  END IF

! GET EPO CONCENTRATIONS
  IF ( T > T_LAST_EPO ) THEN
    EPO = EPO_POST

```

```

ELSE
  CALL CUBIC_GCV (T, X)
  EPO = DEXP(X)      ! BACK-TRANSFORM
END IF

                                IF (DEBUG) EPO = EPO0
! CALCULATE Z(T), EXP(Z(T)), T-EXP(Z(T))
  Z = Y(5)
  EZ = EXP( MIN( Z, MAX_VAL ))
  TMEZ = T - EZ

! GET LAG TIME VALUES
  IF ( TMEZ > T_FIRST_EPO ) THEN ! GET Y3X
    IF ( T - TMEZ < 0.00001 ) THEN
      Y3TMEZ = Y(3)
    ELSE
      Y3TMEZ = YLAG(3,TMEZ)
    ENDIF
  ELSE
    Y3TMEZ = CESS
  ENDIF

  IF (TMB > T_FIRST_EPO ) THEN ! GET Y3TMB
    Y3TMB = YLAG(3,TMB)
  ELSE
    Y3TMB = CESS
  ENDIF

  IF (TMD > T_FIRST_EPO ) THEN ! GET Y3TMD
    Y3TMD = YLAG(3,TMD)
  ELSE
    Y3TMD = CESS
  ENDIF

                                IF (DEBUG) THEN
                                    Y3TMEZ = CESS
                                    Y3TMB = CESS
                                    Y3TMD = CESS
                                ENDIF

! GET HB_STIMULATION RATES
  HBSTIMEZ = HBSTIM(Y3TMEZ, P(3))
  HBSTIMB = HBSTIM(Y3TMB, P(3))
  HBSTIMD = HBSTIM(Y3TMD, P(3))

```

```

! DIFFERENTIAL EQUATIONS
  ! Y(1) = HEMOGLOBIN CONCENTRATION
  ! Y(2) = RETICULOCYTE CELL CONCENTRATION
  ! Y(3) = EFFECT COMPARTMENT EPO CONCENTRATION
  ! Y(4) = BMA(T) VALUE (I.E. B - A(T))
  ! Y(5) = Z(T) ( I.E. LN(B - BMA(T - BMA(T))) )

  YPRIME(3) = KE0*EPO - KE0*Y(3)
  YPRIME(4) = BMAPFUNC( T, T1, T2, COEFFICIENTS )
  YPRIME(5) = ((- BMAPFUNC( TMEZ, T1, T2, COEFFICIENTS ))/(ONE -
BMAPFUNC( TMEZ, T1, T2, COEFFICIENTS ))) * EXP(MIN( -Z, MAX_VAL ))
  YPRIME(1) = ( ONE - YPRIME(5)*EXP(MIN( Z, MAX_VAL )) ) * HBSTIMEZ -
HBSTIMD
  YPRIME(2) = CF*(( ONE - YPRIME(5)*EXP(MIN( Z, MAX_VAL ))
)*HBSTIMEZ - HBSTIMB)

  IF ( Y(3) > CE_MAX ) THEN
    CE_MAX = Y(3)
  ENDIF

!GET A(X(TPHLEB)) IF POST PHLEBOTOMY
INDICATOR='INACTIVE'
IF ( T > TPHLEB ) THEN
  IF ( TPHLEB > T_FIRST_EPO ) THEN
    Y4TMEZ_TPHLEB = YLAG(4,TPHLEB-EZ)
  ELSE
    Y4TMEZ_TPHLEB = BMA0
  ENDIF

  !REDUCTION IN HB LOSS, SINCE LESS BACKGROUND HB IS PRESENT
  POST-PHLEOBOTOMY
  IF ( T < TPHLEB + D - (B - Y4TMEZ_TPHLEB) ) THEN
    CALL GET_VALUE_AT_START_OF_EVENT (1, START_VALUE)
    CALL GET_VALUE_AT_STOP_OF_EVENT (1, STOP_VALUE)
    YPRIME(1) = YPRIME(1) + ((START_VALUE-
STOP_VALUE)*(HBSTIMD))/START_VALUE
  ENDIF

  !REDUCTION IN RETICULOCYTE LOSS, SINCE LESS BACKGROUND
  RETICULOCYTES ARE PRESENT POST-PHLEOBOTOMY
  IF ( T < TPHLEB + Y4TMEZ_TPHLEB ) THEN
    INDICATOR='ACTIVE'
    CALL GET_VALUE_AT_START_OF_EVENT (2, START_VALUE)
    CALL GET_VALUE_AT_STOP_OF_EVENT (2, STOP_VALUE)
    YPRIME(2) = YPRIME(2) + ((START_VALUE-
STOP_VALUE)*(CF*HBSTIMB))/START_VALUE

```

```

    ENDIF
  ENDIF
END IF

```

```
CONTAINS
```

```

  ! ARBITRARY FUNCTION THAT DETERMINES THE BMAPRIME VALUE
  REAL*8 FUNCTION BMAPFUNC (T, T1, T2, P)
  IMPLICIT NONE
  REAL*8, INTENT (IN) :: T, T1, T2
  REAL*8, DIMENSION(0:5), INTENT (IN) :: P
  IF ( ( T < T1 ) .OR. ( T > T2 ) ) THEN
    BMAPFUNC = ZERO
  ELSE
    BMAPFUNC = P(1) + 2*P(2)*T + 3*P(3)*T**2 + 4*P(4)*T**3 + 5*P(5)*T**4
  ENDIF
END FUNCTION BMAPFUNC

```

```
END SUBROUTINE USERMODEL_ODE
```

```
SUBROUTINE USERMODEL(T,Y,P,NP,IFUN)
```

```

  USE EVENTSTRUCTURE
  IMPLICIT NONE
  INTEGER                :: NP, IFUN, JFUN, NUM
  REAL*8                :: T, Y, P(*)

  SAVE                  T_FIRST
  INTEGER,PARAMETER     :: NEQN = 5, NEVENTS = 2, LENSTRING =
180, MAX_NXY = 200, NPS=180
  INTEGER                :: NOBS, J, LUN, NSIGDIGITS
  REAL*8                :: YZERO(NEQN), B, DMA0, HBSTIM, VM, KM, N, SS,
KE0, HBDROP, HB_INITIAL, CE_INITIAL, &
    T_FIRST, EPO, EPO_FIRST, EPO0, ZERO = 0D0, ONE =
1D0, START_VALUE, STOP_VALUE, TPHLEB, &
    T_FIRST_EPO, EPO_HAT_T_FIRST_EPO, T_LAST_EPO,
EPO_HAT_T_LAST_EPO, TWO = 2D0, CE_MAX, &
    TOBS(MAX_NXY), COBS(MAX_NXY), CLN(MAX_NXY),
DT, TC(NPS), CC(NPS), FACTOR, TIME_VAR, &
    CF, BMA0, BMA_END, T1, T2, P1, P2

  LOGICAL                :: DEBUG = .FALSE.

  LOGICAL, SAVE          :: SHOWIT, PLOTSAVED
  CHARACTER (LEN=256)    :: ID, DATAFILENAME
  CHARACTER (LEN=20)     :: PNAME, REPLY
  CHARACTER (LEN=LENSTRING) :: STRING

```

TYPE (EVENTTYPE) EVENT(NEVENTS)
 COMMON /EPO_INFO/T_FIRST_EPO, EPO_HAT_T_FIRST_EPO, T_LAST_EPO,
 EPO_HAT_T_LAST_EPO, CE_MAX
 EXTERNAL HBSTIM

B = P(1) ! LAG TIME (B) BETWEEN EPO ACTIVATION AND
 MATURATION OF RETICULOCYTES INTO RBCS
 DMA0 = P(2) ! BASELINE HB BLOOD RESIDENCE TIME (D - A0)
 KM = P(3) ! PARAMETER USED TO DEFINE THE ACTIVATION RATE
 OF HB BY EPO (SIGMOID EMAX MODEL)
 VM = P(4) ! MAXIMUM HB ACTIVATION RATE BY EPO (SIGMOID
 EMAX MODEL)
 N = P(5) ! SIGMOIDICITY PARAMETER TO DEFINE THE
 ACTIVATION RATE OF HB BY EPO (SIGMOID EMAX MODEL)
 SS = P(6) ! INDICATOR VARIABLE:
 ! SS=1.0 INDICATES STEADY-STATE INITIAL CONDITIONS
 ASSUMED FOR HB AND CE.
 ! BACKGROUND HB A FUNCTION OF CESS (REDUCED
 MODEL)
 ! SS=2.0 INDICATES STEADY-STATE INITIAL CONDITIONS
 ASSUMED FOR CE BUT INITIAL CONDITIONS FOR HB ESTIMATED.
 ! BACKGROUND HB A FUNCTION OF CESS
 ! OTHERWISE:
 ! INDICATES STEADY-STATE INITIAL CONDITIONS
 ASSUMED FOR HB BUT INITIAL CONDITIONS FOR CE ESTIMATED.
 ! BACKGROUND HB A FUNCTION OF CE_INITIAL
 KE0 = P(7) ! RATE CONSTANT FROM EFFECT COMPARTMENT
 HBDROP = P(8) ! PARAMETER THAT DETERMINES (IN
 TRANSFORMED FORM) THE FRACTIONAL DROP IN HB DUE TO
 PHLEBOTOMY
 TPHLEB = P(9) ! TIME FOR PHLEBOTOMY (MEASURED ON SAME
 TIME SCALE AS TFIRST = 1ST OBSERVATION TIME)
 HB_INITIAL = P(10) ! ESTIMATED INITIAL HB CONCENTRATION
 CE_INITIAL = P(11) ! ESTIMATED INITIAL EFFECT COMPARTMENT
 CONC.
 TIME_VAR = P(12) ! INDICATOR VARIABLE:
 ! TIME_VAR=1.0 INDICATES TIME VARIANT A
 ! OTHERWISE, TIME INVARIANCE OF A IS ASSUMED
 ! RETICULOCYTE/HEMOGLOBIN LIFESPAN PARAMETERS
 CF = P(13) ! CONVERSION FACTOR FROM HEMOGLOBIN TO
 RETICULOCYTES
 BMA0 = P(14) ! INITIAL OR BASELINE (SS) BMA VALUE
 BMA_END = P(15) ! FINAL (SS) BMA VALUE
 T1 = P(16) ! TIME OF THE START OF DEVIATION FROM SS BMA
 VALUES (BMA0)
 T2 = P(17) ! TIME OF THE RETURN TO SS BMA VALUE (BMA_END)

P1 = P(18) ! ALPHA4 COEFFICIENT
 P2 = P(19) ! ALPHA5 COEFFICIENT

! START OF PRELIMINARY PREPARATION SECTION =====

IF (IFUN == - 1000) THEN

IF(NP /= 19) STOP ' USERMODEL: INCONSISTENT NUMBER OF
 PARAMETERS IN PARAMETER FILE'

EVENT(1).START = TPHLEB ! EVENT 1: DROP IN HEMOGLOBIN
 DUE TO PHLEBOTOMY

EVENT(1).STOP = TPHLEB

EVENT(1).LINK_TO_VARIABLE = 1

EVENT(1).ZERO_TO_ONE_TRANSFORM = .TRUE. ! ENSURES WE DO NOT
 GET A DROP THAT RESULT IN A NEGATIVE VARIABLE VALUE

EVENT(2).START = TPHLEB ! EVENT 1: DROP IN
 RETICULOCYTES DUE TO PHLEBOTOMY

EVENT(2).STOP = TPHLEB

EVENT(2).LINK_TO_VARIABLE = 2

EVENT(2).ZERO_TO_ONE_TRANSFORM = .TRUE. ! ENSURES WE DO NOT
 GET A DROP THAT RESULT IN A NEGATIVE VARIABLE VALUE

CALL REGISTER_ALL_EVENTS(EVENT, NEVENTS) !* <= REGISTER THE
 EVENTS BEFORE THE START OF THE FITTINGS (IFUN=-1000)

! THIS SECTION IS SWITCHING THE DEBUG MODEL ON AND OFF WHEN
 DOING THE STATISTICAL CALCULATIONS

PRINT*, 'SWITCH DEBUG MODE WHEN DOING STATS CALCULATIONS
 (YES/NO)?'

READ*, REPLY

IF (REPLY(1:1) == 'Y' .OR. REPLY(1:1) == 'y') THEN

CALL SWITCH_NMQUAD_DEBUG_ON (6)

ELSE

CALL SWITCH_NMQUAD_DEBUG_OFF

ENDIF

CALL SETFUNFITPARAMETERNAME(1, " B (DAYS)")

CALL SETFUNFITPARAMETERNAME(2, " D-A0 (DAYS)")

CALL SETFUNFITPARAMETERNAME(3, " KM (MU/ML)")

CALL SETFUNFITPARAMETERNAME(4, " VM (G/DL/DAY)")

CALL SETFUNFITPARAMETERNAME(5, " N")

CALL SETFUNFITPARAMETERNAME(6, " SS (1.0=SS)")

CALL SETFUNFITPARAMETERNAME(7, " KE0 (1/DAY)")

CALL SETFUNFITPARAMETERNAME(8, " DROP")

CALL SETFUNFITPARAMETERNAME(9, " TPHLEB (DAYS)")

```

CALL SETFUNFITPARAMETERNAME(10, " HB_INITIAL (G/DL)")
CALL SETFUNFITPARAMETERNAME(11, " CE_INITIAL (MU/ML)")
CALL SETFUNFITPARAMETERNAME(12, " TIME_VAR (1.0=VAR)")
CALL SETFUNFITPARAMETERNAME(13, " CF (DL*1000C/G*UL)")
CALL SETFUNFITPARAMETERNAME(14, " BMA(0)")
CALL SETFUNFITPARAMETERNAME(15, " BMA(END) ")
CALL SETFUNFITPARAMETERNAME(16, " T1 ")
CALL SETFUNFITPARAMETERNAME(17, " T2 ")
CALL SETFUNFITPARAMETERNAME(18, " P1 ")
CALL SETFUNFITPARAMETERNAME(19, " P2 ")

! PRINT*
! PRINT*,'CHOOSE INTEGRATOR TO USE (RKF45
=1,LSODA=2,RK78_FIXED=3,DOP853R=4)?'
! READ*,NUM
! CALL SET_INTEGRATOR(NUM) ! THIS CALL TELLS WINFUNFIT
WHICH INTEGRATOR TO USE

NOBS = MAX_NXY
CALL GET_XY_DATA_FROM_FUNFIT_FILE (TOBS,COBS,NOBS) ! GET EPO
DATA
PRINT*
PRINT*,' IT IS ASSUMED THAT:'
PRINT*,' 1. EPO CONCENTRATIONS ARE IN MILLIUNITS/MILLILITER'
PRINT*,' 2. HEMOBLOBIN CONCENTRATIONS ARE IN
GRAMS/DECILITER'
PRINT*,' 3. RETICULOCYTE CONCENTRATIONS ARE IN 1000
CELLS/MICROLITER'
PRINT*,' 4. TIME IS GIVEN IN DAKYS'
PRINT*
PRINT*
PRINT*,' ***** PLEASE NOTE *****'
PRINT*,' SS (STEADY-STATE) IS AN INDICATOR VARIABLE THAT
CONTROLS INITIAL CONDITIONS'
PRINT*,' FOR HEMOGLOBIN AND EFFECT COMPARTMENT (Y1(0)=HB
AND Y3(0)=CE, RESPECTIVELY)'
PRINT*,' AND BACKGROUND HEMOGLOBIN (HB) RESPONSE
ASSUMPTIONS:'
PRINT*
PRINT*,' SS=1.0 INDICATES STEADY-STATE INITIAL CONDITIONS
ASSUMED FOR HB AND CE,'
PRINT*,' BACKGROUND HB A FUNCTION OF CESS (REDUCED
MODEL)'
PRINT*,' SS=2.0 INDICATES STEADY-STATE INITIAL CONDITIONS
ASSUMED FOR CE BUT'

```

```

PRINT*,'      INITIAL CONDITIONS FOR HB ESTIMATED, BACKGROUND
HB A FUNCTION OF'
PRINT*,'      CESS'
PRINT*,'    OTHERWISE:'
PRINT*,'      INDICATES STEADY-STATE INITIAL CONDITIONS
ASSUMED FOR HB BUT'
PRINT*,'      INITIAL CONDITIONS FOR CE ESTIMATED, BACKGROUND
HB A FUNCTION OF'
PRINT*,'      CE_INITIAL'
PRINT*
READ*
CALL GET_GLOBAL_XMIN(T_FIRST)           ! GET THE EARLIEST
OBSERVATION TIME
T_FIRST_EPO = TOBS(1)
T_LAST_EPO = TOBS(NOBS)
CLN(1:NOBS)=DLOG(COBS(1:NOBS))         ! TRANSFORM EPO DATA
BEFORE GCV FIT
CALL CUBIC_GCV_FIT(TOBS, CLN, NOBS)     ! GCV FIT TO LOG-
TRANSFORMED EPO DATA
CALL CUBIC_GCV(T_FIRST_EPO, EPO_HAT_T_FIRST_EPO)
EPO_HAT_T_FIRST_EPO = DEXP(EPO_HAT_T_FIRST_EPO)
CALL CUBIC_GCV(T_LAST_EPO, EPO_HAT_T_LAST_EPO)
EPO_HAT_T_LAST_EPO = DEXP(EPO_HAT_T_LAST_EPO)
DT = (TOBS(NOBS) - TOBS(1))/(NPS-1)
DO J = 1, NPS
  TC(J) = TOBS(1) + DT*(J-1)
  CALL CUBIC_GCV(TC(J), CC(J))
  CC(J) = DEXP(CC(J))
ENDDO

      !|-----SELECT CHOICE OF NUMERICAL
INTEGRATION -----
      CALL USE_DELAY_ODE_SOLVER  !| * <= I M P O R T A N T TO SPECIFY
      THAT THE ODE ARE DELAY ODE TYPE, WHICH
      !|                      REQUIRES SPECIAL NUMERIAL
INTEGRATION (USING THE
      !|                      THE DELAY ODE SOLVER LIBRARY OF
SUBROUTINES.

      ! THE FOLLOWING "FACTOR" ALLOWS US TO
INCREASE/DECREASE THE INTERNAL DEFAULT
      ! VALUE USED FOR THE MAXIMUM NUMBER OF OBJ FUNCTION
EVALAUTIONS. IN THIS CASE
      ! WE INCREASE IT BY A FACTOR OF 2 BECAUSE WE ARE DEALING
WIHT THE NUMERICAL
      ! SOLUTION TO ODEs

```

```

CALL SET_ODE_TOLERANCE(1.0D-7) ! THIS SETS REL.TOL = ABS.TOL =
1.0D-7 FOR THE INTEGRATION OF ODE's. DO NOT USE TOO
      ! SMALL VALUES (LESS THAN 1.0D-8 IS NOT
RECOMMENDED). TOO LARGE VALUES (E.G. LARGER
      ! THAN 1.0D-5) WILL REDUCE THE NUMBER OF STEPS
TAKEN IN THE INTEGRATION AND BE
      ! FASTER, BUT WILL PRODUCE LESS ACCURATE AND
MORE ERRATIC INTEGRATIONS. THE LATTER
      ! MAY RESULT IN CONVERGENCE PROBLEMS IF
CONVERGENCE TOLERANCE IS SET QUITE STRICT,
      ! I.E. IF NSIGDIGITS IS REALATIVE HIGH.

```

```

FACTOR = 2D0
CALL SET_MAX_OBJFUN_EVALUATIONS_FACTOR(FACTOR) ! <=
INCREASES (OR DECREASES) THE DEFAULT VALUE BY "FACTOR".

```

```

NSIGDIGITS = 5 !* <= CONVERGENCE CRITERION. THE VARIATION OF
OBJFUN VALUES LIES WITHIN 10**(-NSIGDIGITS)
      ! AT CONVERGENCE. DO NOT USE A TOO HIGH NSIGDIGITS
VALUE WHEN FITTING THE NUMERICAL
      ! SOLUTION OFODE's.

```

```

CALL SET_NSIGDIGITS_OBJFUN_FOR_CONVERGENCE(NSIGDIGITS)

```

```

CE_MAX = ZERO
END IF
! END OF PRELIMINARY PREPARATION SECTION =====

```

```

CALL SET_EVENT_VALUE(1, HBDROP)           ! SET BOLUS DROP
PARAMETER
CALL SET_EVENT_START_AND_STOP (1, TPHLEB, TPHLEB)

```

```

CALL SET_EVENT_VALUE(2, HBDROP)           ! SET BOLUS DROP
PARAMETER
CALL SET_EVENT_START_AND_STOP (2, TPHLEB, TPHLEB)

```

```

!INITIAL CONDITIONS
      ! Y(1) = HEMOGLOBIN CONCENTRATION
      ! Y(2) = RETICULOCYTE CELL CONCENTRATION
      ! Y(3) = EFFECT COMPARTMENT EPO CONCENTRATION
      ! Y(4) = A VALUE (I.E. A(T))
      ! Y(5) = X(T) ( X(T) = T - A(X(T)) )
EPO0 = EPO_HAT_T_FIRST_EPO      ! SET EPO ZERO = VALUE OF FITTED
CUBIC SPLINE AT FIRST EPO OBSERVATION

```

```

IF ( SS == ONE) THEN
  YZERO(3) = EPO0
  YZERO(1) = HBSTIM(YZERO(3), P(3))*DMA0

```

```

ELSE IF ( SS == TWO ) THEN
  YZERO(3) = EPO0
  YZERO(1) = HB_INITIAL
ELSE
  YZERO(3) = CE_INITIAL
  YZERO(1) = HBSTIM(YZERO(3), P(3))*DMA0
END IF

YZERO(4) = BMA0
YZERO(5) = LOG(B - BMA0)
YZERO(2) = CF*(HBSTIM(YZERO(3), P(3)))*BMA0

IF (IFUN == 1 .OR. IFUN == 2) THEN
  JFUN = IFUN
  CALL
INTEGRATE_USERMODEL_ODE(T,Y,P,NP,IFUN,T_FIRST,YZERO,NEQN,JFUN)
END IF

! USER OUTPUT SECTION
=====
IF (IFUN == 0) THEN
  DO J = 1,NP
    CALL GETFUNFITPARAMETERNAME (J,PNAME)
    WRITE(*,"(1X,I2,1X,A,G14.4)")J, PNAME, P(J)
  ENDDO
!   WRITE (*,"('CE(ZERO): ', G12.6, '   CE_MAX: ', G12.6)") YZERO(3), CE_MAX
  CALL PROMT(SHOWIT)
  IF (SHOWIT) THEN
    DO J = 1,NP
      CALL GETFUNFITPARAMETERNAME (J,PNAME)
      WRITE(LUN,"(' PAR',I2,1X,A,G14.4)")J, PNAME, P(J)
    ENDDO
    CALL GETDATAFILENAME(DATAFILENAME)
    CALL ADDMARGINTEXT(DATAFILENAME)
    CALL ADDOBSERVATIONSLEFT(1)           ! HB DATA
    CALL ADDFITTEDCURVELEFT(1)           ! FITTED HB CURVE
    CALL LEFTLABEL ('HEMOGLOBIN (G/DL)')
    CALL RIGHTLABEL ('+ PLASMA EPO (MILLI U/ML)')
    CALL ADDPOINTSRIGHT_D (TOBS, COBS, NOBS)           ! EPO DATA
    CALL ADDCURVERIGHT_D (TC,CC,NPS)           ! GCV FITTED EPO
  CURVE
    CALL XLABEL('TIME (DAYS)')
    CALL DISPLAYPLOT
  ! RECORD PLOT ID IF PLOT IS SAVED
    CALL RECORDPLOTIFSAVED(3)

```

```

CALL RECORDPLOTIFSAVED(LUN)

CALL GETDATAFILENAME(DATAFILENAME)
CALL ADDMARGINTEXT(DATAFILENAME)
CALL ADDOBSERVATIONSLEFT(1)           ! HB DATA
CALL ADDFITTEDCURVELEFT(1)           ! FITTED HB CURVE
CALL LEFTLABEL ('HEMOGLOBIN (G/DL)')
CALL RIGHTLABEL ('+ RETICULOCYTES (1000 CELLS/UL)')
CALL ADDOBSERVATIONSRIGHT(2)         ! RETIC DATA
CALL ADDFITTEDCURVERIGHT(2)         ! FITTED RETIC CURVE
CALL XLABEL('TIME (DAYS)')
CALL DISPLAYPLOT
! RECORD PLOT ID IF PLOT IS SAVED
CALL RECORDPLOTIFSAVED(3)
CALL RECORDPLOTIFSAVED(LUN)

!!*****NEW
PLOTS*****

CALL GETDATAFILENAME(DATAFILENAME)
CALL ADDMARGINTEXT(DATAFILENAME)
CALL RIGHTLABEL ('PLASMA EPO (MILLI U/ML)')
CALL INCLUDEPOINTSRIGHT_D (TOBS, COBS, NOBS, 5)      ! EPO
DATA
CALL ADDCURVERIGHT_D (TC,CC,NPS)           ! GCV FITTED EPO
CURVE
CALL BEGINRIGHTAT(0.0)
CALL ENDRIGHTAT(600.0)
CALL XLABEL('TIME (DAYS)')
CALL DISPLAYPLOT
! RECORD PLOT ID IF PLOT IS SAVED
CALL RECORDPLOTIFSAVED(3)
CALL RECORDPLOTIFSAVED(LUN)

! CALL GETDATAFILENAME(DATAFILENAME)
! CALL ADDMARGINTEXT(DATAFILENAME)
! CALL LEFTLABEL ('RETICULOCYTES (1000 CELLS/UL)')
! CALL ADDOBSERVATIONSLEFT(2)           ! RETIC DATA
! CALL ADDFITTEDCURVELEFT(2)           ! FITTED RETIC
CURVE
! CALL XLABEL('TIME (DAYS)')
! CALL DISPLAYPLOT
! ! RECORD PLOT ID IF PLOT IS SAVED
! CALL RECORDPLOTIFSAVED(3)
! CALL RECORDPLOTIFSAVED(LUN)

```

```

CALL GETDATAFILENAME(DATAFILENAME)
CALL ADDMARGINTEXT(DATAFILENAME)
CALL ADDOBSERVATIONSLEFT(1)           ! HB DATA
CALL ADDFITTEDCURVELEFT(1)           ! FITTED HB CURVE
CALL BEGINLEFTAT(3.0)
CALL ENDLEFTAT(12.0)
CALL LEFTLABEL ('HEMOGLOBIN (G/DL)')
CALL RIGHTLABEL ('+ RETICULOCYTES (1000 CELLS/UL)')
CALL ADDOBSERVATIONSRIGHT(2)         ! RETIC DATA
CALL ADDFITTEDCURVERIGHT(2)         ! FITTED RETIC CURVE
CALL BEGINRIGHTAT(0.0)
CALL ENDRIGHTAT(800.0)
CALL XLABEL('TIME (DAYS)')
CALL DISPLAYPLOT
! RECORD PLOT ID IF PLOT IS SAVED
CALL RECORDPLOTIFSAVED(3)
CALL RECORDPLOTIFSAVED(LUN)

CALL ISPLOTSAVED(PLOTSAVED)         ! CHECKS IF PLOT IS
SAVED
IF(PLOTSAVED) THEN
  DO J = 1, NP
    CALL GETFUNFITPARAMETERNAME(J,PNAME)
    WRITE(3 ,"(1X,A,G14.4)") PNAME, P(J)
  ENDDO
END IF
END IF
END IF
!
=====
=====

RETURN
ENTRY MODELID(ID) !***** N O N OPTIONAL DEFINITION
SECTION *****
ID = 'HB_PROD_V5.14' ! RECORD THE ID FOR THE MODEL USED IN THE
FITTING :
RETURN
END SUBROUTINE USERMODEL
!-----

! EXTERNAL FUNCTION THAT DETERMINES THE PRODUCTION RATE OF
HEMOGLOBIN BY CE
REAL*8 FUNCTION HBSTIM (C, P)
IMPLICIT NONE
REAL*8, INTENT (IN) :: C, P(*)

```

```

HBSTIM = ((P(2))*(C**(P(3))))/(((P(1))**(P(3))) + (C**(P(3))))
END FUNCTION HBSTIM
! END OF FILE ---

```

E.2.2 EVENT STRUCTURE MODULE.F90

```

!=====
MODULE EVENTSTRUCTURE ! MODULE FOR EVENT STRUCTURE (TYPE)
SPECIFICATION
SAVE

TYPE EVENTTYPE
REAL*8      :: START = HUGE(0D0)      !* START OF EVENT
EQUIVALENT TO START OF CONDITION
REAL*8      :: STOP  = HUGE(0D0)      !* STOP OF EVENT
EQUIVALENT TO STOP OF CONDITION
! NOT START AND STOP MAY BE THE
SAME (E.G. BOLUS INPUT)

REAL*8      :: VALUE = 0D0            !* REAL VALUE OF EVENT. E.G.
MG

LOGICAL      :: ZERO_TO_ONE_TRANSFORM = .FALSE. !* APPLIES
ONLY TO "BOLUS EVENT" CASES WHERE START = STOP
! IN THESE CASES THE VARIABLE
REFERRED TO BY "LINK_TO_VARIABLE"
! IS TRANSFORMED ACCORDING TO:
! NEW VALUE = OLD VALUE *
ABS(VALUE)/(1 + ABS(VALUE))

REAL*8      :: START_VALUE= HUGE(0D0)      !* APPLIES ONLY TO
"BOLUS EVENT" CASES WHERE START = STOP.
! START_VALUE IS THE CALCULATED
VALUE OF THE VARIABLE REFERRED TO BY
! "LINK_TO_VARIABLE" BEFORE THE
PERTUBATION (VALUE) IS APPLIED.

REAL*8      :: STOP_VALUE = HUGE(0D0)      !* APPLIES ONLY TO
"BOLUS EVENT" CASES WHERE START = STOP
! STOP_VALUE IS THE CALCULATED
VALUE OF THE VARIABLE REFERRED TO BY
! "LINK_TO_VARIABLE" AFTER THE
PERTUBATION (VALUE) IS APPLIED.

INTEGER      :: LINK_TO_VARIABLE = 0      !* INTEGER VALUE
POINTING TO THE Y-VARIABLE THAT IS PERTURBED

```

```

! BY EVENT.VALUE.
!
! THERE ARE TWO TYPES OF EVENTS:
!
! 1. "BOLUS EVENTS" (EVENT.START =
EVENT.STOP)
!
! THE VARIABLE REFERRED TO BY
"LINK_TO_VARIABLE" IS SUBJECT
! TO A DISCONTINUOUS CHANGE (INCREASE
OR DECREASE)
! IMMEDIATELY AFTER EVENT.START:
!
!     NEW VALUES = OLD VALUE +
VALUE
!
! ,WHERE VALUE MAY BE BEPOSETIVE OR
NEGATIVE.
! ALTERNATIVELY, IF
EVENT.ZERO_TO_ONE_TRANSFORM = .TRUE.
! WE HAVE:
!
!     NEW VALUE = OLD VALUE *
ABS(VALUE)/(1 + ABS(VALUE))
!
! 2. "NON BOLUS EVENTS" (EVENT.START <
EVENT.STOP)
!
!     NEW VALUES = OLD VALUE +
VALUE
!
! ,WHERE VALUE MAY BE BEPOSETIVE OR
NEGATIVE, IS APPLIED TO
! THE VARIABLE REFERRED TO BY
"LINK_TO_VARIABLE"

    CHARACTER (len=3) :: STATUS = "  "      !* "ON" OR "OFF". REFERS
TO THE STATUS OF THE EVENT CONDITION
! THAT IS TURNED "ON" AT "START" AND
"OFF" AT "STOP"

    CHARACTER (len=79) :: NAME = ""        !* NAME OF EVENT, E.G.
"INFUSION", "OUT OF HOSPITAL"

    CHARACTER (len=15) :: START_STOP_UNITS = ""    !* E.G.
(MILLI)SECONDS, MINUTES, HOURS, DAYS, YEARS

```

```

CHARACTER (len=15) :: VALUE_UNITS = ""           !* E.G. MG,
MICROGRAM, MICROGRAM/HOURS, NO UNITS
END TYPE EVENTTYPE

```

```

END MODULE EVENTSTRUCTURE

```

```

!=====

```

E.2.3 SUBROUTINE CALC POLY TERMS.F90

```

SUBROUTINE CALC_POLY_TERMS ( BMA1, BMA2, T1, T2, P1, P2, ALPHA)

```

```

!PURPOSE:

```

```

! TO CALCULATE THE COEFFICIENTS OF A 5TH ORDER POLYNOMIAL, X(T),
GIVEN THE PARAMTERS P1 AND P2 AND THE CONSTRAINTS THAT

```

```

! X(T1)=BMA1, X(T2)=BMA2, XPRIME(T1)=0, AND XPRIME(T2)=0.

```

```

!

```

```

!

```

```

! RECORDE OF REVISIONS

```

```

! DATE      PROGRAMER      DESCRIPTION OF CHANGE

```

```

! =====

```

```

! 14JUL06   K. FREISE      ORIGINAL CODE.

```

```

!

```

```

IMPLICIT NONE

```

```

REAL*8, INTENT (IN) :: BMA1, BMA2, T1, T2, P1, P2

```

```

REAL*8, DIMENSION(0:5), INTENT (OUT) :: ALPHA

```

```

REAL*8 :: ONE=1D0, TWO=2D0, THREE=3D0, FOUR=4D0, FIVE=5D0

```

```

INTEGER :: i

```

```

ALPHA(5) = P2

```

```

ALPHA(4) = P1

```

```

ALPHA(3) = (ONE/(((THREE*T2**TWO - ((T2**THREE - T1**THREE)/(T2 - T1)) /
(TWO*T2 - ((T2**TWO - T1**TWO)/(T2 - T1)))) - ((THREE*(T1**TWO -
T2**TWO))/(TWO*(T1 - T2)))))) * &

```

```

( (((BMA1 - BMA2)/(T2 - T1))/(TWO*T2 - ((T2**TWO - T1**TWO)/(T2 -
T1)))) &

```

```

- ALPHA(4)*(((FOUR*T2**THREE - ((T2**FOUR - T1**FOUR)/(T2 -
T1)))/(TWO*T2 - ((T2**TWO - T1**TWO)/(T2 - T1))) - ((FOUR*(T1**THREE -
T2**THREE))/(TWO*(T1 - T2)))) &

```

```

- ALPHA(5)*(((FIVE*T2**FOUR - ((T2**FIVE - T1**FIVE)/(T2 -
T1)))/(TWO*T2 - ((T2**TWO - T1**TWO)/(T2 - T1))) - ((FIVE*(T1**FOUR -
T2**FOUR ))/(TWO*(T1 - T2)))) )

```

```

ALPHA(2) = (-ONE/(TWO*(T1 - T2)))*((THREE*ALPHA(3)*(T1**TWO -
T2**TWO)) + (FOUR*ALPHA(4)*(T1**THREE - T2**THREE)) +
(FIVE*ALPHA(5)*(T1**FOUR - T2**FOUR)) )
ALPHA(1) = (ONE/(T2 - T1))*(BMA2 - BMA1 - ALPHA(2)*(T2**TWO - T1**TWO)
-ALPHA(3)*(T2**THREE - T1**THREE) - ALPHA(4)*(T2**FOUR - T1**FOUR) -
ALPHA(5)*(T2**FIVE - T1**FIVE))
ALPHA(0) = BMA1 - ALPHA(1)*T1 - ALPHA(2)*T1**TWO -
ALPHA(3)*T1**THREE - ALPHA(4)*T1**FOUR - ALPHA(5)*T1**FIVE

END SUBROUTINE CALC_POLY_TERMS

```

E.2.4 SUB..._SET_EVENT_START_AND_STOP.F90

```

SUBROUTINE SET_EVENT_START_AND_STOP(NUMBER, START_VAL,
STOP_VAL)

```

!Purpose:

! To simultaneously set event start and stop values. This subroutine is useful to use when the value of start and

! stop events are both changing with each iteration, however, this subroutine can be used when both are fixed.

!

! NOTE: When one or both values are fixed it is computationally more efficient to just set both event values

! directly with the SET_EVENT_START and SET_EVENT_STOP subroutines.

!

!

! Record of revisions:

! Date	! Programmer	! Description of change
! =====	! =====	! =====
! 23FEB06	! K. Freise	! Original code.

!

IMPLICIT NONE

INTEGER, INTENT (IN) :: NUMBER !Event number start and stop values are associated with

REAL*8, INTENT (IN) :: START_VAL !Event start value

REAL*8, INTENT (IN) :: STOP_VAL !Event stop value

REAL*8 :: OLD_STOP_VAL=0 !Previous event stop value

!Get previous event stop value

CALL GET_EVENT_STOP_VALUE (NUMBER, OLD_STOP_VAL)

!Determine if the new event start value is greater than the previous event stop value. Then set the new event start and

!stop values in the appropriate order.

```
IF (START_VAL > OLD_STOP_VAL) THEN
  CALL SET_EVENT_STOP (NUMBER, STOP_VAL)
  CALL SET_EVENT_START (NUMBER, START_VAL)
ELSE
  CALL SET_EVENT_START (NUMBER, START_VAL)
  CALL SET_EVENT_STOP (NUMBER, STOP_VAL)
END IF
```

END SUBROUTINE SET_EVENT_START_AND_STOP

E.3 Fortran subroutines for Chapter 3.

E.3.1 TIME_VAR_DIST_V2.14.F90

```
! FILENAME = TIME_VAR_DIST_V2.14.F90
!
! PURPOSE: ANALYSIS HEMOGLOBIN PRODUCTION RATE AND
! RETIULCOCYTE RESIDENCE TIME RELATIVE TO EPO CONCENTRATIONS
! POST-PHLEBOTOMY
!
! MODEL: EPO CONCENTRATIONS MODELED USING A GCV CUBIC SPLINE
! FIT AND STIMULATES HB PRODUCTION USING AN EFFECT COMPARTMENT
! MODEL. EFFECT COMPARTMENT CONCENTRATION IS LINKED TO HB
! STIMULATION USING AN EMAX MODEL. HB APPEARS IN THE
! BLOOD "A" DAYS AFTER STIMULATION AND DISAPPEARS "D" DAYS
! AFTER STIMULATION. RETICULOCYTES
! APPEAR IN THE BLOOD "A" DAYS AFTER STIMULATION AND
! DISAPPEAR FROM THE BLOOD "B" DAYS AFTER STIMULATION. "A" IS
! MODELED BY A WEIBULL DISTRIBUTION AND IS TIME-VARIANT.
!
!
!
! NOTES
! IT IS ASSUMED THAT:
! 1. EPO CONCENTRATIONS ARE IN MILLIUNITS/MILLILITER
! 2. RBCS ARE IN 1000 CELLS/MICROLITER
! 3. RETICULOCYTES ARE IN 1000 CELLS/MICROLITER
! 4. TIME IS GIVEN IN DAYS
!
! CHANGES/REVISIONS:
! VERSION 1.0 MAY 22, 2007 MODIFIED FROM 'HB_PROD_V5.14.F90.' THE
! DIFFERENTIAL EQUATIONS TO CALCULATE THE EFFECT-SITE EPO CONC.
! WERE REPLACED BY AN ANALYTICAL SOLUTION.
```

! VERSION 1.1 MAY 23, 2007 DIFFERENTIAL EQUATIONS WERE REMOVED AND REPLACED BY DIRECT NUMERICAL INTEGRATION OF THE INTEGRAL

! FORM OF THE MODEL FOR THE HEMOGLOBIN AND RETICULOCYTES. HOWEVER, THE DIFFERENTIAL EQUATION FOR Z(T) WAS KEPT TO DEFINE THE UPPER BOUND IN THE NUMERICAL INTEGRATION OF THE MODEL (I.E. TO AVOID SINGULARITIES/DISCONTINUITIES).

! VERSION 1.2 MAY 25, 2007 REMOVED ALL DIFFERENTIAL EQUATIONS AND CHANGED FROM A SINGLE RELEASE AGES (AT A GIVEN POINT IN TIME)

! TO A DISTRIBUTION OF RELEASE AGES OF RETICULOCYTES. THE RELEASE AGES WERE MODELED BY A WEIBULL DISTRIBUTION.

! VERSION 2.0 MAY 31, 2007 REWROTE CODE FOR THE DOUBLE PHLEBOTOMY EXPERIMENTS MEASURING H, M, AND L RETICULOCYTES (ABSOLUTE

! COUNTS) AND RBCS, AND ALSO ACCOUNTING FOR BLOOD VOLUME EXPANSION DUE TO GROWTH. TIME VARIANCE OF THE DISTRIBUTION OF RELEASE AGES OF RETICULOCYTES WAS REMOVED.

! VERSION 2.1 JUNE 6, 2007 ADDED TIME VARIANCE OF THE DISTRIBUTION OF RELEASE AGES OF RETICULOCYTES. TIME VARIANCE OF THE

! LAMBDA (SCALE) PARAMETER OF THE WEIBULL DISTRIBUTION WAS SEMIPARAMETRICALLY MODELED AS A QUADRATIC SPLINE FUNCTION WITH NODES AT THE PHLEBOTOMIES, PLUS 15 DAYS AFTER THE SECOND PHLEBOTOMY.

! VERSION 2.2 JUNE 7, 2007 MODIFIED MODELING THE TIME VARIANCE OF THE LAMBDA (SCALE) PARAMETER OF THE WEIBULL DISTRIBUTION TO EXPONENTIATED 5TH ORDER SPLINE FUNCTION

! VERSION 2.3 JUNE 8, 2007 MODIFIED TO ONLY FIT TO TWO RETICULOCYTE FRACTIONS, THE MATURE (L) AND IMMATURE (H+M) RETICULOCYTES,

! DUE TO THE VERY LOW H AND M COUNTS AND DIFFICULTY IN GETTING THE CORRECT SHAPE OF THE DISTRIBUTION WITH THE TIME VARIANCE. ALSO CHANGED UNITS FOR RBC FROM 10^6 CELLS/UL TO 10^3 CELLS/UL.

! VERSION 2.4 JUNE 8, 2007 MODIFIED FROM A WEIBULL DISTRIBUTION TO A SHIFTED AND SCALED KUMARSWAMY DISTRIBUTION WITH THE TIME VARIANCE ONE OF THE DISTRIBUTION PARAMETERS (I.E. THE SECOND SHAPE PARAMETER OR THE LOWER SUPPORT POINT OF THE DISTRIBUTION).

! VERSION 2.5 JUNE 11, 2007 CHANGED TIME VARIANCE TO TWO CONTINUOUS SPLINES THAT AFFECT THE LOWER SUPPORT POINT

```

!           OF THE KUMARASWAMY DISTRIBUTION.
!  VERSION 2.6 JUNE 12, 2007 CHANGED BACK TO A TIME VARIANT 3-
PARAMETER WEIBULL DISTRIBUTION WITH A TIME VARIANT LOCATION
!           PARAMETER (THETA) AND MODIFIED THE EXTERNAL
FUNCTIONS TO ONLY HAVE A SINGLE FCONTRIB FUNCTION.
!  VERSION 2.7 JUNE 20, 2007 CHANGED BACK TO SHIFTED AND SCALED
KUMARASWAMY DISTRIBUTION WITH AN EPO STIMULATED
ACCELERATION
!           OF THE PROBABILITY OF RELEASE OF THE CELL.
!  VERSION 2.8 JUNE 20, 2007 REMOVED STIMULATED ACCELERATION
OF CELL AND WENT TO TIME VARIANCE IN THE SECOND SHAPE
!  VERSION 2.9 JUNE 25, 2007 CHANGED TIME VARIANCE FROM THE
SHAPE PARAMETER TO THE LOCATION PARAMETER OF THE WEIBULL
DISTRIBUTION
!           AND CHANGED FROM FITTING TWO GROUPS OF
RETICULOCYTES TO JUST THE TOTAL RETICULOCYTE COUNT
!  VERSION 2.10 JUNE 27, 2007 MODIFIED TO ESTIMATE THE BLOOD
VOLUME FROM THE NUMBER OF RBC REMOVED DURING THE
PHLEBOTOMY
!  VERSION 2.14 JULY 26, 2007 CHANGED FROM A LINEAR TO A
QUADRATIC SPLINE TO MODEL THE TIME VARIANCE
!
!
!

```

```

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

```

```

! MODEL STATEMENTS
SUBROUTINE USERMODEL(T,Y,P,NP,IFUN)
  USE SHARED_DATA
  USE NUMERICAL_LIBRARIES
  USE LS_LINEAR_FITTING
  IMPLICIT NONE
  INTEGER                :: NP, IFUN, NUM
  REAL*8, PARAMETER      :: INTERVAL=0.1, ABSERR = 0D0, RELERR =
0.001 ! ADJUST APPROX. RELATIVE ERROR OF THE NUMERICAL
INTEGRATION
  REAL*8                 :: T, Y, P(*)

  INTEGER,PARAMETER      :: NEQN = 1, LENSTRING = 180, NPS = 500
  INTEGER                :: NOBS, J, LUN, NSIGDIGITS, NOBSW, I, L, TEMPN
  REAL*8                 :: B, DMA, E_MAX, EC_50, N, KE0, HBDROP, F1, F2,
THETA, SLOPE, INTERCEPT, &

```

TPHLEB1, TPHLEB2, TOBS(MAXNODES),
 COBS(MAXNODES), DT, TC(NPS), CC(NPS), ECC(NPS), &
 LB, UB, MP1, MP2, Y1, Y2, Y3, Y4, Y5, D, ESTERR, WT,
 VOL, K, LAMBDA, &
 TOBSW(MAXNODES), COBSW(MAXNODES), A0, V,
 CV_VALUE = 15D0, P1, P2, TMCL(NPS), MCL(NPS), &
 FCONTRIB, MEANRES(NPS), TIMEVAR(NPS),
 PRODRATE(NPS), PHLEB1, PHLEB2, TEMPX(NPS), TEMPY(NPS), &
 T1, T1ADD, T2ADD, T3ADD, T4ADD, T5ADD, T6ADD,
 T7ADD, NPP1, NPP2, THETA_S, PRR(NPS), &
 BETAS1, BETAS2, BETAS3, BETAS4, BETAS5, BETAS6,
 BETAS7, LAST_TIME, TMAX, RBCMAX, RETMAX, EPOMAX

LOGICAL, SAVE :: SHOWIT, PLOTSAVED
 CHARACTER (LEN=256) :: ID, DATAFILENAME
 CHARACTER (LEN=20) :: PNAME, REPLY
 CHARACTER (LEN=LENSTRING) :: STRING
 CHARACTER (LEN=1) :: RESPONSE
 EXTERNAL FCONTRIB

DMA = P(1) ! BASELINE HB BLOOD RESIDENCE TIME (D - 'THE
 MEAN "A" TIME 0')
 EC_50 = P(2) ! PARAMETER USED TO DEFINE THE ACTIVATION RATE
 OF RED CELLS BY EPO (SIGMOID EMAX MODEL)
 E_MAX = P(3) ! MAXIMUM RED CELL ACTIVATION RATE BY EPO
 (SIGMOID EMAX MODEL) BY KG OF BW
 N = P(4) ! SIGMOIDICITY PARAMETER TO DEFINE THE
 ACTIVATION RATE OF RED CELLS BY EPO (SIGMOID EMAX MODEL)
 KE0 = P(5) ! RATE CONSTANT FROM EFFECT COMPARTMENT
 PHLEB1 = P(6) ! NUMBER OF RBCS REMOVED FROM PHLEBOTOMY 1
 PHLEB2 = P(7) ! NUMBER OF RBCS REMOVED FROM PHLEBOTOMY 2
 TPHLEB1 = P(8) ! TIME OF 1ST PHLEBOTOMY (MEASURED ON SAME
 TIME SCALE AS TFIRST = 1ST OBSERVATION TIME)
 TPHLEB2 = P(9) ! TIME OF 2ND PHLEBOTOMY (MEASURED ON SAME
 TIME SCALE AS TFIRST = 1ST OBSERVATION TIME)
 V = P(10) ! BLOOD VOLUME PER KG OF BW
 THETA = P(11) ! LOCATION PARAMETER OF WEIBULL DISTRIBUTION
 LAMBDA = P(12) ! SCALE PARAMETER OF WEIBULL DISTRIBUTION
 K = P(13) ! SHAPE PARAMETER OF WEIBULL DISTRIBUTION
 B = P(14) ! CUTOFF AGE BETWEEN RETICULOCYTES AND MATURE
 RBCS
 P1 = P(15) ! UNUSED PARAMETER
 P2 = P(16) ! UNUSED PARAMETER
 T1 = P(17) ! TIME OF NODE1 OF THE SPLINE FUNCTION
 T1ADD = P(18)
 T2ADD = P(19)

T3ADD = P(20)
 T4ADD = P(21)
 T5ADD = P(22)
 T6ADD = P(23)
 T7ADD = P(24)
 BETAS1 = P(25)
 BETAS2 = P(26)
 BETAS3 = P(27)
 BETAS4 = P(28)
 BETAS5 = P(29)
 BETAS6 = P(30)
 BETAS7 = P(31)
 LAST_TIME = P(32)

! START OF PRELIMINARY PREPARATION SECTION =====

IF (IFUN == - 1000) THEN

IF (NP /= 32) STOP ' USERMODEL: INCONSISTENT NUMBER OF
PARAMETERS IN PARAMETER FILE'

CALL SETFUNFITPARAMETERNAME(1, " D - A (DAYS)")
 CALL SETFUNFITPARAMETERNAME(2, " EC50 (MU/ML)")
 CALL SETFUNFITPARAMETERNAME(3, " EMAX (CELLS/D/KG)")
 CALL SETFUNFITPARAMETERNAME(4, " N")
 CALL SETFUNFITPARAMETERNAME(5, " KE0 (1/DAY)")
 CALL SETFUNFITPARAMETERNAME(6, " PHLEB1 (# RBCS)")
 CALL SETFUNFITPARAMETERNAME(7, " PHLEB2 (# RBCS)")
 CALL SETFUNFITPARAMETERNAME(8, " TPHLEB1 (DAYS)")
 CALL SETFUNFITPARAMETERNAME(9, " TPHLEB2 (DAYS)")
 CALL SETFUNFITPARAMETERNAME(10, " VOLUME (ML/KG)")
 CALL SETFUNFITPARAMETERNAME(11, " THETA (DAYS)")
 CALL SETFUNFITPARAMETERNAME(12, " LAMBDA")
 CALL SETFUNFITPARAMETERNAME(13, " K")
 CALL SETFUNFITPARAMETERNAME(14, " B (DAYS)")
 CALL SETFUNFITPARAMETERNAME(15, " UNUSED PARAMETER")
 CALL SETFUNFITPARAMETERNAME(16, " UNUSED PARAMETER")
 CALL SETFUNFITPARAMETERNAME(17, " T1 (DAYS)")
 CALL SETFUNFITPARAMETERNAME(18, " T1ADD (DAYS)")
 CALL SETFUNFITPARAMETERNAME(19, " T2ADD (DAYS)")
 CALL SETFUNFITPARAMETERNAME(20, " T3ADD (DAYS)")
 CALL SETFUNFITPARAMETERNAME(21, " T4ADD (DAYS)")
 CALL SETFUNFITPARAMETERNAME(22, " T5ADD (DAYS)")
 CALL SETFUNFITPARAMETERNAME(23, " T6ADD (DAYS)")
 CALL SETFUNFITPARAMETERNAME(24, " T7ADD (DAYS)")
 CALL SETFUNFITPARAMETERNAME(25, " BETAS1")
 CALL SETFUNFITPARAMETERNAME(26, " BETAS2")
 CALL SETFUNFITPARAMETERNAME(27, " BETAS3")

```

CALL SETFUNFITPARAMETERNAME(28, " BETAS4")
CALL SETFUNFITPARAMETERNAME(29, " BETAS5")
CALL SETFUNFITPARAMETERNAME(30, " BETAS6")
CALL SETFUNFITPARAMETERNAME(31, " BETAS7")
CALL SETFUNFITPARAMETERNAME(32, " LAST TIME")

! GET EPO DATA
NOBS = MAXNODES
PRINT*
PRINT*,' EPO CONCENTRATION DATA:'
CALL GET_XY_DATA_FROM_FUNFIT_FILE (TOBS,COBS,NOBS)
IF ( NOBS > MAXNODES ) STOP ' TOO MANY EPO DATA POINTS. ADJUST
THE MAXNODES APPROPRIATELY'

! GET WEIGHT DATA
NOBSW = MAXNODES
PRINT*,' BODY WEIGHT DATA:'
CALL GET_XY_DATA_FROM_FUNFIT_FILE (TOBSW,COBSW,NOBSW)
IF ( NOBSW > MAXNODES ) STOP ' TOO MANY WEIGHT DATA POINTS.
ADJUST THE MAXNODES APPROPRIATELY'

PRINT*
PRINT*,' IT IS ASSUMED THAT:'
PRINT*,' 1. EPO CONCENTRATIONS ARE IN MILLIUNITS/MILLILITER'
PRINT*,' 2. RETICULOCYTE COUNTS ARE IN  $1*10^3$ 
CELLS/MICROLITER'
PRINT*,' 3. RBC ARE IN  $1*10^3$  CELLS/MICROLITER'
PRINT*,' 4. TIME IS GIVEN IN DAYS AND THE FIRST OBSERVATION
IS TIME 0'
PRINT*
PRINT*
READ*

PRINT*,' WOULD YOU LIKE TO SET THE CV VALUE FOR THE CUBIC GCV
FIT?'
READ (*,*) RESPONSE
PRINT*
IF ( RESPONSE == 'Y' .OR. RESPONSE == 'y' ) THEN
PRINT*,' PLEASE ENTER THE CV VALUE FOR THE CUBIC GCV FIT:'
READ(*,*) CV_VALUE
CALL SET_CV_VALUE_FOR_SPLINE_FIT(CV_VALUE)
PRINT*
END IF

! FIT TO EPO DATA
CALL CUBIC_GCV_FIT(TOBS, COBS(1:NOBS), NOBS)

```

```

CALL SET_CUBIC_GCV_CONSTANT_EXTRAPLOATION

! FIT TO WEIGHT DATA (NOTE LOG TRANSFORMATION - IMPLICITY FITTING
AN EXPONENTIAL REGRESSION MODEL)
  CALL CALC_LS_COEF(TOBSW, DLOG(COBSW(1:NOBSW)), NOBSW)

!   CALL GET_LS_COEF(SLOPE, INTERCEPT)
!   WRITE(*,"(1X,'SLOPE:',1X,G18.8)") SLOPE
!   WRITE(*,"(1X,'INTERCEPT:',1X,G18.8)") INTERCEPT

! GET COEFFICIENTS/NODES OF POLYNOMIAL SPLINE FIT TO EPO DATA AND
STORE IN A SHARED ARRAY
  TK(1:NOBS) = TOBS(1:NOBS)
  NS = NOBS - 1
  DO J = 1, NS
    CALL
CUBIC_GCV_COEFFICIENTS(TK(J),COEF(1,J),COEF(2,J),COEF(3,J),COEF(4,J))
  END DO

! CALCULATE SHARED CONSTANTS
  TBLEED1 = TPHLEB1
  TBLEED2 = TPHLEB2

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

! UPDATE SHARED/SECONDARY PARAMETERS ON EACH ITERATION

  A0 = (LAMBDA*(DGAMMA(1 + 1/K))) + THETA
  D = DMA + A0
  SHAPE = K
  SCALE = LAMBDA

  A(NEXP) = KE0
  ALPHA(NEXP) = KE0
  EC50 = EC_50
  EMAX = E_MAX
  SF = N
  TVAL = T

! UPDATE COEFFICIENTS AND NODES OF SPLINE SEGEMENTS
  TIMES = (/0D0, T1, T1+T1ADD, T1+T1ADD+T2ADD,
T1+T1ADD+T2ADD+T3ADD, T1+T1ADD+T2ADD+T3ADD+T4ADD, &
  T1+T1ADD+T2ADD+T3ADD+T4ADD+T5ADD,
T1+T1ADD+T2ADD+T3ADD+T4ADD+T5ADD+T6ADD, &

```

T1+T1ADD+T2ADD+T3ADD+T4ADD+T5ADD+T6ADD+T7ADD,
MAXVAL/)

BETAS(0,2) = 0D0

DO J = 1,7

BETAS(J,2) = P(J+24)

END DO

BETAS(0,8) = 0D0

BETAS(0,1) = THETA

BETAS(0,3) = (BETAS(0+1,2) - BETAS(0,2))/(2D0*(TIMES(0+1)-TIMES(0)))

DO J=1,7

BETAS(J,1) = BETAS(J-1,1) + BETAS(J-1,2)*(TIMES(J)-TIMES(J-1)) +
BETAS(J-1,3)*((TIMES(J)-TIMES(J-1))**2)

BETAS(J,3) = (BETAS(J+1,2) - BETAS(J,2))/(2D0*(TIMES(J+1)-TIMES(J)))

END DO

BETAS(8,1) = BETAS(8-1,1) + BETAS(8-1,2)*(TIMES(8)-TIMES(8-1)) +
BETAS(8-1,3)*((TIMES(8)-TIMES(8-1))**2)

BETAS(8,3) = 0D0

! DETERMINE NUMBER OF RBCS IMMEDIATELY PRIOR TO THE
PHLEBOTOMITES IN ORDER TO ESTIMATE
! THE FRACTION OF CELLS REMAINING AFTER EACH PHLEBOTOMY (F1
AND F2)

! PHLEBOTOMY 1

TVAL = TPHLEB1

LB = TPHLEB1 - D

UB = TPHLEB1

CLASS = 1

CALL DQDAGS(FCONTRIB, LB, UB, ABSERR, RELERR, NPP1, ESTERR)

F1 = 1D0 - (PHLEB1/NPP1)

! PHLEBOTOMY 2

TVAL = TPHLEB2

LB = TPHLEB2 - D

UB = TPHLEB2

MP1 = TPHLEB1

CLASS = 2

CALL DQDAGS(FCONTRIB, LB, MP1, ABSERR, RELERR, Y1, ESTERR)

CLASS = 3

CALL DQDAGS(FCONTRIB, LB, MP1, ABSERR, RELERR, Y2, ESTERR)

CLASS = 1

CALL DQDAGS(FCONTRIB, MP1, UB, ABSERR, RELERR, Y3, ESTERR)

NPP2 = F1*Y1 + Y2 + Y3

F2 = 1D0 - (PHLEB2/NPP2)

```

TVAL = T

! WRITE(*,"(1X,'TIME:',1X,G14.4)") T
! WRITE(*,"(1X,'FRACTION 1:',1X,G14.4)") F1
! WRITE(*,"(1X,'FRACTION 2:',1X,G14.4)") F2
! READ(*,*)

! MODEL FITTING
IF (IFUN == 1 .OR. IFUN == 2) THEN
  CALL LS_FITTED_VAL(T, WT)
  VOL = (DEXP(WT))*V*1000
  IF ( IFUN == 1 ) THEN
    LB = T - D
    UB = T
    IF ( (UB <= TPHLEB1) .OR. (LB > TPHLEB2) .OR. ((LB > TPHLEB1) .AND.
(CORRECTION NEEDED)
    CLASS = 1
    CALL DQDAGS(FCONTRIB, LB, UB, ABSERR, RELERR, Y1, ESTERR)
    Y = Y1/VOL
    ELSE IF ( (LB <= TPHLEB1) .AND. (UB <= TPHLEB2) ) THEN
SCENARIO 2 (CORRECTION FOR 1ST PHLEBOTOMY NEEDED)
    MP1 = TPHLEB1
    CLASS = 2
    CALL DQDAGS(FCONTRIB, LB, MP1, ABSERR, RELERR, Y1, ESTERR)
    CLASS = 3
    CALL DQDAGS(FCONTRIB, LB, MP1, ABSERR, RELERR, Y2, ESTERR)
    CLASS = 1
    CALL DQDAGS(FCONTRIB, MP1, UB, ABSERR, RELERR, Y3, ESTERR)
    Y = (F1*Y1 + Y2 + Y3)/VOL
    ELSE IF ( (LB > TPHLEB1) ) THEN
SCENARIO 3
(CORRECTION FOR 2ND PHLEBOTOMY NEEDED)
    MP1 = TPHLEB2
    CLASS = 4
    CALL DQDAGS(FCONTRIB, LB, MP1, ABSERR, RELERR, Y1, ESTERR)
    CLASS = 5
    CALL DQDAGS(FCONTRIB, LB, MP1, ABSERR, RELERR, Y2, ESTERR)
    CLASS = 1
    CALL DQDAGS(FCONTRIB, MP1, UB, ABSERR, RELERR, Y3, ESTERR)
    Y = (F2*Y1 + Y2 + Y3)/VOL
    ELSE
SCENARIO 4 (CORRECTION FOR
1ST AND 2ND PHLEBOTOMY NEEDED)
    MP1 = TPHLEB1
    MP2 = TPHLEB2

```

```

CLASS = 2
CALL DQDAGS(FCONTRIB, LB, MP1, ABSERR, RELERR, Y1, ESTERR)
CLASS = 3
CALL DQDAGS(FCONTRIB, LB, MP1, ABSERR, RELERR, Y2, ESTERR)
CLASS = 4
CALL DQDAGS(FCONTRIB, MP1, MP2, ABSERR, RELERR, Y3,
ESTERR)
CLASS = 5
CALL DQDAGS(FCONTRIB, MP1, MP2, ABSERR, RELERR, Y4,
ESTERR)
CLASS = 1
CALL DQDAGS(FCONTRIB, MP2, UB, ABSERR, RELERR, Y5, ESTERR)
Y = (F1*F2*Y1 + F2*Y2 + F2*Y3 + Y4 + Y5)/VOL
ENDIF
ELSE                                     ! *** RETICULOCYTES ***
LB = T - B
UB = T
IF ( (UB <= TPHLEB1) .OR. (LB > TPHLEB2) .OR. ((LB > TPHLEB1) .AND.
(UB <= TPHLEB2))) THEN
! SCENARIO 1 (NO PHLEBOTOMY
CORRECTION NEEDED)
CLASS = 1
CALL DQDAGS(FCONTRIB, LB, UB, ABSERR, RELERR, Y1, ESTERR)
Y = Y1/VOL
ELSE IF ( (LB <= TPHLEB1) .AND. (UB <= TPHLEB2) ) THEN      !
SCENARIO 2 (CORRECTION FOR 1ST PHLEBOTOMY NEEDED)
MP1 = TPHLEB1
CLASS = 2
CALL DQDAGS(FCONTRIB, LB, MP1, ABSERR, RELERR, Y1, ESTERR)
CLASS = 3
CALL DQDAGS(FCONTRIB, LB, MP1, ABSERR, RELERR, Y2, ESTERR)
CLASS = 1
CALL DQDAGS(FCONTRIB, MP1, UB, ABSERR, RELERR, Y3, ESTERR)
Y = (F1*Y1 + Y2 + Y3)/VOL
ELSE IF ( (LB > TPHLEB1) ) THEN                                ! SCENARIO 3
(CORRECTION FOR 2ND PHLEBOTOMY NEEDED)
MP1 = TPHLEB2
CLASS = 4
CALL DQDAGS(FCONTRIB, LB, MP1, ABSERR, RELERR, Y1, ESTERR)
CLASS = 5
CALL DQDAGS(FCONTRIB, LB, MP1, ABSERR, RELERR, Y2, ESTERR)
CLASS = 1
CALL DQDAGS(FCONTRIB, MP1, UB, ABSERR, RELERR, Y3, ESTERR)
Y = (F2*Y1 + Y2 + Y3)/VOL
ELSE                                     ! SCENARIO 4 (CORRECTION FOR
1ST AND 2ND PHLEBOTOMY NEEDED)

```

```

    MP1 = TPHLEB1
    MP2 = TPHLEB2
    CLASS = 2
    CALL DQDAGS(FCONTRIB, LB, MP1, ABSERR, RELERR, Y1, ESTERR)
    CLASS = 3
    CALL DQDAGS(FCONTRIB, LB, MP1, ABSERR, RELERR, Y2, ESTERR)
    CLASS = 4
    CALL DQDAGS(FCONTRIB, MP1, MP2, ABSERR, RELERR, Y3,
ESTERR)
    CLASS = 5
    CALL DQDAGS(FCONTRIB, MP1, MP2, ABSERR, RELERR, Y4,
ESTERR)
    CLASS = 1
    CALL DQDAGS(FCONTRIB, MP2, UB, ABSERR, RELERR, Y5, ESTERR)
    Y = (F1*F2*Y1 + F2*Y2 + F2*Y3 + Y4 + Y5)/VOL
    ENDIF
  ENDF
END IF

! USER OUTPUT SECTION
IF (IFUN == 0) THEN
  DO J = 1, NP
    CALL GETFUNFITPARAMETERNAME (J, PNAME)
    WRITE(*, "(1X, I2, 1X, A, G14.4)") J, PNAME, P(J)
  ENDDO
  CALL PROMT(SHOWIT)
  IF (SHOWIT) THEN
    DO J = 1, NP
      CALL GETFUNFITPARAMETERNAME (J, PNAME)
      WRITE(LUN, "(' PAR', I2, 1X, A, G14.4)") J, PNAME, P(J)
    ENDDO
  ! CREATE DATA FILE FOR PLOTTING FITTED EPO CONCENTRATIONS
  DT = (LAST_TIME - TOBS(1))/(NPS-1)
  DO J = 1, NPS
    TC(J) = TOBS(1) + DT*(J-1)
    CALL CUBIC_GCV(TC(J), CC(J))
  END DO
  ! CREATE DATA FILE FOR PLOTTING EFFECT SITE CONCENTRATIONS
  DO J = 1, NPS
    CALL CONV_POLYSP_POLYEXP(COEF, NCOEF, NS, TOBS, KE0, KE0,
1, TC(J), ECC(J))
  END DO
  ! CREATE DATA FILE FOR PLOTTING TIME VARIANT PARAMETER OF
  WEIBULL DISTRIBUTION
  DO J = 1, NPS
    DO L=0,8

```

```

        I = L
        IF ( TIMES(I+1) > TC(J) ) EXIT
    END DO
    TIMEVAR(J) = BETAS(I,1) + BETAS(I,2)*(TC(J) - TIMES(I)) +
BETAS(I,3)*((TC(J) - TIMES(I))**2)
    TIMEVAR(J) = MAX(TIMEVAR(J), 0D0)
    END DO
! CREATE DATA FILE FOR PLOTTING PRODUCTION RATE (NORMALIZED
FOR BODYWEIGHT)
    DO J = 1, NPS
        PRODRATE(J) = ((EMAX*(ECC(J)**SF))/((EC50**SF) + (ECC(J)**SF)))
    END DO
! CREATE DATA FILE FOR MEAN CIRUCLATING LIFESPAN AND
PROPORTION RELEASED AS RETICULOCYTES
    TMCL(1) = TPHLEB1 - 10D0

    DO J = 2, NPS
        TMCL(J) = TMCL(J-1) + INTERVAL
    END DO

    DO J = 1, NPS
        DO L=0,8
            I = L
            IF ( TIMES(I+1) > TMCL(J) ) EXIT
        END DO
        THETA_S = BETAS(I,1) + BETAS(I,2)*(TMCL(J) - TIMES(I)) +
BETAS(I,3)*((TMCL(J) - TIMES(I))**2)
        THETA_S = MAX(THETA_S, 0D0)
        MCL(J) = (B - THETA_S) &
            - ( (LAMBDA*(DGAMI(1D0 + 1D0/K, ((B-THETA_S)/LAMBDA)**K))) /
(1D0 - DEXP(-(((B - THETA_S)/LAMBDA)**K))) )
        PRR(J) = 1D0 - DEXP(-(((B - THETA_S)/LAMBDA)**K))
    END DO

! CREATE FITTED PLOTS FOR PUBLICATIONS
    PRINT*,' WOULD YOU LIKE TO CREATE FIITED PLOTS FOR
PUBLICATION?'
    READ (*,*) RESPONSE
    PRINT*
    IF ( RESPONSE == 'Y' .OR. RESPONSE == 'y' ) THEN
        PRINT*,' WHAT IS THE DESIRED MAXIMUM TIME VALUE FOR THE
PLOT?'
        READ (*,*) TMAX
        PRINT*
    
```

```

PRINT*, 'WHAT IS THE DESIRED MAXIMUM EPO VALUE FOR THE
PLOT?'
READ (*,*) EPOMAX
PRINT*
PRINT*, 'WHAT IS THE DESIRED MAXIMUM RBC VALUE FOR THE
PLOT?'
READ (*,*) RBCMAX
PRINT*
PRINT*, 'WHAT IS THE DESIRED MAXIMUM RETICULOCYTE VALUE
FOR THE PLOT?'
READ (*,*) RETMAX
PRINT*
! EPO PLOT
CALL GETDATAFILENAME(DATAFILENAME)
CALL ADDMARGINTEXT(DATAFILENAME)
CALL BEGINLEFTAT_D(0D0)
CALL ENDLEFTAT_D(EPOMAX)
CALL BEGINXAT_D(0D0)
CALL ENDXAT_D(TMAX)
CALL LEFTLABEL ('PLASMA EPO (MU/ML)')
CALL INCLUDEPOINTSLEFT_D(TOBS,COBS,NOBS,2)           ! EPO
DATA
CALL ADDCURVELEFT_D(TC,CC,NPS)                       ! GCV
FITTED EPO CURVE
CALL XLABEL('TIME (DAYS)')
CALL DISPLAYPLOT
! RECORD PLOT ID IF PLOT IS SAVED
CALL RECORDPLOTIFSAVED(3)
CALL RECORDPLOTIFSAVED(LUN)

! RBC AND RETICULOCYTE PLOT
CALL GETDATAFILENAME(DATAFILENAME)
CALL ADDMARGINTEXT(DATAFILENAME)
CALL BEGINLEFTAT_D(0D0)
CALL ENDLEFTAT_D(RBCMAX)
CALL BEGINRIGHTAT_D(0D0)
CALL ENDRIGHTAT_D(RETMAX)
CALL BEGINXAT_D(0D0)
CALL ENDXAT_D(TMAX)
CALL GET_XYOBS(1,TEMPX,TEMPY,TEMPN)
CALL INCLUDEPOINTSLEFT_D(TEMPX,TEMPY,TEMPN,1)       !
RBC DATA
CALL ADDFITTEDCURVELEFT(1)                           ! FITTED RBC
CURVE
CALL LEFTLABEL ('RBC (1*10^3 CELLS/UL)')
CALL GET_XYOBS(2,TEMPX,TEMPY,TEMPN)

```

```

                CALL INCLUDEPOINTSRIGHT_D(TEMPX,TEMPY,TEMPN,5)      !
RETIC DATA
                CALL ADDFITTEDCURVERIGHT(2)                        ! FITTED RETIC
CURVE
                CALL RIGHTLABEL ('RETICS (1*10^3 CELLS/UL) ')
                CALL DISPLAYPLOT
                ! RECORD PLOT ID IF PLOT IS SAVED
                CALL RECORDPLOTIFSAVED(3)
                CALL RECORDPLOTIFSAVED(LUN)
            END IF

```

```

! CREATE TIME VARIANT MEAN CIRCULATING LIFESPAN OF
RETICULOCYTES PLOTS AND OUTPUT RESULTS

```

```

    PRINT*,' WOULD YOU LIKE TO CREATE A PLOT OF THE MEAN
CIRCULATING LIFESPAN OF'

```

```

    PRINT*,' RETICULOCYTES AND SAVE TO FILE?'

```

```

    READ (*,*) RESPONSE

```

```

    PRINT*

```

```

    IF ( RESPONSE == 'Y' .OR. RESPONSE == 'y' ) THEN

```

```

        CALL GETDATAFILENAME(DATAFILENAME)

```

```

        CALL ADDMARGINTEXT(DATAFILENAME)

```

```

        CALL LEFTLABEL ('MEAN LIFESPAN (DAYS)')

```

```

        CALL ADDCURVELEFT_D(TMCL,MCL,NPS)

```

```

        CALL XLABEL('TIME (DAYS)')

```

```

        CALL DISPLAYPLOT

```

```

        ! RECORD PLOT ID IF PLOT IS SAVED

```

```

        CALL RECORDPLOTIFSAVED(3)

```

```

        CALL RECORDPLOTIFSAVED(LUN)

```

```

                WRITE(9, "(' TIME (DAYS)   TIME SINCE 1ST PHLEBOTOMY (DAYS)
MCL (DAYS)')")

```

```

                DO J = 1,NPS

```

```

                    WRITE(9, "(1X,F10.5,5X,F10.5,24X,F10.5)") TMCL(J), TMCL(J)-
TPHLEB1, MCL(J)

```

```

                ENDDO

```

```

            END IF

```

```

! CREATE PLOT OF PROPORTION OF CELLS RELEASED AS RETICULOCYTES
AND OUTPUT RESULTS

```

```

    PRINT*,' WOULD YOU LIKE TO CREATE A PLOT OF THE PROPORTION
OF CELLS RELEASED AS'

```

```

    PRINT*,' RETICULOCYTES AND OUTPUT RESULTS TO A FILE?'

```

```

    READ (*,*) RESPONSE

```

```

    PRINT*

```

```

    IF ( RESPONSE == 'Y' .OR. RESPONSE == 'y' ) THEN

```

```

        CALL GETDATAFILENAME(DATAFILENAME)

```

```

CALL ADDMARGINTEXT(DATAFILENAME)
CALL LEFTLABEL ('PROPORTION AS RETICS')
CALL ADDCURVELEFT_D(TMCL,PRR,NPS)
CALL XLABEL('TIME (DAYS)')
CALL DISPLAYPLOT
! RECORD PLOT ID IF PLOT IS SAVED
CALL RECORDPLOTIFSAVED(3)
CALL RECORDPLOTIFSAVED(LUN)

WRITE(9, '(' TIME (DAYS)   TIME SINCE 1ST PHLEBOTOMY (DAYS)
PRR (DAYS)')")
DO J = 1,NPS
WRITE(9, "(1X,F10.5,5X,F10.5,24X,F10.5)") TMCL(J), TMCL(J)-
TPHLEB1, PRR(J)
ENDDO
END IF

! CREATE FITTED PLASMA EPO PLOT WITH RETICULOCYTES
PRINT*, ' WOULD YOU LIKE AN OBSERVED AND FITTED PLASMA EPO
PLOT?'
READ (*,*) RESPONSE
PRINT*
IF ( RESPONSE == 'Y' .OR. RESPONSE == 'y' ) THEN
CALL GETDATAFILENAME(DATAFILENAME)
CALL ADDMARGINTEXT(DATAFILENAME)
CALL LEFTLABEL ('PLASMA EPO (MU/ML) [I]')
CALL ADDPOINTSLEFT_D (TOBS, COBS, NOBS)           ! EPO DATA
CALL ADDCURVELEFT_D (TC,CC,NPS)                   ! GCV FITTED
EPO CURVE
PRINT*, ' WOULD YOU LIKE OBSERVED AND FITTED
RETIUCOLYCTES ADDED TO THE PLOT?'
READ (*,*) RESPONSE
PRINT*
IF ( RESPONSE == 'Y' .OR. RESPONSE == 'y' ) THEN
CALL ADDOBSERVATIONSRIGHT(2)           ! RETICULOCYTE
DATA
CALL ADDFITTEDCURVERIGHT(2)
CALL RIGHTLABEL ('RETICS (1*10^3 CELLS/UL) +')
END IF
CALL XLABEL('TIME (DAYS)')
CALL DISPLAYPLOT
! RECORD PLOT ID IF PLOT IS SAVED
CALL RECORDPLOTIFSAVED(3)
CALL RECORDPLOTIFSAVED(LUN)
END IF

```

! CREATE BODY WEIGHT NORMALIZED PRODUCTION RATE AND EFFECT
SITE EPO PLOT

PRINT*,' WOULD YOU LIKE A PREDICTED BODY WEIGHT
NORMALIZED PRODUCTION RATE PLOT?'

READ (*,*) RESPONSE

PRINT*

IF (RESPONSE == 'Y' .OR. RESPONSE == 'y') THEN

CALL GETDATAFILENAME(DATAFILENAME)

CALL ADDMARGINTEXT(DATAFILENAME)

CALL LEFTLABEL ('PROD. RATE (1*10³ CELLS/D/KG)')

CALL ADDCURVELEFT_D (TC,PRODRATE,NPS) !

CALCULATED PRODUCTION RATE

PRINT*,' WOULD YOU LIKE PREDICTED EFFECT SITE EPO ADDED
TO THE PLOT?'

READ (*,*) RESPONSE

PRINT*

IF (RESPONSE == 'Y' .OR. RESPONSE == 'y') THEN

CALL RIGHTLABEL ('EFFECT SITE EPO (MU/ML)')

CALL ADDCURVERIGHT_D (TC,ECC,NPS) !

CALCULATED EFFECT SITE CONCENTRATION

END IF

CALL XLABEL('TIME (DAYS)')

CALL DISPLAYPLOT

! RECORD PLOT ID IF PLOT IS SAVED

CALL RECORDPLOTIFSAVED(3)

CALL RECORDPLOTIFSAVED(LUN)

END IF

! CREATE TIME VARIANT PARAMETER PLOT

PRINT*,' WOULD YOU LIKE PREDICTED THETA (LOCATION)
PARAMETER PLOT?'

READ (*,*) RESPONSE

PRINT*

IF (RESPONSE == 'Y' .OR. RESPONSE == 'y') THEN

CALL GETDATAFILENAME(DATAFILENAME)

CALL ADDMARGINTEXT(DATAFILENAME)

CALL ADDCURVELEFT_D (TC,TIMEVAR,NPS) !

CALCULATED TIME VARIANT LOCATION PARAMETER OF WEIBULL
DISTRIBUTION

CALL LEFTLABEL ('LOCATION PARAMETER (DAYS)')

CALL XLABEL('TIME (DAYS)')

CALL DISPLAYPLOT

! RECORD PLOT ID IF PLOT IS SAVED

CALL RECORDPLOTIFSAVED(3)

CALL RECORDPLOTIFSAVED(LUN)

END IF

```

! CREATE RBC AND RETICULOCYTE PLOT
  PRINT*,' WOULD YOU LIKE AN OBSERVED AND FITTED RBC AND
RETICULOCYTE PLOT?'
  READ (*,*) RESPONSE
  PRINT*
  IF ( RESPONSE == 'Y' .OR. RESPONSE == 'y' ) THEN
    CALL GETDATAFILENAME(DATAFILENAME)
    CALL ADDMARGINTEXT(DATAFILENAME)
    CALL ADDOBSERVATIONSLEFT(1)           ! RBC DATA
    CALL ADDFITTEDCURVELEFT(1)           ! FITTED RBC
CURVE
    CALL LEFTLABEL ('RBC (1*10^3 CELLS/UL) []')
    CALL ADDOBSERVATIONSRIGHT(2)         ! RETICULOCYTE
DATA
    CALL ADDFITTEDCURVERIGHT(2)
    CALL RIGHTLABEL ('RETICS (1*10^3 CELLS/UL) +')
    CALL XLABEL('TIME (DAYS)')
    CALL DISPLAYPLOT
    ! RECORD PLOT ID IF PLOT IS SAVED
    CALL RECORDPLOTIFSAVED(3)
    CALL RECORDPLOTIFSAVED(LUN)
  END IF

! SAVE PARAMETERS WITH NAMES TO OUTPUT FILE
  PRINT*,' WOULD YOU LIKE PARAMETERS WITH NAMES SAVED TO A
FILE?'
  READ (*,*) RESPONSE
  PRINT*
  IF ( RESPONSE == 'Y' .OR. RESPONSE == 'y' ) THEN
    DO J = 1, NP
      CALL GETFUNFITPARAMETERNAME(J,PNAME)
      WRITE(3 ,"(1X,A,G14.4)") PNAME, P(J)
    ENDDO
  END IF
END IF
END IF
! END USER OUTPUT SECTION

RETURN
  ENTRY MODELID(ID) !***** N O N  OPTIONAL DEFINITION
SECTION *****
  ID = 'TIME_VAR_DIST_V2.14' ! RECORD THE ID FOR THE MODEL USED
IN THE FITTING :
  RETURN
END SUBROUTINE USERMODEL

```

```

! ***** EXTERNAL FUNCTION
*****

! EXTERNAL FUNCTION THAT DETERMINES THE CONTRIBUTION OF CELLS
FROM THE PREVIOUS TIME (U)
! AT THE CURRENT TIME (TVAL)
  ! CLASS = 1 : ALL CELLS
  ! CLASS = 2 : CELLS THAT WERE RELEASED BEFORE THE 1ST
PHLEBOTOMY (TBLEED1)
  ! CLASS = 3 : CELLS THAT WERE RELEASED AFTER THE 1ST
PHLEBOTOMY (TBLEED1)
  ! CLASS = 4 : CELLS THAT WERE RELEASED BEFORE THE 2ND
PHLEBOTOMY (TBLEED2)
  ! CLASS = 5 : CELLS THAT WERE RELEASED AFTER THE 2ND
PHLEBOTOMY (TBLEED2)
REAL*8 FUNCTION FCONTRIB (U)
  USE SHARED_DATA
  USE LS_LINEAR_FITTING
  IMPLICIT NONE
  REAL*8, INTENT (IN) :: U
  INTEGER :: J, I
  REAL*8 :: CE, BW, FSTIM, EXPONENT, WEIGHT, EXPONENT1, WEIGHT1,
EXPONENT2, WEIGHT2, LOC_THETA

  CALL CONV_POLYSP_POLYEXP(COEF, NCOEF, NS, TK, A, ALPHA, NEXP, U,
CE)
  CALL LS_FITTED_VAL(U, BW)
  FSTIM = (DEXP(BW))*((EMAX*(CE**SF))/((EC50**SF) + (CE**SF)))

  DO I=0,8
    J = I
    IF ( TIMES(J+1) > U ) EXIT
  END DO

  LOC_THETA = BETAS(J,1) + BETAS(J,2)*(U - TIMES(J)) + BETAS(J,3)*((U -
TIMES(J))**2)
  LOC_THETA = MAX(LOC_THETA, 0D0)

  IF ( CLASS == 1) THEN
    IF ( TVAL - U >= LOC_THETA ) THEN
      WEIGHT = CDF(TVAL)
    ELSE
      WEIGHT = 0D0
    ENDIF
  
```

```

ELSE IF ( CLASS == 2 ) THEN
  IF ( U > TBLEED1 ) THEN
    WEIGHT = 0D0
  ELSE
    IF ( TBLEED1 - U >= LOC_THETA ) THEN
      WEIGHT = CDF(TBLEED1)
    ELSE
      WEIGHT = 0D0
    ENDIF
  ENDIF
ELSE IF ( CLASS == 3 ) THEN
  IF ( U > TBLEED1 ) THEN
    WEIGHT = 0D0
  ELSE
    IF ( TVAL - U >= LOC_THETA ) THEN
      WEIGHT1 = CDF(TVAL)
    ELSE
      WEIGHT1 = 0D0
    ENDIF
    IF ( TBLEED1 - U >= LOC_THETA ) THEN
      WEIGHT2 = CDF(TBLEED1)
    ELSE
      WEIGHT2 = 0D0
    ENDIF
    WEIGHT = WEIGHT1 - WEIGHT2
    WEIGHT = MAX(WEIGHT, 0D0)
  ENDIF
ELSE IF ( CLASS == 4 ) THEN
  IF ( U > TBLEED2 ) THEN
    WEIGHT = 0D0
  ELSE
    IF ( TBLEED2 - U >= LOC_THETA ) THEN
      WEIGHT = CDF(TBLEED2)
    ELSE
      WEIGHT = 0D0
    ENDIF
  ENDIF
ELSE
  IF ( U > TBLEED2 ) THEN
    WEIGHT = 0D0
  ELSE
    IF ( TVAL - U >= LOC_THETA ) THEN
      WEIGHT1 = CDF(TVAL)
    ELSE
      WEIGHT1 = 0D0
    ENDIF
  ENDIF

```

```

      IF ( TBLEED2 - U >= LOC_THETA ) THEN
        WEIGHT2 = CDF(TBLEED2)
      ELSE
        WEIGHT2 = 0D0
      ENDIF
      WEIGHT = WEIGHT1 - WEIGHT2
      WEIGHT = MAX(WEIGHT, 0D0)
    ENDIF
  ENDIF

  FCONTRIB = FSTIM*WEIGHT

CONTAINS
! FUNCTION THAT DETERMINES THE CDF VALUE
  REAL*8 FUNCTION CDF(UPPERBOUND)
    IMPLICIT NONE
    REAL*8, INTENT(IN) :: UPPERBOUND
    REAL*8      :: EXPONENT
    EXPONENT = (((UPPERBOUND-U-LOC_THETA)/SCALE)**SHAPE)
    CALL CONST_PLUS_EXP(1D0, -1D0, EXPONENT, CDF)    ! CDF = 1.0 -
    DEXP(-(((UPPERBOUND-U-LOC_THETA)/SCALE)**SHAPE))
    RETURN
  END FUNCTION CDF
END FUNCTION FCONTRIB

! ***** END OF FILE
*****

```

E.3.2 CONST_PLUS_EXP.FOR

```

!***** COPYRIGHT PV-P 2001
! LAST EDITION 2-9-02
  SUBROUTINE CONST_PLUS_EXP (A,B,C,VALUE)
    IMPLICIT REAL*8 (A-Z)

!-----
! THIS ROUTINE EVALUATES:
!   VALUE = A+B*EXP(-C)
! FOR ANY VALUES (POSITIVE, ZERO OR NEGATIVE) OF A,B AND C
! TO ENABLE MAXIMUM ACCURACY BY AVOIDING CANCELLATION
! ERRORS.
!
!----- ALGORITHM -----
! IT IS NOTED THAT:
!
!   1 - EXP(X) = 2*SINH(-X/2)*EXP(X/2)
!

```

```

! AND FOR SMALL ABS(X) VALUES:
!
! 1 - EXP(X) = -(X + X*X/2 + X*X*X/6)
!
! WHICH IS USED TO AVOID CANCELLATION ERRORS
! WHEN SIGN OF A AND B DIFFERS THEN:
!
! A + B*EXP(-C) = A*(1-(-B/A)*EXP(-C)) = A(1-EXP(-C+LN(-B/C))
!
!NOTE: THE TRANSFORMATION WILL ONLY TAKE PLACE WHEN
! A AND B HAVE DIFFERENT SIGNS. THE ALGORITHM ALSO
! CONSIDERS THE CASE WHEN C IS NEGATIVE.
!-----
PARAMETER (TOL = 1D-2,SMALL = 1D-4)
!
!** CURRENT VALUE OF MACHINE DEPENDENT "SMALL" ENSURES FULL
!** PRECISION FOR MACINES WITH EPSILON NOT SMALLER THEN 1E-15
!
DATA ZERO,HALF,ONE,TWO,SIX/0D0,0.5D0,1D0,2D0,6D0/
X = - C
E = B*DEXP(X)

VALUE = A+E
IF(DABS(VALUE).LT.TOL*(DABS(A)+DABS(E)))THEN
IF(DABS(A).EQ.ONE.AND.DABS(B).EQ.ONE)THEN
IF(DABS(X).GT.SMALL)THEN
VALUE = TWO*DSINH(-HALF*X)*DEXP(HALF*X)
IF(A.LT.ZERO) VALUE = - VALUE
ELSE
VALUE = -(X+X*(X*(HALF+X/SIX)))
IF(A.LT.ZERO)VALUE = - VALUE
ENDIF
ELSE
X = X + DLOG(-B/A)
IF(DABS(X).GT.SMALL)THEN
VALUE = TWO*A*DSINH(-HALF*X)*DEXP(HALF*X)
ELSE
VALUE = - A*(X+X*(X*(HALF+X/SIX)))
ENDIF
ENDIF
ENDIF
RETURN
END
!*****

```

E.3.3 CONV_POLYSP_POLYEXP_VER2.FOR

```

!
! CONVOLUTION OF POLYNOMIAL SPLINE AND POLYEXPONENTIAL.
COPYRIGHT PV-P 2002
!
! VERSION 2.0: MODIFIED TO ASSUME CONSTANT VALUE OF
POLYNOMIAL SPLINE
! AT T < TK(1) AND T > TK(NS+1), WITH THE CONSTANT VALUE SET TO
THE FIRST
! AND LAST VALUE OF THE SPLINE (25MAY2007, KJF).
!
!
SUBROUTINE CONV_POLYSP_POLYEXP
& (COEF,NCOEF,NS,TK,A,ALPHA,NEXP,T,VALUE)

!
! VALUE (OUTPUT) = CONVOLUTION OF A POLYNOMIAL SPLINE AND A
POLYEXPONENTIAL
! EVALUATED AT T (INPUT)
!
! THE POLYNOMIAL SPLINE (INPUT) IS GIVE BY THE SPLINE COEFFICIENTS,
COEF, AND
! THE KNOTS, TK. THERE ARE NS SPLINE SEGMENTS STARTING AND
ENDING
! AT TK(J) AND T(J+1), J = 1,2,...,NS. EACH SPLINE SEGMENT IS A
POLYNOMIAL:
!
! SEGMENT(J) = COEF(1,J) + COEF(2,J)*X + COEF(3,J)*X*X ... +
COEF(NCOEF,J)*X**(NCOEF-1)
! WHERE X = T - TK(J) WHEN T LIES IN THE INTERVAL FROM TK(J) TO
TK(J+1)
!
! THE POLYEXPONENTIAL IS GIVEN BY SUM OF A(J)*EXP(-ALPHA(J)*T)
!
! NOTE: THAT THE CALLING PROGRAM MUST ASSIGN TK A DIMENSION OF
AT LEAST NCOEF+1
! AND SHOULD ASSIGN THE 2ND DIMENSION OF THE COEFFICIENT
MATRIX
! TO BE AT LEAST NS+1 I.E. COEF(NCOEF,NS+1). (THE + 1 IS TO BE USED
FOR
! FUTURE EXPANSIONS)
!
IMPLICIT NONE
INTEGER NCOEF,NS,NEXP,I,K,J,L
REAL*8 COEF(NCOEF,*),TK(*),A(*),ALPHA(*),T,VALUE,ZERO,

```

```

&   V,TT,SJ,SL
      DATA ZERO / 0D0 /
      VALUE = ZERO
      IF ( T < TK(1) ) THEN
DO I = 1,NEXP
      VALUE = VALUE + COEF(1,1)*(A(I)/ALPHA(I))
      ENDDO
      ELSE
      DO I = 1,NEXP
      VALUE = VALUE+COEF(1,1)*(A(I)/ALPHA(I))*DEXP(-ALPHA(I)*(T-
TK(1)))
      ENDDO
      DO K = 1,NS
      IF(T.LT.TK(K))EXIT
      SJ = ZERO
      DO J = 1,NEXP
      SL = ZERO
      DO L = 0,NCOEF-1
      TT = MIN(TK(K+1)-TK(K),MAX(ZERO,T-TK(K)))
      CALL CONV_POWR_SEXP(L,ALPHA(J),TT,V)
      SL = SL + COEF(L+1,K)*V
      ENDDO
      IF(T.GT.TK(K+1))THEN
      SJ = SJ + A(J)*DEXP(-ALPHA(J)*(T-TK(K+1)))*SL
      ELSE
      SJ = SJ + A(J)*SL
      ENDIF
      ENDDO
      VALUE = VALUE + SJ
      ENDDO
      IF ( T > TK(NS+1) ) THEN
DO I = 1,NEXP
      DO L = 0, NCOEF-1
      VALUE = VALUE+COEF(L+1,NS)*((TK(NS+1)-
TK(NS))*L)*(A(I)/ALPHA(I))*
&   (1.0 - DEXP(-ALPHA(I)*(T-TK(NS+1))))
      ENDDO
      ENDDO
      ENDIF
      ENDIF
      RETURN
      END
!----

```

E.3.4 CONV_POWR_SEXP.FOR

```

! COPYRIGHT PV-P 2002  V1.0
!
  SUBROUTINE CONV_POWR_SEXP(N,ALPHA,T,VALUE)
!
! VALUE = CONVOLUTION OF T**N AND EXP(-ALPHA*T)
! INPUT: N,ALPHA,T
! OUTPUT: VALUE
!
  IMPLICIT NONE
    REAL*8 ALPHA,T,VALUE,S,V,ZERO,ONE
  INTEGER*4 IP
    INTEGER  K,N
    DATA ZERO, ONE / 0D0, 1D0 /
    IF (T.NE.ZERO) THEN
      IF (ALPHA.NE.ZERO) THEN
        IP = 1
        S = ZERO
        DO K = 1,N
          IP = IP*K
          S = S + (T**K)/(DFLOAT(IP)*((-ALPHA)**(N-K)))
        ENDDO
        CALL CONST_PLUS_EXP (ONE,-ONE,ALPHA*T,V)
        VALUE = DFLOAT(IP)*(V/(-ALPHA)**N + S)/ALPHA
      ELSE
        VALUE = (T**(N+1))/DFLOAT(N+1)
      ENDIF
    ELSE
      VALUE = ZERO
    ENDIF
    RETURN
  END
!----

```

E.3.5 LEAST SQUARES FIT.F90

```

! FILENAME = LEAST SQUARES FIT.F90
!
! PURPOSE: TO DETERMINE THE LEAST SQUARES LINEAR REGRESION FIT
! TO (X,Y) DATA
!   OF LENGTH N AND RETURN THE FITTED VALUE WHEN REQUESTED
!
!
! CHANGES/REVISIONS:
!  VERSION 1.0  MAY 31, 2007  ORIGINAL CODE

```

! VERSION 1.1 FEB 05, 2008 ADDED GET_LS_COEF SUBROUTINE

MODULE LS_LINEAR_FITTING

```

IMPLICIT NONE
PRIVATE
PUBLIC CALC_LS_COEF, LS_FITTED_VAL, GET_LS_COEF
REAL*8, SAVE  :: C1 = 0D0, C2 = 0D0

```

CONTAINS

```

SUBROUTINE CALC_LS_COEF(X, Y, N)
  IMPLICIT NONE
  INTEGER, INTENT (IN)  :: N
  REAL*8, INTENT (IN)  :: X(*), Y(*)

  REAL*8                :: SUMY = 0D0, SUMX = 0D0, SUMX2 = 0D0, SUMXY =
0D0
  INTEGER                :: J

  DO J=1, N
    SUMY = SUMY + Y(J)
    SUMX = SUMX + X(J)
    SUMX2 = SUMX2 + X(J)*X(J)
    SUMXY = SUMXY + X(J)*Y(J)
  END DO

  C2 = (N*SUMXY - SUMX*SUMY)/(N*SUMX2 - SUMX*SUMX)
  C1 = (SUMY - C2*SUMX)/N
END SUBROUTINE CALC_LS_COEF

```

```

SUBROUTINE LS_FITTED_VAL(X, VALUE)
  IMPLICIT NONE
  REAL*8, INTENT(IN)  :: X
  REAL*8, INTENT(OUT) :: VALUE

```

```

  VALUE = C1 + C2*X
END SUBROUTINE LS_FITTED_VAL

```

```

SUBROUTINE GET_LS_COEF(M,B)
  IMPLICIT NONE
  REAL*8, INTENT(OUT)  :: M, B

  M = C2
  B = C1
END SUBROUTINE GET_LS_COEF

```

END MODULE LS_LINEAR_FITTING

E.3.6 SHARED_DATA_V2.14.F90

```
! FILENAME = SHARED_DATA_V2.14.F90
!
! PURPOSE: TO SHARE DATA BETWEEN SUBROUTINES AND FUNCTIONS IN
! THE 'TIME VARIANT DISTRIBUTION' MODEL OF THE POLYNOMIAL AND
! POLYEXPONENTIAL COEFFICIENTS IN ORDER TO CALCULATE THE
! EFFECT-SITE COMPARTMENT CONCENTRATIONS
!
!
! CHANGES/REVISIONS:
! VERSION 1.0 MAY 23, 2007 ORIGINAL CODE (FOR
TIME_VAR_DIST_V1.1)
! VERSION 1.1 MAY 31, 2007 MODIFIED FOR USE WITH
TIME_VAR_DIST_V2.0
! VERSION 1.2 JUNE 6, 2007 MODIFIED FOR USE WITH FOR
TIME_VAR_DIST_V2.1
! VERSION 1.3 JUNE 7, 2007 MODIFIED FOR USE WITH FOR
TIME_VAR_DIST_V2.2
! VERSION 1.4 JUNE 8, 2007 MODIFIED FOR USE WITH FOR
TIME_VAR_DIST_V2.3
! VERSION 1.5 JUNE 8, 2007 MODIFIED FOR USE WITH FOR
TIME_VAR_DIST_V2.4
! VERSION 1.6 JUNE 11, 2007 MODIFIED FOR USE WITH FOR
TIME_VAR_DIST_V2.5
! VERSION 1.7 JUNE 12, 2007 MODIFIED FOR USE WITH FOR
TIME_VAR_DIST_V2.6
! VERSION 1.8 JUNE 20, 2007 MODIFIED FOR USE WITH FOR
TIME_VAR_DIST_V2.7
! VERSION 1.9 JUNE 20, 2007 MODIFIED FOR USE WITH FOR
TIME_VAR_DIST_V2.8
! VERSION 1.10 JUNE 25, 2007 MODIFIED FOR USE WITH FOR
TIME_VAR_DIST_V2.9
! JUNE 26, 2007 AND TIME_VAR_DIST_V2.10
! VERSION 1.14 JULY 26, 2007 MODIFIED FOR USE WITH FOR
TIME_VAR_DIST_V2.14
```

MODULE SHARED_DATA

```
IMPLICIT NONE
SAVE
```

```
INTEGER, PARAMETER :: NCOEF = 4, NEXP = 1, MAXNODES = 100
```

```

INTEGER                :: NS = 0, CLASS = 1
REAL*8                :: A(NEXP), ALPHA(NEXP), TK(MAXNODES), &
                      EC50 = 0D0, EMAX = 0D0, SF = 1D0, &
                      TVAL = 0D0, TBLEED1, TBLEED2, &
                      SHAPE, SCALE
REAL*8, PARAMETER     :: MAXVAL = 500D0, DELTA=0.0001
REAL*8, DIMENSION(NCOEF,MAXNODES) :: COEF
REAL*8, DIMENSION(0:8,1:3)    :: BETAS
REAL*8, DIMENSION(0:9)       :: TIMES

END MODULE SHARED_DATA

```

E.3.7 GCV.obj

Also need to include the object file “GCV.obj” with subroutine calls described below:

```

CALL CUBIC_GCV_FIT(X, Y, N)
CALL SET_CUBIC_GCV_CONSTANT_EXTRAPLOATION
CALL CUBIC_GCV_COEFFICIENTS(X, A, B, C, D)
CALL CUBIC_GCV(X, Y)

```

E.4 Fortran subroutines for Chapter 4.

E.4.1 ENVIRON_EFFECTS_V2.2.F90

```

! FILENAME = ENVIRON_EFFECTS_V2.2.F90
!
! PURPOSE: TO SIMULATE THE EFFECT OF CHANGES IN THE RELATIVE
ENVIRONMENTAL STRESS
!     ON CELLULAR RESPONSE USING EITHER AN 'ACCELERATED DEATH'
OR A 'RELATIVE
!     RISK' MODEL FORMULATION
!
! MODEL:  THE BASLINE LIFESPAN DISTRIBUTION IS ASSUMED TO
FOLLOW A TWO PARAMETER
!     WEIBULL DISTRIBUTION PARAMETERIZED IN TERMS OF THE MEAN
AND STANDARD
!     DEVIATION. THE RELATIVE ENVIRONMENTAL EFFECTS CAN ENTER
USING EITHER AN
!     'ACCELERATED DEATH' OR A 'RELATIVE RISK' MODEL. THE
RELATIVE ENVIRONMENTAL
!     EFFECT CHANGES ACCORDING TO THE 'MULTIPLE' AND 'TAU'
PARAMETERS,
!     AS DETAILED IN THE CODE.
!
! NOTES:  NONE

```

```

!
! REVISIONS:
!   VERSION 1.0  MAR 12, 2008  ORIGINAL CODE-KJF.
!   VERSION 2.0  MAR 26, 2008  MODIFIED FROM A FITTING TO A
SIMULATION DRIVEN
!
!           MODEL AND ADDED 'RELATIVE RISK' MODEL-KJF
!   VERSION 2.1  MAR 27, 2008  MODIFIED TO SIMULTANEOUS
SIMULATE CELLULAR RESPONSE
!
!           PROFILES FOR UP TO 5 DIFFERENT MULTIPLE
VALUES-KJF
!   VERSION 2.2  MAR 28, 2008  MODIFIED TO ALLOW FOR MORE
FLEXIBLE PROFILES OF THE
!
!           RELATIVE ENVIRONMENTAL EFFECTS
!

! ===== MODEL START =====
SUBROUTINE USERMODEL(T,Y,P,NP,IFUN)
  USE SHARED_DATA_AND_ROUTINES
  USE NUMERICAL_LIBRARIES

  IMPLICIT NONE
  INTEGER, PARAMETER :: NEQN = 1, NPS=1000, NE=2, ITMAX=1000, NO=5
  REAL*8, PARAMETER :: ABSERR = 0D0, RELERR = 0.001, ERRREL = 0.0001,
&
&           PERCENTILE=0.9999, TOLERANCE = 0.025, PI =
3.14159265358979323846
  INTEGER           :: NP, IFUN, J, I, LUN, JOB, NSUBDIV, K
  REAL*8           :: T, Y, P(*), MU, SIGMA, MULTNO, FPRODSS, MODEL, &
  LB, UB, ESTERR, XMAX, XMIN, DT, XTEMP(NPS), ETEMP(NPS),
&
&           AVGEFFECT, X(NE), XGUESS(NE), NOCELLSAD, NOCELLSRR,
NORM, &
&           MULT1, MULT2, MULT3, MULT4, MULT5, MULTIPLE,
MULTIPLES(NO), &
&           MAXY, SSDUR, DUR1, DURR, YMIN, YMAX, DTEMP(NPS),
LTEMP(NPS), &
&           DELTA, LOGDTEMP, TX, TY, TZ, S, E, INTEGRAL, DMAX,
ZVALUES(NPS)
  LOGICAL, SAVE    :: SHOWIT, PLOTSAVED
  CHARACTER (LEN=256) :: ID, DATAFILENAME, TITLENAME
  CHARACTER (LEN=20) :: PNAME, TEMPTTEXT2
  CHARACTER (LEN=4)  :: TEMPTTEXT
  CHARACTER(LEN=1), SAVE :: RESPONSE
  EXTERNAL NOCELLSAD, NOCELLSRR, LK_FUNC

```

```

! PARAMETER DECLARATIONS
MU      = P(1) ! MEAN POTENTIAL LIFESPAN
SIGMA   = P(2) ! STANDARD DEVIATION OF POTENTIAL LIFESPANS
MULTNO  = P(3) ! NUMBER OF MULTIPLES FROM 1.0 THAT RELATIVE
ENVIRONMENTAL EFFECTS CHANGE TO SIMULATED
SSDUR   = P(4) ! TIME DURATION THAT THE RELATIVE ENVIRONMENTAL
STAY AT THE MULTIPLE VALUE
FPRODSS = P(5) ! STEADY-STATE CELLULAR PRODUCTION RATE
MODEL   = P(6) ! INDICATOR VARIABLE: >= 0.0 'ACCELERATED DEATH'
MODEL
          !
          < 0.0 'RELATIVE RISK' MODEL
MULT1   = P(7) ! MULTIPLE 1 (CORRESPONDS TO IFUN = 1 IN DATA FILE)
MULT2   = P(8) ! MULTIPLE 2 (CORRESPONDS TO IFUN = 2 IN DATA FILE)
MULT3   = P(9) ! MULTIPLE 3 (CORRESPONDS TO IFUN = 3 IN DATA FILE)
MULT4   = P(10) ! MULTIPLE 4 (CORRESPONDS TO IFUN = 4 IN DATA FILE)
MULT5   = P(11) ! MULTIPLE 5 (CORRESPONDS TO IFUN = 5 IN DATA FILE)
DURI    = P(12) ! TIME DURATION THAT THE RELATIVE ENVIRONMENTAL
EFFECTS INITIAL CHANGE TO M FROM 1.0
DURR    = P(13) ! TIME DURATION THAT THE RELATIVE ENVIRONMENTAL
EFFECTS RETURN TO 1.0 FROM M

```

```

! ===== START OF PRELIMINARY PREPARATION SECTION
=====

```

```

IF (IFUN == - 1000) THEN
  IF(NP /= 13) STOP ' USERMODEL: INCONSISTENT NUMBER OF
PARAMETERS IN PARAMETER FILE'

  CALL SETFUNFITPARAMETERNAME(1, " MU (DAYS)")
  CALL SETFUNFITPARAMETERNAME(2, " SIGMA (DAYS)")
  CALL SETFUNFITPARAMETERNAME(3, " MULTIPLE NO.")
  CALL SETFUNFITPARAMETERNAME(4, " SS DURATION (DAYS)")
  CALL SETFUNFITPARAMETERNAME(5, " FPRODSS (10^9 CELLS/DAY)")
  CALL SETFUNFITPARAMETERNAME(6, " MODEL (IND)")
  CALL SETFUNFITPARAMETERNAME(7, " MULTIPLE 1")
  CALL SETFUNFITPARAMETERNAME(8, " MULTIPLE 2")
  CALL SETFUNFITPARAMETERNAME(9, " MULTIPLE 3")
  CALL SETFUNFITPARAMETERNAME(10, " MULTIPLE 4")
  CALL SETFUNFITPARAMETERNAME(11, " MULTIPLE 5")
  CALL SETFUNFITPARAMETERNAME(12, " INITIAL DUR. (DAYS)")
  CALL SETFUNFITPARAMETERNAME(13, " RETURN DUR. (DAYS)")

```

```

END IF

```

```

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

```

```

! UPDATE SHARED DATA

```

```

MEAN = MU
STDEV = SIGMA
XGUESS = (/ MEAN, 4D0/)
CALL DNEQNF(LK_FUNC, ERRREL, NE, ITMAX, XGUESS, X, NORM)
LAMBDA = X(1)
KW = X(2)
TVAL = T
PRODSS = FPRODSS

! CREATE AN ARRAY OF SPLINE NODES
KNOTS = (/ -1000D0, 0D0, DUR1, DUR1 + SSDUR, DUR1 + SSDUR + DURR/)

! CREATE AN ARRAY OF SPLINE COEFFICIENTS
MULTIPLES = (/MULT1, MULT2, MULT3, MULT4, MULT5/)
IF ( ( IFUN > INT(0) ) .AND. ( IFUN <= INT(MULTNO) ) ) THEN
  MULTIPLE = MULTIPLES(IFUN)
ELSE
  MULTIPLE = 1D0
END IF
COEFF(0,0) = 1D0
COEFF(1,0) = 1D0
COEFF(2,0) = MULTIPLE
COEFF(3,0) = MULTIPLE
COEFF(4,0) = 1D0

COEFF(0,1) = 0D0
DO J=1, N-1
  COEFF(J,1) = (COEFF(J+1,0) - COEFF(J,0))/(KNOTS(J+1) - KNOTS(J))
END DO
COEFF(N,0) = COEFF(N-1,0) + COEFF(N-1,1)*(KNOTS(N) - KNOTS(N-1))
COEFF(N,1) = 0D0

IF ( ABS((LAMBDA*DGAMMA(1D0 + (1D0/KW)) - MU)/MU) >= TOLERANCE )
THEN
  WRITE(*, "(' WARNING: ACCURACY OF LAMBDA AND K CALCULATIONS
POOR!!!)')")
  ELSE IF ( ABS( ((LAMBDA**2D0)*DGAMMA(1D0 + (2D0/KW)) - MU**2D0 -
SIGMA**2D0)/(SIGMA**2D0) ) >= TOLERANCE ) THEN
    WRITE(*, "(' WARNING: ACCURACY OF LAMBDA AND K CALCULATIONS
POOR!!!)')")
  END IF

! MODEL FITTING
IF ( (IFUN == 1) .OR. (IFUN ==2) .OR. (IFUN==3) .OR. (IFUN==4) .OR.
(IFUN==5) ) THEN
  UB = T

```

```

      IF ( MODEL >= 0D0 ) THEN
        LB = T - ((LAMBDA/(MIN(1D0,MULTIPLE)))**((LOG(1D0/(1D0 -
PERCENTILE))))**((1D0/KW)))
        CALL DQDAGS(NOCELLSAD, LB, UB, ABSERR, RELERR, Y, ESTERR)
      ELSE
        LB = T - LAMBDA*(((1D0/(MIN(1D0,MULTIPLE)))*(LOG(1D0/(1D0 -
PERCENTILE))))**((1D0/KW)))
        CALL DQDAGS(NOCELLSRR, LB, UB, ABSERR, RELERR, Y, ESTERR)
      ENDIF
    END IF

! ===== USER OUTPUT =====
    IF (IFUN == 0) THEN
      DO J = 1, NP
        CALL GETFUNFITPARAMETERNAME (J, PNAME)
        WRITE(*, "(1X, I2, 1X, A, G14.4)") J, PNAME, P(J)
      ENDDO
      WRITE(*, "(1X, 'LAMBDA', 17X, G14.4)") LAMBDA
      WRITE(*, "(1X, 'K', 22X, G14.4)") KW

      WRITE(*, *)
      DO I=1, INT(MULTNO)
        MULTIPLE=MULTIPLES(I)
        COEFF(0,0) = 1D0
        COEFF(1,0) = 1D0
        COEFF(2,0) = MULTIPLE
        COEFF(3,0) = MULTIPLE
        COEFF(4,0) = 1D0
        COEFF(0,1) = 0D0
        DO J=1, N-1
          COEFF(J,1) = (COEFF(J+1,0) - COEFF(J,0))/(KNOTS(J+1) - KNOTS(J))
        END DO
        COEFF(N,0) = COEFF(N-1,0) + COEFF(N-1,1)*(KNOTS(N) - KNOTS(N-1))
        COEFF(N,1) = 0D0

        TVAL = KNOTS(3)

        UB = TVAL
        IF ( MODEL >= 0D0 ) THEN
          LB = TVAL - ((LAMBDA/(MIN(1D0,MULTIPLE)))**((LOG(1D0/(1D0 -
PERCENTILE))))**((1D0/KW)))
          CALL DQDAGS(NOCELLSAD, LB, UB, ABSERR, RELERR, Y, ESTERR)
        ELSE
          LB = TVAL - LAMBDA*(((1D0/(MIN(1D0,MULTIPLE)))*(LOG(1D0/(1D0 -
PERCENTILE))))**((1D0/KW)))
          CALL DQDAGS(NOCELLSRR, LB, UB, ABSERR, RELERR, Y, ESTERR)
        ENDIF
      END DO
    END IF
  END SUBROUTINE

```

```

ENDIF
WRITE(*,"(' Y(' I2,') EXTREMUM: ', G12.6)") I, Y
END DO
WRITE(*,*)

CALL PROMT(SHOWIT)
IF (SHOWIT) THEN
  DO J = 1,NP
    CALL GETFUNFITPARAMETERNAME (J,PNAME)
    WRITE(3,"(' PAR',I2,1X,A,G14.4)")J, PNAME, P(J)
  ENDDO
  WRITE(3,"(1X,'LAMBDA',17X, G14.4)") LAMBDA
  WRITE(3,"(1X,'K',22X,G14.4)") KW

  ! USER PLOTS - PRELIMINARY INFORMATION/CALCULATIONS
  CALL GET_GLOBAL_XMIN(XMIN)
  CALL GET_GLOBAL_XMAX(XMAX)
  CALL GETDATAFILENAME(DATAFILENAME)
  DT = (XMAX - XMIN)/(NPS-1)
  DO J = 1, NPS
    XTEMP(J) = XMIN + DT*(J-1)
  END DO
  MULTIPLE = MINVAL(MULTIPLES)
  COEFF(0,0) = 1D0
  COEFF(1,0) = 1D0
  COEFF(2,0) = MULTIPLE
  COEFF(3,0) = MULTIPLE
  COEFF(4,0) = 1D0
  COEFF(0,1) = 0D0
  DO J=1, N-1
    COEFF(J,1) = (COEFF(J+1,0) - COEFF(J,0))/(KNOTS(J+1) - KNOTS(J))
  END DO
  COEFF(N,0) = COEFF(N-1,0) + COEFF(N-1,1)*(KNOTS(N) - KNOTS(N-1))
  COEFF(N,1) = 0D0
  MAXY = MEAN
  DO J = 1, NPS
    TVAL = XTEMP(J)

    UB = TVAL
    IF ( MODEL >= 0D0 ) THEN
      LB = TVAL - ((LAMBDA/(MIN(1D0,MULTIPLE)))**((LOG(1D0/(1D0 -
PERCENTILE)))**((1D0/KW)))
      CALL DQDAGS(NOCELLSAD, LB, UB, ABSERR, RELERR, Y,
ESTERR)

```

```

ELSE
  LB = TVAL -
LAMBDA*(((1D0/(MIN(1D0,MULTIPLE))))*(LOG(1D0/(1D0 -
PERCENTILE))))*(1D0/KW))
  CALL DQDAGS(NOCELLSRR, LB, UB, ABSERR, RELERR, Y,
ESTERR)
  ENDIF
  MAXY = MAX(Y, MAXY)
END DO

! PRINT*
! PRINT*,' WOULD YOU LIKE INDIVIDUAL PLOTS OF EACH MULTIPLE'
! PRINT*,' ALL ON ONE PAGE?'
! READ (*,*) RESPONSE
!
! ! CREATE USER PLOTS OF CELLULAR RESPONSE FOR EACH
MULTIPLE OF ENVIRONMENTAL EFFECT ON ONE PAGE
! IF ( RESPONSE == 'Y' .OR. RESPONSE == 'y' ) THEN
! DO I = 1, INT(MULTNO)
! WRITE(TEMPTEXT,'(F4.2)') MULTIPLES(I)
! TITLENAM='MULTIPLE = '//TEMPTEXT
! CALL TITLE(TITLENAM)
! CALL BEGINXAT_D(XMIN)
! CALL ENDXAT_D(XMAX)
! CALL XLABEL('TIME (DAYS)')
! CALL BEGINLEFTAT_D(0D0)
! CALL ENDLEFTAT_D(MAXY)
! CALL LEFTLABEL ('CELLS (10^9)')
! CALL ADDFITTEDCURVELEFT(I)
! CALL PLOTINAREA(I,6)
! END DO
! CALL TITLE('SIMULATED RELATIVE ENVIRONMENT')
! CALL BEGINXAT_D(XMIN)
! CALL ENDXAT_D(XMAX)
! CALL XLABEL('TIME (DAYS)')
! CALL BEGINLEFTAT_D(0D0)
! CALL LEFTLABEL ('RELATIVE EFFECT')
! DO I=1, INT(MULTNO)
! MULTIPLE = MULTIPLES(MIN(I,INT(MULTNO)))
! COEFF(0,0) = 1D0
! COEFF(1,0) = 1D0
! COEFF(2,0) = MULTIPLE
! COEFF(3,0) = MULTIPLE
! COEFF(4,0) = 1D0
! COEFF(0,1) = 0D0
! DO J=1, N-1

```

```

!           COEFF(J,1) = (COEFF(J+1,0) - COEFF(J,0))/(KNOTS(J+1) -
KNOTS(J))
!           END DO
!           COEFF(N,0) = COEFF(N-1,0) + COEFF(N-1,1)*(KNOTS(N) - KNOTS(N-
1))
!           COEFF(N,1) = 0D0
!           DO J = 1, NPS
!             CALL SPLINE_VALUE(XTEMP(J), ETEMP(J))
!           END DO
!           CALL ADDCURVELEFT_D(XTEMP,ETEMP,NPS)
!         END DO
!         CALL PLOTINAREA(6,6)
!         CALL DISPLAYPLOT
!         CALL RECORDPLOTIFSAVED(3)
!         CALL RECORDPLOTIFSAVED(LUN)
!       END IF
!
!       PRINT*
!       PRINT*,' WOULD YOU LIKE INDIVIDUAL PLOTS OF EACH MULPTILE'
!       PRINT*,' ON SEPARATE PAGES WITH THE ENVIRONMENTAL
EFFECTS?'
!       READ (*,*) RESPONSE
!
!       ! CREATE USER PLOTS OF CELLULAR RESPONSE FOR EACH
MULTIPLE OF ENVIRONMENTAL EFFECT
!       ! ALONG WITH THE ENVIRONMENTAL EFFECT ON SEPARATE PAGES
!       IF ( RESPONSE == 'Y' .OR. RESPONSE == 'y' ) THEN
!         DO I=1, INT(MULTNO)
!           CALL BEGINXAT_D(XMIN)
!           CALL ENDXAT_D(XMAX)
!           CALL BEGINLEFTAT_D(0D0)
!           CALL ENDLEFTAT_D(MAXY)
!           CALL LEFTLABEL ('CELLS (10^9)')
!           CALL ADDFITTEDCURVELEFT(I)
!           CALL PLOTINAREA(1,2)
!           WRITE(TEMPTEXT,'(F4.2)') MULTIPLES(I)
!           TITLENAM='MULTIPLE = '//TEMPTEXT
!           CALL TITLE(TITLENAM)
!           CALL BEGINXAT_D(XMIN)
!           CALL ENDXAT_D(XMAX)
!           CALL BEGINLEFTAT_D(0D0)
!           CALL ENDLEFTAT_D(MAXVAL(MULTIPLES))
!           MULTIPLE = MULTIPLES(MIN(I,INT(MULTNO)))
!           COEFF(0,0) = 1D0
!           COEFF(1,0) = 1D0
!           COEFF(2,0) = MULTIPLE

```

```

!          COEFF(3,0) = MULTIPLE
!          COEFF(4,0) = 1D0
!          COEFF(0,1) = 0D0
!          DO J=1, N-1
!              COEFF(J,1) = (COEFF(J+1,0) - COEFF(J,0))/(KNOTS(J+1) -
KNOTS(J))
!          END DO
!          COEFF(N,0) = COEFF(N-1,0) + COEFF(N-1,1)*(KNOTS(N) - KNOTS(N-
1))
!          COEFF(N,1) = 0D0
!          DO J = 1, NPS
!              CALL SPLINE_VALUE(XTEMP(J), ETEMP(J))
!          END DO
!          CALL LEFTLABEL ('RELATIVE ENVIRON. EFFECT')
!          CALL ADDCURVELEFT_D(XTEMP,ETEMP,NPS)
!          CALL XLABEL('TIME (DAYS)')
!          CALL PLOTINAREA(2,2)
!          CALL DISPLAYPLOT
!          CALL RECORDPLOTIFSAVED(3)
!          CALL RECORDPLOTIFSAVED(LUN)
!      END DO
!  END IF
!
!      PRINT*
!      PRINT*,' WOULD YOU LIKE PLOTS OF EACH MULTIPLE OVERLAYED'
!      PRINT*,' ON SAME PLOT?'
!      READ (*,*) RESPONSE
!
!      ! CREATE USER PLOTS OF CELLULAR RESPONSE FOR EACH
MULTIPLE OF ENVIRONMENTAL EFFECT OVERLAYED ON ONE PLOT
!      IF ( RESPONSE == 'Y' .OR. RESPONSE == 'y' ) THEN
!
!          PRINT*
!          PRINT*,' WOULD YOU LIKE A CUSTOM TITLE FOR YOUR PLOTS?'
!          READ (*,*) RESPONSE
!          IF ( RESPONSE == 'Y' .OR. RESPONSE == 'y' ) THEN
!              PRINT*
!              PRINT*,' PLEASE ENTER A TITLE FOR THE PLOTS'
!              READ (*,*) TITLENAM
!          ELSE
!              TITLENAM='SIMULATED ENVIRON. EFFECTS'
!          END IF
!          CALL TITLE(TITLENAM)
!          CALL BEGINXAT_D(XMIN)
!          CALL ENDXAT_D(XMAX)
!          CALL XLABEL('TIME (DAYS)')

```

```

!      CALL BEGINLEFTAT_D(0D0)
!      CALL LEFTLABEL ('CELLS (10^9)')
!      DO I=1, INT(MULTNO)
!          CALL ADDFITTEDCURVELEFT(I)
!      END DO
!      CALL DISPLAYPLOT
!      CALL RECORDPLOTIFSAVED(3)
!      CALL RECORDPLOTIFSAVED(LUN)
!
!      IF ( RESPONSE == 'Y' .OR. RESPONSE == 'y' ) THEN
!          TEMPTEXT2 = TITLENAM
!          TITLENAM = TRIM(TEMPTEXT2)//'-/'/'RESPONSE'
!      ELSE
!          TITLENAM='SIMULATED RESPONSE'
!      END IF
!      CALL TITLE(TITLENAM)
!      CALL BEGINXAT_D(XMIN)
!      CALL ENDXAT_D(XMAX)
!      CALL XLABEL('TIME (DAYS)')
!      CALL BEGINLEFTAT_D(0D0)
!      CALL LEFTLABEL ('CELLS (10^9)')
!      DO I=1, INT(MULTNO)
!          CALL ADDFITTEDCURVELEFT(I)
!      END DO
!      CALL PLOTINAREA(1,2)
!
!      CALL TITLE('SIMULATED EFFECT')
!      CALL BEGINXAT_D(XMIN)
!      CALL ENDXAT_D(XMAX)
!      CALL XLABEL('TIME (DAYS)')
!      CALL BEGINLEFTAT_D(0D0)
!      CALL LEFTLABEL ('RELATIVE EFFECT')
!      DO I=1, INT(MULTNO)
!          MULTIPLE = MULTIPLES(MIN(I,INT(MULTNO)))
!          COEFF(0,0) = 1D0
!          COEFF(1,0) = 1D0
!          COEFF(2,0) = MULTIPLE
!          COEFF(3,0) = MULTIPLE
!          COEFF(4,0) = 1D0
!          COEFF(0,1) = 0D0
!          DO J=1, N-1
!              COEFF(J,1) = (COEFF(J+1,0) - COEFF(J,0))/(KNOTS(J+1) -
KNOTS(J))
!          END DO
!          COEFF(N,0) = COEFF(N-1,0) + COEFF(N-1,1)*(KNOTS(N) - KNOTS(N-
1))

```

```

!       COEFF(N,1) = 0D0
!       DO J = 1, NPS
!           CALL SPLINE_VALUE(XTEMP(J), ETEMP(J))
!       END DO
!       CALL ADDCURVELEFT_D(XTEMP,ETEMP,NPS)
!       END DO
!       CALL PLOTINAREA(2,2)
!
!       CALL DISPLAYPLOT
!       CALL RECORDPLOTIFSAVED(3)
!       CALL RECORDPLOTIFSAVED(LUN)
!   END IF
!
!   PRINT*
!   PRINT*,' WOULD YOU LIKE CUSTOM PLOTS OF EACH MULTIPLE
OVERLAYED'
!   PRINT*,' ON SAME PLOT FOR PUBLICATION?'
!   READ (*,*) RESPONSE
!
!       ! CREATE CUSTOM USER PLOTS FOR PUBLICATION OF CELLULAR
RESPONSE FOR EACH MULTIPLE OF ENVIRONMENTAL
!       ! EFFECT OVERLAYED ON ONE PLOT
!       IF ( RESPONSE == 'Y' .OR. RESPONSE == 'y' ) THEN
!           PRINT*
!           PRINT*,' PLEASE ENTER THE X-AXIS START AND STOP VALUES AS:
START, STOP?'
!           READ (*,*) XMIN, XMAX
!           PRINT*
!           PRINT*,' PLEASE ENTER THE Y-AXIS START AND STOP VALUES AS:
START, STOP?'
!           READ (*,*) YMIN, YMAX
!           PRINT*
!           PRINT*,' PLEASE ENTER A TITLE FOR THE PLOTS'
!           READ (*,*) TITLENAM
!           CALL TITLE(TITLENAM)
!           CALL BEGINXAT_D(XMIN)
!           CALL ENDXAT_D(XMAX)
!           CALL XLABEL('TIME (DAYS)')
!           CALL BEGINLEFTAT_D(YMIN)
!           CALL ENDLEFTAT_D(YMAX)
!           CALL LEFTLABEL ('CELLS (10^9)')
!           DO I=1, INT(MULTNO)
!               CALL ADDFITTEDCURVELEFT(I)
!           END DO
!           CALL DISPLAYPLOT
!           CALL RECORDPLOTIFSAVED(3)

```

```

!      CALL RECORDPLOTIFSAVED(LUN)
!      END IF

      PRINT*
      PRINT*,' WOULD YOU LIKE CUSTOM PLOTS OF OBSERVED LIFESPAN
WEIBULL P.D.F.'
      PRINT*,' OF EACH MULTIPLE OVERLAYED ON SAME PLOT FOR
PUBLICATION?'
      READ (*,*) RESPONSE

      ! CREATE CUSTOM USER PLOTS FOR PUBLICATION OF OBSERVED
CELLULAR LIFESPAN P.D.F. FOR EACH MULTIPLE OF ENVIRONMENTAL
      ! EFFECT OVERLAYED ON ONE PLOT (NOTE: MULTIPLES ARE
ASSUMED CONSTANT FOR ALL T AND Z)
      IF ( RESPONSE == 'Y' .OR. RESPONSE == 'y' ) THEN
        PRINT*
        PRINT*,' PLEASE ENTER THE X-AXIS START AND STOP VALUES AS:
START, STOP?'
        READ (*,*) XMIN, XMAX
        PRINT*
        PRINT*,' PLEASE ENTER THE Y-AXIS START AND STOP VALUES AS:
START, STOP?'
        READ (*,*) YMIN, YMAX
        CALL BEGINXAT_D(XMIN)
        CALL ENDXAT_D(XMAX)
        CALL XLABEL('LIFESPAN (DAYS)')
        CALL BEGINLEFTAT_D(YMIN)
        CALL ENDLEFTAT_D(YMAX)
        CALL LEFTLABEL ('DENSITY')
        DELTA = (XMAX-XMIN)/(NPS-1)
        LTEMP(1) = XMIN
        DO J = 2, NPS
          LTEMP(J) = LTEMP(J-1) + DELTA
        END DO
        DO I=1, INT(MULTNO)
          IF (MODEL >= 0D0 ) THEN
            DO J = 1, NPS
              DTEMP(J) =
MULTIPLES(I)*(KW/LAMBDA)*(((MULTIPLES(I)*LTEMP(J))/LAMBDA)**(KW-
1))*DEXP(-(((MULTIPLES(I)*LTEMP(J))/LAMBDA)**KW))
            END DO
            CALL TITLE("ACCELERATED_LIFE")
          ELSE
            DO J = 1, NPS
              IF (LTEMP(J) <= 0D0) THEN

```

```

        DTEMP(J) =
MULTIPLES(I)*(KW/LAMBDA)*((LTEMP(J)/LAMBDA)**(KW-1))*DEXP(-
((LTEMP(J)/LAMBDA)**KW))*((DEXP(-
((LTEMP(J)/LAMBDA)**KW)))*(MULTIPLES(I) - 1D0))
        ELSE
        LOGDTEMP = DLOG(MULTIPLES(I)) + DLOG(KW) -
LOG(LAMBDA) + (KW - 1D0)*DLOG(LTEMP(J)) - (KW - 1D0)*DLOG(LAMBDA)
&
        - (LTEMP(J)/LAMBDA)**KW - (MULTIPLES(I) -
1D0)*((LTEMP(J)/LAMBDA)**KW)
        DTEMP(J) = DEXP(LOGDTEMP)
        END IF
    END DO
    CALL TITLE("RELATIVE_RISK")
    END IF
    CALL ADDCURVELEFT_D(LTEMP,DTEMP,NPS)
    END DO
    CALL DISPLAYPLOT
    CALL RECORDPLOTIFSAVED(3)
    CALL RECORDPLOTIFSAVED(LUN)
    END IF

    PRINT*
    PRINT*,' WOULD YOU LIKE CUSTOM PLOTS OF OBSERVED LIFESPAN
NORMAL P.D.F.'
    PRINT*,' OF EACH MULTIPLE OVERLAYED ON SAME PLOT FOR
PUBLICATION?'
    READ (*,*) RESPONSE

    ! CREATE CUSTOM USER PLOTS FOR PUBLICATION OF OBSERVED
CELLULAR LIFESPAN P.D.F. FOR EACH MULTIPLE OF ENVIRONMENTAL
! EFFECT OVERLAYED ON ONE PLOT (NOTE: MULTIPLES ARE
ASSUMED CONSTANT FOR ALL T AND Z)
    IF ( RESPONSE == 'Y' .OR. RESPONSE == 'y' ) THEN
        PRINT*
        PRINT*,' PLEASE ENTER THE X-AXIS START AND STOP VALUES AS:
START, STOP?'
        READ (*,*) XMIN, XMAX
        PRINT*
        PRINT*,' PLEASE ENTER THE Y-AXIS START AND STOP VALUES AS:
START, STOP?'
        READ (*,*) YMIN, YMAX
        CALL BEGINXAT_D(XMIN)
        CALL ENDXAT_D(XMAX)
        CALL XLABEL('LIFESPAN (DAYS)')
        CALL BEGINLEFTAT_D(YMIN)

```

```

CALL ENDLEFTAT_D(YMAX)
CALL LEFTLABEL ('DENSITY')
DELTA = (XMAX-XMIN)/(NPS-1)
LTEMP(1) = XMIN
DO J = 2, NPS
  LTEMP(J) = LTEMP(J-1) + DELTA
END DO
DO I=1, INT(MULTNO)
  IF (MODEL >= 0D0 ) THEN
    DO J = 1, NPS
      DTEMP(J) = (MULTIPLES(I)/(SIGMA*SQRT(2D0*PI)))*DEXP(-
(((MULTIPLES(I)*LTEMP(J) - MU)**2D0)/(2D0*(SIGMA**2D0))))
    END DO
    CALL TITLE("ACCELERATED_LIFE")
  ELSE
    DO J = 1, NPS
      IF ( (LTEMP(J) <= 0D0) .OR. (DNORDF((LTEMP(J) - MU)/SIGMA)
>= 1D0) ) THEN
        DTEMP(J) = (MULTIPLES(I)/(SIGMA*SQRT(2D0*PI)))*
(DEXP(-(((LTEMP(J) - MU)**2D0)/(2D0*(SIGMA**2D0))))/(1D0 -
DNORDF((LTEMP(J) - MU)/SIGMA))) &
          *((1D0 - DNORDF((LTEMP(J) -
MU)/SIGMA))**MULTIPLES(I))
      ELSE
        LOGDTEMP = DLOG(MULTIPLES(I)) - DLOG(SIGMA) -
0.5*DLOG(2D0*PI) - (((LTEMP(J) - MU)**2D0)/(2D0*(SIGMA**2D0))) &
          - DLOG(1D0 - DNORDF((LTEMP(J) - MU)/SIGMA)) +
MULTIPLES(I)*DLOG(1D0 - DNORDF((LTEMP(J) - MU)/SIGMA))
        DTEMP(J) = DEXP(LOGDTEMP)
      END IF
    END DO
    CALL TITLE("RELATIVE_RISK")
  END IF
  CALL ADDCURVELEFT_D(LTEMP,DTEMP,NPS)
END DO
CALL DISPLAYPLOT
CALL RECORDPLOTIFSAVED(3)
CALL RECORDPLOTIFSAVED(LUN)
END IF

PRINT*
PRINT*,' WOULD YOU LIKE CUSTOM 2D PLOTS OF OBSERVED
LIFESPAN P.D.F.'
PRINT*,' OF TAU VS. Z FOR PUBLICATION?'
READ (*,*) RESPONSE

```

```

! CREATE CUSTOM USER 2D PLOTS FOR PUBLICATION OF OBSERVED
CELLULAR LIFESPAN P.D.F. OF TAU
! VS. Z FOR EACH MULTIPLE OF ENVIRONMENTAL EFFECT
IF ( RESPONSE == 'Y' .OR. RESPONSE == 'y' ) THEN
  PRINT*
  PRINT*,' PLEASE ENTER THE TAU START AND STOP VALUES AS:
START, STOP?'
  READ (*,*) XMIN, XMAX
  PRINT*
  PRINT*,' PLEASE ENTER THE DENSITY START AND STOP VALUES
AS: START, STOP?'
  READ (*,*) YMIN, YMAX
  PRINT*
  PRINT*,' PLEASE ENTER THE NUMBER OF SUBDIVISIONS OF Z?'
  READ (*,*) NSUBDIV
  PRINT*
  PRINT*,' PLEASE ENTER THE Z -VALUES TO EVALUATE FROM
LOWEST TO HIGHEST?'
  DO J = 1, NSUBDIV
    WRITE(*, "(ENTER ZVALUE ', I2, ':')") J
    READ (*,*) ZVALUES(J)
  END DO

  DELTA = (XMAX-XMIN)/(NPS-1)
  LTEMP(1) = XMIN
  DO J = 2, NPS
    LTEMP(J) = LTEMP(J-1) + DELTA
  END DO

  DO I=1, INT(MULTNO)
    MULTIPLE = MULTIPLES(MIN(I,INT(MULTNO)))
    COEFF(0,0) = 1D0
    COEFF(1,0) = 1D0
    COEFF(2,0) = MULTIPLE
    COEFF(3,0) = MULTIPLE
    COEFF(4,0) = 1D0
    COEFF(0,1) = 0D0
    DO J=1, N-1
      COEFF(J,1) = (COEFF(J+1,0) - COEFF(J,0))/(KNOTS(J+1) - KNOTS(J))
    END DO
    COEFF(N,0) = COEFF(N-1,0) + COEFF(N-1,1)*(KNOTS(N) - KNOTS(N-
1))
    COEFF(N,1) = 0D0
    IF (MODEL >= 0D0 ) THEN
      DO K = 1, NSUBDIV
        DO J = 1, NPS

```

```

CALL SPLINE_VALUE(ZVALUES(K)+LTEMP(J), S)
IF ( ZVALUES(K)+LTEMP(J) /= ZVALUES(K) ) THEN
  CALL SPLINE_INTEGRAL(ZVALUES(K),
ZVALUES(K)+LTEMP(J), INTEGRAL)
  E = INTEGRAL/LTEMP(J)
ELSE
  E = S
END IF
DTEMP(J) = S*(
(KW/LAMBDA)*((E*LTEMP(J))/LAMBDA)**(KW - 1D0))*DEXP(-
((E*LTEMP(J))/LAMBDA)**KW)) )
END DO
CALL BEGINXAT_D(XMIN)
CALL ENDXAT_D(XMAX)
CALL XLABEL('LIFESPAN (DAYS)')
CALL BEGINLEFTAT_D(YMIN)
CALL ENDLEFTAT_D(YMAX)
CALL LEFTLABEL ('DENSITY')
CALL ADD_CURVE(LTEMP, DTEMP, NPS)
CALL TITLE_WITH_VALUE_ADDED("Z =", ZVALUES(K))
CALL PLOT_IN_AREA(K, NSUBDIV)
END DO
CALL
ADD_MARGIN_TEXT_WITH_FORMATTED_VALUE_ADDED("ACCELERATED
LIFE, M =", MULTIPLE, 3)
CALL DISPLAYPLOT
CALL RECORDPLOTIFSAVED(3)
CALL RECORDPLOTIFSAVED(LUN)
ELSE
DO K = 1, NSUBDIV
DO J = 1, NPS
CALL SPLINE_VALUE(ZVALUES(K)+LTEMP(J), S)
CALL SPLINE_PROD_INTEGRAL(ZVALUES(K),
ZVALUES(K)+LTEMP(J), INTEGRAL)

IF ( LTEMP(J) > 0D0 ) THEN
DTEMP(J) = DEXP( DLOG(S) + DLOG(KW) -
KW*DLOG(LAMBDA) + (KW - 1D0)*DLOG(LTEMP(J)) -
(KW/LAMBDA)*((1D0/LAMBDA)**(KW-1D0))*INTEGRAL )
ELSE
DTEMP(J) = S*(KW/LAMBDA)*((LTEMP(J)/LAMBDA)**(KW
- 1D0))*DEXP(-(KW/LAMBDA)*((1D0/LAMBDA)**(KW-1D0))*INTEGRAL)
END IF
END DO
CALL BEGINXAT_D(XMIN)
CALL ENDXAT_D(XMAX)

```

```

CALL XLABEL('LIFESPAN (DAYS)')
CALL BEGINLEFTAT_D(YMIN)
CALL ENDLEFTAT_D(YMAX)
CALL LEFTLABEL ('DENSITY')
CALL ADD_CURVE(LTEMP, DTEMP, NPS)
CALL TITLE_WITH_VALUE_ADDED("Z =", ZVALUES(K))
CALL PLOT_IN_AREA(K, NSUBDIV)
END DO
CALL
ADD_MARGIN_TEXT_WITH_FORMATTED_VALUE_ADDED("RELATIVE RISK,
M =", MULTIPLE, 3)
CALL DISPLAYPLOT
CALL RECORDPLOTIFSAVED(3)
CALL RECORDPLOTIFSAVED(LUN)

END IF

END DO
END IF

PRINT*
PRINT*,' WOULD YOU LIKE CUSTOM 3D PLOTS OF OBSERVED
LIFESPAN P.D.F.'
PRINT*,' OF TAU VS. Z FOR PUBLICATION?'
READ (*,*) RESPONSE

! CREATE CUSTOM USER 3D PLOTS FOR PUBLICATION OF OBSERVED
CELLULAR LIFESPAN P.D.F. OF TAU
! VS. Z FOR EACH MULTIPLE OF ENVIRONMENTAL EFFECT
IF ( RESPONSE == 'Y' .OR. RESPONSE == 'y' ) THEN
PRINT*
PRINT*,' PLEASE ENTER THE TAU START AND STOP VALUES AS:
START, STOP?'
READ (*,*) XMIN, XMAX
PRINT*
PRINT*,' PLEASE ENTER THE Z START AND STOP VALUES AS:
START, STOP?'
READ (*,*) YMIN, YMAX

DO I=1, INT(MULTNO)
MULTIPLE = MULTIPLES(MIN(I,INT(MULTNO)))
COEFF(0,0) = 1D0
COEFF(1,0) = 1D0
COEFF(2,0) = MULTIPLE
COEFF(3,0) = MULTIPLE
COEFF(4,0) = 1D0

```

```

        COEFF(0,1) = 0D0
        DO J=1, N-1
            COEFF(J,1) = (COEFF(J+1,0) - COEFF(J,0))/(KNOTS(J+1) - KNOTS(J))
        END DO
        COEFF(N,0) = COEFF(N-1,0) + COEFF(N-1,1)*(KNOTS(N) - KNOTS(N-
1))
        COEFF(N,1) = 0D0
        IF (MODEL >= 0D0 ) THEN
            CALL DEFINE_2D_GRID_FOR_FUNCTION_EVALUATION(XMIN,
XMAX, NPS/10, YMIN, YMAX, NPS/10)
            JOB = 0
            DO
                CALL CONSTRUCT_3D_POINTS_OF_FUNCTION(TX, TY, TZ,
JOB)

                IF (JOB /= 1) EXIT
                CALL SPLINE_VALUE(TX+TY, S)

                IF ( TX+TY /= TY ) THEN
                    CALL SPLINE_INTEGRAL(TY, TX+TY, INTEGRAL)
                    E = INTEGRAL/TX
                ELSE
                    E = S
                END IF

                TZ = S*( (KW/LAMBDA)*(((E*TX)/LAMBDA)**(KW -
1D0))*DEXP(-(((E*TX)/LAMBDA)**KW)) )
            END DO
            CALL
TITLE_WITH_FORMATTED_VALUE_ADDED("ACCELERATED LIFE, M =",
MULTIPLE, 3)
            CALL ADD_MARGIN_TEXT_WITH_VALUE_ADDED("uSS =",
MU/MULTIPLE)
            CALL INCLUDE_SURFACE(45D0,75D0)
            CALL DISPLAYPLOT
            CALL RECORDPLOTIFSAVED(3)
            CALL RECORDPLOTIFSAVED(LUN)
        ELSE
            CALL DEFINE_2D_GRID_FOR_FUNCTION_EVALUATION(XMIN,
XMAX, NPS/10, YMIN, YMAX, NPS/10)
            JOB = 0
            DO
                CALL CONSTRUCT_3D_POINTS_OF_FUNCTION(TX, TY, TZ,
JOB)

                IF (JOB /= 1) EXIT
                CALL SPLINE_VALUE(TX+TY, S)
                CALL SPLINE_PROD_INTEGRAL(TY, TY+TX, INTEGRAL)

```

```

        IF ( TX > 0D0 ) THEN
            TZ = DEXP( DLOG(S) + DLOG(KW) - DLOG(LAMBDA) + (KW -
1D0)*DLOG(TX) - (KW - 1D0)*DLOG(LAMBDA) - ((TX/LAMBDA)**KW) &
                + ((TX/LAMBDA)**KW) -
(KW/LAMBDA)*((1D0/LAMBDA)**(KW-1D0))*INTEGRAL )
        ELSE
            TZ = S*(KW/LAMBDA)*((TX/LAMBDA)**(KW - 1D0))*DEXP(-
((TX/LAMBDA)**KW))*DEXP(((TX/LAMBDA)**KW)) &
                *DEXP(-(KW/LAMBDA)*((1D0/LAMBDA)**(KW-
1D0))*INTEGRAL)
        END IF
    END DO
    CALL TITLE_WITH_FORMATTED_VALUE_ADDED("RELATIVE
RISK, M =", MULTIPLE, 3)
    CALL INCLUDE_SURFACE(45D0,75D0)
    CALL DISPLAYPLOT
    CALL RECORDPLOTIFSAVED(3)
    CALL RECORDPLOTIFSAVED(LUN)
    CALL TITLE("RELATIVE_RISK")
    END IF
    END DO
    END IF
    END IF
    END IF
! ===== END OF USER OUTPUT =====

    RETURN
    ! RECORD THE ID FOR THE MODEL USED IN THE FITTING
    ENTRY MODELID(ID)
    ID = 'ENVIRON_EFFECTS_V2.2.F90'
    RETURN

END SUBROUTINE USERMODEL

! ===== MODEL END =====

! ===== EXTERNAL FUNCTIONS =====
! FUNCTION THAT CALCULATES THE NUMBER OF CELLS PRODUCED AT
TIME U
! THAT ARE CURRENTLY PRESENT IN THE SAMPLING COMPARTMENT
USING AN
! ACCELERATED DEATH MODEL
REAL*8 FUNCTION NOCELLSAD(U)
    USE SHARED_DATA_AND_ROUTINES
    USE NUMERICAL_LIBRARIES
    IMPLICIT NONE

```

```

REAL*8, INTENT(IN) :: U
INTEGER          :: J
REAL*8          :: E, PROB

! CALCULATE THE CUMULATIVE ENVIRONMENTAL EFFECT OF CELLS
PRODUCED AT TIME U
CALL SPLINE_INTEGRAL(U, TVAL, E)
IF ( TVAL /= U ) THEN
  E = E/(TVAL - U)
ELSE
  CALL SPLINE_VALUE(TVAL,E)
END IF

! CALCULATE THE PROBABILITY THAT CELLS PRODUCED AT TIME U ARE
CURRENTLY PRESENT IN THE
! SAMPLING COMPARTMENT
PROB = DEXP(-(E*(TVAL - U))/LAMBDA)**KW)

! CALCULATE THE NUMBER OF CELLS PRODUCED AT TIME U THAT ARE
CURRENTLY PRESENT IN THE
! SAMPLING COMPARTMENT
NOCELLSAD = PRODSS*PROB
END FUNCTION NOCELLSAD

! FUNCTION THAT CALCULATES THE NUMBER OF CELLS PRODUCED AT
TIME U
! THAT ARE CURRENTLY PRESENT IN THE SAMPLING COMPARTMENT
USING A
! RELATIVE RISK MODEL
REAL*8 FUNCTION NOCELLSRR(U)
  USE SHARED_DATA_AND_ROUTINES
  USE NUMERICAL_LIBRARIES
  IMPLICIT NONE
  REAL*8, INTENT(IN) :: U
  INTEGER          :: J
  REAL*8          :: PROB, INT

! CALCULATE THE PROBABILITY THAT CELLS PRODUCED AT TIME U ARE
CURRENTLY PRESENT IN THE
! SAMPLING COMPARTMENT
CALL SPLINE_PROD_INTEGRAL(U, TVAL, INT)
PROB = DEXP(-(KW/LAMBDA)*((1D0/LAMBDA)**(KW-1D0))*INT)

! CALCULATE THE NUMBER OF CELLS PRODUCED AT TIME U THAT ARE
CURRENTLY PRESENT IN THE
! SAMPLING COMPARTMENT

```

```

    NOCELLSRR = PRODSS*PROB
END FUNCTION NOCELLSRR

```

```

! SUBROUTINE THAT CALCULATES THE VALUE OF THE SYTEM OF
EQUATIONS DEFINED BY LAMBDA AND K
! OF THE WEIBULL DISTRIBUTION IN TERMS OF THE MEAN AND STANDARD
DEVIATION

```

```

SUBROUTINE LK_FUNC (X, F, NE)
  USE SHARED_DATA_AND_ROUTINES
  USE NUMERICAL_LIBRARIES
  IMPLICIT NONE
  INTEGER, INTENT(IN) :: NE
  REAL*8, INTENT(IN) :: X(NE)
  REAL*8, INTENT(OUT) :: F(NE)
  ! NOTE: X(1) IS LAMBDA AND X(2) IS K OF THE WEIBULL DISTRIBUTION

```

```

  F(1) = X(1)*DGAMMA(1D0 + (1D0/X(2))) - MEAN
  F(2) = ((X(1))**2D0)*DGAMMA(1D0 + (2D0/X(2))) - MEAN**2 - STDEV**2

```

```

  RETURN
END SUBROUTINE LK_FUNC

```

```

! ===== END EXTERNAL FUNCTIONS/SUBROUTINES =====

```

```

! ===== END OF FILE =====

```

E.4.2 SHARED_DATA_AND_ROUTINES_V2.0.F90

```

! FILENAME = SHARED_DATA_AND_ROUTINES_V2.0.F90
!
! PURPOSE: TO SHARE DATA BETWEEN SUBROUTINES AND FUNCTIONS IN
THE TIME VARIANT
!   ENVIRONMNETAL EFFECTS MODEL
!
!
! REVISIONS:
! VERSION 1.0 MAR 13, 2008 ORIGINAL CODE-KJF.
! VERSION 2.0 MAR 26, 2008 MODIFIED FOR ENVIRON_EFFECTS_V2.0
MODEL.
!
! M: HIGHEST ORDER OF THE POLYNOMIAL SPLINE (E.G. A QUADRATIC
SPLINE IS SECOND ORDER)
! N: NUMBER OF KNOTS
! KNOTS: A VECTOR OF LENGTH N+1 CONTAINING THE VALUES OF THE
KNOTS
! COEFF: A N+1 BY M ARRAY CONTAINING THE COEFFICIENTS OF THE

```

```

!   CORRESPONDING KNOTS

MODULE SHARED_DATA_AND_ROUTINES
  IMPLICIT NONE
  SAVE

  INTEGER, PARAMETER      :: M=1, N=4
  REAL*8                  :: MEAN, STDEV, PRODSS, TVAL, LAMBDA, KW
  REAL*8, DIMENSION(0:N,0:M) :: COEFF
  REAL*8, DIMENSION(0:N)  :: KNOTS

CONTAINS
  SUBROUTINE SPLINE_INTEGRAL (LB, UB, INTEGRAL)
    ! PURPOSE: TO CALCULATE A FINTE OF INTEGRAL OF A GIVEN
    POLYNOMIAL SPLINE.
    !
    ! LB:   LOWER BOUND OF THE INTEGRAL TO BE CALCULATED
    ! UB:   UPPER BOUND OF THE INTEGRAL TO BE CALCULATED
    ! INTEGRAL: VALUE OF THE INTEGRAL COMPUTED FROM THE LOWER
    BOUND TO THE
    !       UPPER BOUND ACROSS THE SPLINE
    !
    ! NOTE: FOR INTEGRALS CALCULATED BELOW THE FIRST KNOT (I.E.
    KNOTS(0)) USES THE COEFFICIENTS
    !   FROM THE FIRST KNOT TO CALCULATE THE INTEGRAL ACROSS
    THAT PORTION

    IMPLICIT NONE

    REAL*8, INTENT(IN)          :: LB, UB
    REAL*8, INTENT(OUT)         :: INTEGRAL

    INTEGER                    :: I, J, K, L
    REAL*8                     :: X, TUVAL, TLVAL, INTEGRAL1, INTEGRAL2,
    INTEGRAL3

    ! DETERMINE SPLINE SEGMENT LOWER BOUND IS CONTAINED IN
    DO J=1, N
      K=J-1
      IF ( KNOTS(J) > LB ) EXIT
      K=J
    END DO

    ! DETERMINE SPLINE SEGMENT UPPER BOUND IS CONTAINED IN
    DO J=1, N
      L=J-1

```

```

      IF ( KNOTS(J) > UB ) EXIT
      L=J
    END DO

    ! CALCULATE INTEGRAL FROM THE LB TO FIRST KNOT PAST LB OR UB,
    WHICHEVER IS SMALLEST
    INTEGRAL1 = 0D0
    IF ( K == L ) THEN
      X = UB - KNOTS(K)
    ELSE
      X = KNOTS(K+1) - KNOTS(K)
    END IF
    TUVAL = 0D0
    DO J=0, M
      TUVAL = X*((COEFF(K,M-J)/(M-J+1)) + TUVAL)
    END DO
    X = LB - KNOTS(K)
    TLVAL = 0D0
    DO J=0, M
      TLVAL = X*((COEFF(K,M-J)/(M-J+1)) + TLVAL)
    END DO
    INTEGRAL1 = TUVAL - TLVAL

    ! CALCULATE INTEGRAL FROM FIRST KNOT PAST LB TO KNOT
    IMMEDIATELY PRIOR TO UB (IF > 1 KNOT INBETWEEN)
    INTEGRAL2 = 0D0
    IF ( L > K + 1 ) THEN
      DO I = K+1, L-1
        X = KNOTS(I+1) - KNOTS(I)
        TUVAL = 0D0
        DO J=0, M
          TUVAL = X*((COEFF(I,M-J)/(M-J+1)) + TUVAL)
        END DO
        INTEGRAL2 = INTEGRAL2 + TUVAL
      END DO
    END IF

    ! CALCULATE INTEGRAL FROM KNOT IMMEDIATELY PRIOR TO UB TO
    THE UB (IF UB IS NOT IN THE SAME SEGMENT AS LB)
    INTEGRAL3 = 0D0
    IF ( L > K ) THEN
      X = UB - KNOTS(L)
      TUVAL = 0D0
      DO J=0, M
        TUVAL = X*((COEFF(L,M-J)/(M-J+1)) + TUVAL)
      END DO

```

```

    INTEGRAL3 = TUVAL
END IF

! CALCULATE THE TOTAL INTEGRAL
INTEGRAL = INTEGRAL1 + INTEGRAL2 + INTEGRAL3
RETURN

END SUBROUTINE SPLINE_INTEGRAL

SUBROUTINE SPLINE_VALUE (T, VALUE)
! PURPOSE: TO CALCULATE THE VALUE OF A GIVEN POLYNOMIAL
SPLINE.
!
! T:    THE DEPENDENT VARIABLE TO EVALUATE THE SPLINE AT
! VALUE: VALUE OF THE SPLINE AT T
!
! NOTE: FOR VALUES CALCULATED PRIOR TO THE FIRST KNOT (I.E.
KNOTS(0)) USES THE COEFFICIENTS
! FROM THE FIRST KNOT

IMPLICIT NONE

REAL*8, INTENT(IN)    :: T
REAL*8, INTENT(OUT)  :: VALUE

INTEGER              :: J, K
REAL*8               :: X

! DETERMINE SPLINE SEGMENT T IS CONTAINED IN
DO J=1, N
    K=J-1
    IF ( KNOTS(J) > T ) EXIT
    K=J
END DO

! CALCULATE VALUE OF SPLINE AT T
VALUE = 0D0
X = T - KNOTS(K)
DO J=0,M-1
    VALUE = X*(COEFF(K,M-J) + VALUE)
END DO
VALUE = COEFF(K,0) + VALUE
RETURN

END SUBROUTINE SPLINE_VALUE

```

```

SUBROUTINE SPLINE_PROD_INTEGRAL (LB, UB, INTEGRAL)
! PURPOSE: TO CALCULATE A FINTE OF INTEGRAL OF A GIVEN
POLYNOMIAL SPLINE MULIPLIED
!     BY A KERNAL OF THE RATIO OF A WEIBULL (PDF/(1-CDF)).
!
! LB:   LOWER BOUND OF THE PRODUCT INTEGRAL TO BE
CALCULATED
! UB:   UPPER BOUND OF THE PRODUCT INTEGRAL TO BE CALCULATED
! INTEGRAL: VALUE OF THE PRODUCT INTEGRAL COMPUTED FROM THE
LOWER BOUND
!     TO THE UPPER BOUND ACROSS THE SPLINE
!
! NOTE: FOR INTEGRALS CALCULATED BELOW THE FIRST KNOT (I.E.
KNOTS(0)) USES THE COEFFICIENTS
!     FROM THE FIRST KNOT TO CALCULATE THE INTEGRAL ACROSS
THAT PORTION
!
! NOTE: *** ONLY WRITTEN FOR A LINEAR POLYNOMIAL SPLINE ***

IMPLICIT NONE

REAL*8, INTENT(IN)           :: LB, UB
REAL*8, INTENT(OUT)          :: INTEGRAL

INTEGER                       :: I, J, K, L
REAL*8                        :: SLB, SUB, TUVAL, TLVAL, INTEGRAL1,
INTEGRAL2, INTEGRAL3

! ENSURE SPLINE IS NOT HIGHER ORDER THAN LINEAR
IF ( M > 1 ) STOP "SUBROUTINE: 'SPLINE_PROD_INTEGRAL' ONLY
WRITTEN FOR LINEAR OR LOWER ORDER SPLINES"

! DETERMINE SPLINE SEGMENT LOWER BOUND IS CONTAINED IN
DO J=1, N
  K=J-1
  IF ( KNOTS(J) > LB ) EXIT
  K=J
END DO

! DETERMINE SPLINE SEGMENT UPPER BOUND IS CONTAINED IN
DO J=1, N
  L=J-1
  IF ( KNOTS(J) > UB ) EXIT
  L=J

```

```

END DO

! CALCULATE INTEGRAL FROM THE LB TO FIRST KNOT PAST LB OR UB,
WHICHEVER IS SMALLEST
INTEGRAL1 = 0D0
IF ( K == L ) THEN
  SUB = UB
ELSE
  SUB = KNOTS(K+1)
END IF
TUVAL = (COEFF(K,0) - COEFF(K,1)*KNOTS(K))*(((SUB-LB)**KW)/KW) +
(COEFF(K,1)*((SUB-LB)**KW)*(LB + KW*SUB))/(KW*(KW + 1D0))
TLVAL = 0D0
INTEGRAL1 = TUVAL - TLVAL

! CALCULATE INTEGRAL FROM FIRST KNOT PAST LB TO KNOT
IMMEDIATELY PRIOR TO UB (IF > 1 KNOT INBETWEEN)
INTEGRAL2 = 0D0
IF (L > K + 1 ) THEN
  DO I = K+1, L-1
    SUB = KNOTS(I+1)
    SLB = KNOTS(I)
    TUVAL = (COEFF(I,0) - COEFF(I,1)*KNOTS(I))*(((SUB-LB)**KW)/KW) +
(COEFF(I,1)*((SUB-LB)**KW)*(LB + KW*SUB))/(KW*(KW + 1D0))
    TLVAL = (COEFF(I,0) - COEFF(I,1)*KNOTS(I))*(((SLB-LB)**KW)/KW) +
(COEFF(I,1)*((SLB-LB)**KW)*(LB + KW*SLB))/(KW*(KW + 1D0))
    INTEGRAL2 = INTEGRAL2 + TUVAL - TLVAL
  END DO
END IF

! CALCULATE INTEGRAL FROM KNOT IMMEDIATELY PRIOR TO UB TO
THE UB (IF UB IS NOT IN THE SAME SEGMENT AS LB)
INTEGRAL3 = 0D0
IF (L > K ) THEN
  SUB = UB
  SLB = KNOTS(L)
  TUVAL = (COEFF(L,0) - COEFF(L,1)*KNOTS(L))*(((SUB-LB)**KW)/KW) +
(COEFF(L,1)*((SUB-LB)**KW)*(LB + KW*SUB))/(KW*(KW + 1D0))
  TLVAL = (COEFF(L,0) - COEFF(L,1)*KNOTS(L))*(((SLB-LB)**KW)/KW) +
(COEFF(L,1)*((SLB-LB)**KW)*(LB + KW*SLB))/(KW*(KW + 1D0))
  INTEGRAL3 = TUVAL - TLVAL
END IF

! CALCULATE THE TOTAL INTEGRAL
INTEGRAL = INTEGRAL1 + INTEGRAL2 + INTEGRAL3
RETURN

```

```

      END SUBROUTINE SPLINE_PROD_INTEGRAL

END MODULE SHARED_DATA_AND_ROUTINES

```

E.4.3 DUMMY_USERMODEL_ODE.F90

```

! DUMMY (DO NOTHING) ROUTINE TO BE USED IF THERE ARE NO
! ODE TO NE FITTED BY WINFUNFIT
!

```

```

SUBROUTINE USERMODEL_ODE (T, Y, YPRIME, P, NP, IFUN)
  IMPLICIT NONE
  INTEGER,INTENT (IN)      :: NP, IFUN
  REAL*8, INTENT (IN)     :: T, P(*), Y(*)
  REAL*8, INTENT (OUT)    :: YPRIME(*)
  REAL*8, PARAMETER      :: ONE = 1D0
  YPRIME(1) = ONE
END SUBROUTINE USERMODEL_ODE
!

```

E.4.4 DUMMY_USERMODEL_ODE_JACOBIAN.F90

```

! THIS IS A DUMMY ROUTNE TO BE USED IN THE WINFUNFIT LINKAGE IF
THE

```

```

! JACOBIAN OF THE ODE'S IS NOT TO BE USED
!

```

```

SUBROUTINE USERMODEL_ODE_JACOBIAN (T,Y,DFDT,DFDY, N, P,NP,IFUN)
  IMPLICIT NONE
  INTEGER,          INTENT (IN) :: N, NP, IFUN
  DOUBLE PRECISION, INTENT (IN) :: T
  DOUBLE PRECISION, DIMENSION (N), INTENT (IN) :: Y
  DOUBLE PRECISION, DIMENSION (N), INTENT (OUT) :: DFDT
  DOUBLE PRECISION, DIMENSION (N,N), INTENT (OUT) :: DFDY
  DOUBLE PRECISION, DIMENSION (NP), INTENT (IN) :: P
! LOCALS
  DOUBLE PRECISION, PARAMETER      :: ONE = 1D0
  DFDT(1) = ONE
  DFDY(1,1) = ONE
END SUBROUTINE USERMODEL_ODE_JACOBIAN

```

E.5 Fortran subroutines for Chapter 6.

E.5.1 VLBW_V2.2.1.F90

```

! FILENAME = VLBW_V2.2.1.F90

```

!

! PURPOSE: TO DETERMINE THE PRODUCTION RATE OF HEMOGLOBIN IN VERY LOW BIRTH WEIGHT (VLBW)

! NEONATAL INFANTS WHILE ACCOUNTING FOR INFANT GROWTH, PHLEBOTIMES, AND TRANSFUSIONS

!

! MODEL: 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

! RBC 10^6 /UL

! 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

!

! NOTES: TO NOT USE ALL 14 STIMULATION RATE KNOTS JUST READ IN A FSTIM KNOT VECTOR WITH LESS THAN

! 14 KNOTS AND FIX THE VALUE OF THE EXCESS KNOTS TO 0.0 FOR THE FITTING.

!

! REVISIONS:

! VERSION 1.0 APRIL 14, 2008 ORIGINAL CODE-KJF.

! VERSION 1.1 MAY 1, 2008 REMOVED THE STIMULATION FACTOR FROM THE MODEL, ADDED A

! FRACTION DAMAGED OF THE TRANSFUSED CELLS THAT ARE IMMEDIATELY

! REMOVED UPON TRANSFUSION, AND INCREASED THE NUMBER OF FSTIM

! KNOTS TO 14-KJF.

! VERSION 1.2 JUNE 5, 2008 MODIFIED TO REMOVE EXPLICIT BACK EXTRAPOLATION OF TOTAL BODY

! STIMULATION RATE BEYOND TZERO. INSTEAD THE REMAINLING LIFESPAN

```

!           DISTRIBUTION AT TZERO REPRESENTED AS A
CONSTRAINED POLYNOMIAL
!           DENSITY IS USED TO ACCOUNT FOR THE PRIOR
HISTORY BEFORE BIRTH-KJF.
!           VERSION 1.2.1 JULY 18, 2008   MODIFIED TO WORK WITH
PHLEBOTOMY_TRANSFUSION_REMAINING_LIFESPAN_MODULE_V1.1.F90,
!           WHICH IS MATHEMATICALLY IDENTICAL TO THE
PREVIOUS VERSION (V1.0), BUT
!           OF A SIMPLER MATHEMATICAL FORM AND
EASIER TO IMPLEMENT NUMERICALLY-KJF.
!           VERSION 2.0 DEC. 15, 2008   MODIFIED TO WORK WITH
FSTIM_MODULE_V2.0 THAT ESTIMATES FSTIM AS A FUNCTION
!           OF EPO CONCENTRATION-KJF.
!           VERSION 2.1 JAN. 14, 2009   ADDED A SIMULTANEOUS FIT TO RBC
DATA AND TWO MCH PARAMETERS, 1 FOR
!           ENDOGENOUSLY PRODUCED RBCS AND 1 FOR
TRANSFUSED RBCS-KJF.
!           VERSION 2.2 FEB. 6, 2009   MODIFIED TO WORK WITH FILE
'FSTIM_EPO_MODULE_V2.1.F90'. THAT IS, MODIFIED TO
!           ESTIMATE THE IN UTERO STIMULATION RATE
INSTEAD OF IT BEING A FUNCTION OF THE
!           BACK EXTRAPOLATED PLASMA EPO
CONCENTRATIONS-KJF.
!           FEB. 16, 2009   CORRECTED MISTAKE IN FILE-KJF.
!           VERSION 2.2.1 MAR. 11, 2009   ADDED OUTPUT OF MODEL FITS TO
FILE FOR PLOTTING PURPOSES

```

```

! ===== 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
  IMPLICIT NONE

  INTEGER, PARAMETER :: MAXCOEFF = 100, MAXEVENTS = 250
  INTEGER             :: NP, IFUN, NO_PHLEBOTOMIES, NOCOEFF, J, NPHLEB
  REAL*8              :: T, Y(*), YPRIME(*), P(*), A, B, CORRECTION_TERM, &
                       TRANSFUSION_TERM, TEMPA, &
                       TEMPY, TEMPF, ENDO_FRAC, TRANS_FRAC
  LOGICAL             :: EVENT_IS_ACTIVE
  REAL*8, EXTERNAL   :: TOTAL_BODY_STIM_RATE

  IF (IFUN == 1) THEN
    ! ===== PARAMETER DECLARATIONS =====
    A = P(1)

```

```

B = P(1) + P(2)
LT = P(3)
FDAMAGED = 1D0 - P(4)
MRS = P(6)
GESTATIONAL_AGE_AT_BIRTH = P(7)
VMKM = P(8)
IKM = P(9)

CALL GET_TOTAL_NUMBER_PHEBOTOMIES(NPHLEB)

! ===== CALCULATE PHEBOTOMY CORRECTION AND
TRANSFUSION TERM =====
CALL GET_NUMBER_PHEBOTOMIES(T, NO_PHEBOTOMIES)
IF ( NO_PHEBOTOMIES > 0 ) THEN
  IF ( EVENT_IS_ACTIVE(NO_PHEBOTOMIES) ) THEN
    CALL GET_PHEBOTOMY_AMOUNT_VALUE(NO_PHEBOTOMIES,
TEMPA)
    CALL
UPDATE_FRACTION_REMAINING_VALUE(NO_PHEBOTOMIES, 1D0 -
TEMPA/(Y(1) + Y(2)))
    ENDO_FRAC = Y(1)/(Y(1) + Y(2))
    TRANS_FRAC = 1D0 - ENDO_FRAC
    CALL SET_EVENT_VALUE(NO_PHEBOTOMIES, -
ENDO_FRAC*TEMPA)
    CALL SET_EVENT_VALUE(NPHLEB+NO_PHEBOTOMIES, -
TRANS_FRAC*TEMPA)
    END IF
  END IF
  CALL GET_PHEBOTOMY_CORRECTION_TERM(T - (B - A), T,
CORRECTION_TERM)
  CALL GET_TRANSFUSION_TERM(T, TRANSFUSION_TERM)

! ===== DIFFERENTIAL EQUATION(S) =====
! YPRIME(1) = ENDOGENOUSLY PRODUCED HGB
! YPRIME(2) = TRANSFUSED HGB
YPRIME(1) = TOTAL_BODY_STIM_RATE(T-A) -
TOTAL_BODY_STIM_RATE(T-B)*CORRECTION_TERM
YPRIME(2) = - TRANSFUSION_TERM
END IF
END SUBROUTINE USERMODEL_ODE

! ===== MODEL (EXCLUDING DIFFERENTIAL EQUATIONS(S))
=====
SUBROUTINE USERMODEL(T,Y,P,NP,IFUN)
  USE PHEBOTOMY_TRANSFUSION_MODULE

```

```

USE FSTIM_EPO_MODULE
USE BODYWEIGHT_MODULE
USE NUMERICAL_LIBRARIES
IMPLICIT NONE

INTEGER, PARAMETER :: NEQN = 2, NPAR=12, MAXN = 500, MAXCOEFF =
100, LUN = 3
REAL*8, PARAMETER :: FACTOR = 2D0, TOLERANCE = 1.0D-7, TIMEZERO
= 0D0, ABSERR = 0D0, &
    RELERR = 0.001
INTEGER          :: NP, IFUN, JFUN, NSIGDIGITS, NUM, NOEVENTS, NPHLEB,
NTRANS, &
    TEMPN, NOCOEFF, J, SUBNO, JOB, TN, K, KLAST, NEPO
REAL*8          :: T, Y, AMT(NEQN), 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, &
    MAXHBAMT, TX1(MAXN), TX2(MAXN), TY1(MAXN),
TY2(MAXN), TYMAX, DELTA, TEMP(4), &
    MONTHHBTOTALPROD, FUNDAMAGED, XEPO(MAXN),
YEPO(MAXN), MCHE, MCHT, CV, AVGSTIMRATE
LOGICAL, SAVE   :: SHOWIT, PLOTSAVED
CHARACTER (LEN=256) :: ID, DATAFILENAME
CHARACTER (LEN=20)  :: PNAME
CHARACTER (LEN=1)  :: RESPONSE
REAL*8, EXTERNAL  :: TOTAL_BODY_STIM_RATE, STIM_RATE

! ===== 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, " RBC LIFESPAN (DAY)")
    CALL SETFUNFITPARAMETERNAME(3, " LIFESPAN TRANS (DAY)")
    CALL SETFUNFITPARAMETERNAME(4, " FRACTION UNDAMAGED")
    CALL SETFUNFITPARAMETERNAME(5, " BLOOD VOL (DL/KG)")
    CALL SETFUNFITPARAMETERNAME(6, " METAB RATE SCALAR")
    CALL SETFUNFITPARAMETERNAME(7, " GA AT BIRTH (DAYS)")
    CALL SETFUNFITPARAMETERNAME(8, " VM/KM
(G*ML/DAY*MU*(KG**MRS))")
    CALL SETFUNFITPARAMETERNAME(9, " 1/KM (ML/MU)")
    CALL SETFUNFITPARAMETERNAME(10, " MCH ENDOGENOUS (PG)")

```

```

CALL SETFUNFITPARAMETERNAME(11, " MCH TRANSFUSED (PG)")
CALL SETFUNFITPARAMETERNAME(12, " IN UTERO STIMULATION
RATE (G/DAY*(KG**MRS))")

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

TEMPN = MAXN
PRINT*
PRINT*,' TRANSFUSION AMOUNT-TIME DATA:'
CALL GET_XY_DATA_FROM_FUNFIT_FILE (TEMPX,TEMPY,TEMPN)
IF ( TEMPN > MAXN ) STOP ' TOO MANY TRANSFUSION DATA POINTS.
ADJUST THE MAXN APPROPRIATELY'
CALL SET_TRANSFUSION_TIME_VECTOR(TEMPN, TEMPX)
CALL SET_TRANSFUSION_AMOUNT_VECTOR(TEMPN, TEMPY)

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'
WRITE(*, "(/, ' WOULD YOU LIKE TO FIX THE C.V. FOR THE CUBIC SPLINE
FIT (Y/N)?)")
READ(*,*) RESPONSE
IF ( (RESPONSE=='Y') .OR. (RESPONSE=='y')) THEN
WRITE(*, "(/, ' PLEASE ENTER THE C.V. VALUE:)")

```

```

    READ(*,*) CV
    CALL SET_CV_VALUE_FOR_SPLINE_FIT(CV)
END IF
CALL CUBIC_GCV_FIT(TEMPX, TEMPY(1:TEMPN), TEMPN)
XEPO = TEMPX
YEPO = TEMPY
NEPO = TEMPN

! ===== DECLARE EVENTS =====
CALL GET_TOTAL_NUMBER_PHLEBOTOMIES(NPHLEB)
CALL GET_TOTAL_NUMBER_TRANSFUSIONS(NTRANS)
NOEVENTS = NPHLEB + NTRANS
WRITE(*,"(/,' NUMBER PHLEBOTOMIES: ', G12.6)") NPHLEB
WRITE(*,"(' NUMBER TRANSFUSIONS: ', G12.6)") NTRANS
WRITE(*,"(' TOTAL NUMBER OF PHLEBOTOMIES AND TRANSUFSSIONS: ',
G12.6)") NOEVENTS
WRITE(*,"(' TOTAL NUMBER OF EVENTS TO BE REGISTERED: ', G12.6)")
2*NPHLEB + NTRANS
READ(*,*)
FUNDAMAGED = P(4)
IF ( NPHLEB > 0 ) THEN
    DO J = 1, NPHLEB
        CALL GET_TIME_OF_PHLEBOTOMY(J, TEMPT)
        CALL REGISTER_EVENT(J, TEMPT, TEMPT, 0D0, 1)
        CALL REGISTER_EVENT(NPHLEB+J, TEMPT, TEMPT, 0D0, 2)
    END DO
END IF
IF ( NTRANS > 0 ) THEN
    DO J = 2*NPHLEB+1, 2*NPHLEB+NTRANS
        CALL GET_TIME_OF_TRANSFUSION(J-2*NPHLEB, TEMPT)
        CALL GET_TRANSFUSION_AMOUNT_VALUE(J-2*NPHLEB, TEMPA)
        CALL REGISTER_EVENT(J, TEMPT, TEMPT,
+TEMPA*FUNDAMAGED, 2)
    END DO
END IF
CALL COMPLETE_THE_EVENT_REGISTRATION

! ===== SELCECT THE ODE SOLVER AND PLOTTING OPTIONS
=====
CALL USE_DELAY_ODE_SOLVER
CALL DO_NOT_USE_DELAY_ODE_PLOTS
END IF

! ===== PARAMETER DECLARATIONS =====
A = P(1)
B = P(1) + 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)
MCHE = P(10)
MCHT = P(11)
INUTEROSTIM = P(12)

! ===== UPDATE EVENT VALUES =====
CALL GET_TOTAL_NUMBER_PHEBOTOMIES(NPHLEB)
CALL GET_TOTAL_NUMBER_TRANSFUSIONS(NTRANS)
NOEVENTS = NPHLEB + NTRANS
IF ( NTRANS > 0 ) THEN
  DO J = NPHLEB+1, NOEVENTS
    CALL GET_TRANSFUSION_AMOUNT_VALUE(J-NPHLEB, TEMPA)
    CALL SET_EVENT_VALUE(J, +TEMPA*FUNDAMAGED)
  END DO
END IF

! ===== DIFFERENTIAL EQUATION INTITIAL CONDITIONS
=====
TZERO(1) = TIMEZERO
TZERO(2) = TIMEZERO
CALL DQDAGS(TOTAL_BODY_STIM_RATE, TZERO(1) - B, TZERO(1) - A,
ABSERR, RELERR, YZERO(1), ESTERR)
YZERO(2) = 0D0

! ===== MODEL FITTING SECTION =====
IF ((IFUN == 1) .OR. (IFUN == 2)) THEN
  CALL
USERMODEL_ODE_INTEGRATED(T,AMT,P,NP,1,TZERO,YZERO,NEQN)
  IF ( IFUN == 1 ) THEN
    Y = (AMT(1) + AMT(2))/(BVOL*BODYWEIGHT(T))
  ELSE
    Y = (((AMT(1)/MCHE) +
(AMT(2)/MCHT))*10D0)/(BVOL*BODYWEIGHT(T))
  END IF
END IF

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

```

```

! ===== SECONDARY PARAMETER CALCULATIONS AND
WRITE STATEMENTS =====
CALL GET_GLOBAL_XMAX(XMAX)
CALL DQDAGS(TOTAL_BODY_STIM_RATE, TZERO(1) - A, TZERO(1) - A +
XMAX, ABSERR, RELERR, HBTOTALPROD, ESTERR)
CALL DQDAGS(TOTAL_BODY_STIM_RATE, TZERO(1) - A, TZERO(1) - A +
30D0, ABSERR, RELERR, MONTHHBTOTALPROD, ESTERR)
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
CALL DQDAGS(STIM_RATE, TZERO(1), TZERO(1) + 30D0, ABSERR,
RELERR, AVGSTIMRATE, ESTERR)
AVGSTIMRATE = AVGSTIMRATE/30D0
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
WRITE(*,"(' AVG. STIM. RATE OVER 30 DAYS (G/DAY*(KG**MRS)):
',G12.4)") AVGSTIMRATE

```

```

! ===== 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,"(/, ' INFANT 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,"(' AVG. STIM. RATE OVER 30 DAYS (G/DAY*(KG**MRS)):
',G12.4)") AVGSTIMRATE
  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('-
INFANT:', 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_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 AND STIMULATION RATE =====
! ===== AND ENDOGENOUSLY PRODUCED AND
TRANSFUSED TOTAL AMOUNTS OF HEMOGLOBIN =====
WRITE(*, "(/, ' WOULD YOU LIKE PLOTS OF BACK-EXTRAPOLATED
BODY WEIGHT AND STIMULATION RATE', /, &
' AND ENDOGENOUSLY PRODUCED AND TRANSFUSED TOTAL
AMOUNTS OF HB PRESENT?")")
READ(*, *) RESPONSE
IF ( RESPONSE == 'Y' .OR. RESPONSE == 'y' ) THEN
    CALL ADD_MARGIN_TEXT_WITH_INTEGER_NUMBER_ADDED('-
INFANT:', 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('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)

TX = TZERO(1)
MAXHBAMT = YZERO(1)
DO
  TX = TX + (XMAX - TZERO(1))/REAL(MAXN,8)
  IF ( TX > XMAX ) EXIT
  CALL
USERMODEL_ODE_INTEGRATED(TX,AMT,P,NP,1,TZERO,YZERO,NEQN)
  TY = AMT(1) + AMT(2)
  MAXHBAMT = MAX(TY, MAXHBAMT)
END DO
CALL ADD_MARGIN_TEXT_WITH_INTEGER_NUMBER_ADDED('-
INFANT:', SUBNO)

  CALL TITLE_WITH_INTEGER_NUMBER_ADDED('ENDO. PROD. AMT
IN INFANT', 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
USERMODEL_ODE_INTEGRATED(TX,AMT,P,NP,1,TZERO,YZERO,NEQN)
    TY = AMT(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, HBTOTALPHLEB,
HBTOTALTRANS, MAXHBAMT))
  CALL BEGIN_X_AT(TZERO(1))
  CALL END_X_AT(XMAX)
  CALL ADD_CONSTRUCTED_CURVE
  CALL PLOT_IN_AREA(2,4)

```

```

        CALL TITLE_WITH_INTEGER_NUMBER_ADDED('TRANS. AMT IN
INFANT', 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
USERMODEL_ODE_INTEGRATED(TX,AMT,P,NP,1,TZERO,YZERO,NEQN)
            TY = AMT(2)
        END DO
        CALL LEFT_LABEL('HEMOGLOBIN AMT. (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_CONSTRUCTED_CURVE
        CALL PLOT_IN_AREA(4,4)

        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
USERMODEL_ODE_INTEGRATED(TX,AMT,P,NP,1,TZERO,YZERO,NEQN)
            TY = AMT(1) + AMT(2)
            MAXHBAMT = MAX(TY, MAXHBAMT)
        END DO
    
```

```

CALL ADD_MARGIN_TEXT_WITH_INTEGER_NUMBER_ADDED('-
INFANT:', SUBNO)

CALL TITLE_WITH_VALUE_ADDED('TOTAL AMT. REMOVED
=', HBTOTALPHLEB)
IF ( NPHLEB > 0 ) THEN
  TEMPSUM = 0D0
  DO J = 1, NPHLEB
    CALL GET_TIME_OF_PHEBOTOMY(J, TEMPT)
    CALL GET_PHEBOTOMY_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(TEMPX, TEMPY, NTRANS*2+1)
CALL PLOT_IN_AREA(2,4)

CALL TITLE_WITH_VALUE_ADDED('TOTAL AMT. PRODUCED
=',HBTOTALPROD)
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, HBTOTALPHLEB,
HBTOTALTRANS, 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 INFANT', 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
USERMODEL_ODE_INTEGRATED(TX,AMT,P,NP,1,TZERO,YZERO,NEQN)
TY = AMT(1) + AMT(2)
END DO
CALL LEFT_LABEL('HEMOGLOBIN AMT. (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_CONSTRUCTED_CURVE
CALL PLOT_IN_AREA(4,4)

CALL DISPLAY_PLOT
CALL RECORDPLOTIFSAVED(LUN)
END IF

! ===== CONSTRUCTION OF PLOTs OF OBSERVED AND
FITTED HEMOGLOBIN, RBC, EPO CONCENTRATIONS AND BODYWEIGHTS
=====
WRITE(*,"(/, ' WOULD YOU LIKE A PLOT OF THE OBSERVED AND
FITTED HEMOGLOBIN, RBC, EPO CONCENTRATIONS AND BODWEIGHTS?')")
READ(*,*) RESPONSE
IF ( RESPONSE == 'Y' .OR. RESPONSE == 'y' ) THEN
CALL ADD_MARGIN_TEXT_WITH_INTEGER_NUMBER_ADDED('-
INFANT:', SUBNO)
CALL TITLE('HEMOGLOBIN PROFILE')
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
USERMODEL_ODE_INTEGRATED(TX,AMT,P,NP,1,TZERO,YZERO,NEQN)
      TY = (AMT(1) + AMT(2))/(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 PLOT_IN_AREA(1, 4)

      CALL TITLE('RBC PROFILE')
      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
USERMODEL_ODE_INTEGRATED(TX,AMT,P,NP,1,TZERO,YZERO,NEQN)
      TY = (((AMT(1)/MCHE) +
(AMT(2)/MCHT))*10D0)/(BVOL*BODYWEIGHT(TX))
      END DO
      CALL LEFT_LABEL('RBC (10^6/UL)')
      CALL X_LABEL('TIME (DAYS)')
      CALL BEGIN_LEFT_AT(0D0)
      CALL BEGIN_X_AT(TZERO(1))
      CALL END_X_AT(XMAX)
      CALL ADDOBSERVATIONSLEFT(2)
      CALL ADD_CONSTRUCTED_CURVE
      CALL PLOT_IN_AREA(2, 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(3, 4)

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(4, 4)

CALL DISPLAY_PLOT
CALL RECORDPLOTIFSAVED(LUN)
END IF

! ===== CONSTRUCTION OF OUTPUT FILE OF FITTED
HEMOGLOBIN, RBC, EPO CONCENTRATIONS AND BODYWEIGHTS
=====
WRITE(*, "(/, ' WOULD YOU LIKE TO CREATE AN OUTPUT FILE OF THE
FITTED VALUES?')")
READ(*, *) RESPONSE
IF ( RESPONSE == 'Y' .OR. RESPONSE == 'y' ) THEN
  WRITE(9, "( ' AGE(DAYS)  HB(G/DL)  RBC(10^6/UL)  EPO(MU/ML)
BODYWEIGHT(KG)')")
  DELTA = XMAX/MAXN
  TX = 0D0
  DO J = 1, MAXN+1
    CALL
USERMODEL_ODE_INTEGRATED(TX, AMT, P, NP, 1, TZERO, YZERO, NEQN)
    TEMP(1) = (AMT(1) + AMT(2))/(BVOL*BODYWEIGHT(TX))
    TEMP(2) = (((AMT(1)/MCHE) +
(AMT(2)/MCHT))*10D0)/(BVOL*BODYWEIGHT(TX))
    TEMP(3) = CEPO(TX)
    TEMP(4) = BODYWEIGHT(TX)
  
```

```

        WRITE(9,"(1X,F10.5,5X,F10.5,F10.5,5X,F10.5,5X,F10.5)") TX, TEMP(1),
TEMP(2), TEMP(3), TEMP(4)
        TX = TX + DELTA
    END DO

```

```

        END IF
    END IF
END IF
RETURN

```

```

! ===== RECORD THE ID FOR THE MODEL USED IN THE FITTING
=====
    ENTRY MODELID(ID)
    ID = 'VLBW_V2.2.1.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
    TOTAL_BODY_STIM_RATE = ((BODYWEIGHT(X)**MRS)*FSTIM(X)
END FUNCTION TOTAL_BODY_STIM_RATE

```

```

REAL*8 FUNCTION STIM_RATE(X)
! PURPOSE: TO CALCULATE THE STIMULATION RATE OF HEMOGLOBIN
AT TIME X
    USE FSTIM_EPO_MODULE
    IMPLICIT NONE
    REAL*8 :: X
    STIM_RATE = FSTIM(X)
END FUNCTION STIM_RATE

```

```

! ===== END OF FILE =====

```

E.5.2 BODYWEIGHT_MODULE_V1.5.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
!
! REVISIONS:
! VERSION 1.0  APR 21, 2008  ORIGINAL CODE-KJF.
!       1.1  MAY 1, 2008  MODIFIED THE REPRESENTATION OF BODY
WEIGHT FROM
!
!           LINEAR INTERPOLATION FUNCITON TO A SMOOTHED
!           CUBIC SPLINE FUNCTION BASED ON THE COEFFICIENT
!           OF VARIATION-KJF.
!       1.2  MAY 1, 2008  MODIFIED THE REPRESENTATION OF THE BODY
WEIGHT DATA
!
!           TO A 3RD ORDER POLYNOMIAL-KJF
!       1.3  JUN 5, 2008  MODIFIED TO REMOVE BACK EXTRAPOLATION
BEYOND
!
!           BIRTHWEIGHT-KJF
!       1.4  JUL 20, 2008  MODIFIED THE REPRESENTATION OF THE BODY
WEIGHT DATA
!
!           FROM A 3RD ORDER TO A 4TH ORDER POLYNOMIAL-
KJF.
!       1.5  DEC 17, 2008  REMODIIFIED TO DO BACK EXTRAPOLATION TO
IN UTERO BODY WEIGHTS.
!
! 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
  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'
  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! INCONSISTENT 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 = 3.4483 ! C2: EXPONENT OF BODY WEIGHT CURVE FROM
22 TO 32 WEEKS OF GESTATIONAL AGE
REAL*8 :: T, X, INTERCEPT, DERIVATIVE, BIRTHBW
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 ) THEN

```

```

INTERCEPT = BWCOEF(1) + BWCOEF(2)*BWT(1) +
BWCOEF(3)*(BWT(1)**2) + BWCOEF(4)*(BWT(1)**3) +
BWCOEF(5)*(BWT(1)**4)
DERIVATIVE = BWCOEF(2) + 2D0*BWCOEF(3)*BWT(1) +
3D0*BWCOEF(4)*(BWT(1)**2)+ 4D0*BWCOEF(5)*(BWT(1)**3)
BIRTHBW = INTERCEPT + DERIVATIVE*(0D0 - BWT(1))
X = GESTATIONAL_AGE_AT_BIRTH + T
BODYWEIGHT =
(C1*(X**C2))*(BIRTHBW/((C1*(GESTATIONAL_AGE_AT_BIRTH**C2))))
ELSE IF ( T < BWT(1) ) THEN
INTERCEPT = BWCOEF(1) + BWCOEF(2)*BWT(1) +
BWCOEF(3)*(BWT(1)**2) + BWCOEF(4)*(BWT(1)**3) +
BWCOEF(5)*(BWT(1)**4)
DERIVATIVE = BWCOEF(2) + 2D0*BWCOEF(3)*BWT(1) +
3D0*BWCOEF(4)*(BWT(1)**2)+ 4D0*BWCOEF(5)*(BWT(1)**3)
BODYWEIGHT = INTERCEPT + DERIVATIVE*(T - BWT(1))
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

```

E.5.3 FSTIM_EPO_MODULE_V2.1.F90

```

! FILENAME = FSTIM_EPO_MODULE_V2.1.F90
!
! PURPOSE: TO STORE PARAMETERS AND ROUTINES TO CALCULATE
! THE STIMULATION RATE AND EPO CONCENTRATION
!
! REVISIONS:
! VERSION 2.0 DEC 15, 2008 ORIGINAL CODE-KJF.
! VERSION 2.1 FEB 6, 2009 MODIFIED SO EPO IS ALWAYS NON-
NEGATIVE USING A FLOOR FUNCTION
! AND SUCH THAT THE IN UTERO STIMULATION RATE IS
A PARAMETER
! (I.E. NO LONGER A FUNCTION OF BACK-EXTRAPOLATED
TO BIRTH EPO
! CONCENTRATION)
!
! 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
! INUTEROSTIM: IN UTERO STIMULATION RATE
!

```

```

MODULE FSTIM_EPO_MODULE

```

```

  IMPLICIT NONE

```

```

  SAVE

```

```

  REAL*8          :: VMKM, IKM, MRS, INUTEROSTIM

```

```

  REAL*8, PARAMETER :: T_ZERO = 0D0

```

```

CONTAINS

```

```

  REAL*8 FUNCTION CEPO(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)

```

```

    ELSE

```

```

        CEPO = 0D0

```

```

    END IF

```

```

    CEPO = MAX(CEPO, 0D0)

```

```

    END FUNCTION CEPO

```

```

  REAL*8 FUNCTION FSTIM(T)

```

```

    ! PURPOSE: TO CALCULATE THE VALUE OF THE STIMULATION RATE

```

```

  FUNCTION

```

```

    IMPLICIT NONE

```

```

    REAL*8 :: T

```

```

    IF ( T < T_ZERO ) THEN

```

```

        FSTIM = INUTEROSTIM

```

```

ELSE
  FSTIM = VMKM*CEPO(T)/(IKM*CEPO(T) + 1D0)
END IF
END FUNCTION FSTIM

END MODULE FSTIM_EPO_MODULE

```

E.5.4 PHLEB..._TRANSFUSION_MODULE_V1.2.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-KJF.
!   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-KJF.
!   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
    END DO
  END SUBROUTINE GET_NUMBER_PHLEBOTOMIES

```

```

    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)
! PURSPOSE: 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
  AP(J) = A(J)
END DO
IF ( N < MAXPHLEB ) THEN
  DO J = N+1, MAXPHLEB
    AP(J) = 0D0
  END DO
END IF
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 PHLEBOTIMIES'
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 EQAUL TO THE STOP TIME'
CALL GET_NUMBER_PHEBOTOMIES_PRECEEDING(TSTART, NSTART)
CALL GET_NUMBER_PHEBOTOMIES_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_PHEBOTOMY_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
AMOUNTNT VECTOR LARGER THAN MAXIMUM SIZE'
IF (N /= NOTRANS ) STOP ' ERROR! INCONSITENT 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 TRANSUFIONS
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, EXTERNAL :: 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

```

E.5.5 DUMMY_USERMODEL_ODE_JACOBIAN.F90

```

! THIS IS A DUMMY ROUTNE TO BE USED IN THE WINFUNFIT LINKAGE IF
THE
! JACOBIAN OF THE ODE'S IS NOT TO BE USED
!

```

```

SUBROUTINE USERMODEL_ODE_JACOBIAN (T,Y,DFDT,DFDY, N, P,NP,IFUN)
  IMPLICIT NONE
  INTEGER,          INTENT (IN) :: N, NP, IFUN
  DOUBLE PRECISION, INTENT (IN) :: T
  DOUBLE PRECISION, DIMENSION (N), INTENT (IN) :: Y
  DOUBLE PRECISION, DIMENSION (N,N), INTENT (OUT) :: DFDT
  DOUBLE PRECISION, DIMENSION (N,N), INTENT (OUT) :: DFDY
  DOUBLE PRECISION, DIMENSION (NP), INTENT (IN) :: P
! LOCALS
  DOUBLE PRECISION, PARAMETER          :: ONE = 1D0
  DFDT(1) = ONE
  DFDY(1,1) = ONE
END SUBROUTINE USERMODEL_ODE_JACOBIAN

```

**APPENDIX F. LIST OF PEER REVIEWED PUBLICATIONS FROM THE
UNIVERSITY OF IOWA BY KEVIN J. FREISE**

- K. J. Freise**, J. A. Widness, and P. Veng-Pedersen. Erythropoietic response to endogenous erythropoietin in premature very low birth weight infants. Submitted to *J Pharmacol Exp Ther* (2009).
- K. J. Freise**, R.L. Schmidt, J. A. Widness, and P. Veng-Pedersen. Pharmacodynamic modeling of the effect of changes in the environment on cellular lifespan and cellular response. *J Pharmacokinet Pharmacodyn* **35**: 527-552 (2008).
- P. Veng-Pedersen, **K. J. Freise**, R.L. Schmidt, and J. A. Widness. Pharmacokinetic Differentiation of Drug Candidates using System Analysis and Physiological-based Modeling. Comparison of C.E.R.A. and Erythropoietin. *J Pharm Pharmacol* **60**: 1321-1334 (2008).
- K. J. Freise**, J. A. Widness, R.L. Schmidt, and P. Veng-Pedersen. Modeling time variant distributions of cellular lifespans: increases in circulating reticulocyte lifespans following double phlebotomies in sheep. *J J Pharmacokinet Pharmacodyn* **35**: 285-323 (2008).
- K. J. Freise**, R.L. Schmidt, E. L. Gingerich, P. Veng-Pedersen, and J. A. Widness. The effect of anticoagulant, storage temperature, and dilution on cord blood hematology indices over time. *Int J Lab Hematol*, In press (2008).
- K. J. Freise**, J. A. Widness, R.L. Schmidt, and P. Veng-Pedersen. Pharmacodynamic analysis of time-variant cellular disposition: reticulocyte disposition changes in phlebotomized sheep. *J Pharmacokinet Pharmacodyn* **34**: 519-547 (2007).
- K. J. Freise**, J. A. Widness, J. A. Segar, R.L. Schmidt, and P. Veng-Pedersen. Increased erythropoietin elimination in fetal sheep following phlebotomy. *Pharm Res* **24**: 1653-1659 (2007).
- J. A. Widness, R.L. Schmidt, R. J. Hohl, F. D. Goldman, N. H. Al-Hunti, **K. J. Freise**, and P. Veng-Pedersen. Change in erythropoietin pharmacokinetics following hematopoietic transplantation. *Clin Pharmacol Ther* **81**: 873-879 (2007).

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2. S. T. Callender, E. O. Powell, and L. J. Witts. The life span of the red cell in man. *J Pathol Bacteriol* **57**: 129 (1945).
3. G. M. Brown, O. C. Hayward, E. O. Powell, and L. J. Witts. The destruction of transfused erythrocytes in anemia. *J Pathol Bacteriol* **56**: 81 (1944).
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