Title: The natural history of *HFE* simple heterozygotes for C282Y and H63D: a twelve year study

S.G. Zaloumis[1], K.J. Allen[2,3,4], N.A. Bertalli[2], L. Turkovic[1], M.B. Delatycki[2,3,5], A.J. Nicoll[6], C.E. McLaren[7], D.R. English[1,8], J.L. Hopper[1], G.G. Giles[8], G.J. Anderson[9], J.K. Olynyk[10], L.W. Powell[9,11] and L.C. Gurrin[1] for the HealthIron Study Investigators.*

[1] Centre for MEGA Epidemiology, School Population Health, The University of Melbourne, Victoria, Australia

[2] Murdoch Childrens Research Institute, Victoria, Australia

[3] Department of Paediatrics, University of Melbourne, Royal Children's Hospital, Victoria, Australia

[4] Department of Gastroenterology, Royal Children's Hospital, Victoria, Australia

[5] Austin Health, Heidelberg, Victoria, Australia

[6] The Royal Melbourne Hospital, Melbourne, Victoria, Australia

[7] Department of Epidemiology, University of California, Irvine, USA

[8] Cancer Epidemiology Centre, The Cancer Council Victoria, Melbourne, Australia

[9] Queensland Institute of Medical Research and the University of Queensland, Brisbane, Australia

[10] Department of Gastroenterology, Fremantle Hospital, Fremantle, Australia; School of Medicine and Pharmacology, University of Western Australia, Nedlands, Australia; Western Australian Institute of Medical Research, Perth, Australia.

[11] The University of Queensland and the Royal Brisbane and Women's Hospital, Brisbane, Australia

*The HealthIron Study Investigators not already listed above are:
M. Bahlo (The Walter & Eliza Hall Institute for Medical Research, Melbourne, Australia), C.D. Vulpe (University of California, Berkeley, USA), S.M. Forrest (Australian Genome Research Facility, Melbourne, Australia) and A. R. Fletcher (Cancer Council Victoria, Australia).

Corresponding Author

Associate Professor Lyle Gurrin, Centre for MEGA Epidemiology, Melbourne School of Population and Global Health, The University of Melbourne, , Victoria, Australia.
Telephone +61 3 8344 0731 Fax +61 3 9349 5815
Email: katie.allen@rch.org.au

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Abstract

Background: The risk of hemochromatosis-related morbidity among *HFE* simple heterozygotes for either C282Y or H63D substitutions in the HFE protein was assessed in a prospective population-based cohort study.

Methods: *HFE* genotypes were measured in 31,192 persons of northern European descent between the ages of 40 and 69 years who participated in the Melbourne Collaborative Cohort Study and were followed for an average of 12 years. In a random sample of 1438 subjects stratified according to *HFE* genotype and selected for invitation to participate, the following data were obtained: two sets of biochemical iron indices performed 12 years apart and, at follow-up only, the presence/absence of six disease features associated with hereditary hemochromatosis. Summary measures for 257 (139 female) C282Y and 123 (74 female) H63D simple heterozygotes were compared with 330 (181 female) controls with neither *HFE* mutation.

Results: At baseline, men, pre-menopausal women and post-menopausal women had, on average, clinically normal transferrin saturation (TS) levels irrespective of *HFE* simple heterozygosity. Mean TS (95% confidence interval) and prevalence of TS > 55% was 35.14% (33.25,37.04) and 3/112(3%); 33.03% (29.9,36.15) and 0/39(0%); and 29.67% (27.93,31.4) and 3/135(2%) for C282Y, H63D, and wild-type male participants, respectively. At follow-up, mean TS levels remained similar to baseline levels for men, pre- and post-menopausal women who were simple heterozygotes for either C282Y or H63D. No *HFE* C282Y or H63D simple heterozygotes had documented iron overload (based on hepatic iron measures or serum ferritin greater than 1000μg/L at baseline with documented therapeutic venesection). After stratifying by sex, the disease features of both C282Y and H63D simple heterozygotes were similar to those of *HFE* wild-type participants.

Conclusion: No documented iron overload was observed for *HFE* simple heterozygotes for either C282Y or H63D, and morbidity for both *HFE* simple heterozygote groups was similar to that of the *HFE* wild-type participants.

Introduction

Hereditary hemochromatosis (HH) is a genetic predisposition to iron overload which, if not prevented or treated, increases the risk of diseases including liver cirrhosis, arthritis, fatigue and diabetes [1]. Two mutations in the *HFE* gene, C282Y and H63D, are responsible for the majority of clinical cases of iron overload [2]. In particular two *HFE* genotypes, C282Y homozygosity and C282Y/H63D compound heterozygosity, confer an increased risk of iron overload-related disease [2, 3]. Both of these *HFE* genotype groups have been studied extensively in both population and clinical studies, and their epidemiological profile is well established [4, 5] add Gurrin et al. (Hepatology 2009).

Two *HFE* genotype groups that have received less attention are the simple heterozygotes for either the C282Y or the H63D mutation. In populations of northern European descent, H63D simple heterozygosity is more prevalent (23.6% to 31.1%) [6-8] than C282Y simple heterozygosity (8.6% to 11.9%) [5-7, 9]. Despite these high prevalences the population risk of *HFE* C282Y simple heterozygotes and *HFE* H63D heterozygotes developing HH-associated clinical signs and symptoms or iron overload-related disease has not been widely examined. If this risk is increased compared to that of the general community then it would have an immediate implication for population genetic screening for *HFE* mutations, since such screening would potentially label a large proportion of the population as both being at increased risk of disease, and as carriers of a disease causing mutation.

Large cross-sectional population-based studies show that, on average, serum ferritin concentration (SF) and transferrin saturation (TS) levels for C282Y simple heterozygotes are

within their respective clinically normal reference ranges, but tend to be higher compared to that those with a lack of a detectable C282Y or H63D mutation, designated as *HFE* wild-type, for both sexes [5, 6, 9, 10]. Similarly, mean SF and TS levels for H63D simple heterozygotes are within their respective clinically normal ranges, and comparable to *HFE* wild-types for both males and females [6]. Male C282Y simple heterozygotes have been reported to have 0.81-fold decrease (95%CI: 0.71-0.94) in the odds of diabetes compared to *HFE* wild-types [6] although the prevalence of diabetes in this study (11.5%) and our own cohort (2%) is low [5, 11]. An Australian study found no evidence that the presence of the H63D mutation resulted in an increased risk of clinically significant iron overload [8]. In the work-place setting the prevalence of self-reported tiredness, abdominal pain, joint pain and previous diagnosis of diabetes, arthritis and liver disease in simple C282Y heterozygotes was comparable to the prevalence of these symptoms/diseases in *HFE* wild-type individuals [12].

These previous studies [4, 8-10] experience a number of shortcomings. They did not record and, therefore, could not stratify by women's menopausal status, and none have measured iron indices in the same participants at two or more time points. Participants were examined by medical practitioners who were not blinded to their *HFE* genotype status. In some studies, *HFE*-associated signs, symptoms and features of disease were not examined; only iron indices were assessed [9, 10]. Findings from the study in the work-place setting may not be generalizable to the wider population due to the possibility of selection bias [12].

The "HealthIron" study, results of which we present in this paper, does not suffer from the deficiencies outlined in the previous paragraph. We examined clinical and epidemiological data from *HFE* C282Y and H63D simple heterozygotes and wild-type individuals who were followed over a 12-year period and at ages when those at risk of iron overload would have been expected to develop iron overload-related disease (from 40-69 years at baseline to 54-

83 years at follow-up). We describe the natural history of serum iron indices and iron overload-related disease signs and symptoms using this large population-based sample of well-characterised subjects.

Study Methods

The Melbourne Collaborative Cohort Study (MCCS)

Between 1990 and 1994, the Melbourne Collaborative Cohort Study (MCCS) recruited 41,514 people (24,469 females) aged between 27 and 75 years (99% were aged 40 to 69 years) [13]. At baseline, participants attended a study centre where they completed a questionnaire about dietary and lifestyle factors, underwent a physical examination and provided a blood sample.

The HealthIron Study

Beginning in 2004, 31,192 MCCS participants of northern European descent (born in Australia, the United Kingdom, Ireland or New Zealand) were genotyped for the C282Y *HFE* mutation using stored baseline blood samples. Participants of southern European descent (n=10,336) were excluded due to the low prevalence of *HFE* mutations. Those with one copy of the C282Y mutation were then genotyped for H63D to determine whether they were simple (one copy of the C282Y mutation) or compound heterozygotes (one copy of each of the C282Y and H63D mutations).

All participants homozygous for the C282Y mutation (n=203) plus a random sample stratified by the other three *HFE* genotypes (C282Y/H63D compound heterozygote, C282Y simple heterozygote, no C282Y mutation) that were selected for invitation to attend follow-up clinics between 2004 and 2006 as part of the HealthIron study. H63D simple heterozygotes were identified by genotyping of those with no C282Y mutation who attended the follow-up clinic. Of the 1,438 people invited to participate in the HealthIron study, 107 were deceased and 277 were lost to follow-up, leaving 1,054 who participated. The overall participation by those

invited was 73.3 per cent (79.2 per cent excluding those already deceased) with no significant variation in participation when stratified by genotype (data not shown).

At baseline, participants had a fasting blood sample taken and completed questionnaires that included information about diet, alcohol intake and medical history. Follow-up clinics were held between 2004 and 2006. As part of the study, participants completed a computer-assisted personal interview that included information about medical history, blood donation, had a fasting blood sample taken for iron studies and liver enzymes, were examined by a medical practitioner blinded to genotype and had a cheekbrush swab taken to confirm the original *HFE* genotype from their baseline blood sample.

All participants gave written informed consent to participate in both the MCCS and the HealthIron sub-study. Both protocols were approved by the Cancer Council Victoria's Human Research Ethics Committee.

Definitions of biochemical / clinical exposures and outcomes

Iron indices

Elevated SF was defined for males and post-menopausal females as >300 µg/L and for pre-menopausal females >200 µg/L. Elevated transferrin saturation (TS) was defined for males as >55% and for females >45%. When examining the prevalence of disease by elevated iron indices, SF was considered to be elevated if the SF value exceeded the specified threshold on at least one occasion. We defined normal SF as having values below these thresholds at both baseline and follow-up.

Disease features and iron overload-related disease (IORD)

We investigated the prevalence of six disease features associated with hereditary hemochromatosis (HH): Abnormal (i.e. presence of bony spur, effusion or tenderness)

second and/or third metacarpophalangeal (MCP) joints on either hand (MCP 2/3), use of arthritis medication, self reported fatigue, raised aspartate aminotransferase or raised alanine aminotransferase levels (raised AST or raised ALT), self reported history of liver disease and/or hepatomegaly. With the exception of the use of arthritis medication, which was assessed at baseline and follow-up, all disease features were measured at follow-up alone.

Iron overload-related disease (IORD) was defined as the presence of one of the following five features: Hepatocellular carcinoma, liver cirrhosis or fibrosis, abnormal $2^{nd}/3^{rd}$ MCP, raised aminotransferases or physician-diagnosed HH due to symptoms in the context of either provisional or documented iron overload (see footnotes to Table 6 for definition) [5].

Menopausal status and blood donation history

Menopausal status for women was measured at baseline and classified as pre-menopausal or post-menopausal. Blood donation history was classified at baseline as never, former (ceased before baseline) or current (still donating at baseline).

Statistical analysis of biochemical and clinical outcomes

The Mann-Whitney U test was used to detect differences in both location and spread of age, BMI and alcohol intake between groups. For all analyses SF levels were natural log transformed. Comparisons of mean log SF and TS measurements between groups at either baseline or follow-up were made using the two sample t-test and comparisons within groups comparing baseline and follow-up were made using the paired t-test. Two-sided p-values are presented. No correction for multiple testing was made since we present a relatively small number of comparisons in the content of a genetic association study.

The prevalence of elevated iron indices and disease features was estimated as the observed proportions at a single time point, and comparisons between groups were made using Pearson's chi-squared test. If cell counts were less than 5 then Fisher's exact test was performed. Some subjects did not participate in all components and as a result did not contribute data to the prevalence calculation for every disease feature.

We examined the influence of co-morbid factors on liver enzymes by conducting separate analyses, excluding participants with a body mass index (BMI) greater than 30 kg/m^2 or alcohol intake greater than 60g/day for men and greater than 40g/day for women when calculating the prevalence of raised AST/ALT. Increased BMI and alcohol intake are common causes of raised iron indices and abnormal serum transaminase levels.

Participants who were diagnosed and treated for HH and those with any SF>1000 µg/L were included in the analysis. Their inclusion avoids a downward bias of the estimated prevalence of disease features at follow-up due to the exclusion of cases with clinical symptoms.

Results

HFE genotyping was successful for 29,676 of 31,192 (95%) subjects of whom 3295 (11.1%) were C282Y simple heterozygotes. There were 337 C282Y simple heterozygotes and 621 participants with no copies of the C282Y mutation who were selected for invitation to the HealthIron study. Of these, 257 C282Y simple heterozygotes and 469 without the C282Y mutation attended the follow-up clinic (response fractions of 76.3% and 75.5% respectively). Subsequent genotyping revealed that of those without the C282Y mutation attending the clinic, 123 (74 women) were simple heterozygotes for H63D and 330 (181 women) were wild-type for *HFE*. The remaining 16 participants (10 women) attending the clinic were homozygous for the H63D mutation and were excluded from further consideration in this study. Close to or more than half of all women in each of the three *HFE* genotype groups

were post-menopausal at baseline (63/139 (45%) C282Y simple heterozygotes, 44/74 (59%) H36D simple heterozygotes and 110/181 (61%) *HFE* wild-types).

For men and women, there was little difference in the median and IQR of age, BMI and daily alcohol intake for C282Y and H63D simple heterozygotes compared to *HFE* wild-types (Table 1). The Mann-Whitney U test identified a statistically significant difference in the median and spread of the distribution of daily alcohol intake for female C282Y simple heterozygotes (median: 4.3 g/day; IQR: 0-16.5) compared to *HFE* wild-types (median: 1.0 g/day; IQR: 0-8.6), and the distribution of BMI for female C282Y simple heterozygotes (median: 24.5 g/day; IQR: 22.1-27.1) compared to *HFE* wild-types (median: 25.3 g/day; IQR: 23.2-28.5), although the median and spread of BMI appeared to be similar for both of the latter genotype groups. These differences were, however, small in magnitude and therefore not clinically relevant. The blood donation history of HFE simple heterozygotes for either C282Y or H63D was comparable to wild-types for men, pre-menopausal women and post-menopausal women (Table 2).

Iron indices

C282Y and H63D simple heterozygotes had, on average, normal serum ferritin and transferrin saturation (TS) levels at baseline irrespective of sex and menopausal status (Table 3). At follow-up, geometric mean SF levels and mean TS levels remained within the normal range and similar to baseline levels for men and post-menopausal women regardless of whether they had the C282Y or H63D substitution in the HFE protein (Table 4). For pre-menopausal women, the onset of menopause increased the geometric mean SF levels of C282Y simple heterozygotes (Baseline: 36.3µg/L (95% CI: 28.3,46.5); Follow-up: 87.1µg/L (95% CI: 69.3,109.5)) and H63D simple heterozygotes (Baseline: 57.34µg/L (95% CI: 36.14,90.97); Follow-up: 80.4µg/L (95% CI: 51.45,125.64)). For both C282Y and H63D

simple heterozygotes, the prevalence of elevated SF or TS was comparable to *HFE* wild-types at both time points and particularly low for women (Table 5).

Two C282Y simple heterozygotes (one man and one woman), two H63D simple heterozygotes (one man and one woman) previously reported having been diagnosed with haemochromatosis, but none reported being treated with therapeutic venesection during the study period. None of them had elevated transferrin saturation and three had BMI greater than 30 kg/m^2, so although all four had elevated SF at either baseline or follow-up, this is likely to be an incidental finding that was incorrectly diagnosed by their primary care physicians as being consistent with hereditary haemochromatosis. Also incidental is the fact that all four gave different reasons for diagnosis (routine blood examination, genetic test, presence of symptoms, and "other/don't know"). Two male C282Y simple heterozygotes and one male *HFE* wild-type had baseline SF>1000 µg/L and baseline TS<55%. At follow-up, one male H63D simple heterozygote had SF>1000 µg/L and TS<55% and one male *HFE* wild-type had SF>1000 µg/L and TS>55%. All had BMI greater than 30 kg/m^2, with the exception of the male H63D simple heterozygote, whose BMI was 25.8 kg/m^2 and they were abstinent from alcohol. We note in passing that *HFE* C282Y homozygotes with SF>1000 µg/L rarely have TS < 55% (Allen et al. (2008), Gurrin et al. (Gastroenterology 2008)) whereas of the five participants in the current study with SF>1000 µg/L only one had TS > 55%, further suggesting that elevated serum ferritin is attributable to co-morbid factors rather than iron overload. *What about ferroportin diseaseIn such individuals if you measure hepatic iron concentration there is no increase above general population levels – for reference see our Clin Gastroenterol Hepatol paper 2009 and our 2013 paper that shows the reference range for ferritin in Australian probably needs a significant upward adjustment JO???*

Prevalence of disease features

The estimated prevalence of the six disease features for each sex and *HFE* genotype group are given in Table 6. After stratifying by sex, the prevalence of disease features of both C282Y and H63D simple heterozygotes was similar to wild-types, with the exception of a lower prevalence of raised AST or ALT for male C282Y simple heterozygotes (5%) compared to wild-types (14%). After individuals who were obese (BMI>30 kg/m^2) or who had high alcohol intake (>60 g/day for men or >40 g/day for women) were excluded, abnormal liver enzymes were significantly less prevalent for male C282Y simple heterozygotes (3/93 (3.2%)) relative to wild-types (15/105 (14%)). For male and female C282Y and H63D simple heterozygotes, the observed prevalence of disease was similar for those with elevated SF and those with normal SF (Table 7). Excluding those participants who were obese or with a heavy alcohol intake did not alter this finding. The small absolute numbers suggest that this is not clinically relevant.

Table 8 presents the prevalence of iron overload-related disease for C282Y and H63D simple heterozygotes. No male and no female C282Y or H63D simple heterozygotes had documented iron overload-related disease. Two male C282Y simple heterozygotes, one female C282Y simple heterozygote pre-menopausal at baseline and one male H63D simple heterozygote fitted the criteria of provisional iron overload (as defined in [5] and at the bottom of Table 6), but had none of the six disease features associated with HH. Two male *HFE* wild-types fitted the criteria of provisional iron overload, only one of whom reported experiencing disease features associated with HH; arthritis medication and fatigue.

Discussion

This study is the first to describe the natural history of serum iron indices, and to determine whether HH-associated features and iron overload-related disease develop in *HFE* C282Y and H63D simple heterozygotes. Iron indices remained stable, and, on average, within their

clinically normal ranges in middle age for C282Y and H63D simple heterozygotes, so these individuals are unlikely to be candidates for therapeutic venesection for iron overload. Menopause resulted in increased mean SF for pre-menopausal female C282Y and H63D simple heterozygotes and wild-type participants, but the iron indices, on average, remained within their normal ranges. No documented iron overload was observed for *HFE* simple heterozygotes for either C282Y or H63D, and iron overload related disease morbidity for both *HFE* simple heterozygote groups was similar to *HFE* wild-types.

We note that the thresholds used to define elevated SF and elevated TS differ between cross-sectional population studies, and some thresholds are sex dependent while others are not [6, 8-10]. Our results on the prevalence of elevated iron indices are in line with those from other studies so it is unlikely that our conclusions are sensitive to the choice of threshold for the clinically normal range of iron indices such as TS and SF. See my earlier comment on our recent study on what should be the appropriate reference range for ferritin
Lyle

In the introduction we outlined the advantages of the longitudinal design over cross-sectional clinical studies. There is only one other prospectively-recruited, population-based study that focussed specifically on hereditary haemochromatosis. Our findings are consistent with the results of that study, but extend their findings by reporting on serial iron indices and clinical examination at follow-up more than a decade after recruitment. Nevertheless, our study does have some weaknesses. The majority of participants were recruited in middle age so our conclusions do not necessarily apply to younger age groups. Clinical examination for haemochromatosis-associated features was not conducted at baseline, so we relied on self-reported information from questionnaires and interviews. Data on magnetic resonance imaging or liver biopsy to quantify hepatic iron content were not part of the protocol for clinical examination at follow-up, and was only performed when clinically indicated by the

participants' physician independently of their involvement in this study. However, in a separate study we have shown that such subjects do not have iron loading above general population levels (Olynyk et al Clin Gastroenterol Hepatol 2009). For participants who had therapeutic venesection or who engaged in voluntary (or altruistic) blood donation, quantitative phlebotomy was self-reported although, where possible, records were linked to the Australian Red Cross Blood Service to establish the validity of the self-reported number of episodes of blood removal [14].

In conclusion, *HFE* simple heterozygotes for either C282Y or H63D are at low risk of developing iron overload in middle age, and rarely exhibit any of the symptoms of HH. We found no evidence that *HFE* simple heterozygotes would benefit from more frequent medical examination than the general population.

Table 1: Participant information at baseline: age, BMI and alcohol intake stratified by genotype and sex.

	n	Age (years) mean (sd) [median;IQR*]	p@	BMI (kg/m^2) mean (sd) [median;IQR]	p@	Alcohol (g/day) mean (sd) [median;IQR]	p@
Male							
C282Y Simple Heterozygote	118	53.7 (9.2) [52.5;45.5-61.5]	0.67	26 (3.2) [25.6;23.8-27.3]	0.17	18.2 (17.8) [13.9;2.5-29.8]	0.76
H63D Simple Heterozygote	49	54.4 (9.2) [52.8;46.9-63.9]	0.91	27.4 (4.1) [26.7;24.6-28.9]	0.32	15.2 (17.4) [12.6;.2-19.6]	0.29
***HFE* Wild-type**	149	54.2 (9.1) [54.2;45.3-61]		26.8 (4.2) [25.9;24.3-28.3]		19.4 (23.3) [13.5;3-24.2]	
Female							
C282Y Simple Heterozygote	139	52.5 (8.8) [50.4;44.6-59.9]	0.29	25.1 (4.1) [24.5;22.1-27.1]	0.01	11 (14.1) [4.3;0-16.5]	<0.01
H63D Simple Heterozygote	74	55.2 (9.9) [56;44.1-64.4]	0.25	25.3 (4) [24.8;22.6-27.2]	0.15	7.6 (11.9) [1.7;0-12.9]	0.90
***HFE* Wild-type**	181	53.7 (9.1) [53.5;45.2-61.7]		26.3 (4.6) [25.3;23.2-28.5]		7.4 (12.9) [1;0-8.6]	

*IQR – inter-quartile range

@Two-sided p-value derived from Mann-Whitney U test comparing C282Y simple heterozygotes to *HFE* wild-types, and H63D simple heterozygotes to *HFE* wild-types.

Table 2: Blood donation history at baseline for simple heterozygotes and *HFE* wild-types by sex and, for women, menopause status.

			Blood donation at baseline			
Men		**n**	**Never**	**Former**	**Current**	**p[#]**
	C282Y Simple Heterozygote	118	51(43%)	44(37%)	23(19%)	0.67
	H63D Simple Heterozygote	49	25(51%)	16(33%)	8(16%)	0.83
	***HFE* Wild-type**	149	71(48%	48(32%)	30(20%)	
Women						
Pre-menopausal	**C282Y Simple Heterozygote**	76	33(43%)	28(37%)	15(20%)	0.78
	H63D Simple Heterozygote	30	12(40%)	6(20%)	12(40%)	0.21
	***HFE* Wild-type**	71	31(44%)	23(32%)	17(24%)	
Post-menopausal	**C282Y Simple Heterozygote**	63	43(68%)	10(16%)	10(16%)	0.41
	H63D Simple Heterozygote	44	27(61%)	12(27%)	5(11%)	0.85
	***HFE* Wild-type**	110	67(61%)	27(25%)	16(15%)	

[#] p-values comparing C282Y simple heterozygotes to *HFE* wild-types, and H63D simple heterozygotes to *HFE* wild-types.

Table 3: Geometric mean serum ferritin (SF) and mean transferrin saturation (TS) values stratified by *HFE* genotype and sex. Baseline SF and TS for women are stratified according to menopausal status. All women had become post-menopausal by follow-up.

Baseline iron indices:						
	n	**Baseline SF (µg/L) [@] geometric mean (95% CI)**	**p[#]**	**n**	**Baseline TS (%) ** mean (95% CI)**	**p[#]**
Men						
C282Y Simple Heterozygote	112	179.11 (151.31,212.02)	0.14	112	35.14 (33.25,37.04)	<0.01
H63D Simple Heterozygote	38	214.01 (168.66,271.53)	0.03	39	33.03 (29.9,36.15)	0.07
***HFE* Wild-type**	133	150.81 (128.59,176.86)		135	29.67 (27.93,31.4)	
Pre-menopausal women						
C282Y Simple Heterozygote	71	37.23 (29.49,46.99)	0.73	72	29.97 (27.16,32.78)	<0.01
H63D Simple Heterozygote	26	53.36 (35.3,80.66)	0.06	27	26.82 (22.49,31.14)	0.05
***HFE* Wild-type**	65	35.16 (27.93,44.26)		65	22.48 (20.27,24.69)	
Post-menopausal women						
C282Y Simple Heterozygote	51	109.18 (86.73,137.45)	0.09	51	29.1 (26.24,31.96)	0.04
H63D Simple Heterozygote	37	100.38 (73.27,137.52)	0.31	37	28.16 (25.58,30.75)	0.16
***HFE* Wild-type**	95	83.01 (68.3,100.9)		95	26.06 (24.51,27.62)	
Follow-up iron indices:						
		Follow-up SF (µg/L) † geometric mean (95% CI)			**Follow-up TS (%) * mean (95% CI)**	
Men						
C282Y Simple Heterozygote	107	159.65 (131.53,193.79)	0.18	107	33.04 (30.7,35.38)	0.01
H63D Simple Heterozygote	46	141.17 (107.22,185.88)	0.76	46	31.61 (28.33,34.89)	0.15
***HFE* Wild-type**	139	134.16 (113.63,158.38)		140	29.14 (27.5,30.78)	
Women‖						
C282Y Simple Heterozygote	125	97.61 (83.71,113.82)	0.07	127	31.13 (29.28,32.98)	<0.01
H63D Simple Heterozygote	66	87.36 (66.81,114.22)	0.54	66	26.83 (24.66,29)	0.14
***HFE* Wild-type**	169	80.08 (69.29,92.55)		169	24.94 (23.62,26.26)	

[@] 46 (19 C282Y simple heterozygotes (10 males, 6 pre-menopausal females and 3 post-menopausal females), 9 H63D simple heterozygotes (1 male, 3 pre-menopausal females and 5 post-menopausal) and 18 *HFE* wild-types (8 males, 3 pre-menopausal females and 7 post-menopausal females)) had SF measures available at baseline but not follow-up.

** 1 pre-menopausal female C282Y simple heterozygote, 1 pre-menopausal female H63D simple heterozygote, 1 male H63D simple heterozygote and 2 male *HFE* wild-types had a baseline TS measure but no baseline SF measure.

† 99 (39 C282Y simple heterozygotes (17 males, 6 pre-menopausal and 16 post-menopausal females) 22 H63D simple heterozygotes (11 males, 4 pre-menopausal females and 7 post-menopausal females) and 38 *HFE* wild-types (17 males, 6 pre-menopausal and 15 post-menopausal females)) had SF measures available at follow-up but not baseline.

* 2 female C282Y simple heterozygotes and 1 male *HFE* wild-type had a follow-up TS measure but no follow-up SF measure.

‖ 3 C282Y simple heterozygotes, 1 H63D simple heterozygote and 3 *HFE* wild-type females were pre-menopausal at follow-up.

p-values comparing C282Y simple heterozygotes to *HFE* wild-types, and H63D simple heterozygotes to *HFE* wild-types.

Table 4: Geometric mean serum ferritin (SF) and mean transferrin saturation (TS) values stratified by *HFE* genotype and sex in participants with both baseline and follow-up iron measures.

Men	**n**	**Baseline SF (µg/L) geometric mean (95% CI)**	**Follow-up SF (µg/L) geometric mean (95% CI)**	**p#**
C282Y Simple Heterozygote	90	180.37 (148.26,219.43)	148.26 (119.2,184.42)	0.02
H63D Simple Heterozygote	35	216.59 (169.17,277.29)	136.32 (95.83,193.91)	<0.01
***HFE* Wild-type**	122	149.75 (126.76,176.92)	134.69 (112.15,161.77)	0.2
Pre-menopausal women†				
C282Y Simple Heterozygote	62	36.27 (28.27,46.54)	87.1 (69.29,109.48)	<0.01
H63D Simple Heterozygote	23	57.34 (36.14,90.97)	80.4 (51.45,125.64)	0.21
***HFE* Wild-type**	62	34.95 (27.69,44.13)	63.31 (48.32,82.94)	<0.01
Post-menopausal women‡				
C282Y Simple Heterozygote	41	106.48 (80.42,140.99)	107.13 (82.76,138.66)	0.95
H63D Simple Heterozygote	32	104.27 (72.77,149.41)	91.1 (59.76,138.89)	0.51
***HFE* Wild-type**	86	81.7 (66.36,100.58)	85.88 (71.44,103.25)	0.63
Men	**n**	**Baseline TS (%) mean (95% CI)**	**Follow-up TS (%) mean (95% CI)**	**p#**
C282Y Simple Heterozygote	90	34.93 (32.75,37.11)	33.69 (31.03,36.35)	0.36
H63D Simple Heterozygote	36	32.75 (29.49,36.01)	31.47 (27.67,35.27)	0.55
***HFE* Wild-type**	125	29.72 (27.94,31.5)	29.29 (27.49,31.08)	0.67
Pre-menopausal women†				
C282Y Simple Heterozygote	63	29.62 (26.91,32.33)	32.78 (29.98,35.57)	0.08
H63D Simple Heterozygote	24	27.25 (22.38,32.12)	28.5 (24.66,32.34)	0.63
***HFE* Wild-type**	62	22.5 (20.23,24.77)	23.35 (21.08,25.63)	0.54
Post-menopausal women‡				
C282Y Simple Heterozygote	41	29.68 (26.25,33.11)	29.29 (26.19,32.4)	0.84
H63D Simple Heterozygote	32	28.47 (25.57,31.37)	25.03 (21.64,28.42)	0.08
***HFE* Wild-type**	86	26.59 (24.97,28.22)	25.06 (23.29,26.83)	0.14

† Women classified as pre-menopausal at baseline.

‡ Women classified as post-menopausal at baseline.

p-values comparing C282Y simple heterozygotes to *HFE* wild-types, and H63D simple heterozygotes to *HFE* wild-types.

Table 5: Prevalence of elevated baseline and follow-up serum ferritin (SF) and elevated baseline transferrin saturation (TS) for C282Y simple heterozygotes, H63D simple heterozygotes and *HFE* wild-types.

		[@]Elevated Baseline SF	p[#]	[@]Elevated Follow-up SF	p#
Men					
	C282Y Simple Heterozygote	33/112(29%)	0.41	33/107(31%)	0.07
	H63D Simple Heterozygote	14/38(37%)	0.14	6/46(13%)	0.24
	***HFE* Wild-type**	33/133(25%)		29/139(21%)	
Women					
	C282Y Simple Heterozygote	7/122(6%)	*0.22	10/125(8%)	0.25
	H63D Simple Heterozygote	7/63(11%)	*0.01	6/66(9%)	0.2
	***HFE* Wild-type**	4/160(3%)		8/169(5%)	
		[@]Elevated Baseline TS		[@]Elevated Follow-up TS	
Men					
	C282Y Simple Heterozygote	3/112(3%)	*1.00	5/107(5%)	*0.24
	H63D Simple Heterozygote	0/39(0%)	*1.00	2/46(4%)	*0.26
	***HFE* Wild-type**	3/135(2%)		2/140(1%)	
Women					
	C282Y Simple Heterozygote	7/123(6%)	*0.02	9/127(7%)	*0.08
	H63D Simple Heterozygote	2/64(3%)	*0.20	1/66(2%)	*1.00
	***HFE* Wild-type**	1/160(1%)		4/169(2%)	

[@] Elevated SF for males and post-menopausal was defined as SF>300 µg/L and for pre-menopausal women was defined as SF>200 µg/L. Elevated TS for males was defined as TS>55% and for females>45% (irrespective of whether they were pre- or post- menopausal).

* Fisher's exact test

p-values comparing C282Y simple heterozygotes to *HFE* wild-types, and H63D simple heterozygotes to *HFE* wild-types.

Table 6: Prevalence of disease features in simple heterozygotes and *HFE* wild-types by sex.

		Men	p#	Women	p#
MCP 2/3[1]	**C282Y Simple Heterozygote**	11/94(12%)	0.43	8/103(8%)	0.38
	H63D Simple Heterozygote	7/41(17%)	0.81	9/55(16%)	0.32
	***HFE* Wild-type**	18/116(16%)		16/144(11%)	
Arthritis medicine[2]	**C282Y Simple Heterozygote**	4/161(2%)	*0.72	10/176(6%)	0.46
	H63D Simple Heterozygote	2/54(4%)	*0.6	9/76(12%)	0.27
	***HFE* Wild-type**	3/164(2%)		15/197(8%)	
Fatigue[3]	**C282Y Simple Heterozygote**	9/115(8%)	0.47	25/139(18%)	0.86
	H63D Simple Heterozygote	7/46(15%)	0.38	11/70(16%)	0.57
	***HFE* Wild-type**	15/144(10%)		33/176(19%)	
Raised AST/ALT[4]	**C282Y Simple Heterozygote**	5/107(5%)	0.02	9/127(7%)	*0.11
	H63D Simple Heterozygote	4/46(9%)	0.38	0/66(0%)	*0.33
	***HFE* Wild-type**	19/139(14%)		5/169(3%)	
Liver disease[5]	**C282Y Simple Heterozygote**	3/116(3%)	*1.00	13/140(9%)	0.23
	H63D Simple Heterozygote	4/46(9%)	*0.1	5/69(7%)	0.65
	***HFE* Wild-type**	4/142(3%)		10/175(6%)	
Hepatomegaly[6]	**C282Y Simple Heterozygote**	3/91(3%)	*0.73	1/98(1%)	*0.64
	H63D Simple Heterozygote	0/41(0%)	*0.32	2/54(4%)	*0.63
	***HFE* Wild-type**	5/113(4%)		3/135(2%)	

* Fisher's exact test

[1]Presence of bony spur, tenderness or effusion of the 2nd and 3rd MCP joints on either hand. Examination conducted by physicians blinded to genotype and HH status.

[2] Self reported answer to the questions "Has a doctor ever told you that you have arthritis or rheumatism?" followed by "If you have arthritis or rheumatism, do you take aspirin?"

[3]Self reported answer to the question "Have you ever sought medical attention because of fatigue?"

[4]Aspartate aminotransferase > 45 IU/L or alanine aminotransferase >40 IU/L

[5] Self reported answer to the question "Has a doctor ever told you that you have liver disease?"

[6] Liver enlargement defined as a liver span of 13cm or more. Examination conducted by physicians blinded to genotype and HH status.

p-values comparing C282Y simple heterozygotes to *HFE* wild-types, and H63D simple heterozygotes to *HFE* wild-types.

Table 7: Prevalence of disease in male simple heterozygotes with elevated iron indices compared with those with non-elevated iron indices.

	Men			Women		
	Elevated SF†	**Normal SF**	***p#**	**Elevated SF†**	**Normal SF**	***p#**
C282Y Simple Heterozygotes						
MCP 2/3	5/37(14%)	4/48(8%)	0.49	1/9(11%)	5/76(7%)	0.5
Arthritis medicine	0/48(0%)	3/56(5%)	0.25	1/12(8%)	6/95(6%)	0.58
Fatigue	6/43(14%)	3/54(6%)	0.18	2/12(17%)	12/94(13%)	0.66
Raised AST/ALT	2/41(5%)	3/56(5%)	1.00	2/11(18%)	5/95(5%)	0.15
Liver disease	1/43(2%)	0/55(0%)	0.44	1/12(8%)	12/94(13%)	1.00
Hepatomegaly	0/34(0%)	3/48(6%)	0.26	0/8(0%)	0/74(0%)	-
H63D Simple Heterozygotes						
MCP 2/3	2/13(15%)	4/20(20%)	1.00	2/6(33%)	5/39(13%)	0.23
Arthritis medicine	2/15(13%)	./22(.%)	0.16	2/10(20%)	5/46(11%)	0.6
Fatigue	1/14(7%)	4/22(18%)	0.63	1/10(10%)	6/44(14%)	1.00
Raised AST/ALT	1/14(7%)	2/22(9%)	1.00	0/10(0%)	0/46(0%)	-
Liver disease	2/14(14%)	1/22(5%)	0.55	0/10(0%)	3/43(7%)	1.00
Hepatomegaly	0/13(.%)	0/20(.%)	-	0/6(0%)	1/38(3%)	1.00

† 6 male C282Y simple heterozygotes had elevated SF and elevated TS at either baseline or follow-up.

* Fisher's exact test

p-values comparing C282Y simple heterozygotes to *HFE* wild-types, and H63D simple heterozygotes to *HFE* wild-types.

Table 8: Prevalence of iron-overload-related disease in *HFE* simple heterozygotes.

	Simple heterozygotes					
	Male			**Female**		
	HH-associated disease	**No HH-associated disease**	**Total**	**HH-associated disease**	**No HH-associated disease**	**Total**
Documented iron overload[1]	0	0	0	0	0	0
Provisional iron overload[2]	0	2	2	0	1	1
No iron overload[3]	16	99	115	16	117	133
Total	16	101	117	16	118	134
	H63D heterozygotes					
	Male			**Female**		
Documented iron overload[1]	0	0	0	0	0	0
Provisional iron overload[2]	0	1	1	0	0	0
No iron overload[3]	9	37	46	9	65	74
Total	9	38	47	9	65	74

Iron overload is categorized as one of the following
1. Documented iron overload: Increased iron content shown by hepatic iron staining 3 or 4, iron concentration >90 µmol/g, or HII >1.9 (Whitlock14) or SF >1000 µg/L at baseline with documented therapeutic venesection.
2. Provisional iron overload: Raised SF (>300 µg/L for males and post-menopausal women, >200 µg/L pre-menopausal women) in association with raised TS (>55% males, >45% females).
3. No evidence of iron overload: Normal SF or elevated SF but in the context of normal TS during study period.

Iron overload-related disease is defined as occurrence of at least one of the following five conditions in the context of documented iron overload as defined above:
1. Hepatocellular carcinoma.
2. Cirrhosis or fibrosis on percutaneous liver biopsy.
3. Bony, tenderness or effusion of both of the second and third metacarpophalangeal joints on examination by study physician blinded to genotype.
4. Raised serum aspartate aminotransferase (AST >45 IU/L) or serum alanine, aminotransferase (ALT >40 IU/L).
5. Physician diagnosis due to presentation with HH-associated symptoms.

Documented iron overload-related disease was considered present if participants had BOTH documented iron overload AND evidence of iron overload-related disease.

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