

Hemochromatosis and Iron Overload Screening Study

Study Design/Protocol

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Version 1.2

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1. Introduction and Background

Iron overload is a common health problem mistakenly believed by many to be quite rare. The disease is insidious in onset, and many or even most individuals diagnosed with this disorder are not identified until advanced organ damage is present. Excess iron is deposited in body tissues, and can accumulate to toxic levels over time, leading to cirrhosis of the liver, hepatocellular carcinoma, congestive heart failure and arrhythmias, diabetes, arthritis and sexual dysfunction. Men tend to exhibit complications at an earlier age than women since the latter maintain naturally lower iron levels through menstruation and pregnancy. While patients may manifest signs and symptoms of these complications, the underlying iron overload may not be recognized. Iron overload is relatively easy to treat by removing the excess iron through repeated phlebotomy. In addition, evidence suggests that early diagnosis and treatment can prevent disease manifestations and enable normal life expectancy. Thus, hemochromatosis may be suitable for detection and intervention through primary care screening strategies because: 1) it is relatively common; 2) it is asymptomatic in its early stages; 3) screening methods are reliable; 4) standard diagnostic methods are widely available in developed countries and are relatively inexpensive; 5) it is easily and safely treatable; and 6) if untreated, the subsequent burden of morbidity and mortality is substantial. However, there are a number of uncertainties about screening for iron overload and hereditary hemochromatosis, including the prevalence of iron overload in diverse communities, the penetrance of genetic variations, and the optimal care and treatment of asymptomatic and/or genetically at-risk individuals. Consequently, the feasibility and benefits of such a program remain to be assessed. It is also unclear whether to base early screening/diagnostic efforts on serum iron assay indicators (phenotypic measurement), genotyping assays, or a combination of both. The Hemochromatosis and Iron Overload Screening (HEIRS) Study will examine these and related issues.

Iron overload can be the end result of a wide range of genetic, hepatic, and blood diseases. Hereditary forms of iron overload have been identified in Caucasian and African populations. The most common type of iron overload seen in people of northern European ancestry is *HFE*-linked hemochromatosis, named after the discovery of the *HFE* gene (chromosome 6) in 1996. As a result of this discovery, a DNA-based genetic blood test became available and provides an opportunity for early and rapid genetic identification of individuals at risk for development of hereditary hemochromatosis. Much remains to be learned about the penetrance and expression of these alleles, including their relevance to the full spectrum of clinical disease. While 80-90% of Caucasian hemochromatosis patients have *HFE* abnormalities, there are hetero- and homozygotes that do not manifest any evidence of disease, or manifest disease at different ages and with different outcomes, implying the existence of other genetic or environmental factors. Similarly, not all hemochromatosis patients have *HFE* abnormalities. Other genes yet to be discovered are also likely to be involved in pathogenesis of iron overload and familial hemochromatosis, particularly in non-Caucasian populations. *HFE* genotyping at an early age is not a definitive indicator of later disease. Labeling a patient as having a genetic disease or being at genetic risk or susceptibility to a disease also may have discrimination ramifications in terms of denial of medical and life insurance and related employment issues. Thus, consensus panels to date have recommended that universal screening should not be initiated until more is understood

about the relationship between genotype, phenotype and hemochromatosis-related disease outcomes. Further study of minority patients is also essential in order to plan screening /early diagnostic programs appropriate for all groups.

The HEIRS Study is designed to study the prevalence, expression, genetic and environmental determinants and modifiers, and potential clinical, personal, and societal impact of iron overload and hereditary hemochromatosis, in a multi-center, multi-ethnic, primary care-based sample of 100,000 adults over a 5 year period. This information will be used to determine the feasibility and potential individual and public health benefits and risks of primary care-based screening and intervention for iron overload and hereditary hemochromatosis. Estimating the burden of preventable illness from unrecognized hemochromatosis is one of the most important of these needs. Determination of specific and sensitive early signs and symptoms of iron overload and hemochromatosis will enable earlier diagnosis and initiation of care to prevent sequelae. Comparing the relative value and acceptability of diagnosis and screening by genotype vs phenotype means is also important, and may be relevant to other adult-onset genetic disorders as well. In particular, differences by racial/ethnic group, age and other characteristics will need to be examined to be sure the resulting recommendations are appropriate for all patients.

The specific objectives of the HEIRS Study are to:

- a) Determine the prevalence in a primary care population, by race/ethnicity, of: 1) iron overload, defined as confirmed elevation of transferrin saturation and ferritin, and hereditary hemochromatosis; 2) demonstrable clinical and pathological abnormalities related to iron overload and hereditary hemochromatosis; and 3) genetic variants related to iron overload and hereditary hemochromatosis including the recently identified C282Y and H63D mutations of the *HFE* gene.
- b) Identify risk factors influencing the phenotypic expression of iron overload and hereditary hemochromatosis in regard to demonstrable clinical and pathological abnormalities, and examine interactions between risk factors to determine the relationship between genotype and phenotype. Risk factors could include genetic factors (co-modifying genes), or non-genetic factors such as diet, gender, age, alcohol intake or hepatitis B and C viruses.
- c) Examine ethical, legal and social issues related to the possibility of implementation of primary care-based screening for iron overload and hereditary hemochromatosis, including identification of appropriate health care delivery models and potential personal, societal, or family-related impact of and barriers to primary care- or population-based screening.
- d) Estimate the heritability of iron overload and hemochromatosis, and initiate linkage studies to identify main effect and modifier genetic variants associated with iron overload and hemochromatosis.

2. Study Organization and Summary

2.1 Study Organization

The HEIRS Study was initiated and funded by the Genetic Epidemiology Scientific Research Group, Epidemiology and Biometry Program, Division of Epidemiology and Clinical Applications, NHLBI, in conjunction with the Blood Diseases Program, Division of Blood Diseases and Resources, NHLBI, and the Ethical, Legal and Social Implications (ELSI) Research Program, Division of Extramural Research, NHGRI, through a contract mechanism. There are five Clinical Field Centers, a Central Laboratory and a Coordinating Center. An organizational chart of the HEIRS Study and a list of participating centers appears in Appendices A and B.

The HEIRS study has brought together a large team of experts in diverse disciplines such as clinical medicine including hematology and gastroenterology, clinical genetics, population genetics, laboratory medicine, epidemiology, statistics, ethics, law, anthropology, psychology, and genetic counseling, to develop the protocol over a series of meetings that have taken place since the inception of the study in February 2000. The depth of this project is reflected in the detail of this protocol document and is evidence of the collective efforts of all of the HEIRS study team. A companion Manual of Procedures provides specific operational details of the project for all study personnel.

The HEIRS Steering Committee, composed of the Principal Investigators from each participating center, the ELSI Subcommittee Chair, the Project Officer (NHLBI) and a Steering Committee Chair, has responsibility for developing the protocol of the study, assisting the participating centers in the conduct of the study, tracking study progress and resolving operational issues. Specific protocol and operational issues are dealt with by the relevant Subcommittee. A list of Committee and Subcommittees, as well as their members, is provided in Appendix B.

In accordance with NHLBI policy, a Monitoring Board consisting of experts in hemochromatosis/clinical medicine, genetic epidemiology, genetic screening, ELSI research, clinical laboratory issues, and epidemiology is being put together. The Monitoring Board will review and approve the HEIRS protocol and provide oversight of the study.

2.2 Study Summary

This section provides a summary of the main components of the HEIRS study; an outline of study flow appears in Appendix C. Further details of the protocol, with specific hypotheses for each study component are found in the following sections. Operational details of the study are provided in the companion Manual of Procedures.

The HEIRS Study data collection will take place over three years and involve 100,000 male and female patients aged 25 and older, recruited from primary care clinics associated with five field centers in: Birmingham, Alabama; Irvine, California; London, Ontario, Canada; Portland, Oregon; and Washington, D.C (see Appendix B). These clinics serve an ethnically

and socioeconomically diverse sample of the primary care patient community; the recruitment goal is for inclusion of 50% minority patients. The chosen clinics represent a variety of medical care systems including a health maintenance organization, a clinical blood collection center, public and private primary care provider offices and walk-in primary care clinics. Thus, resulting recommendations on early diagnostic screening for iron overload and hemochromatosis should be applicable to a wide range of patients in a variety of care settings.

During the initial stage of the study, planned to begin in February 2001 and last two years, primary care patients will volunteer to have blood drawn and answer a brief questionnaire on their demographics, reasons for participation and attitudes towards genetic testing (ELSI assessment). The blood specimens will be assayed for transferrin saturation, serum ferritin and *HFE* C282Y and H63D genotype. If other relevant polymorphisms, particularly those associated with African iron overload or other minority-prevalent iron disorders, are identified during the course of this study, additional genetic assays may be considered. Phenotypic and genotypic results will be provided to patients, and if they have so consented, directly with their primary care providers. While this study will focus on excess iron, it is recognized that these screening assays may also lead to detection of previously undiagnosed iron deficiencies and anemias.

Patients identified as having genetic or phenotypic evidence of iron overload and/or risk of hemochromatosis will be invited back for further diagnostic testing during the comprehensive clinical examination (CCE) phase. It is estimated that 1000 such patients will be identified. In addition approximately 1000 patients without iron overload or hereditary hemochromatosis, but who may have other types of disorders will be invited to the CCE so as to ascertain which signs, symptoms, biochemical and genetic assays and complications are more commonly associated with hemochromatosis, and thus contribute to the recommendations on early diagnostic screening and testing. The CCE will assess iron stores, attempt to distinguish between primary and secondary causes of iron overload and examine the associated hepatic, endocrinologic, hematologic and cardiovascular disease correlates and sequelae of hemochromatosis. Blood specimens will be used for a variety of diagnostic biochemical assays including liver enzymes, insulin, glucose, inflammatory markers, relevant viruses and complete blood count (CBC). A detailed family and medical history will be obtained. Additional questionnaires will be used to determine lifestyle characteristics such as smoking status, alcohol intake, and diet assessments (particularly iron supplements and inhibitors and promoters of iron absorption) which may explain the range of expression of disease and pattern of complications in those genetically susceptible. An extended ELSI assessment of issues related to genetic screening and testing will be conducted via mail after the CCE. This same ELSI assessment will be mailed to a group of patients with *HFE* variants other than C282Y homozygotes. CCE results will be provided to the patients, and again, if they have so consented, directly with their primary care providers.

Because of the genetic etiology of hemochromatosis, family members may also be at risk of disease. Thus, as examinees with evidence of primary iron overload are identified, 2000 of their family members will be invited to receive a CCE. Besides directly ascertaining the disease status of these individuals both phenotypically and genotypically and referring for

treatment as appropriate, their DNA samples will be used to identify modifier genetic variants related to the expression of iron overload and hereditary hemochromatosis disorders via genome scan genotyping and linkage analyses. If any additional relevant gene variants are found, additional patients at risk may be identified and will receive genetic counseling as appropriate.

Ethical considerations mandate that patients found to have evidence of iron overload or deficiency be treated to prevent or attempt to reverse clinical disease. As such, throughout the study, clinically relevant results will be provided to the patients, along with genetic counseling as appropriate. Patients will be encouraged to discuss these results and follow-up treatment options with their primary care provider. Definitive diagnosis of hemochromatosis may necessitate liver biopsy and/or quantitative phlebotomy. The decision for these tests should be made on an individual basis, involving the patient and his/her primary care provider, and will not be a part of this study. However, with the patient's permission, the results of these procedures will be obtained annually for inclusion in study analyses. Similarly, a one-time follow-up ELSI assessment will be sent to the CCE hemochromatosis patients. While treatment will occur outside of the study, the field center organizations are committed to providing these tests and follow-up care in the advent that the patient does not have adequate medical insurance.

2.3 Description of targeted population

The targeted population is primary-care patients who are 25 years of age or older. The Field Centers' approaches to this targeted patient population differ, and involve approaching:

- (a) Patients presenting at primary-care clinics (Howard University, University of Alabama at Birmingham, University of California at Irvine)
- (b) Patients presenting at clinical laboratories for a blood draw (London Health Sciences Centre)
- (c) Primary-care patients from a health-plan membership (Kaiser Permanente)

The only other inclusion criterion is ability to give informed consent for participation in this research study.

Due to the sparseness of iron overload data in non-Caucasians, the study was designed to include a greater proportion of minority individuals than are present in the general population of the U.S. and Canada. Of the 100,000 participants, it is expected that about 52,000 will be of African, Asian, Hispanic, Native American, or Pacific Island ancestry. The Field Centers represent diverse populations, and are focusing on certain populations. The table below describes each Field Center's ancestry goals and study population in more detail.

Field Center	Ancestry projections	Study population
Howard University (Washington, D.C.)	79% Black/African 20% Hispanic 1% White/European	Howard University Hospital: Primary care blood draw. HUMED: Primary care blood draw. La Clinica del Pueblo: Primary care blood draw. D.C. General Hospital: Primary care blood draw.
Kaiser Permanente (Northwest and Hawaii)	1% Black/African 20% Asian/Pacific Islander 3% Hispanic 1% Native American 75% White/European	Portland, Oregon, metropolitan area and Oahu island, Hawaii: KP members with a primary-care visit.
London Health Sciences Centre (Ontario, Canada)	20% Asian 80% White/European	Primary care patients from London area sent to central lab for blood draw. Patients seen by Chinese Canadian MDs in Toronto, recruited in practices and/or blood lab.
University of Alabama at Birmingham	50% Black/African 50% White/European	Primary care clinics Family Practices State employees health screening clinics
University of California at Irvine	30% Asian 30% Hispanic 5% Black/African 35% White/European	UCI: 5 ambulatory care clinics. UCLA: Family practice.

The age criterion is based on existing information about the age-of-onset of clinical manifestations of iron overload and hemochromatosis. It is very unlikely to observe clinical problems associated with iron overload in individuals younger than 25 years. Individuals with such conditions as acute or chronic infections and pregnancy may be part of a general population screening program for hemochromatosis or iron overload and are not excluded. The impact of such participants on the screening will be assessed. The age criterion will be decreased to 19 (or the age of majority) for the family study.

The selected targeted population, primary-care patients, and implementation strategies (e.g., approaching patients when they present for a clinic visit) reflect likely scenarios for general population screening. Other population definitions were considered, such as more population-based strategies. Only one site could have implemented such a strategy. Although the results would, most likely, better represent actual population parameters (e.g., prevalence of iron overload), they may differ from what one may observe in screening populations. Volunteers outside of the targeted population are not being sought; however, people who volunteer, but are not part of the targeted population, will be accepted into the study. For example, a person who accompanies a friend or family member to a primary-care visit will be eligible to participate.

3. Recruitment and Retention

3.1 Hypotheses

Hypotheses RR1-RR3 are specific to issues of recruitment, retention, and the sampled population, whereas hypotheses RR4 and RR5 are somewhat more general in that they overlap with screening hypotheses.

RR1: The prevalence of HFE mutations (i.e., C282Y and H63D), screen positive rates for TS and SF, clinical penetrance of iron overload given HFE genotype, and prevalence of specific medical conditions associated with iron overload will differ with respect to prevalent health conditions and motivations for participating in this research project.

RR2: The prevalence of HFE mutations (i.e., C282Y and H63D), screen positive rates for TS and SF, clinical penetrance of iron overload given HFE genotype, and prevalence of specific medical conditions associated with iron overload will differ between targeted and intentional volunteers.

RR3. Retention rates will differ between individuals who screen positive (i.e, those who have genotype C282Y/C282Y or C282Y/H63D, and/or have elevated TS and SF) and those who do not.

RR4. The prevalence of HFE mutations (i.e., C282Y and H63D) will differ by race and ethnicity.

RR5. Screen positive rates for TS and SF, clinical penetrance of iron overload given HFE genotype, and prevalence of specific medical conditions associated with iron overload will differ by age, gender, race, and ethnicity.

3.2. General recruitment and retention strategies

Effective recruitment and retention strategies are key to the success of this study. Success will depend on HEIRS study staff, as well as physicians and clinic staff. Effective recruitment methods include: developing recruitment materials that are socially and culturally appropriate for the groups being recruited; pilot-testing recruitment materials (e.g., brochures, consent forms, advertisement, video); doing advance educational work in communities (e.g., local media, community leaders and organizations, community-gathering places, in-clinic posters); working with clinic staff and physicians to provide information about iron overload and this study, to address logistical issues (e.g., patient flow), and to get “buy-in; providing written, and, if possible video, information about iron overload and this study; using a “train-the-trainer” model, providing training for recruiters that includes study explanation (plus FAQs), importance of respect for clinicians/staff and participants, and cultural awareness, as well as technical aspects of recruiting (such as subjective assessment of ability to give informed consent).

3.3. Recruitment and retention methods

Retention is related to respondent burden, defined as inconvenience (time, bother, expenses), discomfort (physical and emotional), and intrusiveness (collection of highly personal or embarrassing information). We plan to minimize respondent burden the following ways. Inconvenience can be minimized by defraying expenses for parking, transportation, etc., by reducing duplicate data collection, by incorporating standardized data collection formats (e.g. consistent question response sets, etc.), by spreading data collection over time to the extent possible, and by minimizing unnecessary duplicated data collection. Physical discomfort (e.g. blood draws) can be minimized by reassurance and training of phlebotomists; emotional discomfort should be minimized through access to someone who can answer participants' questions or concerns. Intrusion can be minimized by reducing questions of a highly personal or threatening nature (e.g., sexual function and behavior, substance use), by deferring them until rapport and trust have developed, and by having a quiet private place to discuss personal concerns.

Retention is also related to intensity and quality of follow-up, defined as the regularity and professionalism with which initial participants are re-contacted in subsequent stages of the project. Intensive follow-up will be aided by: 1) contact at regular intervals; 2) adequate resources to allow multiple attempts; and 3) multiple information sources to aid tracking (e.g. names and addresses of more than one person who will know the whereabouts of study participants). Professionalism includes issues of rapport and compassion, which build trust on the part of participants. This will be aided by staff training and, wherever possible, having one person re-contact the same participants rather than having numerous study personnel unfamiliar to the participant contact them.

3.4. Educational and training materials to be developed.

Recruitment methods will involve approaching potential participants in-person, by phone, and/or by mail, providing written and/or audio-visual information about hemochromatosis, iron overload, and the HEIRS Study, and answering questions about the study. We are targeting primary-care patients, and do not plan to seek non-targeted volunteers. However, other volunteers will not be excluded (e.g., a person who accompanied a primary-care patient to her/his visit and who is interested in participating).

Recruitment and retention methods for each Field Center are summarized in the table below. Recruitment letters, scripts and logs are in Appendix D.

Field Center	How	Who	Recruitment	Retention
Howard University	Approach patients in clinics	Research nurse/counselor. Research phlebotomist.	Community awareness campaign to be developed Pre-consent education at primary care facility. Return visit: local travel funds. Family: local travel funds.	Travel funds for return visits. Follow-up by telephone or home visits if necessary.
Kaiser Permanente	Mail study information in advance, and Meet patient in the clinic Talk with people who respond by mail or phone	Research recruiters.	Awareness campaign, members & staff Mailed study info (use communications & marketing principles) Family: mailed study information.	Clinical assessment: reimbursement for expenses and retention items.
London Health Sciences Centre	Approach patients in clinical laboratory (London and Toronto). Approach patients in clinics (Toronto).	Trained experienced recruiters with private interview area in blood lab. Asian-Canadian recruiter (Toronto site).	Physician education Physician admin. fee. Given HH brochure at blood drawing (Canadian Liver Foundation). Family: brochure from proband.	Clinical assessment: parking expenses provided.
University of Alabama at Birmingham	Approach patients in clinics	Clinic personnel. Recruiters/ interviewers assigned to each clinic.	Academic detailing at each clinic for Physicians & staff.	Evaluated at same local clinic. Phone follow-up.
University of California at Irvine	Call patients in advance of appointment, then meet them in the clinic	Research project coordinator–phone contact prior to visit. Clinical Nurse Coordinator–in clinic.	Clinician education. Videotape for patients. Patient info booklet (goes over in person while in clinic).	Contact by Clinical Nurse for appointment. Reminder call and postcard. Evaluated at usual clinic.

3.5. Monitoring recruitment and retention

Monitoring recruitment progress through accrual targets, participation rates, and retention rates will provide information to evaluate the success of our methods and identify potential problems. Accrual targets will be agreed upon by the Steering Committee, and monitored by each Field Center, the Coordinating Center (CC), and the R&R subcommittee. Each Field Center has primary responsibility to monitor their own recruitment and retention, and solve any problems that may arise. The CC will assist by providing reports. The R&R subcommittee will provide advice should problems persist. If necessary secondary recruitment strategies will be implemented, with any substantive changes in design reviewed and approved by the Steering Committee. Secondary recruitment strategies may include: developing better methods to emphasize study objectives and value to potential participants and clinicians and increasing the number of participating clinics and physicians

3.6 HEIRS Study Recruitment Logs

People who decide not to participate or to discontinue participation can provide valuable information for assessing potential recruitment and retention problems. For people who express interest in the study, as evidenced by starting through the informed consent process, or who begin participation, we will collect qualitative information about reasons for not (or no longer) participating. The recruiter will record this information on the Daily Recruitment Log or Participant Follow-up Contact Log (see Appendix D). These reasons will be reviewed at each site to determine whether there are issues that we can address to make study participation more feasible or desirable.

The primary aim of collecting and summarizing participation status data is to monitor the recruitment process. Two forms will be used for this purpose: (1) the Daily Recruitment Log and (2) the Weekly Recruitment Summary. In the case of the Daily Recruitment Log, each recruiter would fill out a log to record participation information on individuals that he/she approached that day. If a recruiter works at more than one clinic on a given day, then separate logs will be filled out for different clinics. Data from the Daily Recruitment Log will not be entered into the computer. A Weekly Recruitment Summary form would be filled out weekly for each clinic based on the previous week's logs within the clinic. Data from the Weekly Recruitment Summary would be submitted electronically to the CC for Recruitment and Retention status reports.

The data from these summaries will be used to estimate weekly participation rates. For purposes of monitoring recruitment, the participation rate may be defined as $\text{New Participants} / (\text{New Participants} + \text{New Non-Participants} + \text{New Undecideds})$. If the previous status of New Participants is recorded on the Participant form (e.g., answer to the question: 'Is this the first time you have been asked to participate in this study?'), then this information might be used to 'correct' denominators of participation rate estimates over longer time periods.

4. Screening and Comprehensive Exam

4.1. Initial screening

4.1.1 Overview

The HEIRS study will screen 100,000 participants during an initial screening visit to a primary care clinic or blood collection facility. Participants will be eligible for the screening phase of the study if they are age 25 or older and able to provide informed consent. Patients will be approached and asked to give informed consent. If they appear to have difficulty reading, reading of an oral script will be offered (see Appendix E). If they do not appear to comprehend the oral script, they will not be eligible. As a part of this visit, each participant will complete a Contact Information Form and an Initial Screening Form and have blood drawn. Clinic staff will review these forms for completeness and conduct phlebotomy. (Note: at the Kaiser Permanente sites, phlebotomy will be performed by non-study staff) Both forms will be data entered locally. Blood will be shipped to the Central Lab, where values for transferrin saturation (TS) and serum ferritin (SF) will be obtained, a screen of the HFE gene for C282Y and H63D mutations will be performed, and blood will be stored for future study related use. The Central Lab will transmit data on TS, SF and HFE mutations to the Coordinating Center (CoC), and the CoC will make them available to the Field Centers (FC). (Note: at the London site, TS will be performed locally with Central Laboratory quality control.)

4.1.2 Hypotheses

SE1. A higher proportion of cases with iron overload, as defined by the combination of sustained elevations in TS and SF in the absence of anemia, inflammation, transfusion or liver disease, will be identified by genotypic screening for HFE mutations than by phenotypic screening. (Witte et al., 1996; Gordeuk et al., 1998; Phatak et al., 1998; McDonnell et al., 1999; Adams 2000; Barton and Acton, 2000; Beutler et al., 2000).

SE2. HFE mutations account for greater than 90% of IO. (Witte et al., 1996; Gordeuk et al., 1998; Phatak et al., 1998; McDonnell et al., 1999; Adams 2000; Barton and Acton, 2000; Beutler et al., 2000).

SE3. Greater than 75% of C282Y homozygotes have elevated TS. (McLaren, Gordeuk, et al. 1995; Witte et al., 1996; Bulaj et al., 1996; Gordeuk, McLaren et al., 1998).

SE4. Among African-Americans and Asians, homozygotes for C282Y will be found in less than 5 per 10,000. (Barton, Shih, et al., 1997; Roth et al., 1997; Barton, Acton, Edwards, et al., 1998; McNamara et al., 1998; Monaghan et al., 1998; Jeffery et al., 1999; Marshall et al., 1999; Acton and Barton 2000; Acton, Barton, Bell et al., 2000; Bloom et al., 2000; MacClenahan et al., 2000).

SE5. Non-HFE primary iron loading exists in 0.5 to 1% of the primary care population. (Barton, Alford, et al., 1995; Barton, Edwards, et al., 1995; Wurapa et al., 1996; Barton and Acton 2000)

Secondary hypotheses will include testing the above hypotheses in racial and ethnic subgroups. (Barton, Edwards, et al., 1995; Wurapa et al, 1996; Beutler and Gelbart 1997; Barton, Preston et al., 1999; MMWR 1995; Barton and Acton 2000) For hypothesis SE1, using the study definition for IO, the number of IO cases identified by the HFE screen but not by the phenotypic screen will be compared to the number of IO cases identified by the phenotypic screen but not by the HFE screen (using McNemar's test). For hypothesis SE2, the proportion of primary IO cases who have HFE mutations will be computed. For hypothesis SE3, among participants with an HFE mutation, the percentage with and without elevated TS will be estimated. In existing studies, the percentage of expressing HFE homozygotes has ranged from around 65% (Beutler 2000, Adams 2000, McDonnell 1999) to 90% (Olynyk 1999). To test hypothesis SE4, the proportion of C282Y homozygotes will be computed. For hypothesis SE5, the rate of primary IO for which no HFE mutation is identified will be computed. Details of statistical methods are in Appendix F.

4.1.3 Eligibility for the Comprehensive Clinical Exam: Potential Cases

Two possibly overlapping groups will be invited to the CCE: (1) all C282Y homozygotes and (2) other participants whose TS and SF values exceed both cutoffs in Table 1 below (McLaren, Gordeuk, et al. 1995; Gordeuk, McLaren et al., 1998). Throughout the screening phase, heterozygotes and compound heterozygotes will not be invited unless their levels of TS and SF are above cutoffs. An important concern with this protocol is the recall rate; that is, how many people would be eligible for the CCE. Cutoffs may be adjusted as the study progresses so that this number is controlled. Separate "alert" cutoffs may be specified which would trigger notification to clinic staff of participants who have a TS or SF value requiring clinical follow-up.

Table 1. Cutoffs for TS and SF

	Males	Females
TS	50	45
SF	300	200

Current recruitment targets call for the Initial Screening visit to involve approximately 47,000 Caucasian, 11,000 Hispanic, 27,000 African American (AA), and 15,000 Asian participants, or 100,000 total. Based on existing literature, for the purposes of projecting numbers eligible for the CCE, we have assumed a C282Y homozygote prevalence of 5 per 1000 among Caucasians and Hispanics and 5 per 10,000 among AA and Asians. In addition, using NHANES III data, some initial estimates for the proportion of participants eligible by gender and race/ethnicity has been estimated (Table 2). We expect 290 (95% confidence interval: 256 to 324) C282Y homozygotes among the Caucasians and Hispanics and 21 among the AA and Asians, for a total of 311. In addition, based on the percentages in Table 2, we expect a total of 1156 participants to exceed the TS/SF cutoffs. This number is likely

to include at least 65% of the 311 C282Y homozygotes, so that 202 of the 1156 will be C282Y homozygotes and 954 will not. Thus the predicted total number eligible for the CCE will be 1265, and assuming that 80% agree to participate, the number who will actually attend will be approximately 1012. Tables 3 and 4 contain more detailed projections for each category of CCE participant separately for each racial/ethnic group and gender.

Table 2. Estimates of proportions (numbers) exceeding SF and TS cutoffs based on NHANES III.

	<i>SF/TS</i>	<i>Caucasian</i>	<i>Hispanic</i>	<i>AA and Asian</i>
Men	300/50	1.30% (306)	2.10% (116)	1.40% (294)
Women	200/45	1.20% (282)	0.60% (33)	0.60% (126)

Table 3. Projections for the number eligible by category and racial/ethnic group.

	<i>Number eligible</i>					<i>Number participating (80%)</i>				
	<i>C</i>	<i>H</i>	<i>AA</i>	<i>As</i>	<i>Total</i>	<i>C</i>	<i>H</i>	<i>AA</i>	<i>As</i>	<i>Total</i>
C282Y only	82	19	5	2	109	66	15	4	2	87
C282Y+TS+SF	153	36	9	5	202	122	29	7	4	162
TS+SF only	435	113	260	146	954	348	90	208	117	763
Total	670	168	274	153	1265	536	134	219	123	1012

Table 4. Projections for the number eligible by category, racial/ethnic group and gender.

	<i>Number eligible</i>								<i>Number participating (80%)</i>							
	<i>C</i>		<i>H</i>		<i>AA</i>		<i>As</i>		<i>C</i>		<i>H</i>		<i>AA</i>		<i>As</i>	
	<i>M</i>	<i>F</i>	<i>M</i>	<i>F</i>	<i>M</i>	<i>F</i>	<i>M</i>	<i>F</i>	<i>M</i>	<i>F</i>	<i>M</i>	<i>F</i>	<i>M</i>	<i>F</i>	<i>M</i>	<i>F</i>
CY only	41	41	10	9	3	2	1	1	33	33	8	7	2	2	1	1
CY+TS+SF	80	73	28	8	6	3	4	1	64	58	22	6	5	2	3	1
TS+SF	226	209	88	25	182	78	102	44	181	167	70	20	146	62	82	35
Total	347	323	126	42	191	83	107	46	278	258	100	33	153	66	86	37

The approximate number of exams by site are estimated to be: Howard University, 177; Kaiser, 213; LHSC, 214; UAB, 194; UCI, 212.

Beutler (2000, Genetic Screening) reports that the proportion of patients with primary IO but do not have HFE mutations is 10% in northern Europe and 30% in southern Europe. Assuming these same proportions in our study are 20% of Caucasians attending the CCE and 30% of other racial/ethnic groups, we may expect that 31 of the 348 TS/SF only Caucasians and 17 of the 415 TS/SF only participants in other racial/ethnic groups will prove to have non-HFE primary IO. This estimate does not account for C282Y and H63D heterozygotes and compound heterozygotes.

4.1.4 Eligibility for the Comprehensive Clinical Exam: Potential Controls

Those participants in the Initial Screening Visit who are not eligible for the CCE as potential cases may be eligible as potential controls if their TS and SF values fall in the ranges specified in Table 5 and have no C282Y or H63D mutations. These ranges represent the first and third quartiles across all racial/ethnic groups in NHANES. Potential controls will be selected on a regular basis to reflect the Field Center, gender, racial/ethnic and age distribution of the potential cases.

Table 5. Eligible ranges for potential controls

	Males	Females
TS	20 - 334	16 - 28
SF	87 - 247	29 - 121

4.1.5 Alert values for transferrin saturation and serum ferritin

Table 6 contains percentiles of TS and SF for the US population based on NHANES III data. Participants with values below the 2.5th or above the 97.5th percentiles will receive a recommendation to contact their primary care physician as part of their results report.

Table 6. Percentiles of transferrin saturation (TS %) and serum ferritin concentration (SF g/dL) for males and females based on weighted data.

<i>Laboratory Test</i>	<i>Gender</i>	<i>Low</i>	<i>High</i>
Transferrin Saturation	Men	15	50
	Women	15	50
Serum Ferritin	Men	25	530
	Women	15	300

4.1.6 Results reporting

All participants in the screening exam will be notified of the results of their TS, SF and HFE screen. The letters to be sent to various outcome categories are in Appendix G. Site-specific variations in reporting procedures are identified in Appendix H.

4.2. Comprehensive Clinical Exam

4.2.1 Overview

Data collected for the Comprehensive Clinical Exam (CCE) will be used for several analyses, including the Case-Control Study, the Family Study, and parts of the Ethical, Legal and Social Implications (ELSI) Study. Participants completing the Initial Screening visit will be eligible for the CCE if they qualify as potential cases or are selected as potential controls. The invitation to participate in the CCE will be made at the same time as results are given.

Those participants who agree to attend the CCE will be mailed an Informed Consent form, a Food Frequency Questionnaire (FFQ), a Family History Form, a plastic medications bag, and instructions. At the CCE, participants will be asked to complete a Medical History Form. Clinic staff will verify informed consent, review all forms for completeness, complete the Medications section of the Clinical Assessment Form, draw blood and perform the Physical Exam section of the Clinical Assessment Form. All forms will be data entered locally (except the FFQ, which will be scanned), and blood will be shipped to the Central Lab.

It is expected that the CCE will include approximately:

- 87 C282Y homozygotic participants without elevated SF and TS
- 162 C282Y homozygotic participants with SF and TS above cutoff,
- 763 non-C282Y homozygotic participants with SF and TS above cutoff,
- or 1012 total potential cases.

In addition, there will be approximately 1000 controls. Family members of cases taking part in the Family Study, approximately 2000, will also undergo the CCE.

4.2.2 Study Case definition.

Study cases will be participants who are C282Y homozygotes, or who have evidence of primary iron overload. Primary iron overload will be defined as having elevated TS and SF, and no evidence of secondary IO or elevated TS and SF due to hepatocellular dysfunction or elevated SF due to inflammation. Evidence of secondary IO will include: lifetime history of blood transfusions more than 10 units or anemia. Evidence of hepatocellular dysfunction will include elevations in ALT and/or AST. Evidence of inflammation will include inflammation present by history or elevated CRP.

Specifically, for a participant who presents with elevated serum ferritin concentration and transferrin saturation, the four questions posed below will be asked. The first two address IO versus elevations in SF due to other causes. The second two address primary IO versus secondary IO. If the answer to all questions is no, then the subject likely has primary iron overload. If there are yes answers, further evaluation is needed to determine if patient may have primary iron overload.

1. Is inflammation present by history (e.g. active cancer, rheumatoid arthritis, SLE, other connective tissue disease, active or chronic infection), elevated ESR (>50 mm/hr), or C-reactive protein (> 2 mg/dl)? If no, there is possible primary iron overload. If yes, there is possible increased serum ferritin concentration due to inflammation. Note that the presence of both elevated serum ferritin concentration and transferrin saturation will tend to rule out inflammation, as inflammation tends to cause an increased serum ferritin concentration along with a decreased transferrin saturation.
2. Is hepatocellular dysfunction present (ALT or AST > 60 IU/L)? If no, there is possible primary iron overload. If yes, there is possible raised serum ferritin concentration and transferrin saturation due to hepatocellular damage. Note that further clarification of hepatocellular dysfunction should be pursued if ALT or AST is elevated. This includes

history of alcohol abuse; positive hepatitis B surface antigen or antibody to hepatitis C; tests for Wilson's disease, alpha-1 antitrypsin deficiency, auto-immune hepatitis, sclerosing cholangitis; drug toxicity; metastatic disease; infection. If these are all negative, the hepatocellular dysfunction may be secondary to iron overload.

3. Is lifetime history of blood transfusions (not given for hemorrhage) more than 10 units? If no, there is possible primary iron overload. If yes, there is possible secondary iron overload.

4. Is anemia present (hemoglobin concentration < 13 g/dL in men or 11 g/dL in women)? If no, there is possible primary iron overload. If yes, there is possible secondary iron overload or inflammation. The anemia should be further clarified. Elevated reticulocytes with elevated indirect bilirubin, LDH or low haptoglobin indicates hemolytic anemia. Normal reticulocytes with elevated indirect bilirubin, LDH, or low haptoglobin indicates possible secondary iron overload (ineffective erythropoiesis). Normal reticulocytes with normal indirect bilirubin, LDH and haptoglobin and history of chemotherapy, radiation therapy, aplastic anemia, pure red cell aplasia, or myelodysplasia indicates possible secondary iron overload (shift from red cell compartment). Normal reticulocytes with normal indirect bilirubin, LDH and haptoglobin and evidence of chronic inflammation indicates possible raised serum ferritin concentration due to inflammation.

4.2.3 Study Control definition

As described in Section 4.1.4, study controls will have TS and SF values in the ranges specified in Table 5 and have no C282Y or H63D mutations. Potential controls will be selected to reflect the Field Center, racial/ethnic and age distribution of the potential cases. Frequency matching on agegroups stratified by Field Center and racial/ethnic group will be performed on a regular basis as primary iron overload cases are identified.

4.2.4 Hypotheses for the Case-Control Study.

Participants who are C282Y homozygotes or who are classified as having primary IO after results from the Comprehensive Exam have been obtained will be 'cases'. The Case-Control Study will examine a variety of hypotheses.

CC1. The prevalence of some symptoms and signs will be increased in cases relative to controls. (Gordeuk et al., 1992; Barton, Edwards, et al., 1995; Witte et al., 1996; Wurapa et al., 1996; Bloom et al., 2000) The symptoms and signs of primary interest are:

- (a) fatigue,
- (b) joint stiffness,
- (c) shortness of breath,
- (d) change in skin color.

CC2. The prevalence of some medical conditions will be increased in cases relative to controls. (Gordeuk et al., 1992; Barton, Edwards, et al., 1995; Witte et al., 1996; Wurapa et al., 1996; Bloom et al., 2000) The medical conditions of primary interest are:

- (a) diabetes,
- (b) liver disease,
- (c) heart failure and arrhythmias.

CC3. The prevalence of some environmental factors will be increased in cases relative to controls. (Gordeuk et al., 1992; Barton, Edwards, et al., 1995; Witte et al., 1996; Wurapa et al., 1996; Bloom et al., 2000) The environmental factors of primary interest are:

- (a) use of iron supplements,
- (b) use of alcohol,
- (c) younger age at menopause.

CC4. There will be a variety of genetic and gene-environment interactions (Gordeuk et al., 1992; Barton, Edwards, et al., 1995; Witte et al., 1996; Wurapa et al., 1996; Bloom et al., 2000). The hypotheses of primary interest are:

- (a) Dietary iron modifies the effect of genotype on serum ferritin and transferrin saturation.
- (b) Patterns of dietary intake will modify clinical features of IO.
- (c) Age at menopause modifies the effect of genotype on serum ferritin and transferrin saturation.
- (d) Alcohol consumption modifies the effect of genotype on liver damage.
- (e) The hepatitis C virus modifies the effect of genotype on liver damage.

4.2.5 Description of forms

Forms collected during the CCE include the FFQ, the Family History Form, the Medical History Form, and the Clinical Assessment Form. Data collection for the Family Study is described in Section 7. The Medical History Form includes information on current symptoms and signs, medical history, reproductive history for women, blood transfusion and donation, and lifestyle. The Clinical Assessment form includes information on current medications, both prescription and over-the-counter, demographics, and physical exam. A list of variables appears in Appendix I. All forms will be translated into Spanish, Vietnamese and Mandarin, except the FFQ, which will be translated into Spanish only. Data will be entered using a web-based system, except for the FFQ, which will be scanned at a central location.

4.3 Follow-up of Comprehensive Clinical Exam participants.

One year following the CCE and annually thereafter until the end of the study, CCE participants' Primary Care Physicians will be contacted and asked to complete a Clinical Follow-up Form to determine the course of clinical treatment and appearance of new manifestations in primary iron overload among their patients who attended the CCE. In addition, CCE participants will be mailed an ELSI follow-up forms one week and one year following the exam (see Section 5).

5. Ethical, Legal, and Social Implications (ELSI)

5.1 Overview

The overarching aim of the ELSI research protocol is to provide data to assess the psychological and social effect of screening for hereditary hemochromatosis (HH) as it is implemented in the HEIRS study. In this the ELSI studies are an integral part of the HEIRS study. The general questions of interest in the ELSI protocol are: (a) psychosocial impact of being screened for HH for all possible screening outcomes; (b) level of comprehension of information conveyed to participants about HH; (c) the extent of, attitudes toward, and reasons for sharing genetic risk information about HH with family members and others; (d) adherence to medical recommendations in regard to HH; and (e) evidence of stigmatization and discrimination in regard to screening for HH. These areas will be investigated through self-administered questionnaires. Baseline data will be collected on the Initial Screening form at the screening visit. All participants completing the Comprehensive Clinical Exam will be mailed the either the Post Result Form or, for family members of probands, the Family Post-Result form one week after notification of their exam results. A sample of participants not attending the Comprehensive Clinical exam will also be mailed the Post Result form. One year after the Comprehensive Clinical Exam, participants will be mailed either the One Year Follow-up

5.2 Specific Aims and Hypotheses

Aim 1: Assess the psychological impact of screening for iron overload and hereditary hemochromatosis on individuals from diverse populations.

EL1: Compared to individuals with negative test results, individuals with positive genetic test results will show poorer perceived health, increased endorsement of both the risks and benefits of testing, increased endorsement of heredity as a reason why people get sick, a transient reduction in well-being--followed by a return to baseline at follow-up, greater knowledge about iron overload and HH and its genetic contribution, and more information seeking.

EL2: Compared to individuals with definitive (positive or negative) test results, individuals with ambiguous test results will show reduced psychological well-being as evidenced by: decreased psychological well-being, increased endorsement of the risks of testing, decreased endorsement of the benefits of testing, reduced satisfaction with counseling, no difference is expected in knowledge about iron overload and HH and its genetic contribution or in perceived health.

Aim 2: Assess awareness, knowledge, and attitudes about iron overload, hereditary hemochromatosis, and genetic testing. Examine the effectiveness of knowledge transfer and satisfaction during screening, results disclosure, clinical exam, and counseling experiences.

EL3: Baseline awareness and knowledge about iron overload and hereditary hemochromatosis will be poor, but will improve significantly for those who are given positive test results for either elevated iron or HH mutations (both homozygotes and heterozygotes). Level of knowledge will also be related to education level.

EL4: Participants will be generally positive about genetic testing and the ability of genetic information to help improve their health. The positive feelings will diminish over time as more is learned about the limitations of access to genetic information.

EL5: Participants will be generally satisfied with their screening experiences. Less satisfaction will be observed in those who test positive in any of their tests (phenotype or genotype, including both homozygotes and heterozygotes). The least satisfied will be those who have ambiguous test results and those who are mutation carriers, but show no evidence of iron overload.

Aim 3: Compare the predictors of intention to share the results of screening and clinical exam with actual sharing of information with others during the one year follow up. Examine the reasons for and attitudes toward sharing of genetic risk information.

EL6: Individuals who are homozygous for C282Y will be more likely to intend to share their test results with family members, than those who are heterozygotes, compound heterozygotes, or who have ambiguous genetic test results. Those who phenotypically test positive will be the least likely to intend to share their test results with family members.

EL7: Attitudes toward information sharing and concerns about confidentiality will be patterned according to educational level – more educated participants will have more concerns about confidentiality and discrimination.

Aim 4: Examine the factors that predict adherence to follow up recommendations.

EL8: Comprehension of test results, understanding of the disease, hemochromatosis, increased self efficacy for following recommendations, satisfaction with screening, decreased distress, perceived health and well being will be positively associated with increased intention to adhere and real actions related to adherence.

Aim 5: Identify evidence of stigmatization or discrimination as a result of participation in screening for iron overload and hereditary hemochromatosis.

EL9: Participants who are genotype-positive will perceive themselves as more stigmatized (more worried and perceived to be ill) and will be more likely to experience discrimination.

5.3 Discussion of Aims and Hypotheses

Aim 1 is a key research aim in almost all ELSI-based, psychosocial genetic studies, since the earliest studies of Huntington disease (HD). Although HD was one of the earliest disorders studied, it is, in many ways, an extreme case: A neurodegenerative, adult onset, uniformly fatal, disorder with virtually 100% penetrance and no available treatment. Finding out that one had the HD mutation was found often to be associated with severe depression, including suicide and suicidal ideation. Later studies of adult onset disorders (e.g. breast and colon cancer), in general found less serious sequelae – anxiety and depression but, in most cases, not of a degree to interfere with daily functioning. HH is, again, a different case – a disorder where treatment is available and of a less invasive nature than screening and surgical modalities involved with cancer. For this reason we are hypothesizing even less severe psychological reactions associated with HH. However, there is an interesting and important countervailing trend. As HH is an autosomal recessive condition, it is possible that individuals and families will be confronted with their risk for this condition without a prior family history. The unexpectedness of this diagnosis or risk and of the disease itself, may have its own sequelae of anxiety, confusion, and denial. Since denial may lead to lack of compliance with screening and treatment regimens, it would be an important finding.

Stigma is another issue prominent in discussions of genetic information. Stigma is a social as well as a psychological phenomenon, since it relates to ways one fears one is regarded by others. Stigma associated with HH, as a disease of the blood, may be particularly influenced by two variables: (a) culture (ethnic/race variation) and (b) phenotypic versus genotypic information. The first of these will be studied through the inclusion of ethnic minorities within the ELSI sample; the second will be studied through naturally occurring variations in screening results (it will also be the focus of the qualitative interview substudy, described elsewhere).

Aim 1 will be met using both standardized and investigator-devised instruments. Questions about perceptions of health and well being, psychological distress, and perceptions about stigmatization will be asked at baseline, after receiving screening or comprehensive clinical exam results, and one year follow up in HEIRS/ELSI cases and controls.

Data will be analyzed by analysis of covariance on change scores from the baseline assessment, with initial scores as a covariate. The independent variable will be test result (i.e., Group in Table 1). For variables on which there are no baseline data (e.g., information seeking) data will be analyzed by analysis of variance. For hypothesis EL1, Groups 1 and 7 will be compared to Group 12. For Hypothesis EL2, Groups 1, 7, and 12 will be compared to Groups 2-5. In a separate analysis of participants not receiving a comprehensive clinical exam, Hypothesis EL3 will be further tested by comparing Groups 8-11 to Group 12.

Aim 2 will be met by asking awareness and attitude questions at baseline. Knowledge, attitudes and information seeking questions will be asked at baseline, pre-exam, post-exam, and one year follow up in HEIRS/ELSI cases and controls. The questions will pertain both to HH and to general attitudes toward genetics and genetic testing.

Hypothesis EL4 will be analyzed by analysis of variance with education as a covariate. Hypothesis EL5 will be analyzed by examining changes in attitudes toward genetic testing across the three assessment points. Hypothesis EL6 will be analyzed by comparing satisfaction with testing among groups receiving different types of test results as described in EL2.

Aim 3 will be met by asking a series of questions regarding participants' perceived obligations to share information and their intentions to share information with family members in the post-result assessment. These same questions will be asked again at the one year follow up to assess their actual sharing of information with family members. Attitudes toward physician disclosure of information will also be assessed in the post-result assessment and again at the one-year follow-up.

Hypothesis EL7 will be tested by repeated measures analysis of variance within the participants who have elevated iron levels. Specifically, Group 1 participants will be compared to a pooled group of ambiguous results (Groups 2-5), and to Group 6 in a repeated measures ANOVA with one factor (test result, including 3 levels: positive, ambiguous, and negative), and time of assessment. A further analysis will compare Groups 1 and 7 in their intention to share information with family members and their subsequent sharing of information. Hypothesis EL8 will be analyzed by examining the correlation of attitudes about sharing information with education level. In addition, sharing of information will be analyzed by multiple regression with education level, race, test result, age, and gender as predictors.

Aim 4 will be met by asking questions regarding their recall of the follow up recommendations given to them, their understanding of the signs and symptoms of hemochromatosis, their confidence that they can follow the recommendations (self-efficacy), their expectation that following the recommendations will improve their health (outcome expectancy) and their resultant confidence that they will actually adhere to screening recommendations made at the post-exam assessment. At the one-year follow up their actual follow through on the screening recommendations will be assessed along with their plans to continue in the future. This hypothesis will be analyzed by multiple regression.

Aim 5 will be met by asking questions at the one year follow up about experiences with employers and insurers since screening. These data will be compared with information collected about insurance and employment status at the comprehensive exam. Their worry about illness will also be measured before being fully informed about results (at the pre-exam) and at the one year follow up. These data will be analyzed by analysis of variance.

5.4 Definition of ELSI cases and controls. Table 1 describes the ELSI cases and controls.

Table 1. ELSI Cases (including CCE cases) and controls.

ELEVATED IRON LEVELS	NORMAL IRON LEVELS
GROUP 1 C282Y/C282Y (estimated N » 201) N=200	GROUP 7 C282Y/C282Y (estimated N»109) N=109
GROUP 2 C282Y/H63D (estimated N » 58) N=58	GROUP 8 C282Y/H63D (estimated N » 1102) N=200
GROUP 3 H63D/H63D (estimated N » 19) N=19	GROUP 9 H63D/H63D (estimated N » 1257) N=200
GROUP 4 C282Y/+ (estimated N »238) N=200	GROUP 10 C282Y/+ (estimated N » 6, 258) N=200
GROUP 5 H63D/+ (estimated N » 151) N=151	GROUP 11 H63D/+ (estimated N » 12, 493) N=200
GROUP 6 +/+ (estimated N » 716) N=200	GROUP 12 +/+ (estimated N > 93.000) N=200

The ELSI protocol will seek subjects in all of these categories. The numbers of subjects in each cell is an estimate; the actual number will not be known until the study is underway. Clearly some of these cells are underpowered. If adequate numbers of participants prove not to exist, cells will be collapsed for analysis. Specifically, the boxes representing groups 2, 3, 4, and 5 may have to be collapsed. Groups 1, 2, 3, 4, 5, 6, and 7 will be eligible for the comprehensive clinical exam. Groups 8, 9, 10, and 11 will only be ELSI cases. Group 12 will constitute controls for the ELSI. The eligibility of controls for the ELSI study will be the same as for the CCE and will be similarly frequency matched as to age, gender, and ethnicity.

5.5 Family Study.

The ELSI protocol will also involve all participants in the Family Study. The family study participants will go directly to the comprehensive clinical exam, rather than beginning with the screening visit, so that they will not complete the baseline form. The family study instruments were shortened by removing items on which probands would provide sufficient data and where it was not expected that recruitment as a family member would affect responses.

5.6 ELSI Study Instruments.

The ELSI study instruments are: Initial Screening; Post Result Form; One Year Follow-Up Form; Family Post Result Form; Family One Year Follow-Up. The Initial Screening Form is handed to the participant and completed during the initial screening visit. All other instruments are mailed with stamped, self-addressed envelopes. All instruments are self-administered. We will take a passive, inclusive approach to literacy. That is, those participants who request that the information be read to them will be provided with that help and will not be excluded from the study. We expect, however, that most individuals with low levels of literacy will self-select not to participate in the study. Some of the field centers will be recruiting non-English speaking individuals, specifically, individuals who may be monolingual in Spanish, in Vietnamese or in Mandarin. All ELSI instruments will be translated into those three languages.

5.7 Study Flow.

As participants are recruited, they will be given the Initial Screening Form. Half of the information on this form constitutes baseline psychosocial data for the ELSI protocol. It is estimated that the ELSI-specific part of the form requires less than 3 minutes to complete. It will be filled out by all 100,000 participants. After the screening tests have been completed, all 100,000 participants will be informed of their screening test results (see Appendix C).

Those who screen positive, plus a frequency matched set of controls, will be invited to the comprehensive clinical exam. Of the remaining participants, approximately 800 will be ELSI cases who will **not** undergo the comprehensive clinical exam. Approximately one week after receiving their screening results by letter, those participants who are ELSI cases but not invited to the comprehensive clinical exam, will receive the Post-Result Form in the mail with instructions to fill it out and return it in the enclosed, stamped, self-addressed envelope. Approximately one week after receiving their comprehensive clinical exam results, by phone or by mail, CCE participants will receive the Post Result Form in the mail with instructions to fill it out and return it in the enclosed, stamped, self-addressed envelope.

If the Post Result Form for a particular participant is not received back within one month, a single mailing, containing a reminder letter, a new copy of the Post Result Form, and another stamped, self-addressed envelope will be mailed. Failure to return the assessment at this point will lead to the participant being considered lost to follow-up. One year after the Post Result Form is returned, all ELSI cases and controls will receive the One Year Follow-Up. All procedures, including the second, reminder mailing will follow the protocol of the Post Result Form.

5.8 Substudies

Two ELSI substudies will be performed. These are described in Appendices K and L.

6. Genetics Education and Counseling

6.1 Initial Recruitment and Screening Results Notification

Recruitment materials regarding the HEIRS study will have an educational component regarding iron overload and hemochromatosis. These will be developed jointly between the ELSI Genetic Counseling Subcommittee and the Recruitment and Retention Committee.

6.1.1 Initial Recruitment and Education Regarding Iron Overload and Hemochromatosis

Individuals will be approached in the clinic by trained recruiters to determine if they would be interested in participating in the HEIRS study. Geneticist(s) and/or genetic counselor(s) will be involved in training study recruiters so that they will be able to respond to basic questions regarding the genetics and other aspects of iron overload and hemochromatosis. In this way, the recruiters will be able to respond to simple and straightforward questions which may arise during the informed consent process about iron overload, hereditary hemochromatosis, and the screening tests to be conducted as a part of this study.

6.1.2 Screening Results Notification

All research participants will be notified of the results of their initial screening tests. However the manner of notification will differ depending on the actual results of the tests and also by field center (see Appendix H). Generally speaking, those individuals in whom no genotype variations are identified and whose iron studies all fall within the reference range of the central laboratory will be sent the results of their tests by letter. A small fraction of those individuals will be invited to continue their participation in this study as controls.

It is expected that all field centers will contact subjects qualifying for the Comprehensive Clinical Exam (CCE) both by telephone and by letter. During the course of the initial telephone call/letter, individuals will be told the results of their screening tests and that they qualify for the CCE. Those qualifying for the second stage of the study will be invited in for the CCE. It has been decided that the order of notification can vary from field center to field center because there is no evidence that it is more effective or acceptable to receive results by letter or telephone first. Satisfaction with how individuals are initially notified of their test results will be assessed during the course of this study.

Individuals whose test results fall outside the reference range (e.g. those with iron deficiency or mild evidence of iron overload), but who do not qualify for the CCE of this study will be notified of these findings. Some field centers may call individuals with their results while others will send letters summarizing these results. In each case, the participants will be encouraged to discuss their results with their primary care physician. The results letter will discuss the potential health implications of the results of the screening tests and will strongly urge them to share their results with their primary care physician so that he/she can further evaluate any potential health risks and provide treatment if needed.

6.2 Notification Letters: Content, Rationale, and Educational Materials

The draft results letters are included in this protocol. Each contains a brief description of the results genotype testing and also the iron studies and the results and whether they will be invited (or have been invited to participate in the second stage of this study). Each letter will also contain a laboratory report which individuals will be encouraged to share with their primary care physician.

The individuals receiving letters for those in whom no variations in genotype have been identified and whose iron studies all fall within the reference range will be those in whom no abnormalities were identified in the screening tests. They will be sent a letter specifically reporting that they have no genotypic variations noted in their HFE genes. They will also be told that their iron studies all fall within the reference range of the Central Screening Laboratory. They will be provided with a laboratory report to share with their primary care physician if they wish. They will be thanked for their participation in the study and will also be informed that they may be randomly selected to continue participating as a control. They will be provided with the name of the study coordinator and a telephone number to reach him/her should they have questions.

Those qualifying for the CCE will include those individuals who have C282Y homozygosity (with or without evidence of iron overload) and also those who have any other genotypes and also evidence of iron overload (TS>45 in women and >50 in men and serum ferritin >200 in women and >300 in men). In addition to their discussing their specific laboratory test results in the letter, these individuals will also be sent a confirmation of their appointment time for the CCE (if they have agreed to continue in the study) and instructions for the clinic visit. If they have not agreed to continue in the second stage of this study they will be strongly encouraged to share these results with their primary care physician as they will have a high risk to already have iron overload or to develop iron overload at some time in the future. These individuals will also be given the name and telephone number of the study coordinator should they or their physician have any questions about their test results.

Individuals receiving letters for those who do not qualify for the CCE, but in whom (a) genotype variation(s) is (are) identified and/or whose iron studies fall outside the reference range in one or more of the tests carried out, will be those in whom a genotypic variation has been identified or an iron test has fallen outside the reference range (resulting in the need to notify them of a alert value). These individuals will be given their actual genotype results: that the tests did not detect any variations in the HH genes tested in this study; that the tests revealed a single variation in one of their two HH genes (C282Y/+ or H63/+), a situation which is frequently encountered in individuals with normal iron levels; or that the tests identified two 'variations' in their HH genes (C282Y/H63D or H63D/H63D), a situation which is often encountered in individuals with normal iron levels, but which may slightly increase the chance of iron overload in the future.

Participants will also be notified if they have iron test results that exceed alert values, as determined by the screening and exam committee. This will include individuals who have either decreased or increased transferrin saturation or serum ferritin, suggesting possible iron

deficiency, early signs of iron overload, inflammation, or any number of possible health risks. In either of these cases, participants will be encouraged to follow up with their primary care physician and to share these results with him/her.

The rationale behind disclosing all test results was based on the conclusion that it was not appropriate to withhold such information from individuals participating in this study. It was researchers' conclusion that the participants and their primary care physician should be informed that genetic testing was done in the course of this study and that only *certain* possible 'variations' were tested. They further concluded that individuals should be informed which (if any) genotype variations were identified. Researchers suggested that it was important to inform individuals of their genotype test results because those individuals, particularly from Caucasian and Hispanic populations, have an increased risk to have at risk or affected family members (parents, siblings, and children). Regarding the iron studies test results, it was the researchers' conclusion that the participants' primary care physicians will have an interest in knowing the actual transferrin saturation and serum ferritin levels, particularly in contrast to the reference range of the central laboratory. Such specific information will be needed in order to inform decisions about further diagnostic evaluation.

Educational materials will be developed or identified (e.g., recruitment materials or CDC educational materials under development) that will review iron overload, hereditary hemochromatosis, genetics, and other relative information. The materials will include the clinical manifestations, future health implications, available treatment for iron overload and hereditary hemochromatosis, and genetic and other contributions to developing disease. The likelihood of relatives being at risk to develop disease will also be included. These materials will be included in the results informing letter, along with the name and telephone of the study coordinator if they or their physician have additional questions regarding their test results.

6.3 Family History, Pedigree Construction, and Genetic Consultation for Those Qualifying for the Comprehensive Clinical Exam (CCE)

All CCE participants will receive a family history form with their results notification and appointment confirmation letter. They will be asked to complete the family history information form (with help from other family members if necessary) prior to their CCE visit. When they arrive for their appointment, the information contained in the family history form will be reviewed and clarified by a genetic specialist or another trained health professional. A pedigree will be constructed from the information provided and a determination will be made regarding the appropriateness of the family structure for inclusion in the family study.

Genetic consultation will be conducted by a geneticist, a genetic counselor, or a health professional trained and supervised by a genetics specialist. This consultation will include a review of the family history form, a review of the pedigree constructed, a discussion of the potential availability and willingness of specific family members to participate in a family study, and the provision of information about their own test results, what is known (and what is not known) about genetic and other contributions to iron overload and hereditary

hemochromatosis, and a discussion of future health and possible reproductive implications for themselves and other family members.

Participants will be asked whether they have any specific concerns and feelings regarding the above information and if they have any questions about the possible family study if indicated from above. Some centers intend to give participants letters regarding the study for them to forward to relatives, with instructions as to how the relative can contact the study coordinator. Others plan to ask for permission and contact information of family members so that the field center can contact the relative directly. It is anticipated that the same information initially used to recruit participants into this study will be appropriate to include in letters inviting relative participation in the family study.

Telephone contacts and/or letters will be constructed to inform individuals of the results of test conducted in the CCE.

6.4 Family Study Participation

Family study subjects will also receive a family history form with their appointment confirmation letter. As with other study participants, they will be asked to complete this form in advance of the CCE. When the family member arrives for their appointment, the family history information will be reviewed and clarified by a genetic specialist or another trained health professional before their physical examination. During the exam, the family history information will be compared with that in the family's previously constructed pedigree, and any discrepant information will be noted for clarification and correction if needed.

Since family members will come directly to the CCE without an initial screen, no genotype or phenotype information will be available at the time of their CCE. Consequently, only a brief consultation will be needed with the genetic counselor or geneticist to review general information about genetic and other contributions to iron overload and hereditary hemochromatosis, possible outcomes of their test results, including limitations of knowledge regarding other genetic and environmental influences and interactions in the development of iron overload and hereditary hemochromatosis. The genetics specialist will also assess how they reacted to being contacted (if many family members express that they were alarmed or dissatisfied with how they were contacted, modifications in this protocol may need to be made), and their feelings about being 'at-risk' for iron overload or hereditary hemochromatosis.

Individualized telephone contacts and/or letters will be constructed to inform individuals of the results of test conducted in the CCE. It is not anticipated that family members will return to the field center for face-to-face genetic counseling, unless they seem distressed or specifically ask for further discussion. A genetic specialist will be available by telephone for subjects and family members who have questions about the meaning of their genotype results and will refer them to clinical counseling services should further counseling be needed.

7. Family Study

7.1 Introduction

Hemochromatosis, a disorder that can cause iron overload (IO), is a common autosomal recessive disorder of iron metabolism occurring with a prevalence of 0.2-0.5% in Caucasians of central, northern and western European descent (EASL 2000, Witte 1996). Recent identification of a hemochromatosis predisposing gene, designated HFE and located on chromosome 6p approximately 4 Mb from HLA-A, allows for presymptomatic diagnosis (Feder 1996). The HFE gene encodes a protein that interacts with the transferrin receptor to regulate iron absorption (Feder 1998). About 9 mutations have been identified in the HFE gene, including C282Y and H63D (Feder 1996). Most Caucasian individuals with hemochromatosis (80-100%) are homozygous for the transition mutation C282Y (Feder 1996, 1998; Barton 1997). A small number of compound heterozygotes (C282Y/H63D) may develop clinical IO (Barton 1997). Some persons with hemochromatosis have novel mutations of HFE (Barton 1999, Pipereno 2000). Armed with the frequency of HFE mutations and the genotype status of an affected individual, one can predict the probability of family members possessing HFE mutations. These family members can be advised to obtain phenotypic screening that can detect IO.

Although mutations within the *HFE* gene account for most of hemochromatosis that occurs in Caucasians, there is evidence that there may be other genes on chromosome 6p that modify the extent of iron overload. Data from at least three groups of investigators suggest that the severity of iron overload is greater in individuals who possess HLA-A3 compared to those who are HLA-A3 negative (Crawford 1995, Simon 1987, Barton 1996). This difference in the propensity to absorb iron cannot be explained by *HFE* mutations alone (Whitfield 2000). Thus, genes on chromosome 6p or on other chromosomes may be involved.

There is considerable variation in the frequency of *HFE* mutations among different racial and ethnic populations (Merryweather-Clark 1997, Chang 1997, Sohda 1999, Cullen 1998, Monaghan 1998, Marshall 1999, Acton 2000). The frequency of *HFE* mutations in different racial/ethnic populations appears to correlate with the prevalence of hemochromatosis. However, it is clear that other genes are involved in hemochromatosis and in IO. One example is a recently described mutation in the transferrin receptor gene 2 (*TFR2*), which has been observed segregating with hemochromatosis in two Sicilian families (Camaschella 2000).

IO was originally described to occur among sub-Saharan Africans (Strachan 1929); this form of siderosis is referred to as African IO. While original observations attempted to link the long-term drinking of traditional beers containing large quantities of absorbable iron to IO, no correlation was demonstrated with the behavior (Bloom 2000, Gordeuk 1992). Clinical complications of African IO are similar to those of hemochromatosis, which occurs in Caucasian populations (Bloom 2000, Gordeuk 1992). These include hepatic cirrhosis, primary liver cancer, hypogonadism, diabetes mellitus, heart injury (cardiomyopathy), and joint disease. Estimates of sub-Saharan regions have suggested as much as 40% of sub-Saharan Africans are affected with IO (Bloom 2000).

Studies by other investigators have revealed evidence that there is a strong genetic susceptibility component that is important in the development of African IO (Gordeuk 1992, Moyo 1998). The data appear to support the hypothesis that African IO is attributable to a common, co-dominant autosomal mutation. However, common *HFE* mutations and HLA types associated with hemochromatosis in most western Caucasian peoples are uncommon or rare in persons with African IO. These observations, including the differences in cell deposition of excess iron in African IO and Caucasian hemochromatosis, led to the general hypothesis that mutation of an iron-associated gene other than *HFE* must be responsible for most cases of primary iron overload in persons of African descent. Although a genetic susceptibility locus has not been identified for African IO, primary IO among African Americans has been described in a few reports of single cases. In 1995, a series of cases was reported from Alabama (Barton 1995) and from the Midwest (Wurapa 1996). Taken together, these reports indicate that African American IO is the result of increased absorption of dietary iron, is associated with injury to the liver and other organs attributed to iron overload, sometimes affects multiple individuals in the same kinship, and is probably relatively common (Barton 1995, Wurapa 1996,). A review of the limited number of reported cases suggests that the serum iron concentration and transferrin saturation values are sometimes lower than those in Caucasian hemochromatosis, that Kupffer cell iron deposition in the liver is often prominent, and that the disorder is not linked to HLA loci (Barton 1995, Wurapa 1996, Monaghan 1998, Barton 1998, Acton, 2000). These results indicate that this disorder is similar to African IO, except that the affected persons diagnosed in the United States have not apparently consumed traditional beer or other food/drink items that contain great quantities of iron.

Common *HFE* mutations (C282Y and H63D) and uncommon *HFE* mutations (S65C, I105T, G93R) occur infrequently in African Americans with or without IO (Barton JC and Rothenberg BE, personal communication). However, the degree of Caucasian admixture in African Americans varies considerably throughout the United States (Barton 1998, Acton 2000). Approximately 5% of African Americans with primary IO could have inherited *HFE* mutations due to Caucasian admixture. Other pertinent observations indicate that frequencies of HLA markers, including HLA-A3, vary significantly among African Americans in different regions of the United States (Acton 1993). Thus, the frequency of African American IO may also vary significantly according to geographic location, indicating that an assessment of demographic factors pertinent to African American subpopulations may be important in planning screening and diagnosis programs for this disorder.

There is also a paucity of information on the frequency of *HFE* mutations and IO in Hispanic and Asian populations. The overall goal of the HEIRS Family Study will be to identify families with hemochromatosis and/or IO that can be used to more clearly define the genetics of these disorders.

7.2 Rationale

Family studies characterize heritability and utilize methods involving segregation and linkage analyses in order to identify, delineate and provide genetic evidence for causation. In order to carry out the aforementioned types of studies, a family history of disease assessment is a necessary and important part of the history and physical workup of patients. Given that other races of non-Caucasian descent rarely carry the *HFE* allele, the explainable level of ethnic admixture with Caucasians and *HFE* genotype, the HLA diversity of African Americans and the preliminary observations of sub-Saharan African IO families support a non-HLA linked modifier (Gordeuk 1992, Moyo 1998), the information obtained during the family study will allow for the potential determination of risk for IO, the diagnoses of hereditary IO and the determination of who would best benefit from genetic testing to refine risk. This information is also a mandatory element of research studies whose aim is to decipher the genetic features of a disease state.

7.3 Hypotheses

The hypotheses of the HEIRS Family Study are as follows:

- FS1. In Caucasians and Hispanics there is at least one gene responsible for modifying risk or expression of hemochromatosis and related complications.
- FS2. In African Americans one or more genes contribute to the risk of iron overload.
- FS3. In other racial and ethnic groups other genes contribute to the risk of iron overload.
- FS4. A small number of African Americans and Asians will develop hemochromatosis due to *HFE* mutations.
- FS5. Among families selected through probands with primary iron overload, the heritability of iron parameters is at least 40% and is similar for Caucasians and non-Caucasians for which data is available.
- FS6. Among families selected through probands with specific *HFE* mutations, variation in iron parameters is primarily due to variation in *HFE* genotype.

7.4 Study Aims

The primary aims of the HEIRS Family Study are as follows:

1. To estimate heritability of iron overload and heritability of the related phenotypes transferrin saturation (TS) and serum ferritin (SF);
2. To localize or identify genes that modify the risk or expression of iron overload, hemochromatosis, or related complications in individuals who carry the C282Y allele or another known *HFE* mutation;
3. To localize non-*HFE* genes that influence iron overload, TS or SF.

7.5 Methods

7.5.1 Overview of Protocol

The HEIRS Family Study is designed to identify probands from among comprehensive clinical exam (CCE) examinees, and to enroll approximately 2,000 relatives of probands for participation in the CCE. CCE participants who are not relatives of a proband will be eligible to be probands if they meet the Family Study Eligibility Criteria (detailed below in 7.5.3). Table 1 presents the schedule for administering Family Study instruments.

Table 1. Schedule for Administering Family Study Instruments

Instrument	Administered To	Administration	Center's Time to Administer
Family History of Disease	All subjects scheduled for CE & controls.	Self administered. Sent to subject before CE. Checked by interviewer at CE. Info passed along to physician at CE and person conducting genetic counseling.	10 - 15 min.
Pedigree Structure	All subjects undergoing CE.	Interviewer constructs at CE. (Optional). Info passed along to physician at CE and person conducting genetic counseling.	10 - 15 min.
Participant Interview	All subjects undergoing CE.	Interviewer obtains info from subjects whose family structure meets the criteria for entry into the family study.	10 - 20 min.
Reminder to Proband to Contact Family Members	Proband whose family meets the criteria for entry into the family study.	Given to proband at time decision is made to enter family members into study.	5 min.
Letter to Proband's Family Member from Center	Proband's family members eligible for entry into family study.	Composed & mailed by each Center after decision made to enter family members into study.	5 - 10 min.
Letter to Proband's Family Member from Proband	Proband's family members eligible for entry into family study.	Composed and mailed by each Center after proband signs and decision made to enter family members into study. (Optional).	5 - 10 min.
Script for Family Member's Phone Call	Proband's family members eligible for entry into family study after members have had time to receive letters.	Interviewer at each Center.	10 - 20 min.

7.5.2 Proband's CCE Pedigree Assessment

Family history of disease information will be collected by use of a self-administered form, the Family History Form, which the proband will complete before the CCE. The HEIRS staff at each field center will use the information obtained on the Family History Form to construct a pedigree on the Pedigree Form. HEIRS staff will assess the pedigree information and a decision will be made as to whether the family meets the Family Study eligibility criteria.

Each potential proband will be given a Family History Form to complete before the time of the CCE. During the CCE visit, clinic staff will construct a pedigree for the potential proband, and will assess the availability of age-eligible relatives; this information will be recorded on the Pedigree Form. If the pedigree meets eligibility criteria, then the clinic staff will obtain permission from the potential proband to contact the available age-eligible relatives; this information will be recorded on the Family Contact Form. Either during the CCE or within 1-2 weeks afterward, HEIRS staff will determine whether the potential proband meets all Family Study eligibility criteria by consulting the Pedigree Form, the screening test results, and the CCE results if necessary. The proband and proband's relatives will be invited to participate in the Family Study when all eligibility criteria are met.

The initial use of the information collected will be to identify families whose structure would allow heritability, segregation and linkage studies to be conducted. The families identified in this manner will be contacted and recruited to participate in the study.

Many of the diseases, on which information will be collected in the HEIRS study, have been previously reported (most reports are of Caucasian subjects) to be associated with hemochromatosis or iron overload. However, there is a paucity of data on the associated disease prevalence in iron overload affected individuals and whether there is racial/ethnic variation. Although there is evidence that iron overload can damage various tissues, which often results in some of the diseases for which information will be obtained, it is not clear how much of the occurrence of the disorders in a patient or family members is coincidental. Many of the associated diseases are rather common in the general population. Thus, family history of disease information from iron overload affected and normal control individuals will provide an opportunity to address these issues.

There is data available suggesting that individuals heterozygous for C282Y are at risk for heart disease. The family history of disease information collected will also allow one to assess the relationship of various *HFE* genotypes with the disease states in family members and in those subjects who are not iron overloaded but carry a mutation associated with hemochromatosis or iron overload.

There is data available which suggests that individuals who participate in family studies are those who have the disease in their family. There is a general sense that hemochromatosis and iron overload may not be as recognizable as other common disorders, i.e. heart disease, cancer, diabetes. The family members will be recruited based on having a member phenotypically affected or genotypically at risk for hemochromatosis or iron overload.

Healthy controls will be recruited who are not at risk for hemochromatosis or iron overload. Thus, the data collected will provide an opportunity to determine if there is a selection bias in the subjects recruited for the family study due to their recognizing that the disorder runs in their family.

There is speculation, which suggests that individuals with a positive family history of a disease may be more proactive in learning about the disease than those who have no family history. The data we propose to collect will allow one to test this hypothesis.

There is data which suggests that family history of disease information obtained from a member of a family by use of self administered forms or by interview is highly sensitive and specific for a number of diseases, i.e. heart disease, cancer and diabetes. In that the family members who agree to participate in this study will be interviewed and a comprehensive history and physical performed, there will be the opportunity to validate the family history of disease information obtained from the proband and to determine the sensitivity and specificity of the information for identifying diseases in family members.

Timing of the study will follow the Schedule for Administering Family Study Instruments (summarized in Table 1.).

Table 1. Timing of Family Study Protocol Events.

	<i>Proband Group</i>	<i>Contact</i>	<i>Ascertainment</i>	<i>Interview/Pedigree</i>	<i>Counsel</i>
Proband	C82Y/C282Y	Mail prior to CCE	Mail prior to CCE	Interview during CCE	Interview during CCE
	Primary IO	Mail prior to CCE	Final CCE lab results	Interview during CCE	After final CCE results
Family members	C82Y/C282Y	After proband's CCE	After proband's CCE	Interview during CCE	After final CCE results
	Primary IO	After proband's CCE	After proband's CCE	Interview during CCE	After final CCE results

* **Proband groups:** (1) C282Y/C282Y genotype (regardless of 1° iron overload status); (2) 1° Iron Overload regardless HFE status.

7.5.3 Family Study Eligibility Criteria

Comprehensive Clinical Exam participants who are not relatives of a proband will be eligible to be probands if they have either a) C282Y/C282Y genotype or b) HEIRS Study criteria for primary iron overload, regardless of *HFE* mutation status. In addition, they must have family members, all 19 years of age or older and available for study, which include a group of at least one of the following: a) Three offspring of the proband and the non-proband parent of these offspring; b) Two offspring of the proband, the non-proband parent of these offspring and one full sibling of the proband; c) Two full siblings of the proband and both of the proband's parents.

In the case of those with C282Y/C282Y genotype, families will be used to map modifier genes, and in the case of primary iron overload, families will be used to map new genes for iron overload. In each case, the pedigree structures are the minimal criteria for families. The optimal family structure consist of a nuclear family of at least four age-eligible individuals, of whom at least one is a parent, and at least eight additional age-eligible first-degree relatives of the nuclear family members. Spouses/partners will be included if they are parents of a family member. The Family Study Subcommittee will review the structures of enrolled families and will consider changes to the above criteria if warranted after the first six months of the Family Study.

7.5.4 Contacting Relatives of the Proband

If the family meets the study criteria, permission will be obtained from the proband to contact each family member using the Family Contact Form. This form will also be used to collect family member contact information and information on race/ethnicity. The proband will be asked to inform family members of the study, and will be given a reminder to do so, the Reminder to Proband (see Appendix D). The proband will also be asked to either give permission to the HEIRS field center to send a letter to each family member for which contact permission was given, the Family Letter from Field Center, or, if they prefer, a letter will be generated by each HEIRS field center for the proband to sign, the Letter from Proband. After sufficient time has passed to allow the letters to reach family members, HEIRS field center staff will place a call to each family member using the Phone Script for Family (see Appendix D).

7.5.5 Relatives' Comprehensive Clinical Exam

Approximately 2000 family members of probands who take part in the Family Study will undergo the CCE.

7.6 Other Aspects of Design

A case control study is also proposed. One approach could be to only use *HFE* C282Y/C282Y positive Caucasians and to search for modifier genes by the candidate gene approach or through global mapping studies. Data from mapping studies might also be used in conjunction with HLA typing data if collected. Present funding will not allow all these studies to be conducted. A decision can be made as the study progresses if supplemental funding should be sought to pursue some of these approaches.

8. Laboratory Procedures

The HEIRS Central Laboratory at the Fairview-University Medical Center is responsible for developing the protocols for blood collection, processing, shipping, and storage; writing manuals for operations; implementing laboratory testing quality control measures; training and certifying field center staff on these protocols; and performing assays and reporting results as specified in the contract. The blood samples of HEIRS will be collected and processed using a modified version of the protocol used for the NHLBI ALLHAT Study over the past 6 years. This protocol allows for efficient specimen collection, handling, storage, and shipment.

Written protocols will be provided for the Initial Screening Visit blood collection. This is a very straightforward collection provided by clinical laboratories on a daily basis. The Central Laboratory will train the Study Coordinator for each Field Center in the protocols for processing and shipping of samples. For the Comprehensive and Family Study Exam blood collections, the Central Laboratory will provide training for Field Center technicians both centrally and locally at the sites. All technicians will be trained, evaluated, and certified prior to participation in this part of the study. Recertification will take place periodically throughout the study. Performance will be monitored by the Central Laboratory and the Coordinating Center. Additional training will be initiated as necessary.

8.1 Blood Collection for All Visits

Blood collection will be performed using standard venipuncture (antecubital fossa in the arm) by trained staff. A total of 20 - 56 mL (equivalent of one to three tablespoons) of whole blood will be collected into two to five different collection tubes. Blood samples from the screening visit will be used for measurement of iron overload and genotyping for known hemochromatosis mutation genotypings. Serum and buffy coat will be stored in a long-term repository for future analyses. Blood samples from the comprehensive and family studies visits will be assayed for measures of liver function, alcohol consumption, diabetic status, inflammation, and viral markers (if liver function tests are greater than 1.5 times the upper reference limit. Serum and buffy coat will be stored in long-term repository for future analyses. DNA may be isolated from blood cells collected at both the screening and comprehensive/family study visits and stored for future genetic testing related to iron overload disorders. Cells (i.e. lymphocytes) from the comprehensive and family study visit may be cryopreserved and used to establish cell lines for future genetic testing related to iron overload disorders. The average length of time required for phlebotomy is expected to be 5 minutes.

8.1.1 Blood Collection Instructions

The Test Request Form will be completed during the participant's visit. A number of checkpoints related to QC will be addressed on the Test Request Form for each blood draw. Before starting the venipuncture, the phlebotomist must check to ascertain that the participant has signed the study consent form. This form explains the reason for the blood samples and states the risks associated with the blood collection process.

Prior to venipuncture, the participant should be seated in the phlebotomy chair. The phlebotomist should assure the participant that this is a simple procedure, that only about one to three tablespoons will be collected and that no problems are anticipated. The tourniquet is then applied and venipuncture is performed as per instructions in the Manual of Operations. After venipuncture, the venipuncture site is bandaged and the participant should rest in the chair. Any deviations to protocol should be noted on the forms either in response to specific questions or in the section for comments.

8.2 Laboratory Analyses

Screening Visit Blood Testing (20 mL blood): Transferrin saturation, ferritin, genotyping for C282Y and H63D.

Comprehensive Clinical Exam Blood Testing (56 mL blood): Transferrin saturation, ferritin, glucose, insulin, ALT, AST, GGT, CRP, CBC with reticulocyte count, haptoglobin, LDH, bilirubin (total, direct and indirect), hemoglobin identification, hemoglobin A2 quantitation, HBsAg, HCV antibody, other polymorphism detections to be determined, lymphocyte cryopreservation. Hepatitis B surface antigen and hepatitis C antibody will be done only on those participants with elevated ALT. Reticulocyte count, haptoglobin, LDH, bilirubin (total, direct and indirect) will be done if hemoglobin is depressed.

Family Study Blood Testing (56 mL blood): same as Comprehensive Clinical Exam tests, plus a genome screen.

Transferrin Saturation

Transferrin saturation and ferritin are the most common screening test used to evaluate iron status. Iron analysis and unbound iron binding capacity (UIBC) are required for the determination of transferrin saturation. For screening testing, the participant samples from the London Ontario Field Center will be analyzed at the MDS Laboratory by a ferrozine based colorimetric iron and UIBC method from Roche Diagnostics/Boehringer Mannheim on a Hitachi 917 analyzer (Roche Diagnostics/Boehringer Mannheim Corp., Indianapolis, IN). All other samples will be analyzed at Fairview-University Medical Center Clinical Laboratory using a ferrozine based colorimetric iron and UIBC method from Roche Diagnostics/Boehringer Mannheim on a Hitachi 911 analyzer. In order to determine comparability between testing sites, quality control comparisons will be analyzed at the Fairview-University Laboratory on 2% of the samples tested at MDS Laboratory. In addition, the method performed at the Fairview-University Laboratory will be correlated with the 1990 ICSH ferrozine-based method (Iron Panel of the International Committee for Standardization of Hematology, 1978 and 1990).

Serum Ferritin

Ferritin is a high molecular weight iron-containing protein that functions as a storage compound. Approximately 2/3 of the body's iron stores exist in the form of ferritin.

Although the bulk of the ferritin in the serum is present as apoferritin and contains no iron, there is nevertheless a direct relationship between measured serum ferritin concentration and total body iron stores. Every ng/mL of serum ferritin represents approximately eight mg of stored iron in normal volunteers (Walters et al., 1973). Unfortunately, a wide variety of inflammatory conditions such as rheumatoid arthritis, infections, some malignancies, especially lymphomas, leukemias, and breast cancer, as well as any hepatic insult such as viral or alcoholic hepatitis cause serum ferritin to rise far out of proportion to the body iron stores. Most authorities agree that serum ferritin is less sensitive and less specific for diagnosis of hemochromatosis and significant iron overload states in Caucasians (Fairbanks and Klee, 1999). However, most of the studies directly comparing transferrin saturation to ferritin for diagnosis of hemochromatosis were limited to relatively small geographic and ethnic groups. Thus, we recommend serum ferritin measurement as part of this study to clarify better its diagnostic value. Ferritin will be analyzed on the Hitachi 911 analyzer using a turbidimetric antibody method by Roche Diagnostics. In this assay, ferritin antibody bound to latex forms an antigen-antibody complex with ferritin in the sample. Turbidity measured at 700 nm is directly proportional to the concentration of ferritin.

C282Y, H63D, and Other Polymorphism Detection

The C282Y and H63D mutations are found in the HLA region of chromosome 6 and both have been associated with a high risk for development of hereditary hemochromatosis. While C282Y mutation is found more frequently than H63D, both mutations are present in approximately 80-90% of Caucasian hemochromatosis patients. With time it is likely that other candidate genes associated with hereditary hemochromatosis and iron overload disorders will be discovered.

C282Y and H63D genotyping will be performed using the Third Wave Technologies Invader assay (Third Wave Technologies, Inc. Madison, WI.). In this assay, two oligonucleotides (wildtype or mutant probe and Invader oligo) hybridize in tandem to a specific region of DNA generating a structure that is recognized and cleaved by the Cleavase VIII enzyme. This structure includes an unpaired "flap" on the 5' end of the wildtype or mutant probe. Cleavage releases the 5' flap, which serves as the Invader oligo in the second cleavage reaction on a FRET oligonucleotide probe, which is tagged, with fluorescein quenched by an internal dye. Upon cleavage, the 5'-fluorescein labeled product is detectable using a fluorescence plate reader. Other mutations or polymorphisms yet to be determined could be analyzed by this same approach.

Serum Glucose

Hemochromatosis is known to cause damage to the pancreatic beta cells and impair insulin secretion (Phelps et al., 1989). Therefore, fasting glucose analysis is typically used for assessment of diabetes. According to the recommendations of the Expert Committee on Diagnosis and Treatment of Diabetes Mellitus, a fasting glucose of ≥ 126 mg/dL is diagnostic criteria of diabetes mellitus. This Committee has also recommended that epidemiological studies of diabetes prevalence and incidence should use this criteria (Expert Committee Report, 1997). Glucose will be analyzed on the Hitachi 911 analyzer using a

hexokinase method by Roche Diagnostics/Boehringer Mannheim (Roche Diagnostics/Boehringer Mannheim Corp., Indianapolis, IN). In this assay, hexokinase catalyzes the phosphorylation of glucose by ATP. G-6-P is oxidized to 6-phosphogluconate in the presence of NADP by G-6-PDH. Formation of NADPH measured at 304nm is directly proportional to the concentration of glucose.

Serum Insulin

In this study, the major reason to measure insulin would be to estimate pancreatic insulin secretory reserve in that hemochromatosis is known to destroy islet as well as exocrine pancreatic cells. Analysis of insulin will be performed on the Beckman Sanofi Access (Beckman Coulter, Inc. Chaska, MN) using an immunoenzymatic insulin method with little proinsulin cross-reactivity. In this immunoenzymatic sandwich assay, insulin binds to antibody on a solid phase and anti-insulin alkaline phosphatase conjugate reacts with a different antigenic site on the insulin molecule. Light generated by the reaction of a chemiluminescent substrate added to the bound antigen-antibody-conjugate complex is proportional to the concentration of insulin.

Serum ALT and AST

Both alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are found in the liver, skeletal muscle, and heart. Additionally, AST is found in kidney and erythrocytes. Clinically, ALT and AST are good markers of liver disease with elevations as high as 100 times the upper limit of the reference range in viral hepatitis. More moderate elevations of both ALT and AST are observed in extrahepatic cholestasis, varying levels are seen in cirrhosis depending on the severity, and little to as much as 5 to 10-fold increases are observed in liver cancer. Ingestion of certain drugs such as alcohol, opiates, salicylates, or ampicillin has been associated with slightly elevated levels of ALT. ALT is a more liver-specific enzyme because elevations are rarely found other than in parenchymal liver disease and ALT levels remain elevated longer than AST levels. ALT and AST will be analyzed on the Hitachi 911 analyzer using a colorimetric method by Roche Diagnostics/Boehringer Mannheim (Roche Diagnostics/Boehringer Mannheim Corp., Indianapolis, IN). In these assays, alpha-ketoglutarate reacts with L-aspartate or L-alanine in the presence of AST or ALT, respectively, to form L-glutamate and oxaloacetate or pyruvate, respectively. An indicator reaction utilizes the oxaloacetate or pyruvate for a kinetic determination of NADH consumption, which is directly proportional to the enzyme activity.

Serum Gamma Glutamyltransferase (GGT)

While GGT is a very sensitive indicator of hepatobiliary disease, it does not discriminate well between the different types of liver disease. Elevated GGT can be found in intra- or extra-hepatic biliary obstruction, infectious hepatitis, acute and chronic pancreatitis, and liver cancer. Patients with fatty livers may have increased GGT and transient increases are known to be caused by ingestion of many reasonably commonly used drugs (e.g., anticonvulsants) and heavy alcohol consumption. Though not very specific for any particular liver disease, it is unusual to find normal GGT levels in the presence of even mild liver disease potentially

making this a good marker for this study. GGT will be analyzed on the Hitachi 911 analyzer using a colorimetric method by Roche Diagnostics/Boehringer Mannheim (Roche Diagnostics/Boehringer Mannheim Corp., Indianapolis, IN). In this assay, L-gamma-glutamyl-3-carboxy-4-nitroanilide reacts with glycylglycine in the presence of GGT to form 5-amino-2-nitrobenzoate and L-gamma-glutamyl-glycylglycine. The photometric determination of the rate of 5-amino-2-nitrobenzoate production is directly proportional to the GGT activity.

C-Reactive Protein (CRP)

CRP is a protein produced by the liver and is used as a marker for acute inflammation and/or tissue necrosis. Levels of CRP are elevated in acute rheumatic fever, bacterial or viral infections, myocardial infarctions, rheumatoid arthritis, carcinomatosis, gout and post surgery. Because serum transferrin and ferritin concentrations both tend to change in response to acute inflammatory states or tissue necrosis, CRP levels have been proposed as a means to normalize ferritin and transferrin saturation for inflammatory status (Witte, 1991; Baynes et al., 1986). CRP will be analyzed on the Hitachi 911 analyzer using a turbidimetric immunoprecipitation method by Roche Diagnostics/Boehringer Mannheim (Roche Diagnostics/Boehringer Mannheim Corp., Indianapolis, IN). In this assay, antibody to human CRP forms insoluble antigen-antibody complexes with CRP in the sample. Turbidity measured at 340nm is directly proportional to the concentration of CRP.

CBC with reticulocyte count

An automated complete blood count will be performed using the Cell-Dyne 4000 analyzer from Abbott Laboratories, Inc. Vernon Hills, IL. This analysis will include WBC, RBC, hemoglobin, hematocrit, MCV, MCH, MCHC, RDW. Reticulocyte count will be analyzed if the MCV or hemoglobin values are below the lower limit of the reference range.

Haptoglobin

Haptoglobin is an acute phase reactant that binds free hemoglobin. The haptoglobin-hemoglobin complex is removed from circulation by the reticuloendothelial cells within minutes of its formation. Thus, decreased levels of haptoglobin are associated with intravascular hemolysis or hemoglobin turnover as seen in hemolytic anemias. For this reason, haptoglobin can be used in this study as a marker of decreased red blood cell life span and will be analyzed if the MCV or hemoglobin values are below the lower limit of the reference range. Haptoglobin will be analyzed on the Hitachi 911 analyzer using a turbidimetric antibody method by Roche Diagnostics/Boehringer Mannheim (Roche Diagnostics/Boehringer Mannheim Corp., Indianapolis, IN). In this assay, antibody to human haptoglobin forms insoluble antigen-antibody complexes with haptoglobin in the sample. Turbidity measured at 340nm is directly proportional to the concentration of haptoglobin.

Lactate dehydrogenase (LDH)

Lactate dehydrogenase is present in virtually all cells of the body and the activity in tissues is many-fold greater than found in serum. Therefore, leakage from damaged tissue cells will cause increased serum levels. Because hepatocytes and red cells contain very large amounts of LDH, this enzyme will be used for detection of possible hemolysis and liver disease. LDH will be analyzed if the MCV or hemoglobin values are below the lower limit of the reference range. LDH will be analyzed on the Hitachi 911 analyzer using a colorimetric method by Roche Diagnostics/Boehringer Mannheim (Roche Diagnostics/Boehringer Mannheim Corp., Indianapolis, IN). In this assay, NAD and lactate are converted to pyruvate and NADH in the presence of LDH. The rate of NADH formation is directly proportional to LDH activity.

Bilirubin, total, direct and indirect

Approximately 80% of the bilirubin formed daily is derived from the degradation of hemoglobin. When hemoglobin is degraded, the iron enters the iron stores for reuse. Disturbances in the bilirubin metabolism can be caused by a number of diseases and depending upon the disorder, results in elevation of either glucuronide-conjugated or unconjugated bilirubin or both. The liver in normal individuals has capacity to metabolize four to five times as much bilirubin as is normally formed by normal heme turnover. Thus, disorders that cause only mild increases in heme turnover lead to no change in serum bilirubin. With more severe hemolytic or dyserythropoetic disorders, unconjugated bilirubin rises. In liver diseases or obstructions of the biliary outflow process, conjugated bilirubin tends to rise. Bilirubin will be analyzed if the MCV or hemoglobin values are below the lower limit of the reference range. Total and direct bilirubin will be analyzed on the Hitachi 911 analyzer using a colorimetric method by Roche Diagnostics/Boehringer Mannheim (Roche Diagnostics/Boehringer Mannheim Corp., Indianapolis, IN). In the total bilirubin assay, bilirubin is coupled with a diazonium ion in the presence of a solubilizing agent and a strongly acid medium. The production of azobilirubin is measured photometrically and is directly proportional to the concentration of total bilirubin. In the direct bilirubin assay, diazotized sulfanilic acid without an accelerator converts primarily conjugated bilirubin, but little unconjugated bilirubin, to an azobilirubin isomer, which is measured photometrically. Indirect bilirubin is calculated by subtracting the direct bilirubin from the total bilirubin.

Hemoglobin Identification and Hemoglobin A2 Quantitation

Anemia is a secondary cause of iron overload. Both beta thalassemia, which is a reduction in the synthesis of the beta chain of hemoglobin and certain hemoglobinopathies, which are structural abnormalities of the globin chain can result in anemia. For this reason, participants with low hemoglobin or low MCV will be evaluated for presence of genetic variants of hemoglobin and for hemoglobin A2 quantitation, which is generally elevated in beta thalassemia. These tests will be performed using high performance liquid chromatography.

Viral Markers

Viral hepatitis is an infection of the liver that is most commonly caused by hepatitis A virus (HAV), hepatitis B virus (HBV), or hepatitis C virus (HCV). Because HAV is typically a fairly mild self-limited disease, we recommend only monitoring for current or past infection by HBV and HCV using the hepatitis B surface antigen (HBsAg) test and hepatitis C antibody test (anti-HCV). These tests will only be performed if the ALT is greater than 1.5-fold upper limit of the reference range.

In acute HBV infection, HBsAg appears prior to clinical symptoms and remains present through the course of the active disease. Most patients infected with HBV eventually clear the virus from their body, but a certain percentage go on to develop chronic HBV infection. It is thought that antibody to HBsAg must develop in order to clear the body of the HBV. Without the development of antibody to HBsAg, the patient becomes a chronic carrier of the HBV in their hepatocytes with continued HBV replication. Individuals with on-going HBV chronic hepatitis continue to test positive for HBsAg. HbsAg will be analyzed using an ELISA immunoassay from Abbott Laboratories, Vernon Hills, IL. In this method, sample is added to beads that are coated with mouse monoclonal antibody to hepatitis B surface antigen. Antigen present in the sample binds to the antibody. Then, mouse monoclonal antibody to hepatitis B surface antigen conjugated with horseradish peroxidase is added. This reacts with the antigen-antibody complex. When o-phenylenediamine and hydrogen peroxide are added to the reaction mixture, a yellow-orange color develops proportional to the amount of HbsAg and is read spectrophotometrically at 492 nm. Results are reported as positive or negative.

Acute hepatitis C infection is somewhat different from hepatitis B infection in that, once infected, patients rarely, if ever, clear the disease. Measurement of antibodies to HCV is the test typically used to detect HCV infection. Unfortunately, development of anti-HCV does not lead to elimination of the virus from the body. Abbott Laboratories has a newer version of their EIA test for anti-HCV (version 2.0), which is approximately 98% specific, thus eliminating the need for a RIBA confirmation. Anti-HCV will be analyzed using an ELISA assay from Abbott Laboratories. In this method, sample is incubated with beads that are coated with recombinant HCV antigens C100-3, HC-31, and HC-34. If antibody is present in the sample, human immunoglobulins in the sample bind to the coated bead forming a bead-antigen-antibody complex. This complex is incubated with a solution containing horseradish peroxidase labeled goat antibodies directed against human immunoglobulins. When o-phenylenediamine and hydrogen peroxide are added to the reaction mixture, a yellow-orange color develops proportional to the amount of anti-HCV and is read spectrophotometrically at 492 nm. Results are reported as positive or negative.

Lymphocyte cryopreservation

Cyopreservation of lymphocytes involves isolation of peripheral blood mononuclear cells (PBMC) from anticoagulated whole blood using density gradient centrifugation (Ting A, et al. 1971) (Mittal KK). Sample layering with Ficoll-Hypaque utilizes differences in density between mononuclear cells and other blood sample elements to harvest the mononuclear

component. We have also used a new Vacutainer CPT tube method that utilizes a collection tube containing density adjusted solution, anticoagulant, and gel barrier. The CPT tube affords faster and more convenient separation. After separation, the PBMC are washed in Hanks Balanced Salt Solution (HBSS). Then, the PBMCs are suspended in a protein-rich media consisting of 20% fetal calf serum in RPMI-1640 + hepes. A cell count is made using a hemocytometer. Either cells are diluted 1:1 with DMSO and frozen in liquid nitrogen or cells are directly incubated with Epstein-Barr Virus (EBV) for transformation.

Genome Screen (Family Study only)

It is planned to apply to the Marshfield Genotyping Services (MGS) for performance of a 10cM density genome-wide polymorphism screen on the participants of the Family Study. Dr. James Weber, Director of MGS is a consultant on this study and will participate in planning this study. Use of this data to determine modifier genes and new genotypes is described in Section 7.

9. Quality Control

9.1 Quality Assurance

Quality Assurance activities are those performed before the data are collected, to minimize the number of data errors that occur. Primary steps in assuring good quality of study data are adequate training and periodic observation of study personnel. A highly motivated, conscientious staff may be the best guarantee of data quality. Other key considerations include adequate monitoring of technician performance by supervisory staff at the Field Centers and support units. Such monitoring can identify and correct problems weeks or months before they would become apparent from Quality Control activities such as statistical analyses performed by the Coordinating Center.

Quality Assurance activities in HEIRS will include: (1) a well-documented, standard protocol to be performed at all sites in an identical manner; (2) centralized training of key clinic staff so that all clinics perform HEIRS procedures in the same way; (3) requirements regarding demonstrated proficiency in administering questionnaires/interviews and minimum number of procedures required to obtain and maintain certification; (4) routine observation of clinic staff to verify adherence to protocol; and (5) routine calibration of equipment used during the screening and comprehensive examinations.

9.2 Centralized training of key clinic staff

An approach toward training that should be most efficient for HEIRS is the “train the trainer” model. In this model, each Field Center appoints a Training and Quality Assurance liaison who assumes overall responsibility for training (and re-training) of other clinic staff and verifying that all staff adhere to established study protocols. In general, training on specific components of the HEIRS protocol will consist of a detailed process review, an item-by-item review of the relevant forms(s) including rationale for inclusion and instructions on proper form completion, demonstration of techniques where applicable, and practice sessions. Training of Non-Field Center Training and Quality Assurance (QUALITY ASSURANCE (QA) staff will be conducted by Field Center Training and QUALITY ASSURANCE (QA) liaisons following protocols, guidelines and standards established for Centralized training. Appropriate documentation of training completion should be received by the CC before any Non-Field Center Training and QUALITY ASSURANCE (QA) staff member can be authorized to conduct study procedures.

9.3 Certification and Re-certification

9.3.1 Field Center Training and Quality Assurance (QA) liaisons

Certification of Field Center Training and Quality Assurance (QA) liaisons will occur during centralized training for physical examination components of the comprehensive examination based on certification standards established by the Steering Committee. Although initial certification for questionnaires and interviews will be done during centralized training, final certification will be given following submission and review of 1-2 audio taped interview

sessions. Re-certification of Field Center Training and Quality Assurance (QA) liaisons and other staff will occur periodically as specified by the HEIRS Study Protocol or as necessary as evidenced by QC results.

9.3.2 Non-Field Center Training and Quality Assurance (QA) staff

Certification of Non-Field Center Training and Quality Assurance (QA) staff will be conducted on-site by Field Center Training and Quality Assurance (QA) liaisons following the protocol and standards established in the Centralized Training session. Appropriate documentation of certification should be received by the CC before any Non-Field Center Training and Quality Assurance (QA) staff member can be authorized to conduct study procedures.

9.4 Quality Control

Quality Control activities are those performed during or after data are collected, to identify any errors which have occurred. Quality control in a large study such as HEIRS has two major purposes: (1) to identify problems in data collection and measurement in time to institute appropriate corrections; and (2) to quantify the quality of data collected over the course of the study so as to provide information necessary to interpret study results. To accomplish the first goal, adequate data must be accumulated to enable valid analyses to be performed within a brief period after initiation of data collection. To accomplish the second goal, sufficient data must be compiled throughout the study to detect any drift or deterioration in data quality over time. Because of finite resources, both in staff and in acceptable burden on participants, each component of a quality control program must be selected on the basis of assessing the need, feasibility, and overall importance to the main goals of HEIRS.

Data from the Central and local laboratories are among the most important collected by HEIRS. High quality data must be obtained from these units in order to fulfill the primary goals of the study. For these reasons, the Quality Control Committee will place special emphasis on quality control of these units.

For the other study components (e.g. data management), the Coordinating Center can provide considerable quality control information by relatively simple analyses of data acquired from all sites. Monitoring of the distribution of individual values and of mean or median values by center and clinic may identify many problems. Because of the large numbers available, this will be a particularly useful way of detecting many problems. Some of this information, such as noting problems with blood processing at a certain clinic, may be reviewed by a central unit.

9.5 Laboratory quality control

9.5.1 Central Laboratory

As part of the overall quality control program for laboratory analyses, duplicate specimens are sent to the laboratory, with one half of each specimen pair sent under the participant's regular HEIRS Laboratory ID number, and the other half under a HEIRS Quality Control Phantom Participant (QC) Laboratory ID number. The HEIRS QC Laboratory ID numbers are not distinguishable from other HEIRS Laboratory ID numbers so that this forms a blinded external quality control program monitoring measurement variability. To create the phantom duplicates a second set of tubes is collected from the approximate 50th participant at each site and sent out under HEIRS QC Laboratory ID numbers. Results on each laboratory measurement are matched to the appropriate participant results at the coordinating center. The HEIRS Field Center laboratory technicians maintain a weekly checklist of the QC samples to be collected during the week. As soon as each sample is drawn and processed, the corresponding labels are placed on the chart, and it is checked off. An example of the checklist is given below: After the list is completed the original form is sent to the coordinating center and a copy is kept at the Field Center.

9.5.2 Collecting and Processing QC Samples

Every 50th HEIRS Laboratory ID label is highlighted with a colored marker to remind the technician that QC samples need to be collected. (The participant who is assigned the highlighted labels does not necessarily have to be the volunteer for the QC samples.) When a participant is selected for the QC sample collection, select a HEIRS Laboratory ID number that is NOT sequential with the HEIRS Laboratory ID number that is being assigned to the participant. QC blood samples are processed along with the regular blood samples.

10. Statistical Considerations

10.1 Power for risk factors and gene-environment interactions

As indicated in Appendix F, using a conservative estimate of yield from the screening process of 300 cases, and assuming we have 600 controls, HEIRS will have over 80% power for a wide range of alternate hypotheses for both main effects of risk factors and gene-environment interactions.

10.2 Estimation of prevalences

Prevalences of primary iron overload as determined at the CCE will not be estimated by simple proportions. As described by Phatak et al (1998), estimation of prevalences from data collected in a multi-stage screening study requires the use of survival techniques, since dropouts may occur at each stage. Kaplan-Meier estimates (Kaplan 1958) which allow for censoring at each stage, are valid in this situation. Estimates of prevalence will be done for: primary iron overload by study definition; related clinical and pathological abnormalities; and genetic variants, including allele frequencies for HFE mutations C282Y and H63D and others which may be determined. Models allowing for changes in rates over the recruitment period and for adjustment by variables used to stratify sampling (Field Center, age, race/ethnicity) will also be fit.

10.3 Statistical analysis of case-control data

The data obtained for the case-control study will consist of genotypes at candidate loci, risk factors (measured as categorical or continuous variables) and the case/control status variable. Several types of statistical analyses will be performed to assess the relationships between the outcome (case/control status) and risk factors (both genetic and environmental). Variables (FC, race, gender, age) used to sample the controls, there have no distributional differences between cases and controls on these variables by design. Primary iron overload cases will be compared to controls with respect to other variables (e.g. serum ferritin, alcohol usage, iron intake) using chi-square tests for categorical variables and t-tests for continuous. For assessment of multivariate relationships, we will fit multiple logistic regression (Hosmer 1989) models. These models also allow us to examine interactions among the different risk factors for predicting case/control status. Sample size and power for these analyses is considered in Appendix F.

A second series of analyses relate to the distribution of clinical complications and manifestations of IO as outcomes among the cases. Each subject will be characterized by the mutations found at HFE, considered as binary variables. In order to evaluate the relationships between inherited susceptibility (genotype) and environmental exposures (physiologic blood loss, iron intake, etc.), several strategies can be employed. One approach is stratification by genotype, with analysis of outcomes related to environmental exposures. A second approach is to use multiple and logistic regression for continuous outcomes within genotype strata. Multiple and logistic regression can be used to predict group membership based upon demographic variables, environmental risk factors that appear significant in

univariate analyses, and genotype, with first-order interaction terms of genotype with environment.

10.4 Analysis of ELSI data

Various quantitative analyses, appropriate to the information generated and the questions posed, will be conducted to explore ELSI aims (see Section 5). Methods for the quantitative analyses will include analysis of variance and covariance, logistic regression (e.g. for analysis of factors predicting the decision to participate in screening), paired t-tests and McNemar's tests for comparing variables measure pre-post, and regression models for covariate (race, age, gender) adjustments.

10.5 Analysis of genetic data from the Family Study

One goal of this study is to detect genes outside of the HFE region that contribute to the iron overload phenotype and lead to hemochromatosis. This search for linkage to hemochromatosis (and other associated phenotypes) with polymorphic DNA markers will occur in several steps. The full effort will comprise a comprehensive genome wide search based upon a 10 cM map. There are two important components to this element of the study. First is the choice of markers and genotyping strategy, including the technical approach to be used in order to achieve high efficiency and accuracy. The second is the analytic capabilities and experience in genetic data management. In order to facilitate the identification of genes predisposing to hemochromatosis, we will perform a combination of a qualitative and quantitative trait analysis of data generated by the systematic genome wide search and a consideration of analyses conditional on the amino acids present in HFE.

Potential genotyping errors will be evaluated and identified both in individual families for single markers and across all the patient set for problem markers. This will provide high density and high quality data for analysis. Although the number of families to be evaluated by the genome screen may be small (perhaps only 100 in subpopulations), the actual number will only be determined after screening for HFE mutations occurs. Thus, formal power analyses are not feasible at this time; however, it is certain that the power to identify genes for a qualitative (hemochromatosis) trait will be low, while the power to map quantitative trait loci (QTLs) will be much larger. Guidelines for identification, ascertainment and collection of family materials are described in Chapter 7. Although neither the family structures or the heritability of the traits are known at this time, we know from simulation studies that collection of 100 families each with 15 measured relatives in three generations can have 80% power to detect a QTL that accounts for 30% of the genetic variance in a trait with 40% heritability. It may be possible to combine HEIRS data with data from other population based genetic studies of hemochromatosis, such as the ongoing studies at CDC (Karen Steinburg, PI) and the University of Rochester (Prad Phatak, PI).

Linkage analysis quantifies the evidence for linkage of a genetic marker locus with a putative disease susceptibility locus either by model-dependent or by model-independent methods. For the proposed studies of hemochromatosis and iron overload in families without an HFE mutation, this evidence is accumulated by statistically significant deviations from expected

proportions of marker allele sharing (25% sharing 2, 50% sharing 1, and 25% sharing 0; equivalent to an excess of sharing 2 alleles) among affected sib pairs or, in the case of discordant sib pairs (DSP), the deviation is in the excess of sharing 0 alleles. With larger families, the expectations are based upon relative pair sharing statistics. These methods may include those available in GeneHunter, but also in model-dependent (LINKAGE) and novel methodologies (Conditional linkage analysis using GeneHunter-plus, transmission disequilibrium tests using GASSOC, and logistic regression approaches to model gene-environment interaction). A variety of statistical genetic methods to detect and localize quantitative trait loci that influence variation in iron overload (using SOLAR) will be used.

The current proposed design should provide reasonable power to detect linkage for a quantitative trait, given the available sample size and the substantial underlying genetic contribution to the phenotypes under study. A three step analysis approach will be used: (1) single marker testing; (2) map generation; and (3) multipoint analysis. As detailed in Appendix F, data will be available for markers that are multiplexed to minimize size overlap, rather than all markers of a chromosome performed at once. Thus, the data for any one chromosome will be incomplete until late in the study. In this regard, the genome scan data initially will be checked for inconsistencies and linkage using pairwise methods implemented in SOLAR (MLE estimation of ethnic- and site-specific allele frequencies) will provide an additional check on Mendelian inheritance. Once appropriate maps have been formalized and estimated allele frequencies are determined, multipoint linkage analyses using SOLAR (for quantitative traits) and GeneHunter-plus (for qualitative traits) will be performed. Examples of analytic issues and methods are provided in Appendix F.

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