A prospective study of coronary heart disease and the hemochromatosis gene (HFE) C282Y mutation: the Atherosclerosis Risk in Communities (ARIC) study

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Abstract

Increased iron stores may play a role in the development of coronary heart disease (CHD) by increasing lipoprotein oxidation. Recently, mutations have been discovered in the gene (HFE) for hereditary hemochromatosis, an autosomal recessive condition of disordered iron metabolism, absorption, and storage. It is possible that people who carry HFE mutations have increased risk of CHD. We used a prospective case-cohort design (243 CHD cases and 535 non-cases) to determine whether the HFE C282Y mutation was associated with incident CHD in a population-based sample of middle-aged men and women. The frequencies of homozygosity and heterozygosity for the C282Y mutation in the ARIC study population were 0.2% (one homozygous person) and 6%, respectively. The C282Y mutation was associated with nonsignificantly increased risk of CHD (relative risk 1.60, 95% CI 0.9–2.9). After adjusting for other confounding risk factors (age, race, gender, ARIC community, smoking status, diabetes status, hypertension status, LDL cholesterol, HDL cholesterol, and triglycerides), the association became stronger (relative risk 2.70, 95% CI 1.2–6.1). However, a sensitivity analysis showed that this estimate of relative risk was somewhat unstable due to few subjects in some strata. Our prospective findings suggest that individuals carrying the HFE C282Y mutation may be at increased risk of CHD. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Hemochromatosis; Coronary disease; Prospective study

1. Introduction

Coronary heart disease (CHD) has a multifactorial etiology with both genetic and nongenetic causes. Sullivan hypothesized that the sex difference in CHD is due to iron excess in men and postmenopausal women compared to premenopausal women [1]. A prospective study by Salonen and colleagues [2,3] in Eastern Finnish men suggested high serum ferritin, a marker of iron excess, is associated with increased risk of CHD. However, the majority of prospective epidemiologic research found no association between serum ferritin [4–7], or other measures of iron level [4,8–14], and CHD. In the ARIC study, there was no association between serum ferritin levels and carotid wall thickness [15].

Iron is thought to catalyze the formation of free radicals [16–20] and convert poorly reactive radicals (e.g. hydrogen peroxide) into highly reactive ones (e.g. the hydroxyl radical). The hydroxyl radical can initiate lipid peroxidation, causing LDL to undergo an oxidative modification that targets it for uptake by macrophages [20–22]. These macrophages form lipid rich foam cells in the endothelium of arteries, leading to
the development of fatty streaks, the precursor to atherosclerotic plaques. It is also believed that free radicals may damage arterial endothelium directly [23] and interfere with normal vasomotor regulation [24].

Hereditary hemochromatosis, a relatively common autosomal recessive condition, is a disease of disordered iron metabolism causing the body to absorb and store excess iron. In 1996, Feder and colleagues [25] identified and cloned a gene for hemochromatosis at the HLA locus termed \( HFE \). Amino acid substitutions caused by two missense mutations Cys282Tyr (C282Y) and His63Asp (H63D) in the gene have been reported [25]. The C282Y mutation, which causes a substitution of a tyrosine for a cysteine, provides a more consistent association with the disease across studies [26–31] and has a higher penetrance than H63D [32]. Compound heterozygosity for C282Y and H63D is associated with increased hemochromatosis risk. Depending on ethnic backgrounds, the frequencies of homozygosity and heterozygosity for the C282Y mutation are \( \approx 0\%\) to \( 1\%\) and \( 2\%\)–\( 14\%\), respectively [33].

Tissue damage from iron overload in homozygous hemochromatosis results in various clinical manifestations including cirrhosis, cardiomyopathy, diabetes, skin pigmentation and various cancers [33,34]. Heterozygosity for the C282Y mutation could increase risk of CHD by increasing iron stores and lipid oxidation. To date, two studies have suggested that heterozygosity for the C282Y mutation is associated with CHD [35,36], and four have not [30,37–39]. The objective of this study was to determine if there is an association between the presence of the C282Y mutation of the \( HFE \) gene and incident CHD in a population-based cohort study.

2. Methods

2.1. Study population

Between 1987 and 1989, the ARIC Study recruited a cohort totalling 15,792 persons aged 45–64 years. Population samples were selected by probability sampling of Forsyth County, North Carolina; Jackson, Mississippi (black people only); the northwest suburbs of Minneapolis, Minnesota; and Washington County, Maryland. Participants underwent reexamination in 1990 through 1992 (93% return rate), in 1993 through 1995 (86% return rate), and in 1996 through 1998 (80% return rate).

2.2. Baseline measurements

After an 8-h fasting period blood was drawn with minimal trauma from an antecubital vein. Serum, plasma, and buffy coat were obtained and stored at \( -70^\circ \text{C} \) until analysis. Plasma total cholesterol [40] and triglycerides [41] were measured by enzymatic methods, and LDL cholesterol was calculated [42]. HDL cholesterol was measured after dextran-magnesium precipitation of non-HDL lipoproteins [43]. Hemoglobin and hematocrit were measured on fresh blood by hospitals in each field center.

Current use of medications, vitamins, or supplements was assessed by reviewing pill bottles. Three blood pressure measurements were taken with a random-zero sphygmomanometer and the last two measurements averaged. Hypertension was defined as systolic blood pressure \( \geq 140 \text{ mmHg} \) or diastolic blood pressure \( \geq 90 \text{ mmHg} \) or currently taking antihypertensive medication. Diabetes was defined as a physician diagnosis of diabetes, fasting serum glucose of \( \geq 126 \text{ mg/dl} \), a nonfasting glucose \( \geq 200 \text{ mg/dl} \), or pharmacologic treatment for diabetes. Smoking status was ascertained by asking subjects whether they currently or had ever smoked cigarettes to define never smokers, former smokers, and current smokers. Parental history of myocardial infarction (yes, no) was based on participant report. Women reported their menstrual and pregnancy history in a standardized interview.

For exclusion, prevalent cardiovascular disease at baseline was defined as a reported history of a physician-diagnosed heart attack, stroke, or TIA; prior myocardial infarction by electrocardiogram; or prior cardiovascular surgery or coronary angioplasty.

2.3. Ascertainment and classification of incident CHD cases

For this paper, CHD events occurring between ARIC Visit 1 and December 31, 1991 were included. The median follow-up time was 2.9 years (maximum = 5 years). Incident CHD was defined as (1) a definite or probable myocardial infarction, (2) a silent MI between examinations by electrocardiogram, (3) a definite CHD death, or (4) a coronary revascularization.

All CHD events in the ARIC cohort were ascertained [44]. ARIC interviewers contacted participants annually by telephone to identify all hospitalizations and deaths. Death certificates and discharge lists from local hospitals were also surveyed to detect additional cardiovascular events. ARIC investigated out-of-hospital deaths by means of the death certificate and, in most cases, an interview with next of kin and questionnaires completed by the patients’ physicians. When available, coroner reports and autopsy reports were also obtained for use in validation. For hospitalized patients, trained abstractors recorded all discharge diagnoses and procedure codes, the presenting signs and symptoms including chest pain, cardiac enzymes, and related clinical information. Technicians visually coded up to three 12-lead electrocardiograms using the Minnesota Code
and evaluated wave-form evolution using side-by-side comparisons. An ARIC Morbidity and Mortality Classification Committee then reviewed and adjudicated all potential clinical CHD events using published criteria [44]. Coronary revascularization was defined as being present if hospital procedure codes included coronary bypass, coronary angioplasty, or coronary atherectomy.

2.4. Cohort sample

A case-cohort design was employed for this investigation. Hemochromatosis genotypes were determined for all incident CHD cases and a stratified random sample of all ARIC participants free of CHD and stroke at baseline. Sampling was stratified on average carotid intima-media thickness measurements at baseline (≤30 percentile, >30 percentile), age (<55 years, ≥55 years), and sex, for a total of eight strata. To account for our sampling design, weighted analyses were performed with weights inversely proportional to the sampling fraction.

2.5. Genotyping methods

In 1995 after ARIC had identified the incident cases and cohort random sample, these participants’ baseline frozen buffy coat samples that had been frozen between 1987–1989 were retrieved. The samples were thawed, genomic DNA extracted by a salt precipitation procedure, treated with a proteinase K (1.9 mg/ml), and then frozen at −70°C.

Hemochromatosis genotyping for the C282Y mutation was done in the ARIC Clinical Chemistry Laboratory at the University of Minnesota using the method of Jouanolle et al. [26]. A 387 base-pair fragment of the \(HFE\) gene was amplified by polymerase chain reaction (PCR). The PCR product was then digested using the restriction enzyme Rsa I. The digestion products were electrophoresed and the fragments identified by silver staining. Two bands, one of 247 bp and the other of 140 bp, are found in DNA from individuals not carrying the mutation. For individuals carrying the mutant allele, the 140 bp band is further digested to 111 and 29 bp. Thus, heterozygous carriers show four bands and those who are homozygous for the mutant allele show three bands of 247, 111, and 29 bp.

To assess laboratory reliability, ARIC also included split-specimen, blinded duplicates prepared at the time of baseline blood drawing. \(HFE\) genotyping on these specimens yielded 100% agreement (5 of 5 heterozygous genotypes identified, 33 of 33 normal genotypes identified). In addition, because of recent widespread concern that the primers may occasionally misclassify heterozygous individuals as homozygous, the University of Minnesota verified that its \(HFE\) testing was accurate.

2.6. Data analysis

Prior to selection of the case-cohort sample, exclusions to the ARIC cohort included participants identified as having unknown or prevalent CVD at baseline, and non-whites in Minneapolis and Washington County. Further exclusions were made for participants who did not want their DNA used for analysis. For this analysis, there were 64 participants heterozygous and one homozygous for the \(HFE\) C282Y mutation. Seven hundred-thirteen subjects had the normal genotype. The single homozygote was grouped with the heterozygotes for the analysis.

Mean levels or percentages of the exposure variable and baseline characteristics in CHD cases, the cohort random sample non-cases, and the ARIC population were computed, as well as mean levels or percentages of the covariates in the C282Y positive and C282Y negative groups, by applying estimation procedures for linear and logistic regression models which accounted for the case-cohort sampling design.

Relative risks and their 95% confidence intervals of CHD in relation to the exposure variable were computed using weighted proportional hazards regression models, using Barlow’s method to account for the stratified random sampling and the case-cohort design [45]. Initially a crude relative risk and an age-, gender-, race- (white, black), and ARIC community-adjusted relative risk were computed. The final multivariate model also adjusted for other major CHD risk factors chosen a priori: smoking status (current, former, never), hypertension (yes, no), diabetes (yes, no), LDL cholesterol (continuous), HDL cholesterol (continuous), and triglycerides (continuous).

3. Results

3.1. Sample characteristics

The sample included 243 incident CHD cases (157 definite or probable MI, 20 silent MI, 26 definite fatal CHD, and 40 revascularization procedure) and a reference cohort sample of 535. Due to the case-cohort design, 10 individuals in the reference cohort sample eventually became CHD cases. Approximately 25% of the CHD cases were black, and 72% were men.

Based on the cohort random sample, we estimated that the frequency of homozygosity for the C282Y mutation in the entire ARIC population was 0.2% (one homozygous person) and the frequency of heterozygosity was 6.0%. The heterozygous frequencies were 5.0 and 6.6% for men and women, respectively, and 2.5 and 7.1% for black and white people, respectively.
3.2. Mean differences between CHD cases and non-cases

As expected, compared to non-cases, participants who developed CHD had significantly higher levels of LDL cholesterol and triglycerides, lower levels of HDL cholesterol, and were more often current smokers, hypertensive, diabetic, older and male (data not shown). The HFE C282Y mutation was found in 9.9% of the cases compared to 6.1% of the cohort random sample ($P = 0.09$).

3.3. Mean differences between genotypes

Compared to participants with the normal genotype, those with the HFE C282Y mutation were significantly older and less likely to be diabetic (Table 1). The mean age of the C282Y positive group was 56.8 years compared to 53.7 years in the C282Y negative group ($P = 0.02$). One percent of the C282Y positive group were diabetic whereas 8.1% of the C282Y negative group were ($P < 0.001$). Although not statistically significant, those with the C282Y mutation had higher HDL cholesterol ($P = 0.15$), lower LDL cholesterol ($P = 0.23$), and were less likely to be current smokers ($P = 0.18$) than those with the normal genotype. There were no significant differences between C282Y carriers and non-carriers on parental history of myocardial infarction, hemoglobin, hematocrit, menopausal status, or use of hormone replacement, vitamins, or iron supplements. Similarly, among women, age at menarche and the number of years they menstruated did not differ by C282Y status (data not shown).

3.4. Relative risk of CHD

The crude relative risk of CHD in those with versus without the C282Y mutation was 1.60 (95% CI 0.9–3.0) (Table 2); this relative risk was 1.72 (95% CI 0.9–3.3) in white people and 0.92 (95% CI 0.2–5.8) in black people. Adjusting for age, sex, race, and ARIC community decreased the relative risk to 1.32 with a 95% CI of 0.7–2.6. Adding current and former smoking to the previous model slightly increased the relative risk to 1.43 (95% CI 0.7–3.0). Including diabetes status and hypertension status in the model increased the relative risk to 1.83 (95% CI 0.9–3.9). Finally, adding lipids (LDL cholesterol, HDL cholesterol, and triglycerides) to the model greatly increased the relative risk to 2.70 with a 95% confidence interval of 1.2–6.0.

Because the number of black people was few, we repeated the final model for white people only. The fully-adjusted relative risk in white people only was 3.4 with a 95% confidence interval of 1.3–9.4.

Interactions of the C282Y mutation with gender, race, LDL cholesterol, HDL cholesterol, triglycerides, current or former smoking, and hypertension status were not statistically significant. For example, the model 7 relative risks for the C282Y mutation among those with LDL cholesterol $<130$ mg/dl was 2.0 and among those with LDL $\geq130$ mg/dl was 2.1. The model 8 relative risk of CHD for the C282Y mutation was 4.4 for those in the upper quartile of hemoglobin versus 2.0 for those in the lower three quartiles, although this interaction also was not statistically significant ($P > 0.05$). There was however a significant

### Table 1

<table>
<thead>
<tr>
<th>Baseline characteristic</th>
<th>C282Y positive* ($n = 65$)</th>
<th>C282Y negative* ($n = 713$)</th>
<th>Entire ARIC sample ($n = 14215$)</th>
<th>$p^{b}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>56.8</td>
<td>53.7</td>
<td>53.9</td>
<td>0.02</td>
</tr>
<tr>
<td>Race (% black)</td>
<td>13.8</td>
<td>25.2</td>
<td>24.5</td>
<td>0.26</td>
</tr>
<tr>
<td>Gender (% male)</td>
<td>35.2</td>
<td>43.7</td>
<td>43.2</td>
<td>0.39</td>
</tr>
<tr>
<td>Current smoker (%)</td>
<td>14.0</td>
<td>24.2</td>
<td>23.6</td>
<td>0.18</td>
</tr>
<tr>
<td>Former smoker (%)</td>
<td>22.9</td>
<td>30.8</td>
<td>30.3</td>
<td>0.39</td>
</tr>
<tr>
<td>LDL-C (mg/dl)</td>
<td>126.6</td>
<td>136.6</td>
<td>136.0</td>
<td>0.23</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>62.0</td>
<td>53.7</td>
<td>54.3</td>
<td>0.15</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>131.2</td>
<td>122.8</td>
<td>123.3</td>
<td>0.48</td>
</tr>
<tr>
<td>Diabetic (%)</td>
<td>1.0</td>
<td>8.1</td>
<td>7.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hypertensive (%)</td>
<td>42.0</td>
<td>28.9</td>
<td>29.7</td>
<td>0.19</td>
</tr>
<tr>
<td>Parental history of MI (%)</td>
<td>42.1</td>
<td>37.5</td>
<td>37.8</td>
<td>0.67</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>13.9</td>
<td>13.8</td>
<td>13.8</td>
<td>0.97</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>41.9</td>
<td>41.7</td>
<td>41.7</td>
<td>0.74</td>
</tr>
<tr>
<td>Multivitamin use (%)</td>
<td>28.8</td>
<td>25.3</td>
<td>25.5</td>
<td>0.73</td>
</tr>
<tr>
<td>Antioxidant vitamin use (%)</td>
<td>6.4</td>
<td>14.5</td>
<td>14.0</td>
<td>0.19</td>
</tr>
<tr>
<td>Iron supplements (%)</td>
<td>1.1</td>
<td>4.0</td>
<td>3.8</td>
<td>0.23</td>
</tr>
<tr>
<td>Postmenopausal women (%)</td>
<td>41.2</td>
<td>34.5</td>
<td>35.0</td>
<td>0.37</td>
</tr>
<tr>
<td>Current hormone replacement (%)</td>
<td>19.8</td>
<td>12.9</td>
<td>13.3</td>
<td>0.36</td>
</tr>
</tbody>
</table>

*a Includes both incident CHD cases and non-cases.

*b Test of difference between the C282Y positive and C282Y negative groups.
interaction with diabetes status. The pattern of association in non-diabetics \((n = 674)\) was similar to that of the whole sample. The association in diabetics \((n = 102)\) was difficult to evaluate due to small numbers, but the C282Y mutation appeared to increase CHD risk more so than in non-diabetics. Of the eight diabetics with the mutation, seven (87.5%) developed CHD. In contrast, 58 of 94 of diabetics without the mutation (61.7%) developed CHD.

3.5. Sensitivity analysis

Concerns regarding the potential for unreliable estimation of the relative risk due to the small number of C282Y mutation carriers and large sampling weights for some strata led us to examine the influence of these factors on the stability of the estimated relative risks. We used a resampling method, known as the jackknife [46], to judge the reliability of our observed estimate of relative risk. The method consists of forming new samples by omitting, in turn, one of the observations of the original sample. The relative risk is computed for each of the samples generated, and the resulting distribution of the estimates allows one to draw conclusions about the sensitivity of the relative risk to individual observations. The jackknife procedure yielded fully-adjusted (Model 8) relative risk estimates in the range of 2.0–2.6, with mean 2.15 and associated 95% confidence interval of 2.08–2.22. Although the mean jackknife relative risk estimate was slightly lower that the relative risk based on the observed data \((RR = 2.70; 95\% \text{ CI} 1.20–6.07)\), the results were consistent with a positive association between C282Y mutation and CHD risk.

4. Discussion

In this prospective study, we found that individuals who carried the \(HFE\) C282Y mutation may be at greater risk of developing CHD than those without the mutation. The crude association was positive but relatively weak \((RR = 1.60)\) and not statistically significant. After adjusting for confounding variables the association became very strong \((RR = 2.70)\). This was explained by the surprising fact that C282Y carriers had lower CHD risk factors than non-carriers: less diabetes, less smoking, lower LDL cholesterol, and higher HDL cholesterol. These risk factor differences, which may partly be due to chance from the low number of participants \((n = 65)\) carrying the mutation, tended to mask a relatively strong association detected after adjustment. Given the degree of change in the relative risk with adjustment, our results should be interpreted cautiously.

Our results appear to corroborate Sullivan’s 1990 hypothesis that ‘heterozygosity for hemochromatosis increases the susceptibility of the carrier to premature myocardial infarction’ [47], and two other prospective studies recently linking C282Y heterozygosity with an approximately doubling of cardiovascular disease risk [35,36]. In contrast, four cross-sectional studies reported no association between hemochromatosis gene mutations and coronary artery disease [30,37–39], and the majority of prospective studies have not shown an association of serum ferritin, or other iron measures, with CHD [4–14]. In a meta-analysis, Danesh and Appleby [48] estimated the pooled relative risk for CHD in individuals with serum ferritin > 200 versus < 200 \(\mu g/l\) to be 1.03 \((95\% \text{ CI} 0.83–1.29)\). Comparing the top third versus bottom third of transferrin saturation, total iron binding capacity, and serum iron, they found the pooled relative risks for CHD were 0.92 \((95\% \text{ CI} 0.74–1.14)\), 0.98 \((95\% \text{ CI} 0.66–1.46)\), and 0.83 \((95\% \text{ CI} 0.67–1.03)\), respectively.

Sullivan’s hypothesis was partially based on the assumption that subjects heterozygous for hemochromatosis have higher iron stores than normal subjects. It has been reported that stored iron and plasma ferritin increase with age [49], and that iron stores in older subjects not carrying the mutation are similar to iron stores in younger heterozygotes [50]. Bulaj and colleagues [51] reported that mean values for serum iron, transferrin saturation, and ferritin were higher in heterozygous subjects compared to normal subjects and
only ferritin levels increased with age (in both groups). We did not have direct measures of iron stores in ARIC. Hemoglobin and hematocrit were not different between C282Y carriers and non-carriers, nor were use of vitamins, iron supplements, or menstrual history.

Although there was a suggestion that the relative risk of CHD associated with the C282Y mutation was somewhat higher in participants with higher versus lower hemoglobin, this interaction was not statistically significant.

Although some literature suggests that C282Y carriers are more likely than non-carriers to be diabetic [52,53], other studies have found no difference in prevalence of diabetes between the two groups [54–57]. In our sample, C282Y positive subjects were significantly less likely than C282Y negative subjects to be diabetic. Our data suggested further that diabetes status may be an effect modifier of the association between the C282Y mutation and CHD, however the small numbers made this hard to evaluate.

The homozygous and heterozygous frequencies of the C282Y mutation in this population-based study were 0.2 and 6%, respectively; the homozygous frequency is similar to those reported in other US and European studies (0–1%) [25,26,33,35,36,58–65]. Buetler and colleagues [58] reported a heterozygous frequency of 14% in a US study of persons of European origin. Barton and colleagues [59] and Feder and colleagues [25] reported heterozygous frequencies in white US individuals of 10.6 and 6.4%, respectively. Heterozygosity estimates from recent European studies ranged from 7–15% [35,36,63–66]. The heterozygous frequency of 6% in our study is lower than that in the literature and may be due to the inclusion of black participants. An international study reported that the C282Y mutation was absent in African, Asian, and Australian subjects and was most common in persons of Northern European descent [67]. Black people in the United States may have Caucasian admixture, so finding some black people with the C282Y mutation might be expected. In our case-cohort study, 8 of 190 black participants and 57 of 588 white participants had the C282Y mutation.

Strengths of this study include its prospective, population-based, cohort design as well as uniform and complete CHD event ascertainment. Limitations include the small number of participants with the mutation in some sampling strata, decreasing precision and possibly distorting the adjusted estimates. The sensitivity analysis suggested that the C282Y mutation may increase CHD risk, but the estimate of risk was somewhat unstable. Serum ferritin and other markers of iron status were not available for analysis. Only one of the two HFE missense mutations was examined, potentially leading to incomplete classification of true mutation status of the gene. Finally, as with all prospective studies, participants with prevalent cardiovascular disease at baseline were excluded. However, if the C282Y mutation led to CHD at an age younger than the ARIC cohort, our results could be biased.

In conclusion, our findings suggest that individuals carrying the HFE C282Y mutation may be at increased risk of CHD, although the association was only apparent and statistically significant after adjustment for confounding risk factors. Alternatively, it is possible that the association between the C282Y mutation and CHD is due to linkage disequilibrium with other biologically important variant(s). Further studies involving simultaneous measurement of biochemicals, such as serum ferritin and iron, will be needed to differentiate between these two possibilities.

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