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The role of HFE (hemochromatosis) gene mutations in sporadic Alzheimer disease

by

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ABSTRACT

Although a central etiology for Alzheimer disease (AD) has not yet been determined, support has amassed for the notion that oxidative stress may be involved in the pathogenesis of AD. The disruption of iron homeostasis and iron's excessive deposition in AD brain tissues has received increased attention due to the metal's capacity to promote the production of harmful free radicals. Several studies have recently examined whether DNA mutations involved in the iron overload disorder, hemochromatosis, pose an increased risk of acquiring AD. However, the small sample size and low generalizability of previous studies have warranted further investigation. We genotyped 213 AD patients, 106 Mild Cognitively Impaired (MCI) individuals, and 63 Normal Elderly Control (NEC) subjects for the H63D and C282Y HFE mutations to examine whether a relationship exists between HFE gene status and AD presentation in our patient population. DNA analysis was conducted by polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP). We did not find any statistically significant associations between HFE gene status and the clinical, demographic, or neuropsychological aspects of AD in our patient population. Interesting trends that fell short of statistical significance included: a) a deleterious effect of HFE mutations on motor performance, b) an influence of H63D homozygosity on an earlier onset of cognitive decline, and c) an influence of H63D homozygosity on an accelerated progression from MCI to AD.

RÉSUMÉ

Malgré le fait qu'une étiologie centrale n'a pas encore été déterminée pour la maladie d'Alzheimer (AD), les données accumulées indiquent que le stress oxidatif pourrait être impliqué dans la pathogénèse de cette maladie. La perturbation de l'homéostase du fer et le dépôt excessif de fer dans les tissus du cerveau atteint de AD ont attiré l'attention dû à la capacité de ce métal à promouvoir la production de radicaux libres nocifs. Récemment, plusieurs études ont examiné si les mutations du gène HFE impliqué dans la maladie causée par la surcharge de fer, l'hémochromatose, pouvaient augmenter le risque de contracter la AD. Cependant, l'échantillonnage limité des études antérieures justifient des recherches additionelles. Nous avons génotypé 213 patients atteints de la AD, 106 individus ayant de léger désordres cognitifs (MCI), et 63 sujets normaux agés utilisés témoins (NEC), pour les mutations H63D et C282Y du gène HFE afin d'examiner s'il existe une relation entre le statut de gène HFE et la présence de la AD dans notre population de patients. L'ADN de nos patients a été analysé par PCR suivi de RFLP. Nous basant sur nos resultats, nous concluons que le statut du gène HFE ne contribue pas de façon significative aux aspects cliniques, démographiques, ou neuropsychologiques chez nos patients atteints d'AD. Des tendances intéressantes, qui ne sont cependant pas statistiquement significatives, incluaient: a) l'effet nuisible des mutations HFE sur la performance motrice, b) l'influence de l'homozygosité de l'alléle H63D sur un début prématuré de déclin cognitif, et c) l'influence de l'homozygosité de l'alléle H63D sur la progression accélérée de MCI à AD.

To my grandparents, parents, and sisters for their endless support and love

In memory of my beloved grandfather, Dr. Shlomo Kats, z''l.

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I would also like to extend my gratitude to Dr. George Chong, of the Molecular Diagnostics laboratory of the Jewish General Hospital (JGH), for introducing me to the PCR-RFLP technique. His helpfulness, advice, and support are well appreciated. I gratefully acknowledge the staff of the McGill Memory Clinic for providing me access to blood samples, medical records, and neuropsychological testing results of all subjects used in this study. I would also like to thank Dr. Natalie Philips for assisting me with the statistical analysis of the neuropsychological data. In addition, I thank Mrs. Adrienne Liberman for helping me construct the figures presented in this thesis and for her sound advice concerning all aspects of laboratory management.

Contributions to Original Knowledge

Two previous studies have examined whether the HFE (hemochromatosis) mutations might influence the natural history of Alzheimer disease (AD). One of these studies suggested that the H63D mutation may be involved in precipitating an earlier symptoms onset in sporadic AD. To our knowledge, we are the first to examine the effects of these mutations among AD, MCI, and NEC subjects. Our findings indicate that the HFE mutations do not significantly contribute to the clinical, demographic, or neuropsychological aspects of sporadic AD. The discrepancy between our study and the earlier work demonstrating an effect of the H63D mutation on AD symptoms onset, suggests that the HFE mutations may have population-specific effects. Herein, we report three novel trends that were detected. These include: a) a deleterious effect of HFE mutations on motor skills, b) an influence of H63D homozygosity on an earlier onset of cognitive decline, and c) an influence of H63D homozygosity on an accelerated progression from MCI to AD.

Preface

The author was solely responsible for the HFE genotyping, hospital records research, patient database construction, and statistical analyses described in the body of this thesis. DNA extraction and apoE genotyping were performed by the Molecular Diagnotics laboratory of the Jewish General Hospital (JGH). Neuropsychological evaluations of our subjects were conducted by Drs. Nora Kelner and Lennie Babins, neuropsychologists affiliated with the McGill Memory Clinic (Jewish General Hospital).

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INTRODUCTION

Alzheimer's disease

Alzheimer's disease (AD) is named after the German neuropathologist and psychiatrist, Alois Alzheimer, who in 1907 first described the clinical and pathological characteristics of the disorder that now bears his name (Alzheimer, 1907). AD is a progressive and debilitating neurological disease that largely affects the basal forebrain, hippocampus, and association cortices. The molecular hallmarks of AD are extracellular β-amyloid-containing (Aβ) senile plaques and intracellular neurofibrillary tangles that are comprised of hyper-phosphorylated tau. The loss of synapses and neurons in the AD brain ultimately results in a progressive deterioration in cognitive functioning (reviewed in Cummings et al., 1998 and Vickers et al., 2000). The earliest symptom is memory impairment, followed by deficits in language functions, praxis, visuospatial abilities, and the capacity to independently conduct activities of daily living (Honig and Mayeux, 2001).

It is generally thought that the clinical symptoms of AD manifest themselves in response to a critical accumulation of brain lesions over one's lifetime. Appropriately, aging is considered a major risk factor for AD. Evidence exists that brain damage leading to AD may accumulate for decades before symptoms present themselves or deficits in neuropsychological measures are observed (Morris, 1997). The brain's capacity to sustain and buffer such long-term injury in AD is similar to how in Parkinson's disease (PD) abnormalities are noted only after 80% of dopaminergic neurons in the substantia nigra pars compacta are lost (Langston et al., 1992). As the brain's pathological burden increases, the threshold for clinical dementia is crossed and impairments become progressively more severe until patients are no longer able to speak,

walk, recognize faces, or feed themselves. Death following this global cognitive deterioration is often due to infection, typically pneumonia (Honig and Mayeux, 2001).

The Amyloid Hypothesis of AD

Although a central etiology for AD has not yet been determined, one well recognized explanation for neuronal degeneration is the amyloid hypothesis. According to this theory, Aβ, a proteolytic product of the amyloid precursor protein (APP), deposits in the brain's parenchyma and initiates a neurotoxic cascade that eventually leads to cell death. APP is a membrane-anchored glycoprotein that is expressed on most mammalian cells. Transfection experiments reveal that APP is commonly cleaved by a protease referred to as 'α-secretase', resulting in the release of its soluble extracellular domain termed APP_s (Esch et al., 1990). Neuroprotective roles have been established for APP_s and include its ability to protect against excitotoxic and hypoglycemic damage (Mattson et al., 1993). Alternatively, APP processing by proteases 'β-secretase' and 'γ-secretase' results in the release of a 40-42 residue Aβ peptide (Shoji et al., 1992). The amyloid hypothesis of AD posits that an abnormal production and aggregation of Aβ peptides results in the accumulation of reactive oxygen species (ROS), the recruitment of microglia, and culminates in the destabilization of the surrounding cells' cytoskeleton networks by the hyper-phosphorylation of tau.

Free radicals and AD

Support has amassed for the notion that free radical damage may be intimately involved in the pathogenesis of AD (Markesbery, 1997; Nunomura et al., 2001; Perry and Smith, 1998). Free radicals are species that contain one or more unpaired valence-shell

electrons. These highly reactive agents oxidize (abstract electrons from) or reduce (donate electrons to) other molecules in order to reach a more favorable and stable energy state. Oxidative stress results from an increased formation of free radicals and/or a decreased functioning of anti-oxidant defense systems (Sies, 1985). The brain is especially prone to oxidative damage due to its high oxygen consumption rate, its abundance of redox-active metals such as iron, its large lipid content, and its relative shortage of antioxidant defenses compared with other tissues (Coyle, 1993).

The extent of oxidative damage to biological molecules in the AD brain is far greater than that in the normal elderly brain. Oxidative damage to nuclear DNA (Gabbita et al., 1998) and mitochondrial DNA (Mecocci et al., 1994), as measured by the presence of oxidatively modified bases, is significantly increased in the AD brain. Such alterations result in DNA strand breakage and base substitutions (Halliwell and Gutteridge, 1999). Although AD is foremost a disease of the brain, the detection of oxidatively damaged DNA in blood lymphocytes derived from AD patients is suggestive of a peripheral component to this disorder (Mecocci et al., 1998). Furthermore, relative to normal elderly controls, proteins and lipids in the AD brain are subjected to increased oxidative damage, leading to amino acid abnormalities and decreased membrane fluidity, respectively (Halliwell and Gutteridge, 1999). It is still unknown whether oxidative radical generation in AD is a primary (causative) event or a secondary result of other underlying pathologies.

The strong association between oxidative stress and the cytopathological lesions in AD supports the assertion that oxidative damage is central to the disorder's pathogenesis. Heme oxygenase-1 (HO-1), a member of the stress protein superfamily, is robustly up-regulated in response to the presence of ROS and is therefore considered a

sensitive marker of oxidative stress (Tyrrell et al., 1993). HO-1 is massively overexpressed in the neurons and astrocytes of the AD hippocampus and cerebral cortex relative to age-matched controls (Schipper et al., 1995). In addition, senile plaques and neurofibrillary tangles are both immunoreactive for HO-1 (Schipper et al., 1995, Smith et al., 1994). Furthermore, *in-vitro* experiments demonstrate that oxidative stress increases Aβ production (Zhang et al., 1997) and that Aβ itself advances free radical generation (Behl et al., 1992). Interestingly, Hensley and colleagues (1994) reported that the Aβ peptide exhibits a chemistry that facilitates its fragmentation into neurotoxic oligopeptide radicals. They suggest that Aβ-derived radical species may promote peptide aggregation, a process which is considered to initiate the sequence of neuropathological events in AD. Moreover, the development of animal models of AD has allowed investigators to examine the *in-vivo* effects of Aβ overexpression. Transgenic mice harboring APP mutations demonstrate abundant Aβ deposition and pervasive oxidative damage (Smith et al., 1998).

With relevance to this dissertation, the disruption of iron homeostasis and iron's excessive deposition in AD brain tissues (Connor et al., 1992) have received increasing attention due to the metal's ability to catalyze the production of ROS (Halliwell and Gutteridge, 1999). Ferrous iron is capable of reducing hydrogen peroxide by the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$) and generating the toxic hydroxyl radical, a species that contributes to a range of biological damage (Meneghini, 1997). Iron's close association with AD pathology has been demonstrated by its localization to senile plaques and neurofibrillary tangles (Smith et al., 1997). *In-vitro*, iron-catalyzed oxidation systems have been shown to accelerate the aggregation of β-amyloid into its toxic fibrillar form (Kontush, 2001; Mantyh et al., 1993), whereas radical scavengers such as

ascorbic acid prevent the aggregation process (Dyrks et al., 1992). Our laboratory has provided evidence that HO-1 over-expression in the AD brain may contribute to iron mismanagement in this disease. Schipper et al. have shown that HO-1 over-expression in rat astroglial cultures significantly augments sequestration of iron by the mitochondrial compartment. In addition, others have noted that APP mRNA contains a region that is homologous in sequence to an iron response element (Tanzi and Hyman, 1991). The possibility that iron may directly influence the regulation of APP production further implicates iron in the pathogenesis of AD. That elevated brain iron concentrations may have deleterious effects in AD was further suggested in one study where desferrioxamine, an iron-chelating agent, appeared to slow the clinical progression of this disease (Crapper McLachlan et al., 1991).

AD: Therapeutic considerations

In the past decade, considerable progress has been made in understanding the molecular basis of AD. These advances have facilitated research into various therapies that may act by preventing disease occurrence, deferring its onset, or slowing its progress (Cummings et al., 1998). Currently available treatments for AD are of two types: one focuses on symptom alleviation through the enhancement of cholinergic functioning, and the second centers more on neuroprotection by alleviating oxidative damage.

The standard treatment for AD are cholinesterase inhibitors (Cummings and Cole, 2002). In the normal brain, the major source of acetylcholine is the nucleus basalis of Meynert. Since the basal forebrain is affected early in AD, cholinesterase inhibitors are used to enhance residual cholinergic activity. Currently, four of these inhibitors are available for clinical use: tacrine, donepezil, rivastigmine, and galantamine. These drugs

are the only medications approved by the US Food and Drug Administration for the treatment of AD. Tacrine, a first-generation cholinergic enhancer, is now rarely prescribed due to its significant toxicity profiles. These agents have been shown to improve cognitive functioning and prolong a patient's ability to perform activities of daily living, although efficacy varies between patients.

Antioxidant treatments are sometimes employed to counter the harmful effects of ROS. Potent antioxidants such as alpha-tocopherol (vitamin E), and selegiline, a monoamine oxidase inhibitor, have been reported to slow the progression of AD when administered to patients with moderate disease severity (Sano et al., 1997). Other studies have suggested that antioxidant therapy may be a promising avenue for delaying disease onset (Zaman et al., 1992). Such findings underscore the significance of oxidative stress in AD and suggest that an early antioxidant intervention may be particularly advantageous.

Biological markers of AD

Early treatment of AD is important since brain cells lost in the process of the disease cannot be replaced. It is for this reason that biological and genetic markers that allow for a positive diagnosis at an early stage of AD are currently in demand (reviewed in Engelborghs and De Deyn, 2001). One particularly powerful diagnostic test that has been proposed for AD involves an assay for elevated tau and reduced Aβ-42 levels in the cerebrospinal fluid (CSF) (Hulstaert et al., 1999). Others have suggested using redox-related biochemical markers such as HO-1 (Schipper et al., 2000) and oxidized guanosine (8-hydroxy-2-deoxyguanosine) to aid in the disorder's diagnosis (Lovell and Markesbery, 2001). With the advent of cDNA microarray technology, the expression of a wide

spectrum of genes derived from AD patients can be compared against normal elderly controls in order to identify markers that confer an increased risk for AD (Pasinetti, 2001). Furthermore, single nucleotide polymorphisms (SNPs) in human genes related to oxidative stress have been associated with an increased risk for the development of certain neurodegenerative disorders such as PD and motor neuron disease (Forsberg et al., 2001). It is possible that SNPs present in redox-associated genes may contribute to AD pathology and influence the disease's presentation.

While diagnostic markers are used to detect disease presence, risk factors indicate the probability of disease development. Expansion in the assortment and quality of both of these tools would permit a more sensitive diagnosis and bolster predictive capabilities. Considering that AD is a large and growing health problem worldwide, it is critical to uncover new and promising means to diagnose and treat this disorder. Without such intervention, AD is poised to afflict as many as 14 million people in the United States by the year 2050 (Scinto and Daffner, 2000).

Epidemiology of AD

The burden that AD places on our society includes the suffering of patients and their families, the enormous costs associated with the disease, and the overshadowing threat that worries the growing elderly population. AD is the most common aging-related neurodegenerative disorder and poses direct and indirect costs of roughly 10 billion dollars per year in Canada (100 billion dollars annually to the United States).

Measures of AD frequency can be described in terms of prevalence or incidence. Prevalence denotes the proportion of people within a population that have AD at a specific point in time. Prevalence studies are therefore important for examining the

burden of the disease. In North America, two-thirds of patients with dementia have AD (McDowell, 2001). Approximately 4 million Americans and roughly 400,000 Canadians are presently diagnosed with AD. The prevalence of this disorder rises exponentially with age, such that its rate doubles every five years after age 65 (McDowell, 2001). Moreover, these figures are considered underestimates since many individuals live with dementia for years in their community before being diagnosed.

Incidence expresses the number of new cases of AD that develop in a population of individuals at risk during a specified time interval. Studies of incidence are of more clinical value since they are crucial for identifying risk factors (McDowell, 2001). The incidence rate for AD is similar between European, American, and Canadian populations. In 1997, there were 360,000 new cases of AD in the US. The incidence is anticipated to rise by more than three times to 1.14 million new cases in 2047 (Brookmeyer et al., 1998).

The tremendous public health impact of AD is largely a result of recent demographic trends (McDowell, 2001). This century's advancements in medicine and technology have increased life expectancy and resulted in a rapid growth of the elderly population. Given that age is a major risk factor for AD, several studies have projected a sharp growth in the prevalence of the disease (Brookmeyer and Gray, 2000; Jorm and Korten, 1988). One American study suggests a quadrupling of AD prevalence in the next 50 years (Brookmeyer et al., 1998). These staggering figures signal the need for interventions that can mitigate the projected burden of AD. Even modest delays in disease onset can have a dramatic effect. If AD onset could be delayed by 1 year, following 50 years of intervention, there would be 770,000 fewer people afflicted with the disease than currently projected (Brookmeyer et al., 1998). Furthermore, a 1 year

delay in disease onset would result in an annual savings of roughly 36 billion dollars 50 years after the start of the intervention (Brookmeyer et al., 1998). Longitudinal studies have demonstrated that early-onset AD is typically characterized by a more rapid cognitive and functional decline (Lucca et al., 1993; Jacobs et al., 1994). Therefore, in some cases, delaying symptoms onset may extend patients' lives and improve their quality of living. The most effective preventative strategy will utilize sensitive preclinical markers and risk factors to identify those people who are most likely to develop AD and begin treatment before considerable brain damage is done.

Genetic risk factors for early-onset AD

AD is a complex and heterogeneous disorder. Between 5% and 10% of cases occur before the age of 65 and are designated early-onset AD. These cases are often referred to as familial AD (FAD) since the majority of them display an autosomal dominant inheritance with high penetrance. Late-onset AD, comprising the majority of AD cases, is also termed sporadic AD since most of these individuals do not have any of the genetic abnormalities associated with FAD. In the past 15 years, defects in three genes have been shown to cause FAD. These include mutations in APP on chromosome 21, presenilin 1 (PS-1) on chromosome 14, and presenilin 2 (PS-2) on chromosome 1 (reviewed in Sorbi et al., 2001). The finding that mutations in all three of these genes result in an increased production of Aβ-42 consolidates the peptide's close ties to AD's pathogenesis (Scheuner et al., 1996). The discovery of these point mutations has also allowed for the development of various animal models of AD (reviewed in Emilien et al., 2000).

The first gene to be identified as a strong risk factor for AD was the APP gene (Goate et al., 1991). APP mutations account for only 2-3% of FAD cases (Tanzi et al., 1996). There are six different missense mutations in proximity to the Aβ peptide coding domain that alter the processing of the precursor protein and result in an increased release of Aβ (Tanzi et al., 1996). Transgenic mice expressing the APP mutations develop profuse Aβ-containing senile plaques and neuronal loss, although their reliability as an AD model is compromised by their lack of neurofibrillary tangle pathology (Games et al., 1995).

The pivotal role of genetics in FAD was supported further by the association of PS-1 and PS-2 gene mutations with FAD. These genes were both identified by a positional cloning technique (Sherrington et al., 1995; Rogaev et al., 1995) and were found to be causative in about 50% of individuals with FAD. There are currently over 50 different missense mutations known in PS-1 and six possible mutations in PS-2, indicating the relative rarity of the latter. PS-1 is an integral membrane protein with eight transmembrane domains, resembling the structure of a cell-surface receptor. Speculation has arisen that the presenilin proteins may play a role in the proteolytic processing of APP and somehow cause a selective increase in the production of the Aβ-42 peptide. Transgenic mice mutant for PS-1 exhibit a plethora of Aβ-42 but their validity as an AD model is questionable as they exhibit neither plaques nor tangles (Duff et al., 1996). Analysis of brain tissue derived from human patients bearing the PS-1 mutation also demonstrates an abundance of Aβ-42 (Lemere et al., 1996).

It is widely considered that mutations and polymorphisms in other genes, not yet identified, may also result in an increased susceptibility to FAD. Although the literature implies that APP, PS-1, and PS-2 mutations are fully penetrant, some evidence has

indicated otherwise (personal communication from M. Percey, University of Toronto). Incomplete penetrance by these mutations supports the possibility that protective genes exist and modulate the effect of the known familial mutations. The valuable knowledge that has been acquired from the discovery of the FAD mutations reaffirms the significance of the Aβ hypothesis. However, the inability to generate an animal model of AD that accurately reproduces the disorder's pathological lesions suggests that other factors besides Aβ over-production are also responsible for AD development.

Genetic risk factors for late-onset AD

Those who develop AD after the age of 65 are said to suffer from late-onset or sporadic AD. Late-onset AD is the predominant form of the disease, affecting 90% of all AD patients. It is considered to be far more genetically complex than the early-onset variety. Although there is no evidence of Mendelian inheritance in late-onset AD, several studies have indicated that genes play an important role in it's etiology. It has been shown that sporadic AD cases often have a positive family history of dementia (Frisoni and Trabucchi, 1997). In addition, a twin study demonstrated that the AD concordance rate among monozygotic twins is significantly higher than that for dizygotic twin pairs. The authors concluded that heredity is the major causal factor in late-onset AD (Bergem et al., 1997). The majority of sporadic AD cases can presumably be attributed to the effects of several genes (reviewed in Richard and Amouyel, 2001).

The only established genetic risk factor for late-onset AD is the E-4 allele of the Apolipoprotein E (ApoE) gene, located on chromosome 19. The three alleles of the apoE gene are designated E-2, E-3, and E-4. ApoE is synthesized in astrocytes and microglia and is implicated in the regulation of lipid metabolism in the brain. The association of

apoE-4 with sporadic AD was first reported by Strittmatter and colleagues (1993) and subsequently confirmed by other groups (Saunders et al., 1993; Slooter et al., 1998). These studies demonstrated that the apoE-4 allele is over-represented in both early and late-onset AD compared to age-matched controls. A meta-analysis conducted by Farrer and colleagues (1997) revealed that homozygosity for apoE-4 is associated with a 12.5 times increased risk of AD, while possession of one E-4 allele is associated with a 2.7 times increased risk, indicative of a dose dependent response. It should be noted that between 35% and 50% of individuals that suffer from AD do not carry an E-4 allele and many individuals who possess the E-4 allele will never develop AD (Blacker et al., 1997). Since apoE-4 is neither necessary nor sufficient for the development of the disease it is not recommended for routine use in clinical diagnosis or for predictive testing (ACMG/ASHG, 1995).

Importantly, apoE-4 has been shown to be responsible for lowering the age of AD symptoms onset. Onset has consistently been shown to occur earlier in individuals who are homozygous for apoE-4 than in those lacking the E-4 allele (Corder et al., 1993; Blacker et al., 1997; Meyer et al., 1998). While some studies have demonstrated that the inheritance of one E-4 allele also lowers the age of symptoms onset (Corder et al., 1993; Meyer et al., 1998), others did not show this effect (Blacker et al., 1997).

The association between apoE-4 and AD prompted research into the molecular mechanisms that may account for this allele-specific effect. The apoE-4 allele seems to facilitate Aβ deposition and fibrillation in the brain (Sanan et al., 1994). Neuropathological studies have revealed that AD patients possessing even one E-4 allele exhibit an increased density of Aβ-containing senile plaques (Berr et al., 1994). The E-4 allele has also been linked to the development of neurofibrillary tangles (Ohm et al.,

1995). Furthermore, the apoE protein has been shown to possess an antioxidant activity. The protein's antioxidant effect is proposed to be a result of its ability to bind metals such as iron and copper that might otherwise mediate the production of ROS. ApoE's iron-binding capacity is highest in the E-2 allele and lowest in the E-4 allele, reiterating the important role of oxidative stress in AD pathogenesis (Miyata and Smith, 1996).

Primarily two types of approaches have been used to identify novel risk factors for sporadic AD. The first, genomic scanning, studies the distribution of alleles within families and identifies loci that segregate with the disease phenotype. This strategy has implicated chromosomes 1, 4, 6, 9, 10, 12, 19, and 20 as potential sites of additional AD susceptibility genes (Kehoe et al., 1999). The more commonly conducted study is the case-control association. These studies select a gene based on its functional relationship to AD and determine whether its expression is altered in the disease state compared to controls matched for age and other salient variables.

Case-control studies have implicated a range of genes to be associated with sporadic AD. However, only a few of these studies have been replicated with consistent results. A polymorphism in the α2-macroglobulin (A2M) gene on chromosome 12 was found to be genetically associated with late-onset AD (Blacker et al., 1998; Alvarez et al., 1999). A2M is a serum protease inhibitor that normally binds Aβ and mediates its clearance and degradation. This function is presumed to be compromised by certain polymorphisms in the A2M gene. Although biologically plausible, other studies were not able to show this effect (Wang et al., 2001). The transferrin C2 allele (TfC2) on chromosome 3 has also been associated with late-onset AD (van Rensburg et al., 1993; Namekata et al., 1997). Transferrin is the major serum protein responsible for iron transport in the circulation. TfC2 has a decreased iron binding capacity compared to

other transferrin alleles (Wong and Saha, 1986). As described earlier, excess iron could potentially promote ROS formation and endanger neurons. Although the association of TfC2 with late-onset AD is intriguing, some studies were unable to confirm this result (Hussain et al., 2002). Furthermore, the human leukocyte antigen-A2 (HLA-A2) allele has been reported to lower the age of onset of AD (Ballerini et al., 1999; Zareparsi et al., 2002). Other genes that have been reported to be associated with sporadic AD include the low density lipoprotein receptor-related protein (LRP) (Kang et al., 1997) and the very low density lipoprotein receptor-related protein (VLDL) (Okuizumi et al., 1995), both encoding neuronal receptors for ApoE. All of these genes deserve further investigation to fully understand their roles in AD.

In recognition of iron's notable involvement in AD, several studies have recently examined whether DNA mutations involved in the iron overload disorder, hemochromatosis, pose an increased risk of acquiring AD (Moalem et al., 2000; Sampietro et al., 2001; Connor et al., 2001b).

Hereditary Hemochromatosis: A disorder of systemic iron overload

Hereditary Hemochromatosis (HC) is the most common autosomal recessive disorder in caucasians (Merryweather-Clarke et al., 1997). Approximately 1 out of every 200 people of European origin are affected. HC is characterized by an excess absorption of dietary iron, primarily by the duodenal enterocytes, and results in the metal's pathological deposition into principally the liver, pancreas, heart, and pituitary gland (reviewed in Jazwinska, 1998 and Lyon and Frank, 2001). Symptoms of HC include fatigue, arthritis, cardiac disorders, diabetes mellitus, hepatic cirrhosis, hyperpigmentation, and hypogonadism (Fairbanks, 1999).

Ever since the term 'hemochromatosis' was first introduced in 1889, this disorder has been known to affect various organs, although the brain's involvement has received only sparse attention. Neurological symptoms related to HC have been considered to be rare (Nielsen et al., 1995). Recent findings of an association between DNA mutations in the HC gene (termed HFE) and an earlier onset of sporadic AD (Sampietro et al., 2001) is intriguing and suggests that HC-related excess brain iron may contribute to neuronal deterioration.

In 1996, Feder and colleagues discovered two missense DNA mutations in the HFE gene that are often present in HC patients: a guanine to adenine transition at nucleotide position 845 (G845A) and a cytosine to guanine transversion at nucleotide 187 (C187G). G845A causes a cysteine to tyrosine substitution at amino acid position 282 (C282Y) of the HFE protein and C187G results in a histidine to aspartic acid substitution at amino acid position 63 (H63D). Epidemiological studies were undertaken to uncover the origin of these mutations and determine their worldwide prevalence. Meanwhile, molecular biologists have focused on elucidating the chemical and biological properties of the HFE protein.

The C282Y mutation is considered to be of Celtic origin, since the geographical distribution of this allele matches the migration of the Celts (Simon et al., 1980). The H63D mutation is thought to have arisen from the Mediterranean region because it is found highest in those of Basque lineage (Merryweather-Clarke et al., 1997). These mutations may have been sustained through time due to the selective advantage that they would confer in times of malnourishment (Jazwinska, 1998). Their worldwide prevalence is estimated at 1.9% for C282Y and 8.1% for H63D (Merryweather-Clarke et al., 1997). The clinical penetrance of these two mutations varies depending on an

individual's genotype. The highest penetrance is exhibited by C282Y homozygotes, followed by compound heterozygotes (those who are heterozygous for both mutations), then H63D homozygotes, C282Y heterozygotes, and finally H63D heterozygotes (Burke et al., 1998).

The protein product of the HFE gene is a transmembrane glycoprotein that is homologous to HLA class I molecules (Fig 1). Immunohistochemical studies have detected HFE protein presence throughout the gastrointestinal tract. Particularly intense staining was demonstrated in the deep crypts of the duodenum (Byrnes et al., 2000). The HFE protein is also found on Kupffer cells in the liver (Bastin et al., 1998) and on the capillary endothelial cells of the brain (Bastin et al., 1998; Connor et al., 2001b).

The HFE protein is thought to interact with the TfR and attenuate its capacity to mediate intracellular iron delivery, although the exact molecular mechanism is debated. One model purports that the wildtype HFE protein decreases TfR affinity for Tf-bound iron (Feder et al., 1998), another proposes that the HFE protein blocks the binding of Tf to TfR (Lebron et al., 1999), and yet a third contends that the HFE protein prevents the release of iron from the TfR/Tf-Fe complex within the endosome (Roy et al., 1999) (Fig. 2). The more penetrant HFE gene mutation, G845A, impairs the protein's processing and hinders its ability to bind the β_2-microglobulin protein, an event critical for transport of the HFE protein to the cell surface (Waheed et al., 1997). The less severe C187G mutation permits HFE's membrane localization but results in an aberrant interaction with the TfR (Feder et al., 1998). Loss of HFE protein function is assumed to be responsible for excess intracellular iron absorption in HC.

The development of an HFE gene knockout mouse has confirmed the importance of the HFE protein in the regulation of iron homeostasis (Zhou et al., 1998). Even on a

standard diet, mice lacking the HFE protein exhibit an excessive iron accumulation in the liver and an elevated transferrin saturation. Iron deposition is also seen in the spleen and small intestine. The HFE knockout mouse may prove to be useful in testing the efficacy of experimental treatments for iron overload in human HC.

Currently, little is known concerning the regulation of HFE protein expression. Transcription factors that bind elements on the HFE gene promoter include activator protein 1 (AP1), activator protein 2 (AP2), and c-AMP response element binding protein (CREB) (Sanchez et al., 1998). AP1 and CREB are generally considered to promote basal gene transcription. AP2 has also been shown to stimulate the transcription of another gene involved with metal metabolism (Mitchell et al., 1987). It is not known whether intracellular iron levels influence the transcription of the HFE gene. The signaling molecules and transcriptional elements involved in HFE gene expression are presently under investigation.

Early detection for HC is emphasized since treatment by phlebotomy (blood-letting) can result in increased survival (Witte et al., 1996) and the avoidance of serious complications such as liver and heart failure. Genetic testing for the HFE mutations is commonly used to supplement the traditional diagnostic criteria of elevated transferrin saturation, iron levels, and ferritin concentration in the blood.

Iron regulation in the normal brain

Iron is the most abundant trace metal in the brain (Beard et al., 1993). As in other tissues, its cycling redox-state is essential for ATP production and lipid biosynthesis. Brain-specific functions include its involvement in neurotransmitter synthesis, myelin production, and axonal growth (Beard et al., 1993). Iron uptake into the brain is highest

during rapid brain growth, suggestive of the metal's significance in brain development (Taylor and Morgan, 1990). Additionally, iron is important for normal cognition and behavior. Iron deficient children are known to have shortened attention spans and lowered intelligence scores (Dobbing, 1990). For all of the metal's importance, stringent regulation of brain iron is necessary to avoid iron's excessive accumulation and the production of excess ROS.

In general, intracellular iron homeostasis is maintained in a coordinated manner by the differential expression of the membrane-associated transferrin receptor (TfR), the gateway for transferrin (Tf)-bound iron entry, and ferritin, the protein responsible for the metal's storage and detoxification (reviewed in Aisen et al., 2001). In brain cells, as in peripheral organs, TfR and ferritin synthesis are regulated at the post-transcriptional level based on intracellular iron levels. When cytoplasmic iron levels are low, iron regulatory proteins (IRPs) bind stem-loop structures (termed iron response elements (IREs)) within the 3' region of the TfR mRNA and confer stability to this nucleic acid. TfR mRNA translation is thereby promoted. IRPs concurrently repress the translation of ferritin mRNA by binding to IREs within the 5' region of the ferritin transcript. Conversely, elevated iron levels result in the absence of an IRP/IRE interaction, increased ferritin mRNA translation, and TfR mRNA degradation.

Iron is most commonly considered to enter the brain through endocytosis of the Tf-bound iron/TfR complex into capillary endothelial cells that comprise the blood-brain barrier (Crowe and Morgan, 1992). The clathrin-mediated uptake of the complex into early endosomes is followed by an acidification of the vesicle and the release of iron via the divalent metal transporter protein (DMT1) into the cytoplasm. In a small proportion of cases, Tf-bound iron undergoes transcytosis through the capillary endothelium and is

delivered directly to the brain parenchyma (Fishman et al., 1987). TfR levels on the brain's microvasculature are thought to be modulated regionally based on local iron requirements (Connor et al., 2001a). Another route by which iron is transported to the brain is through the choroid plexus epithelial cells. In this case, the Tf-bound iron is circulated through the ventricular system by the cerebrospinal fluid (CSF) and homogeneously distributed throughout the brain (Connor et al., 2001a).

Accumulating evidence suggests that Tf-independent mechanisms are also involved in iron's entry into the brain (reviewed in Qian and Wang, 1998). The findings that hypotransferrinemic mice exhibit substantial iron uptake into the brain (Dickinson and Connor, 1995) and that iron transport into the brain exceeds that of transferrin (Banks et al., 1988) support the existence of non-Tf mediated iron transport. Melanotransferrin (MTf) and the lactoferrin receptor (LfR) are iron-binding proteins that are expressed on the brain's capillary endothelium. The former has aroused interest due to its alleged role in AD iron overload (Jefferies et al., 1996a), and the latter has been implicated in raising intraneuronal iron levels in Parkinson's disease (Faucheux et al., 1995).

Iron dysregulation in the AD brain

In the normal human brain, iron concentrations generally increase until age 40 and then remain at a steady state (Bartzokis et al., 1997). In 1986, Ehmann and colleagues performed quantitative studies on iron levels in normal and Alzheimer diseased human brains. They concluded that iron presentation in AD brain tissue was 67% higher in gray matter and 27% higher in white matter compared to age-matched controls. Subcortical nuclei that display elevated iron levels include the globus pallidus (Loeffler et al., 1995), hippocampus, amygdala, and the nucleus basalis of Meynert

(Thompson et al., 1988). Additionally, iron exhibits considerable accumulations in the frontal cortex of AD patients (Loeffler et al., 1995).

The manner by which iron homeostasis is disturbed in AD is presently inconclusive. Multiple groups have investigated TfR and ferritin levels in normal and AD brain to determine if these proteins are involved in iron's dysregulation. The results depict a trend toward decreased TfR and increased ferritin in AD-affected brain tissue, as would be expected under conditions of heightened iron concentrations (Connor et al., 1992; Kalaria et al., 1992). The overall reduction in TfR suggests that the Tf pathway of iron transport is not the main contributor to the pathological deposition of iron in the AD brain (Schipper, 1999). Nonetheless, some irregularities in the expression of ferritin and TfR have been described (Morris et al., 1987; Conner et al., 1995; Pinero et al., 2000).

MTf may be responsible for abnormal iron levels in AD, as suggested by its elevated concentration in the serum of AD patients (Kennard et al., 1996). A correlation was shown to exist between the increase in serum MTf and the progression of AD. Furthermore, while TfR and ferritin levels are regulated by iron bioavailability, the mechanism that manages intracellular MTf levels is presently unknown. The absence of a negative feedback system in the regulation of MTf may account for the protein's elevated levels in AD patients. This may result in an increased uptake of MTf-bound iron.

HFE mutations: Implications for AD

The notion that HC entails a neurological component has been promulgated. There are several descriptions of HC patients presenting with dementia (Jones and Hedley-Whyte, 1983) and Parkinsonian symptoms (Nielsen et al., 1995). Autopsy

findings have revealed that iron deposition in the brains of HC patients was mainly localized to the choroid plexus, the pituitary gland, and in perivascular spaces (McDougal and Adams, 1950). In addition, lesions of the lenticular nucleus have been observed by MRI and CT scans of affected individuals (Berg et al., 2000). HFE protein is found on brain capillaries, choroid plexus, and ependymal cells in proximity to TfRs (Connor et al., 2001b). Such placement makes it available to limit iron traffic into the brain, a task that may be compromised in those with HFE mutations.

It has been hypothesized that the possession of one or more HC mutations may influence AD development (Connor et al., 2001b). In 2000, Moalem and colleagues reported the first evidence of an association between HFE mutations and AD. They proposed that a complex gender-based relationship exists between HFE mutations and the apoE4 allele in familial AD. They suggested that HFE mutations might be predisposing for FAD in apoE4 negative males and protective against FAD among apoE4 negative females. Presently, these complex findings are difficult to interpret. In addition, this study's small FAD patient population (n=26) urges us to question the reliability of their results.

In the following year, Sampietro and colleagues (2001) evaluated the influence of HFE mutations on the development of sporadic AD in an Italian population. They reported that patients who carried one or two copies of the H63D mutation developed AD, on average, five years earlier than those with the wildtype HFE genotype. Although H63D mutation frequency was similar between patients and controls, stratification of the patients based on age of symptoms onset demonstrated that the mutation was five times more frequent in those presenting with symptoms before age 70 compared to those presenting after the age of 80. Sampietro et al. (2001) formulated their conclusions based

on the analysis of the H63D mutation's effect on AD patients of Italian origin. World-wide H63D allele frequency exhibits considerable variation, with Italians having a 12.6% frequency, while those of African and Middle Eastern background having a 2.6% expression (Merryweather-Clarke et al., 1997). The mutation's variable prevalence and penetrance (Aguilar-Martinez et al., 2001) raises concern that the Sampietro et al. (2001) findings may only be generalizable to the Italian population.

The pioneering studies by Moalem et al. (2000) and Sampietro et al. (2001) have begun to illuminate the putative relationship between HFE mutations and AD. Further research is warranted to validate their findings and to ascertain whether they are population-specific. The relatively high prevalence of the HFE mutations in the general population indicates that many AD patients may unknowingly carry these common polymorphisms. The HFE mutations may be distinguished from other genetic factors linked to late-onset AD because the biological effects of the former can be readily modified by minimizing exposure to iron (Connor et al., 2001). If the HFE-AD relationship is confirmed, iron chelation therapy and the control of dietary iron intake may delay the development and/or progression of this neurodegenerative disorder.

OBJECTIVES

The **primary objective** of this thesis is to determine if the earlier finding of an association between HFE mutations and sporadic AD (Sampietro et al., 2001) holds true in our patient population recruited from the Jewish General Hospital/McGill University Memory Clinic in Montreal, Canada. Our Montreal-based patient population is fairly heterogeneous, composed of individuals from diverse backgrounds. Therefore, our results may be of global significance.

Specifically, we will ascertain whether: a) HFE mutation frequency is similar among AD patients, mild cognitively impaired (MCI) patients, and normal elderly control (NEC) subjects; b) HFE mutation frequency is similar between genders in the AD cohort, c) HFE mutations interact with the apoE-4 allele to increase the probability of developing AD; d) HFE mutations are associated with an earlier AD symptoms onset or disease diagnosis; e) HFE mutations are associated with specific neuropsychological deficits in sporadic AD; f) the H63D mutation affects cognitive decline in MCI and AD patients; and g) the H63D mutation accelerates the progression from MCI to AD.

The hypothesis is that HFE mutations are partly responsible for excessive iron deposition in the AD brain and contribute to the disease's clinical manifestations. To test this hypothesis, 213 sporadic AD patients, 106 MCI patients, and 63 NEC were genotyped for the two most common HC mutations and the data were then statistically analyzed to determine the impact, if any, of these mutations on the clinical, neuropsychological, and demographic features of sporadic AD.

MATERIALS AND METHODS

Materials

The QIAamp DNA blood minikit and the PCR amplification kit were purchased from Qiagen (Mississauga, ON, Canada). PCR was performed on an MJ Research PTC 200 PCR machine (Watertown, MA). PCR primers and the 100kb DNA ladder were obtained from Invitrogen, Canada. RSA I and DPN II restriction enzymes were purchased from New England Biolabs (Beverly, MA). Metaphor agarose was obtained from FMC Bioproducts (Rockland, ME).

Human Subjects

This study was approved by the Research and Ethics Committee of the Sir Mortimer B. Davis Jewish General Hospital (JGH) in Montreal, Canada. Two hundred and thirteen late-onset AD patients (100 males and 113 females) meeting the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria for probable AD (McKhann et al., 1984) were recruited from the JGH/McGill University Memory Clinic. One hundred and six patients with mild cognitive impairment (MCI) meeting the World Health Organization criteria for age-associated cognitive decline (AACD) (Levy, 1994) and sixty-three normal elderly control subjects were recruited from the same clinic. Blood samples were originally procured from each of the 382 subjects for the purpose of apoE genotyping. Peripheral leukocytes were isolated from whole blood by the Molecular Diagnostics laboratory of the JGH and used as the DNA source. Following the apoE genotyping, the subjects' DNA samples were stored at -20°C and later utilized for the HFE genotyping described herein.

DNA extraction

DNA extraction from blood samples was performed by the Molecular Diagnostics laboratory of the Jewish General Hospital. Whole blood was collected by phlebotomy in heparinized tubes. Each blood sample was centrifuged at 1800 rpm for 10 minutes and the leukocyte buffy layer was collected for further processing. DNA was isolated from this fraction using the QIAamp DNA blood minikit from Qiagen.

HFE genotyping

Genomic DNA analysis for the G845A and C187G HFE mutations was conducted by polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP). The restriction patterns, visualized by gel electrophoresis and ethidium bromide staining, confirmed the presence or verified the absence of the HFE mutations.

The relevant region of DNA for the G845A mutation was amplified with the following primer sequences (designed by Feder et al., 1996): TGG CAA GGG TAA ACA GAT CC (sense) and CTC AGG CAC TCC TCT CAA CC (antisense). The primer sequences used for the DNA region containing the C187G mutation were ACA TGG TTA AGG CCT GTT GC (sense) and GCC ACA TCT GGC TTG AAA TT (antisense). The PCR reaction was carried out with the following parameters: an initial denaturing sequence at 95°C for 5min, followed by 35 cycles of denaturing (94°C for 30sec), annealing (55°C for 30sec), and extension (72°C for 30sec). A final 10min extension period at 72°C was performed following the final PCR cycle. The PCR products were then incubated overnight with the appropriate restriction enzymes at 37°C.

The Rsa I restriction enzyme was employed to digest the 388bp PCR product of the G845A-region amplification. Addition of Rsa I to DNA containing the wildtype HFE gene generated two fragments of 248bp and 140bp (Fig 3a). In individuals homozygous for the G845A mutation (i.e. those who carried two copies of the G845A mutation) an additional restriction site was created, producing three fragments of 248bp, 111bp, and 29bp (Fig 3a). In heterozygotes (i.e. those who carried one copy of the G845A mutation), four fragments of 248bp, 140bp, 111bp, and 29bp were generated due to the presence of normal and mutated DNA.

DpnII was used to digest the 208bp product of the C187G-region amplification. In this case, the enzyme cut the wildtype HFE gene into two pieces of 138bp and 70bp (Fig 3b). In the C187G homozygote (i.e. those who carried two copies of the C187G mutation), the mutation resulted in the loss of the normal restriction site and consequently, the expression of the full 208bp DNA template (Fig 3b). In heterozygotes (i.e. those who carried one copy of the C187G mutation), three fragments of 208bp, 138bp, and 70bp were generated due to the presence of both normal and mutated DNA.

The digested products were size-separated on a 3% metaphor gel by electrophoresis and visualized by ethidium bromide staining. The DNA band patterns on the gel indicated whether an individual was normal, heterozygous, or homozygous for the G845A and C187G HFE mutations (Fig. 4).

ApoE Genotyping

ApoE genotyping was performed independently of this study by the Molecular Diagnostics laboratory of the Jewish General Hospital in Montreal, Canada. The PCR primers and assay conditions were those previously described by Hixson et al. (1990).

Neuropsychological Testing

Neuropsychological Testing of all subjects was performed by Dr. Nora Kelner and Dr. Lennie Babins, psychologists affiliated with the JGH Memory Clinic. The global severity of cognitive impairments was examined using the Folstein Mini-Mental State Examination (MMSE) (Folstein, 1975). More intricate tests used to assess the effects of the HFE mutations on cognitive decline were: clock design, logical memory, trail making, oral fluency, Boston naming, and digit symbol tasks. These tests were chosen because they evaluate a range of cognitive dimensions and are considered sensitive indicators of cognitive deterioration. In the clock design test, the subject is asked to draw a clock and set the time for 10 past 11. This task, scored out of 10, assesses the subject's time orientation and the capacity to form time-number relationships. In the logical memory test, the examiner reads a short story to the subject, who is then asked to recall as many ideas from the story as possible. This memory task, scored out of 25, evaluates the quantity of information that is retained during immediate free recall. The trail making test is a timed visuo-motor tracking task that is comprised of two parts. The first part asks the subject to draw lines connecting consecutive numbers randomly arranged inside a box. The more challenging second part involves connecting consecutive numbers and letters that are arranged within a box while alternating between the two sequences. The subject's performance is evaluated based on the time it takes to complete both tasks. Oral Fluency tests the subject's vocabulary by asking them to produce as many words as possible that start with the letters "F" or "S" within 120 seconds. Alternatively, this task may ask the subject to name as many animals as they can in 60 seconds. In both cases, the subject is awarded one point for each word they produce. Boston naming is another test of verbal expression. It consists of 60 ink drawings of items ranging from a pencil to

a sphinx. The subject is asked to name each object shown. They are awarded one point for each correct response. Finally, the Digit Symbol test evaluates a subject's psycho-motor abilities. Small blank squares are randomly assigned a number from one to nine. A legend pairs each number with a given symbol. The subject is given 90 seconds to fill in as many blank spaces as possible with the appropriate symbols and is scored on the number correctly completed.

Database Construction

Due to the retrospective nature of this study, it was necessary to obtain all relevant background information on each subject from his or her hospital records. As a standard, all of the AD patients' cognitive testing scores and biochemical laboratory results were obtained from the hospital visit when they were first diagnosed with AD. The relevant information for patients identified as MCI subjects (at the time of this study) was obtained from the hospital visit when they were first diagnosed as such. Data on our NEC subjects was obtained from their most recent visit to the Memory Clinic.

In the process of reviewing their hospital charts, a data form was filled out for each of the 382 individuals in our subject population (Fig. 5). Each subject's gender, year of birth, education, diagnosis, family history of AD, Mini Mental Status Exam (MMSE) scores, hemoglobin (HGB), year of symptoms onset (when applicable), year of AD diagnosis (when applicable), year of MCI diagnosis (when applicable), and medications used were documented in a personal data form. The age of symptoms onset was defined as the age when memory impairment was first noted by relatives, whereas the age of AD diagnosis denoted the age when the patient was first clinically diagnosed as having AD by a neurologist or geriatrician. For those AD patients who were

previously diagnosed with MCI, the time span between the two diagnoses was noted and this interval was termed the MCI-AD interval. In addition, each subject's apoE and HFE genotyping results were recorded. All of this information, along with each subject's neuropsychological testing scores, were entered into a comprehensive subject database to facilitate statistical analysis.

Statistical Analysis

To determine whether the subject groups differed significantly in their education levels, MMSE scores, or age at neuropsychological evaluation, a between-subjects analysis of variance (ANOVA) was conducted in each case. Once a significant main effect was established, Tukey's post-hoc test was used to conduct pairwise comparisons between the means of the subject groups.

A chi-squared test was employed to compare the observed and expected values for the HFE and apoE genotype distributions between the AD, MCI, and NEC subject groups. It was also used to compare the HFE genotype distributions between genders in the AD group and to determine whether an interaction existed between HFE mutations and the apoE-4 allele. For each subject group, we calculated the proportion of individuals that possessed the H63D mutation or the C282Y mutation (in the heterozygous or homozygous state) in combination with the apoE-4 allele and the proportion of individuals who possessed these mutations alone. We then compared our observed percentages with the expected values.

Kaplan-Meier (KM) survival analysis was used to analyze the effects of the HFE mutations and apoE-4 allele on the age of AD symptoms onset and the age of AD diagnosis. KM Survival analysis was also employed to ascertain whether the H63D

mutation accelerated the progression from MCI to AD. MCI subjects who did not develop AD (at the time of this study) were censored in this analysis. In all cases, the log-rank test was utilized to determine whether the genotype-specific survival functions (curves) were significantly different from one another.

A between-subject multivariate analysis of variance (MANOVA) was conducted to determine whether the three subject groups' mean neuropsychological testing scores were significantly different from one another. Once an overall effect was confirmed, Tukey's post-hoc test was used to assess for between-subject differences for each testing measure. A between-subject MANOVA was also performed to determine whether the HFE mutations measurably impacted the testing scores of AD patients. Factors that would potentially confound the results, such as education level and MMSE performance, were co-varied out where appropriate. A multivariate analysis was used, as opposed to univariate tests, to better control for type I errors. In all cases, p-values less than 0.05 indicated significance. Statistical analyses were performed using SPSS Software, version 10.1 (Chicago, IL).

RESULTS

Subject demographics

Dementia in the AD cohort was mild to moderate in severity, with a mean MMSE score of 22.8. The MCI and NEC subject groups averaged MMSE scores of 27.9 and 29.0, respectively. Significant differences in MMSE scores were present between the AD and NEC groups ($p<0.001$), and the AD and MCI groups ($p<0.001$), as expected. Years of formal education in the AD, MCI, and NEC subject groups averaged 10.7, 11.9, and 12.8 years, respectively. The AD cohort had significantly fewer years of education than the NEC group ($p<0.001$) and the MCI group ($p<0.05$). This is notable since a lower education has been considered to increase the risk of AD development by decreasing cognitive reserve (Katzman, 1993). The mean age at neuropsychological evaluation did not significantly differ between the subject groups ($p>0.05$) (Fig. 6).

Genotype frequencies of the HFE mutations in AD, MCI, and NEC subjects

When stratified by H63D genotype status, the AD, MCI, and NEC subject groups displayed an almost identical frequency distribution of individuals who were wildtype, heterozygous, and homozygous for this mutation (Fig. 7a). Amongst the three subject groups, between 66% and 74% of individuals were wildtype for H63D, between 25% and 30% were heterozygous, and between 1% and 4% were homozygous. Between subject groups, the proportion of individuals possessing each genotype was not significantly different from one another ($\chi^2(4)=3.83$, $p=0.43$).

Similarly, when the three subject groups were stratified by C282Y genotype status, there was no significant between-group difference in the percentage of people who were wildtype, heterozygous, or homozygous for this mutation ($\chi^2(4)=4.59$, $p=0.33$) (Fig.

7b). Amongst the three subject groups, the percentage of individuals who were wildtype for C282Y ranged from 90% to 95% and the percentage who were heterozygous ranged from 5% to 10%. Only one person in the entire subject population (an MCI patient) was homozygous for the C282Y mutation. H63D and C282Y allele frequencies within our subject population fell within worldwide ranges that have previously been published (Merryweather-Clarke et al., 1997).

Genotype frequencies of the HFE mutations between male and female AD subjects

In the general population, females are considered to be at a higher risk for AD than males (Jorm, 1987). We sought to determine whether this gender difference may in part be due to an over-representation of the H63D or C282Y HFE mutations in women as compared to men. Of all females in the AD patient group, 67% were H63D wildtype, 31% were heterozygotes, and 2% were homozygotes. Of all males, 64% were H63D wildtype, 29% were heterozygotes, and 7% were homozygotes (Fig. 8a). The proportion of males and females within each H63D genotype category were not significantly different from one another ($\chi^2(2)=3.59$, $p=0.17$). Similarly, when males and females were stratified based on their C282Y genotype status, the two genders' distribution patterns were not significantly different ($\chi^2(1)=0.01$, $p=0.92$) (Fig. 8b). In fact, 95% of both genders were C282Y wildtype and 5% of both sexes were heterozygous for C282Y. There were no C282Y homozygotes in the AD patient group.

Relationship between the HFE mutations and the apoE-4 allele

Since the apoE-4 allele is the only confirmed genetic risk factor for sporadic AD, we sought to determine whether an interaction was present between the apoE-4 allele and

the HFE mutations. No statistically significant association existed between the apoE-4 allele and the H63D mutation in our AD cohort ($\chi^2(2)=4.04$, $p=0.13$). A similar analysis was conducted to determine whether an interaction was present between the apoE-4 allele and the C282Y mutation. Once again, no statistically significant allelic association was detected ($\chi^2(2)=4.80$, $p=0.09$).

Effect of the HFE mutations on age of symptoms onset and age of AD diagnosis

There was no statistically significant difference in the age of symptoms onset between AD patients who were wildtype (n=140), heterozygous (n=64), or homozygous (n=9) for the H63D mutation ($\chi^2(2)=0.67$, $p=0.72$). The three overlapping age of onset distribution curves, each representing a particular genotype, indicated this fact (Fig. 9a). The median ages of symptoms onset for the wildtype, heterozygous, and homozygous AD patients were 76, 74, and 76, respectively. In addition, there was no significant difference in the age of AD diagnosis among patients who were wildtype, heterozygous, or homozygous for the same mutation ($\chi^2(2)=1.79$, $p=0.41$) (Fig. 9b). The median ages of AD diagnosis for these three groups were 79, 77, and 80, respectively. The curves depicting H63D mutation effect on age of AD diagnosis were similar in shape to those demonstrating the H63D effect on symptoms onset, only shifted slightly to the right because diagnosis follows symptoms onset.

Furthermore, there was no significant difference in the age of symptoms onset between individuals who were wildtype (n=202) or heterozygous (n=11) for the C282Y mutation ($\chi^2(1)=0.01$, $p=0.93$) (Fig. 10a). The median age of symptoms onset for both groups was 75. Additionally, there was no significant difference in the age of AD diagnosis between individuals who were wildtype or heterozygous for the C282Y

mutation ($\chi^2(1)=0.57$, $p=0.45$) (Fig. 10b). The median ages of AD diagnosis for these two groups were 79 and 77, respectively. As before, the survival functions depicting the effect of C282Y on age of AD diagnosis were similar in shape to those illustrating the C282Y effect on age of symptoms onset, only shifted slightly to the right because diagnosis follows symptoms onset.

Effect of the HFE mutations on neuropsychological testing scores of AD patients

To ensure that our patient population displayed an inverse relationship between cognitive decline and neuropsychological testing performance, we compared the mean scores of the AD, MCI, and NEC subject groups for each administered test (Fig. 11). In each case, the AD patients' testing scores were significantly worse than that of NEC subjects ($p<0.001$). In addition, the testing scores of MCI patients were in all cases intermediate relative to the other two groups. After verifying these relationships, we set out to determine whether the possession of the HFE mutations would exacerbate any specific cognitive deficits in the AD patients.

There was no significant difference between the mean neuropsychological testing scores of AD patients who were wildtype, heterozygous, or homozygous for the H63D mutation ($F(14,388)=0.344$, $p=0.98$) (Fig. 12a). Additionally, there was no significant difference between the mean neuropsychological testing performances of AD patients who were wildtype or heterozygous for the C282Y mutation ($F(7,205)=0.30$, $p=0.95$) (Fig. 12b). These findings suggest that HFE mutations do not impact the cognitive functions that were measured in our AD population.

Effect of the H63D mutation on the progression from MCI to AD

The term MCI refers to a cognitive phase when deficits in memory, attention, and planning abilities are first noted. MCI patients have a 50% probability of developing AD within 4 years of their initial diagnosis (Peterson et al., 1999). We sought to determine whether the H63D mutation might act to accelerate the progression from MCI to AD. KM survival analysis demonstrated that there was no significant difference in the MCI-AD time interval among individuals who were wildtype (n=115), heterozygous (n=41), or homozygous (n=5) for the H63D mutation ($\chi^2(2)=3.61$, p=0.16) (Fig. 13). The median MCI-AD time interval was 4 years for individuals who were H63D wildtype, 3 years for heterozygotes, and 1 year for homozygotes. Inspection of the survival functions revealed that the wildtype and heterozygote curves were overlapping, whereas the homozygote curve was shifted left of the other two. This trend in our data is noteworthy and suggests that homozygosity for H63D may effect the rate of conversion from MCI to AD. The absence of a statistically significant difference among the three curves may be due to the small sample of individuals who were homozygous for H63D.

Effect of H63D on age of symptoms onset in MCI and AD subjects

We next sought to determine whether the H63D mutation was associated with an earlier onset of cognitive decline. All MCI and AD subjects were stratified based on whether they were wildtype, heterozygous, or homozygous for the H63D mutation. There was no significant difference in the age of symptoms onset between the three genotype groups ($\chi^2(2)=0.32$, p=0.85). The KM age of onset distribution curves for the wildtype and heterozygous subjects were almost completely overlapping (Fig. 14). The first half of the survival function representing the homozygous subjects was shifted left of

the other two curves, while the second half was superimposed over them. This suggests that between the ages of approximately 55 to 75, H63D homozygous patients may have a higher probability of developing earlier cognitive deficits than their counterparts in the other two groups. Additionally, between the ages of 75 and 85, all three groups display similar probabilities of developing cognitive decline. The median age of symptoms onset was 75 for wildtype patients, 75 for heterozygotes, and 68 for homozygotes. Although, overall, the three curves were not significantly different from each other, the trend noted in the H63D homozygotes suggests that a certain window of time exists when these individuals may be susceptible to an earlier cognitive decline.

Effect of the apoE-4 allele in AD, MCI, and NEC subjects

To verify that apoE-4 was associated with AD in our patient population, we set out to determine whether the apoE-4 allele was over-represented in our AD patients and whether it influenced the age of AD symptoms onset. To determine if the apoE-4 allele frequency was elevated in AD patients relative to the MCI and NEC groups, all subjects were stratified based on the number of E-4 alleles they possessed (Fig. 15). There was a highly significant difference between the AD, MCI, and NEC subject groups in their proportions of individuals who possessed zero, one, or two E-4 alleles ($\chi^2(4)=26.94$, $p<0.001$). The percentages of the AD, MCI, and NEC subjects who were apoE-4 homozygous were 9%, 4%, and 2%, respectively. Proportions of the groups who were apoE-4 heterozygous were 44%, 27%, and 19%, respectively. In addition, the NEC group had the highest percentage of individuals who were devoid of the E-4 allele (79%), whereas the AD group had the lowest (47%). Therefore, the E-4 allele was clearly over-represented in our AD cohort.

To determine whether the apoE-4 allele influenced the age of AD symptoms onset we compared the symptoms onset distribution curves of AD patients who possessed zero (n=95), one (n=91), or two (n=18) E-4 alleles (Fig. 16). The median ages of symptoms onset for these three distributions were 71, 75, and 75, respectively. Although the difference in the survival functions was not statistically significant ($\chi^2(2)=3.92$, $p=0.14$), the survival curve representing the E-4 homozygotes was noticeably shifted left of the other two. This suggests that homozygosity for the apoE-4 allele might play a role in precipitating an earlier symptoms onset in our patient population. The absence of a statistically significant effect may be due to the relatively small sample of E-4 homozygotes. The distribution curves representing patients who were heterozygous for apoE-4 and patients who did not possess the E-4 allele were overlapping, indicating that one E-4 allele did not reduce the age of symptoms onset.

DISCUSSION

In order to establish a definitive association between a particular gene and AD, multiple studies that corroborate this putative relationship are necessary. The association between the apoE-4 allele and AD underwent tremendous scrutiny before this allele became recognized as a bona fide genetic risk factor for late-onset AD. Combining the search terms, "apolipoproteins E" and "Alzheimer disease" in the MEDLINE database currently results in over 1,500 journal article hits. A variety of other genes are presently being evaluated to determine whether they might be considered risk factors for AD. Our belief that oxidative stress is closely involved in the pathogenesis of AD has compelled us to examine whether mutations in the HFE gene might influence the presentation of this disease.

Two previous case-control studies were published that investigated whether the H63D and C282Y HFE mutations were involved in AD. The first reported that a complex gender-based relationship existed between the HFE mutations and the apoE-4 allele in FAD (Moalem et al., 2000). The second demonstrated that sporadic AD patients carrying the H63D mutation were more likely to develop an earlier onset of symptoms (Sampietro et al., 2001). In the present study, we examined several possible ways in which these two common HFE mutations might affect the presentation of sporadic AD. In order to verify that our clinical cohort was representative of AD cohorts in other studies, we demonstrated that the E-4 allele was over-represented in our AD patients relative to MCI and NEC subjects and that homozygosity for the E-4 allele resulted in a tendency for an earlier age of AD symptoms onset.

We first demonstrated that the genotype frequencies of the HFE mutations were similar among the AD, MCI, and NEC subject groups. The H63D mutation was

previously shown to be common in both sporadic AD patients and age-matched controls in an Italian population (Sampietro et al., 2001). To our knowledge, our findings are the first to demonstrate that the H63D and C282Y HFE mutations appear to be equivalently distributed among AD, MCI, and NEC subjects. The presence of the HFE polymorphisms among MCI and NEC subjects and their absence among some AD patients suggests that they are neither necessary nor sufficient for the development of the disease. Nonetheless, the HFE mutations may have the capacity to modify the clinical appearance of AD (see below).

In examining the distribution of the HFE genotypes between males and females in our AD cohort, we were interested to determine whether an over-representation of these mutations in women might partially account for the higher prevalence of AD in this group. One meta-analysis suggested that the female to male ratio of AD prevalence was 1.30 to 0.77 (Jorm, 1987). It remains unclear why women are more likely to suffer from AD than men. Some have attributed this phenomenon to the greater longevity of women. Since the probability of developing AD increases with age, the longer life span of women would increase their probability of developing the disease. However, even after adjustments were made to account for this survival effect, women were still at a slightly greater risk for AD than men (Rocca et al., 1986). We demonstrated that H63D and C282Y HFE mutations were equally represented between males and females in the AD cohort, suggesting that they are not involved in the differential prevalence of the disease between genders. Our finding is in agreement with the earlier work of Sampietro et al. (2001) but is in contradistinction to the results of Moalem et al. (2000). The latter demonstrated that, in combination, HFE mutations were over-represented in males and under-represented in females suffering from FAD, relative to age-matched controls.

We also analyzed whether an interaction was present between the HFE and apoE genes. The term "interaction" describes a situation in which two or more factors modify each others' effects to increase or decrease the probability of an occurrence. Previous work examining the influence of gene-gene interactions on the development of AD suggested that an interaction between the apoE and APP genes shaped the final pathological phenotype of AD (St George-Hyslop et al., 1994). Our findings indicate that there was no significant interaction between the HFE and apoE genes in patients with sporadic AD. The lack of an association between these two loci was also demonstrated by Sampietro et al. (2001). Contrary to these findings, Moalem et al. (2000) determined that gender-specific associations were present between the HFE mutations and the apoE-4 allele in FAD.

We next investigated whether the HFE mutations were associated with a lower age of AD symptoms onset and/or a lower age of AD diagnosis. The mutations' effects on age of symptoms onset is more biologically relevant since this is the time when the disease pathology reaches it's clinical threshold. On the other hand, an analysis that examines the mutations' effects on age of AD diagnosis would likely be more accurate since the diagnosis is made in a more standardized and objective fashion. We took advantage of the merits of each of these analyses and demonstrated that neither the H63D nor the C282Y mutations were responsible for a lower age of symptoms onset or a lower age of diagnosis in our AD cohort. Conversely, Sampietro et al. (2001) reported that patients in their study who were heterozygous or homozygous for H63D experienced, on average, a five year earlier symptoms onset than those with a wildtype HFE genotype.

Several factors may account for the inconsistencies between our findings and the results of the two previous studies that examined the effect of the HFE mutations on AD.

Moalem et al. (2000) based their conclusions on a small sample size of familial AD patients (n=26). Therefore, some of their observed associations may represent false positives simply due to chance. In addition, HFE mutations may have differential effects on familial AD patients versus sporadic AD patients. These two forms of the disease are likely to be subject to different genetic influences.

To reconcile our finding that the H63D mutation does not lower the age of AD symptoms onset with the positive association of Sampietro et al. (2001), we surmised that other factors might be responsible for the earlier symptoms onset among patients in their population. We hypothesized that the genetic disorder, thalassemia, a disease that is fairly common in Italy and the Mediterranean region as a whole, may have been present in a proportion of those AD cases that presented with an earlier symptoms onset. Individuals with thalassemia exhibit a decreased production of normal hemoglobin (Hgb) and therefore require frequent blood transfusions to maintain an optimum Hgb level. Thalassemic patients are at a risk for iron overload due to the abundance of exogenous iron they acquire through blood transfusions. We speculated that this origin of potential iron toxicity may have accounted for a proportion of the AD cases that presented with an earlier symptoms onset in the Sampietro et al. (2001) study. We later discovered that few, if any, of the patients in the Sampietro et al. study were thalassemic and that when thalassemia is found in association with the H63D mutation, this rarely alters one's iron levels significantly (personal communication from M. Sampietro, University of Milan).

Another explanation that may account for the discrepancy between the findings of our study and that of Sampietro et al. (2001) is the fact that HFE mutations may influence the age of AD symptoms onset in some patient populations but not in others. The Sampietro et al. study was conducted using a patient population that was recruited from

the urban area of Milan, Italy. Our Montreal-based study utilized a more diverse patient population that represented a variety of nationalities. A gene's influence on a disease may vary between patient populations. A gene may be associated with AD in a particular population due to an interactive effect with other genetic and/or environmental elements that are present in that population, but not in others. It is likely that most genes that confer a risk for AD effect only a specific subset of individuals (Lehmann et al., 2001). The population-specific effects of most risk genes may explain why our results are at odds with those of Sampietro et al.

To our knowledge, we are the first to analyze the effects of the HFE mutations on the neuropsychological testing performance of AD patients. Others have similarly investigated the impact of the apoE-4 allele on cognitive functioning in sporadic AD. While some studies have demonstrated that AD patients who were homozygous for apoE-4 were characterized by a more severe memory loss (Lehtovirta et al., 1996), others have not shown this effect (Rasmusson et al., 1996). A recent study suggested that the apoE-4 allele may influence some cognitive functions of AD patients, such as delayed recall and recognition, but not others (Kim et al., 2002). We hypothesized that the HFE mutations might have an impact on the neuropsychological testing scores of our AD patients. Specifically, we suspected that the H63D and/or C282Y mutations might result in lower test scores in tasks that evaluated one's motor performance. Although iron is found throughout the normal elderly brain, its levels are known to be highest in the basal ganglia, a group of nuclei that play a major role in the control of movement (Hallgren and Sourander, 1958). Since the basal ganglia are a major repository of excess iron under various neuropathological conditions (Schipper, 1999), we hypothesized that possession of HFE mutations may result in an additional and preferential deposition of iron in these

subcortical nuclei. Abnormal changes to the basal ganglia have been described in patients with HC (Berg et al., 2000). In addition, HC patients have been reported to present with movement disorders that may be attributed to excess brain iron (Demarquay et al., 2000). We determined that the testing performance of AD subjects that possessed either of the HFE mutations did not markedly differ from patients who were wildtype. Nonetheless, a trend was noted regarding patient performance on the trail making task, a visuo-motor tracking test. Testing performance was best in patients who were HFE wildtype and grew increasingly worse from H63D heterozygotes to H63D homozygotes. In the same task, patients who were HFE wildtype also outperformed those who were C282Y heterozygous. Although the testing scores were not significantly different from one another, the possibility that HFE mutations deleteriously affect motor skills is intriguing and deserves further attention.

Based on our analyses, we conclude that in our patient population the HFE mutations are evenly distributed among subject groups, are equally present in males and females, do not interact with the apoE-4 allele, and do not significantly affect the age of disease onset or any specific cognitive domains. To explain the lack of an association between the HFE mutations and AD, we draw attention to the mechanism by which excessive iron is deposited in the brains of AD patients. Ample evidence exists that the excessive sequestration of iron in the brains of AD patients occurs via non-transferrin mediated pathways. Indeed, multiple studies support a role for the iron binding protein, melanotransferrin (p97), in excess brain iron accumulation in AD patients (Jefferies et al., 1996b; Kennard et al., 1996; Rothenberger et al., 1996). It is believed that the normal HFE protein attenuates the affinity of the transferrin receptor for diferric transferrin, thereby limiting iron entry into cells. In HC, HFE mutations cause a decreased inhibition

of the transferrin receptor/transferrin interaction, promoting an excessive iron loading of tissues. Therefore, if pathological brain iron deposition in AD is independent of the transferrin receptor, then the HFE mutations would not markedly influence brain iron stores in this disease.

Nevertheless, we presently report three novel trends in our data that suggest a role for the HFE mutations in advancing cognitive decline. We suggest that between the ages of 55 and 75, individuals who are homozygous for the H63D mutation have a higher probability of developing an earlier onset of cognitive decline than individuals who are H63D heterozygous or wildtype. The selective effect on the 55-75 age range may be explained by the fact that individuals older than 75 are typically affected by numerous aging-related factors that might obscure the effect of the H63D mutation. We additionally suggest that homozygosity for H63D may accelerate the rate of progression from MCI to AD. We also suggest that HFE mutations may act to increase iron levels in the basal ganglia and deleteriously affect patients' motor skills. Larger-scale prospective studies must be conducted to further examine these putative effects. Confirmation of these findings would indirectly emphasize the importance of iron in the pathogenesis of AD. It would also support the use of iron chelation therapy and possibly phlebotomy to delay the onset of AD in MCI patients who are H63D homozygous.

CONCLUSION

The burden of AD is continually increasing due to the constant rise in the elderly population. To minimize the future prevalence of this disease, it is important to discover new genetic risk factors for AD that may aid in diagnosis and treatment. We examined whether a relationship exists between mutations in the hemochromatosis gene and sporadic AD. Previous studies have suggested that excess brain iron resulting from the H63D and C282Y HFE mutations may influence AD development. We did not find any statistically significant associations between HFE gene status and the clinical, demographic, or neuropsychological aspects of AD in our patient population. Larger-scale studies are warranted to further examine this relationship and to validate trends that we have discovered; namely, that the HFE mutations may deleteriously affect motor performance and that H63D homozygosity may influence the onset of cognitive decline and accelerate the conversion from MCI to AD. Future studies must be conducted to determine whether these common HFE polymorphisms might render individuals susceptible to Parkinson's disease, multiple sclerosis, Hallervorden-Spatz syndrome, or other neurological disorders characterized by central iron toxicity.

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Figure 1: Hypothetical structure of the HFE protein. The HFE protein is a single polypeptide with a transmembrane domain that resembles the structure of MHC class I molecules. The amino terminus is located in the extracellular space while the carboxyl terminal lies in the intracellular space. The protein is comprised of three extracellular domains: α1, α2, and α3 loops. On the plasma membrane, it is found in association with the β_2-microglobulin protein (β2-m) which is involved in the transport of the HFE protein to the cell surface. The sites of the H63D and C282Y HFE mutations are indicated. (Modified after Jazwinska, 1998)

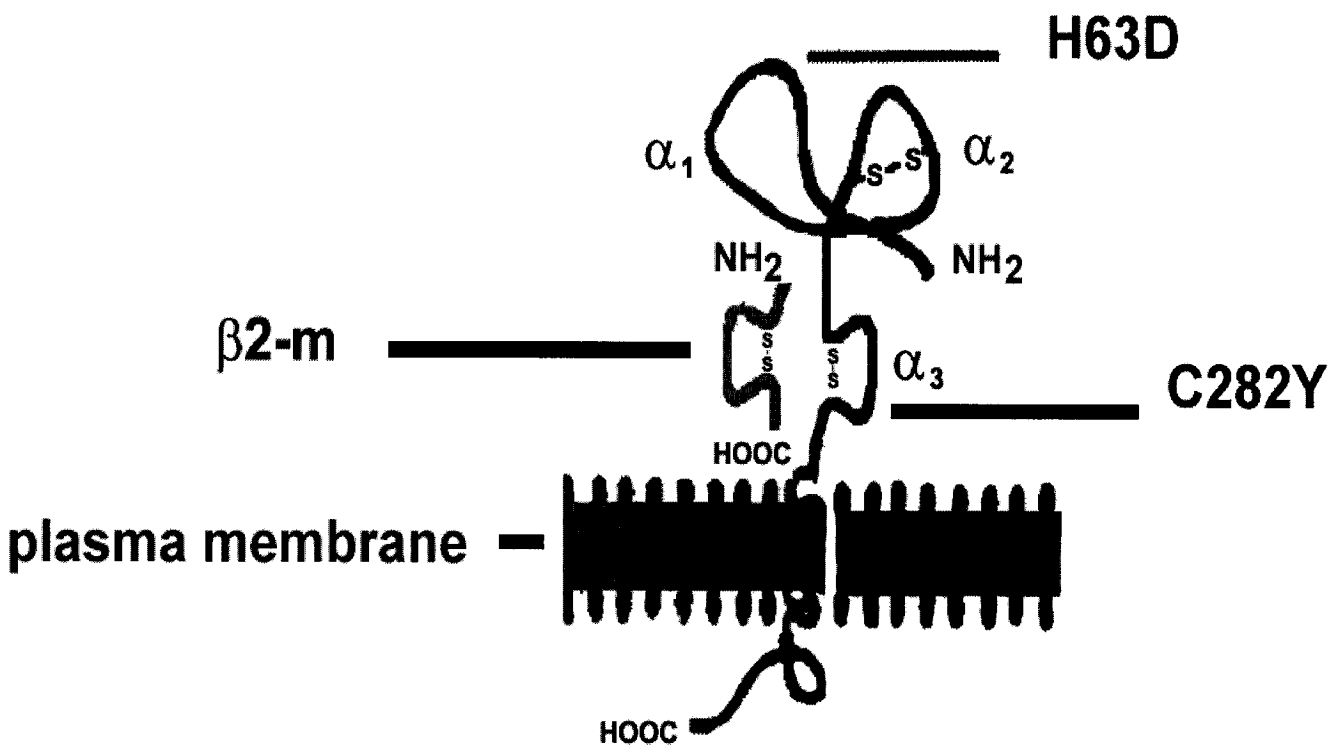

H63D
α1
α2
S-S
NH2
NH2
β2-m
S-S
S-S
α3
C282Y
HOOC
plasma membrane
HOOC

Figure 2: Three models by which the HFE protein may attenuate intracellular iron entry. (A) HFE protein binds to the transferrin receptor and decreases the receptor's binding affinity for diferric transferrin. (B) HFE protein binds to the transferrin receptor in proximity to the transferrin binding site and competitively inhibits the receptor-ligand interaction. (C) HFE protein allows entry of the transferrin receptor-diferric transferrin complex into the cell but prevents the release of iron from the early endosome. (Modified after Lyon and Frank, 2001)

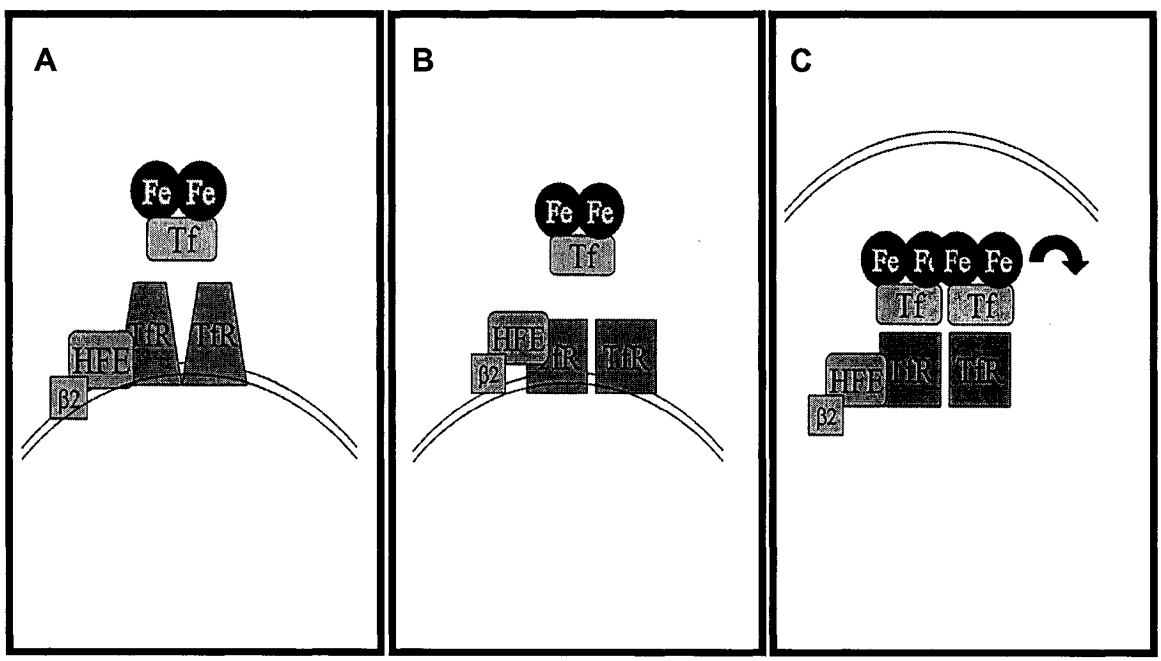
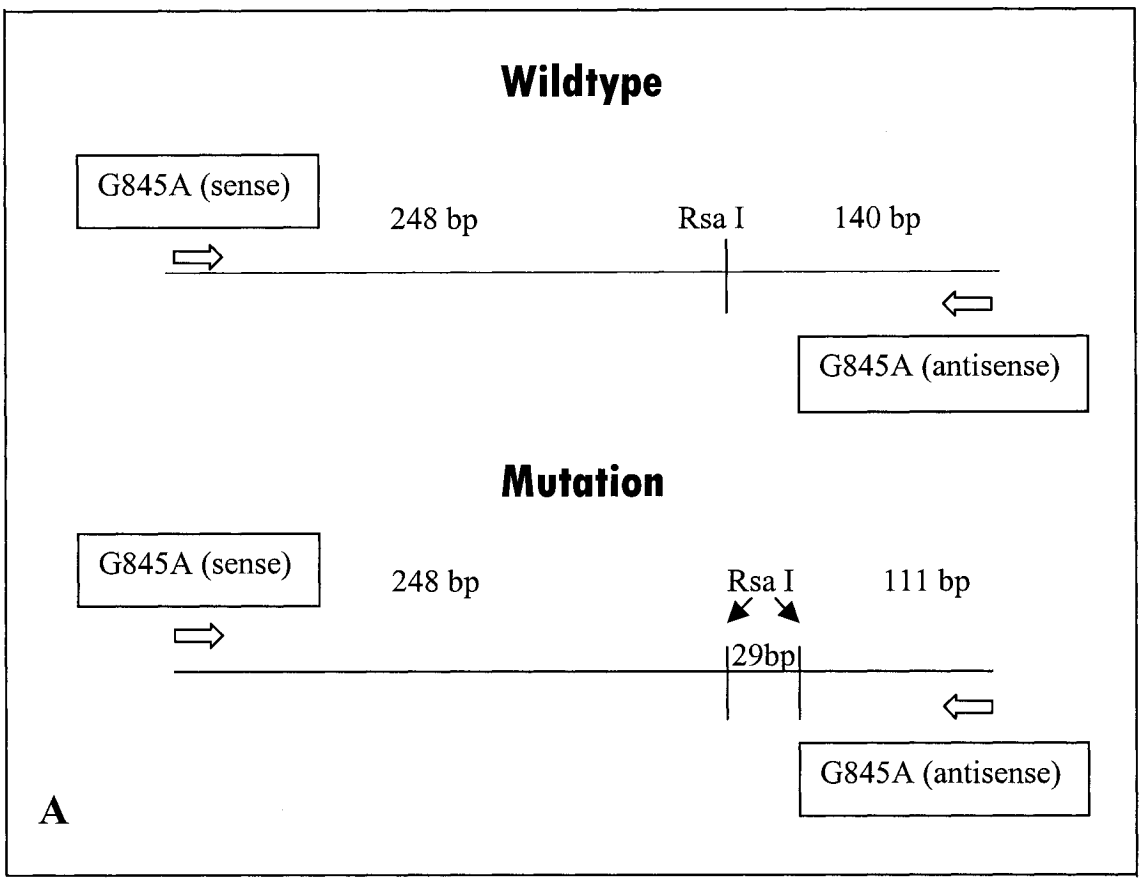

A
Fe Fe
Tf
HFE
β2
TfR
TfR
B
Fe Fe
Tf
HFE
β2
TfR
TfR
C
Fe Fe Fe Fe
Tf
Tf
HFE
β2
TfR
TfR

Figure 3: Patterns of DNA fragmentation following restriction enzyme digestion depend on whether individuals are wildtype or mutant at nucleotide positions 845 (A) and 187 (B). (A) RSA I was employed to digest the 388bp product of the G845A-region amplification. The generation of three DNA fragments (248bp, 111bp, 29bp) indicates that the G845A mutation is present. The production of two DNA fragments (248bp, 140bp) signifies that an individual possesses at least one chromosome that is wildtype at this locus. (B) DPN II was employed to digest the 208bp product of the C187G-region amplification. Detection of the full DNA template (208bp) signifies that the C187G mutation is present. The production of two DNA fragments (138bp, 70bp) indicates that an individual possesses at least one chromosome that is wildtype at this locus. See text for further details.


Wildtype
G845A (sense)
248 bp
Rsa I
140 bp
G845A (antisense)
Mutation
G845A (sense)
248 bp
Rsa I
111 bp
29bp
G845A (antisense)
A

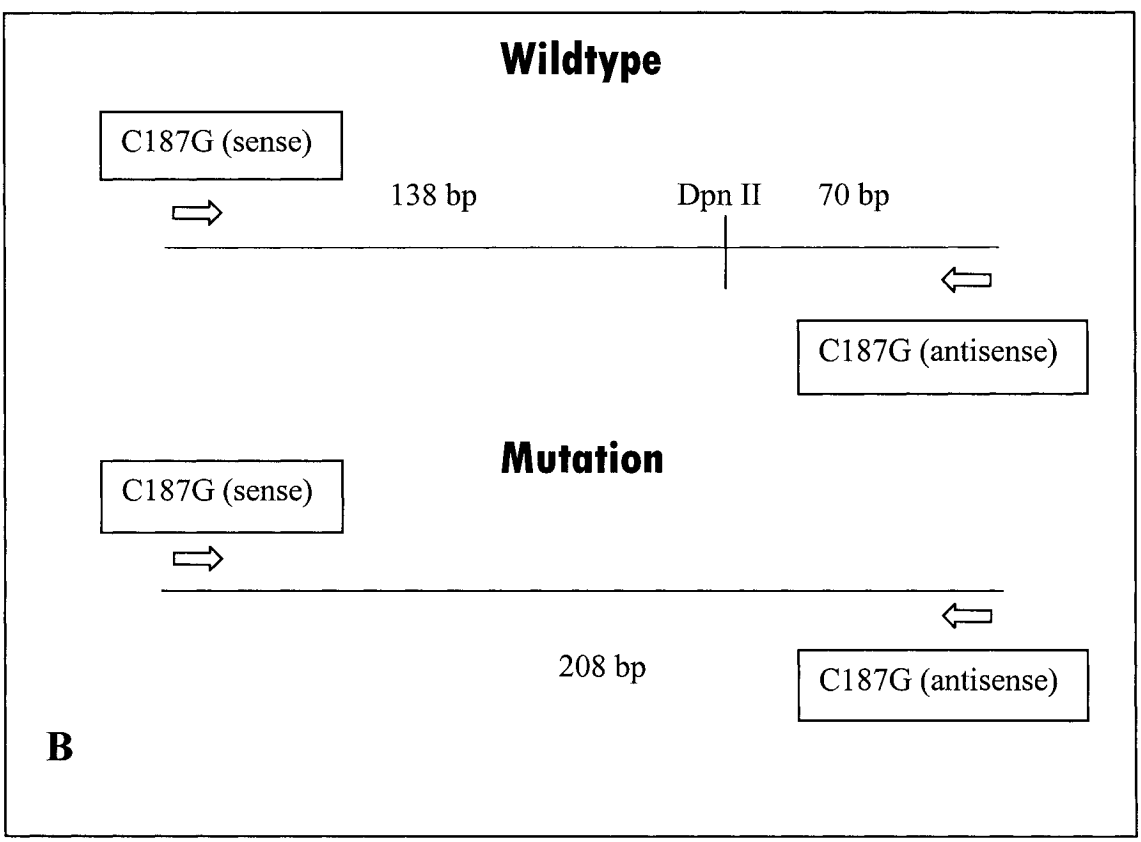

Wildtype
C187G (sense)
138 bp
Dpn II
70 bp
C187G (antisense)
Mutation
C187G (sense)
208 bp
C187G (antisense)
B

Figure 4: Representations of gels that were used to determine whether individuals were homozygous, heterozygous, or wildtype for the C282Y (A) and H63D HFE mutations (B). (A) Lane 1 shows the 100kb DNA ladder; lane 2 depicts the absence of bands when no DNA is added to the PCR mix, controlling for contamination; lanes 8, 9, and 10 are controls included in each gel to illustrate the band patterns of individuals who are heterozygous, homozygous, and wildtype, respectively, for the C282Y mutation; lanes 3-7 depict the DNA band patterns of five different subjects that were genotyped for the C282Y mutation as part of this study. Lanes 3, 4, 6, and 7 represent individuals who do not possess the C282Y mutation and lane 5 represents a person who is C282Y heterozygous. (B) Lane 1 shows the 100kb DNA ladder; lane 2 depicts the absence of bands when no DNA is added to the PCR mix, controlling for contamination; lanes 8, 9, and 10 are controls included in each gel to illustrate the band patterns of individuals who are heterozygous, homozygous, and wildtype, respectively, for the H63D mutation; lanes 3-7 depict the DNA band patterns of five different subjects that were genotyped for the H63D mutation as part of this study. Lanes 3, 5, and 7 represent individuals who do not possess the H63D mutation and lanes 4 and 6 represent individuals are H63D heterozygous.

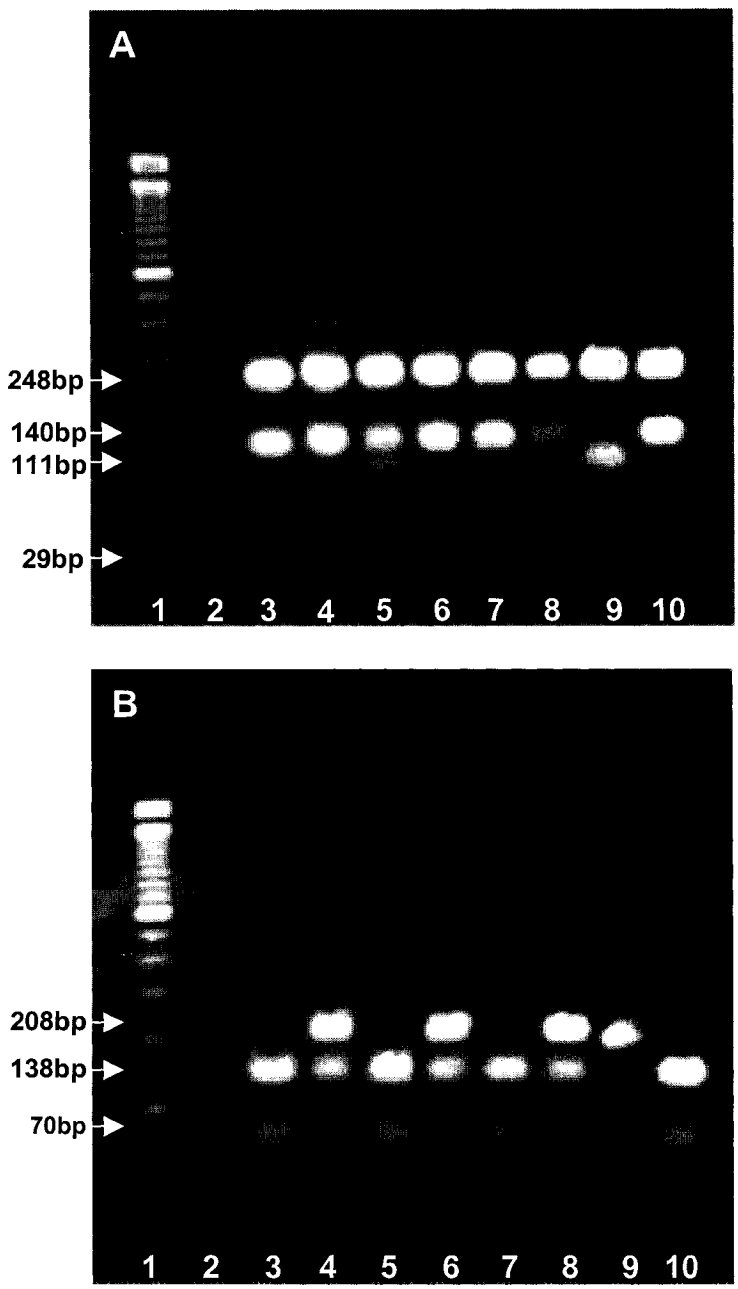

A
248bp
140bp
111bp
29bp
1 2 3 4 5 6 7 8 9 10
B
208bp
138bp
70bp
1 2 3 4 5 6 7 8 9 10

Figure 5: A sample patient data form. All relevant patient information was obtained from hospital records and documented in patient data forms. Each subject's gender, year of birth, education, diagnosis, family history of AD, MMSE scores, hemoglobin (HGB), year of symptoms onset (when applicable), year of AD diagnosis (when applicable), year of MCI diagnosis (when applicable), and medications used were recorded. These forms were used to construct a comprehensive patient database. See text for further details.

Patient Data Form

Role of HFE (Hemochromatosis) mutations in sporadic Alzheimer disease (AD)

1) Name: A.B. ID number: 351 U number: 719013

2) Gender: [√] Male [ ] Female

2) Year of birth: 1920

3) Most recent diagnosis: [ ] Normal [ ] MCI [√] AD

4) Major mutation status (G845A): [√] -/- [ ] +/- [ ] +/+

5) Minor mutation status (C187G): [√] -/- [ ] +/- [ ] +/+

6) APOE genotype: [ ] 2,2 [ ] 2,3 [√] 3,3 [ ] 2,4 [ ] 3,4 [ ] 4,4

7) Year of symptoms onset: 1994

8) Year of AD diagnosis: 2000

9) Year of MCI diagnosis: 1999

10) MMSE scores:

	Year	Diagnosis	Score
1	1999	MCI	28
2	2000	AD	28
3	2001	AD	27
4			
5			

11) HGB: 138

12) Cholinesterase Inhibitors: [√] Aricept [ ] Reminyl [ ] Exelon

13) Antioxidants: none

14) Education: 12 years

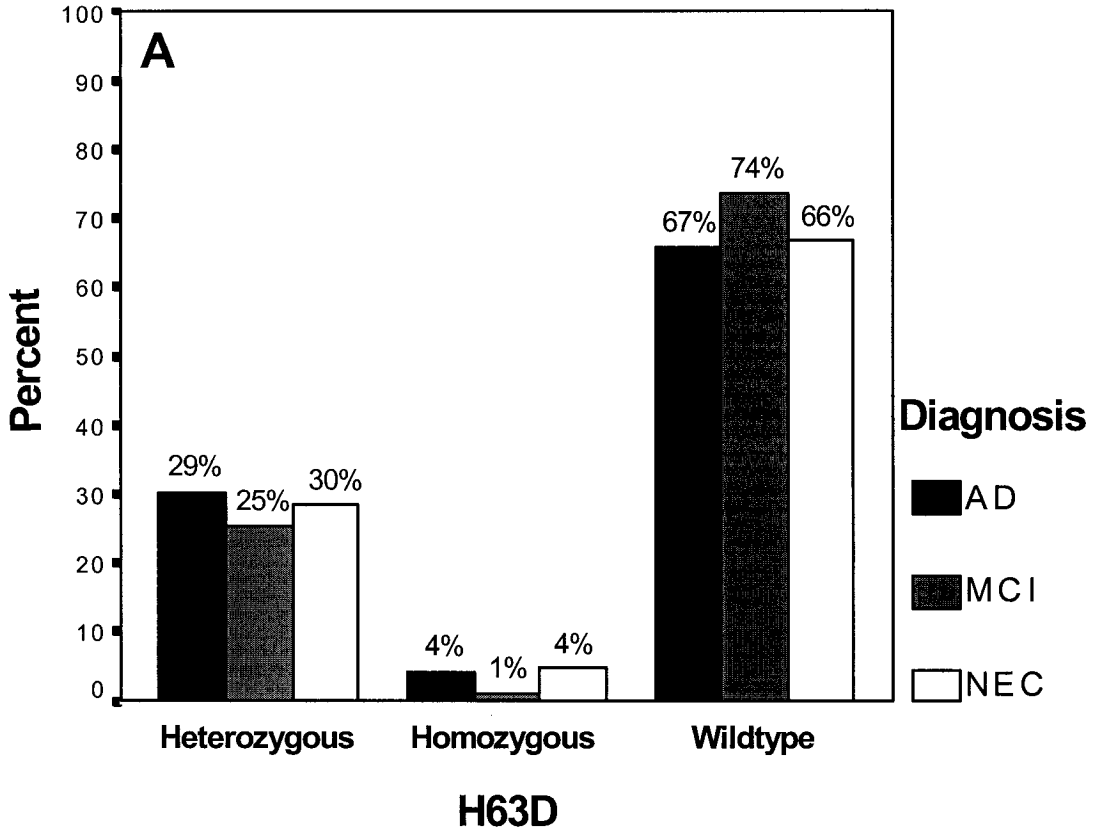
15) Family History: none

16) Nationality/Ethnicity: British

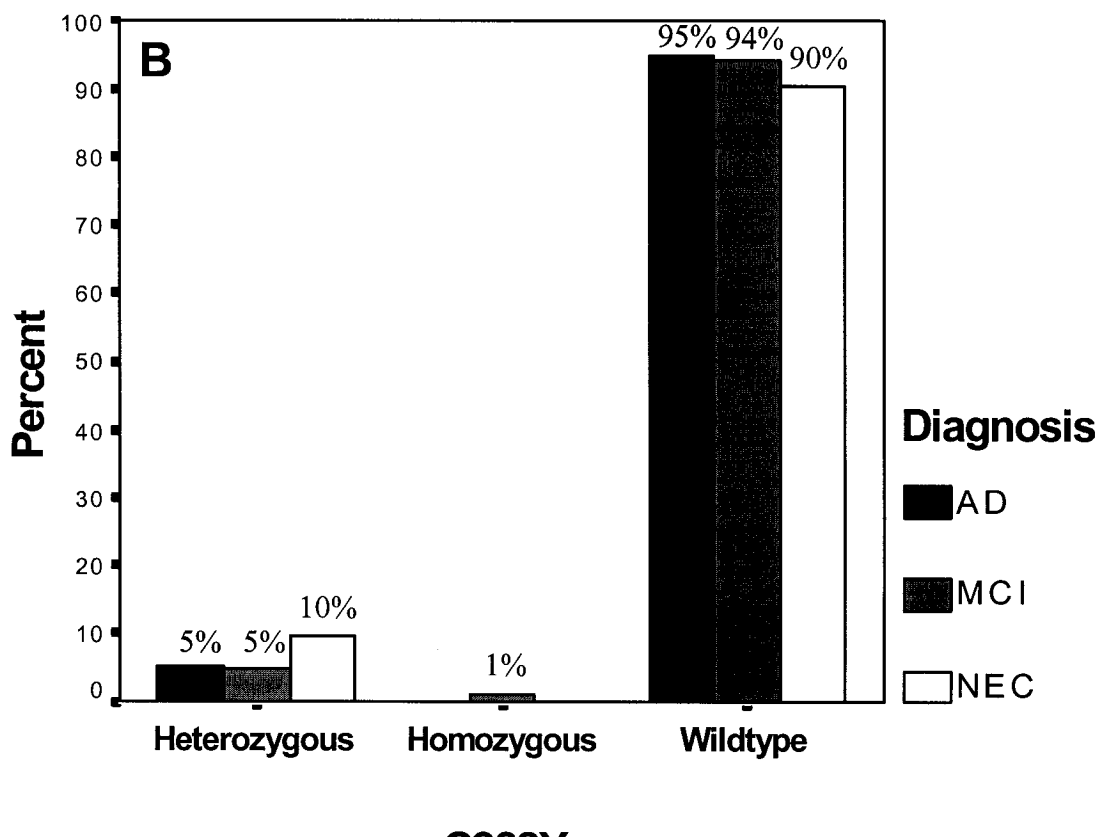
Figure 6: Subject demographics. 213 AD patients, 106 MCI patients, and 63 NEC subjects were recruited for use in this study. Significant differences in MMSE scores are present between the AD and NEC groups ($p<0.001$), and the AD and MCI groups ($p<0.001$). The AD cohort has significantly fewer years of education than the NEC group ($p<0.001$) and the MCI group ($p<0.05$). The mean age at neuropsychological evaluation does not significantly differ between the subject groups ($p>0.05$). The data represent mean ± standard deviation (S.D.).

Subjects	N	MMSE	Education (years)	Age (years)
AD	213	22.8 ± 3.7	10.7 ± 3.9	76.5 ± 7.6
MCI	106	27.9 ± 1.6	11.9 ± 3.8	75.4 ± 6.8
NEC	63	29.0 ± 1.0	12.8 ± 2.8	75.0 ± 5.5

Figure 7: Genotype frequencies of the H63D (A) and C282Y (B) mutations between AD, MCI, and NEC subjects. (A) When stratified by H63D genotype status, there are no significant differences between the three subject groups in the percentages of people who are wildtype, heterozygous, or homozygous for this mutation ($\chi^2(4)=3.83$, $p=0.43$). (B) When stratified by C282Y genotype status, there are no significant differences between the three subject groups in the percentages of people who are wildtype, heterozygous, or homozygous for this mutation ($\chi^2(4)=4.59$, $p=0.33$).

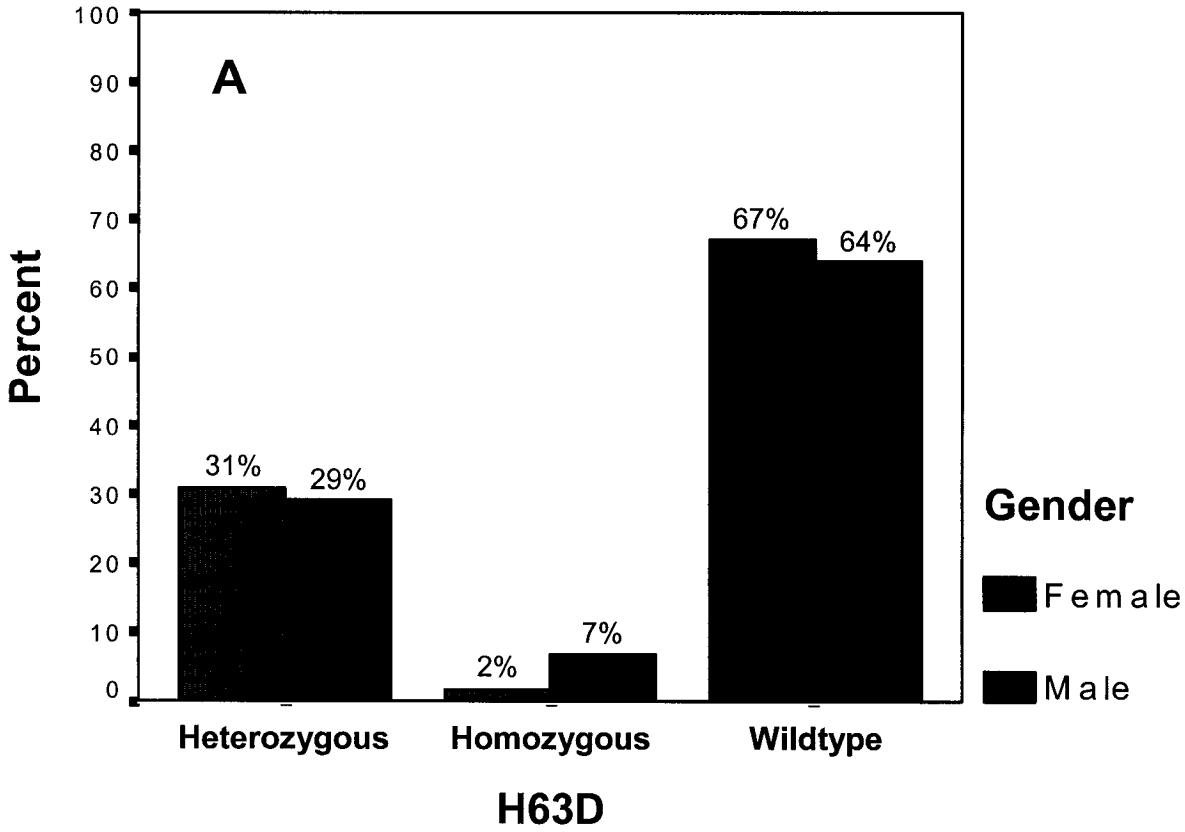

A
Percent
100
90
80
70
60
50
40
30
20
10
0
29%
25%
30%
4%
1%
4%
67%
74%
66%
Heterozygous
Homozygous
Wildtype
Diagnosis
AD
MCI
NEC

H63D


B
Percent
100
90
80
70
60
50
40
30
20
10
0
5%
5%
10%
1%
95%
94%
90%
Heterozygous
Homozygous
Wildtype
Diagnosis
AD
MCI
NEC

C282Y

Figure 8: Genotype frequencies of the H63D (A) and C282Y (B) mutations between male and female AD subjects. (A) The proportions of males and females within each H63D genotype category are not significantly different from one another ($\chi^2(2)=3.59$, $p=0.17$). (B) The proportions of males and females within each C282Y genotype category are not significantly different from one another ($\chi^2(1)=0.01$, $p=0.92$).


A
Percent
100
90
80
70
60
50
40
30
20
10
0
31%
29%
2%
7%
67%
64%
Heterozygous
Homozygous
Wildtype
Gender
Female
Male
H63D

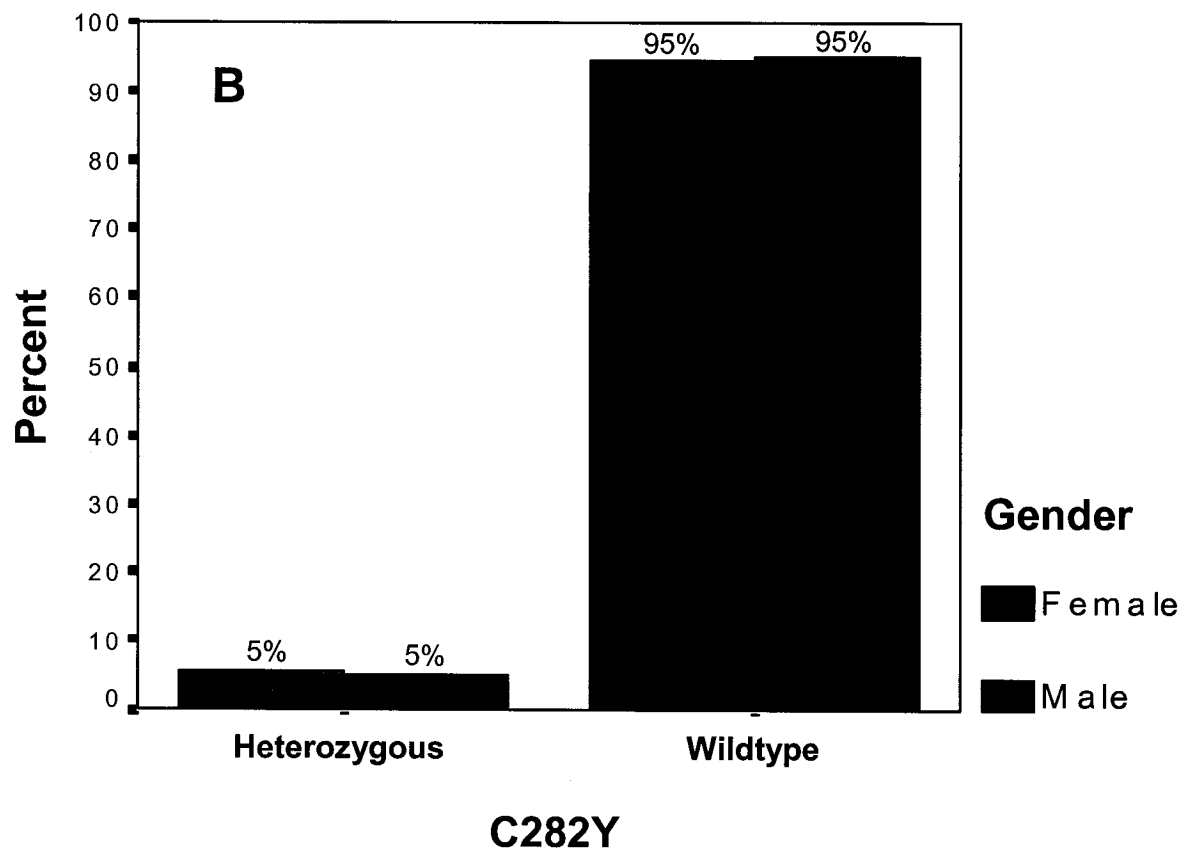
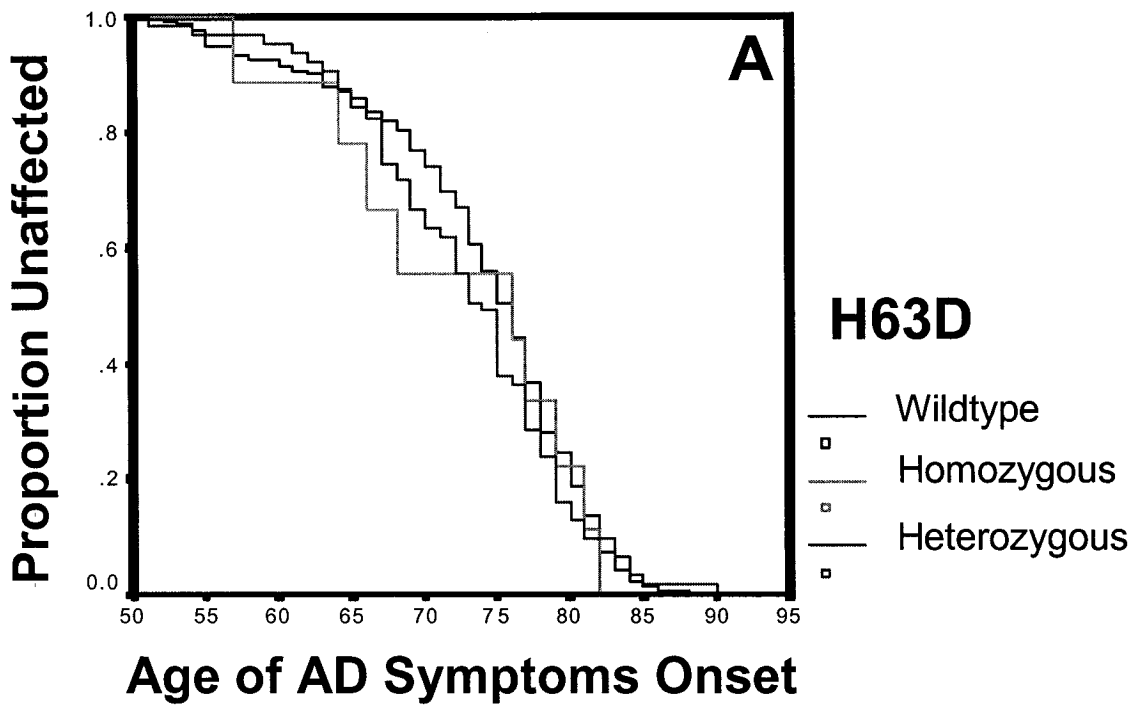

B
Percent
100
90
80
70
60
50
40
30
20
10
0
5%
5%
95%
95%
Heterozygous
Wildtype
Gender
Female
Male
C282Y

Figure 9: Effect of the H63D mutation on age of AD symptoms onset (A) and age of AD diagnosis (B). (A) There are no statistically significant differences in the age of AD symptoms onset between patients who are wildtype (n=140), heterozygous (n=64), or homozygous (n=9) for the H63D mutation ($\chi^2(2)=0.67$, p=0.72). The three overlapping KM survival curves, each representing a specific genotype, indicate this fact. The median ages of symptoms onset for the wildtype, heterozygous, and homozygous AD patients are 76, 74, and 76, respectively. (B) There are no significant difference in the age of AD diagnosis among patients who are wildtype, heterozygous, or homozygous for the H63D mutation ($\chi^2(2)=1.79$, p=0.41). The three overlapping KM survival curves, each representing a specific genotype, indicate this fact. The median ages of AD diagnosis for these three groups are 79, 77, and 80, respectively.


A
Proportion Unaffected
1.0
.8
.6
.4
.2
0.0
50
55
60
65
70
75
80
85
90
95
Age of AD Symptoms Onset
H63D
Wildtype
Homozygous
Heterozygous

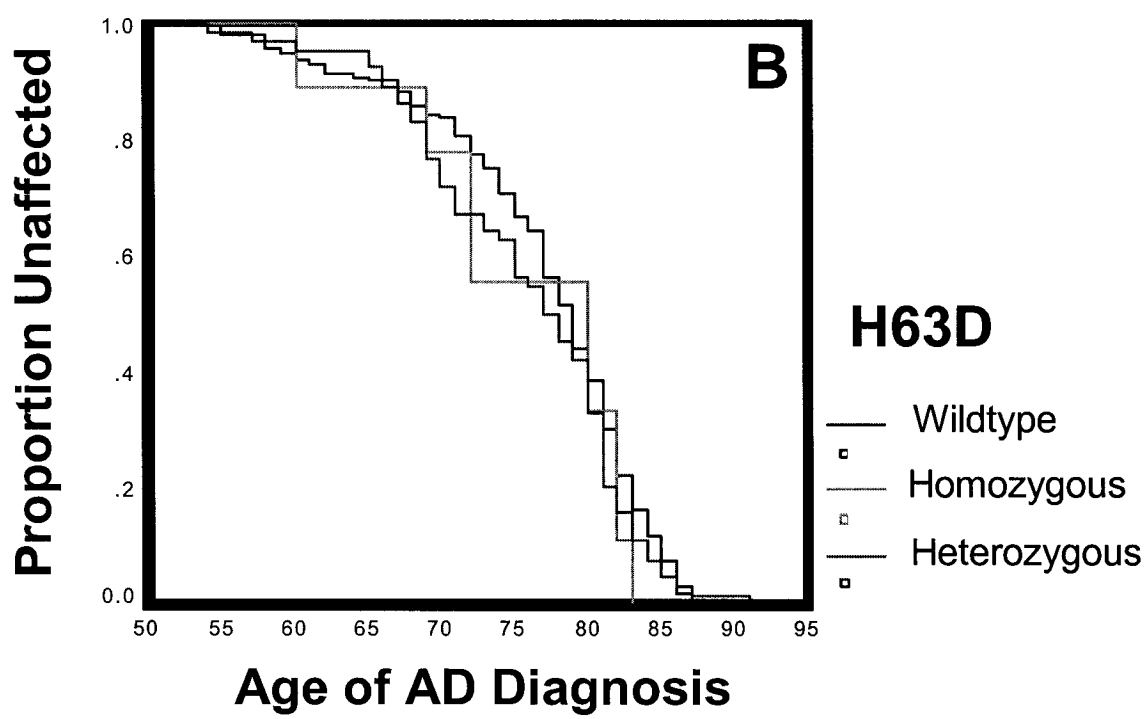
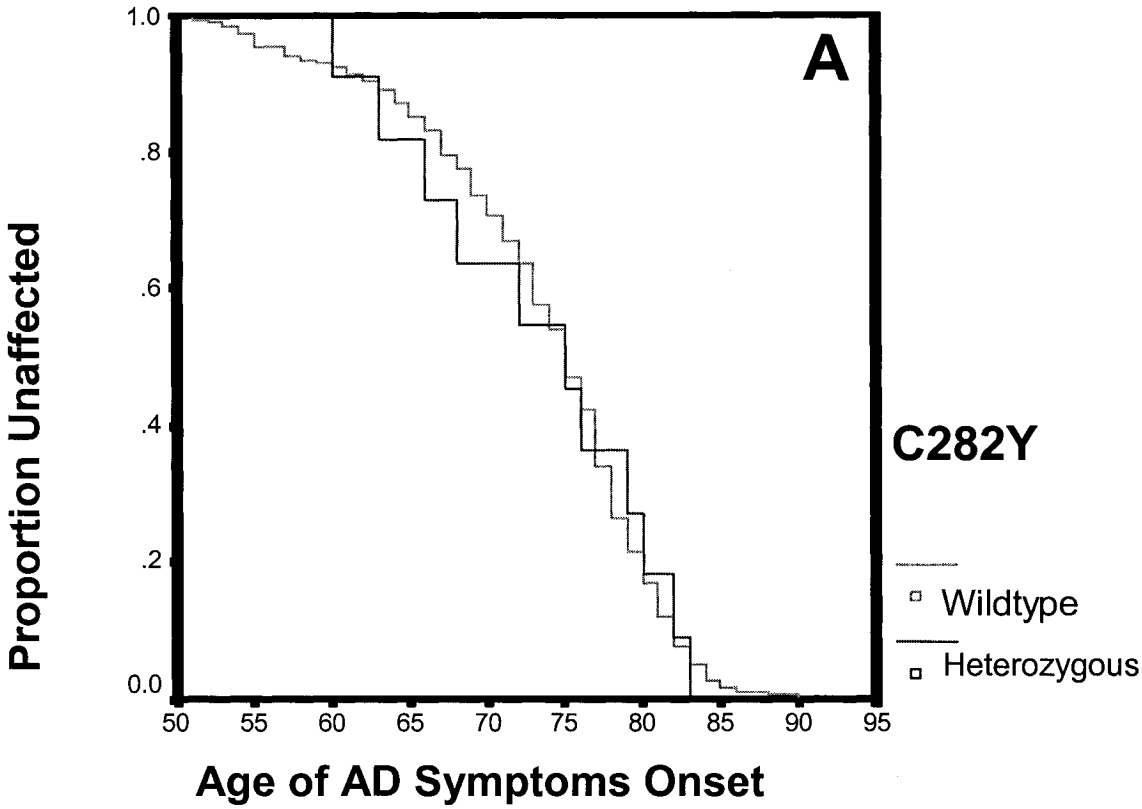

B
Proportion Unaffected
1.0
.8
.6
.4
.2
0.0
50
55
60
65
70
75
80
85
90
95
Age of AD Diagnosis
H63D
Wildtype
Homozygous
Heterozygous

Figure 10: Effect of the C282Y mutation on age of AD symptoms onset (A) and age of AD diagnosis (B). (A) There are no significant differences in the age of symptoms onset between individuals who are wildtype (n=202) or heterozygous (n=11) for the C282Y mutation ($\chi^2(1)=0.01$, p=0.93). The two overlapping KM survival curves, each representing a specific genotype, indicate this fact. The median age of symptoms onset for both groups is 75. (B) There are no significant differences in the age of AD diagnosis between individuals who are wildtype or heterozygous for the C282Y mutation ($\chi^2(1)=0.57$, p=0.45). The two overlapping KM survival curves, each representing a specific genotype, indicate this fact. The median ages of AD diagnosis for these two groups are 79 and 77, respectively.


A
Proportion Unaffected
1.0
.8
.6
.4
.2
0.0
50
55
60
65
70
75
80
85
90
95
Age of AD Symptoms Onset
C282Y
Wildtype
Heterozygous

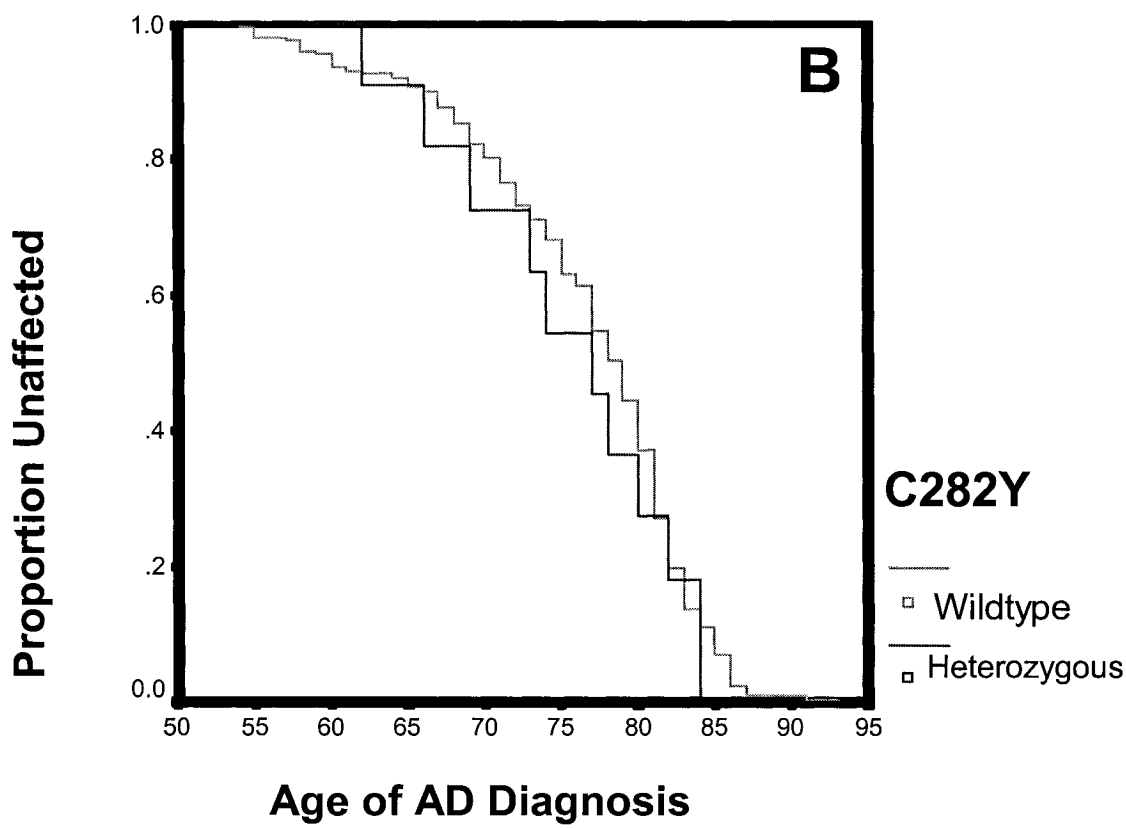

B
Proportion Unaffected
1.0
.8
.6
.4
.2
0.0
50
55
60
65
70
75
80
85
90
95
Age of AD Diagnosis
C282Y
Wildtype
Heterozygous

Figure 11: Mean neuropsychological testing scores of AD, MCI, and NEC subject groups. For each test administered, the AD patients' testing scores are significantly worse than those of NEC subjects ($p<0.001$). In addition, the testing scores of MCI patients are in all cases intermediate relative to the other two groups. This demonstrates that an inverse relationship exists between cognitive decline and neuropsychological testing performance in our patient population. The data represent mean scores ± S.D.

Test	AD	MCI	NEC
Clock (/10)	5.8 ± 2.0	7.9 ± 1.7	8.9 ± 1.0
Logical Memory (/25)	0.9 ± 1.6	5.0 ± 3.7	10.6 ± 3.7
Trail Making (sec)	101.5 ± 52	59.0 ± 38	45.0 ± 23
F+S Fluency	16.2 ± 7.0	21.8 ± 7.8	29.8 ± 8.8
Animal Fluency	8.0 ± 3.3	12.4 ± 4.6	16.8 ± 4.5
Boston Naming (/60)	32.6 ± 13.5	44.7 ± 9.1	53.3 ± 8.2
Digit Symbol	23.0 ± 10.5	35.8 ± 9.3	48.0 ± 12

Figure 12: Effect of the H63D (A) and C282Y (B) mutations on neuropsychological testing scores of AD patients. (A) There are no significant differences between the mean neuropsychological testing scores of AD patients who are wildtype, heterozygous, or homozygous for the H63D mutation ($F(14,388)=0.344$, $p=0.98$). (B) There are no significant differences between the mean neuropsychological testing performances of AD patients who are wildtype or heterozygous for the C282Y mutation ($F(7,205)=0.30$, $p=0.95$). In all cases, the data represent mean scores ± S.D.

A

Test	H63D Wildtype	H63D Heterozygous	H63D Homozygous
Clock (/10)	5.8 ± 2.0	5.8 ± 1.9	5.9 ± 1.6
Logical Memory (/25)	0.8 ± 1.5	0.9 ± 1.7	1.4 ± 2.7
Trail Making (sec)	100 ± 48	104 ± 61	108 ± 33
F+S Fluency	16.0 ± 7.0	16.9 ± 7.2	14.7 ± 5.5
Animal Fluency	7.9 ± 3.0	8.1 ± 3.1	8.0 ± 3.7
Boston Naming (/60)	32.7 ± 13	32.8 ± 15	29.8 ± 17
Digit Symbol	22.4 ± 10.5	24.7 ± 9.9	20.3 ± 13

B

Test	C282Y Wildtype	C282Y Heterozygous
Clock (/10)	5.8 ± 2.0	5.9 ± 1.7
Logical Memory (/25)	0.9 ± 1.6	1.1 ± 1.8
Trail Making (sec)	102 ± 52	114 ± 38
F+S Fluency	16.0 ± 7.0	16.7 ± 7.3
Animal Fluency	7.93 ± 3.0	7.8 ± 2.7
Boston Naming (/60)	32.5 ± 13	33.3 ± 14
Digit Symbol	22.7 ± 10.5	24.0 ± 8.5

Figure 13: Effect of the H63D mutation on progression from MCI to AD. There are no significant differences in the MCI-AD time interval among individuals who are wildtype (n=115), heterozygous (n=41), or homozygous (n=5) for the H63D mutation ($\chi^2(2)=3.61$, $p=0.16$). Nonetheless, inspection of the survival functions reveals that the wildtype and heterozygote curves are overlapping, whereas the homozygote curve is shifted left of the other two. This trend suggests that homozygosity for H63D may effect the rate of conversion from MCI to AD. The median MCI-AD time interval is 4 years for individuals who are H63D wildtype, 3 years for heterozygotes, and 1 year for homozygotes. The turquoise blue, red, and dark blue symbols denote individuals from the wildtype, heterozygous, or homozygous groups, respectively, who are censored at the specified time points.

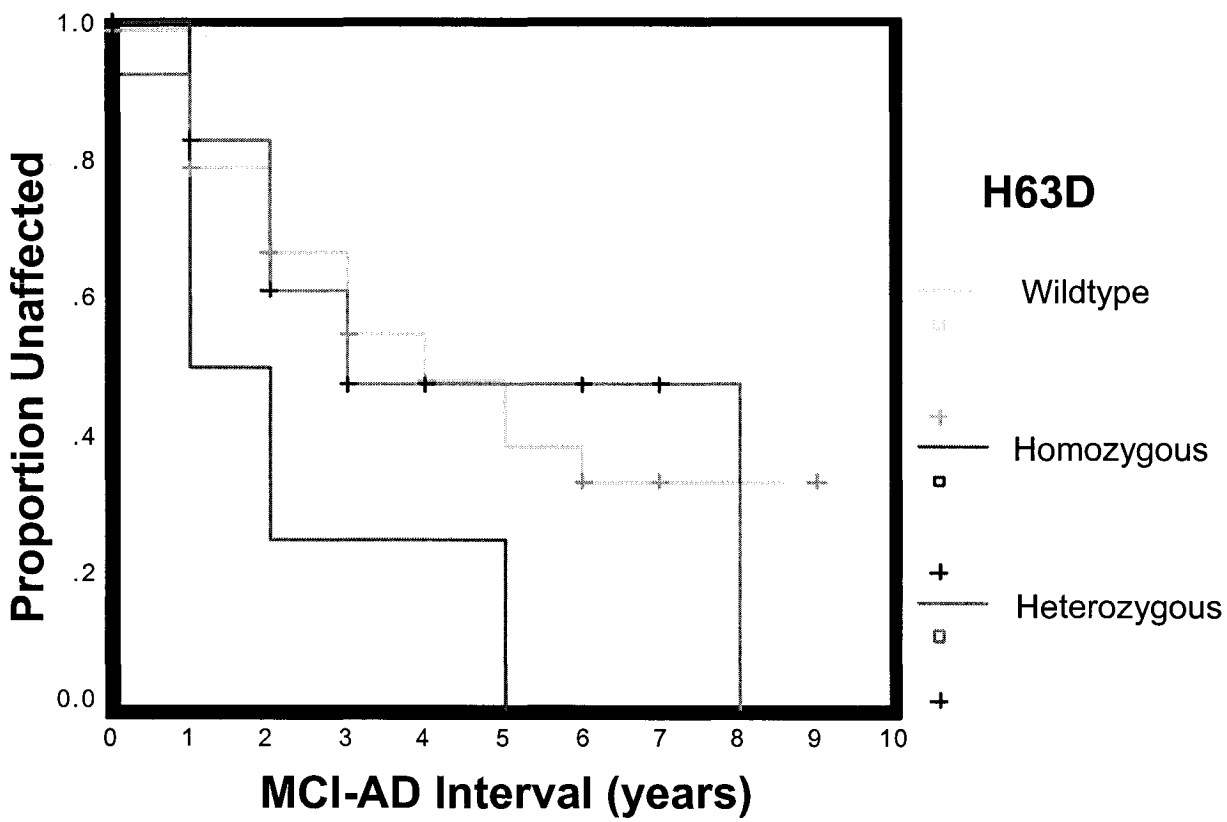

1.0
.8
.6
.4
.2
0.0
Proportion Unaffected
0
1
2
3
4
5
6
7
8
9
10
MCI-AD Interval (years)
H63D
Wildtype
Homozygous
Heterozygous

Figure 14: Effect of the H63D mutation on age of symptoms onset in MCI and AD subjects. All MCI and AD patients were stratified based on whether they were H63D wildtype, heterozygous, or homozygous. There are no significant differences in the age of symptoms onset between the three genotype groups, as indicated by KM survival analysis ($\chi^2(2)=0.32$, $p=0.85$). Nonetheless, the first half of the survival function representing the homozygous subjects is shifted left of the other two curves, while the second half is superimposed over them. This suggests that between the ages of approximately 55 to 75, H63D homozygous patients may have a higher probability of developing earlier cognitive deficits than their counterparts in the other two groups. The median age of symptoms onset is 75 for wildtype patients, 75 for heterozygotes, and 68 for homozygotes.

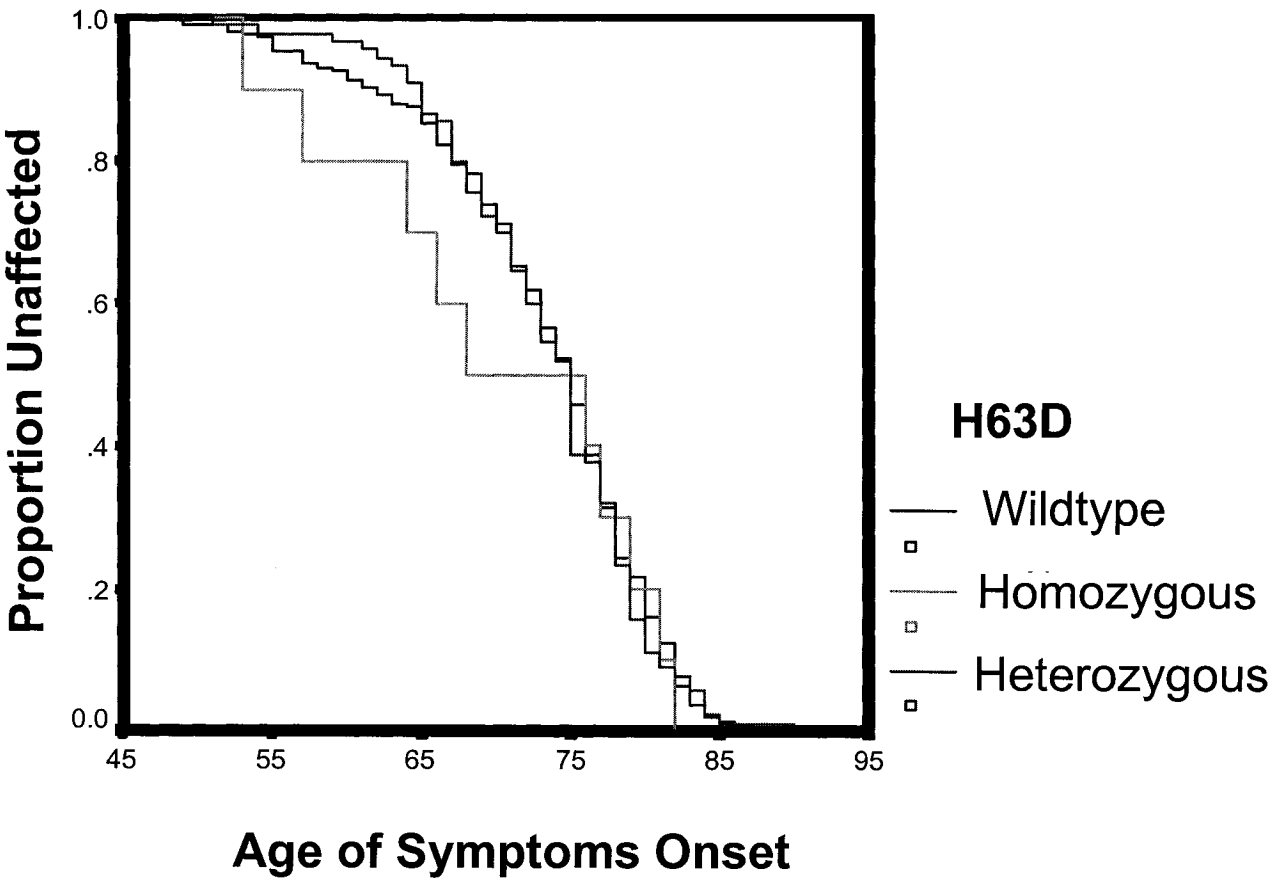

Proportion Unaffected
1.0
.8
.6
.4
.2
0.0
45
55
65
75
85
95
Age of Symptoms Onset
H63D
Wildtype
Homozygous
Heterozygous

Figure 15: Genotype frequencies of the apoE-4 allele between AD, MCI, and NEC subjects. There is a highly significant difference between the AD, MCI, and NEC subject groups in their proportions of individuals who possess zero, one, or two E-4 alleles ($\chi^2(4)=26.94$, $p<0.001$). The E-4 allele is clearly over-represented in our AD cohort.

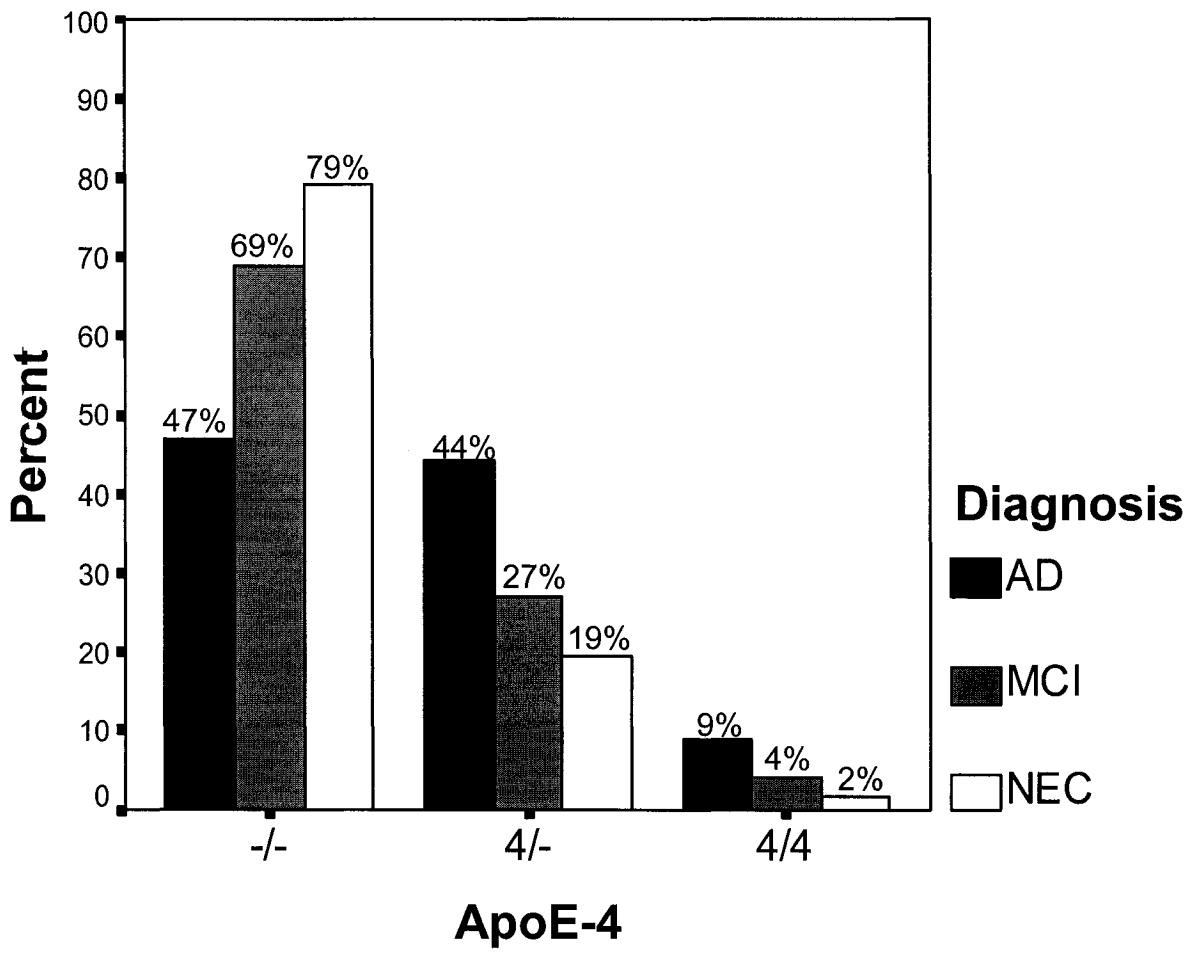
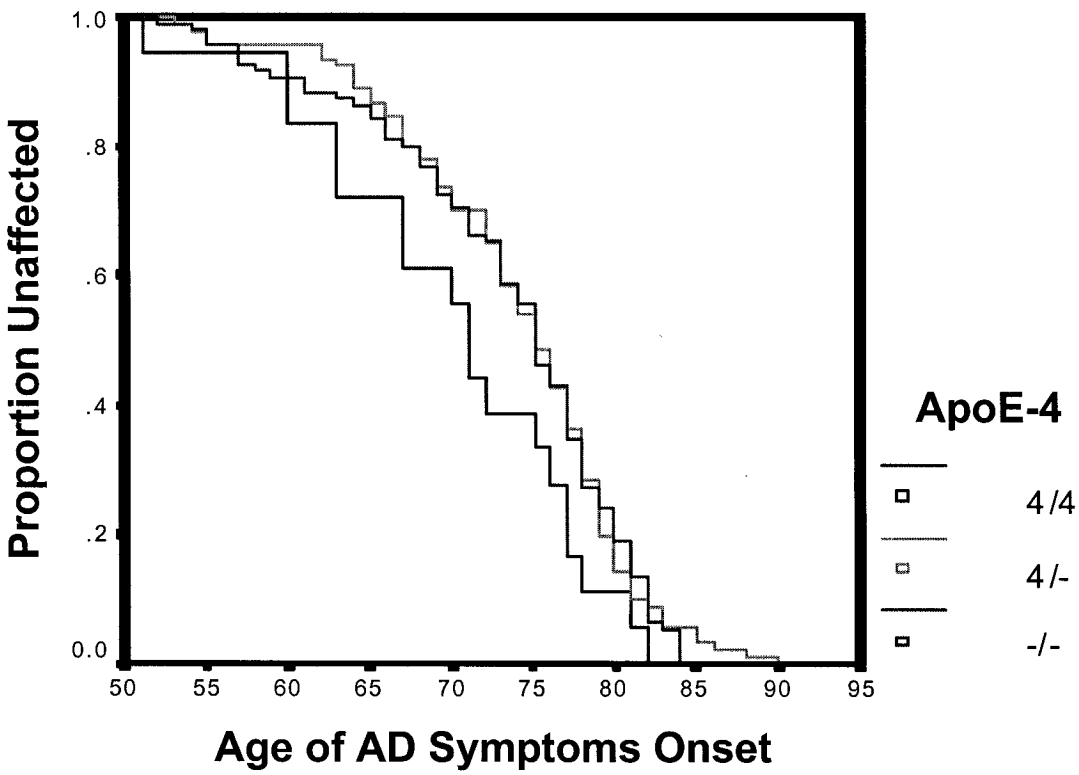

100
90
80
70
60
50
40
30
20
10
0
Percent
47%
69%
79%
44%
27%
19%
9%
4%
2%
-/-
4/-
4/4
ApoE-4
Diagnosis
AD
MCI
NEC

Figure 16: Effect of the apoE-4 allele on age of AD symptoms onset. Although there are no statistically significant differences between the survival functions of AD patients possessing zero (n=95), one (n=91), or two (n=18) E-4 alleles ($\chi^2(2)=3.92$, p=0.14), the survival curve representing the E-4 homozygotes is noticeably shifted left of the other two. This suggests that homozygosity for the apoE-4 allele might play a role in precipitating an earlier symptoms onset in our patient population. The distribution curves representing patients who are heterozygous for apoE-4 and patients who do not possess the E-4 allele are overlapping, indicating that one E-4 allele does not reduce the age of symptoms onset.


Proportion Unaffected
1.0
.8
.6
.4
.2
0.0
50
55
60
65
70
75
80
85
90
95
Age of AD Symptoms Onset
ApoE-4
4/4
4/-
-/-