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## Extrinsic factors modifying expressivity of the *HFE* variant C282Y, H63D, S65C phenotypes in 1,294 Danish men

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**Abstract** This study analysed the influence of extrinsic factors on the phenotypic expression of *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. Serum iron, serum transferrin, transferrin saturation, and serum ferritin were analysed in 1,452 men and 1,294 men completed a questionnaire on factors, which could influence iron balance. The C282Y allele was present in 5.6%, H63D in 12.8% and S65C in 1.8% of the men. In the entire series, 3% had elevated iron status markers (transferrin saturation  $\geq 50\%$ , ferritin  $\geq 300 \mu\text{g/L}$ ). Self-reported liver disease had an elevating effect and peptic ulcer a lowering effect on iron status markers. Age increased the fraction of men with elevated ferritin from 8.3% at 32–38 years to 16.2% at 46–53 years of age ( $p=0.002$ ). Blood donation had a lowering effect on iron status markers ( $p=0.0001$ ). Alcohol consumption elevated serum iron and serum ferritin ( $p=0.001$ ). Meat consumption had an elevating effect ( $p=0.02$ ) and milk consumption a lowering effect ( $p=0.03$ ) on serum ferritin. There was no influence of vitamin-mineral tablets on iron status markers. In adjusted logistic regression analysis, the *HFE* genotype had the highest impact on iron status markers; high alcohol consumption was significantly associated with elevated transferrin saturation. High age and high alcohol consumption were significantly associated with elevated ferritin and high egg consumption and blood donation was significantly associated with normal ferritin levels. In conclusion, the expressivity of *HFE* variant phenotypes in Danish men was

enhanced by alcohol and meat consumption and decreased by milk and egg consumption and blood donation.

**Keywords** Expressivity · Genotypes · Hemochromatosis · Mutations · Penetrance · Scandinavia

Genetic *HFE* hemochromatosis is inherited as an autosomal recessive trait [1–3] and the phenotypic penetrance of the most important *HFE* mutation or variant, i.e. C282Y, has been assessed in various studies using phenotypic, clinical and genetic criteria [4–11]. A longitudinal study of C282Y homozygous individuals, followed up for more than 10 years, assessed the fraction, who developed disease due to iron overload [12].

The expressivity of the *HFE* variant C282Y (c845 G→A), H63D (c187 C→G) and S65C (c193 A→T) phenotypes also depend on extrinsic factors or modifiers of iron balance, such as dietary iron intake, bioavailability of dietary iron, blood losses at menstruation and pregnancy as well as blood donation. The aim of the present study was to analyse environmental extrinsic factors, which might influence the phenotypic expression of the *HFE* variants on iron status markers.

### Methods

#### Participants and study design

The study was performed in 2000–2001 and described in previous papers [11, 13, 14]. It was approved by The Danish Scientific Ethical Committee and fulfilled The Declaration of Helsinki. Ethnic Danish men aged 30–53 years ( $n=10,993$ ) living in Næstved and Vordingborg municipalities were drawn from the Census Registry and invited to participate.

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**Table 1** Data in 1,294 men having iron status markers analysed

	Yes (n)	No (n)	Total (n)	No data (n)
Fasting blood sample	1,202	250	1,452	1
Blood donation	448	837	1,285	168
Self-reported disease				
Diabetes	28	1,266	1,294	159
Arthritis	82	1,212	1,294	159
Liver disease	15	1,279	1,294	159
Anemia	17	1,277	1,294	159
Iron deficiency	20	1,274	1,294	159
Hemochromatosis	3	1,291	1,294	159
Peptic ulcer	57	1,237	1,294	159
Heart disease	32	1,262	1,294	159
Iron intake				
Vitamin-mineral tablets	401	842	1,243	210
Iron tablets	29	871	900	553

In the first phase of the study, *HFE* genotypes were assessed on saliva samples from the participants. In the second phase, participants with *HFE* variants plus participants in whom the genotype could not be assessed on saliva, were invited to genetic retesting and analysis of iron status markers. Participants with elevated iron status markers were invited to a reanalysis.

#### Iron status

Blood samples were drawn in the fasting state (except in 250 men) between 0800 and 1000 hours. Analyses of iron status markers, i.e. serum iron, serum transferrin and serum ferritin were performed on Dimension® RxL Clinical Chemistry System with heterogeneous Immunoassay Module (Dade Behring Inc., Deerfield, Illinois, USA, now merged with Siemens Healthcare Diagnostics GmbH, Eschborn, Germany) [11]. The serum transferrin saturation percent (TSAT) was calculated from serum iron and serum transferrin concentrations as: serum iron ( $\mu\text{mol/L}$ )  $\times$  100/serum transferrin ( $\mu\text{mol/L}$ )  $\times$  2. Transferrin saturation values  $\geq 50\%$

**Table 2** Iron status markers in the study population, median (95% interval)

	All n=1452	Fasting 1202	Non-fasting 250	P-value*
Ferritin ( $\mu\text{g/L}$ )	153 (25-531)	153 (23-527)	152 (31-681)	1.0
Transferrin ( $\mu\text{mol/L}$ )	29 (22-40)	30 (22-40)	29 (23-39)	0.3
Iron ( $\mu\text{mol/L}$ )	18 (8-33)	17 (8-33)	19 (9-36)	0.006
TSAT (%)	29 (12-63)	29 (12-62)	32 (13-82)	0.003

\*) Mann-Whitney's test between fasting and non-fasting men

and serum ferritin values  $\geq 300 \mu\text{g/L}$  were considered elevated [15, 16].

#### Questionnaire

The questionnaire yielded information concerning factors, which might influence iron status, such as blood donation, iron supplements, self-reported disease, alcohol consumption and dietary habits comprising consumption of meat, milk and egg. As previously reported [13], the questionnaire also assessed the psychological consequences of genetic screening.

#### Statistics

Statistical analyses were performed using Statistical Package for the Social Sciences 11.5.1 for Windows. Non-parametric statistics were used for comparison between groups (Kruskal Wallis' test, Mann-Whitney's test, Pearson's  $\chi^2$  test). The 95%

**Table 3** Influence of age on iron status markers, median (95% interval)

Age (y)	n <sup>a</sup>	Ferritin ( $\mu\text{g/L}$ )	Ferritin $\geq 300 \mu\text{g/L}$ (%)	n <sup>b</sup>	TSAT (%)
32-39	363	149 (25-417)	8.3	327	30 (13-68)
40-46	433	153 (25-519)	13.9	301	29 (12-59)
47-53	656	153 (23-566)	16.2	574	28 (12-61)
P-value		0.09*	0.002**		0.07*

a) The 3 groups had similar *HFE* genotype distribution,  $\chi^2$  test,  $p=0.3$

b) Fasting men

\*) Kruskal Wallis' test

\*\*)  $\chi^2$  test

**Table 4** Influence of alcohol consumption on iron status markers, median values

Drinks <sup>a</sup> / week	n <sup>b</sup>	Ferritin ( $\mu\text{g/L}$ )	Transferrin ( $\mu\text{mol/L}$ )	n <sup>c</sup>	Iron ( $\mu\text{mol/L}$ )	TSAT (%)
<5	586	145	29	500	17	29
5-15	481	149	30	404	18	30
16-21	144	156	30	109	17	28
>21	70	225	31	45	20	32
P-value*		0.0001	0.2		0.01	0.3

a) One drink = 12 g ethanol

b) The 4 groups had similar *HFE* genotype distribution,  $\chi^2$  test  $p=0.2$

c) Fasting men

\*) Kruskal-Wallis' test

reference interval was defined as the 2.5-97.5 interpercentile range. The significance level was set at 0.05.

The association between *HFE* genotype and the risk of having elevated transferrin saturation and/or serum ferritin was assessed by conditional logistic regression with adjusted odds ratios (OR) and 95% confidence intervals (CI). *HFE* genotype, age, blood donation, self-reported disease, vitamin mineral tablets containing iron, alcohol, meat, milk and egg consumption were included as covariates.

The predicted phenotypic penetrance of the *HFE* genotypes, adjusted for other covariates, was calculated as previously described [11]. The probability for a specific *HFE* genotype to present elevated transferrin saturation or elevated serum ferritin is an estimate of penetrance when other modifiers are in the reference state. The reference group was wt/wt homozygous men aged 32-39 years, who never had donated blood, had no self-reported disease and low alcohol, meat, milk and egg consumption.

## Results

Salvia samples were obtained from 6,567/10,993 (60%) men. In 1,064/6,567 (16%) men, genotype could not be assessed. They were invited to genetic testing and analysis of iron status markers, which was obtained in 522/1,064 (49%).

**Table 5** Influence of blood donation on iron status markers, median values

a) The 4 groups had similar *HFE* genotype distribution,  $\chi^2$  test  $p=0.3$

b) Fasting men

\*) Kruskal-Wallis' test

Blood donor	n <sup>a</sup>	Ferritin ( $\mu\text{g/L}$ )	Transferrin ( $\mu\text{mol/L}$ )	n <sup>b</sup>	Iron ( $\mu\text{mol/L}$ )	TSAT (%)
No	856	179	29	707	18	30
Yes (donation last 2 years)						
0	153	154	29	130	17	29
1-4	131	93	31	112	18	29
>4	133	44	32	103	15	23
P-value*		0.0001	0.0001		0.01	0.0001

*HFE* genotype was assessed in 6,020 men. The 2,149 men carrying a *HFE* variant were invited to analysis of iron status markers being obtained in 930/2,149 (43%) men. In the entire series, iron status markers were obtained in 1,452/6,020 (24%) men of whom 1,294/1,452 (89%) completed the questionnaire. Data from the questionnaire are summarized in Table 1.

### *HFE* allele frequencies

The *HFE* genotypes C282, H63, and S65 are designated wild type (wt). In the entire series, the allele frequency of C282Y, H63D, and S65C variants was 5.6%, 12.8% and 1.8%, respectively [11, 14].

### Iron status markers

In total 250/1,452 (17%) men were non-fasting. Serum iron and transferrin saturation was significantly lower in fasting than in non-fasting men (Table 2). Consequently, we used only serum iron values in fasting men in the calculation of transferrin saturation and the statistical analyses.

### Age vs iron status markers

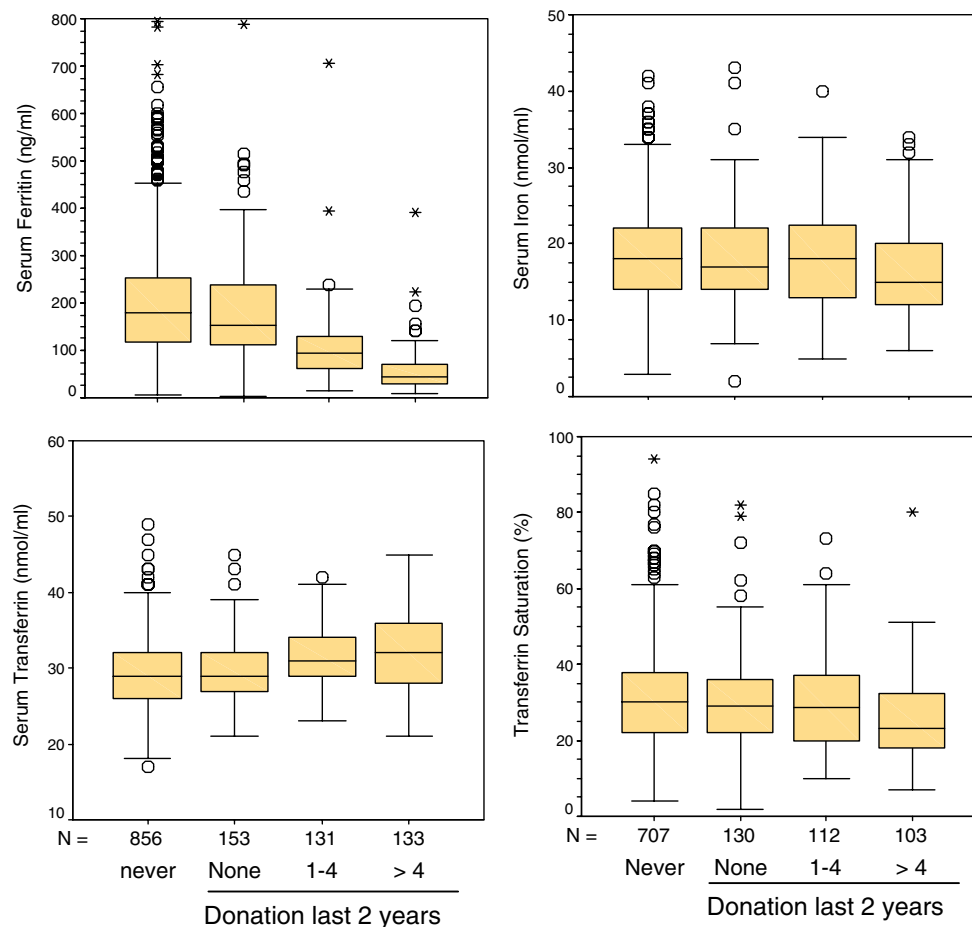
Age had no influence on transferrin saturation values. There was no significant difference in serum ferritin levels in the various age groups (Table 3) and the lower limit of the 95% reference interval for ferritin remained unchanged from 32 to 53 years of age. However, the upper limit for ferritin and the percentage of men having elevated ferritin  $\geq 300 \mu\text{g/L}$  increased gradually with age.

### Alcohol vs iron status markers

There was a significant elevation of serum ferritin and serum iron with increasing alcohol consumption (Table 4).

### Blood donation vs iron status markers

In blood donors, serum ferritin, serum iron and transferrin saturation decreased and serum transferrin increased with the number of blood donations (Table 5 and Fig. 1).



**Fig. 1** Influence of blood donation on iron status markers

Blood donors who were bled more than two years ago had significantly lower median ferritin than non-donors (Mann-Whitney's test,  $p=0.04$ ), but similar serum iron, serum transferrin and transferrin saturation.

Donors bled from one up to more than four times during the last two years had higher serum transferrin than donors who were bled more than two years ago and non-donors (Mann-Whitney's test,  $p<0.001$ ). Donors bled more than 4 times during the last two years had lower serum iron and transferrin saturation than non-donors ( $p=0.01$  and  $p=0.0001$ , respectively).

#### Dietary factors vs iron status markers

Consumption of meat had an elevating effect and consumption of milk a lowering effect on serum ferritin levels (Table 6). There was no significant influence of egg consumption and vitamin-mineral tablets containing iron. A few took oral iron, they had low iron status due to iron deficiency, which obviously was the indication for treatment.

#### Self-reported disease vs iron status markers

Self-reported disease had a significant influence on transferrin saturation (Table 7). Although median serum ferritin level was higher in men with liver disease and lower in men with peptic ulcer, the differences were not statistically significant, probably due to the low number of men in the groups.

#### Logistic regression analysis

C282Y homozygotes had an 86-fold higher risk of elevated ferritin and a 66-fold higher risk of elevated transferrin saturation than wt/wt homozygotes (Tables 8 and 9). C282Y/H63D compound heterozygotes had a 7.2-fold higher risk of having elevated transferrin saturation and a 3.3-fold higher risk of having elevated ferritin than wt/wt homozygotes. Of the remaining genotypes, C282Y heterozygotes and H63D homozygotes had higher risk of having elevated transferrin saturation.

**Table 6** Influence of dietary factors on iron status markers, median values

	n	Ferritin (µg/L)	Transferrin (µmol/L)	n <sup>a</sup>	Iron (µmol/L)	TSAT (%)
Meat (days/week)						
<4	188	132	30	151	17	27
4-6	549	156	30	461	17	28
7	503	153	29	414	18	30
P-value**		0.02	0.3		0.14	0.16
Milk (days/week)						
<4	233	167	30	189	17	27
4-6	189	151	30	156	17	27
Daily	850	147	29	705	17	29
P-value**		0.03	0.5		0.7	0.3
Egg (no./week)						
0-4	1124	153	29	930	17	29
>4	150	144	30	121	18	30
P-value*		0.3	0.4		0.8	0.7
Vitamin-mineral tablets						
No	842	154	30	699	18	29
Yes	401	142	29	329	17	28
P-value*		0.08	0.12		0.01	0.06
Iron tablets						
No	870	151	29	717	17	28
Yes	29	62	30	22	12	22
P-value*		0.0001	0.3		0.03	0.02

a) Fasting men

\*) Mann-Whitney's test

\*\*) Kruskal-Wallis' test

High alcohol consumption was associated with elevated transferrin saturation. High age and high alcohol consumption were significantly associated with elevated ferritin and high egg consumption and blood donation was significantly associated with normal ferritin levels.

## Discussion

In genetic terms, penetrance describes the proportion of individuals carrying a particular variation of a gene, i.e. the genotype that expresses an associated trait, i.e. the

phenotype, while expressivity refers to variations of a phenotype in individuals carrying a particular genotype. The present study assessed the penetrance of the three most frequent *HFE* variants, i.e. the influence of the genotype on the phenotype, as well as the impact of age and environmental factors on the expressivity of the phenotype. Men are more predisposed to iron overload than women, i.e. the influence of the *HFE* variants and modifying factors are more likely to be expressed in men than in women [1, 12]. Due to iron losses at menstruation and pregnancy, iron overload is less frequent in fertile women [17]. Allen et al. [12] followed up 203 C282Y homozygous individuals over 12 years; 28% of men vs 1% of women developed iron overload-related disease.

In our series, the phenotypic penetrance in C282Y homozygous men (n=23) was close to 100% and higher than the penetrance in C282Y/H63D compound heterozygous [11].

*HFE* variant homozygous and compound heterozygous men had significantly higher transferrin saturation than wt/wt homozygous men [11]. However, even among the wt/wt homozygotes, 6% had elevated transferrin saturation, 13% elevated serum ferritin and 1% elevation of both iron status markers [11], suggesting that other genetic factors (besides *HFE*) and/or environmental factors influence iron homeostasis.

**Table 7** Self-reported disease and iron status markers in Danish men, median values

Disease	n	Ferritin (µg/L)	Transferrin (µmol/L)	n <sup>a</sup>	Iron (µmol/L)	TSAT (%)
Liver disease	no	1268	151	30	1048	17
	yes	15	202	28	12	21
Peptic ulcer	no	1226	153	29	1011	18
	yes	57	125	30	49	16*

a) Fasting men

Mann-Whitney's test: \* p&lt;0.05 \*\* p&lt;0.001 compared with the "no" groups

**Table 8** Logistic regression analysis of risk factors associated with elevated transferrin saturation  $\geq 50\%$ 

Independent variable	$\beta$	OR Exp ( $\beta$ )	95% CI	P-value
<i>HFE</i> genotype				0.0001
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
Alcohol (drinks/week)				0.07
6-15	0.095	1.1	0.7-1.8	0.7
16-21	0.578	1.8	0.94-3.4	0.07
>21	0.862	2.4	1.1-5.1	0.03
Egg (no./week)				
>4	-0.772	0.5	0.2-1.04	0.06

Logit-value ( $\alpha$ ) in this model is -3.586.

C282Y homozygous or C282Y/H63D compound heterozygous individuals have an excess dietary iron absorption and may over time develop iron-overload-associated organ damage. However, both the phenotypic penetrance and the expressivity of *HFE* hemochromatosis are incomplete and several homozygotes and compound heterozygotes do not develop clinical iron overload disease [12, 18]. Modifier genes and/or environmental factors may cause this variability in penetrance.

We observed an age-associated increase in high serum ferritin values  $\geq 300 \mu\text{g/l}$ , although there was no significant increase in median serum ferritin levels (Table 3). In H63D heterozygotes, the percent of men with elevated serum ferritin rose significantly more with age than in wt/wt homozygotes ( $\chi^2$  test,  $p=0.004$ ), stressing the impact of H63D on body iron status.

Age-related increase in high serum ferritin values have been reported in other Danish studies [16, 19] and may be

**Table 9** Logistic regression analysis of risk factors associated with elevated serum ferritin  $\geq 300 \mu\text{g/L}$ 

Independent variable	$\beta$	OR Exp ( $\beta$ )	95% CI	P-value
<i>HFE</i> genotype				0.0001
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
Age (years)				0.03
39-45	0.585	1.8	1.03-3.1	0.04
46-53	0.680	2.0	1.2-3.3	0.01
Blood donation last 2 years				0.0001
0	-0.0605	0.5	0.3-0.96	0.04
1-4	-2.032	0.1	0.04-0.4	0.001
>4	-3.450	0.03	0.004-0.3	0.001
Alcohol (drinks/week)				0.0001
6-15	0.271	1.3	0.9-2.0	0.20
16-21	0.777	2.2	1.2-3.8	0.006
>21	1.453	4.3	2.2-8.2	0.0001
Egg (no./week)				
>4	-0.716	0.5	0.3-0.95	0.04

Logit-value ( $\alpha$ ) in this model is -2.66

due to genetic modifiers of iron absorption and/or changes in dietary habits with increased meat [20] and alcohol consumption ([www.sst.dk](http://www.sst.dk)) A similar age dependent effect was not observed for transferrin saturation.

Danish men have a dietary iron intake of median 10.9 mg/day (10-90 percentile 7.2-15.3) [20] with a bioavailability of 15-20%. Cook [21] reported that iron fortification in foods could increase the phenotypic penetrance of hemochromatosis. In Denmark, food iron fortification was stopped in 1987.

The Danish Health Authorities recommended a maximum alcohol intake for men of 21 drinks/week corresponding to 252 g ethanol/week ([www.sst.dk](http://www.sst.dk)). High alcohol consumption (>21 drinks/week) was associated with elevated serum iron. Among Danish men 45-66 years of age, ~20% consume more than 21 drinks per week ([www.sst.dk](http://www.sst.dk)). However, the increase in serum iron was not high enough to elevate transferrin saturation. Likewise, high alcohol consumption was associated with elevated serum ferritin (Table 4). The positive association between alcohol intake and serum ferritin has been observed in previous Danish surveys [22]. The mechanisms by which alcohol increase serum ferritin are not completely understood, but alcohol can induce hepatocellular injury, suppress erythropoiesis, reduce erythrocyte survival [23] enhance iron absorption and possibly also ferritin synthesis [24], all of which contribute to elevated ferritin. Altogether, alcohol promotes and accelerates the phenotypic penetrance of *HFE* variants and the expressivity with development of hepatic damage [25, 26].

Donation of blood has a profound impact on iron status [16, 27–29]. Among Danish men, ~28% are blood donors [27]. With an increasing number of blood donations, serum ferritin and eventually serum iron and transferrin saturation decrease. Blood donation is clearly an effective way to reduce body iron stores and decrease the phenotypic penetrance and expressivity of the *HFE* variants. In patients with *HFE* associated iron overload, phlebotomies are usually performed until serum ferritin reaches a level of 50-100 µg/L.

In our adjusted logistic regression analysis, the *HFE* genotype had the highest impact on iron status markers followed by blood donation, alcohol intake, age, and egg consumption.

Consumption of meat (beef, pork, poultry) has a positive effect on iron status [30, 31] and men with a high meat intake displayed higher serum ferritin compared to those with a low intake. In addition to “the meat factor”, which enhances iron absorption, meat contains heme iron, which has a higher bioavailability than non-heme iron. Therefore, high meat consumption may increase the phenotypic penetrance and expressivity of the *HFE* variants. Danish men consume median 132 g meat (beef, pork, poultry) per day (10-90 percentile 46-158) and ~80% consume meat on a daily basis [20].

Drinking large quantities of milk inhibits iron absorption in a dose related manner, probably due to the calcium content [32]. Danish men consume median 248 g milk/day and ~66% consume milk on a daily basis [20]. Consequently, devoted milk consumption may lower the phenotypic penetrance of the *HFE* variants. Other studies have shown that regular tea drinking causes a 30% decrease in iron absorption in hemochromatosis patients [33].

Egg contains sulphides, which can inhibit iron absorption, but appeared to have no effect on iron status even in a quantity of more than four eggs per week. The recommended intake is 3-4 eggs/week. Danish men consume median 2 eggs/week and ~5% consume more than 7 eggs per week [20].

Multivitamin-multimineral tablets containing 14-20 mg of ferrous iron had no influence on iron status as previously reported in Danish studies [19]. Among Danish men, ~30% use daily vitamin-mineral supplements containing median 14 mg ferrous iron [27]. Obviously, iron absorption from these preparations is very low, probably due to the content of other essential divalent metal ions (zinc, copper, manganese ect.), which compete with iron about the absorption capacity in the gastrointestinal tract [34, 35]. However, inappropriate use of iron tablets on vague indications such as fatigue ect. without an established diagnosis of iron deficiency may promote iron overload in C282Y homozygous individuals [36–39].

Men reporting having peptic ulcers had significantly lower iron status than men without ulcers. *Helicobacter pylori* infection may induce peptic ulcer, has a negative influence on iron status [40] and may thereby reduce the phenotypic penetrance of *HFE* variants. In general, pathological blood losses tend to lower the penetrance and expressivity of the *HFE* variants

In conclusion, the C282Y variant have a high phenotypic penetrance in Danish men [11]. The expressivity of the C282Y and H63D variants may be promoted by high alcohol and meat consumption and decreased by high milk consumption and blood donation.

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