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Heterozygosity for a Hereditary Hemochromatosis Gene Is Associated With Cardiovascular Death in Women

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Background—The genetic background of hereditary hemochromatosis (*HH*) is homozygosity for a cysteine-to-tyrosine transition at position 282 in the *HFE* gene. Heterozygosity for *HH* is associated with moderately increased iron levels and could be a risk factor for cardiovascular death.

Methods and Results—We studied the relation between *HH* heterozygosity and cardiovascular death in a cohort study among 12 239 women 51 to 69 years of age residing in Utrecht, the Netherlands. Women were followed for 16 to 18 years (182 976 follow-up years). The allele prevalence of the *HH* gene in the reference group was 4.0 (95% CI 2.9 to 5.4). The mortality rate ratios for *HH* heterozygotes compared with wild types was 1.5 (95% CI 0.9 to 2.5) for myocardial infarction (n=242), 2.4 (95% CI 1.3 to 3.5) for cerebrovascular disease (n=118), and 1.6 (95% CI 1.1 to 2.4) for total cardiovascular disease (n=530). The population-attributable risks of *HH* heterozygosity for myocardial infarction and cerebrovascular and total cardiovascular death were 3.3%, 8.8%, and 4.0%, respectively. In addition, we found evidence for effect modification by hypertension and smoking.

Conclusions—We found important evidence that inherited variation in iron metabolism is involved in cardiovascular death in postmenopausal women, especially in women already carrying classic risk factors. **(*Circulation*. 1999;100:1268-1273.)**

Key Words: atherosclerosis ■ genes ■ genetics ■ cardiovascular diseases ■ cerebrovascular disorders

Atherosclerosis is a multifactorial disease in which iron may play a role by promoting oxygen radical formation and lipid peroxidation.[1,2] The evidence from epidemiological studies linking body iron status to development of cardiovascular disease is inconclusive. Some studies have found an association between high levels of serum ferritin,[3] serum iron,[4] and total iron-binding capacity[5] with cardiovascular disease, whereas others have not found an association.[6–9]

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Studies of levels of serum ferritin and serum iron and total iron-binding capacity in relation to cardiovascular disease have limitations as estimates of body iron load because they are influenced by short-term effects such as inflammation, iron intake, blood loss, and diurnal variation.[9,10] Recently, a G-to-A transition was found in a nonclassic *MHC* class I gene, called *HFE* gene (also referred to as *HLA-H* gene), resulting in a cysteine-to-tyrosine substitution at amino acid 282 (HFE Cys282Tyr).[11] Because homozygosity for HFE Cys282Tyr is the major cause of hereditary hemochromatosis *(HH)*, we will refer to this mutation as *HH* polymorphism. *HH* is characterized by increased iron release from intestinal mucosal cells, resulting in iron deposition in the liver and several other organs. Biochemical parameters of *HH* are increased levels of serum ferritin and serum iron and increased iron saturation of serum transferrin. Heterozygotes do not express clinical signs of *HH* unless combined with disorders such as porphyria cutanea tarda[12,13] or hereditary spherocytosis.[14] Still, heterozygotes have slightly but significantly increased levels of serum ferritin and serum iron, whereas total iron-binding capacity is reduced.[15] Heterozygosity for the *HH* polymorphism may therefore be a common genetic marker of lifelong moderate iron overload. This marker may be used to study the relation of iron overload and cardiovascular death.

We examined prospectively the association between *HH* heterozygosity and cardiovascular death among 12 239 women initially 51 to 69 years of age.

Methods

Population

Between December 1974 and October 1980, all 20 555 women, born between 1911 and 1925 who lived in the city of Utrecht, The Netherlands, were invited for an experimental program for breast

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cancer screening, (the so-called DOM project[16]). The women were invited for repeat examination at 1- to 6-year intervals. The baseline population of our study consisted of 12 239 (60%) who visited the second examination (1976 to 1978) because the first examination did not include a questionnaire on smoking. All women gave oral consent to use their data and urine for future scientific research. The study was approved by the medical ethics committee of the University Hospital Utrecht, the Netherlands.

Risk Factors

At baseline, questionnaires on cardiovascular risk factors, including medication use, prescribed diets, previous or present cardiovascular disease, and smoking were completed, and blood pressure, height (m), and weight (kg) were measured. Women were classified as having diabetes mellitus if they reported use of insulin or oral hypoglycemic drugs or were following a diabetes diet. Women were defined as smokers if they reported that they were current smokers. Body mass index (kg/m^2) was calculated as weight (kg) divided by height squared (m^2). Obesity was defined as body mass index $\geq$30 kg/m^2. Hypertension was defined as systolic blood pressure $>$160 mm Hg and/or diastolic blood pressure $>$95 mm Hg.

End Points

Municipal registries informed the Department of Epidemiology (presently called the Julius Center for Patient Oriented Research) about migration and death of cohort members. Cause of death was inquired from the general practitioners. The 9062 surviving women had a median follow-up time of 17 years, with a maximum of 18 years. One thousand four hundred sixty-three (12.0%) women had moved outside the recruitment area and had a median follow-up of 10 years, with a maximum of 18 years. During follow-up (182 976 women-years), 1714 women died: 608 of cardiovascular diseases (codes 390 to 459 of the International Classification of Diseases, Ninth Revision; ICD), 601 of neoplasms (ICD 140 to 239), 299 of other causes, and 206 of unknown causes.

Design

Full cohort analysis on the effects of DNA polymorphisms on cardiovascular death is both expensive and labor intensive. We therefore quantified the effect of *HH* heterozygosity on cardiovascular death by using a nested case-referent approach, which is an alternative name for a nested case-control approach.[17] The cases were 608 women who died of cardiovascular disease and the reference group was composed of a random sample of 618 of 11 631 women who did not die of cardiovascular disease (sampling fraction 1: 18.8). Urine samples of 77 cardiovascular death cases and of 63 women of the reference group were not collected at baseline or were not suitable for DNA analyses, and these women were therefore excluded from the study. The final study group comprised 531 cardiovascular death cases and 555 women of the reference group.

DNA Isolation and Polymerase Chain Reaction

DNA was isolated from 50-mL urine samples with the use of a DNA isolation kit (Puregene, Gentra Systems). DNA was stored in 40 mL of 10 mmol/L TRIS, 1 mmol/L EDTA (TE), pH 7.6. A 268-bp fragment of the *HFE* gene, which contained nucleotide 845, was amplified in 20 mmol/L TRIS/HCl, pH 8.0, 2.5 mmol/L $MgCl_2$, 50 mmol/L KCl, 0.1 mg/mL BSA, 0.4 pmol of 5′-primer (5′-CCTCCTTTGGTGAAGGTGACA-3′) and 0.4 pmol 3′-primer (5′-CACAATGAGGGGCTGATCCA-3′), 0.42 mmol/L of each nucleotide (Pharmacia, Biotech), 0.075 U of super-TAQ polymerase (HT Biotechnology Ltd), and 5 mL of DNA, with the use of an MJ Research PTC200 multicycler. Temperature cycles were 4 minutes at 94°C, 33 cycles of 40 seconds at 94°C, 40 seconds at 55°C, and 2 minutes at 72°C. The reaction was terminated with 10 minutes of incubation at 72°C.

Detection

The amplified 268-bp fragment of each polymerase chain reaction was dotted on 2 separate blot membranes (Hybond N+ nylon transfer membranes, Amersham), following the manufacturer's protocol. Antigen-specific oligonucleotides (ASO) were labeled with γ-^{32}P-ATP (Amersham) with the use of a T4-polynucleotide kinase kit (New England Biolabs). One set of dot blots was hybridized with γ^{32}P-labeled ASO for mutated DNA (MUTHLA; 3′-GATATACGTGCCAGGTGGA-5′) and the set of dot blots were hybridized with γ^{32}P-ATP–labeled ASO for wild-type DNA (OLHLA: 3′-GATATACGTACCAGGTGGA-5′). Dot blots were washed twice for 30 minutes in 2× SSC, 0.1% SDS at room temperature. A specific binding of MUTHLA to wild-type polymerase chain reaction products was removed by washing the blots for 30 minutes at 51°C in 2× SSC, 0.1% SDS; a specific binding of OLHLA to mutated DNA was removed at 55°C. Dots were visualized on x-ray films (DuPont) after overnight radiation. Mutation analyses were performed with samples blinded for case or reference group status.

Data Analysis

Means and proportions of baseline cardiovascular risk factors and presence of *HH* genotype were computed for wild types, heterozygotes, and homozygotes. Difference in means was tested by ANOVA, whereas differences in proportions were tested by χ^2 statistics. Allele frequencies were calculated by the law of Hardy-Weinberg.[18,19] The χ^2 goodness-of-fit test was used to determine whether the observed number of genotypes were in equilibrium.

The nested case-referent approach enabled us to study mortality rates and rate ratios (RR) of cardiovascular disease for *HH* heterozygosity compared with wild types.[20] Because our reference group was a random sample of the total cohort of noncases, multiplication of the person-years in the reference group with 18.8 (the inverse of the sampling fraction) enabled us to analyze the nested case-referent approach exactly as a full cohort analysis, in which the person-years are unbiased estimates of the true person-years. Poisson regression was used to estimate mortality rates and risk ratios; 95% CIs were calculated with Huber's method.[21] Similarly, crude relative risks, mortality rates, and RRs were estimated for women with myocardial infarction (ICD 410 to 414), cerebrovascular disease (ICD 430 to 438), and other cardiovascular disease (all remaining ICD codes between 390 to 459), separately.

Potential confounding by age, smoking, obesity, and hypertension was analyzed in a multivariate model. Effect modification of smoking was investigated by comparing the mortality rates in 4 subgroups: nonsmokers and non–*HH* carriers (reference group), smokers and non–*HH* carriers, nonsmokers and *HH* carriers, and smokers and *HH* carriers. Similarly, effect modification for age (above or below the median), obesity (yes/no), and hypertension (yes/no) was studied. Effect modification by smoking and hypertension was also studied in one model. Cardiovascular mortality rates were estimated for *HH* carriers and non-*HH* carriers classified in subgroups of nonsmokers and nonhypertensives, either smokers or hypertensives, and both smokers and hypertensives.

The proportion of all cases occurring in our population that is attributable to *HH* heterozygosity is expressed as population-attributable risk.[22]

Homozygous subjects were analyzed as separate groups; however, the number of homozygous subjects was too low to draw any conclusions.

Results

Baseline Statistics

As expected, the mean age, the percentages of women with hypertension, history of cardiovascular disease, or diabetes mellitus, and women who were current smokers and obese did not differ between *HH* heterozygotes and women who were wild type (Table 1). The number of homozygotes (n=4) was too small for meaningful statistical analysis.

TABLE 1. Population Characteristics Among HH Genotypes

Characteristics	Wild-type Cys282	Heterozygotes Cys282/Tyr282	Homozygotes Tyr282
n, total	986	96	4
Cardiovascular cases (% of total)	474 (89.3)	56 (10.5)	1 (0.1)
Random sample of noncases (% of total)	512 (92.3)	40 (7.2)	3 (0.5)
Mean age at entry (SD), y	58.2 (4.3)	58.8 (4.0)	62.3 (4.3)
Hypertension, n	669 (67.8%)	63 (65.6%)	3 (75%)
History of cardiovascular diseases, n			
Myocardial infarction	30 (3.0%)	2 (2.1%)	0
Stroke	21 (2.1%)	4 (4.2%)	0
Venous thrombosis	25 (2.5%)	3 (3.1%)	0
Diabetes mellitus, n	74 (7.5%)	8 (8.3%)	0
Smokers, n	324 (32.9%)	28 (29.2%)	2 (50%)
Obesity, n	172 (17.4%)	22 (22.9%)	1 (25%)

Allele Frequencies

The allele prevalence of the *HH* polymorphism in the reference group of 555 women was 4.1% (95% CI 2.9 to 5.4). The prevalence of *HH* heterozygotes in the reference group was 40 (7.2%) and the prevalence of *HH* homozygotes was 3 (0.5%). The number of homozygotes was higher than expected from previously reported data[12]; our population was therefore not in Hardy-Weinberg equilibrium ($\chi^2=4.56$; 1 *df*, $P=0.033$). One woman, who was homozygous for the *HH* polymorphism, died of unspecified heart failure.

Mortality Rates

The mortality rate for cerebrovascular disease was significantly higher in *HH* heterozygotes than in wild types (Table 2); the RR was 2.4 (95% CI 1.3 to 4.4), whereas a borderline-significantly increased RR of 1.5 (95% CI 0.9 to 2.5; $P=0.135$) was found for death as the result of myocardial infarction. The overall cardiovascular death risk was significantly higher for *HH* heterozygotes than for wild types: RR of 1.6 (95% CI 1.1 to 2.4; $P=0.028$). Population-attributable risks of myocardial infarction, cerebrovascular, other cardiovascular, and total cardiovascular deaths were 3.3%, 8.8%, 1.4%, and 4.0%, respectively (Table 2). The RR of *HH* heterozygosity for cardiovascular death compared with wild types was not changed when age, smoking, obesity, and hypertension were included in a multivariate model, suggestive of heterozygosity for the *HH* polymorphism to be an independent risk factor for cardiovascular death.

Adjustment for age, smoking, and hypertension had minor effects on the cardiovascular death ratio. The age-, smoking-, and hypertension-adjusted cardiovascular death ratio between *HH* heterozygotes and *HH* wild types remained 1.6 (95% CI 1.0 to 2.5), for myocardial infarction 1.5 (0.9 to 2.7), for stroke 2.6 (1.4 to 4.9), and for other cardiovascular death 1.3 (0.7 to 2.7).

Effect modification by smoking is presented in Table 3. In the subgroup of nonsmokers, the risk of cardiovascular death is similar for *HH* carriers as for noncarriers. In smokers, however, cardiovascular mortality rate was higher for *HH* carriers than for noncarriers (Table 3). Similarly, cardiovascular mortality rate was highest when women were both an *HH* carrier and hypertensive. Women who were either hypertensive and were not a carrier or *HH* carrier and not hypertensive had a moderately higher risk of cardiovascular death than did women who were not a carrier and not hypertensive (Table 3). The risk of cardiovascular death in subgroups of age and weight, higher or lower than the median, were not different from population risks.

Further subgroup analysis is presented in Table 4. *HH* carriership was not associated with cardiovascular death in women who were nonsmokers and nonhypertensives (RR 1.41, 95% CI 0.67 to 2.95). In women who were either smokers or hypertensives, *HH* carriers had a moderately increased risk of cardiovascular death (RR 1.78, 95% CI 0.95 to 3.32), whereas in women who were both smokers and hypertensives, *HH* heterozygotes had a strongly increased

TABLE 2. Incidence Rates, Rate Ratios, and Population-Attributable Risks for Cardiovascular Death for *HH* Heterozygotes Compared With Wild Types

		Cardiovascular Death			
	Person-Years	Myocardial Infarction	Cerebrovascular	Other	Total
Incidence rates, 1000 y					
Wild type	154 302	1.4 (1.2–1.6)	0.7 (0.5–0.8)	1.0 (0.9–1.2)	3.1 (2.8–3.4)
Heterozygotes	11 483	2.1 (1.3–2.9)	1.6 (0.8–2.3)	1.2 (0.6–1.9)	4.9 (3.6–6.2)
Rate ratio (95% CI)		1.5 (0.9–2.5)	2.4 (1.3–4.3)	1.2 (0.7–2.3)	1.6 (1.1–2.4)
PAR, %		3.3	8.8	1.4	4.0

PAR indicates population-attributable risk.

TABLE 3. Cardiovascular Death (Incidence/1000 Person-Years) for *HH* Carriers and Non–*HH* Carriers Classified in Smoker or Nonsmoker and Hypertensive/Nonhypertensive Subgroups

Subgroup	n (Cases)	Follow-Up, y	Cardiovascular Death			
			Total	Myocardial	Cerebrovascular	Other
Nonsmoker/non–*HH* carrier	312	109 075	2.9 (2.5–3.3)	1.3 (1.1–1.6)	0.6 (0.5–0.8)	0.9 (0.7–1.1)
Smoker/non–*HH* carrier	162	45 227	3.6 (2.9–4.4)	1.6 (1.2–2.1)	0.7 (0.5–1.1)	1.3 (1.0–1.7)
Nonsmoker/*HH* carrier	36	9488	3.8 (2.4–6.0)	1.8 (1.0–3.3)	1.3 (0.7–2.6)	0.8 (0.3–1.9)
Smoker/*HH* carrier	20	1994	10.0 (4.2–22)†	3.5 (1.2–10.1)	3.0 (1.0–9.4)*	3.5 (1.2–10.1)†
Nonhypertensive/non—*HH* carrier	128	56 758	2.3 (1.8–2.8)	1.0 (0.8–1.4)	0.5 (0.3–0.7)	0.7 (0.5–1.0)
Hypertensive/non–*HH* carrier	346	97 544	3.5 (3.1–4.1)	1.6 (1.4–2.0)	0.8 (0.6–1.0)	1.2 (0.9–1.4)
Nonhypertensive/*HH* carrier	12	6091	2.0 (1.0–4.1)	0.8 (0.3–2.6)	0.7 (0.2–2.4)	0.5 (0.1–2.4)
Hypertensive/*HH* carrier	44	5391	8.2 (4.8–13.6)§	3.5 (1.9–6.6)‡	2.6 (1.3–5.2)§	2.0 (1.0–4.4)

*$P<0.05$ compared with nonsmoker/non–*HH* carrier.
†$P<0.01$ compared with nonsmoker/non–*HH* carrier.
‡$P<0.05$ compared with nonhypertensive/non–*HH* carrier.
§$P<0.01$ compared with nonhypertensive/non–*HH* carrier.

risk of cardiovascular death (RR 18.85, 95% CI 8.38 to 42.37). The findings of the highest risk associated with the combination of *HH* carriership plus 2 conventional risk factors was not confined to 1 particular subgroup of cardiovascular death (Table 4). The Figure illustrates the effect modification of smoking and hypertension on the relation between *HH* carriership and cardiovascular death.

Discussion

We studied the relation between *HH* heterozygosity and cardiovascular death among 12 239 postmenopausal women living in the city of Utrecht who were prospectively followed for 15 to 18 years. The allele prevalence of the *HH* polymorphism was 4.0% (95% CI 2.9 to 5.4). Compared with wild types, *HH* heterozygotes had a statistically significant RR for total cardiovascular death of 1.6 (95% CI 1.1 to 2.4) and for cerebrovascular death of 2.4 (95% CI 1.3 to 4.3) and a borderline-significant RR of 1.5 (0.9 to 2.5) for myocardial infarction.

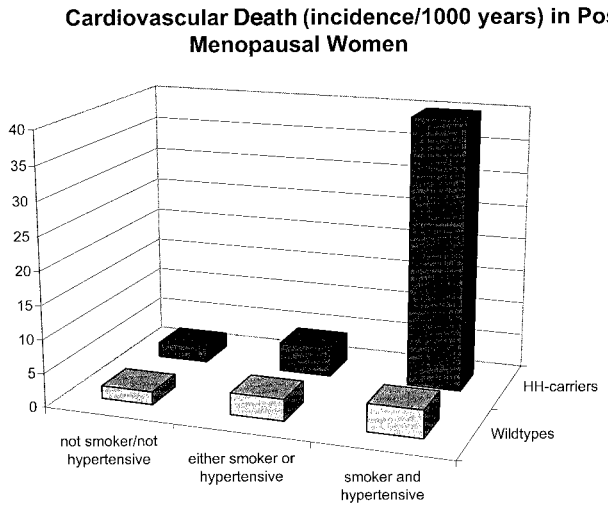
We found strong evidence for effect modification by both smoking and hypertension. The association between *HH* heterozygosity and cardiovascular death appeared to be stronger in women who were hypertensive or current smokers. Women who were smokers, hypertensive, and heterozygous for *HH* had an 18.85-fold increased risk of cardiovascular death compared with nonsmokers, nonhypertensives, and noncarriers. The RR (95% CI) for fatal myocardial infarction was 19.93 (5.55 to 71.52); for cerebrovascular death it was 35.35 (8.31 to 150.46), and for the rest of the group it was 33.38 (9.78 to 113.87). Smokers and hypertensives who did not carry the *HH* polymorphism had an RR (95% CI) of 2.06 (1.39 to 3.03) compared with the group of nonsmokers, nonhypertensives, and noncarriers. The RRs for fatal myocardial infarction, cerebrovascular death, and the rest group of cardiovascular deaths were in the same range.

Our findings suggest that *HH* heterozygosity is associated with an increased risk of cardiovascular death. This result provides support for the view that iron overload may play a role in cardiovascular disease.[2,23] Moderately excessive iron may be involved in oxygen radical formation, which may initiate peroxidation of LDL. Oxidized LDL is recognized by the scavenger receptor and taken up by macrophages in the

TABLE 4. Cardiovascular Death (Incidence/1000 Person-Years) for *HH* Carriers and non–*HH* Carriers Classified in Subgroups of Nonsmokers and Nonhypertensives, Either Smokers or Hypertensives, and Both Smokers and Hypertensives

Subgroup	n	Follow-Up, y	Cardiovascular Death			
			Total	Myocardial	Cerebrovascular	Other
Wild type						
Nonsmoker, nonhypertensive*	71	36 983	1.9 (1.4–2.6)	1.0 (0.7–1.4)	0.4 (0.2–0.7)	0.6 (0.4–1.0)
Either smoker or hypertensive	298	91 867	3.2 (2.8–3.8)†	1.5 (1.2–1.8)†	0.8 (0.6–1.0)	1.0 (0.8–1.3)†
Both smoker and hypertensive	105	25 452	4.1 (3.1–5.5)†	1.9 (1.3–2.7)†	0.7 (0.5–1.3)	1.5 (1.0–2.2)†
HH carrier						
Nonsmoker, nonhypertensive	10	4547	2.2 (1.0–5.0)	0.9 (0.3–3.3)	0.7 (0.2–3.3)	0.7 (0.2–3.3)
Either smoker or hypertensive	28	6487	4.3 (2.5–7.5)	2.2 (1.1–4.3)	1.5 (0.7–3.4)*	0.6 (0.2–2.3)
Both smoker and hypertensive	18	450	40.0 (15.5–91.1)‡	13.3 (4.3–41.2)‡	11.1 (3.3–38.5)‡	15.6 (5.2–44.9)‡

*Reference group.
†$P<0.05$ compared with nonsmoker/nonhypertensive/wild types.
‡$P<0.01$ compared with nonsmoker/nonhypertensive/wild types.



Cardiovascular death, death due to myocardial infarction, cerebrovascular death, and all other forms of cardiovascular death in subgroups: *HH* carrier/not *HH* carrier by nonhypertensives, nonsmoker/either hypertensive or smoker/hypertensive and smoker (2 by 3). Smoking was defined as reported to be current smoker in the baseline questionnaire. Hypertension was defined as diastolic pressure >95 and/or systolic pressure >160 mm Hg.

intima of the arterial wall, leading to transformation of tissue macrophages into foam cells, the most important cells of the fatty streak.[1] The iron necessary for catalyzing lipid peroxidation can be derived from ferritin,[24] heme,[25] and plasma iron, either transferrin bound[26] or not.[27] Although the LDL-oxidation pathway appears to be very plausible, iron may be involved in several other processes that promote cardiovascular disease.

The recently discovered *HFE* gene may play a role in the iron release from intestinal mucosal cells and macrophages to the plasma. Homozygotes for the common *HFE* mutation show clinical expression of *HH*. *HFE* has a disulfide bridge in its α3 domain, necessary for association of β2-microglobulin with MHC class I molecules. The Cys282Tyr mutation in *HFE* probably interferes with the formation of the disulfide bridge, thus impairing association with β2-microglobulin and eliminating cell-surface presentation. Results of a study of mice lacking the gene coding for β2-microglobulin, which also develop hemochromatosis,[28] support this hypothesis. Heterozygotes for the *HH* mutation do not develop hemochromatosis but have slightly increased levels of serum ferritin and serum iron and a reduced iron-binding capacity. Heterozygosity for the *HH* mutation may therefore be a genetic indicator of lifelong exposure to a moderate excess of iron not sufficient to lead to clinical signs and symptoms of iron overload.

From our data, we cannot explain the functional mechanism that explains the effect modification by smoking and/or hypertension on the relation between *HH* carriership and cardiovascular death. A possible explanation is the combined oxidative effect of increased body iron caused by *HH* carriership and increased oxidative stress from smoking, which may lead to an overexposure of oxygen radicals, which may be involved in lipid peroxidation and therefore increase the risk of cardiovascular death. An alternative explanation is that lifelong exposure to moderately increased iron levels in *HH* carriers may lower the threshold for cardiovascular disease. *HH* carriers may therefore be more sensitive to the effects of smoking and hypertension on cardiovascular disease than women who do not carry the mutation.[29]

This is the first large follow-up study to detect a significant association between a single genetic polymorphism and cardiovascular death in women. Similar to our findings, an increased risk of cardiovascular disease for *HH* carriers was found in a cohort study among Finnish men.[30] The population-attributable risk represents the proportion of women who died of a cardiovascular event that was attributable to a specific risk factor. In our study, the population-attributable risk of *HH* heterozygosity for cerebrovascular death was 8.8%, which was comparable to the population-attributable risk of smoking (7.4%) and obesity (6.1%) but not as high as the population-attributable risk of hypertension (27.2%). The population-attributable risk of *HH* heterozygosity for total cardiovascular death (4.0%) was comparable to the population-attributable risk of obesity (4.4%) but not as high as population-attributable risks of smoking (7.9%) and hypertension (30.4%).

Epidemiological studies on genetic markers have the advantage of not being biased by storage and handling procedures of biological samples, seasonal variability, or intrasubject and intersubject variability. Data were analyzed with the use of a nested case-referent approach, which is an alternative term for a nested case-control-study. Nested analysis of prospectively collected material has several advantages compared with normal case-control studies. First of all, population-attributable risks can be calculated. Second, control subjects are not biased by selection because they are randomly selected from the entire cohort and are therefore representative for the entire cohort. Third, follow-up time can be included in the data analysis. Moreover, the prospective approach enabled us to study cardiovascular death because DNA was collected before the event occurred. In case-control studies, DNA will be collected after the event, precluding the study of acute cardiovascular death.

A limitation of our study is that no blood samples were collected at baseline. We were therefore not able to study whether *HH* heterozygotes indeed had increased iron parameters as intermediate steps in the association between *HH* genotype and cardiovascular death. For the same reason, we could not measure lipid peroxidation by moderately increased iron exposure or lipid levels to study effect modification in hyperlipidemic patients.

We measured *HH* genotype in cardiovascular death cases and a random sample of the rest of the cohort only and do not have genetic data of all noncardiovascular death cases such as cancer. If women died of a cause other than cardiovascular disease, then they were censored from the follow-up and treated similarly to the rest of the cohort. The *HH* gene may be associated with increased risk of cancer; therefore excluding cancer cases from the cohort may lead to an even stronger cardiovascular death ratio between *HH* heterozygotes and wild types.

Our subjects were members of a normal population, and causes of death were obtained from the general practitioner. We expect some misclassification between ischemic and hemorrhagic fatal stroke because the diagnosis was not routinely confirmed by computed tomographic scan or magnetic resonance imaging. Moreover, the number of subjects in both subgroups become very small when analyzed separately. It was not possible to obtain a reliable distinction between ischemic or hemorrhagic fatal stroke in this study.

HH genotyping may play an important role in predicting the risk of cardiovascular death in postmenopausal women, especially when women have increased risks of cardiovascular disease such as hypertension and smoking, whereas effect modification by lipid levels needs to be delineated. Recent data from a Finnish cohort study are suggestive of a similar role of *HH* genotype in men.[30]

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