

Risk Factors for Development of Dementia in a Unique Six-Year Cohort Study. I. An Exploratory, Pilot Study of Involvement of the E4 Allele of Apolipoprotein E, Mutations of the Hemochromatosis-HFE Gene, Type 2 Diabetes, and Stroke

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Accepted 19 August 2013

Abstract. Risk factors for dementia development are not well-defined. We evaluated several factors alone and in combination in a unique cohort of Caucasian volunteers over an approximately 6-year observation window using a nested case/control design. Factors included: apolipoprotein E (ApoE) gene variants (the E4 allele is the strongest confirmed genetic predisposing factor for Alzheimer's disease), the hemochromatosis-HFE gene mutations (H63D and C282Y), diabetes, and stroke. At study entry, subjects were ≥ 65 years of age ($M \pm SD = 73.0 \pm 4.9$), had an MMSE score ≥ 24 , and no evidence of cerebrovascular disease or current depression. Genotyping was completed on 163 available DNA samples from three different groups at the study end: those who still had normal cognitive function; those who had developed dementia; and those with Mild Cognitive Impairment (MCI). Analyses were interpreted at the 95% confidence level without Bonferroni corrections. In the subgroup with dementia, all cases of diabetes were type 2 and present at study entry, whereas all strokes occurred during the study. The results highlight apparently synergistic interactions between genetic and medical risk factors for dementia development, gender differences in risk factors, and involvement of HFE mutations. Having E4 (i.e., either of E3/4 or E4/4), C282Y, H63D, diabetes, or stroke alone did not attain significance. Significant predisposing factors with *post-hoc* power $\geq 80\%$ were: E4 homozygosity (E4/4)males+females, odds ratio (OR) = 56.0; E4+diabetes (males+females, OR = 13.7; E4+H63D+diabetes (females, OR = 52.0); E4+stroke (males, OR = 46.5). The importance of preventing diabetes and stroke to ward off dementia and the possible role of iron dysmetabolism in dementia are discussed.

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Keywords: Alzheimer's disease, apolipoprotein E, C282Y, cohort study, dementia, HFE mutations, H63D, hemochromatosis, iron, mild cognitive impairment, risk factors, stroke, type 2 diabetes mellitus

INTRODUCTION

Factors contributing to the development of dementia are not well defined. However, various forms of dementia and mild cognitive impairment (MCI), a precursor of dementia, increase in prevalence with aging [1–3]. The burden on caregivers and the economic burden of dementias continue to grow as life expectancy rises in most countries [4]. Many case/control studies have evaluated risk factors for dementia and Alzheimer's disease (AD), the most common form of progressive dementia. However, our understanding of the etiology, pathophysiology, and prevention of these disorders remains incomplete [5–7]. In the present paper, we evaluated involvement of the polymorphisms of the apolipoprotein E (ApoE) and hemochromatosis HFE (C282Y and H63D) genes, as well as two common medical conditions affecting the elderly—diabetes and stroke—in the development of dementia in a cohort study. The following text provides background information about the putative risk factors and the rationale for the study.

Apolipoprotein E (ApoE)

ApoE is a multifunctional protein. In the blood, it binds lipids in order to solubilize them for transport [8]. In the brain, ApoE transports cholesterol to neurons [9]. It has also been implicated in immunoregulation, the innate immune response [10, 11], and cerebrovascular integrity [12]. There are three common alleles of ApoE (E2, E3, and E4) which give rise to three different ApoE protein isoforms with different functional properties [8, 9, 11]. The three common alleles give rise to six different genotypes: E2/2, E2/3, E2/4, E3/3, E3/4, and E4/4. E3 is the most prevalent and considered to be the “wild type” allele, whereas E4 is associated with elevated cholesterol levels and heart disease [13]. E4 is the strongest confirmed genetic risk factor for AD, with homozygotes at higher risk than heterozygotes [14–16]. The risk attributable to E4 varies by geographical region and by race/ethnicity [17]. Studies of E4 in dementing disorders other than AD have been conflicting [18]. However, associations between E4 and all-cause dementia [19, 20], dementia with Lewy bodies (with or without features of AD) and Parkinson's disease [21], vascular dementia [22–24], and the temporal variant of frontotemporal lobe dementia [25]

have been reported. Whether E4 is a risk factor for MCI and/or for conversion to AD remains controversial. For example, Heun and colleagues reported no significant involvement of E4 in MCI [26], while Brainerd et al. found that “E4 was a reliable predictor of MCI” and “was not a risk factor for other forms of cognitive impairment without dementia” [27]. In a recent meta-analysis, E4 was found to be a significant risk factor for conversion from MCI to AD [28].

Diabetes mellitus

Diabetes mellitus refers to a group of metabolic disorders associated with elevated blood sugar [29]. As of 2013, approximately 285 million people worldwide are affected by diabetes [29, 30]. In type 1 diabetes, insulin deficiency results from loss of the insulin-producing pancreatic beta cells [29]. In type 2 diabetes, insulin resistance may be accompanied by reduced insulin secretion [29, 30]. In insulin resistance, cells do not respond appropriately to circulating insulin that is produced. Approximately 90% of people with diabetes have type 2 [29]. Over the last decade, the prevalence of type 2 diabetes has increased dramatically as the result of changes in human behavior and lifestyle. Type 2 diabetes in children, youth, and young adults is being called a “new epidemic” [31, 32]. Persons affected by diabetes are at increased risk from well-known complications including heart disease and stroke [29], and large epidemiological studies have also demonstrated that type 2 diabetes is a risk factor for both vascular dementia and AD [33, 34]. Diabetes and prediabetes have consistently been shown to be risk factors for cognitive decline, MCI, and dementia, though the mechanisms by which diabetes impairs brain function and cognition are not fully understood [35, 36]. Diabetes may not only increase the risk of dementia and MCI but also the risk of progression from MCI to dementia [37]. Type 1 diabetes is associated with a greater risk for dementia than type 2 [33]. Evidence for insulin resistance in autopsied brains of persons with AD has led some investigators to refer to AD as type 3 diabetes [38, 39].

Stroke

A stroke of the brain is a condition in which brain cells die because of a lack of oxygen after a vascular event. This can be caused by a block in the blood flow

(ischemic stroke) or the rupture of an artery that feeds the brain (hemorrhagic stroke). A transient ischemic attack is a transient episode of neurologic dysfunction caused by ischemia that does not result in permanent brain damage [40]. Approximately 87% of strokes result from ischemia or infarction with the remainder resulting from intracranial hemorrhages [41]. In the United States, the prevalence of stroke based upon data collected between 2006 and 2010 was 8.3% for those ≥ 65 years of age, compared to 0.7% in those aged 18–44 [42]. Stroke is a confirmed risk factor for dementia [43, 44] and dementia predisposes to stroke [43], but the pathological mechanisms involved are not well understood [45, 46]. Stroke has been reported to occur more frequently and earlier in males than females [47] and to be a “male-specific” risk factor for dementia [48].

*HFE mutations*¹

Hemochromatosis refers to a spectrum of disorders, acquired or genetic, in which iron progressively accumulates to toxic levels [49]. The HFE gene was implicated in hemochromatosis in 1996 when Feder and colleagues identified two polymorphic variants (C282Y and H63D) in a series of patients with an inherited form of hemochromatosis common in persons of northern or western European ancestry [50]. Wild-type HFE protein is required for the negative regulation of iron entry into cells; the binding of HFE protein to transferrin receptors reduces their affinity for iron-loaded transferrin, the primary iron-carrier in blood [51]. The HFE protein is primarily expressed on liver and intestinal cells and also on some immune system cells [52]. In addition to interacting with proteins on the cell surface to detect the amount of iron in the body, HFE protein increases the level of hepcidin, the master iron regulatory hormone [53]. Hepcidin inhibits iron absorption by intestinal enterocytes and iron export from monocytes by binding and inactivating the iron exporter, ferroportin-1 (Fp-1). C282Y homozygosity, C282Y/H63D compound heterozygosity, and H63D homozygosity are all associated with hemochromatosis, though not everyone with these genetic factors develops hemochromatosis [54].

The C282Y mutation alters HFE protein structure, preventing its association with β_2 microglobulin (β_2 M) [55, 56]. As a result, mutant HFE protein is degraded before it can be incorporated into the cell

membrane and regulate iron uptake, leading to iron overload at the cellular level. Hemochromatosis associated with C282Y is now called hemochromatosis 1 [52, 54]. In hemochromatosis 1, it is believed that low circulating levels of hepcidin are responsible for unregulated iron absorption by the gut and low macrophage iron [53].

The H63D mutation does not affect association of HFE protein to β_2 M or its cell surface expression. However, unlike wild-type HFE protein which decreases the affinity of transferrin receptor for transferrin, overexpressed H63D protein does not have this effect, indicating a functional consequence of the H63D mutation [51]. As well, H63D is associated with “iron dyshomeostasis, increased oxidative stress, glutamate release, tau phosphorylation, and an altered inflammatory response, each of which is under investigation as a contributing factor to neurodegenerative diseases” [57]. H63D is also associated with prolonged endoplasmic reticulum stress and chronically increased neuronal vulnerability [58]. Homozygosity for H67D, the mouse analogue of H63D, results in hepatic iron overload [59]. Mice carrying H67D are reported to have altered brain iron profiles and oxidative stress, as well as the induction of adaptive mechanisms to these metabolic perturbations [60].

On the basis of reports that iron metabolism was abnormal in aging and AD, members of our group previously investigated involvement of the HFE hemochromatosis mutations in AD. In 2000, we reported involvement of HFE gene polymorphisms in a series of individuals with familial AD (average age at onset, 63.0 ± 9.4 years) [61]. In this study, the absence of C282Y and/or H63D in combination with absence of E4 was found to be more protective against AD than absence of E4 or HFE mutations alone in males; this interaction was not evident in females. In 2008, we reported evidence for involvement of H63D in sporadic AD (average age at diagnosis, 74.1 ± 9.7 years) [62]. In females, E4 significantly predisposed to AD in the presence of H63D but this effect was not evident in males. Over the last decade, interest in aberrant iron metabolism in AD, aging, and other neurodegenerative diseases has continued to increase [63, 64]. However, significant involvement of hemochromatosis HFE mutations in AD alone or in combination with E4 has not been reported consistently, possibly due to complex gene-environment interactions [64]. Two separate studies have revealed synergistic interaction between C282Y and the transferrin C2 polymorphism in the development of AD among northern Europeans [65].

¹ According to nomenclature convention, the gene and the protein product are both called HFE.

Interactions between diabetes, stroke, and E4 in dementia

It is unclear whether diabetes and stroke act independently or synergistically with E4 as a risk factor for dementia. Previous studies of involvement of E4 and diabetes in AD indicate that the two factors in combination are a stronger risk factor than either factor alone, though it is not clear if they act synergistically [34, 66, 67]. Studies of the involvement of E4 and stroke in dementia are suggestive that the two factors act independently [68, 69].

Rationale and objectives of the present study

Although the C282Y and H63D HFE mutations resulting in classical hemochromatosis have been studied in AD, to our knowledge they have not been evaluated in cohort studies of the development of dementia. There also is no information about whether these HFE gene variants interact with E4, diabetes, or stroke in the development of dementia. In 1997, one of our group (AG) initiated a longitudinal study of elderly volunteers to study the relation between blood markers of cobalamin metabolism and the development of cognitive impairment [70, 71]. Because exclusion criteria for this study were quite stringent and the cohort was unique, we reasoned that an exploratory "add-on" nested case/control study might shed new light on the involvement of E4 and the hemochromatosis-associated HFE alleles, as well as of diabetes and stroke, in the development of dementia. In the present paper we have systematically evaluated involvement of these factors alone and in combination as risk factors for dementia.

MATERIALS AND METHODS

Ethics approval

The study was approved by research ethics boards at Queen's University and St. Mary's of the Lake Hospital, Kingston, Ontario. Procedures involving experiments on human subjects were done in accord with the Helsinki Declaration of 1975. Informed signed consent was obtained from all volunteers before entry into the study [70].

Participants

Independently-living volunteers aged 65 years and older were recruited from the Kingston area through

the use of flyers and announcements at senior community group meetings and activities. Recruitment was conducted between 1997 and 2001; follow-up continued until mid-2006 [70]. The study began at approximately the time that folic acid fortification of food was made mandatory in Canada [70].

The stringent exclusion criteria for this cohort have been published previously [70]. These included having: a score of less than 24 out of 30 on the Mini-Mental State Examination (MMSE); history of cerebrovascular or neurological disease; excessive oral supplementation with B12 at dosages higher than 25 mcg daily, or any injected dose; renal failure; history of ileal (pertaining to the small intestine)/gastric surgery; hospitalization during the three months before testing; current depression; and any acute medical condition. Of 317 persons who volunteered for the study, 36 were excluded because they had a low MMSE score (<24/30), history of stroke, Parkinson's disease, severe renal, cardiac or respiratory disease, or psychiatric diagnosis. The average age ($M \pm SD$) of the 281 study entrants was 73.0 ± 4.9 years (73.0 ± 4.8 for females, 73.0 ± 5.2 for males).

Participant evaluation, classification, and diagnosis

Evaluation procedure

Participants were evaluated at study entry and on up to three more occasions at intervals of approximately 18 months over the approximately 6-year observation window. Demographic and medical history information were systematically collected. During each visit, neurocognitive function was evaluated using a battery of tests. These included the MMSE, the Stroop Neuropsychological Screening Inventory (Stroop), the California Verbal Learning Test (CVLT), and the Mattis Dementia Rating Scale (Mattis DRS). (See reference [70] for additional details of these tests.) Participants who could not attend the last assessment of the study were administered the Telephone Interview for Cognitive Status (TICS) [72].

Diagnosis/classification

At the study end, each participant was assigned to one of seven subgroups based upon all available evidence: 1) still cognitively normal; 2) affected by dementia; 3) having cognitive impairment but not dementia (CIND); 4) having some cognitive decline; 5) having another diagnosis; 6) unable to classify; or 7) lost to follow-up. DSM-IV-TR criteria were used for

diagnosis of dementia [73, 74]. Participants in group 3 were said to have CIND rather than MCI because classification of these individuals did not follow standard practice. Classification was based upon multiple longitudinal evaluations using a research battery of cognitive tests and the TICS was used for evaluation of some participants (see above). From the medical history, we noted if and when individuals were diagnosed with diabetes or stroke. A diagnosis of diabetes was based on current Canadian guidelines [29]. Note that Canadian Guidelines for diabetes are not identical to those used in the United States or Europe [29, 30]. A diagnosis of stroke included evidence for ischemic stroke, hemorrhagic stroke, or a transient ischemic attack [75].

The nested case/control study

At the end of the observation period (i.e., at the study end), two subgroups of participants were selected for a case/control study: those who still were cognitively normal and those who had developed dementia. For comparison, we included the subgroup of patients who had developed CIND by the study end. For genetic studies, blood samples were obtained from 170 available participants in these three subgroups for whom clinical data were largely complete. Genomic DNA was isolated, and ApoE and HFE typing was completed for 163 of the 170 individuals. (In a few cases, the DNA yield was too low for genetic analysis to be completed.)

Genetic studies

Genomic DNA was isolated from peripheral blood samples using QIAamp DNA Blood Mini Kits (Qiagen). ApoE typing was conducted using a standard RFLP-PCR protocol previously described [63]. The ApoE PCR procedure distinguishes the six different ApoE genotypes resulting from the three common ApoE alleles (E2, E3, and E4): E2/2, E2/3, E2/4, E3/3, E3/4, and E4/4. Because the sample size was small, in most evaluations of E4 effects, we pooled individuals carrying either E3/4 or E4/4; in the text, the term "E4" denotes having either the E3/4 or E4/4 genotype. Screening for the HFE C282Y and H63D mutations was done using the simplex PCR procedures described by Feder et al. [50] and the multiplex PCR procedure described previously [62]. In the text, the term "H63D" denotes having one or two copies of H63D and "C282Y" denotes having one or two copies of C282Y.

Statistical analysis

The null hypotheses were that the distributions of E4, C282Y, H63D, diabetes, or stroke alone or in various combinations were the same in those who were still cognitively normal at the study end compared to those who had developed dementia or CIND in the same time period. Contingency table and odds ratio (OR) analysis were used to compare frequencies of risk factors in these groups. Analyses were conducted with and without segregation by gender. We also asked if age and level of education affected the results.

The significance of associations between putative genetic or medical explanatory variables (alone or in combination) and dementia or CIND were determined using Fisher's Exact Test [76]. Two tailed analyses were conducted in all cases. Strengths of associations between genetic and medical factors and dementia (or CIND) were evaluated using OR analysis. ORs and their 95% confidence intervals (95% CIs) were calculated using SAS[®] 9.3. For these determinations, the frequencies of different risk factors in persons who developed dementia (or CIND) by the study end were compared with the corresponding frequencies in the persons who remained cognitively normal. Differences between means were evaluated using Student's *t*-test (Excel 2010; two-tailed, unequal variance). Bonferroni corrections were not applied because the study was exploratory. Results of analyses were interpreted at the 95% level of confidence ($\alpha = 0.05$). Because the conversion rate to dementia in this study was low, in order to facilitate comparison of the study results with published information, we have reported some *p* values that were >0.05 . To aid with interpretation of results, *post-hoc* power calculations were conducted using the procedure of Kane [77].

RESULTS

Results are summarized in Tables 1 through 5. With the exception of Table 1, which includes results based upon all available data, results are based on data for the 163 individuals represented in Table 2.

Characteristics of study participants

A summary of demographic and medical information for the participants is given in Table 1. After approximately 6 years of follow-up ($M \pm SD$: 6.39 ± 1.27 years), 192 of the 281 participants (68.3%) remained cognitively normal, 26 (9.3%) were diag-

Table 1
Summary of demographic and medical information for participants based upon all available data*

	Females (F)		Males (M)		Comments
	Normal**	CIND	Normal**	CIND	
Participants admitted to study (n)	212	69			Of 317 individuals who volunteered for the study, 36 were excluded and 281 were admitted
Classification of participants after approximately 6 years of follow-up	Normal** 147	CIND 18	Normal** 45	CIND 4	In addition to the 240 persons classified as either still cognitively healthy, or having CIND or dementia at the study end, 2 (1F, 1M) had severe cognitive decline, 13 (10F, 3M) could not be classified, and 26 (21F, 5M) were lost to follow-up. The proportion of males who developed dementia by the study end (11/69; 15.9%) was significantly higher than for females (15/212; 7.1%). ($p = 0.0333$; $OR = 2.49$ (1.09–5.72); <i>post-hoc</i> power = 57.7%)
Average age (in years) at study entry $M \pm SD$	72.4 \pm 4.5	74.8 \pm 6.3	71.6 \pm 4.7	70.0 \pm 3.1	Participants (M+F) who developed dementia were significantly older at study entry than those who remained cognitively normal. ($p = 0.00250$)
Average years of education $M \pm SD$	13.4 \pm 3.1	11.3 \pm 2.4	13.5 \pm 4.2	15.3 \pm 3.9	There were no significant differences in average years of education for participants developing CIND or dementia compared to those who remained cognitively normal.
Fraction with 11 or fewer years of education (%)	37/147 (25.2)	11/18 (61.1)	19/45 (42.2)	1/4 (25.0)	A significantly higher proportion of participants who developed CIND in the 6-year observation window (12/22; 54.6%) had 11 or fewer years of education than those who remained cognitively normal (56/192; 29.2%). ($p = 0.0271$; $OR = 2.91$ (1.19–7.13); <i>post-hoc</i> power = 66.9%)
Fraction with type 2 diabetes (%)	12/147 (8.2)	4/18 (22.2)	4/45 (8.9)	0/4 (0)	The frequency of type 2 diabetes was somewhat higher in participants with dementia ($p = 0.146$), and in females with CIND ($p = 0.0781$) than in the corresponding groups that remained cognitively normal. All cases of diabetes in the subgroup with dementia were present at the study start (i.e., pre-existing)
Fraction with stroke occurring during the study*** (%)	9/123 (7.3)	0/7 (0)	3/43 (7.0)	0/4 (0)	The frequency of stroke occurring during the study was somewhat higher in males who developed dementia than in males who remained cognitively normal. ($p = 0.0568$)
Number (fraction:%) of admitted participants who were ApoE and HFE typed (see Table 2)	105 (105/147:71.4)	5 (5/18:27.8)	36 (36/45:80.0)	3 (3/4:75.0)	Genetic testing was completed on a somewhat higher proportion of male than female participants (47/60:78.3% versus 116/180:64.4%) ($p = 0.0552$)

* p values and odds ratios were calculated using all available data. **The designation "normal" refers to individuals who still had normal cognitive function at the study end. ***Medical record information about occurrence of stroke during the study was not complete for all those receiving a classification of cognitively normal, CIND, or dementia at the study end.

Table 2

Co-distribution of ApoE and HFE variants in three groups of participants at the study end for whom clinical information was largely complete and DNA was available*

Cognitively Still Normal		Females (105)						Males (36)					
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													
-,-			8	33	1	15			1	19		6	
-,C282Y			3	11		1				1			
-,H63D			6	16	1	7			5	2		2	
C282Y,C282Y						1							
C282Y, H63D				1									
H63D,H63D						1							
Dementia													
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						3		2	
-,C282Y						1				1			
-,H63D				2		1	1						
C282Y,C282Y													
C282Y, H63D									1				
H63D,H63D													1
CIND													
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		2	1					1	
-,C282Y										1			
-,H63D				1								1	
C282Y,C282Y													
C282Y, H63D													
H63D,H63D													

*The average age (M ± SD) of these 163 participants at the study end (i.e., at the time of diagnosis/classification) was 79.0 ± 4.9 years (79.1 ± 4.8 for females, 78.7 ± 5.1 for males). Information about diabetes and stroke for these individuals was generally available. See Table 4 for the co-distribution of genetic and medical risk factors in the individuals who developed dementia by the study end.

nosed with dementia, 22 (7.8%) were considered to have CIND, 2 (0.7%) had some cognitive decline, 13 (4.6%) could not be classified, and 26 (9.3%) were lost to follow-up. A higher proportion of males (11/69:15.9%) than females (15/212:7.1%) developed dementia by the study end ($p=0.0333$). Persons who developed dementia were older at study entry (75.9 ± 5.2 years) than those who remained cognitively normal (72.3 ± 4.6 years) ($p=0.00250$). In the subgroup with dementia, all instances of diabetes in the participants were type 2 and present at the study start. Type 2 diabetes was more frequent in those with dementia (5/26:19.2%) and in females with CIND (4/18:22.2%) than in the corresponding groups who remained cognitively normal (16/192:8.3% overall; 12/147:8.2% for females). All participants were stroke-free at the study start. Stroke occurred in both males and females during the study, but it was a risk factor for dementia only in males (no females who developed dementia suffered a stroke). The frequency of stroke occurring during the study was greater in males who developed dementia (3/9:33.3%) than in

those who were still cognitively normal at the end of the study (3/43:7.0%), though this difference did not attain significance ($p=0.0568$). Having 11 or fewer years of education significantly predisposed to CIND overall ($p=0.0271$; OR = 2.91 (95% CI: 1.19–7.13); *post-hoc* power = 66.9%) but not to dementia (see Table 1 for details).

Genetic risk factors in dementia development

The co-distribution of ApoE and HFE polymorphisms in the 163 participants from whom sufficient DNA was available and clinical information was largely complete is given in Table 2. Frequencies of the three ApoE and two HFE alleles in this group are given in Table 3. The co-distribution of the genetic and medical risk factors evaluated in this study in individuals who developed dementia by the study end are given in Table 4. P values for associations between different risk factors and dementia and the corresponding ORs are given in Table 5.

Table 3
Allele frequencies (%) of the ApoE and HFE polymorphisms*

Gene**	Allele 1***	Allele 2***	Allele 3***
ApoE	E2	E3	E4
M+F (n = 326)	26 (8.0)	251 (77.0)	49 (15.0)
F (n = 232)	19 (8.2)	178 (76.7)	35 (15.1)
M (n = 94)	7 (7.5)	73 (77.7)	14 (14.9)
HFE	C282Y	WT	
M+F (n = 326)	22 (6.8)	304 (93.3)	
F (n = 232)	19 (8.2)	213 (91.8)	
M (n = 94)	3 (3.2)	91 (96.8)	
HFE	H63D	WT	
M+F (n = 326)	51 (15.6)	275 (84.4)	
F (n = 232)	38 (16.4)	194 (83.6)	
M (n = 94)	13 (13.8)	81 (86.2)	

*Data in this table are based upon data in Table 2 (i.e., a total of 163 participants: 116 females and 47 males). **Values of n in column 1 denote the total number of chromosomes in each category (i.e., twice the number of participants). ***Unbracketed numbers in columns 2, 3, and 4 denote the numbers of each allele in each category; adjacent bracketed numbers indicate the % frequency of each allele (i.e., allele frequency****). **** Allele frequency (%): The proportion of all copies of a gene that is made up of a particular gene variant (i.e., number of a particular allele/total number of chromosomes) × 100).

Allele frequencies of ApoE and HFE polymorphisms

As shown in Table 3, among the 163 participants with genetic data, frequencies of the ApoE alleles were similar for males and females. Frequencies of the C282Y and H63D alleles were somewhat higher for females than males, but these differences were not significant. Frequencies of the different ApoE and HFE alleles in the dementia or CIND groups did not differ from frequencies for those who remained cognitively normal, overall or for males and females separately (results of analyses not shown).

Involvement of E4

We first considered E4 homozygosity (i.e., E4/4) as a risk factor for dementia without considering other genetic or medical information that was available. E4 homozygosity was strongly associated with dementia in males and females combined ($p=0.00760$; $OR=56.6$ (95% CI: 2.57–1250); *post-hoc* power = 80.6%), but was not represented among those remaining cognitively normal (Tables 2 and 5). E3/4 heterozygosity showed no significant association with dementia in males and females combined, but the “E4” genotype (i.e., having either of E3/4 or E4/4) was somewhat more common than among those with dementia than those who were still cognitively normal at the study end ($p=0.118$, Table 5). Note from the

Table 4
Co-distribution of genetic variants and medical conditions in individuals with dementia

Participant number	ApoE	C282Y*	H63D**	Diabetes***	Stroke****
Females					
1	3/3	0	1	0	0
2	3/3	0	1	0	0
3	3/3	0	0	0	0
4	3/4	1	0	0	0
5	3/4	0	1	1	–
6	4/4	0	1	1	0
Males					
1	2/3	1	1	0	0
2	3/3	0	0	0	1
3	3/3	0	0	1	0
4	3/3	1	0	0	0
5	3/3	0	0	0	0
6	3/4	0	0	0	0
7	3/4	0	0	1	1
8	4/4	0	2	0	1

*: 0, wild type; 1, C282Y heterozygote. **: 0, wild type; 1, H63D heterozygote; 2, H63D homozygote. ***: 0, not affected with type 2 diabetes; 1, affected at study start. ****: 0, not affected with stroke; 1, affected during study; –, no information available.

distribution of risk factors in individuals with dementia (Table 4) that in 5 of 6 persons carrying one or two E4s, one or more of the other factors under evaluation are present in addition to E4.

Involvement of HFE mutations

There were gender differences in the involvement of HFE mutations with dementia. Having either H63D homozygosity or compound heterozygosity (H63D/C282Y) was strongly associated with dementia in males ($p=0.0296$; $OR=28.1$ (95% CI: 1.20–655); *post-hoc* power = 72.3%) (Tables 2 and 5). (The third genotype associated with hemochromatosis—homozygosity for C282Y—was not represented in the sample.) In contrast, H63D homozygosity and compound heterozygosity were represented in the females who remained cognitively normal, but not in females who developed dementia. These gender differences are consistent with the hypothesis that H63D homozygosity or compound heterozygosity exerts deleterious effects earlier in males than females.

Among females, neither E4, H63D, nor C282Y alone were significantly associated with dementia (Table 5). However, E4 plus H63D and/or C282Y in combination was significantly associated with dementia in females ($p=0.0208$; $OR=9.50$ (95% CI: 1.69–53.3); *post-hoc* power = 75.2%).

Table 5
Explanatory factors for dementia or CIND in participants who were genotyped*

Outcome	Gender	Explanatory factors	Data used for estimation of p values, ORs, and post-hoc power**	p values (Fisher's exact Test; two-tailed)	Odds ratio (95% CI) (SAS)	Post-hoc power (%)
Dementia	M+F	E4 homozygosity (E4/4) (E4/4)	0/141 versus 2/14 (0:141 versus 2:12)	0.0076	56.6 (2.57–1250)	80.6
	M+F	E4 (i.e., E3/4 or E4/4)*** E3/4	33/141 versus 6/14 (33:108 versus 6:8)	0.1180	2.46 (0.795–7.58)	37.5
	M+F	Diabetes	14/141 versus 4/14 (14:127 versus 4:10)	0.0609	3.63 (1.05–13.1)	53.4
	M+F	E4 + diabetes	4/141 versus 4/14 (4:137 versus 4:10)	0.0024	13.7 (2.97–63.1)	86.8
	M+F	Stroke****	12/139 versus 3/13 (12:127 versus 3:10)	0.1210	3.18 (0.768–13.1)	41.7
	F	E4	25/105 versus 3/6 (25:80 versus 3:3)	0.1680	3.20 (0.607–16.9)	32.3
	F	H63D	32/105 versus 4/6 (32:73 versus 4:2)	0.0805	4.56 (0.795–26.2)	45.3
	F	E4 + H63D	8/105 versus 2/6 (8:97 versus 2:4)	0.0905	6.06 (0.956–38.3)	54.4
	F	E4 + H63D &/or C282Y	10/105 versus 3/6 (10:95 versus 3:3)	0.0208	9.50 (1.69–53.5)	75.2
	F	Diabetes	11/105 versus 2/6 (11:94 versus 2:4)	0.1450	4.27 (0.700–26.1)	42.7
	F	E4 + diabetes	3/105 versus 2/6 (3:102 versus 2:4)	0.0228	17.0 (2.19–132)	75.6
	F	H63D + diabetes	4/105 versus 2/6 (4:101 versus 2:4)	0.0334	12.6 (1.76–90.5)	71.4
	F	E4 + H63D + diabetes	1/105 versus 2/6 (1:104 versus 2:4)	0.0072	52.0 (3.86–700)	83.9
	M	Stroke****	3/36 versus 3/8 (3:33 versus 3:5)	0.0632	6.60 (1.03–42.2)	56.5
	M	E4	8/36 versus 3/8 (8:28 versus 3:5)	0.3910	2.10 (0.410–10.8)	16.6
	M	E4 + stroke	0/36 versus 3/8 (0:36 versus 3:5)	0.0042	46.5 (2.10–1027)	85.6
	CIND	M	H63D/H63D or H63D/C282Y	0/36 versus 2/8 (0:36 versus 2:6)	0.0296	28.1 (1.20–655)
M		E4-ve/C282Y-ve	27/36 versus 3/8 (27:9 versus 3:5)	0.0869	0.200 (0.0397–1.01)	53.9
M+F		E4 (i.e., E3/4 or E4/4)	33/141 versus 5/8 (33:108 versus 5:3)	0.0260	5.46 (1.24–24.1)	67.7
M+F		E4-ve/C282Y-ve	90/141 versus 2/8 (90:51 versus 2:6)	0.0544	0.189 (0.0368–0.971)	60.5
F		E3/3 + diabetes	6/105 versus 2/5 (6:99 versus 2:3)	0.0417	11.0 (1.53–78.9)	69.1

*Data used in estimation of the p, OR, and post-hoc power values were derived from information on participants whose genetic data are given in Table 2. **The unbracketed fractions in column 4 indicate the proportions of persons with the given explanatory factor who were still cognitively normal at the study end versus those with dementia or CIND. The bracketed ratios in column 4 denote the corresponding numbers of individuals with/without the given explanatory factor. ***As explained in the text, the term "E4" denotes having either of E3/4 or E4/4. ****As shown in Table 4, no females diagnosed with dementia had a stroke during the study.

Genetic risk factors in combination with medical conditions in dementia development

The co-occurrence of genetic and medical risk factors in individuals with dementia is given in Table 4. Results given in Table 5 support the hypothesis that there is synergy between genetic factors and type 2 diabetes or stroke in dementia development.

Genetic factors plus diabetes

The combination of E4 and diabetes was associated with a significant risk of dementia overall ($p=0.0024$; OR = 13.7 (95% CI: 2.97–63.1); *post-hoc* power = 86.8%) though neither factor on their own attained significance. This apparent synergistic effect was stronger in females ($p=0.0228$; OR = 17.0 (95% CI: 2.19–132); *post-hoc* power = 75.6%). In females, having H63D and diabetes was also significantly associated with dementia ($p=0.0334$; OR = 12.6 (95% CI: 1.76–90.5); *post-hoc* power = 71.4%), whereas H63D or diabetes on their own were not. In females, having E4, H63D, and diabetes was associated with a higher risk of dementia ($p=0.0072$; OR = 52.0 (95% CI: 3.86–700); *post-hoc* power = 83.9%) than the combination of E4 and diabetes alone (see above). These observations suggest that H63D and/or diabetes interact synergistically with E4 to accelerate dementia development. Lack of involvement of H63D with E4 and diabetes in males reflects the low frequency of H63D in males with dementia compared with females.

Genetic factors plus stroke

An over-representation of stroke in males with dementia compared to males who remained cognitively normal was apparent in the subset of 163 who were genotyped (Table 5) as well as in the full data set (Table 1). Having E4 and developing stroke during the study was strongly associated with dementia in males ($p=0.0042$; OR = 46.5 (95% CI: 2.10–1027); *post-hoc* power = 85.6%). These findings, along with absence of stroke in females with dementia (Tables 1 and 4), indicate that stroke occurring during the study had a more deleterious effect in males than females.

Genetic and medical risk factors for CIND

Risk factors for CIND were evaluated by comparing their frequencies in the cognitively normal and the CIND subgroups (Table 5). E4 (i.e., having either of E3/4 or E4/4) significantly predisposed to CIND in males and females combined. Having E3/3 in combination with type 2 diabetes significantly predisposed

to CIND in females. (Having E2/3 in combination with diabetes was not associated with CIND, data not shown.) The absence of E4 in combination with absence of C282Y was marginally protective against CIND in males and females combined. The *post-hoc* power values in the CIND group were $\geq 67.7\%$ but $\leq 80\%$. The CIND and dementia groups were not large enough to test the hypothesis that the risk factors for dementia and CIND were different.

DISCUSSION

Major findings

The results of this small study of dementia development pertain to a group of individuals with an average age of 79.0 ± 4.9 years at the study end. They highlight the potential importance of: evaluating risk factors alone and in combination to identify those of most importance (especially in dementia prevention strategies); involvement of HFE mutations; and segregating by gender in risk factor analysis.

The analysis provided confirmation of the involvement of several factors previously implicated in dementia development: increased age (Table 1); E4 homozygosity; E4 plus type 2 diabetes; and E4 plus stroke (Table 5; Introduction). The strengths of the ORs for associations between E4 and diabetes or E4 and stroke are suggestive that the medical conditions interact synergistically with E4 in dementia development (Table 5). To our knowledge, our study is the first to demonstrate involvement of HFE hemochromatosis mutations in a cohort study of the development of dementia: E4 plus H63D and/or C282Y (females); E4 plus H63D plus pre-existing type 2 diabetes (females); and H63D homozygosity or compound heterozygosity (males) (Table 5). Gender differences in predisposing risk factors were noted. In addition to the different involvement of HFE mutations in males and females (Table 5), a higher proportion of males than females developed dementia (Table 1). Furthermore, E4 plus stroke was a strong predisposing factor only in males (Table 5). While determination of risk factors for CIND was not the study focus, and the sample size was small, factors that predisposed to CIND—11 or fewer years of education, having either E3/4 or E4/4 (males and females combined), and having E3/3 plus pre-existing type 2 diabetes (females)—were qualitatively different from the factors predisposing to development of dementia in the approximately 6-year observation window (Table 5).

Interpretation

The finding that E4 homozygosity was a strong predisposing factor for dementia, but that E3/4 heterozygosity alone, or having either E3/4 or E4/4 were not, supports a gene dosage effect of E4 on the rate of dementia development.

The finding that E4 interacted with H63D and diabetes and also with stroke as a risk factor for dementia development, raises the question of whether either genetic factor might be a risk factor for diabetes or stroke as well as for dementia. In our sample, there was evidence for a weak association between E4 and diabetes ($p = 0.161$), suggestive that E4 might predispose to diabetes as well as to dementia. However, there was no evidence for an association between H63D and diabetes, and no evidence for an association between either E4 or H63D and stroke (data not shown).

Although markers of body iron status were not measured in this study, the finding that HFE mutations affected the risk of dementia development (Table 5) and were present in 5 of the 6 females and 3 of the 8 males with dementia (Table 4), raises the question of whether high iron load resulting at least in part from the HFE polymorphisms might be contributing to pathogenic processes in the brain.

Though the small sample size of the study precluded testing of the hypothesis that risk factors for dementia and CIND development are different, the results in Table 5 support a model in which the nature and number of risk factors affecting individuals, as well as gender, affect outcome within a given time period. Because diabetes and stroke are inherently preventable, and the strength of their interactions with the genetic factors were striking, the study results are relevant to the prevention of dementia.

Relation of findings to previous studies

Our confirmation of previous observations that E4 homozygosity, E4 plus diabetes, and E4 plus stroke predispose to dementia (Introduction) serve as a reference for gauging the relative strengths of the new combinations of factors associated with significant risk that are highlighted in this study.

There is published evidence to support the idea that E4 and H63D each predispose to type 2 diabetes. For example, others have noted: an association between E4 and type 2 diabetes with or without co-morbid coronary artery disease [78]; a marginal association between E4 and type 2 diabetes [79]; and an association between H63D and type 2 diabetes [80, 81]. There

is evidence for an association between E4 and ischemic stroke [82, 83], though not in the very elderly [84]. Evidence for involvement of HFE mutations in stroke is controversial [85–87]. Others have shown that E4 and hemochromatosis HFE mutations are associated with cardiovascular disease [88], suggesting a possible association with vascular dementia.

Our observation that the two hemochromatosis genotypes are associated with dementia in males but not females is in accord with the fact that males suffer iron overloading consequences earlier than females, because females tend to be spared as the result of blood loss through menstruation and childbirth [54]. The finding that the combination of H63D and diabetes, or H63D, E4, and diabetes predisposed to dementia in females but not males (Table 5), may reflect the possibility that males are affected by H63D earlier than females, rendering them not able to participate in the study. Involvement of the HFE mutations in the development of dementia continues to support the conclusion of Nandar and Connor that the hemochromatosis HFE variants are “genetic modifiers or a risk factor for neurodegenerative diseases by establishing an enabling milieu for pathogenic agents” [57]. The HFE-related study findings thus add to existing evidence for a key role of iron dyshomeostasis in dementia (Introduction).

Because of the design of the present study, our results cannot be compared to other models for dementia development that are based upon population studies [89]. There is information in the literature relevant to the prevention of type 2 diabetes [90] and primary prevention of stroke [91] but it is beyond the scope of this paper to discuss these strategies. Strategies also are available for treatment and prevention of iron overload [92–94] and for potentially ameliorating harmful effects of E4 by exercise [95].

Strengths and limitations of the study

Strengths of this study are: the relatively stringent inclusion and exclusion criteria; the thorough neurocognitive evaluation conducted on a research basis enabling persons with CIND to be distinguished from those with dementia and those who were still cognitively normal at the end of the study; evaluation of risk factors separately in males and females as well as singly and in combination to better identify predisposing factors. In addition, the relatively homogeneous ethnic background of the participants (Caucasian and not typical of the ethnic heterogeneity characteristic of some larger cities in Ontario or Canada), the nature

of the panel of risk factors, and the approximately 6-year observation window, are unique to this study. Because study hypotheses were not predefined, the study must be considered exploratory. The low rate of dementia (9.3% overall) and unavailability of blood samples from all participants at the study end resulted in a small data set for analysis. Thus the study must also be considered “pilot”. The possibility that mandatory folic acid supplementation of food, in effect by January 1998, may have influenced classification of some of participants by the study end has not been excluded [70, 96]. Finally, the study sample is one of convenience and not representative of ethnically diverse populations in some large cities in Ontario or Canada.

Future research

Observations from this small study provide rationale for future activity in several areas. First, they underscore the potential importance of strategies to prevent type 2 diabetes and stroke in order to prevent dementia. Education and outreach activities communicating the research findings should be promoted to encourage individuals to take initiative for diabetes and stroke prevention in collaboration with their physicians even in the absence of clinical prevention trials. It also is crucial to raise awareness about hemochromatosis and the importance of maintaining normal levels of body iron. Second, our finding that 8 of 14 participants with dementia carried at least one of the two common hemochromatosis HFE mutations (Table 4), provides rationale for systematic examination of the role of body iron metabolism in dementia development in a larger cohort study. The C282Y and H63D HFE variants are only two of several genetic polymorphisms known to be involved in hemochromatosis. Future studies might also evaluate mutations associated with hemochromatosis in non-HFE genes [97, 98], the transferrin C2 variant which synergistically interacts with C282Y in some AD patients [65], as well as variants of certain other genes involved in iron metabolism (e.g., see references [99–103]). Future research should include measurement of blood markers of body iron status including levels of hepcidin and a complete blood cell count, as well as standard blood indicators of organ and endocrine function and extensive medical history information. In addition to consideration of the strategies described in this paper, small future cohort studies would benefit from the application of full genome sequencing which is rapidly decreasing in cost [104]. Full genome sequencing should enable identification of known variants of, as well as new

mutations in, virtually any gene suspected of affecting dementia (or MCI) development and facilitate the prioritization of risk factors to be addressed in prevention strategies.

ACKNOWLEDGMENTS

We thank: Dr. George Perry and the anonymous reviewers of this paper for their critical input and helpful suggestions; and Dr. Laurel Duquette, Director, Statistical Consulting Services, Statistical Sciences, University of Toronto, for conducting odds ratio analysis using SAS. We apologize to authors whose work could not be cited because of space limitations. The following organizations provided in kind support: Surrey Place Centre, Toronto; Best Research Institute, University of Toronto; University of Toronto High School Mentorship Program; Department of Medical Genetics, University of Alberta. Grant sponsors included: Ontario Mental Health Foundation (A.G.); Queen’s University Initiation Grants (A.G.).

Authors’ disclosures available online (<http://www.j-alz.com/disclosures/view.php?id=1916>).

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