Association Between Increased Iron Stores and Impaired Endothelial Function in Patients With Hereditary Hemochromatosis

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OBJECTIVES
We studied associations between iron status and early functional and structural vascular abnormalities in patients with hereditary hemochromatosis (HH).

BACKGROUND
Iron may be involved in atherogenesis, and patients bearing a genetic mutation associated with HH are possibly at risk of developing coronary heart disease.

METHODS
We studied the vascular properties of 41 HH patients who had homozygosity for the C282Y mutation, along with 51 age-matched control subjects, by determination of endothelium-dependent dilation (EDD) of the brachial artery and intima-media thickness (IMT) of the carotid artery.

RESULTS
Male HH patients who were not receiving phlebotomy therapy showed a reduced EDD and increased IMT compared with controls and HH patients receiving therapy. In female HH patients, irrespective of treatment status, vascular parameters were not different from those of controls, and none of these patients had severe iron overload. In HH patients, increased iron load was significantly associated with reduced EDD and increased IMT. Moreover, we found a positive correlation between body iron stores and indicators of oxidative stress. When previously untreated male HH patients were re-investigated after intensive phlebotomy therapy, a significant improvement in EDD was observed (2.6 ± 1.3% before vs. 5.5 ± 2.1% after treatment, p = 0.0015).

CONCLUSIONS
Impaired endothelial function and increased IMT are associated with iron overload, with subsequent induction of oxidative stress, and are not linked to a genetic disability in HH patients. Consequent iron-depletion therapy normalizes endothelial function and may thus reduce the increased risk of cardiovascular events. Female patients may be at a reduced risk, presumably due to continuous iron loss by menstruation. (J Am Coll Cardiol 2002;40:2189–94) © 2002 by the American College of Cardiology Foundation

Iron is a transition metal that catalyzes the formation of reactive oxygen species (ROS) by the Fenton reaction (1). Oxygen radical formation and subsequent lipid peroxidation are postulated to be involved in the pathogenesis of atherosclerosis (2). Several epidemiologic studies have investigated the role of iron as a potent risk factor in coronary heart disease (CHD) (3–9). Elevated stores of iron in the body were associated with an increased risk of CHD-related death or myocardial infarction (MI) in some (3–5) but not all (6–9) studies. Recent studies in subjects heterozygous for a cysteine-to-tyrosine mutation at amino acid position 282 (C282Y) within the hemochromatosis gene (HFE), associated with hereditary hemochromatosis (HH), identified those persons to be at an increased risk of cardiovascular death and MI (10,11).

An increased intima-media thickness (IMT) of carotid arteries has proven to be a reliable marker reflecting early structural vascular pathology associated with cardiovascular risk factors and CHD prevalence (12). Despite impressive correlations between increased body iron stores and early atherosclerotic lesions in some studies (13,14), another report failed to confirm this association (15). Endothelial dysfunction, preceding the appearance of structurally evident atherosclerosis, has been recognized as an important early functional abnormality in atherogenesis (16) and accepted as a surrogate marker of vascular pathology leading to atherosclerosis (17).

In patients with iron overload due to HH, these functional and structural markers of increased cardiovascular risk had not been assessed thus far. To this aim, we investigated in the present study the inter-relationship between brachial artery endothelial function and early structural changes in carotid arteries, and parameters of iron overload and oxidative stress in HH subjects with and without iron-depletion therapy, along with age- and gender-matched controls.

METHODS

Subjects. From a list of 1,797 subjects genotyped for the C282Y and H63A mutations of the HFE gene, according to Simonsen et al. (18) at the Department of Internal Medicine in Innsbruck, Austria, between March 1996 and

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December 1999, we found 119 of these patients to be homozygous for the C282Y mutation. These subjects were invited to participate in this study. Of 105 subjects who were willing to participate, 64 were excluded because they met at least one of the following criteria: age >65 or <18 years, clinical evidence of HH-related advanced disease (e.g., liver cirrhosis, cardiomyopathy, diabetes mellitus) or cardiovascular disease, and any type of medical treatment, including intake of anti-oxidant agents. Presence of cardiovascular disease was determined by taking a history, physical examination, and rest electrocardiogram (ECG); presence of diabetes mellitus was determined by measuring fasting plasma glucose. Subjects homozygous or heterozygous for the H63A mutation of the HFE gene were excluded, as the clinical role of this mutation in the pathophysiology of HH is not very well understood (19). Fifty-one healthy subjects were willing to participate, 64 were excluded because they were homozygous or heterozygous for the C282Y mutation. These subjects were matched for age, gender, blood pressure, body mass index, and nicotine consumption, recruited from hospital staff members, served as control subjects. None of the control subjects was either homozygous or heterozygous for the respective HH-associated HFE mutations, C282Y or H63A. All participants gave written, informed consent. This study conformed to the principles outlined in the Declaration of Helsinki.

**Study protocol.** Blood samples were drawn after an overnight fast and 12-h abstinence from smoking. Thereafter, all subjects were given a typical continental breakfast and refrained from further food intake and smoking. At 1:00 PM, vascular studies were performed after taking a medical history and measuring resting pulse and blood pressure in subjects in a supine position. Previously untreated patients with HH were subjected to regular phlebotomy, according to standard guidelines (20), after the baseline examination and were re-examined after three to six months of therapy.

**Brachial artery study.** Endothelium-dependent and -independent dilation (EDD and EID, respectively) of the brachial artery were determined as described by Celermajar et al. (17). In brief, this vessel was scanned 2 to 15 cm above the elbow with the use of a 13.0-MHz, linear-array transducer and a standard Acuson Sequoia 512 system (Acuson, Mountain View, California). After recording a rest scan, a pneumatic cuff was placed around the forearm and inflated to a pressure of 250 mm Hg for 4.5 min. Pressure release resulted in reactive hyperemia, which is the stimulus for flow-mediated EDD. A scan of the brachial artery was performed within 45 to 90 s after cuff deflation. Thereafter, a period of 10 min was allowed for recovery of the vessel. Sublingual glyceryl trinitrate was then administered (400 μg) to induce EID, and 3 to 4 min later the scan was taken.

The vessel diameter was measured by two independent investigators who were unaware of the subjects’ clinical details and stage of study. The technique for diameter measurement was highly reproducible in our laboratory and showed a coefficient of variation of <3%, based on measurements taken from the same subjects on separate days (21). Both EDD and EID were determined as the percentage of diameter change relative to the mean value of the corresponding baseline measurements.

**Carotid artery study.** Longitudinal B-mode scans of the common carotid artery were obtained immediately after the studies of brachial artery reactivity, using the same ultrasound system and a 9.0-MHz, linear-array transducer. The far wall was assessed just proximal to the carotid bifurcation (last 2 cm) to identify the maximal IMT, defined as the distance between the junction of the lumen and intima and that of the media and adventitia (12). Three measurements were made in the right and left carotid arteries and were averaged to determine the IMT for each side.

**Chemical analyses.** Thiobarbituric acid–reactive substance (TBARS) levels in the serum samples were measured spectrophotometrically at 535 nm, exactly as described by Buege and Aust (22), using 1,1,3,3-tetramethoxypropane (Sigma Chemical Co., Munich, Germany) as a standard. Plasma levels of total cholesterol, low-density and high-density lipoprotein cholesterol, and triglycerides were measured by standard automated enzymatic or turbimetric assays. Serum iron was measured by a ferrozine-based spectrophotometric assay; the transferrin concentration by a turbimetric method; and ferritin by an enzyme-linked immunosorbent assay (23). Gluthathione levels in plasma were determined by means of a commercially available assay (Calbiochem, Darmstadt, Germany) with a detection limit of 5 μmol/l. Ferroxidase activity was measured as a parameter of increased iron turnover and measured spectrophotometrically, according to Erel (24).

**Statistical analysis.** Analyses were performed by using the statistical software package SYSTAT, version 7.01 (SPSS, Chicago, Illinois). Continuous variables were compared by using the Student t test. Proportions were compared by using the Fisher exact test. Because iron parameters (ferritin) followed skewed rather than gaussian distributions, they were also evaluated by non-parametric statistical analyses (Kruskal-Wallis), and a Bonferroni correction was applied when p values were calculated. Correlations among various measures were assessed by the Spearman rank correlation technique.
Table 1. Baseline Clinical and Biochemical Characteristics in Treated and Untreated Patients With Hereditary Hemochromatosis and Control Subjects

<table>
<thead>
<tr>
<th></th>
<th>Control Subjects</th>
<th>Treated Patients With HH</th>
<th>Untreated Patients With HH</th>
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<tbody>
<tr>
<td></td>
<td>Women (n = 14)</td>
<td>Men (n = 37)</td>
<td>Women (n = 6)</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>32.0 ± 8.4</td>
<td>40.1 ± 10.0</td>
<td>38.3 ± 11</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>21.7 ± 1.9</td>
<td>24.9 ± 2.9</td>
<td>23.9 ± 6.1</td>
</tr>
<tr>
<td>Smokers</td>
<td>0 (5.1%)</td>
<td>0 (13.5%)</td>
<td>0 (15.8%)</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>122 ± 9</td>
<td>122 ± 11</td>
<td>117 ± 13</td>
</tr>
<tr>
<td>No. of phlebotomies</td>
<td>0</td>
<td>0</td>
<td>1.9 ± 1.5</td>
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Baseline vascular characteristics. Brachial artery EDD was significantly reduced in untreated male HH patients compared with controls and treated HH patients, with no difference between treated HH patients and controls (Table 2). In women, no difference in EDD was observed between HH patients and controls, irrespective of treatment status. Baseline vessel diameter, baseline blood flow, reactive hyperemia, and EID were not different between HH patients and controls, for both genders. An increased IMT of the common carotid artery was observed in untreated male HH patients compared with controls and treated patients. In women, no differences in IMT were observed between the different groups.

Changes in clinical, laboratory, and vascular parameters after phlebotomy in previously untreated hemochromatotic patients. Among the 41 HH patients studied, 10 male and 6 female individuals were not yet receiving phlebotomy treatment at study entry. Although none of these female patients had evidence of severe iron overload in serum, which would have justified the initiation of regular phlebotomy treatment, all male patients had severe iron overload and were assigned to treatment with intensive phlebotomy. Furthermore, Tables 1 and 2 show the effect of phlebotomy therapy on the clinical, biochemical, and vascular characteristics of these 10 male HH patients. Not surprisingly, parameters of iron overload (e.g., ferritin, iron, transferrin saturation) were reduced after phlebotomy therapy. Moreover, in parallel, we found that TBARS became

RESULTS

Baseline clinical and laboratory data. Of the 41 HH subjects investigated, 25 were already receiving phlebotomy therapy at study entry, whereas 16 were untreated. Male patients receiving therapy had significantly more phlebotomies performed per year than female patients (Table 1). The baseline clinical and biochemical characteristics of control subjects and treated and untreated HH patients are shown in Table 1, with separate analyses for men and women. As expected, parameters of iron overload were higher in subjects with HH compared with controls and revealed impressive differences when comparing male and female groups. Untreated male HH patients showed a higher level of iron overload than male patients already receiving phlebotomy treatment at study entry, a difference that was not evident between untreated and treated female HH patients. We also determined fasting plasma glucose and glycosylated hemoglobin, but no differences were found between the respective groups (details not shown).

Serum levels of TBARS were significantly higher in HH patients compared with controls, irrespective of gender, but again, the levels were highest in untreated male patients. Reduced levels were also observed for the radical scavenger glutathione in subjects with HH compared with controls.
Functional measures of the brachial artery
Structural measures of the common carotid artery
for untreated male patients before versus after phlebotomy therapy. Data are presented as the mean value
significantly ameliorated with iron-depletion therapy. During therapy, hemoglobin concentrations and hematocrit levels declined, but no patient became anemic.
Most interestingly, EDD significantly improved after phlebotomy therapy and was, at this time, not different from that of previously treated HH subjects and even controls (Tables 1 and 2, respectively). All other vascular parameters, including IMT, did not change within this observation period.

Inter-relationship between vascular parameters, iron metabolism, and oxidative stress. To see whether EDD, IMT, iron burden, and oxidative stress are in a relationship together, we calculated Spearman rank correlations among these respective parameters (Table 3). When investigating treated HH patients, untreated HH patients, and controls separately, the correlations among the parameters remained the same within all groups (details not shown).

Table 3, showing the results obtained in all HH patients, suggests that increased iron burden, as estimated by higher ferritin levels and transferrin saturation, is associated with impaired EDD and increased IMT. In contrast, neither hemoglobin nor hematocrit showed a significant relationship to EDD or IMT.
Moreover, impaired EDD and increased IMT were correlated with TBARS levels, and this association was even more pronounced when investigating all subjects (both controls and HH patients), which can be related to the higher number of individuals (n = 92) involved in this analysis (p = 0.003 for EDD and TBARS; p = 0.064 for IMT and TBARS). As a parameter of oxidative stress, TBARS was also positively associated with increased iron concentrations in serum (Table 3), again with the correlation being more significant when the analysis included all subjects participating in the study (p = 0.001 for ferritin and TBARS; p < 0.001 for transferrin saturation and TBARS). Finally, IMT was inversely related to EDD.

DISCUSSION

Our study shows that endothelial function is impaired in HH patients with profound iron overload. Because reduced EDD was closely associated with increased levels of ferritin and transferrin saturation, we consider that endothelial dysfunction is linked to iron overload. This assumption is supported by various findings of our study.
First, untreated male HH patients with excessive iron overload were the only group with EDD impairment. Second, we found that a reduction in iron burden by

| Table 2. Baseline Vascular Characteristics in Treated and Untreated Patients With Hereditary Hemochromatosis and Control Subjects |
|---|---|---|---|---|
| Functional measures of the brachial artery | \[\text{Control Subjects} \quad \text{Treated Patients With HH} \quad \text{Untreated Patients With HH} \quad \text{Men (n = 10)} |
| Women (n = 14) | Men (n = 37) | Women (n = 6) | Men (n = 19) | Women (n = 6) | Before Phlebotomy | After Phlebotomy |
| Baseline diameter (mm) | 3.8 ± 0.4 | 4.5 ± 0.5 | 3.6 ± 0.3 | 4.8 ± 0.6 | 3.9 ± 0.3 | 4.5 ± 0.5 | 4.6 ± 0.3 |
| Baseline blood flow (mL/s) | 138 ± 19 | 141 ± 32 | 138 ± 52 | 136 ± 43 | 145 ± 29 | 145 ± 50 | 132 ± 55 |
| Reactive hyperemia (%) | 389 ± 71 | 384 ± 76 | 443 ± 76 | 420 ± 99 | 353 ± 43 | 409 ± 47 | 398 ± 38 |
| EDD (%) | 8.9 ± 5.5 | 6.2 ± 3.2 | 6.6 ± 2.4 | 5.2 ± 2.9 | 8.9 ± 1.1 | 2.6 ± 1.3† | 5.5 ± 2.1¶ |
| EID (%) | 21.1 ± 6.1 | 14.9 ± 4.3 | 19.6 ± 4.8 | 14.5 ± 6.1 | 21.7 ± 4.5 | 12.9 ± 5.1 | 12.4 ± 2.6 |

Structural measures of the common carotid artery
Intima-media thickness (mm)

- Right artery
  - Women: 0.41 ± 0.05, 0.55 ± 0.12
  - Men: 0.47 ± 0.07, 0.56 ± 0.16
  - Change: 0.06 ± 0.07, 0.68 ± 0.08‡¶, 0.68 ± 0.07

- Left artery
  - Women: 0.44 ± 0.06, 0.57 ± 0.14
  - Men: 0.48 ± 0.06, 0.58 ± 0.20
  - Change: 0.04 ± 0.03, 0.71 ± 0.15§, 0.71 ± 0.13

- Combined arteries
  - Women: 0.42 ± 0.05, 0.56 ± 0.13
  - Men: 0.47 ± 0.05, 0.57 ± 0.18
  - Change: 0.05 ± 0.04, 0.70 ± 0.11§, 0.70 ± 0.10

| Table 3. Inter-relationship Between Serum Concentrations of Iron Metabolism Parameters, Oxidative Stress, and Endothelial Function in Patients With Hereditary Hemochromatosis |
|---|---|---|---|---|
| Ferritin | TS | TBARS | EDD | IMT |
| TS | 0.664 (<0.001) | — | — | — |
| TBARS | 0.455 (0.03) | 0.389 (0.07) | — | — |
| EDD | -0.730 (<0.001) | -0.530 (0.004) | -0.382 (0.07) | — |
| IMT | 0.544 (0.002) | 0.403 (0.06) | 0.316 (NS) | -0.617 (<0.001) |
| Hct | 0.261 (NS) | 0.183 (NS) | 0.217 (NS) | -0.267 (NS) |

Data are presented as correlation coefficients with p values (Bonferroni-corrected) in parentheses. NS = not significant at p > 0.1. The results for patients with hereditary hemochromatosis, irrespective of therapy status, are shown (n = 41). EDD = endothelium-dependent dilation; Hct = hematocrit; IMT = intima-media thickness; TBARS = thiobarbituric acid-reacting substances; TS = transferrin saturation.
initializing phlebotomy therapy in previously untreated male HH patients led to both a reduction in the parameters of iron overload and a significant improvement of endothelial function. Third, EDD was not impaired in male HH patients treated with phlebotomy at study entry; they demonstrated only moderate iron overload at this time. Fourth, in females, we were unable to reveal any difference in endothelial function between controls and HH patients, irrespective of treatment with phlebotomy. This is in agreement with the observation that even untreated women with HH have an almost balanced iron status, which could be explained, in part, by the fact that there is an additional iron loss in women due to menstruation. This notion is supported by the finding of significantly less phlebotomies per year in women compared with men.

Endothelium-independent dilation induced by application of the exogenous nitric oxide (NO) donor glyceryl trinitrate, and considered to reflect vascular responsiveness independent of endogenous NO production, was unaffected in patients with HH. Thus, iron impairs EDD by modulating endothelial function. Although iron can reduce the formation of NO in the endothelium (25), thereby affecting endothelial dilation, it is more likely that impaired EDD in iron overload is linked to the capacity of the metal to catalyze the formation of ROS by the Haber-Weiss reaction (1). This leads to oxidative stress, as reflected by our finding of an association between increased TBARS and reduced glutathione levels with iron overload and impaired EDD, which is also confirmed by a recent report studying the effects of iron infusion on EDD (26). Oxidative stress may cause lipid peroxidation (2) or lead to impairment of endothelium-dependent signaling processes (1,27), with a subsequent reduction of endothelial relaxation.

Endothelium-dependent dilation could be improved after phlebotomy therapy in previously untreated patients (Tables 1 and 2) or by application of the iron chelator deferoxamine in patients with CHD (28). Thus, endothelial function is not generally impaired in patients with HH as a function of genetics, as determined by mutations within the HFE gene, but rather is a consequence of iron overload (29). From our calculations, it is further suggestive that EDD is primarily influenced by iron burden and not directly by hemoglobin or hematocrit (Table 3).

We found an association between iron overload and early structural atherosclerotic changes, as reflected by increased IMT in the group of previously untreated male HH patients. In contrast, in patients receiving phlebotomy therapy and in women, IMT was not significantly different from that of controls. Thus, prolonged iron overload and subsequent oxidative stress may ultimately result in increased IMT, but consequent iron-depletion therapy may prevent the development of early atherosclerotic changes. A recent study indicated that prolonged and consequent iron-depletion therapy may reduce previously increased radial artery wall thickness in subjects with HH (30), which could not be confirmed by our study within a short treatment period of three months.

Because impaired EDD and increased IMT reflect early functional and structural abnormalities in atherogenesis and are thus accepted as surrogate indicators of an increased cardiovascular risk (12,17), our data support the hypothesis that iron is a risk factor of CHD. In 1981, Sullivan (31) proposed that the difference in the incidence of CHD between men and women could be explained by differences in stored iron. He argued that the physiologic blood loss by menstruation represents the underlying mechanism for protective iron depletion. Consequently, iron depletion by regular blood loss decreases the risk of MI (32), whereas iron supplementation, especially in patients with renal disease, is associated with severe coronary events (33).

However, data on the risk of CHD in subjects with iron overload are conflicting and contradictory (3–9,13–15). From our data showing a linkage of EDD and IMT to iron overload, but not to the C282Y mutation of the HFE gene, it is suggestive that one reason for the conflicting data among these studies is that the patients investigated were heterogeneous in terms of iron burden and effectiveness of iron-depletion therapy (34).

Study limitations. The number of untreated HH patients investigated was small. This bears the potential bias of results by a few outliers and chance findings. However, even when comparing the small number of untreated male patients before and after phlebotomy therapy, we found significant differences with both parametric and non-parametric tests, even with a Bonferroni correction. We were unable to recruit untreated female subjects with severe iron overload, and we did not study postmenopausal women with or without HH. Thus, we cannot provide information on the chances of EDD and IMT and their relationship to iron overload in such patients. Our study also had the limitation that the intervention portion of the previously untreated patients was not randomized. We used indirect indicators of oxidative stress to verify the inter-relationship between iron, oxidative stress, and endothelial dysfunction. Although the TBARS assay lacks some specificity to reflect lipid peroxidation, it is a widely used method to monitor oxidative stress. Moreover, our TBARS level findings are supported by our measurement of glutathione levels, which followed iron-modulated changes in a similar way. Finally, we cannot rule out the contribution of environmental influences or genetic backgrounds, other than HFE mutations, to endothelial function or handling of iron or detoxification of radicals within the body. Although we have good evidence pointing to the cause-effect relationship between iron and EDD, we cannot rule out that factors other than modulation of iron homeostasis may be influenced by phlebotomy therapy, thus altering EDD. Moreover, it is also possible that the effect of iron on EDD is indirect, as iron may alter immune function, oxidative phosphorylation, NO production, susceptibility to infections, or the availability and function of micronutrients and vitamins (35,36).
Conclusions. Our results demonstrate that impaired EDD and increased IMT in untreated male HH patients are due to excessive iron overload and are not linked to a genetic disability. Endothelial dysfunction may be a reflection of iron induced oxidative stress, with both parameters improving with induction of iron-depletion therapy by phlebotomy. Whether the change in endothelial function may indeed reduce the risk of cardiovascular events has to be verified by prospective, randomized clinical studies.

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