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Involvement of ApoE E4 and H63D in Sporadic Alzheimer's Disease in a Folate-Supplemented Ontario Population

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Abstract. Dysregulation of iron homeostasis is implicated in Alzheimer's disease (AD). In this pilot study, common variants of the apolipoprotein E (APOE) and HFE genes resulting in the iron overload disorder of hereditary hemochromatosis (C282Y, H63D and S65C) were evaluated as factors in sporadic AD in an Ontario sample in which folic acid fortification has been mandatory since 1998. Laboratory studies also were done to search for genetic effects on blood markers of iron status, red cell folates and serum B12. Participants included 58 healthy volunteers (25 males, 33 females) and 54 patients with probable AD (20 males, 34 females). Statistical analyses were interpreted at the 95% confidence level. Contingency table and odds ratio analyses supported the hypothesis that in females of the given age range, E4 significantly predisposed to AD in the presence but not absence of H63D. In males, E4 significantly predisposed to AD in the absence of H63D, and H63D in the absence of E4 appeared protective against AD. Among E4+ AD patients, H63D was associated with significant lowering of red cell folate concentration, possibly as the result of excessive oxidative stress. However, folate levels in the lowest population quartile did not affect the risk of AD. A model is presented to explain the experimental findings.

Keywords: APOE, folate, HFE, iron status, sporadic Alzheimer's disease

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[1]Deceased; this paper is dedicated to his memory.

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INTRODUCTION

There is evidence from a number of different sources that iron homeostasis is dysregulated in Alzheimer's disease (AD), although it is not clear how such dysregulation originates [4,7,10,12,13,19,20,45,48,49,52,56, 60,89,96]. Furthermore, there is much interest in using metal ion chelators and antioxidants in the treatment and prevention of AD [4,21,25]. Thus allelic variants of genes known to be associated with altered iron homeostasis should be considered as factors in AD. Following the discovery that two mutations of a class I-like major histocompatibility gene called HFE (C282Y and H63D) resulted in the iron overload disorder of hereditary hemochromatosis now called type 1 [1,15,30,66], we characterized involvement of C282Y and H63D in early onset familial AD (EOFAD) [60]. Because the E4 allele of apolipoprotein E (APOE) is the major genetic susceptibility factor for AD in Caucasians [24,75], HFE mutation involvement was evaluated with stratification for the presence or absence of E4. The joint absence of E4 and HFE mutations was found to be a significant protective factor against EOFAD in males but not females. Following a subsequent study implicating H63D as a factor in sporadic AD [86], a number of other studies of HFE involvement in sporadic AD yielded variable results (reviewed in [21]; see also [15, 17,51,79]).

In the current paper, we have evaluated involvement of E4 and common variants of HFE associated with hemochromatosis in sporadic AD in an Ontario sample. Two approaches were used: 1) comparison of the distributions and co-distributions of the genetic variants in AD patients and controls and calculation of odds ratios to evaluate effects of genotype on AD risk; and 2) an exploratory study of genotype effects on blood markers of iron status, red cell folates and serum B12 in AD patients. Markers of iron status were included because of the known function of HFE in iron homeostasis [1,15, 21,30,50,66,82–85,97]. Red cell folate and B12 also were measured since these vitamins are inherently susceptible to oxidative degradation [45,53,62,68]. Additionally, research on B vitamins and their relationship to homocysteine is very topical in field of AD among others [47,57,65,71,72,80]. A model was developed to account for the experimental findings. Of note, folic acid fortification of certain grain products has been mandatory in Canada since 1998 [9,35,78] and some of the study participants were taking vitamin supplements. See the following references for additional information about: hemochromatosis [1,11,15,30,61,66]; AD [8, 16,18,22,25,34,40,64,93]; APOE function [27,31,41, 54,55,68]; HFE function [30,50,81–85].

MATERIALS AND METHODS

Human subjects and diagnosis

Research protocols were approved for ethical acceptability by the Baycrest Centre for Geriatric Care/University of Toronto and Queen's University Research Ethics Review Boards. All participants were Caucasian. Patients with probable sporadic AD (N = 54; mean age, 74.1 ± 9.7 years) were recruited from clinics in Toronto and Kingston, Ontario. The age range was restricted in this study to ensure representation of the E4 allele of APOE which is reported to maximally affect AD risk between the ages of 60 and 75 [34]. Diagnosis of AD was made using NINCDS-ADRDA criteria [58]. Almost all patients had undergone CT scanning. Information from the scans enabled patients with AD to be distinguished from those with vascular dementia or "mixed" dementia syndrome arising from the combination of AD and vascular injuries who were excluded from the study. Patients from the Toronto clinic were largely of eastern European origin whereas those from the Kingston clinic were largely of western European origin. Healthy volunteers (also denoted as controls) (N = 58) with a similar gender distribution and age range as the AD patients were recruited from several community organizations in Toronto, Ontario. At the time of recruitment, a health status questionnaire was completed for each AD patient and control. Information collected included age at diagnosis of AD and entry into the study, current medications (prescription and non-prescription, including vitamin and mineral supplements), and health status including arthritis, cancer, cardiovascular disease, diabetes, hypertension, thyroid disorders, major operations and smoking history. No participant was included in the study if they were suffering from a current infection (e.g., cold, cough, sore throat, influenza). No participant was excluded on the basis of having major health issues. Controls were not screened for cognitive function, but were excluded if they had been diagnosed with any form of dementia. Mini Mental State Examination (MMSE) scores corresponding to the recruitment date were available for the Kingston AD patients; MMSE scores are inversely correlated with AD severity. Most AD patients were on pharmacological treatment for AD (Aricept, Exelon or Reminyl). Eight AD patients with a known B12 deficiency (4 males, 4 females) were being treated either with an oral supplement or by injection. None were taking a folate supplement exclusively; 18 others (3 males, 15 females) were taking supplements of vi-

tamins C, D, E, and/or multivitamins. Of the controls, 1 (female) was taking only a B12 supplement and 15 (4 males, 11 females) were taking one or more other forms of vitamin supplement including multivitamins and vitamins C, D or E. Participants were recruited over the time period April 2000 to March 2003.

Blood sampling

At the time of entry into the study, non-fasting blood samples were collected from each participant at convenience by the collaborating hospital laboratories (Kingston General Hospital and Baycrest Centre for Geriatric Care/Mount Sinai Hospital) or by a mobile phlebotomist. These were aliquoted for DNA extraction by the Surrey Place Centre Neurogenetics Laboratory and for standard hematological and biochemical analyses by the collaborating hospital laboratories. Serum was recovered within 30 min of blood collection using a standard clinical procedure (room temperature for 30 min after blood collection followed by centrifugation at 1,000 x g for 10 min). Non-fasting samples were taken because these have been shown to be informative for the diagnosis of homocysteinemia [33], as well as for studies of serum total homocysteine and related metabolites, including red cell folates, in aging [35].

DNA extraction

DNA samples were extracted from blood samples collected in heparin or EDTA tubes using QIAamp DNA Mini Kits (QIAGEN, Mississauga, ON, Canada). These were stored in 200 μL aliquots in 500 μL polypropylene microfuge tubes at concentrations of about 10 ng/μL in 1X Tris-EDTA buffer at $-70\,^{\circ}$C.

Genotyping

Genotyping for the E2, E3 and E4 variants of APOE was done using the PCR-RFLP protocol of Wenham et al. [100]. Each sample was APOE typed at least twice. HFE typing to identify C282Y, H63D and a third hemochromatosis-associated variant called S65C [91] was done using two different approaches. The multiplex PCR-RFLP procedure of Stott et al. [92] to detect C282Y and H63D was applied in the Surrey Place Centre Neurogenetics Laboratory. An allelic discrimination assay was designed for array-based detection of C282Y, H63D and S65C and applied at the Stollery Children's Hospital Molecular Diagnostic Laboratory. Briefly, all analyses were carried out using primer and TaqMan® probe sets for:

C282Y – GGCTGGATAACCTTGGCTGTA, CACAATGAGGGGCTGATCC, VIC-CACCTGGCACGTATA-MGBNFQ, 6FAM-TCCACCTGGTACGTATA-MGBNFQ;
H63D – GAAGCTTTGGGCTACGTGGAT, CCACATCTGGCTTGAAATTCTACTG, 6FAM-CGACTCTCATGATCATAGA-MGBNFQ, VIC-CGACTCTCATCATCATAGA-MGBNFQ;
S65C– GACCAGCTGTTCGTGTTCTATGAT, TTCTACTGGAAACCCATGGAGTT, VIC-CGGCGACTCTCAT-MGBNFQ, 6FAM-ACGGCGACACTCA-MGBNFQ.

Each variant locus was amplified in 3 separate reactions using 75 ng of each DNA sample, 200 nM of forward and reverse primer, 900nM of wildtype and mutant probe in TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA), using conditions slightly modified from standard conditions (50 °C for 2 min, 95°C for 3 min, followed by 35 cycles of 95°C for 15 sec, 60°C for 1 min). All reactions were run and analyzed in 384-well plates in duplicate. Plates were scanned using an AB 7900HT Sequence Detection System (Applied Biosystems). Fluorescence data were compiled and genotyped based on slopes of data points relative to blanks and genotype controls using an Excel macro. For samples that amplified across both the H63D and C282Y loci, results from the two laboratories were 100% concordant.

Blood analyses

Complete blood cell counts

These were done using the Beckman-Coulter LH780 Hematology System (Beckman Coulter, Inc., Carlsbad, CA).

Markers of iron status

These included serum ferritin, iron and transferrin, % transferrin saturation, and total iron binding capacity (TIBC). Serum ferritin was measured using a monoclonal sandwich principle. Serum iron was measured using the FerroZine method without deproteinization. Serum transferrin was measured using an immunoturbidimetric assay. Measurements were done on the Hitachi 917 analyzer (Roche Diagnostics Canada, Laval PQ). Measurement of serum unsaturated iron binding capacity (UIBC) involved the addition of excess iron at alkaline pH prior to measurement of iron. The percentage of transferrin saturated with iron was calculated as $(100 - (\mathrm{UIBC/TIBC})) \times 100\%$. TIBC was calculated from total iron plus unsaturated iron binding capacity (UIBC). See [43] for a review of the interpretation of iron studies.

Red cell folate and serum B12

Concentrations of red cell folates and serum B12 were measured using ruthenium chelate electrochemiluminescence assays on the Roche Elecsys 2010 immunoassay analyzer (Roche Diagnostics). Red cell folates rather than serum folate was chosen for investigation because the former parameter is not usually affected by rapid dietary influxes [6,32]. Folate assays on the Kingston patients were done on fresh blood samples decoagulated with EDTA; in such assays the concentration of folates in whole blood is considered equivalent to the concentration of red cell folates. Folate assays on the Toronto patients and the healthy normals (controls) were done on once-thawed red blood cell fractions of blood decoagulated with sodium heparin that had been stored at −70°C. In the folate assays, blood lysates remained at 25°C for 2 hr before analysis.

Statistical analysis

Subjects were classified as E4+ if they had the E(3,4) or E(4,4) genotype or E4- if they were E(2,2 or 2,3) or E(3,3). Subjects were classified as C282Y+ if they carried one or two C282Y alleles, and H63D+ if they carried one or two H63D alleles. The term H63D- denotes absence of H63D but not necessarily of C282Y. The S65C allele was present in too low a frequency to be informative so this marker was not considered in analyses. Allele frequencies and genotype distributions in different groups were compared using contingency table analysis [28,39]. Odds ratios and 95% confidence intervals were calculated as described by Hutchon [42]. Means of biological variables in different groups were evaluated by the independent samples t test (Microsoft Office Excel 2003, two tailed test, unequal variance). To identify H63D specific effects on biological variables, analyses were conducted on pooled samples, on females separately, or after removal of those taking any form of extra vitamin supplement from the pool. To ensure that results attributed to H63D and/or E4 heterozygosity were not affected by presence of the E2 allele of APOE which is protective against AD [31], by E4 homozygosity which has a greater effect on age at onset than E4 heterozygosity [24], or by C282Y in combination with E4 [87], effects of removing participants with these genotypes on results also were examined. Analyses were interpreted at the 95% level of confidence. Because sample sizes were small, retrospective power analysis was conducted after statistical analysis to determine the power of the original analysis to detect the observed difference with the given sample size [44]. Bonferonni corrections for multiple analyses were not applied.

RESULTS

Results of the study are given in Tables 1 to 7. Because the frequencies of E4 and HFE mutations are reported to vary from one geographical region to another [1,7,34], allele frequency data in Tables 1 through 3 are shown separately for patients from the Kingston and Toronto sites since their ethnic backgrounds were largely western European or eastern European, respectively. The study findings highlight interaction between the E4 allele of APOE and the H63D allele of HFE and that this interaction does not result in significantly different serum iron indices in AD patients and controls. Rather, interaction had an apparent effect on the concentration of red cell folates.

Demographic data

Table 1 lists the mean age at entry into the study for the AD patients and the healthy volunteers (controls), overall and with stratification for gender and clinic site. Also shown are the mean ages at entry for the AD patients and the controls with stratification for the presence or absence of H63D. To note is that the average age range of the participants is relatively restricted (71.5 ± 9.5 years).

Genetic studies

Allele frequencies of E4 and the HFE variants

Allele frequencies of E4 and the three HFE variants in the participants are given in Table 2 with and without stratification for gender and clinic site. Consistent with its confirmed status as a strong risk factor in AD in Caucasians, the allele frequency of E4 was greater in the pooled AD patients than in the controls (0.389 vs. 0.121, respectively). Retrospective power analysis indicated that the frequencies of the C282Y allele in AD patients and controls and corresponding frequencies of the H63D allele with/without stratification for APOE type were too low for meaningful intergroup statistical comparisons, as done by some other groups (e.g., see [21]). As detailed below, however, examination of the co-distribution of E4 and HFE mutations in individuals revealed some striking differences in the frequencies of compound genotypes in AD patients and controls. Only one individual in the entire sample carried an S65C mutation. Hence in subsequent analyses, the involvement of S65C was not considered.

Table 1
Demographic data

Participants	Category	Age at Entry M ± SD (years)
Healthy volunteers (controls)	All	72.5 ± 9.6 (58)
	H63D+	72.1 ± 9.5 (19)
	H63D-	72.7 ± 9.8 (39)
	M	71.4 ± 8.0 (25)
	H63D+	69.1 ± 7.2 (13)
	H63D-	73.1 ± 8.5 (12)
	F	73.3 ± 10.7 (33)
	H63D+	77.2 ± 11.4 (6)
	H63D-	72.5 ± 10.5 (27)
AD patients	All	74.1 ± 8.7 (54)
	H63D+	73.2 ± 10.8 (18)
	H63D-	75.6 ± 6.7 (36)
	M	74.0 ± 9.6 (20)
	H63D+	75.1 ± 9.2 (5)
	H63D-	73.4 ± 10.0 (15)
	Kingston site	65.4 ± 8.7 (9)
	Toronto site	74.3 ± 8.7 (11)
	F	74.9 ± 8.1 (34)
	H63D+	73.2 ± 10.8 (11)
	H63D-	75.6 ± 6.7 (23)
	Kingston site	68.8 ± 9.0 (18)
	Toronto site	76.1 ± 6.9 (16)

M: male; F: female; M ± SD: mean ± standard deviation; integers in round brackets: sample size (see Table 3).

Table 2
Frequencies of APOE and HFE alleles in healthy volunteers (controls, C) and Alzheimer patients (AD)

Gene variant	Overall		Males		Females	
	C	AD	C	AD	C	AD
E4	0.121 (58)	0.389 (54)	0.08 (25)	0.389* (9) 0.273** (11)	0.152 (33)	0.500* (18) 0.344** (16)
C282Y	0.0517 (58)	0.0463 (54)	0.020 (25)	0* (9) 0** (11)	0.0758 (33)	0.0556* (18) 0.0938** (16)
H63D	0.190 (58)	0.158 (54)	0.300 (25)	0.111* (9) 0.182** (11)	0.106 (33)	0.139* (18) 0.188** (16)
S65C	0 (58)	0.00926 (54)	0 (25)	0* (9) 0** (11)	0 (33)	0* (18) 0.0313** (16)
H63D in the E4+	0.107 (14)	0.162 (37)	0.250 (4)	0.136 (11)	0.0500 (10)	0.173 (26)
H63D in the E4-	0.216 (44)	0.152 (17)	0.310 (21)	0.167 (9)	0.130 (23)	0.125 (8)

Integers in brackets: number of subjects from which data were derived (see Table 3); *Kingston site; **Toronto site.

Co-distribution of E4, C282Y and H63D in individuals

The co-distribution of the three common variants of APOE, and of the C282Y and H63D variants of HFE in individuals in the AD and control groups, is given in Table 3. As C282Y was not represented in males with AD, and the frequency of C282Y was very low in females with AD, subsequent statistical evaluation focused on involvement of E4 and H63D in AD (Table 4 and below). Only 4 controls and 1 AD patient had an HFE genotype commonly found in type 1 hemochromatosis (see Table 4).

Contingency table and Odds Ratio analysis of different genotypes in AD patients and controls

Contingency table analysis was used to compare the distributions of different genotypes in AD patients and controls. Odds Ratio (OR) analysis also was used to evaluate genotype effects on AD risk. As evident from Table 4, results from both types of analysis were concordant. Retrospective power analysis also was conducted to provide information about sample size adequacy.

Table 3
Co-distribution of APOE and HFE variants in individuals in the participant groups

Healthy normals	Males (25)						Females (33)					
APOE	2,2	2,3	3,3	2,4	3,4	4.4	2,2	2,3	3,3	2,4	3,4	4,4
HFE												
—/—		2	7		2			5	10		8	
—/C282Y		1							3		1	
—/H63D			9		2				3	1		
C282Y/C282Y												
C282Y/H63D									1			
H63D/H63D		1	1						1			
Alzheimer's disease, Kingston	Males (9)						Females (18)					
APOE	2,2	2,3	3,3	2,4	3,4	4,4	2,2	2,3	3,3	2,4	3,4	4,4
HFE												
—/—			2	1	4				3		6	2
—/C282Y											1	1
—/H63D					2					1	4	
C282Y/C282Y												
C282Y/H63D												
H63D/H63D												
Alzheimer's disease, Toronto	Males (11)						Females (16)					
APOE	2,2	2,3	3,3	2,4	3,4	4,4	2,2	2,3	3,3	2,4	3,4	4,4
HFE												
—/—		1	4		1	1				3	4	
—/C282Y						1	1		2			
—/H63D			1	1					2	1	3	
C282Y/C282Y												
C282Y/H63D												
H63D/H63D			1									

The integers in the table indicate the number of males or females in each category with the given HFE and APOE genotypes. There are three common APOE alleles: 2, 3 and 4. The six corresponding APOE genotypes are shown in the APOE row. The horizontal dashes in the HFE column denote the wild-type HFE gene. HFE genotypes predisposing to hereditary hemochromatosis are: C282Y homozygosity; C282Y/H63D compound heterozygosity; and H63D homozygosity.

Without consideration for HFE type, the OR for E4 on its own was 5.56 (1.62–19.1) in males and 6.86 (2.40–16.2) in females ($P < 0.05$; retrospective power = / > 73% in both cases). Corresponding values for H63D on its own were not significant. However, in females, the OR for the H63D+/E4+ genotype was 7.13 (2.07–24.6; retrospective power = 84%) and for the absence of this genotype was 0.175 (0.0644–0.477; retrospective power = 91%). Thus in the study females, the joint presence of H63D and E4 (in 26.5% of female patients) appears to be significantly predisposing to AD and their joint absence (in 17.6% of female patients) significantly protective. In contrast, there was no obvious involvement of the H63D-/E4+ or H63D+/E4- genotypes in females. OR and retrospective power analysis suggests that these latter results are negative because the risks of AD associated with these genotypes are lower and cannot be determined with the small sample size. In contrast, in males, the H63D+/E4+ genotype had little effect, but the H63D-/E4+ genotype (in 40% of male patients) appeared predisposing to AD and the H63D+/E4- genotype (in 10% of male patients) appeared protective. Of note is that the latter finding results from a higher frequency of individuals with the H63D+/E4- genotype in the control than the patient group. Thus, joint consideration of the presence or absence of H63D and E4 has revealed differences in AD involvement in males and females, at least over the given age range of the study participants. As shown in Table 4, the findings noted above for females are not substantially affected by the presence or absence of C282Y, E2, or E4 homozygosity. In males, the possibility that E2, C282Y and/or E4 homozygosity is affecting the risk of the H63D-/E4+ genotype for AD cannot be excluded.

Biochemical studies

Effects of Alzheimer disease and gender on iron status, hematological indices and white blood cell counts without stratification for genotype

In order to understand how H63D and E4 might interact in AD patients, we first examined blood markers in

Table 4
Effects of the presence or absence of H63D and E4 on AD risk

Males			Females		
Genotype	P	OR (95% confidence interval)	Genotype	P	OR (95% confidence interval)
E4+ (55%)*	0.010 [73%]	5.56 (1.62–19.1)	E4+ (76.5%)*	0.000 [96%]	6.86 (2.40–16.2)
H63D+ (25%)*	0.078 [42%]	0.333 (0.102–1.09)	H63D+ (32.4%)*	0.262 [20%]	2.10 (0.701–6.23)
H63D+/E4+ (15%)*	0.642 [7%]	2.00 (0.314–12.7)	H63D+/E4+ (26.5%)*	0.003 [84%] 0.009 [74%]	7.13 (2.07–24.6) 10.2 (2.07–50.5)**
H63D-/E4+ (40%)*	0.014 [69%] 0.202 [25%]	6.11 (1.51–24.8) 3.97 (0.771–20.4)**	H63D-/E4+ (50.0%)*	0.080 [42%]	2.57 (0.968–6.81)
H63D+/E4- (10%)*	0.020 [64%] 0.035 [56%]	0.198 (0.055–0.715) 0.210 (0.0534–0.828)**	H63D+/E4- (5.9%)*	0.259 [20%]	0.377 (0.0797–1.78)
H63D-/E4- (35%)*	0.767 [5%]	0.812 (0.245–2.70)	H63D-/E4- (17.6)*	0.001 [91%] 0.020 [64%]	0.175 (0.0644–0.477) 0.195 (0.055–0.691)**

Data in this table were derived from information in Table 3. (male controls, N = 25; male AD patients, N = 20; female controls: N = 33; female AD patients: N = 34); E4+/E4-: having/not having the E4 allele of APOE; H63D+/H63-: having/not having H63D; *: percentage of AD patients with the designated genotype; **: after removal of individuals carrying C282Y, E2 and two copies of E4 from the samples; P: probability that the difference in number of individuals with the given genotype versus all others in AD patients and controls is unlikely to be a coincidence; Percentages in square brackets: retrospective power of the statistical test corresponding to each P value; OR: Odds ratio and (95% confidence interval) corresponding to each P value.

AD patients and controls stratified by gender (Table 5). Data for the Toronto and Kingston sites are shown separately. Mean values of markers of iron status fell within the laboratory reference ranges for both AD patients and controls and no significant differences in means for AD patients and controls were observed. None of the controls or AD patients had iron indices consistent with a diagnosis of hemochromatosis (transferrin saturation > 45% in conjunction with elevated ferritin). No AD patient or control had low hemoglobin, or an abnormal mean corpuscular volume (MCV). However, not known is the extent to which iron-withholding as the result of inflammation, infection or chronic disease has modulated blood markers of iron status in either the AD patients or controls [43,61]. As evident from Table 5, there was no evidence for significant effects of AD or gender on mean hematological indices or white cell counts (Table 5).

Genotype effects on blood markers in AD patients

Involvement of the H63D+/E4+ genotype in AD would be supported by the finding of relevant biochemical changes associated with this marker. Mean values of serum iron and ferritin, red cell folates and serum B12, stratified for genotype, are given in Table 6. Also shown in Table 6 are corresponding data for lymphocyte counts, age at diagnosis of AD and entry into the study, as well as for MMSE scores. Intergenotype comparisons were restricted to the Kingston AD patients because numbers of participants with or without H63D among the E4+ in this group were adequate for statistical comparisons. Furthermore, data from the two sites could not be pooled as distributions of blood marker measurements for the two patient groups were not superimposable. Analysis of variance revealed an apparent effect of genotype only on red cell folates that was significant in females ($P = 0.012$; retrospective power = 64%). This effect was largely due to an association between H63D and lowered folate concentration among the E4+ ($P = 0.00826$). This phenomenon was apparent in AD patients overall ($P = 0.0132$), and was not substantially affected by removal of those carrying C282Y (2 cases) and E2 (2 cases) or taking extra vitamin supplements (5 cases) ($P = / < 0.009$; retrospective power $= / >$ 74%, in all comparisons). Correlational analysis showed that the folate phenomenon was not an effect of patient age; plots of folate concentration versus age at entry into the study showed positive but insignificant correlations between these variables in both males ($r^2 = 0.0167$; $N = 13$; nondirectional $P = 0.674$) and females ($r^2 = 0.0164$; $N = 22$; nondirectional $P = 0.570$). The sample size was too small to determine if there was an effect of being a current smoker on folate levels in AD patients, as the majority of previous smokers were no longer smoking.

There was no significant effect of the genotypes shown in Table 6 on the markers of iron status (serum iron and serum ferritin) or on serum B12 among the patients.

AD patients with the H63D+/E4+ genotype (pooled, and females only) had mean ages at diagnosis of AD that were approximately 11% higher than those with H63D-/E4+ genotype, an observation suggesting that H63D possibly may be delaying the onset of AD di-

Table 5
Blood test results for Alzheimer patients (AD) and healthy volunteers (C)

	Males		Females	
Blood test (Reference ranges) N: Toronto site (N): Kingston site	AD M ± SD 11 (9)	C M ± SD 25	AD M ± SD 16 (18)	C M ± SD 33
Iron status				
Serum ferritin (30–300 μg/L; 30–100 for females)	220 ± 402 (145 ± 111)	103 ± 78.9	71.8 ± 44.4 (61.0 ± 41.8)	77.1 ± 63.0
Serum iron (10–28 μmol/L)	19.7 ± 7.61 (19.7 ± 14.0)	18.9 ± 11.9	13.8 ± 3.19 (13.6 ± 3.91)	15.0 ± 4.73
TIBC (38–76 μmol/L)	55.4 ± 8.81 (50.7 ± 5.62)	60.7 ± 9.30	61.9 ± 9.02 (58.2 ± 9.22)	62.9 ± 10.3
Tf receptor (1.9–5.0 mg/L)	3.13 ± 1.03	3.17 ± 2.04	3.71 ± 0.863	3.55 ± 1.47
% Tf saturation (20–55)	34.9 ± 11.2 (32.1 ± 9.57)	25.6 ± 6.42	22.7 ± 5.63 (24.8 ± 6.80)	24.9 ± 9.87
Tf (1.51–3.19 g/L)	2.40 ± 0.400 (2.37 ± 0.305)	2.62 ± 0.350	2.66 ± 0.506 (2.92 ± 0.470)	2.75 ± 0.533
Hematological indices				
ERCS (4.5–6.5 × 10^{12}/L; 3.8–5.8 for females)	4.63 ± 0.349 (4.55 ± 0.442)	4.80 ± 0.500	4.45 ± 0.364 (4.39 ± 0.283)	4.37 ± 0.482
Hemoglobin (120–180 g/L; 115–160 for females)	142 ± 12.0 (142 ± 10.5)	143 ± 8.92	136 ± 8.69 (135 ± 8.30)	131 ± 9.61
Hematocrit (0.40–0.54 L/L; 0.35–0.47 for females)	0.427 ± 0.0394 (0.419 ± 0.0261)	0.431 ± 0.0366	0.405 ± 0.0298 (0.406 ± 0.0239)	0.396 ± 0.0356
MCV (80–100 fL)	93.0 ± 5.00 (92.3 ± 4.22)	90.7 ± 5.30	91.3 ± 4.02 (92.5 ± 4.69)	90.9 ± 4.56
RDW (11.5–14.5)	12.9 ± 1.30 (13.2 ± 0.469)	12.5 ± 0.660	12.7 ± 0.566 (13.7 ± 1.30)	13.0 ± 1.01
Platelet count (150–450 × 10^9/L)	215 ± 49.9 (256 ± 66.8)	243 ± 40.2	276 ± 75.5 (256 ± 63.0)	252 ± 81.7
White cell counts				
Leukocytes (4.0–10 × 10^9/L)	5.99 ± 1.34 (6.45 ± 1.25)	6.87 ± 1.84	6.94 ± 1.80 (7.03 ± 2.00)	7.21 ± 1.65
Neutrophils (1.5–7.8 × 10^9/L)	3.97 ± 0.959 (3.87 ± 1.07)	4.53 ± 1.63	4.64 ± 1.63 (4.60 ± 1.53)	4.50 ± 1.40
Lymphocytes (1.5–4.0 × 10^9/L)	1.34 ± 0.422 (1.69 ± 0.525)	1.81 ± 0.519	1.64 ± 0.434 (1.69 ± 0.587)	2.20 ± 1.00
Monocytes (0.20–0.80 × 10^9/L)	0.556 ± 0.235 (0.563 ± 0.121)	0.336 ± 0.150	0.524 ± 0.138 (0.563 ± 0.243)	0.450 ± 0.500
Eosinophils (0–0.70 × 10^9/L)	0.122 ± 0.0441 (0.132 ± 0.0642)	0.146 ± 0.0522	0.110 ± 0.109 (0.153 ± 0.122)	0.190 ± 0.130
Basophils (0–0.10 × 10^9/L)	0 ± 0 (0.0322 ± 0.0228)	0.0546 ± 0.0686	0.0286 ± 0.0561 (0.0278 ± 0.0199)	0.0870 ± 0.110
Folate & B12 status				
Red cell folates (750–1800 nmol/L)	883 ± 263 (1315 ± 546)	987 ± 267	897 ± 345 (1280 ± 355)	977 ± 270
Serum B12 (165–740 pmol/L)	354 ± 364 (387 ± 157)	250 ± 133	409 ± 308 (418 ± 305)	286 ± 178

Unbracked data are from the Toronto site; bracketed data are from the Kingston site.

agnosis among E4+ patients in the study sample. Of note is that the age at diagnosis of AD should not be equated to age at onset of significant cognitive and/or behavioral symptoms associated with AD, which often preceed the age of diagnosis by a number of years [11]. Age at diagnosis of AD is more related to the need for care than the age at onset. Mean MMSE scores revealed that E4 or H63D did not substantially affect the stage of AD which on average was mild in all genotypes (mean scores ranged from 20.1 to 22.2). Yet small intergeno-

Table 6
Selected genotype effects in Alzheimer patients from the Kingston site

Parameter	H63D+/E4+ M ± SD (N)	H63D-/E4+ M ± SD (N)	H63D-/E4- M ± SD (N)
Serum iron	16.0 ± 5.07 (7)	16.1 ± 2.28 (12)	12.9 ± 2.80 (5)
(10–28 μmol/L)	16.0 ± 4.53 (5)	16.0 ± 2.00 (6)	n.d.
	14.8 ± 4.44 (6)	16.6 ± 2.2 (9)	n.d.
Serum ferritin	78.1 ± 78.6 (7)	110 ± 109 (12)	83.6 ± 52.1 (5)
(30–300 μg/L)	35.8 ± 22.4 (5)	70.3 ± 41.7 (6)	n.d.
	91.8 ± 89.8 (6)	110 ± 112 (9)	n.d.
Red cell folates	992 ± 294 (7)	1503 ± 521 (12)	1244 ± 92.8 (5)
(750–1800	922 ± 306 (5)*	1547 ± 307 (6)	n.d.
nmol/L)	930 ± 267 (6)*	1513 ± 608 (9)	n.d.
	978 ± 320 (6)*	1629 ± 448 (10)	1244 ± 92.8 (5)
Serum B12	352 ± 110 (7)	443 ± 184 (12)	465 ± 553 (5)
(165–740 pmol/L)	335 ± 92.8 (5)	412 ± 171 (6)	n.d.
	377 ± 97.6 (6)	364 ± 130 (9)	n.d.
Lymphocyte count	2.13 ± 0.625 (7)	1.56 ± 0.521 (12)	1.51 ± 0.384 (5)
(1.5–4.0 × 10^9/L)	2.27 ± 0.709 (5)	1.47 ± 0.285 (6)	n.d.
	2.17 ± 0.720 (6)	1.66 ± 0.526 (9)	n.d.
Age at diagnosis	71.2 ± 8.4 (7)	64.1 ± 9.0 (10)	70.1 ± 7.7 (5)
(years)	72.2 ± 8.4 (5)	66.5 ± 10.4 (6)	n.d.
	71.7 ± 9.2 (6)	64.1 ± 9.9 (7)	n.d.
Age at entry	74.0 ± 8.8 (7)	66.3 ± 9.2 (10)	72.9 ± 8.7 (5)
(years)	75.0 ± 8.3 (5)	68.7 ± 10.9 (6)	n.d.
	74.2 ± 9.6 (6)	66.7 ± 10.1 (7)	69.3. ± 7.6 (5)
MMSE scores	21.2 ± 4.9 (5)	20.2 ± 3.8 (11)	22.2 ± 2.9 (5)
	n.d.	20.9 ± 4.3 (6)	n.d.
	21.1 ± 4.9 (5)	20.7 ± 4.1 (9)	n.d.

M ± SD: mean ± standard deviations; N: sample size; n.d.: M ± SD was not determined when $N<4$. In this table, patients homozygous for E4 (N = 3) were eliminated from analyses to avoid possible confounding effects; Within each cell, data for different groups of patients are shown on separate lines: Line 1: all patients; 2: females only; 3: all patients minus those taking any form of vitamin supplements; 4. all patients after removal of individuals carrying E2 (N = 3) and C282Y (N = 2) from the sample;$^{*}P =/< 0.009$; retrospective power = / > 74% (H63D-/E4+ vs. H63D+/E4+).

Table 7
Distribution of genotype by folate quartile in Alzheimer patients

Folate quartile	C282Y+/E4-	C282Y+/E4+	H63D+/E4+	H63D-/E4+	H63D+/E4-	H63D-/E4-
Lowest	3 (23.1%)	0 (0%)	5 (38.5%)	3 (23.1%)	1 (7.7%)	1 (7.7%)
	0 (0%)	2 (25.0%)	4 (50.0%)	2 (25.0%)	0	0
Others	1 (2.1%)	2 (4.3%)	9 (19.1%)	23 (48.9%)	3 (6.4%)	9 (19.1%)
	2 (8.3%)	1 (4.2%)	5 (20.8%)	12 (50.0%)	0	4 (16.7%)

Line 1: all patients; 2: females only. See Results for additional detail.

type changes in mean MMSE scores fluctuated in the same directions as intergenotype differences in age at diagnosis and age at entry into the study (see Table 6) suggesting effects of genotype on all of these variables. The sample size of males was too small for an analysis of genotype effects as was done for females.

Effects of red cell folate concentration on AD risk

Because low folate is currently under investigation as a possible contributing factor to AD and/or dementia (e.g., see [56]), we asked if there was any evidence that low red cell folate levels associated with the H63D+/E4+ genotype were affecting the risk of AD. Folate levels for patients from the Kingston and Toronto sites were stratified by centile, information was pooled, and the genotype distribution among different folate quartiles was tabulated (Table 7). Among AD patients in the lowest folate quartile, 38.5% had the H63D+/E4+ genotype; in contrast, among AD patients in the other quartiles combined, this genotype was present only in 19.1%. When the analysis was restricted to female AD patients, among those in the lowest quartile, 50% had the H63D+/E4+ genotype; in contrast, among those in the other quartiles combined,

only 20.8% had this genotype. Thus folate levels in the lowest quartile were preferentially though not exclusively associated with the H63D+/E4+ genotype. Three patients carrying C282Y also fell into the lowest folate quartile, suggesting that low folate is characteristic of C282Y (in the absence of E4) as well as of H63D (in the presence of E4). We next asked if folate levels in the lowest quartile versus those in other quartiles had any effect on AD risk. This issue was examined in pooled Toronto AD patients ($N = 24$) and the controls ($N = 28$) since their folate levels were determined by the same laboratory. Findings were negative with or without elimination of the participants taking any vitamin supplement (13 patients, 12 normals) ($P > 0.05$). However, in the subgroup not taking extra vitamin supplements, folate levels in the highest quartile appeared protective compared to those in the other quartiles ($P = 0.012$; retrospective power = 64%).

Of note is that a high proportion of AD patients were on pharmacological treatment for AD (see Methods). However, analysis of variance showed that these three drugs did not affect folate or B12 levels differently.

DISCUSSION

This study has revealed two main findings that require a biological explanation: 1) In the study age range, H63D interacts with E4 as a predisposing factor for AD in females, increasing the risk of AD relative to E4 alone; and 2) Among E4+ AD patients, H63D is associated with a lowering of the concentration of red cell folates that did not affect the risk of AD in the sample.

We propose that the genetic effects associated with H63D among the E4+ result from increased oxidative stress and/or an increased labile iron pool. There is evidence from multiple sources that H63D and E4 are each associated with excessive oxidative stress [46,50, 76,93]. One source of oxidative stress may be from changes in the primary structures of the H63D and E4 proteins relative to the wild-type HFE and E3 isoforms (a histidine to aspartic acid at position 63 in the former [30] and a cysteine to arginine substitution at position 112 in the latter [24]). As the result of these substitutions, the variant isoforms should be more resistant to oxidation and less able to buffer against oxidative stress than the wild-type [60,98]. There is evidence from clinical studies [15,60] and an animal model [97] that the H63D mutation does result in increased body iron, though the abnormality is mild in comparison to effects of C282Y. In transfection experiments with a neuroblastoma cell line established from a female, Lee et al. demonstrated that relative to wild-type HFE, H63D was associated with increased baseline oxidative stress and lowered mitochondrial potential without a significant increase in the labile iron pool [50]. Because there were no significant effects on markers of iron status in our sample, effects of H63D in our sample may be mediated by oxidative stress, although effects of iron cannot be ruled out. Such effects may include increased translation of the amyloid-β protein precursor (AβPP), since this is known to be regulated by a functional iron response element (IRE) that interacts with the iron responsive proteins (IRPs) in its 5'untranslated region [10], and IRE/IRP-regulated expression increases in response to iron and reactive oxygen species [69]. Additionally, increased oxidative stress associated with H63D may result in increased production of amyloid-β [59]. Amyloid-β interaction with mitochondria in turn might lower their energy potential [5]. Excessive oxidative stress also may result in changes in expression of other genes that predispose to AD development and/or pathogenesis [2,84].

Increased oxidative stress associated with H63D and E4 may also result in the destruction of folate in red cells [41,71,72]. In turn, this could affect other molecules and processes and compromise the ability of red cells to scavenge free radicals as well as their vital role in oxygen and carbon dioxide transport [48]. Thus red cell dysfunction might contribute to the etiology and/or pathogenesis of AD. Although low red cell folates appear to be only a genetic marker in our sample, excessively lowered folate might predispose to AD in at least three different ways. First, because folic acid can scavenge free radicals [47,65,71,72], lowered folate might result in less free radical scavenging. Second, because folic acid can oxidize ferrous iron (Fe2+) in the absence of strong reducing agents such as ascorbic acid, thereby attenuating the Fenton reaction [72], lowered folate might directly compromise this protective effect. Finally, lowered folate would affect homocysteine metabolism [67]. Somewhat lowered folate might actually promote production of glutathione, the most important antioxidant in the body, via the transsulfuration pathway through reduction of the homocysteine remethylation reaction. Perhaps such enhanced glutathione production explains the H63D protective effect on AD risk in E4-males (Table 4) and the nonsignificant delay in age at AD diagnosis in E4+ females (Table 6) in our Ontario sample. However, excessively low levels would be expected to seriously compromise

the homocysteine remethylation cycle and the reactions that lead from this. As a result of the high hemoglobin content of red cells, Fe2+ is continually being generated and fuelling the oxidizing Fenton reaction [90].

Of note is that the level of oxidative stress may be independent of, or dependent upon, the presence of iron. This also could be aggravated by smoking and the presence of other metals acting alone or in combination with iron [29,70]. It is suggested that differing levels of oxidative stress and differing degrees of protection against oxidative stress, including folate status, might explain at least in part why different research groups have found different HFE effects on the risk of AD and its age at onset, and on the involvement of homocysteine in AD (see Introduction). Additionally, diverse effects of HFE in AD may reflect population differences in the frequencies of the HFE and/or APOE gene variants, as well as different frequencies of other gene variants including those affecting folate and homocysteine metabolism [1,23,36]. Finally, in the Canadian population, mandatory folic acid fortification has increased the mean folate level substantially since 1998. Prior to 1998, the geometric mean red cell folate concentration for Canadians was reported to be 527 nmol/L; two years later, it had risen to 741 nmol/L [9]. See also [35]. Mean folate levels in our AD patients and controls are now above this value (Table 5). Thus, mandatory folate supplementation since 1998 may have offset previous overt folate deficiencies. Not known is if this practice has affected the risk of AD or its age at diagnosis or onset. Also not known is whether pharmacological treatment of AD is affecting the fate of folate.

In male AD patients, excessive mortality associated with the E4 genotype may explain the gender differences in genetic effects (Table 4) in our sample [26, 32]. Females may be spared as the result of their lower iron levels and estrogen protective effects. Because 17β-estradiol is able to scavenge free radicals [14], females may have better antioxidant defense than males. Females also tend to have lower circulating iron levels than males. During the reproductive period of their lives, they have lower body iron stores than males as the result of blood loss through menstruation and childbirth [15,60,66].

The first study of HFE mutations in sporadic AD published by Sampietro et al described an age lowering effect of H63D homozygosity compared to wild-type HFE homozygosity on age at onset of sporadic AD and a five-fold decrease in frequency of the H63D allele in AD patients over 80 years old compared to those under 70 [86]. A recent paper has now confirmed an effect of H63D homozygosity on age at onset of AD in a cohort study [3]. Results from our study cannot be directly compared to those of Sampietro et al. for several reasons. First, we evaluated different genotypes as risk factors for probable AD. Second, the frequency of H63D homozygosity was too low in our population for evaluation (see Table 2). Finally, because the age range of participants was relatively narrow in our study, it was not possible to compare allele frequencies in patients over 80 with those in other age ranges. In the Sampietro et al. study, the apparent failure to identify interaction between E4 and H63D may be a consequence of the broad age range of their participants, since the maximal effect of E4 in sporadic AD is reported to occur under age 70 [34]. Of note is that results from the previous study of HFE and APOE gene variants in EOFAD [60] should not be directly compared with the present study of sporadic AD because the definition of sporadic AD implies a lack of history of AD in relatives of the probands. Although lack of representation of H63D homozygosity in our control sample may in part be a consequence of small sample size, we question if lack of representation among AD patients might also reflect our exclusion of patients with vascular or mixed dementia from the study since an association of H63D homozygosity with ischemic stroke has been reported [27].

The elegant study of Morris et al. concluded that dietary intakes of folate, B12 and B6 were not associated with incident AD over a 4-year period [62]. Although we also found no effect of low folate on the risk of AD, it was suggested that this possibility was not eliminated since mandatory folic acid fortification might be masking a previous contribution of low folate to AD in our populations. However, our study and that of Morris et al. cannot be directly compared for two reasons. First, participants in the latter were biracial whereas those in ours were Caucasian. Because allele frequencies of C282Y and H63D are much lower in African Americans than in Caucasians [1], frequency of the H63D+/E4+ genotype in the Morris et al. study may have been very low in comparison to ours. Second, because AD is a heterogeneous disorder with age at onset spanning many decades, risk factors for incident AD developing in a 4-year time interval in a cohort likely are not the same as for probable sporadic AD in the age range in our study. Because folate concentration is affected not only by oxidative degradation as this paper proposes, but also by genetic differences in enzymes involved in folate and homocysteine metabolism [23,36, 67], findings by different groups may not necessarily be comparable.

Because Bonferroni corrections were not applied for multiple comparisons and the sample size is small, the study must be considered pilot. Application of retrospective power analysis showed that the small sample size affects the power of the conclusions. The fact that information from patients from the two clinics could not always be pooled further reduces the power. Additionally, the pooling of two ethnically diverse groups of patients may confound the identification of genetic factors that affect AD risk. Furthermore, the findings apply only to the study age range. Also to note is that we compared the prevalence of each genotype in prevalent (not incident) cases of probable AD and controls. Greater prevalence of a given genotype among cases could be because of an increased risk of developing the disease, or because of greater survival, or a greater likelihood of reaching a hospital for diagnosis. Selection of a control series also may have biases [60]. Thus larger independent studies in males and females in discrete age intervals over a wide age range are warranted. The importance of longitudinal cohort studies of incident AD cannot be overemphasized.

In the future, it will be of particular interest to examine HFE and APOE variants in combination with variants of other genes involved in iron metabolism, inflammation and oxidative stress as risk factors. For example, combinations of C282Y, the C2 allele of transferrin, and E4 may be associated with a very high risk of AD [51,79]. Possibly as the result of the restricted age range, the frequency of C282Y was too low in the present study to evaluate a joint effect of C282Y and C2 on AD risk. However, in females, there is pilot evidence for synergy between H63D, E4 and C2. The odds ratio of these three gene variants together was 1.25 times higher than that for H63D and E4, though C2 did not affect the risk of AD on its own (Schwarz and Percy, unpublished).

Although multiethnic studies and the international pooling of samples have been encouraged in the past, careful consideration should be given to possible complicating effects of such practices on interpretation and generalization of results [1]. Future genetic association studies also should consider possible complicating effects of geographical location even within a population that is relatively ethnically homogeneous, since north to south gradients have been reported for some genes [23, 37]. See also [60,73] for reviews of other limitations in genetic association studies. Biochemical analyses are not without complication. Some markers are known to show diurnal or seasonal variation [74,95]. The issue of whether samples should be fasting or non-fasting also merits consideration, as there are pros and cons for either metabolic state (see Methods). For example, although red cell folate levels may be informative in non-fasting samples, this may not be the case for levels of serum B12 and markers of iron status. Furthermore, when samples from different sites need to be pooled for analysis, it is preferable that biochemical analyses be done by one collaborating laboratory to minimize interassay variation from environmental factors such as prolonged storage at any temperature or excessive exposure to light which can accelerate the oxidative destruction of biological molecules *in vitro* [74].

Finally, insights from the present study may aid other research areas as well as AD. For example, studies of HFE function and of HFE variant involvement in other disorders should consider possible modulating effects of folate. Although it is clear from the present study that patients with sporadic AD of the given age range are not affected by iron overload as in hemochromatosis (see pages 19 and 23) the question of whether AD is a consequence of hemochromatosis remains unanswered, as does the issue of APOE variant involvement in hemochromatosis.

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