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Hereditary hemochromatosis has been classified as the most common autosomal recessive disorder in the United States. However, there exists no documentation of the frequency of screening and/or diagnosis in the military. Through a review of clinical records, it was found that very few military personnel and dependents receive iron-status screening. In addition, the actual number of clinical referrals and subsequent diagnoses at the Air Force medical genetics center is quite low. Overall, more males were genetically screened for hemochromatosis. However, females had a higher percentage of individuals homozygous for the C282Y mutation. More males were referred with abnormal iron status, while more females reported a family history of hemochromatosis. Patients at smaller medical treatment facilities and primary care providers more often included a family history of hemochromatosis in their referrals. Blood and serum were obtained from over 2,000 previously collected samples at a large military medical center. The importance of

population specific reference ranges was demonstrated by age and sex differences in several of the biochemical parameters measured. The use of the transferrin saturation percentage was shown to be effective in identifying individuals homozygous for the C282Y mutation. The mean corpuscular volume, a calculated parameter of the complete blood count, was shown to be significantly higher in C282Y homozygotes and affected heterozygotes. Liver function tests were found to be too non-specific to use routinely in screening for hemochromatosis. However, affected females had a higher activity level of ALT and AST to reference value ratio when compared to males. Iron alone correlated reasonably well with transferrin saturation to be potentially useful as a stand-alone screening test. Few active duty males were found to have presumptive iron deficiency anemia. Anemia in general was fairly common among all females and older males. Presumptive iron deficiency anemia was found in many females. Implementation of a hemochromatosis-screening program will require additional education of clinicians in order to be effective. Such a program will identify many individuals with hemochromatosis and iron deficiency anemia.

IDENTIFICATION, BIOCHEMICAL EFFECTS, AND
MOLECULAR DETERMINANTS OF IRON
TOXICITY IN A MILITARY SETTING

By

Nathan Hacker Johnson

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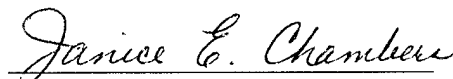
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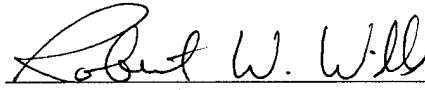
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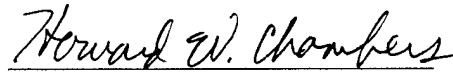
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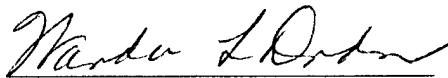
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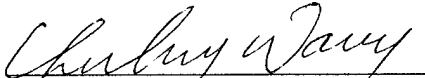
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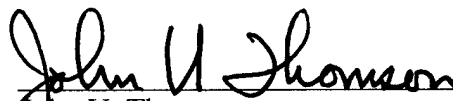
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DEDICATION

I would like to dedicate this work and its successful completion to my father and mother, whose gave me the opportunitites, morals, and work ethic to succeed in life, my children who make my life complete, my brother . who showed me that one can persevere, and my brother . who was my first example of excellence.

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CHAPTER I

Introduction

Hemochromatosis is a disorder that has both a long and short history. While many individuals have been affected over time, it has only been recently that clinical diagnosis has been possible.¹ Hemochromatosis is an autosomal recessive disorder.² There are numerous autosomal recessive disorders. Some common examples are cystic fibrosis, sickle cell anemia, and Tay Sachs disease. However, hemochromatosis is the most common of them all.² Up to 0.5 percent of Americans are homozygous for the gene that causes hemochromatosis and can be considered at risk for developing it.² Approximately ten percent of the population is heterozygous for the gene. Hemochromatosis putatively causes a large number of pathologic conditions.² The wide variety of diseases can make the diagnosis of hemochromatosis especially troublesome. In all but the very late stages of the disorder, the only way for diagnosis to occur is by biochemical screening of affected individuals.² Unlike many other genetic disorders, there is an effective treatment for hemochromatosis. Iron can be removed from the affected individual's body by therapeutic phlebotomy.²

The possibility of mass screenings for hemochromatosis is indeed an interesting concept. Historically, due to a variety of reasons, hemochromatosis has been thought of as a rare disorder.³ This misinformation has been passed down over the years and brings the medical community to an interesting crossroad.^{4,5} Hereditary hemochromatosis will only be found if screened for. In that respect, only medical providers with "recent" knowledge about hemochromatosis are likely to screen for it.⁵ It is therefore imperative to produce sound evidence that hemochromatosis is present in any population being examined. Due to the high medical compliance rate of military personnel and detailed medical records that are kept on individuals in the military, the military is an ideal organization to perform mass screenings. There are also a wide array of problems that must be overcome before such aggressive screening programs become a reality.

Definition

Hemochromatosis is a genetic disorder that results in excess iron accumulation in the body.^{4,6} This accumulation of iron can eventually lead to premature death.⁴ One of the most critical components of understanding hemochromatosis is the definition used in describing it. Many of the first diagnosed cases of hemochromatosis were found at autopsy in older men who were alcoholics. Identification of these hemochromatosis cases was perhaps due to the fact that these men were more likely to have a liver biopsy that revealed elevated hepatic iron and that alcohol consumption can exacerbate the clinical symptoms of hemochromatosis.⁴ The early definition of hemochromatosis being an "alcoholic disorder of older men"

was passed down to countless clinicians.⁴ As time went by, a newer definition of hemochromatosis was adopted. This newer definition stated that hemochromatosis was the presence of massive iron overload with accompanying iron-related organ injury.² This definition of hemochromatosis, although more accurate than earlier ones, was also lacking. By the 1960s, it was suspected that hemochromatosis was an inherited condition.¹ The prior definitions of hemochromatosis did not address this genetic component. The discovery of the major hemochromatosis gene (HFE) in 1996 caused the definition to be revisited. Hemochromatosis is now defined as "a disorder of iron metabolism that is inherited as an autosomal recessive trait due to two mutant hemochromatosis (HFE) alleles".⁷ One major difference from earlier definitions of hemochromatosis is the lack of reliance on overt iron overload and more emphasis on the genetic predisposition.⁷ As will be discussed later, the semantics of the hemochromatosis definition is extremely important when ethical issues are considered.

History

Although not definitive, recent genetic advances have caused many scientists to speculate on when and where mutations in the HFE gene were first introduced. The major mutation (C282Y) is thought to have been introduced in northern Europe about 800 to 1,200 years ago.⁸ There is debate as to the ethnic origin of the C282Y mutation. The most plausible explanation is a mutation occurred in a single (founder effect) or few individuals in the Viking population. The main Viking invasions of northern Europe occurred during the time that the C282Y mutation was thought to

occur.⁸ This massive migration along with the possible genetic advantage of increased iron absorption is the reason this hypothesis explains why such a high allelic expression could occur in such a short period of time.

Frequency of Occurrence/ Racial Differences

The frequency that hemochromatosis occurs in a given society is determined primarily by the demographics of that society. Given the fact that hemochromatosis is generally considered to have arisen from descendants of individuals of northern European origin, it is not surprising that societies that have a higher percentage of those individuals have higher incidences of hemochromatosis.^{2,9}

There are other forms of hemochromatosis for which the molecular mechanisms have not been determined. Sub-Saharan Africans have a type of hemochromatosis that is not linked with the HFE gene.¹⁰ This form of hemochromatosis is also exacerbated by the fact that Sub-Saharan Africans individuals drink a homemade beer that is very high in iron content.² There are also individuals who have idiopathic hemochromatosis.

The gene for the most common mutation of hemochromatosis is found on the short arm of chromosome six.¹¹ The majority of hemochromatosis is also associated with Human Lymphocytic Antigen (HLA)-A3, but not vice versa.¹¹ It is interesting to examine the frequency of HLA-A3 among various populations. The frequency of HLA-A3 is as low as one percent for Japanese individuals and up to twenty-six percent for Caucasians of European descent.² Other ethnic groups have HLA-A3 frequencies somewhere in between one and twenty-six percent. With these

frequencies in mind, it would be reasonable to expect that North Americans of African ancestry would have a rather low incidence of hemochromatosis due to HFE mutations. However, over time, 25 to 45% of the genes found in North Americans of African ancestry have been shown to be of Caucasian derivation¹¹ This explains why hemochromatosis may be seen in an otherwise unsuspected population. However, some North Americans of African ancestry have hemochromatosis that is similar to that seen in sub-Saharan Africans.¹¹ Some feel that the iron overload seen in North Americans of African ancestry is not related to the type seen in Caucasians.¹² The overall frequency of homozygotes in the Caucasian population of the United States is estimated at five per thousand.² Other ethnic groups have a much lower frequency.

Signs and Symptoms of Overt Disease

Although debatable, most feel that individuals with hemochromatosis will develop signs and symptoms related to the disorder.² There are many examples of signs and symptoms associated with hemochromatosis.¹³ Several signs and symptoms appear prominently. Arthritis, including osteoarthritis, is one of the more common symptoms.^{14, 15} The longest association of any disease with hemochromatosis, recognized over a century ago, is diabetes mellitus.¹⁶ These symptoms of hemochromatosis are caused by excess iron in the pancreas and the liver.¹⁶ There is debate regarding the association of mutations of the HFE gene and diabetes mellitus, as some feel this is an artifact of the increasing rates of diabetes mellitus in industrialized countries due to other causes.¹⁷ Often associated with diabetes, skin pigmentation is also often seen in hemochromatosis.¹⁸ Non-diabetic

endocrinopathy (i.e. hypogonadism) has also been reported in hemochromatosis.¹⁹ Excess iron has been strongly associated with hepatocellular carcinoma.²⁰ Iron dependent cirrhosis can occur with or without hepatocellular carcinoma.²¹ Cardiac involvement, to include cardiomyopathy, may occur later in course of hemochromatosis.²² Excess iron can impair immune function and has been shown to be associated with an increased risk of colon cancer and possibly Alzheimer's disease.^{23 24, 25} Hemochromatosis, along with other iron loading disorders, can cause increased susceptibility to bacterial infections.²⁶ Iron overload has been associated with increased risk for hepatitis B and C.²⁷ One interesting paradox is that individuals with hemochromatosis can have anemias.²⁸ Not all anemias are caused by iron deficiency which is a common misconception.^{4,29} Other less common diseases (e.g. porphyria cutanea tarda) are also known to occur in individuals either homozygous or heterozygous for the C282Y mutation.^{30, 31} The most important aspect of the wide variety of signs and symptoms of hemochromatosis is the unique ability of this disorder to masquerade itself.⁴ Without biochemical screening, most affected individuals are treated for the signs and symptoms and not for hemochromatosis itself.² For example, an individual may be treated for diabetes for many years and eventually die without ever being diagnosed with hemochromatosis.⁴ There are also those who feel that the signs and symptoms attributed to hemochromatosis occur so commonly in the general population that great care must be used when evaluating epidemiological data associated with hemochromatosis.^{32, 33}

One overlooked aspect of hemochromatosis is the possible potentiation with other environmental factors.³⁴ This is particularly important due to the high allele

frequency seen with this disease.¹¹ Although limited in scope, there are several examples where this potentiation has been demonstrated. Most of the known examples involve hepatic diseases. Excess iron has been shown to enhance the development of carbon tetrachloride induced liver fibrosis.³⁵ Acceleration of liver fibrosis is seen in patients with both hepatitis C and hemochromatosis.³⁶ Alcohol consumption in hemochromatosis patients dramatically increases the risk of liver cirrhosis.³⁷ It is probable that there are many other environmental factors that may be important with hemochromatosis.³⁴ It is possible that individuals with mutations in the HFE gene may be considered a "sensitive" population in the future.

Differences Between Sexes

One of the more puzzling aspects of hemochromatosis is the characterization of it as a "male" disorder. This characterization as a "male" disorder would normally typify an X-linked disorder. However, hemochromatosis is autosomal recessive in nature.² Nevertheless, more men are diagnosed with hemochromatosis than women.² Why is this so? Due to the autosomal recessive nature of hemochromatosis, women should genotypically suffer from this disorder with the same prevalence as men. It has been shown that the major difference in the clinical symptoms of males and females is a later onset in females.² This difference in the onset of signs and symptoms is easily explained. Females typically lose blood throughout their adult life during menstruation and pregnancy.² Because of this blood loss, the typical time of onset of clinical symptoms for females is several years later than men.² This delayed onset is not universal as some women suffer iron overload very early in life.²

Screening - Biochemical

It must be noted that no screening program guarantees a beneficial outcome. The goal of clinical screening programs should be to provide clinicians with a valuable tool to aid in the diagnosis and management of disease.² Early identification of hemochromatosis can reduce the likelihood of organ related injury and will undoubtedly extend lives. In addition, a major benefit of screening for hemochromatosis is the reduction in long-term medical treatment costs to the affected individual and to society at large.² A side benefit to screening for hemochromatosis is the capability to detect individuals with iron deficiency anemia.²

The mechanics of biochemical screening for hemochromatosis is fairly straightforward.² There are three components to a routine iron status profile.² Two of the components of biochemical screening are directly measured and one is calculated. The two measured components are serum iron and serum total iron binding capacity.² The most useful aspect of the iron profile is the calculated transferrin saturation. Transferrin saturation is calculated by dividing the total serum iron by the total serum iron binding capacity.² In general, the higher the transferrin saturation, the greater the individual is at risk for hemochromatosis. Setting absolute "abnormals" and screening algorithms is difficult.² Technical and methodological variations require each testing laboratory to determine what is an "abnormal" transferrin saturation. In general, values greater than fifty percent are considered abnormal.²

As with any biochemical screening, careful attention must be paid to any variation that may exist in the test or testing system. Tests for iron stores are no exception. Tests for iron stores are subject to analytical, preanalytical, and intra-

individual variation.² Recent ingestion of iron, which can elevate short-term iron stores, is one of the major factors that must be dealt with. It is therefore recommended that screening tests be performed on individuals who are fasting.² Total iron binding capacity and ferritin do not seem to be affected by fasting status.² There are also variations in iron status tests that exist in the methodology employed by the laboratory. Most clinical laboratories have coefficients of variation of under ten percent for iron status tests.² However, these small variations can have a profound impact when setting "concrete" cut-off values for screening tests. One manageable way around this problem is to avoid such "concrete" cut-off values. Rather, by investigating individuals in upper percentiles, a common screening approach may be maintained. Intraindividual variation of iron and total iron binding capacity has been estimated to be five and fifteen percent respectively.²

Individuals with elevated transferrin saturation should have a serum ferritin concentration performed.² Ferritin is a large iron storage protein. It can hold as many as 4,500 atoms of iron.³⁸ The purpose of ferritin analysis is to estimate the body iron burden. In individuals with hemochromatosis, there is great variability in the amount of measurable ferritin. This variability in measurable ferritin is due to variables such as age and sex.² Older individuals have more time to store iron in ferritin and females tend to have lower ferritin levels due to blood loss from menstruation and childbirth.² Intraindividual variation of ferritin has been estimated to be above twenty-eight percent.² Many physiological events, such as recent infection, can occur that will raise ferritin values.² Liver enzymes, such as aspartate amino transferase (AST) and alanine amino transferase (ALT), have been used as an initial screening tool for hemochromatosis.²

For individuals with hemochromatosis, a combination of an elevated ferritin and AST can aid in the prediction of cirrhosis.³⁹ The non-specific nature of liver enzyme elevations have cast doubt upon their ability to accurately discriminate between normal and disease states.²

One of the keys to the future success of biochemical screening for hemochromatosis, which will be the primary screening tool for the foreseeable future, is the ability to communicate to the general public the importance to "know your number".² This type of public education is similar to what has been done with cholesterol screening programs. This public education effort would help individuals who are both iron deficient and overloaded. This knowledge of individual iron status will also prompt individuals to ask their primary care health care provider to be screened more completely for hemochromatosis. Currently, this screening is rarely done.

The general consensus is the cost of mass screenings for hemochromatosis may be substantial. However, the cost for mass screenings for hemochromatosis should be no more than similar screening programs (i.e. cholesterol). Mathematical analysis, under a wide array of assumptions, has concluded that mass screening for hemochromatosis is cost-effective.⁴⁰ The major determinants of cost-effectiveness are the prevalence, penetrance, and disease burden of hemochromatosis, the performance characteristics of the screening tests, the cost of screening, and compliance with diagnosis and therapy.⁴¹ One large multi-center screening study calculated the cost per diagnosis of hemochromatosis at less than \$9,000 per case.⁴² However, key questions regarding disease expression, the natural history of the

disorder, and penetrance still remain regarding hemochromatosis.^{20, 40, 43-50} Some studies differentiate between the biochemical and clinical penetrance of hemochromatosis.^{51, 52} The key to the different presumptive penetrance rates found in the literature is the use of differing definitions of “a case” and possible ascertainment biases.^{44, 53} Other studies have found lower allele frequencies as age increases which indicates that individuals with the allele may have died at a higher rate than those without the allele.⁵⁴ Some studies support a low penetrance with equal allele frequencies in both older and younger subjects.³³ The only definitive method to answer the question of penetrance is a randomized, controlled trial. With a disorder such as hemochromatosis, such a study is neither possible nor ethical due to ramifications such as insurance cancellation.⁵⁵ Even with unanswered questions regarding hemochromatosis, biochemical screening should not be ignored. Over 80 percent of recently diagnosed patients referred to a large medical center with a diagnosis of hemochromatosis came to medical attention by virtue of biochemical screening.⁵⁶ Most of these recently diagnosed individuals had normal physical examinations, which underscores the importance that biochemical screening plays in the diagnosis of hemochromatosis.

Screening - Genetic

One of the more controversial issues surrounding hemochromatosis is genetic screening. It has been known for more than 25 years that hemochromatosis has a strong genetic component.^{57,58,59} More recently, in 1996, Feder discovered a 250-kilobase region on chromosome six that is associated with iron absorption. This

gene was named HFE.⁶⁰ This gene produces a protein that can bind to transferrin receptors.⁶¹

There have been many genetic variations of the HFE gene reported.⁶² Two of these variations appear to have clinical significance.⁶³ The most important of these variations is the C282Y mutation. This mutation is due to a single base substitution at nucleotide 845 of HFE gene. This base substitution is guanine for adenine.⁶³ The result of this base substitution is the replacement of the normal amino acid cysteine with tyrosine.⁶³ The second major mutation is the H63D mutation. This mutation is due to a single base substitution at nucleotide 187 of HFE gene. This base substitution is guanine for cytosine.⁶³ The result of this base substitution is the replacement of the normal amino acid aspartate with histidine.⁶³ The C282Y mutation appears to alter the protein product.⁶⁴ The H63D mutation does not seem to alter the product to such a great degree.⁶⁵ There is little doubt that this alteration of the protein product affects iron absorption. Studies with HFE-deficient mice have also supported its importance in regulating iron absorption.⁶⁶

Genetic testing has been rapidly incorporated into clinical practice by some physicians.⁴⁸ One study found that approximately 31 percent of referred hemochromatosis samples to a genetic testing center were "positive".⁶⁷ These were cases in which the patient was either homozygous for the C282Y mutation, a compound heterozygote (heterozygous for both mutations), or homozygous for the H63D mutation. It is rare that a patient will be homozygous for one mutation and heterozygous for the other.⁶⁸ There have been no documented cases where an individual is homozygous for both mutations. Clinically, there is agreement that the

C282Y mutation is the more important and the H63D mutation alone is not significant in most cases.^{44, 69} It is thought that compound heterozygotes are more affected than individual heterozygotes.⁷⁰

When Should Screening Occur?

The appropriate time to screen for hemochromatosis is debatable.² Most of this timing debate is based on the "definition" that one uses for hemochromatosis. If one considers only the clinical signs and symptoms, screening for hemochromatosis at mid-life would be preferable. Mid-life is when the obvious signs and symptoms appear in the majority of individuals.² However, these symptoms of hemochromatosis may occur much earlier.⁴ If one uses the modern genetic definition of hemochromatosis, difficult questions arise based on one's opinion of genetic screening. Opinions of genetic screening fall into three distinct groups. First, there are those who feel everyone should have hemochromatosis genetic screening at birth.⁷¹ Second, there are those who feel that genetic screening should occur only after "abnormal" biochemical tests. Hemochromatosis genetic screening would occur only after iron had time to accumulate and produce elevated results. Screening under these circumstances could begin around age twenty in males and later in females.² Lastly, there are those individuals who think only biochemical testing should be performed to screen for hemochromatosis. The rationale behind this approach is that a small percentage of individuals with hemochromatosis will not test "positive" for the current genetic markers. Thus, these individuals with a false-negative test will be given a false "clean bill of health". Proponents of biochemical only testing suggest

treatment of all individuals with elevated iron stores.

Who Should Be Screened?

A basic question that must be answered is who should be screened for hemochromatosis? This is not easily answered. Theoretically, it is possible for anyone to carry an abnormal gene mutation and have hemochromatosis.⁴ This was shown by early studies demonstrating a wide variety of HLA antigens in hemochromatosis patients.⁷² Given the fact that anyone can potentially have hemochromatosis, an argument can be made that all individuals should be screened. Complicating the question of who should be screened is the fact that resources are limited in most situations. Limited resources may prevent universal screening. In situations where resources are limited, many consider utilitarianism, providing the greatest good to the greatest number of people, the right approach. Utilitarianism may be appropriate when screening for hemochromatosis because current screening techniques cannot characterize all individuals with the disorder. However, scientific attempts can be made to maximize the number of individuals diagnosed with hemochromatosis.²

Who then should be clinically investigated for the presence of hemochromatosis? Because of the genetic nature of hemochromatosis, all individuals of blood relation to anyone homozygous for the abnormal mutation of the HFE gene should be screened.² Currently, because of the small percentage of patients diagnosed with hemochromatosis, few blood relatives are tested by this rationale. The next

group of individuals who should be investigated for hemochromatosis are those who exhibit signs and symptoms of overt disease. The number tested because of signs and symptoms is also small. Most signs or symptoms of hemochromatosis are either silent or attributed to other diseases or conditions.^{2, 73, 74} Historically, most of these signs or symptoms will not be linked to hemochromatosis.² The last major group of individuals who should be investigated are those who have abnormal iron status tests.² Most individuals have iron status testing to rule in or out iron deficiency anemia. Few people currently have iron status testing for potential iron overload.

Currently, the Hemochromatosis and Iron Overload Screening Study (HEIRS) is attempting to answer some of the questions surrounding who should be screened. The purpose of this five-year, \$30 million dollar study is to determine the genetic and environmental determinants, prevalence and potential personal, clinical and societal impact of iron overload and hemochromatosis.⁷⁵ It will consist of a primary care-based sample of 100,000 adults.⁷⁵ The HEIRS study is conducted by the Division of Epidemiology and Clinical Applications of the National Heart, Lung, and Blood Institute (NHLBI), the Division of Blood Diseases and Resources of the NHLBI, and the Ethical, Legal, and Social Implications Research Program of the National Human Genome Research Institute.⁷⁵ Until the lingering questions about who should be screened are hopefully answered, it is considered wise to continue to biochemically screen for hemochromatosis, particularly into periodic health examinations and surveys.⁴⁴⁻⁴⁷

Is Screening Being Performed In The Military?

In the civilian community, it is documented that American physicians have inadequate knowledge of hemochromatosis and the majority of civilian physicians have not recently screened for hemochromatosis in their clinical practice.⁵ In addition, older physicians are less likely to be informed about hemochromatosis.⁵ Due to the nature of the military, there are a higher percentage of young physicians who provide medical service. The higher percentage of younger physicians should impact the number of individuals screened in a positive manner. However, there has been no documented study of hemochromatosis screening in the military. There are individuals who are diagnosed with hemochromatosis in the military. However, it is very probable that the number diagnosed with hemochromatosis pales in comparison with the actual number who have the disorder. For example, in calendar year 2000, nineteen individuals were confirmed by DNA analysis to be homozygous for the C282Y mutation at the United States Air Force medical genetics center. During this time, well over one million beneficiaries were eligible for care by virtue of their service to the military. An estimate of 0.4% of the military population being homozygous for this mutation would place well over 4,000 individuals at risk. If ten percent, or 400, of those were already diagnosed with hemochromatosis, that would leave 3,600 individuals undiagnosed. Based on these estimates, 19/3600, or 0.5% of those with undiagnosed disease were diagnosed in calendar year 2000. Therefore, it is safe to say that while some are being diagnosed, the great majority with hemochromatosis are not.

Military Importance

The importance of identifying hemochromatosis in a military setting is difficult to ignore. Many of the signs and symptoms of hemochromatosis may cause problems in military readiness². However, there are only a few cases of military diagnosed hemochromatosis in the literature.^{76, 77} While it is true that the majority of individuals with hemochromatosis will be affected after their time of active military service, this is not always the case.² It has been noted, much like the situation seen outside of the military, that it is very difficult for military health care providers to accurately diagnose hemochromatosis without biochemical screening measures.⁷⁸ Undiagnosed hemochromatosis could lead to problems in military readiness and decreased morale of service members with undiagnosed family members. Screening for hemochromatosis, and subsequent diagnosis, should also reduce future health care costs to the Department of Defense.

Other Conditions Where Iron Overload Is Observed

Although this study focuses on hemochromatosis, it should be noted that it is not the only condition that causes iron overloading. While not a complete list, some of the more familiar conditions where iron overload is observed include sideroblastic anemia, porphyria cutanea tarda, β -thalassemia, medicinal iron overload, and transfusional iron overload. Each of these iron overloading conditions has a unique complement of signs and symptoms that can be used to differentiate it from hemochromatosis.² It is also possible that some conditions, such as β -thalassemia,

may act in a synergistic manner with hemochromatosis.⁷⁹ In addition, there is a form of juvenile hemochromatosis that develops earlier than traditional hemochromatosis.⁸⁰

Therapy

The treatment for hemochromatosis is therapeutic phlebotomy. In general, there are three major variables that determine how long an individual who has hemochromatosis will survive. These variables are the timeliness of initiation of phlebotomy therapy, the presence of diabetes, and the presence of major organ damage.² This is especially true when the organ damage includes hepatic cirrhosis.² It is crucial that identification and treatment of hemochromatosis be initiated as soon as possible. It has been shown that patients with hemochromatosis who undergo iron-depletion phlebotomy before the onset of diabetes mellitus or hepatic cirrhosis can have a normal life expectancy.⁷⁴

There is no set phlebotomy schedule for all patients to follow during iron-depletion therapy. Rather, each phlebotomy schedule must be individually determined.² The goal of iron-depletion therapy is for the patient to develop a mild blood loss anemia.² One key feature of this phlebotomy therapy is the monitoring of the hemoglobin and hematocrit before each procedure.² Frequent monitoring can assist in ensuring the anemia is mild. The hemoglobin and hematocrit should be just low enough to stimulate red blood cell production.² Lowering the hemoglobin and hematocrit to lower levels does not increase red blood cell production and may subject the patient to symptoms of severe anemia.² The exact number of therapeutic

phlebotomies that will be performed on a patient depends on their tolerance to the procedure. Some individuals can tolerate one or more phlebotomy per week.² The exact volume of blood withdrawn from the patient is variable. Elderly and small individuals may have less blood withdrawn.² While there is some debate concerning the timing of initiation of phlebotomy, a reasonable option is initiation based on serum ferritin levels.² The degree of ferritin elevation can aid in estimating the number and duration of therapeutic phlebotomy treatment.² Ferritin values of less than 500 ng/ml will usually require ten or less therapeutic phlebotomies.² Ferritin values greater than 1,000 ng/ml may require in excess of twenty-five phlebotomies.² Initial therapeutic phlebotomy usually ceases when serum ferritin falls below 10 to 20 ng/ml.² When the initial therapeutic phlebotomy has ended, it is very important that monitoring be continued. The next phase of therapy is "maintenance phlebotomy".² In most cases, therapeutic phlebotomy will be required between two and six times per year.² Serum ferritin should be monitored at regular intervals for the rest of the patient's life. Serum ferritin should not be allowed to rise above 100 ng/ml in hemochromatosis patients.² Blood from hemochromatosis patients is safe to use for transfusion and hemochromatosis patients are more likely to be repeat blood donors.⁸¹

If diagnosed with hemochromatosis early enough, therapeutic phlebotomy can improve life expectancy. In some cases, patients with hemochromatosis will have a normal life expectancy.² Some complications of hemochromatosis are not improved by phlebotomy. Hepatocellular carcinoma, which causes thirty percent of all hemochromatosis deaths, can not be reversed by therapy.² Arthritis and sexual dysfunction are usually not reversible.² In patients without an intact hematopoietic

system, chelation therapy may hold future promise for hemochromatosis treatment.⁸²

Dietary Recommendations

Individuals with hemochromatosis should eat foods high in iron (e.g., red meat) in moderation and should not use iron containing vitamin supplements.⁸³ Other dietary restrictions in hemochromatosis patients are not recommended because the amount of excess iron absorbed from the diet per day pales in comparison with the amount of iron removed by phlebotomy.⁸³ Two other dietary considerations in hemochromatosis patients are the limitation of alcoholic beverages which may increase iron absorption, and elimination of shellfish from the diet which may be contaminated by the iron loving *Vibrio vulnificus*.^{9, 83}

Mechanisms of Absorption and Toxicity

The toxicity of hemochromatosis arises from a lifelong abnormal absorption of dietary iron.⁸⁴ This increased iron absorption results in excessive body iron loading.⁸⁵ Normal iron absorption consists of three distinct phases.⁸⁴ The first phase of iron absorption involves iron presentation and uptake by mucosal cells.⁸⁴ The second phase of iron absorption involves transcellular transport and processing of iron.⁸⁴ The last phase of iron absorption involves the transfer of iron out of the mucosal cell and into the portal venous circulation.⁸⁴ There are differences in the first phase of iron absorption between non-heme and heme iron.⁸⁴ Normal adults have around four grams of total body iron compartmentalized between erythrocytes, body stores, myoglobin and enzymes, and serum.⁸⁶ Iron is heavily conserved. An adult

male will lose about one milligram of iron per day due to defoliated epithelium and secretions from the gut and skin.⁸⁶ Women of childbearing age lose an average of an additional five milligrams per menstrual cycle and about 500 milligrams with each pregnancy.⁸⁶ Loss of iron signals the body to absorb more iron. The body has no mechanism to "rid itself" of excess iron.⁸⁶

Individuals with hemochromatosis absorb roughly 4 milligrams of iron per day.⁸⁶ This iron builds up over time. Other non-ferrous metals, such as lead, have also been shown to be absorbed at increased levels in hemochromatosis.⁸⁷ In fact, lead absorption has been shown to be fifty percent higher in hemochromatosis patients when compared to normal controls.⁸⁸

The liver plays a vital role in the uptake, distribution and storage of iron.⁸⁹ The importance of the liver is a major reason that it is one of the common organ dysfunctions seen in hemochromatosis. However, it is now known that iron accumulation also occurs in many other tissues and organs.⁸⁹ Excess dietary iron can exacerbate tissue damage occurring in the liver.⁹⁰ Cellular damage has been attributed to excess iron catalyzing the formation of oxyradicals or creating lysosomal injury.⁹¹ Alcohol exacerbates iron absorption.²

Heterozygotes

The issue of heterozygous individuals for the C282Y mutation is controversial.^{92, 93} The frequency of heterozygosity is about twelve percent among Caucasians of European ancestry.⁹² Most individuals who are heterozygous for the C282Y mutation will not suffer any adverse effects.² In fact, there may be some

protection from iron deficiency anemia in heterozygotes.² The most common signs seen in heterozygotes are a slight increase in serum iron concentration, transferrin saturation, and serum ferritin.^{92, 94, 95} However, not all individuals with hemochromatosis will have elevated iron stores.^{94, 95} Abnormalities in iron status are seen at a higher rate in compound heterozygotes.⁹⁵ Heterozygotes have only a slightly higher relative risk for such diseases as cancer, heart attack, stroke, hypertension, and diabetes mellitus.^{96, 97} Iron overload in these individuals should be treated in a manner similar to those who are homozygous for the C282Y mutation.

Ethical Issues

There are inherent ethical issues that accompany the diagnosis of hemochromatosis.⁹⁸ One of the more troublesome ethical issues involves insurance related risk. Other hemochromatosis related ethical issues involve such events, as pre-employment physicals like seen in the military. In order to ascertain the risk a prospective client may present, it is common practice for individuals to answer questions about their present health. Many times, these questions pertaining to health are open-ended. For example, such a question may read, "Do you have any medical conditions for which you are under a physician's care"? For those individuals who have been labeled with having hemochromatosis, this can present a serious dilemma. An individual may have been diagnosed with hemochromatosis at an early stage and treated with no expected negative outcome and thus honestly answer that they are not under a physicians care. However, with the newer genetic definition of hemochromatosis, some individuals may not be able to answer in a negative manner.

Insurers, like most health care providers, are unaware of the pervasive nature of hemochromatosis. Therefore, most do not include iron status tests in the battery of tests that is conducted when one applies for health or life insurance. It is possible in the future that insurance companies could use such iron status testing for genetic discrimination purposes. Although such testing may have ethical concerns, it would identify many undiagnosed individuals with hemochromatosis.

Another ethical issue is the potential to identify individuals with a disorder they would otherwise never know about. False positives, depending on the definition of penetrance, can also occur. The psychological issue of genetic testing is often ignored by scientific personnel.⁹⁹ There has been one study that has addressed these issues.⁹⁹ Surprisingly, this study found no negative psychological effects associated with a diagnosis of hemochromatosis.⁹⁹

Population Based Screening

There are six principles in determining the efficacy of population based screening for early diagnosis and prevention.¹⁰⁰ These principles are: early detection should improve the clinical outcome, adequate resources must be present, the therapy must be acceptable in such a manner that high compliance is expected, studies of efficacy point to a positive outcome, there must be a high probability of disability and/or death, and the sensitivity and specificity of testing methodologies must be acceptable and at a low cost.¹⁰⁰ Each of these principles is fulfilled with hemochromatosis with the possible exception of conclusive studies of efficacy, which requires more study.² The frequency of heterozygosity in the United States

population is estimated to be near twelve to thirteen percent.² This means that about 1.7 percent of marriages in the United States will be between Caucasians of European descent who are both heterozygous for the HFE allele. Simple math dictates twenty-five percent of offspring in such marriages will be homozygous for the C282Y mutation. In addition, there is a thirteen percent chance that a known homozygote will marry a heterozygote.² Fifty percent of offspring from such marriages will be homozygotes. The mendelian nature of hemochromatosis and the relatively high frequency make it a prime candidate for population based screening.²

Computer simulations have shown that the societal cost of prevention screening is much less than that of treating symptomatic hemochromatosis.² These types of computer simulations do not take into account the alleviation of pain and suffering that such screening will prevent. One approach that may be appropriate is to include iron status tests with all routine clinical chemistry panels.² However, routine inclusion of iron status tests with other traditional chemistry tests has been administratively troublesome. Medicare has ruled indiscriminate iron status testing of samples "non-reimbursable". One major laboratory, while adding only pennies per test and making no profit, was charged with fraud for adding transferrin saturation to their basic chemistry panel. Therefore, population based hemochromatosis screening must be done "clinically" with individual health care providers ordering such testing on an "individual" basis.

Histopathology in Hemochromatosis

Before genetic testing was available, histopathology (liver biopsy) was crucial in the final diagnosis of hemochromatosis.¹⁰¹ Liver biopsies were performed after abnormal iron status tests were obtained.² Liver biopsies are still of value today for individuals suspected of having hemochromatosis but lacking specific genetic mutations.¹⁰² Of special importance in liver biopsies is the total hepatic iron.¹⁰³ Although new methods of early detection of hemochromatosis are being investigated, such as computed tomography, hepatic iron is still considered the gold standard.¹⁰⁴ However, this does not mean that all individuals should receive a liver biopsy, as there are inherent risks involved with the procedure.¹⁰²

CHAPTER II

CURRENT TRENDS OF IRON STATUS TESTING IN A MILITARY POPULATION

Introduction

The significance of identifying the status of screening programs for disorders in which biochemical abnormalities are present is universally understood.² Such is the case with screening for hereditary hemochromatosis. Since there is not a universally accepted military or civilian program, evaluation of hemochromatosis screening can only be accomplished by retrospective analysis of clinical records. The number of individuals identified with hemochromatosis by civilian biochemical screening is known to be very low.⁴ It is assumed the low number of individuals identified with hemochromatosis is reflective of the infrequency of biochemical screening by primary care providers.

Although several studies have demonstrated that prospective biochemical screening for hemochromatosis can be effective, there has been no study of what the actual frequency of biochemical screening is in outpatient settings.¹⁰⁵ This was verified by Dr. Wylie Burke of the University of Washington Medical School, one of the foremost experts on genetic and hemochromatosis screening.¹⁰⁶ The military health care system is a good environment to study screening frequencies due to the

"cradle to grave" nature of care given. In addition, several interesting factors may influence the frequency of biochemical screening for hemochromatosis in the military setting. Among the factors that may influence the frequency of biochemical screening for hemochromatosis is a different mix of medical providers than seen in non-military settings. Due to the significant number of physicians who leave the military medical health care system each year, the average military physician is younger than non-military counterparts. This leads to a provider pool with more recent medical training that may include screening for hemochromatosis. The military also uses a greater proportion of non-traditional providers such as physician assistants and nurse practitioners. These are individuals who possess a Bachelor or Master of Science degree in physician assisting or nurse practitioner. Physician assistants and nurse practitioners have less formal medical training than their physician counterparts. However, many physician assistants and nurse practitioners have significant medical experience. This experience may influence, negatively or positively, their inclination to screen for hemochromatosis.

Another relevant reason to use a military setting to determine the frequency of hemochromatosis screening is the completeness of the military medical record. The military medical record is the property of the United States government and is used for many important reasons, one of which is the determination of fitness for military service. Therefore, good record keeping is mandated. In theory, all military medical care received is included in the military medical record. Most civilians do not have a consolidated medical record that represents their complete adult medical history.

Examination of military medical records should reveal not only the frequency of common iron status tests, but also the rationale for such testing.

An additional reason for examination of screening trends in a military setting is the universal availability of health care. Each individual in the military, more or less, has health care available "at will". In this setting, there is no financial incentive to "stay away" from the health care provider. No payment is required by the individual who is seen at military medical treatment facilities and no sick or vacation time is lost for such visits. Although military medical facilities are required to operate in a fiscally responsible manner, no pressure is put on health care providers to cut back on inexpensive procedures such as iron status screening. Each patient has equal access to medical care and each medical provider has the discretion to request iron status testing on any patient under his or her oversight.

There are also other important factors that should be examined from this data set. One of the most important is a comparison of the frequency of iron status testing to longstanding markers of health. Two such common markers, picked for their common inclusion in most health screening programs, are glucose and cholesterol. Some in the medical community would rank iron status screening just as important as screening using glucose or cholesterol.² Continuation of care is another important factor that warrants examination. Although universal health care is available to each service member, there is no guarantee that the same health care provider will see an individual patient in a recurrent manner. More importantly, when a move is made between bases or posts, it is almost a certainty that a different health care provider will be seen at the new assignment. There is also the possibility that one may transfer

from an installation with a large medical center to one with a small clinic or vice versa. A transfer to a different size of medical treatment facility could have a dramatic impact on screening as medical centers have the reputation as being more aggressive in adopting new screening techniques. A final benefit from examination of iron status testing is the determination of the documented frequency of iron related disorders other than hemochromatosis such as iron deficiency anemia.

Methods

The methods used for this research were approved by the Mississippi State University Institutional Review Board. Medical records were obtained from 249 individuals who currently receive medical treatment at Columbus Air Force Base, Mississippi. Every 25th record was selected for evaluation from a population of over 6,000 medical records. The duty status distribution of these medical records included 124 active duty male, 109 dependent female, and 16 active duty female. Retired individuals were not included in this sample because the active duty portion of their medical record is located in Saint Louis, Missouri. Therefore, retired individuals do not have a “complete” medical record that is easily accessible. Information from the medical records was entered into Access, an electronic database (Microsoft, Redmond, Washington). After the data were collected, all unique identifiers were removed and the data were untraceable to the medical record from which it came. The specific data collected included gender, military status (active duty or dependent), rank, number of years that military health care was provided, obvious diagnosis of iron related disorders (e.g., iron deficiency anemia, hemochromatosis,

etc...), the number and type of iron status testing performed, the clinical indication for any iron status testing performed, number and type of liver profile testing performed, number of complete blood counts performed, and number of comparison tests performed. Statistical analyses included descriptive statistics, Students t-test, Fisher's exact test and chi square using Number Cruncher Statistical System (NCSS, Kaysville, Utah).

Results

The documented frequency of various biochemical analyses, to include iron status testing, is listed in Table 2.1. Due to the limited number of active duty females included in the records review (n=16), statistical significance was determined using active duty males (n=124) and dependent females (n=109). Iron and ferritin analyses were found to be requested in no active duty male or female patients. Iron and ferritin were requested in a small percentage (11.9% and 6.4% respectively) of dependent females. This difference in proportions was highly statistically significant using Fisher's exact test for iron ($p<0.0001$) and ferritin ($p<0.003$). A statistical difference was also seen in glucose ($p=0.004$) and liver function tests ($p<0.0001$). However, there was no statistical difference in proportions of complete blood counts or cholesterol.

The test per patient-year is listed in Table 2.2. Because there was great variation in the number of years of medical care provided (1-30 years for active duty and 1-36 years for spouses), raw data were standardized by dividing by the

corresponding years of medical service provided. The dependent spouses had an average of 11.16 years of medical service provided versus 6.96 years for active duty. Seventy-five percent of dependent spouses, all of whom were female, had a complete blood count performed at some point while under military care. Ninety-five percent of females with over five years of care had a complete blood count. The overall average for dependent females was 0.45 CBC per year of medical care provided. Using the standardized data, the overall screening rate for CBCs, glucose, cholesterol, and liver function tests were all statistically higher in the dependent population ($p < 0.0001$ for each) using Fisher's exact test.

Overall, the rate of iron status specific testing (iron, total iron binding capacity, transferrin saturation, or ferritin) was found in 12 dependent records (10.5% of the dependent spouse records reviewed). Each of these 12 was performed to investigate suspected anemia. Eleven of these requests were ordered by their primary care provider. There was no evidence in any record of screening for iron overload. In addition, liver function tests were rarely ordered on the active duty population (0.03 per year of medical service provided).

Table 2.1: Frequency of Biochemical Analysis

	Active Duty Male		Active Duty Female	Dependent Female
Number	124		16	109
Years Medical Service Provided	7.35		4.00	11.47
% Iron	0.00 (0/124) $p<0.0001$		0.00 (0/16)	11.93 (13/109) $p<0.0001$
% Ferritin	0.00 (0/124) $p=0.003$		0.00 (0/16)	6.42 (7/109) $p=0.003$
% CBC	66.94 (83/124)		37.50 (6/16)	74.31 (81/109)
% Glucose	54.84 (68/124) $p=0.004$		37.50 (6/16)	73.39 (80/109) $p=0.004$
% Cholesterol	77.42 (96/124)		87.50 (14/16)	79.82 (87/109)
% Liver Function Test	17.74 (22/124) $p<0.0001$		12.50 (2/16)	66.97 (73/109) $p<0.0001$

Table 2.2: Test per Patient Year

	Active Duty Male		Active Duty Female		Dependent Female
Number	124		16		109
Years Medical Service Provided	7.35		4.00		11.47
Patient Years	911.40		64.00		1250.23
Iron	0.00 (0/911.40) $p < 0.0001$		0.00 (0/64)		0.02 (30/1250.23) $p < 0.0001$
Ferritin	0.00 (0/911.40) $p = 0.003$		0.00 (0/64)		0.01 (14/1250.23) $p = 0.003$
CBC	0.20 (183/911.40) $p < 0.0001$		0.22 (14/64)		0.45 (561/1250.23) $p < 0.0001$
Glucose	0.115 (105/911.40) $p < 0.0001$		0.34 (22/64)		0.48 (600/1250.23) $p < 0.0001$
Cholesterol	0.24 (218/911.40) $p < 0.0001$		0.48 (31/64)		0.42 (530/1250.23) $p < 0.0001$
Liver Function Test	0.04 (33/911.40) $p < 0.0001$		0.05 (3/64)		0.32 (401/1250.23) $p < 0.0001$

Discussion

The percentage of active duty individuals who are screened for iron related disorders is shockingly low. In fact, none of the 124 records from active duty males and less than twelve percent of dependent females indicated iron status testing. The higher percentage for females, although considered by itself is very low, is because some were screened for iron deficiency. It is noteworthy that none of the 249 records reviewed had any indication of biochemical screening for iron overload. Therefore, it is safe to assume that most individuals with hemochromatosis will not receive a correct diagnosis due to a lack of biochemical screening.

The paucity of iron status testing is highlighted when compared to standard biochemical tests of wellness such as glucose and cholesterol. Although not found in all records, documentation of glucose and cholesterol screening are found in the majority of medical records. This is not surprising due to the emphasis of preventive medicine practiced in the military. This “preventative medicine” mentality and paradigm also underscores the potential of military medicine to screen for other biochemical-based disorders such as hemochromatosis. If iron status tests were requested at the same frequency as glucose or cholesterol, many with iron related disorders could be found. In men, this biochemical screening need not occur very often, as only several tests in a lifetime would provide their medical provider with sufficient diagnostic information. Females, due to menstruation, would need more frequent testing.²

Complete blood counts are fairly common in both men and women. Liver function tests are more common in the dependent female population. Liver function

tests are part of some routine screening, however, these tests are more commonly associated with acute medical conditions. The frequency of liver function tests is important because these tests may be used as a surrogate or in adjunct screening methodologies for hemochromatosis. One aspect of any potential iron status-screening program that must be addressed in the active-duty population is the apparent under-utilization of medical services by males. This can be seen in the test per patient year for active-duty males. Reasons for such under-utilization, when compared to the dependent female population, may be complex and beyond the scope of this project. Potential variables that may influence under-utilization in active duty males could include the younger age of the active-duty males, higher physical fitness requirements, and an unwillingness to seek medical attention. When examining cholesterol screening, which has a periodic mandatory requirement for active duty personnel, the difference in rates between males and females is not as dramatic. It may be sound clinical practice to include iron-status testing in a manner similar to mandatory periodic cholesterol screening.

Of the twelve females that had documentation of iron-status testing in their medical record, all were requested for suspected iron deficiency. The high percentage of requests for suspected iron deficiency is not surprising due to the awareness that exists in the medical community about the great importance of iron deficiency. In eleven of these cases, the requesting medical provider was not a specialist. The fact that non-specialists represented the majority of clinical requisitions is important in relation to iron-status screening. Since the majority of individuals do not see a specialist except for chronic illness, screening is most appropriate to occur at the

primary care provider level. Although specialists should suspect hemochromatosis in those with advanced clinical symptoms, the majority of those diagnosed with hemochromatosis would best be uncovered at an earlier age and stage. The few individuals who are screened for iron-deficiency can be used as an example of proper primary care screening for iron overload. In addition, the well-accepted iron deficiency-screening paradigm can be used as a lever to hopefully jump start iron overload screening. Emphasizing the benefits of iron-status testing for both iron deficiency and iron overload may be a vehicle to reach out to medical providers who are screening for neither. It is noteworthy that 95 percent of dependent females who have been in the military medical system for over five years had a complete blood count at some point. This indicates the military medical system is directly or indirectly doing an excellent job at screening for anemia in general. The next step must occur with adding iron status to the screening protocol.

One of the limitations of this study, as may occur in any retrospective record review, is the possibility of non-inclusion of laboratory reports or physicians notes in the military medical record. This may occur due to oversights in those generating or filing the reports.

CHAPTER III

ANALYSIS OF CURRENT GENETIC TESTING FOR HEMOCHROMATOSIS IN THE UNITED STATES AIR FORCE

Introduction

One of the many important characterizations that are needed in relation to the current state of hemochromatosis in the military is the number of diagnoses annually made. Because the total number of individuals with genotypic hemochromatosis can be reasonably estimated, elucidation of the current diagnoses can give an indication of “how we are doing”. The best estimate of current diagnoses of hemochromatosis comes from the Air Force medical genetics laboratory. This estimation of hemochromatosis diagnoses may be slightly lower than the actual number due to diagnoses made without genetic confirmation, confirmatory testing sent to a non-military lab, or individuals who have a form hemochromatosis but lack the genetic mutation. However, it is probable that the vast majority of Air Force hemochromatosis cases are included in this data set.

Another important aspect is the current trend of clinical requests for hemochromatosis analysis. An examination of clinical requests for hemochromatosis analysis can give insight into the type of clinician ordering the analysis, the type of

patient receiving the analysis, and current clinical predictors of hemochromatosis. It is known that most individuals with hereditary hemochromatosis are Caucasian, and more specifically of northern European descent. Therefore, if clinicians have a pool of patients that include individuals of northern European descent, the vast majority of clinical requests should be for individuals of northern European descent. Since the military has a representative mix of different ethnic groups, the military population should be ideal to evaluate if individuals of northern European descent are actually receiving the majority of analyses. If it is found that individuals of northern European descent are not overly represented, it could mean that clinicians are uninformed about the racial component of hereditary hemochromatosis.

In an ideal situation, there should be no major sex-based differences in diagnosed hemochromatosis cases. Due to the genetic nature of hemochromatosis, the actual percentage of diagnosed genotypic hemochromatosis should mirror the population (i.e. 50% male, 50% female). One would find a representative ratio if prospective genetic screening were occurring. However, in practice, some clinicians screen for hemochromatosis on the basis of symptomology. Screening using symptomology could slightly skew more requests to males as females have a delayed phenotypic expression of hemochromatosis. An evaluation of the gender of those for whom clinical requests for hemochromatosis analysis are made could aid in the understanding of diagnostic rationales.

One of the most effective ways to screen for individuals with a genetic disorder is to know if a family member is affected with the same disorder. In this way, diagnosis of one individual can lead to the subsequent diagnosis of many

others. An examination of the clinical requests for hemochromatosis can aid in the understanding of the importance that family history has on the diagnosis of hemochromatosis in the military. It is also useful to examine the gender of the affected family member when a patient indicates a family history of hemochromatosis. In theory, we should see a slightly higher number of male requests and male affected family members due to earlier clinical presentation. However, this number should not be excessive (i.e. 55-60%).

Many signs and symptoms have been reported to be possessed by individuals with hemochromatosis. To date, there has been little information regarding the clinical picture of individuals in the military with hemochromatosis. One of the required items on the Air Force medical genetics laboratory test requisition is clinical indication. This information should give a broad picture of the various signs and symptoms the affected military beneficiaries have. It will also be helpful to know which, if any, type of clinical indication is a better predictor of disease. In addition, there may be trends that may point to knowledge deficiencies of hemochromatosis in military clinicians.

The final area of examination is the influence that the size of the medical treatment facility has on the ultimate diagnosis of hemochromatosis. It is suspected that larger facilities, with their in-house specialists, may be more proficient in diagnosing hereditary hemochromatosis. An analysis of the requesting patterns for hemochromatosis analysis of large and small facilities should provide information that can be used to target their respective clinicians to aid in future diagnosis.

Methods

The methods used for this portion of research were approved by the Mississippi State University Institutional Review Board. The data for this objective came from all records obtained from the Air Force medical genetics laboratory located at Keesler Air Force Base Mississippi, for the years 2000, 2001, and the first half of 2002. The following data were obtained from this laboratory: sex, race, age, the clinical indication for genetic analysis provided by the requesting clinician (family history, lab results, etc...), base of origin, medical treatment facility (small clinic, medium hospital, or large medical center), clinic of origination, indication of family history, and the results of the genetic analysis. The genetic analysis included both major mutations of the HFE gene, the C282Y and H63D mutations. No identifying information was gathered. All information was entered into Access, an electronic database (Microsoft, Redmond Washington). Statistical analysis was performed using Number Cruncher Statistical System (NCSS, Kaysville Utah) and included descriptive statistics, chi square, and Fisher's exact test, and logistic regression.

Results

There were a total of 45 patients who were homozygous for the C282Y mutation during the 2.5-year period of evaluation. Approximately 77 percent of the requests for hemochromatosis genetic analysis, where the race was indicated by the requesting provider, were on patients classified as being of European (Caucasian) descent (Figure 3.1). By the total number and percentage of samples ordered,

Caucasians were not preferentially represented in the total caseload. Surprisingly, nearly twenty-five percent of referred cases did not include the race with the test requisition. In those samples where the race was not indicated with race being the dependent variable, logistic regression revealed that samples where the clinical indication was also not included in the test requisition were statistically significant (compared to those with a family history of hemochromatosis, $p \leq 0.003$, odds ratio 4.45). The sex of the patient, the facility size, and the type of specialist demonstrated no significant association. Of those identified as homozygous for the C282Y mutation, 95% were Caucasian, 2.5% native-American, and 2.5% black. Of those identified as heterozygous for the C282Y mutation, 93% were white, 4.5% black, and 2.5% Hispanic. Smaller facilities had a statistically non-significant ($p=0.08$) higher percentage of referrals of individuals of European descent.

There was a disproportionate percentage ($p \leq 0.001$) of referrals from males (Figure 3.2). Approximately 71 percent of the documented requests for hemochromatosis genetic analysis were performed on males. In those samples where the race was indicated as male, logistic regression using race as the dependent variable revealed samples with a clinical indication of abnormal lab results ($p \leq 0.001$, odds ratio 4.9), screening ($p \leq 0.05$, odds ratio 2.7), and clinical disease ($p \leq 0.02$, odds ratio 2.8), as statistically significant when compared to those with a family history of hemochromatosis. The sex of the patient, the facility size, and the type of specialist demonstrated no significant association. The overall percentage of females who were homozygous for the C282Y mutation was significantly higher (21.2%, $p=0.02$) when

compared to males (11.3%). The percentage of individuals of European descent homozygous for the C282Y mutation was significantly higher (22.1%, $p \leq 0.001$) when compared to all others (6.2%). The percentage of homozygous individuals whose referral came from specialists was significantly higher (18.0%, $p \leq 0.002$) when compared to those whose referral came from primary care providers (8.2%). An analysis of individuals homozygous for the C282Y mutation by logistic regression revealed several statistically significant independent variables. These variables included a sex of female (compared to male, $p \leq 0.05$, odds ratio 2.1), a race of other ($p \leq 0.02$, odds ratio 0.14) or unknown ($p \leq 0.04$, odds ratio 0.32) compared to European, and referral from primary care provider ($p \leq 0.02$, odds ratio 0.18) compared to specialist. The clinical indication and facility size were not significant. A total of 36 males and 29 females reported a family history of hemochromatosis.

In the period of evaluation, 309 samples were submitted for evaluation. A total of 66 samples had a family history of hemochromatosis. Of these 66, 18 were heterozygous for the C282Y mutation (27%), 16 were homozygous for the C282Y mutation (24%), 7 were heterozygous for the H63D mutation (10%), 5 were compound heterozygotes (8%), and 1 was homozygous for the H63D mutation (2%). 19 had no mutation (29%). Samples identified as homozygous for the C282Y mutation or heterozygous for both the C282Y and H63D mutation were classified as “positive”. The overall percentage of “positive” samples with a family history, as compared to those without a family history was statistically different ($p = 0.05$) when compared to all other samples. In those samples where the result was “positive”,

logistic regression using a positive result as the dependent variable revealed an unknown clinical indication (i.e. not included in the test requisition) as statistically significant (compared to those with a family history of hemochromatosis, $p \leq 0.01$, odds ratio 0.22) and other race ($p \leq 0.04$, odds ratio 0.21) and unknown race ($p \leq 0.03$, odds ratio 0.41) as statistically significant when compared to those of European descent. The facility size and the type of specialist demonstrated no significant association. There was a highly significant difference ($p = 0.0002$) in the percentage of females (33.7%) who indicated a family history of hemochromatosis when compared to males (16.9%). Logistic regression of those who indicated a family history of hemochromatosis revealed five statistically significant independent variables which included sex of female (compared to male, $p \leq 0.001$, odds ratio 2.96), other race (compared to European, $p \leq 0.004$, odds ratio 0.11), small medical facility (compared to large facility, $p \leq 0.03$, odds ratio 2.75), medium medical facility size (compared to large facility, $p \leq 0.04$, odds ratio 2.29), and primary care provider (compared to specialist, $p \leq 0.02$, odds ratio 2.67). When broken down into individual mutations, only C282Y homozygous individuals were statistically associated ($p = 0.017$) by a family history. When other variables are included in the logistic regression model, the C282Y mutation lost statistical significance ($p = 0.078$, odds ratio 2.07). As illustrated in Figure 3.3, there was no statistical difference in the percentage homozygous females with history (27.6%) and without history (19.2%). There was a statistical difference ($p = 0.03$) in the percentage of homozygous males with history (24.2%) and without history (9.1%). In males with a family history of

hemochromatosis, logistic regression using family history as the dependent variable, revealed C282Y homozygotes (compared to all others, $p \leq 0.02$, odds ratio 4.17), referral from a primary care provider (compared to specialist, $p \leq 0.003$, odds ratio 6.01) and referral from an unknown provider (compared to specialist, $p \leq 0.05$, odds ratio 3.42) as statistically significant. The facility size and race were not statistically different. There was no statistical difference in homozygous percentages between males and females who reported a family history. Females without a family history had a higher homozygous percentage when compared to males. This difference approached significance ($p = 0.055$). An analysis of the sex distribution of affected family members revealed a statistical significant difference ($p = 0.01$) in the proportion of males and females.

Table 3.1. lists the clinical indications for genetic analysis for hemochromatosis mutations. A total of 19 different clinical reasons, among them several disease states, were listed as rationale for testing. The most prominent reason, by group, is abnormal iron status testing followed by familial history of hemochromatosis (Figure 3.4). Table 3.2 lists the clinical indications and the corresponding genetic result for hemochromatosis mutations at Keesler AFB in 2000-2002. A familial history of hemochromatosis resulted in the most C282Y mutations, while abnormal laboratory findings resulted in the most "positive" results. Males were far more likely ($p = .003$) to have some type of abnormal iron status test when compared to females. Logistic regression using documented abnormal iron status results as the dependent variable revealed several statistically significant independent

variables including sex of female (compared to males, $p \leq 0.001$, odds ratio 0.33), referral from a primary care provider (compared to specialist, $p \leq 0.04$, odds ratio 0.41), and referral from a unknown provider (compared to specialist, $p \leq 0.006$, odds ratio 0.19). Logistic regression using specialist as the dependent variable revealed an indication of abnormal lab results (vs. family history, $p = 0.004$, odds ratio 3.50), and small facility (vs. large facility, $p \leq 0.001$, odds ratio 0.04) and medium (vs. large facility, $p \leq 0.004$, odds ratio 0.12) as statistically significant. There were no statistical sex based differences in specialists compared to sex.

Air Force medical treatment facilities can generally be divided into 3 groups: large (medical centers), medium (hospitals) and small (clinics). The distribution of referrals from each type of medical treatment facility is shown in Figure 3.5. In general, fewer referrals originate from primary care clinics at larger facilities (4%) compared to larger facilities (55%). Familial history of hemochromatosis is more important ($p = 0.0001$) for referrals at smaller facilities (33%) when compared to larger facilities. (12%). When the type of provider, gender, race, and sex are included as independent variables using logistic regression, the size of the medical treatment facility is statistically important for both small facilities (compared to large, $p \leq 0.04$, odds ratio 2.57) and medium facilities (compared to large, $p \leq 0.05$, odds ratio 2.27). In addition, for those with a family history of hemochromatosis, logistic regression revealed referrals from primary care providers (compared to specialist, $p \leq 0.01$, odds ratio 3.10) as statistically different. There was no statistical difference in the number of “positive” samples from smaller facilities (23%) when compared to larger facilities

(25%). A summary of the univariable and multivariable analyses can be found in Tables 3.3 – 3.7.

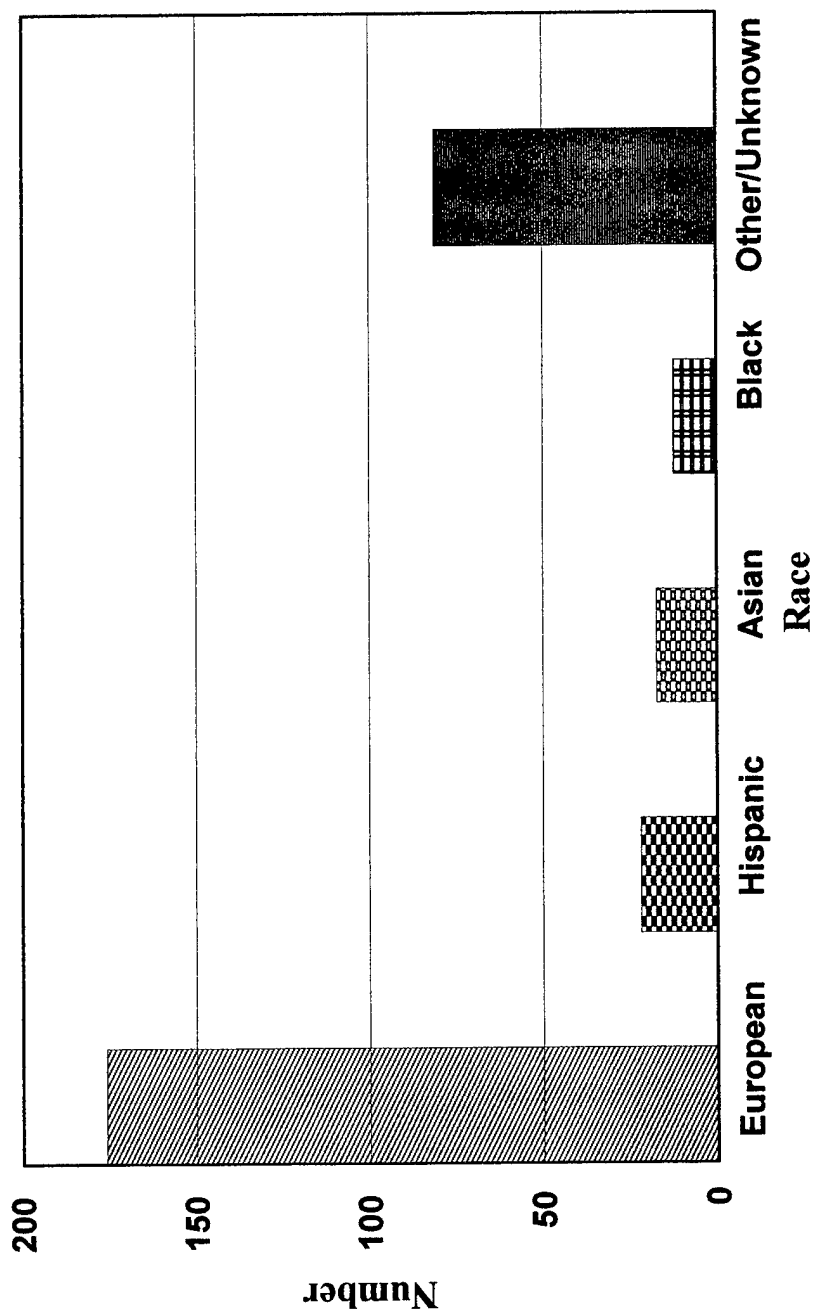


Figure 3.1: Total number of hemochromatosis genetic profiles requested 2000-2002, Keesler AFB, Mississippi

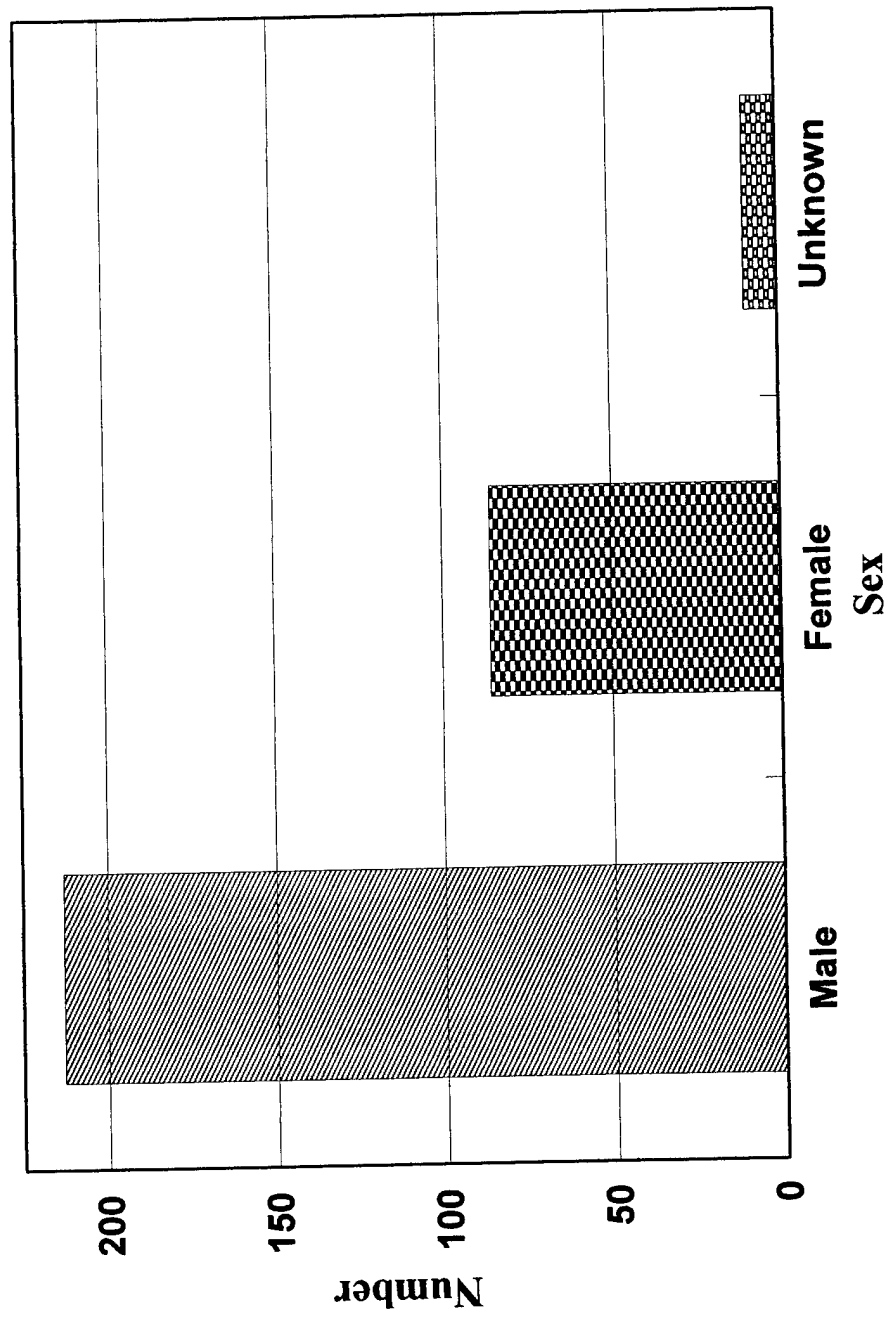


Fig 3.2: Total number of hemochromatosis genetic profiles from male and female beneficiaries 2000-2002, Keesler AFB, Mississippi

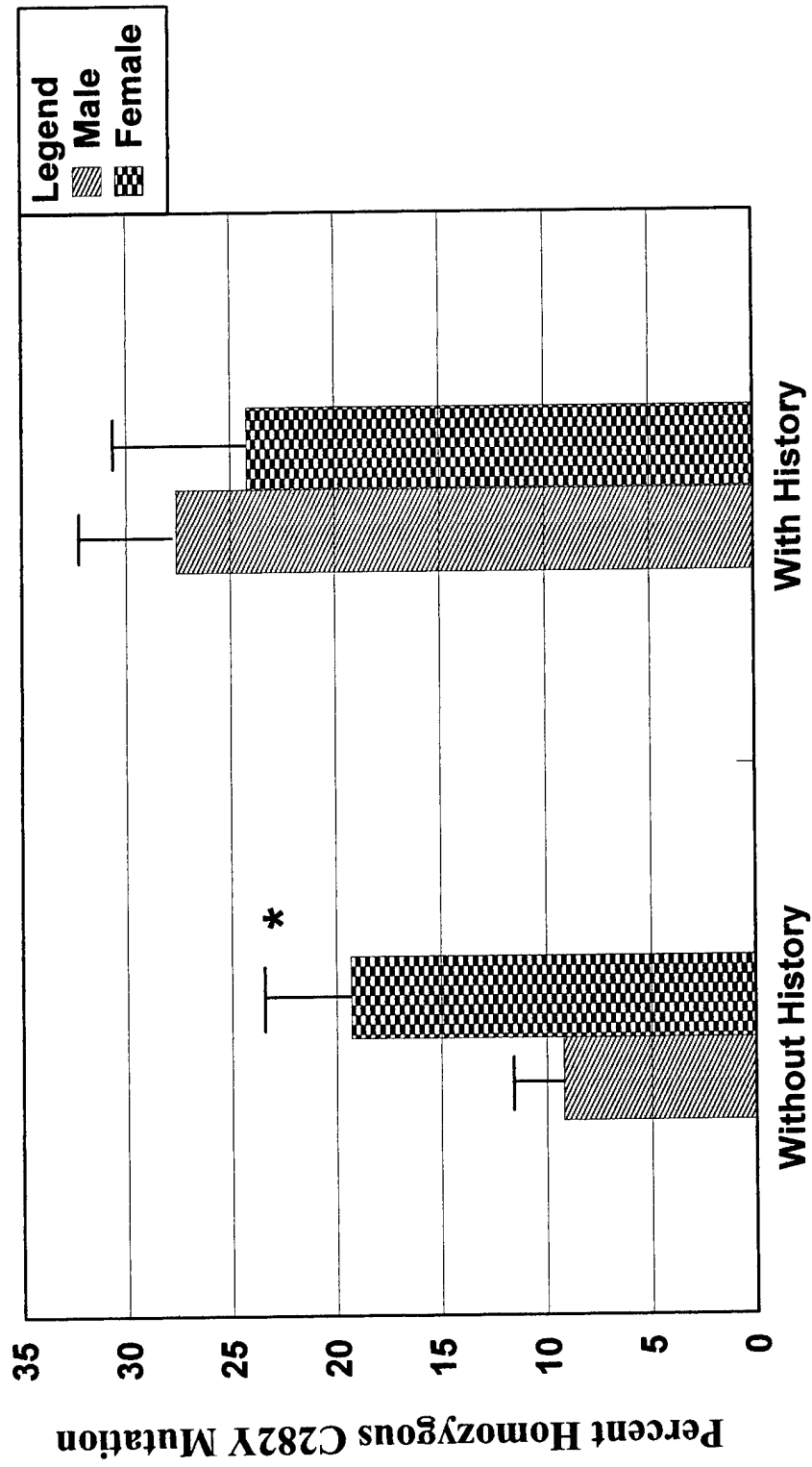


Fig 3.3: Total percentage of male and female homozygous C282Y patients with and without family history of hemochromatosis

Table 3.1: Clinical indications for genetic analysis

Non Specific Reasons	Number	Specific Medical Conditions	Number
Family History	66	Hepatitis	7
Abnormal Ferritin	64	Hemochromatosis	6
Abnormal Transferrin Saturation	29	Diabetes Mellitus	5
Rule Out Hemochromatosis	20	Skeletal Dysplasia	1
Liver Damage (Elevated Liver Function Test)	16	Atria Enlargement	1
Iron (Non Specific)	10	Arthropathy	1
Liver Biopsy	10	Gulf War Syndrome	1
Suspected	4	Budd-Chiari Syndrome	1
Hemoglobin	1	Chronic Pain	1
Screening	1		

Table 3.2: Clinical indications vs.genetic result for hemochromatosis mutations

Indication	C282Y +	H63D +	Compound Heterozygote	Wild Type
History	16	1	5	44
Abnormal Labs	12	5	8	77
Screening	3	1	3	28
Disease	11	3	3	47
Unknown	3	1	1	36

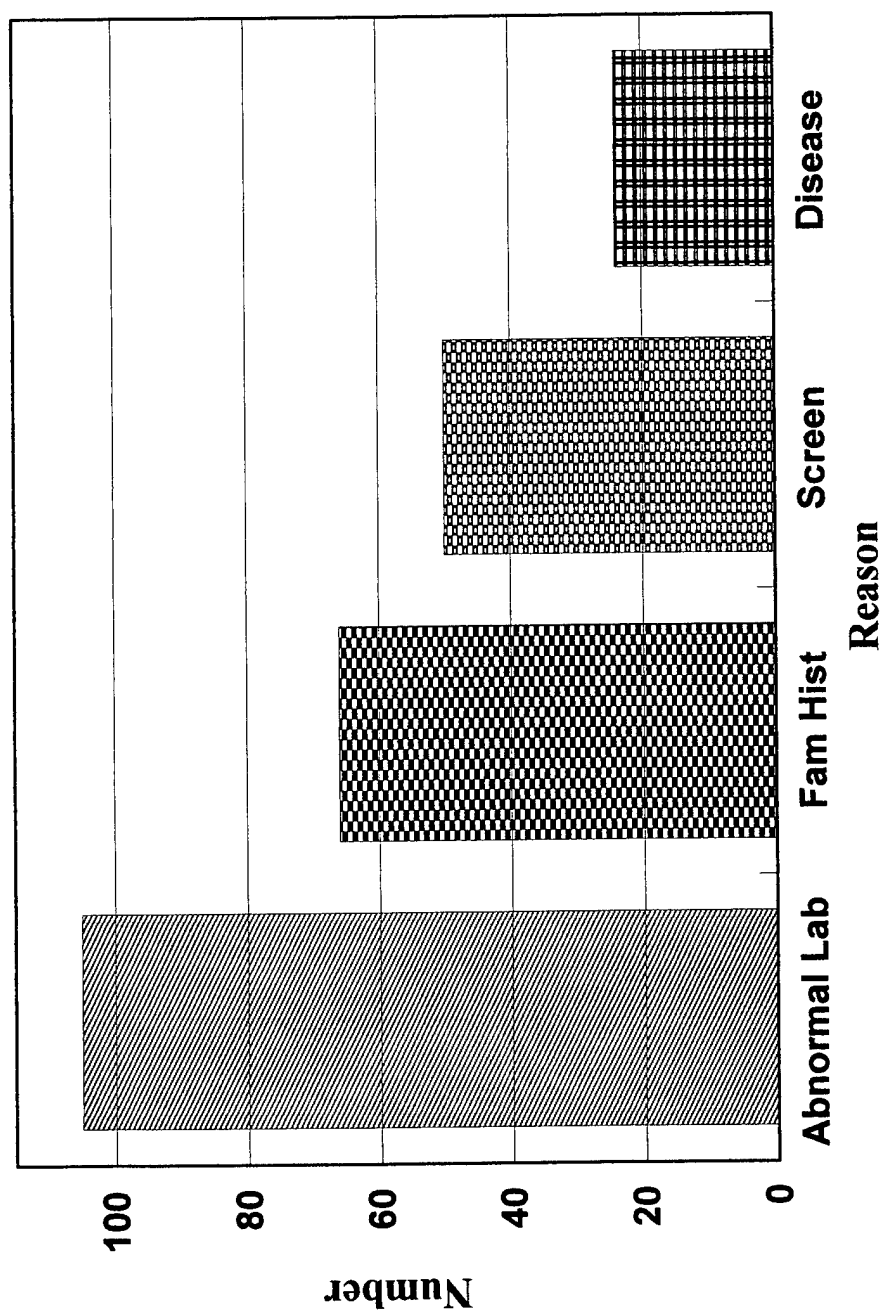
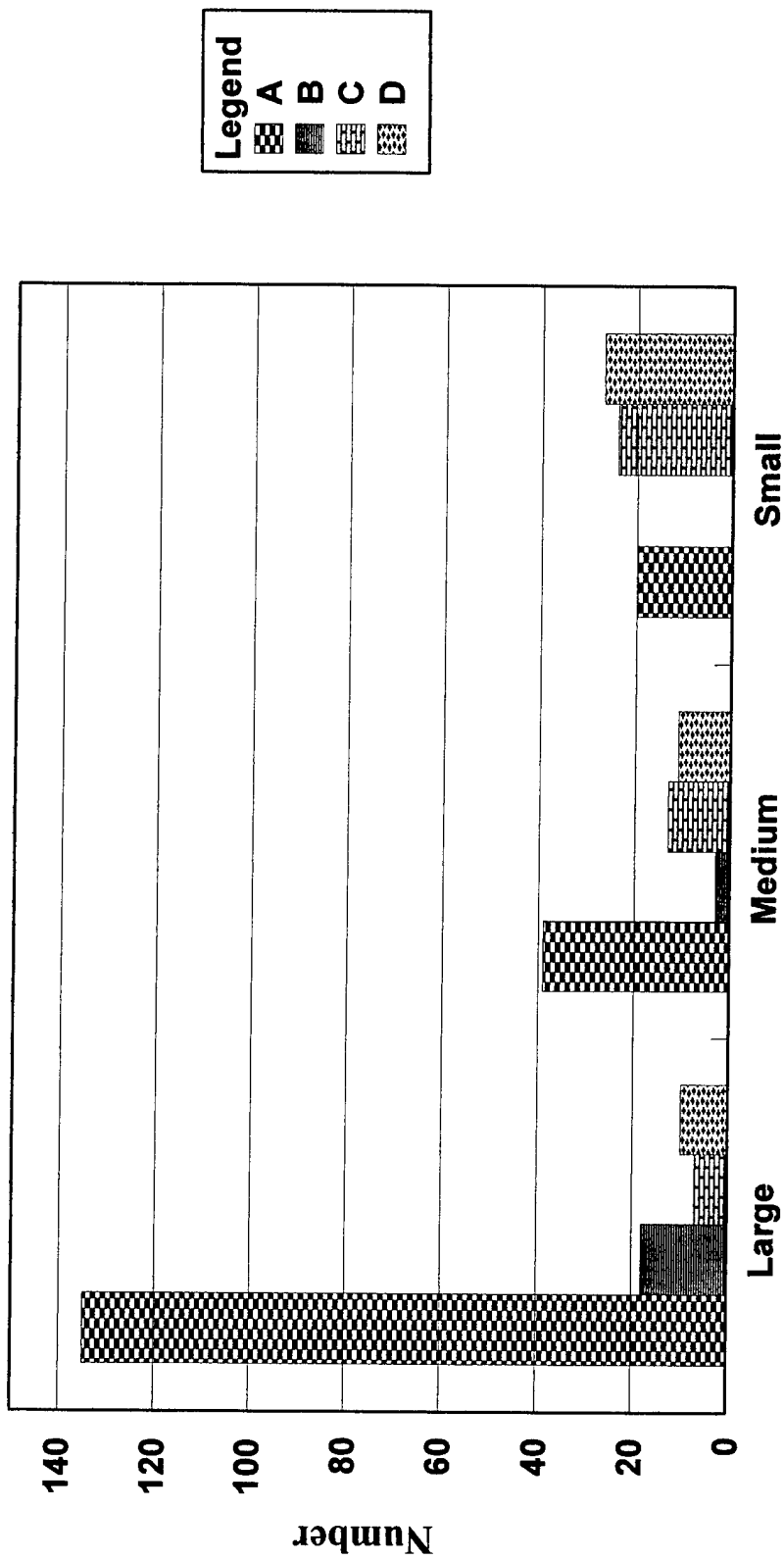


Fig 3.4: Clinical indications of genetic testing for hemochromatosis



A=gastroenterology, internal medicine, and hematology/oncology B =other specialty clinics C=primary care D=unknown
 Fig 3.5: Referring clinics of genetic testing for hemochromatosis

Table 3.3: Univariable Analysis of Independent Variables

Dependent Variable	Independent Variable	Pr > Likelihood Ratio ChiSq
C282Y mutation	Sex -Male	0.0200
C282Y mutation	Race - Caucasian	0.0003
C282Y mutation	Race – other	0.0142
C282Y mutation	Race – unknown	0.0488
C282Y mutation	Indication - Family History	0.0082
C282Y mutation	Indication – Screen	0.5119
C282Y mutation	Indication – Lab	0.3518
C282Y mutation	Indication – Specific Disease	0.5471
C282Y mutation	Indication – Unknown	0.1605
C282Y mutation	Facility – Small	0.6522
C282Y mutation	Facility –Medium	0.2745
C282Y mutation	Facility -Large	0.7736
C282Y mutation	Primary Care Provider	0.1505
C282Y mutation	Unknown Provider	0.1663
C282Y mutation	Specialist Provider	0.0265

Table 3.4: Multivariable analysis of independent variables - I

Data Set	All data		
Dependent Variable	Race Included		
Independent Variable	Level	Odds Ratio	p > z
C282Y	Homozygous vs Other	0.41	0.10
Sex	Female vs Male	0.70	0.30
Indication	Abnormal Lab vs Family History	0.60	0.26
Indication	Screening vs Family History	0.82	0.73
Indication	Unknown vs Family History	4.45	< 0.01
Indication	Specific Disease vs Family History	0.81	0.66
Facility Size	Small vs. Large	1.86	0.13
Facility Size	Medium vs. Large	0.71	0.43
Provider Type	Primary Care vs Specialist	1.76	0.19
Provider Type	Unknown vs Specialist	0.78	0.58

Data Set	All data		
Dependent Variable	Sex - Male		
Independent Variable	Level	Odds Ratio	p > z
Race	Other vs Caucasian	0.76	0.46
Indication	Abnormal Lab vs Family History	4.86	< 0.01
Indication	Screening vs Family History	2.67	0.05
Indication	Unknown vs Family History	1.35	0.49
Indication	Specific Disease vs Family History	2.78	0.01
Facility Size	Small vs. Large	1.28	0.55
Facility Size	Medium vs. Large	0.74	0.42
Provider Type	Primary Care vs Specialist	1.05	0.91
Provider Type	Unknown vs Specialist	1.22	0.65

Table 3.5: Multivariable analysis of independent variables - II

Data Set	All data		
Dependent Variable	C282Y Homozygous Mutation		
Independent Variable	Level	Odds Ratio	p > z
Sex	Female vs Male	2.13	0.04
Race	Other vs Caucasian	0.15	0.01
Race	Unknown vs Caucasian	0.32	0.03
Indication	Abnormal Lab vs Family History	0.41	0.07
Indication	Screening vs Family History	0.27	0.07
Indication	Unknown vs Family History	0.26	0.11
Indication	Specific Disease vs Family History	0.70	0.47
Facility Size	Small vs. Large	1.95	0.21
Facility Size	Medium vs. Large	1.18	0.72
Provider Type	Primary Care vs Specialist	0.18	0.01
Provider Type	Unknown vs Specialist	0.30	0.07

Data Set	All data		
Dependent Variable	Homozygous/Compound Mutation		
Independent Variable	Level	Odds Ratio	p > z
Sex	Female vs Male	1.50	0.21
Race	Other vs Caucasian	0.21	0.01
Race	Unknown vs Caucasian	0.41	0.31
Indication	Abnormal Lab vs Family History	0.61	0.23
Indication	Screening vs Family History	0.50	0.22
Indication	Unknown vs Family History	0.22	0.03
Indication	Specific Disease vs Family History	0.69	0.40
Facility Size	Small vs. Large	0.97	0.95
Facility Size	Medium vs. Large	0.95	0.90
Provider Type	Primary Care vs Specialist	0.60	0.30
Provider Type	Unknown vs Specialist	0.91	0.84

Table 3.6: Multivariable analysis of independent variables - III

Data Set	All data		
Dependent Variable	Indication – Family History		
Independent Variable	Level	Odds Ratio	p > z
Sex	Female vs Male	2.96	< 0.01
Race	Other vs Caucasian	0.11	< 0.01
Race	Unknown vs Caucasian	0.62	0.19
Facility Size	Small vs. Large	2.57	0.04
Facility Size	Medium vs. Large	2.27	0.05
Provider Type	Primary Care vs Specialist	2.68	0.02
Provider Type	Unknown vs Specialist	1.06	0.90
C282Y Homozygous	Homozygous vs. Negative	2.07	0.08
H63D Homozygous	Homozygous vs. Negative	0.21	0.20
Compound Heterozygote	Compound Heterozygote vs Neg	1.28	0.69

Data Set	Male only data set		
Dependent Variable	Indication – Family History		
Independent Variable	Level	Odds Ratio	p > z
C282Y Mutation	Homozygous vs Other	4.17	0.01
Race	Other vs Caucasian	0.15	0.08
Race	Unknown vs Caucasian	0.66	0.38
Facility Size	Small vs. Large	1.44	0.52
Facility Size	Medium vs. Large	1.41	0.54
Provider Type	Primary Care vs Specialist	6.02	< 0.01
Provider Type	Unknown vs Specialist	3.43	0.04

Data Set	All data		
Dependent Variable	Indication – Abnormal Lab		
Independent Variable	Level	Odds Ratio	p > z
Sex	Female vs Male	0.33	< 0.01
Race	Other vs Caucasian	1.13	0.72
Race	Unknown vs Caucasian	0.59	0.11
Facility Size	Small vs. Large	1.79	0.13
Facility Size	Medium vs. Large	1.28	0.50
Provider Type	Primary Care vs Specialist	0.41	0.04
Provider Type	Unknown vs Specialist	0.29	0.01

Table 3.7: Multivariable analysis of independent variables - IV

Data Set	All data		
Dependent Variable	Provider - Specialist		
Independent Variable	Level	Odds Ratio	p > z
Sex	Female vs Male	1.06	0.87
Race	Other vs Caucasian	1.30	0.58
Race	Unknown vs Caucasian	0.86	0.68
Indication	Abnormal Lab vs Family History	3.50	0.004
Indication	Screening vs Family History	3.30	0.05
Indication	Unknown vs Family History	0.44	0.13
Indication	Specific Disease vs Family History	1.53	0.37
Facility Size	Small vs. Large	0.04	≤ 0.001
Facility Size	Medium vs. Large	0.12	≤ 0.001

Discussion

The total number of patients diagnosed with hemochromatosis during the period of evaluation (45 total or 18/year) is very low considering the total number of probable individuals with genotypic hemochromatosis. It is possible the number of individuals diagnosed may be slightly higher due to use of laboratories other than the Air Force medical genetics laboratory. However, it is expected that most Air Force labs use this service because of the cost involved (free to the requester), reputation, and marketing of the lab. The small number of individuals diagnosed with genotypic hemochromatosis represents only a small fraction of those affected and underscores the need for better screening and prevention.

Because hemochromatosis is found primarily in individuals with northern European ancestry, it would seem logical that most individuals screened would be classified as Caucasian. Not all Caucasians are of northern European descent, but most individuals who are of Northern European descent are Caucasian. The military is often described as the "ultimate melting pot". While the majority of clinical requests are for individuals of European ancestry, the actual percentage tested is not unlike the active duty force composition. It is somewhat surprising that the percentage of specimens from individuals of african-americans, asian, or hispanic descent mirror the active duty force composition. This would suggest that Air Force clinicians are not using race as a selective criterion when requesting genetic analysis for hemochromatosis. It is also noteworthy that there were many requests that listed "other/unknown" or left the race blank on the requisition. This could be caused by a variety of reasons. First, it may be true that the clinician did not know the race of the patient. However, since every military

member/dependent must classify themselves in some manner, this could possibly be a failure of the clinician to ask. It could also represent individuals who are of mixed race. This could represent clinicians that are unaware of the important link between race and hereditary hemochromatosis. Logistic regression revealed an unknown clinical indication as an important independent variable for those samples with no race indicated. This may indicate carelessness by the part of the requesting provider if both race and clinical indication are not included in the request. Ultimately, the vast majority of C282Y mutations, both homozygous and heterozygous, were identified in Caucasians. This suggests that targeted screening may be most effective in a Caucasian population. It is somewhat surprising that the smaller medical treatment facilities had a higher percentage of Caucasian referrals. This could indicate that clinicians at these facilities were more appropriately screening. While some "non-Caucasians" should be analyzed, there should be more Caucasians analyzed, percentage wise, if the population is drawn from mixed-ethnicity.

There were 43 samples that were homozygous for the C282Y mutation where sex was determinable. Fifty-six percent were from males and forty-four percent were from females. However, the total number of males screened was almost 2.5 times the number of females screened. On the surface, the disproportionate percentage of males tested is not unexpected since males develop symptoms at an earlier age and there are more males on active duty. On the other hand, this viewpoint does not consider the increasing female percentage of the active duty force, the large number of dependent females, and the fact that women tend to outlive men. The actual percentage of males eligible for medical care is more evenly balanced than most realize. For example, in 2002, 47 percent of eligible

beneficiaries at Columbus AFB, MS were female. Therefore, one might expect only a slightly higher number of males screened. In addition, the biochemical elevation of iron status is somewhat delayed in females due to menstruation. However, this delay in iron status elevation should be counter-balanced by more females being screened for iron deficiency (Chapter II), thus revealing abnormalities. However, abnormal iron status seems to play a far less significant role in patient referral of females. Specialists are more likely to document iron status testing. However, the proportion of males seen by specialists is not statistically significant when compared to females. Since many more men are referred for genetic analysis and those women who are referred are not likely to have abnormal iron status lab results strongly suggest that many genetic analyses of females are requested based on symptomology. Symptomology has not been shown to be a reliable indicator for hemochromatosis due to the variable nature of symptoms that the disorder produces. In addition, one might suspect that the percentage of females screened might be higher at smaller facilities where there are fewer specialists. However, this is not the case. These data indicate that females may be at a disadvantage in some ways in regard to genetic screening for hemochromatosis.

Seventy one percent of samples from individuals who had a self-reported family history of hemochromatosis had at least one mutation. Twenty four percent of samples were homozygous for the C282Y mutation. When compared to samples without a family history of hemochromatosis, samples with a family history were dramatically more likely to possess the C282Y mutation. Family history had little effect on diagnosis with other mutations. The importance of family history magnifies the necessity of patients reporting a family history and conversely clinicians asking patients if they have a history. It can be

argued that family history should be a requirement on patient intake questionnaires. One very unexpected finding was the highly significant difference in the percentage of females who reported a family history of hemochromatosis. The difference in the total number who indicated a family history, which is reflective of the greater number of males screened, is not as great (male 55%, female 45%) and not vastly different from the population of interest. This leads to some very interesting questions. Are males less likely to know if they have a family history? Are males less informed about the genetic nature of the disorder? Do females volunteer this information? Do clinicians neglect to ask this information from men? These are questions that can not be answered from the data collected in this project. However, these types of questions need to be addressed if a screening program is implemented.

Further comparison of homozygous males and females revealed interesting characteristics. Since family history is statistically significant for C282Y mutations and females are more likely to report family history, it may be expected that family history may impact the higher C282Y homozygous rate for females. However, there is no statistical difference in C282Y homozygous rates for females with or without family history. However, in males, the difference is significant with family history playing an important role. In addition, examination of homozygous rates for those who did not report a history found a homozygous rate more than double for females. This rate approached statistical significance. Therefore, the higher homozygous rate in females does not seem to be due to reporting family history. There may be some other factor(s) involved in females causing them to have a higher homozygous rate. Logistic regression did not reveal this factor. Although far fewer males report a family history, it is an important

factor in homozygous males. Another interesting finding is that there is almost a 2:1 ratio of males to females of affected family members (i.e. the family member with hemochromatosis who caused the patient to seek treatment). This ratio may be expected to be slightly higher in males due to the earlier presentation of the disorder. However, this dramatic ratio further validates the idea that females are not diagnosed at appropriate rates.

A better understanding of the rationale for individuals referred for genetic testing could assist in providing information for military clinicians. It is not surprising that abnormal lab results and familial history constitute the majority of referrals for mutation analysis. This is valuable because both of these are "potential point of entries" available to clinicians at most military medical treatment facilities. Therefore, proper diagnosis should in theory be available to patients at all military medical treatment facilities if clinicians are properly educated about hemochromatosis. Recognition of the wide variety of clinical disease states associated with requests for genetic analysis is also useful. Of the 18 samples in which a specific disease state was listed with no mention of family history or abnormal iron status, only one was homozygous for the C282Y mutation. Interestingly, the suspected condition for this patient was "Gulf-War Syndrome". Symptomology should not be used alone when screening for hemochromatosis.

There is large variation in the type of medical care provided "on-base" in the United States Air Force. Bases with medical centers will have many specialists, hospitals will have some, and clinics will have few. Therefore, it is of use to examine the distribution of referrals for genetic testing from the various size medical treatment facilities. From large medical facilities, the largest number of referrals is from specialized departments such as internal medicine, gastroenterology, and hematology/oncology.

primary care (i.e., family practice) is a larger contributor at smaller facilities. At the larger facilities, this does not necessarily mean that screening is occurring infrequently during primary care appointments. Rather, it could be that these patients are referred "in-house" to specialists after presumptive diagnosis. It does, however, bring up the possibility that screening issues may need to be addressed in primary care clinics in larger facilities. One area that can be examined is the impact of familial history of hemochromatosis for screening at both the large and small medical treatment facilities. Nearly one-third of all referrals for genetic analysis at small facilities includes a familial history of the disorder. This is statistically different from larger facilities where only about one of eight have such a familial history. Again, this strongly suggests that clinical symptomology is more important at the larger facilities. The smaller percentage of familial history could be due to the robust diagnostic resources available at larger facilities. However, the total number of "positive" samples is not statistically different when comparing genetic analysis between small and large facilities. This suggests that identification of affected individuals may be accomplished at both small and large facilities. Improvement could be targeted at seemingly underutilized screening strategies such as clinical observation at smaller facilities and intake of family history at larger facilities.

CHAPTER IV

ANALYSIS OF SCREENING TECHNIQUES THAT MAY ALLOW FUTURE PROSPECTIVE SCREENING FOR HEMOCHROMATOSIS

Introduction

A practical demonstration of mass screening for hemochromatosis has yet to be accomplished in a military setting. Few of the estimated five to fifteen thousand Air Force beneficiaries with this disorder have been identified. The primary reason for this fact has been a failure to screen. Therefore, the primary significance of this chapter is to demonstrate the potential of effective screening for hemochromatosis, which could result in saving thousands of lives.

Subservient to the primary goal of potentially saving lives is the development of practical methods that may be useful in screening for hemochromatosis in a military setting. These screening methods need to be user-friendly for the primary care provider and communicated to the clinicians in such a way that they will manifest themselves in everyday clinical practice.

In any type of screening procedure, reference ranges appropriate for the population need to be determined. This is very important because percentiles, derived from the reference ranges, can be used in the development of novel algorithms for screening. These reference ranges can also be broken up into various sub-groups if statistical analysis reveal significant differences between sub-groups.

It would be beneficial if commonly ordered laboratory tests could be used as surrogate markers for a portion of hemochromatosis cases. Specifically, liver enzymes and indices from the complete blood count are potentially promising. These are common laboratory tests available at each medical treatment facility. It has long been known that liver is one the primary accumulators of excessive iron in the body.² This can cause an elevation in liver enzymes. However, there are numerous diseases and conditions that can cause an elevation of enzymes.¹⁰⁷ Careful attention must be paid to such results to ascertain the potential causes.¹⁰⁷ One study demonstrated that over three percent of individuals with elevated activity of serum liver enzymes were shown to have hemochromatosis.¹⁰⁸ This represents a six-fold increase in risk for hemochromatosis as compared to normal enzyme levels. It must be noted that some report that up to 35 percent of individuals with hemochromatosis do not have elevated liver enzymes.¹⁰⁹ However, most physicians ignore elevated liver enzymes as a possible indication of hereditary hemochromatosis despite the increased risk.¹¹⁰ Some of this ignorance may be due to variation in reference ranges between clinical laboratories. The reference range of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), like many enzymes, is variable according to the methodology used.¹⁰⁷

Recently, certain red blood cell indices have been suggested to aid in the identification of individuals with hemochromatosis.¹¹¹ In particular, mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), hemoglobin concentration, and hematocrit have been studied and found to be significantly different in individuals with hemochromatosis.¹⁰³ The MCV calculation is based on the red blood cell count and hematocrit, the MCH calculation is based on the red blood cell count and hemoglobin, and the MCHC is based on the red blood cell count, hematocrit, and hemoglobin. It has long been established that these indices could be of use in the diagnosis of individuals with anemia, including iron deficiency anemia.¹¹² The significance of possibly using these indices in a military setting to aid in the diagnosis of hemochromatosis is one that warrants investigation. More patients receive a complete blood count than any other test parameter that has been mentioned in conjunction with iron overload screening. If determined to be useful, it would open the door to screening for hemochromatosis as many health care providers already use the complete blood count in every day clinical practice. To date, complete blood count indices have not been used to evaluate a large scale-screening program.

The laboratory tests with the longest history with respect to hemochromatosis are the traditional iron status tests, which include total iron, total iron binding capacity, calculated transferrin saturation, and ferritin. These tests have been extensively studied in non-military settings. Although dramatic differences may not be found in military personnel, there are unique aspects of the military lifestyle and culture that may influence the use of these traditional tests. First, the military offers an environment where few individuals are nutritionally lacking. Diets that are low in bioavailable iron is one of the

causes of iron deficiency.¹¹³ Conversely, this diet may also affect hemochromatosis patients. In the United States, most individuals with iron deficiency are women of childbearing age and infants.¹¹³ Iron deficiency is less common among adult men.¹¹³ However, while it is expected that rate of iron deficiency in the military setting will be lower (i.e., middle class population, frequent health visits, nutrition education) than seen in the general public, these same facts make it possible that there may be a higher "reference" range in the general military population. This could invalidate traditional "cut-off" values that are commonly used for screening. Another factor that may influence the military, and counter act the aforementioned rationale, is increased blood loss due to volunteer blood donations. The military is known for its volunteer spirit. This spirit is also seen when the call for blood donations is made. For example, in Washington D.C., the American Red Cross receives approximately ten percent of its blood donations from military bases/posts. This is despite the fact that there are two military-only blood donation centers located on the military installations. This type of donation pattern is seen throughout the military both in military and civilian blood donation centers. While one time donations have little effect on individuals with hemochromatosis, this is not the case with individuals with "normal" iron status.¹¹⁴ Repeated donations over a period of time by the military population could decrease reference ranges. There are also many unknown variables that could also play a role in shaping iron status in the military population. It is also possible the military environment, with the before-mentioned qualities, may also affect phenotypic expression in heterozygotes.

There are several other important research questions that this project may help answer. One of these questions is identification of specific patterns in identifying

individuals heterozygous for the C282Y mutation that have significant iron overload. It has been documented that a small percentage of heterozygotes may develop significant iron overload and most will have elevated iron stores.² These individuals will not be identified without biochemical testing. In addition, the chance of one of the parents of a heterozygote being a homozygote is one in ten. This may lead to increased awareness and testing among family members.

Another interesting analysis is the incidence of mutant HFE alleles in the non-Caucasian military population. Although the common mutations are sometimes seen in non-Caucasian populations, it is at a much lower rate. In the future, it could be possible to estimate the number of homozygous non-Caucasian individuals based on the number of mutant HFE alleles in the different ethnic groups in the military.

In addition, it is worth considering the corollary benefits of identifying individuals with iron deficiency anemia in a hemochromatosis-screening program. Due to the significant number of women of childbearing age that would be included in hemochromatosis screening programs, it would be expected that many women would be identified with iron deficiency anemia. In addition, communication with uninformed clinicians about the benefits of a hemochromatosis-screening program could be aided with the inclusion of the well-accepted benefits of iron deficiency screening.

Methods

The methods used for this portion of research were approved by the Mississippi State University and Keesler Air Force Base Institutional Review Board.

Ethylenediaminetetraacetic Acid (EDTA) preserved blood and serum samples, previously

collected for clinical analysis, were obtained from 2,341 presumably healthy individuals. These individuals were patients at a large military medical center who had both a lipid profile (serum sample collected in a eight mL evacuated blood collection tube with clot activator and double gel for transport, Becton Dickinson, Franklin Lakes, New Jersey) and complete blood count (whole blood sample collected in a 5 mL evacuated blood collection tube with the anticoagulant potassium ethylenediaminetetraacetic acid, Becton Dickinson, Franklin Lakes, New Jersey) collected at the same time. The samples for analysis were identified by running a "pending report" from the hospital information system (Composite Healthcare Information System, San Diego, California). A "pending report" is a specialized query that allows the determination of which samples were requested on a particular day given a set of circumstances (e.g., "all individuals who received both a lipid panel and complete blood count on March 20, 2003").

Each of the identified samples had corresponding red blood cell indices entered onto a logsheet. These indices include mean corpuscular hemoglobin concentration, mean corpuscular volume, mean corpuscular hemoglobin concentration, and total red blood cell count. These indices were obtained from the clinical sample that was performed on a MAXM hematology analyzer (Beckman-Coulter, Inc., Fullerton, California).

Both the left over serum from the lipid profile and the whole blood from the complete blood count were taken from the clinical laboratory to the clinical research laboratory. At this point, the samples were still identifiable. Each sample was given to a coder. The coder assigned each sample a new number that was associated with the sample throughout the project. In addition, demographic information from each sample was obtained from the hospital information system. The information entered into the computer

system consisted of sex, age, race, and duty status. After the demographic information was collected, all information that could possibly link the patient with the sample was destroyed. In summary, before any additional analysis was undertaken, all linkable data was destroyed. The information available at this point was the new identifier (non-linkable to original sample), demographic information, and selected components of the complete blood count originally performed on the whole blood sample.

Next, deoxyribonucleic acid (DNA) was isolated from whole blood. This step was completed within two weeks of initial collection of the blood sample. Isolation of DNA was accomplished using a Puregene DNA isolation kit (Gentra Systems, Minneapolis, Minnesota). Briefly, one milliliter of whole blood was added to three milliliters of red blood cell lysis solution. After a ten-minute incubation, the sample was centrifuged at $2,000 \times g$ for 10 minutes and the supernatant was removed. A small pellet remained. After vortexing the pellet, one milliliter of cell lysis solution was added to the resuspended pellet. Next, 333 microliters of protein precipitation solution was added to the cell lysate and this mixture was centrifuged at $2,000 \times g$ for ten minutes. The supernatant of this process contained the DNA. This supernatant was transferred to a tube containing one milliliter of isopropanol. This mixture was centrifuged at $2,000 \times g$ for three minutes. The DNA was now visible as a white pellet. The supernatant was poured off and the DNA was washed in one milliliter of 70 percent ethanol. This mixture was centrifuged at $2,000 \times g$ for one minute. The ethanol supernatant was poured off and replaced with 100 microliters of DNA hydration solution which left a concentration of approximately 30 micrograms of DNA. The isolated DNA samples were stored at two to eight degrees centigrade.

After DNA isolation, the corresponding serum sample was tested for iron status. Iron status tests include total iron, total iron binding capacity, and transferrin saturation. As previously mentioned, all serum samples in this project were obtained from fasting individuals (lipid profile). This is important because total iron can be falsely elevated by non-fasting specimens. All iron status tests were performed on an Express 550 spectrophotometric chemistry analyzer (Ciba Corning, East Walpole Massachusetts). All iron status testing was analyzed in conjunction with appropriate control material (QCS1 and QCS2, Ciba Corning, East Walpole Massachusetts) and standards (multi-cal 1 and multi-cal 2, Ciba Corning, East Walpole Massachusetts). Reagent grade water was used as the blank in all reactions. The iron assay is a modification of the method reported by Persing using the chromogenic compound ferrozine.¹¹⁵ Neocuproine is also used to prevent copper interference. The serum iron bound to transferrin is reduced to the ferrous form by hydroxylamine in an acidic medium. This iron then reacts with ferrozine to form a violet color, which absorbs light at 560 nm. Total iron binding capacity (TIBC) is measured indirectly by measuring unsaturated iron binding capacity (UIBC). This technique involves the addition of excess ferrous ions in a slightly alkaline medium. The ferrous ion will bind specifically to transferrin at iron binding sites. After all the unsaturated iron-binding sites have been saturated with ferrous ions, the remaining unbound ferrous ion is measured by reacting with Ferrozine. The UIBC is the difference in iron concentration between the known excess and that obtained in the assay. Therefore, the TIBC is the sum of total iron plus UIBC. Transferrin saturation is calculated by dividing the total iron by the TIBC.

Liver enzyme analysis consisted of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) determinations. All liver enzyme analysis was performed on an Express 550 spectrophotometric analyzer (Ciba Corning, East Walpole Massachusetts). All testing was analyzed in conjunction with appropriate control material (QCS1 and QCS2, Ciba Corning, East Walpole Massachusetts) and standards (multi-cal 1 and multi-cal 2, Ciba Corning, East Wamplpole Massachusetts). The ALT and AST assays are based on a standardized procedure. Briefly, AST catalyzes the transfer of the amino group from L-aspartate to 2-oxoglutarate resulting in the formation of oxaloacetate and L-glutamate. Malate dehydrogenase (MDH) catalyzes the reduction of oxaloacetate with the simultaneous oxidation of NADH to NAD. The rate of NAD formation, indicated by the rate of decrease in absorbency at 340 nm, is directly proportional to AST activity. ALT catalyzes the transfer of the amino group from L-alanine to 2-oxoglutarate resulting in the formation of pyruvate and L-glutamate. Lactate dehydrogenase (LDH) catalyzes the reduction of pyruvate with the simultaneous oxidation of NADH to NAD. The rate of NAD formation, indicated by rate of decrease in absorbency at 340 nm, is directly proportional to the ALT activity. Hemolyzed samples can falsely elevate results of both ALT and AST assays. Therefore, hemolyzed samples were not included in this project.

Serum samples with elevated transferrin saturation were analyzed for ferritin. All ferritin analysis was performed on an AxSYM chemistry system (Abbott Laboratories, Abbott Park, Illinois). The ferritin assay is based on the microparticle enzyme immunoassay (MEIA) technology. In this method, the ferritin in the sample is incubated in a reaction cell with diluent, anti-ferritin alkaline phosphatase (ALP) conjugate, and antiferritin coated microparticles (mouse, monoclonal). An ALP-antibody-antigen-

antibody:microparticle complex is formed. An aliquot of this complex is transferred to the glass fiber matrix to which the microparticles bind irreversibly. The matrix is then washed to remove unbound materials, and the substrate 4-methylumbelliferyl phosphate is added to the matrix and the fluorescent product is measured by the MEIA optical assembly. Appropriate calibrators (AxSYM master calibrator, Abbott Laboratories, Abbott Park, Illinois) and control samples (Biorad Immunology TDM level 1 and 2, Hercules, California) are used in conjunction with all analysis.

Determination of genetic mutations in the DNA samples was determined by polymerase chain reaction (PCR). Two mutations were screened for in selected samples. These are the C282Y and H63D mutations. This analysis was accomplished in accordance with laboratory operating instruction 160-137 that was furnished by the Air Force Medical Genetics Flight at Keesler AFB.¹¹⁶ Briefly, genomic DNA from the previously mentioned sample was amplified using approximately 200 ng of DNA in 30 μ l reaction volumes. After amplification, specific restriction endonucleases (Rsa I for C282Y and MBO I for H63D, Life Technologies, Chicago, Illinois) were used to digest the amplified DNA. This sample is then run on an agarose electrophoresis system at 100 volts for 30 minutes. After the cut DNA has been electrophoretically separated, it is visualized under an ultraviolet transilluminator. For the C282Y mutation, the wild type fragment will have an expected size of 140 base pairs and the mutant fragment will have an expected size of 111 base pairs. For the H63D mutation, the wild type fragment will have an expected size of 130 base pairs while the mutant fragment will have an expected

size of 200 base pairs. Molecular weight markers and control samples were run with each PCR analysis.

Population based reference ranges are needed to evaluate the various parameters in this study. Determination of population based reference ranges was accomplished by using Number Cruncher Statistical System (NCSS, Kaysville Utah). Statistical analysis of this research consisted of descriptive statistics, paired t-test, analysis of variance, and Fisher's exact test. The control group consists of 114 samples, picked at random using a random number generator (www.randomizer.org), from the available pool of samples for specific comparison purposes. These statistical comparisons are used in the evaluation of screening paradigms. All statistical analysis were conducted using Number Cruncher Statistical System (NCSS, Kaysville Utah).

Results

The calculated reference ranges (mean and 95% confidence interval) for each analyte are listed in table 4.1 (male) and table 4.2 (female). Each sex is broken down into active duty, non-active duty under age 40, and non-active duty over age 40. One-way analysis of variance with Fisher's least significance difference test was performed on each analyte across the different groups. For males, each complete blood count parameter was significantly different in non-active duty over age 40 ($p < 0.0001$) with the exception of MCHC which was significantly different in the under age 40 group ($p < 0.0001$). In addition, the MCV was different across all groups ($p = 0.0001$). Iron ($p < 0.0001$), transferrin saturation ($p < 0.0001$), and TIBC ($p = 0.008$) were significantly different in non-active duty over age 40. Among females, some parameters were significantly different in

the non-active duty over age 40 group. The MCV, MCH ($p<0.0001$) and TIBC ($p=0.003$) were significantly lower than both other groups. The RBC and MCHC ($p<0.0001$) was significantly different from the active duty group.

Genetic analysis was performed on all samples with elevated transferrin saturations which was defined as greater than 51 percent for males and greater than 44 percent for females.² Of the 46 male samples, 28 were negative for mutations, 15 were heterozygous for the C282Y mutation, and 3 were homozygous for the C282Y mutation. Six out of 10 heterozygous samples tested for the H63D mutation were found to be compound heterozygotes. Of 45 female samples, 34 were negative for mutations, 8 were heterozygous for the C282Y mutation, and 3 were homozygous for the C282Y mutation. 2 out of 6 heterozygous samples tested for the H63D mutation were found to be compound heterozygotes. The allele frequency for randomized control samples is shown in Figure 4.1. The overall allele frequency from random control samples was 0.039, for white only controls was 0.048, for non-white/unknown controls 0.029, for females with elevated transferrin saturation was 0.1956 and male with elevated transferrin saturation 0.250.

A summary of each of 6 homozygote cases is listed in Table 4.3. There were 3 male and 3 female cases. Only one case was from an active duty member. The percentile of each measured and calculated analyte is listed in Table 4.4 (vs. overall distribution of reference ranges) and Table 4.5 (vs. group distribution of reference ranges).

A comparison of results between affected individuals, which are classified as homozygous for the C282Y mutation or heterozygotes for the C282Y mutation with elevated transferrin saturations, and all other samples is listed Table 4.6 (male) and Table 4.7 (female). The only significant difference between the male groups was MCV

($p=.037$) with MCH approaching significance ($p=0.06$). In females, the RBC was significantly lower ($p=0.001$) and the MCV ($p=0.0002$) and MCH ($p=0.003$) were significantly higher. Table 4.8 includes compound heterozygotes as affected individuals. Using this definition, hemoglobin approaches significance ($p=0.058$) and MCH ($p=0.038$) is significant in males and RBC ($p=0.06$) is only approaching significance in females.

Figure 4.2 illustrates the summary of liver function tests (LFT) in affected male and females, homozygotes and heterozygotes with elevated transferrin saturation, and other individuals. Affected males had slightly lower LFTs than other males. Females had higher ($p=0.096$) ALT (27.1 U/L) than non-affected (18.5 U/L) and higher AST ($p=0.11$) ALT (30.3.1 U/L) than non-affected (23.6 U/L). Figure 4.3 illustrates the ratio of individual liver function test/reference mean from affected male and female samples. Females had significantly higher individual ratios ($p=0.03$) when compared to affected male ratios.

Figure 4.4 is the correlation between iron and transferrin saturation in male samples. These samples are highly correlated with a R^2 of 0.8195. Figure 4.5 is the correlation between iron and transferrin saturation in female samples. These samples are highly correlated with a R^2 of 0.8343. Figure 4.6 is presumptive anemia, based on the Center for Disease Control and Prevention criteria (< 12.0 g/dl Hg female, < 13.5 g/dL male), in male and female samples. A total of 12.6% of females and 13.2% of males were anemic. There were no significant differences across age groups in females. In males, there was a significant difference ($p=0.002$) for those under age 40 and ($p=0.028$) over age 40 when compared to the overall mean. Figure 4.7 is the percentage of presumptive iron deficiency anemia (transferrin saturation <15 percentile) in the anemia cases from Figure

4.6. Overall, 33 percent of females and 32 percent of males who had anemia were presumptively found to be iron deficient. Males under age 40 had only 9 percent ($p=0.017$) presumptively identified as iron deficient. A summary of the heterozygote (C282Y) control cases is presented Table 4.9. Percentile based results for these heterozygote controls are presented in Table 4.10. Table 4.11 lists statistical comparisons between male heterozygote and wild type male Caucasian controls. Iron, transferrin saturation, MCH and ALT were significantly higher in male heterozygote controls.

Table 4.1: Reference mean and range for analytes - male

Analyte	Overall Group Distribution	Active Duty Distribution	Other Age Less Than 40 Years	Other Age More Than 40 Years
Hemoglobin (g/dL)	14.81 (12.15 – 17.50)	15.11 (13.05 – 17.17)	15.2 (12.98 – 17.42)	14.52(11.54 – 17.50)
Hematocrit (%)	43.93 (35.88 – 51.98)	44.85 (38.57 – 51.13)	45.46 (38.46 – 52.46)	42.95(34.19 – 51.71)
RBC (M/ μ L)	4.86 (3.89 – 5.83)	5.02 (4.24 – 5.80)	5.05 (4.21 – 5.89)	4.71(3.69 – 5.73)
MCV (fL)	90.63 (80.60 – 100.66)	89.43 (80.81 – 98.05)	90.25 (80.49 – 100.01)	91.47(80.79 – 102.15)
MCH (pg)	30.58 (25.73 – 35.43)	30.13 (26.83 – 33.43)	30.17 (26.57 – 33.77)	30.97(26.87 – 35.07)
MCHC (g/dL)	33.71 (31.86 – 35.56)	33.7 (31.82 – 35.58)	33.45 (31.53 – 35.37)	33.79(31.99 – 35.59)
Iron (mg/dL)	73.33 (13.41 – 133.25)	76.9 (16.9 – 136.9)	80.5 (22.3 – 138.7)	69.1(9.9 – 128.3)
TIBC (μ g/dL)	261.25 (172.55 – 349.95)	261.6 (178.4 – 344.8)	268.3 (182.3 – 354.3)	259.1(166.7 – 351.5)
Transferrin Saturation (%)	28.47 (4.83 – 52.11)	29.8 (6.2 – 53.4)	30.6 (6.4 – 54.8)	27.05(3.93 – 50.17)
ALT (U/L)	22.23 (0 – 48.28)	24.5 (0 – 56.1)	21.9 (0 – 47.7)	20.95(0 – 42.43)
AST (U/L)	22.44 (3.45 – 45.43)	25.5 (0 – 57.1)	24.2 (2.6 – 45.8)	23.85(4.85 – 42.85)
Ferritin (μ g/L)	(30-300)	(30-300)	(30-300)	(30-300)

RBC = red blood cell count, MCV = mean corpuscular volume, MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration, ALT = alanine aminotransferase, AST = aspartate amino transferase

Table 4.2: Reference mean and range for analytes – female

Analyte	Overall Group Distribution	Active Duty Distribution	Other Age Less Than 40 Years	Other Age More Than 40 Years
Hemoglobin (g/dL)	13.22 (10.71-15.73)	13.16 (10.66 – 15.66)	13.23 (11.11 – 15.35)	13.21 (10.57 – 15.85)
Hematocrit (%)	39.10 (31.75 – 46.45)	38.73 (30.69 – 46.77)	39.21 (33.11 – 45.31)	39.08 (31.46 – 46.70)
RBC (M/ μ L)	4.40 (3.33 – 5.46)	4.51 (2.49 – 6.53)	4.44 (3.68 – 5.20)	4.35 (3.47 – 5.23)
MCV (fL)	89.41 (76.57 – 102.25)	87.65 (69.29 – 106.01)	88.55 (77.81 – 99.29)	90.13 (77.99 – 102.27)
MCH (pg)	30.20 (25.53-34.87)	29.56 (24.44 – 34.68)	29.87 (25.61 – 34.13)	30.48 (25.82 – 35.14)
MCHC (g/dL)	33.79 (31.91-35.67)	34.61 (14.95 – 54.27)	33.80 (31.88 – 35.72)	33.81 (32.15 – 35.47)
Iron (mg/dL)	62.45 (0-125.16)	67.0 (0 – 136.6)	64.50 (0 – 135.3)	60.67 (3.83 – 117.51)
TIBC (μ g/dL)	278.75 (176.00 – 381.50)	289.0 (181.4 – 396.6)	283.60 (179.4 – 387.8)	274.3 (173.9 – 374.7)
Transferrin Saturation (%)	23.16 (0 – 47.91)	23.80(0 – 48.8)	23.60 (0 – 50.8)	22.90 (0 – 46.50)
ALT (U/L)	17.74 (0 – 40.90)	15.60 (0 – 32.2)	17.10 (0 – 39.3)	18.40 (0 – 43.00)
AST (U/L)	22.80 (0-48.02)	21.10 (4.1 – 38.1)	20.50 (0 – 34.9)	24.10 (0 – 53.70)
Ferritin (ug/L)	(30-300)	(30-300)	(30-300)	(30-300)

RBC = red blood cell count, MCV = mean corpuscular volume, MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration, ALT = alanine aminotransferase, AST = aspartate amino transferase

Table 4.3: Summary of homozygous cases

Case	Age	Sex	Race	Active Duty	Hg	Hct	RBC	MCV	MCH	MCHC	Iron	TIBC	TS%	ALT	AST	Ferritin
1	54	F	C	N	14.7	41.9	4.53	92	32	35	169	252	67	16	21	67.77
2	42	M	C	N	16.1	46.4	4.74	98	34	35	102	179	57	30	34	704.13
3	37	M	C	Y	14.9	43.1	4.78	90	31	35	187	215	87	29	24	771.74
4	70	F	C	N	N/A	N/A	N/A	N/A	N/A	N/A	163	164	99	70	64	3177.9
5	38	F	C	N	12.7	37.9	3.55	107	36	34	221	263	84	19	25	455.18
6	57	M	C	N	10.2	30.6	3.4	90	30	33	127	209	61	16	21	190.43

Age= years, Hg=hemoglobin (g/dL), Hct=hematocrit(%), RBC=red blood cell count (M/ μ L), MCV = mean corpuscular volume (fL), MCH = mean corpuscular hemoglobin (pg), MCHC = mean corpuscular hemoglobin concentration (g/dL), Iron= mg/dL, TS%=%, ALT = alanine aminotransferase (U/L), AST =aspartate aminotransferase(U/L), Ferritin= μ g/L

Table 4.4: Percentile of measured and calculated analyte vs. overall distribution

Case	Age	Sex	Race	Active Duty	Hg	Hct	RBC	MCV	MCH	MCHC	Iron	TIBC	TS%	ALT	AST	Ferritin
1	54	F	C	N	90	81	66	69	81	90	99	32	99	59	56	0.23
2	42	M	C	N	86	73	36	95	97	91	85	2	98	83	91	2.35
3	37	M	C	Y	47	38	39	44	59	90	99	13	99	81	61	2.57
4	70	F	C	N	N/A	N/A	N/A	N/A	N/A	N/A	99	1	99	99	98	10.59
5	38	F	C	N	31	34	3	99	79	58	99	41	99	71	78	1.52
6	57	M	C	N	1	1	1	45	35	24	96	10	99	33	41	0.63

Age= years, Hg=hemoglobin (g/dL), Hct=hematocrit(%), RBC=red blood cell count (M/ μ L), MCV = mean corpuscular volume (fL), MCH = mean corpuscular hemoglobin (pg), MCHC = mean corpuscular hemoglobin concentration (g/dL), Iron= mg/dL, TS%=%, ALT = alanine aminotransferase (U/L), AST =aspartate aminotransferase(U/L), Ferritin= multiples of the upper reference range

Table 4.5: Percentile of measured and calculated analyte vs.group distribution

Case	Age	Sex	Race	Active Duty	Hg	Hct	RBC	MCV	MCH	MCHC	Iron	TIBC	TS%	ALT	AST	Ferritin
1	54	F	C	N	89	80	69	64	78	90	99	34	99	55	52	0.23
2	42	M	C	N	87	79	50	91	94	89	88	2	99	86	91	2.35
3	37	M	C	Y	38	28	25	54	70	90	99	12	99	75	56	2.57
4	70	F	C	N	N/A	N/A	N/A	N/A	N/A	N/A	99	1	99	99	97	10.59
5	38	F	C	N	31	34	1	99	99	58	99	35	99	73	83	1.52
6	57	M	C	N	1	1	1	36	28	21	96	12	99	35	44	0.63

Age= years, Hg=hemoglobin (g/dL), Hct=hematocrit(%), RBC=red blood cell count (M/ μ L), MCV = mean corpuscular volume (fL), MCH = mean corpuscular hemoglobin (pg), MCHC = mean corpuscular hemoglobin concentration (g/dL), Iron= mg/dL, TS%=%, ALT = alanine aminotransferase (U/L), AST =aspartate aminotransferase(U/L), Ferritin= multiples of the upper reference range

Table 4.6: Comparison between male homozygotes/heterozygotes with elevated transferrin saturation percentage

	Homozygotes/Heterozygotes (↑ TS%) n=14	Other Samples n=1288	Significance (p)
Hg	15.17	14.81	0.15
Hct	44.85	43.92	0.20
RBC	4.83	4.86	0.56
MCV	93.00	90.60	0.037
MCH	31.36	30.57	0.06
MCHC	33.86	33.70	0.60
ALT	20.86	22.21	0.35
AST	23.57	24.86	0.40

Hg=hemoglobin (g/dL), Hct=hematocrit(%), RBC=red blood cell count (M/ μ L), MCV = mean corpuscular volume (fL), MCH = mean corpuscular hemoglobin (pg), MCHC = mean corpuscular hemoglobin concentration (g/dL), Iron= mg/dL, TS%=%, ALT = alanine aminotransferase (U/L), AST =aspartate aminotransferase(U/L)

Table 4.7: Comparison between female homozygotes/heterozygotes with elevated transferrin saturation percentage

	Homozygotes/Heterozygotes (↑ TS%) n=11	Other Samples n=917	Significance (p)
Hg	13.06	13.21	0.65
Hct	38.64	39.11	0.66
RBC	4.04	4.38	0.001
MCV	95.8	89.4	0.0002
MCH	32.20	30.22	0.003
MCHC	33.70	33.80	0.37
ALT	23.90	18.10	0.20
AST	27.80	23.70	0.33

Hg=hemoglobin (g/dL), Hct=hematocrit(%), RBC=red blood cell count (M/ μ L), MCV = mean corpuscular volume (fL), MCH = mean corpuscular hemoglobin (pg), MCHC = mean corpuscular hemoglobin concentration (g/dL), Iron= mg/dL, TS%=%, ALT = alanine aminotransferase (U/L), AST =aspartate aminotransferase(U/L)

Table 4.8: Comparison between male homozygotes/heterozygotes with elevated transferrin saturation percentage/compoundheterozygotes

	Male Homozygotes/ Heterozygotes (↑ TS%) n=14	Other Samples n=1288	Significance (p)	Female Homozygotes/ Heterozygotes (↑ TS%) n=11	Other Samples n=917	Significance (p)
Hg	15.25	14.80	0.058	13.30	13.21	0.55
Hct	45.93	43.91	0.055	39.51	39.07	0.59
RBC	4.89	4.86	0.37	4.15	4.40	0.06
MCV	92.68	90.59	0.026	95.45	89.33	0.001
MCH	31.09	30.56	0.038	32.00	33.88	0.005
MCHC	33.64	33.71	0.35	33.63	33.90	0.19
ALT	22.72	22.19	0.57	23.18	18.44	0.13
AST	24.09	24.86	0.27	27.18	23.64	0.23

Hg=hemoglobin (g/dL), Hct=hematocrit(%), RBC=red blood cell count (M/ μ L), MCV = mean corpuscular volume (fL), MCH = mean corpuscular hemoglobin (pg), MCHC = mean corpuscular hemoglobin concentration (g/dL), Iron= mg/dL, TS%=%, ALT = alanine aminotransferase (U/L), AST =aspartate aminotransferase(U/L)

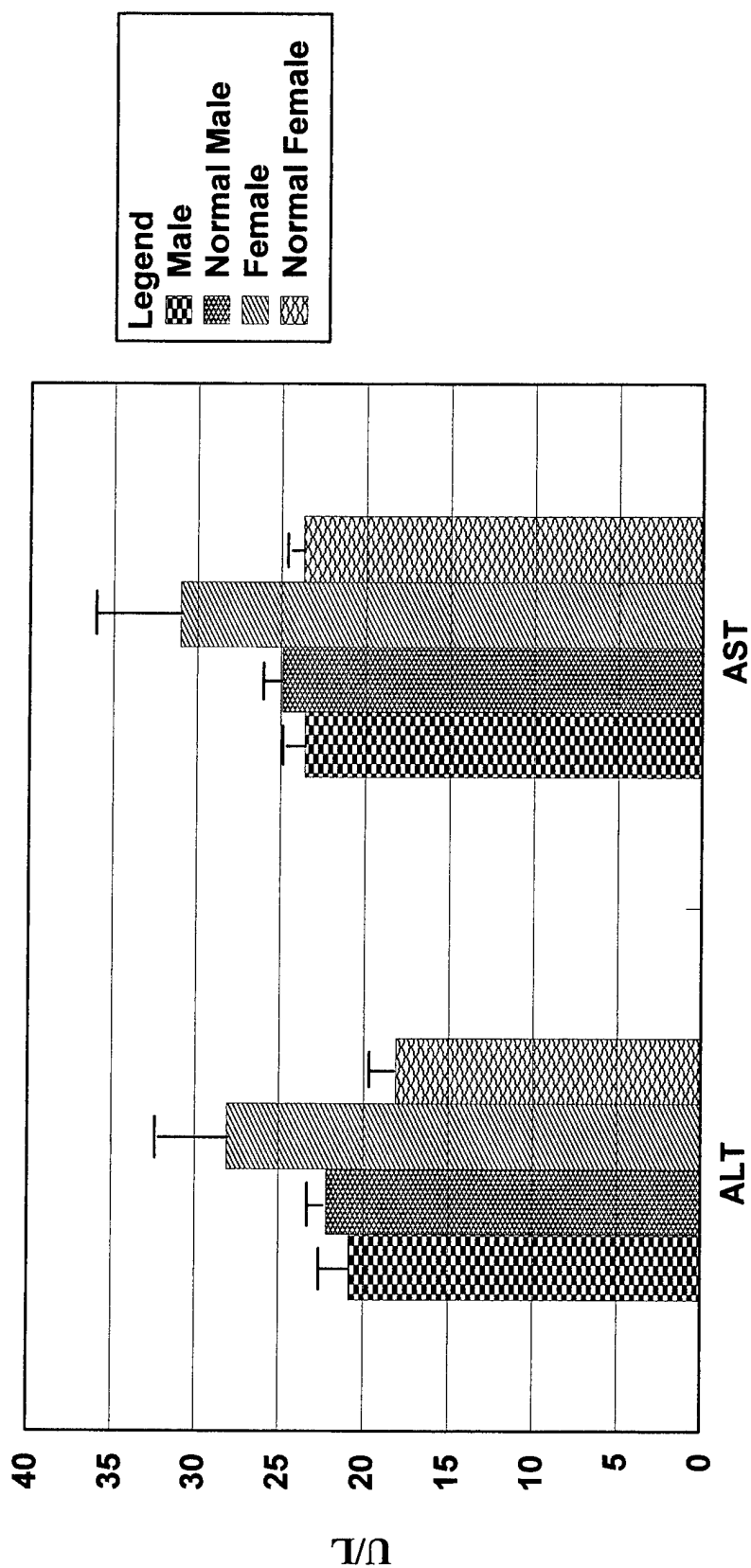


Figure 4.2: Summary of liver function tests in male and female samples from homozygous or heterozygous with elevated transferrin saturation percentage

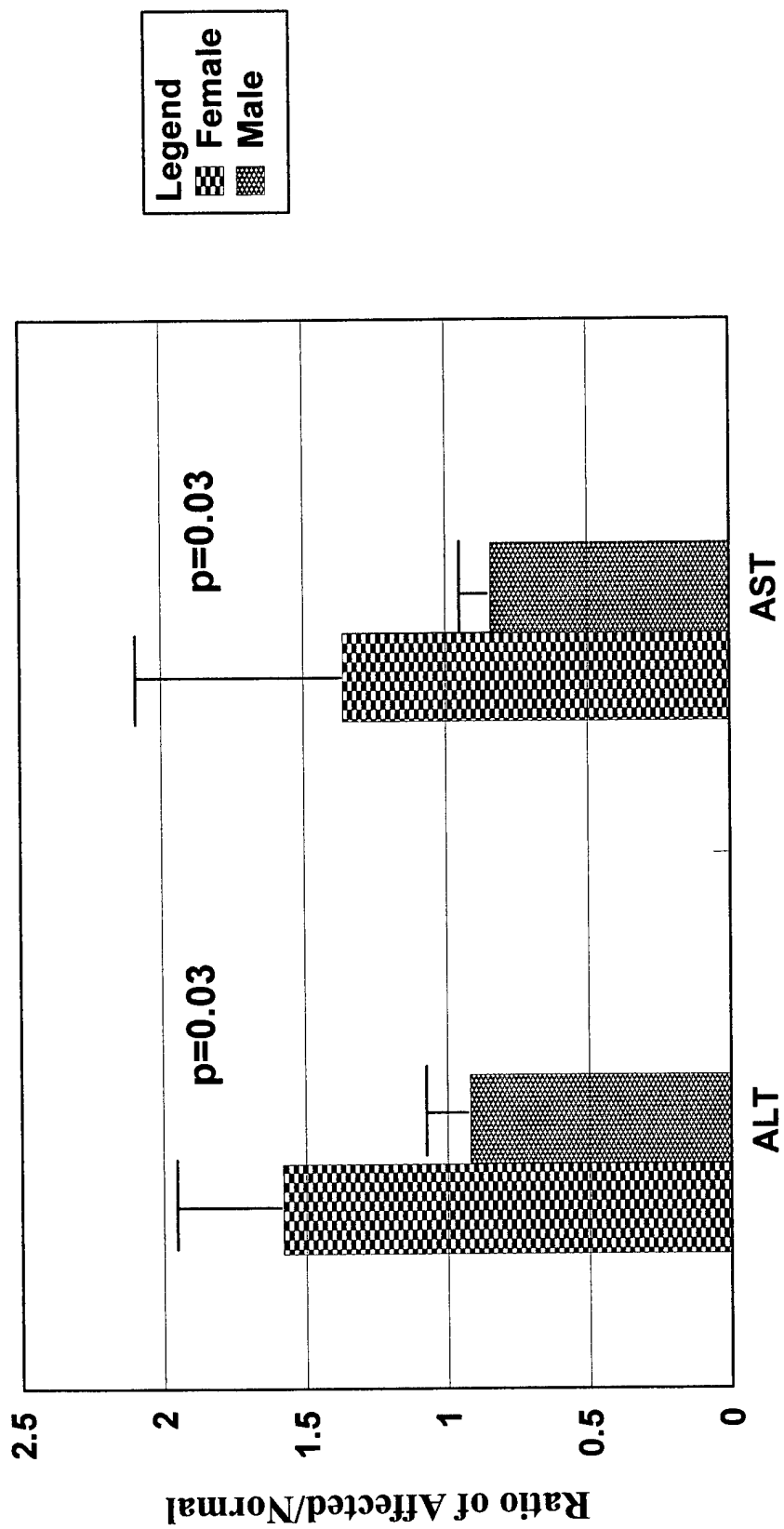


Figure 4.3: Ratio of liver function tests from affected male and female samples vs. other samples

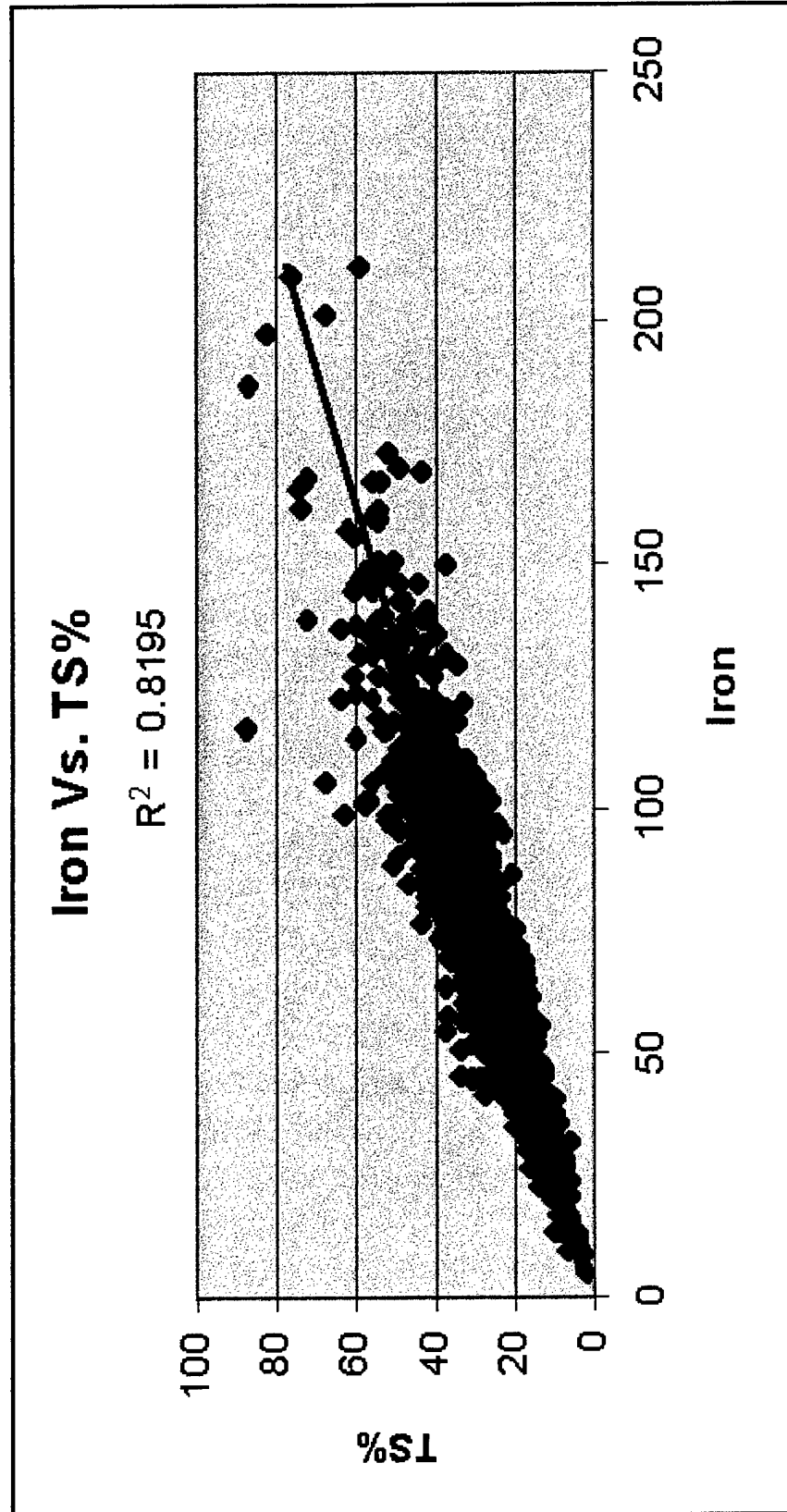


Figure 4.4: Correlation between iron and transferrin saturation in male samples

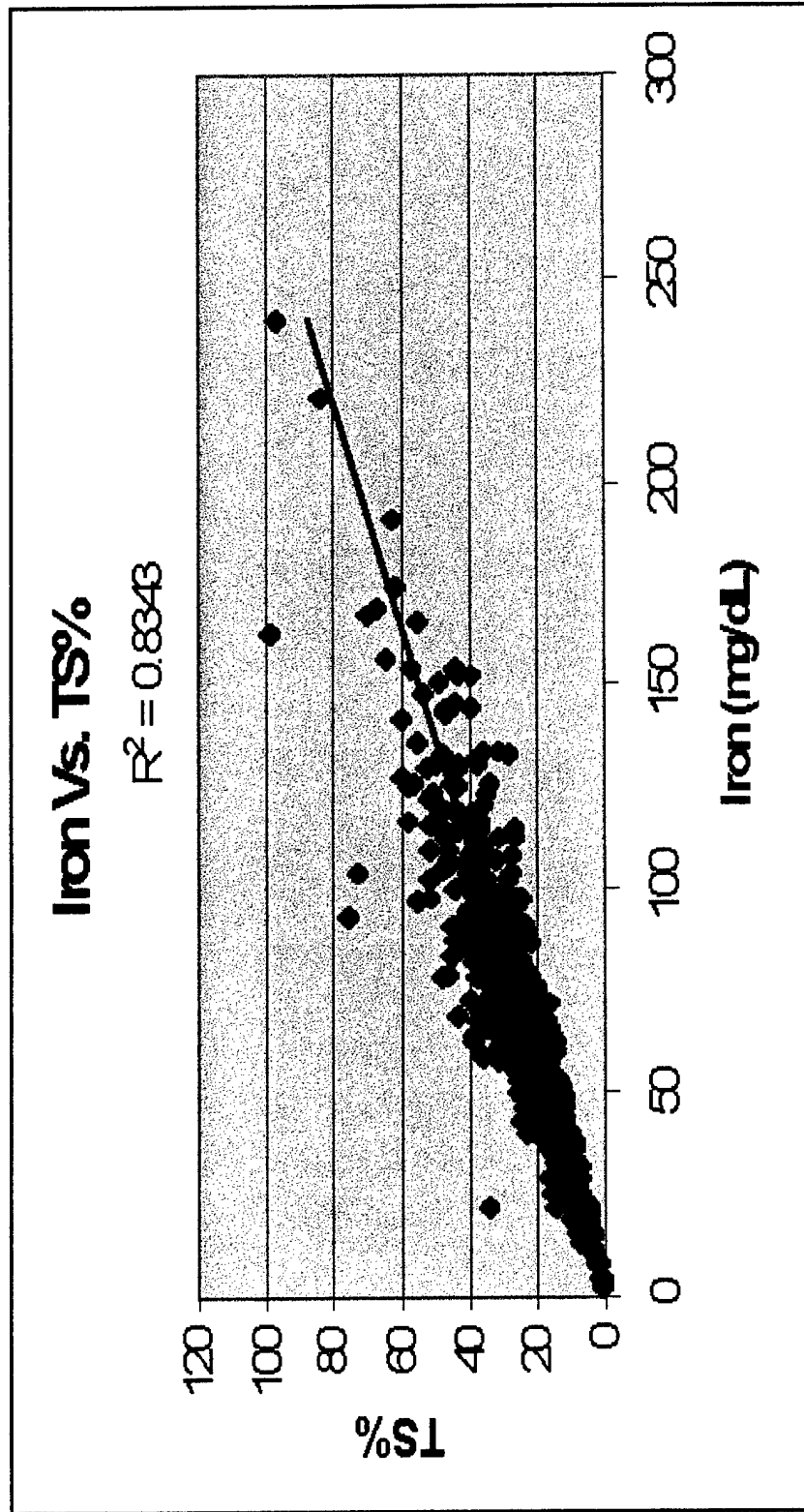


Figure 4.5 Correlation between iron and transferrin saturation in female samples

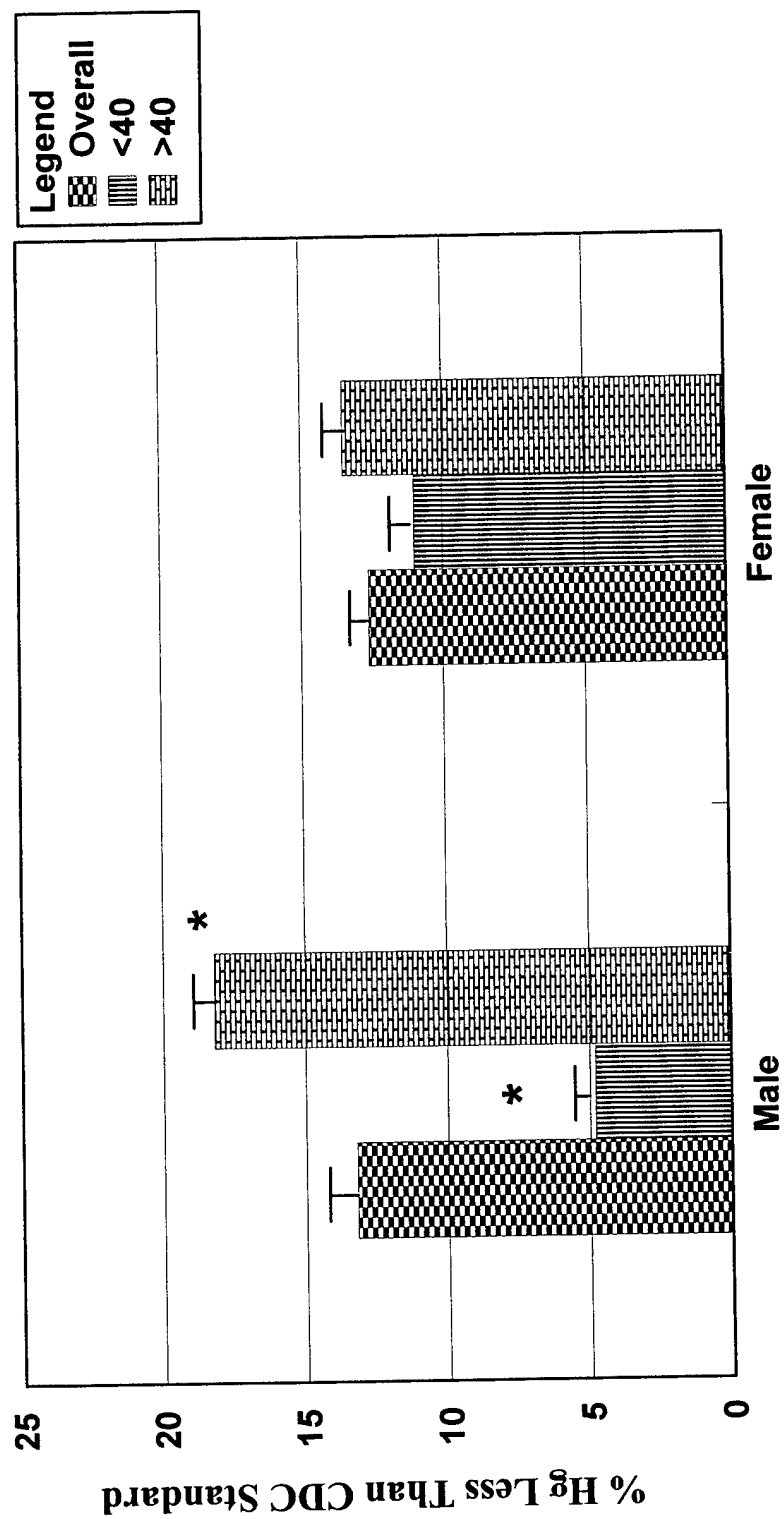


Figure 4.6: Presumptive anemia in male and female samples

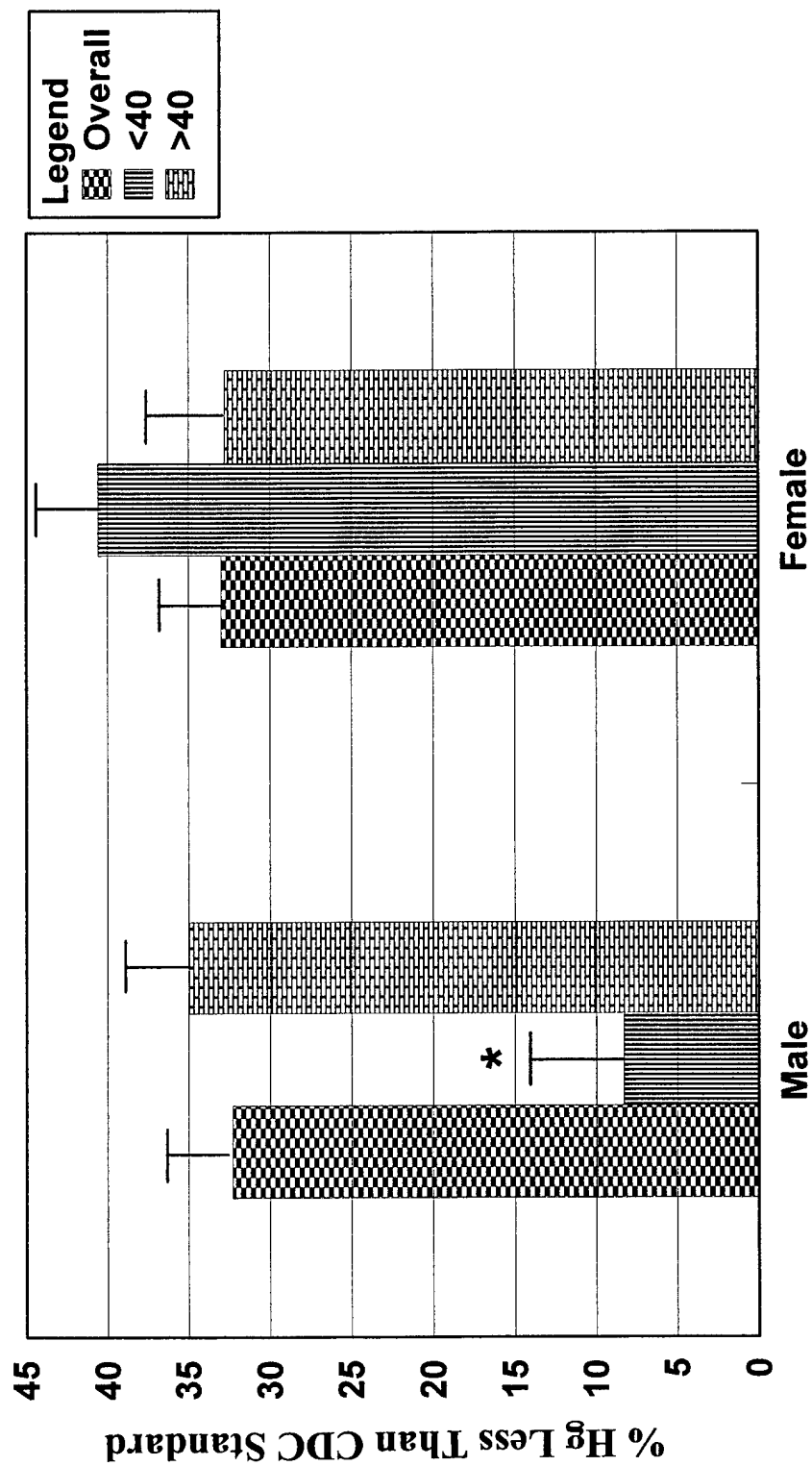


Figure 4.7: Presumptive iron deficiency anemia in male and female samples

Table 4.9: Summary of heterozygous control cases

Case	Age	Sex	Race	Active Duty	Hg	Hct	RBC	MCV	MCH	MCHC	Iron	TIBC	TS%	ALT	AST
1	67	M	C	N	14.8	43.1	4.31	100	34	34	98	247	40	15	26
2	81	M	C	N	15.2	45.6	4.88	93	31	33	131	293	45	19	19
3	56	M	?	N	17.6	50	5.63	89	31	35	105	208	50	64	29
4	39	M	C	N	15.3	45.7	N/A	N.A	N/A	N/A	61	282	22	35	23
5	61	M	C	N	16.1	47.7	5.1	94	32	34	162	219	74	19	21
6	48	F	?	N	14.9	42.9	4.56	94	33	35	43	285	15	23	25
7	27	F	?	Y	14.9	43.6	4.43	98	34	34	85	283	30	10	18

Age= years, Hg=hemoglobin (g/dL), Hct=hematocrit(%), RBC=red blood cell count (M/ μ L), MCV = mean corpuscular volume (fL), MCH= mean corpuscular hemoglobin (pg), MCHC = mean corpuscular hemoglobin concentration (g/dL), Iron= mg/dL, TS%=%, ALT = alanine aminotransferase (U/L), AST =aspartate aminotransferase(U/L), Ferritin= μ g/L

Table 4.10: Percentile of measured and calculated analyte vs. overall distribution in heterozygous controls

Case	Age	Sex	Race	Active Duty	Hg	Hct	RBC	MCV	MCH	MCHC	Iron	TIBC	TS%	ALT	AST
1	67	M	C	N	45	40	15	96	97	72	81	40	86	25	71
2	81	M	C	N	61	67	52	73	60	28	95	77	91	52	27
3	56	M	?	N	99	97	97	29	63	97	86	10	95	99	83
4	39	M	C	N	64	68	N/A	N/A	N/A	N/A	43	69	32	91	54
5	61	M	C	N	86	86	71	77	72	44	99	15	99	52	41
6	48	F	?	N	93	86	70	83	90	86	29	57	26	81	77
7	27	F	?	Y	93	90	57	96	85	64	81	57	77	15	33

Age= years, Hg=hemoglobin (g/dL), Hct=hematocrit(%), RBC=red blood cell count (M/ μ L), MCV = mean corpuscular volume (fL), MCH = mean corpuscular hemoglobin (pg), MCHC = mean corpuscular hemoglobin concentration (g/dL), Iron= mg/dL, TS%=%, ALT = alanine aminotransferase (U/L), AST =aspartate aminotransferase(U/L)

Table 4.11: Comparison between male heterozygote and other wild type male controls

	Male Heterozygotes Controls n=5	Wild Type Controls Samples n=35	Significance (p)
Hg	15.8	14.7	0.07
Hct	46.4	43.5	0.10
RBC	4.98	4.80	0.28
MCV	93.9	90.8	0.09
MCH	32.1	30.6	0.05
MCHC	33.8	33.7	0.41
Iron	111.4	75.3	0.01
TIBC	249.8	258.4	0.67
TS%	46.2	30.5	0.02
ALT	30.4	20.9	0.04
AST	23.6	25.6	0.69

Units: Age= years, Hg=g/dL, Hct=%, RBC=M// μ L, MCH= fL, MCHC= g/dL, Iron= mg/dL, TS%=%, ALT, AST = U/L

Discussion

The importance of establishing accurate reference ranges should not be dismissed. As was seen in the results section, there are some statistically significant differences in several parameters of both male and females across the groups examined. Of special importance is the difference in the red blood cell indices. As will be discussed later, these indices may be useful as an indicator to investigate potential iron overload. Without accurate local reference ranges, attempts to use and interpret result may be cumbersome. Therefore, before any local facility implements an iron overload-screening program, it would be wise to produce local reference ranges.

Traditionally, iron status testing has been the primary biochemical method to screen for iron overload. Reference ranges for iron status vary from location and specific methodology used. In the literature, there are many iron status algorithms used to screen for iron overload. From the local reference ranges calculated for this study, a transferrin saturation of greater than 51 percent for males and greater than 44 percent for females was used to define excess iron in the serum. Using these criteria, a total of 46 males and 45 females were identified as potentially having hemochromatosis. From these samples, a total of 3 males were identified as homozygous for the C282Y mutation and 15 were heterozygotes. A total of 3 females were identified as homozygous for the C282Y mutation and 3 were heterozygotes. Therefore, a minimum of 6 individuals would be identified with probable phenotypic hemochromatosis if these samples had come from an actual screening program. It is most probable that these people will never be diagnosed

unless actual screening patterns are altered. In addition, half of the heterozygotes tested were compound heterozygotes. This means they also are heterozygous for the H63D mutation. These individuals may also be at greater risk for iron overload. The C282Y allele frequency for individuals with elevated transferrin saturation was approximately six times that found in controls for males and five times that found in controls for females. This further validates the usefulness of screening those with elevated transferrin saturation for the C282Y mutation.

It is also useful to examine the six cases of hemochromatosis identified. The most striking characterization of this group is the percentile ranking when compared to their respective group. Each of these six cases had transferrin saturations at greater than 99 percentile. The other measured analytes were varied upon observation, with the red blood cell indices trending to elevated percentiles. One case had extremely low hemoglobin, hematocrit, red blood cell count, and red blood cell indices. These observations suggest that this individual also had blood loss from another cause. Ironically, this severe blood loss may be protective in this individual as indicated by his normal ferritin. In addition, all individuals except the individual with the suspected blood loss, had liver function tests in the upper 50 percentile with the majority greater than 80 percentile. The individual with the greatest ferritin, which suggests the greatest body iron burden, was a 70-year-old female, which had an ALT greater than 99 percentile and AST greater than 97 percentile. This is very suggestive of massive liver damage. Four of the 6 cases had ferritins outside the normal reference range. All those identified were Caucasian with one individual being a 37-year-old active duty male. This is important because one common

misconception is that only older individuals have hemochromatosis. This example can be used to help communicate the fact that younger active duty individuals do have the disorder. In this case, this person's ferritin is elevated to an extent where damage is already probable.

Despite the recent questions that have arisen concerning the actual penetrance of hemochromatosis, there is still no debate that all individuals homozygous for the mutation should prophylactically donate blood. It is also reasonable to assume that those who are found to be heterozygous for the C282Y mutation along with elevated transferrin saturation should donate. In addition, compound heterozygotes may be advised to donate blood regardless of their iron status. Therefore, every effort should be made to identify these individuals. The complete blood count and liver function tests were selected to investigate their possible use in assisting in the diagnosis of these individuals. Fourteen males were found to be either homozygous or heterozygous for the C282Y mutation with elevated transferrin saturation. These individuals were considered "affected". Comparisons of these individuals with all other males demonstrated a statistically higher MCV ($p=0.037$) and approaching a statistically higher MCH ($p=0.06$). There were 11 females who met the same criteria. These females had highly significant statistical differences in the RBC (lower, $p=0.001$), MCV (higher, $p=0.0002$), and MCH (higher, $p=0.003$). The liver function tests were actually non-statistically significantly lower in males and higher in females. The females were somewhat higher, but due to the variation in liver enzymes, the difference was not statistically significant ($p=0.20$ for ALT and $p=0.33$ for AST). A similar comparison included compound heterozygotes in the "affected"

group. This increased the number of affected males to 22 and affected females to 13. Inclusion of these additional samples in male samples revealed both elevated hemoglobin ($p=0.058$) and hematocrit ($p=0.055$) that approached significance. In addition, the elevated MCV ($p=0.026$) and MCH ($p=0.038$) were both significant. Inclusion of compound heterozygotes for females decreased the significance of the red blood cell count ($p=0.06$). Overall, the hemoglobin and hematocrit seemed to be more asignificant in affected males. The MCV was significant in both males and females, although females were affected to a greater extent. The MCH was also affected in females and one of the groupings of males. The MCHC was not affected in either group. The liver function tests were not statistically significant in the expanded groups, although closer to significance in the female groups. Since the red blood cell indices are included in all modern automated complete blood counts, it is only necessary to pick the one calculated parameter that best suits the task at hand. Therefore, the MCV seems to be the best choice. It is clear that initiation of iron status testing and or genetic analysis upon identification of an increased MCV should aid in the identification of such patients. It must be noted that in no way should the MCV replace traditional iron status tests. Although the means of the groups are different, there will always be some affected individuals who have low MCHs. This is not often seen with transferrin saturation. However, more clinicians routinely order the complete blood count. The use of increased MCV may be a realistic conduit to increasing hemochromatosis identification and awareness.

Although abnormal liver function tests should include hemochromatosis as a possible cause in the diagnostic workup, these tests are non-specific enough to

preclude them from use as a screening tool in the military population. However, interesting responses are seen in affected individuals. The ALT and AST of affected females are generally higher than their group mean. This is not seen in males. One way of comparing differences between affected males and females is to analyze individual multiple of the means. A multiple of the mean is calculated by dividing the individual result by the group mean. The average of the individual multiple of the means is approximately 1.5 times the mean for female ALT and 1.4 times the mean for female AST. These same values for males are well under 1 times the mean. The differences in these multiple of the means is significant for both ALT and AST ($p=0.03$). This information may provide clues to the pathogenesis of the disorder. The liver may be targeted at an earlier stage in females. This topic will need further investigation in the future.

The gold standard in biochemical surrogate markers for hemochromatosis is the traditional iron status panel. Usually, this consists of an initial screen of serum total iron combined with serum total iron binding capacity or unbound iron binding capacity that results in a calculated transferrin saturation. Abnormal iron status testing may be followed a serum ferritin. Abnormal results may then be followed by genetic analysis or liver biopsy. The major problem with this type of screening is that it is not performed, as discussed in Chapter I. Part of the problem may be the failure of these iron status tests to be included in the common biochemical profile requested by and offered by clinical laboratories. For the greater part of the twentieth century, requests for biochemical analysis were often only requested when absolutely necessary. Testing was performed using tedious manual methods. This caused fewer

screening type tests to be performed with more focus on acute medical problems. As rudimentary automation was introduced into the clinical chemistry laboratory in the late 1960s and early 1970s, the technology was still limited such that analysis was usually batched and a limit on the number of routine tests offered was usually the rule. However, more tests could be performed in a shorter period of time. A panel of what was thought of the 20 most important clinical chemistry tests was developed. Iron status testing was not included in this panel. While technology has advanced dramatically over the past 35 years, the chemistry panel paradigm has remained. Therefore, most providers today routinely request many of the "20-test panels" that originated in the early 1970s. In many instances, the extra effort to order individual tests may dissuade the clinician from ordering the test. If transferrin saturation were included in such panels, there is no doubt that many more individuals would be diagnosed with hemochromatosis. Some clinical laboratories may be hesitant to include such iron status tests on routine panels due to ignorance or the fact that two tests are required to obtain the calculated transferrin saturation. However, in the military population, the total iron is correlated very well with both male and female transferrin saturation. Although it would be preferable to include transferrin saturation on routine panels, it may be easier to start with just total iron. Inclusion of total iron in the routine biochemical panel would produce abnormal results that would in theory be followed by the complete complement of iron status testing. The correlation of iron to transferrin saturation was shown to be sufficient enough to justify substitution of iron alone for the transferrin saturation when required by the testing laboratory.

While most heterozygotes will not suffer any known ill effects, some may have iron overload due to environmental factors. In addition, it is also possible that this group might be a sensitive sub-population to environmental toxins. These individuals would also generate a good supply of blood donors. Therefore, it may also be advantageous to examine methods to identify these individuals through screening methodologies. A total of 7 C282Y heterozygotes were identified from the random controls analyzed. Five of these heterozygotes were male. In contrast to the homozygotes, the MCH ($p=0.05$) seemed to be more prognostic than the MCV. Although not as dramatic or consistent, the transferrin saturation ($p=0.02$) and total iron ($p=0.01$) were elevated. Interestingly, the ALT in the males was significantly higher than the wild type controls ($p=0.04$). This was not seen in the affected homozygotes and heterozygotes. This may also require further investigation.

One final benefit of screening for hemochromatosis is the ability to identify those with iron deficiency. Iron deficiency has been labeled one of the world's most important nutritional deficiencies. The very same tests that are used to biochemically screen for hemochromatosis are also used for iron deficiency. Using the Center for Disease Control and Prevention's diagnosis criteria, it is estimated around 11 percent of military women under age 40 and 14 percent of military women over age 40 met the criteria as having anemia. Surprisingly, almost 18 percent of men over age 40 met the criteria. Less than 5 percent of men under age 40, like those who comprise the majority of those on active duty, were found to have anemia. However, 5 percent would still represent an enormous threat to military readiness. No matter what the cause, these cases of anemia should be investigated. Using low transferrin saturation

as a indication of possible iron deficiency as a cause for anemia, it was found that over 40 percent of these anemias were due to iron deficiency in women under age 40 and 33 percent due to iron deficiency in women over age 40. The attributable cause of anemia due to iron deficiency is somewhat lower than that found in the civilian community. The lower rate of anemia in military is most likely due to the sufficient nutritional intake of military females. In males over age 40, 35 percent of anemia was attributable to iron deficiency. However, only 8 percent of anemic males under the age of 40 had iron deficiency. It is likely that many older males are iron deficient and require pharmacological intervention. Therefore, although younger males seem to have fewer cases of iron deficiency anemia, there are no age groups that are risk free.

CHAPTER V

SUMMARY, IMPLICATIONS, AND CONCLUSIONS

Hemochromatosis is a genetic disorder that affects many military personnel, active-duty and retired, and their families. It is imperative that appropriate screening techniques be used to identify and treat affected individuals. It was with this understanding that this project was conducted.

One of the first questions that must be addressed in any evaluation of future screening programs is the current status of screening. It was found that very few individuals are being screened for iron status. This percentage was especially low in males. Iron deficiency, not overload, is the primary reason for such screenings. Most screening is performed by primary care providers. This research illustrates the dire need for better education among health care providers. One possible way to emphasize the need for screening for iron overload is the corollary benefit of also detecting those who have iron deficiency.

Since it has been demonstrated that few are being screened for hemochromatosis, it is therefore desirable to quantify the number of individuals actually diagnosed. The number of hemochromatosis cases submitted to the Air Force medical genetics center is rather low and the subsequent number of diagnoses

is low. It was also notable that the demographics of the requests mirrored the military population, thus seemingly ignoring the strong association of the disorder from those of northern European descent. The vast majority of the diagnoses were made in individuals of Northern European descent. Smaller medical treatment facilities, which have a higher proportion of primary care providers, had a similar success rate in detecting C282Y mutations and also were more likely to include a family history with the request for analysis. Many more males are annually screened, yet an almost equal number of homozygous C282Y mutations are uncovered in males and females. A higher percentage of males are referred for genetic analysis with documented abnormal iron status. A higher percentage of women referred for genetic analysis with documented family history. Although family history is important in the overall identification of C282Y mutations, a breakdown by sex revealed only important distinctions among males. There are very important facets of this research. First, the low number of diagnoses reveals a direct indication of the failure to identify the majority of individuals with the disorder. This research also brings forth interesting questions about diagnosed hemochromatosis and females. Additional research needs to be performed to uncover additional reasons why females have a higher percentage of homozygous C282Y mutations while receiving far fewer requests. Compounding these results is the fact that females are more likely to have a history and less likely to have abnormal iron status tests.

This research also demonstrated the importance of establishing accurate reference ranges in the military population. If successful screening programs are to exist, accurate reference ranges must be the cornerstone they are built upon.

Differences in several key parameters across age groups were uncovered by performing reference range analysis. Elevated transferrin saturation percentage was also shown to be sufficient to identify individuals with hemochromatosis. The mean corpuscular volume (MCV), a calculated component commonly requested laboratory value, was shown to be statistically higher in C282Y homozygotes and heterozygotes. This could lead to clinical inroads for providers who do not routinely use iron status tests in their clinical practice. Liver function tests, while elevated in some with hemochromatosis, is not specific enough for routine screening. However, as a teaching point, providers should be aware that iron status tests should be included in the differential diagnosis of those abnormal liver function tests. Affected females seemed to have a larger elevation in ALT and AST when compared to their respective reference range. This elevation should be investigated more thoroughly in the future. Because transferrin saturation percentage is a calculation based on two measured tests, it may be problematic to persuade clinical laboratories to include these tests on routine clinical chemistry panels. However, this research has demonstrated that iron alone correlates reasonably well with transferrin saturation percentage. With this knowledge, iron may be included in panels with abnormal results followed up by a complete iron status panel. A large-scale screening program, in combination with routine complete blood counts, will also identify many individuals with iron deficiency anemia. This research demonstrated that a noticeable number of females and older males have anemia, many of which are presumptively due to iron deficiency.

As with any exempt type of research, there are limitations that should be addressed. The sample of clinical records to determine the frequency of iron status testing was drawn from one base. While the majority of these records were from individuals who have served at more than one military installation, it is possible that these results may be somewhat skewed due to the fact that all these individuals have received care at one smaller base. The review of records from the Air Force medical genetics center is dependent upon good record keeping at both the submitting facility and the genetics center. It is possible that information commonly known was left out of the record. The collection and testing of the clinical samples from a presumed healthy population carries the risk that some of these individuals had medical conditions that may have affected the results of tests. Since this was an exempt research project, there is no allowable mechanism to contact the individuals from which the records were reviewed or samples obtained to collect more or verify data.

In summary, the military should do a better job at identifying those individuals who have genotypic hemochromatosis. Efforts should be made to implement screening programs that would support the goal of increased identification. Although such programs are susceptible to being sidetracked by past practices and faulty paradigms, marketing of these programs must highlight the intertwined corollary benefits. It is foreseen that implementation of iron status screening programs will identify many previously undiagnosed cases of hemochromatosis.

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