CLINICAL VALIDITY

Question 18: How often is the test positive when the disorder is present?
Question 19: How often is the test negative when the disorder is not present?
Question 20: Are there methods to resolve clinical false positive results in a timely manner?
Question 21: What is the prevalence of the disorder in this setting?
Question 22: Has the test been adequately validated on all populations to which it may be offered?
Question 23: What are the positive and negative predictive values?
Question 24: What are the genotype/phenotype relationships?
Question 25: What are the genetic, environmental or other modifiers?

CLINICAL VALIDITY

Question 18: How often is the test positive when the disorder is present?

Summary

- Among non-Hispanic Caucasians, clinical sensitivity of C282Y homozygosity for detecting individuals with primary iron overload and associated morbidity is estimated to be at least 87 percent (95 percent CI 80 to 94%).
  - It is based on four studies totaling 247 individuals, not all of whom were symptomatic
  - When the disorder is defined more rigorously in clinical terms, clinical sensitivity of the test increases
- Actual clinical sensitivity is likely to be slightly lower, because analytic sensitivity is less than 100 percent (estimated in Question 9 to be 98.4 percent).
- It is not possible to confidently estimate clinical sensitivity among other racial/ethnic groups because little, if any, data are published
- Among other racial/ethnic groups, the sensitivity appears to be lower
  - 0% among Hispanic Caucasians according to 6 cases reported in 1 studies
  - 0% among Black/African Americans according to 14 cases reported in 1 studies
  - 0% among Asians/Asian Americans according to 23 cases reported in 2 studies

Introduction

The definitions of clinical sensitivity (Question 18) and clinical specificity (Question 19) can be derived using a two-by-two contingency table for data from case/control or cohort studies. If the data are from a general population cohort, both positive predictive and negative predictive values (Question 23) can also be directly computed. In Table 3-1, the rows are defined by the *HFE* gene test results, stratified into two categories; C282Y homozygosity and all other test results. The *HFE* gene has been identified as the major genetic factor leading to iron overload in the Caucasian population, and the C282Y mutation is, by far, the most important mutation in this gene. The columns are defined by the specific clinical disorder that the screening test aims to detect – in this instance, primary iron overload with associated morbidity. The first column contains all individuals with primary iron overload sufficient to cause significant morbidity and mortality, and the second column contains all individuals who do not have the clinical manifestations of iron overload.

Table 3-1 A Two-by-Two Contingency Table for Deriving the Four Major Clinical Performance Parameters

	Clinically Manifest Primary Iron Overload*		
	Yes	No	Totals
C282Y Homozygosity			
Yes	A	B	A+B
No	C	D	C+D
Totals	A+C	B+D	A+B+C+D

* primary iron overload of adult onset associated with significant morbidity

- Clinical sensitivity [A / (A + C)] is the proportion of individuals with clinical manifestations of primary iron overload (A+C) who are correctly identified as being C282Y homozygotes (A) by the screening test.
- Clinical specificity [D / (B + D)] is the proportion of individuals not affected with clinical manifestations of primary iron overload (B+D) who are correctly identified as not being C282Y homozygotes (D) by the screening test.
- Positive predictive value [A / (A + B)] is the proportion of positive tests (A + B) that correctly identify individuals with clinical manifestations of primary iron overload (A). This can only be directly derived if the table is derived from a population-based cohort study.
- Negative predictive value [D / (C + D)] is the proportion of negative tests (C + D) that correctly identify unaffected individuals/controls (D). This also can only be directly derived if the table is derived from a population-based cohort study.

Definition of clinical phenotype

In Question 1, a general definition of the disorder being screened for is stated as: primary iron overload of adult onset sufficient to cause significant morbidity and mortality. This definition includes individuals who will develop clinical manifestations of iron overload in their adult life. A more specific definition of the clinical phenotype for primary iron overload is needed before selecting studies that provide data appropriate for assessing clinical validity.

The primary iron overload phenotype will be defined as:

- biochemical evidence of iron overload that includes two or more of the following indices:
  - excess hepatic iron of 80 μmol/g or more
  - hepatic iron index greater than 1.9
  - histologic stainable iron 3-4+
  - removal of 4 to 5 or more grams of iron by quantitative phlebotomy
- and clinical manifestations associated with progressive organ damage (e.g., liver disease, cardiomyopathy, and arthropathy associated with specific radiological changes).

Clinical sensitivity of C282Y testing for primary iron overload

Clinical sensitivity refers to the proportion of individuals who have, or who are destined to develop, the primary iron overload phenotype who have a positive test result for C282Y homozygosity. In contrast, analytic sensitivity describes how often the laboratory correctly

identifies C282Y homozygosity. The penetrance of this genotype – or the proportion of individuals homozygous for C282Y who will progress to the primary iron overload phenotype - is not yet precisely known, but is recognized to be considerably below 100 percent.

The ideal study to assess clinical performance

The ideal study to assess clinical performance of *HFE* testing as a way to detect the primary iron overload phenotype would be to perform DNA testing in a large population-based cohort of young adults. This entire population would then be followed at intervals to determine in whom, and at what age, the phenotype of interest developed. At the conclusion of the study, it would be possible to fill in the four critical numbers in Table 3-1. Such a study would not provide the information in a timely manner and also would not be considered ethically acceptable.

A realistic study to assess clinical sensitivity

A more realistic approach would be to first identify a group of individuals who have the primary iron overload phenotype (A+C from Table 3-1), and then determine the proportion who are C282Y homozygotes (A from Table 3-1). This would provide an estimate of clinical sensitivity, but this design does not allow for the computation of the positive predictive value (the penetrance) of the genotype. Case or case-control studies can be used to determine the proportion of individuals clinically affected with the primary iron overload phenotype who are C282Y homozygotes. Limitations of this approach include:

- some studies do not provide information about whether cases might be from the same family
- some studies do not provide adequate information about race/ethnicity (most provide race but few stratify by ethnicity)
- studies vary widely in their definitions of both clinical phenotype and iron overload
- some studies may not have ruled out secondary causes of the iron overload phenotype (e.g., chronic anemia)
- some studies may have selection biases (e.g., if C282Y homozygotes are routinely identified and classified as having the phenotype, they may be over-represented among individuals recruited into the study)

Clinical sensitivity in non-Hispanic Caucasians in the United States

Initially, the focus of this analysis is the non-Hispanic Caucasian population, because most of the iron overload in this group is associated with the *HFE* gene. This review uses the term 'non-Hispanic Caucasian' as a surrogate for the more common designation of 'northern European Caucasian'. Few, if any, studies published in the U.S. collect information about country of origin, but many collect information about race/ethnicity. A total of 10 studies report the frequency of the C282Y homozygous genotype in non-Hispanic Caucasian individuals previously classified as having the primary iron overload phenotype, based on biochemical and/or clinical evidence. Definitions of the primary iron overload phenotype are variable. Appendix A contains a summary table of all 10 studies. Overall, the clinical sensitivity ranges from 32 to 91 percent (consensus 69%) and is highly heterogeneous ($\chi 2=155$, $p< 0.001$). Appendix A has complete information on these estimates.

In order to properly examine the relationship between C282Y homozygosity and the clinical phenotype, we found it necessary to exclude some of these studies. Two studies were removed

because they did not rule out secondary causes of iron overload that were likely to be common in their subjects (Bartolo et al, 1998; Press et al, 1998). A third study was removed because the population was restricted to iron-overloaded individuals without manifestations and also included first-degree relatives of probands (Sham et al., 2000). Two additional studies were removed because they might have included cases reported in an earlier data set (Bacon et al., 1999; Barton et al., 2000) and/or because the inclusion of some HLA-identical siblings of probands could affect genotype frequencies (Bacon et al., 1999). Once these studies were removed, heterogeneity was greatly reduced. Table 3-2 shows the remaining five studies, one of which (Beutler et al., 1996) probably had defined cases adequately, but the manuscript was not sufficiently clear to be sure. The four remaining study estimates were homogeneous, and the summary estimate of the clinical sensitivity was 87 percent (95 percent CI 80 to 94%). Raw data from all 10 studies are available in Appendix A. Exact confidence intervals for individual studies were computed using the binomial distribution (True Epistat, Texas).

Table 3-2 Studies That Can be Used to Compute Clinical Sensitivity of *HFE* Testing for the Iron Overload Phenotype among Non-Hispanic Caucasians in the U.S.

		Initial Clinical Sensitivity (%) [a]		Revised Clinical Sensitivity (%) [b]	
Study No.	Author,Year	Suspected IO[a] (N)	(95% CI)	Defined IO [b] (N)	(95% CI)
3	Barton *et al*, 97	74	60 (47-71)	44	82(67-92)
4	Sham *et al*, 97	61	67 (54-79)	27	82 (62-94)
7	Beutler *et al*, 96	147	82 (74-88)		--
8	Feder *et al*, 96	178	83 (77-88)	178	83 (77-88)
9	Brandhagen *et al*, 00	82	85 (76-92)	69	94 (86-98)
ALL		542		247	87 (80-94)

χ^2 test for heterogeneity = 2.2, p = 0.14

[a] Definitions of cases:

- Study 1 = Elevated transferrin saturation at least twice in the absence of other causes of iron overload
- Study 2 and study 3 = defined only by elevated transferrin saturation and serum ferritin
- Study 4 = All study subjects satisfied at least 2 of the following 4 criteria (hepatic iron concentration >4,500 ug/g, hepatic iron index >2.0, 3-4+ stainable iron, removal of at least 4 grams of mobilizable iron).
- Study 5 = All study subjects were classified by liver biopsy or quantitative phlebotomy (hepatic iron index >1.9 or removal of at least 5 grams of mobilizable iron).

[b] Definitions of cases:

- Study 1 = subset of probands satisfying at least 2 of the following 4 criteria (hepatic iron concentration >4,500 ug/g, hepatic iron index >2.0, 3-4+ stainable iron, removal of at least 4 grams of mobilizable iron).
- Study 2 = confirmed by liver biopsy or therapeutic phlebotomy
- study 3 = not possible to determine which results were derived from the group with liver biopsy and quantitative phlebotomy
- Study 4 = All study subjects satisfied at least 2 of the following 4 criteria (hepatic iron concentration >4,500 ug/g, hepatic iron index >2.0, 3-4+ stainable iron, removal of at least 4 grams of mobilizable iron).
- Study 5 = All study subjects were classified by liver biopsy or quantitative phlebotomy (hepatic iron index >1.9 or removal of at least 5 grams of mobilizable iron).

Among the five studies shown in Table 3-2, none stratify their results according to whether or not the primary iron overload phenotype was present. Instead, cases were defined according to more limited criteria; namely, measurement of iron indices that confirmed iron overload biochemically. Column 3 of Table 3-2 lists the clinical sensitivity as reported in each of the five studies. It was possible to perform further calculations in four of these studies using supplementary data from the study to more rigorously define the clinical phenotype. Column 5 shows the revised clinical sensitivity for the four remaining studies. In the three where more rigorous definitions were used in this analysis (Studies 3, 6 and 5), the revised clinical sensitivity was always higher.

Earlier in this section, an "ideal study" was described that would provide data to define clinical sensitivity. That study would follow a population-based genotyped cohort through life to determine the proportion of C282Y homozygotes that develops clinical manifestations. Such a study is not possible. Instead, the available studies (Table 3-2) use a combination of biochemical and tissue analyses to characterize the extent of iron overload as a surrogate for the clinical phenotype. In addition, many of the individual studies did report some clinical manifestations, but no study separately provided the proportion of these that was homozygous. The current analysis demonstrates that the more rigorously the extent of iron overload is defined, the closer its relationship to C282Y homozygosity becomes. Clinical sensitivity is known to be less than 100 percent, because a small proportion of individuals with the clinical phenotype is known not to be homozygous for C282Y mutation. Thus, clinical sensitivity of C282Y homozygosity for the clinical phenotype is likely to be at least as high as 87 percent but also must be several percentage points less than 100 percent.

Limitations and strengths of this analysis

The reliability of estimating clinical sensitivity of C282Y homozygosity for the primary iron overload phenotype is limited, because the number of acceptable studies (four) and the number of patients studied (318) is small. In addition, all four studies include some individuals who did not have clinical manifestations (e.g., liver damage), a key component of the clinical phenotype. No study included only clinically affected individuals, and none provided a separate estimate for the clinically affected subset. The strength of this analysis is in showing that when the studies are restricted to a more rigorous definition of iron overload, the clinical sensitivity increases. The clinical sensitivity of C282Y homozygosity may even higher than that found in the present analysis.

> **Gap in knowledge The clinical sensitivity of C282Y homozygosity in individuals with clinical manifestations and documented iron overload has not yet been defined.** Currently, our estimate of clinical sensitivity of C282Y homozygosity testing is based mainly on individuals with documented biochemical iron overload who may, or may not, have clinical manifestations. While this is likely to a reasonable approximation, it would be worthwhile to attempt to obtain a more appropriate group for analysis to confirm our estimate.

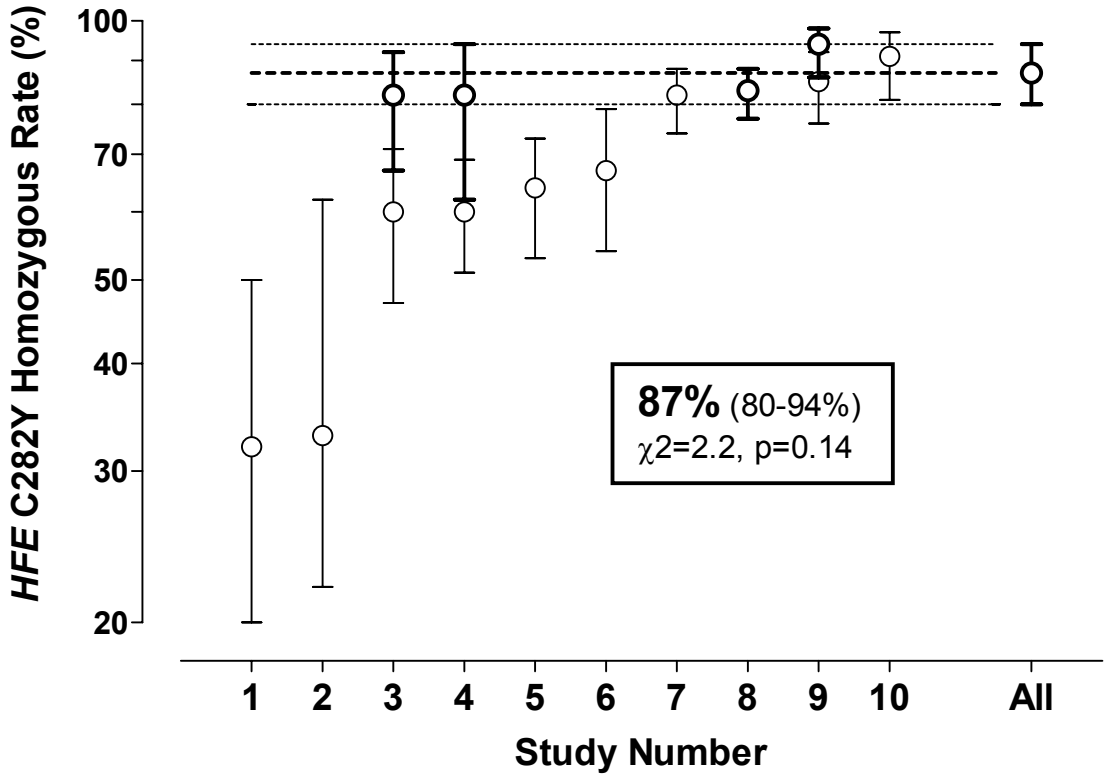

Figure 3-1 Estimated Clinical Sensitivity of C282Y Homozygosity in Non-Hispanic Caucasians in the U.S. The 10 studies identified in our literature search and summarized in Appendix A, are ordered from lowest to highest clinical sensitivity. The study number (from Appendix A, Table 3-3) is located on the horizontal axis, and the clinical sensitivity (open circle) and associated 95 percent confidence intervals (thin vertical lines) are shown on the vertical-axis. Only four of these studies are used in computing the revised estimates (bolded circles and thick vertical lines). In three instances, the estimates are revised (studies 3,4 and 9), and both the original (thin) and revised (thick) estimates are provided. The horizontal dashed lines indicate the overall revised consensus estimate of the clinical sensitivity (bold horizontal line) and 95 percent confidence intervals (thin horizontal lines).

Clinical Specificity in Other Racial/Ethnic Groups

Hispanic Caucasians There is limited genotype information for Hispanic Caucasians with a clinical diagnosis of HHC. One study from Mexico (Ruiz-Arguell *et al.,* 2000) identified six individuals, none of whom were homozygous for C282Y (two were heterozygotes).

Blacks/African Americans There is limited genotype information for Blacks/African Americans with a clinical diagnosis of HHC. One study from Zimbabwe (Gangaidzo *et al.,* 1999) identified 14 suspected cases of HHC by autopsy. None of the 28 chromosomes carried a C282Y mutation.

Asians/Asian Americans There is limited genotype information for Asians/Asian Americans with a clinical diagnosis of HHC. Two studies (Tsui *et al*, 2000 and Shiono *et al*., 2001) identified 12 and 11 cases with clinical findings suggestive of HHC. None of the 46 chromosomes studies carried a C282Y mutation.

Appendix A

Table 3-3. Studies Reporting Frequencies of the C282Y Homozygous Genotype in Non-Hispanic Caucasians in the United States with Primary Iron Overload.

Study Number	Author & Date	Number of Cases[a]	C282Y Homozygote Frequency (%) In Cases (95% CI)
1	Press et al., 1998	37	32 (18-50)
2	Bartolo et al., 1998	15	33 (12-62)
3	Barton et al., 1997	74	60 (47-71)
4	Sham et al., 2000	123	60 (51-69)
5	Barton et al., 2000	94	64 (53-73)
6	Sham et al., 1997	61	67 (54-79)
7	Beutler et al., 1996	147	82 (74-88)
8	Feder et al., 1996	178	83 (77-88)
9	Brandhagen et al., 2000	82	85 (76-92)
10	Bacon et al., 1999	66	91 (81-97)
ALL		877	69 (64-73)

χ^2 test for heterogeneity = 155, $p < 0.001$

[a] Definitions of cases
Study 1 = liver biopsy with hepatic stainable iron of 2+
Study 2 = liver biopsy with elevated hepatic stainable iron
Study 3 = Elevated TS at least twice in the absence of other known causes of IO.
Study 4 = Ranges from elevated TS and serum ferritin to confirmation by liver biopsy or therapeutic phlebotomy
Study 5 = Elevated TS at least twice in the absence of other known causes of IO.
Study 6 = Ranges from elevated TS and serum ferritin to confirmation by liver biopsy or therapeutic phlebotomy
Study 7 = Ranges from elevated TS and serum ferritin to confirmation by liver biopsy or therapeutic phlebotomy.
Study 8 = At least 2 of 4 IO criteria (HIC >4,500 ug/g, HII >2.0, 3-4+ stainable iron, >4g mobilizable iron).
Study 9 = Liver biopsy or quantitative phlebotomy (HII >1.9 or removal of >5g mobilizable iron).
Study 10 = Liver biopsy with 3-4+ hepatic stainable iron or HII >1.9 or HLA identity to a proband.

Reference numbers used for Tables 3-2 and 3-3 and Figure 3-1.

1. Press et al., 1998
2. Bartolo et al., 1998
3. Barton et al., 1997
4. Sham et al., 2000
5. Barton et al., 2000
6. Sham et al., 1997
7. Beutler et al., 1996
8. Feder et al., 1996
9. Brandhagen et al., 2000
10. Bacon et al., 1999

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Sham RL, Raubertas RF, Braggins C, Cappuccio J, Gallagher M, Phatak PD. 2000. Asymptomatic hemochromatosis subjects: genotypic and phenotypic profiles. *Blood* **96:**3707-3711.

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Question 19: How often is the test negative when the disorder is not present?

Summary

- Clinical specificity is estimated to be 99.6 percent and is dependent upon
  - The proportion of homozygotes who develop the iron overload phenotype
  - The analytic specificity and the impact of subsequent confirmatory testing
- Clinical specificity is likely to be somewhat higher in other racial/ethnic groups, mainly because few C282Y homozygous individuals occur in these populations. Based on allele frequencies the estimates are:
  - 99.8% among Hispanic Caucasians (allele frequency of 2.0%, N=2,778, 4 studies)
  - 99.8% among Black/African Americans (allele frequency of 1.0%, N=3,572, 5 studies)
  - 99.8% among Asians/Asian Americans (allele frequency of 0.034%, N=1,489, 6studies)

Definition of clinical specificity

Clinical specificity refers to the proportion of individuals who do not have, and are not destined to develop, the primary iron overload phenotype (B+D from Table 3-1) and have a negative test result for C282Y homozygosity (B from Table 3-1). An alternative way to view clinical specificity is to consider the clinical false positive rate (1-clinical specificity). Individuals with clinical false positive results are C282Y homozygotes who do not ever develop serious clinical manifestations (in the absence of treatment), whether or not there is biochemical evidence of iron overload. Clinical false positives will usually be due to incomplete penetrance of the genotype, but analytic errors might also lead to occasional misclassification as a C282Y homozygote (i.e., analytic false positive).

Table 3-1. A Two-by-Two Contingency Table for Deriving the Four Major Clinical Performance Parameters (reprinted for convenience)

	Clinically Manifest Primary Iron Overload*		
	Yes	**No**	**Totals**
C282Y Homozygosity			
Yes	A	B	A+B
No	C	D	C+D
Totals	A+C	B+D	A+B+C+D

* primary iron overload of adult onset associated with significant morbidity

Estimating clinical specificity

As described in the previous section, determining the clinical specificity of C282Y homozygote screening to identify the primary iron overload phenotype requires data on both clinical and analytic false positives. As with estimating clinical sensitivity (Question 18), the ideal study design would involve genotyping a general population cohort and collecting long term follow-up information. Such a study is unlikely to be performed because of the cost, lengthy follow-up and biases introduced by routine medical care. Existing case-control and population genotyping studies provide an indirect method to estimate clinical specificity by determining the frequency

of C282Y homozygosity in an unbiased sampling of the general healthy population in the United States. The rate of homozygosity in the general population can then be used to place limits around the false positive rate. For example, if the rate of homozygosity were found to be 50 per 10,000, then the false positive rate must be no more than 0.5 percent (assuming 0% penetrance and no analytic false positive results). If the penetrance were 10 percent, then the false positive rate would be 0.45 percent (5 of the 50 are true positives, the remaining 45 are false positives). Although this methodology is crude, it is sufficient to provide for modeling the positive and negative predictive values. Limitations of existing published data include:

- it is not possible to determine the racial/ethnic distribution in some studies (many provide race but few stratify by ethnicity)
- some studies may not reflect the general population
- the clinical specificity is likely to be underestimated using this approach, because some homozygous individuals who are destined to develop the phenotype of interest are likely to be "healthy" earlier in life.

Genotype Misclassification - According to the analysis shown earlier (Question 9), the analytic specificity is estimated to be 99.8 percent in the absence of confirmatory testing (i.e., one in 1000 individuals is incorrectly reported as being a C282Y homozygote). This rate is derived from external proficiency testing and, therefore, may not reflect the checks and balances routinely in place in the clinical laboratory. Routine confirmatory procedures might identify and correct many of these errors (Question 14).

***HFE* Allele frequencies in healthy non-Hispanic Caucasians in the U.S.** A total of 18 studies report genotyped Caucasian individuals in the United States. Five of these studies are rejected in the present analysis because they: 1) studied a group that might not represent general population *HFE* frequencies (Bacon et al., 1999, tested only patients at a liver clinic); 2) reported on an earlier published dataset (Koziol *et al.,* 2001 includes data from Beutler *et al.,* 2000); or 3) included data from primarily African American (Barton *et al.,* 2001) or Ashkenazi Jewish populations (Beutler *et al.,* 1997). Table 3-4 lists the remaining 13 reports, sorted by allele frequency. All report frequencies for the C282Y mutation (11 also report H63D mutation frequencies - Appendix B). Genotype frequencies satisfy the Hardy-Weinburg assumption in 12 of the 13 studies. The thirteenth study (Brandhagen *et al*., 2000) was barely statistically significant (chi-squared = 5.0, p=0.03). Raw data from these studies are available in Appendix B. In Table 3-4, exact confidence intervals are computed using the binomial distribution (True Epistat, Texas). According to the DerSimonian & Laird random effects model (Berlin et al., 1989) the overall estimate for C282Y allele frequency is 6.4 percent (95 percent CI 6.0-7.6%). However, there is considerable heterogeneity (chi-squared = 34, $p < 0.001$). When the highest and lowest reports are excluded (Figure 3-4), the allele frequency changes only slightly to 6.8 percent (95 percent CI 6.1 to 6.7%), and the heterogeneity is essentially eliminated. Using the Hardy-Weinburg assumption, this allele frequency of 6.8 percent will yield a homozygous rate of 41/10,000. The final estimate of clinical specificity will need to take into account two additional factors: 1) some of these 41 homozygotes will develop clinical manifestations of iron overload thereby increasing clinical specificity, and 2) analytic false positives will occur at a rate of about 10/10,000, thereby decreasing clinical specificity. These two factors tend to cancel each other out. Under the assumption of a 25 percent lifetime penetrance among homozygotes, clinical specificity would be 99.6 percent. If more than 25 percent of homozygotes developed the iron

overload phenotype, or confirmatory testing reduced the analytic false positive rate, the clinical specificity would increase. The clinical specificity will be higher among males than females, because a greater proportion of homozygous males develop the iron overload phenotype.

Table 3-4. Thirteen Studies Reporting C282Y Allele and C282Y Homozygous Genotype Frequencies in non-Hispanic Caucasians From Healthy Populations in the U.S.

					C282Y	
Study Number	**First Author**	**Year**	**Number Of Study Subjects**	**Source of Study Subjects**	**Allele Freq. (%) (95% CI)**	**Homozygote Freq. (per 10k) (95% CI)**
1	Feder	1996	155	Random/CEPH	3.2 (1.6- 5.9)	0 (0-235)
2	Marshall	1996	100	Hospital patients	5.0 (2.4- 9.0)	100 (25-545)
3	Garry	1997	287	Healthy elderly	6.1 (4.3- 8.4)	0 (0-128)
4	McDonald	1999	1,450	HMO employees	6.1 (5.2- 7.0)	41 (15- 90)
5	Press	1998	127	Blood donors	6.3 (3.6-10.0)	0 (0-286)
6	Beutler	2000	7,864	Health appraisal clinic	6.3 (5.9- 6.7)	48 (34- 66)
7	Steinberg	2001	2,016	General population	6.4 (5.6- 7.1)	30 (11- 65)
8	Bartolo	1998	23	Unspecified	6.5 (1.4-17.9)	0 (0- 15)
9	Bradley	1998	1,001	Pregnant couples	6.6 (5.6- 7.8)	70 (28-144)
10	Beutler	1996	193		7.5 (5.1-10.6)	0 (0-189)
11	Barton	1997	142	Random recruits	7.7 (4.9-11.5)	70 (20- 39)
12	Brandhagen	2000	81	Blood donors	8.0 (4.3-13.3)	247 (30-864)
13	Barton	2000	132	General population	14.4 (10-19.2)	0 (0- 28)
ALL			13,571		6.8 (6.0-7.6)*	45 (34- 58)*
Final Consensus					**6.4 (6.1- 6.7)****	**41 (37- 45)****

χ^2 test for heterogeneity = 3.4, p = 0.6

* The weighted average

** The final consensus estimate for the allele frequency was computed using a random effects model and after trimming two outlying studies (numbers 1 and 13). The final consensus estimate for the rate of homozygosity is estimated using the 6.4 percent allele frequency and assumes Hardy-Weinberg equilibrium.

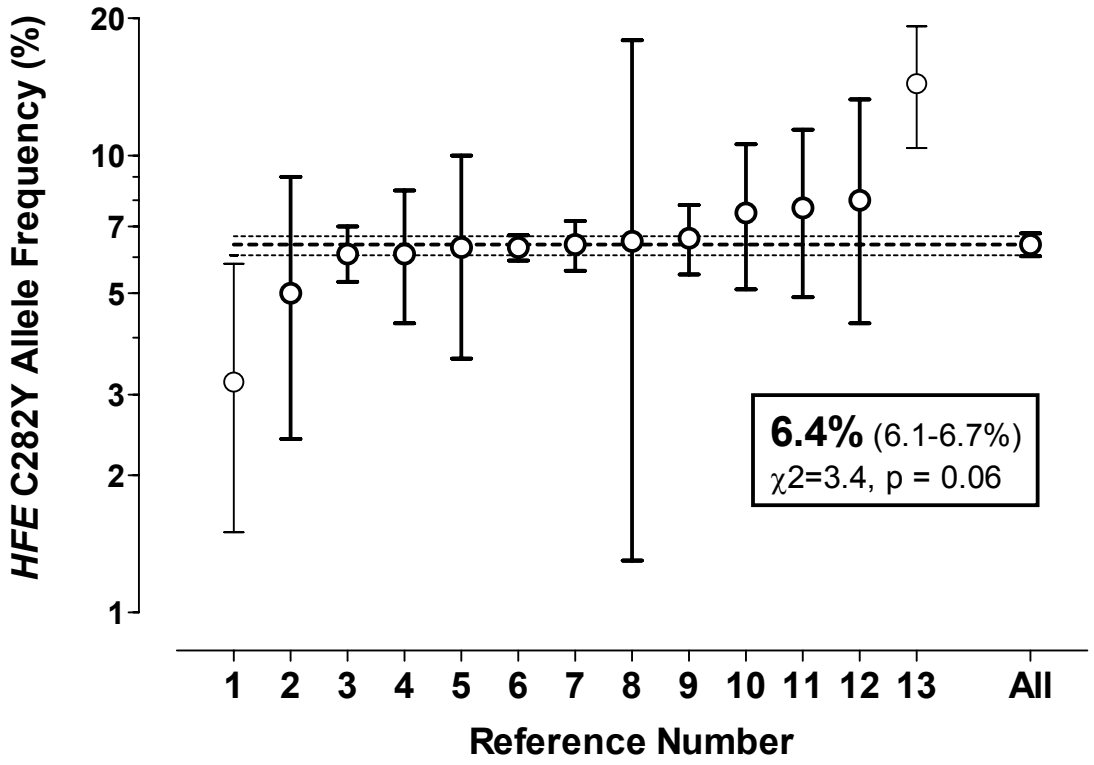

Figure 3-2. *HFE* C282Y Allele Frequency in 13 Studies of Healthy Non-Hispanic Caucasians in the U.S. The figure graphically represents the 13 studies in Table 3-3. These are arranged from lowest to highest allele frequency (vertical axis). The allele frequency is indicated by an open circle, and the 95 percent confidence intervals are shown by thin vertical lines. The respective study number from Table 3-3 is indicated on the horizontal axis. The horizontal dashed bold line indicates the overall consensus, and the vertical dashed thin lines indicate the 95 percent confidence intervals. The studies are highly heterogeneous, due mainly to the effect of two studies (one high estimate and one low estimate). If these two observations are excluded, the overall estimate of allele frequency is only slightly lower (6.4 versus 6.8%), and the 11 remaining studies are, essentially, homogeneous.

References for Table 3-4 and Figure 3-2

1. Feder 1996
2. Marshall 1996
3. Garry 1997
4. McDonald 1999
5. Press 1998
6. Beutler 2000
7. Steinberg 2001
8. Bartolo 1998
9. Bradley 1998
10. Beutler 1996
11. Barton 1997
12. Brandhagen 2000
13. Barton 2000

Clinical Specificity in Other Racial/Ethnic Groups

Hispanic Caucasian Individuals Three studies from the United States (Beutler et al., 2000, Marshall et al., 1996 and Steinbert et al., 2001) and one study from Mexico (Ruiz-Arguell et al., 2000) reported the *HFE* mutation status of Hispanic Caucasians (Table 3-5). Among the 2,778 Hispanic Caucasians studied, the C282Y allele frequency was 2.0 percent (95 percent CI 1.3 to 2.8%). A total of five homozygotes were identified (four from study 2 and 1 from study 4). Study 2 did not satisfy the Hardy-Weinberg assumption (χ^2 goodness of fit = 15, $p < 0.001$) and this is probably responsible for the high rate of homozygosity found for that study, and overall (18 per 10,000). A more reliable estimate of the homozygous rate can be made, based on the C282Y allele frequency. Thus, the expected homozygosity rate among Hispanic Caucasians is 4 per 10,000. Again, this provides a starting point for estimating the clinical false positive rate, assuming low penetrance and no analytic false positives. Earlier, the analytic false positive rate was found to be about 20 per 10,000 (analytic specificity of 99.8%); a rate that is five times higher than the rate of C282Y homozygosity in this population. Under the assumption of a 25 percent lifetime penetrance among homozygotes, clinical specificity would be 99.8 percent. If more than 25 percent of homozygotes developed the iron overload phenotype, or confirmatory testing reduced the analytic false positive rate, the clinical specificity would increase.

Table 3-5. Studies Reporting C282Y Allele and C282Y Homozygous Genotype Frequencies in Hispanic Caucasians from the General Population

Study Number	First Author	Year	Number	Source	C282Y Allele Frequency (%) (95% CI)	C282Y Homozygote Frequency Per 10,000 (95% CI)
1	Ruiz-Arguell	2000	153	Blood Donors	1.3 (0.4-3.3)	0 (0-238)
2	Steinberg	2001	1,555	General Population	1.6 (1.2-2.1)	6 (1- 36)
3	Marshall	1996	100	Hospital Patients	2.0 (0.6-5.0)	0 (0-362
4	Beutler	2000	970	Health Clinic	2.7 (2.1-3.6)	41 (11-105)
ALL			2,778		2.0 (1.3-2.8)	18 (6- 42)
Final Consensus					**2.0 (1.3-2.8)**	**4 (1.6–7.8)**

χ^2 test for heterogeneity = 8.2. p = 0.004

Black/African American Individuals Nine studies reported the *HFE* mutation status of Black/African Americans (Table 3-6). Three were from Africa (Roth et al., 1997; Jeffery et al., 1999 and DeVillievs et al., 1998) and only one heterozygote was identified among 667 individuals tested. Of more relevance, were six additional studies from outside of Africa. Four were from the United States (Barton et al., 2001, Beutler et al., 2000, Marshall et al., 1996 and Steinbert et al., 2001). One other study was from France (Aguilar-Martinez et al., 2001) and one from Brazil (Pereira et al., 2001). Among the 3,572 Black/African Americans studied, the C282Y allele frequency was 1.0 percent (95 percent CI 0.8 to 1.3%). Only one homozygote was identified (homozygous rate of 2.8 per 10,000). A more reliable estimate of the homozygous rate can be made, based on the C282Y allele frequency. Thus, the expected homozygosity rate among Black/African Americans is 1 per 10,000. Again, this provides a starting point for estimating the clinical false positive rate, assuming low penetrance and no analytic false positives. Earlier, the analytic false positive rate was found to be about 20 per 10,000 (analytic specificity of 99.8%); a rate that is 20 times higher than the rate of true homozygosity in this population. Under the assumption of a 25 percent lifetime penetrance among homozygotes, clinical specificity would be 99.8 percent. If more than 25 percent of homozygotes developed the iron overload phenotype, or confirmatory testing reduced the analytic false positive rate, the clinical specificity would increase.

Table 3-6. Studies Reporting C282Y Allele and C282Y Homozygous Genotype Frequencies in Black/African Americans from the General Population

Study Number	First Author	Year	Number	Source	C282Y Allele Frequency (%) (95% CI)	C282Y Homozygote Frequency Per 10,000 (95% CI)
1	Roth	1997	370	General Population	0.0 (0.0-0.5)	0
2	Jeffery	1999	97	Healthy Volunteers	0.0 (0.0-1.9)	0
3	De Villievs	1998	200	Unspecified	0.3 (0.0 1.4)	0
4	Pereira	2001	101	Blood Donors	0.5 (0.1-2.7)	0
5	Marshall	1996	56	Hospital Patients	0.9 (0.0-4.9)	0
6	Aguilar-Martinez	2001	171	Neonates	0.9 (0.2-2.5)	0
7	Beutler	2000	371	Health Appraisal	1.1 (0.5-2.1)	0
8	Barton	2001	1373	Newborn/Other	1.1 (0.7-1.6)	0
9	Steinberg	2001	1600	General Population	1.3 (0.9-1.8)	1
4-9			3572		1.0 (0.8-1.3)	2.8 (0.0-16)
Final Consensus					**1.0 (0.8-1.3)**	**1.0 (0.6-1.7)**

For studies 4 through 9: χ^2 test for heterogeneity = 1.3. p = 0.25

Asian/Asian American Individuals Seven studies reported the *HFE* mutation status of Asians/Asian Americans (Table 3-7). Two were from Japan (Shiono *et al*, 2001 and Sohda *et al*., 1999) and the rest were from countries outside of Asia, including the United States. Only one heterozygote was identified among 667 individuals tested. Of more relevance, were six additional studies from outside of Africa. Four were from the United States (Barton et al., 2001, Beutler et al., 2000, Marshall et al., 1996 and Steinberg et al., 2001). One other study was from France (Aguilar-Martinez et al., 2001) and one from Brazil (Pereira et al., 2001). Among the 3,572 Asian/Asian Americans studied, the C282Y allele frequency was 1.0 percent (95 percent CI 0.8 to 1.3%). Only one homozygote was identified (homozygous rate of 2.8 per 10,000). A more reliable estimate of the homozygous rate can be made based on the C282Y allele frequency. Thus, the expected homozygosity rate among Asian/Asian Americans is 1 per 10,000. Again, this provides a starting point for estimating the clinical false positive rate, assuming low penetrance and no analytic false positives. Earlier, the analytic false positive rate was found to be about 20 per 10,000 (analytic specificity of 99.8%); a rate that is 20 times higher than the rate of true homozygosity in this population. Under the assumption of a 25 percent lifetime penetrance among homozygotes, clinical specificity would be 99.8 percent. If more than 25 percent of homozygotes developed the iron overload phenotype, or confirmatory testing reduced the analytic false positive rate, the clinical specificity would increase.

Table 3-7. Studies Reporting C282Y Allele and C282Y Homozygous Genotype Frequencies in Asian/Asian Americans from the General Population

Study Number	First Author	Year	Number	Source	C282Y Allele Frequency (%) (95% CI)	C282Y Homozygote Frequency Per 10,000 (95% CI)
4	Distante	2000	127	Hospital Patients	0.0 (0.0-1.4)	0 (0-140)
3	Rochette	1999	137	Immigrants	0.0 (0.0-1.3)	0 (0-130)
1	Shiono	2001	151	Healthy Volunteers	0.0 (0.0-1.2)	0 (0-120)
7	Cullen	1998	158	Unspecified	0.0 (0.0-1.2)	0 (0-116)
5	Beckman	1997	203	Blood Donors	0.0 (0.0-0.9)	0 (0- 90)
2	Sohda	1999	252	Unrelated Volunteers	0.0 (0.0-0.7)	0 (0- 70)
6	Beutler	2000	445	Health Appraisal	0.2 (<0.1-0.8)	0 (0- 40)
ALL			1480		0.034 (0.001-0.2)	0.0 (0.0- 14)
Final Consensus					**0.034 (0.001-0.2)**	**0.001**

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CLINICAL VALIDITY

Question 20: Are there methods to resolve false positive results in a timely manner?

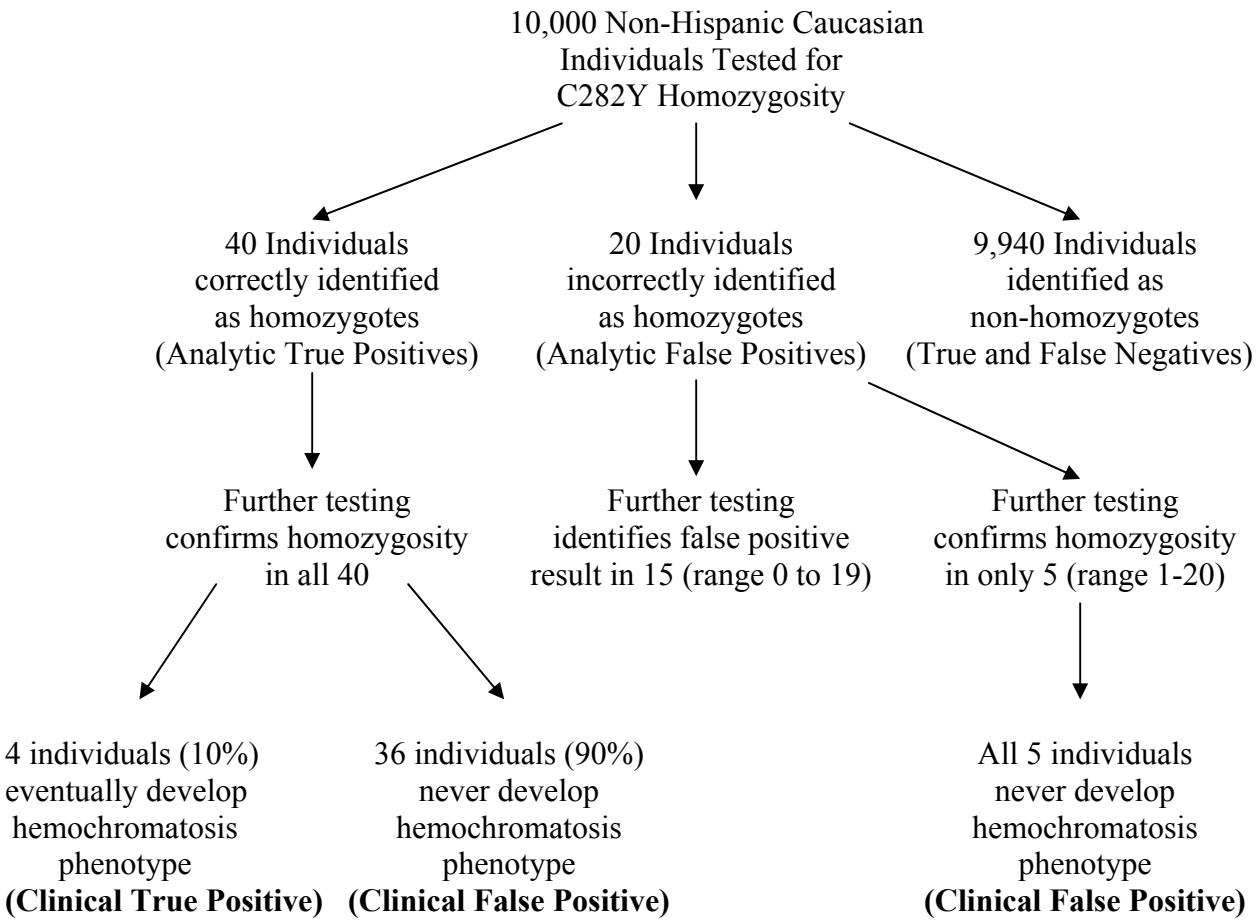
Summary

- Clinical false positives occur when:
  - An individual homozygous for C282Y is correctly genotyped, but that individual will not develop the iron overload phenotype. There are currently no definitive methods to identify which homozygous individuals will develop serious clinical manifestations.
  - An individual who is not homozygous for the C282Y mutation is incorrectly identified as being homozygous by DNA testing. Confirmatory testing may correct this type of false positive in most instances.
- The short term chances of developing serious clinical manifestations can be predicted to some extent by measurements of serum transferrin saturation and serum ferritin**, as follows:**
  - If neither **is** elevated, the likelihood of developing serious manifestations is low
  - If both are elevated, the likelihood of developing serious manifestations **is** increased

In the absence of confirmatory *HFE* testing, the analytic false positive rate for C282Y homozygosity is estimated to be 2 per 1,000 individuals tested in the general population (Question 10 – analytic specificity of 99.8, 95 percent CI 99.4 to 99.9%). Confirmatory testing, on either the same or a second sample, might reduce this rate substantially, though definitive data are not currently available. Approximately 4 per 1000 non-Hispanic Caucasians are homozygous for C282Y, but only some of these will develop serious manifestations. Figure 3-3 shows the relationship between analytic false positives and clinical false positive**s** that might occur in the general population. Among the 100,000 non-Hispanic Caucasians tested, 60 will be identified as homozygotes – 40 of these are true analytic positive results, and 20 are false positive analytic results. The third row shows the impact of subsequent confirmatory testing. The actual impact of confirmatory testing is unknown. The table assumes that virtually all true positives will remain positive (40) but that 75 percent of the false positive test results will be identified and corrected (15). The wide range of false positive results corrected (numbers in parentheses) is a direct consequence of the gap in knowledge about the impact of confirmatory testing. Under these assumptions, 45 homozygotes are confirmed among the 10,000 individuals tested. The fourth row shows the hypothetical outcome among these 45 individuals under the assumption that 10 percent of true positives will develop serious manifestations associated with hemochromatosis (penetrance). According to the figure, most clinical false positives will be among true homozygotes who never develop disease, rather than due to individuals incorrectly classified as being homozygous for C282Y.

Gap in Knowledge: The Extent to Which Confirmatory Testing Identifies False Positive Analytic Test Results. It is not yet known to what extent analytic false positive test results will be correctable because**:** 1) confirmatory testing of homozygous test results is not routinely performed in all laboratories, 2) different types of confirmatory testing will have different rates of identifying errors (e.g., testing the same sample on the same methodology versus testing a new sample on a different methodology) and 3) the types of errors responsible for false positives are not known.

Figure 3-3 Diagram Showing the Causes and Frequencies of Clinical False Positive *HFE* Testing Results.



Subsequent Biochemical Testing. Among individuals confirmed to be homozygous for the C282Y mutation after screening, most do not have current clinical manifestations (As**b**erg et al., 2001; Beutler et al., 2002). However, biochemical testing might be undertaken to determine whether any of the individuals are iron loaded. This can be accomplished by obtaining serum transferrin saturation and serum ferritin measurements. Given what is known about the natural history of hemochromatosis, iron loading is a necessary prerequisite for the development of clinical manifestations (Burke et al., 1998). Virtually all individuals with clinically diagnosed hemochromatosis have elevated transferrin saturation and serum ferritin measurements. Such elevations are almost a prerequisite for establishing the clinical diagnosis, but this cannot be taken as evidence that all individuals with hemochromatosis have elevated values of these analytes. Guidelines have been published providing reasonable cut-off levels (Witte et al., 1996). For example, the transferrin saturation cut-off level is usually between 50 to 60 percent in males and lower in females. The serum ferritin cut-off level is usually 300 or 400 ug/L or higher in males and lower in females. However, given the variability of laboratory assays and methodologies, such cut-off levels often vary from study to study.

Results from Two Published Studies

The results from two cohort studies trials (Burt et al., 1998; McDonnell et al., 1999) are summarized in Table 3-8. These provide further documentation of the usefulness of biochemical testing after genotyping. In both studies, all individuals had both biochemical (at least transferrin saturation) and genetic testing performed. The population was 79 percent female**,** and over 97 percent were non-Hispanic Caucasian. The combined genotype frequency for C282Y homozygosity (44 per 10,000, 95 percent CI 22 to 78 per 10,000) is consistent with the consensus estimates of 41 per 10,000 (Table 3-4). There were 11 C282Y homozygotes identified; 9 of the 11 (82**%**) had persistently elevated transferrin saturation test results. Five of the nine also had elevated serum ferritin, all of whom had additional evidence of iron loading based on diagnostic test results (e.g., liver iron or mobilizable iron – the same definition used in Question 18). C282Y homozygotes accounted for 35 percent of all persistently elevated transferrin saturation results (9/26), 46 percent of all elevated serum ferritin measurements (5/11) and 83 percent of iron loaded individuals (5/6). No individual in the two studies was considered to have clinical evidence of hemochromatosis. Among the six C282Y homozygotes who did not have evidence of iron loading, most were premenopausal women. The two homozygotes with neither elevated transferrin saturation nor elevated serum ferritin measurements are the least likely to develop clinical manifestation in the near future. On the other hand, the five individuals whose serum measurements both are elevated and whose tissue iron measurements indicate iron loading are at the highest risk of developing clinical manifestations.

Table 3-8. Summary of Transferrin Saturation Screening Results and *HFE* Genotypes in Two Population-Based Studies Involving Non-Hispanic Caucasians

HFE **Genotype**	**Number (rate per 10,000)**	**Elevated Transferrin Saturation N (%)[1]**	**Elevated Serum Ferritin N (%)[2]**	**Iron Overload[c] N (%)[3]**
282/282	**11 (44)**	**9 (81.8)**	**5 (45.5)**	**5 (45.5)**
282/63	54 (215)	3 (5.6)	0 (0.0)	0 (0.0)
63/63	74 (294)	2 (2.7)	1 (0.0)	1 (2.0)
282/wild	250 (994)	3 (1.2)	3 (1.2)	0 (0.0)
63/wild	587 (2335)	5 (0.9)	2 (0.3)	0 (0.0)
wild/wild	1538 (6118)	4 (0.3)	0 (0.0)	0 (0.0)
All	2514 (10,000)	26 (1.0)	11 (0.4)	6 (0.2)

[1] Burt *et al*., initial fasting transferrin saturation of 55% or more, repeat of 50.5% or more (97.5th centile of nm/nm). McDonnell *et al*., females ≥50%, males ≥60% on both fasting samples.

[2] Burt et al., serum ferritin of > 160 ug/L (females) or > 300 ug/L (males). McDonnell et al., > 95th sex-adjusted centile (referred to as NHANES III ranges – found to be > 200 for females, > 400 for males).

[3] At least 2 of the following 4 criteria: hepatic iron concentration >4,500 ug/g, hepatic iron index >2.0, 3-4+ stainable iron, removal of at least 4 grams of mobilizable iron

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