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ORIGINAL ARTICLE

Genetic screening for *HFE* hemochromatosis in 6,020 Danish men: penetrance of C282Y, H63D, and S65C variants

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Abstract The aim of this epidemiologic population survey was to assess the penetrance of the most frequent hemochromatosis (*HFE*) gene variants in ethnic Danish men. A cohort of 6,020 men aged 30–53 years was screened for *HFE* C282Y, H63D, and S65C variants by restriction fragment length polymorphism analysis. Subsequently, iron status markers (serum transferrin saturation, serum ferritin) were analyzed in 1,452 men. The C282Y allele was present in 5.6%, H63D in 12.8%, and S65C in 1.8% of the men. We found 23 out of 6,020 (0.38%) C282Y homozygotes, of whom two had been treated with phlebotomy. Among untreated C282Y homozygotes (n=21) with available iron status markers (transferrin saturation n=18, ferritin n=16), 89% had elevated transferrin saturation ≥50%, 94% had elevated ferritin ≥300 μg/L, and 88% had elevation of both iron status markers; seven out of 16 (44%) had ferritin values >800 μg/L. One C282Y homozygote had normal iron status markers possibly due to nonexpressivity. Among C282Y/H63D compound heterozygotes (n=66), 23% had elevated transferrin saturation, 27% elevated ferritin, and 9% elevation of both iron status markers. Among H63D/H63D homozygotes (n=74), 15% had elevated transferrin saturation, 19% elevated ferritin, and 5.4% elevation of both iron status markers. Among C282Y/wild type (wt) heterozygotes (n=255), 9% had elevated transferrin saturation, 9% elevated ferritin, and 1.2% elevation of both iron status markers. Among H63D/wt heterozygotes (n=600), 8% had elevated transferrin saturation, 12% elevated ferritin, and 2% elevation of both iron status markers. None of the men with the S65C variant displayed elevation of both iron status markers. In conclusion, this study demonstrates a high penetrance of the C282Y variant in Danish men followed by the H63D variant while the S65D variant had no significant impact on iron status markers.

Keywords Genotypes · Hemochromatosis · Mutations · Penetrance · Prevalence · Scandinavia

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Introduction

Hereditary *HFE* hemochromatosis is a frequent genetic disorder in individuals of northern European heritage with a significant morbidity and mortality unless adequately treated [1]. Since the discovery of the *HFE* gene, *HFE* allelic frequencies have been analyzed in most European populations [2] and screening for hemochromatosis based on phenotypic or genetic testing has been discussed. *HFE* hemochromatosis is inherited as an autosomal recessive trait [3] and the penetrance of the most significant *HFE* mutation, i.e., C282Y, has been assessed using phenotypic [4–8] or clinical criteria [9, 10]. Three longitudinal studies have estimated the proportion of C282 homozygous subjects who develop disease due to iron overload [5, 11, 12]. Two studies were underpowered to assess disease prevalence [5, 11]. The largest prospective study comprised ~200 homozygous individuals followed up for more than 10 years [12].

The aim of this study was to assess the penetrance of the *HFE* mutations or variants C282Y (c845 G→A), H63D

(c187 C→G), and S65C (c193 A→T) on the phenotypic expression of iron status markers in Danish men.

Materials and methods

Participants and study design

This prospective, epidemiologic population survey was performed in 2000–2001 [13, 14]. It was approved by the Scientific Ethical Committee and fulfilled the Declaration of Helsinki. All men of ethnic Danish origin aged 30–53 years (n=10,993) being residents in Næstved and Vordingborg municipalities in the southern part of Zealand were drawn from the Census Registry and invited by letter to participate. Written informed consent was obtained from all participants.

In the first screening, *HFE* genotypes were assessed on mailed saliva samples from the participants. The second screening comprised: (1) participants in whom the genotype could not be determined on saliva samples, they were invited to genetic testing and assessment of iron status markers; (2) participants with established *HFE* variants, they were invited to assessment of iron status markers. Subsequently, participants with elevated iron status markers were invited to a repeat analysis.

HFE variant analysis

Extraction of DNA from saliva and blood was performed by restriction fragment length polymorphism [13–15]. Three cases of C282Y and H63D in "cis" phase have been reported [16–18]. These genotypes were not found in our series and we assume that all examined chromosomes carry only one of the three *HFE* variants. The three most frequent *HFE* genotypes C282, H63, and S65 are designated wild type (wt).

Iron status

Blood samples were drawn in the fasting state (except in 250 men) between 0800 and 1000 hours. Serum was stored at 4°C and analyzed within 4 days. Analyses of iron status markers were performed according to the manufacturers' instructions on Dimension® RxL Clinical Chemistry System with heterogeneous Immunoassay Module (Dade Behring, Deerfield, IL, USA, now merged with Siemens Healthcare Diagnostics, Eschborn, Germany). Serum iron was analyzed with Dimension® IRN Flex reagent catalogue number DF49A using calibrator catalogue number DC21. The normal range for serum iron in men is 13–36 μmol/L. Serum transferrin (molecular weight 78,000 Da) was analyzed with Dimension® TRNF Flex reagent catalogue number DF103 using calibrator catalogue number DC51. The calibrator is standardized according to the International Federation of Clinical Chemistry, International Reference Preparation for Plasma Proteins, the Community Bureau of Reference, and the College of American Pathologists. The normal range for serum transferrin is 24–41 μmol/L.

The serum transferrin saturation percent (TSAT) was calculated by the equation:

$$\frac{\text{Serum iron } (\mu\text{mol/L}) \times 100}{\text{Serum transferrin } (\mu\text{mol/L}) \times 2}.$$

The normal range for transferrin saturation is 16–49%; we consider values ≥50% to be elevated [19].

Serum ferritin was analyzed with Dimension® FERR Flex reagent catalogue number RF440 using calibrator catalogue number RC440. The calibrator is standardized according to the World Health Organization (WHO) ferritin standard, 3rd IS, 95/572 [20]. We used the following cut-off values: normal range for ferritin, 15–299 μg/L; elevated ferritin, ≥300 μg/L; moderate iron overload for ferritin, 300–800 μg/L; major iron overload for ferritin, >800 μg/L [19, 21].

Statistics

Statistical analyses were performed using the Statistical Package for the Social Sciences 11.5.1 for Windows. Due to the non-normal distribution of several variables including serum ferritin, nonparametric statistics were used for comparison between groups. Hardy–Weinberg equilibrium in genotype distribution was assessed with chi-square goodness-of-fit test. The 95% reference interval was defined as the 2.5–97.5 interpercentile range. Significance level was chosen at $p<0.05$.

The association between *HFE* genotype and the risk of having elevated transferrin saturation and/or serum ferritin was assessed using conditional logistic regression to obtain adjusted odds ratios (OR) with 95% confidence interval (95%CI). The predicted phenotypic penetrances of *HFE* genotypes were calculated by the equation:

$$P(\text{response}|X_1, \ldots\ldots, X_n) = \frac{e^{\alpha+\sum_{j=1}^{n}\beta_j X_j}}{1+e^{\alpha+\sum_{j=1}^{n}\beta_j X_j}}.$$

The probability of having elevated transferrin saturation or serum ferritin for a specific *HFE* genotype is an estimate of penetrance when other modifiers in the model are in the reference state (X_i=0). The reference group was wt/wt homozygous participants aged 32–39 years who never donated blood, had no self-reported disease, and had minimal alcohol, meat, milk, and egg consumption.

Table 1 Iron status markers in the study population (median and 95% interval)

	All (n=1,452)	Fasting (n=1,202)	Nonfasting (n=250)	p value[a]
Ferritin (μg/L)	153 (25–531)	153 (23–527)	152 (31–681)	1.0
Transferrin (μmol/L)	29 (22–40)	30 (22–40)	29 (23–39)	0.3
Iron (μmol/L)	18 (8–33)	17 (8–33)	19 (9–36)	0.006
TSAT (%)	29 (12–63)	29 (12–62)	32 (13–82)	0.003

[a] Mann–Whitney's test between fasting vs. nonfasting values
TSAT transferrin saturation

Results

Among the 10,993 invited men, saliva samples were obtained from 6,567 out of 10,993 (60%) men. In 1,064 out of 6,567 (16%) men, the *HFE* genotype could not be assessed on saliva. They were invited for genetic testing and analyses of iron status markers; this was obtained in 522 out of 1,064 (49%) men of whom 331 out of 522 (60%) had *HFE* wild type. In total, *HFE* genotype was determined in 6,020 out of 10,993 (55%) men.

The 2,149 out of 6,020 (36%) men carrying a *HFE* variant were invited for analyses of iron status markers. Blood samples were drawn from 930 out of 2,149 (43%) men. Iron status markers were analyzed in 1,452 out of 6,020 (24%) men.

HFE allele frequencies

The *HFE* allele distributions were in Hardy–Weinberg equilibrium (χ^2=5.4, p=0.8). In the entire series (n=6,020), the allele frequency of C282Y, H63D, and S65C variants was 5.6%, 12.8%, and 1.8%, respectively. We found 1.4% C282Y/H63D, 0.1% C282Y/S65C, and 0.4% H63D/S65C compound heterozygotes [14].

Iron status markers

At blood sampling, 250 out of 1,452 (17%) men were nonfasting. Serum iron and transferrin saturation were significantly lower in fasting than in nonfasting men (Table 1). Consequently, we used only fasting serum iron values in the calculation of transferrin saturation and in the statistical analyses. There were no significant differences between fasting and nonfasting values of serum transferrin and serum ferritin, so these data were pooled.

HFE variants and iron status markers

Table 2 shows the association between *HFE* genotypes and iron status markers. C282Y and H63D homozygotes as well as compound heterozygotes had significantly higher iron status markers than wt/wt homozygotes. In contrast, S65C heterozygotes had significantly lower serum ferritin than wt/wt homozygotes.

Box plots of iron status markers in different *HFE* genotypes are displayed in Fig. 1, and box plots of serum ferritin and transferrin saturation illustrating the separate effects of C282Y and H63D genotypes in Fig. 2.

Table 3 and Fig. 3 show the impact of the various *HFE* genotypes on iron status markers. Among untreated C282Y homozygotes (n=21) with iron status markers (transferrin saturation n=18, serum ferritin n=16), 89% had elevated transferrin saturation ≥50%, 94% had elevated serum ferritin ≥300 μg/L, and 88% had elevation of both iron status markers; seven out of 16 (44%) had serum ferritin values >800 μg/L (Table 6). Three C282Y homozygotes had normal iron status markers. Two were treated with phlebotomy. The third may have nonexpressivity of the

Table 2 *HFE* genotypes and iron status markers (median values) in 1,452 men

Genotype	n	Iron[a] (μmol/L)	Transferrin (μmol/L)	TSAT[a] (%)	Ferritin (μg/L)
wt/wt	331	16	30	25	151
H63D/wt	600	17*	30	28*	148
S65C/wt	84	15	30	25	125*
C282Y/wt	255	18**	29	30**	147
H63D/H63D	74	19**	29	33**	173
H63D/S65C	17	21***	27*	35**	204
C282Y/S65C	8	21	25***	38***	245
C282Y/H63D	66	20**	26**	37**	183*
C282Y/C282Y	17	25**	24**	56**	505**

*p<0.05, **p<0.001, ***p<0.01; Mann–Whitney's test, differences between *HFE* variants and wt/wt men
[a] Fasting values

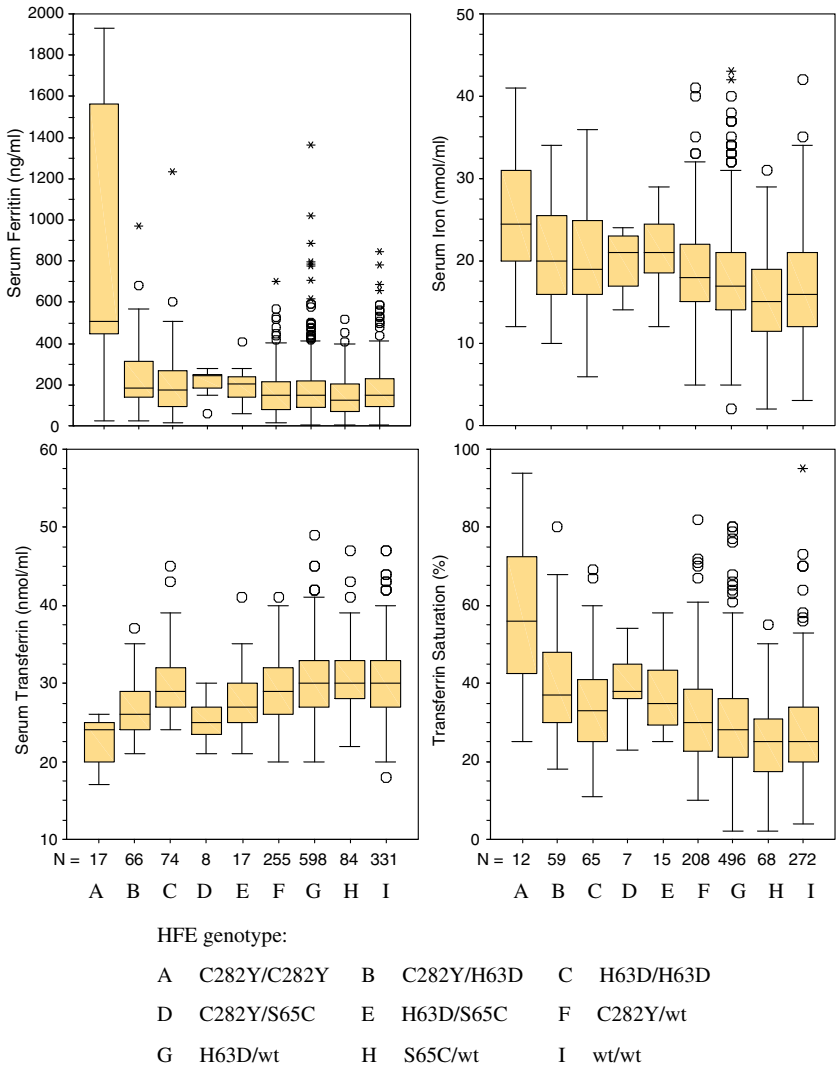


Fig. 1 Influence of *HFE* genotypes on iron status markers in Danish men. *HFE* genotype: *A* C282Y/C282Y, *B* C282Y/H63D, *C* H63D/H63D, *D* C282Y/S65C, *E* H63D/S65C, *F* C282Y/wt, *G* H63D/wt, *H* S65C/wt, *I* wt/wt

C282Y variant, but repeat analysis of iron status markers was not available to make a reliable confirmation. Assuming that the two treated men initially had elevated iron status markers, the C282Y variant showed penetrance in 22 out of 23 men.

In addition, Table 3 displays the calculated penetrance, i.e., the effect of the *HFE* genotype on iron status markers, when other modifying factors in the model are in the reference state (see the "Statistics" section).

Logistic regression analysis

Tables 4 and 5 show the results of logistic regression used to estimate the risk of elevated transferrin saturation and serum ferritin associated with specific *HFE* genotypes. In C282Y homozygotes, the risk of elevated transferrin saturation was 86-fold higher and of elevated serum ferritin 66-fold higher than in wt/wt homozygotes. C282Y/H63D compound heterozygotes had a 7.2- and 3.3-fold higher risk of having elevated iron status markers than wt/wt homozygotes. Of the remaining genotypes, C282Y heterozygotes and H63D homozygotes had higher risk of having elevated transferrin saturation.

Table 6 shows details on the C282Y homozygotes. Iron status markers were available in 20 out of 23 men. One died during the study period, one moved to another district, and four did not respond to the follow-up invitation. Median age of the C282Y homozygotes at detection was 49 years.

Discussion

The present study assessed the penetrance of the three most frequent *HFE* variants on iron status markers. To our knowledge, this is the first study designed to perform primarily genetic screening for *HFE* hemochromatosis followed by secondary phenotypic evaluation. Consequently, the results of our study are not directly comparable with

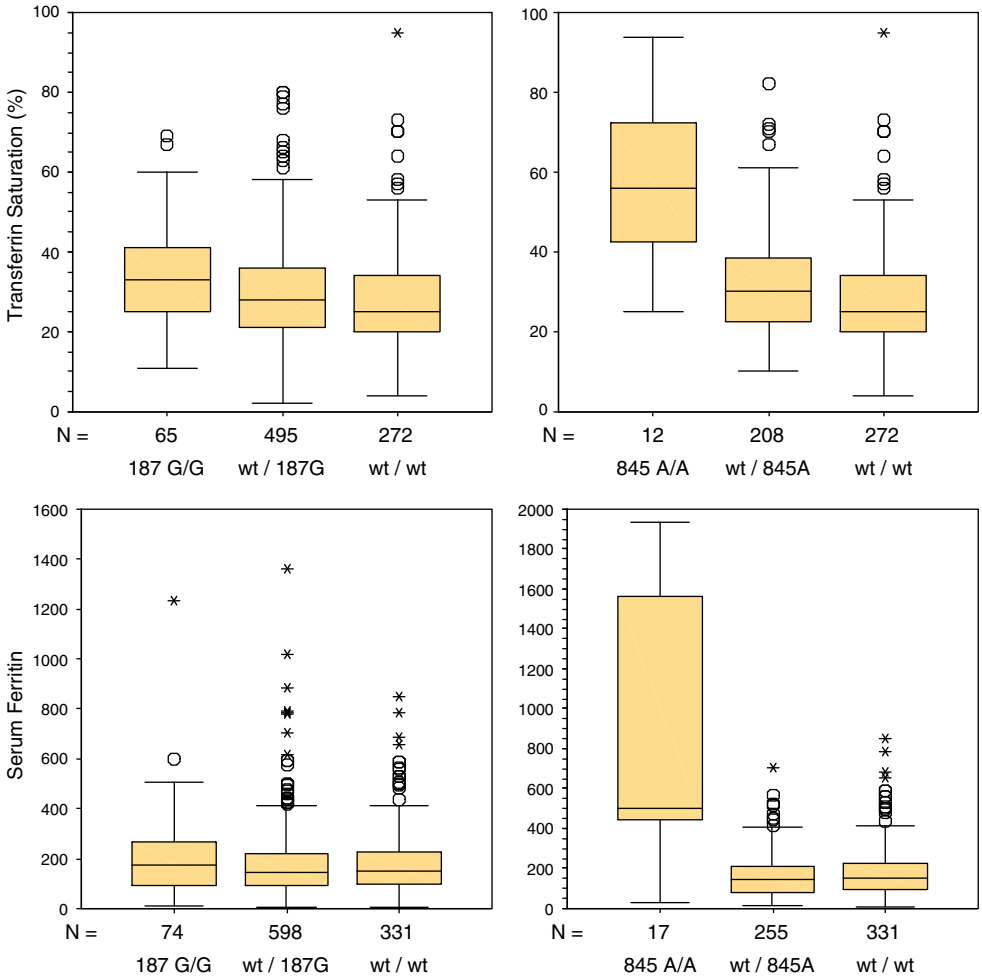


Fig. 2 Influence of *HFE* C282Y (845A) and H63D (187G) variants on serum transferrin saturation and serum ferritin in men

other penetrance studies. We decided that the screening population should consist of men who are more predisposed to develop iron overload than women, i.e., the influence of *HFE* variants is more likely to be expressed in men than in women [1, 12]. Women are partly protected against iron overload due to iron losses at menstruation and pregnancy [22]. In men, body iron reserves accumulate from adolescence to 30–35 years of age and subsequently

Table 3 *HFE* genotypes: influence on iron status markers and calculated penetrance

Genotype	*n*	TSAT ≥50% (%)	Penetrance[a] (%)	Ferritin ≥300 μg/L (%)	Penetrance[a] (%)	TSAT ≥50% and ferritin ≥300 μg/L (%) (*n*)
C282Y/C282Y	17[b]	88.9[c]	82.5	93.8[d]	85.2	87.5[e] (14)
C282Y/H63D	66	22.7	16.7	27.3	19.0	9.1 (6)
C282Y/S65C	8	12.5		0		0 (0)
H63D/S65C	17	5.9		5.9		0 (0)
H63D/H63D	74	14.9	13.2	18.9		5.4 (4)
C282Y/wt	255	9.4	8.3	8.6		1.2 (3)
H63D/wt	600	8.0		12.2		2.0 (12)
S65C/wt	84	2.4		11.9		0 (0)
wt/wt	331	5.7		13.3		0.9 (3)
Total	1452	9.2		13.6		2.8 (41)

[a] Penetrance of elevated transferrin saturation/ferritin calculated by the equation in the "Statistics" section using α and β values from Tables 4 and 5
[b] Untreated men with iron status markers
[c] 16 out of 18 men
[d] 15 out of 16 men
[e] 14 out of 16 men

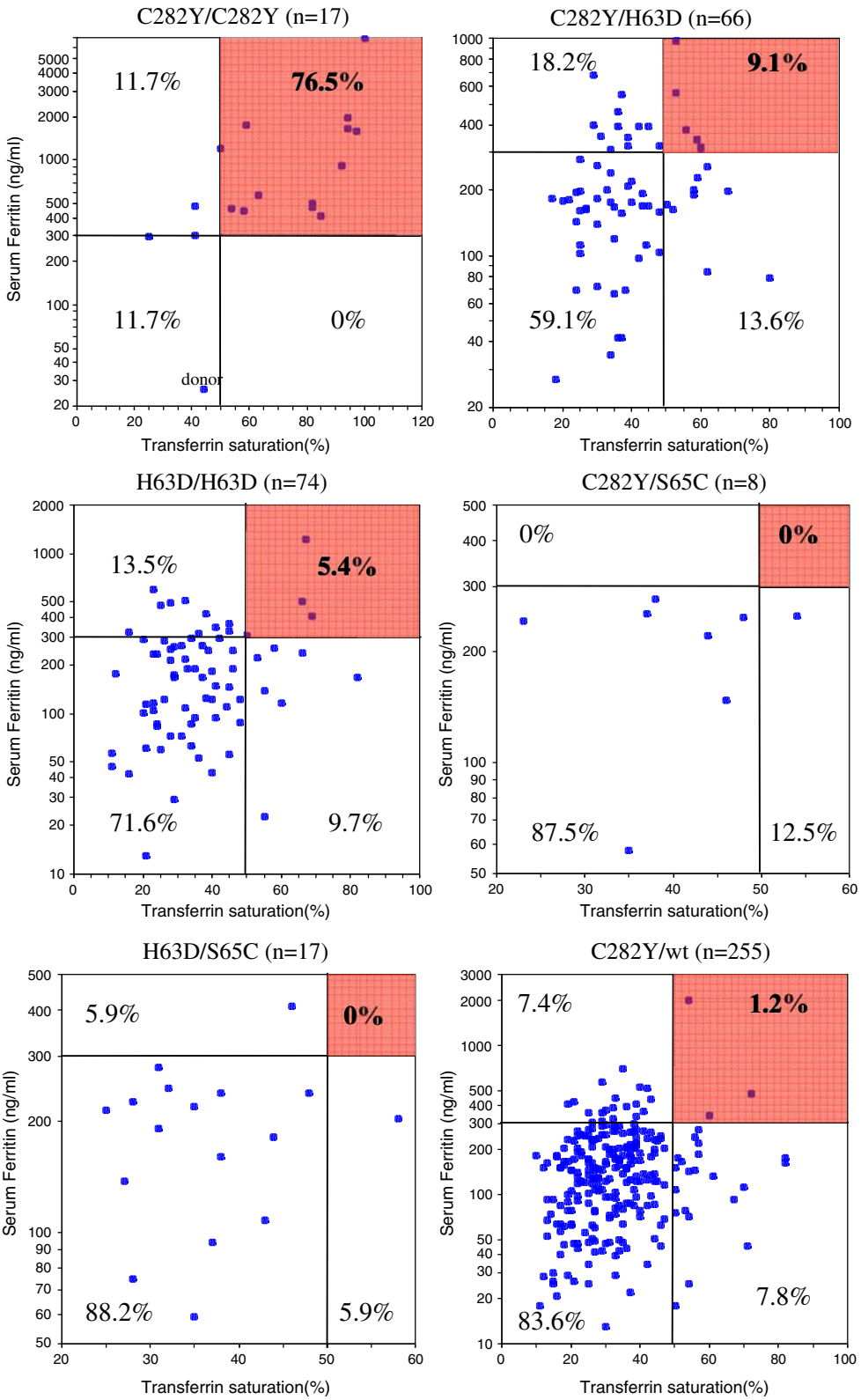
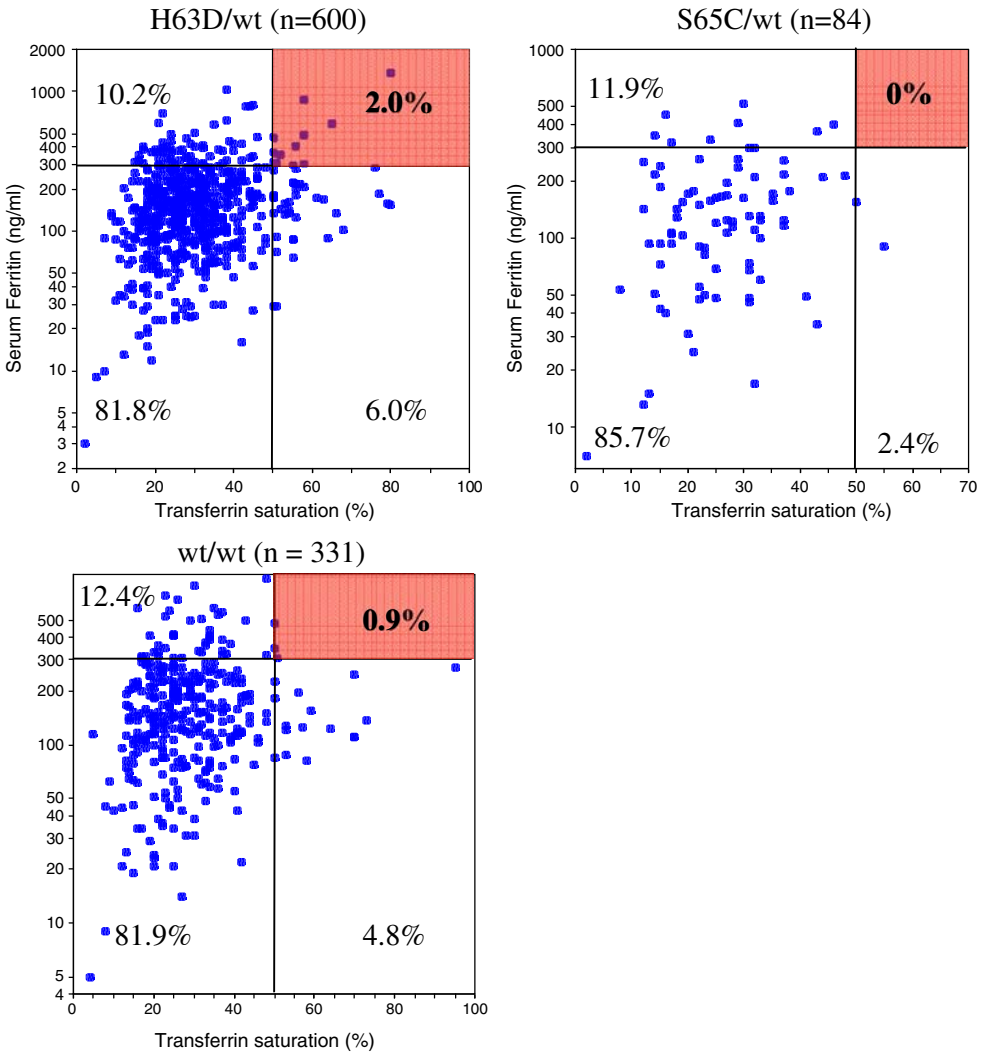


Fig. 3 Impact of *HFE* genotypes on iron status markers in men. Subjects in the *red squares* have transferrin saturation ≥50% and serum ferritin ≥300 μg/L

remain at a stable level, which is individual and determined by genetic and environmental factors [23, 24]. In Danish patients with clinical hemochromatosis, the median age at diagnosis is ~56 years [1]. Therefore, we chose to screen men 30 to 53 years of age. In order to evaluate a genetic effect, the population should be as homogeneous as possible, so we included men exclusively of Danish heritage.

Fig. 3 (continued)



In this series, the phenotypic penetrance in the 23 C282Y homozygous men was close to 100%, when the two phlebotomy-treated men were included in the estimates. Only one man had probable nonexpressivity. Unfortunately, we had no opportunity to evaluate the extent of iron-induced organ involvement in these men. The penetrance in C282Y/H63D compound heterozygotes was significant, but lower than in C282Y homozygotes.

HFE variant homozygous and compound heterozygous genotypes displayed significantly higher transferrin saturation than wt/wt homozygous genotype. Among wt/wt homozygotes, 5.7% had elevated transferrin saturation, 13.3%

Table 4 Logistic regression analysis of *HFE* genotype associated risk for elevated transferrin saturation ≥50%

Independent variable	β	OR exp (β)	95%CI	p value
Genotype				
C282Y/C282Y	4.456	86.1	21.6–343.5	0.0001
C282Y/H63D	1.977	7.2	2.6–19.9	0.0001
H63D/H63D	1.700	5.5	2.0–14.9	0.001
H63D/S65C	0.498	1.6	0.2–14.6	0.7
C282Y/wt	1.186	3.3	1.4–7.9	0.01
H63D/wt	0.968	2.6	1.2–6.0	0.02
S65C/wt	−0.177	0.8	0.2–4.2	0.8

Logit value (α) of this model is −3.586

Table 5 Logistic regression analysis of *HFE* genotype associated risk for elevated serum ferritin ≥300 μg/L

Independent variable	β	OR exp (β)	95%CI	p value
Genotype				
C282Y/C282Y	4.189	66.0	10.7–404.9	0.0001
C282Y/H63D	1.209	3.3	1.6–7.1	0.002
H63D/H63D	0.682	2.0	0.9–4.2	0.08
H63D/S65C	−0.825	0.4	0.05–3.7	0.5
C282Y/wt	−0.308	0.7	0.4–1.4	0.4
H63D/wt	0.084	1.1	0.7–1.8	0.8
S65C/wt	0.231	1.3	0.5–2.9	0.6

Logit value (α) of this model is −2.660

elevated serum ferritin, and 0.9% elevation of both iron status markers. These findings suggest that other genetic factors besides *HFE* variants play a role for body iron homeostasis.

We observed that the S65C variant had no significant influence on iron status markers compared with the C282Y and H63D variants (Table 3). S65C/wt heterozygotes actually had a lower frequency of elevated iron status markers than wt/wt heterozygotes. H63D/S65C compound heterozygotes had similar frequency of elevated iron status markers as wt/wt homozygotes but lower frequency than H63D/wt heterozygotes. Furthermore, C282Y/S63D compound heterozygotes had similar or lower frequency of elevated iron status markers than C282/wt heterozygotes. These results contrast an Australian study, which concluded that C282Y/S65C compound heterozygotes had an increased risk of phenotypic hemochromatosis [25] but is in accordance with a Norwegian study reporting that, although some C282Y/S65C compound heterozygotes have elevated iron status markers, the overall penetrance of this genotype is low [26]. Screening for S65C may be useful in individuals with iron overload who are not C282Y homozygous or C282Y/H63D compound heterozygous.

The majority of subjects of northern European descent with hemochromatosis have *HFE* hemochromatosis due to C282Y homozygosity [27]. The penetrance of C282Y homozygosity in various populations range from 0.7% [10] to 100% [4], depending on the criteria used. Most studies have included both genders despite differences in penetrance [4–10, 12]. Allen et al. [12] followed up 203 C282Y homozygotes during a 12-year period; 28% of men

Table 6 Data on C282Y homozygotes

Age (years) n=23	TSAT (%) first/second n=20	Ferritin (μg/L) first/second n=18	Symptoms[a]
38	59/43	1,731/987	Arthritis
50	94/86	1,629/1,580	NR
50*	93		Arthritis
47*	66		Cancer
49			Asthma
33	85/76	413/438	NR
51	82	505	NR
47			Arthritis, heart disease, deceased
50	92/86	919/1,358	Skin pigmentation, renal transplant
52	41/105	302/320	NR
39	100/104	6,847/5,478	General weakness
46	94/100	1,931/2,165	Cardiac arrhythmia
36	50	1,209	Spondylolisthesis
49*	44	131	Treated, phlebotomy
39			NR
46	82	474	NR
48	97/94	1,561/1,608	Arthritis, lactose intolerance
51	63	577	NR
48	41	479	NR
39	58	446	NR
44	54/86	462/526	General weakness
45	25	297	NR
50	44	26	Treated, blood donor

Persons without iron status markers did not respond to invitation

NR not recorded

[a] Information from medical records

and 1% of women developed clinically overt iron overload. C282Y homozygotes with a serum ferritin level of >1,000 μg/L were at increased risk of hemochromatosis-associated signs and symptoms, when compared with either homozygotes with a serum ferritin level of ≤1,000 μg/L or individuals with other *HFE* genotypes [12].

The earliest phenotypic sign of *HFE* hemochromatosis is an elevated transferrin saturation [28]. The serum ferritin concentration reflects body iron accumulation over time and in the early stage of hemochromatosis serum ferritin is usually within normal range. We used a serum ferritin limit of 800 μg/L in the definition of major iron overload as we have experienced that nearly all such subjects have elevated serum transaminases [19].

The frequency of the C282Y allele in Denmark is ~5.6%, corresponding to a homozygosity frequency of ~0.31% [14]. According to calculations based on the National Census Registry in 2002 (population in Denmark ~5,000,000) the estimated number of C282Y homozygotes in Denmark was ~16,900, comprising ~3,088 men aged 30–54 years.

In the logistic regression analysis, men with an expected higher risk had a significantly elevated calculated risk of having elevated iron status markers. In C282Y homozygotes, the OR for having elevated transferrin saturation was 86 and for having elevated serum ferritin it was 66 (Tables 4 and 5). In C282Y/H63D compound heterozygotes, the corresponding OR were 7.2 and 3.3, respectively.

The calculated penetrance of *HFE* genotypes on iron status markers was highest for C828Y homozygotes and C282Y/H63D compound heterozygotes (Table 3). However, the statistics should be interpreted with caution due to the small number of C282 homozygotes.

Previously, a diagnosis of hereditary hemochromatosis was based on the presence of clinical symptoms due to iron overload in combination with elevated iron status markers. In families with hemochromatosis, elevated iron status markers in parents/siblings to the probands were sufficient to make the diagnosis. In the screening situation, the diagnosis has so far been based on phenotypic expression of elevated iron status markers [28, 29].

With DNA-based technology, the diagnosis can be confirmed in the asymptomatic, preclinical stage of the disorder, and preventive measures can be initiated before organ damage occurs. *HFE* hemochromatosis has variable penetrance, and there is an ongoing discussion whether we should initiate screening programs in populations with high frequencies of *HFE* variants [12]. Although *HFE* hemochromatosis is the most prevalent genetic disorder in northern European populations, there have been raised doubts about whether it is appropriate to use genetic screening to diagnose preclinical cases [10].

HFE hemochromatosis fulfills the majority of the WHO criteria for population screening, yet several issues have to be more clarified before screening programs can be initiated, e.g., age- and gender-related penetrance of different *HFE* genotypes, interactions between *HFE* genotypes and environmental modifiers, and psychosocial consequences of screening [13, 30].

Serum iron displays significant 24-h and day-to-day variation and is influenced by meals. In order to reduce the number of high false-positive values, participants were told to be fasting at blood sampling [31]. Transferrin saturation has been widely used as initial screening procedure in *HFE* hemochromatosis with a cut-off value ranging from 45% to 60% [4–10, 27]. However, due to the variation in serum iron, transferrin saturation can be unreliable as demonstrated in some of our C282Y homozygotes (Table 6), which stresses the importance of repeated analyses of transferrin saturation when hemochromatosis is suspected. Serum ferritin is often included in the screening and there is consensus that the upper limit in men is ~300 μg/L [21, 27, 32].

In conclusion, this study has combined initial genetic screening with subsequent phenotypic screening in *HFE* hemochromatosis. The C282Y variant is frequent in Danes and appears to have a high phenotypic penetrance in men, being close to 100%. From a cost-effective point of view, our results are in favor of screening Danish men above ~40 years of age for hemochromatosis [33]. *HFE* hemochromatosis is a frequent disorder; phlebotomy treatment is cheap, effective, prevents organ damage, and ensures a normal lifespan and quality of life [1]. The potential psychological consequences of screening have raised much anxiety [30], which appears to be exaggerated. In Denmark, the population has a positive, practical, and cooperative attitude toward genetic screening surveys [13].

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