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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. 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, serum iron, and total iron-binding capacity with cardiovascular disease, whereas others have not found an association.

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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. 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). 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 or hereditary spherocytosis. Still, heterozygotes have slightly but significantly increased levels of serum ferritin and serum iron, whereas total iron-binding capacity is reduced. 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.

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 project16). 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/m²) was calculated as weight (kg) divided by height squared (m²). Obesity was defined as body mass index ≥30 kg/m². 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), and 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 HFE 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 who did not die of cardiovascular disease (sampling fraction 1:18.8) were 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 non–smokers and non–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.
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; 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 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 risk.

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 risk.
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

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

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>n (Cases)</th>
<th>Follow-Up, y</th>
<th>Total</th>
<th>Myocardial</th>
<th>Cerebrovascular</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonsmoker/non--<strong>HH</strong> carrier</td>
<td>312</td>
<td>109,075</td>
<td>2.9 (2.5–3.3)</td>
<td>1.3 (1.1–1.6)</td>
<td>0.6 (0.5–0.8)</td>
<td>0.9 (0.7–1.1)</td>
</tr>
<tr>
<td>Smoker/non--<strong>HH</strong> carrier</td>
<td>162</td>
<td>45,227</td>
<td>3.6 (2.9–4.4)</td>
<td>1.6 (1.2–2.1)</td>
<td>0.7 (0.5–1.1)</td>
<td>1.3 (1.0–1.7)</td>
</tr>
<tr>
<td>Nonsmoker/<strong>HH</strong> carrier</td>
<td>36</td>
<td>9,488</td>
<td>3.8 (2.4–6.0)</td>
<td>1.8 (1.0–3.3)</td>
<td>1.3 (0.7–2.6)</td>
<td>0.8 (0.3–1.9)</td>
</tr>
<tr>
<td>Smoker/<strong>HH</strong> carrier</td>
<td>20</td>
<td>1994</td>
<td>10.0 (4.2–22)</td>
<td>3.5 (1.2–10.1)</td>
<td>3.0 (1.0–9.4)</td>
<td>3.5 (1.2–10.1)</td>
</tr>
<tr>
<td>Nonhypertensive/non--<strong>HH</strong> carrier</td>
<td>128</td>
<td>56,758</td>
<td>2.3 (1.8–2.8)</td>
<td>1.0 (0.8–1.4)</td>
<td>0.5 (0.3–0.7)</td>
<td>0.7 (0.5–1.0)</td>
</tr>
<tr>
<td>Hypertensive/non--<strong>HH</strong> carrier</td>
<td>346</td>
<td>97,544</td>
<td>3.5 (3.1–4.1)</td>
<td>1.6 (1.4–2.0)</td>
<td>0.8 (0.6–1.0)</td>
<td>1.2 (0.9–1.4)</td>
</tr>
<tr>
<td>Nonhypertensive/<strong>HH</strong> carrier</td>
<td>12</td>
<td>6,091</td>
<td>2.0 (1.0–4.1)</td>
<td>0.8 (0.3–2.6)</td>
<td>0.7 (0.2–2.4)</td>
<td>0.5 (0.1–2.4)</td>
</tr>
<tr>
<td>Hypertensive/<strong>HH</strong> carrier</td>
<td>44</td>
<td>5,391</td>
<td>8.2 (4.8–13.6)</td>
<td>3.5 (1.9–6.6)</td>
<td>2.6 (1.3–5.2)</td>
<td>2.0 (1.0–4.4)</td>
</tr>
</tbody>
</table>

*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.

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**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**

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>n</th>
<th>Follow-Up, y</th>
<th>Total</th>
<th>Myocardial</th>
<th>Cerebrovascular</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>71</td>
<td>36,983</td>
<td>1.9 (1.4–2.6)</td>
<td>1.0 (0.7–1.4)</td>
<td>0.4 (0.2–0.7)</td>
<td>0.6 (0.4–1.0)</td>
</tr>
<tr>
<td>Either smoker or hypertensive</td>
<td>298</td>
<td>91,867</td>
<td>3.2 (2.8–3.8)</td>
<td>1.5 (1.2–1.8)</td>
<td>0.8 (0.6–1.0)</td>
<td>1.0 (0.8–1.3)</td>
</tr>
<tr>
<td>Both smoker and hypertensive</td>
<td>105</td>
<td>25,452</td>
<td>4.1 (3.1–5.5)</td>
<td>1.9 (1.3–2.7)</td>
<td>0.7 (0.5–1.3)</td>
<td>1.5 (1.0–2.2)</td>
</tr>
<tr>
<td><strong>HH</strong> carrier</td>
<td>10</td>
<td>4,547</td>
<td>2.2 (1.0–5.0)</td>
<td>0.9 (0.3–3.3)</td>
<td>0.7 (0.2–3.3)</td>
<td>0.7 (0.2–3.3)</td>
</tr>
<tr>
<td>Either smoker or hypertensive</td>
<td>28</td>
<td>6,687</td>
<td>4.3 (2.5–7.5)</td>
<td>2.2 (1.1–4.3)</td>
<td>1.5 (0.7–3.4)</td>
<td>0.6 (0.2–2.3)</td>
</tr>
<tr>
<td>Both smoker and hypertensive</td>
<td>18</td>
<td>4,500</td>
<td>40.0 (15.5–91.1)</td>
<td>13.3 (4.3–41.2)</td>
<td>11.1 (3.3–38.5)</td>
<td>15.6 (5.2–44.9)</td>
</tr>
</tbody>
</table>

*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.

Cardiovascular Death (incidence/1000 years) in Post Menopausal Women

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, 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.

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. 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 intra-subject and inter-subject 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 becomes 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

**Acknowledgment**

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**References**