Prevalence, Characteristics, and Prognostic Significance of HFE Gene Mutations in Type 2 Diabetes

The Fremantle Diabetes Study

OBJECTIVE — To examine the relationship between iron status, hereditary hemochromatosis (HFE) gene mutations, and clinical features and outcomes of type 2 diabetes in a well-characterized representative sample of community-based patients.

RESEARCH DESIGN AND METHODS — HFE genotype data were available for 1,245 type 2 diabetic patients from the longitudinal observational Fremantle Diabetes Study (FDS), representing 96.2% of the total FDS type 2 diabetes cohort. Data were collected at recruitment between 1993 and 1996 and annually until the end of June 2001. Hospitalization and mortality data were available until the end of June 2006. The presence of the C282Y HFE mutation was determined in all subjects and H63D in C282Y heterozygotes. Fasting serum iron, transferrin, and ferritin were measured in all C282Y homozygotes and C282Y/H63D heterozygotes and in 286 randomly selected wild-type subjects. Multiple logistic regression analysis was performed to determine independent baseline associates of prevalent complications (myocardial infarction, cerebrovascular disease, retinopathy, neuropathy, and nephropathy), as was Cox proportional hazards modeling to determine predictors of incident complications and mortality.

RESULTS — Although there were expected positive associations between HFE gene mutations and serum iron and transferrin saturation, there were no independent positive associations between HFE gene status and either microvascular or macrovascular complications in cross-sectional and longitudinal analyses. HFE gene status did not independently predict cardiac or all-cause mortality. Measures of iron metabolism including serum ferritin were not associated with combined microvascular or macrovascular end points.

CONCLUSIONS — Directed screening for iron overload and/or HFE mutations appears unwarranted in patients with type 2 diabetes.

Although early reports suggested that hemochromatosis protects against the chronic complications of diabetes (1), recent studies have identified adverse metabolic and vascular effects that could be associated with iron overload (2–11). Serum ferritin correlates positively with insulin resistance and glycated hemoglobin (2) and has been suggested to be an additional component of the metabolic syndrome (3). Hyperglycemia, and other effects of excess tissue iron including oxidant stress, angiogenesis, and fibrosis (4–6), could promote the development of complications such as nephropathy (7,8). The C282Y and H63D variants of the hemochromatosis (HFE) gene product are important determinants of iron storage. HFE-related hemochromatosis is considered to include C282Y homozygous and compound C282Y/H63D heterozygous genotypes (9). H63D and C282Y have been reported to be independent risk factors for diabetic nephropathy (10) and proliferative retinopathy (11), respectively.

Because most published studies have been small-scale and cross-sectional, with a restricted number of potentially explanatory variables, there is a need for detailed large-scale longitudinal studies examining the relationship between iron metabolism, HFE mutations, and the clinical features and complications of diabetes (4). Such studies are essential before directed screening for iron overload can be recommended in diabetic patients (12). We have, therefore, analyzed data from the observational Fremantle Diabetes Study (FDS) to assess the effects of iron status and HFE mutations on the characteristics and outcome of type 2 diabetes.

RESEARCH DESIGN AND METHODS — The FDS took place in a postal code–defined urban community of 120,097 people in the state of Western Australia. Descriptions of recruitment and details of nonrecruited patients have been published (13). Of 2,258 diabetic patients identified between 1993 and 1996, 1,426 (63%) entered the FDS and 1,294 had type 2 diabetes based on age at diagnosis, history of insulin treatment, adiposity, and other features including islet autoantibody status if required (13). Eligible patients who were not recruited were a mean of 1.4 years older than participants, but their sex distribution and the distributions of diabetes types and treatment modalities were similar (13,14). The FDS protocol was approved by the Fremantle Hospital Human Research Ethics Committee, and all subjects gave informed consent.
HFE gene mutations in type 2 diabetes

Clinical assessment
At baseline and annual reviews up to November 2001, a questionnaire was completed, and fasting biochemical tests were carried out in a single laboratory using standard methods (13). Assessments in a small minority of patients (≤3%) with self-reported acute intercurrent illness or with clinical signs of an acute infection were deferred until after recovery. Ethnic background was assessed as northern European (principally Anglo-Celts), southern European, Asian, African, Aboriginal, or other (13). Identification of the metabolic syndrome was by the definitions of the World Health Organization, the National Cholesterol Education Program’s Adult Treatment Panel III, and the International Diabetes Federation Consensus Group (14).

Complications were identified using standard criteria (15). Peripheral neuropathy was defined as a score ≥2 of 8 on the Michigan Neuropathy Screening Instrument clinical portion. Retinopathy was taken as any grade detected by direct/indirect ophthalmoscopy and/or ophthalmologist assessment. Self-reported stroke/transient ischemic attack was amalgamated with prior hospitalizations to define baseline cerebrovascular disease (CVD) status. Patients were classified as having coronary heart disease (CHD) if there was a self-reported history of/hospitalization for myocardial infarction (MI), angina, revascularization, and/or definite MI on a Minnesota-coded electrocardiogram. Peripheral arterial disease was considered present when the ankle-to-brachial index was ≤0.9. Nephropathy was defined as a first-morning urinary albumin-to-creatinine ratio ≥3.0 mg/mmol.

Mortality and hospital morbidity data
A government register records all hospitalizations in Western Australia and, with the death register, is part of the Western Australia Data Linkage System (WADLS) (16). The WADLS was linked to the FDS database to provide morbidity/mortality data from the beginning of the study until the end of June 2006. Causes of death were classified independently by two investigators (T.M.E.D. and D.G.B.) as cardiovascular/other (15), with consensus if required. Hospitalizations were categorized using International Classification of Diseases 9-CM and 10-AM codes (17).

Laboratory measurements
HFE gene mutations were determined in buffy-coat DNA by PCR amplification using published primers (18), followed by restriction enzyme cleavage and analysis on a 3% agarose gel. All subjects were assessed for C282Y using the unique Rsal digestion site. Because it is not clinically significant in the absence of C282Y (9,19), H63D was determined only in C282Y heterozygotes using the unique MboI digestion site. We divided subjects into 1) wild types, 2) C282Y/wild-type heterozygotes, 3) compound C282Y/H63D heterozygotes, and 4) C282Y homozygotes.

Fasting serum iron, transferrin, and ferritin were measured in available samples from C282Y homozygotes and heterozygotes and in 286 randomly selected wild-type subjects, using established assays (18). All sera were stored at −80°C until analysis. Serum iron was measured, after separation from transferrin in an acidic guanidine chloride solution and reduction with ascorbic acid, from the absorbance of the colored complex formed with ferrozine (Hitachi 917 analyzer; Boehringer Mannheim, Sydney, Australia). Serum transferrin was determined by rate immunoturbidity (Hitachi 917 analyzer). Transferrin saturation was calculated as [serum iron/(2 × serum transferrin)] × 100. Serum ferritin was measured using a two-site chemiluminescent immunoassay (ACS-180; Ciba, Coming, MA).

Data analysis
Pancreatic β-cell function (%B) and tissue insulin sensitivity (%S) were estimated from fasting serum glucose and insulin using homeostasis model assessment (20). Statistical analysis was performed using SPSS for Windows (version 14.0) and parametric or non-parametric tests as appropriate. Multiple linear regression analysis was used to examine relationships between indexes of iron status and %B and %S. Multiple logistic regression (forward conditional entry at P < 0.05, removal at P > 0.10) was performed to determine independent associates of prevalent baseline complications. Because of limited numbers, C282Y homo- and heterozygotes were pooled for comparison with wild-type subjects. Survival curves for all-cause mortality, cardiac death, or first occurrence of complications defined by HFE status were constructed using Kaplan-Meier estimates and compared by a log-rank test. Cox proportional hazards modeling (forward conditional entry at P < 0.05, removal at P > 0.05) was used to determine independent predictors of these events, with the validity of the proportional hazards assumption assessed from ln[−ln(survival)] curves and time-dependent covariates. All clinically plausible univariate variables with P < 0.20 were considered for entry.

RESULTS

Baseline patient characteristics
The characteristics of the 1,245 type 2 diabetic patients with complete HFE genotype data (96.2% of the total type 2 diabetes cohort) are summarized in Table 1. Major mutations were most frequent among the 786 patients of Anglo-Celt ethnicity and least in the 229 Southern Europeans. The significant relationship between education and HFE genotype reflects the fact that Anglo-Celts were more likely to have progressed beyond primary school. Apart from weak associations between serum lipids and genotype, there were no significant between-group differences in a range of demographic, biophysical, and diabetes-specific variables.

Of the eight C282Y homozygotes, two males were known to have hereditary hemochromatosis and were managed by regular venesection. The remaining six were previously undiagnosed—five were females aged 47–79 years with a serum ferritin ≤752 μg/l and the other was a 79-year-old male with a serum ferritin of 2,476 μg/l. All except the 79-year-old female were Anglo-Celt. None of the 14 C282Y/H63D heterozygotes had been diagnosed previously with hemochromatosis.

Baseline measures of iron and glucose metabolism
Valid indexes of iron metabolism were obtained from 430 patients including 286 who were wild type (Table 2). There were stepwise increases in serum iron and transferrin saturation from wild-type patients to C282Y homozygotes, especially in males. The same pattern was evident for serum ferritin, but there was relatively large within-group variability and no significant differences emerged. Although postmenopausal women had higher serum ferritin concentrations than those who were premenopausal, there were no significant differences between HFE genotype groups in either case (P > 0.68).

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There were no significant differences in ln(%B) and ln(%S) by HFE genotype for 386 non–insulin-treated patients (Table 2). In addition, there were no significant associations between serum iron, serum ferritin, or transferrin saturation and either ln(%B) or ln(%S) in multiple linear regression analyses that included ethnicity and type of oral hypoglycemic therapy as potential independent variables (P > 0.21).

**Prevalent complications and HFE status**

At baseline, there were no significant differences in the unadjusted prevalence of CHD, CVD, neuropathy, or nephropathy by HFE gene status (wild-type versus any
C282Y mutation; P = 0.083), but retinopathy was more common among wild-type patients (16.7 vs. 7.4%, P = 0.022). After adjustment for univariate associates of each complication in individual multiple logistic regression models, HFE gene status remained a nonsignificant associate of CHD, nephropathy, and neuropathy (P > 0.55) and became nonsignificant for retinopathy (P = 0.07). Non–wild-type patients were, however, half as likely to have CVD as wild-type patients after adjustment for age, diabetes duration, and systolic blood pressure (odds ratio [OR] 0.46 [95% CI 0.22–0.94]; P = 0.034).

Incident complications, mortality, and HFE status
The number of complication-free patients at baseline, the patient-years of follow-up, and the percentages of patients developing end points by HFE status are shown in Table 3. There were no differences between wild-type and non–wild-type patients in the time to hospitalization with death from MI or stroke or to the development of peripheral neuropathy, retinopathy, or nephropathy by log-rank test. After adjustment for the most parsimonious Cox proportional hazards model (the proportional hazards assumption was valid in all cases), HFE status was not associated with time to event for any outcome (Table 3). There was a similar finding when the small numbers of C282Y/H63D heterozygotes and C282Y homozygotes were pooled (n = 22) and compared with wild-type patients (data not shown).

Complications, mortality, and iron metabolism
Of 423 patients with follow-up data but without retinopathy, neuropathy, or nephropathy at entry, 139 had baseline measures of iron metabolism. Of these, 113 (81.3%) had a first microvascular complication during 425 patient-years (mean 3.1 ± 2.1 years) of follow-up. In Cox proportional hazards models, there was no association between serum iron, ferritin, or transferrin saturation and the first occurrence of microangiopathy (P > 0.32). Similarly, there were 1,255 type 2 diabetic patients without prior MI or stroke at baseline and 418 of these had measures of iron metabolism. Of these, 129 (30.9%) had a first MI, stroke, or cardiac/cerebrovascular death during 3,757 patient-years (mean 9.0 ± 3.9 years) of follow-up. In Cox models, there was no association between serum iron, ferritin, or transferrin saturation and the first occurrence of this combined macrovascular end point (P > 0.51).

CONCLUSIONS — The present data do not suggest that iron overload or the major HFE gene mutations have important pathophysiological consequences in community-based type 2 diabetic patients. There were the expected associations between HFE gene status and serum iron and transferrin saturation but no differences in either diabetes treatment or in

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**Table 2**—Serum iron, serum ferritin, transferrin saturation, β-cell function, and insulin sensitivity in the subgroups of patients with type 2 diabetes classified according to the major mutations

|                | Wild-type | Heterozygous C282Y | Compound heterozygous C282Y/H63D | Homozygous C282Y/C282Y | Trend P |
|----------------|-----------|--------------------|----------------------------------|------------------------|---------|
| n              | 286       | 123                | 13                               | 8                      |
| Serum iron (µg/l) |           |                    |                                  |                        |
| All            | 16.6 ± 5.4| 19.2 ± 7.5*        | 21.5 ± 5.0*                      | 26.1 ± 14.6            | <0.001  |
| Male           | 17.1 ± 5.2| 20.7 ± 8.0†        | 24.6 ± 4.9†                      | 39.6 ± 8.3†            | <0.001  |
| Female         | 16.1 ± 5.6| 17.3 ± 6.3         | 18.0 ± 1.4                       | 23.6 ± 14.9*           | 0.038   |
| Serum ferritin (µg/l) |         |                    |                                  |                        |
| All            | 111 (41–297) | 129 (51–329)  | 180 (68–478)                     | 136 (22–845)           | 0.20    |
| Male           | 139 (55–351) | 184 (82–409)†    | 225 (63–804)                     | 263 (35–1,980)         | 0.09    |
| Female         | 85 (32–231) | 85 (34–212)       | 139 (90–216)                     | 91 (15–559)            | 0.70    |
| Transferrin saturation (%) |     |                    |                                  |                        |
| All            | 22.9 ± 8.1| 27.5 ± 11.2†      | 34.9 ± 10.4†                     | 56.3 ± 30.7†           | <0.001  |
| Male           | 24.3 ± 8.3| 30.1 ± 11.8†      | 41.9 ± 8.6†                      | 71.3 ± 15.1            | <0.001  |
| Female         | 21.3 ± 7.6| 24.4 ± 9.5‡       | 26.8 ± 4.9                       | 47.2 ± 35.5            | <0.001  |
| n§             | 237       | 107                | 11                               | 5                      |
| β-Cell function (%) |       |                    |                                  |                        |
| All§           | 45 (18–108) | 43 (16–114)      | 34 (15–76)                       | 42 (7–271)             | 0.76    |
| Diet           | 54 (28–107) (85) | 65 (33–129) (41) | 32 (24–42) (2)                  | 109 (89–135) (4)       | NV      |
| Metformin alone| 32 (9–114) (24) | 40 (22–72) (20) | 46 (30–71) (2)                  | —                     | NV      |
| Sulfonylurea alone | 47 (21–106) (81) | 46 (27–78) (22) | 50 (22–113) (4)                | 41 (1)                | NV      |
| Combination therapy | 36 (17–77) (47) | 37 (22–61) (24) | 32 (19–52) (3)                 | —                     | NV      |
| Insulin sensitivity (%) |       |                    |                                  |                        |
| All§           | 38 (16–90) | 33 (14–77)        | 39 (20–78)                       | 39 (6–232)             | 0.36    |
| Diet           | 39 (20–79) (85) | 32 (15–65) (41) | 41 (22–77) (2)                  | 21 (16–29) (4)         | NV      |
| Metformin alone| 42 (14–122) (24) | 35 (22–55) (20) | 46 (30–71) (2)                  | —                     | NV      |
| Sulfonylurea alone | 41 (19–89) (81) | 35 (20–62) (22) | 39 (17–93) (4)                  | 22 (1)                | NV      |
| Combination therapy | 39 (18–84) (47) | 30 (17–52) (24) | 31 (22–44) (3)                 | —                     | NV      |

Data are means ± SD, geometric means (SD range), or means ± SD (n). *P < 0.01 vs. wild type; †P < 0.001 vs. wild type (uncorrected for multiple comparisons); §P < 0.05 vs. wild type; §data are from 362 non–insulin-treated patients. NV, not valid because of low numbers in some groups.
In addition, despite a higher prevalence of CVD in wild-type patients at entry, there were no associations between HFE gene status and either microvascular or other macrovascular complications in cross-sectional and longitudinal analyses, and incident CVD was similar in patients with or without HFE mutations. Similarly, HFE gene status was not an independent predictor of cardiac or all-cause mortality. In a subset of patients, indexes of iron status including serum ferritin were not associated with combined microvascular or macrovascular end points. These findings imply that directed screening for iron overload and/or HFE mutations is unwarranted in type 2 diabetes.

Because serum ferritin reflects inflammatory processes as well as total body iron stores (2), and it may cluster with other vascular risk factors (3), its possible role in the development of cardiovascular disease was first raised 25 years ago (25). However, we found no association between serum ferritin and combined complications in type 2 diabetes.

Table 3—Results of analysis of incident complications by HFE status

| Complication              | Follow-up patient-years (Mean ± SE) | Total Wild type | Total Non–wild type | P for HFE status |
|---------------------------|--------------------------------------|-----------------|---------------------|-----------------|
| Myocardial infarction     | 11,398 (9.4 ± 3.7)                   | 261 of 1,212 (21.5%) | 232 of 1,061 (21.9%) | 0.56            |
| Stroke                    | 11,961 (9.7 ± 3.5)                   | 127 of 1,242 (10.2%) | 110 of 1,089 (10.1%) | 0.69            |
| Peripheral neuropathy     | 2,617 (3.7 ± 2.2)                    | 438 of 700 (62.6%) | 377 of 615 (62.4%) | 0.68            |
| Retinopathy               | 3,723 (4.4 ± 2.3)                    | 277 of 848 (32.7%) | 236 of 731 (32.3%) | 0.91            |
| Microalbuminuria          | 2,537 (4.2 ± 2.3)                    | 255 of 610 (41.5%) | 221 of 535 (41.3%) | 0.51            |

ACR, albumin-to-creatinine ratio; PAD, peripheral arterial disease.
and macrovascular end points. The numbers of patients in these analyses were limited, and it is possible that, in larger cohorts and those with higher serum ferritin levels, such pathophysiological relationships exist. Nevertheless, the highest serum ferritin concentrations are in newly diagnosed diabetic patients (2), implying that glycemic and other vascular risk factor management has a normalizing effect. In addition, recent epidemiologic studies have not found an independent association between serum ferritin and cardiovascular disease (26). The present data are consistent with this finding in the high-risk context of type 2 diabetes.

Because 1) there were only 22 C282Y homozygous or C282Y/H63D heterozygous patients, 2) significantly elevated serum iron concentrations were observed in C282Y heterozygotes (without H63D), and 3) the published observation that the C282Y allele is associated with microangiopathy (11), we pooled all patients with C282Y for multivariate analysis of complications. Despite contrary evidence (10,11), the associations between HFE angio-pathy in our patients paralleled those for serum ferritin. We had sufficient numbers of patients to detect ORs of >1.80 and >2.0 for prevalent CHD and CVD, respectively, in non–wild-type patients with >80% power and to detect an OR >1.60 for incident MI and stroke with >90% power. Thus, if there is an effect of HFE mutations, and by implication increased tissue iron, on diabetes-related macroangiopathy, it appears limited.

In a cross-sectional study of 233 selected clinic-based type 2 diabetic patients (11), the presence of C282Y was associated with an adjusted OR (95% CI) for proliferative retinopathy of 6.1 (1.2–30.5). There were no between-group differences by H63D status, and no biochemical markers of serum iron status were provided (11). We had few patients with proliferative retinopathy in our larger community-based cohort but found no association between C282Y and prevalent or incident retinopathy of any grade. Our patient numbers were sufficient to detect an OR >1.80 for prevalent and >1.60 for incident retinopathy with 80% power, implying that a mildly increased C282Y-associated risk is still possible. However, the first study to examine the effect of hemochromatosis on diabetic microangiopathy found a protective effect (1). In addition, Peterlin et al. (11) postulate iron overload–related retinal damage to be the mechanism underlying their findings, but the lack of a relationship between iron status and microangiopathy in the present study does not support this contention.

A second microvascular complication with a possible association with an HFE mutation is nephropathy. Moczulski et al. (10) studied 424 type 2 diabetic clinic patients with varying degrees of urinary protein loss and 196 healthy control subjects. There were no differences in C282Y prevalence between normoalbuminuric and nephropathic patients, but there was an increased frequency of H63D mutations in the latter group (OR 1.8 [95% CI 1.2–2.8]) that persisted after adjustment for A1C and blood pressure (10). No biochemical measures of iron status were provided (10). Although the authors postulate that their findings reflect iron-related kidney damage, the H63D mutation only determines iron storage when in the H63D/C282Y heterozygous state (18,19). The relationship of iron-related tissue damage in hereditary hemochromatosis to that in hypertransfused patients with β-thalassemia (8) is also uncertain. In the latter situation, glycemic control but not serum ferritin is associated with microalbuminuria, whereas regular desferoxamine chelation and antioxidant vitamin supplementation may themselves affect nephropathy (8).

Cardiovascular end points are the major cause of death in type 2 diabetes (27). Importantly, the present study has shown no association between HFE mutations and cardiac or all-cause mortality over a mean follow-up of 10 years. Indeed, we had sufficient numbers of patients to detect an OR >1.60 for both mortality end points in non–wild-type patients with >90% power. In a similar long-term study (28), diabetic patients with hemochromatosis had reduced survival compared with nondiabetic hemochromatotic patients and subjects from the normal population. However, without a diabetic nonhemochromatotic group, this can be interpreted as showing diabetes-associated reduced life expectancy alone rather than a synergistic effect of iron overload and diabetes. Alternatively, diabetes may be a relatively late complication of hereditary hemochromatosis with a consequently minimal effect on longevity.

The present analyses represent the largest study of the relationship between iron metabolism, HFE gene mutations, and the clinical features and complications of diabetes, but they have limitations. Because of the observational, “usual care” nature of the FDS, complete data on the management of patients with known hemochromatosis, and its effect on indexes of iron storage and outcome, were not available. The FDS sample size was smaller than in other studies such as the Hemochromatosis and Iron Overload Screening (HEIRS) study, which had ~100,000 participants, but HEIRS was conducted in the general population and relied on self-report for diabetes diagnosis, and no complication or mortality data have been reported (21). Interestingly, the HEIRS study did not show that the main HFE mutations were associated with diabetes (21). Indeed, their distribution in the FDS (87.7% homozygous wild type, 10.5% heterozygous C282Y/wild type, 1.1% heterozygous C282Y/H63D, and 0.6% homozygous C282Y) was similar to that in a population-based study in a large rural Western Australian center (85.4, 11.9, 2.2, and 0.5%, respectively) (18). In both cases, the proportions of heterozygotes were in Hardy-Weinberg equilibrium, suggesting there was no detrimental effect of inheriting C282Y heterozygosity on life expectancy. Other strengths of the present study are the WADLS capture of both public and private hospital admissions in Western Australia (16) and the low rates of coding errors and migration out of Western Australia (14).

The present findings, those of studies such as HEIRS (21), and the results of a recent systematic review (12) do not provide evidence that enhanced case finding strategies for hemochromatosis should extend to diabetes. Nevertheless, because the consequences of delayed diagnosis of hemochromatosis can be significant (28), patients with weakness, fatigue, joint or abdominal pain, liver or cardiac disease, impotence, infertility, or menstrual disturbance that is atypical or unexplained irrespective of the presence or absence of diabetes should have biochemical and genetic testing in the same way as individuals who have a close blood relative with hemochromatosis (9).

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