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

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Abstract

Risk of fractures may be increased in individuals with iron deficiency, iron overload, and/or *HFE* hemochromatosis. To test this hypothesis, we followed 142,146 Danish general population individuals for a median of 11 years (range:0-41) after study enrolment for hospital and emergency room admissions with fractures. All individuals had blood samples drawn at study enrolment. We measured iron, transferrin saturation, and ferritin in 136,611, 136,555, and 37,990 individuals, respectively, while 132,499 individuals were genotyped for the *HFE* C282Y and H63D variants. We found a U-shaped relationship between fracture risk and concentrations of plasma iron and transferrin saturation when studying all individuals irrespective of genotype. When studied according to plasma ferritin, fracture risk was increased in individuals with low ferritin concentrations, while risk was not increased in individuals with high concentrations. When compared to non-carriers, *HFE* C282Y homozygotes had increased risk of any fracture (hazard ratio[HR]:1.38;95%CI:1.09-1.75;p=0.008), and risk was increased even in C282Y homozygotes with normal ferritin concentrations (HR:2.89;95%CI:1.50-5.56), which is important as these individuals would not usually be recommended for *HFE* genotyping according to clinical guidelines. When compared to non-carriers, risk of fracture of the hip and femur was increased in C282Y homozygotes (HR:1.78;95%CI:1.17-2.70;p=0.007) but surprisingly also in H63D homozygotes (HR:1.21;95%CI:1.00-1.47;p=0.04), C282Y heterozygotes (HR:1.10;95%CI:1.00-1.21;p=0.04), and C282Y/H63D compound heterozygotes (HR:1.23;95%CI:1.00-1.51;p=0.05). The markedly increased fracture risk in C282Y homozygotes with normal ferritin may challenge the presumption that systemic iron accumulation is the primary mechanism causing their increased fracture risk. Further studies are needed to examine whether phlebotomy reduces fracture risk.

Introduction

Iron is a mineral essential for life due to its role in several fundamental processes, including transport of oxygen, DNA synthesis, and ATP generation.[1] Systemic iron regulation is orchestrated by hepcidin, a liver-produced peptide that binds ferroportin, inhibiting iron export from macrophages and hepatocytes and reducing intestinal absorption.[2] Its expression is primarily stimulated by elevated plasma iron and high transferrin saturation.[2] In clinical settings, plasma iron and transferrin saturation are often measured to evaluate the amount of iron in the bloodstream, while ferritin is a marker of overall iron storage.[3] Notably, both plasma iron and transferrin saturation are very labile and may fluctuate during common clinical conditions such as acute infections, acute or chronic inflammation, liver disease, kidney disease, blood loss, pregnancy, and when taking certain medications.[1,2,4]

Hereditary hemochromatosis is one of the most common genetic diseases in Northern Europe and is primarily caused by variants in the *HFE* gene.[3] It is generally considered an iron-overload disorder, characterized by low hepcidin expression, even when iron stores are high, causing high iron uptake with the potential of iron accumulation and progressive organ damage.[4] In Northern Europe, the allele frequency of the C282Y variant is around 5-10%, and among individuals diagnosed with hereditary hemochromatosis, more than 80% are homozygous for the C282Y variant.[3,5,6] The penetrance of clinically apparent hemochromatosis in C282Y homozygotes varies according to previous studies, depending on the definitions of evident disease, with estimates ranging from more than 80% to less than 1%.[7–10] The H63D variant has a broader distribution globally, with allele frequencies ranging from 10-20%,[6] but H63D homozygosity or isolated H63D heterozygosity have generally not been found to cause hemochromatosis-related complications,[11] while the clinical effects of C282Y/H63D compound heterozygosity remains controversial.

Iron overload has previously been associated with increased risk of osteoporotic bone loss and fractures in some studies;[12–14] however, results have been conflicting.[15] It is therefore unclear whether general population individuals with low or high concentrations of plasma iron, transferrin saturation, or ferritin are at increased risk of fractures. Previous population-based studies have found increased risk of fractures[16,17] in individuals with C282Y homozygosity. Although most clinical guidelines are based on the presumption that complications from hereditary hemochromatosis are primarily a result of systemic iron overload, the exact mechanism causing increased risk of fractures in C282Y homozygotes is unknown.

Most current guidelines recommend that testing for *HFE* variants should primarily be performed in individuals with high ferritin and high transferrin saturation (except for testing family members of patients already diagnosed with *HFE* hemochromatosis).[3,18–21] However, recent studies from our group have found increased risk of infections in C282Y homozygotes despite normal iron, transferrin saturation, or ferritin, and increased risk of diabetes in C282Y homozygotes with normal transferrin saturation and/or ferritin, which are individuals not usually recommended for *HFE* genotyping according to clinical guidelines.[22,23]

In this study, we therefore tested the hypotheses that risk of fractures is increased in individuals with low or high plasma iron, transferrin saturation, or ferritin. Furthermore, we tested whether risk of fractures is increased in hemochromatosis *HFE* C282Y homozygotes overall and specifically in C282Y homozygotes with normal concentrations of iron, transferrin saturation, or ferritin. To do so, we studied 142,146 individuals from the Danish general population who had blood samples drawn at study enrolment, after which they were followed prospectively for a median of 11 years.

Methods

Participants

We studied 142,146 individuals aged 20-100 years from three general population cohort studies: 13,663 individuals from the Copenhagen City Heart Study,[22,24] examined between 1981-1983 or 1991-1994, 108,051 individuals from the Copenhagen General Population Study,[25] examined between 2003-2015, and 20,432 individuals from the Danish General Suburban Population Study,[26] examined between 2010-2013. At study enrollment, all individuals attended a health examination where they filled out a questionnaire regarding lifestyle and health, had a physical examination, and blood samples drawn. Due to the completeness of the Danish registries, no individuals were lost to follow-up. From the Copenhagen City Heart Study, we included individuals at two different examinations: 9,002 individuals with ferritin measurements from the 1981-1983 examination and 9,727 individuals with iron, transferrin saturation, or *HFE* genotype data from the 1991-1994 examination. 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 further details, see Supplemental Methods.

The studies received approval from Danish ethics committees and all individuals supplied written informed consent.

Covariates and comorbidities

All covariates for the multivariable adjusted models were chosen *a priori* based on previous reports of an association with iron, transferrin saturation, ferritin, and/or risk of fractures.[27–29] For details, see Supplemental Methods.

Plasma iron, transferrin saturation, and ferritin

Blood samples used for measuring plasma iron, transferrin saturation, and ferritin were collected at study enrollment. In total, 136,611 individuals had plasma iron measurements, 136,555 had transferrin saturation measurements, and 37,990 individuals had ferritin measurements. For details, see Supplemental Methods.

Genotypes

The *HFE* gene was genotyped for the C282Y and H63D variants in 132,499 individuals using blood samples collected at study enrolment. Details on genotyping are described in our previous publications.[22,23] For details, see Supplemental Methods.

Fractures, vital status, and diagnoses of hemochromatosis

We used the national Danish Patient Register[30], 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. We defined fracture events as any inpatient or emergency room admissions due to fractures, categorized using WHO's International Classification of Diseases (ICD)-8 and ICD-10 codes (Supplemental Table 1).[31] Information on emigration and vital status until December 31st, 2021, was obtained from the Danish Civil Registration system,[32] with 100% complete information. 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 obtained 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. For details, see Supplemental Methods.

Statistical analyses

We used Stata version 18.0, and all statistical tests were two-tailed. Risks of fractures were modelled using Cox proportional hazards regression, adjusting for age by using left-truncated age as the timescale. We chose this approach that is often recommended for general population studies, as the date of study enrollment is chosen at random, and therefore, age is generally a stronger predictor for risk of future disease than time since study enrollment. Furthermore, when using this type of age-adjustment, the impact of age on fracture risk is not assumed to follow a specific pattern, meaning that the method efficiently adjusts for age at any given age during follow-up without underlying assumptions regarding linearity or other specific patterns of the relationship between age and risk of fractures. To estimate the cumulative incidence of any fracture, we used the competing risk model by Fine and Gray,[33] taking competing risk of death from any cause into consideration.
All age and sex-adjusted models were also adjusted for study cohort. We decided *a priori* to study risk of fractures according to *HFE* genotype using the age and sex-adjusted model, and to study risk of fractures according to concentrations of iron, transferrin saturation, and ferritin, using a multivariable adjusted model additionally adjusting for alcohol consumption, body mass index, smoking status, cumulative smoking in pack-years, menopausal status (women only), plasma C-reactive protein, and Charlson comorbidity index. As a sensitivity analysis, we also studied risk of fractures according to *HFE* genotype using the multivariable adjusted model. Further details in Supplemental Methods.

Results

Baseline characteristics for 142,146 individuals according to study cohort are presented in **Table 1**, while baseline characteristics according to *HFE* genotype are presented in Supplemental Table 2.

Plasma iron, transferrin saturation, and ferritin and risk of fractures

When studying risk of fractures according to plasma iron, transferrin saturation, and ferritin in all individuals irrespective of *HFE* genotype, follow-up began at time of study enrolment, giving a median

follow-up of 11 years (range:0-41), during which 11,549 individuals were hospitalized due to any fracture. For individuals diagnosed with fractures during follow-up, the median time from study enrollment to fracture diagnose was 6 years for individuals with plasma iron≤5th percentile, 6 years for individuals with plasma iron in the 26-74th percentile, and 7 years for individuals with plasma iron≥95th percentile. Similar, the median time to fracture diagnose was 6 years for individuals with transferrin saturation≤5th, in the 26-74th, and≥95th percentile, and 7, 7, and 8 years for individuals with ferritin≤5th, in the 26-74th, and≥95th percentile, respectively. When performing univariate analyses, each of the variables included in the multivariable adjusted analyses (age, sex, menopausal status, smoking status, alcohol consumption, body mass index, C-reactive protein level, and comorbid disease) were univariately associated with risk of fractures(Supplemental Figure 1).

Risk of any fracture was increased in individuals with low plasma iron≤5th percentile and high plasma iron≥95th percentile(hazard ratios are listed below the splines in **Figure 1)**. Similarly, risk of any fracture was increased in individuals with low transferrin saturation≤5th percentile, high transferrin saturation≥95th percentile, and low ferritin≤5th percentile, whereas we found no association between high ferritin≥95th percentile and risk of any fracture(**Figure 1**).

Results on plasma iron and transferrin saturation were similar to those presented in **Figure 1** when only investigating individuals who were non-carriers for C282Y and H63D, while risk of any fracture was not increased in non-carrier individuals with low ferritin or high ferritin (Supplemental Figure 2). As anemia and comorbid diseases may affect risk of fractures, we also performed analyses excluding all individuals with anemia(Supplemental Figure 3) or excluding individuals with any comorbid disease at study enrollment(Supplemental Figure 4), giving results similar to those presented in **Figure 1**.

Fractures of the hip and femur were the most common type of fractures, and also represents clinically important fracture types, as 1-year mortality after hip fractures is often reported to be 15-25%.[34,35] Therefore, risk of fracture of the hip and femur is presented in **Figure 2**, showing increased risk in individuals with low iron≤5th percentile, high iron≥95th percentile, low transferrin saturation≤5th percentile, high transferrin saturation≥95th percentile, or low ferritin≤5th percentile. When examining other specific fractures, risk of fracture of the axial skeleton was increased in individuals with high iron (1.22;1.02-1.46), low transferrin saturation (1.23;1.02-1.49), and high transferrin saturation (1.27;1.08-1.51)(Supplemental Figure 5). Furthermore, risk of fracture of the humerus was increased in

individuals with low iron (1.24;1.02-1.50), low transferrin saturation (1.25;1.03-1.53), high transferrin saturation (1.35;1.10-1.66), and high ferritin (1.53;1.10-2.13), while concentrations of iron, transferrin saturation, or ferritin were not associated with risk of fracture of the wrist(Supplemental Figure 5). When stratifying by sex, relative risk estimates for any fracture according to concentrations of iron, transferrin saturation, and ferritin were similar for women and men(Supplemental Figure 6).

As serum iron, transferrin saturation, and ferritin may change over time, we did a sensitivity analysis examining risk of any fracture separately in the first five years after blood sampling, and from five years after blood sampling and onwards. Generally, we found similar risk estimates for risk of fractures according to concentrations of iron, transferrin saturation, and ferritin during the first five years after blood sampling as in the time period from 5 years after blood sampling and onwards (Supplemental Figures 7-9).

Hemochromatosis *HFE* genotypes and risk of fractures

132,499 individuals were genotyped for the *HFE* C282Y and H63D variants and 422 were homozygous for the C282Y variant. Since *HFE* genotype is constant throughout life, follow-up for the analyses 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. During a median follow-up of 42 years (range:0-45), 17,474 of the genotyped individuals were hospitalized due to any fracture. C282Y homozygotes had increased risk of any fracture (age and sex adjusted HR 1.38;95%CI:1.09-1.75;p=0.008 when compared to non-carriers for C282Y and H63D) and fracture of the hip and femur (1.78;1.17-2.70;p=0.007)(**Figure 3**). Notably, C282Y homozygotes had fractures at an earlier age than non-carrier individuals (mean age at any fracture: 60.6 years in C282Y homozygotes vs. 64.9 years in non-carriers;p=0.03)(**Figure 3**). When examining risk of other specific fractures in C282Y homozygotes, we did not find convincingly increased risk of fractures of the wrist (1.35;0.95-1.91), axial skeleton (1.17;0.69-1.97), or humerus (1.29;0.75-2.22)(Supplemental Figure 10). Risk of any fracture was not increased in H63D heterozygotes, H63D homozygotes, C282Y heterozygotes, or C282Y/H63D compound heterozygotes. Importantly, however, risk of fracture of the hip and femur was increased to a moderate extent in H63D homozygotes (1.21;1.00-1.47;p=0.04), C282Y heterozygotes (1.10;1.00-1.21;p=0.04), and C282Y/H63D compound heterozygotes (1.23;1.00-1.51;p=0.05)(**Figure 3**).

Most clinical guidelines recommend testing for the *HFE* genotype primarily in individuals with high concentrations of ferritin and high transferrin saturation.[3,18–21] To test if these guidelines are sufficient for identifying C282Y homozygotes at high risk of fractures, we studied risk of any fracture in C282Y homozygotes according to concentrations of iron, transferrin saturation, and ferritin. As therapeutic phlebotomy affects concentrations of iron, transferrin saturation, and ferritin, we excluded individuals diagnosed with hemochromatosis before study enrollment from these stratified analyses as they may have been treated with phlebotomy before blood samples were drawn. Notably, C282Y homozygotes with normal concentrations of ferritin had high risk of any fracture (HR 2.89;95%CI:1.50-5.56, for C282Y homozygotes with normal ferritin vs. non-carriers with normal ferritin). In contrast, risk of any fracture was not increased in C282Y homozygotes with normal plasma iron (1.27;0.97-1.68) or normal transferrin saturation (0.86;0.49-1.52)(**Figure 4**). Results were similar to those presented in Figure 4 when performing sensitivity analyses on risk of any fracture according to concentrations of iron, transferrin saturation, and ferritin using the multivariable adjusted model(Supplemental Figure 11). To investigate if the increased fracture risk in C282Y homozygotes with normal concentrations of ferritin is due to slow iron accumulation occurring after study enrollment, we performed additional analyses restricted to only study risk of fractures occurring before blood samples was obtained at study enrollment(**Figure 5**). When doing this, the hazard ratio for any fracture in C282Y homozygotes with normal ferritin was 3.27(95%CI:1.36-7.87), similar to the risk estimate in Figure 4 for investigating the total follow-up time. This implies that the increased fracture risk in C282Y homozygotes with normal concentrations of ferritin is not a result of iron accumulation occurring after study enrollment.

When stratifying according to whether or not C282Y homozygotes had been diagnosed with hemochromatosis at any time, risk of any fracture was similar in C282Y homozygotes never diagnosed with hemochromatosis (HR 1.37;95%CI:1.05-1.79 when compared to non-carrier individuals) and C282Y homozygotes diagnosed with hemochromatosis (1.42;0.84-2.40)(**Figure 6**). Likewise, relative risk estimates for any fracture were similar for C282Y homozygotes never diagnosed with liver disease and/or diabetes (1.33;1.02-1.73 when compared to non-carriers without liver disease and/or diabetes) and for C282Y homozygotes diagnosed with liver disease and/or diabetes (1.58;0.91-2.73 when compared to non-carriers with liver disease and/or diabetes)(Supplemental Figure 12). When stratifying by sex, relative risk estimates for any fracture and fracture of the hip and femur were similar for C282Y

homozygous men and women when compared to non-carrier men and women, respectively (p-values for interaction between genotype and sex 0.09 and 0.37 for risk of any fracture and fracture of the hip and femur, respectively)(Supplemental Figure 13). Likewise, when compared to non-carriers of the same age, relative risk estimates for any fracture and fracture of the hip and femur were similar for C282Y homozygotes <60 years and for C282Y homozygotes ≥60 years of age (Supplemental Figure 14).

Cumulative incidence of any fracture

Cumulative incidence of any fracture according to age was analyzed using Fine-Gray competing risk models to take into account the competing risk of death from any cause. For the overall group of C282Y homozygotes, the cumulative incidence of any fracture was 8% at age 60 years and 24% at age 80 years, while the corresponding cumulative incidences were 6% and 18% in non-carriers aged 60 and 80 years, respectively. Notably, C282Y homozygotes with normal ferritin had a substantially higher cumulative incidence of any fracture, as their cumulative incidences were 19% at age 60 and 49% at age 80 years (**Figure 7**).

Discussion

In this prospective study of 142,146 individuals from the general population, risk of any fracture and risk of fracture of the hip and femur were increased in individuals with low or high plasma iron, low or high transferrin saturation, or low ferritin when studying all individuals irrespective of *HFE* genotype. Individuals with *HFE* C282Y homozygosity had increased risk of any fracture, and fracture risk was markedly increased even in C282Y homozygotes with normal ferritin, which is important as these individuals would not usually be recommended for *HFE* genotyping according to most guidelines. Risk of fracture of the hip and femur was increased in *HFE* C282Y homozygotes but surprisingly also in H63D homozygotes, C282Y heterozygotes, and C282Y/H63D compound heterozygotes, which is novel.

Our finding that risk of any fracture was increased in individuals with low or high plasma iron, low or high transferrin saturation, or low ferritin, irrespective of *HFE* genotype, indicates that fracture risk may be increased in both iron deficiency and iron overload. These results are to some extent supported

by previous studies.[12,13] Kim et al. investigated 14,017 individuals from the Korean National Health and Nutrition Examination Survey and found an inverse association between serum ferritin and bone mineral density scores in women ≥45 years of age, and an increased prevalence of fractures in women in the highest vs. lowest ferritin quartile.[12] No previous studies have investigated fracture risk in individuals with low plasma iron, transferrin saturation, or ferritin, underlining the novelty of these results.

Mechanistically, both low and high iron may hypothetically affect bone remodeling, which could potentially explain our findings. Bones constantly undergo remodeling involving osteoclasts that resorb bone tissue and osteoblasts that actively synthesize new bone.[36] Iron overload facilitates bone resorption by promoting osteoclastogenesis and increasing osteoclast activity, both *in vitro*[37] and *in vivo*.[38,39] In addition, high iron suppresses osteoblast function.[40] Low iron may also affect bone remodeling, as iron is involved in collagen synthesis,[41] vitamin D hydroxylation,[42] and as hypoxia, which may be seen in anemia, also stimulates bone resorption.[43] However, in our study, risk of fractures remained high in individuals with low concentrations of plasma iron, transferrin saturation or ferritin after excluding individuals with anemia, indicating that anemia is not a likely explanation for the high risk of fractures.

Our finding of an overall increased risk of any fracture and fracture of the hip and femur in C282Y homozygotes is partly supported by findings from the UK biobank.[16,17] Banfield et al. investigated 451,143 UK general population individuals and found increased risk of hip and femur fractures in C282Y homozygous men, but not in women, while they did not find an overall increased risk of any fracture in women and men.[17] In contrast, we observed an overall increased risk of any fracture and fracture of the hip and femur in all C282Y homozygous individuals combined, without convincing evidence for interaction between genotype and sex on risk of fractures. Importantly, and a novel finding, we found a markedly increased risk of any fracture even in C282Y homozygotes with normal concentrations of ferritin, which has not been studied in other large general population cohorts such as the UK Biobank, as ferritin was not measured in that cohort. The reliability of our results is further illustrated by the fact that fractures occurred at a younger age in C282Y homozygous individuals compared to non-carrier individuals. Another surprising finding is our novel observation of a moderately increased risk of fracture of the hip and femur in H63D homozygotes, C282Y

heterozygotes, and C282Y/H63D compound heterozygotes. Although the magnitude of the risk estimates for fracture of the hip and femur in individuals with the above mentioned genotypes were lower than for C282Y homozygotes, the finding of an increased risk in H63D homozygotes and C282Y heterozygotes is particularly surprising, as previous studies have found that individuals with these genotypes are not at increased risk of other hemochromatosis-related complications such as liver disease, infections, or diabetes.[16,22,23] While this is interesting from a mechanistic point of view, the moderately increased risk may be of limited clinical relevance, and we do not believe our findings should cause changes in the current clinical practice for these intermediate genotypes.

Mechanistically, common signaling pathways involved in bone remodeling and hepcidin expression may potentially explain the increased fracture risk in *HFE* hemochromatosis, including the increased risk of fractures in C282Y homozygotes with normal ferritin. The expression of hepcidin in hepatocytes is controlled by signaling through the bone morphogenetic protein pathway, which is also a critical regulator of bone metabolism.[44,45] Furthermore, *HFE* is highly expressed in both osteoblasts and osteoclasts.[46] *HFE* is located on chromosome 6 within the extended HLA class I region,[47] and the HLA locus also contains genes encoding major histocompatibility complex (MHC) proteins and tumor necrosis factor-α (TNF-α).[47] Notably, TNF-α might be implicated in the risk of fractures by promoting bone resorption through osteoclast activation,[48] but we cannot make any conclusions on the exact mechanisms underlying our findings due to the observational nature of our study.

Our finding of a markedly increased risk of fractures in individuals with C282Y homozygosity and normal concentrations of ferritin may challenge the presumption that systemic iron accumulation is the primary mechanism causing increased fracture risk in C282Y homozygotes.[14,45] Although our observational study cannot determine the potential effect of lowering ferritin through therapeutic phlebotomy, the high risk of any fracture in C282Y homozygotes with normal ferritin questions whether phlebotomy aimed at reducing iron storage is sufficient for reducing fracture risk in C282Y homozygotes, underlining the need for further studies.

Given the high morbidity and mortality associated with hip and femur fractures,[34] interventions aimed at reducing fracture risk may possibly improve quality of life while also reducing health care spending.[49] Several prophylactic measures, such as smoking and alcohol cessation, regular weight-bearing exercise, or treatment with antiresorptive agents, including calcium and vitamin D, either alone

or in combination with anabolic agents, have been found to reduce fracture risk in other high-risk populations.[50] Further studies are needed to determine whether similar interventions could effectively mitigate fracture risk in *HFE* hemochromatosis.

Strengths of this study include the large general population cohort of 142,146 individuals who were invited based on age and geography without taking prior health conditions or healthcare utilization into account. Another strength is the extensive national Danish registries with detailed information on hospitalizations due to fractures, comorbidities, and vital status without any losses to follow-up. Among the limitations of our study is that we do not know whether concentrations of iron, transferrin saturation, or ferritin might have changed between study enrollment and the time of fracture. However, we have previously investigated the change in plasma iron and transferrin saturation in repeat blood samples after a median of 10 years in 89 of the same C282Y homozygotes that were included in our present study. At study enrolment, 76 of the 89 C282Y homozygotes had normal plasma iron, and in 67 of these (88.2%) plasma iron were persistently normal after a median of 10 years. Among 24 C282Y homozygotes with normal transferrin saturation at study enrolment, 15 (62.5%) persistently had normal transferrin saturation in the repeat blood samples.[22,23] Another limitation is that more than 99% of individuals in our study were white and of Danish descent, making us unable to examine whether our findings are generalizable to other ethnic groups.

Concludingly, risk of any fracture and fracture of the hip and femur was increased in individuals with low or high iron, low or high transferrin saturation, or low ferritin when studying all individuals irrespective of *HFE* genotype. *HFE* C282Y homozygotes had increased risk of any fracture and fracture of the hip and femur. Importantly, even C282Y homozygotes with normal concentrations of ferritin had increased risk of any fracture, highlighting that this subgroup, typically not recommended for *HFE* genotyping under current clinical guidelines, remains at considerable fracture risk with a cumulative incidence of any fracture of 49% at age 80 years.

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Table 1. Baseline characteristics of 142,146 Danish general population individuals divided by study cohort

	General population cohort		
	Copenhagen City Heart Study	**Copenhagen General Population Study**	**Danish General Suburban Population Study**
Individuals, n.	13,663	108,051	20,432
Plasma iron measurements*, n. (%)	8,882 (65)	107,334 (99)	20,395 (100)
Plasma transferrin saturation*, n. (%)	8,869 (65)	107,294 (99)	20,392 (100)
Plasma ferritin*, n. (%)	9,002 (66)	8,633 (8)	20,355 (100)
HFE* genotyped individuals*, n. (%)	9,172 (67)	103,270 (96)	20,057 (98)
Age, years (IQR)	61 (49-70)	58 (48-67)	57 (46-67)
Male sex, n. (%)	6,439 (47)	48,634 (45)	9,333 (46)
Pre-menopausal women, n. (% of all women)	1,907 (26)	19,299 (32)	3,958 (36)
Ever smokers (current or former), n. (%)	10,880 (80)	62,495 (58)	11,639 (57)
Cumulative smoking†, pack-years (IQR)	25 (12-40)	16 (6-30)	18 (8-32)
Alcohol consumption above 168/84 g/week‡, n. (%)	3,945 (29)	40,934 (38)	4,835 (24)
Body mass index, kg/m^2 (IQR)	24.9 (22.5-27.9)	25.6 (23.2-28.4)	26.1 (23.5-29.2)
C-reactive protein level, mg/L (IQR)	1.7 (1.3-3.0)	1.4 (0.9-2.3)	1.4 (0.7-3.0)
Any comorbid disease§, n. (%)	2,195 (16)	22,867 (21)	4,285 (21)

Values are median (IQR) for continuous variables and number of individuals (%) for categorical variables.
IQR, interquartile range.
*From the Copenhagen City Heart Study, we included individuals at two different examinations; in 1981-1983 and 1991-1994. We included 9,002 individuals with ferritin measurements from the 1981-1983 examination (out of 12,696 attending [71%]) and 9,727 individuals with iron, transferrin saturation, or HFE genotype measured from the 1991-1994 examination, of whom 8,882 out of 10,133 attending (88%) had iron measurements, 8,869 out of 10,133 attending (88%) had transferrin saturation measured, and 9,172 out of 10,133 attending had *HFE* genotyping performed (91%). Of note, 5,063 individuals attended both examinations in the Copenhagen City Heart Study.
† Only ever smokers
‡ 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.
§ As defined by the Charlson comorbidity index

Figure Legends

Figure 1. Risk of any fracture according to iron, transferrin saturation, and ferritin at study enrolment. Based on risk estimates from Cox proportional hazards regression, cubic splines were fitted, and hazard ratios presented by a solid red line and 95% confidence intervals by a dashed black line. 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. For the categorical analyses and when modeling the splines, all individuals with available measurements of iron, transferrin saturation, or ferritin were included, however, for graphical presentation of the splines, the x-axis was limited to the 99.9th percentile for iron and transferrin saturation, and the 99th percentile for ferritin. Reference levels for the graphical splines were set at the median values of iron, transferrin saturation, or ferritin. n., number; ref., reference group; sat., saturation; CI, confidence interval.

Figure 2. Risk of fracture of the hip and femur according to plasma iron, transferrin saturation, and ferritin at study enrollment. Based on risk estimates from Cox proportional hazards regression, cubic splines were fitted, and hazard ratios presented by a solid red line and 95% confidence intervals by a dashed black line. 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. For the categorical analyses and when modeling the splines, all individuals with available measurements of iron, transferrin saturation, or ferritin were included, however, for graphical presentation of the splines, the x-axis was limited to the 99.9th percentile for iron and transferrin saturation, and the 99th percentile for ferritin. Reference levels for the graphical splines were set at the median values of iron, transferrin saturation, or ferritin. n., number; ref., reference group; sat., saturation; CI, confidence interval.

Figure 3. Risk of any fracture (upper panel) and fracture of the hip and femur (lower panel) 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. n., number; CI, confidence interval.

Figure 4. 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. Follow-up began at the time of creation of the Danish National Patient register in 1977 or each individual's 20th birthday, whichever came last. 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 enrolment where blood samples were obtained. C282Y/C282Y, homozygous for C282Y; Non-carrier/non-carrier, non-carrier for both C282Y and H63D. n., number; CI, confidence interval.

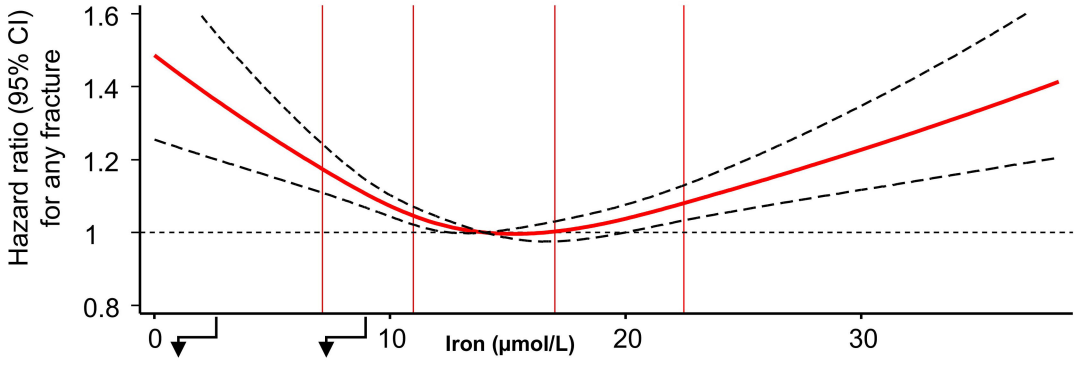
Figure 5. Risk of any fracture for *HFE* C282Y homozygotes vs. non-carriers for the C282Y and H63D variants stratified by normal vs. high concentrations of plasma iron (upper panel), transferrin saturation (middle panel), and ferritin (lower panel) when restricting the analyses to only study risk of fractures occurring before study enrollment (where blood samples were obtained). 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. Follow-up began at the time of creation of the Danish National Patient register in 1977 or each individual's 20th birthday, whichever came last. 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 enrolment where blood samples were obtained. C282Y/C282Y, homozygous for C282Y; Non-carrier/non-carrier, non-carrier for both C282Y and H63D. n., number; CI, confidence interval.

Figure 6. Risk of any fracture for *HFE* C282Y homozygotes vs. non-carriers for the C282Y and H63D variants stratified according to whether or not C282Y homozygotes had been diagnosed with hemochromatosis at any time. Diagnoses of hemochromatosis were obtained from the Danish National Patient Register, which covers all hospitals in Denmark. C282Y/C282Y, homozygous for C282Y; Non-carrier/non-carrier, non-carrier for both C282Y and H63D, number; CI, confidence interval.

Figure 7. Cumulative incidence of any fracture in *HFE* C282Y homozygotes and non-carriers for C282Y and H63D variants as a function of age. Fine-Gray competing risk regression was used to calculate cumulative incidence of any fracture, taking death from any cause as competing event into consideration. Presented are cumulative incidences of any fracture for all C282Y homozygotes and non-carriers, and specifically for C282Y homozygotes and non-carriers with normal plasma iron, normal transferrin saturation, or normal ferritin at study enrolment. 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. For the analyses on individuals with normal concentrations of plasma iron, transferrin saturation, and ferritin, C282Y homozygous individuals were excluded if they had been diagnosed with hemochromatosis before the day of study enrolment where blood samples were obtained, as therapeutic phlebotomy can affect plasma iron, transferrin saturation, or ferritin. The cumulative incidence curves for C282Y homozygotes and non-carriers with normal transferrin saturation overlap in their entire range, and may therefore appear graphically as a single curve, but are indeed two separate curves. Log-rank p values are for comparing C282Y homozygotes with non-carrier individuals. C282Y/C282Y, homozygous for C282Y; Non-carrier/non-carrier, non-carrier for both C282Y and H63D. n., number.

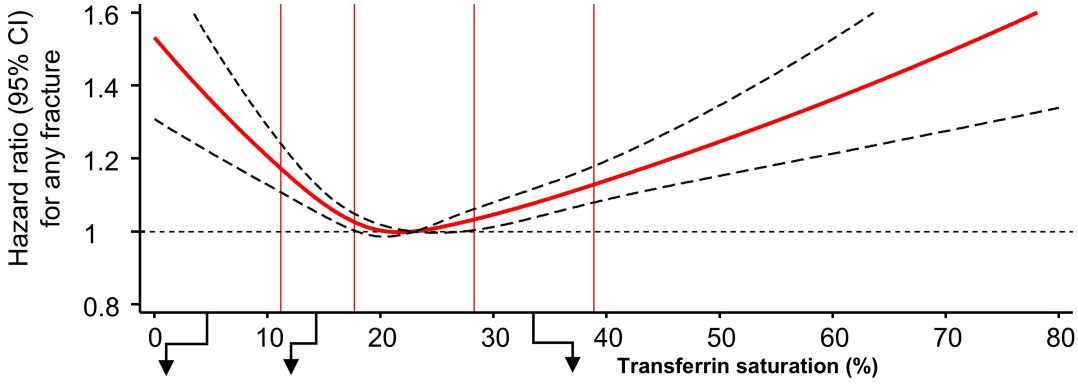
Figure 1

Iron



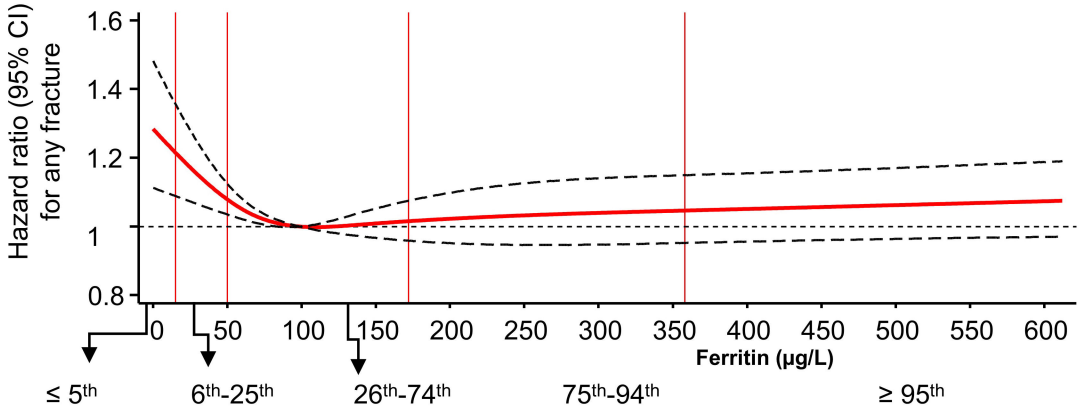
Percentiles	≤ 5th	6th-25th	26th-74th	75th-94th	≥ 95th
Iron (µmol/L)	0-7.1	7.2-11.0	11.1-16.9	17.0-22.4	22.5-130.2
Individuals, n.	7,231	29,803	63,546	28,853	7,178
Fractures, n.	631	2,622	4,898	2,060	483
Hazard ratio	1.19	1.10	1.00	1.04	1.14
95% CI	(1.09-1.29)	(1.05-1.16)	(ref.)	(0.98-1.09)	(1.04-1.25)
p	9×10^{-5}	9×10^{-5}		0.18	0.006

Transferrin saturation



Percentiles	≤ 5th	6th-25th	26th-74th	75th-94th	≥ 95th
Transferrin sat. (%)	0-11.2	11.3-18.0	18.1-28.2	28.3-38.8	38.9-194.6
Individuals, n.	6,827	27,594	67,713	27,525	6,896
Fractures, n.	557	2,353	5,200	2,032	549
Hazard ratio	1.22	1.09	1.00	1.04	1.25
95% CI	(1.11-1.33)	(1.04-1.14)	(ref.)	(0.99-1.10)	(1.14-1.36)
p	2×10^{-5}	0.0007		0.10	1×10^{-6}

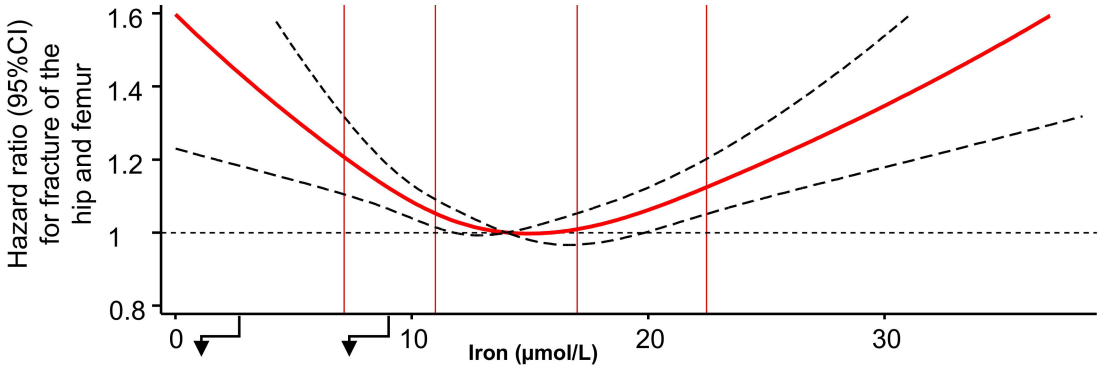
Ferritin



Percentiles	≤ 5th	6th-25th	26th-74th	75th-94th	≥ 95th
Ferritin (µg/L)	0-15.0	15.1-50.0	50.1-172.1	172.2-358.1	358.2-17,200
Individuals, n.	1,900	7,599	18,805	7,788	1,898
Fractures, n.	188	853	2,118	703	151
Hazard ratio	1.22	1.14	1.00	0.99	1.08
(95% CI)	(1.05-1.43)	(1.05-1.24)	(ref.)	(0.91-1.08)	(0.91-1.28)
p	0.01	0.001		0.86	0.37

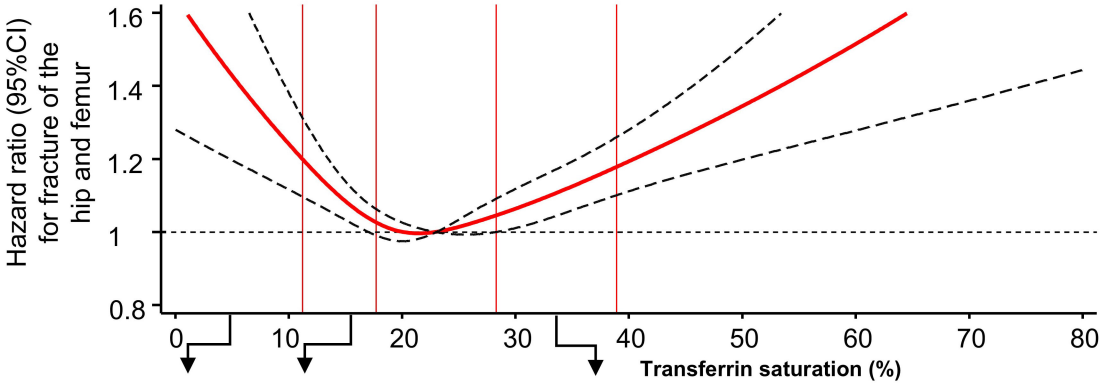
Figure 2

Iron



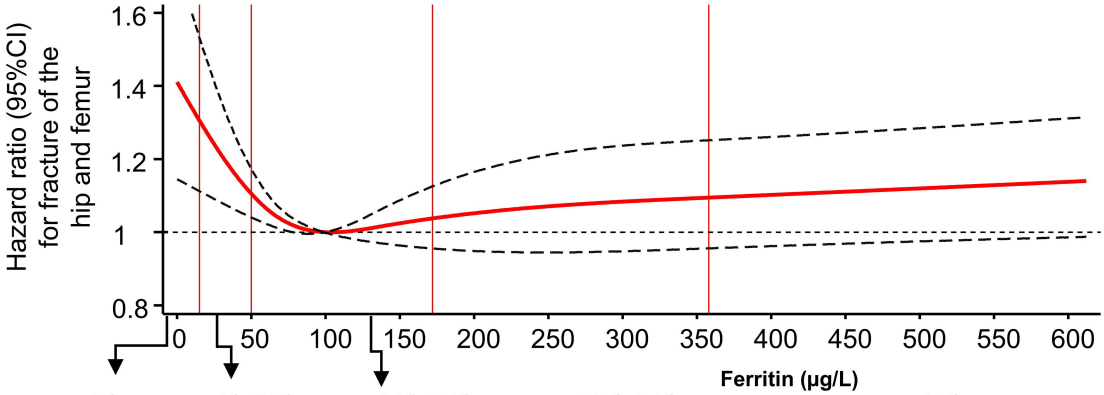
Percentiles	≤ 5th	6th-25th	26th-74th	75th-94th	≥ 95th
Iron (µmol/L)	0-7.1	7.2-11.0	11.1-16.9	17.0-22.4	22.5-130.2
Individuals, n.	7,231	29,803	63,546	28,853	7,178
Fractures, n.	262	1,067	2,012	809	211
Hazard ratio	1.20	1.10	1.00	1.01	1.29
95% CI	(1.05-1.37)	(1.02-1.19)	(ref.)	(0.93-1.09)	(1.12-1.49)
p	0.007	0.01		0.86	5×10^{-4}

Transferrin saturation



Percentiles	≤ 5th	6th-25th	26th-74th	75th-94th	≥ 95th
Transferrin sat. (%)	0-11.2	11.3-18.0	18.1-28.2	28.3-38.8	38.9-194.6
Individuals, n.	6,827	27,594	67,713	27,525	6,896
Fractures, n.	225	918	2,108	864	245
Hazard ratio	1.27	1.08	1.00	1.07	1.32
95%CI	(1.11-1.47)	(1.00-1.17)	(ref.)	(0.98-1.15)	(1.16-1.51)
p	8×10^{-4}	0.05		0.12	5×10^{-5}

Ferritin

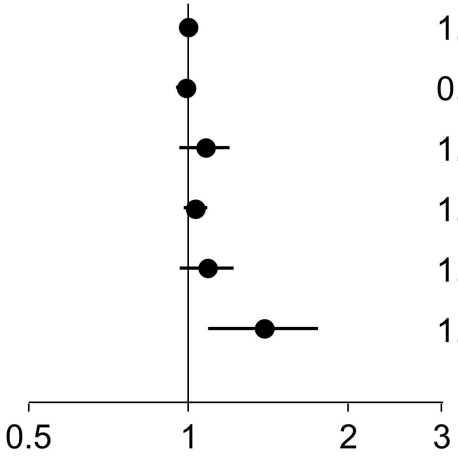


Percentiles	≤ 5th	6th-25th	26th-74th	75th-94th	≥ 95th
Ferritin (µg/L)	0-15.0	15.1-50.0	50.1-172.1	172.2-358.1	358.2-17,200
Individuals, n.	1,900	7,599	18,805	7,788	1,898
Fractures, n.	79	395	1,033	340	79
Hazard ratio	1.28	1.16	1.00	0.98	1.20
(95%CI)	(1.01-1.62)	(1.03-1.31)	(ref.)	(0.86-1.11)	(0.95-1.51)
p	0.04	0.01		0.71	0.13

Figure 3

Any fracture

	Individuals, n.	Fractures, n.	Mean age at fracture, years	Age and sex adjusted hazard ratio (95 % CI)	p
Non-carrier/non-carrier	87,286	11,537	64.9	1.00 (reference)	
H63D/non-carrier	27,811	3,580	64.9	0.98 (0.95 to 1.02)	0.42
H63D/H63D	2,357	331	64.3	1.07 (0.96 to 1.20)	0.21
C282Y/non-carrier	12,508	1,667	64.4	1.03 (0.98 to 1.09)	0.25
C282Y/H63D	2,115	291	64.2	1.08 (0.96 to 1.22)	0.18
C282Y/C282Y	422	68	60.6	1.38 (1.09 to 1.75)	0.008



Fracture of the hip and femur

	Individuals, n.	Fractures, n.	Mean age at fracture, years	Age and sex adjusted hazard ratio (95% CI)	p
Non-carrier/non-carrier	87,286	3,387	75.8	1.00 (reference)	
H63D/non-carrier	27,811	1,090	76.2	1.03 (0.97 to 1.11)	0.33
H63D/H63D	2,357	110	77.2	1.21 (1.00 to 1.47)	0.04
C282Y/non-carrier	12,508	513	73.9	1.10 (1.00 to 1.21)	0.04
C282Y/H63D	2,115	96	72.8	1.23 (1.00 to 1.51)	0.05
C282Y/C282Y	422	22	72.0	1.78 (1.17 to 2.70)	0.007

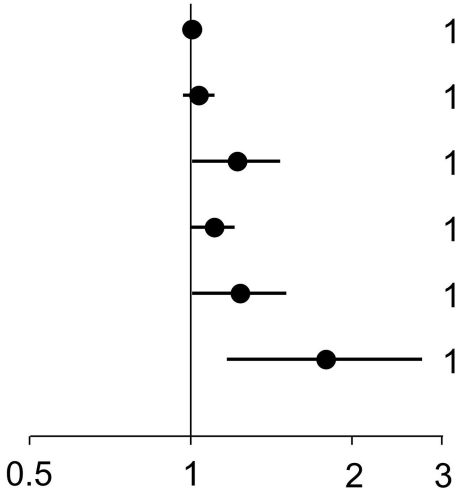


Figure 4

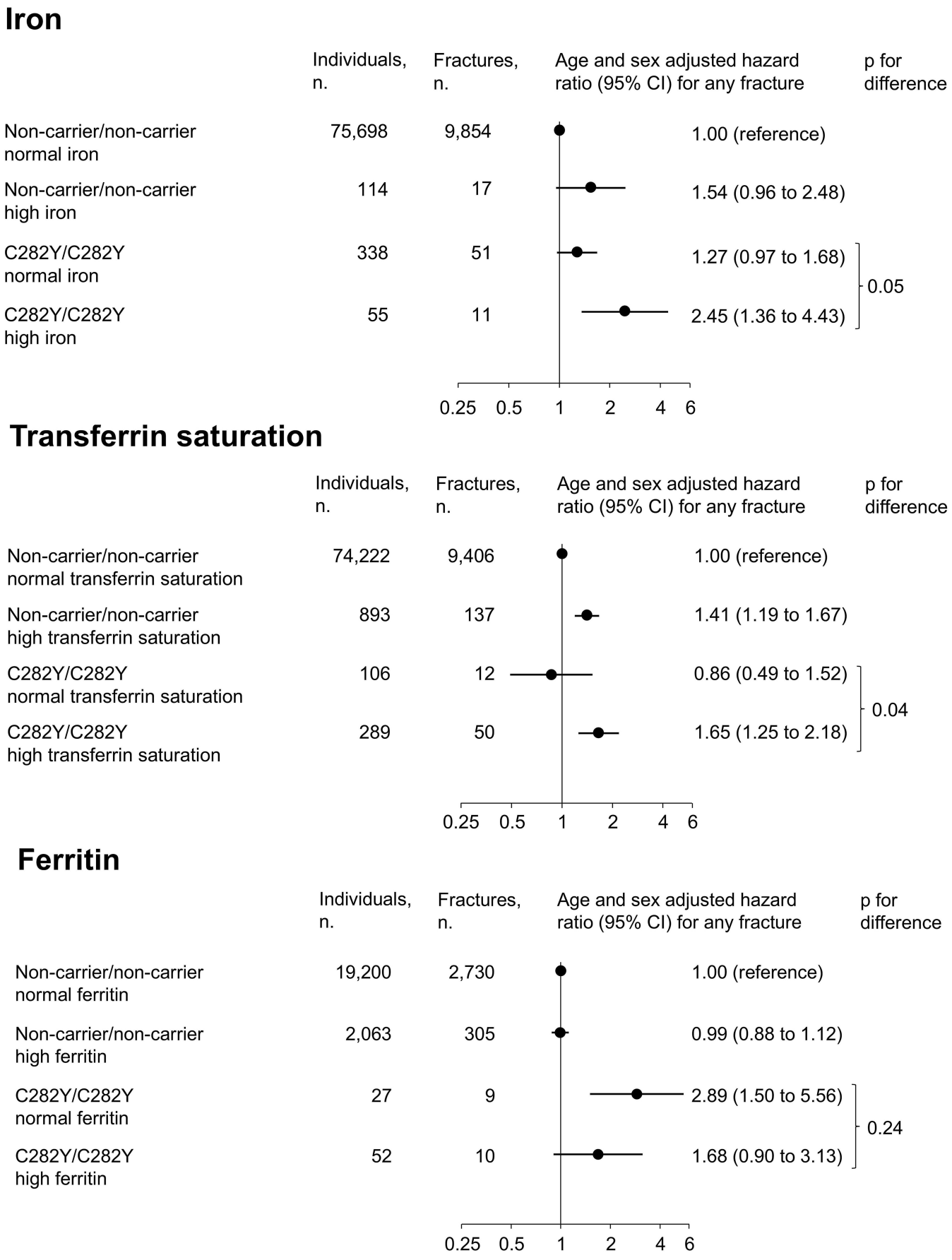
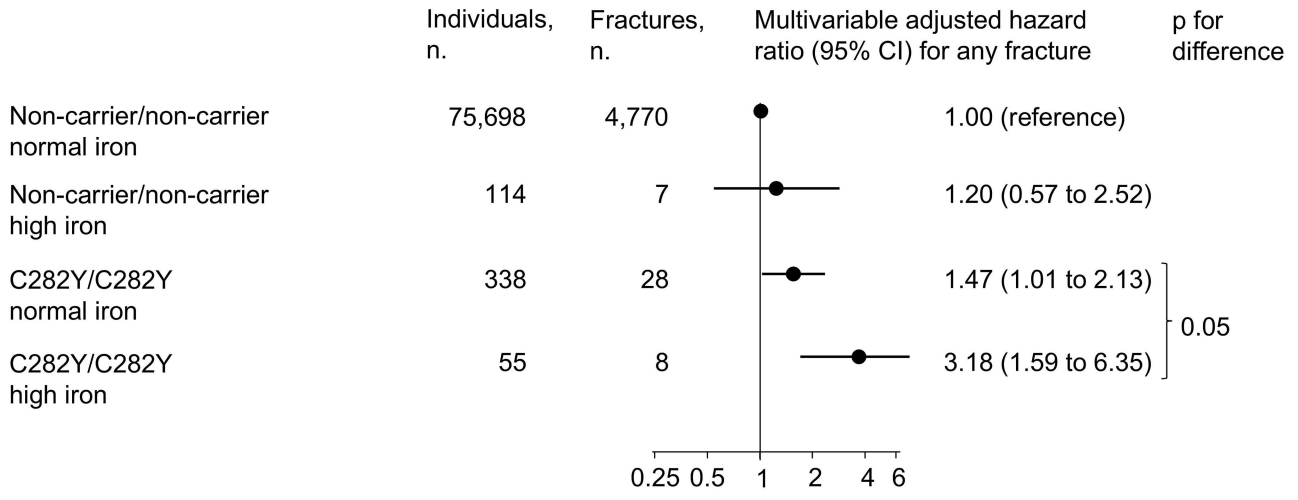
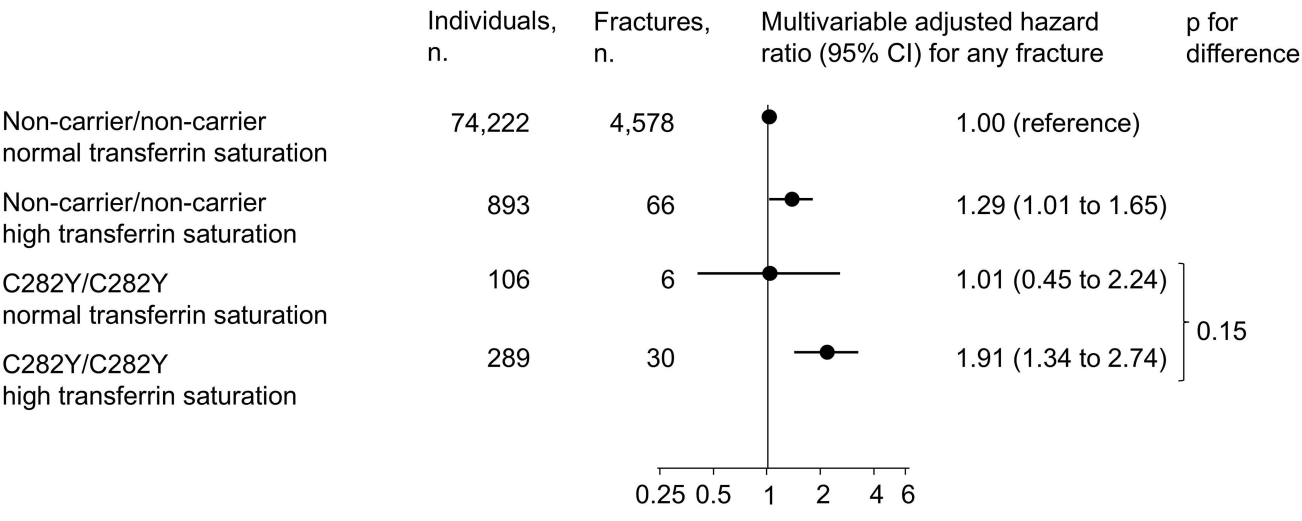

Iron
Individuals, n.
Fractures, n.
Age and sex adjusted hazard ratio (95% CI) for any fracture
p for difference
Non-carrier/non-carrier normal iron
75,698
9,854
1.00 (reference)
Non-carrier/non-carrier high iron
114
17
1.54 (0.96 to 2.48)
C282Y/C282Y normal iron
338
51
1.27 (0.97 to 1.68)
C282Y/C282Y high iron
55
11
2.45 (1.36 to 4.43)
0.05
0.25 0.5 1 2 4 6
Transferrin saturation
Individuals, n.
Fractures, n.
Age and sex 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
1.41 (1.19 to 1.67)
C282Y/C282Y normal transferrin saturation
106
12
0.86 (0.49 to 1.52)
C282Y/C282Y high transferrin saturation
289
50
1.65 (1.25 to 2.18)
0.04
0.25 0.5 1 2 4 6
Ferritin
Individuals, n.
Fractures, n.
Age and sex 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
0.99 (0.88 to 1.12)
C282Y/C282Y normal ferritin
27
9
2.89 (1.50 to 5.56)
C282Y/C282Y high ferritin
52
10
1.68 (0.90 to 3.13)
0.24
0.25 0.5 1 2 4 6

Figure 5

Iron



Transferrin saturation



Ferritin

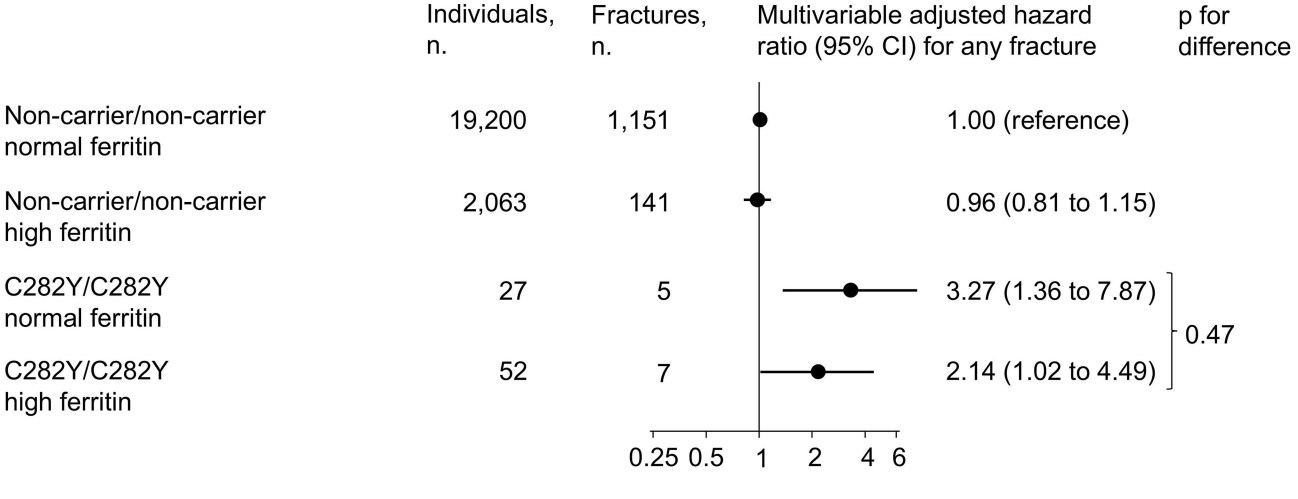


Figure 6

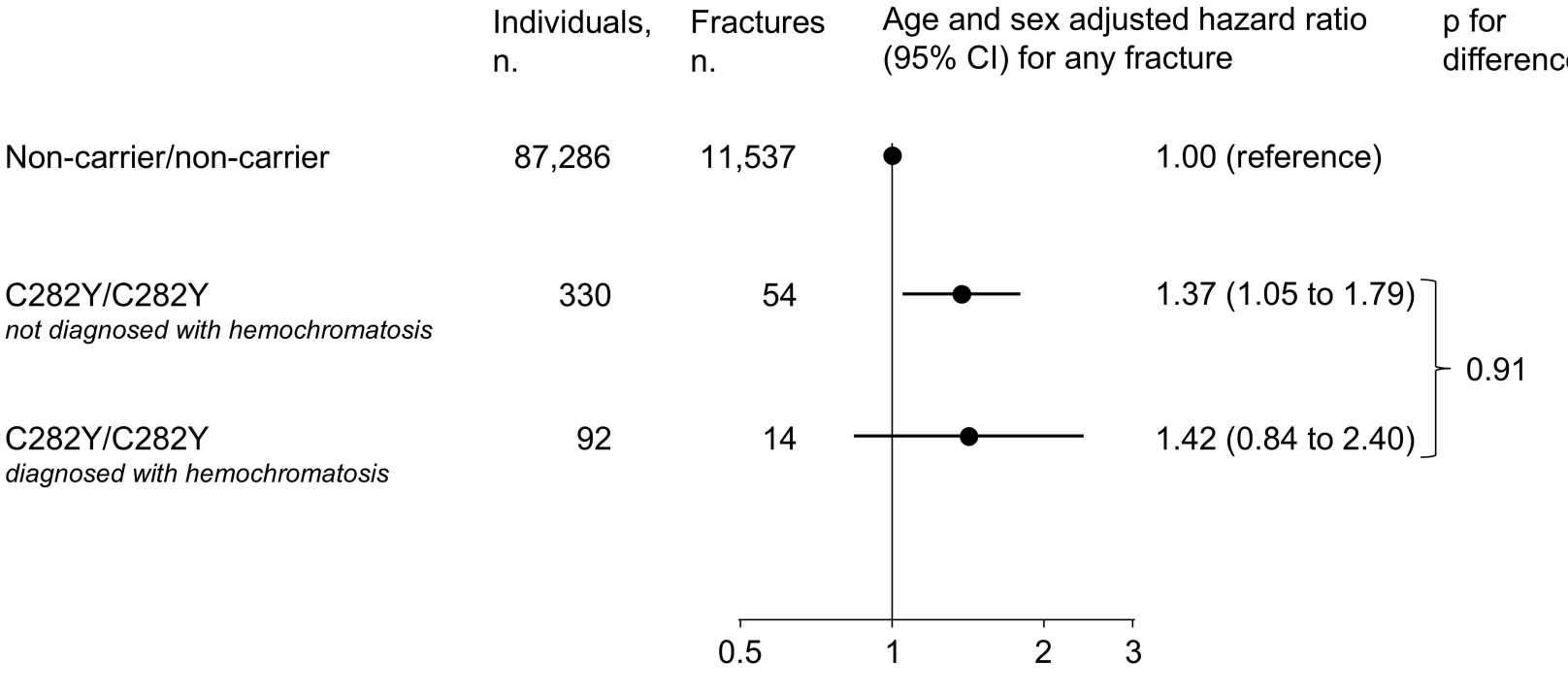
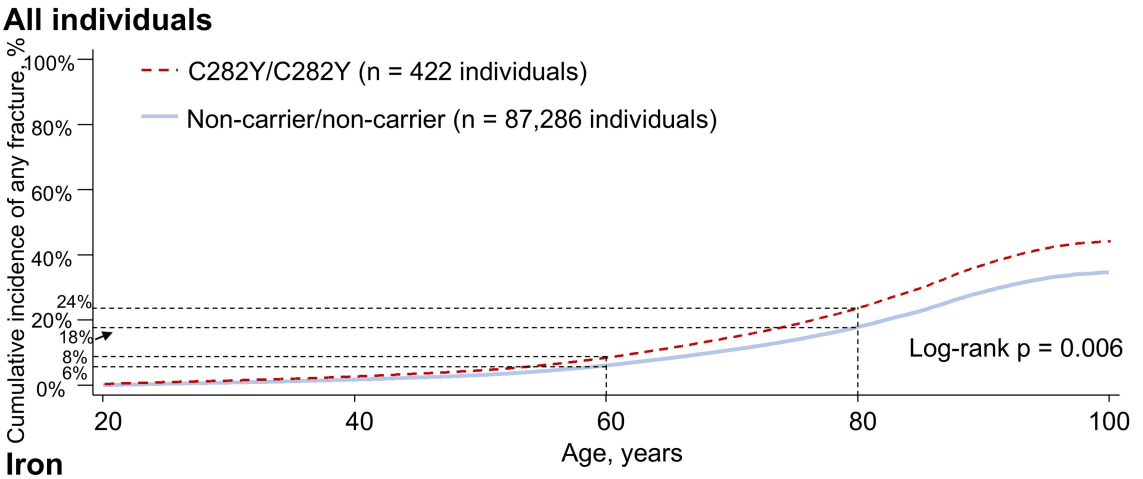
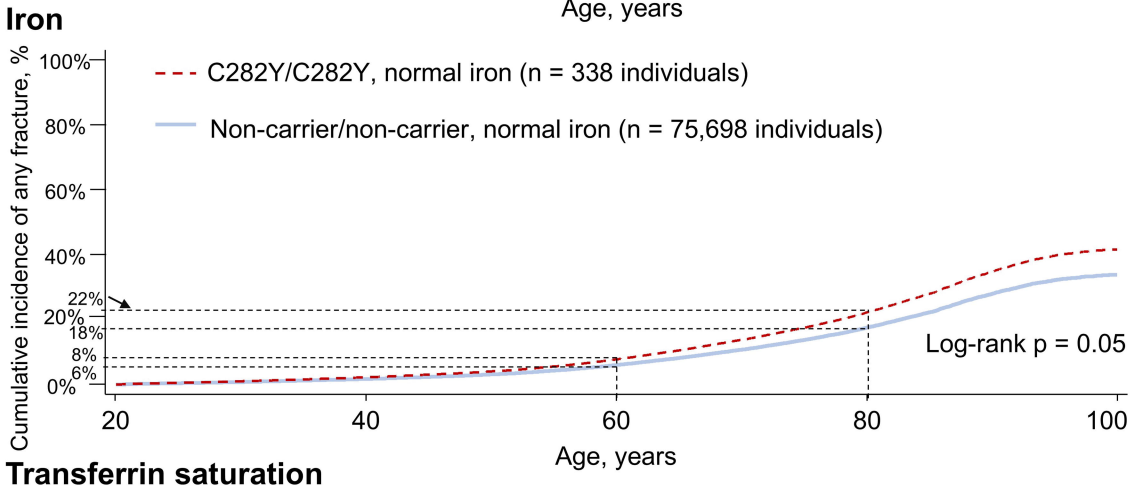
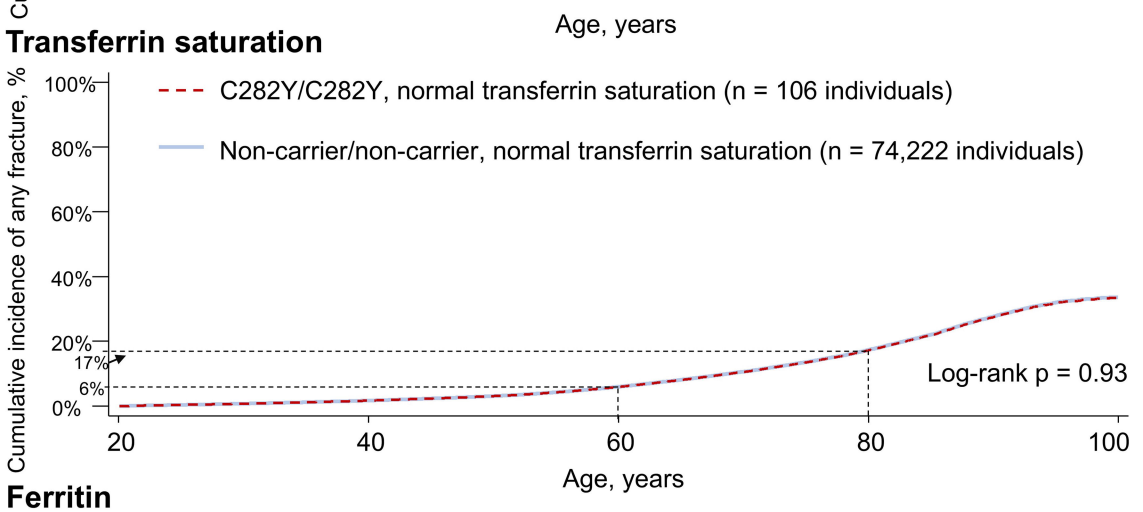
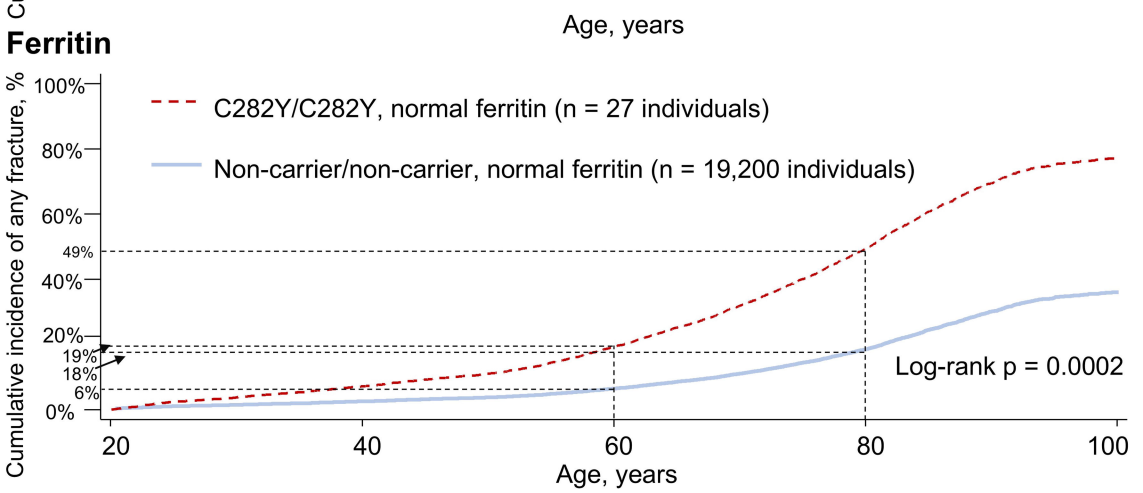

Individuals, n.
Fractures n.
Age and sex adjusted hazard ratio (95% CI) for any fracture
p for difference
Non-carrier/non-carrier
87,286
11,537
1.00 (reference)
C282Y/C282Y
not diagnosed with hemochromatosis
330
54
1.37 (1.05 to 1.79)
0.91
C282Y/C282Y
diagnosed with hemochromatosis
92
14
1.42 (0.84 to 2.40)
0.5
1
2
3

Figure 7


All individuals
C282Y/C282Y (n = 422 individuals)
Non-carrier/non-carrier (n = 87,286 individuals)
Cumulative incidence of any fracture, %
100%
80%
60%
40%
24%
20%
18%
8%
6%
0%
Log-rank p = 0.006
20
40
60
80
100
Age, years


Iron
C282Y/C282Y, normal iron (n = 338 individuals)
Non-carrier/non-carrier, normal iron (n = 75,698 individuals)
Cumulative incidence of any fracture, %
100%
80%
60%
40%
22%
20%
18%
8%
6%
0%
Log-rank p = 0.05
20
40
60
80
100
Age, years


Transferrin saturation
C282Y/C282Y, normal transferrin saturation (n = 106 individuals)
Non-carrier/non-carrier, normal transferrin saturation (n = 74,222 individuals)
Cumulative incidence of any fracture, %
100%
80%
60%
40%
20%
17%
6%
0%
Log-rank p = 0.93
20
40
60
80
100
Age, years


Ferritin
C282Y/C282Y, normal ferritin (n = 27 individuals)
Non-carrier/non-carrier, normal ferritin (n = 19,200 individuals)
Cumulative incidence of any fracture, %
100%
80%
60%
49%
40%
20%
19%
18%
6%
0%
Log-rank p = 0.0002
20
40
60
80
100
Age, years

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

Authors: Marie Warny, Andreas Glenthøj, Børge Grønne Nordestgaard, Mathis Mottelson, Christina Ellervik, Jesper Petersen, Stig Egil Bojesen, and Jens Helby

Supplemental material

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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

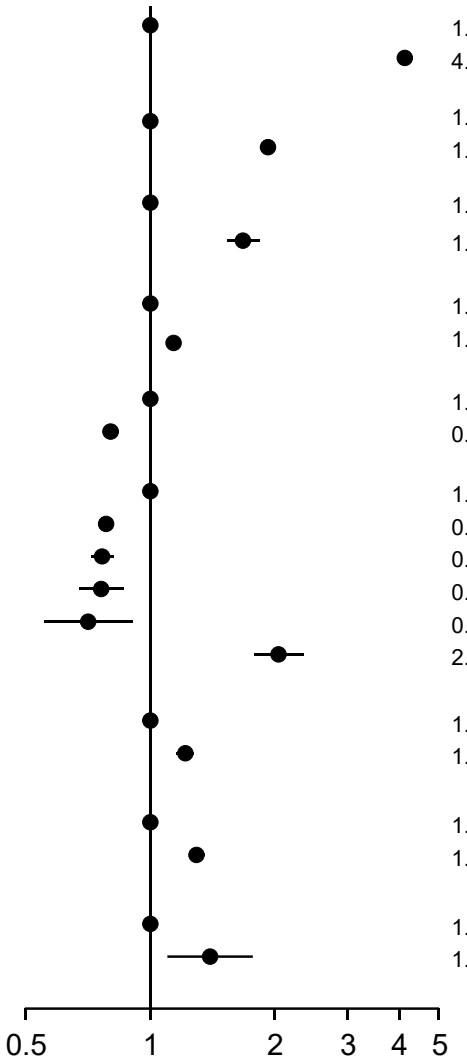
Supplemental Table 1: WHO's International Classification of Diseases, revision 8 (ICD8) and revision 10 (ICD10) codes for categories of fracture types.

	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

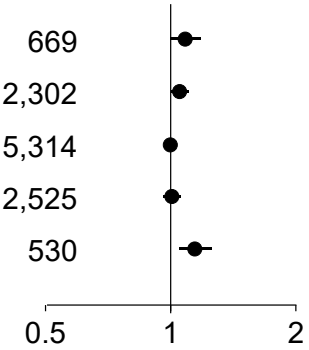
	Individuals, n.	Fractures, n.	Hazard ratio (95% CI)	p
Age, years				
≤ 60	76,802	3,336	1.00 (reference)	
> 60	64,103	8,213	4.15 (3.98 to 4.32)	$< 10^{-300}$
Sex				
Men	63,872	3,449	1.00 (reference)	
Women	77,033	8,100	1.91 (1.84 to 1.99)	7×10^{-223}
Menopause*				
Before	24,891	781	1.00 (reference)	
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.

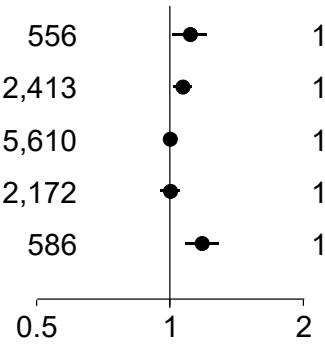
Iron

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



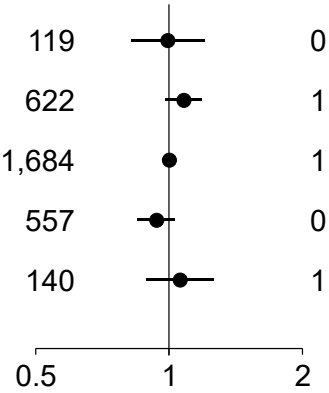
Transferrin saturation

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

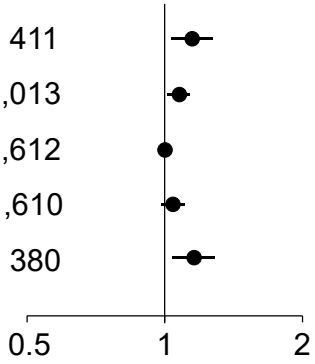
Percentiles of ferritin	Individuals, n.	Fractures, n.	Multivariable adjusted hazard ratio (95% CI) for any fracture	p
≤ 5th	1,105	119	0.99 (0.82 to 1.20)	0.93
6-25th	4,417	622	1.08 (0.98 to 1.19)	0.12
26-74th	11,034	1,684	1.00 (reference)	
75-94th	4,418	557	0.94 (0.85 to 1.03)	0.19
≥ 95th	1,106	140	1.06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

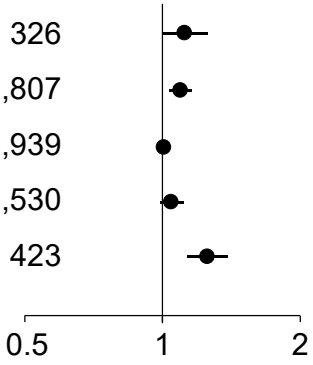
Iron

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



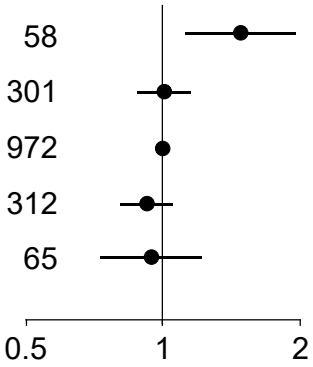
Transferrin saturation

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

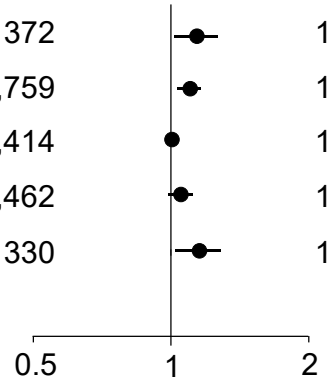
Percentiles of ferritin	Individuals, n.	Fractures, n.	Multivariable adjusted hazard ratio (95% CI) for any fracture	p
≤ 5th	1,149	58	1.48 (1.12 to 1.95)	0.006
6-25th	5,507	301	1.01 (0.88 to 1.15)	0.90
26-74th	13,656	972	1.00 (reference)	
75-94th	5,657	312	0.92 (0.81 to 1.05)	0.24
≥ 95th	1,358	65	0.94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

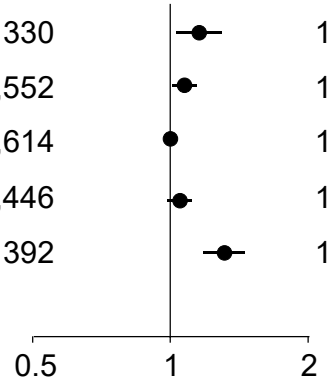
Iron

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



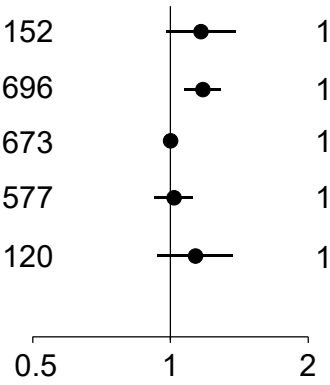
Transferrin saturation

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}

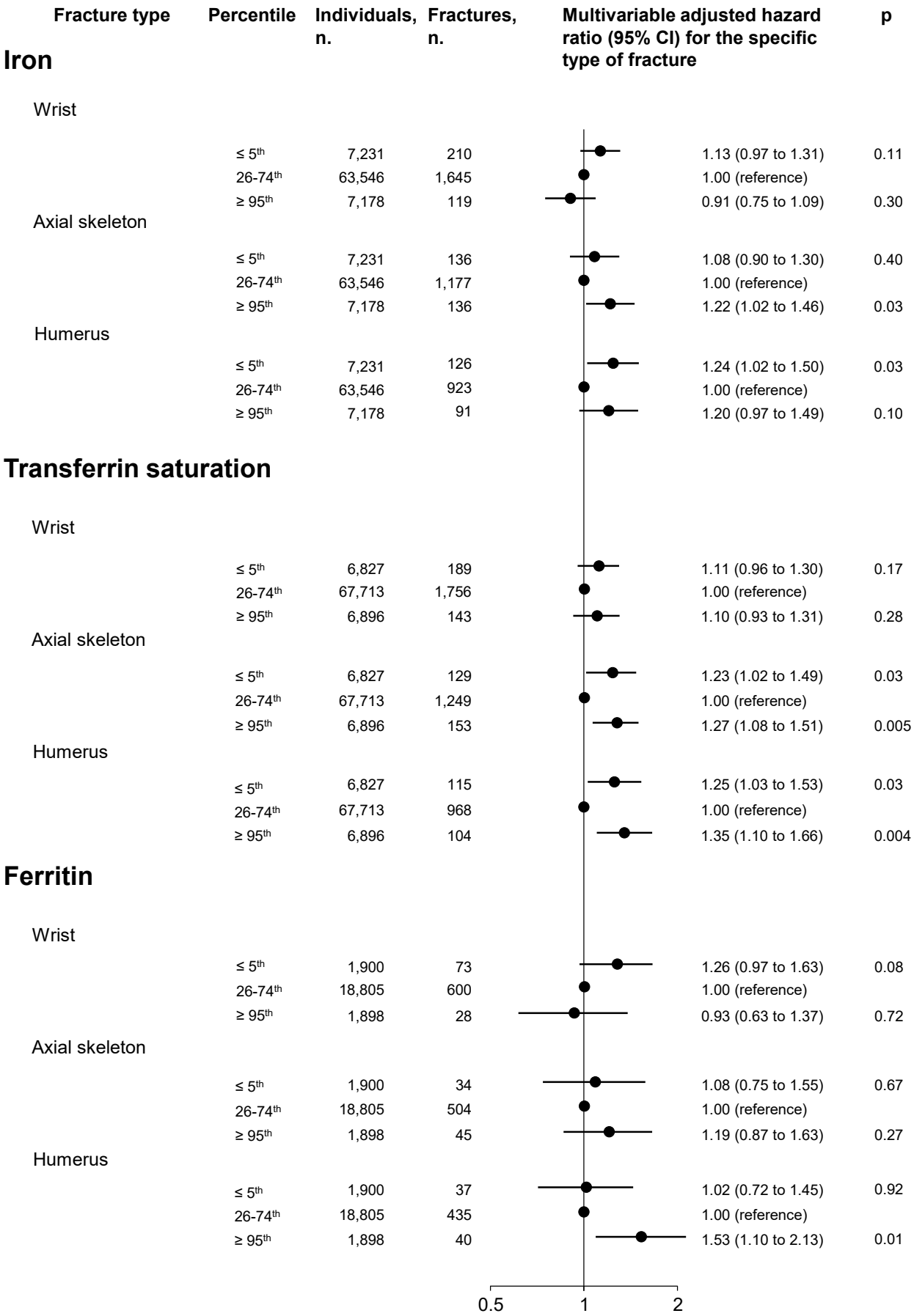


Ferritin

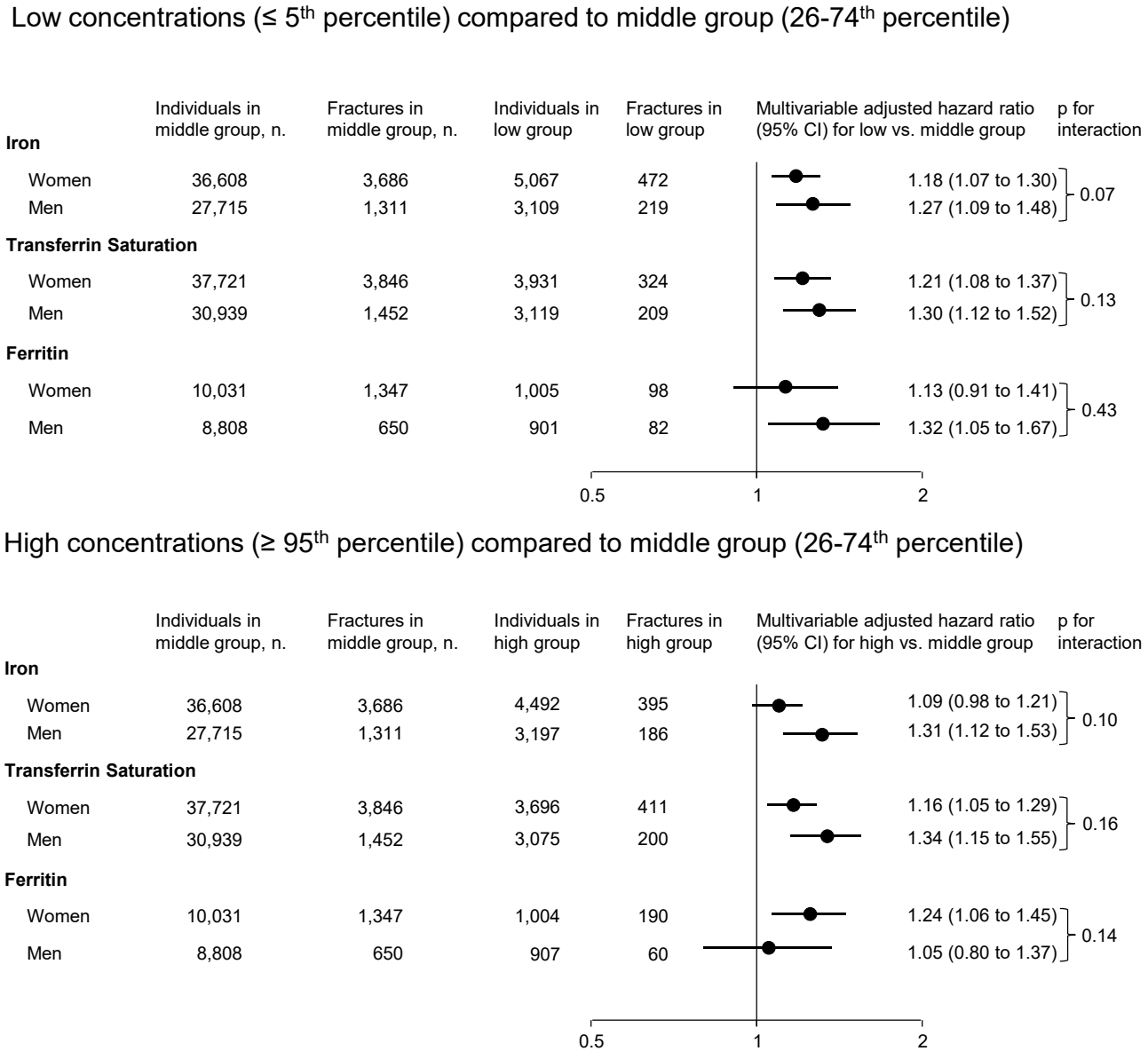
Percentiles of ferritin	Individuals, n.	Fractures, n.	Multivariable adjusted hazard ratio (95% CI) for any fracture	p
≤ 5th	1,662	152	1.16 (0.98 to 1.39)	0.09
6-25th	6,350	696	1.18 (1.07 to 1.29)	6×10^{-4}
26-74th	15,135	1,673	1.00 (reference)	
75-94th	6,458	577	1.02 (0.92 to 1.12)	0.73
≥ 95th	1,513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



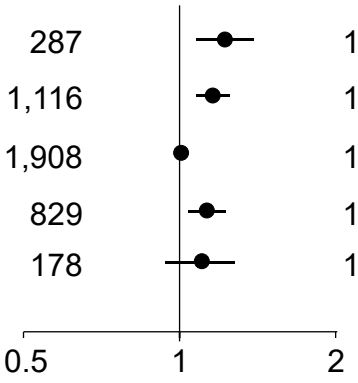
Supplemental Figure 6: Risk of any fracture according to iron, transferrin saturation, and ferritin, stratified by sex. Percentiles of iron, transferrin saturation, and ferritin were calculated for each sex separately. Based on the sex-specific percentiles, we calculated sex specific cut-offs for low 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

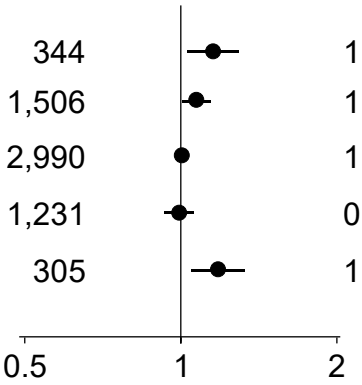
First 5 years after study enrollment

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

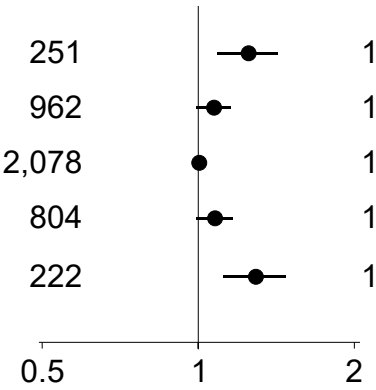


Supplemental Figure 7: Risk of any fracture according to concentrations of iron when each individual's follow-up time was split into the first five years after study enrollment (upper panel) and from 5 years after study enrollment and onwards (lower panel). For this analysis, individuals were included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

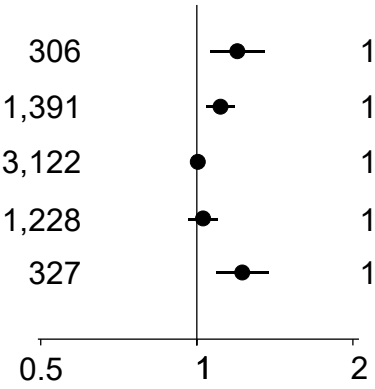
First 5 years after study enrollment

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

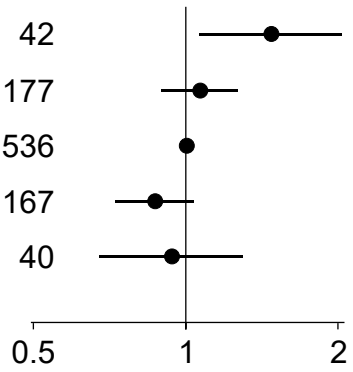


Supplemental Figure 8: Risk of any fracture according to concentrations of transferrin saturation when each individual's follow-up time was split into the first five years after study enrollment (upper panel) and from 5 years after study enrollment and onwards (lower panel). For this analysis, individuals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

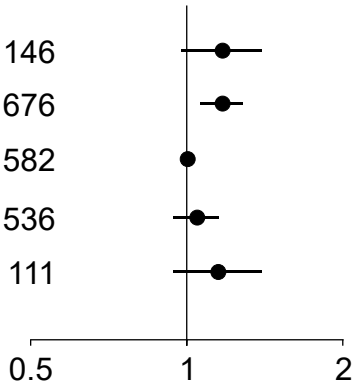
First 5 years after study enrollment

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

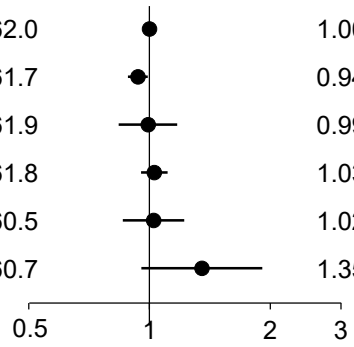
Percentiles of ferritin	Individuals, n.	Fractures, n.	Multivariable adjusted hazard ratio (95% CI) for any fracture	p
≤ 5th	1,801	146	1.17 (0.98 to 1.40)	0.09
6-25th	7,159	676	1.17 (1.06 to 1.28)	0.001
26-74th	17,452	1,582	1.00 (reference)	
75-94th	7,235	536	1.04 (0.94 to 1.15)	0.41
≥ 95th	1,725	111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

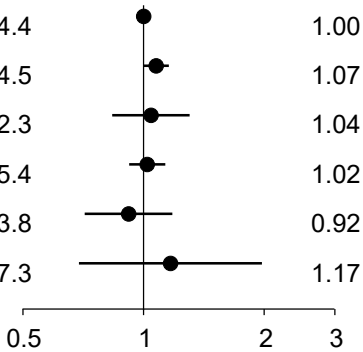
Fracture of the wrist

	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



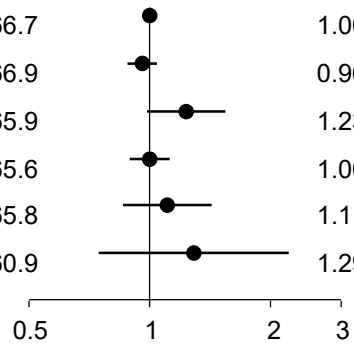
Fracture of the axial skeleton (vertebral, ribs, pelvic)

	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

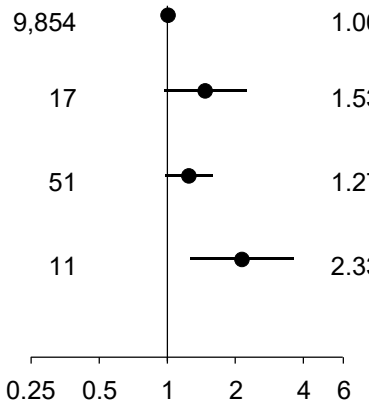
	Individuals, n.	Fractures, n.	Mean age at fracture, years	Age and sex adjusted hazard ratio (95% CI)	p
Non-carrier/non-carrier	87,286	2,337	66.7	1.00 (reference)	
H63D/non-carrier	27,811	705	66.9	0.96 (0.88 to 1.04)	0.33
H63D/H63D	2,357	78	65.9	1.23 (0.98 to 1.55)	0.07
C282Y/non-carrier	12,508	328	65.6	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.

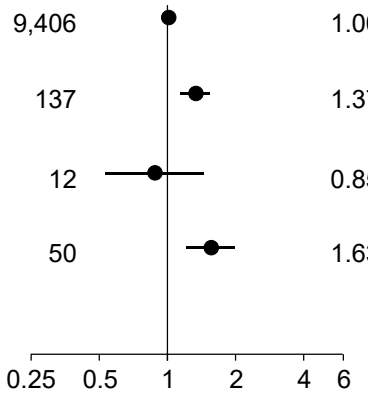
Iron

	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)	



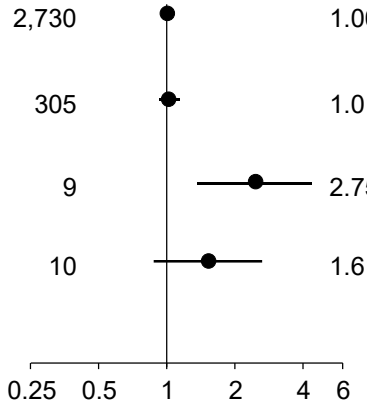
Transferrin saturation

	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)	

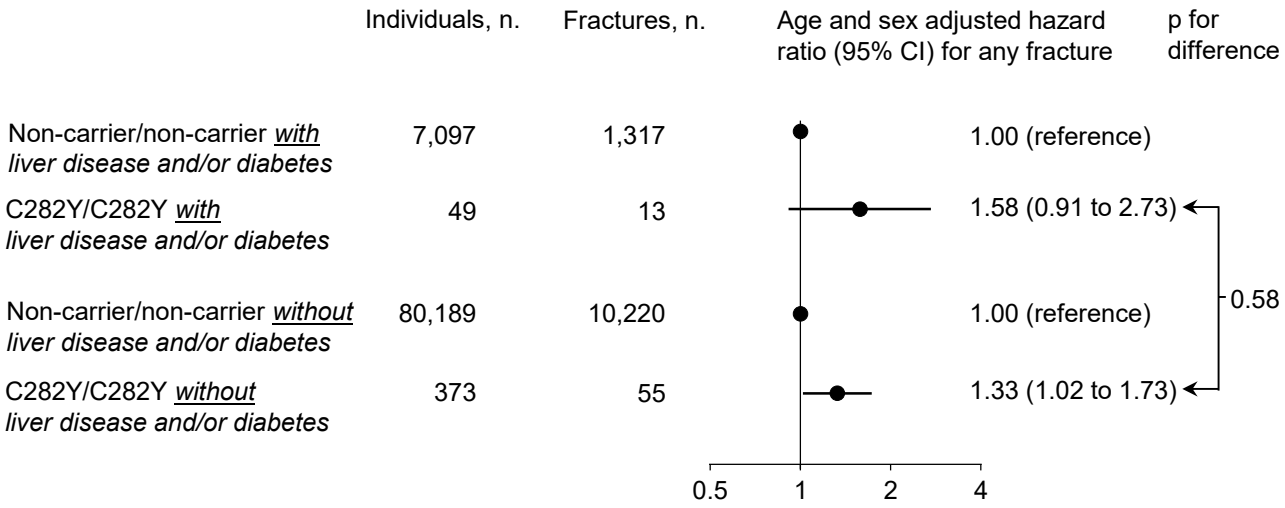


Ferritin

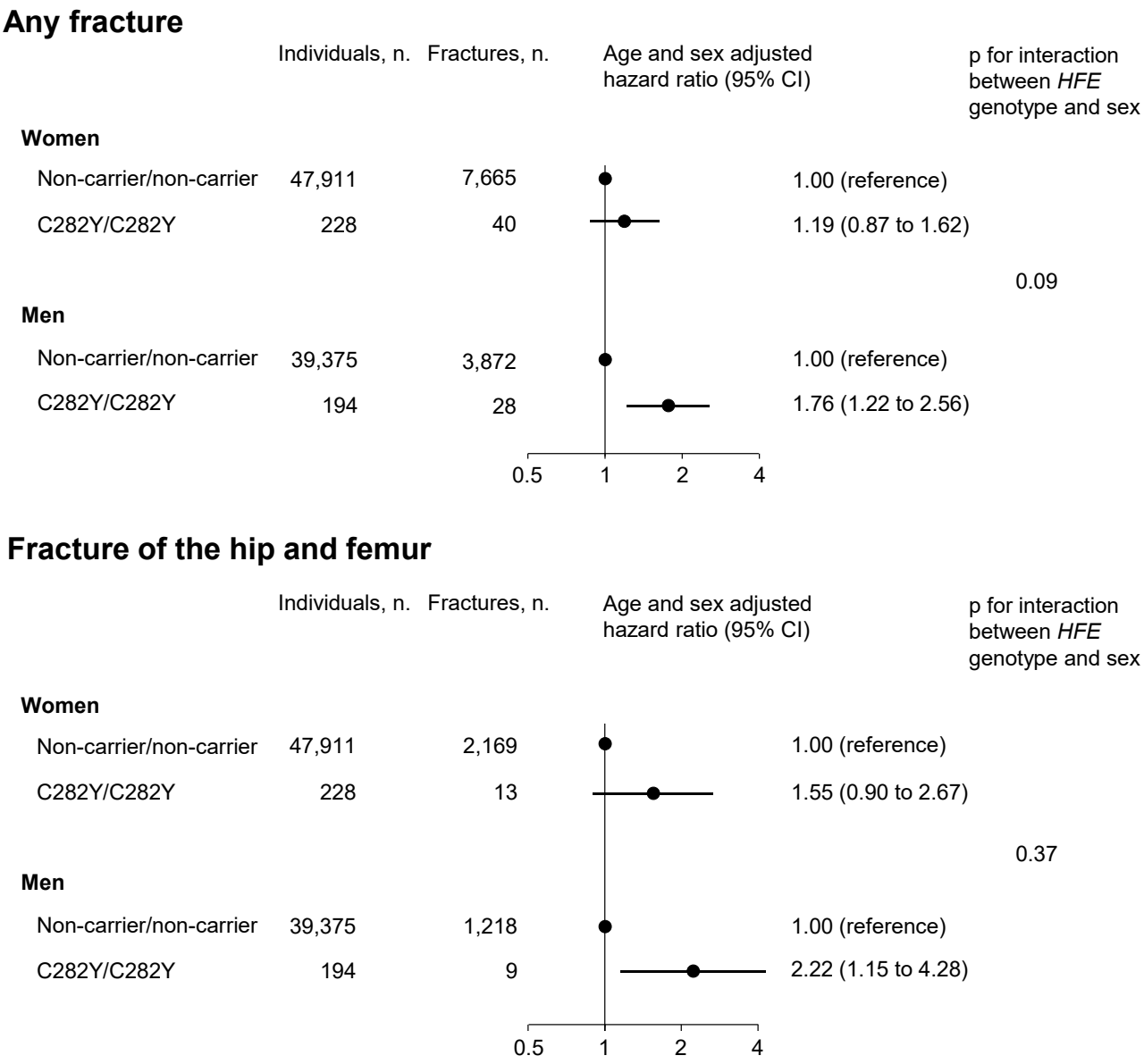
	Individuals, n.	Fractures, n.	Multivariable adjusted hazard ratio (95% CI) for any fracture	p for difference
Non-carrier/non-carrier normal ferritin	19,200	2,730	1.00 (reference)	
Non-carrier/non-carrier high ferritin	2,063	305	1.01 (0.90 to 1.14)	
C282Y/C282Y normal ferritin	27	9	2.75 (1.43 to 5.30)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



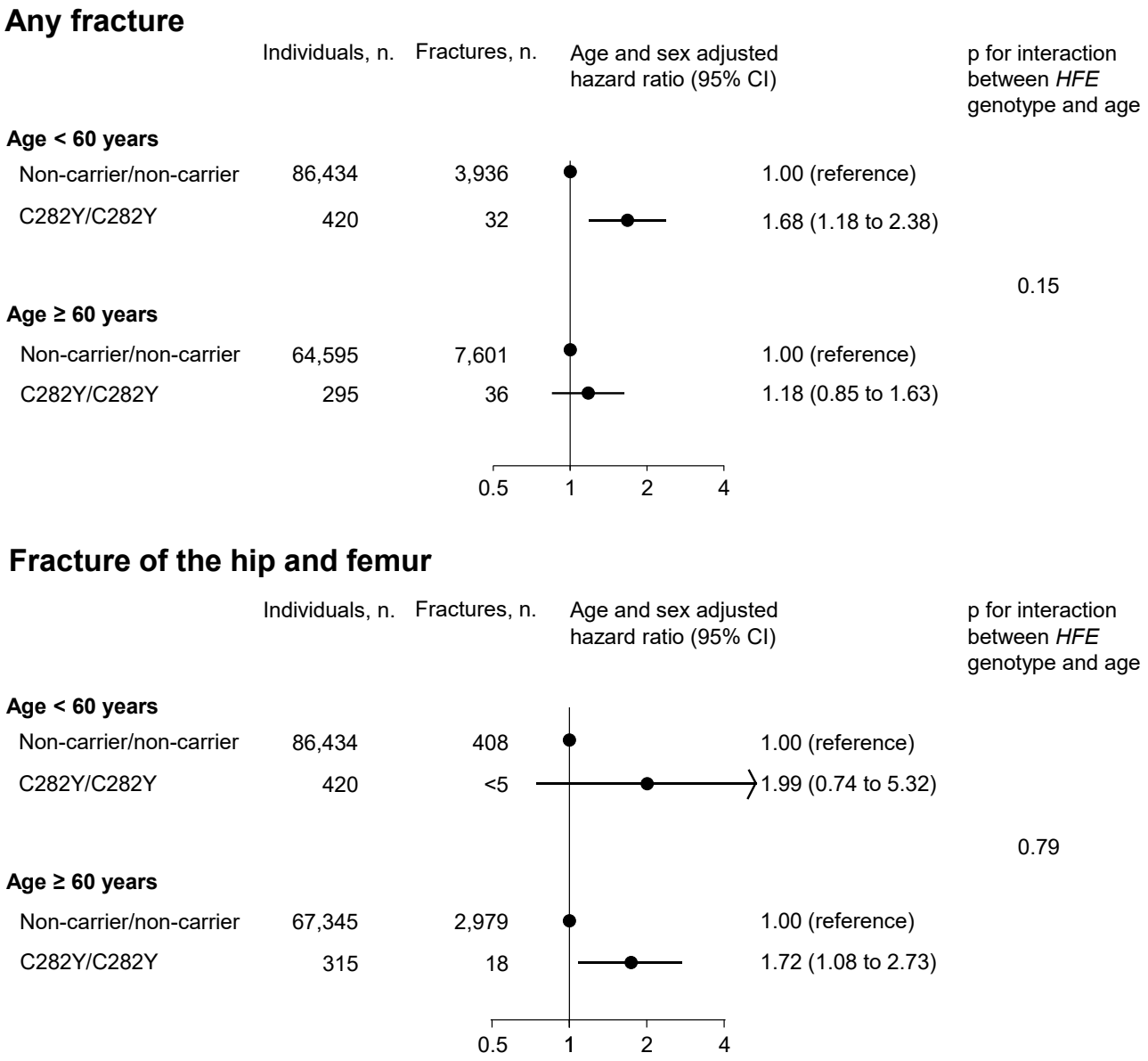
Supplemental Figure 11: Multivariable adjusted risk of any fracture for *HFE* C282Y homozygotes vs. non-carriers for the C282Y and H63D variants stratified by normal vs. high concentrations of plasma iron (upper panel), transferrin saturation (middle panel), and ferritin (lower panel) at study enrollment.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