REDS-II Donor Iron Status Evaluation (RISE) Study

For the National Heart, Lung, and Blood Institute
Retrovirus Epidemiology Donor Study-II

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REDS-II DONOR IRON STATUS EVALUATION (RISE) STUDY

SUMMARY

Iron depletion is a known consequence of blood donation. Although the overall health significance of iron depletion in blood donors is uncertain, iron depletion leading to iron deficient erythropoiesis and lowered hemoglobin levels results in donor deferral and, occasionally, in mild iron deficiency anemia. Hemoglobin deferrals represent more than half of all donor deferral, deferring 16% of women.

Several cross sectional studies of blood donors, using older measures of iron status in blood donors have indicated that female gender, frequent donation and not taking iron supplements are predictors of iron depletion. However, none of these studies have included racial/ethnic, anthropomorphic, or behavioral factors and none have evaluated the impact of newly discovered iron protein polymorphisms.

Among the six REDS-II centers, we propose a 19-20 month longitudinal study of iron status in two cohorts of blood donors: a first time/reactivated donor cohort of 800 enrolled donors (450 with at least one return visit) in which baseline iron and hemoglobin status can be assessed without the influence of previous donations, and a frequent donor cohort of 1,440 enrolled donors (1080 with at least one return visit) , where the cumulative effect of additional frequent blood donations can be assessed. Each cohort will donate blood and test samples frequently during the study period.

Hemoglobin levels and a panel of iron protein and red cell and reticulocyte indices will be measured at baseline and at a final follow-up visit 15-24 months after the baseline visit (average 19-20 months). Donors will also complete a self-administered survey assessing past blood donation, smoking history, use of vitamin/mineral supplements, iron supplements, aspirin, frequency of heme rich foods intake, and, for females, menstrual status and pregnancy history at these two time points. A DNA sample will be obtained at the baseline visit to assess three key iron protein polymorphisms. Further, fingerstick hemoglobin or hematocrit values will be obtained at each interim visit between the baseline and final follow up visits and plasma will be collected for selected measurements of ferritin, and sTfR levels at each study visit. Test results and survey data will be combined with demographic, anthropomorphic, racial/ethnic, and residence (altitude) data routinely compiled at all REDS-II centers. Finally, a plasma and DNA linked repository will be established to allow the future assessment of new genetic, chemical or cellular markers of iron status, as related to blood donation behavior and other measured parameters. A simplified flow chart for the proposed study is presented in Figure 1 below, whereas Figure 2 provides a summary of the data collection plan at each visit.

The three primary objectives of this study are to:

1. Evaluate the effects of blood donation intensity on iron and hemoglobin status and assess how these are modified as a function of baseline iron/hemoglobin measures, demographic factors, and reproductive and behavioral factors.

2. Identify the optimal laboratory measures that would predict the development of iron depletion, hemoglobin deferral, and/or iron deficient hemoglobin deferral in active whole blood and double red cell donors at subsequent blood donations.

3. Provide data to help formulate optimal whole blood donation frequency guidelines by establishing a model that predicts the development of iron depletion, hemoglobin deferral and/or iron deficient hemoglobin deferral in whole blood donors.

This study will develop better predictive models for the development of iron depletion and hemoglobin deferral (with or without iron deficiency) in blood donors. It will allow improved strategies for donor screening and open the possibility for customized donation frequency guidelines for individuals or classes of donors. It will provide important baseline information for the design of targeted iron supplementation strategies in blood donors, and improved counseling messages to blood donors regarding diet or supplements. Finally the elucidation of the effect of genetic iron protein polymorphisms on the development of iron depletion will enhance the understanding of the role of these proteins in states of iron stress, using frequent blood donation as a model.

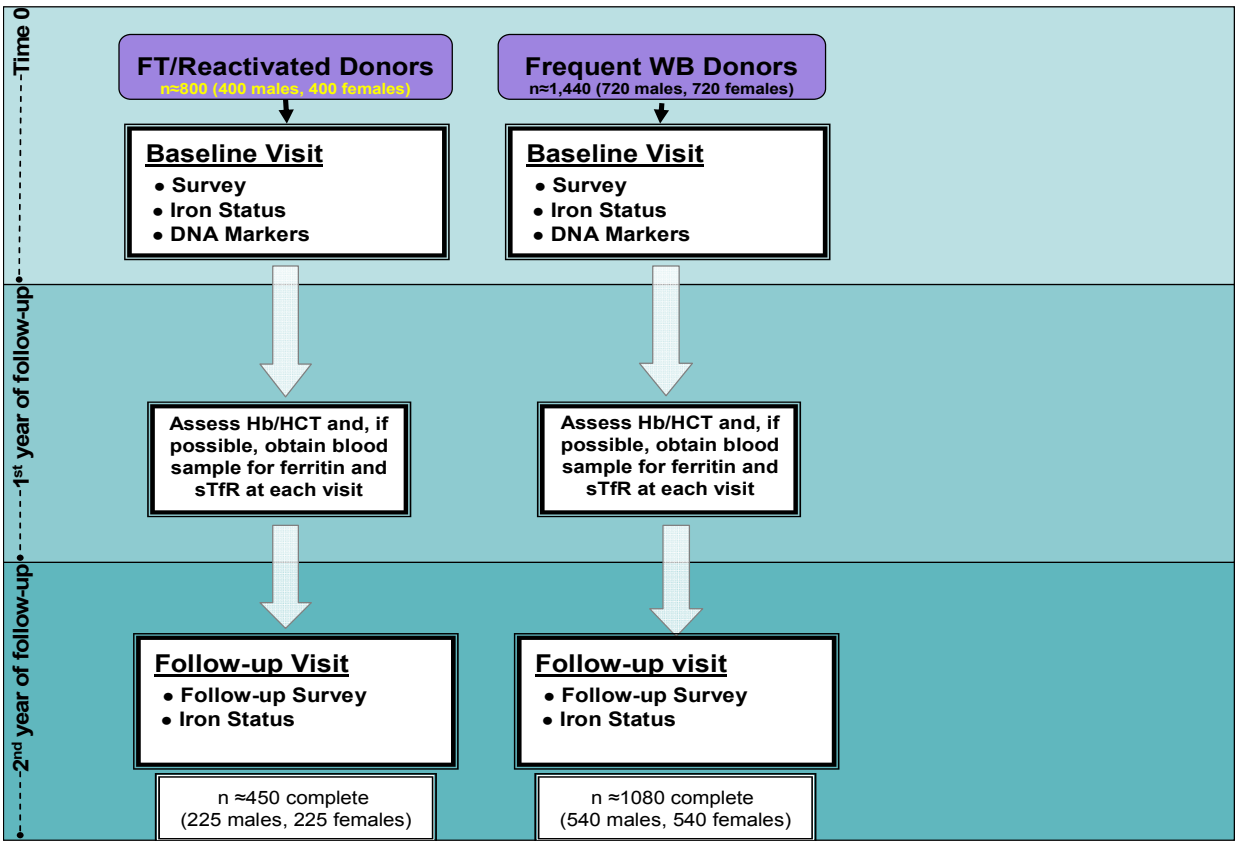


Figure 1. Study Flow Chart

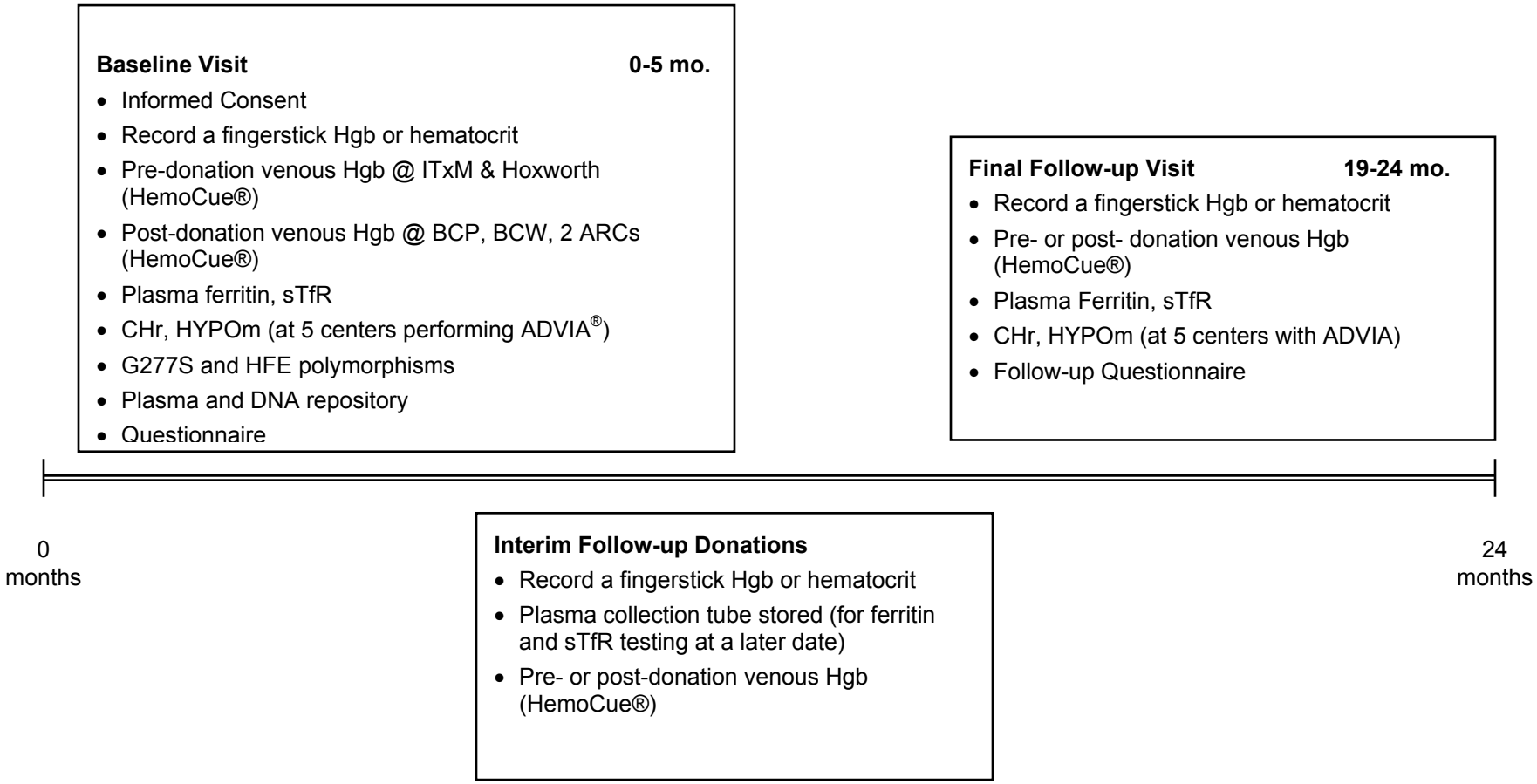


Figure 2. Activities for Each Study Visit

A. BACKGROUND AND SIGNIFICANCE

Hemoglobin Deferrals – A Major Problem for the Blood Supply

Deferral for low hemoglobin levels is the most common cause of presenting donor loss, particularly in females. Within the New England Region they represent more than half of all deferrals,[1] nearly all being women. Sixteen percent of women presenting at blood drives are deferred for their hemoglobin level, in contrast to 1% of male blood donors. The deferral of large numbers of presenting female donors, many of whom do not return and are again deferred on repeat presentation, leads to significant problems maintaining, let alone increasing the blood supply –a principal goal of REDS II.

Furthermore, this deferral is not uniformly distributed across all donor groups. For instance, in the Southeast Michigan Region of the Red Cross, the deferral rate for black women is approximately 30% (personal communication, Bruce Newman, Medical Director). Based on analysis of NHANES data, hemoglobin levels in Black men and women were found to be 0.8 g/dL lower on average than Caucasians. In addition, analysis of Red Cross data shows that deferral rates are lower at higher altitudes, explained by higher normal hemoglobin levels.[2]

Blood donors are required to have a hemoglobin level of at least 12.5 g/dL in the United States.[3,4] The National Health and Nutritional Evaluation Survey (NHANES) has identified the distribution of hemoglobin levels for healthy United States residents by periodic surveys.[5] An analysis of a subset of the NHANES II data for subjects shown to have adequate iron stores[6] shows that 12.5 g/dL is not an appropriate lower limit of normal for men or women of any race. It is clear that some iron-replete women are deferred, while some anemic men are allowed to donate.

Blood Donors and Iron Depletion

Regular blood donation, particularly every 8 weeks, is known to deplete blood donor iron stores.[7] The original description by Finch et al. used the newly available ferritin assay on blood donors to show that blood donation was associated with a significant drop in ferritin levels, which was related to the intensity of blood donation over the previous 4-5 years. [8]

The relationship of blood donation to iron stores was further elucidated by Simon, et al. in an elegant cross-sectional observational study of blood donors.[7] The frequency of iron depletion as measured by serum ferritin was 12% in female first time donors, but <3% in male first time donors, reflecting the impact of menstrual blood loss. Each successive lifetime donation reduced the mean ferritin values, particularly in men so that both sexes reached apparent iron equilibrium after approximately 5-6 lifetime donations. The overall frequency of iron depletion was 8% in male blood donors and 23% in female blood donors. Iron deficiency (as reflected by both a low ferritin and a transferrin saturation of < 16%) was found in 13% of female and 2% of male donors. Post-menopausal women had higher ferritin levels than pre-menopausal women but lower values than male donors. Pre-menopausal women taking iron supplements had improved iron stores approaching those of male donors. No change in hemoglobin levels were seen with blood donation. Furthermore, among donors with hemoglobin levels higher than required for blood donation, only a slight increase in ferritin levels was observed, confirming the insensitivity of one-time hemoglobin measurement as an assessment of body iron status.

Current Unstudied Aspects of Iron Depletion in Blood Donors

The Finch and Simon studies were conducted in the late 70's and early 80's. Since that time a reduction in the required hemoglobin level for blood donation in men from 13.5 g/dL to 12.5 g/dL and an increase in the nominal blood container volume from 450 to 500 mL have increased the likelihood that blood donors may develop depleted iron stores with regular donation. Some of these donors eventually are deferred for low hemoglobin, although we do not have validated models to predict which donors will be deferred. The increased use of intensive donor recruitment such as professional telerecruitment and direct mail marketing campaigns has probably also contributed to an increased frequency of blood donation since that time, which has likely increased the potential for iron depletion. Further, the study by Simon et al. was conducted in Albuquerque NM which has an altitude of 5000 feet[7] and in the early 1980's, when smoking rates were higher. These two factors would tend to increase blood donor hemoglobin levels which also may influence the prevalence of iron depletion.

Furthermore, other demographic variables such as race and behavioral variables such as use of mineral supplements in the blood donor population will have important influences on the prevalence of iron depletion in various donor groups and have not been adequately studied. The recent description of a polymorphism in transferrin, which increases the likelihood a menstruating women will be iron deficient,[9] and description of several genes for hemochromatosis, at least one of which (C282Y) has been shown in heterozygous first time blood donors to be associated with increased iron stores,[10] leads to the possibility that genetic markers might define "at risk" and "protected" donor groups with respect to iron depletion in frequent donors. Finally, the availability of new tests of body iron status (see below) that were not available at the time of Simon's study leads to the question of their utility to detect or to prevent the development of iron depletion and subsequent hemoglobin deferral in blood donors by identifying "at risk" donors.

The recent implementation of double red cell collections in most blood centers has the potential to substantially increase the intensity of blood donation in eligible donors: most men and larger women. Although the hemoglobin or hematocrit and weight requirements are generally higher than whole blood donation (and vary by machine manufacturer's 510k application to FDA) and the inter-donation interval is 16, rather than 8 weeks,[11] the goal is to obtain a greater number of red cells from desirable donors while making it more convenient. Thus, although the iron loss at maximum donation frequency is the same as for whole blood donation, double red cell donation is likely to increase the iron depletion potential, particularly for men who are the primary eligible population.

Iron Physiology in Men and Women

Iron is an essential element in many physiologic processes. In association with heme, it is involved in the reversible binding of oxygen by red blood cells, the myoglobin of muscle, and mitochondrial cytochromes. Non-heme iron plays a key role in the activity of many enzymatic reactions. Iron may also be toxic when present in excess: Absorption is tightly regulated because there is no mechanism for excretion. Dietary iron is absorbed in the proximal small intestine. Iron from animal sources (heme iron) has greater bioavailability (~35% absorbed) than nonheme (i.e., plant products) iron (~10% absorbed). Men normally absorb ~ 1 mg/day, equaling basal losses primarily from the gastrointestinal tract. Iron absorption in premenopausal women is greater, ~ 1.5 mg/day, because of additional losses from menstruation. Absorption capacity increases proportionate to the level of iron deficiency, up to 3-4 mg/day. Absorbed iron binds to transferrin and is transported to transferrin receptors located on early erythroid and all other nucleated cells. Iron not directly utilized in physiologic pathways

is stored in tissues as ferritin; small amounts present in blood are in equilibrium with tissue ferritin, the plasma level of which is considered a reliable indicator of available storage iron. Molecular defects have been described that impact iron homeostasis. In hereditary hemochromatosis, a protein that normally down regulates iron absorption is defective, leading to excessive accumulation of iron in the body. In addition, a common transferrin polymorphism (G277S mutation) has been elucidated that predisposes individuals to the development of iron deficiency.[9]

Total iron stores in men average approximately 50 mg /kg whereas women have 35 mg/kg. The majority of the total exists in red blood cells in the form of hemoglobin. Cook, et al., estimated tissue iron stores of 776 $\pm$ 313 mg in men and 309 $\pm$ 346 mg in women.[12] The loss of approximately 230 mg iron with each whole blood donation along with a limited absorption capacity leads to a high incidence of iron deficiency in frequent donors, especially women.

Factors Influencing the Development of Iron Depletion

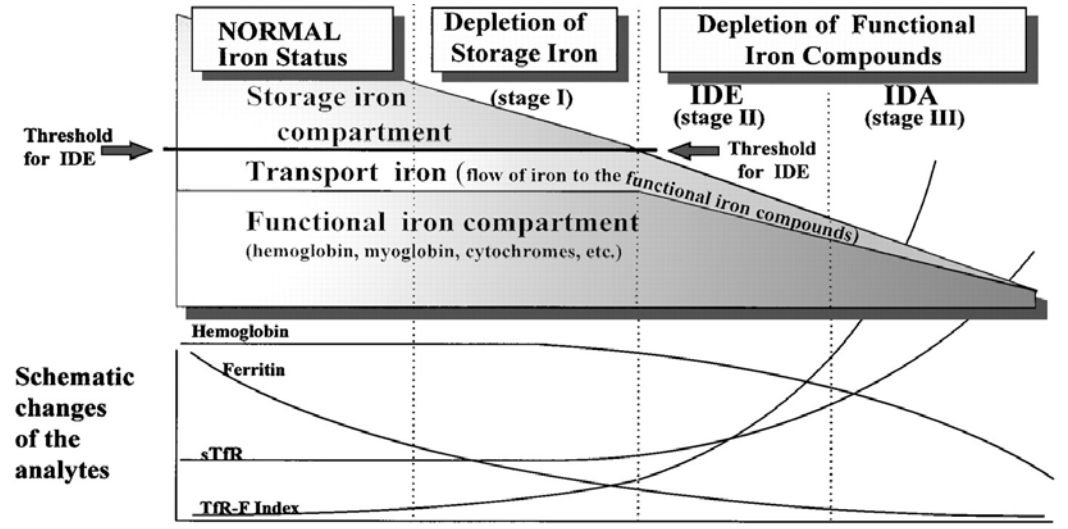
Other than the well described effects of donation frequency and menstrual/pregnancy status, little is known about the influence of other factors on the probability of iron depletion and iron deficiency developing in blood donors. Although donor size (and implied red cell volume) also affects how a donation of a fixed volume will cause iron depletion, most studies have ignored this variable and there is no useful quantitative data in the literature. The intensity of blood donation can be better measured as a percentage of RBC volume lost per time period, than as reported in previous studies as the number of donations per year or as the interval between donations. We therefore anticipate using this definition of donation intensity in our analysis.

Demographic variables (e.g., age, gender, race/ethnicity), genetic factors (e.g., transferrin G277S polymorphism, HFE markers), behavioral factors (e.g., mineral supplements, smoking) are all known to impact iron or hemoglobin status. The study proposed here will investigate how these factors interact with one another to influence the development of iron depletion and iron deficiency in blood donors.

Use of Peripheral Blood Tests to Identify Current and Predict Future Iron Depletion and Iron Deficient Erythropoiesis in Blood Donors

Measurement of hemoglobin is a poor indicator of iron depletion and iron deficient erythropoiesis (IDE). Thus, improved measures to identify current and predict future iron depletion and IDE are needed. Although it is believed that hemoglobin screening is intended, among other benefits, to prevent iron depletion in donors, we hypothesize those donors with higher, rather than lower baseline (first time donor) hemoglobin levels are at greater risk of developing iron deficient erythropoiesis when put under the stress of a regular donation program. This is because, on the one hand, iron replete donors with sufficient iron who fail the hemoglobin screen (women), do not donate and thus are not made iron deficient, and, on the other hand, frequent donors with higher baseline hemoglobin (say 16 g/dL) will be eligible to donate even though they may have IDE, with a hemoglobin of 13g/dL, fully 3 g/dL lower than normal for them. Note that donors with increased hemoglobin also donate more red cell hemoglobin per donation and therefore lose more iron per donation. The use of serial hemoglobin determinations at repeat donations, as opposed to only day-of-donation hemoglobin testing, may better identify donors who are at risk of developing iron deficiency anemia.

Iron depletion occurs in progressive stages, beginning with the gradual loss of storage iron, followed by the development of iron-deficient erythropoiesis (IDE), and culminating in iron-deficiency anemia (IDA), (see Figure A-1).[13] Recent studies have performed ferritin assays using both serum and plasma and have established that the plasma sample gave consistently lower values than serum samples. Using the obtained conversion factor, plasma as well as serum may be used for ferritin determinations.[34] Older studies of iron status in blood donors used various biochemical markers including serum iron, serum transferrin, transferrin saturation, and serum ferritin levels, as discussed above.[7,8] For these studies, iron depletion was defined as a ferritin concentration below 12ug/dl. This cutoff is a highly specific indicator of iron deficiency, but lacks sensitivity.[14, 15] A recent study found that this cutoff failed to identify iron deficiency in over 1/3 of cases in blood donors[16], and another found a higher ferritin level (22ug/dl) more indicative of functional iron depletion.[13] Both investigations based their findings on more sensitive measures of iron status based on serum (soluble) transferrin receptor (sTfR) levels, which reflect the functional iron compartment and have been shown to correlate with depleted iron stores in bone marrow preparations.[14] Serum TfR levels also show excellent correspondence to oral iron treatment in otherwise healthy anemic females.[17] The transferrin receptor is expressed primarily on the surface of erythroid cells. Reduced iron levels leads to increased TfR synthesis and shedding into circulating blood. Levels greater than 2.4-4.0 mg/L have been used to suggest tissue iron deficiency. In addition, ferritin measurements (which reflect storage iron) and sTfR values (which reflect functional iron) have been combined into a ratio, log (sTfR/ferritin), as a derived measurement. Use of the two reciprocally regulated measurements in this way appears to provide excellent discrimination of clinical IDA and early experience in blood donors suggests high sensitivity in the detection of iron depletion.[16]



Note: "Our Population" in Figure A-1 refers to the cited publication, not to this protocol

Figure A-1.[13] Iron Depletion, Iron Deficient Erythropoiesis (IDE) and Iron Deficiency Anemia (IDA)*

Although biochemical tests of iron status are useful as part of investigational studies, they are not suitable for blood donor screening because of their expense and the difficulty of performing them with rapid turn around in a blood center laboratory. More recent advances in the rapid assessment of functional iron status by measuring red blood cell indices have been made using a new generation of hematology analyzer (ADVIA® 120). In functional iron deficiency, a reduction in hemoglobinization of red blood cells results from an imbalance between iron supply and iron requirements of erythropoiesis. Analysis of the fraction of individual red blood cells with deficient hemoglobinization by laser scatter reflects recent changes in erythropoiesis, which may be at least as good and possibly superior to biochemical markers.[18] CHr (reticulocyte hemoglobin content) represents an indicator of iron availability to reticulocytes (i.e., iron incorporation within the 3 day lifespan of these cells), whereas the proportion of HYPOm (hypochromic mature red blood cells) is a time-averaged marker (iron availability within the 3 month lifespan of mature RBCs). These measurements have been likened to glucose and HbA1c levels in diabetics, respectively. These RBC indices have been found to be more sensitive detectors of functional iron deficiency than biochemical iron tests in renal failure patients receiving r-huEPO. In a study of female students with iron deficiency anemia, Kotisaari, et al found excellent correlation between sTfR and HYPOm (Area under Receiver Operating Characteristic (ROC) curve = 0.98), as well as correction in all iron-deficient subjects after oral elemental iron replacement.[19] In the only study to date employing the ADVIA® 120 analyzer in the evaluation of blood donor iron status, Radke, et al, found reasonable sensitivity of CHr and HYPOm individually (57%), and combined (69%) in the identification of iron-deficient erythropoiesis. Since biochemical measures such as sTfR and the hematologic indices CHr and HYPOm reflect iron currently available for erythropoiesis, normal values may be seen in a blood donor with absent iron stores, who is maintaining sufficient dietary iron intake. Thus, performance of these tests may be useful in assessing the ability of some donors who continue to successfully donate (without deferral) or in predicting future donor deferrals (at risk donors).

Currently at the 6 REDS-II centers, whole blood donors are deferred for a hemoglobin < 12.5 mg/dL by CuSO4 screening followed by HemoCue® hemoglobin or microhematocrit confirmation; or by microhematocrit testing only (using the equivalent hematocrit cut-off of 38%), performed on a finger stick blood sample. Additional iron depletion/IDE measures may be helpful if they could be implemented with relative ease at blood centers. Although RBC indices, Fe/TIBC, ferritin and EPP have classically been used in patients and in research studies to determine iron status and blood centers have routinely used only hemoglobin or hematocrit to evaluate donors, the newer markers sTfR, HYPOm, and CHr may be valuable for the blood center setting but need further evaluation. One or several of these tests may predict which donors are at high risk for hemoglobin deferral at their next donation. Although the EPP test was considered, it will not be conducted in this study due to methodological testing issues, lack of improved sensitivity/specificity compared to the other chemistry tests, and cost.

Significance

Clearly, a study conducted to better define the effects of blood donation intensity, demographics and behavioral characteristics on the development of iron depletion and the likelihood of being deferred for low hemoglobin is needed.

A model that predicts the development of iron depletion and likelihood of hemoglobin-deferral in previously acceptable whole blood donors can be developed and may help policy makers better define standards that determine which donors should be deferred or donate more or less frequently. Such a model can be developed around the variables of baseline and pre-donation hemoglobin and other

laboratory indices, donation history, donor demographics, donor estimated red cell volume, recent menstrual/pregnancy status, dietary habits, mineral supplement use and smoking. Such a model can also be used by blood centers to tailor recruitment strategies likely to be both safe for donors, avoiding iron depletion, and effective for the blood center, by lowering unnecessary donor deferrals.

We anticipate that the development of iron depletion in first-time or repeat blood donors will be more frequent in donors with high donation intensity, irrespective of baseline hemoglobin levels. As stated above, we also hypothesize that the development of iron depletion or deficiency in first time donors will be more frequent in donors with high baseline hemoglobin levels.

Further, identifying the optimal laboratory measures (newer and older markers of iron status, and hemoglobin levels) that predict the development of iron depletion in blood donors (we anticipate that most donors will give whole blood although some may give double red cell donations) or predict low hemoglobin deferral at subsequent blood donations will be of great interest. While all the available tests mentioned above can be added to the model, a simplified model using only more readily available laboratory indices (such as changes in hemoglobin) will be of even greater practical value.

Ordinarily all donors are encouraged to donate whole blood every eight weeks or double red cells every 16 weeks without knowledge of their iron status. If donors with low iron stores could be prospectively identified and asked to return at a date later than 8 or 16 weeks, deferral at the time of donation could be prevented. Thus, this model could be effectively used by blood centers to tailor individualized recruitment/donation schedules strategies that will prevent low hemoglobin deferral. This would result in increased safety for blood donors by preventing development of anemia/iron deficiency and increased blood collections for the blood center by preventing loss of blood donation by these willing donors. Thus, this proposal addresses two key concerns in blood banking: increasing the safety of donors, and increasing availability of the blood supply.

The information obtained from this study will also help tailor management of hemoglobin/iron-deferred donors. For example: When will it be safe and effective to recommend that a particular deferred donor returns to donate? How often should a hemoglobin/iron deferred donor donate in the future? Is the donor's status compatible with the developed model for normal donors, or does the donor have an abnormality which should be evaluated/treated by his/her doctor?

Such data will also improve donor education and counseling messages. Currently there is no consensus in the Blood Bank community about the specific steps that should be taken to prevent donors from developing iron deficiency or to educate blood donors about the effect of blood donation on their iron status. For example, we propose to inquire about general dietary habits (although we do not propose to conduct extensive dietary surveys), and through this, we may be able to test the apparent benefit of the advice some blood centers give to regular or deferred blood donors to eat red meat frequently. As another example of a benefit from the model we propose to develop, if mineral supplements are shown to be helpful in preventing iron depletion and resulting blood donor deferral in a subset of donors, these donors can be targeted for education/counseling messages about taking supplements.

Elucidation of the influence of the known genetic polymorphisms in iron proteins on blood donor iron status will enhance understanding of the role of these proteins in states of iron stress, using frequent blood donation as a model. It will also provide potentially useful markers in identifying donors at particular risk or protection from iron depletion.

Although the study is not powered to derive highly accurate normal hemoglobin values (or reference ranges for the studied iron tests) for men and women donors of different ages and races, a comparison of the blood donor population with the general United States population is planned; any extreme findings related to the selected nature of the blood donor population would be ascertained.

Finally, data obtained from the study will be invaluable in designing appropriate candidate iron supplementation strategies for study.[20] Such a study could be an important future REDS II initiative.

B. OBJECTIVES

The main objectives of the proposed study are delineated below:

B.1 Primary Objectives

1. Evaluate the effects of blood donation intensity on iron and hemoglobin status and assess how these are modified as a function of baseline iron/hemoglobin measures, demographic factors, and reproductive and behavioral factors.

2. Identify the optimal laboratory measures that would predict the development of iron depletion, hemoglobin deferral, and/or iron deficient hemoglobin deferral in active whole blood and double red cell donors at subsequent blood donations.

3. Provide data to help formulate optimal whole blood donation frequency guidelines by establishing a model that predicts the development of iron depletion, hemoglobin deferral and/or iron deficient hemoglobin deferral in whole blood donors.

There are limited studies assessing the relationship of iron depletion and hemoglobin among blood donors, let alone any compound effect due to donation intensity. Further, few studies have considered possible alternative practical predictors of iron stores, other than hemoglobin and hematocrit. Hence, the analyses addressing the primary objectives are to be taken as exploratory. Descriptive models will be developed and guidelines/recommendations obtained from them are meant to be suggestive. Important new observations may require additional study for confirmation.

B.2 Secondary Objectives

1. Evaluate hemoglobin distributions in the blood donor population (eligible and deferred donors) and compare with NHANES data.

2. Elucidate key genetic influences on hemoglobin levels and iron status in a donor population as a function of donation history.

3. Establish a plasma and DNA archive to evaluate the potential utility of future iron studies and genetic polymorphisms

C. STUDY OVERVIEW

Study Visits

The study is proposed as an approximately 19-20 month longitudinal study of regularly recruited blood donors. Two cohorts will be established and followed through time (minimum of 15 and maximum of 24 months of follow-up):

1. A first time/reactivated donor (no donations for 2 years) cohort (FT) and

2. A frequent donor cohort (men ≥ 3 whole blood donations in the last year; women ≥ 2 whole blood donations in the last year – or equivalent double red cell donations) (RPT).

Baseline Visit. Both cohorts will provide baseline laboratory iron, hemoglobin, and red cell indices studies, genetic markers, and information on demographic, menstrual/pregnancy, smoking, mineral supplement, and dietary habits variables.

During the enrollment donation, approximately 12 – ml of EDTA whole blood will be collected in 2 – lavender top vacuum tubes for DNA and iron testing. At baseline, hemoglobin/hematocrit levels will be measured in three ways: a fingerstick quantitative hemoglobin or hematocrit at all centers, a post-donation venous hemoglobin at 4 centers, and a pre-donation venous hemoglobin at 2 centers. The choice of having a post- or pre- donation venous hemoglobin sample is dictated by the method used to collect blood at each center. Venous hemoglobin will be measured by the ADVIA® hematology analyzer at 5 centers, as well as by the HemoCue® method at all centers. The numbers of subjects will be sufficient to define the relationship between these measures. (See Section H.2.1)

Further, a plasma and DNA repository will be established at baseline to evaluate the utility of potential future iron tests and genetic polymorphisms.

Follow-up Period and Interim Study Visits. During the 15-24 month (average 19-20 months) study period, first time/reactivated (FT) and frequent (RPT) donor cohorts will be recruited per routine blood center procedures. However, these donors will have been encouraged during the consent process and through newsletters to donate 2 or 3 whole blood donations per year - or the equivalent in double red cell donations. It is anticipated that this frequency of donation is more likely to be achieved in the frequent donor cohort. We also expect that about 10% of the donations given in this study will be double red blood cells while the large majority will be whole blood.

At enrollment in the study, donors will be asked to return to donate at the original sites of recruitment (primarily fixed donor centers) for approximately the next 2 years. During that period, a fingerstick quantitative hemoglobin or hematocrit will be performed, following approved center operational procedures, on all donors (including study enrollees) presenting to donate at these recruitment sites to determine donor eligibility and to evaluate quantitative hemoglobin or hematocrit levels of study participants. This will require several centers to modify current operational procedures of first performing a copper sulfate hemoglobin screen for donor assessment at these sites. In an effort to obtain a quantifiable fingerstick hemoglobin value consistently at all centers, it would be desirable for all centers to use the HemoCue® at all recruitment sites for a period of 2 years. If this is impossible operationally, centers will use their current confirmatory quantitative method to perform fingerstick quantitative hematocrit on all donors during the study period at the participating donation sites. Also, as an operational

alternative, those collection sites with online access to the donor data base, and an ability to flag research subjects in the data base, can develop approved procedures to reliably identify research subjects and obtain the required quantitative hematocrit/hemoglobin only on these research donors.

We also propose obtaining ferritin and sTfR levels on as many enrolled donors as possible at interim study visits by collecting and freezing plasma from an EDTA tube for selected analysis (see below). This will be easily accomplished at some centers by utilizing already available "retention tubes" but at other centers will require operational staff to identify study participants and collect an additional tube.

Enrolled donors deferred for hemoglobin or hematocrit during study visits will be asked at the deferral visit or, if operational constraints prevent this, within 4 weeks of deferral to provide a blood sample for iron studies and a repeat fingerstick hemoglobin (or hematocrit). Deferred donors will be encouraged to continue donation per participating center operating procedures. Similarly, medical management of hemoglobin-deferred donors will be per routine center operating procedures.

Final Follow-up Visit. At the end of the study period, all donors will be asked to return to the blood center to provide, similar to the enrollment visit, a full set of iron, hemoglobin, and red cell tests and to complete a follow-up survey. Genetic tests will not be performed at this visit. These blood samples and survey will usually be obtained during a blood donation visit but could be otherwise scheduled.

Data Compilation and Analysis

All data obtained at baseline and during interim and final follow-up will be compiled in a dataset. Using baseline visit data, we will

- Characterize donors' iron status by donation history, demographic characteristics, reproductive and behavioral factors;
- Evaluate hemoglobin distributions in first-time/reactivated donors and compare these distributions to those of the general population using available NHANES data;
- Evaluate normal hemoglobin levels in iron-replete donors to update previous analyses;[6]
- Characterize key genetic influences on hemoglobin status and iron status; and
- Characterize relationships between newer and older measures of iron status.

Further, at completion of the study, we will develop models that identify the iron, hemoglobin, and red cell laboratory measures and other factors such as blood donation intensity, baseline iron/hemoglobin measures, and demographic, reproductive and behavioral factors that predict the subsequent development of iron depletion and hemoglobin deferral (with or without iron deficient erythropoiesis).

Major Variable Definitions

In order to develop a model of factors leading to iron deficiency and depletion among blood donors, we intend to use the following definitions:

- **Iron depletion** will be defined in two alternate ways: As serum ferritin < 12 ng/mL or as ferritin < 22 ng/mL. Although serum ferritin < 12 ng/mL is not considered an adequately sensitive indicator of iron status, it is the marker which has been most frequently used in studies on blood donors[7]. It thus has the advantage of allowing comparison to most previous studies. More recent studies[13] suggest that a ferritin cut-off < 22 ng/mL provides better correlation with bone marrow iron status. Therefore, this cut-off will be used as a separate indicator of iron depletion.

- **Iron deficient erythropoiesis (IDE)** will be defined as log (sTfR/ferritin) exceeding the 97.5th percentile of the gender specific distribution obtained for first time donors in the study.

- **Hemoglobin deferral** will be defined as fingerstick hemoglobin <12.5 g/dL or as fingerstick microhematocrit < 38% performed on a presenting donor (depending on test of record for center).

- **Iron deficient hemoglobin deferral** will be defined as hemoglobin deferral in association with IDE.

- **Donation intensity** will be defined as the percent red cell volume lost per month. Further detail of defining donation intensity is given in section H.

Additional variable definitions are in section H.

D. STUDY POPULATION

D.1 Inclusion Criteria

To evaluate various donation intensity levels, the following eligible whole blood donors will be enrolled and followed for up to two years:

- First-time and reactivated donors (FT) (donors whose last donation was > 2 years ago) ≥ 18 yo presenting to give a whole blood or double red cell donation at enrollment.
- Frequent repeat (RPT) donors, ≥18 yo, with a history of ≥3 annual whole blood donations in the last year for men and ≥2 annual whole blood donations in the last year for women – or double red cell equivalent, presenting to give a whole blood or double red cell donation at enrollment.
- FT/reactivated donors meeting the above criteria who will commit to 2 additional donations or more per year for two years and to give a final blood sample and complete a follow-up survey about 19-20 months after the baseline visit.
- Repeat donors meeting the above criteria who will commit to maintain or exceed their current (last year) donation frequency for two more years and to give a final blood sample and complete a follow-up survey about 19-20 months after the baseline visit.
- Donors in either cohort who are deferred for whole blood donation by hemoglobin or hematocrit during the course of the study after the enrollment visit.
- Recruitment will generally occur only at fixed donation sites but may occur at mobile sites scheduled ≥ 3 times per year if necessary for recruitment of subjects.

D.2 Exclusion Criteria

- Donors unwilling to commit to their assigned donation frequency
- Donors deferred at the enrollment visit.
- Enrolled donors whose enrollment samples cannot be completely tested or who do not provide an enrollment survey within time requirements.

E. STUDY SIZE

E.1 Sample Size Considerations

This section defines five sets of hypotheses related to the primary objectives delineated in B.1. Each of these hypotheses is already generally accepted among the Blood Bank community, thus the sample sizes and statistical power are meant to attest that the exploratory analyses to address the primary objectives will be constructive. These five sets of hypotheses are detailed below. Hypotheses 1 and 2 address the likelihood of being hemoglobin-deferred, whereas hypotheses 3, 4 and 5 concern changes over time in ferritin. Further the hypotheses are divided among FT/reactivated donors (hypotheses (1, 3, and 4) and repeat donors (hypotheses 2 and 5). Sample sizes are driven by the ferritin changes in FT/reactivated female donors (hypothesis 3), and the ferritin changes in repeat female donors (hypothesis 5).

E.1.1 First-time/Reactivated Donor Sample Size Based on First Hypothesis

> ***Hypothesis 1*:** The likelihood of hemoglobin deferral in male and female first time blood donors is more frequent in donors with high donation intensity, irrespective of baseline hemoglobin levels.

We restrict attention to female donors and we will draw an equal size sample of male donors. Based on NEARC data, we assumed that the likelihood of being hemoglobin-deferred at least once during the study period would be 25% and 1.3% for first-time female and male donors, respectively (we used 7/2004 to 12/2005 data from NEARC with hemoglobin-deferral defined as any hemoglobin-deferred donation occurring in the 18 months following a first-time donation).

FT/reactivated donors can be categorized as high or low donation intensity at the end of the study period. Donation intensity is defined as the percent red cell lost per month and will be categorized as high (top 50^{th} percentile) or low (bottom 50^{th} percentile) based on its distribution among participants in the study. We assume 16% of low donation intensity FT/reactivated female donors and 32% of high donation intensity FT female donors will be hemoglobin-deferred at least once during the study period, thus, a risk ratio of 2.0.

A sample of 225 female FT/reactivated donors will have 88% power in a two-tailed 0.05 level test to detect a risk ratio of 2.0 between the prevalence of hemoglobin deferral in women with high donation intensity and the prevalence of hemoglobin deferral in women with low donation intensity. Assuming 57% of donors will be successfully followed (i.e. give at least one additional successful sample beyond the enrollment donation) then we would need to have 400 FT/reactivated eligible female donors fully enrolled in the study (i.e., with baseline laboratory data) for 88% power.

Hemoglobin deferral among male FT/reactivated donors is rare (i.e. 1.3%), hence a relationship between hemoglobin deferral and donation intensity among male FT/reactivated donors is not expected. Nonetheless, if we enroll an equal sample of male FT/reactivated donors, and we assume a 4% loss because of an inability to collect a sample, inadequate volume, broken vial, etc at baseline, then we need to enroll 840 FT/reactivated donors (420 males, 420 females) to have about 800 FT/reactivated eligible fully enrolled donors.

E.1.2 Repeat Donor Sample Size Based on Second Hypothesis

> ***Hypothesis 2*:** The likelihood of hemoglobin deferral in male and female repeat blood donors is more frequent in donors with high donation intensity, irrespective of baseline hemoglobin levels.

A sample of male and female RPT donors will be enrolled and followed for the course of the study period. RPT donors will be categorized as high (top 50th percentile) or low (bottom 50th percentile) donation intensity at the end of the study period. We assumed that the likelihood of hemoglobin-deferral in repeat female and male donors during study period would be 16.8% and 1.8%, respectively (NEARC 7/2004 to 12/2005 data).

Further, assume 11% of low donation intensity female RPT donors are hemoglobin deferred at least once during the study period and 22% of high donation intensity donors are hemoglobin deferred at least once during the study period. Thus, we assume a risk ratio of 2.0. A sample of 540 RPT female donors will have 94% power in a two-tailed 0.05 level test to detect a risk ratio of 2.0. Assuming 75% of donors will be successfully followed then a sample of about 720 eligible RPT female donors should be enrolled (and have baseline data).

Despite an expected low hemoglobin deferral rate among male RPT donors (1.8%), an equal sample of successfully followed RPT male donors (i.e., n=540) is planned. Assuming 75% of RPT donors will be successfully followed then a sample of about 720 eligible RPT male donors should be enrolled (and have baseline data). Assuming a 4% loss because of an inability to collect a sample, inadequate volume, broken vial, etc, we plan on enrolling 750 RPT female donors and 750 RPT male donors to have a total of 720 RPT female donors and 720 RPT male donors with baseline data.

E.1.3 First-time/Reactivated Donor Sample Size Based on Third Hypothesis

> ***Hypothesis 3:*** The development of iron depletion in male and female first time blood donors is more frequent in donors with high donation intensity, irrespective of baseline hemoglobin levels.

Simon et al[7] found that 12% of first-time (FT) female donors were measured as iron depleted (observed serum ferritin <12 ng/mL), whereas 24% of repeat (RPT) female donors were measured as iron depleted. Thus, for the purpose of our sample size computation, we surmise that 12% of FT/reactivated female donors will be measured as iron depleted at baseline, and an additional 12% of FT/reactivated female donors (not deemed iron depleted at baseline, nor deferred for low hemoglobin at baseline) will be measured as iron depleted within the study period.

First time/reactivated donors will be categorized as having had a high (top 50th percentile) or low (bottom 50th percentile) donation intensity at the end of the study period. We assume 6% of low donation intensity donors will develop iron depletion and 18% of high donation intensity donors will develop iron depletion, thus, a risk ratio of 3.0.

A sample of 225 female FT/reactivated donors will yield a sample of about 200 FT/reactivated female donors who are not iron depleted at baseline. A sample of 200 female FT/reactivated donors will have 85% power in a one-tailed 0.05 level test to detect a risk ratio of 3.0 between the development of iron-depletion in women with high donation intensity and the development of iron-depletion in women with low donation intensity.

An equal sample of 225 FT/reactivated male donors is planned. Assuming 57% of donors will be successfully followed (i.e. give at least one additional successful sample beyond the enrollment donation), and assuming a 4% loss because of an inability to collect a sample, inadequate volume, broken vial, etc at baseline, then we need to enroll 840 FT/reactivated donors (420 males, 420 females) to have about 800 FT/reactivated eligible fully enrolled donors.

E.1.4 First-time/Reactivated Donor Sample Size Based on Fourth Hypothesis

Hypothesis 4: The prevalence at the end of study of iron depletion in male and female first time/reactivated blood donors is more frequent in donors with high baseline hemoglobin levels.

As in hypothesis 3, we assume that 24% of FT/reactivated female donors be iron depleted by the end of the study period (i.e. by the end of the study period, these FT/reactivated female donors characterize like repeat female donors). FT/reactivated donors can be categorized by gender as high (top 50^{th} percentile of the baseline hemoglobin distribution) or low (bottom 50^{th} percentile) baseline hemoglobin level. We assume 16% of low baseline hemoglobin donors will be iron depleted and 32% of high baseline hemoglobin donors will be iron depleted, a risk ratio of 2.0.

A sample of 225 female FT/reactivated donors will have 88% power in a one-tailed 0.05 level test to detect a risk ratio of 2.0 between the prevalence of iron-depletion in women with high baseline hemoglobin and the prevalence of iron-depletion in women with a low baseline hemoglobin.

An equal sample of FT/reactivated male donors is planned. Assuming 57% of donors will be successfully followed (i.e. give at least one additional successful sample beyond the enrollment donation), and assuming a 4% loss because of an inability to collect a sample, inadequate volume, broken vial, etc at baseline, then we need to enroll 840 FT/reactivated donors (420 males, 420 females) to have about 800 FT/reactivated eligible fully enrolled donors.

E.1.5 Repeat Donor Sample Size Based on Fifth Hypothesis

Hypothesis 5: The development of iron depletion in male and female repeat blood donors is more frequent in donors with high donation intensity.

A sample of male and female RPT donors will be enrolled and followed for the course of the study period. RPT donors will be categorized as high (top 50^{th} percentile) or low (bottom 50^{th} percentile) donation intensity at the end of the study period. Results from Simon et al [7] indicate that 24% of RPT female donors measure low ferritin and that 7% of RPT male donors measure low ferritin.

Assume 20% of low donation intensity female RPT donors are measured as iron depleted at the end of the study period and 30% of high donation intensity donors are measured as iron depleted at the end of the study. Thus, we assume a risk ratio of 1.5. A sample of 540 RPT female donors will have 85% power in a one-tailed 0.05 level test to detect a risk ratio of 1.5. Assuming 75% of donors will be successfully followed then a sample of about 720 eligible RPT female donors should be enrolled (and have baseline data).

An equal sample of successfully followed RPT male donors (i.e., n=540) means that we will have 87% power in a one-tailed 0.05 level test to detect a risk ratio of 2.5, assuming that 4% of low donation intensity male RPT donors are iron depleted at the end of the study period and 10% of high donation intensity donors are iron depleted at the end of the study.

Assuming 75% of RPT donors will be successfully followed then a sample of about 720 eligible RPT male donors should be enrolled (and have baseline data). Assuming a 4% loss because of an inability to collect a sample, inadequate volume, broken vial, etc, we plan on enrolling 750 RPT female donors and 750 RPT male donors to have a total of 720 RPT female donors and 720 RPT male donors with baseline data.

E.2 Other Considerations

Ferritin and hemoglobin levels are higher among males than females, hence the hypotheses as stated will have low statistical power to detect male specific relationships. However, to address the objectives in section B various gender specific models will be developed. Further, generally the models will incorporate continuous variables pertaining to ferritin, hemoglobin, and such, rather than the qualitative measures outlined in the hypotheses. Therefore, requiring equal size samples of male donors seems practical.

E.3 Final Study Size

As previously stated, there are limited studies assessing the relationship of iron depletion and hemoglobin among blood donors, let alone any compound effect due to donation intensity. Further, few studies have considered possible surrogate measures for iron stores other than hemoglobin and hematocrit. Hence, the analyses addressing the primary objectives are to be taken as exploratory. Descriptive models will be developed and guidelines/recommendations obtained from them are meant to be suggestive. Important new observations may require additional study for confirmation.

It is understood that measurement errors and the multiplicity of hypotheses used in the above power calculations may degrade the actual statistical power achieved compared to the calculated values. The robust nature of several of the power calculations provides reassurance that these concerns are unlikely to seriously compromise the primary objectives of the study. Making reasonable assumptions for deferral rates (20% in FT/reactivated, 15% in RPT females, and 5% in RPT males), non-deferred donor consent rates (30% for FT/reactivated donors, 50% for repeat donors), loss at enrollment for specimen loss and incomplete blood unit drawn (4%), and donor compliance with the protocol requirements (57% FT donor cohort, 75% RPT donor cohort), we estimate a need to initially approach approximately 1750 FT/reactivated non-deferred male donors and 1750 FT/reactivated female donors (292 males and 292 females per center) to identify 1400 male and 1400 female non-deferred FT/reactivated donors, enroll about 420 males and 420 females and have final follow-up data on 225 male and 225 female FT/reactivated donors. Similarly, for RPT donors, we estimate that 1770 female and 1580 male donors will need to be evaluated (295 female and 264 male RPT donors per center) so that 1500 female and 1500 male RPT donors who are not deferred can be approached for possible enrollment. We would then expect to enroll 750 female and 750 male RPT donors and have final follow-up data for 540 female and 540 male RPT donors. These estimates are presented in Table **E.3-1** below.

Table E.3-1. Approximate Number of Donors*

	Approximate # of FT/Reactivated Donors		Approximate # of Frequent RPT Donors			
	# Females	# Males	# Females	# Males	Total #	Approximate # per center (equal distribution among 6 centers)
Total donors required (20% deferral FTD ½ being for Hb reasons; 15% deferral RPT female; 5% deferral RPT male)	1750	1750	1770	1580	6850	1142 donors 292 FT/react females 292 FT/react males 295 RPT females 264 RPT males
Estimated Number of Non-deferred Donors to be Approached	1400	1400	1500	1500	5800	967 donors 40 FT/react non-deferred males/month 40 FT/react non-deferred females/month 42 RPT non-deferred males/month 42 RPT non-deferred females/month
Estimated Number of Consenting Donors (Consent rate: 30% FT, 50% RPT; 30% deferred)	420	420	750	750	2340	390
Estimated Number of Successfully Enrolled Donors providing a whole blood unit, specimens and survey at enrollment (assuming 4% loss)	400	400	720	720	2240	375
Estimated Target Sample Size with at least two data collection time points (Compliance rate: 57% FT, 75% RPT)	225	225	540	540	1530	255

*Estimates were rounded up for a conservative estimate.

F. STUDY DESIGN

F.1 Donor Enrollment and Retention Plan

Eligible donors will be approached primarily at fixed sites by research staff or specially trained operational staff (if necessary, donors who give at blood drives scheduled ≥ 3 times/year can also be approached). Appropriate educational/marketing materials will be developed to assist in recruitment and help achieve donation return. Research staff will administer informed consents at time of enrollment that includes all elements required (see Section I) including significance and objectives of the study; the need for enrollees to state their intent to return at least at their assigned donation frequency; and what is expected of enrollees at each visit. Donors will also be given specific instructions and a study card to facilitate their identification when returning to the recruitment sites during follow-up.

Each site is required to develop Center specific SOP's that will detail how the center will incorporate the following recruitment and blood drive study requirements into operational activities. These SOP's will be reviewed and approved by Westat and the PI and archived at Westat.

We anticipate that enrollment can be achieved within 5 months at each of the 6 centers. The study schedule will allow for an additional 1-2 month enrollment period if necessary, by reducing the final visit period by an equal amount. (Follow-up time per donor will be unaffected.) Each REDS-II center has assessed their numbers of FT/reactivated donors (since these donors are in the minority), has identified fixed and, if necessary, mobile sites that meet all study and staffing requirements, and has demonstrated that there is an adequate donor flow to support recruitment for the study, assuming consent rates of 30% for the FT/reactivated donors and 50% for the repeat donor cohort. Lower consent rates and other logistic barriers may require other-than-even distribution of recruitment targets to each of the 6 REDS-II centers.

F.1.1 Recruitment Staffing and Sites

The baseline (enrollment) and final follow-up visit will require a few hours of research staff time for completing forms, accessioning, processing, and shipping specimens, etc. Dedicated research staff will be needed for most of these functions. During the study period, operation staff will be asked to perform quantitative fingerstick hemoglobin or hematocrit per the center's operational test-of-record on all donors presenting to donate at the recruitment sites; and, for centers that cannot collect retention tubes, to draw a lavender-top tube on study enrollees who return to donate. At centers that can collect retention tubes, research staff will need to identify which tubes were provided by study enrollees so they can be selectively saved and processed into aliquots. Research staff will be responsible for entering all specimen information in the sample tracking system provided by Westat.

Given 1-1.5 FTE research staff required for the recruitment visit, each blood center will likely need to limit recruitment to 2-3 fixed sites (each site on a less than 5 day-a-week basis) or combinations of fixed sites and large/frequent mobiles. Ideally, fixed sites will have a small research area where study donors can be welcomed, complete the questionnaire at the baseline and at the final follow-up visit, and await donation. This area should allow for privacy during the consent process, etc. Mobile sites selected by the blood centers as study recruitment sites need to be large and frequent so that donors can return to the same mobile for subsequent donations and so research staff can properly conduct the enrollment and final follow-up visit.

F.1.2 Donor Enrollment Plan

a. Eligibility

The Research staff needs to determine the eligibility of donors based on the inclusion and exclusion criteria presented in section D of the protocol. Three options are proposed:

1. **Real-time:** The preferred option is for research staff to have real-time access to the center's operational database (e.g., via a laptop) so they can check past 12 month donation histories as donors present to the registration desk. Some centers said that operational registration staff can generally access first time/repeat status but do not have detailed donation history data. Therefore research staff may need a different level of access to the blood center's computer database.

2. **Night Before:** Alternatively, research staff can check the donation history of scheduled donors the night before and "flag" donors who are eligible for recruitment. The drawback of this option is that the study population, especially reactivated donors, may be biased by the exclusion of "walk-ins." However, we do not believe such a bias would impact study results.

3. **Database Review:** A final option is for recruitment to be based on mining the center's database for eligible donors and recruiting them "offline" by letter or telephone to come in specifically for the study. By definition this will work only for the repeat and possibly the reactivated donors but not for the FT group. Potentially, there is a greater risk of bias in that recruitment itself may influence the donor's decision to donate.

b. Enrollment Visit

Each donor meeting the eligibility criteria will be approached by the research staff. The optimal timing for recruitment would be after the routine pre-donation questioning and health screening but before donation. Alternatively, it could occur before the health screen begins. Regardless, donors expressing interest in study participation will be escorted to a private area by research staff. The study will be explained in detail and the Informed Consent reviewed. The donor will be given ample opportunity to ask questions. If the donor agrees to participate after being fully informed, he/she will be asked to sign the consent form.

The placement of the research recruitment activity before or after the health screen will influence the efficiency of the process. If a donor is recruited before the health screen but is then deferred based on health history/screen, the research staff will be required to remove that donor from the study Hence, recruiting donors after completion of the health screen will be a more efficient process, whenever possible. However, the analysis of FT/reactivated hemoglobin levels for comparison with NHANES (See secondary objective B.#) will require that a sufficient and representative number of hemoglobin/hematocrit values from deferred donors be recorded at the same collection site. (See analysis section below). Each center will be required to develop specific procedures for review by the coordinating center and the PI prior to initiating the study.

During the enrollment visit, the staff needs to give the donor the self-administered baseline questionnaire; ensure the short-form questions have been completed; complete the Subject Locator Form

which includes the subject's name, address, phone number, and other contact information, (see Appendix 1) and file it locally; explain study procedures for subsequent donations; and finally, give the donor a study membership card. The recruiter will also need to accompany the donor to the donation area and ensure that the operational phlebotomists have the tubes needed to collect the necessary blood samples. At the end of the donation and after the questionnaire is completed (if done in the canteen), research staff will thank the donor, remind them of their next target donation date, and ensure that the research blood tubes have been routed to the research processing lab. It is estimated that this process (eligibility screen, consent, questionnaire, explain/schedule return donation and sample collection) will take about 1 hour from a research staff for each enrolled donor. There would be an additional 10-15 minutes required to screen the additional donors who decline enrollment (it is estimated that 1-2 donors will decline enrollment for each donor enrolled), plus down time while waiting for eligible donors to appear.

Data from the Subject locator form which includes all identifiers will be entered and managed in a confidential data base at each center. All identifying information will be maintained confidential and will not be accessible to another REDS-II center or to Westat.

c. Recruitment Materials

Information will be posted at the participating/selected donor centers and mobiles to introduce and advertise the upcoming REDS II Donor Iron Status Evaluation (RISE) study as this should facilitate enrollment of repeat donors. This can be done using posters and brochures in the reception and canteen areas of the research sites of each blood center. A draft brochure is presented in Appendix 2. Information should be available 8 to 12 weeks prior to start of enrollment. At time of recruitment, research staff will also provide each approached donor with the study brochure.

Centers may wish to recruit donors by phone or mail who have not donated in over 2 years to return to active donation by enrolling in the study as a reactivated donor. This effort will have the effect of increasing the efficiency of enrollment into the FT/reactivated donor cohort at the designated fixed sites. Such recruitment plans and materials will be locally developed and approved as a protocol modification by each center's IRB.

Each enrolled donor will be given a study membership card (see Appendix 1) and will be instructed to show this card when they return for all subsequent visits so they can be appropriately managed by operational staff. For example, at centers that cannot collect retention tubes, the membership card will indicate to operational staff that this donor is enrolled in the study and needs to have an EDTA lavender-top plasma tube drawn at time of donation.

F.1.3 Donor Retention Plan and Follow-up Visits

Donors will be recruited for subsequent donations based on center-specific operating procedures. At enrollment, donors will be told who they are to contact to schedule repeat donations if the visit is not scheduled on the day of enrollment. Participating donors will be instructed to come to a designated fixed site research location for any drop-in donations. Instructions regarding this will be printed on the study membership card. Since regular recruitment is important to the study and also to center operations, in some centers, specially trained recruiters may call these donors to donate. As with recruitment procedures in F.1.2 above, each center will be required to develop local procedures for

recruiting and tracking enrolled subjects. The procedures will need to be reviewed and approved by the coordinating center and blood center PI and archived at Westat.

a. Interim Follow-up Visit(s)

As mentioned above, all donors will have been instructed to show their study membership card when donating so that donors can be properly managed by operational staff when they return to the center. Further, the research staff each day will review the next day's scheduled appointments to prospectively identify enrolled donors scheduled for donation and thus alert operational staff and better plan next day's activities. Alternatively, centers with collection site online access and an ability to flag research donors in the center's data base may use this method to develop approved procedures to manage research subjects.

Information on the quantitative hemoglobin or hematocrit needs to be recorded for each enrollee at each follow-up visit and sent to Westat for compilation. At the time of donation, operational staff will record the hemoglobin or hematocrit result on the blood donation record (BDR). Research staff will then retrieve the operational hemoglobin or hematocrit value from the center's paper or computer records and compile this information in the study data management system. Another alternative is for centers to modify their REDS-II monthly donation/deferral data deliveries and include information on the hemoglobin or hematocrit in these deliveries.

We will attempt to collect plasma on each study participant at each of their interim follow-up visit in an effort to evaluate ferritin and sTfR at selected visits. For centers that can collect retention tubes, research staff will be charged with identifying each day the study enrollees who presented to donate the day before and selectively retrieve and process the corresponding retention tubes. Resulting aliquots will be stored frozen either at each center or at the NHLBI repository. For centers that cannot routinely collect retention tubes, the study is relying on 1) research staff identifying the day before those donors scheduled to donate on the next day and alert the operational staff that these donors are study participants and 2) donors showing their study membership card to the collection site staff or being identified by staff using online data base query so they can be identified as study participants who need to have an EDTA lavender-top plasma tube drawn.

Similarly, donors who show their study membership card but are hemoglobin-deferred will have blood drawn for an evaluation of their iron status. Hemoglobin deferred donors who do not self-identify with the study membership card or otherwise do not give samples can be identified by the research staff the day after their visit. The research staff can then contact the deferred donor and ask if they can return within 4 weeks of their deferral to provide a sample to evaluate their iron levels. This return visit can be combined with another blood donation attempt, consistent with center donor requirements.

To maximize study participant identification, we also propose that participating fixed sites or mobiles have reminder materials posted at each recruitment site to further remind donors to identify themselves as study participants. These may include posters such as those described for exhibit prior to the start of recruitment, as well as a table tent at the check-in table asking donors to alert the staff if they are participating in the study. While participating donors should be flagged in the blood center donor database as participants in the study, it may not always be feasible to rely on computerized information. Probing of the donor through reminder materials at the site will help minimize the likelihood that donors will make a donation without operational staff being aware of their visit.

Research staff will need to access the center's operational database on a daily basis to assess who of the enrolled donors have donated or attempted to donate the day before at recruitment sites. Access to the next day's schedules for the recruitment sites would also help better plan daily activities. The blood center database, if used for this purpose, should therefore include a "flag" for donors enrolled in the research protocol so that the research staff can identify who, among presenting donors, are study enrollees.

In summary, the research staff's function during the interim follow-up period will be to identify research donors who presented to donate the day before and those who are scheduled to donate on the next day (to alert the operational staff). For donors who donated the day before, they will obtain the operational hemoglobin or hematocrit from records per center-specific procedures, obtain and process all blood samples (see below), follow-up on missing samples for donors deferred for hemoglobin or hematocrit and enter all information in the study data management/tracking system.

b. Final Follow-up Visit

As a reminder, we plan on sending a letter to all study participants a few months prior to their final follow-up visit. The final follow-up visit will be scheduled and similar to the enrollment visit although expected to be shorter. The research staff will need to ensure that blood samples have been collected and appropriately processed. At the end of the donation and after the final questionnaire is completed, research staff will thank the donor for their participation in the study. It is estimated that this process (sample collection and questionnaire) will take about ½-1 hour from a research staff for each enrolled donor.

F.1.4 Hemoglobin and Hematocrit assays: Description of Available Tests, Integration into Operations, and Analysis Issues

A key variable in this study is donor hemoglobin. The "baseline hemoglobin" or hemoglobin on enrollment is particularly important to several of the study objectives and hypotheses. Because of the integration of this study into the operations of six blood centers, with somewhat different operating environments, it is necessary to allow some variation in center testing methods. In order to put these approaches into context it is first necessary to review background information on hemoglobin and hematocrit testing at blood centers, then to review differences in hemoglobin and hematocrit testing, and finally to review sample source issues that are important in this protocol design.

a. Hemoglobin testing

Hemoglobin is classically assayed as cyanomethemoglobin by a chemical method, well standardized with available standardized control material. In clinical environments, it is most commonly analyzed using a hematology analyzer. Such devices typically claim a precision on venous samples with coefficient of variation (CV) of around 1.2% with relatively minor between-instrument variability (According to 2005 College of American Pathologists - CAP – proficiency survey reports, between-lab CVs are 1-2% for the commonly used analyzers). Blood donor screening for hemoglobin is performed in two ways. Classically the copper sulfate density method is performed as a screen using a copper sulfate solution calibrated to identify hemoglobin levels below 12.5 g/dL, the FDA requirement for blood

donation. Five of the REDS-II centers currently use this method as their primary donor screening test. This method provides only pass/fail output. REDS-II centers that use copper sulfate screening all perform a second quantitative test on donors who fail the copper sulfate screen. The protocol will require that centers adopt a quantitative hemoglobin or hematocrit screening test for donors in the study – copper sulfate will not be used. During the interim period when no research staff will attend mobiles, centers will be required to use a quantitative screening test for all donors donating at the sites used for study recruitment or have robust computer methods to identify research donors among other donors so that they can be separately managed (see above). This is to ensure that donors in the study will not have missing quantitative data due to failure to identify research donors at these collection sites.

The most widely available and precise field quantitative hemoglobin screening assay is the HemoCue® B-Hemoglobin system (1.5% CV is claimed by the manufacturer on venous samples and CAP survey results demonstrate a 2.0-2.5% between-lab CV on typical proficiency samples). There are other available hemoglobin screening methods, but none are widely used in the US. Recently the HemoCue® B Hemoglobin system has been replaced by the chemically identical HemoCue® Hb 201 analyzer. The latter will be used for venous hemoglobin testing.. The HemoCue® system consists of disposable microcuvettes with reagent in dry form and a single purpose designed photometer. The microcuvette is used for measuring the sample, as a reaction vessel and as a measuring cuvette. No dilution is required. After conversion of the hemoglobin to azidemethemoglobin the reading takes place in the photometer, which measures the light absorption at two wavelengths. The photometer follows the reaction and presents the result only when the reaction has stopped. The photometer is calibrated at the factory against the cyanomethemoglobin (HiCN) method, which is the international reference method for the determination of the total hemoglobin concentration in blood.

An alternative HemoCue® method, designed for blood donor screening by fingerstick, and recently introduced into the United States (previously used in Europe), is the HemoCue® Donor Hb Checker. This device and cuvettes are essentially similarly to the B-Hemoglobin device except that the absorbance of hemoglobin rather than azidemethemoglobin is measured at two wavelengths. This modification allows marketing of this device at a significantly lower cost. The Donor Hb Checker has similar performance characteristics to the B-Hemoglobin device at hemoglobin levels around 12.5 g/dL (CV 1.0%.; Correlation coefficient 0.99 with HemoCue® B-Hemoglobin device and 0.98 with ICSH standard method) except that its linearity is restricted to the range 10.5-19.0 g/dL. The REDS-II donor iron status evaluation (RISE) study laboratory committee has determined that the HemoCue® Donor Hb checker can be used interchangeably with the HemoCue® B-Hemoglobin and the HemoCue® Hb201 analyzer in this protocol (hereafter all abbreviated as HemoCue®). Currently only ITxM uses the HemoCue® B-Hemoglobin to re-test copper sulfate failures, and may convert to the Donor Hb Checker before or during the initiation of the protocol Two other centers, Hoxworth and NE ARC, have committed to introduce the HemoCue® Donor Hb Checker into operational use before the initiation of the protocol. Other centers may also do so, but we anticipate that at least one and maybe up to three centers will continue to use the fingerstick hematocrit as their quantitative donor hemoglobin method.

b. Hematocrit Testing

Hematocrit is not measured directly by hematology analyzers. Rather, it is derived by multiplying the red cell count by the mean corpuscular volume (MCV) both of which are both directly measured.

Fingerstick hematocrit is performed at blood collection sites using variations of the classic microhematocrit method. Microhematocrit testing is generally regarded as less precise and more subject to operator subjectivity than quantitative hemoglobin testing. The CV for the most common microhematocrit version, the HemataStat (which performs the microhematocrit more quickly than other methods) is cited as 3.6%, significantly worse than HemoCue®.

Five REDS-II centers currently use variations of the microhematocrit method, one center as the primary screening test, and four centers as a quantitative backup to copper sulfate. For the protocol, these centers will be encouraged but not required to switch to HemoCue® testing for routine donor screening (two have committed to do so). If they do not do so they will be required to use their current microhematocrit test as the primary screening method for the duration of the study.

c. Use of Hemoglobin and Hematocrit to Accept Donors and to Identify Iron Deficiency

The relationship between the hemoglobin and hematocrit is defined by the mean corpuscular hemoglobin concentration (MCHC) which is defined as MCHC = (Hemoglobin g/dL) x100/ (hematocrit %). The normal range is generally considered to be 32-36 g/DL. In iron deficiency, the MCHC is reduced. Thus, hemoglobin drops earlier than hematocrit when people develop iron deficiency, and hemoglobin is a more sensitive measure of iron deficiency. While both hemoglobin and hematocrit can be used for blood donor screening, the FDA required levels of 12.5 g/dL for hemoglobin and 38% for hematocrit, corresponding to an MCHC of 32.9 g/dL.

d. Sample Source, Venous Hemoglobin, and Related Issues

The fingerstick will be the sample source used for donor acceptance at all visits. Fingerstick values may be slightly lower than the venous values and will be more variable, although this has not been carefully studied previously with pre-donation venous samples.[21]

At the enrollment and final visits, venous samples will be analyzed as well, using the HemoCue® Hb 201 cuvette discussed above on a venous EDTA sample analyzer. In addition 5 centers will use the ADVIA® Hematology Analyzer (see below). At the Southern ARC center, it was not possible to obtain ADVIA® testing in a cost-effective manner.

At two centers we expect that pre-donation venous samples will be available without an additional donor phlebotomy, as these centers (ITxM and Hoxworth) have or will implement routine use of pre-donation sampling for all blood donor testing. The ability to do so is dependent on the availability of blood component sets allowing such samples to be collected safely and sterilely, and the samples validated as suitable for viral and other testing. At the 4 other REDS-II centers, post-donation samples will be collected. Post-donation samples have been shown in small studies to result in somewhat lower hemoglobin values, as donors assume the recumbent position and then tissue fluid is mobilized because of position and the collection of a unit of blood.[22]

Because baseline hemoglobin is a critical variable, we would optimally like to obtain a pre-donation venous sample on all donors. However, this would necessitate an additional phlebotomy in all donors enrolling at the recruitment sites of the 4 centers using post-donation sampling. Because such a phlebotomy would be an unacceptable barrier to study recruitment and would significantly impact

operations, we will instead use the venous HemoCue® value as a unifying hemoglobin measurement whose relationship with pre-donation venous hemoglobin and post-donation venous hemoglobin will be defined using published data as well as data obtained in a small study of blood donors conducted at 2-4 REDS centers (see section H.2.1). We will also obtain the quantitative fingerstick hematocrit performed at the baseline visit for operational purposes by the centers not using HemoCue®.

e. Plan to Integrate the Protocol and Operations Hemoglobin/Hematocrit Testing Procedures

All donors enrolled in the protocol will have a research hemoglobin test by the HemoCue® system from a venous sample at all visits.

In an effort to obtain a quantifiable and accurate hemoglobin value consistently at all centers, the preferred approach is for centers to operationally incorporate the HemoCue® B-Hemoglobin or Donor Hb Checker as their test of record for donors who give at each of the recruitment sites for the study period. It is recognized that operational changes will be necessary in order to achieve this level of standardization.

Although centers will be asked and strongly encouraged to use one of the HemoCue® methods during the follow-up period for all donors at those sites recruiting donors into the study (or for research donors only for those centers with approved procedures to use a collection site online donor data base), a center may not be able to do so because of expense or operational considerations. In this case, the center's current operational quantitative test (a quantitative microhematocrit) will be used instead of one of the HemoCue® methods. No changes in the operational screening methodology will be allowed during the study period for study donors, so that each donor and center will have a consistent methodology.

F.1.5 Donor Deferral and Communication

a. **Deferral at the enrollment visit:** Donors deferred at the enrollment visit will not be entered into the study.

b. **Deferral during follow-up:** Enrolled donors who are deferred for reasons other than hemoglobin during the study will be removed from the study at that point and their previous data included as is the case for donors who drop-out of the study or choose to discontinue. An EDTA lavender top plasma tube and a hemoglobin/hematocrit test of record (if not already performed) will be requested at the deferral visit. No further follow-up will be conducted.

Enrolled donors who are deferred for hemoglobin during follow-up will be recruited to donate again per routine blood center operating procedures. They will be requested to provide an EDTA lavender top tube at the time of deferral, or within 4 weeks of their deferral. The lavender top tube can be collected at a repeat donation within 4 weeks if allowed by center procedures. Deferred donors will be followed up at the final follow-up visit as will donors who have not been deferred. Research staff will follow-up on all deferrals of study recipients the next day. They will be responsible for obtaining the hemoglobin or hematocrit test of record, properly processing the collected plasma sample, recruiting hemoglobin deferred donors without samples to give a sample within 4 weeks, and entering all appropriate information into the study data management/tracking systems.

c. Deferred donor counseling: Deferred donor counseling, recommendation for changes in diet or supplements, referral for medical care if necessary and subsequent donor eligibility will be per center operating protocol. Donors will be notified of low screening hemoglobin (deferral) per routine center operational procedures to prevent clinically relevant iron deficiency. Further, the Center Medical Director will routinely review abnormal relevant hematology test results and notify donors/donors' physicians as needed (See Section I, Human Subjects Considerations below). Donors will not otherwise be notified of their laboratory tests, except upon request. (In any case, most results will not be available in a clinically relevant time frame.) On request, test results will be communicated when available to the donor/donor's physician by a blood center physician.

F.1.6 Recruitment and Retention Materials

As discussed above, several items will be developed for the purpose of recruiting and retaining donor subjects during the development phase of the study. These will include *posters* and a study information brochure for the purpose of advertising the study prior to recruitment, as well as reminder posters once recruitment has started to ensure that returning subjects are identified and properly managed when they return to donate.

When being approached for enrollment, donor subjects will also be provided with *the study information brochure*. As shown in Appendix 2, the brochure will summarize the study; outline what is expected from participants; provide contact information for the center research staff should they have any questions or want to schedule or change an appointment; and give a list of the participating fixed or mobile sites where they can donate as a research donor.

A wallet sized *study membership card* (Appendix 1) will also be provided to each enrolled donor. The card will include the donor's unique Study/Participant ID, research staff contact information, and a summary of the number of donations expected over the next two years. On the back of the card will be a grid that can be used by the donor to write his/her next scheduled donation. The donors will be instructed to show this card each time they come in to donate.

F.1.7 Tracking Systems

Westat will provide a study management system (SMS*)* and a specimen tracking system (STS) to help each center's research staff manage subject retention and study activities, as well as to accession, track, and ship study specimens for testing and storage.

The SMS will allow the centers to monitor their recruitment and retention activities and generate mailing lists for the reminder letter/postcard that will need to be sent prior to the final follow-up visit. The subject tracking system will be based upon the unique Study/Participant ID assigned to each donor.

The STS is a web based system that will be used by research laboratory staff to accession and process samples collected for the protocol. It will also be used for the shipment of specimens for testing. The key identifiers will be the Study/Participant ID, the BUI, and the specimens IDs. See Section F.2.2 for additional information on the features of this system.

F.2 Data and Specimen Collection Plan

F.2.1 Dependent and Independent Variables

Baseline Data

a. Laboratory Test Data

At baseline, the following analytes will be measured:

- Fingerstick whole blood hemoglobin or hematocrit used routinely in operations (HemoCue® preferred)
- Pre-donation HemoCue® venous hemoglobin on all donors at centers with that capability (currently only ITxM/Hoxworth will perform exclusively pre-donation sampling as part of routine operations)
- Post-donation HemoCue® venous hemoglobin (except centers with routine pre-donation samples)
- Plasma ferritin
- Soluble TfR (sTfR)
- CHr, HYPOm, and other newer red cell indices available from the ® 120 Analyzer – See Table F.2.2.6 – 1. Southern ARC Center will not perform this assay. (The ADVIA® 120 Analyzer also provides data on white cell count, platelet count and reticulocyte count.)
- G277S human transferrin polymorphism (predisposes to IDE)
- HFE polymorphisms C282Y and H63D (predisposes to iron overload).

In addition a plasma aliquot and a DNA repository will be established linked to the donor, for future studies of hemoglobin and iron protein/GI absorption/iron control mechanism polymorphisms.

b. Donation and Donor Characteristics (Demographic, Anthropomorphic, Behavioral, Donation History, Data Visit, Menstrual/Pregnancy History)

- Date of visit i.e., donation date or date when sample was collected
- Donation type (whole blood or 2-RBC, sample only)
- Assumed donation/sample red cell volume (per center procedures)
- Sex
- Date of birth (to calculate age at time of donation)
- Race/ethnicity
- Education
- Height
- Weight
- Estimated subject/participant's Red Cell Volume (from height, weight, hemoglobin, using established algorithms, see Appendix 4)
- Cigarette smoking history in the last 3 months – yes or no and amount
- Regular iron supplements – yes or no? and frequency? (over the last 12 months)
- Vitamin/Mineral supplement – yes or no? and frequency? (over the last 12 months)
- Dietary habits (# foods with significant heme iron per week over the last 12 months)
- Current Aspirin intake

- Altitude of residence (from ZIP Code of residence)
- Life time donation estimate, 2-year donation estimate, date of last donation.
- First time donor ever status y/n
- Menstrual history (females): Pre- or post-menopausal, date of last menses, menstrual flow estimate
- Pregnancies (females): Total, live births, and date of last birth

Interim Follow-up Donations or Deferral Data

- Date of donation
- Donation type (whole blood or 2-RBC, sample)
- Assumed donation /sample red cell volume (per center procedures)
- Current Height, Weight and Estimated subject/participants Red Cell Volume
- Fingerstick whole blood hemoglobin or hematocrit (HemoCue® preferred)
- HemoCue® venous hemoglobin (pre- or post-donation per center operating procedures)
- Plasma samples collected and frozen: Plasma ferritin and Soluble TfR (sTfR) will be done on either all or a portion of these samples based on budgetary considerations (see F.2.2.5).

Final Follow-up Visit Data

a. Laboratory Test Data

- Fingerstick whole blood hemoglobin or hematocrit (HemoCue® preferred)
- Pre-donation HemoCue® venous hemoglobin (by centers with that capability)
- Post-donation HemoCue® venous hemoglobin (by the other centers)
- Plasma ferritin
- Soluble TfR (sTfR)
- CHr, HYPOm, and other newer red cell indices available from the ADVIA® 120 Analyzer – See Table F.2.2.6-1.

b. Updated Demographic, Anthropomorphic, Behavioral, and Menstrual/Pregnancy History Data

- Date of visit i.e. donation date or date when sample was collected
- Donation type (whole blood or 2-RBC, sample)
- Assumed donation /sample red cell volume (per center procedures)
- Current Height, Weight and Estimated subject/participants Red Cell Volume
- Current cigarette smoking (Last 30 days)
- Current iron supplementation and vitamins/minerals intake
- Current aspirin intake
- Current menstruation history
- Live birth since enrollment?

F.2.2 Specimen and Laboratory Test Data Collection Procedures

F.2.2.1 Sample Collection Procedures

At the time of donor enrollment and at the final visit, one 7-mL and one 4.5-mL EDTA lavender top tube will be collected from each donor. During the interim follow-up visits, a 7-ml EDTA retention tube will be collected at centers for whom this is routinely available. For those centers that do not have retention tubes, a dedicated tube will be collected if the donor is known to be enrolled in the RISE study. If the donor is not identified as a study participant, there will be no sample collected for that particular donation.

After verifying that consent has been obtained, a blood collection kit will be sent with the donor as appropriate for the visit (e.g., baseline, interim, or follow-up). The kit will contain the necessary vacuum tubes as appropriate and the labels to be placed on the blood tubes and on the forms for documentation and linkage.

Each center will need to designate a blood unit identifier (BUI)/Whole Blood number barcode label per donation (visit) for the study. Research staff will be required at baseline and the final visit to apply a series of labels on a *Subject Visit Log Form*, including a Study Participant ID (provided by Westat), a BUI, and a Tube ID label (Appendix 1). The log form will also indicate the date of the collection and have a checkbox for the research staff to indicate that the consent was collected or, for interim and final visits, to verify the presence of consent on file for the donor. This series of labels affixed to the Subject Visit Log Form will serve two critical purposes:

1. It will allow the laboratory staff to verify that research samples sent to the lab are only processed and tested on subjects with consent on file.

2. It provides the essential mechanism that allows for the linking of research information stored in the specimen tracking system and the subject management system, and the REDS-II donation data.

F.2.2.2 Sample Processing Overview

Research staff at each blood center is responsible for obtaining and processing all identified donor samples according to the procedures outlined in this protocol section and in the corresponding Manual of Operations and Procedures (MOP). As research blood samples are collected during the donation process, they will be processed by the REDS-II research staff and, when appropriate, sent to the laboratories or repository under storage and transportation conditions stipulated by the study protocol (See Table **F.2.2.2-1**). The research specimen processing staff will obtain from the blood center operations staff or from the REDS-II research recruiter who is monitoring the process, the enrollment), interim visits, or final follow-up visit blood collection tubes that are required. Once in the laboratory, the barcodes on the blood tubes are to be scanned into the specimen tracking system to electronically link the subject, BUI and the specimen tubes. This process for the baseline and follow-up visits must proceed in a timely manner at centers using the ADVIA® (or other hematology analyzers) as the sample required for the red cell indices must be collected, processed, shipped and tested within 24 hours of collection.

CBC Analytes Specimen

For the centers performing ADVIA® (or other hematology analyzers) the 4.5-mL EDTA tube (study tube –001 collected at baseline and during the final follow-up visit) must be transferred to and then maintained at refrigerated temperatures of 4 – 8°C within 4 hours of collection. These tubes must be transported via courier to the local ADVIA® service provider so that they can be tested within the required 24 hours of collection. This transfer will be recorded in the specimen tracking system by scanning the barcode of each sample (or a surrogate such as a paper tracking log with the barcode) and generating a shipping list (both electronic and hard copy) within the specimen tracking system to show that the samples have been collected and are enroute or have been shipped to the ADVIA® testing facility.

Frozen Specimens

The 7-mL EDTA lavender-top whole blood tube (study tube -002 collected at all visits) is to be refrigerated at 4 – 8°C as soon as feasible. Upon receipt in the REDS-II processing area, these tubes will be logged into the specimen tracking system for reconciliation through this system that the donor has consented. Prior to processing tube -002, a HemoCue® venous hemoglobin will be performed using the HemoCue® Hb 2001 analyzer and results will be recorded in the STS.

Specimens will then be centrifuged at 2250xg for 10 minutes, followed by separation into plasma and cells, aliquoted into the appropriate cryovials and frozen at the blood center within 24 hours of collection, if 24 hours is unrealistic for retention tube method a maximum of up to 72 hours from time of collection is allowable. See Table F.2.2.2–1 for handling and processing specifications. All sites are required to have a –70 to -80°C freezer designated and available for temporary storage of freezer boxes containing donor cryovials. As appropriate, frozen specimens will be shipped approximately once a month on dry ice from the blood center to Blood Systems Research Institute (BSRI) in San Francisco, CA, and ARUP, the reference laboratory for iron assays. Those specimens designated for long term repository storage will be held at the blood centers until such time as it is determined the study usage is finalized and they can then be transferred to the NHLBI long-term storage facility SeraCare BioServices in Gaithersburg, MD. The MOP contains more detailed information on the equipment, collection, storage, shipping processes and schedule of shipments.

F.2.2.3 Specimen Identification and Labeling

As the first of the blood Tube IDs are scanned into the specimen tracking system to link with the consent, another label with the Specimen ID will be applied to the first blood tube and scanned into the system as well. These Specimen ID labels will be provided to each site by the NHLBI designated repository, SeraCare BioServices. This will allow the specimens held in the repository or sent to other laboratories for analysis to be identified by a Specimen ID and not the BUI. The link between the Study/Participant ID, Tube ID, BUI and the Specimen ID will be maintained at both the originating blood center and the coordinating center but only the Specimen ID will be found on tubes and on documentation leaving the blood center. Each Specimen ID will have a "root" ID number with multiple suffixes to indicate a specific blood tube or aliquot of the blood tube (i.e. "root" = RL00569; suffix = 001, 002, 003 etc. See Example1 below). Sheets of labels with the same "root" (AABBBBB-) but with sequential suffixes (-CCC) of -001 to -010 will be removed or torn from the roll to place on the blood tubes and aliquots that will be prepared from the blood collection tubes.

Table F.2.2.2-1. Blood Specimen Specifications

Collection tube number	-001	-002 (and -003 at NEARC)	
Tube Type	4.5-ml K2 or K3 EDTA (Lavender top)	7-ml K2 or K3 EDTA (Lavender top)	
Collected at donation	Baseline/Enrollment and final Follow-up Visit	At all visits	
Purpose	ADVIA® (CBC and reticulocyte) Assays	Iron assays, DNA polymorphisms& Plasma Repository	
Handling	Room temperature* to refrigeration** within 4 hours of collection.	Room temperature* to refrigeration** within 4 hours of collection or as soon as feasible. Perform HemoCue® venous hemoglobin after thorough mixing and before centrifugation.	
Centrifugation	None	2250xg for 10 minutes	
Processing	None	Baseline/Enrollment	Interim & Final Visits
		1-ml plasma into -004 1-ml plasma into -005 1-ml plasma into -006 residual plasma into -007 0.5-ml cells into -008 0.5-ml cells into -009 0.5-ml cells into -010	1-ml plasma into -004 1-ml plasma into -005 residual plasma into -006
Storage	Room temperature to refrigeration within 4 hours of collection or as specified in manufacturers' instructions. Test within 24 hours.	Freeze within 24 hours (up to 72 hours allowable) –70 to -80°C freezer	
Shipping	Courier to local ADVIA® testing facility by Close of Business(COB) for testing to occur within 24 hours of specimen collection	For iron assays: - Baseline/Enrollment and Final Donation ship to BSRI (or directly to other designated testing facility such as ARUP for iron assays) Interim samples: TBD hold at Blood Center until directed to ship For repository: - Hold at Blood Center until directed to ship to the NHLBI repository, SeraCare BioServices in Gaithersburg, MD	

NOTE: Room Temperature (RT) is defined as 18° - 28°C (64° – 82°F); Refrigerated is defined as 4° – 8°C (35° - 46°F)

Example 1:

A set of Specimen ID labels is provided for each repository blood sample. The format for the cryovial labels is "AABBBBB - CCC," where:

AA = Blood Center
RI = BCSEW
RJ = BCP
RK = Emory/SRARC
RL = Hoxworth/HBC
RM = ITxM
RN = NERARC
BBBBB = Sequential Specimen ID / Cryovial number
00001 – 99999

CCC = Sample type 001 = EDTA lavender top Tube 1
(For ADVIA® testing or substitute CBC – baseline & final)

002 = EDTA lavender top Tube 1
(at all visits)

004 = Plasma from 002 (first cryovial)
005 = Plasma from 002 (second cryovial)
006 = Plasma from 002 (third cryovial)
007 = Plasma from 002 (fourth cryovial)
008 = Cells from 002 (first cryovial)
009 = Cells from 002 (second cryovial)
010 = Cells from 002 (third cryovial)

Examples of Lab ID Labels

RK00002-001 indicates that the tube:

- Was collected at ARC – Southern Region;
- Is the sequential root/Specimen ID number 00002 and
- Is the EDTA Blood Tube 1 collected for performing ADVIA assays.

As the blood collection tubes are scanned into the tracking system where they are linked to consent and to each of the aliquot tubes and given an individual specimen ID. The first EDTA tube, -001 is sent via courier for ADVIA® testing within 24 hours of collection., EDTA tube -002 is brought to room temperature, mixed by inversion, a HemoCue Hgb reading is taken and recorded in the STS. The EDTA tube is then placed in a centrifuge and spun at 2250xg for 10 minutes for separation into their plasma and cellular components, within 24 -72 hours of collection. While the tubes are centrifuging, transfer tubes and cryovials should be labeled with the corresponding labels containing the suffixes -002 through -010 and placed in racks prearranged to allow verifiable transfer of the sample components. Using transfer pipettes, 1-mL plasma will be placed into each of four cryovials (-004, -005, -006, and -007). The cells

found in the bottom of the EDTA lavender-top tube will be gently resuspended to mix the buffy coat and the packed red blood cells. This will be accomplished by drawing the blood up into and expelling it from the pipette a minimum of five to six times. A volume of 0.5 mL will then be transferred into the remaining three cryovials labeled with the suffixes -008 to -010. When the cryovials have each been filled and capped, they will be scanned one at a time into the specimen tracking system and placed into the specimen tracking system designated freezer box locations. Transfer tube -004 will be scanned, and placed into the first open slot in the freezer box designated by the tracking system for the tubes to be sent to the iron assay reference laboratory for the plasma iron studies. The same process will occur for tube -005, which will be placed into the same freezer box in the next open freezer box cell designated by the tracking system. The remaining cryovials will each be scanned into the system in turn and each will be placed in freezer box (es) that is(are) designated for shipment to BSRI in San Francisco or to be held for long term storage.

F.2.2.4 Tracking of Specimens

Each specimen will be entered and tracked by the specimen tracking system (STS) beginning with participant consent, through specimen processing, linking all ID codes, storage location and ending with the shipment to a testing or storage facility that maintains their own system of tracking specimens. The specimen tracking system will be used to monitor each process and action, and will retain a history of each blood sample and aliquot that has been collected, processed and tested for this study. When possible a downloaded file from or to the specimen tracking system or the NHLBI repository inventory system BSI-II will be used to reconcile the transfer or shipment of specimens from the custody of the blood centers to the repository. In the event there are additional laboratories such as the ADVIA® testing sites, that have the capability of providing an electronic receipt of shipment, this information too can be downloaded into the specimen tracking system for reconciliation purposes.

F.2.2.5 Interim Plasma Samples

The interim plasma samples will be temporarily stored at each center until testing occurs at the end of the follow-up period. While all blood samples obtained during the baseline and final follow-up visit will be tested (see below), we will evaluate whether all interim samples can be tested (ferritin and sTfR) at the end of the follow-up period based on budget considerations. If not all samples can be tested, the retained frozen plasma samples collected during the follow-up period will be prioritized for testing as follows:

1a. All retained samples from participants deferred for hemoglobin (hematocrit) during the Interim Follow-up visits (estimated 20% of samples).

1b. All donors who were found to be iron deficient or iron depleted at the final follow-up visit (1a+1b estimated at 25% of samples).

2. All female donors under 50 (#'s 1+2 estimated at 50% of samples).

3. All female donors over 50 (estimated #'s 1+2+3 at 60% of samples).

4. All samples.

Interim and other samples that are not selected for testing will be retained by the centers until the completion of the study and will be transferred to the NHLBI repository.

The final decision on sample selection for testing will be based on actual budget/expenditure status at the end of the interim follow-up period. At a minimum, samples from hemoglobin (hematocrit) deferred donors and donors who are iron deficient/depleted at follow-up (categories 1a and 1b above) will be tested. This is included in the budget in Table K.1.

F.2.2.6 Formation of a Repository

To allow for future testing of donor samples, portions of the baseline donor visit specimen will be placed into long-term storage. The amount of specimen saved will be approximately 2.0-43.0 - mL of plasma and 1.2 - 2.0-mL of frozen red cells (that can be used for PCR/DNA extraction and analysis). Approximately half of the collected volume is anticipated to be used for protocol defined testing such as the DNA extraction and analysis described in section F.2.2.6, with half reserved for long-term repository use.

F.2.2.7 Laboratory Testing

The primary assays of interest that will be used to measure the iron status in blood donors are described in Table F.2.2.7-1.

Plasma Ferritin and Soluble Transferrin Receptor (sTfR)

The assays which will be used to measure iron levels will include plasma Ferritin and Soluble Transferrin Receptor (sTfR). See Table **F2.2.7-1.** The testing facility and methodologies for these assays are still undetermined; selection will be based on scientific and financial considerations. Specifications for specimens collected for this protocol are such that they should be adequate for any assay eventually selected. Hence, all specimens will be processed within 24 (if at all possible) up to a maximum of 72 hours post collection. They may be held at room temperature for a period of time but should be placed under refrigerated conditions as soon as is feasible. Each blood collection tube should be handled per the manufacturer's direction and then be spun at approximately 2,000 – 2,250xg for 5 to 10 minutes prior to transfer into the cryovials for frozen storage. It is critical for the iron assays that the specimens not have hemolysis as this causes the release of hemoglobin from the red blood cells and can cause false elevation of the iron parameters being measured for this study. To address this, specimens will be graded 0 to 4+ hemolysis, which will be recorded in the STS.

%HYPOm, CHr, and other RBC parameters (ADVIA® tests)

The REDS-II Donor Iron Status Evaluation (RISE) Study will take advantage of the more recent advances in the rapid assessment of functional iron status by measuring red blood cell indices using the new generation of hematology analyzer (ADVIA® 120 or a model with similar capabilities). Based on practical and budgetary considerations, it is expected that 3-5 REDS-II blood centers will be able to contract, within the overall protocol budget, with a local facility with an expertise in utilization of the ADVIA® analyzer in a research capacity. The 4.5-mL EDTA blood collection tube drawn will be sent via

courier to a local laboratory where testing can be completed on donation samples within 24 hours of collection. The parameters measured utilizing the ADVIA® assays include: the %HYPOm and CHr described in Table **F.2.2.7-1**, a number of additional RBC parameters listed in Appendix 5 and a WBC, platelet and reticulocyte counts. Primary analyses will be conducted using %HYPOm and CHr although secondary analyses of additional RBC parameters and other CBC results may be conducted.

Table F.2.2.7-1. Assays to be Performed to Measure Iron Status in Blood Donors

Test*	Assay and laboratory	Reference range(s)**	Explanation
Plasma ferritin	BSRI or subcontract	15 – 150 ug/L (F); 30 – 350 ug/L (M)[18]	Major Fe storage protein. Level is directly proportional to Fe stores in healthy subjects. Levels below 12 ng/mL and more recently below 22 ng/mL have been proposed as indicative of iron depletion.[13]
sTfR Soluble transferrin receptor)	BSRI or subcontract	Depends on lab	Increases in response to Iron deficient erythropoiesis and increased erythroid proliferative activity. Values for the Log[sTFR/ferritin] above the >97.5 percentile represent Iron Deficient Erythropoiesis (IDE) in this protocol.
%HYPOm (% Hypochromic mature RBCs)	ADVIA® 120 (Bayer)[3]	$\leq$ 5%[19]	Percentage of mature RBC population with hemoglobin concentration less than 280 g /L[23] Early indicator of Iron deficient erythropoiesis. May not be as sensitive as ferritin, but may be useful as a blood center test in donor management. May be affected by other disorders that lead to decreased RBC hemoglobinization, i.e., thalassemia. There have been only limited studies in blood donors.
CHr (Cellular hemoglobin of reticulocytes)	ADVIA® 120 (Bayer)[3]	$\geq$ 28 pg[19]	Cellular hemoglobin of reticulocytes is the mean hemoglobin concentration for the reticulocyte population and is therefore a measure of "hemoglobinization" of immature red blood cells. This measure used in conjunction with HYPOm may improve the ability to predict the development of Iron deficient erythropoiesis. There have been only limited studies in blood donors.

* The Erythrocyte Free Protoporphyrin (EPP) test[24] (or zinc protoporphyrin ZPP test[25]) which is an indicator of recent inadequate iron incorporation into RBCs will not be included due to methodology issues, lack of improved sensitivity/specificity compared to other tests, and cost.

** Published Normal Ranges. Normal ranges for the study will be confirmed in the testing laboratory from first-time donors' results.

To assist in comparison of hemoglobin values across different centers and hematology analyzers, all centers will also perform venous hemoglobin, using the HemoCue® Hb201 analyzer on a room temperature well mixed 7-mL EDTA tube -002 prior to the aliquoting and processing procedures.

The ADVIA® analyzer will be utilized in the "raw data" research mode allowing for compilation of results on all samples tested for the REDS-II study in one data file and comparison of test data accounting for intra- and inter-laboratory differences and variance of control parameters. Hard copy results will be forwarded to the Blood Centers to be reviewed by the research staff within 72 hours of testing. Results requiring donor notification will be flagged for donor notification. Each center will be responsible to specify responsibility between the contract lab staff and Center Research staff for this flagging and include this in their research procedures, which will be reviewed by the PI and Westat and archived at Westat. The process for notification of donors with abnormal test results is described in the Human Subject Considerations Section of this protocol (Section I).

Electronic ADVIA® data files will be sent to the REDS-II Coordinating Center in a batch mode at time periods negotiated with the testing facilities and Bayer/Siemens HealthCare. Results will be identified by BUI or specimen ID. Personal donor information such as the donor's name, address, etc. will NOT be available to the testing lab or the coordinating center (see above).

DNA Extraction and Analysis: HFE and Transferrin Gene Mutations

DNA will be tested for the presence of mutations of the HFE gene (predisposes to hemochromatosis), and for a mutation on the transferrin gene (predisposes to IDE).

Cells will be extracted from a 0.5ml sample of frozen whole blood stored at -80C. The sample will be thawed and centrifuged at 2,000 x g for 5 minutes to generate leukocyte pellets. Any residual red blood cells will be lysed by the addition of a lysing solution. The preparation will then be centrifuged at 4,000 x g for 5 minutes and the pellet washed. A crude DNA lysate will be prepared from this cell pellet and amplified using SyBr green based real-time polymerase chain reaction (PCR). In brief, real-time PCR quantitatively measures the amount of PCR product at the end of each cycle of the reaction either by binding of a double strand-specific fluorescent dye, SyBr green, to the double-stranded DNA product or by hybridization of a sequence-specific, dual-labeled fluorogenic oligonucleotide.

The BSRI laboratory will screen for three single nucleotide mutations using sequence specific primers. Two mutations on the HFE gene: 1) a guanine to adenine mutation at position 845 resulting in cystein to tyrosine change at position 282 (C282Y), 2) a cytosine to guanine mutation in position 187 resulting in a change from histidine to aspartate at position 63 (H63D) and a third mutation on the transferrin gene, a guanine to adenine change at position 829 (G277S), will be amplified. Simultaneously, wild type primers corresponding to each mutation will also be used. Each sample will be amplified in duplicate for each 3 wild type and 3 mutations. In all, 14 PCR reaction wells will be amplified for each sample, including 2 DQ-α reaction wells, which will be used to evaluate DNA input. If the sample is positive for wild type and negative for mutation, the sample will be evaluated as homozygous for wild type. If the sample is positive for both wild type and mutation, the sample will be evaluated as heterozygous. If the sample is positive for the mutation and negative for wild type, the sample will be evaluated as homozygous for the mutation.

F.2.2.8 Laboratory Testing Data Entry and Transmission to the Coordinating Center

Electronic data transmission to the Coordinating Center via a secure web site, such as the REDS-II web site at https://www.red-ii.org, will be the method most often used. There will be

standardized formats such as comma separated values (.csv files) with variables agreed upon by the Coordinating Center and the testing facility prior to the transmission of any data. Electronic data files will be sent to the REDS-II Coordinating Center in a batch mode at time periods negotiated with the testing facilities. The maximum identifiers to be included in the electronic file will be the Study/Participant ID, BUI, Specimen ID and the specimen collection date. Usually the identifiers will be limited to either specimen ID or BUI or collection date. If a hard copy of the results is the only option, the results will be sent to the research staff at the blood center which will then be responsible for removing any personally identifying information and then transferring copies of the information to the Coordinating Center through the US Mail or a courier service such as Federal Express.

F.2.3 Donation and Donor Characteristics Data Collection Procedures

There are two major sources for the non laboratory test data to be collected. Demographic, anthropomorphic and donation data will be extracted from the REDS-II donation/donor database. Behavioral, donation, iron supplements, and menstrual/pregnancy histories will be collected through the administration of a baseline and a final questionnaire. The baseline questionnaire will also inquire into dietary habits.

a. Questionnaire Development

Cognitive testing was performed on the surveys described below to ensure respondent understanding of what was being asked. The testing was performed among both male and female first-time and repeat blood donors at two of the REDS-II blood centers (8 donors participated). Testing resulted in some minor modifications to the surveys. Appendices 6 and 7 show the final versions of the baseline and final questionnaires, respectively.

Baseline Questionnaire. The baseline questionnaire is shown in Appendix 6. It is a self-administered instrument, consisting of 22 questions. The questionnaire is divided in five sections to collect information on 1) blood donation history; 2) smoking history; 3) dietary habits; 4) use of vitamins and supplements; and 5) for females, reproductive and menstrual history. Most of the questions were adopted from previously validated surveys. The blood donation history questions were taken from previous NHLBI REDS surveys; the smoking history questions were taken from the California Smoking Survey;[26] the vitamins/supplement questions were taken from the NIH Diet History Questionnaire;[27] and menstrual and pregnancy history questions were adopted from the National Health and Nutrition Examination Survey (NHANES)[28] and menstrual flow from the Mansfield-Voda-Jorgensen Menstrual Bleeding Scale.[29]

Final Follow-up Questionnaire. The final questionnaire (Appendix 7) consists of nine questions inquiring into any changes since the last study visit in the donor's smoking habits, use of vitamins and supplements, and for females, in their reproductive history. Donors will be asked to complete this questionnaire during their final follow-up visit.

b. OMB Submission - Completed

The coordinating center is responsible for securing approval of the surveys that have been developed for this protocol. The protocol was submitted following the required procedures for posting on the *Federal Register* and review by the Office of Management and Budget.

c. Questionnaire Administration

The questionnaires will be self-administered with the research staff available if questions arise. Administration of the questionnaires should be done at a time least disruptive to donor flow. On mobiles, the questionnaire may best be completed prior to donation while at fixed sites it may be easier to have the donor complete it in the canteen. Final decision on timing of administration will be at each center's discretion; these decisions will be detailed in each center's procedures. The questionnaire will be identified by Study/Participant ID (these IDs will have been provided by Westat and will be unique to each donor), BUI and date, and the study recruiter will be responsible for ensuring that the appropriate label(s) are affixed to the questionnaire. If the research staff/research coordinator is not physically present, center operation staff trained to backup the research coordinator (Team Leaders, charge person) could be responsible for the completion of the questionnaires and be able to refer questions by phone to the research staff.

Standard answers to frequently asked questions (FAQs) will be developed by Westat and provided to each center. Questions asked will be collated by the Center Coordinator and provided periodically to Westat so that additional FAQs may be included, as needed.

In extenuating circumstances, donors may take the surveys home for completion (this option is discouraged as completion of the survey is less likely); in this case, the staff will need to give an envelope and instructions for the donor subject to send completed questionnaires back to research staff. Baseline questionnaires will be required for entry into the study, and must be completed within two weeks of the entry blood donation.

Questionnaires will be returned to the research coordinator to be reviewed before data entry is conducted or questionnaires are sent to Westat (see below).

d. Questionnaire Data Scanning/ Entry and Transmission to the Coordinating Center

It is anticipated that the Subject Management System (subject tracking system) that will be provided by Westat will allow each center with the ability to enter questionnaire data. Quality control procedures will be developed for this function and could include double key entry or a QC of some threshold level of questionnaires. Data captured in this manner could be transmitted to the coordinating center on a "real-time" basis. Alternatively, questionnaires could be shipped to the coordinating center for entry. A routine shipment schedule would be developed for centers to follow. Centers would be asked to keep copies of the questionnaires on file at the center in the event that shipments were lost or damaged. Once keyed and all QC performed on the questionnaire data, the centers would be able to destroy their copies of the questionnaires.

G. DATA MANAGEMENT

The most likely scenario is that all donor data collected at baseline and during follow-up will be electronically transmitted from the centers and laboratories to the coordinating center for compilation in a centralized study SAS database. In the event that questionnaires are sent to Westat for data entry, these data will first be compiled in a questionnaire database and then compiled with the remaining data to form the centralized study database. The centralized study SAS database will include laboratory test results, quantitative hemoglobin and hematocrit values, questionnaire data, and relevant information from the REDS-II donation database, the study management system, and the specimen tracking system.

All data obtained on each donor through time will be linked through the use of the Study/Participant ID (the ID includes as its first characters a code that identifies the Center). Compilation of study data and REDS-II donation database data will be done through the use of the links between [study data, donation date, BUI, Specimen ID, Study/Participant ID] and [donation database data, donation date, BUI, Center-ID, and Donor-ID] maintained by Westat. Only Centers will have access to the link between the [Study/Participant ID, BUI, Donor-ID] and identifying information (name, address, etc.). This procedure will help ensure the confidentiality of all data collected for this study as the coordinating center will not have access to identifying information.

The coordinating center intends to compile and QC study data after the baseline visit to allow for formation of a baseline dataset that can be used to perform analysis of these cross-sectional data. After completion of all data collection, a comprehensive "final" analytical dataset will be created that will include baseline and all follow-up data. Data frequency dictionaries will be produced to evaluate the data for possible outlying observations necessitating further evaluation.

H. DATA ANALYSIS

Analyses will primarily be performed to address the objectives given in Section B, although analyses directed by the hypotheses outlined in section E will also be performed.

H.1 Variable Definitions

Iron Status Variables

The key outcome variables are iron depletion (see section C), plasma ferritin (a continuous variable expressed in ng/mL), hemoglobin deferral (see section C), and pre-donation venous hemoglobin (a continuous variable expressed in g/dL). Iron depletion will be defined in two alternate ways: a primary variable (as in Simon et al, and used in testing hypotheses presented in section E) will be defined as "yes" if plasma ferritin is <12 ng/mL, and as "no" if ≥12 ng/mL, and a secondary variable will be defined as "yes" if serum ferritin is <22 ng/mL, and as "no" if ≥22 ng/mL.

Several other dichotomous variables will be defined. For example, iron deficient erythropoiesis (IDE, a "yes/no" variable) will be defined as "yes" if log(sTfR/ferritin) >97.5th percentile of the sex-specific distribution obtained among first time donors in the study and as "no" if equal or below this cutoff. Iron deficient donor deferral will be defined as IDE plus a fingerstick operational test of record that results in deferral. Another hemoglobin-based dichotomous variable of interest among first-time and reactivated donors will be 'baseline hemoglobin'. Baseline hemoglobin will be defined as "high" if the baseline pre-donation venous hemoglobin is greater than the median baseline pre-donation venous hemoglobin (as determined from the sex-specific distribution of baseline pre-donation venous hemoglobin at the enrollment visit in first-time and reactivated donors), and will be defined as "low" otherwise. Since some centers (expected 4 centers) will obtain baseline post-donation venous hemoglobin rather than baseline pre-donation venous hemoglobin, the baseline pre-donation venous hemoglobin will be estimated at these centers using a conversion formula outlined in section H.2.1.

Some of these variables (e.g., hemoglobin, ferritin, and log(sTfR/ferritin)) will probably be evaluated both as dichotomized variables and as continuous variables in the analyses.

Donation Intensity and Donation Red Cell Percentage. The donation rate will be defined as the number of follow-up donations (not including the baseline donation) divided by the time period between baseline donation date and last study donation date. More refined measures of donation frequency (see definition of donation intensity below) are planned. Nonetheless, the simplicity of donation rate may warrant its use over donation intensity.

Based on the blood donation volume and the blood donation type (whole blood or double red cell donation), an estimate of the volume in mL of red cells donated (including the volume collected from the samples) can be derived. Second, based on the donor's hemoglobin and anthropomorphic characteristics, an estimate of the donor's total red cell volume in mL can be derived (See Appendix 4). The donation red cell percentage is the volume of red cells donated (including samples) divided by the donor's total red cell volume and can be assessed at each visit. We will also define an end-of-study measure for each donor, namely *donation intensity* (percent red cell lost per month). Donation intensity will be defined as the donor's average donation red cell percentage, excluding the last donation (or sample), divided by their average inter-donation interval (expressed in months) which includes the interval to the last visit. In some analyses, this variable will be dichotomized. "Low" donation intensity

will be defined as less than or equal to the median donation intensity, and "high" donation intensity will be defined as greater than the median donation intensity. The "median" value for the donation intensity will be based on different cohort and sex-specific distributions depending on the objective of the analysis. For example, when conducting the analysis directed at hypotheses 1 and 3 (Sections E.1.1 and E.1.3), the median will be determined from the sex-specific distribution of donation intensity among first-time/reactivated donors successfully followed, whereas for hypotheses 2 and 5 (Section E.1.2 and E.1.5), the distributions of interest will be the sex-specific distributions obtained among repeat donors who are successfully followed.

Donation and Donor Characteristics. Variables will be defined using donation and donor characteristic data collected at the baseline and final follow-up visit. Some variables such as gender and race/ethnicity will be fixed and will be based on the information obtained at baseline visit. For smoking, iron supplementation, and menstrual status, we will inquire into changes since enrollment and will therefore use the follow-up data for these variables, when available. Dietary habits will only be assessed at baseline.

H.2 Baseline Analyses

We will use descriptive statistics to evaluate the distributions of all variables. We will use log-likelihood χ^2 statistics (or exact tests if cell sizes are too small) to evaluate if the distribution of a categorical characteristic (e.g., high vs. low baseline hemoglobin in first-time/reactivated donors; high vs. low HYPOm) is significantly different among groups (e.g., gender, race/ethnicity, iron supplementation vs. not). For comparison of continuous characteristics among groups, we will compare means among several groups by conducting t-test (two groups) or analysis of variance (> 2 groups); or if a non-parametric method is more appropriate by conducting a Wilcoxon rank-sum test (two groups) or a Kruskal-Wallis test (> 2 groups). Correlations and coefficients of determination may also be used to evaluate the association between two continuous variables. To evaluate whether two continuous variables are equivalent (such as evaluating if the hemoglobin level obtained by fingerstick HemoCue® is similar to that obtained from a pre-donation venous draw), a paired t-test would be suggested.

H.2.1 Hemoglobin Measures

Several analyses are tentatively planned that will use a quantitative measure of hemoglobin/hematocrit.

a. Conversion of Fingerstick Hematocrit and Fingerstick Hemoglobin Measurements Into Pre-Donation Venous Hemoglobin Estimates

The pre-donation venous hemoglobin result is anticipated to be an unbiased measure of the donor's hemoglobin pre-donation. The fingerstick HemoCue® result is also anticipated to be an essentially unbiased measure of the donor's pre-donation hemoglobin, albeit a more variable measure. However, to ensure that these 2 values (pre-donation venous hemoglobin and fingerstick HemoCue®) are similar we will compare the fingerstick HemoCue® results and pre-donation venous hemoglobin results for donations on which both measurements are made. Each center is expected to successfully enroll 375 donors (Table E.3-1). Two of these centers will have performed both a pre-donation venous hemoglobin and a fingerstick HemoCue® at baseline on each enrolled donor. Thus, we will be able to compare the pre-

donation venous hemoglobin to the fingerstick HemoCue® results on 750 donors. If there is a statistically significant difference, we will estimate the bias of the fingerstick HemoCue®, Δ, and will be able to convert HemoCue® results into pre-donation venous hemoglobin equivalents using the following equation:

$$\mathrm{Hb}_{\mathrm{pre}} = \mathrm{Hb}_{\ \mathrm{fingerstick}} + \Delta \qquad \text{(H-1)}$$

A reasonable value of Δ might be 1 or 2% of the mean hemoglobin level (since the CV of the venous and fingerstick tests are supposed to be about 2%). A sample of 375 donors will have greater than 99% power in a two-tailed 0.05 level test to detect an average 1% magnitude difference between the venous and fingerstick hemoglobin results. Further, the sample of 375 donors will have 80% power in a two-tailed 0.05 level test to detect an average 0.29% magnitude difference between the venous and fingerstick hemoglobin results.

Since not all centers use the same operational test of record (fingerstick hematocrit or fingerstick hemoglobin by HemoCue®), a conversion factor for fingerstick hematocrit to pre-donation venous hemoglobin is also planned to be developed. The first step is to convert a fingerstick hematocrit to a pre-donation venous hematocrit (similar to equation H-1):

$$Hct_{pre} = Hct_{fingerstick} + \Delta' \qquad \text{(H-2)}$$

The fingerstick hematocrit and pre-donation venous hematocrit are known to be statistically equivalent.[22] Thus the value of Δ' may be assumed to be zero. If we can estimate Δ' from our data or from a supplemental dataset of REDS-II donors then we will.

The second step is to convert a pre-donation venous hematocrit to a pre-donation venous hemoglobin:

$$Hb_{pre} = \lambda \times Hct_{pre} \qquad \text{(H-3)}$$

The factor in equation H-3 may be assumed to be $\lambda = 0.34$[22] A gender specific conversion factor may be considered for equation H-3. Again, if we can estimate λ from our data or from a supplemental dataset of REDS-II donors then we will. Using REDS-II data may dictate that center specific conversion factors are appropriate. Thus, we will be able to convert fingerstick hematocrit results into pre-donation venous hemoglobin equivalents using the following equation:

$$Hb_{pre} = \left(\lambda \times \left(Hct_{\ fingerstick} + \Delta'\right)\right) \qquad \text{(H-4)}$$

The CV for pre-donation venous hemoglobin is expected to be about 1%. The center specific CV for HemoCue® tests is expected to be about 2%, and the center specific CV for microhematocrit tests is expected to be about 4%. Thus, the CV for pre-donation venous hemoglobin estimated from a HemoCue® test is expected to be about 2.1%. And the CV for pre-donation venous hemoglobin estimated from a microhematocrit test is expected to be about 4.1%.

b. Longitudinal Analyses Involving Fingerstick Hematocrit/Hemoglobin Measurements

Examples of longitudinal analyses to evaluate changes in fingerstick hematocrit/hemoglobin over time as a function of donation intensity are:

1. Analysis where fingerstick hemoglobin (HemoCue®) is the outcome variable of interest; this analysis will be restricted to the centers that will use HemoCue® as their test-of-record.

2. Analysis where fingerstick hematocrit is the outcome variable of interest; this analysis will be restricted to the centers that use a fingerstick hematocrit as their test-of-record.

3. Analysis where pre-donation venous hemoglobin is the outcome variable of interest. In this analysis, we will not use actual observed pre-donation venous hemoglobin values (available at baseline visit for 2 centers, but not at interim visits), but rather transform the fingerstick hemoglobin or hematocrit value obtained at each visit into a pre-donation venous hemoglobin equivalent using the appropriate conversion factors provided in equations (H-1) and (H-3) of section H.2.1.a).

Other longitudinal analyses will be considered. The hypothesis pertaining to hemoglobin changes (E.1.1 and E.1.2) address qualitative changes in hemoglobin (i.e. donor is hemoglobin deferred or not). The analyses proposed in this section concern quantitative changes in hemoglobin. As such, the proposed analyses are deemed exploratory and are meant as an initial endeavor.

The HemoCue® B-Hemoglobin will be considered equivalent to the HemoCue® Donor Hb Checker. While the two methods have effectively equivalent standard deviations, the Donor Hb Checker methodology does not report values below 10.5 g/dL, nor above 19.0 g/dL. In the study, only rarely are donation hemoglobin levels expected to fall outside the range 10.5-19.0 g/dL. Nonetheless, censored regression techniques can be applied to account for the few right and left censored values obtained, as appropriate.

c. Analyses Involving Only Baseline and Final Follow-Up Visit Hemoglobin Levels

We propose to define baseline and final follow-up visit hemoglobin as the observed pre-donation venous hemoglobin value at 2 centers (ITxM and Hoxworth) and projected pre-donation venous hemoglobin value at the other 4 centers. As discussed in section F, ITxM and Hoxworth will obtain a pre-donation venous hemoglobin at baseline and at the time of the final follow-up visit. In contrast, the 4 other REDS-II centers will obtain a post-donation venous hemoglobin. We therefore propose to project pre-donation venous hemoglobin from post-donation venous hemoglobin for those 4 centers. Whereas a post-donation measurement is thought to be about 0.7 g/dL lower than a pre-donation measurement,[22] this conversion factor is expected to vary as a function of a donor's blood volume (blood volume is a function of weight and height as indicated in Appendix 4). A center specific relationship between pre- and post-donation venous hemoglobin can therefore be evaluated using the following regression:

$$Hb_{pre} = Hb_{post} + \alpha_i + \gamma_i(\text{donor blood volume}) \qquad \text{(H-5)}$$

Since pre-donation venous hemoglobin and post-donation venous hemoglobin values are not simultaneously measured at any donation, the parameter values, α_i and γ_i, will be estimated indirectly using the parameters estimated in H.2.1.a).

If a center measures fingerstick HemoCue® and post-donation venous hemoglobin at baseline, then the center's expected enrollment of 375 baseline donors will be used in the following regression;

$$Hb_{fingerstick} + \hat{\Delta} = Hb_{post} + \alpha_i + \gamma_i(\text{donor blood volume}) \quad \text{(H-6)}$$

If a center measures fingerstick hematocrit and post-donation venous hemoglobin at baseline, then the center's expected enrollment of 375 baseline donors will be used in the following regression;

$$(\hat{\lambda}_i \times Hct_{fingerstick}) + \hat{\Delta} = Hb_{post} + \alpha_i + \gamma_i(\text{donor blood volume}) \quad \text{(H-7)}$$

The CV of the venous hemoglobin values is supposed to be about 2%. Thus, the CV of an observed pre-donation venous hemoglobin value will be about 2%. The CV of a projected pre-donation venous hemoglobin value (based on post-donation venous hemoglobin value) will be increased to about 2.1%, due to uncertainty of the parameter estimates $\hat{\alpha}_i$ and $\hat{\gamma}_i$. Hence, the consequence of having some centers obtaining post-donation venous hemoglobin values is expected to be modest.

Once a relationship between pre-donation and post-donation venous hemoglobin levels has been established, we will convert all post-donation venous hemoglobin obtained at baseline and at the final follow-up visits into pre-donation venous hemoglobin equivalents at the four centers that conduct post-donation sampling; and combine these data with the pre-donation venous hemoglobin levels observed at ITxM and Hoxworth. These pre-donation venous hemoglobin values will be used in the following analyses:

1. Analyses where baseline hemoglobin is the variable of interest. For example, sex-specific median pre-donation venous hemoglobin values will be determined from the observed and projected pre-donation venous hemoglobin values among FT/reactivated donors. Baseline hemoglobin will be defined as "high" if the baseline pre-donation venous hemoglobin is greater than the sex-specific median and will be defined as "low" otherwise. This dichotomization is used for hypothesis 4.

2. Analyses comparing hemoglobin distributions among first-time/reactivated donors and NHANES (see section H2.2. below).

3. Analyses that evaluate changes between baseline and final follow-up visit hemoglobin as a function of donation intensity and other variables of interest.

An alternative dichotomization of baseline hemoglobin will also be considered since there are marked differences among centers on how baseline hemoglobin is obtained. Namely, sex-specific median hemoglobin values stratified by centers will be determined from each center's 'best' hemoglobin values among FT/reactivated donors (presumably ITxM and Hoxworth would use pre-donation venous

hemoglobin result and other centers would use post-donation venous hemoglobin result). The resulting dichotomization would be expected to be very similar to the dichotomization described above using observed and projected pre-donation venous hemoglobin values. The latter is expected to be preferred for two primary reasons; 1) adjusting for blood volume is expected to be beneficial, and 2) medians stratified by centers will be expected to have greater variation due to being based on samples $1/6^{th}$ as large as the combined sample.

H.2.2 Comparison to NHANES-III

The baseline venous hemoglobin measures from FT/reactivated donors will be used to determine estimates of the mean hemoglobin levels for the blood donor population by age, gender and race. An adjustment will be necessary to account for deferred FT/reactivated donors. Hemoglobin will not be known for these deferred donors. Instead, hemoglobin will be assumed to be left-censored at 12.5 g/dL (or if available, the center specific quantitative level for the operational test of record). Estimated mean hemoglobins will be found using survival analysis methodology. These means will be compared to the NHANES-III[30] data results as shown in Table H.2.2-1. It should be noted that the NHANES-III analysis includes all subjects, despite their iron status. For additional discussion of these data, see Beutler's recent review.[31]

Initially, a chi-square test will be used to test if any estimated weighted means differ from the NHANES-III means. The Chi-square statistic is defined as;

$$\chi^2 = \sum_{i,j,k} \left(\frac{\hat{\mu}_{ijk} - \mu_{ijk}}{\hat{\sigma}_{ijk}} \right)^2$$

where i represents the age categorization, j represents gender, and k represents race/ethnicity, and $\hat{\mu}_{ijk}$ represents the estimated weighted means, μ_{ijk} represents the NHANES-III means, and $\hat{\sigma}_{ijk}$ represents the estimated weighted standard errors. If mean hemoglobin levels among the blood donor population differ from the U.S. populations (as determined by NHANES-III), then further analysis will be undertaken to demonstrate how the hemoglobin distribution among the blood donor population differs from the distribution among the U.S. population.

Table H.2.2-1. NHANES-III Mean Hemoglobin (g/dL) by age, gender, and race/ethnicity

	Male			Female		
Age (years)	White	Black	Hispanic	White	Black	Hispanic
20-29	15.5	14.8	15.5	13.3	12.4	13.0
30-39	15.3	14.6	15.5	13.4	12.4	13.0
40-49	15.2	14.5	15.4	13.4	12.4	13.0
50-59	15.1	14.3	15.3	13.6	12.9	13.5
60-69	14.8	13.9	15.1	13.5	12.9	13.4
70+	14.4	13.4	14.9	13.4	12.5	13.4

H.3 Iron Status and Donation Intensity End-of-Study Analyses

Hypotheses 1, 2, 3, 4 and 5 will be tested using cross-sectional analyses of end-of-study variables (primarily end-of-study iron status variables and donation intensity). These analyses will be based on the log-likelihood χ^2 statistics associated with the corresponding 2x2 tables. Males and females will initially be analyzed separately. Examples of 2x2 tables of interest include a 2x2 table of 'iron depletion status' by 'donation intensity' among female first-time/reactivated donors and a 2x2 table of 'iron depletion status' by 'donation intensity' among male first-time/reactivated donors. We will be interested in computing four such χ^2 statistics among FT/reactivated donors: two to evaluate iron depletion by donation intensity for females and males separately (hypothesis 3); and two to evaluate iron depletion by baseline hemoglobin for females and males separately (hypothesis 4). Further, two other χ^2 statistics of interest will be conducted in repeat donors to address hypothesis 5 (iron depletion by donation intensity for females and males separately). Next, two χ^2 statistics of interest will be conducted in FT/reactivated donors to address hypothesis 1 (ever Hb deferred by donation intensity for females and males separately). Finally, two χ^2 statistics of interest will be conducted in repeat donors to address hypothesis 2 (ever Hb deferred by donation intensity for females and males separately).

These initial log-likelihood χ^2 statistics can be considered to be the results from corresponding unadjusted binary logistic regressions (e.g.; 'iron depletion' as the binary outcome variable and donation intensity as the single predictor or independent variable). Although adjusted logistic regression models could be considered (e.g., adjust for smoking, mineral supplementation, etc.), they would be only an intermediate step to deriving the models suggested by the objectives in section B and are probably not necessary to the overall analysis.

H.4 Models Predicting Iron Status

The goal of this analysis is to develop models that will accurately predict the development of iron depletion, IDE, and/or iron deficient donor deferral in active whole blood and double red cell donors. Information derived from these models will help suggest potential guidelines to reduce the development of these outcomes among donors.

Logistic models (for binary outcomes) and linear regression models (for continuous variables) will be developed that can be either unadjusted (one independent variable) or adjusted (several independent variables). Covariates such as age, smoking, dietary supplement, menstrual history variables, genetic markers and other laboratory indices will be considered when building the adjusted models. Some continuous outcomes may require transformation (e.g., while ferritin levels tend to be on the order of 30 or 50 ng/mL, some donors have levels of 100, 200 ng/mL, or more; thus ferritin will probably need to be analyzed on the log scale).

Models when the outcome is binary and is defined at times of donations: These will be the models of most interest to us. For dependent variable outcomes such as 'iron depletion status at time of donation' (two different binary outcomes based on a 12 ng/mL or 22 ng/mL cutoff- defined at time of each donation), 'IDE at time of each donation', or 'Iron deficient donor deferral', repeated measures logistic regression models will be used.

To address primary objective 2 (see Section B.1), the various laboratory test results at each successful blood donation for a donor will be independent variables in the regression models. Laboratory

test results can be used in the regression models as either dichotomous or continuous variables. For example, hemoglobin can be included in a regression classified as "hemoglobin eligible: Yes or No" (a binary variable) or using the measured HemoCue® result in g/dL (a continuous variable). Laboratory tests will be dichotomized using clinically meaningful cut-points (e.g., hemoglobin above or below 12.5 g/dL). If no clinically meaningful result is known, then the dichotomization will be according to the sex-specific median result among first-time and reactivated donors. Presumably, an unadjusted regression of hemoglobin on iron depletion will show that donors who are hemoglobin ineligible at the time of donation have a higher probability of being iron depleted. When a regression is attempted using the continuous measure of hemoglobin, the effect on iron depletion will be tested in several ways. For instance, the probability of being iron depleted may increase for every unit g/dL decrease in hemoglobin. Or the probability of being iron depleted may increase for every percentage decrease in hemoglobin. Or the probability of being iron depleted may increase only after hemoglobin decreases below some threshold g/dL level.

Adjusted regressions will include covariates of interest that change over time (smoking, menstrual status, iron supplementation, etc.). Donation intensity (an end-of-study variable) can also be adapted to be a "changing over time" covariate. For example, the history of donation dates and donation red cell percentages can be used as covariates. The form of the relationship between donation dates and donation red cell percentages with an outcome is difficult to ascertain a priori. Although donation red cell percentage is not predictive of iron status prior to the current donation, the donation red cell percentage from previous donations is likely predictive. While the end-of-study analyses done for the four hypotheses should suggest the form of the relationship i.e., whether all previous donations affect iron status or just donations within the last 6 months or the last year, developing an adequate final model of iron status will be an exploratory analysis (i.e. development will be based on a combination of statistical analyses and modeling, physiologic plausibility, and limited published findings). For example, the probability of being iron depleted or having IDE may be increased for the 6 months following a donation. Or maybe the probability of being iron depleted/IDE is increased for a time period proportional to the donation red cell percentage. Alternatively, the probability of being iron depleted/IDE may be increased immediately following the donation and linearly 'recovers' during the year following the donation. These and more complex relationships (involving the multiple donation history) will be investigated. These models will allow us to determine the significant predictors of iron depletion and IDE using information 0collected at each donation.

Models when the outcome is continuous and is defined at time of each donation: A quantitative measure for hemoglobin will be ascertained at each donation as outlined in section H.2.1. Also, ferritin will be measured at the baseline and last donation, as well as at as many as possible intermediate donations. We will use repeated measures linear regression models to measure the rate of hemoglobin decline. Hemoglobin will be the outcome measure in the regression. The regression model will include an indicator for the type of hemoglobin measurement (i.e. fingerstick HemoCue®, etc.). The purpose of the indicator would be to allow for different measurement variation depending on the type of hemoglobin measurement (i.e. pre-donation venous hemoglobin is least variable and fingerstick hematocrit is most variable). A first model will use only time as a covariate (i.e. baseline donation is time zero, and time is measured in years since baseline donation). This first model results will give the overall average rate of hemoglobin decline per year. The regression model will then include donation intensity. This model will test whether the average rate of hemoglobin decline per year is greater among high donation intensity donors. Donation intensity can then be replaced by the history of donation dates and donation red cell percentages. A model using the history (donation dates and donation red cell percentages) as opposed to the static variable (donation intensity) has potential to be more statistically

informative although, as in the repeated measure logistic regressions, modeling the effect of donation red cell percentages may be difficult. A priori we don't know whether the donation red cell percentage has only a transient effect and if it is transient how long the effect persists. For example, after a donation, hemoglobin levels may recover linearly or exponentially back to the level prior to the donation. Or possibly the recovery rises to some asymptotic level lower than the level prior to the donation. Other covariates will be added to the model. These additional predictor variables (covariates) will include various demographic, behavioral, and laboratory test variables. The models will also be constructed in an attempt to model the hemoglobin recovery phase (i.e. the donation causes the level of hemoglobin to decrease but the donor's hemoglobin level recovers over time as a function of diet, enhanced GI absorption, etc.). Similar modeling will be done for ferritin.

Additional repeated measures regression models may be of interest. A possibility would be to consider log (sTfR/ferritin) as a continuous variable (rather than a categorical 'IDE' status variable) if it was found to have high sensitivity and specificity for functional iron status. Although, the limited number of donations with ferritin and/or sTfR measures undertaken may preclude such analyses.

H.5 Sensitivity and Specificity of Iron Measures

To identify optimal surrogate measures of IDE and iron depletion, sensitivity, specificity and Receiver Operating Characteristic (ROC) curves will be considered. Although this is not a completely new approach, results using these relatively large numbers of donors and predicting future outcomes in the cohorts may provide more useful guidelines on what potential screening methods may best predict the presence and future development of iron depletion/IDE. Criteria for useful guidelines will be ensuring blood donors' safety while maximizing the quantity of blood donations and minimizing laboratory screening costs.

We will define the sensitivity and specificity of the iron and hemoglobin laboratory measures to detect IDE or iron depletion at the same donation (or sample) and also, in a separate analysis, to predict iron depletion, IDE, and hemoglobin-deferral at the next donation (or sample). Candidate cut-offs for the various assays will be derived from sex-specific reference intervals obtained at baseline among the first time/reactivated donors. Receiver Operating Characteristic (ROC) curves will be derived for the various assays to portray the various tradeoffs between sensitivity and [1-specificity] for the different cut-offs of each assay. The relative importance of having false positive and false negative results will be evaluated whenever evaluating each assay.

For example, the optimal cut-off for assessing iron depletion by HYPOm may be determined to be 0.3%. The sensitivity of such a HYPOm test may be found to be 60% and the specificity may be 90%. This would be compared to the sensitivity and specificity of a screening Hemoglobin test (estimated sensitivity 25% and specificity 69%). If the costs of the HYPOm and Hemoglobin tests were comparable, then replacing the Hemoglobin test with the HYPOm test could be suggested as a predictor of iron deficiency due to its greater sensitivity and specificity.

I. HUMAN SUBJECT CONSIDERATIONS

The study has been approved by the IRBs of the participating blood centers and the Coordinating Center. To facilitate this process an IRB packet was developed to include a protocol abstract, a draft informed consent document (Appendix 3), procedures for participant, sample, and data coding, and required procedures for blood center follow-up of donor lab results and donor events.

The following are important overall approaches to Human Subjects management of this protocol:

1. The blood donation process at each REDS-II centers follows all requirements of the host blood center and is conducted in accordance with FDA, AABB, and industry standards.

2. The donor and laboratory information will be coded so that only the individual center Research and Medical staff will have access to any identifiable test results or other research data.

3. Research results will be published only in aggregate form. Thus donor identity will not be discernable from the published reports.

4. The study staff will not make any recommendations to donors about changes in donation behavior, response to donor deferral, or wisdom of dietary supplements or dietary changes. Any such recommendations will be managed by blood center operating protocols.

5. Donor hematology and chemistry test results will not be shared with study participants unless:

   - The donor requests the data,

   - The data are inconsistent with similar data obtained by the blood center test of record (e.g., significantly different hemoglobin values are obtained),

   - Clinically relevant linked laboratory abnormalities are reported from use of the ADVIA® autoanalyzer (e.g., abnormalities in platelet or white cell counts that are "by-products" and otherwise unrelated to the protocol), or

   - Subsequent clinically relevant results are discovered by future testing of the plasma repository (see below).

The following will apply to the DNA analysis: The donor will be notified and counseled if the donor is found homozygous or mixed heterozygous for either of the two HFE polymorphisms C282Y and H63D.

1. Subsequent tests performed on linked samples in the DNA repository are found to have clinical significance.

2. The donor will be asked to consent to have his/her samples placed in the plasma/DNA repository which is solely to be used for future research on markers of iron metabolism or homeostasis of hemoglobin levels. Non-consent to the repository samples will eliminate the donor from participation in the protocol. Consideration was given to allowing a separate consent for the repository. But because the cost of entering and testing each donor in the study is substantial, and we do not anticipate difficulty recruiting for the repository as required, we concluded that it is not a reasonable use of research resources to enroll donors in the study who would not, through repository testing, allow the updating of knowledge from new iron/hemoglobin markers which may be discovered.

3. Donor complaints and injuries related to the blood donation process will be referred to each center so they can follow their operating procedures. Donor injury related to sample phlebotomy, donor complaints, and protocol violations that might affect subject's confidentiality and other rights will be managed per institutional IRB procedures.

I.1 Informed Consent

A single Informed Consent is proposed (Appendix 3). The consent form has a substantial background section that describes the nature of hemoglobin, iron, and the effects of blood donation, and other sources of blood loss on the body iron stores. It clarifies that frequent blood donation is currently encouraged by blood centers, and that this research project does not require blood donation above routine frequencies. It clarifies the nature of the testing performed and outlines when and to whom test results will be made available. It describes the repository and the limited uses allowed for the repository.

The consent form has two sections for check-boxes, one to identify the type of donor (FT/reactivated or Repeat), and the second to outline the research blood donation expectations for each type of donor. Use of a single form will simplify administration, ensure document control and availability, and allow the donor to understand the nature of the other donor groups being studied. Consents will not be required for subsequent visits, samples, or surveys.

The consent process will be administered at the enrollment visit only by trained research staff. Donors expressing interest in study participation will be escorted to a private area by research staff. The study will be explained in detail and the Informed Consent reviewed. The donor will be given ample opportunity to ask questions. If the donor agrees to participate after being fully informed, he/she will be asked to sign the consent form. Signed consents will be retained by the Blood Center in a subject-specific file along with the Subject Locator Form and other study related notes, reminders and communications.

I.2 Laboratory Data Reporting Plan

There are four types of laboratory results that will be obtained by this protocol. The management of the 4 types of data with respect to donor notification will vary.

1. **Hemoglobin (Hematocrit) testing.** Hemoglobin (by HemoCue®) or hematocrit (by microhematocrit) screening results will be obtained by fingerstick, as the operational test of record by the blood center. This test, required by the FDA (Hb $\geq$ 12.5 g/dL or

Hct ≥ 38%) will be used to accept or reject the donor, and is not subject to change. The results of these tests will be reported to the donor by operational protocols. Venous hemoglobin will also be obtained on study visits. The HemoCue® or ADVIA® venous hemoglobin results will not be used for donor qualification. It is expected that there will be differences between the fingerstick test of record and these assays. Each Center Medical Director will be responsible to develop procedures to ensure that he/she reviews results which are sufficiently discrepant as to have relevance for the donor's health. Such results will be conveyed to the donor (and donor's physician if appropriate) by the center MD along with recommendations as to the advisability of future blood donation. The PI and Westat will approve and archive these procedures.

2. **Iron markers and markers of IDE.** The study will obtain the results listed in Table F.2.2.7 -1 as primary endpoints. Most of these assays are chemical assays and will be batched tested with at least a 2-3 month delay. The two ADVIA® assays, HYPOm and CHr, are research assays and have no specific health significance, although they are being evaluated for their utility to predict iron depletion or IDE. Because these assays are end-points of the study, because iron depletion is a known result of frequent blood donation, and because the classical measures of iron deficiency are the chemical assays, the protocol will not provide these data to the donors, except per the donor's request. If the donor requests this information, these data will be communicated to the donor (and with their permission, their physician) after completion of the batch chemistry testing. Finally, should an unusually high ferritin value be obtained on the baseline sample (>1000 ng/mL), the donor will be notified in association with their genetic data.

3. **Other hematology tests obtained from the ADVIA® or other hematology analyzer.** Although not part of the primary research data, the ADVIA® or other hematology analyzer can also generate a CBC and a reticulocyte count. Because these data are performed on a healthy blood donor population, we plan to report to the donor only the laboratory values found in Table I.2. These limits were developed in a consensus manner by REDS-II investigators with Hematology expertise. All hematology analyzer laboratory output will be screened for these values and reportable values will be reported to the Center's Medical Director within 72 hours of assay. Each Center must develop a procedure outlining the responsibility for this screening and reporting. The PI and Westat will approve and archive these procedures. The Center Medical Director will be responsible to contact the donor and, at a minimum, repeat the assay in cooperation with the ADVIA® facility, funded by the protocol. Should the result be reproducible, the Center Medical Director will refer the donor to his/her physician or other appropriate medical follow-up at the donor's expense.

Table I.2-1. Reportable Hematology Results*

Assay	Lower limit	Upper Limit
Hemoglobin	11 g/dL	19.0 g/dL
White Count	2 x 109/L	20 x 109/L
Platelet Count	100 x 109/L	750 x 109/L

* Results below the lower limit or above the upper limit will be reported

4. **Genetic Tests.** The donor will be notified and counseled if the donor is found homozygous or mixed heterozygous for either of the two HFE polymorphisms C282Y and H63D. The counseling will have the intent to refer the donor to their physician for follow-up. Since this is a blood donor population, the plasma ferritin may not be useful as a further diagnostic tool for hemochromatosis. However, the donor will be notified if his/her ferritin is > 1000 ng/ mL on the baseline sample. The donor will also be notified if the transferrin polymorphism G277S or subsequent linked tests on the DNA repository are found to have clinical significance. At this time the transferrin polymorphism G277S is not considered to have clinical significance.

J. SCHEDULE

The schedule is delineated in Table J-1 below

Table J-1. Schedule

Step	Date of completion	Comment
OMB appeal	September 2007	
IRB notification	October 2007	
Refresher training of center staff	December 2007	
Donor enrollment and baseline assessment Visit	December 2007-May 2008	Five months allotted to recruitment in the study. However some centers plan to start recruitment in December 2007, while others will start in January 2008.
Baseline Laboratory Testing	June-July 2008	Up to two months allotted for receiving results of tests conducted on all samples taken at baseline
Baseline data compilation and cleaning	August 2008	This step includes development of baseline data frequency dictionaries
Baseline data analyses/interpretation	November 2008-February 2009	Statistical analyses of baseline data
Interim Period	June 2008 – July 2009	Research staff not at blood collection sites.
Follow-up period	December 2007 – May 2008 to February 2009 – July 2009	19-20 months follow-up period (minimum of 15 to maximum of 24 months)
Final donor visit	July 2009 – December 2009	Six months allotted to obtain final data on each donor
Laboratory Testing	January-February 2010	Testing of purple top tubes obtained during follow-up (ferritin/ sTfR) and testing of specimens obtained during the follow-up visit.
Data compilation and cleaning	March 2010	QC and formation of dataset
Data analyses	April–July 2010	Four months for longitudinal data statistical analysis

K. ESTIMATED BUDGET

UNDER REVISION

Appendix 1

Study Forms

REDS - II Donor Iron Status Evaluation (RISE) Study

Subject Locator Form

We would like to be able to contact you regarding your future donations.
Please complete this form and return it to the REDS Donor Iron Status Evaluation (RISE) Study Research Staff.

Today's Date: ____ ____ - ____ ____ - ____ ____
Month Day Year

Name: .....
First Name Middle Name Last Name

Address: .....
Street City State ZIP Code

Phone (Home): .....

Phone (Work): .....

Phone (Mobile): .....

Email: .....

Comments: .....

.....

Sponsored by
National Heart Lung and Blood Institute
National Institutes of Health (NIH)

AFFIX LABEL WITH ID HERE

REDS - II Donor Iron Status Evaluation (RISE) Study

Study Membership Card

Front

< LOGO > REDS Donor Iron Study

< Insert Name of Blood Center >

AFFIX LABEL WITH ID HERE

Name:_____
Birth date:_____
Donor ID:_____

Dear Donor: Please remember that you indicated you would donate at least ___times per year over the two year study period.

Back

Dear Donor: Thank you for joining this important study. When you donate blood again, please call one of our recruitment staff at XXX-XXX-XXXX to make an appointment. You can record your appointments below.

Locations for your donation:
1) _____
2) _____
Blood Center Staff: This donor is part of an iron research study.

REDS - II Donor Iron Status Evaluation (RISE) Study

Subject Visit Laboratory Form

Each center will need to designate a blood unit identifier (BUI) -- or whole blood number -- barcode label per donation (visit) for the study. Research staff will be required at baseline and each subsequent interim visit to apply a series of labels on a *Subject Visit Laboratory Form*, including a Study/Participant ID provided by Westat, a BUI, and a Tube ID label. The form will also indicate the date of the collection and have a checkbox for the research staff to indicate that the consent was collected or, for interim visits, to verify the presence of consent on file for the donor.

TODAY'S DATE: _____ - _____ - _____

Month Day Year

STUDY / PARTICIPANT ID: AFFIX LABEL WITH ID HERE

BUI: AFFIX LABEL WITH ID HERE

TUBE ID: AFFIX LABEL WITH ID HERE

CONSENT FORM:

ENROLLMENT / BASELINE VISIT

☐ **CONSENT OBTAINED TODAY**

INTERIM VISIT

☐ **CONSENT IS ON FILE (VERIFIED)**

Comments: .....

.....

REDS RESEARCH STAFF INITIALS: .....

ATTACH
TUBE / SPECIMEN ID
LABEL SHEET HERE

ATTACH
STUDY / PARTICIPANT ID
LABEL SHEET HERE

Appendix 2

Invitation to Participate Brochure

As a blood donor you already know the vital role that your blood donation plays in saving the lives of others. By participating in the REDS Donor Iron Status Evaluation (RISE) Study, you can also play a vital role in the research that helps keep frequent blood donors healthy. The information contained in this brochure can help you decide if you would like to participate in this important study.

The RISE Study is designed to answer questions about how iron and hemoglobin levels change in blood donors over time. Iron is a necessary part of hemoglobin which is the part of the red blood cell that carries oxygen from your lungs to other parts of the body. This study is designed to look at how blood donation and personal characteristics may affect levels of iron and hemoglobin in a person's blood. Information from the study will also help us evaluate which laboratory tests are best for monitoring donor's iron levels, the best frequency for blood donation, and how genetic factors may influence iron and hemoglobin levels.

Who is being asked to participate in the REDS Donor Iron Status Evaluation (RISE) Study?

Two groups of donors are being asked to participate in the study for 1 ½-2 years. One group consists of frequent donors who have given blood at this center regularly in the past year. The other group includes donors who either have never given blood before or have not done so in the past 2 years but who, we hope, will now agree to give regularly.

We need to study both types of donors in order to understand how blood donation influences iron and hemoglobin levels. You will need to qualify for blood donation today to participate in the study.

What will be required of me if I decide to enroll in the REDS Donor Iron Status Evaluation (RISE) Study?

First you will be required to read and sign a consent form to participate in the study. This consent form will explain in greater detail than this brochure what the study requirements are. It will also contain information on your rights as a study participant and how your privacy is protected. You should read the consent form carefully before enrolling in the study and only sign it if you meet the study requirements, want to participate and have all your questions answered by the study staff.

The main requirement of the study will be to continue donating blood during the next two years. Most people involved in the study will be asked to donate twice a year for the next two years. Men who were frequent donors before enrolling will be asked to donate 3 times per year. This will be explained again in the consent form for the study. Because of the study requirements, you can only donate blood during this study at a limited number of sites, preferably at the donation site where you enroll in the study.

After enrolling, you will be asked to fill out a brief questionnaire about yourself that should take no more than 10 minutes to complete. The questionnaire will ask you about your previous blood donations, dietary habits, whether you take iron supplements or vitamins with iron, and your smoking habits. Women will also be asked about their menstrual cycle and blood loss during menstruation and their history of pregnancy. You will then be asked to donate blood as you normally would. At the time of your donation today and until your final visit, when samples are taken from your donation for routine blood testing, an additional 2-3 teaspoons of your blood may be taken to be used for laboratory tests that measure your iron stores.

After approximately a year and a half you will be recruited by the research staff to give a final set of blood samples which will be used to check your iron levels and complete another survey. You can donate

blood at the same time, but the final visit will need to be specially scheduled. The final visit will need to be scheduled within 2-3 months of your receiving a reminder notification letter.

Before you leave, you will be given a membership card identifying you as a participant in the RISE Study. You can use the contact information on this card to arrange for your next donation. The card will also let you know where you can donate while you are enrolled in the study and remind you (if you already made an appointment) when you agreed to next donate blood. But, first of all, you should show this card to the blood center staff when you come in to donate so they are reminded that you are a study participant!

Please note, that if at any time after you are enrolled and successfully donate, you are asked not to donate blood because your hemoglobin level becomes too low, you are still asked to continue participating in the study. Although you will not be able to donate that day, you will be asked to provide blood samples to check your iron levels on the day you are deferred. The blood center will give you advice on how quickly you can return to donate.

What tests will be performed on my blood sample for the research study?

Your blood samples will be tested for several indicators of your body's iron stores. Your blood will be analyzed using newer testing designed to detect early iron deficiency. Some people, depending on how their body uses available iron, may be more likely to have too little iron while others may be more likely to have too much. Because of this we will also analyze your genes related to iron metabolism to find out how your body uses iron.

A small portion of the blood collected on your visits will be frozen and stored for possible later use. These samples will only be used if other tests for iron status or iron genetic markers are developed. The researchers will not use your blood for any other purpose without your written consent.

Will anyone be able to link my survey answers or blood test results back to me?

Your information will be kept confidential. Details of how we keep your information private are in the consent form.

Who are the REDS-II Donor Iron Status Evaluation (RISE) Study researchers?

The REDS-II Donor Iron Status Evaluation (RISE) Study researchers are part of a larger group of researchers participating in the REDS-II study, sponsored by the National Institutes of Health, National Heart, Lung and Blood Institute. If you have not already received information about the REDS-II study we will be happy to provide it to you now.

Who do I contact if I have questions about the study?

<INSERT BLOOD CENTER NAME AND CONTACT INFORMATION>

Appendix 3

Informed Consent

You are asked to participate in a research study, called the **REDS-II Donor Iron Status Evaluation (RISE) Study**, which is being conducted at the _____ Blood Center under the supervision of Dr. _____. This study is part of a larger network of blood safety research called REDS (Retrovirus Epidemiology Donor Study) funded by the National Heart, Lung and Blood Institute. The **REDS Donor Iron Status Evaluation (RISE) Study** will assess how blood donation and personal characteristics may affect levels of iron and hemoglobin in a person's blood. Information from the study will help us evaluate which laboratory tests are best for monitoring donors' iron and hemoglobin levels, the best frequency for blood donation, and how some personal characteristics such as your diet, use of mineral supplements, or smoking may influence iron levels and the ability to donate blood. We will also assess in women donors how menstrual periods affect their iron levels and ability to donate blood.

Introduction

For a number of years, blood donation has been known to lower body iron stores, although usually not to levels that are believed to be of major health significance. This is because iron in the body is primarily found in the red cells of the blood (actually in the main oxygen carrying protein, hemoglobin, within the red cells). You can lose iron for reasons other than blood donation. For example, before menopause, women lose blood during their menses; pregnant women need to provide iron to their developing child; and some people may lose blood due to health conditions such as intestinal bleeding.

Blood donors who do not have enough iron in their body may have a low hemoglobin level in their blood, a condition called anemia. When you have anemia, you may be tired, have problems exercising, and may have other health problems. It is for this reason that blood centers routinely screen for anemia in persons who try to donate and require all donors to have a minimum hemoglobin level in their blood before they can donate blood.

Whether there is health significance for persons with a low level of iron in their body if this level is not low enough to cause anemia is uncertain. Some research suggests that a slightly low iron level can cause mild problems, such as being tired and difficulty concentrating while other research suggests that having a slightly low iron level may be beneficial and decrease heart and blood vessel disease.

Overview of the Study

Why was I asked to participate?

We are asking for your participation in this study because: [One box below to be checked by Research staff]

_____You are new to blood donation and have never donated blood.

_____You have not donated blood in the last two years before today.

_____You are a man who has donated at least 3 times in the last 12 months (not including today). Double red cell donations count as two donations.

_____You are a woman who has donated at least 2 times in the last 12 months (not including today). Double red cell donations count as two donations.

_____You are new to blood donation or have not donated blood in the last two years, and have a hemoglobin level today that is not high enough for you to give blood.

What do I need to do to participate?

If your hemoglobin level is high enough today for you to donate**,** we are asking you to participate in this study for approximately 2 years during which time we will assess your hemoglobin and/or iron levels each time you come to donate.

For the study to accomplish its goals, it is important that you understand we would like you to donate blood to the _____ Blood Center as frequently as you can over the next two years (you are eligible to donate blood every 8 weeks or double red cells, using a special blood collection method, every 16 weeks). It is also important that you do NOT donate to another blood center during the two year study. You will be given instructions about how you can schedule donations but the donations should be made at the site where you enrolled or another site which is participating in the research. You will also receive reminders from the research staff at _____ Blood Center to donate blood while you are enrolled in the study. You will also receive routine recruitment calls from the Blood Center.

We would like you to donate at least as often as checked below (double red cell donations count as two donations): [Research staff to check the appropriate box below]

_____ If you are new to blood donation and have never donated blood, you agree to donate blood at least twice a year for the next two years (4 more donations after today over the next two years)

_____ If you have not donated blood in the last two years before today, you agree to donate blood at least twice a year for the next two years (4 more donations after today over the next two years)

_____ If you are a man who has donated at least 3 times in the last 12 months (not including today), you agree to continue to donate at least three times a year for the next two years (6 more donations after today over the next two years)

_____ If you are a woman who has donated at least 2 times in the last 12 months (not including today), you agree to continue to donate at least two times a year for the next two years (4 more donations after today over the next two years)

If your hemoglobin level is NOT high enough today for you to donate, we will not be able to enroll you in the study.

What you can expect if you participate in this study

At each donation visit, including today, you will be evaluated as usual by regular Blood Center staff to determine if you are eligible to donate. This will include a hemoglobin screening test to check for anemia.

If you are eligible, you will then donate blood as normal. When samples are taken from your donation for routine blood testing, an additional three teaspoons (15 ccs) of blood will be taken to check your iron and hemoglobin levels. At the donations between the first and last, only two teaspoons (10ccs) of blood will be taken. The samples between the first and last donations may be used to check on your iron levels later, but the decision on whether these will be tested will be made at the end of the study. The iron tests that will be done on the blood samples you provide today when you enroll in the study will include checking your genetic material (your DNA) for genes that may make you likely to have too little iron or too much body iron. (No other genetic tests other than those related to iron or hemoglobin will be done on your DNA). At today's donation and at the end of the study, we will also check your count of red blood cells, white blood cells and platelets (the different cells in your blood).

At today's donation, you will be asked to complete a 10 minute survey about your blood donation history, your diet, your use of iron supplements and aspirin, your smoking history, and, for women, your pregnancy and menstrual history. You will also be asked to complete a shorter survey (5 minutes) at the end of the study to check if there have been any changes in your use of vitamins and iron supplements, your smoking habits, and, for women, your menstrual history. These are all factors that are expected to influence your body's iron stores. Some of these questions may be sensitive, but it is important they be answered fully and accurately for you to participate in the study.

If you are told you cannot donate blood

If you are told you cannot donate blood today because your hemoglobin level is too low, you cannot participate in the study. You should ask the Blood Center staff when you can next try to donate blood.

If you can give blood today but cannot at some point in the next two years because your hemoglobin level is too low, we will ask you at that time to provide three teaspoons of blood for the research tests. You should ask the Blood Center staff when you can next attempt to donate blood. You are still being asked to continue to participate in the study until it ends.

If you cannot donate blood for a reason other than hemoglobin during the next two years, your participation in this study will end but you will be asked to provide a final sample of three teaspoons of blood and to complete the survey one last time. The regular Blood Center staff will provide you further information on why you cannot donate blood at that time, whether you can donate blood in the future and whether this means anything for your health.

Your blood test results

In general, the iron research test results will not be available until late in the study. Since iron loss is a known effect of blood donation in many donors and the _____Blood Center will routinely let you know if your hemoglobin level is too low when you donate, we do not plan to share with you the results of any research test that may show this expected iron loss, although, upon request we will share these results with you and your physician (if you identify one) when they are available.

Certain research test results however may be important to your health. You (and your physician if you identify one) will be notified if these test results are abnormal and may be of potential medical concern

Sample Repository

If you agree to participate in this study, samples of your blood will be frozen and saved indefinitely in a repository for future research on iron stores. Future testing on these saved samples will be done only to check body iron and hemoglobin levels and may include additional tests of your genetic material if new genes are identified that tell us how your body absorbs and keeps iron or sets hemoglobin levels. No other genetic tests other than those related to iron or hemoglobin will be done on your DNA. The testing may be done at other laboratories, but your identity (name, address) will remain coded and only be known to the research staff at the _____ Blood Center. All proposed testing on saved samples will be subject to review and approval by the Blood Center's Institutional Review Board, which has the responsibility to protect the rights of research study subjects, the REDS-II study, and representatives of the National Institutes of Health.

What are the risks and benefits of participating in this study?

Risks: Other than the known risks of blood donation (*Insert individual Center's "What You Must Know" that describe these risks to blood donors)* the only additional risks of participation in this research study are:

1) If extra blood draws are needed: pain, bruising, and rarely infection.
2) Small additional blood loss: Rarely, the extra 2-3 teaspoons of blood drawn for the study at each blood donation could aggravate iron loss.
3) Information risk: If I request my results or am notified of a serious health implication from the testing, this information could be upsetting, although it could also represent a benefit to me.
4) Genetic testing: Knowing that you have a genetic or inherited abnormality in how your body absorbs iron could cause distress to you and your family, although it could also represent a benefit to you or your family.
5) Confidentiality: Participation in research may involve loss of privacy, but information about me will be handled as confidentially as possible by the investigators. My name and address information will be kept locked in a locked file at my local blood center, and other study data will have a code number instead of my name. Representatives from the funding agency, the National Institutes of Health, may review information about me to check on the study. My name will not be used in any published report about this study.

   To further protect your privacy, the study investigators have obtained a Certificate of Confidentiality from the Department of Health and Human Services (DHHS). With this certificate, the investigators may not disclose information (for example by court order or

subpoena) that may identify you in any federal, state or local civil, criminal, administrative, legislative, or other proceedings. Disclosure will be necessary, however, upon request of DHHS for audit or program evaluation purposes. A Certificate of Confidentiality does not prevent you, however, from voluntarily releasing information about yourself or your involvement in this research.

Benefits: Although you will not directly benefit from participating in this study, this study may benefit other donors like you in the future, by helping _____Blood Center develop donor-specific guidelines on how often one can safely donate blood. You will not be paid to participate in the study.

Non Consent/Withdrawal from the study

Whether you choose to participate or not in this study will not affect your opportunity to donate blood today nor any rights or privileges you may have with the _____ Blood Center. If you decide to participate in the **REDS-II Donor Iron Status Evaluation (RISE) Study**, but change your mind later you may withdraw at any time or elect not to provide a study blood sample or complete one of the questionnaires. In the case that you are unwilling to provide samples or complete surveys as outlined in this consent, we may decide to withdraw you from the study. You may also request to have your samples withdrawn from the sample repository. Withdrawal from the research study will not affect your relationship with _____ Blood Center or your previous or future blood donations.

Subjects' Rights

Your decision whether or not to take part in this study is voluntary. It will not change your future relationship with _____Blood Center in any way. You are free to end your participation at any time without harm to your rights or your future relationship with _____Blood Center.

If you are injured

[Each Center to insert their own wording- One example given below]

In the event that you suffer physical injury as a direct result of your participation in this research activity, the _____Blood Center will assume responsibility for making immediate medical care available to you. This care will be provided without charge if you notify Dr. _____ (Principal Investigator's or designee's name and telephone number) within fifteen days of the date of the injury or appearance of symptoms, and consent to the care offered. There is no provision for monetary compensation to you at the expense of _____ Blood Center for such things as lost wages, disability, injury or discomfort resulting to you from such physical injury. Further information concerning treatment and payment of medical expenses in the event of an injury may be obtained from _____ (Principal Investigator's or designee's name and telephone number).

Contact Person

If you have any questions, please ask us now. If you have any additional questions later, contact Dr. _____at_____ who will be happy to answer them. If you have questions about your rights as a research subject, call_____(local IRB). If you decide to participate, you will be given a copy of this form to keep.

Consent Authorization

My signature indicates that I have read the above explanation of this research project. I have been given the opportunity to ask questions of _____ and my questions have been answered. The potential risks and benefits have been explained to me. Based on this information, I have voluntarily decided to participate in this research study. I understand that I have the option to withdraw from the study without penalty at any time after signing this form.

_____

Printed or Typed Name

_____ _____

Signature of the participant **Date**

_____ _____

Witness Name and Signature **Date**

Appendix 4

Algorithm to Determine Estimated Red Cell Volume

Estimated Red Cell Volume will be calculated as described by Mollison[32]

1. Estimated Blood volume (EBV in Liters) is determined according to the following formulas from Nadler:[33]

   For Men: $EBV = 0.3669\ H^3 + 0.03219\ W + 0.6041$
   For Women: $EBV = 0.3561\ H^3 + 0.03308\ W + 0.1833$,
   Where H = height in meters and W = weight in kilograms

2. Estimated Red Cell Volume (RCV in mL) is then derived as:

   $RCV = (EBV/1000) \times PCV \times 0.91$
   Where PCV is the predonation venous hematocrit and 0.91 is a correction for the difference between venous and whole body hematocrit

3. Predonation venous hematocrit will be derived as:
   PCV = venous Hb/MCHC
   Where venous Hb is the predonation venous hemoglobin and MCHC (mean corpuscular hemoglobin concentration) is the pre- or post-donation value from the ADVIA®

4. Finally, venous Hb (predonation) will be derived either directly (from a predonation sample) or a derived value obtained from the predonation fingerstick HemoCue® Hb and the measured ratio of fingerstick to venous hemoglobin obtained at ITxM during the study.

Appendix 5

Indices Measured by the ADVIA® 120 Hematologic Analyzer[19]

Measurement	Index	Abbreviation	Explanation
Cellular hemoglobin	CH (RBC) (pg)	CHm	Cellular hemoglobin is the mean of the RBC hemoglobin concentration for the red blood cell population. CH is comparable with the more conventional MCH (Mean Corpuscular Hemoglobin)
	CH (retic), pg	CHr	Cellular hemoglobin is the mean of the RETIC hemoglobin concentration for the reticulocyte population
	Low CH (RBC) (%)	%LowCHm	Percentage of mature RBC population with cellular hemoglobin less than 27 pg
	Low CH (retic) (%)	%LowCHr	Percentage of reticulocyte cell population with cellular hemoglobin less than 27 pg
Hemoglobin concentration	CHCM (RBC) (g L1)	CHCMm	Cellular hemoglobin concentration mean is the mean of the RBC hemoglobin concentration for the mature RBC population. CHCM is comparable with the more conventional MCHC (mean corpuscular hemoglobin concentration)
	CHCM (retic) (g L1)	CHCMr	Cellular hemoglobin concentration mean is the mean of the RETIC hemoglobin concentration for the reticulocyte population
	Hypochromic (RBC) (%)	%HYPOm	Percentage of mature RBC population with hemoglobin concentration less than 280 g/ L
	Hypochromic (retic) (%)	%HYPOr	Percentage of reticulocyte population with hemoglobin concentration less than 280 g/ L
Reticulocyte count	Reticulocyte count (1012 L1)	Reticulocytes (abs.)	Absolute number of reticulocytes
	Reticulocytes (%)	Reticulocytes (%)	The percentage of reticulocytes
Volume	MCV (RBC) (fL)	MCVm	Mean corpuscular volume is the mean of the RBC volume for the mature RBC population
	MCV (retic) (fL)	MCVr	Mean corpuscular volume is the mean of the RETIC volume for the reticulocyte population
	Microcytic (RBC) (%)	%MICROm	Percentage of mature RBC population with cell volumes less than 60 fL

Appendix 6

Baseline Questionnaires

Donor Iron Status Survey (Cohort version)

This research sponsored by the National Heart, Lung, and Blood Institute of the National Institutes of Health (NIH) will help us better understand iron status in blood donors and contribute valuable information for improving the health of blood donors. This survey will ask you questions about your donation history, smoking history, diet, vitamins and supplements that you take and if you are female, a few questions on your reproductive history. Your answers to all questions will be kept confidential and only be used for the purpose of this research.

Your participation in this survey is voluntary. If you choose not to participate, it will not affect your ability to donate blood again in the future. You will not lose any benefits.

Name: .....

First Name Middle Name Last Name

Today's Date: ____ ____ - ____ ____ - ____ ____
Month Day Year

Blood Center ID: .....

Whole Blood Number (WBN): .....

AFFIX LABEL WITH ID HERE

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National Institutes of Health (NIH)

SECTION A
Your blood donation history:

1. **Is this the first time you have EVER donated blood?**

   ☐ Yes **{SKIP TO SECTION B, QUESTION 7}**
   ☐ No

2. **Including your most recent donation, how many times in your life have you donated blood?**

   ☐ 1 to 2 times
   ☐ 2 to 5 times
   ☐ 5 to 10 times
   ☐ 10 to 20 times
   ☐ More than 20 times
   ☐ Don't Know

3. **Other than today, when was the last time you donated blood?**

   |__|__| |__|__|
   M M Y Y

   ☐ Don't Know

   {IF YOUR LAST DONATION WAS MORE THAN 2 YEARS AGO SKIP TO SECTION B, QUESTION 7}

4. **Please tell us the total number of blood donations you have made in the last 2 years.**

   |__|__|
   NUMBER OF DONATIONS

   ☐ Don't Know

5. **Were any of these donations made through a DIFFERENT blood center?**

   ☐ Yes
   ☐ No
   ☐ Don't Know

6. **Were any of these apheresis donations?** (Apheresis: Donors give only select blood components such as platelets, plasma, red cells, or a combination of these)

   ☐ Yes
   ☐ No

   How many of these where apheresis donations?
   |__|__|
   NUMBER OFAPHERESIS DONATIONS
   ☐ Don't Know

SECTION B
Your smoking history:

7. **Have you smoked at least 100 cigarettes in your entire life?**

   ☐ Yes
   ☐ No
   ☐ Don't know

8. **Did you smoke ANY cigarettes during the last 90 DAYS (3 months)?**

   ☐ Yes
   ☐ No **{SKIP TO SECTION C QUESTION 11}**
   ☐ Don't know

9. **Thinking about the last 30 DAYS (1 month), on how many of these days did you smoke?**

   |___|___|
   NUMBER OF DAYS

   ☐ Don't know

10. **In the LAST 30 DAYS, on the days that you DID smoke, about how many cigarettes did you usually smoke per day?**

    |___|___|
    NUMBER OF CIGARETTES

    ☐ Don't know

AFFIX LABEL WITH ID HERE

SECTION C
Your Diet:

11. Over the LAST 12 MONTHS, about how many times per week did you eat the following foods?

[When thinking about the foods you eat, remember to include soups, stews, sandwiches, lunch meats, casseroles and salads that are made with these food items.]

Foods	How many times?							
	Never	Less than once/ week	Once/ week	Twice/ week	3-4 times/ week	5-6 times/ week	Once every day	2 or more times/day
Liver (any kind)	☐	☐	☐	☐	☐	☐	☐	☐
Beef (including ground Beef)	☐	☐	☐	☐	☐	☐	☐	☐
Lamb, Pork, Chicken, Turkey	☐	☐	☐	☐	☐	☐	☐	☐
Clams	☐	☐	☐	☐	☐	☐	☐	☐
Oysters, Mussels, Shrimp, Sardines	☐	☐	☐	☐	☐	☐	☐	☐
Other Fish	☐	☐	☐	☐	☐	☐	☐	☐
Eggs	☐	☐	☐	☐	☐	☐	☐	☐
Dairy Products (Milk, Yoghurt, Cheese)	☐	☐	☐	☐	☐	☐	☐	☐

SECTION D
Your use of vitamin pills, supplements and aspirin:

12. Over the LAST 12 MONTHS, did you take any multivitamins such as One-A-Day, Theragran, or Centrum type multivitamins (as pills, liquids, or packets) on a regular basis (at least once a week)?

☐ Yes
☐ No
☐ Don't know

How often did you take multivitamins?

☐ Everyday
☐ 4 to 6 days per week
☐ 1 to 3 days per week
☐ Don't know

Does your multivitamin contain iron?

☐ Yes
☐ No
☐ Don't Know

13. Over the LAST 12 MONTHS, did you take any iron supplements other than your multivitamins on a regular basis (at least once a week)?

☐ Yes
☐ No
☐ Don't know

How often did you take iron supplements?

☐ Everyday
☐ 4 to 6 days per week
☐ 1 to 3 days per week
☐ Don't know

AFFIX LABEL WITH ID HERE

14. **Do you currently take Aspirin or Aspirin containing pain relievers daily or nearly everyday?**

- ☐ Yes → **Why?**
  - ☐ For heart or cardiac health
  - ☐ For pain relief
  - ☐ For both
- ☐ No
- ☐ Don't Know

{MALE DONORS SKIP SECTION E AND GO TO END STATEMENT}

SECTION E
FOR FEMALE DONORS ONLY
Your reproductive history:

---

15. **Which of these statements best describes your current menstrual status?**

- ☐ I am still having periods and am NOT going through menopause
- ☐ I am still having periods, but am possibly going through menopause
- ☐ My periods have stopped completely because I have gone through menopause **{SKIP TO QUESTION 19}**
- ☐ I had an operation which stopped my periods **{SKIP TO QUESTION 19}**
- ☐ I am taking a medication that has stopped my periods completely **{SKIP TO QUESTION 19}**
- ☐ My periods have stopped because of other reasons **{SKIP TO QUESTION 19}**

16. **What was the date when your last menstrual period started?**

|__|__| |__|__|
M M Y Y
ENTER DATE OF LAST PERIOD

- ☐ I am having my period now

17. **About how many periods did you have in the last year (12 Months)?**

|__|__|
ENTER NUMBER OF PERIODS

18. **How would you describe your menstrual flow or bleeding?**

- ☐ **Spotting**, a drop or two of blood, not even requiring sanitary protection though you may prefer to use some.
- ☐ **Very light bleeding** (you would need to change the least absorbent tampon or pad one or two times per day, though you may prefer to change more frequently)
- ☐ **Light bleeding** (you would need to change a low or regular absorbency tampon or pad two or three times per day, though you may prefer to change more frequently)
- ☐ **Moderate bleeding** (you would need to change a regular absorbency tampon or pad every 3 to 4 hours, though you may prefer to change more frequently)
- ☐ **Heavy bleeding** (you would need to change a high absorbency tampon or pad every 3 to 4 hours, though you may prefer to change more frequently)
- ☐ **Very heavy bleeding or gushing** (protection hardly works at all; you would need to change the highest absorbency tampon or pad every hour or two)

AFFIX LABEL WITH ID HERE

The next few questions are about your pregnancy history. This information is very important to this study because it will help improve the health of all women. So please take whatever time you need to answer them as accurately and completely as possible.

19. **Have you ever been pregnant? Please include live births, miscarriages, still births, tubal pregnancies and abortions.**

    ☐ Yes
    ☐ No **{SKIP TO END STATEMENT}**
    ☐ Don't know

20. **How many times have you been pregnant in your life? Again, be sure to include live births, miscarriages, still births, tubal pregnancies and abortions.**

    |___|___|
    ENTER NUMBER OF PREGNANCIES

    ☐ Don't know

21. **How many of your pregnancies resulted in a live birth? Please count the number of pregnancies, not number of live-born children. For example, if you had twins or other multiple births, count as a single pregnancy.**

    |___|___|
    ENTER NUMBER OF PREGNANCIES RESULTING IN LIVE BIRTHS

    ☐ No live births **{SKIP TO END STATEMENT}**

22. **When was your last baby born?**

    |___|___| |___|___|
    M M Y Y

END STATEMENT

The survey is now complete. We appreciate you taking the time to complete this survey. Your responses have provided us with valuable information

AFFIX LABEL WITH ID HERE

Appendix 7

Final Questionnaire

Donor Iron Status Follow-up Survey

Thank you for your continued participation in the Donor Iron Status Survey sponsored by the National Heart, Lung, and Blood Institute of the National Institutes of Health (NIH).This follow-up survey will ask you questions about any changes in your smoking history, vitamins and supplements that you take and if you are female, a few questions on your reproductive history. Your answers to all questions will be kept confidential and only be used for the purpose of this research.

Your continued participation is extremely important and will help us better understand iron status in blood donors. Your participation in this survey is voluntary. If you choose not to participate, it will not affect your ability to donate blood again in the future. You will not lose any benefits.

Name: .....

First Name Middle Name Last Name

Today's Date: ____ ____ - ____ ____ - ____ ____
Month Day Year

Blood Center ID: .....

Whole Blood Number (WBN): .....

AFFIX LABEL WITH ID HERE

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National Institutes of Health (NIH)

SECTION A
Your smoking history:

1. **SINCE THE SUMMER OF 2007, WHEN YOU ENROLLED IN THIS STUDY, have you started smoking, stopped smoking, continued to smoke, or still do not smoke? PLEASE CHECK ONE BOX**

- ☐ I started smoking → **Thinking about the last 30 DAYS (1 month), on how many of these days did you smoke?**
  - |___|___| NUMBER OF DAYS
  - ☐ Don't know
- ☐ I stopped smoking
- ☐ I have continued to smoke → **In the LAST 30 DAYS, on the days that you DID smoke, about how many cigarettes did you usually smoke per day?**
  - |___|___| NUMBER OF CIGARETTES
  - ☐ Don't know
- ☐ I still do not smoke

SECTION B
Your use of vitamin pills, supplements and aspirin:

2. ARE YOU CURRENTLY TAKING any multivitamins such as One-A-Day, Theragran, or Centrum type multivitamins (as pills, liquids, or packets) on a regular basis (at least once a week)?

☐ Yes
☐ No
☐ Don't know

When did you start?

|___|___| |___|___|
M M Y Y

How often do you take multivitamins?

☐ Everyday
☐ 4 to 6 days per week
☐ 1 to 3 days per week
☐ Don't know

Does your multivitamin contain iron?

☐ Yes
☐ No
☐ Don't Know

3. ARE YOU CURRENTLY TAKING any iron supplements other than your multivitamins on a regular basis (at least once a week)?

☐ Yes
☐ No
☐ Don't know

When did you start?

|___|___| |___|___|
M M Y Y

How often do you take iron supplements?

☐ Everyday
☐ 4 to 6 days per week
☐ 1 to 3 days per week
☐ Don't know

4. Do you currently take Aspirin or Aspirin containing pain relievers daily or nearly everyday?

☐ Yes
☐ No
☐ Don't Know

Why?

☐ For heart or cardiac health
☐ For pain relief
☐ For both

{MALE DONORS SKIP SECTION C AND GO TO END STATEMENT}

SECTION C
FOR FEMALE DONORS ONLY
Your reproductive history:

5. **Which of these statements best describes your current menstrual status?**

- ☐ I am still having periods and am NOT going through menopause
- ☐ I am still having periods, but am possibly going through menopause
- ☐ My periods have stopped completely because I have gone through menopause
- ☐ I had an operation which stopped my periods
- ☐ I am taking a medication that has stopped my periods completely
- ☐ My periods have stopped because of other reasons

When did you stop having your menstrual period?

|__|__| |__|__|
M M Y Y

ENTER DATE OF LAST PERIOD AND THEN PLEASE SKIP TO QUESTION 8

6. **What was the date when your last menstrual period started?**

|__|__| |__|__|
M M Y Y
ENTER DATE OF LAST PERIOD

- ☐ I am having my period now

7. **How would you describe your MOST RECENT menstrual flow or bleeding?**

- ☐ **Spotting**, a drop or two of blood, not even requiring sanitary protection though you may prefer to use some.
- ☐ **Very light bleeding** (you would need to change the least absorbent tampon or pad one or two times per day, though you may prefer to change more frequently)
- ☐ **Light bleeding** (you would need to change a low or regular absorbency tampon or pad two or three times per day, though you may prefer to change more frequently)
- ☐ **Moderate bleeding** (you would need to change a regular absorbency tampon or pad every 3 to 4 hours, though you may prefer to change more frequently)
- ☐ **Heavy bleeding** (you would need to change a high absorbency tampon or pad every 3 to 4 hours, though you may prefer to change more frequently)
- ☐ **Very heavy bleeding or gushing** (protection hardly works at all; you would need to change the highest absorbency tampon or pad every hour or two)

AFFIX LABEL WITH ID HERE

8. SINCE THE SUMMER OF 2007, WHEN YOU ENROLLED IN THIS STUDY, have you given birth to a baby?

☐ Yes

☐ No

When was this baby born?

|__|__| |__|__|
M M Y Y

END STATEMENT

The follow-up survey is now complete. We appreciate you taking the time to complete this survey. Your responses have provided us with valuable information. THANK YOU!

AFFIX LABEL WITH ID HERE

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