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HFE Gene Mutations Increase the Risk of Coronary Heart Disease in Women

Running head: *HFE* mutations and CHD risk

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Abstract

The purpose of the present study is to examine *HFE* gene mutations in relation to newly diagnosed (incident) coronary heart disease (CHD). In a population-based follow-up study of 7,983 individuals aged 55 years and older, we compared the risk of incident CHD between *HFE* carriers and non-carriers, overall and stratified by sex and smoking status. *HFE* mutations were significantly associated with an increased risk of incident CHD in women but not in men (hazard ratio [HR] for women = 1.7, 95% confidence interval [CI] 1.2 to 2.4 versus HR for men = 0.9, 95% CI 0.7 to 1.2). This increased CHD risk associated with *HFE* mutations in women was statistically significant in never smokers (HR = 1.8, 95% CI 1.1 to 2.8) and current smokers (HR = 3.1, 95% CI 1.4 to 7.1), but not in former smokers (HR = 1.3, 95% CI 0.7 to 2.4). *HFE* mutations are associated with increased risk of incident CHD in women.

Introduction

Hereditary hemochromatosis is a genetic disorder characterized by iron overload [1]. Iron overload in patients with hereditary hemochromatosis results in iron depots in the pancreas, liver, joints and heart. In the heart, such depots have been associated with coronary heart disease (CHD) and shortened life expectancy [2]. The most common cause of hereditary hemochromatosis are two common mutations in the *HFE* gene, C282Y and H63D [3,4]. Over 80% of the patients with hereditary hemochromatosis are homozygous for the C282Y mutation, about 1% are homozygous for the H63D mutation and about 7% are compound heterozygous [1]. In populations of European origin, an estimated 0.4% of the population is homozygous and 9% is heterozygous carrier of the C282Y mutation and 13% is homozygous and 2% heterozygous for the H63D mutation [1].

Complications of iron overload may not only occur to homozygous carriers of the risk alleles. Compound heterozygotes and heterozygotes for the C282Y and H63D mutations also have a subtle increases in serum iron, serum ferritin and transferrin saturation [5,6,7,8]. These slight changes suggest that heterozygotes are more likely to have a slow accumulation of iron, which may lead to pathology later in life**. Iron deposits in the arterial wall trigger the low density lipoprotein cholesterol peroxidation and therefore contributes the formation of atherosclerotic lesions and to the inflammation leading to cardiovascular disease.[1] [2] Some studies also showed that C282Y carriers had an increased risk of myocardial infarction,[3] and coronary heart disease (CHD).[4] Similarly, smoking has also been associated to cardiovascular disease through increased inflammation, thrombosis, and oxidation of low-density lipoprotein cholesterol. [6] Therefore, to properly disentangle the association between *HFE* mutations and cardiovascular disease requires taking into account the effect of smoking. Following this, previous work of our group also showed an increased risk of stroke among *HFE* carriers who smoked.[5]** The aim of the present

study was to examine the effect of the two common *HFE* C282Y and H63D mutations on incident CHD **accounting for the effect of smoking.** Since women are protected early in life from iron-related pathology by menstruation, we examined men and women separately.

Methods

Study population

The present analysis was performed within the Rotterdam Study, an ongoing population-based study on the determinants of disease and disability in 7,983 subjects aged 55 years and older. Design, rationale and details of the study have been described previously [24]. The present analyses used baseline data (collected between 1990 and 1993) and follow-up morbidity and mortality data (collected until December 2001; mean follow-up of 8.3 years, standard deviation (SD) 2.7 years). The Medical Ethics Committee of the Erasmus MC University Medical Center approved the study protocol and all participants provided written informed consent.

Data collection

Baseline data collection

During home visits, a trained interviewer obtained information on health status, medical history, medication use and smoking status at baseline. Subsequently, participants were invited to the study center where they underwent an extensive clinical examination in which height, weight, systolic and diastolic blood pressures were measured. Body mass index (BMI) was computed as weight (kg) divided by height squared (m^2). Systolic and diastolic blood pressures were measured twice in sitting position, after 5 minutes rest, using a random-zero sphygmomanometer. The mean of the two measurements was used for the analysis. Hypertension was defined as systolic blood pressure higher than 160 mm Hg, diastolic blood pressure higher than 100 mm Hg or the use of medication indicated to treat high blood pressure (hypertension grades 2 and 3) [25]. Serum glucose, total cholesterol, high-density lipoproteins (HDL) cholesterol and C-reactive protein levels, as a marker of inflammation, were determined using an automated enzymatic procedure [26,27]. Diabetes was diagnosed

based on the use of medication, and/or a random or post-load glucose levels higher than 11.1 mmol/L [28]. Iron and ferritin levels and transferrin saturation were determined in subgroup as previously described [6].

Follow-up assessment

During follow-up, information on fatal and non-fatal cardiovascular endpoints was obtained from the general practitioners and hospital records. Two research physicians and a cardiologist independently reviewed all information and classified all the events according to the International Classification of Diseases, 10th edition (ICD-10) [29]. Incident CHD was defined as the occurrence of non-fatal myocardial infarction (ICD-10 code I21), revascularization procedure (percutaneous transluminal coronary angioplasty or coronary artery bypass graft) and cardiac death. Cardiac death was defined as death caused by myocardial infarction or other ischemic heart disease (ICD-10 codes I20 - I25), sudden cardiac death (ICD-10 code I46), cardiac arrhythmias (ICD-10 code I49), sudden death undefined (ICD-10 code R96) or death from heart failure (ICD-10 code I50).

Genotyping of HFE mutations

C282Y and H63D mutations were genotyped in a random sample of 3,798 individuals as described in details previously [22]. Baseline characteristics did not differ between randomly selected and non-selected participants.

Statistical analysis

From the 3,798 individuals who had C282Y and H63D genotype available, 3,435 had complete information on cardiovascular risk factors at baseline and were included in the analyses. There were only 10 homozygotes for the C282Y and 80 homozygotes for the H63D

mutation among which 2 and 11 cases of incident CHD respectively. Previous studies on the *HFE* mutations and iron parameters showed that serum iron, serum ferritin and transferrin saturation were similar for C282Y and H63D heterozygotes [5,6]. Therefore, we pooled carriers of C282Y or H63D mutations as *HFE* carriers. Differences in baseline characteristics between the *HFE* carriers and non-carriers were tested using chi-squared statistics (for categorical variables) or t-tests (for continuous variables). Variables that were not normally distributed, are presented as median with interquartile range and analyzed using the Mann-Whitney-U test. CHD incidence rates were calculated as number of events per 1,000 person-years. Risks of incident CHD were quantified as hazard ratios (HRs) from Cox proportional hazards models using age as time scale [30]. Survival time was calculated from age at baseline to age at event. HRs were calculated crude and adjusted for sex, smoking status, hypertension, BMI, total serum cholesterol, HDL cholesterol, diabetes mellitus and CHD at baseline. Further, HRs were additionally adjusted for C reactive protein, to evaluate the role of inflammation in the relationship between *HFE* mutations and incident CHD. The association between *HFE* mutations and the risk of incident CHD was investigated overall and in sex and smoking subgroups given that both are associated with CHD and the clinical manifestations of iron overload in our study [22]. Proportionality of all models was tested by Schoenfeld residuals [31]. P-values lower then 0.05 were considered statistically significant.

Results

Genotypes and allele frequencies were in Hardy-Weinberg equilibrium (C282Y: CC 88.1%, CY 11.6%, YY 0.3%, p = 0.41; H63D: HH 73.2%, HD 24.5%, DD 2.4%, p = 0.25). At baseline, women had lower prevalence of smoking, hypertension and myocardial infarction and a higher mean BMI compared to men. Baseline characteristics by genotype are presented in Table 1. Carriers and non-carriers of the *HFE* mutations did not significantly differ in risk

factors for cardiovascular disease with three exceptions in women. In women, *HFE* carriers had higher median body mass index (27.0 versus 26.6 kg/m^2; p=0.05), higher median C-reactive protein levels than non-carriers (2.0 versus 1.7 mg/dL; p = 0.05) and a lower frequency of diabetes mellitus (10% versus 13%; p=0.05). *HFE* carriers had higher levels of iron and ferritin and higher transferrin saturation than non-carriers (Table 2). This difference was statistically significant for both iron levels and transferrin saturation in men and women. A similar trend was observed within smoking strata, although the differences were not statistically significant (Data not shown). Figure 1 shows that females *HFE* carriers tend to have higher C-reactive protein levels than non-carriers, particularly among current or former smokers (p=0.08). In men, no differences in C-reactive protein levels between *HFE* carriers and non-carriers were observed.

During follow-up, 483 participants developed incident CHD. Men had higher CHD incidence than women (25.3 versus 11.8 cases/1,000 person years, p <0.001; Table 3). *HFE* mutations were associated with an increased risk of incident CHD in women **(HR$_{crude}$ [95% CI] = 1.3 [1.0 to 1.8]), but not in men (HR$_{crude}$ [95% CI] = 0.9 [0.7 to 1.2]). Further adjustment by co-variates including smoking status lead to an increase in the risk of CHD in women (HR$_{adjusted}$ [95% CI] = 1.7 [1.2 to 2.4]), but not in men (HR$_{adjusted}$ [95% CI] = 0.9 [0.7 to 1.2]). Additionally to HFE status, history of myocardial infarction, diabetes mellitus at baseline and smoking were important risk factors for CHD in women. In men, serum cholesterol level and high-density lipoprotein level, diabetes mellitus and history of myocardial infarction were key risk factors for CHD.** This increased CHD risk associated with *HFE* mutations in women was statistically significant in current smokers (HR = 3.1, 95% CI 1.4 to 7.1) and never smokers (HR = 1.8, 95% CI 1.1 to 2.8), but not in former smokers (HR = 1.3, 95% CI 0.7 to 2.4; Table 4). Exclusion of the homozygous and compound heterozygous carriers did not change the results (Data not

shown). **The interaction term HFE status * smoking status was not statistically significant.**

Discussion

We have found that the *HFE* mutations are associated with higher risk of incident CHD in women. C-reactive protein, a strong predictor of CHD risk was not associated with *HFE* mutations in women with current or former smoking history. Our study was embedded in a large follow-up study of Caucasian individuals aged 55 and older. This long follow-up allows us to study outcomes that are the result of lifelong exposures and express at older ages like cardiovascular disease associated with iron overload.

A previous report based on a smaller sample of the Rotterdam study (n = 342) showed that *HFE* genotypes, also those who were heterozygous, were associated with higher levels of transferrin saturation and serum iron levels, albeit that levels in those homozygous for the mutations are exponentially higher [6]. Previous work of our group also showed an increased risk of stroke among *HFE* carriers who smoked [22]. Altogether, these results are in line with the mechanism linking *HFE* mutations and smoking to cardio- and cerebrovascular disease through damage to vessel wall and inflammation [22]. The underlying mechanism linking *HFE* and CHD is thought to be through iron-related oxidative stress and the subsequent damage to vessel wall and inflammation [22,32]. Such mechanism is also supported by endothelial dysfunction observed in normal subjects after receiving intravenous iron sucrose reported recently [33], as well as the positive association between markers of oxidative stress and iron therapy [34]. Despite the solid grounds for this association, our data showed that CRP levels were not an intermediate factor in this association. A previous study on the association of chronic inflammation and hemochromatosis phenotype revealed that CRP levels did not change between homozygotes for the C282Y mutations with high or low iron stores [35]. Most likely, blood CRP level is a biomarker for generalized inflammation not specific to the association *HFE* and CHD. How and through which early intermediate inflammatory pathways *HFE* is related to inflammation, remains to be determined.

Another issue to be explained is the fact we only found significant evidence for association of *HFE* and CHD in women and not in men. While there is an ongoing discussion of the validity of subgroup testing by sex in genetic studies [36], there are reasons to separate men and women for *HFE*. First, phlebotomy is the key strategy for prevention of hemochromatosis and women are therefore naturally protected due to their menstruation [37]. While this is a common explanation for sex-differences in CHD risk associated with *HFE* mutations, in our study an increased risk was found in female carriers rather than in male carriers. This may be related to the study design. We studied elderly people and therefore male carriers may have been selected out from the population due to early mortality related to *HFE*. In women, the gene effect is likely to express clinically later as they are protected by menstruation up to menopause. **Second, it also should be noted that the major risks factors for CHD are smoking, body mass index, diabetes and lipid profile as supported by the effect of these co-variates when adjusting HR for CHD in women and men.** The extent of CHD risk conferred by these factors is of such magnitude that the genetic predisposition associated with *HFE* carrier status is rapidly outweighed as seen in Table 4 where it is shown that greater CHD risk is associated with smoking status rather than with *HFE* mutations. Given the higher prevalence of smoking (current and former) among men compared to women at baseline, it is likely that the modest effect of *HFE* mutations on the risk of CHD is only apparent among women.

Because of their role in iron overload and their impact on CHD risk, *HFE* mutations have been of interest in public health [7,38,39,40,41,42,43], For long, they have been viewed as a model for genetic testing. A genetic-based strategy for prevention of iron overload and its complications has been investigated after the identification of the two common mutations in the *HFE* gene [44]. The small effect size, the lack of consistency in the association between *HFE* mutations and cardiovascular disease and mortality [11,19,20,21], and the

possible effect modification by environmental factors such as smoking [11,17,19,20,21,22] implies that the benefit of such screening program varies between different subgroups, imposing further difficulties to the use of genetic testing for population-based screening. The results of our study confirm these previous findings and do not support genetic screening on *HFE* mutations.

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Table 1. **Baseline characteristics by sex and *HFE* carrier status**

		Women					Men				
		HFE-carriers (n=656)		Non-carriers (n=1,174)		p-Value	*HFE*-carriers (n=617)		Non-carriers (n=988)		p-Value
Age at entry (years)		69.3	(9.0)	69.6	(9.2)	0.46	67.7	(7.8)	68.0	(7.6)	0.39
Smoking status	Current smokers	105	(16%)	242	(21%)		193	(31%)	288	(29%)	
	Former smokers	187	(29%)	333	(28%)	0.43	367	(59%)	638	(65%)	0.46
	Never smokers	364	(55%)	599	(51%)		57	(9%)	62	(6%)	
Systolic blood pressure (mm Hg)		139.9	(21.7)	139.3	(23.2)	0.62	138.4	(21.3)	138.5	(22.5)	0.95
Diastolic blood pressure (mm Hg)		73.6	(11.5)	73.1	(11.6)	0.32	75.1	(11.8)	74.1	(11.7)	0.12
Hypertension		405	(62%)	735	(63%)	0.61	416	(68%)	675	(68%)	0.73
Body mass index (kg/m^2)		27.0	(4.3)	26.6	(3.9)	0.05	25.8	(3.0)	25.7	(3.0)	0.60
Diabetes mellitus		66	(10%)	154	(13%)	0.05	80	(13%)	111	(11%)	0.30
Total serum cholesterol (mmol/L)		6.83	(1.2)	6.85	(1.2)	0.72	6.28	(1.3)	6.35	(1.1)	0.26
HDL cholesterol (mmol/L)		1.44	(0.4)	1.42	(0.4)	0.41	1.19	(0.3)	1.22	(0.3)	0.07
C-reactive protein (mg/L)†		2.0	(0.9-3.7)	1.7	(0.9-3.3)	0.05	1.9	(0.9-3.9)	1.9	(0.9-3.8)	0.72
Myocardial infarction at baseline		66	(11%)	152	(13%)	0.18	147	(25%)	232	(24%)	0.74

Values are means (standard deviations) for continuous variables and numbers (percentages) for categorical variables. P-Values were obtained by t-test for continuous variables, $\chi 2$ for categorical variables, and Mann-Whitney U test for C-reactive protein. † Median (interquartile range).
HDL = High density lipoprotein

Table 2. **Iron parameters by sex and *HFE* carrier status**

	Women					Men				
	HFE-carriers (n=37)		Non-carriers (n=130)		p-Value	*HFE*-carriers (n=35)		Non-carriers (n=124)		p-Value
Iron (umol/L)	18.0	(14.0 – 21.0)	14.0	(13.0 - 18.5)	0.02	20.0	(15.3 – 23.0)	16.0	(13.0 – 20.0)	<0.01
Ferritin (ug/L)	141.5	(79.5 - 218.0)	109.0	(76.5 - 223.0)	0.39	194.0	(107.0 - 358.0)	187.0	(86.0 - 275.0)	0.22
Transferrin saturation (%)	29.9	(23.6 - 35.5)	23.4	(20.1 - 29.1)	<0.01	33.7	(25.4 - 41.8)	27.2	(22.0 - 31.6)	<0.01

Values are medians (interquartile range). P-Values were obtained by Mann-Whitney U test.

Table 3. **Incidence rates and hazard ratios for incident coronary heart disease by sex and *HFE* carrier status**

		CHD	Follow-up (py)	Incidence rate (CHD/1,000 py)	Hazard ratio (95% CI) Crude	Hazard ratio (95% CI) Adjusted*
Women	All	177	15,020	11.8 (10.2 to 13.7)		
	HFE-carriers	73	5,370	13.6 (**10.8** to 17.1)	1.3 (1.0 to 1.8)	1.7 (1.2 to 2.4)
	Non-carriers	104	9,650	10.8 (**8.9** to 13.1)		
Men	All	306	12,093	25.3 (22.6 to 28.3)		
	HFE-carriers	116	4,735	24.5 (**20.4** to 29.4)	0.9 (0.7 to 1.2)	0.9 (0.7 to 1.2)
	Non-carriers	190	7,358	25.8 (**22.4** to 29.8)		

* **Adjusted for body mass index, hypertension, diabetes mellitus, smoking status at baseline, total and high density lipoprotein cholesterol, C - reactive protein and myocardial infarction at baseline. CHD: Coronary heart disease, py: person-years, CI: confidence interval.**

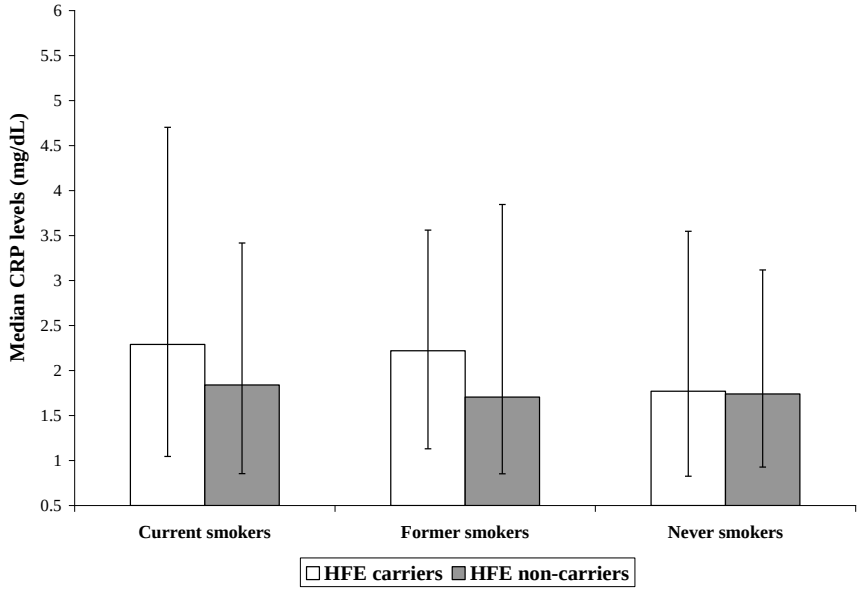
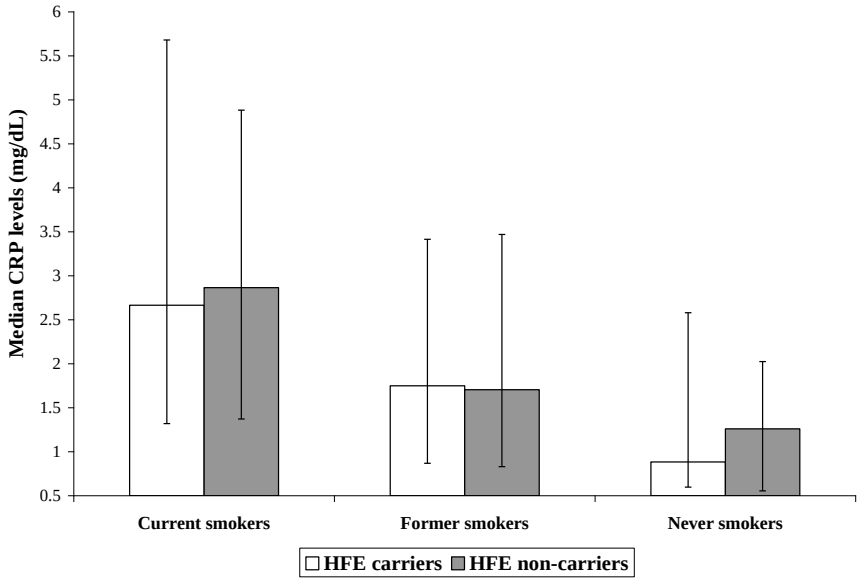
Table 4. **Incidence rates and hazard ratios for incident coronary heart disease by sex, smoking status and *HFE* carrier status**

		CHD	Follow-up (py)	Incidence rate (CHD/1000 py)	Hazard ratio (95% CI) Crude	Adjusted*	Adjusted*
Women							
Never smokers	Non-carriers	50	4.817	10.4 (7.9 to 13.7)	reference	reference	reference
	HFE-carriers	37	2.938	12.6 (9.1 to 17.4)	1.3 (0.9 to 2.0)	1.8 (1.1 to 2.8)	1.8 (1.1 to 2.8)
Former smokers	Non-carriers	33	2.783	11.9 (8.4 to 16.7)	1.6 (1.0 to 2.5)	1.9 (1.1 to 3.1)	reference
	HFE-carriers	20	1.584	12.6 (8.1 to 19.6)	1.7 (1.0 to 3.0)	2.2 (1.2 to 4.0)	1.3 (0.7 to 2.4)
Current smokers	Non-carriers	21	2.05	10.2 (6.7 to 15.7)	1.6 (1.0 to 2.8)	1.4 (0.8 to 2.7)	reference
	HFE-carriers	16	848	18.9 (11.6 to 30.8)	3.1 (1.7 to 5.6)	3.9 (2.1 to 7.3)	3.1 (1.4 to 7.1)
Men							
Never smokers	Non-carriers	9	477	18.9 (9.8 to 36.3)	reference	reference	reference
	HFE-carriers	8	440	18.2 (9.1 to 36.3)	0.9 (0.3 to 2.3)	0.6 (0.2 to 1.8)	0.6 (0.2 to 1.8)
Former smokers	Non-carriers	132	4.801	27.5 (23.2 to 32.6)	1.4 (0.7 to 2.8)	1.2 (0.6 to 2.5)	reference
	HFE-carriers	67	2.902	23.1 (18.2 to 29.3)	1.2 (0.6 to 2.4)	1.0 (0.5 to 2.0)	0.8 (0.6 to 1.1)
Current smokers	Non-carriers	49	2.081	23.5 (17.8 to 31.2)	1.2 (0.6 to 2.5)	1.1 (0.5 to 2.2)	reference
	HFE-carriers	41	1.392	29.4 (21.7 to 40.0)	1.5 (0.7 to 3.2)	1.3 (0.6 to 2.8)	1.2 (0.8 to 1.9)

* Adjusted for body mass index, hypertension, diabetes mellitus, total and high density lipoprotein cholesterol, C-reactive protein and myocardial infarction at baseline. CHD: Coronary heart disease, py: person-years, CI: confidence interval.

Figure 1. **Median C-reactive protein (CRP) levels and interquartile range by *HFE* genotype and smoking status for men and women**

A. Women



B. Men