

Reference Centiles for Serum Ferritin and Percentage of Transferrin Saturation, with Application to Mutations of the *HFE* Gene

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Background: The gene that causes most cases of hereditary hemochromatosis is designated *HFE*. Individuals with mutations in the *HFE* gene may have increased serum iron, transferrin saturation, and ferritin concentrations relative to individuals with the wild-type genotype.

Methods: We generated reference centiles for percentage of transferrin saturation and serum ferritin concentrations in normal (wild-type), healthy Caucasian adults. We then examined transferrin and ferritin concentrations relative to these centiles in 81 individuals homozygous for the major hemochromatosis mutation C282Y and 438 individuals with the compound heterozygous *HFE* genotype C282Y/H63D.

Results: Serum ferritin concentrations, but not percentage of transferrin saturation, in normal, healthy women tended to increase sharply as they progressed through menopause. Transferrin and serum ferritin centiles for normal, healthy females were lower than the corresponding centiles in normal, healthy males. C282Y homozygotes had abnormally high transferrin saturation and serum ferritin values relative to the wild types. Compound heterozygotes appeared to be a mixture of individuals with unexceptional transferrin and ferritin values and those with abnormally large values similar to the homozygotes, with equal proportions of each.

Conclusions: There are age- and sex-related differences in reference centiles for the percentage of transferrin saturation and serum ferritin concentrations in normal, healthy adults. Individuals homozygous for the C282Y mutation in the *HFE* gene have abnormal transferrin saturation and serum ferritin values relative to the

reference population; penetrance with the compound heterozygotes, as reflected by abnormal transferrin and ferritin values, is less than with the homozygotes.

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The development of reference norms for hematologic variables is useful to characterize the health status of a population and to assess individual patients' values relative to a reference population. For example, clinical laboratories commonly report results from various tests or assays together with reference intervals of the variables, within which values for normal (wild-type), healthy individuals might be expected to fall. That a particular value falls outside the reference interval might well be of high relevance and utility to clinicians for purposes of diagnosis or therapy.

Operationally, a reference interval encompasses 95% of the values in the reference population of interest. Reference norms are often presented in the form of centiles: the *n*th centile is the value below which *n* percent of individuals in the reference population fall. Given reference centiles for an individual variable from a "normal" population, comparison of a patient's value for that variable relative to the reference centiles may well be more informative than merely establishing whether that patient is or is not within the (95%) reference interval. (In this regard, the model used to calculate the reference centiles can also be used to calculate the centile for an individual measurement.)

In this report, we generate age-related reference centiles for percentage of transferrin saturation and serum ferritin concentrations from 7250 adults who attended a health appraisal clinic in San Diego. These individuals had consented to DNA examination for hemochromatosis and to participate in an ongoing clinical study, the purposes of which are to determine the gene frequency of the most common mutations of the *HFE* gene that causes most cases of hereditary hemochromatosis and to relate genotypes to various clinical and laboratory variables (1). We demonstrate the usefulness of these reference centiles

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determined from normal (wild-type *HFE* gene) individuals by examining the percentage of transferrin saturation and serum ferritin values for 81 individuals previously undiagnosed for hemochromatosis who were homozygous for the major hemochromatosis mutation, C282Y, and for 438 individuals with the compound heterozygous *HFE* genotype C282Y/H63D.

Materials and Methods

PATIENTS

Patients were recruited as described previously (1) from the Kaiser Permanente San Diego Health Appraisal Clinic, which is available to all Kaiser Health Plan members who wish to have a general health assessment. Beginning in March 1998, all patients older than 24 years who were registered with the clinic were apprised of a research project in which analysis of DNA for mutations of *HFE* and measurement of serum ferritin would be added to the tests usually performed. The study was approved by the Institutional Review Boards of Kaiser Permanente and Scripps Clinic. Through December 2000, there were ~32 000 patients who consented to participate in the study and from whom *HFE* genotype data and demographic data had been obtained. For the purposes of constructing reference centiles, we limited consideration to Caucasian patients with no detectable mutations (i.e., wild type) of the *HFE* gene (17 000 individuals) and who appraised their health status as allowing full activity (the alternative being activity limited to some degree; 3871 males and 3379 females). In addition, we identified 81 individuals (34 males and 47 females) who were homozygous for the C282Y *HFE* mutation and 438 individuals (222 males and 216 females) with the compound heterozygous *HFE* genotype C282Y/H63D; all of these individuals had been previously undiagnosed for hemochromatosis and did not have an unusual number of blood donations.

LABORATORY ANALYSES

Serum iron concentrations and percentage of transferrin saturation were determined at the Kaiser Permanente Regional Reference Laboratory in Burbank, CA. A Hitachi 717 analyzer was used (Hitachi Instruments), with a Roche calibrator for automated systems, Verichem Iron Standard for calibration verification, and Bio-Rad Unassayed Liquichek 1 and 2 and Roche Assayed Precitrol-N and Precitrol-A as controls. The method for iron determination (FE) was Roche Ferrozine without deproteinization, with a CV of <2.0% in repeated within-run precision calculations. The method for total iron-binding capacity was J&S alumina adsorption, with CVs of 3.0% in low-concentration serum pools and 2.5% in high-concentration serum pools in repeated within-run precision calculations. The percentage of transferrin saturation was calculated as: FE/total iron-binding capacity \times 100.

Serum ferritin concentrations were measured at the Kaiser Permanente Regional Endocrinology Laboratory, using an Advia Centaur reagent set (Bayer Diagnostics)

with reagents and calibrators provided by the manufacturer, and Bio-Rad Assayed Tri-level Immunoassay Plus Control as control. The Advia Centaur ferritin assay is a two-site sandwich immunoassay that uses direct chemiluminometric technology with constant amounts of two ferritin antibodies. In repeated within-run precision calculations, the CVs were 1.9–3.6%; in repeated between-run precision calculations, CVs averaged 4.4% for identical samples tested daily for 21–28 days and 6.6% for different levels of controls tested daily for 21–28 days.

With regard to laboratory accuracy, the Kaiser Permanente Regional Reference Laboratory participates in the College of American Pathology Proficiency Testing Survey program, which provides information demonstrating the agreement between measurements achieved at Kaiser against other participating laboratories in the United States that use the same method and instrument system. Results from Kaiser have been consistently acceptable.

Samples were analyzed for the C282Y and other mutations by allele-specific oligonucleotide hybridization using peripheral-blood DNA amplified by PCR assay in a 96-well format (1, 2).

STATISTICAL ANALYSES

We used the LMS method of Cole and Green (3, 4) to generate the age-related reference centiles for transferrin saturation and ferritin. This method assumes that sample values at every age do not necessarily follow a gaussian distribution, but can be transformed to approximate normality via the Box-Cox family of power transformations (5). In this regard, there are three parameters that characterize the Box-Cox family of transformations: the power is λ , the mean is μ , and the coefficient of variation is σ ; the initials of the parameter estimates give the name to the LMS method. L, M, and S are estimated within-age brackets, but are constrained to change smoothly as age changes, i.e., smoothed curves are fitted to the individual L, M, and S estimates to give $L(t)$, $M(t)$, and $S(t)$, where t denotes time (age). Details of the fitting procedure are given in Cole and Green (4).

In brief, the LMS fitting strategy is used to optimize the M curve equivalent degrees of freedom (*edf*) by increasing or decreasing the *edf* until the change in penalized log likelihood is small. Once the M curve is fitted, the process is repeated with the S curve, and then the L curve is fitted. The results we report subsequently use L,M,S values of 1,3,3, respectively, for females, transferrin saturation and males, and serum ferritin; and 1,4,3, respectively, for males, transferrin saturation and females, and serum ferritin. Let y denote the variable of interest (e.g., percentage of transferrin saturation), the distribution of which varies with t . Under the LMS method, the Z score given by:

$$Z = \{[y/M(t)]^{L(t)} - 1\}/[L(t) M(t)]$$

will have approximately a standard gaussian distribution. Furthermore, the 100 α th centile of y at t is estimated by:

$$C_{100\alpha}(t) = M(t)[1 + L(t)S(t)Z_{\alpha}]^{1/L(t)}$$

where Z_{α} is the α percent point of the gaussian distribution. We began with the (untransformed) percentage of transferrin saturation and log ferritin values within each cohort before LMS fitting because these scales are commonly reported and are taken as having near-gaussian distributions. In addition, all percentage of transferrin saturation and log ferritin values in our cohorts were positive valued, so there was no conceptual difficulty in fitting Box–Cox transformations to these datasets. We fit data from males and females separately because our sample sizes were rather large and gender differences might be expected. We used quantile plots to assess graphically the goodness-of-fit of the LMS-transformed data to a gaussian (normal) distribution and considered more formal goodness-of-fit tests as in Royston and Wright (6), with adaptations from Koziol (7, 8).

Results

As described in *Materials and Methods*, using gene sequencing for mutations in the *HFE* gene we identified 3871 Caucasian males (mean age, 57.1 years; SD, 13.2 years; range, 25–92 years) and 3379 Caucasian females (mean age, 56.6 years; SD, 13.4 years; range 25–99 years) whose *HFE* genotype was wild-type/wild-type and who appraised their health status as allowing full activity. In addition, we identified 222 males (mean age, 58.7 years; SD, 13.7 years; range, 26–89 years) and 216 females (mean age, 57.4 years; SD, 13.6 years; range, 26–89 years) with the compound heterozygous *HFE* genotype C282Y/H63D, and 34 males (mean age, 55.8 years; SD, 11.5 years; range, 27–79 years) and 47 females (mean age, 55.8 years; SD, 13.1 years; range, 27–80 years) who were homozygous for the *HFE* mutation C282Y. However, these individuals had not been previously diagnosed with hemochromatosis, nor did they have an unusual number of blood donations.

In Fig. 1 we present reference centiles for percentage of transferrin saturation for the wild-type/wild-type males and females. For both sexes, the median and lower centiles tended to increase slightly with increasing age, whereas the higher centiles tended to decrease slightly. Note that, for both sexes, the centiles were not symmetric around the median. Centiles of percentage of transferrin saturation values in males tended to be uniformly higher than corresponding values in females.

Reference centiles for serum ferritin values for the wild-type/wild-type males and females are presented in Fig. 2. Except for the extreme centiles, the centiles for males tend to be relatively constant with increasing age. On the other hand, the centiles trend upward in females as they progress through menopause. Nevertheless, serum ferritin values for females remain lower than the corresponding values in males for the range of ages depicted.

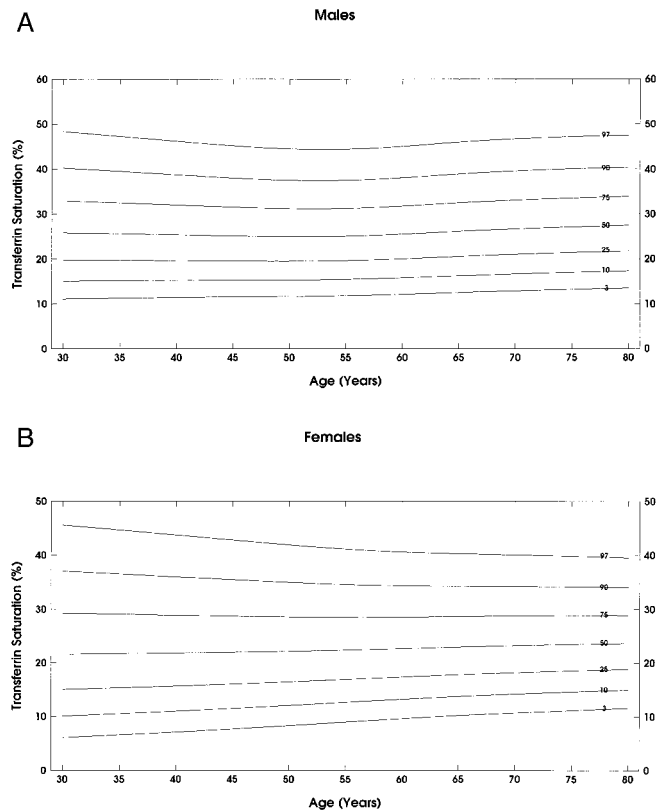


Fig. 1. Seven centiles for percentage of transferrin saturation, derived from a sample of 3871 healthy Caucasian males (A) and 3379 healthy Caucasian females (B) ages 25 years and above, using the LMS method as described in the text.

In Fig. 3, we show quantile plots of the Box–Cox-transformed data compared with an underlying gaussian distribution for the four datasets displayed in Figs. 1 and 2. There is little departure from normality over the central portion of the range of Z values (putatively approximately normally distributed) between -2.0 and 2.0 . Outside this range, however, there is some indication that the tails of the transformed data are somewhat heavier than would be expected under a gaussian distribution. That is, the Box–Cox family of transformations does not necessarily correct for kurtosis in the extreme tails of the observed data.

More formally, we also examined goodness-of-fit with a modification of the Q tests of Royston and Wright (6), i.e., we examined the first four components of a smooth test for univariate normality [according to the method of Koziol (7, 8)], which assess location, scale, skewness, and kurtosis, respectively. From examination of the components of the smooth tests, we found that the Z scores for transferrin saturation in both sexes exhibited high kurtosis, as might be seen in the quantile plots. There was some suggestion that the kurtosis increases with increasing age, but the evidence was weak. There was no indication of disparate values or age dependencies in location, scale, or skewness in any of the sets of Z scores.

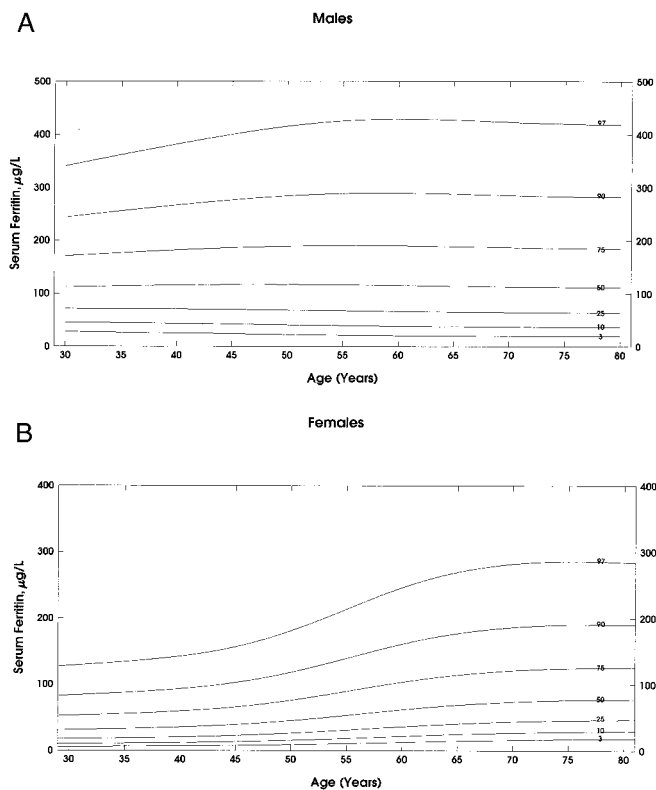


Fig. 2. Seven centiles for serum ferritin, derived from a sample of 3871 healthy Caucasian males (A) and 3379 healthy Caucasian females (B) ages 25 years and above, using the LMS method as described in the text.

Using the LMS fits, we estimated the centiles of percentage of transferrin saturation and serum ferritin concentrations for the 81 individuals homozygous for the *HFE* C282Y mutation and the 438 individuals with the compound heterozygous *HFE* genotype C282Y/H63D, i.e., we estimated values among those with abnormal genotypes relative to the wild-type centiles. We then tabulated the frequency distributions of the estimates; these are displayed in Fig. 4. The distributions for the homozygotes and the compound heterozygotes are clearly distinct, with the distributions for the homozygotes more extreme relative to the reference population than the distributions for the compound heterozygotes, and the percentage of transferrin saturation distributions more extreme relative to the reference population than the serum ferritin distributions. Among the homozygotes, 37 individuals (45.7%) had ferritin Z scores >2.0 and 19 (24.0%) had Z scores >3.0; 51 individuals (63.0%) had percent transferrin Z scores >2.0 and 34 (42.0%) had Z scores >3.0. In contrast, among the compound heterozygotes, 43 individuals (9.8%) had ferritin Z scores >2.0 and only 4 (0.9%) had Z scores >3.0, whereas 84 individuals (19.2%) had percent transferrin Z scores >2.0 and 16 (3.7%) had Z scores >3.0.

Discussion

We constructed reference centiles for the distributions of transferrin saturation and serum ferritin for healthy Caucasian males and females ages 25 and above. The most prominent age-related trend occurs with females, whose serum ferritin values tend to increase sharply as they progress through menopause. This agrees with previous studies (9–11), which typically were based on much smaller samples. In contrast, this trend does not occur with the percentage of transferrin saturation in females, which remains relatively unchanged with increasing age. One might thereby infer that total iron-binding capacity tends to increase proportionately to serum iron in females as they progress through menopause. We did not detect any substantial age-related trends in these variables with males.

There are clear differences between healthy Caucasian males and females in percentage of transferrin saturation and serum ferritin concentrations. The centiles for the percentage of transferrin saturation for females are ~5% lower than the corresponding centiles in males, regardless of age. Similarly, serum ferritin centiles for females are lower than the corresponding centiles in males, but the magnitude of the difference depends on age and which centile is being compared.

The usefulness of reference centiles is demonstrated by considering where patients homozygous for the C282Y mutation of the *HFE* gene and patients compound heterozygous for the *HFE* genotype C282Y/H63D fall with regard to the reference population. Clearly, we can easily distinguish between homozygotes and wild types on the basis of percentage of transferrin saturation or serum ferritin, with transferrin saturation having somewhat larger discriminatory power than serum ferritin. On the other hand, compound heterozygotes appear to be a mixture of individuals with unexceptional values for percentage of transferrin saturation or serum ferritin and individuals with abnormally large values similar to those for the homozygotes, with roughly equal proportions of each (12). A blanket statement that the percentage of transferrin saturation or serum ferritin is increased in compound heterozygotes relative to wild types reflects the contribution of individuals with abnormally large values for percentage of transferrin saturation or serum ferritin, but it obscures the fact that a substantial proportion of compound heterozygotes have percentage of transferrin saturation and serum ferritin values virtually indistinguishable from wild types (13). Penetrance with the compound heterozygotes is less than with the homozygotes.

We note that the reference centiles are not symmetric around the median. This would not be the case had we adopted the usual assumption that the underlying population values for percentage of transferrin saturation or log ferritin followed a gaussian distribution with no variation with age. This assumption would be particularly misleading with serum ferritin values for females, which

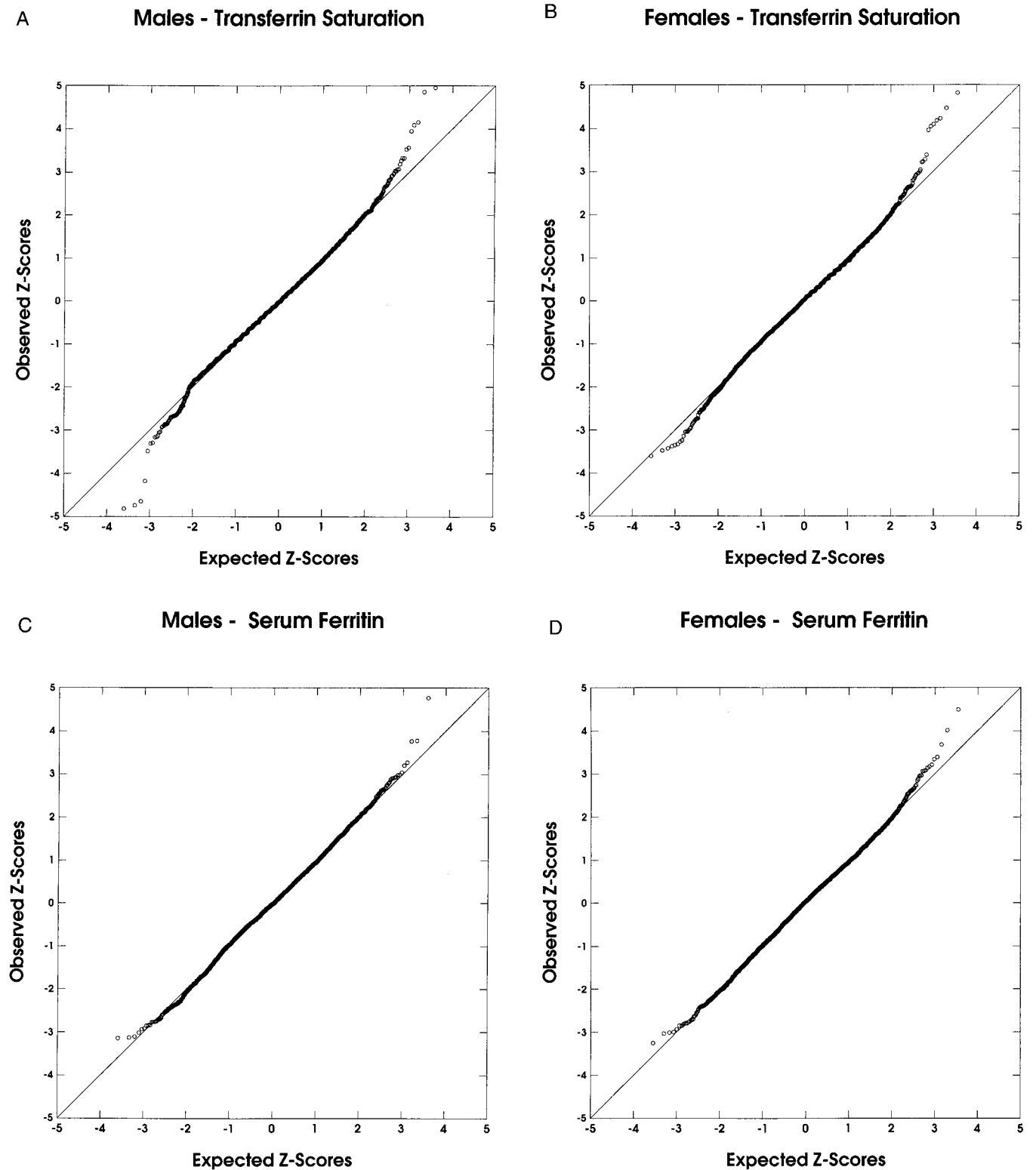
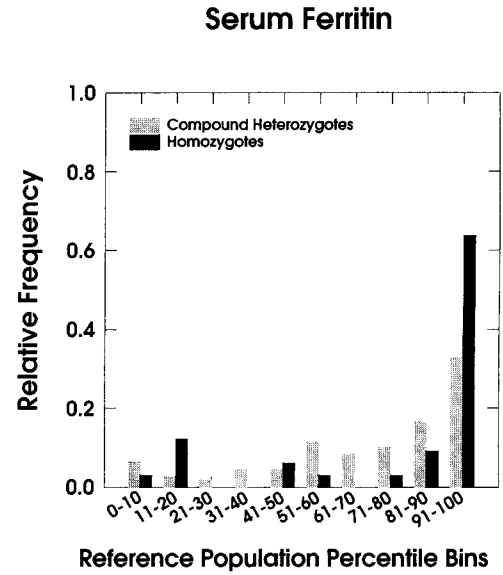
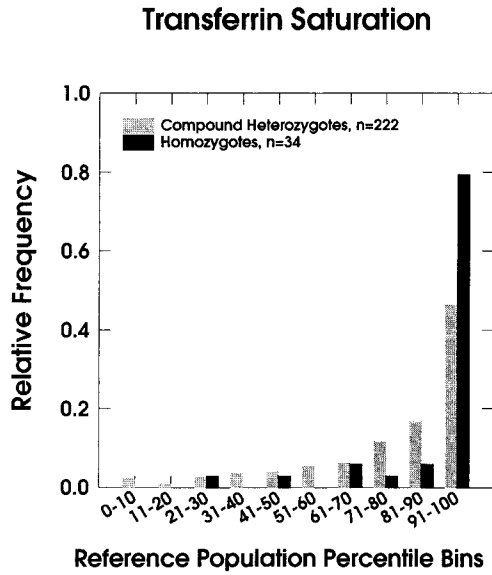


Fig. 3. Quantile-quantile plots of expected vs observed Z scores for the percentage of transferrin saturation data (males, *panel A*; females, *panel B*) and serum ferritin data (males, *panel C*; females, *panel D*) obtained from 3871 healthy Caucasian males and 3379 healthy Caucasian females ages 25 years and above.

The expected Z scores are calculated under the assumption that the LMS-transformed data follow a gaussian distribution; if this assumption holds, then the relationship between the values on the two axes should be linear.

A

Males



B

Females

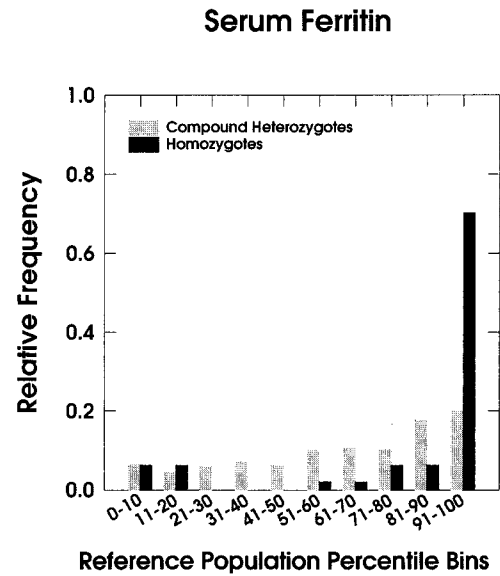
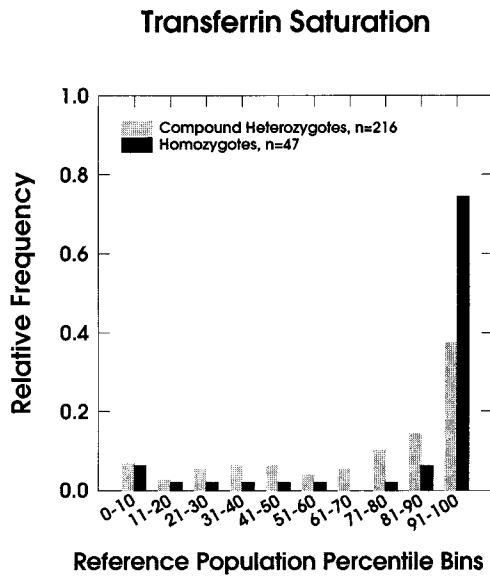


Fig. 4. Relative frequency distributions of the Z scores derived from the percentage of transferrin saturation and the serum ferritin values in 34 males and 47 females homozygous for the C282Y *HFE* mutation and 222 males and 216 females with the compound heterozygous *HFE* genotype C282Y/H63D.

The individual Z scores were converted into centiles with the LMS models that had established the reference centiles from the healthy Caucasian samples. The resulting centiles for the homozygotes and compound heterozygotes were binned into the corresponding reference population percentile intervals; the relative frequencies in these bins are displayed separately for the homozygotes and the compound heterozygotes.

clearly tend to increase as the patients progress through menopause.

There are alternative methods to the LMS method used herein for constructing reference centiles; see Harris and Boyd (14) and Wright and Royston (15) for reviews. An IFCC panel (16) proposed nonparametric and parametric methods to calculate reference intervals for clinical laboratory measurements: the nonparametric procedure uses simple ranking techniques, whereas the parametric method involves transformation when the observations do not follow a gaussian distribution. The Box–Cox family of transformations underlies the LMS method we present here: in the current setting, we would infer from the quantile plots that the LMS method works adequately in the central portion of the distributions of transferrin saturation and serum ferritin, but centiles beyond the lower 3rd percentile or above the 97th percentile would be inaccurate. This is not altogether surprising because the Box–Cox transformations may not necessarily correct for kurtosis in the underlying data.

We caution that our reference values strictly pertain to our reference population, with the laboratory methods we have used. There is ample evidence [e.g., see Refs. (17, 18)] that substantial interlaboratory and intermethod variability exists in the determination of serum ferritin. We have avoided these potential sources of error in our iron determinations by reliance on standardized methodology at one reference laboratory with excellent reproducibility characteristics. Similarly, a key component of the notion of reference centiles is that of the underlying reference population. From the population of individuals attending a health appraisal clinic, we chose to limit consideration to Caucasians, as these accounted for >80% of patients attending the clinic, and with the normal (wild-type) *HFE* gene, as heterozygotes or homozygotes for *HFE* gene mutations might be expected to have abnormal ferritin or percentage of transferrin saturation values. Furthermore, we chose individuals who appraised their current health as allowing full activity vs activity limited to some degree. In this regard, patients were queried concerning potential limitations in activity; the designation of health allowing full activity explicitly excludes limitations because of pain or stiffness of joints; shortness of breath or difficulty breathing; heart problems, including chest pain; fatigue, tiredness, or lack of energy; and depression or feeling blue. Our reference population might therefore be expected to be somewhat “healthier” than individuals whose health limited their activity to some degree; more importantly, exclusions might be more likely to have symptoms indicative of underlying conditions that might cause the percentage of

transferrin saturation or ferritin concentrations to be somewhat depressed or increased.

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