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Serum Ferritin and C282Y Mutation of the Hemochromatosis Gene as Predictors of Asymptomatic Carotid Atherosclerosis in a Community Population

Enrico Rossi, PhD; Brendan M. McQuillan, FRACP; Joseph Hung, FRACP; Peter L. Thompson, MD, FRACP; Conchita Kuek, BSc; John P. Beilby, PhD, FAACB

Background and Purpose—Serum ferritin and heterozygosity for the C282Y mutation of the hemochromatosis gene have both been associated with an increased risk of cardiovascular events. The purpose of the study was to test whether either is a risk predictor for asymptomatic carotid atherosclerosis.

Methods—We assessed carotid intima-media wall thickness (IMT) and focal plaque formation by high-resolution B-mode ultrasound, conventional risk factors, serum ferritin levels, and the C282Y mutation of the hemochromatosis gene in a randomly selected community population of 1098 subjects (545 women and 553 men) aged 27 to 77 years.

Results—After adjustment for conventional risk factors, serum ferritin was not associated with carotid mean IMT. Women with ferritin values over the first quartile (>34 µg/L) had an adjusted odds ratio of 2.1 (95% CI, 1.3 to 3.4; P=0.0016) for carotid plaque compared with the first quartile. Ferritin was not associated with carotid plaque in men. Subjects who were heterozygous for the C282Y mutation constituted 11.4% of the population, and there was no independent association of this genotype with either carotid IMT or focal plaque formation.

Conclusions—we conclude that in our community population, C282Y genotype status was not a risk predictor for either carotid mean IMT or plaque formation. Serum ferritin values in women were independently associated with carotid plaque.

Key Words: atherosclerosis n ferritin n genetics n hemochromatosis n ultrasonics

It has been postulated that increased iron stores promote atherogenesis and coronary heart disease by increasing lipid peroxidation through free radical-mediated mechanisms.1,2 However a meta-analysis of prospective epidemiological studies of iron stores and coronary heart disease concluded that the evidence was inconclusive.3 For example, increased body iron stores as assessed by serum ferritin levels were found to be an independent risk predictor for acute myocardial infarction in one study4 but not in others.5,6 When other markers of iron stores are used, for example, serum iron or transferrin saturation, similar conflicting evidence arises.3 An explanation for these discrepant results may lie in the limitations of these analytes as markers for iron stores, for example, serum iron and transferrin saturation are affected by factors such as inflammation and diurnal variation, and ferritin is affected by inflammation, liver disease, and blood loss.

Other studies have related serum ferritin levels to subclinical carotid atherosclerosis as detected by B-mode ultrasound. The resulting indices of carotid intima-media thickness (IMT) and focal carotid plaque are strong predictors of subsequent vascular events.7 Serum ferritin levels were found to be an independent risk factor for carotid atherosclerosis in a community population in Bruneck, Italy,8 and confirmed in a 5-year follow-up of the same population.9 However, a US matched case-control study10 and a cross-sectional study of Finnish men11 both reported no association of ferritin with carotid IMT after adjustment for major cardiovascular risk factors.

Hereditary hemochromatosis, a common inherited disorder characterized by iron overload and homozygosity for the C282Y mutation of the HFE gene,12 is responsible for 89% of cases in our community.13 Heterozygotes for the C282Y mutation are carriers of hereditary hemochromatosis, and 2 recent prospective population-based studies have reported an association between heterozygotes and vascular events. A study of 12 239 postmenopausal Dutch women showed that C282Y heterozygotes were at a significantly increased risk of mortality from vascular events, either myocardial infarction or cerebrovascular disease.14 Another prospective study of

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1150 Finnish men found that C282Y heterozygosity was associated with a 2.3-fold increased risk of acute myocardial infarction.15

As a result of these findings, we tested for the association of serum ferritin and C282Y heterozygosity with carotid IMT and plaque formation in the Perth Carotid Ultrasound Disease Assessment Study (CUDAS).16 The latter consisted of 1111 male and female subjects, aged 27 to 77 years, randomly selected from the Perth community population, all of whom had high-resolution bilateral B-mode carotid ultrasound examination, serum ferritin, and C282Y mutation status determined as part of a detailed risk factor assessment.

Subjects and Methods

Subjects
Subjects were original participants in the 1989 Australian National Heart Foundation Perth Risk Factor Prevalence Survey.17 This was a random electoral roll survey of 2000 people from the Perth, Western Australia metropolitan area, with equal numbers of men and women and equal numbers of subjects in each age decile between 20 and 70 years. Repeated electoral roll and death record matching in May 1995 established a current address for 1807 living subjects. All of these were invited to attend our study clinic between June 1995 and December 1996, and 1111 subjects (61% of those eligible) agreed to participate. A complete set of data, including ferritin and C282Y mutation results, was available on 1098 subjects (545 women and 553 men). Subjects who had previous carotid artery surgery were excluded. The present study population was predominantly white, with 90% of participants recording Australasian as their country of birth. Their age-adjusted prevalence of risk factors was similar to that reported for the entire 1989 cohort.13 Written informed consent was obtained from all study participants. The study protocol was approved by the Institutional Ethics Committee of the University of Western Australia.

A self-administered questionnaire similar to that used by the 1989 Australian National Heart Foundation Risk Factor Prevalence Survey was used to record a history of hypertension, hyperlipidemia, diabetes, angina pectoris, myocardial infarction, or stroke or a family history of premature onset coronary heart disease by age 55 years in first-degree relatives.17 Smoking lifetime exposure was calculated by pack-years. Anthropomorphic measurements and the lower of 2 separate occasions 7 to 10 days apart. The intraobserver coefficient of variability was 5.9%.

Laboratory Measurements
In all subjects, a fasting venous blood sample was obtained. Total cholesterol, HDL cholesterol, and triglyceride levels were determined enzymatically, and C-reactive protein was determined by nephelometry with a Hitachi 747 autoanalyzer. LDL cholesterol was calculated with the formulas of Friedewald et al.18 Serum ferritin was determined by chemiluminescence immunoassay on an ACS-180 autoanalyzer (Chiron) and homocysteine by high-performance liquid chromatography.19 Genomic DNA was extracted by the salt phenol-chloroform method from the cells of the buffy coat. Polymerase chain reaction amplification of the region containing the C282Y mutation was performed with the use of the published primer sequences of Feder et al20 (GenBank U60319). The C282Y missense mutation was detected by restriction enzyme digestion with RsaI, followed by analysis on a 3% agarose gel.

Carotid Ultrasound
Bilateral carotid B-mode ultrasound was performed by 2 trained sonographers using a 7.5-MHz annular phased-array transducer on an Interspec (Apogee) CX 200 ultrasound machine. Scans were performed according to a standardized protocol similar to that used by Salonen et al20 and previously reported by our group.15 The characteristic echo interfaces on the far wall of the distal common carotid artery were optimized and recorded on super VHS videotape along with an ECG lead for subsequent offline analysis. A thorough search of the distal common carotid, carotid bulb, and internal and external carotid arteries on both sides was also made to determine the presence of focal plaque. Plaque was defined as a clearly identified area of focal increased thickness (>1 mm) of the intima-media layer. The IMT was defined as the distance between the characteristic echoes from the lumen-intima and media-adventitia interfaces.16 End-diastolic images were digitized, and a semiautomated edge-detection software program was used to identify leading-edge echo-interface points from the far wall of the distal 1 cm of the common carotid artery.16 Three end-diastolic images were analyzed from the right and left distal common carotid arteries at a site free of any discrete plaque, and measurements were averaged to give the mean IMT. Repeated measurement of randomly selected scans revealed no significant variation in the IMT measurement obtained during any specific time period of the study. Quality control measures included repeated scans on a subset of 30 subjects on 2 separate occasions 7 to 10 days apart. The intraobserver coefficient of variability was 2.9% for sonographer 1 and 4.8% for sonographer 2. The interobserver coefficient of variability was 5.9%.

Statistical Analysis
Allele frequencies were calculated by the law of Hardy-Weinberg21 to determine whether the observed prevalences of each genotype were in equilibrium. The $\chi^2$ goodness-of-fit test was used to test for equilibrium and to assess for trends across ferritin quartiles. Logarithmic transformation of serum ferritin was performed to normalize the distribution, and all mean ferritin results are obtained from log-transformed data. Results are expressed as mean ± SD. Spearman rank correlation analysis was used to describe the association of continuous vascular risk factors, including plasma homocysteine, with ferritin. Determinants of serum ferritin were assessed by stepwise multiple linear regression.

Carotid mean IMT and serum ferritin were treated as continuous variables for linear regression, while serum ferritin was entered in quartiles for the purpose of logistic regression. Focal carotid plaque was considered a categorical variable. Multivariate linear analysis was used to examine the independent associations of mean IMT with serum ferritin and/or C282Y genotype. Logistic regression was used to test the independent relation between serum ferritin and/or C282Y genotype (independent variable) and focal plaque (dependent variable). The adjusted odds ratios from logistic regression are presented with the 95% CIs. Analysis was performed with SAS statistical software.22 Paired comparisons were tested by Wilcoxon signed rank test. ANOVA was used to compare mean values between groups, and if overall significance was demonstrated, intergroup differences were assessed by multiple range testing. Statistical significance was taken as a 2-sided $P$ value of <0.05.

Results

Characteristics of Subjects
The characteristics of the study population divided by sex and including conventional risk factors, mean serum ferritin results, and C282Y mutation status are shown in Table 1. The prevalence of conventional risk factors was similar between sexes, except that women had lower values for BP, serum triglycerides, homocysteine, alcohol consumption, waist-hip ratio, ferritin, and smoking pack-years and had higher HDL cholesterol values than men.

Ferritin and C282Y Mutation Status
The mean ferritin value was 58±82 µg/L in women and 154±255 µg/L in men. The prevalence of C282Y heterozygotes (CY) in the overall population was 125 (11.4%) and of C282Y homozygotes (YY) was 9 (0.8%). There were no significant differences in the prevalences of either CY or YY
TABLE 1. Characteristics of the Study Population

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Women (n=545)</th>
<th>Men (n=553)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>53±13</td>
<td>52±13</td>
</tr>
<tr>
<td>Systolic BP, mm Hg</td>
<td>127±20</td>
<td>130±17*</td>
</tr>
<tr>
<td>Diastolic BP, mm Hg</td>
<td>79±11</td>
<td>82±9*</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>5.6±1.0</td>
<td>5.5±1.0</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>3.6±0.9</td>
<td>3.7±0.9</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.5±0.4</td>
<td>1.2±0.3*</td>
</tr>
<tr>
<td>Triglyceride, mmol/L</td>
<td>1.1±0.6</td>
<td>1.5±0.8*</td>
</tr>
<tr>
<td>Homocysteine, μmol/L</td>
<td>11.2±3.8</td>
<td>12.9±3.1*</td>
</tr>
<tr>
<td>Smoking, pack-years</td>
<td>7.9±16.3</td>
<td>16.7±24.7*</td>
</tr>
<tr>
<td>Alcohol, g/d</td>
<td>5.3±8.4</td>
<td>12.3±15.6*</td>
</tr>
<tr>
<td>Obese (BMI&gt;30), %</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>Waist-hip ratio</td>
<td>0.77±0.07</td>
<td>0.90±0.05*</td>
</tr>
<tr>
<td>Diabetes, %</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Family history of IHD, %</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>Myocardial infarction, %</td>
<td>2</td>
<td>6*</td>
</tr>
<tr>
<td>Stroke, %</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Serum ferritin, μg/L</td>
<td>58±82</td>
<td>154±255*</td>
</tr>
<tr>
<td>Wild-type (CC) genotype, n (%)</td>
<td>482 (88.4%)</td>
<td>482 (87.2%)</td>
</tr>
<tr>
<td>Heterozygous (CY) genotype, n (%)</td>
<td>61 (11.2%)</td>
<td>64 (11.6%)</td>
</tr>
<tr>
<td>Homozygous (YY) genotype, n (%)</td>
<td>2 (0.4%)</td>
<td>7 (1.3%)</td>
</tr>
</tbody>
</table>

BMI indicates body mass index; IHD, ischemic heart disease. Values are mean±SD.

*P<0.05 for difference between women and men.

Table 1 shows the relationship between ferritin values and continuous variables for both sexes. Ferritin levels were positively correlated with age, mean IMT, triglyceride, and waist-hip ratio. Multiple linear regression demonstrated age, triglyceride, and waist-hip ratio as independent predictors of ferritin in both sexes.

Risk Factors and Ferritin

Table 2 shows the relation between ferritin and continuous risk predictors by Spearman rank correlation in this community population. Only those continuous risk predictors that achieved a significant relationship with ferritin are shown, with the exception of C-reactive protein, which has been included to test for any effect of inflammatory conditions on ferritin values. Ferritin in women was positively correlated with age, mean IMT, systolic and diastolic BP, cholesterol, LDL cholesterol, triglyceride, homocysteine, body mass index, and waist-hip ratio. Analysis by multiple linear regression demonstrated age, triglyceride, and waist-hip ratio as independent predictors of ferritin in women (Table 2). Overall, approximately 21% of the variability in serum ferritin was explained by these factors (model $R^2=0.21$, $P=0.0001$).

In men, ferritin was negatively associated with age and mean IMT and positively associated with alcohol intake, body mass index, and waist-hip ratio. Multiple linear regression selected age, alcohol intake, and triglyceride as independent predictors of ferritin in men (Table 2), and these factors explained approximately 15% of the variability in serum ferritin (model $R^2=0.15$, $P=0.0001$).

Ferritin, C282Y Genotype, and IMT

On univariate analysis serum ferritin showed a significant association with increased mean IMT, defined as values above the 80th percentile of mean IMT for both sexes.
The ferritin levels for subjects with increased IMT compared with those without were 84±99 versus 54±79 μg/L, respectively, for women (P=0.0001) and 131±165 versus 162±433 μg/L, respectively, for men (P=0.007). However, after adjustment for conventional risk factors with the use of the multivariable linear model, there was no independent association of ferritin with IMT in either sex. With regard to the C282Y mutation, heterozygous (CY) genotype status was not associated with increased IMT in either sex with the use of the multivariable linear model. There were insufficient homozygote (YY) subjects to permit meaningful analysis.

**Ferritin, C282Y Genotype, and Carotid Plaque**

Univariate analysis demonstrated a significant association of serum ferritin with the presence of focal plaque in women: ferritin values for subjects with and without plaque were 94±105 and 51±73 μg/L, respectively, for women (P=0.0001) and 144±8 and 159±31 μg/L, respectively, for men (P=0.06). The cutoff thresholds for ferritin quartiles were <34, 34 to 62, 63 to 113, and >113 μg/L for women and <98, 98 to 170, 171 to 270, and >270 μg/L for men. When the number of subjects with focal plaque was calculated across ferritin quartiles, the percentages of women with plaque were 9%, 12%, 30%, and 37% in ascending quartiles of ferritin (P for trend<0.001) compared with 36%, 29%, 21%, and 29% in the men (P for trend=NS).

Table 3 shows ferritin quartiles and heterozygous C282Y genotype as risk predictors of carotid plaque for both sexes, obtained by stepwise logistic regression analysis. Odds ratios were adjusted for age, LDL cholesterol, systolic BP, pack-years of smoking, waist-hip ratio, history of diabetes, and homocysteine. Women with ferritin values in the second or fourth quartile had a significant association with carotid plaque when compared with the first quartile, although there was no significant trend across quartiles. When women with ferritin values in the second, third, and fourth quartiles were combined and the statistical analysis was repeated, they had an adjusted odds ratio of 2.1 (95% CI, 1.3 to 3.4; P=0.0016) for carotid plaque compared with the first quartile. Ferritin was not associated with carotid plaque in men. With regard to the C282Y mutation, heterozygous (CY) genotype status was not associated with carotid plaque in either sex with the use of the multivariate linear model. There were insufficient homozygote (YY) subjects to permit meaningful analysis.

**Discussion**

In our study of asymptomatic carotid atherosclerosis in a community population, serum ferritin levels were an independent risk predictor for focal carotid plaque in women but did not predict carotid mean IMT in either sex. Heterozygosity for the C282Y mutation was common, constituting 11.4% (n=125) of the population, but proved to be unrelated to either carotid mean IMT or the presence of focal carotid plaque.

**Ferritin and C282Y Mutation Status**

Heterozygotes for the C282Y mutation are carriers of hereditary hemochromatosis, and their frequency varies widely according to the population studied, for example, 7.2% and 6.7% in a Dutch study and Finnish community population, respectively, compared with 14.1% in an Australian sample, one of the highest values reported. The present community population had a heterozygote frequency of 11.2% for women and 11.6% for men.

The ferritin values in our C282Y heterozygote and wild-type subjects were not significantly different. We previously reported no significant differences between ferritin levels in heterozygotes and wild-type subjects among a community-based Australian population of premenopausal and postmenopausal women and adult men. Ferritin levels were not quoted in the Dutch study that reported a significant increase in risk of vascular mortality for postmenopausal heterozygote women, but there was no difference in the ferritin levels of heterozygote and wild-type men studied in Finland.

The assertion that heterozygotes have significantly increased serum ferritin compared with wild-type subjects is based on US and Canadian studies conducted before the availability of genotyping for the C282Y mutation. Putative heterozygotes were identified on the basis of HLA typing in family studies of hereditary hemochromatosis probands, and there may have been a selection bias resulting from studying...
hereditary hemochromatosis families rather than a community population.

Ferritin and Cardiovascular Risk Factors

Univariate analysis by Spearman rank correlation (Table 2) shows that ferritin is correlated with several conventional risk predictors specific to each sex. Ferritin may be elevated in infection, inflammation, or malignancy, and this may interfere with its use as a valid measure of body iron stores. The lack of significant correlation of C-reactive protein levels with ferritin indicates that chronic disease did not substantially affect our community population.

Multiple linear regression selected age, serum triglycerides, and waist-hip ratio as independent positive predictors of ferritin levels in women and alcohol intake and serum triglycerides as independent positive predictors of ferritin levels in men. Age was a predictor in women because of the physiological increase in mean ferritin levels that occurs after menopause. Age proved to be a negative predictor in men, whereas a large previous study of Danish men aged 30 to 60 years reported no significant change with age. The same study found that serum triglyceride levels and alcohol intake were independent predictors of ferritin in Danish men and women. However, the clinical significance of these reported associations is uncertain.

Ferritin and Carotid Ultrasound

In the present study univariate analysis demonstrated a positive relationship between serum ferritin concentration and carotid mean IMT in women that was lost after adjustment for conventional risk factors. A US study using a matched case-control design also reported loss of the association of ferritin with carotid IMT after adjustment for major cardiovascular risk factors. In a cross-sectional study of 206 Finnish men, neither ferritin nor dietary iron levels were associated with carotid bifurcation atherosclerosis. The Bruneck study used a composite plaque score as an index of early carotid atherosclerosis, and reported serum ferritin was a strong predictor of the overall progression of atherosclerosis over a 5-year follow-up period.

Although there was no trend across the quartiles, ferritin values in either the second or fourth quartile were independent risk predictors for focal carotid plaque in women (Table 3) compared with the first quartile (<34 µg/L). Women with ferritin values higher than the first quartile (>34 µg/L) had an adjusted odds ratio of 2.1 (95% CI, 1.3 to 3.4; P=0.0016) for carotid plaque compared with the first quartile. The relationship between serum ferritin concentration and focal carotid plaque occurred only in women, and a possible explanation may relate to their lower ferritin levels. When the number of subjects with focal plaque across each ferritin quartile was calculated, we found similar prevalences of plaque formation in women with ferritin values in the fourth quartile (>113 µg/L) and men with ferritin values in the first quartile (<98 µg/L). If we hypothesize that a threshold effect operates in which lower ferritin values are protective against focal carotid plaque formation, then subjects of either sex at higher levels may be equally at risk of developing plaque. A low ferritin threshold for carotid atherosclerosis was reported in the Bruneck study: both men and premenopausal women with ferritin levels <50 µg/L had similar low incidences of plaque score, at 0.3/100 versus 0.2/100 person-years, respectively. The mechanism of the apparent protective effect is unclear but may relate to a decreased susceptibility of LDL cholesterol to oxidative modification where body iron stores are low.

C282Y Genotype and Atherosclerotic Events

Our study finds that C282Y genotype status was not related to either carotid mean IMT or the presence of focal carotid plaque. We could not find references to any previous studies relating C282Y genotype to early atherosclerosis assessed by carotid ultrasound; however, 2 studies using cardiovascular disease as end points have also reported no association with HFE genotype. A study of 265 patients reported that premature (<50 years of age) coronary or peripheral atherosclerosis was not associated with either the C282Y or the H63D mutation of the HFE gene. Another study compared the prevalence of HFE mutations in 2 groups of patients with coronary artery disease of early (<50 years of age) or late onset (>65 years of age) and found similar prevalences in both groups.

However, recent prospective population-based studies have reported an association between heterozygosity for the C282Y mutation and cardiovascular events. A study of 12,239 Dutch women followed for up to 16 to 18 years showed that incidence rate ratios of C282Y heterozygosity were 1.5 for mortality by myocardial infarction, 2.4 for cerebrovascular mortality, and 1.6 for total cardiovascular mortality. A prospective study of 1150 Finnish men followed for a mean period of 9 years found that C282Y heterozygosity was associated with a 2.3-fold increased risk of acute myocardial infarct. Although our results for carotid wall thickening or the presence of focal carotid plaque did not reveal any association with C282Y heterozygosity, this does not necessarily contradict the previous independent findings that heterozygotes are at increased risk of vascular events. Sullivan has speculated that iron may have a role in ischemic events other than initiating or promoting vascular structural lesions and that increased vascular events could conceivably occur without any observed increase in structural lesions.

We conclude that serum ferritin was not related to carotid mean IMT in our community population. Serum ferritin proved to be an independent risk predictor for focal carotid plaque in women, and this may relate to a protective effect of low serum ferritin levels. We report that C282Y genotype status was unrelated to either carotid mean IMT or the presence of focal carotid plaque.

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