Original Article

HFE Genetic Variability, Body Iron Stores, and the Risk of Type 2 Diabetes in U.S. Women

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To determine whether the HFE gene variants H63D and C282Y are associated with body iron stores and the risk of type 2 diabetes, we conducted a nested case-control study of 714 incident cases of type 2 diabetes and 1,120 matching control subjects in a prospective cohort, the Nurses' Health Study. In both healthy control and diabetic case subjects, H63D homozygosity, C282Y, and the compound heterozygotes were associated with significantly higher levels of plasma ferritin and significantly lower ratios of transferrin receptors to ferritin. Such effects were independent of age, BMI, and lifestyle factors. Overall, there were no significant differences in genotypes of H63D and C282Y between the case and control subjects. A meta-analysis of 4,245 case and 5,982 control subjects indicated a null association of C282Y with diabetes risk, whereas carriers of H63D or the compound heterozygotes had marginally increased risk (odds ratio [OR] 1.11 [95% CI 1.00–1.25] and 1.60 [0.99–2.60], respectively). In addition, we found a significant interaction between HFE variants and heme iron intake (*P* for interaction = 0.029). The ORs of type 2 diabetes across increasing quartiles of heme iron were 1.00, 1.21 (0.72–2.01), 1.72 (1.03–2.88), and 1.49 (0.91–2.46) among the participants with either the H63D or C282Y variant, whereas the ORs were 1.00, 0.71 (0.49–1.05), 1.12 (0.76–1.66), and 0.96 (0.65–1.42) among those with wild-type genotypes. Our data indicate significant effects of H63D and C282Y on body iron stores and suggest a potential interaction between HFE genotypes and heme iron intake in relation to the risk of type 2 diabetes. *Diabetes* 54: 3567–3572, 2005

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Received for publication 2 June 2005 and accepted in revised form 6 September 2005.

ADA, American Diabetes Association; CRP, C-reactive protein; HH, hereditary hemochromatosis; NHS, Nurses' Health Study.

Hereditary hemochromatosis (HH), a late-onset autosomal-recessive disorder, results in excess dietary iron absorption and iron deposition in multiple tissues (1). Epidemiological studies have revealed that the variability of the hemochromatosis (HFE) gene, which is localized at the short arm of chromosome 6, contributes to the development of HH. The HFE protein binds to the transferrin receptor and reduces its affinity for iron-loaded transferrin (2,3). Homozygosity for the HFE variant C282Y is the main susceptibility genotype and is highly prevalent (50–100%) in HH patients, and the homozygosity for variant H63D and the compound heterozygotes of H63D and C282Y also succumb to HH with a lower penetrance (3–6). The observed genetic effects are likely caused by the predisposition to iron overload (7,8). In the U.S. population, the prevalence estimates for HFE variants are 5.4% for C282Y and 13.5% for H63D (9).

Diabetes is commonly manifested in HH (10). In addition, body iron stores have been associated with abnormal glucose tolerance and the risk of diabetes (11–13). A number of studies have examined the associations between variants H63D and C282Y and the risk of type 2 diabetes (14–29). Frequency of C282Y or H63D was observed to be higher in diabetic patients compared with nondiabetic control subjects in some (18,21,24) but not all of the studies. Pooled analysis of some previous studies tends not to support a major role of HFE variants in diabetes (16,17). Nevertheless, none of these studies have considered the potential modification effects of diet and lifestyle factors. In earlier analyses, we found that dietary iron intakes contributed to body iron stores and strongly predicted risk of type 2 diabetes (30,31). Therefore, we hypothesize that the effects of HFE variants on diabetes may be modified by dietary iron intake, and vice versa.

To address these issues, we examined the relationship between HFE variants C282Y and H63D and the risk of type 2 diabetes in the thus far largest prospective nested case-control study from Nurses' Health Study (NHS) cohort. We also examined the effects of genetic variability of the HFE gene on body iron stores. Taking advantage of the detailed measurements of dietary intake (especially heme iron intake), we examined in detail the potential interactions between HFE variants and iron intake. In addition, we conducted a meta-analysis of the genetic variability of HFE gene and risk of type 2 diabetes to pool estimates from this and previous studies.

RESEARCH DESIGN AND METHODS

The NHS cohort was established in 1976, when 121,700 female registered nurses aged 30–55 years and residing in 11 large U.S. states completed a mailed questionnaire on their medical history and lifestyle (32). The lifestyle factors, including smoking, menopausal status and postmenopausal hormone therapy, and body weight, have been updated by validated questionnaires every 2 years. Samples for the present case-control study were selected from a subcohort of 32,826 women who provided a blood sample between 1989 and 1990 and were free from diabetes, cardiovascular disease, stroke, or cancer at the time of blood collection. Incident cases were defined as self-reported diabetes confirmed by a validated supplementary questionnaire and diagnosed at least 1 year after blood collection through 2000. The supplementary questionnaire obtained information on symptoms, diagnostic tests, and hypoglycemic therapy used to define type 2 diabetes cases. Medical record review confirmed the diagnosis of type 2 diabetes using this questionnaire for 98% of cases using the National Diabetes Data Group criteria (33). We used the American Diabetes Association (ADA) diagnostic criteria for diagnosis of diabetes cases during the 1998 and 2000 cycles (34).

There were 714 incident case subjects diagnosed at least 1 year after blood collection through 2000 that were matched to 1,120 control subjects who did not report physician-diagnosed diabetes. Case and control subjects were matched on age, month and year of blood draw, and fasting status. For the case subjects diagnosed in 1996 or earlier, two control subjects were matched to each case subject. One of the two control subjects was also matched according to BMI ($\pm$1 kg/m^2). For the case subjects diagnosed after 1996, one control subject was matched to each case subject. To improve statistical control for obesity at the upper extreme of the distribution, control women were also matched on BMI to case subjects in the top 10% of the BMI distribution.

Assessment of dietary intakes and covariates. Detailed dietary information was obtained through the use of semiquantitative food frequency questionnaires. Participants were asked to report their average frequency of consumption of selected foods and beverages with a specified commonly used unit or portion size during the previous year. The reproducibility and validity of the dietary questionnaires were assessed by comparing nutrient intake from the food frequency questionnaire with two 1-week diet records spaced 6 months apart (35). The correlation coefficient for energy-adjusted total dietary iron intake was 0.60 after adjustment for within-person variability in daily intake. To reduce within-person variability and obtain the best estimate of long-term dietary intake, we used the average intakes from the 1986 and 1990 questionnaires, which are the closest to the baseline of this study.

Anthropometric data and lifestyle factors were derived from the baseline questionnaires (1990). BMI was calculated as weight (in kilograms) divided by the square of height (in meters). Physical activity, which was measured in 1988, was expressed as metabolic equivalent task hours based on self-reported types and durations of activities over the previous year.

Assessment of iron stores and other markers. Blood was collected between 1989 and 1990. Concentrations of ferritin and transferrin receptors were measured by a particle-enhanced immunoturbidimetric assay using a Hitachi 911 analyzer (Roche Diagnostics, Indianapolis, IN). C-reactive protein (CRP) concentrations were measured via a highly sensitive latex-enhanced immunonephelometric assay on a BN II analyzer (Dade Behring, Newark, DE). Plasma insulin and proinsulin were determined by radioimmunoassay using a commercial kit (Linco Research, St. Charles, MO). The coefficients of variation were: ferritin: 3.75%; transferrin receptors: 8.4%; CRP: 3.8%; fasting insulin: 13.9%; and proinsulin: 7.3% (30,36,37).

Genotype determination. DNA was extracted from the buffy coat fraction of centrifuged blood using a QIAmp blood kit (Qiagen, Chatsworth, CA). Two single nucleotide polymorphisms at the HFE locus, H63D and C282Y, were genotyped using Taqman single nucleotide polymorphism allelic discrimination by means of an ABI 7900HT (Applied Biosystems, Foster City, CA). Replicate quality control samples were included and genotyped with 100% concordance.

Statistical analyses. A χ^2 test was used to assess whether the genotypes were in Hardy-Weinberg equilibrium and to compare the genotype and allele frequencies between case and control subjects. Odds ratios (ORs) were calculated using unconditional logistic regression, adjusting for type 2 diabetes risk factors, including age, physical activity (<1.5, 1.5–5.9, 6.0–11.9, 12–20.9, and 21.0 metabolic equivalent task hours/week), smoking (never, past, and current), alcohol intake (nondrinker and drinker [0.1–4.9, 5–10, or >10 g/day]), menopausal status (pre- or postmenopausal [never, past, or current hormone use]), aspirin use (yes or no), BMI (<23, 23–24.9, 25–29.9, 30–34.9, or 35 kg/m^2), and dietary factors. Interactions were assessed using a likelihood ratio test. We created three interaction terms for quartiles 2–4 of heme iron and used a 3-df (degrees of freedom) test. The geometric mean levels of the markers of iron stores were compared among genotype or allele groups using ANOVA, adjusting for covariates. An SAS statistical package was used for the analyses (version 8.2 for UNIX; SAS Institute, Cary, NC).

A meta-analysis was conducted using STATA (version 7.0; College Station, TX). Relevant studies were identified by searching the Medline, PubMed, and OMIM (Online Mendelian Inheritance in Man) databases for all published genetic association studies up to May 2005. In general, we included observational studies that provided estimates of relative risks or ORs or frequency data that permitted estimation of these parameters. Formal tests of heterogeneity were assessed by a χ^2 statistic. Because no heterogeneity was detected, we used fixed-effect models, and the summary ORs were obtained by averaging the natural logarithms of the ORs from individual studies, weighted by the inverses of their variances (38). All P values are two sided.

TABLE 1
Age-standardized diabetes risk factors in case and control subjects in U.S. women

	Case subjects	Control subjects	P
n	714	1120	
Age (years)	54.4	54.5	0.83
BMI (kg/m^2)	30.4	27.2	<0.001
Physical activity (metabolic equivalent task hours/week)	12.2	14.9	<0.001
Alcohol consumption (g/day)	2.9	5.2	<0.001
Ethnicity (% white)	95.1	95.6	0.60
Current smoker (%)	14.5	12.0	0.11
Postmenopausal status (%)	75.6	75.3	0.52
Family history of diabetes (%)	46.8	22.3	<0.001
Biomarkers			
Insulin (μU/ml)*	13.8	10.5	<0.001
Proinsulin (fmol/ml)	26.0	11.7	<0.001
CRP (mg/dl)	0.52	0.23	<0.001
Ferritin (ng/ml)	69.4	46.5	<0.001
Ratio of transferrin receptor to ferritin	46.1	64.1	<0.001
Dietary intakes			
Total iron (mg/day)	17.3	17.4	0.90
Nonsupplemental iron (mg/day)	12.3	12.5	0.39
Supplemental iron (mg/day)	4.9	4.9	0.90
Heme iron (mg/day)	1.12	1.06	0.001
Red meat (servings/day $\times$ 1,000 kcal)	1.21	1.12	0.002

*Insulin and proinsulin were measured in fasting samples only (insulin in 415 case subjects and 570 control subjects and proinsulin in 200 case subjects and 306 control subjects).

RESULTS

The allele frequency was 0.16 for H63D and 0.07 for C282Y, and the distribution of the two variants were in Hardy-Weinberg equilibrium ($P > 0.05$). Characteristics of the case and control subjects are presented in Table 1. The case subjects had higher BMI, engaged in less physical activity, and drank less alcohol than the control subjects. The levels of insulin, CRP, and ferritin were significantly higher in the case than the control subjects, whereas the ratio of transferrin receptor to ferritin was lower in the case subjects. In addition, case subjects had higher intakes of heme iron than the control subjects.

In the control subjects, homozygosity of H63D, the allele of C282Y, and the compound heterozygotes were associated with significantly higher levels of serum ferritin and with significantly lower levels of the ratio of trasferrin

receptor to ferritin, after adjusting for age and BMI (Table 2). Further adjustment for smoking, alcohol consumption, physical activity, aspirin use, and postmenopausal hormone use did not appreciably change the associations. Adding dietary factors (dietary glycemic load, cereal fiber, vitamin C, phyrate, calcium, polyunsaturated-to-saturated ratio, trans fat, and coffee) to the models also did not significantly alter the associations. The significant associations of C282Y and the compound heterozygotes with body iron stores were also observed in the diabetic patients, among whom the associations of H63D were somewhat attenuated.

The frequencies of H63D homozygosity, C282Y, and the compound heterozygotes were slightly higher in diabetic case subjects than in the control subjects. However, none of these associations reached statistical significance. After adjustment for covariates, only the homozygosity of H63D showed a marginal association (OR 1.84 [95% CI 0.96–3.53]) (Table 3). Restricting analysis to the postmenopausal women did not appreciably change the associations. In the meta-analysis of 4,245 case subjects with type 2 diabetes and 5,982 control subjects from 17 association studies (14–29), including the current study, C282Y was not associated with the disease risk (overall OR 1.03 [95% CI 0.89–1.20]; P for heterogeneity >0.05). However, the carriers of H63D (1.11 [1.0–1.25]; P for heterogeneity >0.05) or the compound heterozygotes (1.60 [0.99–2.60]; P for heterogeneity >0.05) showed a modestly increased risk compared with those with the wild-type genotypes (Fig. 1).

We also examined the interactions of HFE variability and dietary heme iron in relation to the risk of type 2 diabetes. Because both variants H63D and C282Y showed associations with body iron stores, we grouped the subjects with either H63D or C282Y together as the "risk genotypes" to improve the power. We found a significant interaction between the risk genotypes and the intake of dietary heme iron (P for interaction = 0.029) (Fig. 2). After adjustment for covariates, the OR of type 2 diabetes across increasing quartiles of heme iron intake were 1.00, 1.21 (95% CI 0.72–2.01), 1.72 (1.03–2.88), and 1.49 (0.91–2.46) among the participants with either variants, whereas the ORs were 1.00, 0.71 (0.49–1.05), 1.12 (0.76–1.66), and 0.96 (0.65–1.42) among those with the wild-type genotypes (Fig. 2). The linear trend of heme iron and diabetes risk was significant (P = 0.04) in the variants group (HFE H63D or C282Y) but not significant in the wild-type group, after adjusting for age and BMI.

TABLE 2
Body iron stores by HFE variants H63D, C282Y, and the compound heterozygotes*

	H63D			P		C282Y			The compound heterozygotes		
	HH	HD	DD	DD vs. HH	DD vs. HD	CC	CY + YY	P	HHCC	HDCY	P
Ferritin (ng/ml)											
Control	782 (44.7)	302 (48.4)	24 (82.3)	0.03	0.06	982 (45.2)	137 (58.0)	0.03	669 (43.4)	23 (73.7)	0.03
Diabetic	498 (69.4)	184 (68.0)	23 (86.5)	0.32	0.28	616 (67.4)	94 (87.4)	0.03	423 (67.4)	21 (121.5)	0.01
Total	1,280 (55.1)	486 (57.4)	47 (85.6)	0.01	0.02	1,598 (54.6)	231 (72.2)	0.0006	1,092 (53.5)	44 (99.5)	0.0002
Ratio of transferrin receptor to ferritin											
Control	782 (66.7)	302 (60.9)	24 (32.1)	0.02	0.03	982 (66.7)	137 (47.9)	0.006	669 (70.1)	23 (38.5)	0.02
Diabetic	498 (46.5)	184 (45.2)	23 (30.9)	0.07	0.11	616 (47.5)	94 (34.5)	0.01	423 (48.9)	21 (22.4)	0.002
Total	1,280 (56.3)	486 (52.5)	47 (31.2)	0.001	0.005	1,598 (56.8)	231 (40.0)	<0.0001	1,092 (59.1)	44 (27.7)	<0.0001

Data are n (means). *Adjusting for age, BMI, alcohol consumption, smoking, physical activity, aspirin use, family history of diabetes, and postmenopausal hormone use.

DISCUSSION

In this prospective nested case-control study of U.S. women, we observed that HFE variants H63D and C282Y were associated with significantly higher serum ferritin levels and lower ratios of transferrin receptor to ferritin. Such associations were independent of age, BMI, race, lifestyle, and dietary factors that may affect iron absorption. Overall, there was no significant difference in genotypes of H63D and C282Y between the case and control subjects. The meta-analysis of 4,499 case and 6,323 control subjects indicates a null association of C282Y with the risk of type 2 diabetes, whereas carriers of H63D or the compound heterozygotes had a modestly increased risk. We found a significant interaction between HFE variants and heme iron intakes. Increased risk of type 2 diabetes was observed along with increasing intake of heme iron

TABLE 3
Associations of HFE variants H63D, C282Y, and the compound heterozygotes with the risk of type 2 diabetes among U.S. women

Variants	Control subjects	Case subjects	OR	
			Age and BMI adjusted	Multivariate adjusted*
H63D				
HH	782 (70.6)	489 (70.6)	1.0	1.0
HD	302 (27.3)	184 (26.1)	0.97 (0.78–1.22)	0.95 (0.75–1.20)
DD	24 (2.1)	23 (3.3)	1.68 (0.91–3.12)	1.84 (0.96–3.53)
C282Y				
CC	982 (87.8)	616 (86.8)	1.0	1.0
CY + YY	137 (12.2)	94 (13.2)	1.13 (0.84–1.52)	1.16 (0.85–1.59)
Compound heterozygotes				
HHCC	669 (96.7)	423 (95.3)	1.0	1.0
HDCY	23 (3.3)	21 (4.7)	1.61 (0.86–3.02)	1.57 (0.80–3.09)

Data are *n* (%) or OR (95% CI). *Adjusting for age, BMI, alcohol consumption, smoking, physical activity, family history of diabetes, race, and postmenopausal hormone use.

among women with either H63D or C282Y but not among those with the wild-type genotypes.

In the healthy women, the homozygosity of H63D, C282Y, and the compound heterozygotes showed clear effects on the body iron stores, represented by elevated serum ferritin levels and lowered ratio of transferrin

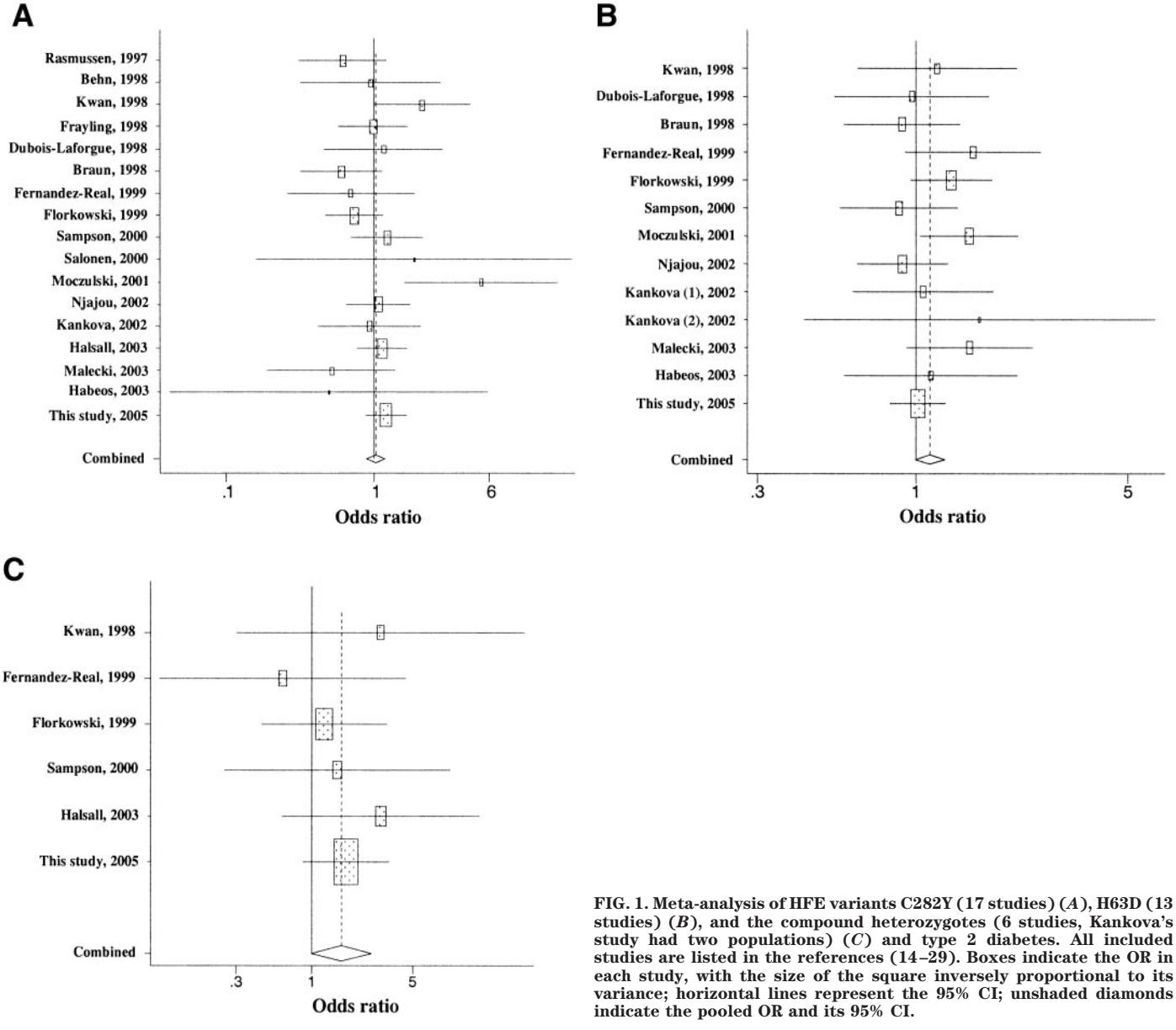

FIG. 1. Meta-analysis of HFE variants C282Y (17 studies) (*A*), H63D (13 studies) (*B*), and the compound heterozygotes (6 studies, Kankova's study had two populations) (*C*) and type 2 diabetes. All included studies are listed in the references (14–29). Boxes indicate the OR in each study, with the size of the square inversely proportional to its variance; horizontal lines represent the 95% CI; unshaded diamonds indicate the pooled OR and its 95% CI.

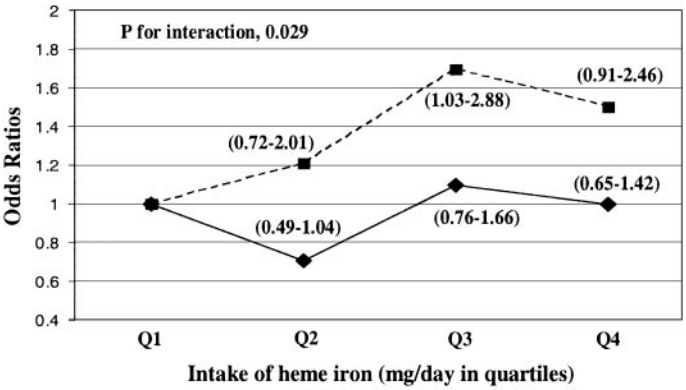

FIG. 2. The modification effects of carrying either H63D or C282Y on the associations between intake of heme iron (in quartiles) and the risk of type 2 diabetes. The corresponding 95% CIs are presented in parenthesis. ■, ORs among subjects with either variant H63D or C282Y; ♦, ORs among subjects with the wild-type genotypes.

receptor to ferritin. The effects of C282Y and the compound heterozygotes persisted, whereas the effects of H63D appear to be attenuated in diabetic patients. Our findings of H63D and C282Y on body iron stores are highly consistent with previous evidence from both animal and human studies. Both C282Y and H63D have been associated with iron overload with different magnitudes (39). The HFE protein is a 343-residue type I transmembrane protein bound to β2-microglobulin, and it may modify the affinity of transferrin for its receptor (40). Mutated HFE protein at position 63 or 282 disrupts the association with β2-microglobulin and blocks the association of HFE with transferrin receptor (3,41). The defective gene product may cause increased iron absorption (1,42). Because absorption is the main regulatory step in iron homeostasis in humans, the genetically caused overabsorption of iron may lead to a chronic iron overload.

The null association of C282Y and diabetes risk are in agreement with the results of previous meta-analysis by others (16,17), although increased frequency of C282Y in patients with type 2 diabetes was observed in some studies (18,24). Our meta-analysis also indicated that carrying H63D or the compound heterozygotes tended to be associated with a modestly increased risk of diabetes, but such associations need to be confirmed in studies with much larger sample sizes.

In earlier studies, we have found that intake of heme iron was a major dietary determinant for body iron stores (31) and strongly predicted an increased risk of diabetes in U.S. women (30). Because of the potential roles of HFE variants in iron homeostasis, we suspected that HFE genotypes might interact with dietary heme iron in determining the risk of type 2 diabetes. We found that the high intake of heme iron increased the risk of type 2 diabetes only in the presence of HFE variants (either H63D or C282Y). Heme iron, which is present in red meat, fish, and poultry, is highly bioavailable and contributes ~50% of the total bioavailable iron in the typical U.S. diet (43). Our findings suggest a potential synergetic effect of genetic predisposition and heme iron on type 2 diabetes.

A unique advantage of this study is that we were able to adjust for important lifestyle and dietary covariates that were not considered by most previous studies. Heme iron intake was assessed multiple times, and using the average intake during 1986 and 1990 could reflect long-term dietary intake and reduce measurement error. Considering that genetic effects of HFE on diabetes risk may differ by race, population stratification may cause spurious associations. However, the vast majority of women included in this study were Caucasians (~96%). We also examined the associations in Caucasian women separately and did not find any appreciable change in the results (data not shown). Nevertheless, the interactions of the HFE genotypes and iron intake need to be replicated in other populations.

One potential limitation of our study is that some of our control subjects may have undiagnosed diabetes, which may bias the results toward the null. However, our previous validation study (44) suggested that the prevalence of undiagnosed diabetes was much lower than that in the general population because our participants were all health professionals. The diagnostic criteria for type 2 diabetes were changed in 1997 such that lower fasting glucose levels (>126 mg/dl) would now be considered diagnostic. Thus, we have used the ADA criteria for diagnosis of diabetes cases after 1998. If the new criteria were used for earlier years, some control subjects might have been reclassified as case subjects, but the number of patients meeting ADA but not NDDG (National Diabetes Data Group) criteria was very small (<5% of case subjects). Thus, the change in diagnostic criteria are unlikely to affect our results.

In summary, we found that HFE variants H63D, C282Y, and the compound heterozygotes were associated with higher body iron stores in U.S. women. Our data, combined with other epidemiological data, support the hypothesis that HFE variability itself has moderate effects, but it may interact with heme iron intake in determining the risk of type 2 diabetes.

ACKNOWLEDGMENTS

This study was supported by National Institutes of Health Grants DK58845 and CA87969.

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