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Biological variability of transferrin saturation and unsaturated iron binding capacity

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Abstract

Background—Transferrin saturation is widely considered the preferred screening test for hemochromatosis. Unsaturated iron binding capacity has similar performance at lower cost. However, the within-person biological variability of both these tests may limit their ability at commonly used cut points to detect *HFE* C282Y homozygous patients.

Methods—The Hemochromatosis and Iron Overload Screening (HEIRS) Study screened 101,168 primary care participants for iron overload using transferrin saturation, unsaturated iron binding capacity, ferritin and *HFE* C282Y and H63D genotyping. Transferrin saturation and unsaturated iron binding capacity were performed at initial screening and again when selected participants and controls returned for a clinical examination several months later. A missed case was defined as a C282Y homozygote who had transferrin saturation below cut point (45 % women, 50 % men) or unsaturated iron binding capacity above cut point (150 μ mol/L women, 125 μ mol/L men) at either the initial screening or clinical examination, or both, regardless of serum ferritin.

Results—There were 209 C282Y previously undiagnosed homozygotes with transferrin saturation and unsaturated iron binding capacity testing done at initial screening and clinical examination. Sixty-eight C282Y homozygotes (33%) would have been missed at these transferrin saturation cut points (19 men, 49 women, median SF 170 μ g/L, first and third quartiles 50 and 474 μ g/L), and 58 homozygotes (28 %) would have been missed at the unsaturated iron binding capacity cut points (20

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men, 38 women, median SF 168 µg/L, quartiles 38 and 454 µg/L). There was no advantage to using fasting samples.

Conclusions—The within-person biological variability of transferrin saturation and unsaturated iron binding capacity limit their usefulness as an initial screening test for expressing C282Y homozygotes.

Introduction

The diagnosis of hemochromatosis was previously based on a combined clinical and laboratory assessment that included history and physical examination, elevated transferrin saturation and serum ferritin, liver biopsy, iron removed by phlebotomy, and pedigree studies identifying other family members with iron overload¹. Since the discovery of the hemochromatosis gene (*HFE*) in 1996² most studies from referral centers have shown that > 90 % of typical hemochromatosis patients are homozygous for the C282Y mutation of the *HFE* gene³. Prior to DNA-based testing, it was assumed that most hemochromatosis patients have elevated transferrin saturation. However, population screening studies have shown that many C282Y homozygotes have a normal transferrin saturation, and may never develop clinical signs and symptoms related to iron overload^{4–9}. Transferrin saturation has been recommended in many studies to be the ideal screening test for hemochromatosis because it is widely available and may be increased even in young adults with a genetic predisposition to hemochromatosis. It has been suggested that transferrin saturation is preferable to DNA-based testing as an initial screening test because it may detect other types of iron overload besides those associated with *HFE* mutations, and iron deficiency. Screening for iron overload with transferrin saturation may reduce the risks of potential genetic discrimination that some authors suggest is associated with identification of a C282Y homozygote with normal serum iron tests^{10–12}. Important characteristics of a screening test are its reproducibility over time and diagnostic sensitivity.

In this study we sought to determine the variability of transferrin saturation and unsaturated iron binding capacity as well as the impact on their use as a practical and sensitive screening test for hemochromatosis.

Methods

The study design and overall results of the Hemochromatosis and Iron Overload Screening Study have been previously reported^{13–14}. Participants were recruited from five Field Centers that serve ethnically and socio-economically diverse populations. The study recruited all participants ≥ 25 years of age who gave informed consent and was approved by all local IRBs. All participants had random testing for serum unsaturated iron binding capacity, serum iron, serum ferritin (without intentional fasting) and were genotyped for the C282Y and H63D mutations of the *HFE* gene. In this analysis, participants that reported a previous diagnosis of hemochromatosis or iron overload (treated or untreated), were excluded because of the potential effects of phlebotomy or other interventions on the serum transferrin saturation and unsaturated iron binding capacity.

Transferrin saturation was calculated using the serum iron / (serum iron + unsaturated iron binding capacity) and expressed as a percentage. Serum iron and unsaturated iron binding capacity were measured using a ferrozine based colorimetric assay (Hitachi 917 analyzer, Roche Diagnostics/Boehringer Mannheim Corp., Indianapolis, IN). Initial transferrin saturation samples from field centers located in the US were tested at the University of Minnesota Medical Center -Fairview, Minneapolis, MN and those from London, Ontario were tested at MDS Laboratory Services, Toronto, Canada. Initial samples from Toronto, Ontario (n = 2,000) and all follow up Canadian samples were tested in Minneapolis. Method biases were assessed three times yearly using external proficiency testing samples provided by the

College of American Pathologists Surveys (Northfield, IL) and using blind replicate samples that were collected from 2% of all participants and analyzed in both laboratories. In addition, comparisons between MDS Laboratory Services and the Central Laboratory were conducted before starting the testing, and 2% of the MDS samples were repeated at the Central Laboratory throughout the study. Internal quality control pools at normal and high iron levels were included with each analytical batch and methods were calibrated using Roche calibrator materials and instructions.

HFE C282Y and H63D were detected in DNA obtained from whole blood ethylenediaminetetraacetic acid (EDTA) samples using a modification of the Invader assay (Third Wave Technologies, Madison, WI) that increases the allele-specific fluorescent signal by including 12 cycles of locus-specific polymerase chain reaction before the cleavage reaction¹³.

Transferrin saturation and unsaturated iron binding capacity were performed at initial screening and again when selected participants and controls returned for a clinical examination several months later. Clinical examinations were performed on participants with an elevated transferrin saturation and ferritin, all C282Y homozygotes, and control participants (matched for age, gender and race) without *HFE* mutations and normal transferrin saturation and ferritin (n = 2,285). Initial screening specimens were obtained randomly throughout the day (i.e., without intentional fasting); samples for transferrin saturation and unsaturated iron binding capacity measurements at clinical examination were obtained after fasting (mean time since last meal 13 hours). This study represents modeling based on the HEIRS data; the actual cut points and detection of C282Y homozygotes have been previously reported¹⁴.

At cut points for transferrin saturation and unsaturated iron binding capacity (transferrin saturation > 45 % for women, > 50 % for men, unsaturated iron binding capacity is < 150 $\mu\text{mol/L}$ in women, < 125 $\mu\text{mol/L}$ % in men), the potential number of missed C282Y homozygotes was modeled at initial screening and at the clinical examination (Figure 3). A missed case was defined as a C282Y homozygote who had transferrin saturation below cut point or unsaturated iron binding capacity above cut point at either initial screening or at the clinical examination, or both, regardless of serum ferritin. This assumes that for an ideal screening test, a case will have a positive test at the time of each test, and a non-case will have a negative test at the time of each test. Missed cases were expressed as patients missed divided by total patients, rather than total number of tests.

Control participants for the clinical examination had neither C282Y or H63D *HFE* mutations detected and a transferrin saturation between the 25th and 75th percentile. This selection criteria for the controls results in an apparent “gap” in the transferrin saturation distribution around 40 % (Figure 1).

Fasting and non-fasting samples were compared using Receiver Operating Characteristic (ROC) curve analysis and comparisons of area under the curve.

Results

The HEIRS Study recruited 101,168 participants from February 2001- February 2003. There were 1,261 participants (97 C282Y homozygotes) excluded from this analysis, 1,216 because of a previous diagnosis of hemochromatosis or iron overload and an additional 45 with a missing unsaturated iron binding capacity. There were 236 undiagnosed C282Y homozygotes in this analysis (91 men, 145 women). Non-C282Y homozygotes included 37,004 men and 62,667 women. The median age of all participants in this study was 50 years (range 25 – 100). By self-identified race/ethnicity, the sample included 44 % Caucasian, 27 % African-American, 13 % Asian, 13 % Hispanic, 0.7 % Pacific Islander, 0.7 % Native American and 2

% mixed or unknown race. The C282Y homozygotes were 94 % Caucasian. There were 5 Hispanic and 3 African American C282Y homozygotes. An elevated serum ferritin was found in 86 % of the male C282Y homozygotes ($> 300 \mu\text{g/L}$) and in 58 % of the female homozygotes ($> 200 \mu\text{g/L}$).

Analytical variation estimated from a sample of blind replicates at the initial screening visit comprised 1.3% of the total variation in transferrin saturation and 4.4% of the total variation in unsaturated iron binding capacity, and the correlation between replicates was 0.98 for transferrin saturation and 0.99 for unsaturated iron binding capacity. Figures 1 and 2 display substantial variability from the initial screening to clinical exam visit among both C282Y homozygotes and non-C282Y homozygotes. Since the analytic variability is a small percentage, the observed visit-to-visit variability is primarily within-person biological variability. The correlation between initial screening and exam visit values among non-C282Y homozygote men was 0.47 for transferrin saturation and 0.58 for unsaturated iron binding capacity; the corresponding values for C282Y homozygote men were 0.62 and 0.49 respectively. Results for women and estimates from the subset who were fasting at both visits were similar.

There were 209 previously undiagnosed C282Y homozygotes with transferrin saturation and unsaturated iron binding capacity testing done at initial screening and at the clinical examination (Figures 1 and 2). The number of missed homozygotes at a clinically relevant cutpoint for transferrin saturation and unsaturated iron binding capacity was assessed (transferrin saturation $> 45\%$ women, transferrin saturation $> 50\%$ men, unsaturated iron binding capacity < 150 , $< 125 \mu\text{mol/L}$ men). Sixty-eight C282Y homozygotes (33%) would have been missed at these transferrin saturation cut points (19 men, 49 women, median serum ferritin at initial screening = $170 \mu\text{g/L}$, first and third quartiles 50 and $474 \mu\text{g/L}$), and 58 homozygotes (28 %) would have been missed at the unsaturated iron binding capacity cut points (20 men, 38 women, median serum ferritin at initial screening = $168 \mu\text{g/L}$, quartiles 38 and $454 \mu\text{g/L}$). The percentage of missed homozygotes increases as the transferrin saturation cutpoint increases or the unsaturated iron binding capacity cutpoint decreases. Forty-nine percent of transferrin saturation values increased and 55 % of unsaturated iron binding capacity values decreased with the second fasting sample.

A sub-analysis was done which excluded participants initially tested at MDS Laboratories to remove all inter-laboratory variability. All of these participants had their testing done at the Minneapolis site ($n = 126$ homozygotes). In this sub-analysis, the percentage of missed homozygotes by transferrin saturation (34 %) and unsaturated iron binding capacity testing (29 %) did not differ from the larger sample ($n = 199$ homozygotes).

The random testing included 29,994 participants who did provide fasting samples. The sensitivity and specificity of a fasting transferrin saturation and unsaturated iron binding capacity (> 8 h since eating) compared to a non-fasting transferrin saturation and unsaturated iron binding capacity for the detection of C282Y homozygotes ($>45\%$ women, $> 50\%$ men) is shown in Table 1. The area under the ROC curves (AUC) were compared for men and women using fasting and non-fasting samples. For men, the AUC for fasting samples was 0.96 (0.92 – 1.0, 95 % confidence interval) and for non-fasting samples was 0.93 (0.89 – 0.97). For women, the AUC for fasting samples was 0.89 (0.83 – 0.95) and for non-fasting samples was 0.91 (0.87 – 0.95). There were no significant differences between fasting and non-fasting samples.

Discussion

Many advisory documents on screening for hemochromatosis recommend that the initial screening test should be the transferrin saturation^{6,10,15,16}. This is based on the assumption

that most iron loaded hemochromatosis patients will have elevated transferrin saturation. Screening with a phenotypic test will detect primarily iron loaded cases requiring treatment regardless of genotype and will also detect iron deficiency. The initial studies on transferrin saturation were performed in tertiary referral centers and subsequent population based studies demonstrated a much lower sensitivity of transferrin saturation for the detection of C282Y homozygotes^{4,17}. Furthermore, elevated transferrin saturation has been often embedded in the case definition which does not allow for an independent evaluation. The unsaturated iron binding capacity has been shown in population studies to be similar in screening performance to the transferrin saturation and can be performed at a lower cost¹⁷. However, both of these tests are subject to analytical and biological variability which limit their utility as screening tests for C282Y-linked hemochromatosis. In this study we have demonstrated that at a clinically relevant cutpoint for transferrin saturation and unsaturated iron binding capacity, initial phenotyping with transferrin saturation or unsaturated iron binding capacity could miss approximately 30 % of cases and 40 % of these missed cases had an elevated ferritin. Therefore, it is not simply a matter of non-expressing cases being missed by phenotypic screening.

Within-person biological variability in iron tests has been previously described and diurnal fluctuations have been described primarily for serum iron^{18–22}. Transferrin saturation is a calculated value determined from the serum iron divided by one of the following: total iron binding capacity, unsaturated iron binding capacity plus serum iron, or serum transferrin multiplied by a constant. The higher variability in transferrin saturation compared to unsaturated iron binding capacity may be related to the fact that it is a two step test rather than the single step unsaturated iron binding capacity test. It has been reported that most C282Y homozygotes have persistent elevations in transferrin saturation and false positive tests in non-homozygotes would likely return to normal on the second test²³. This was the rationale for two transferrin saturation tests (first random, second test fasting) before proceeding to more diagnostic tests including DNA-based testing or liver biopsy. However, this study and other previous studies have failed to confirm the added value of fasting iron tests compared to random iron tests^{18,22} and variability in this study was similar between homozygotes and non-homozygotes. Fasting adds a level of complexity and inconvenience to a screening program. As illustrated in this study, the second fasting value is as likely to increase as decrease and regression to the mean is the most likely explanation. Any biochemical test with such wide biological variation is unlikely to be an ideal screening test. In this study, we assume that most of the observed variability was biological rather than analytical based on our laboratory analysis of blind replicate samples.

If the goal of a screening project is to detect C282Y homozygotes (regardless of expressivity), it would seem most advisable to utilize the DNA-based test directly, rather than to perform indirect iron tests to guide the definitive DNA testing. Large population studies in several countries have demonstrated that documented instances of genetic discrimination are essentially nonexistent and the genotypic *HFE* test is well accepted by participants without long term psychosocial consequences^{24–26}. The cost of the DNA-based test can be relatively modest because it only tests for a single mutation and, given the large sample volumes, can be performed on an automated instrument platform.

Determining the value of screening iron tests for the detection of non-*HFE*-related iron overload was outside the focus of the present analyses, but other studies have suggested that transferrin saturation is more commonly elevated in C282Y-linked than in non-*HFE* hemochromatosis¹. The latter group of conditions are relatively uncommon and phenotypically and genetically heterogeneous; many of the associated mutations are rare. As a consequence, it is unlikely that genetic tests for non-*HFE* hemochromatosis-associated genes will become commercially available. Therefore, assessment of non-*HFE* linked hemochromatosis will likely depend on phenotypic testing and clinical assessment. The design of this study does not

allow for an assessment of the operating characteristics of transferrin saturation and unsaturated iron binding capacity for the assessment of iron overload, because this would require a study in which all participants, including those with normal screening transferrin saturation and unsaturated iron binding capacity, would undergo quantitative phlebotomy or liver biopsy. Similarly, estimation of the degree of iron overload by means other than measurement of serum ferritin concentration was beyond the scope of the HEIRS Study.

In summary, both the transferrin saturation and unsaturated iron binding capacity have significant within-person biological variation in C282Y homozygotes discovered through a primary care screening program. This limits their utility as ideal screening tests for HFE-associated hemochromatosis. Other screening approaches such as *HFE* genotyping followed by measurement of serum ferritin require further evaluation.

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Figure 1.

A comparison of the transferrin saturation at initial screening (random) and at the clinical examination (fasting) in all participants recalled for a clinical exam (●= C282Y homozygotes, ○ = non-C282Y homozygote, n = 2,145). The apparent gap around 40 % is related to the requirement of control participants to have a transferrin saturation between the 25th and 75th percentile.

Figure 2.

A comparison of the unsaturated iron binding capacity at initial screening (random) and at the clinical examination (fasting) in all participants recalled for a clinical exam (● = C282Y homozygotes, ○ = non-C282Y homozygote, n = 2,145).

Figure 3.

A comparison of the lowest transferrin saturation and serum ferritin in male (○) and female (●) C282Y homozygotes that would have been missed at the cutoffs of TS < 45 % for women and < 50 % for men at initial screening or at the clinical examination). The serum ferritin represents the serum ferritin at initial screening.

Table 1
The effect of fasting on detection of C282Y homozygotes by transferrin saturation and unsaturated iron binding capacity.

	Non- homozygote	C282Y homozygote	Sensitivity (%; 95 % CI)	Specificity (%; 95 % CI)	PPV (%; 95% CI)
Fasting TS normal *	29495	16			
Fasting TS elevated *	2301	53	76.8 (64.8, 85.8)	92.8 (92.5, 93.0)	2.3 (1.7, 3.0)
Fasting UIBC normal	29994	14			
Fasting UIBC decreased **	1802	55	79.7 (68.0, 88.1)	94.3 (94.1, 94.6)	3.0 (2.3, 3.9)
Non-Fasting TS normal	60895	40			
Non-Fasting TS elevated	3335	119	74.8 (67.2, 81.2)	94.8 (94.6, 95.0)	3.5 (2.9, 4.1)
Non Fasting UIBC normal	61281	33			
Non Fasting UIBC decreased	2949	126	79.3 (71.9, 85.1)	95.4 (95.2, 95.6)	4.1 (3.4, 4.9)

Fasting refers to a blood sample drawn at least 8 hours after eating. Participants with missing values for 'hours since last food' were excluded.

PPV = positive predictive value for detecting a C282Y homozygote.

- * An elevated TS was > 45 % for women, > 50 % for men
- ** A decreased UIBC is < 150 µmol/L in women, < 125 µmol/L % in men

TS = transferrin saturation
UIBC = unsaturated iron binding capacity