Risk of fractures according to iron parameters and hemochromatosis *HFE* genotype in 142,146 general population individuals

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Supplemental material

Table of contents Page No.

Supplemental Methods 3

Supplemental Table 1: WHO's International Classification of Diseases, revision 8 (ICD8) and revision 10 (ICD10) codes for categories of fracture types. 8

Supplemental Table 2: Baseline characteristics of general population individuals (n=132,499) according to *HFE* genotypes. 9

Supplemental Figure 1: Risk of any fracture when investigating all variables included in the multivariable adjusted model. 10

Supplemental Figure 2: Risk of any fracture according to concentrations of iron, transferrin saturation, and ferritin, when only investigating non-carriers for C282Y and H63D. 12

Supplemental Figure 3: Risk of any fracture according to concentrations of iron, transferrin saturation, and ferritin, when only investigating individuals without anemia. 14

Supplemental Figure 4: Risk of any fracture according to concentrations of iron, transferrin saturation, and ferritin, when only investigating individuals without any comorbidity at study enrollment. 16

Supplemental Figure 5: Risk of fractures of the wrist, axial skeleton, and humerus according to iron, transferrin saturation, and ferritin. 18

Supplemental Figure 6: Risk of any fracture according to concentrations of iron, transferrin saturation, and ferritin, stratified by sex. 20

Supplemental Figure 7: Risk of any fracture according to concentrations of iron when each individual's follow-up time was split into the first five years after study enrollment (upper panel) and from 5 years after study enrollment and onwards (lower panel). 22

Supplemental Figure 8: Risk of any fracture according to concentrations of transferrin saturation when each individual's follow-up time was split into the first five years after study enrollment (upper panel) and from 5 years after study enrollment and onwards (lower panel). 23

Supplemental Figure 9: Risk of any fracture according to concentrations of ferritin when each individual's follow-up time was split into the first five years after study enrollment (upper panel) and from 5 years after study enrollment and onwards (lower panel). 24

Supplemental Figure 10: Risk of fractures of the wrist, axial skeleton, and humerus according to *HFE* genotype. 25

Supplemental Figure 11: Multivariable adjusted risk of any fracture for *HFE* C282Y homozygotes vs. non-carriers for the C282Y and H63D variants stratified by normal vs. high concentraions of plasma iron (upper panel), transferrin saturation (middle panel), and ferritin (lower panel) at study enrollment. 26

Supplemental Figure 12: Risk of any fracture for *HFE* C282Y homozygotes vs. non-carriers for the C282Y and H63D variants, stratified according to whether or not individuals had been diagnosed with liver disease and/or diabetes at any time before or after enrolling into the study. 28

Supplemental Figure 13: Risk of any fracture (upper panel) and fracture of the hip and femur (lower panel) for *HFE* C282Y homozygotes vs. non-carriers for the C282Y and H63D variants, stratified by sex. 29

Supplemental Figure 14: Risk of any fracture (upper panel) and fracture of the hip and femur (lower panel) for *HFE* C282Y homozygotes vs. non-carriers for the C282Y and H63D variants, stratified by age. 30

References for supplemental material 31

Supplemental Methods

Participants

We studied 142,146 individuals aged 20-100 years from three general population cohort studies. Among individuals invited for the Copenhagen City Heart Study, 61% participated, while 43% of the invited individuals participated in the Copenhagen General Population Study and the Danish General Suburban Population Study. More than 99% of individuals were white and of Danish descent. The three cohort studies used similar study procedures, and individuals were only included in one of the studies.

Covariates and comorbidities

All included covariates were chosen *a priori* based on previous reports of an association with iron, transferrin saturation, ferritin, and/or risk of fractures.[1–4] Information on date of birth and sex was obtained from the Danish Civil Registration System, whereas information on alcohol consumption (none/moderate/heavy, with heavy defined as >168 g/week for men and >84 g/week for women, which was the recommendation from The Danish State Health Authority at the end of follow-up for this study), smoking status (current/former/never), cumulative smoking in pack-years (defined as 20 cigarettes or equivalent smoked per day for a year), and menopausal status (premenopausal/postmenopausal in women only) was obtained from the questionnaire, and body mass index (measured weight in kilograms divided by the measured height in meters squared, categorized into six groups: <18.5/18.5-24.9/25-29.9/30-34.9/35-39.9/≥40) from the physical examination. Plasma C-reactive protein levels were measured in blood samples drawn at study enrollment using standard high-sensitivity hospital assays. For the statistical analyses, plasma C-reactive protein levels were dichotomized into < 3 or ≥ 3 mg/L.

As comorbid disease may affect concentrations of plasma iron, transferrin saturation, and ferritin, but also potentially affect risk of fractures, we used the Charlson comorbidity index and retrieved information from the national Danish Patient Register on comorbidities at study enrollment using previously published ICD-8 and ICD-10 codes.[5] The Charlson Comorbidity Index[6] considers both the number and severity of comorbid diseases, and is a severity-weighted index that has been validated for its capacity to predict mortality.[5,7] The Charlson Comorbidity Index categorizes comorbidities into the following 17 categories: HIV/AIDS, any malignancy, cerebrovascular disease, chronic pulmonary disease, congestive heart failure, dementia, diabetes without chronic

complications, diabetes with chronic complications, hemiplegia/paraplegia, metastatic solid tumor, mild liver disease, moderate/severe liver disease, myocardial infarction, peptic ulcer disease, peripheral vascular disease, renal disease, and rheumatic disease. For the statistical analyses, the Charlson comorbidity index was categorized into any comorbidity or no comorbidity.

Measurements of plasma iron, transferrin saturation, and ferritin

On the day of study enrollment, all individuals in the three general population cohorts had blood samples drawn. In total, 136,611 individuals had plasma iron measurements, 136,555 had transferrin saturation measurements, and 37,990 individuals had ferritin measurements. From the Copenhagen City Heart Study, we included individuals at two different examinations: in 1981-1983 and 1991-1994. Hence, we included 9,002 individuals with ferritin measurements from the 1981-1983 examination of the Copenhagen City Heart Study and 9,727 individuals with iron, transferrin saturation, or *HFE* genotype measured from the 1991-1994 examination. Of note, 5,063 individuals attended both examinations in the Copenhagen City Heart Study and had ferritin measured in the blood samples obtained in 1981-1983, while their plasma iron, transferrin saturation, and/or *HFE* genotype were measured using the blood samples obtained in the 1991-1994 examination.

For the Copenhagen City Heart Study and the Copenhagen General Population Study, a Konelab autoanalyzer (ThermoFisher Scientific) was used to measure plasma iron and transferrin (for calculation of transferrin saturation), and an Advia Centaur (Siemens) was used for ferritin measurements. For the Danish General Suburban Population Study, a Cobas 6000 (Roche) was used for iron, transferrin, and ferritin measurements.

Precision of the assays was tested on a daily basis using internal control samples (coefficients of variation were typically around 1-3% for plasma iron, 4-8% for transferrin, and 4-8% for ferritin). Accuracy was monitored using an external quality control program, typically on a monthly basis. As we intended to produce an operative single-calibrator measurement, we normalized the raw measurements of plasma iron, transferrin, and ferritin across analysis platforms and calibrator lots, as previously described in detail, along with details on sample storage, assays, and calibration.[8,9]

Categorization of plasma iron, transferrin saturation, and ferritin

For the analyses on risk of fractures according to concentrations of plasma iron, transferrin saturation, and ferritin, our focus was primarily to examine risk of fractures in individuals with the lowest or highest concentrations of the three biomarkers. Therefore, we used five categories of

plasma iron, transferrin saturation, and ferritin: ≤ 5th percentile, 6th-25th percentile, 26th-74th percentile, 75th-94th percentile, and ≥ 95th percentile, with the middle category (26th-74th percentile) defined as the reference group for the statistical analysis.

In contrast, when studying risk of any fracture in C282Y homozygotes stratified by concentrations of plasma iron, transferrin saturation, and ferritin, our focus was to examine whether current clinical guidelines on hemochromatosis are sufficient for identifying C282Y homozygotes at high risk of fractures. Therefore, we used clinically relevant definitions obtained from our local hospital laboratory (for plasma iron) or from clinical guidelines on hemochromatosis (for transferrin saturation and ferritin).[10,11] Thus, definitions were as follows: Normal iron was defined as 9-34 μmol/L and high iron as >34 μmol/L. For transferrin saturation, the reference range varies according to age and sex, and therefore normal transferrin saturation was defined as 10-45% for women ≤50 years of age and 15-45% for women >50 years of age and men of any age. High transferrin saturation was defined as >45% for men and women of any age. Normal ferritin was defined as 12-200 μg/L for women and 12-300 μg/L for men, and high ferritin as >200 μg/L for women and >300 μg/L for men.

Genotypes

The *HFE* gene was genotyped for the C282Y and H63D variants in 132,499 individuals using blood samples collected at study enrollment. We tested for Hardy-Weinberg equilibrium using the χ^2 test, which was done separately for the C282Y variant and the H63D variant. P-values for Hardy-Weinberg equilibrium for the C282Y variant were 0.28, 0.28, and 0.58 in the Copenhagen City Heart Study, the Copenhagen General Population Study, and the Danish General Suburban Population Study, respectively. Corresponding p-values for the H63D variant were 0.35, 0.03, and 0.68. When taking the three multiple comparisons into account by using the Bonferroni method (as we included three general population cohorts), none of the above-mentioned p-values were significant at the $p < 0.05$ level, as it would require a p-value < 0.017 (=0.05/3).

Fractures, vital status, and diagnoses of hemochromatosis

We used the national Danish Patient Register[12] which covers all Danish hospitals, to obtain information on all inpatient admissions from January 1st, 1977, until December 31st, 2021, and information on all outpatient and emergency room visits from January 1st, 1994, until December 31st, 2021. Diagnoses were coded using WHO's International Classification of Diseases (ICD)-8

codes from January 1st, 1977, which was directly replaced by ICD-10 on January 1st, 1994. We defined fracture events as any inpatient or emergency room admissions due to fractures, categorized using ICD-8 and ICD-10 codes as presented in Supplemental Table 1.[13] Information on emigration and vital status until December 31st, 2021, was obtained from the Danish Civil Registration system,[14] which contains 100% complete information for all individuals with permanent residence in Denmark.

National Danish clinical guidelines state that hereditary hemochromatosis must be treated in public hospitals only.[15] To perform stratified analyses on whether or not C282Y homozygotes were diagnosed with hereditary hemochromatosis and had therefore potentially been treated with therapeutic phlebotomy, we used the national Danish Patient Register to obtain information on hospital contacts due to hereditary hemochromatosis (ICD-8 code 27329 and ICD-10 code E831A) from January 1st, 1977, until December 31st, 2021.

Statistical analyses

When risk of any fracture and fracture of the hip and femur were modelled using Cox regression according to continuous concentrations of iron, transferrin saturation, and ferritin, results were presented using restricted cubic splines. The number of knots for all cubic splines presented in this study was four, which was chosen based on Akaike's information criteria[16] combined with visual inspection of the splines in case an underfitted model was suspected. For the categorical analyses on risk of fractures according to plasma iron, transferrin saturation, and ferritin, we used five categories of plasma iron, transferrin saturation, and ferritin: ≤5th percentile, 6th-25th percentile, 26th-74th percentile, 75th-94th percentile, and ≥95th percentile, with the middle category (26th-74th percentile) defined as the reference group. Since *HFE* genotype is not influenced by comorbid diseases or lifestyle factors, we decided *a priori* to study risk of fractures according to *HFE* genotype using the age and sex-adjusted model, and to use the multivariable models only when studying risk of fractures according to concentrations of iron, transferrin saturation, and ferritin. However, as a sensitivity analysis, we also studied risk of fractures according to *HFE* genotype using the multivariable adjusted model.

When analyzing risk of fractures according to concentrations of iron, transferrin saturation, and ferritin, follow-up began at the date of study enrollment (where blood samples were drawn) and ended on date of hospitalization due to a fracture, death due to any cause (n=28,342), emigration (n=545), or December 31st, 2021, whichever came first. When analyzing risk of fractures according

to *HFE* genotype, follow-up began at age 20 or January 1st, 1977, whichever came last, and ended on the date of hospitalization due to a fracture, death, emigration, or December 31st, 2021, whichever came first. Fractures prior to the start of follow-up were ignored in all analyses.
For the Cox proportional hazard models, no major violations of the proportional hazards assumption were observed when tested using visual plotting of -ln(-ln(survival)) against ln(analysis time) and further using formal testing based on Schoenfeld residuals if non-parallel curves were suspected visually.

All age and sex-adjusted models were also adjusted for study cohort to account for potential differences between the three cohorts. Multivariable adjusted models were likewise adjusted for age, sex, and study cohort, and were additionally adjusted for values at study enrollment of alcohol consumption, body mass index, smoking status, cumulative smoking in pack-years, menopausal status (women only), plasma C-reactive protein, and Charlson comorbidity index.

Information on age, sex, study cohort, and Charlson comorbidity index was available on all individuals with no missing data. For the remaining variables in the multivariable adjusted models, information was missing for an average of 3% of individuals. Missing data for the categorical variables smoking status, alcohol consumption, body mass index, C-reactive protein, and menopausal status were coded as a missing category, while missing values for the continuous variable cumulative smoking were imputed based on age, sex, study cohort, and smoking status using linear regression. However, results were similar to those presented if only individuals with complete information on all covariates were included.

Fracture type	ICD8 codes	ICD10 codes
Hip and femur	82000-82003, 82008, 82009, 82109-82111, 82118, 82119, 82199	S720, S721, S721A-C, S722-724, S724A-C, S727, S728, S728A, S729
Wrist	81320, 81321, 81328, 81329	S525, S525A-C, S526
Axial skeleton		
• **Vertebral**	80500, 80501, 80508-80511, 80519	S120, S121, S121A, S121B, S122, S122A-E, S127, S129, S220, S220A-L, S221, S320, S320A-E
• **Ribs**	80709	S223
• **Pelvic**	80800	S321-325, S327, S327A-C, S328, S328A-C
Humerus	81200-81202, 81208, 81209, 81219-81222, 81228, 81229, 81299	S422, S422A-C, S423, S423A, S424, S424A-C

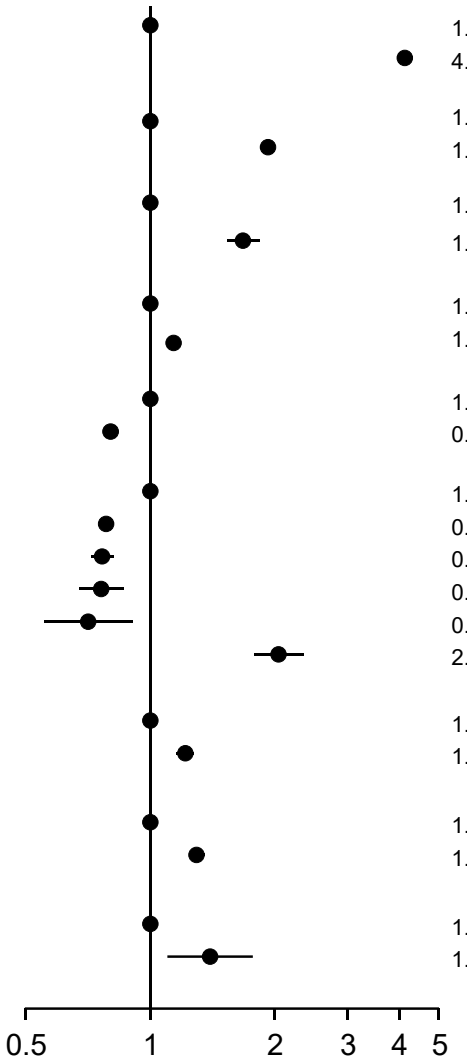
Supplemental Table 1: WHO's International Classification of Diseases, revision 8 (ICD8) and revision 10 (ICD10) codes for categories of fracture types.

	HFE genotype					
	Non-carrier/non-carrier	H63D/non-carrier	H63D/H63D	C282Y/non-carrier	C282Y/H63D	C282Y/C282Y
Individuals, n.	87,286	27,811	2,357	12,508	2,115	422
Plasma iron measurements, µmol/L (IQR)	13.0 (10.6-16.0)	14.6 (11.7-17.7)	16.6 (13.0-20.0)	15.0 (12.0-18.0)	18.0 (15.0-22.0)	25.0 (19.0-31.0)
Plasma transferrin saturation, % (IQR)	21.6 (16.9-26.8)	23.9 (18.9-29.6)	28.3 (22.6-34.9)	25.7 (20.2-31.6)	33.3 (26.7-41.4)	57.5 (42.9-75.0)
Plasma ferritin, µg/l (IQR)	98.0 (49.0-165.0)	103.0 (52.0-175.0)	121.5 (57.4-202.5)	105.7 (51.4-184.0)	124.3 (58.0-241.0)	311.2 (93.5-605.2)
Age, years (IQR)	58 (48-67)	58 (48-67)	58 (48-67)	58 (48-67)	57 (47-67)	57 (47-65)
Male sex, n. (%)	39,375 (45)	12,476 (45)	1,077 (46)	5,668 (45)	961 (45)	194 (46)
General population cohort						
Copenhagen City Heart Study, n. (%)	6,133 (7)	1,883 (7)	158 (7)	844 (7)	131 (6)	23 (5)
Copenhagen General Population Study, n. (%)	67,940 (78)	21,680 (78)	1,846 (78)	9,800 (78)	1,667 (79)	337 (80)
Danish General Suburban Population Study, n. (%)	13,213 (15)	4,248 (15)	353 (15)	1,864 (15)	317 (15)	62 (15)

Supplemental Table 2: Baseline characteristics of general population individuals (n=132,499) according to *HFE* genotypes. C282Y/C282Y, homozygous for C282Y; C282Y/H63D, compound heterozygous for C282Y and H63D; C282Y/non-carrier, heterozygous for C282Y; H63D/H63D, homozygous for H63D; H63D/non-carrier, heterozygous for H63D; Non-carrier/non-carrier, non-carrier for both C282Y and H63D. Values are median (IQR) for continuous variables and number of individuals (%) for categorical variables. n., number; IQR, interquartile range.

Any fracture

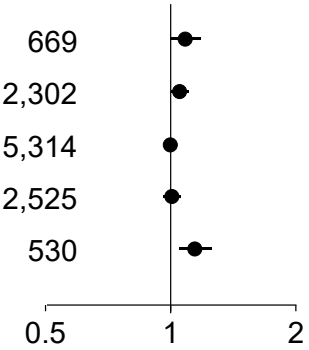
	Individuals, n.	Fractures, n.	Hazard ratio (95% CI)	p
Age, years				
≤ 60	76,802	3,336	1.00 (reference)	
> 60	64,103	8,213	4.15 (3.98 to 4.32)	$< 10^{-300}$
Sex				
Men	63,872	3,449	1.00 (reference)	
Women	77,033	8,100	1.91 (1.84 to 1.99)	7×10^{-223}
Menopause*				
Before	24,891	781	1.00 (reference)	
After	51,563	7,249	1.71 (1.57 to 1.87)	4×10^{-32}
Smoking				
Never smoker	56,184	3,935	1.00 (reference)	
Ever smoker	84,259	7,583	1.13 (1.09 to 1.18)	2×10^{-10}
Alcohol†				
Alcohol consumption ≤ 168/84 g/week	85,384	7,131	1.00 (reference)	
Alcohol consumption > 168/84 g/week	49,315	3,792	0.80 (0.77 to 0.83)	3×10^{-28}
Body mass index, kg/m²				
> 18.5 & ≤ 25	60,330	5,239	1.00 (reference)	
> 25 & ≤ 30	55,465	4,308	0.79 (0.76 to 0.82)	6×10^{-31}
> 30 & ≤ 35	17,836	1,342	0.77 (0.73 to 0.82)	2×10^{-17}
> 35 & ≤ 40	4,115	274	0.77 (0.68 to 0.86)	2×10^{-5}
> 40	1,242	68	0.71 (0.56 to 0.91)	0.006
≤ 18.5	1,400	274	2.07 (1.81 to 2.37)	1×10^{-26}
C-reactive protein level, mg/L				
≤ 3	108,104	7,989	1.00 (reference)	
> 3	25,599	2,613	1.22 (1.16 to 1.27)	5×10^{-18}
Comorbid disease‡				
No	111,786	8,088	1.00 (reference)	
Any	29,119	3,461	1.30 (1.25 to 1.36)	3×10^{-37}
***HFE* hemochromatosis**				
Non-carrier/non-carrier	87,286	11,537	1.00 (reference)	
C282Y/C282Y	422	68	1.39 (1.10 to 1.77)	0.006



Supplemental Figure 1: Risk of any fracture when performing univariate Cox regression investigating each variable included in the multivariable adjusted model independently and when univariately analyzing the effect of *HFE* genotype in the lower part of the figure (C282Y homozygotes versus non-carriers). For the analyses on age, Cox regression used time since study enrollment as timescale. For the rest of the analyses, left-truncated age was used as timescale. To be consistent with the analyses in the main manuscript, follow-up for the analysis on risk of fractures according to *HFE* genotype began at the time of creation of the Danish National Patient Register in 1977 or each individual's 20th birthday, whichever came last. For all the remaining univariate analyses, follow-up began at the date of study enrollment. Number of individuals in the different univariate analyses varies slightly, due to varying number of individuals with missing values. For the analyses of age, sex, and comorbid disease, all individuals with either an iron, transferrin saturation, or ferritin measurement were included, and therefore the number of included individuals in these analyses are slightly higher than number of individuals included in Figure 1. * Women only. † Alcohol consumption ≤ 168 g/week for men and ≤ 84 g/week for women as was the recommendation from The Danish State Health Authority at the end of follow-up for this study. ‡ Any comorbid disease at study enrollment as defined by the Charlson comorbidity index.

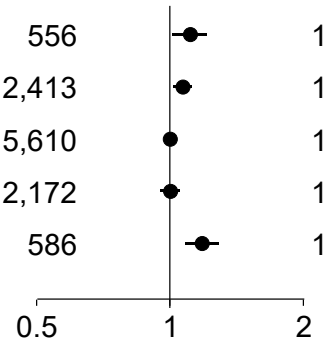
Iron

Percentiles of iron	Individuals, n.	Fractures, n.	Multivariable adjusted hazard ratio (95% CI) for any fracture	p
≤ 5th	4,689	669	1.08 (1.00 to 1.18)	0.05
6-25th	15,922	2,302	1.05 (1.00 to 1.11)	0.04
26-74th	40,386	5,314	1.00 (reference)	
75-94th	20,805	2,525	1.01 (0.96 to 1.06)	0.72
≥ 95th	4,445	530	1.14 (1.05 to 1.25)	0.003



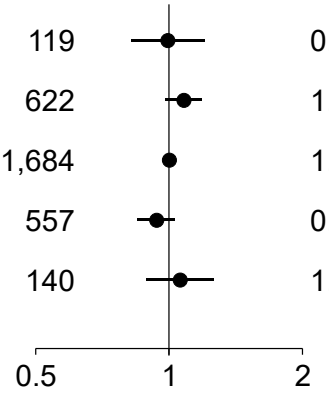
Transferrin saturation

Percentiles of transferrin saturation	Individuals, n.	Fractures, n.	Multivariable adjusted hazard ratio (95% CI) for any fracture	p
≤ 5th	4,296	556	1.11 (1.01 to 1.21)	0.02
6-25th	17,259	2,413	1.07 (1.02 to 1.12)	0.009
26-74th	42,873	5,610	1.00 (reference)	
75-94th	17,437	2,172	1.00 (0.95 to 1.05)	0.94
≥ 95th	4,359	586	1.18 (1.08 to 1.29)	0.0002



Ferritin

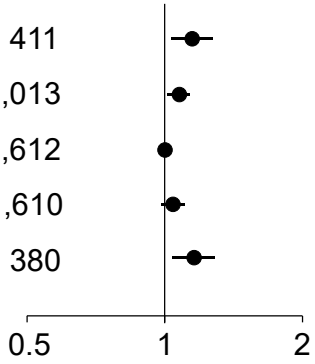
Percentiles of ferritin	Individuals, n.	Fractures, n.	Multivariable adjusted hazard ratio (95% CI) for any fracture	p
≤ 5th	1,105	119	0.99 (0.82 to 1.20)	0.93
6-25th	4,417	622	1.08 (0.98 to 1.19)	0.12
26-74th	11,034	1,684	1.00 (reference)	
75-94th	4,418	557	0.94 (0.85 to 1.03)	0.19
≥ 95th	1,106	140	1.06 (0.89 to 1.26)	0.53



Supplemental Figure 2: Risk of any fracture according to concentrations of iron, transferrin saturation, and ferritin, when only investigating non-carriers for C282Y and H63D and thereby excluding individuals who were H63D homozygous, H63D heterozygous, C282 heterozygous, H63D/C282Y compound heterozygous, or C282 homozygous. Of note, individuals who had not been genotyped were also excluded. Multivariable adjustment included age, sex, study cohort, alcohol consumption, body mass index, smoking status, cumulative smoking in pack-years, menopausal status (women only), Charlson comorbidity index, and C-reactive protein level. n., number; CI, confidence interval.

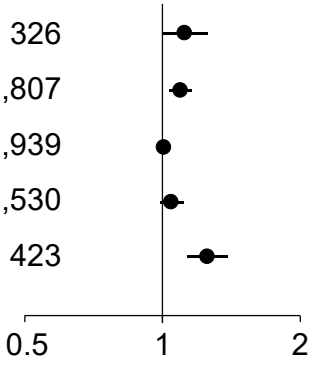
Iron

Percentiles of iron	Individuals, n.	Fractures, n.	Multivariable adjusted hazard ratio (95% CI) for any fracture	p
≤ 5th	5,387	411	1.15 (1.03 to 1.27)	0.01
6-25th	26,451	2,013	1.07 (1.02 to 1.14)	0.01
26-74th	56,576	3,612	1.00 (reference)	
75-94th	26,224	1,610	1.04 (0.98 to 1.11)	0.18
≥ 95th	6,521	380	1.16 (1.04 to 1.29)	0.007



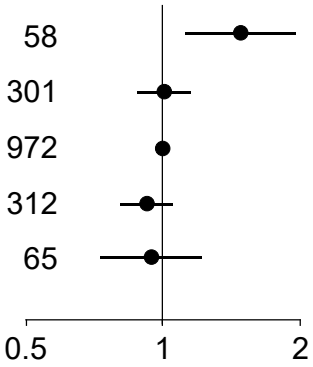
Transferrin saturation

Percentiles of transferrin saturation	Individuals, n.	Fractures, n.	Multivariable adjusted hazard ratio (95% CI) for any fracture	p
≤ 5th	4,990	326	1.11 (0.99 to 1.25)	0.08
6-25th	24,556	1,807	1.09 (1.03 to 1.15)	0.004
26-74th	60,674	3,939	1.00 (reference)	
75-94th	24,717	1,530	1.04 (0.98 to 1.10)	0.22
≥ 95th	6,179	423	1.24 (1.12 to 1.38)	2×10^{-5}



Ferritin

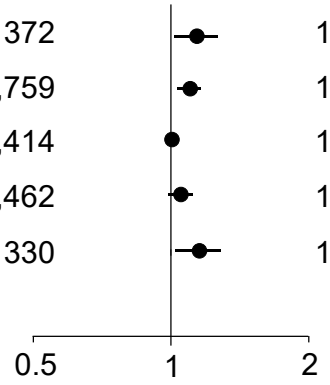
Percentiles of ferritin	Individuals, n.	Fractures, n.	Multivariable adjusted hazard ratio (95% CI) for any fracture	p
≤ 5th	1,149	58	1.48 (1.12 to 1.95)	0.006
6-25th	5,507	301	1.01 (0.88 to 1.15)	0.90
26-74th	13,656	972	1.00 (reference)	
75-94th	5,657	312	0.92 (0.81 to 1.05)	0.24
≥ 95th	1,358	65	0.94 (0.73 to 1.22)	0.67



Supplemental Figure 3: Risk of any fracture according to concentrations of iron, transferrin saturation, and ferritin, when only investigating individuals without anemia, defined as a hemoglobin concentration < 11.8 g/dL for women and < 13.4 g/dL for men, using reference values from our local hospital laboratory. Individuals who did not have a measurement of hemoglobin were also excluded, meaning that all individuals from the Copenhagen City Heart Study were excluded from these analyses, as hemoglobin measurements were not performed in the Copenhagen City Heart Study. Multivariable adjustment included age, sex, study cohort, alcohol consumption, body mass index, smoking status, cumulative smoking in pack-years, menopausal status (women only), Charlson comorbidity index, and C-reactive protein level. n., number; CI, confidence interval.

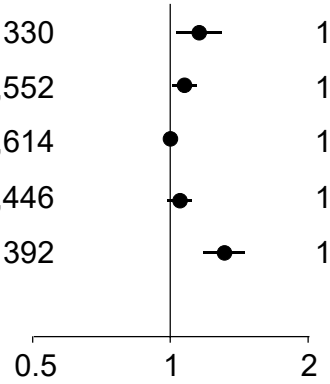
Iron

Percentiles of iron	Individuals, n.	Fractures, n.	Multivariable adjusted hazard ratio (95% CI) for any fracture	p
≤ 5th	5,475	372	1.13 (1.02 to 1.27)	0.03
6-25th	23,310	1,759	1.10 (1.03 to 1.16)	0.002
26-74th	50,438	3,414	1.00 (reference)	
75-94th	23,161	1,462	1.05 (0.98 to 1.11)	0.15
≥ 95th	5,682	330	1.15 (1.02 to 1.29)	0.02



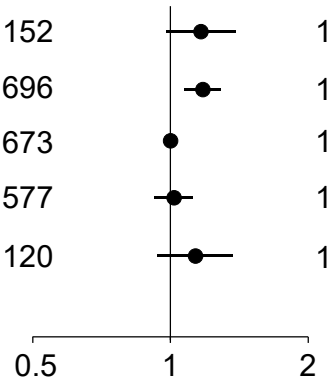
Transferrin saturation

Percentiles of transferrin saturation	Individuals, n.	Fractures, n.	Multivariable adjusted hazard ratio (95% CI) for any fracture	p
≤ 5th	5,293	330	1.15 (1.03 to 1.30)	0.01
6-25th	21,588	1,552	1.07 (1.01 to 1.14)	0.02
26-74th	53,716	3,614	1.00 (reference)	
75-94th	22,018	1,446	1.05 (0.99 to 1.12)	0.12
≥ 95th	5,409	392	1.31 (1.18 to 1.46)	4×10^{-7}

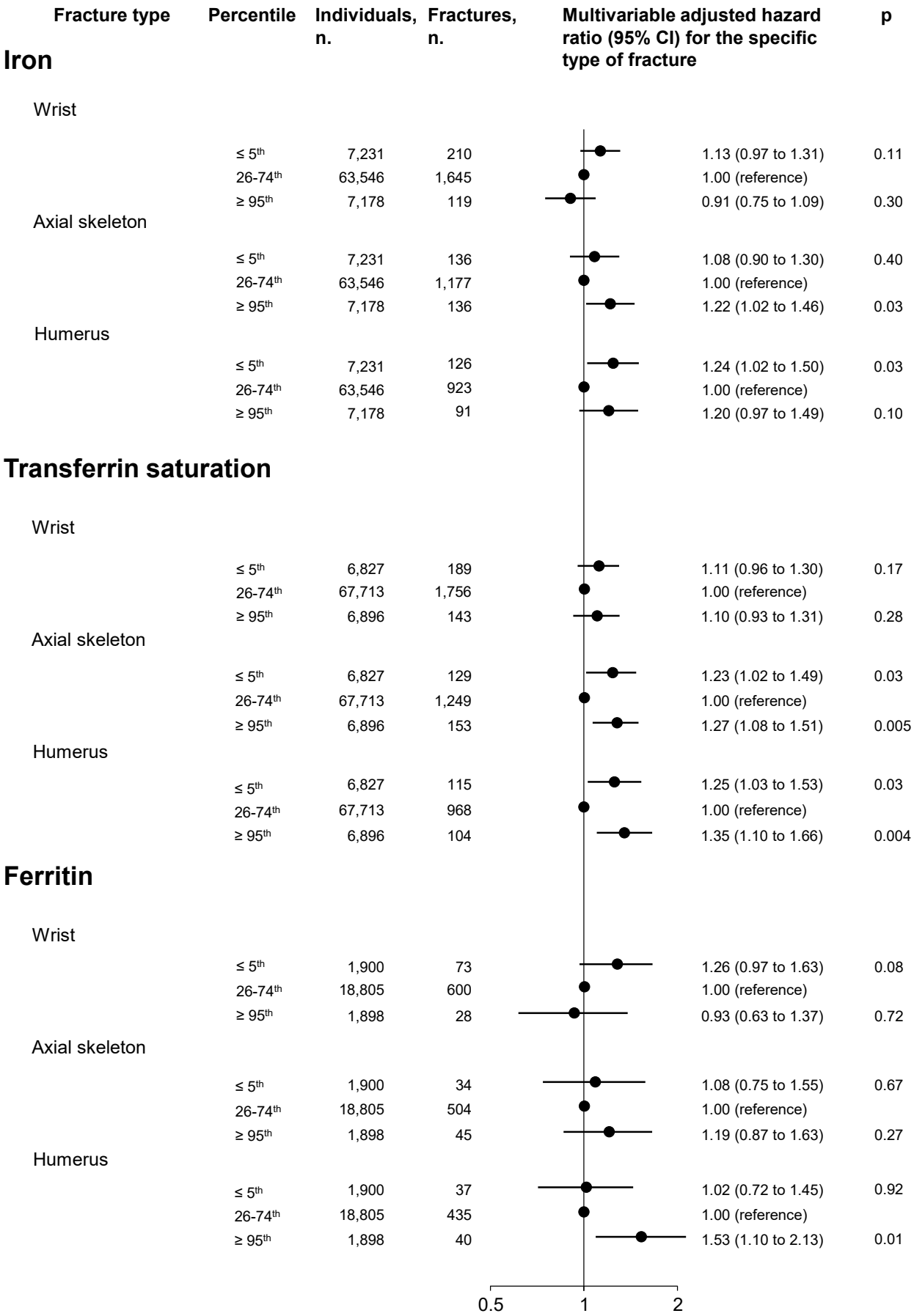


Ferritin

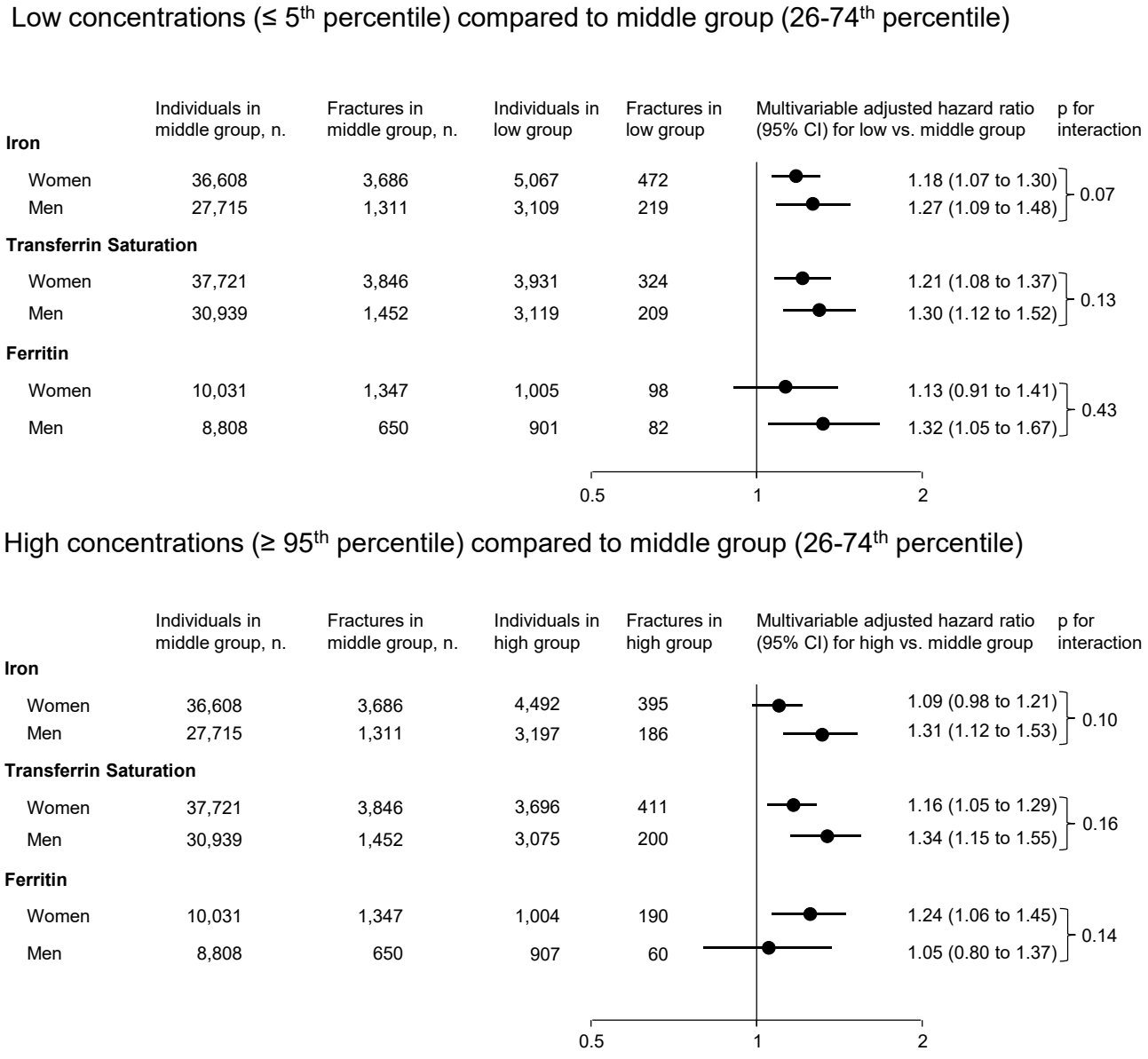
Percentiles of ferritin	Individuals, n.	Fractures, n.	Multivariable adjusted hazard ratio (95% CI) for any fracture	p
≤ 5th	1,662	152	1.16 (0.98 to 1.39)	0.09
6-25th	6,350	696	1.18 (1.07 to 1.29)	6×10^{-4}
26-74th	15,135	1,673	1.00 (reference)	
75-94th	6,458	577	1.02 (0.92 to 1.12)	0.73
≥ 95th	1,513	120	1.13 (0.94 to 1.37)	0.20



Supplemental Figure 4: Risk of any fracture according to concentrations of iron, transferrin saturation, and ferritin, when only investigating individuals without any comorbidity at study enrollment, as defined by the Charlson comorbidity index. Multivariable adjustment included age, sex, study cohort, alcohol consumption, body mass index, smoking status, cumulative smoking in pack-years, menopausal status (women only), and C-reactive protein level. n., number; CI, confidence interval.


Fracture type
Percentile
Individuals, n.
Fractures, n.
Multivariable adjusted hazard ratio (95% CI) for the specific type of fracture
p
Iron
Wrist
≤ 5th 7,231 210 1.13 (0.97 to 1.31) 0.11
26-74th 63,546 1,645 1.00 (reference)
≥ 95th 7,178 119 0.91 (0.75 to 1.09) 0.30
Axial skeleton
≤ 5th 7,231 136 1.08 (0.90 to 1.30) 0.40
26-74th 63,546 1,177 1.00 (reference)
≥ 95th 7,178 136 1.22 (1.02 to 1.46) 0.03
Humerus
≤ 5th 7,231 126 1.24 (1.02 to 1.50) 0.03
26-74th 63,546 923 1.00 (reference)
≥ 95th 7,178 91 1.20 (0.97 to 1.49) 0.10
Transferrin saturation
Wrist
≤ 5th 6,827 189 1.11 (0.96 to 1.30) 0.17
26-74th 67,713 1,756 1.00 (reference)
≥ 95th 6,896 143 1.10 (0.93 to 1.31) 0.28
Axial skeleton
≤ 5th 6,827 129 1.23 (1.02 to 1.49) 0.03
26-74th 67,713 1,249 1.00 (reference)
≥ 95th 6,896 153 1.27 (1.08 to 1.51) 0.005
Humerus
≤ 5th 6,827 115 1.25 (1.03 to 1.53) 0.03
26-74th 67,713 968 1.00 (reference)
≥ 95th 6,896 104 1.35 (1.10 to 1.66) 0.004
Ferritin
Wrist
≤ 5th 1,900 73 1.26 (0.97 to 1.63) 0.08
26-74th 18,805 600 1.00 (reference)
≥ 95th 1,898 28 0.93 (0.63 to 1.37) 0.72
Axial skeleton
≤ 5th 1,900 34 1.08 (0.75 to 1.55) 0.67
26-74th 18,805 504 1.00 (reference)
≥ 95th 1,898 45 1.19 (0.87 to 1.63) 0.27
Humerus
≤ 5th 1,900 37 1.02 (0.72 to 1.45) 0.92
26-74th 18,805 435 1.00 (reference)
≥ 95th 1,898 40 1.53 (1.10 to 2.13) 0.01
0.5
1
2

Supplemental Figure 5: Risk of fractures of the wrist, axial skeleton, and humerus according to iron, transferrin saturation, and ferritin. For all specific fracture types, results are presented only for individuals with concentrations of each biomarker (iron, transferrin saturation, or ferritin) <5th percentile, 26-74th percentile (reference group), or ≥95th percentile. Multivariable adjustment included age, sex, study cohort, alcohol consumption, body mass index, smoking status, cumulative smoking in pack-years, menopausal status (women only), Charlson comorbidity index, and C-reactive protein level. n., number; CI, confidence interval.



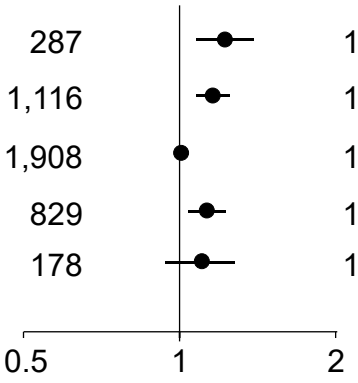
Supplemental Figure 6: Risk of any fracture according to iron, transferrin saturation, and ferritin, stratified by sex. Percentiles of iron, transferrin saturation, and ferritin were calculated for each sex separately. Based on the sex-specific percentiles, we calculated sex specific cut-offs for low iron (<7.0 μmol/L for women, <8.1 μmol/L for men), iron in the middle group (10-16 μmol/L for women, 12-18 μmol/L for men), high iron (>21 μmol/L for women, >24 μmol/L for men), low transferrin saturation (<10.0% for women, <13.6% for men), transferrin saturation in the middle group (16.2-26.1% for women, 19.9-30.6% for men), high transferrin saturation (>35.9% for women, >41.7% for men), low ferritin (<10.7 μg/L for women, <30.0 μg/L for men), ferritin in the middle group (34-117 μg/L for women, 89-227 μg/L for men), and high ferritin (>233 μg/L for women, >438 μg/L for men). The upper panel show risk estimates for comparing low concentrations (≤ 5th percentile) to the middle group (26th-74th percentile), while the lower panel present risk estimates for comparing high concentrations (≥ 95th percentile) to the middle group

(26^{th}-74^{th} percentile). Multivariable adjustment included age, study cohort, alcohol consumption, body mass index, smoking status, cumulative smoking in pack-years, menopausal status (women only), Charlson comorbidity index, and C-reactive protein level. Using a likelihood-ratio test, p for interaction was calculated by comparing two models, one model with and one model without an interaction term between sex and either iron, transferrin saturation, or ferritin. n., number; CI, confidence interval; vs., versus.

Iron

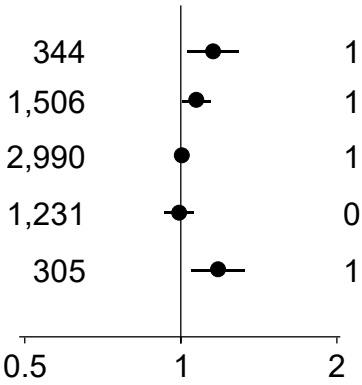
First 5 years after study enrollment

Percentiles of iron	Individuals, n.	Fractures, n.	Multivariable adjusted hazard ratio (95% CI) for any fracture	p
≤ 5th	7,231	287	1.22 (1.08 to 1.39)	0.002
6-25th	29,803	1,116	1.16 (1.08 to 1.25)	0.0001
26-74th	63,546	1,908	1.00 (reference)	
75-94th	28,853	829	1.13 (1.04 to 1.23)	0.004
≥ 95th	7,178	178	1.10 (0.94 to 1.28)	0.25



From 5 years after study enrollment and onwards

Percentiles of iron	Individuals, n.	Fractures, n.	Multivariable adjusted hazard ratio (95% CI) for any fracture	p
≤ 5th	6,492	344	1.15 (1.02 to 1.29)	0.02
6-25th	27,462	1,506	1.07 (1.00 to 1.14)	0.05
26-74th	59,368	2,990	1.00 (reference)	
75-94th	27,019	1,231	0.99 (0.92 to 1.06)	0.73
≥ 95th	6,694	305	1.17 (1.04 to 1.32)	0.009

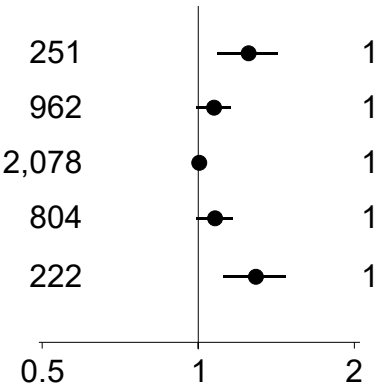


Supplemental Figure 7: Risk of any fracture according to concentrations of iron when each individual's follow-up time was split into the first five years after study enrollment (upper panel) and from 5 years after study enrollment and onwards (lower panel). For this analysis, individuals were included in both intervals of follow-up time if an individual was followed for 5 years or longer after study enrollment. Multivariable adjustment included age, sex, study cohort, alcohol consumption, body mass index, smoking status, cumulative smoking in pack-years, menopausal status (women only), Charlson comorbidity index, and C-reactive protein level. n., number; CI, confidence interval.

Transferrin saturation

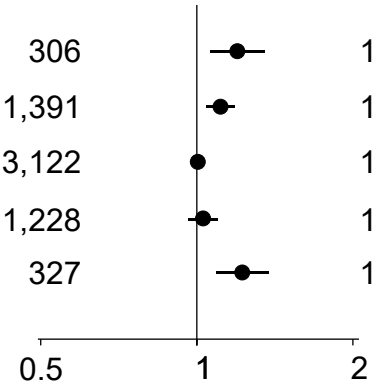
First 5 years after study enrollment

Percentiles of transferrin saturation	Individuals, n.	Fractures, n.	Multivariable adjusted hazard ratio (95% CI) for any fracture	p
≤ 5th	6,827	251	1.24 (1.09 to 1.42)	0.001
6-25th	27,594	962	1.07 (0.99 to 1.15)	0.10
26-74th	67,713	2,078	1.00 (reference)	
75-94th	27,525	804	1.07 (0.99 to 1.16)	0.09
≥ 95th	6,896	222	1.29 (1.12 to 1.48)	0.0004



From 5 years after study enrollment and onwards

Percentiles of transferrin saturation	Individuals, n.	Fractures, n.	Multivariable adjusted hazard ratio (95% CI) for any fracture	p
≤ 5th	6,203	306	1.19 (1.06 to 1.34)	0.004
6-25th	25,543	1,391	1.11 (1.04 to 1.18)	0.002
26-74th	63,186	3,122	1.00 (reference)	
75-94th	25,706	1,228	1.02 (0.96 to 1.09)	0.49
≥ 95th	6,345	327	1.22 (1.09 to 1.37)	0.0008

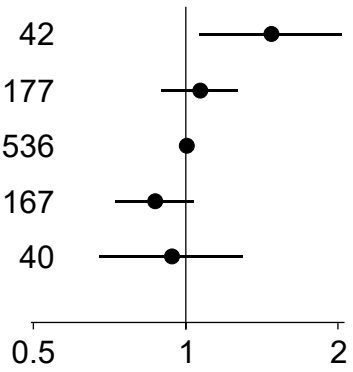


Supplemental Figure 8: Risk of any fracture according to concentrations of transferrin saturation when each individual's follow-up time was split into the first five years after study enrollment (upper panel) and from 5 years after study enrollment and onwards (lower panel). For this analysis, individuals were included in both intervals of follow-up time if an individual was followed for 5 years or longer after study enrollment. Multivariable adjustment included age, sex, study cohort, alcohol consumption, body mass index, smoking status, cumulative smoking in pack-years, menopausal status (women only), Charlson comorbidity index, and C-reactive protein level. n., number; CI, confidence interval.

Ferritin

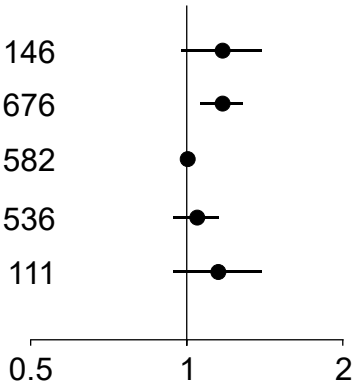
First 5 years after study enrollment

Percentiles of ferritin	Individuals, n.	Fractures, n.	Multivariable adjusted hazard ratio (95% CI) for any fracture	p
≤ 5th	1,900	42	1.47 (1.06 to 2.03)	0.02
6-25th	7,599	177	1.06 (0.89 to 1.27)	0.49
26-74th	18,805	536	1.00 (reference)	
75-94th	7,788	167	0.87 (0.72 to 1.03)	0.11
≥ 95th	1,898	40	0.93 (0.67 to 1.30)	0.69



From 5 years after study enrollment and onwards

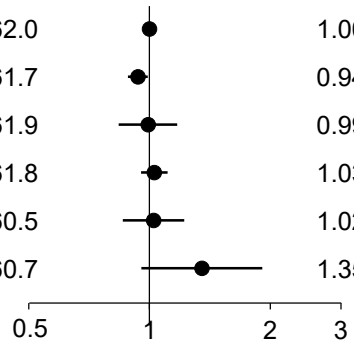
Percentiles of ferritin	Individuals, n.	Fractures, n.	Multivariable adjusted hazard ratio (95% CI) for any fracture	p
≤ 5th	1,801	146	1.17 (0.98 to 1.40)	0.09
6-25th	7,159	676	1.17 (1.06 to 1.28)	0.001
26-74th	17,452	1,582	1.00 (reference)	
75-94th	7,235	536	1.04 (0.94 to 1.15)	0.41
≥ 95th	1,725	111	1.15 (0.94 to 1.39)	0.18



Supplemental Figure 9: Risk of any fracture according to concentrations of ferritin when each individual's follow-up time was split into the first five years after study enrollment (upper panel) and from 5 years after study enrollment and onwards (lower panel). For this analysis, individuals were included in both intervals of follow-up time if an individual was followed for 5 years or longer after study enrollment. Multivariable adjustment included age, sex, study cohort, alcohol consumption, body mass index, smoking status, cumulative smoking in pack-years, menopausal status (women only), Charlson comorbidity index, and C-reactive protein level. n., number; CI, confidence interval.

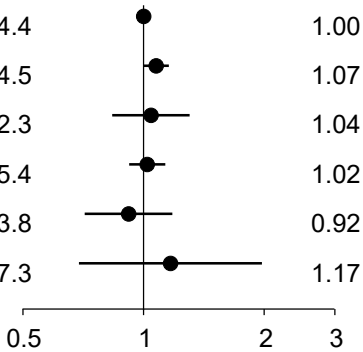
Fracture of the wrist

	Individuals, n.	Fractures, n.	Mean age at fracture, years	Age and sex adjusted hazard ratio (95% CI)	p
Non-carrier/non-carrier	87,286	5,219	62.0	1.00 (reference)	
H63D/non-carrier	27,811	1,552	61.7	0.94 (0.88 to 0.99)	0.02
H63D/H63D	2,357	140	61.9	0.99 (0.84 to 1.17)	0.92
C282Y/non-carrier	12,508	758	61.8	1.03 (0.95 to 1.11)	0.48
C282Y/H63D	2,115	127	60.5	1.02 (0.86 to 1.22)	0.79
C282Y/C282Y	422	32	60.7	1.35 (0.95 to 1.91)	0.09



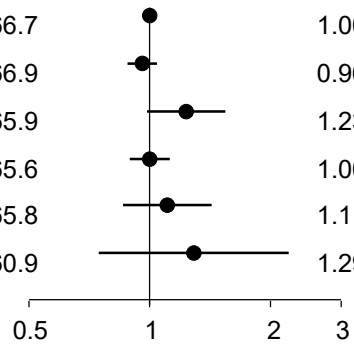
Fracture of the axial skeleton (vertebral, ribs, pelvic)

	Individuals, n.	Fractures, n.	Mean age at fracture, years	Age and sex adjusted hazard ratio (95% CI)	p
Non-carrier/non-carrier	87,286	2,855	64.4	1.00 (reference)	
H63D/non-carrier	27,811	960	64.5	1.07 (1.00 to 1.16)	0.06
H63D/H63D	2,357	80	62.3	1.04 (0.84 to 1.30)	0.71
C282Y/non-carrier	12,508	411	65.4	1.02 (0.92 to 1.13)	0.69
C282Y/H63D	2,115	62	63.8	0.92 (0.71 to 1.18)	0.50
C282Y/C282Y	422	14	57.3	1.17 (0.69 to 1.97)	0.57



Fracture of the humerus

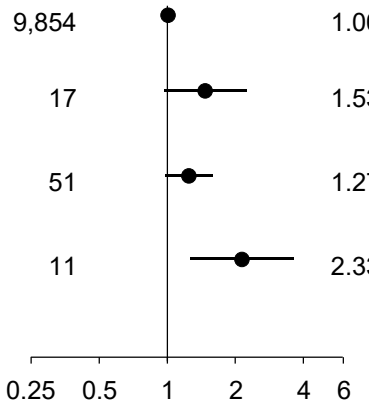
	Individuals, n.	Fractures, n.	Mean age at fracture, years	Age and sex adjusted hazard ratio (95% CI)	p
Non-carrier/non-carrier	87,286	2,337	66.7	1.00 (reference)	
H63D/non-carrier	27,811	705	66.9	0.96 (0.88 to 1.04)	0.33
H63D/H63D	2,357	78	65.9	1.23 (0.98 to 1.55)	0.07
C282Y/non-carrier	12,508	328	65.6	1.00 (0.89 to 1.12)	0.99
C282Y/H63D	2,115	61	65.8	1.11 (0.86 to 1.43)	0.43
C282Y/C282Y	422	13	60.9	1.29 (0.75 to 2.22)	0.36



Supplemental Figure 10: Risk of fractures of the wrist, axial skeleton, and humerus according to *HFE* genotype. C282Y/C282Y, homozygous for C282Y; C282Y/H63D, compound heterozygous for C282Y and H63D; C282Y/non-carrier, heterozygous for C282Y; H63D/H63D, homozygous for H63D; H63D/non-carrier, heterozygous for H63D; Non-carrier/non-carrier, non-carrier for both C282Y and H63D.s C282Y and H63D.

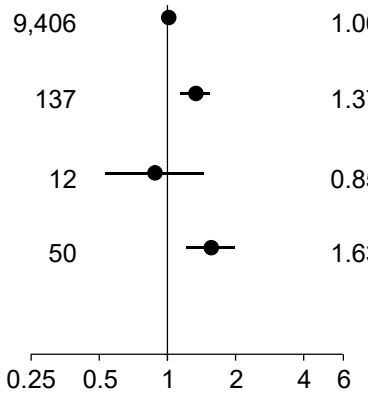
Iron

	Individuals, n.	Fractures, n.	Multivariable adjusted hazard ratio (95% CI) for any fracture	p for difference
Non-carrier/non-carrier normal iron	75,698	9,854	1.00 (reference)	
Non-carrier/non-carrier high iron	114	17	1.53 (0.95 to 2.46)	
C282Y/C282Y normal iron	338	51	1.27 (0.96 to 1.67)	0.07
C282Y/C282Y high iron	55	11	2.33 (1.29 to 4.22)	



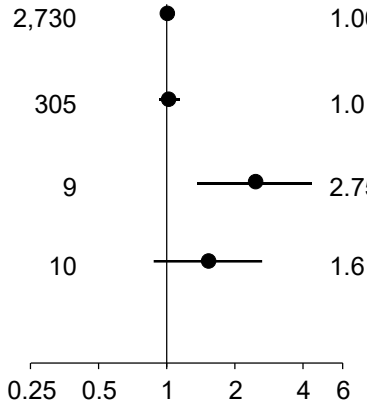
Transferrin saturation

	Individuals, n.	Fractures, n.	Multivariable adjusted hazard ratio (95% CI) for any fracture	p for difference
Non-carrier/non-carrier normal transferrin saturation	74,222	9,406	1.00 (reference)	
Non-carrier/non-carrier high transferrin saturation	893	137	1.37 (1.15 to 1.62)	
C282Y/C282Y normal transferrin saturation	106	12	0.85 (0.48 to 1.50)	0.04
C282Y/C282Y high transferrin saturation	289	50	1.63 (1.23 to 2.15)	

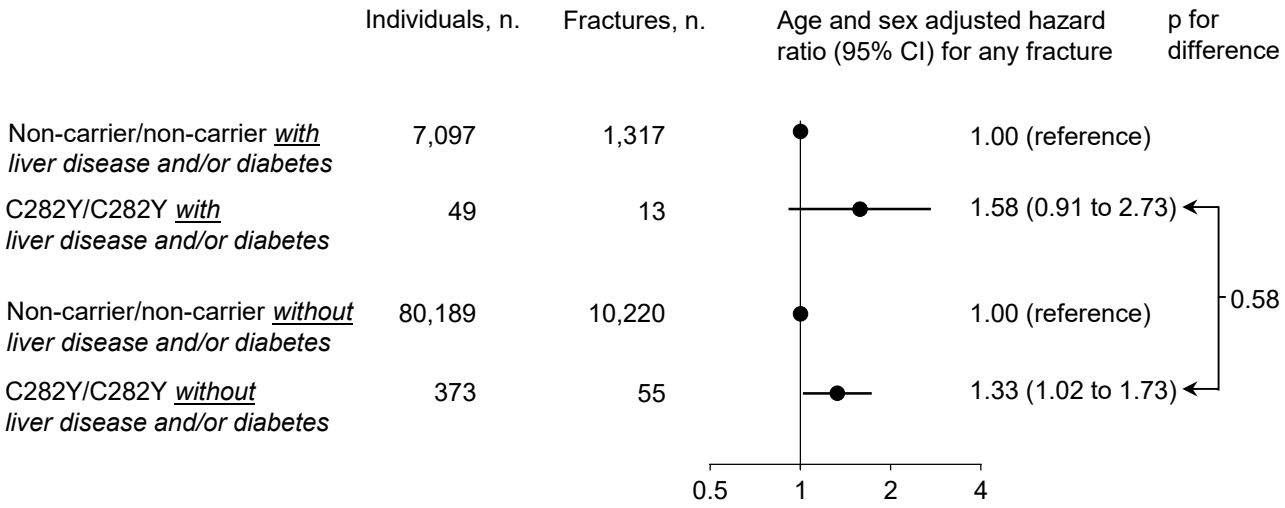


Ferritin

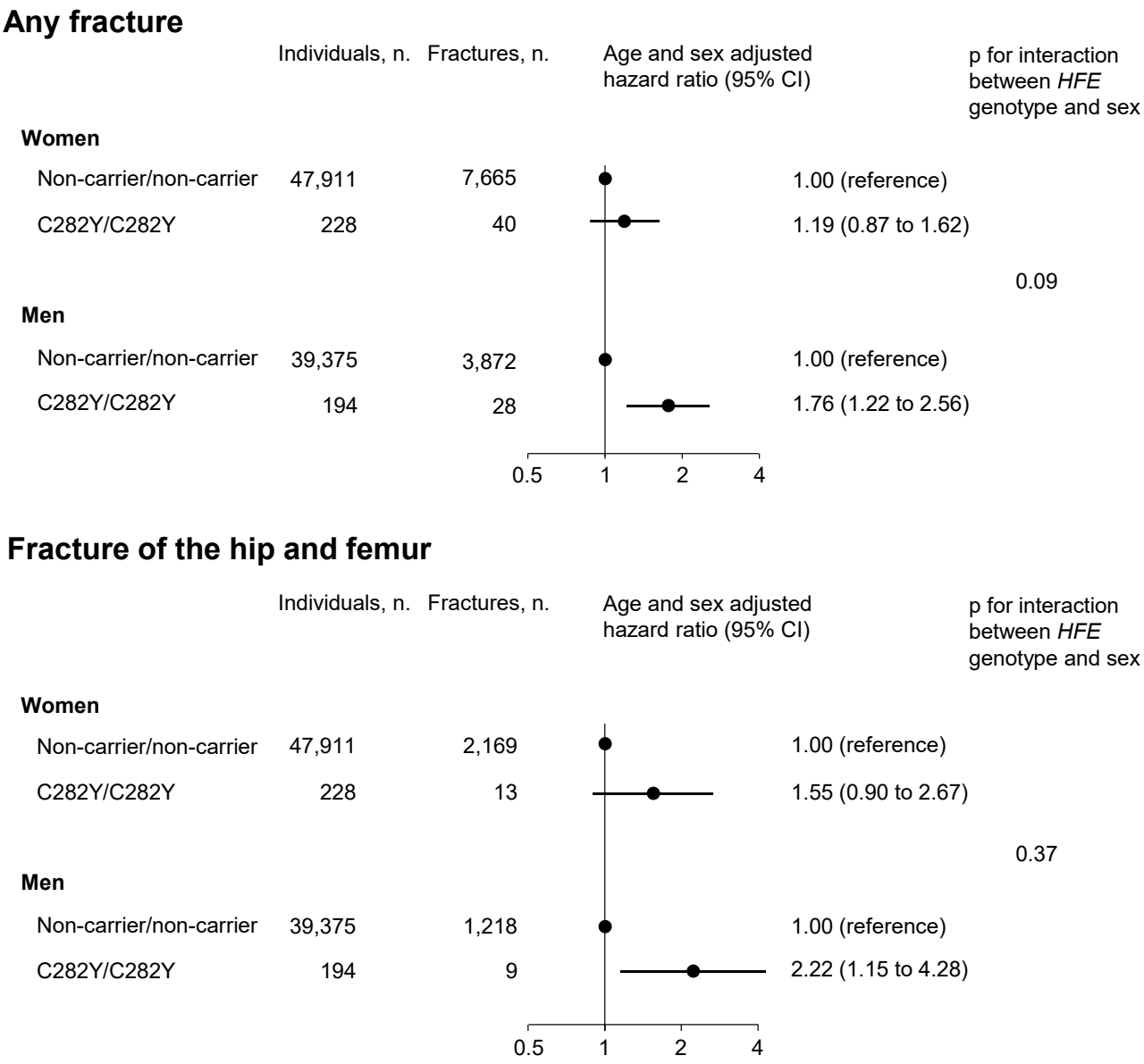
	Individuals, n.	Fractures, n.	Multivariable adjusted hazard ratio (95% CI) for any fracture	p for difference
Non-carrier/non-carrier normal ferritin	19,200	2,730	1.00 (reference)	
Non-carrier/non-carrier high ferritin	2,063	305	1.01 (0.90 to 1.14)	
C282Y/C282Y normal ferritin	27	9	2.75 (1.43 to 5.30)	0.24
C282Y/C282Y high ferritin	52	10	1.61 (0.86 to 2.99)	



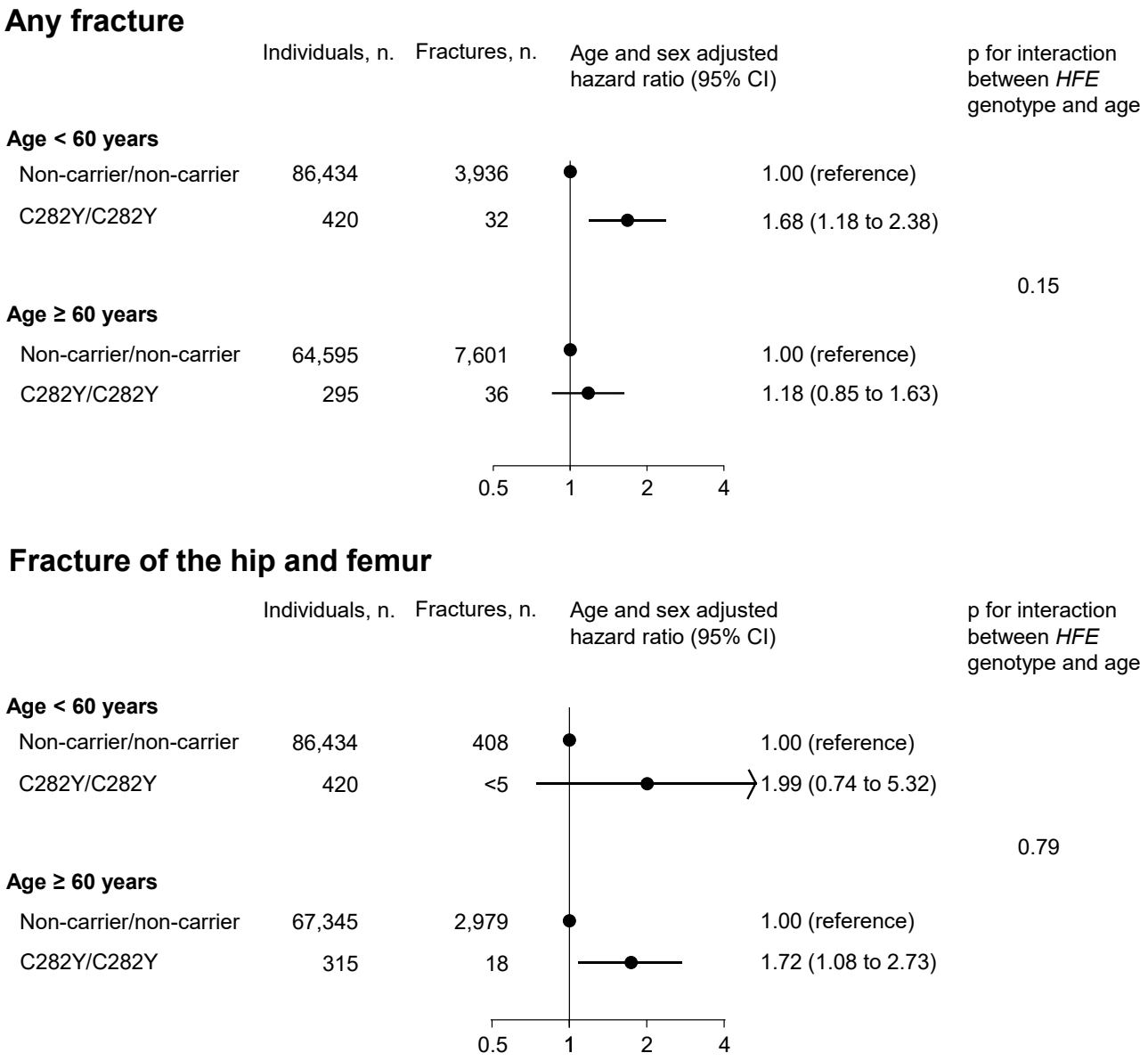
Supplemental Figure 11: Multivariable adjusted risk of any fracture for *HFE* C282Y homozygotes vs. non-carriers for the C282Y and H63D variants stratified by normal vs. high concentrations of plasma iron (upper panel), transferrin saturation (middle panel), and ferritin (lower panel) at study enrollment. Normal iron was defined as 9-34 μmol/L and high iron as >34 μmol/L. For transferrin saturation, the reference range varies according to age and sex, and therefore normal transferrin saturation was defined as 10-45% for women ≤50 years of age and 15-45% for women >50 years of age and men of any age. High transferrin saturation was defined as >45% for men and women of any age. Normal ferritin was defined as 12-200 μg/L for women and 12-300 μg/L for men, and high ferritin as >200 μg/L for women and >300 μg/L for men. As therapeutic phlebotomy can affect plasma iron, transferrin saturation, or ferritin, we excluded 11 C282Y homozygous individuals because they had been diagnosed with hemochromatosis before the day of study enrollment where blood samples were obtained. Multivariable adjustment included age, sex, study cohort, alcohol consumption, body mass index, smoking status, cumulative smoking in pack-years, menopausal status (women only), Charlson comorbidity index, and C-reactive protein level. C282Y/C282Y, homozygous for C282Y; Non-carrier/non-carrier, non-carrier for both C282Y and H63D. n., number; CI, confidence interval.



Supplemental Figure 12: Risk of any fracture for *HFE* C282Y homozygotes vs. non-carriers for the C282Y and H63D variants, stratified according to whether or not individuals had been diagnosed with liver disease and/or diabetes at any time before or after enrolling into the study. P for difference between C282Y homozygotes with liver disease and/or diabetes compared to C282Y homozygotes without liver disease and/or diabetes were calculated using the Z-test described by Altman and Bland. C282Y/C282Y, homozygous for C282Y; Non-carrier/non-carrier, non-carrier for both C282Y and H63D. n., number; CI, confidence interval.



Supplemental Figure 13: Risk of any fracture (upper panel) and fracture of the hip and femur (lower panel) for *HFE* C282Y homozygotes vs. non-carriers for the C282Y and H63D variants, stratified by sex. Using a likelihood-ratio test, p for interaction was calculated by comparing two models, one model with and one model without an interaction term between sex and genotype. C282Y/C282Y, homozygous for C282Y; Non-carrier/non-carrier, non-carrier for both C282Y and H63D. n., number; CI, confidence interval.



Supplemental Figure 14: Risk of any fracture (upper panel) and fracture of the hip and femur (lower panel) for *HFE* C282Y homozygotes vs. non-carriers for the C282Y and H63D variants, stratified by age. For each individual, follow-up time was split into two intervals: the first interval contained each individuals follow-up time before 60 years of age, and the second interval contained each individuals follow-up time from 60 years of age and onwards. Hence, any individual who turned 60 years of age during follow-up was included in both follow-up intervals. Using a likelihood-ratio test, p for interaction was calculated by comparing two models, one model with and one model without an interaction term between age interval and genotype. In compliance with Danish data privacy regulations, <5 is reported instead of the exact number of individuals if less than 5 individuals in a category had a fracture, but all risk estimates were calculated based on the exact numbers. C282Y/C282Y, homozygous for C282Y; Non-carrier/non-carrier, non-carrier for both C282Y and H63D. n., number; CI, confidence interval.

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