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A search for genes influencing late-onset Alzheimer's disease on chromosomes one and six.

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A SEARCH FOR GENES INFLUENCING LATE-ONSET ALZHEIMER'S DISEASE ON CHROMOSOMES ONE AND SIX

by

JULIANNE SHEA COLLINS

A DISSERTATION

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ABSTRACT OF DISSERTATION

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Title A Search for Genes Influencing Late-Onset Alzheimer's Disease on Chromosomes One and Six

A collaborative genome-wide scan for Alzheimer disease (AD) genes in 266 late-onset families implicated a region at chromosome 6p21.3 that includes the tumor necrosis factor (TNF) gene, as well as a region at 1p36 that includes the TNF receptor 2 (TNFR2) gene. TNF, a pro-inflammatory cytokine, may be involved in the pathogenesis of Alzheimer's disease (AD) based upon observations that senile plaques have been found to upregulate pro-inflammatory cytokines. Additionally, nonsteroidal anti-inflammatory drugs have been found to delay and prevent the onset of AD. Three TNF polymorphisms and one TNFR2 polymorphism were typed in 145 families consisting of 562 affected and unaffected siblings. The TNF polymorphisms formed a haplotype that was significantly associated with AD ($P = 0.005$) using the sibling disequilibrium test. Although the results from the TNFR2 polymorphism were not significant, there does seem to be evidence that the region may be harboring a candidate gene or modifier. This TNF association with AD lends further support for an inflammatory process in the pathogenesis of AD.

DEDICATION

I dedicate this dissertation to all those whose family members have suffered from Alzheimer's disease and other forms of dementia.

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LIST OF ABBREVIATIONS

A2M	alpha-2-macroglobulin
aa	amino acid
AD	Alzheimer's disease
ADRDA	Alzheimer's Disease and Related Disorders Association
APM	affected pedigree member
ApoE	apolipoprotein E
APP	amyloid precursor protein
ASP	affected sib pair
Aβ	amyloid beta
cM	centimorgans
CERAD	consortium to establish a registry for Alzheimer's disease
COX-2	cyclo-oxygenase-2
FTDP-17	fronto-temporal dementia and Parkinsonism linked to chromosome 17
HRR	haplotype relative risk
IBD	identical by descent
IBS	identical by state
IL-1	interleukin-1
IL-6	interleukin-6
IL-12	interleukin-12

LRP	low density lipoprotein receptor-related protein
NFTs	neurofibrillary tangles
NIMH	National Institutes of Mental Health
NINCDS	National Institute of Neurological and Communicative Disorders and Stroke
NPL	nonparametric linkage
NPs	neuritic plaques
NSAIDs	nonsteroidal anti-inflammatory drugs
OR	odds ratio
PCR	polymerase chain reaction
PD	Parkinson's disease
PHFs	paired helical filaments
PS1	Presenilin 1
PS2	Presenilin 2
RR	relative risk
SDT	sibship disequilibrium test
S-TDT	sib transmission/disequilibrium test
STM2	second seven transmembrane protein
TDT	transmission/disequilibrium test
TNF	tumor necrosis factor
TNFR2	tumor necrosis factor receptor 2
UAB	University of Alabama at Birmingham

INTRODUCTION

On November 4th 1906, Alois Alzheimer described the case of Auguste D., a 51-year old woman with reduced comprehension and memory, hallucinations, disorientation, and psychosocial impairment. After her death, he found plaques, neurofibrillary tangles, and arteriosclerotic changes in her brain [Alzheimer, 1906]. Subsequently in 1910, Emil Kraepelin first used the term Alzheimer's disease (AD) to describe cases of senile dementia with these distinctive autopsy findings [Maurer et al., 1997; Kraepelin, 1910].

Today AD is a major public health concern. It affects 10% of those over 65 years of age, 50% of those over 85-years of age, and is the leading cause of dementia in the elderly. An estimated $100 billion a year is spent to care for the four million individuals with AD. For the year 2000, over $466 million has been allocated by the federal government of the United States to study AD [Alzheimer's Association, 2000].

It is estimated that 20-40% of all AD cases are familial [Morris, 1994]. Children of affected individuals have a 50% risk of inheriting AD in early-onset (before the age of 60) families, and an 85% risk in late-onset (after the age of 60) families [Farrer et al., 1990; Rao et al., 1994]. Gatz et al. [1997] found a 67% concordance rate of AD among monozygotic twins, and a 22% concordance rate among dizygotic twins, which is representative of a genetic disease as monozygotic twins are genetically identical and dizygotic twins are full siblings. Early-onset families with AD transmit the gene as a

fully penetrant autosomal dominant, while late-onset families show heterogeneous transmission [Rao et al., 1994].

Three genes have been found to cause early-onset AD. They are the Amyloid Precursor Protein (APP), which maps to chromosome 21 [Goate et al., 1991], Presenilin 1 (PS1), localized to chromosome 14 [Schellenberg et al., 1992], and Presenilin 2 (PS2), which is located on chromosome 1 [Levy-Lahad et al., 1995a, 1995b]. In addition, the apolipoprotein E (ApoE) ε4 allele has been identified as a major risk factor [Saunders et al., 1993] for late-onset AD. Together, these genes cause about 50% of all diagnosed familial AD cases [Tanzi et al., 1996]. Currently, other causes of AD are unknown.

Clinical Signs

The clinical course of AD begins with the inability to learn new information and the deterioration of short-term memory [Morris, 1994]. Alzheimer's disease patients also have language problems, or aphasia, which include difficulty in finding words and the deterioration of language comprehension [Price et al., 1993]. Motor skill deficits (apraxia) and deficits in perception (agnosia) such as hallucinations are also common. Another common feature of AD is disturbed executive functioning, or problems with planning, organizing, sequencing, or abstracting [Morris, 1994], which lead to problems with daily living.

Typical psychiatric symptoms of AD are aggression [Hamel et al., 1990], agitation, delusions, hallucinations, wandering, poor hygiene, apathy, insomnia [Teri, Larson, and Reifler, 1988; Cooper, Mungas, and Weiler, 1990], and depression [Merriam et al., 1988; Rovner et al., 1989]. Alzheimer's disease progresses until patients are unable

to care for themselves and eventually become bedridden [Morris, 1994]. The average clinical course is 8-10 years from the onset of AD to death [Walsh et al., 1990].

Neuropathological Changes

Upon external exam of the brain, there is significant atrophy of the frontal, temporal, and parietal cortex [Mirra and Markesbery, 1996]. Much of this atrophy can be attributed to neuronal loss, in which the neurons in these areas become dysfunctional and/or die, leading to synaptic alterations [Troncoso et al., 1996]. There is also granulovacuolar degeneration [Okamoto et al., 1991] and shrinkage of the hippocampus, as well as enlargement of the ventricular system [Mirra and Markesbery, 1996].

Pathological changes in the brain include extracellular neuritic plaques (NPs) in the temporal, parietal, and occipital lobes [Arnold et al., 1991], which are not commonly seen in normal individuals [Haroutunian et al., 1998]. These NPs have a core of fibrillar amyloid beta (Aβ) protein, which is surrounded by lysosomes, mitochondria, and bundles of hyperphosphorylated tau in fibers known as paired helical filaments (PHFs) [Mirra and Markesbey, 1996]. NPs typically have a β-pleated-sheet conformation and stain positively with Congo Red and thioflavine. Diffuse plaques, which have a softer appearance and are more common in the neocortex of normal individuals, do not contain this amyloid core and thus do not stain positively with Congo Red and thioflavine [Afagh et al., 1996].

The cytoplasm of some neurons contains neurofibrillary tangles (NFTs), which are composed of PHFs [Mirra and Markesbey, 1996]. These NFTs are commonly found in the limbic and temporal lobes of the brain [Arnold et al., 1991] and identified by using

Congo Red and thioflavine stains [Mirra and Markesbey, 1996]. PHF components, in addition to tau, are ubiquitin, proteoglycans, apolipoprotein E, amyloid P component, and complement factor C1q [Yen, 1995]. NFTs occur in varying densities in AD patients [Arnold et al., 1991] and can also occur in normal elderly individuals [Mirra and Markesbey, 1996].

The Consortium to Establish a Registry for Alzheimer's disease (CERAD) established a task force in 1991 to standardize the neuropathological evaluation of AD. The evaluation included assessment of plaque and tangle frequency, an age-related plaque score, and the presence of dementia in patients to determine the neuropathological diagnosis of AD [Mirra et al., 1991]. In 1996, these criteria were updated during a meeting sponsored by the National Institute of Aging and the Ronald and Nancy Reagan Research Institute. These new recommendations stated that the likelihood of AD is high when high numbers of NPs and NFTs are in the neocortex, intermediate when there are moderate numbers of NPs and NFTs in limbic regions, and low when NPs and NFTs have a more limited distribution [The NIA-Reagan Working Group, 1997].

Therefore, neuropathological changes in AD patients include atrophy of their frontal, temporal, and parietal cortex as well as their hippocampus [Mirra and Markesbery, 1996]. As the frontal, temporal, and parietal cortex, as well as the hippocampus, are involved in working, or short-term memory, atrophy of these areas in AD patients can account for this neurocognitive deficit [Gazzaniga et al. 1998]. Other affected areas include the amygdala and the olfactory system, which could account for the decreased sense of smell in AD patients. Other areas of substantial neuronal loss also occur in the nucleus basalis, which accounts for the substantial cholinergic deficiencies

seen in AD patients and in the entorhinal cortex, which links the neocortex to the limbic system and is one of the primary sites of tangle formation [Kandel et al., 1991].

NPs and NFTs located in the neocortex are good indicators of dementia [The NIA-Reagan Working Group, 1997]. The number of NPs also increase as the severity of dementia increases, but level off once patients reach the level of moderate dementia [Haroutunian et al., 1998]. However, there seems to be no correlation between severity of NPs or NFTs and duration of illness or age at death [Arnold et al., 1991].

Diagnostic Criteria

In 1984, the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer's Disease and Related Disorders Association (ADRDA) published criteria to standardize the diagnosis of AD into three categories: probable, possible, and definite. Probable AD is characterized by a progressive onset of dementia, which is documented by clinical exam and testing. The age of onset of the patient must be between the ages of 40 and 90 years. The patient may not have any other disorders that could cause dementia, such as vascular dementia or Parkinson's disease [McKhann et al.,1984]. These criteria were found to be 81-88% accurate, depending on the neuropathologic criteria used, in a study of 57 AD cases [Keefover, 1996].

Diagnosis of possible AD occurs when there are variations in the course of the dementia in the absence of other dementing disorders. Possible AD diagnosis may include another systemic or brain disorder that is not considered to cause the patient's dementia. The definite diagnosis of AD is used for cases of clinical AD for which there

is histopathologic confirmation of NPs and NFTs by autopsy, or, in rare cases, a biopsy [McKhann et al., 1984; Khachaturian, 1985].

The DSM-IV criteria for AD must include memory impairment, and at least one of the following, aphasia (language problems), apraxia (motor skill deficits), agnosia (deficits in perception), or disturbed executive functioning. The patient should have a continual cognitive and functional decline, and other causes of dementia should have been excluded [Morris, 1994].

Treatment

As there is often a decreased amount of acetylcholine in the brains of AD patients, it has been hypothesized that higher amounts of acetylcholine in the patient's brains will improve their AD symptoms. In 1993, tacrine (Cognex) was the first drug approved by the FDA to treat AD. It increases levels of the neurotransmitter acetylcholine in the brain by inhibiting acetycholinesterase. Studies have shown that patients using tacrine show improvements in their cognitive functioning [Knapp et al., 1994]. Another AD drug that is currently available is donepezil, which is also an acetycholinesterase inhibitor. Cholinesterase inhibitors have also been shown to improve cooperativeness in patients, decrease apathy, and make them less delusional [Rogers and Friedhoff, 1996].

Selkoe [1997] envisioned many types of AD treatments in the future. The first is protease inhibitors to decrease the cleaving of Aβ. The second is compounds that bind to Aβ and prevent aggregation. The third treatment is anti-inflammatory drugs. Currently nonsteroidal anti-inflammatory drugs (NSAIDs) and prednisone are being used in trials to prevent inflammation [Aisen, 1997] and may be effective in preventing the onset of or

slowing AD progression [Mackenzie and Munoz, 1998]. The fourth treatment Selkoe mentions is antioxidants, which could be beneficial for the brain, as it has a high metabolic rate resulting in the production of free radicals [Behl, 1997]. Dementia has been associated with low levels of the antioxidant vitamins C and E, which act as free-radical scavengers. Vitamin E has been found to protect neurons against Aβ toxicity [Behl, 1992] and slow the progression of AD in a clinical trial [Sano et al., 1997].

Genetics of Early-Onset AD

The protein that forms the core of the NPs in AD is called Aβ protein. It has a partial beta pleated sheet structure and is cleaved from exons 16 and 17 of the APP protein, which is transcribed from the APP gene at chromosome 21q21 [Yoshikai et al., 1990]. APP is a 19 exon membrane spanning protein whose 695 amino acid (aa) form (where exons 7 and 8 are deleted) is expressed in neurons, while other cell types express 750 aa (exon 8 deletion) and 770 aa (full length) forms.

The 39 to 43 aa Aβ (4 kDa) is formed by the cleavage of APP by β-secretase N-terminally and γ-secretase C-terminally. Alpha-secretase cleavage of APP prevents the formation of Aβ, as it cleaves between the other two secretases (Fig. 1). However, once APP is cleaved by β-secretase, it is not susceptible to cleavage by α-secretase. Two forms of the Aβ protein are normally produced by γ-secretase cleavage: Aβ40 and the rarer Aβ42 [Volloch, 1996]. Aβ42 is the most common variant of Aβ found in NPs, and it appears to aggregate and form neurotoxic AD NPs more easily [Yankner et al., 1989] than Aβ40 [Jarrett and Lansbury, 1993].

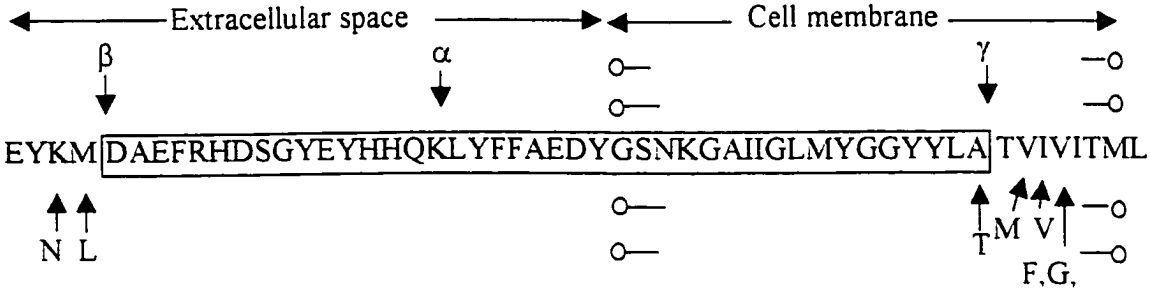


Fig. 1. Depiction of Aβ42 (surrounded by box), with arrows pointing to the secretase cleavage sites and the aa changes of the known AD mutations.

Down syndrome (Trisomy 21) patients usually accumulate Aβ protein deposits in late childhood [Selkoe, 1996] and exhibit clinical signs of AD by middle age [Wisniewski, Wisniewski, and Wen, 1985]. This pathology may be due to a triplication and increased dosage effects of the APP gene. In 1991, Goate et al. sequenced the APP gene and discovered a C to T transition changing valine to isoleucine in exon 17 of the APP gene. This mutation was not found in 100 normal individuals, but it was found in a second unrelated family with AD.

APP mutations account for 2% of all cases of AD [Tanzi et al., 1992], as only 7 AD mutations have been found in the APP gene. These are Val^{717}→Ile, Val^{717}→Phe, Val^{717}→Gly, double mutation Lys^{670}→Asn/Met^{671}→Leu, Ala^{713}→Thr, Ile^{716}→Val [for review see Tanzi et al., 1996], and Val^{715}→Met [Ancolio et al., 1999] (see Figure 1). These mutations increase the production of Aβ42 by affecting the processing of APP so that more Aβ42 is produced [Selkoe, 1996; Ancolio et al., 1999]. For example, patients with the Lys^{670}→Asn/Met^{671}→Leu mutation have significantly ($P < 0.0001$) higher levels of Aβ42 in their plasma than those of the controls [Scheuner et al., 1996].

Transgenic mice overexpressing the heterozygous APP $Val^{717}\rightarrow$Phe AD mutation had deposits of Aβ protein by 9 months of age that resembled the pathology of AD [Games et al., 1995]. Hsiao et al. [1996] generated transgenic mice with the APP AD double mutation ($Lys^{670}\rightarrow$Asn/$Met^{671}\rightarrow$Leu) that showed neurological impairment, increased Aβ production, and positive Congo Red Aβ NPs [Sturchler-Pierrat et al., 1997]. These mice were also examined by Irizarry et al. [1997], who concluded that the "Aβ is not acutely neurotoxic, but can disrupt neuronal processes and provoke an inflammatory response."

Soluble Aβ42 has been found to suppress acetylcholine synthesis in cholinergic neurons [Hoshi et al., 1997] although Aβ40 has no effect. Cholinergic synapses contain nicotinic receptors and muscarinic receptors and use acetylcholine as a transmitter [Voet and Voet, 1990]. Therefore, the increased production of Aβ42 by the APP mutations found in some AD patients could lead to neurotransmitter suppression and an associated loss of memory and cognitive function. However, not all cases of early-onset AD were linked to chromosome 21 [St George-Hyslop et al., 1990].

In 1992, Schellenberg et al. found that an early-onset (mean age of onset was 45 years), autosomal dominant form of AD was localized to marker D14S43 on chromosome 14q24.3. In 1995, Sherrington et al. isolated 19 different transcripts in this region. Five missense mutations in transcript S182 were found in early-onset AD patients. S182 coded for a 6 to 9 transmembrane domain protein and was later renamed PS1 by the Alzheimer's Disease Collaborative Group [1995].

There have been a total of 42 missense mutations found in the PS1 gene [for review see Cruts and van Broeckhoven, 1998]. Interestingly, these mutations have been

found to produce increased amounts of Aβ42 when transfected into cells [Duff et al., 1996; Citron et al., 1997; Mehta et al., 1998; Wisniewski et al., 1998] and may increase apoptosis [Guo et al., 1996]. Alzheimer's disease patients with these mutations also have increased levels of Aβ42 in plasma ($P < 0.0001$) and fibroblasts ($P < 0.0001$) as compared to controls [Scheuner et al., 1996].

The 43rd PS1 mutation, Δ9, splices out exon 9 of PS1 and is believed to be a loss of function variant that displaces the wild type protein [Mehta et al., 1998]. Individuals with the Δ9 mutation also have spastic paraparesis in addition to the classic features of AD [Kwok et al., 1997]. The Δ9 mutation has been found to produce twice as much Aβ42 and more Aβ40 than the other mutations found in the PS1 gene [Mehta et al., 1998]. Mice that contain a mutant PS1 gene have been found to have more Aβ42 in their brains than wild-type mice [Duff et al., 1996; Citron et al., 1997]. This finding suggests that mutated PS1 may cause a gain of function of APP by altering γ-secretase processing and, thus, the production of Aβ42. This is supported by the finding that transgenic mice with mutations in both APP and PS1 had a 41% increase of Aβ42 in their brains and more plaques than mice with mutations only in APP [Holcomb et al., 1998].

An absence of PS1 in knockout mice embryos has been found to prevent cleavage of the APP transmembrane domain by γ-secretase and a drop in production of Aβ. Therefore, inhibition of PS1 may be a possible treatment of AD [de Strooper et al., 1998; Haass and Selkoe, 1998].

In 1995[a], Levy-Lahad et al. reported linkage of early-onset AD with marker D1S479 (mapped to chromosome 1q31-q42) in 7 related Volga German families. Linkage analyses in these families had previously excluded linkage with the known AD

loci on chromosomes 21 and 14. These families had a mean age of onset at 52 years of age and were clinically and pathologically similar to other cases of AD.

Levy-Lahad et al. [1995b] identified a candidate gene (STM2 or second seven transmembrane protein) which was homologous to the PS1 gene. They discovered that a point mutation in this gene was carried in these affected Volga German individuals. The Alzheimer's Disease Collaborative Group [1995] later called this gene PS2 and until now only 3 AD mutations have been found in it [for review see Cruts and van Broeckhoven, 1998].

PS2 is a 6 to 9 transmembrane domain protein that participates in neuronal cell death (apoptosis), especially when overexpressed. It works with Aβ and may actually increase the amount of apoptosis induced by Aβ. An AD associated PS2 mutation constitutively activates PS2 and may make the neurons more susceptible to apoptotic stimuli, such as free-radical oxidation or aggregated Aβ [Wolozin et al., 1996]. This accelerates neurodegeneration, and may explain the earlier age of onset of AD in these families.

PS2 mutations have been found to increase the amount of Aβ42 produced when transfected into cells [Citron et al., 1997]. Furthermore, PS2 mutations found in AD patients significantly increase the level of Aβ42 found in their plasma ($P = 0.009$) and fibroblasts ($P = 0.03$) as compared to controls [Scheuner et al., 1996]. This increase in the level of Aβ42 produced could lead to its increased deposition in the brain.

Mutations in APP, PS1, and PS2 occur in and cause about 50% of early-onset cases of AD [Tanzi et al., 1996]. APP mutations may cause AD by increasing the production of Aβ42 [Selkoe, 1996], which appears to be more prone to aggregation and

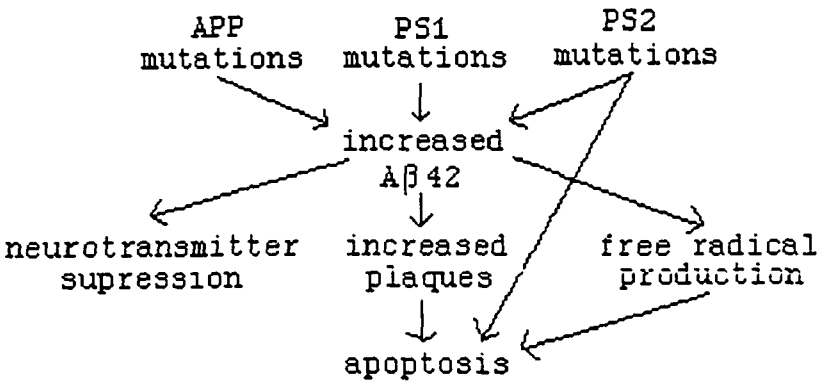


Fig. 2. Early-onset genes and their involvement in AD neurodegeneration.

fibril formation [Jarrett and Lansbury, 1993]. Aβ is also proposed to produce free radicals through interactions with endothelial cells [Thomas et al., 1996] and may over-stimulate the immune system [Behl, 1997; Kaltschmidt et al., 1997]. Alzheimer's disease-causing mutations in the PS1 and PS2 genes increase the production of Aβ42 [Scheuner et al., 1996; Citron et al., 1997] and could make neurons more susceptible to apoptosis [Guo et al., 1996]. It has also been recently hypothesized that PS1 and PS2 are actually γ-secretases, which could explain their role in APP processing [De Strooper, 2000]. Figure 2 depicts schematically how mutations in APP, PS1, and PS2 are interrelated and their probable role in AD pathogenesis.

Genetics of Late-Onset AD

In 1991, Pericak-Vance et al. found linkage in predominantly late-onset (after the age of 60) AD families to chromosome 19q13.2. ApoE mapped to this region and its product bound Aβ [Strittmater et al., 1993], which made it a good candidate gene. ApoE is a major component in lipid transport, and the ε4 allele has been associated with high

serum cholesterol levels and coronary artery disease. ApoE is found in the NPs and NFTs of AD, as well as in vascular deposits. A high atherosclerosis score and 1 to 2 ApoE ε4 alleles have been very strongly associated with vascular dementia (odds ratio (OR) = 19.8) [Hofman et al., 1997], which comprises about 15% of all cases of dementia. However, vascular dementia has a more abrupt onset and stepwise mental deterioration than AD's gradual progression [Morris, 1994].

The ApoE ε4 allele, especially in its homozygous form, has also been identified to be highly associated with AD [Strittmater et al., 1993; Tang et al., 1996; Farrer et al., 1997; Hofman et al., 1997] in familial and sporadic families [Corder et al., 1993; Saunders et al., 1993; Mayeux et al., 1993; Myers et al., 1996]. Bullido et al. [1998] also reported that a polymorphism in the regulatory region of ApoE (which upregulates expression) is associated with risk of AD.

Aβ, which binds to ApoE, has been found to be more efficient at fibril and plaque formation with the ε4 allele, than the ε3 [Weisgraber and Mahley, 1996] or ε2 alleles [Polvikoski et al., 1995]. It has been shown that ApoE ε4/ε4 homozygotes have increased levels of Aβ40 and Aβ42 than individuals with other ApoE genotypes [McNamera et al., 1998]. It is interesting to note that, when the ApoE gene is knocked out in mice that overproduce APP, very few Aβ deposits are formed when compared to mice with an active ApoE gene [Bales et al., 1997].

The ApoE ε2 allele has been found to have greater antioxidant activity than the ε3 allele, which in turn has greater antioxidant activity than the ε4 allele form [Miyata and Smith, 1996]. Therefore, one mechanism for the AD ε4 effect could be the decreased antioxidant capabilities of the ε4 allele leads to more free radical damage, accelerating

the effects of AD. The ApoE ε4 allele definitely plays a role in AD, but it is not necessary, nor sufficient enough, to cause the disease by itself, as there are some ε4 homozygotes that never develop AD [Polvikoski et al., 1995]. It is currently considered an AD risk factor. It's role as a risk factor, and it's effect on age of onset, is further described in the epidemiology section.

In 1997, a genomic screen of 54 families with late-onset AD [Pericak-Vance et al., 1997] showed the strongest linkage to a region of chromosome 12. Other studies have replicated this finding [Rogaeva et al., 1998; Scott et al., 1998; Farrer et al., 1998]. Pericak-Vance [1997] proposed the low density lipoprotein receptor-related protein (LRP) as a candidate gene in this chromosome 12 region, because it acts as an ApoE and APP receptor and mediates clearance of ApoE and Aβ complexes [Hyman et al., 2000].

Kang et al. [1997] looked at an LRP exon 3 marker and found significant differences between AD cases and controls and the number of NPs with different LRP genotypes. Another group, Lambert et al. [1998], confirmed these associations in sporadic late-onset AD patients. However, Small et al. [1997] established that a marker close to the LRP receptor gene was not associated with an increased risk of AD.

Blacker et al. [1998] showed that a deletion in exon 2 of the alpha-2-macroglobulin (A2M) gene on chromosome 12 is associated ($P = 0.00009$) with AD in late-onset AD families who are not ApoE ε4 carriers. Wu et al. [1998] supported this with a lod score of 1.91 at marker D12S1042 (close to the A2M locus) in non ApoE ε4 families. A2M is an LRP ligand and a component of AD NPs [Rebeck et al., 1995; van Gool et al., 1993]. A2M binds to Aβ [Hughes et al., 1998] and plays a role in its

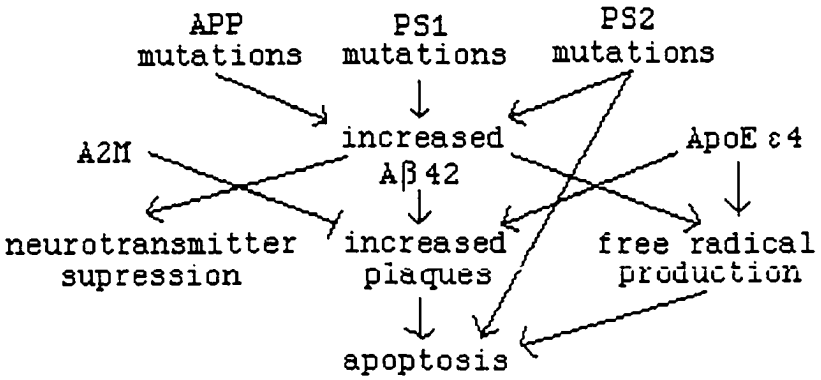


Fig. 3. Early and late-onset gene involvement in AD neurodegeneration.

degradation [Qiu et al., 1996], as well as lessening fibril formation and neurotoxicity [Hughes et al., 1998; Du et al., 1998].

In summary, the ApoE e4 allele is a well-defined risk factor that affects plaque formation and free radical production (Fig. 3). There may be a gene influencing late-onset AD on chromosome 12, possibly LRP or A2M as they are both involved in the clearance of Aβ; however, further studies are needed to describe their effect on AD. Nevertheless, these genes only explain a portion of late-onset AD cases, and there is evidence that 4 additional loci may play a role in late-onset AD [Warwick et al., 2000].

Epidemiology

Age is the most strongly associated AD risk factor. Incidence rates increase with age, although the rate of acceleration declines with increased age [Gao et al., 1998]. Prevalence rates range from 0.2 to 3.0% for those under age 75 and from 7.1 to 47.3% for those over age 85 [Breteler et al., 1992]. However, the prevalence of senile dementia in

the elderly appears to level off at 40% around age 95. Elderly survivors may actually have a decreased risk of dementia [Ritchie and Kildea, 1995].

Tang et al. [1998] found that the prevalence of AD (possible and probable diagnoses) in New York City residents over 65 years of age varied by ethnic group. The Caucasians' prevalence was 9.7%, whereas Hispanics and African Americans had prevalences of 21.9% and 29.3%, respectively. Some ethnic groups, such as Indians and Nigerians, do not have reported cases of AD. Another group, the Cree Indians in Canada, has a low prevalence rate of 0.5% [for review see Keefover et al., 1996].

The ApoE allele ε4 is a major risk factor for AD in different ethnic groups. The ε4/ε4 genotype is most strongly associated with AD in Caucasians (OR = 14.9, 95% CI = 10.8 - 20.6) and Japanese (OR = 33.1, 95% CI = 13.6 - 80.5). Caucasian (OR = 3.2, 95% CI = 2.8 - 3.8) and Japanese (OR = 5.6, 95% CI = 3.9 - 8.0) individuals with a ε3/ε4 genotype were also found to be at increased risk of AD. The ε2/ε3 genotype was found to be protective (OR = 0.6, 95% CI = 0.5 - 0.8) against AD in Caucasians [Farrer et al., 1997].

Farrer et al. [1997] also found a weaker association between AD and the ε4/ε4 genotype in African Americans (OR = 5.7, 95% CI = 2.3 - 14.1). However, the association between AD and the ε4/ε4 genotype in Hispanics (OR = 2.2, 95% CI = 0.7 - 6.7) was also weaker but not significantly. A recent study by Tang et al. [1998] of elderly New York residents showed that the presence of one or more ApoE ε4 alleles did not increase the risk of AD for African Americans (relative risk (RR) = 1.0, 95% CI = 0.6 - 1.6) and Hispanics (RR = 1.1, 95% CI = 0.7 - 1.6). This disparity in these two reports for African Americans could be explained by the fact that one group was looking at an

association with the ε4/ε4 genotype, and the other group was looking at individuals with one or more ε4 alleles. Tang et al. [1998] also discovered that African Americans (RR = 4.4, 95% CI = 2.3 - 8.6) and Hispanics (RR = 2.3, 95% CI = 1.2 - 4.3) over age 65 without ApoE ε4 alleles have a greater risk of AD than Caucasians without ApoE ε4 alleles. These authors hypothesized that other genes or risk factors might cause AD in those ethnic groups.

The ApoE ε4 allele appears to decrease the age of onset of AD [Blacker et al., 1997; Meyer et al., 1998; Corder et al., 1993] and exerts its maximal effect between the ages of 65 and 75 [Blacker et al., 1997; Frisoni et al., 1998]. In the 1997 study by Blacker et al., individuals with 2 copies of the ε4 allele had a significantly earlier age of onset than those with one or no copies of the ε4 allele. In families, the member with the earliest age of onset had significantly more ε4 alleles than the family member with the latest age of onset. Therefore, since Blacker et al. [1997] found the highest ε4/ε4 effect in the 60 to 66 age group, Tang et al. [1998] could have missed seeing the effect of ApoE ε4 in their AD cases over 65 years of age.

ApoE genotype has also been found to modify the risk of AD in Down syndrome patients. In a study of 111 Down syndrome adults by Schupf et al. [1998], individuals with an ε3/ε4 or ε4/ε4 genotype were at a significantly higher risk (RR = 4.1, 95% CI = 1.5 - 11.6) for AD than those with an ε3/ε3 genotype. No patient with an ε2 allele developed AD, signifying that this allele may be protective in this population.

Diet may play a role in AD, as dietary fat could contribute to oxidative stress and inflammation. An ecological-neurological study, using regression analyses [Grant, 1997], found that dietary fat and total caloric supply have the highest correlations with

AD prevalence rates. However, another study [Graff-Radford, Lemke, and Wallace, 1990] found that probable AD patients over 71 years of age are significantly thinner than controls. It has been found that increased dietary cholesterol has an inverse effect on secreted brain Aβ1-42 in normal mice, but no effect on ApoE knockout mice [Howland et al., 1998].

Fish consumption has been found to reduce the prevalence of AD [Grant, 1997]. Incidence of cardiovascular disease, autoimmune disorders, and inflammatory disorders are all decreased in fish consuming populations. Fish oil, which is high in omega-3 fatty acids, may lessen inflammation by reducing the response to, and the production of, pro-inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-1 (IL-1) [Calder et al., 1997; McCarty, 1999].

Dietary antioxidants could be especially beneficial for the brain, as it normally has a low level of endogenous antioxidants [Behl, 1997]. The brain has a high metabolic rate and derives its energy from oxidative phosphorylation in the mitochondria, which results in the production of free radicals. The brain contains high concentrations of polyunsaturated fatty acids which are substrates for lipid peroxidation. Also, the brain is rich in metals which may catalyze free radical formation [Behl, 1997]. Dementia is associated with low levels of the antioxidant vitamins C and E, which act as free-radical scavengers. Vitamin E has also been found to protect neurons against Aβ toxicity [Behl, 1992] and slow the progression of AD [Sano et al., 1997]. The ApoE ε3 and ε2 alleles, superior antioxidants to the ε4 allele [Miyata and Smith, 1996], could also help protect the brain from these free radicals and explain the role of the ε4 allele as an AD risk factor.

AD may also be associated with a chronic inflammatory state, as many proteins associated with inflammation (such as complement and cytokines) have been found in the brain lesions of AD. Aβ and peroxides also induce the activity of transcription factor NF-κB, which activates pro-inflammatory genes [Behl, 1997; Kaltschmidt et al., 1997]. Neuronal damage may be caused by inflammation overstimulating the immune system [McGeer and McGeer, 1995], which is supported by the fact that induced brain inflammation in rats causes neurodegeneration and memory loss [Hauss-Wegrzyniak et al., 1998].

Additional evidence for the involvement of inflammatory processes in AD is the evidence that NSAIDs may be effective in preventing the onset or slowing the progression of AD. In one recent study [Mackenzie and Munoz, 1998], the RR for AD was inversely related with the amount of NSAIDs used. Mackenzie and Munoz [1998] examined brain tissue from 40 non-demented individuals, of whom 19 used NSAIDS for arthritis. They found similar levels of NPs and NFTs in the two groups; however, the NSAID group had fewer activated microglia ($P < 0.01$). The authors believe that NSAID use may suppress the inflammation associated with NPs. In another study, aspirin users were also at a decreased risk of AD [Stewart et al., 1997].

Head injury with loss of consciousness has been found to increase the risk of AD [O'Meara et al., 1997; Schofield et al., 1997] and is associated with an earlier age of onset of AD [Schofield et al., 1997; Nemetz et al., 1999]. In a study of professional boxers, individuals with ApoE ε4 alleles had significantly greater chronic brain injury than those without ε4 alleles [Jordan et al., 1997]. Head injuries increase Aβ deposition and expression [Graham et al., 1995], and more neurotoxic NPs could be formed in

individuals with ε4 alleles [Weisgraber and Mahley, 1996]. However, this is disputed by O'Meara et al. [1997], who found ApoE ε4 and head injury to be independent risk factors for AD.

Head injury causes cerebral damage and swelling, which could initiate or accelerate the onset of AD by slowing cerebral blood flow and delaying the transport of oxygen and glucose [de la Torre, 1997]. Alzheimer's disease patients have significantly decreased standing blood pressures when compared to healthy controls [Jhee et al., 1995]. Together, these studies lead to a hypothesis that decreased cerebral blood flow could be a risk factor for AD.

More highly-educated individuals may have a decreased risk of AD [Stern et al., 1994]. A 1997 study by Evans et al. found that fewer years of formal schooling, lower income, and lower occupational status lead to an increased risk of AD. Evans et al. [1997] also found that the AD risk decreased 17% for each year of formal education. However, this may reflect a general association of poor health with lower socioeconomic status. Katzman [1993] states that mental activity early in life may protect individuals against AD and that low education levels may be a risk factor for AD. Another theory by de la Torre [1997] states that complex thinking patterns could protect the brain against AD by increasing cerebral blood flow.

Some studies have shown that smoking has a protective effect against AD [Grossberg et al., 1989; Ferini-Strambi et al., 1990; van Duijn and Hofman, 1991]. This could be secondary to a decrease of the stimulating effect of nicotine in AD patients. Individuals who smoke show a reduction in their nicotinic acetylcholine binding [Perry et al., 1987]. However, other studies have reported no significant association between

smoking and AD [Shalat et al., 1987; Joya, Pardo, and Londono, 1990; Letenneur et al., 1994], and AD cases tended to use tobacco more frequently. Letenneur et al. [1994] believes that the protective effect can be explained by confounding with education and occupation. It is interesting to note that smoking also appears to have a protective effect in Parkinson's disease (PD) [Riggs, 1996], as approximately 15% of PD patients also exhibit AD brain abnormalities and dementia [Hughes et al., 1993].

There are gender differences in AD. Among individuals with AD, women have significantly ($P < 0.01$) more difficultly than men naming objects [Buckwalter et al., 1996]. Payami et al. [1996] reports that women are more likely to develop AD than men based on a study of 26 Oregon families (OR = 3.24, 95% CI = 1.75 - 6.01) and 32 National Cell Repository families (OR = 2.31, 95% CI = 1.32 - 4.06). Gao et al. [1998] shows in a meta-analysis that females have a higher risk of developing AD than males (OR = 1.56, 95% CI = 1.16 - 2.10). These higher risks could be secondary to the fact that women tend to live longer and are more likely to reach the age of onset of AD [Heun, Maier, and Muller, 1996], or secondary to low estrogen levels in post-menopausal women that increase their AD risk [Tang et al., 1996].

Tang et al. [1996] found a decrease in the risk of AD (RR = 0.40, 95% CI = 0.22 - 0.85) in 156 New York post-menopausal women who used synthetic estrogen when compared to 968 non-users. Kawas et al. [1997] also found that a group of Baltimore women who had taken estrogen were protected from AD (RR = 0.45, 95% CI = 0.21 - 0.98). These studies have been further supported by an Italian study [Baldereschi et al., 1998] which found that estrogen use was higher in women without AD (OR = 0.24, 95% CI = 0.07 - 0.77) than women with AD. This protection could be due to the fact that

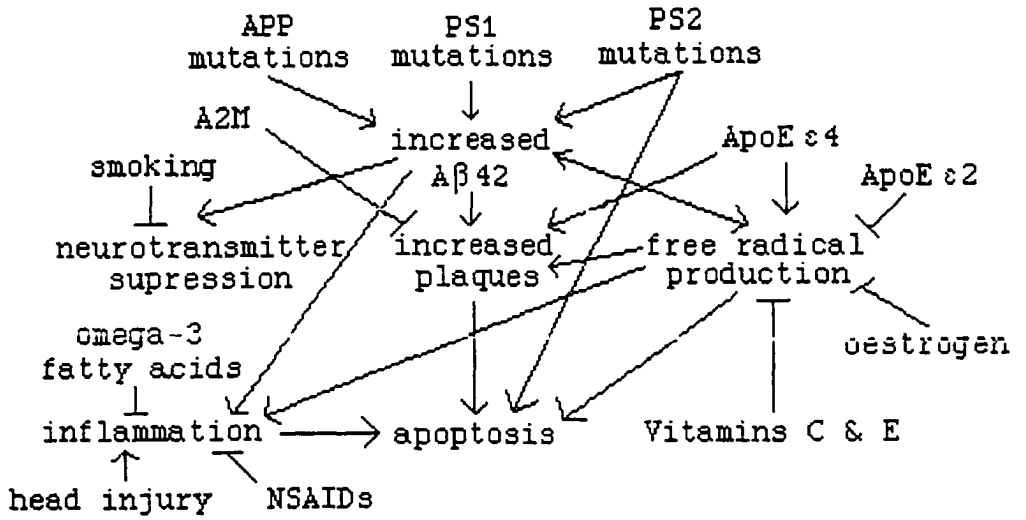


Fig. 4. Genetic and epidemiological factor involvement in AD neurodegeneration.

synthetic estrogen can act as an antioxidant [Sack, Rader, and Canon, 1994], or to the fact that women who take estrogen are healthier and better educated, confounding the results [Yaffe et al., 1998].

In summary, many of these AD risk factors are related to free radical formation or inflammation. Free radicals are hypothesized to cause the cell damage that leads to dementia. They are formed through the normal aging process with the accumulation of Aβ protein, and its interaction with ApoE ε4 alleles. Antioxidants such as Vitamin C, Vitamin E, estrogen, and the ApoE ε2 allele may protect the brain from these free radicals. Head injury may increase inflammation, damaging neurons, while NSAIDs and omega-3 fatty acids may decrease inflammation. See Figure 4 for a visual representation of how these factors interact during AD pathogenesis.

Segregation Analyses

Segregation analysis consists of finding a model to explain the inheritance pattern of genotypes and phenotypes in a pedigree. It is more difficult to find a model to explain the complicated inheritance pattern of complex traits than to explain simple Mendelian traits. According to Lander and Schork [1994], complex traits do not exhibit classic recessive or dominant inheritance that one can attribute to a single gene locus. These diseases can be caused by multiple genes, environmental causes, or a combination of both.

In 1991, Farrer et al. performed a segregation analysis on 232 nuclear families with AD. Their results showed that AD susceptibility is an autosomal dominant trait with an additional multifactorial component. Also, they suggested that there could be phenocopies or more than one major AD gene. In 1994, Rao et al. used logistic regression in the segregation analysis of 401 nuclear AD families. They found that splitting the families into early and late-onset groups fit the model better than a single group. They determined that early-onset families with AD transmit the gene as a fully penetrant autosomal dominant, and late-onset families show possible heterogeneous autosomal dominant transmission. They determined that phenocopies were present and that there was a higher AD risk for females [Rao et al., 1994].

Association Testing

Association tests are often used in studies to determine if a candidate gene is related to a disease. They are easy to perform and generally require a smaller sample size than linkage analysis [Risch and Merikangas, 1996]. One of the most well-known

designs for association testing is the case-control study. The other main study design used for genetic association testing is called family-based association testing.

In case-control studies, cases are selected because they have a disease. Controls are unrelated individuals without the disease who are selected from the same population. These individuals are then examined to see if there is a difference between exposures or alleles of candidate gene markers between the two groups. The statistic produced is the odds ratio, which is a measure of the strength of the association, and indicates whether cases with the disease are more or less likely to have the exposure. Exposures that have an odds ratio with a 95% confidence interval greater than one are considered risk factors, and those with a 95% confidence interval less than one are protective factors [Khoury, Beaty, and Cohen, 1993].

Advantages of a case-control study are that it is inexpensive, quickly performed, and multiple risk factors can be examined. Disadvantages are that it is not suitable for rare diseases, bias may exist due to stratification in the population, and risk factors may be subject to recall bias by the cases [Khoury, Beaty, and Cohen, 1993]. Case-control studies can be a suitable approach for AD genetic studies as genetic markers are not subject to recall bias in elderly patients. However, as AD is a common disease, false-positives could occur due to population stratification, for example,if a subset of the population has a higher prevalence of the disease and the marker allele, this could result in an association even if the two are not associated and are unlinked [Curtis, 1997].

Family-based association studies test for associations between markers and diseases while avoiding the false-positives (due to population stratification) that may be present in case-control studies. The most commonly used forms of family-based

association testing are the haplotype relative risk (HRR) [Falk and Rubinstein, 1987], the affected family-based controls test [Thompson, 1989], and the transmission/ disequilibrium test (TDT) [Spielman, McGinnis and Ewens, 1993]. Newer tests based on TDT, such as Curtis' test [Curtis, 1997], the discordant alleles test [Boehnke and Langefeld, 1998], and the sibship disequilibrium test [Hovarth and Laird, 1998], have also been introduced recently.

The HRR, which is equivalent to an odds ratio [Thompson, 1995], was introduced in 1987 by Falk and Rubinstein. It assumes that alleles or haplotypes transmitted from a parent to affected offspring carry a disease allele, and parental alleles or haplotypes not transmitted to the affected offspring are representative of disease and normal alleles frequencies in the population at large [Falk and Rubinstein, 1987]. Therefore, bias due to sampling and population stratification are eliminated [Thompson, 1995].

The affected family-based controls test is an extention of the HRR for use in nuclear families with at least two affected siblings [Thompson et al., 1989]. Alleles from both parents that are never transmitted to affected children are used to calculate the frequencies in the "control" population, which is then compared to the frequencies of the alleles that are transmitted from the parents to the affected children. Affected parents are excluded, and an HRR is calculated from the case and control allelic information [Thompson, 1995].

A common method of family-based association testing is the TDT, which can be used to detect linkage even in the presence of linkage disequilibrium [Spielman, McGinnis and Ewens, 1993; Clerget-Darpoux, Babron, and Bickeboller, 1995]. The TDT evaluates the frequency at which an allele is transmitted from heterozygous parents

to affected offspring. The statistic produced is equivalent to a χ^2 under a null hypothesis. It does not require typing of multiple affected family members or unaffected siblings. However, it does require the typing of parents, which may not be suitable for a late-onset disease such as AD. It has also been found to be inefficient for recessive diseases and common alleles [Schaid, 1999].

The TDT has been recently modified by Spielman and Ewens [1998] for use in cases in which parental data is unavailable to make it more suitable for use in late-onset diseases. In these cases a test called the sib TDT (S-TDT) uses marker data from unaffected siblings instead of parents. The number of times affected siblings have a specific allele is added to the number of transmissions of this allele from parents to affecteds. The mean is subtracted from this number and then it is divided by the square root of the variance. This produces a Z statistic, which can then be evaluated using Z tables. They also propose using the S-TDT in conjunction with the TDT to test for linkage in presense of association, using the χ^2 test, but this can only be done in families where the parents are typed. Knapp [1999] proposes a modified S-TDT called the RC-TDT. The RC-TDT corrects for bias that may result from the reconstruction of the parental haplotypes during the S-TDT and improves its power.

Curtis [1997] proposes a test using unaffected siblings as controls, which chooses the most genotypically genetically different sibling as the control for the single case chosen from each sibship. This would allow matching with siblings for environmental and other genetic factors, which may not be possible with unrelated controls. If unaffected siblings are genetically the same as affecteds, then that sibship is dropped from the analysis. Counts of the time each allele appears in a case compared to a control

are then evaluated using a χ^2 under the null hypothesis. This test, implemented in the SIBASSOC program, should produce positive results only if the marker is associated with and linked to the disease locus.

Boehnke and Langefeld [1998] recently developed and evaluated many tests of association that used discordant sib-pairs, in which one sibling is affected with the disease and one is not. The most powerful test developed is called the discordant-alleles test. This test compares the number of non-matching alleles between discordant siblings. They feel that it retains this power regardless of the disease model or type of marker.

Another method that uses discordant siblings is the sibship disequilibrium test (SDT), which is a family-based association test that does not require parental genotypes [Hovarth and Laird, 1998]. It compares the average number of common alleles in affected versus unaffected siblings and can be used as both a test for linkage and linkage disequilibrium. It requires discordant sibships with one affected and at least one unaffected sibling, and can use all of the siblings in a sibship.

The SDT calculates a *d* for each sibship, which is the difference of proportion of a specific allele in the affected, and the proportion of the same allele in the unaffected siblings. The number of sibships in which *d* is greater than 0 is subtracted from the number of sibships where it is less than 0. This is squared and divided by the total sibships. This produces the SDT statisic *T* which is a sign test equivalent to a χ^2 under the null hypothesis from which exact *P*-values can be computed. This test can also be extended for situations with multiple alleles. Hovarth and Laird [1998] show that this test has good power when compared to the TDT and Curtis' test, and remains valid even if affection status is misclassified.

Linkage Analyses

Genetic mapping compares the familial transmission of a disease with the segregation of chromosomal regions to locate genes responsible for diseases. These genes are then found through positional cloning, which is the isolation of genes by chromosomal location instead of biochemical function [Lander and Schork, 1994]. This is relatively straight-forward for Mendelian traits, but becomes more complicated when dealing with complex traits such as AD. Large, multi-generational pedigrees are often used to find genes that may cause rare or heterogeneous diseases, as only one genetic cause is usually present within a family. However, it is difficult to identify and genotype multiple generational pedigrees transmitting late-onset diseases, as the older generations are deceased, and the younger individuals have not yet reached the age of onset of the disease.

Two methods are currently used in the analysis of complex traits: parametric and nonparametric linkage analysis. Parametric linkage analysis classically assumes a dominant, codominant, or recessive model, and is more powerful if the mode of inheritance of the disease is known. It also allows for estimation of genetic parameters such as the recombination fraction, penetrance, dominance [Greenberg et al., 1996], and heterogeneity [Terwilliger and Ott, 1994].

Parametric linkage analysis programs such as LINKAGE [Lathrop et al., 1984], and a faster version of LINKAGE called FASTLINK [Cottingham et al., 1993], estimate the recombination fraction (θ) and give a measure of the linkage strength (lod score). Parameters for the mode of inheritance are assumed to be known and are input into these programs; for example, gene frequency, penetrance, and degree of dominance. Linkage

between single markers and a putative disease locus are calculated using two-point lod scores. The HOMOG program can then be used to evaluate the lod scores at specific thetas produced by the LINKAGE or FASTLINK programs to determine if there is locus heterogeneity present in the pedigrees analyzed [Terwilliger and Ott, 1994].

Theta, the probability of a recombination event occurring between two loci, can be used to estimate the genetic distance between each marker and between a marker and disease locus. Since females tend to have higher recombination rates during gametogenesis than males, the effect of this on map distances can be determined by noting if there is a significant difference between varying, constant, or no sex difference models [Terwilliger and Ott, 1994].

Recombination is less common when loci are physically close, and becomes more frequent as marker distances increase. Markers that indicate greater than or equal to 50% recombination on a chromosome independently assort into gametes, just as they would if the markers were on different chromosomes. Linked loci have θs ranging from 0.0 to less than 0.5, and unlinked loci have a θ of 0.5. θ can then be converted into a map distance using the Haldane or Kosambi map functions [Terwilliger and Ott, 1994].

The lod score is defined as the logarithm of the likelihood of the pedigree information at a specific θ divided by the likelihood of the data under the assumption of no linkage. Therefore, a lod score of 3 means that the likelihood at a specific θ is 1000 times more likely than if the markers were unlinked [Terwilliger and Ott, 1994]. A lod score of three or above corresponds to a *P*-value of 0.05 or less [Risch, 1992] and is considered to be significant. A lod score of -2 is generally used to exclude the disease

gene from that region of the chromosome. Lod scores between -2 and 3 are inconclusive [Terwilliger and Ott, 1994].

Multipoint analysis evaluates more than one marker at a time against a putative disease locus and is used to extract all available information from a genetic map. Once the markers are in the correct order, lod scores are calculated for the disease locus at specific intervals between the markers. These features are available in the FASTLINK and LINKAGE programs; however, it is a time consuming process, as the programs must be directed to do each comparison separately [Terwilliger and Ott, 1994].

How might misspecified parameters and heterogeneity affect LINKAGE and FASTLINK results? If gene frequencies, penetrance, or degree of dominance are misspecified, they can overestimate the recombination fraction. The lod score is not affected at all by misspecified gene frequencies, is slightly affected by penetrance, and is definitely affected by the misspecification of degree of dominance [Clerget-Darpoux, Bonaiti-Pellie, and Hochez, 1986].

What if there is heterogeneity in the pedigrees? In 1992, Vieland, Hodge and Greenberg simulated pedigrees in which two disease loci were causing a disease. They found similar lod scores but inflated recombination fractions when analyzing the data under a dominant single gene model with reduced penetrance and the correct two gene model. They concluded that a single locus model is a good approximation, even when there may be more than one disease gene involved. Therefore, misspecified parameters and genetic models tend to affect the recombination fraction more strongly than lod scores.

The other major class of linkage analyses is nonparametric (model-free) linkage analyses, which do not specify a mode of inheritance. These methods are often used for more complex and late-onset diseases. Siblings or relative pairs from many different families are used to examine if the relatives inherit certain chromosomal regions more often than expected by chance. According to Lander and Schork [1994], this is a robust method, as there should be excess allele sharing even with incomplete penetrance, phenocopies, genetic heterogeneity, and high frequency disease alleles. However, they do stress that it is "often less powerful than a correctly specified (parametric) linkage model."

Two main methods of nonparametric linkage analysis are the affected sib pair (ASP) and affected pedigree member (APM) methods. Full siblings are supposed to share one allele identical by descent (IBD or from a common ancestral source) 50% of the time, no alleles IBD 25% of the time, and two alleles IBD 25% of the time. ASP programs test for excess allele sharing at a specific marker across many families analyzed. Excess allele sharing will occur when the marker is close to the disease gene, even if the underlying genetic model is dominant or recessive; however, the power is greater for a recessive disease [Terwilliger and Ott, 1994]. Affected sib pairs are useful in situations where there is a small λ_S (the risk ratio of siblings compared to population prevalence) under a single locus model or a multiple locus model with interaction. Other relative pairs may be more useful with a multilocus model without interaction [Risch, 1990].

The SIBPAL [S.A.G.E. 1997] program is often used for nonparametric linkage analysis of sib-pair families. To detect linkage in qualitative traits, it estimates the

proportion of alleles IBD (mean IBD) and tests whether it is equal to one half. For quantitative traits, it regresses the sib-pair trait squared differences and the proportion of alleles IBD. It has good power in many situations and is considered a "very reliable, versatile choice" by Davis and Weeks [1997]. However, information is lost from other individuals in the pedigree besides siblings [Kruglyak et al., 1996]. Currently, the SIBPAL program only does two-point analyses, but there is a multipoint version of the program being tested.

Affected pedigree member methods examine whether the relative pair has alleles identical by state (IBS), which defines alleles of identical sequence that are not necessarily from the same founder. This is used because of the difficulty in determining IBD in more distantly related pairs. However, IBS methods are generally less powerful than IBD [Krugylak et al., 1996], as the alleles may not be from the same ancestor. It is recommended to use this method only when no mode of inheritance is clear from the pedigree information [Bishop and Williamson, 1990].

A test developed by Weeks and Lange [1988] uses a Z statistic to test for excess allele sharing in APMs, as those individuals will show excess sharing if the marker locus is linked to the disease locus. The power of this test depends on marker polymorphism, probability of IBD, and the recombination fraction between the trait and marker loci [Bishop and Williamson, 1990]. It is less powerful than lod score methods, but no assumptions are made of the mode of inheritance [Weeks and Lange, 1988]. The RELPAL [S.A.G.E., 1997] program can be used for nonparametric linkage analysis of quantitative traits in affected relatives pairs.

The GENEHUNTER [Kruglyak et al., 1996] program performs multipoint parametric analyses based upon the LINKAGE program, as well as multipoint nonparametric linkage (NPL) analyses. This program allows the computation of multipoint lod scores using many polymorphic markers, even when data is missing [Kong and Cox, 1997], and is much easier to use for multipoint analyses than LINKAGE or FASTLINK. It performs interval mapping, which calculate scores at specific distances between markers. GENEHUNTER also helps to determine the intervals that need more markers typed [Kruglyak et al., 1996].

The GENEHUNTER NPL statistic primarily uses information from family members other than affecteds to determine parental and offspring haplotypes and to determine if alleles are IBD rather than IBS. It is the first nonparametric analysis program to add multipoint features and to use all avaliable familial information. The NPL statistic performed as well as parametric methods of analysis, and was found to be more powerful than nonparametric methods such as APM for dominant models and complex traits [Kruglyak et al., 1996; McPeek, 1999]. However, this statistic has been found to be a more conservative test for linkage than SIBPAL, especially when parents are untyped [Davis and Weeks, 1997; Kong and Cox, 1997]. A correction for the conservative nature of this test has been adapted into the GENEHUNTER-PLUS program [Kong and Cox, 1997].

Genome Screening

In 1992, the first major study using genome screening to find genes involved in a complex trait (type 1 diabetes) was published [Davies et al., 1994]. Since then, it has

become a popular technique to dissect complex traits [Rao et al., 1998]. Genome screening is used to scan each chromosome for possible disease or susceptibility genes linked to a grid of genetic markers, and can also be used to exclude chromosomal regions from consideration [Hauser et al., 1996]. The power to detect linkage depends on pedigree structures, the number of affected individuals, and the informativeness of the markers.

During a genomic screen, one or more families with the disease of interest are genotyped with highly polymorphic markers that are evenly spaced in the genome. To increase efficiency and decrease costs, a course grid of markers can be followed up with a finer grid, or the sample can be split to save half of the families for follow up analyses [Elston, Guo, and Williams, 1996; Holmans and Craddock, 1997].

However, when using a *P*-value of 0.05, 1 in 20 markers screened will be positive. When using a screen of 300 markers, this could lead to 15 potential false-positive markers. How can we avoid following up false-positives? A lod score of 2.0 and a *P*-value of 0.001 are the suggested values that signify suggestive linkage in a genomic scan with sib-pairs [Lander and Kruglyak, 1995]. However, Goldin and Chase [1997] developed less stringent criteria by taking into consideration the length of the peak. They found that if 3 of 5 markers in a region have *P*-values of less than 0.01, the power to detect disease genes is similar to Lander and Kruglyak's criteria for sib-pairs. This finding was further supported by Terwilliger et al. [1997].

Hauser et al. [1996] recommends an affected sib-pair screen (with markers spaced at 10-20 cM intervals) using a mode of inheritance free ASP linkage method proposed by Risch in a 1993 presentation. This method is an intermediate method similar to non-

parametric analysis, but one that allows for linkage mapping and exclusion. They recommend investigating further all regions with lod scores greater than 1 and excluding regions with lod scores less than -2. Brown, Gorin, and Weeks [1994] recommend that genomic screens using APMs should start with a 20 cM grid, and further investigation of those markers with APM statistics above 1.67, then 2.00, and lastly 2.26.

Rao [1998] has three recommendations for genomic scans: first, only tolerate one false-positive per scan by using a significance level or α of 0.001; second, calculate sample size based on an α of 0.01 and a β of 0.90; and third, use a significance level of $\alpha = 0.000022$ [as proposed by Lander and Kruglyak, 1995] when pooling multiple scans. It is also important to insure that the affection status of each individual is classified correctly and that quality control is high in the laboratory and database.

Rationale

In 1990, the University of Alabama at Birmingham (UAB) was funded under the NIMH Genetics Initiative, in collaboration with Johns Hopkins University and Massachusetts General Hospital, to collect families of relative pairs with AD (related no further than first cousins). To date, over 470 families have been identified and collected using NINCDS-ADRDA criteria [Tierney et al., 1988]. Blood specimens from these family members were sent to Coriell Institute (Camden, New Jersey), where cell lines were established to provide DNA for current and future studies. The Institutional Review Boards from each site approved the human subject research (see Appendix).

In 1994, the sites were funded to begin genotyping affected and unaffected family members using highly polymorphic microsatellite markers (average heterozygousity $\geq$

0.75) spaced approximately 10 cM apart (Weber set, version 5.0). UAB has currently completed a genome screen of chromosomes 1 and 6 in 266 sib-pair families with 31 and 25 markers, respectively. One hundred forty-five of these 266 families have an age of onset of greater than 70 in at least two siblings. Eighty-four of these families have at least 1 individual who is homozygous for the ApoE ε4 allele.

Using these 266 sib-pair families, markers on chromosomes 1 and 6 have been shown to be associated with AD [Collins et al., 1997a; Collins et al., 1997b; Go et al., 1998]. The original 10cM screen was also followed up by typing dinucleotide and tetranucleotide repeat markers flanking the positive regions at 6p21 and 1p36. Boehnke [1994] has shown that for a study of our size, at an intial grid of 10 cM distances, the minimum distance of the flanking markers should be 0.5 cM. These nonparametric and parametric linkage analyses findings are summarized in Table 1, Figure 5, Table 2, and Figure 6, respectively.

As shown in Table 1 and Figure 5, a region that may harbor an AD gene is the HLA region on chromosome 6p21.3 [Go et al., 1998]. The subset of 84 AD families that contain ApoE ε4 homozygotes and our overall set of 266 AD families are associated with this region. Further evidence for an AD gene in this region was found by Pericak-Vance et al. [1997] who reported that they are following up preliminary findings of linkage to markers on chromosome 6. One marker that they are interested in is D6S1019, which maps close to our marker 9N3 at 39.1 cM.

Additional evidence is given by several reports on associations with HLA-A2. In 1984, Renvoize et al. reported a weak association between AD and the A2 allele of the HLA-A locus, which was confirmed by Payami et al. in 1991. In 1997, Payami et al.

Table 1. Chromosome 6 Single-Point Results

		Stratum	SIBPAL		GENEHUNTER		FASTLINK	
Marker	Distance	(# fams)	Mean	*P*-Value	NPL score	*P*-Value	LOD score	θ
F13A1	2.8 cM	ALL (266)	0.52	0.22	-0.006	0.501	0.00	0.45
F13A1	2.8 cM	ε4/ε4 (84)	0.51	0.30	0.600	0.273	0.02	0.30
D6S263	3.1 cM	ALL (266)	0.53	0.10	-0.024	0.508	-0.02	0.45
D6S263	3.1 cM	ε4/ε4 (84)	0.48	1.00	-0.162	0.563	-0.02	0.45
D6S259	~14 cM	ALL (266)	0.51	0.29	-0.125	0.549	0.01	0.45
D6S259	~14 cM	ε4/ε4 (84)	0.52	0.19	1.028	0.152	0.23	0.20
D6S285	18.8 cM	ALL (266)	0.53	0.11	-0.493	0.688	-0.02	0.45
D6S285	18.8 cM	ε4/ε4 (84)	0.50	0.47	0.101	0.459	0.00	0.35
D6S105	31.9 cM	ALL (266)	0.50	0.52	-0.221	0.586	-0.02	0.45
D6S105	31.9 cM	ε4/ε4 (84)	0.53	0.09	1.029	0.152	0.23	0.20
D6S1051	35.2 cM	ALL (266)	0.52	0.12	0.745	0.226	0.16	0.30
D6S1051	35.2 cM	ε4/ε4 (84)	0.53	0.09	0.877	0.190	0.57	0.10
[a]MIB	37.7 cM	ALL (266)	0.52	0.06	0.873	0.191	0.05	0.35
[a]MIB	37.7 cM	ε4/ε4 (84)	0.55	0.02	1.392	0.082	0.50	0.15
[b]TNFa	37.8 cM	ALL (266)	0.53	0.01	1.146	0.126	0.13	0.30
[b]TNFa	37.8 cM	ε4/ε4 (84)	0.57	0.005	1.571	0.059	0.51	0.15
[a]9N3	38.0 cM	ALL (266)	0.52	0.06	0.655	0.255	0.06	0.35
[b]9N3	38.0 cM	ε4/ε4 (84)	0.57	0.003	1.665	0.049	0.87	0.10
[a]D6S1017	42.3 cM	ALL (266)	0.52	0.06	0.797	0.212	0.14	0.30
[b]D6S1017	42.3 cM	ε4/ε4 (84)	0.56	0.003	1.386	0.083	0.62	0.10
D6S271	42.9 cM	ALL (266)	0.51	0.34	0.082	0.466	0.01	0.40
D6S271	42.9 cM	ε4/ε4 (84)	0.54	0.06	1.491	0.068	0.31	0.20
D6S1280	44.8 cM	ALL (266)	0.52	0.10	0.934	0.17	0.03	0.35
D6S1280	44.8 cM	ε4/ε4 (84)	0.54	0.04	1.089	0.14	0.12	0.25
D6S254	68.4 cM	ALL (266)	0.55	0.01	0.408	0.34	0.00	0.45
D6S254	68.4 cM	ε4/ε4 (84)	0.49	1.00	-0.219	0.59	-0.01	0.45
D6S251	86.2 cM	ALL (266)	0.49	1.00	-1.511	0.93	-0.04	0.45
D6S251	86.2 cM	ε4/ε4 (84)	0.46	1.00	-1.342	0.91	-0.03	0.45
D6S300	107.2 cM	ALL (266)	0.50	0.46	-0.099	0.54	0.00	0.40
D6S300	107.2 cM	ε4/ε4 (84)	0.49	1.00	-0.134	0.55	0.00	0.40
D6S1021	119.7 cM	ALL (266)	0.53	0.09	0.586	0.28	0.80	0.20
D6S1021	119.7 cM	ε4/ε4 (84)	0.54	0.04	1.254	0.11	1.01	0.05
D6S474	123.8 cM	ALL (266)	0.55	0.01	1.029	0.15	0.46	0.25
D6S474	123.8 cM	ε4/ε4 (84)	0.51	0.38	0.301	0.38	0.00	0.45
D6S1040	128.2 cM	ALL (266)	0.53	0.08	-0.274	0.61	-0.02	0.45
D6S1040	128.2 cM	ε4/ε4 (84)	0.48	1.00	-0.391	0.65	-0.01	0.45
D6S1003	145.3 cM	ALL (266)	0.51	0.39	0.003	0.50	0.00	0.45
D6S1003	145.3 cM	ε4/ε4 (84)	0.51	0.39	-0.076	0.53	0.28	0.20
D6S1007	165.3 cM	ALL (266)	0.49	1.00	-1.084	0.86	-0.03	0.45
D6S1007	165.3 cM	ε4/ε4 (84)	0.50	0.46	0.037	0.48	-0.01	0.45
D6S305	170.3 cM	ALL (266)	0.51	0.29	-0.320	0.62	0.18	0.30
D6S305	170.3 cM	ε4/ε4 (84)	0.55	0.02	1.613	0.05	0.83	0.10
D6S264	179.6 cM	ALL (266)	0.54	0.05	0.428	0.33	0.23	0.25
D6S264	179.6 cM	ε4/ε4 (84)	0.53	0.10	0.988	0.16	0.55	0.15
D6S503	179.7 cM	ALL (266)	0.51	0.33	-0.475	0.68	-0.01	0.45
D6S503	179.7 cM	ε4/ε4 (84)	0.51	0.34	0.399	0.34	0.00	0.45
D6S1027	179.8 cM	ALL (266)	0.54	0.07	0.412	0.34	0.02	0.40
D6S1027	179.8 cM	ε4/ε4 (84)	0.54	0.02	1.580	0.06	0.43	0.10

[a]flanking marker

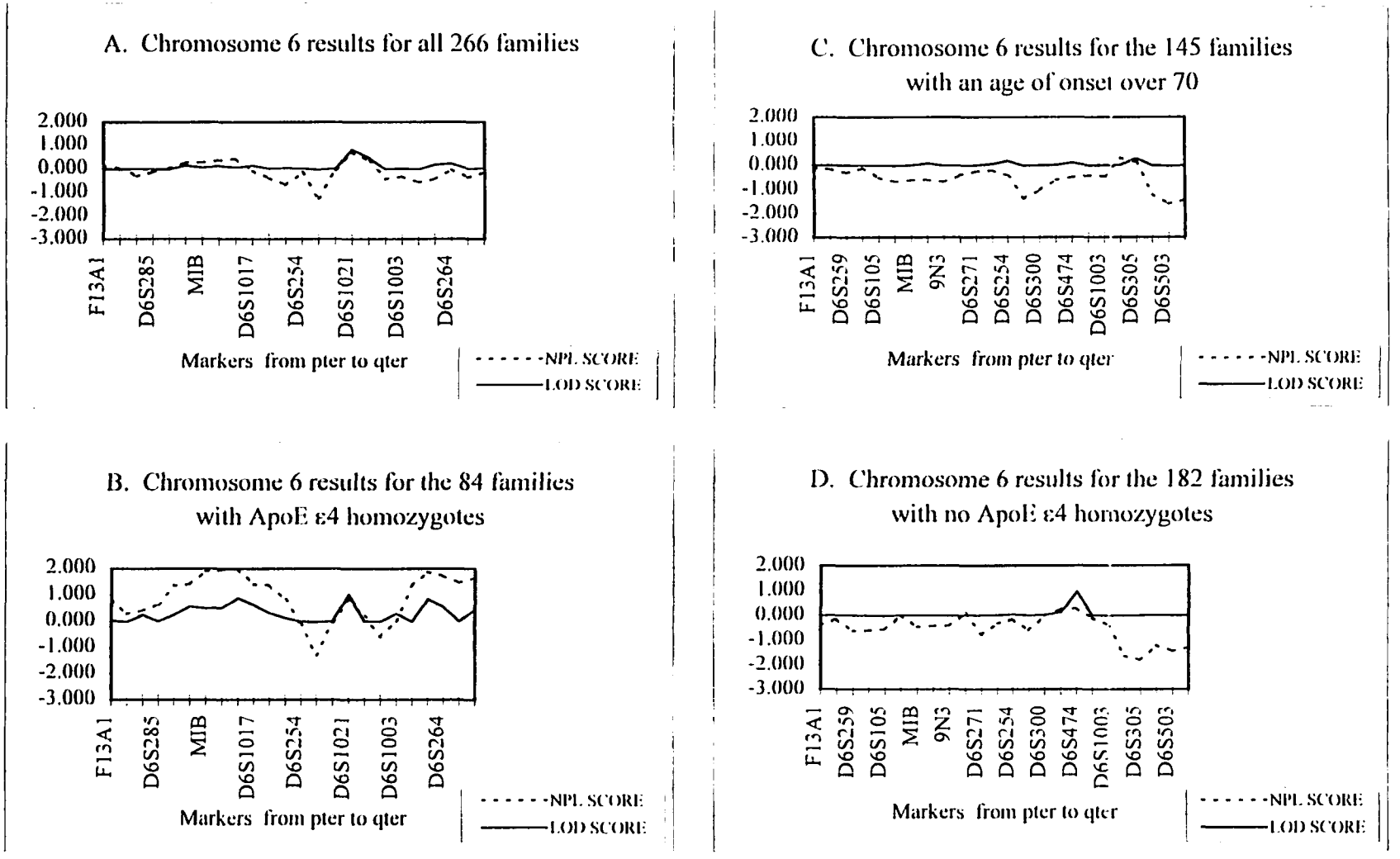


Fig. 5. Chromosome 6 multipoint results with 6p21 flanking markers in the total subset (A), 84 families with ApoE ε4 homozygotes (B), 145 families with an age of onset over 70 (C), and 182 families with no ApoE ε4 homozygotes (D).

Table 2. Chromosome 1 Single-Point Results

		Stratum	SIBPAL		GENEHUNTER		FASTLINK	
Marker	Distance	(# fams)	Mean	*P*-Value	NPL score	*P*-Value	LOD score	θ
D1S468	~0 cM	ALL (266)	0.52	0.19	-0.199	0.58	-0.01	0.45
[a]D1S548	4.2 cM	ALL (266)	0.51	0.21	0.162	0.43	0.00	0.45
[a]D1S548	4.2 cM	AO > 70 (145)	0.53	0.04	1.015	0.15	0.14	0.25
D1S1646	4.4 cM	ALL (266)	0.51	0.17	0.518	0.30	0.06	0.30
D1S1646	4.4 cM	AO > 70 (145)	0.53	0.07	0.971	0.17	0.15	0.25
D1S1597	9.1 cM	ALL (266)	0.52	0.03	1.036	0.15	0.31	0.25
D1S1597	9.1 cM	AO > 70 (145)	0.53	0.04	1.184	0.12	0.19	0.20
D1S1368	~15 cM	ALL (266)	0.52	0.14	1.022	0.15	0.06	0.35
D1S1368	~15 cM	AO > 70 (145)	0.54	0.04	1.429	0.08	0.23	0.20
[a]D1S1592	16.8 cM	ALL (266)	0.51	0.16	0.840	0.20	0.49	0.20
[a]D1S1592	16.8 cM	AO > 70 (145)	0.52	0.12	0.940	0.17	0.35	0.20
D1S552	22.6 cM	ALL (266)	0.50	0.40	0.406	0.34	0.13	0.30
D1S552	22.6 cM	AO > 70 (145)	0.52	0.16	1.227	0.11	0.14	0.25
D1S233	37.7 cM	ALL (266)	0.49	1.00	-0.480	0.68	0.08	0.35
D1S186	37.7 cM	ALL (266)	0.47	1.00	-0.640	0.74	0.28	0.25
MYCL1	46.0 cM	ALL (266)	0.49	1.00	-0.509	0.69	0.44	0.25
D1S162	59.7 cM	ALL (266)	0.49	1.00	-0.140	0.55	0.46	0.25
D1S550	92.2 cM	ALL (266)	0.51	0.35	-1.034	0.85	-0.04	0.45
D1S207	97.6 cM	ALL (266)	0.52	0.23	-1.346	0.91	-0.04	0.45
D1S236	105.0 cM	ALL (266)	0.52	0.16	0.267	0.39	0.00	0.45
D1S1623	107.2 cM	ALL (266)	0.53	0.10	-0.354	0.64	-0.02	0.45
D1S534	125.0 cM	ALL (266)	0.51	0.33	0.043	0.48	0.11	0.30
D1S1595	139.9 cM	ALL (266)	0.53	0.06	0.827	0.20	0.15	0.30
D1S1167	~160 cM	ALL (266)	0.53	0.15	0.910	0.18	0.45	0.25
D1S318	185.2 cM	ALL (266)	0.48	1.00	-1.310	0.90	-0.01	0.45
D1S1589	189.1 cM	ALL (266)	0.54	0.03	0.786	0.22	0.51	0.25
D1S518	194.8 cM	ALL (266)	0.49	1.00	-0.102	0.54	0.00	0.40
D1S1660	204.6 cM	ALL (266)	0.48	1.00	-0.188	0.57	0.02	0.35
D1S249	216.6 cM	ALL (266)	0.50	1.00	0.553	0.29	0.02	0.40
D1S414	230.0 cM	ALL (266)	0.51	0.42	0.498	0.31	0.01	0.35
D1S439	243.2 cM	ALL (266)	0.53	0.13	-0.115	0.54	0.49	0.25
D1S479	243.5 cM	ALL (266)	0.55	0.03	0.748	0.22	0.93	0.15
D1S549	239.5 cM	ALL (266)	0.50	1.00	-0.253	0.60	0.16	0.30
D1S1656	245.0 cM	ALL (266)	0.50	0.49	-1.121	0.87	0.08	0.35
D1S179	250.5 cM	ALL (266)	0.49	1.00	-2.090	0.98	-0.01	0.45
D1S180	260.6 cM	ALL (266)	0.51	0.41	-0.715	0.76	-0.01	0.45
D1S102	261.7 cM	ALL (266)	0.53	0.06	-0.161	0.56	0.07	0.30

[a]flanking marker

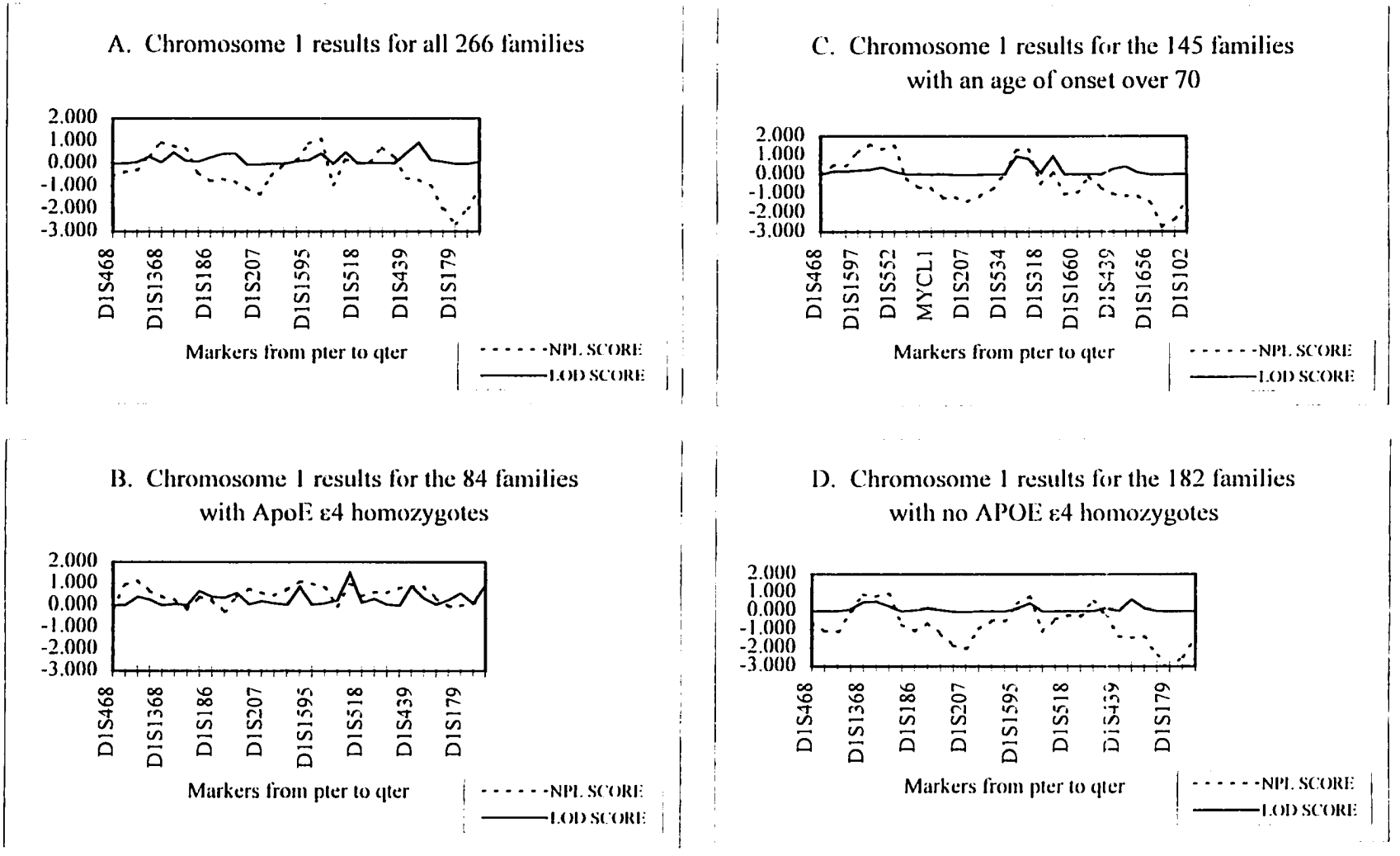


Fig. 6. Chromosome 1 multipoint results in the total subset (A), 84 families with ApoE ε4 homozygotes (B), 145 families with an age of onset over 70 (C), and 182 families with no ApoE ε4 homozygotes (D).

followed this up by reporting that the HLA-A2 allele was associated with a reduced mean age of onset for AD, with a possible additive effect by the ApoE ε4 allele. This was confirmed by Combarros et al. [1998] and Ballerini et al. [1999], but has been disputed by Small et al. [1999].

Besides genes at the HLA-loci, another candidate gene in this 5 cM region at 6p21.3 is TNF (a.k.a. TNFα), which is located at 37.8 cM on our map of markers. TNF produces a pro-inflammatory cytokine that helps initiate and regulate cytokine production [Calder, 1997]. It can affect AD pathology because TNF increases the production of Aβ and inhibits the secretion of amyloid precursor protein [Blasko et al., 1999]. However, conflicting results regarding the levels of TNF in AD patients have been reported [Tarkowski et al., 1999; Lanzrein et al., 1998; Lombardi et al., 1999; Bruunsgaard et al., 1999; Alvarez et al., 1996].

TNF is secreted by macrophages and targets tumor and inflammatory cells. TNF initiates bacterial and tumor cell killing, stimulates T-cells and B-cells, and regulates the production of other pro-inflammatory cytokines [Calder, 1997]. TNF deficient mice and heterozygotes developed by Marino et al. [1997] are very susceptible to infectious agents and have a disorganized inflammatory response, which leads the authors to conclude that TNF has an anti-inflammatory function. Bruce et al. [1996] also postulates that TNF may stimulate antioxidant pathways. TNF's involvement in inflammation, antioxidant pathways, and its effect on Aβ make it an appropriate AD candidate gene.

Polymorphisms in the promoter region and flanking the TNF gene have been shown to either increase its transcription or its secretion. The TNF–308 promoter polymorphism TNF2 (G→A) allele has been shown to have an increased frequency in

autoimmune and inflammatory diseases [Wilson et al., 1995] and is associated with stronger transcriptional activation than the TNF1 allele [Wilson et al., 1997]. The TNF microsatellite TNFa 2 allele (99 basepairs) has been previously associated with higher TNF secretion [Poicot et al., 1993] and susceptibility to rheumatoid arthritis [Mulcahy et al., 1996, Field et al., 1997].

Another candidate region can be found when examining Table 2 and Figure 6, as it becomes apparent that the short arm of chromosome 1 may be involved in late-onset AD [Collins et al., 1997a; Collins et al., 1998]. According to Terwilliger et al. [1997], true positive peaks are longer (broader) than false-positive peaks. Because there is a broad region at 1p36 associated with AD in all subsets of families, this region is suspected to harbor an AD gene or modifier.

One interesting gene at 1p36.2 is tumor necrosis factor receptor 2 (TNFR2), which is expressed on lymphocytes, and is located at 8.1 cM on our map of markers. TNFR2 participates in B-cell activation, is expressed on T-lymphocytes, and is involved in T-Cell apoptosis [Al-Ansari et al., 2000]. Demented patients have more of these receptors than controls, which could indicate an immune response has been initiated [Bongioanni et al., 1997].

Bruce et al. [1996] knocked-out TNFR2 and TNFR1 in a mouse model. These mice showed no adverse changes under normal conditions, but did have some impaired inflammatory responses. Moreover, these knockout mice showed greater neuronal damage from brain injury than normal mice under similar conditions. Oxidative stress was increased and antioxidant enzyme levels were decreased in these mice. Therefore, a

lack of, or mutation in, TNFR2 could cause lower antioxidant levels that lead to more oxidative stress and neuronal damage.

TNFR2 is a 75/80 kDa receptor with 10 exons and 9 introns. Exon 6 codes a portion of the transmembrane region and is a cleavage site for soluble TNFR2. There is an exon 6 676 T→G polymorphism that leads to a position 196 M→R substitution. This polymorphism has been associated with SLE in Japanese populations [Komata et al., 1999], but is not associated with SLE in Caucasian (UK) or Spanish populations [Al-Ansari et al., 2000].

From preliminary studies at UAB during the NIMH Genetics Initiative, two candidate regions have been chosen for further study of late-onset AD. Polymorphisms at chromosome 6p21 in the TNF gene will be studied to determine if they are associated with AD, as this gene is located in the putative AD region from our analyses, and the TNF gene could be biologicially involved in the pathogenesis of AD. A polymorphism and flanking markers at 1p36 will also be studied, concentrating on TNFR2, as it is a receptor for the aforementioned TNF gene, and could thus be involved in AD pathogenesis.

OBJECTIVES

The goal of this proposed project was to identify genes that may influence late-onset AD at chromosome regions 6p21 and 1p36. The following set of objectives were accomplished to achieve this goal:

1. Positive screen results found in the initial genome screen of chromosome 6p21 and 1p36 were further characterized by genotyping and analyzing dinucleotide repeat flanking markers TNFa and D1S507.

2. Polymorphisms at or near candidate genes in the regions of 6p21 (TNF) and 1p36 (TNFR2) were genotyped and analyzed using family-based association tests.

3. Age of onset was studied as a quantitative trait in AD patients to see if it could identify new AD genes or confirm the location of previously identified candidate regions localized using affectation status.

METHODS

Genotyping

Positive findings at 6p21 and 1p36 were followed up by typing dinucleotide repeats TNFa and D1S507 in 266 sib-pair families, and single nucleotide change polymorphisms TNF–238, TNF–308, and TNFR2 in 150 families with affected and unaffected siblings. These markers were chosen by using map data currently available on the Internet and from Medline searches at the library. Useful sites for marker and map information include the Genome Database (http://www.gdb.org), the Genetic Location Database (http://www.gdb.ac.uk), the CEPH server (http://www.cephb.fr), and the Cooperative Human Linkage Center (http://www.chlc.org). These sites were checked periodically to avoid neglecting new and useful information concerning new markers, marker distances, disease associations, or potential candidate genes.

TNFa, which is a dinucleotide repeat with alleles ranging from 97-123 base pairs, maps to chromosome 6p21.3 at 37.8 cM. TNFa primers IR2 (5' gccctctagatttcatccagcca-ca) and IR4 (5' cctctctcccctgcaacacaca) (Genome Database at http://www.gdb.org) were synthesized using a Oligo 1000 DNA synthesizer (Beckman Instruments, Fullerton, CA). Ten pmols of IR4 were end-labeled with 2 μCi of ^{32}P using 0.5 units of *T4* kinase. Polymerase chain reaction (PCR) was performed in an MJ thermocycler (MJ Research, Watertown, MA) using a 96-well microtiter plate format and reaction volumes of 25 μl. One hundred nanograms of DNA were added to 10X reaction buffer containing $MgCl_2$

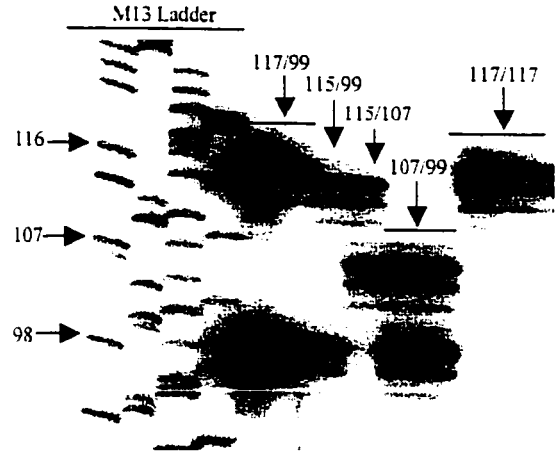


Fig. 7. Example of TNFa typings.

(1.5 mM), dNTPs (250 mM), deionized and distilled water, 10 pmol of each primer (one labeled with ^{32}P), and 0.5 units of *Taq* polymerase. PCR conditions consisted of an initial denaturation at 94°C (90 sec), 30 cycles of denaturation at 94°C (40 sec) and annealing at 60°C (30 sec), followed by a final extension at 72°C (2 min). After PCR, the samples were denatured at 94°C (3 min), and 3-4 µl of each sample was electrophoresed along with M13 sizing ladders on a 7% denaturing polyacrylamide gel for 3 hr at 65 watts. Autoradiography was performed for approximately 24-72 hr at -80°C, after which the film was developed (Fig. 7).

D1S507, which maps to chromosome 1p36 at 12.7 cM, is a dinucleotide repeat with alleles that range from 183 to 203 base pairs. Flanking marker primers AFMa127zc9a (5' aggggatcttggcacttgg) and AFMa127zc9m (5' ctctagggtttctggaaaatgctg) (Genome Database at http://www.gdb.org) were purchased from Research Genetics (Huntsville, AL). Ten pmols of AFMa127zc9a were end-labeled with 1 µCi of ^{32}P using 0.5 units *T4* kinase. PCR was performed in an MJ thermocycler (MJ Research, Watertown, MA) using a 96-well microtiter plate format with reaction volumes of 25 µl.

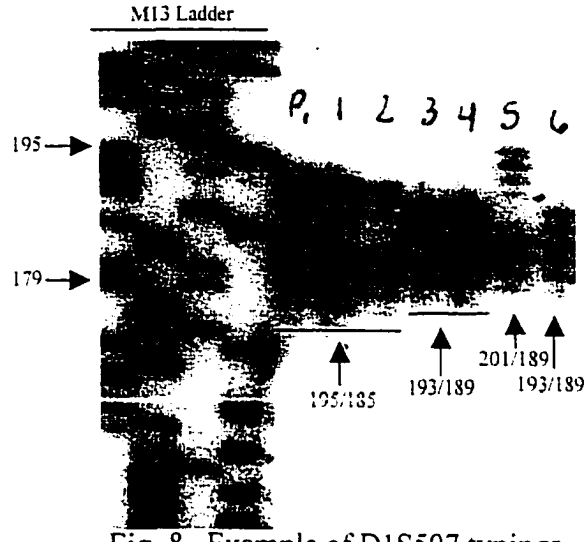


Fig. 8. Example of D1S507 typings.

One hundred nanograms of DNA were added to 10X reaction buffer containing $MgCl_2$ (1.5 mM), 2 µl DMSO, dNTPs (250 mM), deionized and distilled water, 10 pmol of each primer (one labeled with ^{32}P), and 0.5 units of *Taq* polymerase. PCR conditions consisted of an intial denaturation at 94°C (90 sec), followed by 30 cycles of denaturation at 94°C (40 sec), and annealing at 56°C (30 sec), followed by a final extension of 72°C for 2 min. After PCR, the samples were denatured at 94°C (3 min), and 7 µl of each sample was electrophoresed along with M13 sizing ladders on a 6% denaturing polyacrylamide gel for 3 hr at 90 watts. Autoradiography was performed for 24-72 hr at -80°C, after which the film was developed (Fig. 8).

In the TNF gene, G/A transitions at positions -308 and -238 were typed using a modified protocol and primers from Vinasco et al. [1997]. The α1 (5' aggcaataggttttga-gggCcat) and α3 (5' ctGgtccctectacccctcaca) primers (Genosys, the Woodlands, TX) created *NcoI* and *AvaII* sites, respectively. These were amplified together in a 50 µl reaction with 2 units of *Taq* and 500 ng of DNA. Amplification was performed in an MJ

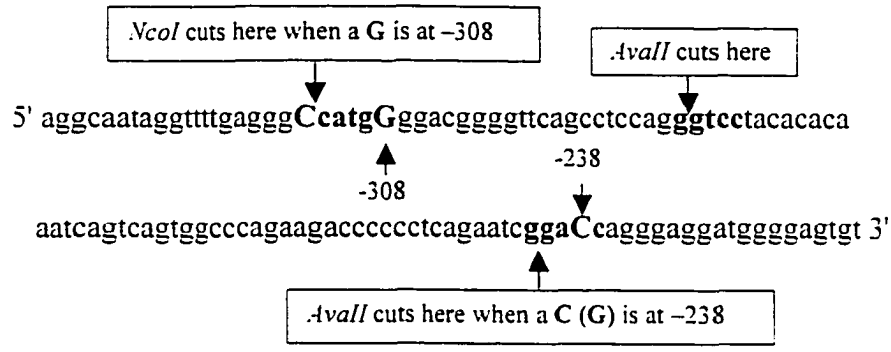


Fig. 9. Sequence of the product created by the α1 and α3 primers showing where the digestions by *NcoI* and *AvaII* take place.

thermocycler (MJ Research, Watertown, MA) with an initial denaturation at 94°C (5 min), 30 cycles of denaturation at 94°C (45 sec), annealing at 58°C (30 sec), and extension at 72°C (45 sec), followed by a final extension at 72°C (7 min).

Figure 9 shows the sequence of the product created by the α1 and α3 primers and where the digestions by NcoI and AvaII take place. The NcoI digestion cuts the 117 length product into 97 and 20 bp fragments when there is a G at position –308, and does not cut when there is an A at position -308. The AvaII digestion cuts the product into 49, 46, and 22 length fragments when there is a G at position –238, and cuts the product into 68 and 49 length fragments when there is an A at position –238.

The amplification of the α2 (5' aggcaataggttttgagggTcat) and α4 (5' ctAgAccctcc-taccctcaca) primers (Genosys, the Woodlands, TX), which created *BspHI* and *BglII* sites, used slightly different conditions, in that the annealing temperature was 55°C and 2.5 mM $MgCl_2$ was added. Figure 10 shows the sequence of the product created by the α2 and α4 primers and where the digestions by *BspHI* and *BglII* take place. The *BspHI* digestion cuts the 117 length product into 97 and 20 bp fragments when there is an A at

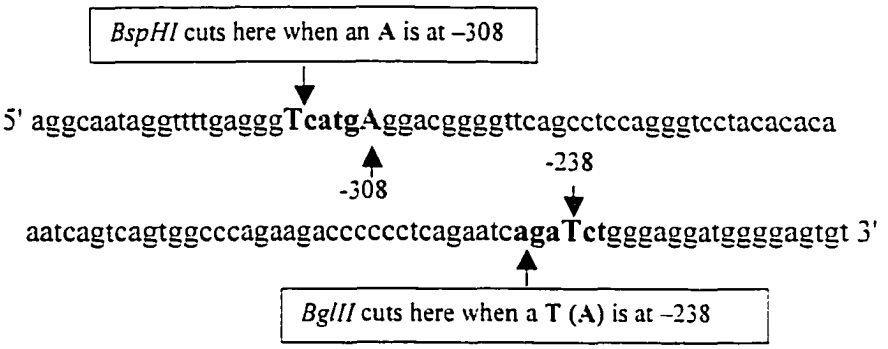


Fig. 10. Sequence of the product created by the α2 and α4 primers showing where the digestions by *BspHI* and *BglII* take place.

position –308, and does not cut when there is a G at position -308. The *BglII* digestion cuts the 117 length product into 97 and 20 bp fragments when there is an A at position –238, and does not cut when there is a G at position -238.

Products were run on agarose gels to check for the full product, which is a 117 bp band (Fig. 11). If it was present, 8 µl of the product was digested overnight at 37°C with 2 units of each enzyme. These digests were electrophoresed on a 3% agarose gel with EtBr and photographed using ultraviolet light. As the α1 and α3 PCR reactions worked better, the *NcoI* (Fig. 11) and *AvaII* digests (Fig. 13) were used for the initial typing of the –308 and –238 polymorphisms. These typings were confirmed by the *BspHI* (Fig. 12)

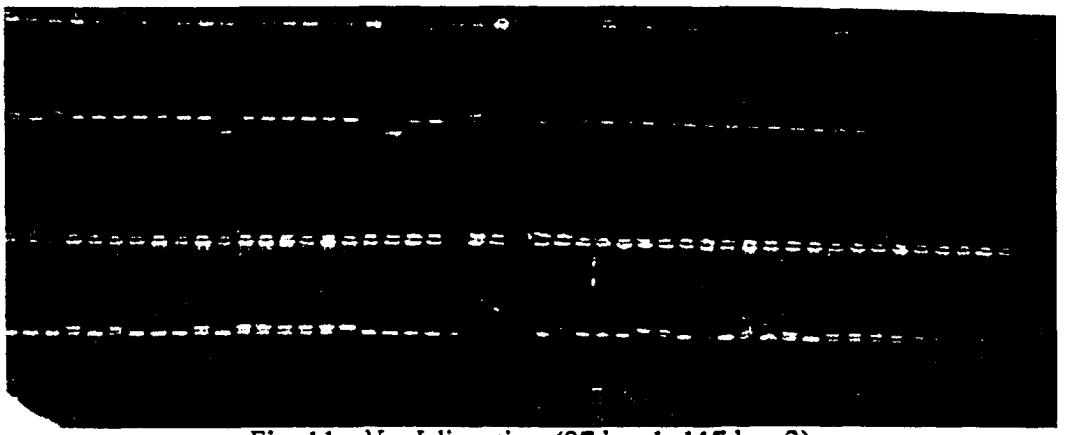


Fig. 11. *NcoI* digestion (97 bp=1, 117 bp=2)

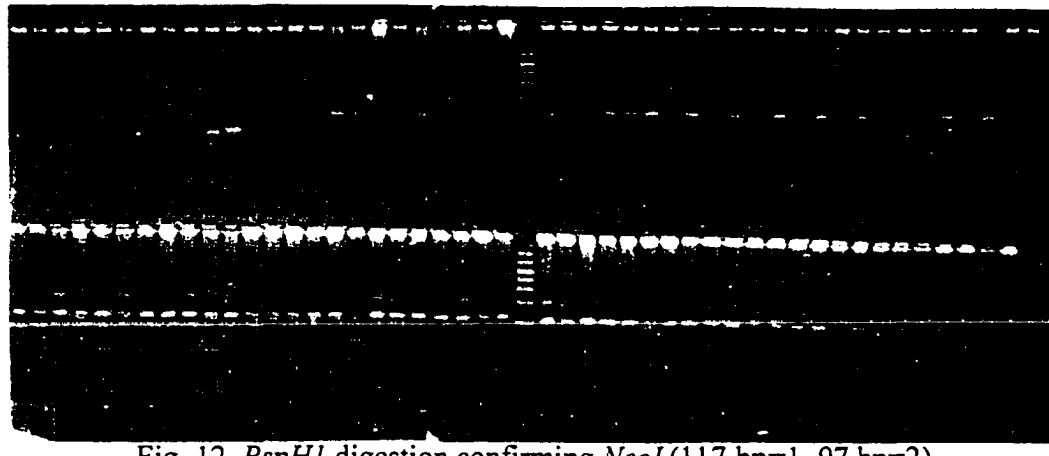


Fig. 12. *BspHI* digestion confirming *NcoI* (117 bp=1, 97 bp=2)

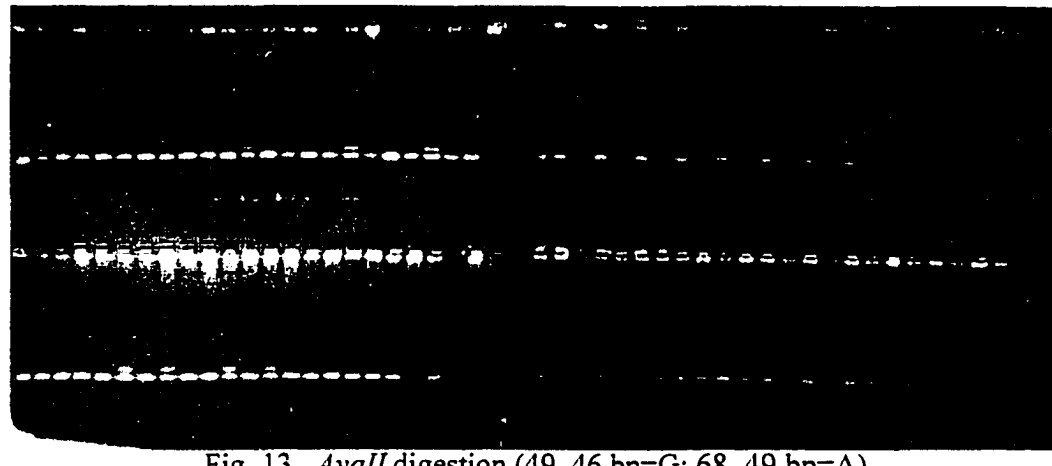


Fig. 13. *AvaII* digestion (49, 46 bp=G; 68, 49 bp=A)

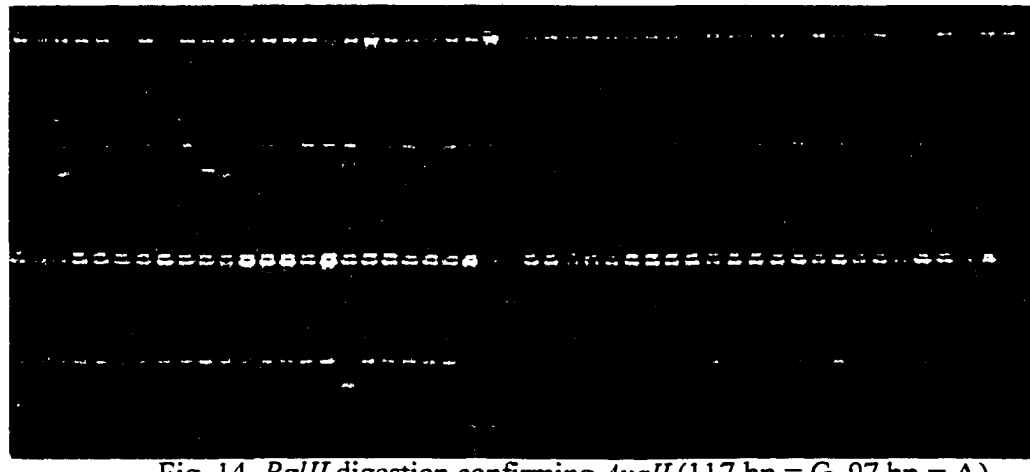


Fig. 14. *BglII* digestion confirming *AvaII* (117 bp = G, 97 bp = A)

and *BglII* (Fig. 14) digests, respectively, and by the digestion of samples that were undigested by the *NcoI* and *AvaII* digestions. For example, the *NcoI* gel would have the opposite pattern of the *BspHI* gel when digesting the same samples.

To type the affected and unaffected siblings for the TNFR2 exon 6 676 T→G (196M→R) polymorphism, a modified protocol from Al-Ansari et al. [2000] was used. TNFR2 forward (5'-actctcctatcctgcctgct) and reverse (5'-ttctggagttggctgcgtgt) primers [Al-Ansari et al. 2000] were synthesized by Genosys (the Woodlands, TX). PCR was performed using a 96-well microtiter plate format and reaction volumes of 25 µl. One hundred nanograms of DNA were added to 10X reaction buffer containing $MgCl_2$ (1.5 mM), dNTPs (250 mM), deionized and distilled water, 10 pmol of each primer, and 0.5 units of *Taq* polymerase (Promega, Madison, WI). Amplification was performed in an MJ thermocycler (MJ Research, Watertown, MA) at an initial denaturation at 95°C (3 min), followed by 35 cycles at 95°C (40 sec) and 57°C (30 sec), and a final extension at 72°C (5 min). After PCR, the products were electrophoresed on a 2% agarose gel containing EtBr to check for amplification of the 242 bp fragment (Fig. 15).

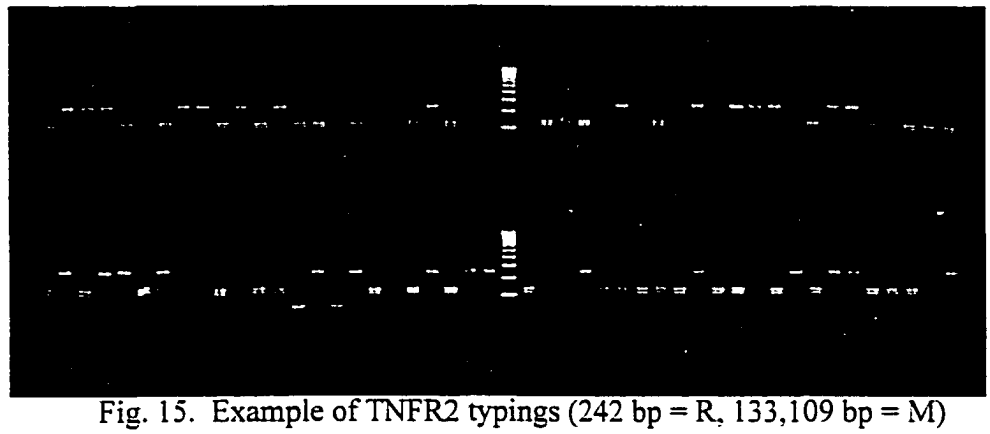


Fig. 15. Example of TNFR2 typings (242 bp = R, 133,109 bp = M)

Eight µl of each product were digested at 37°C with 5 units of the *Nla III* (New England Biolabs) enzyme overnight (total cocktail w/product 15 µl). Loading dye was added and 7 µl of each digest were run with M13 sizing ladders on a 3% agarose gel containing EtBr for 45 min at 120 volts. The gels were scanned and photographed using a Flour-S Multi-Imager (Biorad, Hercules, CA). The 196R allele was uncleaved (242 bp), and the M allele was cleaved into two fragments of 133 and 109 bp (Fig. 15).

Standard quality control procedures were used to assure accuracy of the genotypings and the database. The gels were read by two independent readers who resolved any discrepancies. The genotypes were entered into the database, Labman25 [Adams 1994], and checked by two separate individuals. Samples with mendelization errors, missing typings, or unresolved discrepancies were repeated.

In some of the analytic programs, allele frequencies are needed for its statistical models. Hence, allele frequencies for each marker were calculated by randomly selecting one individual from each family. As these individuals are from the study population, they can be used to accurately determine the frequency of the marker alleles [Terwilliger and Ott, 1994].

Linkage Analyses

Statistical analyses of the marker and clinical data were performed using the FASTLINK [Lathrop et al. 1984; Cottingham et al. 1993], SIBPAL [S.A.G.E. 1997], and GENEHUNTER [Kruglyak et al. 1996] programs.

FASTLINK was used to perform a parametric maximum likelihood log odds (lod) score for two-point linkage analyses under a dominant model with a gene frequency of

0.02 for the AD susceptibility gene with a penetrance of 0.80. A lod score of 1.0 or greater was considered a positive finding. SIBPAL performs two-point nonparametric analysis in sib-pair families and a *P*-value of less than 0.05 was considered a positive finding. SIBPAL was also used to analyze age of onset as a quantitative trait (square of the age of onset difference between two affected siblings) in the 1p36 and 6p21 regions. GENEHUNTER performs parametric and nonparametric multipoint analyses and a NPL statistic with a *P*-value of 0.10 or less was considered a positive finding.

The above liberal criteria were used for the initial follow-up and the analysis of flanking markers to enable the detection of major and/or minor genes that influence AD. This increases the sensitivity of the analysis, but carries a risk of false-positives. One way to eliminate false-positive findings is to follow-up areas where associations are seen with flanking markers, as true-positive peaks are broader than false-positive peaks [Terwilliger et al. 1997; Goldin and Chase 1997]. If the analysis of flanking markers continued to show an AD association, PCR-RFLP polymorphisms at candidate genes in the region were typed.

Family-based Association Testing

Association and linkage analyses of AD with the polymorphisms and haplotype were performed by three family based association tests that do not require parental genotypes. The SIBASSOC [Curtis, 1997] program performs a χ^2 test using the most genotypically distinct unaffected sibling as a control for each case. This produces positive results only if the marker is associated with, and linked to, the disease locus, and is similar to the TDT proposed by Spielman et al. in 1993, which requires parents-child

trios. The S-TDT [Spielman and Ewens, 1998], which eliminates the need for parental genotypes as in the TDT, uses marker information from unaffected siblings to test for linkage in sibships containing at least one affected and one unaffected sibling. The final test, the SDT, which compares transmission of alleles to all affected and unaffected siblings in a sibship [Hovarth and Laird, 1998], is a test for linkage as well as linkage disequilibrium.

RESULTS

The TNF promoter polymorphisms were typed in 145 families (151 sibships) containing 311 affected (69% female; mean age of onset 69.4 years) and 251 unaffected (64% female; mean age at follow up 72.5 years) siblings. Genotype frequencies for the TNF–308 and TNF–238 promoter polymorphisms are listed in Table 3. There were no significant differences found when comparing genotype frequencies between affected and unaffected siblings for TNF–308 ($\chi^2_2 = 0.60$) and TNF–238 ($\chi^2_1 = 0.21$).

As a result of the low heterozygosity of these polymorphisms, they were combined with the previously typed microsatellite, TNFa, to create a haplotype in the order of TNF-308, TNF-238, and TNFa. The haplotypes were determined using maximum likelihood in the GENEHUNTER program and were checked by visual inspection. The 2-1-2 haplotype was found to be significantly associated with AD using the SIBASSOC ($P = 0.005$), S-TDT ($P = 0.02$), and SDT ($P = 0.005$) analysis programs.

Table 3. Distribution of TNF Genotypes.

	Patients	Siblings
TNF-308	n (%)	n (%)
TNF1/1	216 (69.2)	162 (65.6)
TNF1/2	85 (27.2)	75 (30.4)
TNF2/2	11 (3.5)	10 (4.0)
TNF-238		
TNFG/G	281 (91.2)	222 (92.5)
TNFG/A	26 (8.4)	18 (7.5)
TNFA/A	1 (0.3)	0 (0.0)

Table 4. TNF Polymorphism Association Analyses.[a]

Allele	SIBASSOC	S-TDT	SDT
TNF-308 1	$P = 0.55$	$P = 0.08$	$P = 0.17$
TNF-308 2	$P = 0.55$	$P = 0.11$	$P = 0.17$
TNF-238 G	$P = 0.40$	$P = 0.37$	$P = 0.81$
TNF-238 A	$P = 0.40$	$P = 0.91$	$P = 0.81$
TNFa 2	$P = 0.04$	$P = 0.24$	$P = 0.09$
Haplotype 2-1-2	$P = 0.005$	$P = 0.02$	$P = 0.005$

[a] *P*-values not corrected for multiple comparisons

There was also a significant association ($P = 0.04$) between the TNFa 2 allele and AD using the SIBASSOC program (Table 4).

After the AD age of onset distributions were evaluated for normality using the Shapiro-Wilk test, mean ages of onset were compared between affected patients with the TNF 2-1-2 haplotype and those who did not carry this haplotype using the standard t-test. These analyses were performed using SAS Release 8.0 [SAS, 1999]. Age of onset was not significantly lower ($P = 0.32$) in the 51 affected siblings with the TNF 2-1-2 haplotype (mean = 68.0 years) as compared to the 243 affected siblings without the TNF 2-1-2 haplotype (mean = 69.5 years).

To examine TNFR2 as a candidate gene for the 1p36 region, the exon 6 T→G polymorphism was typed in 150 families (156 sibships) containing 324 affected (71% female; mean age of onset 69.5 years) and 253 unaffected (64% female; mean age at follow up 72.5 years) siblings. There was no significant difference ($\chi^2_2 = 2.51$) found when comparing genotype frequencies between affected and unaffected siblings (see Table 5).

There was no significant association between AD and TNFR2 using family-based association testing. Results from the SIBASSOC ($P = 0.71$), S-TDT ($P = 0.28$), and SDT ($P = 0.63$) analysis programs are shown in Table 6.

Table 5. Distribution of TNFR2 Frequencies.

Genotype	Patients n (%)	Siblings n (%)
M/M	191 (59.3)	165 (65.2)
M/R	116 (36.0)	73 (28.9)
R/R	15 (4.7)	15 (5.9)
Allele		
M	(77.3)	(79.6)
R	(22.7)	(20.4)

Table 6. TNFR2 196 M→R Substitution Association Analyses.[a]

Allele	SIBASSOC	S-TDT	SDT
TNFR2 M	$P = 0.71$	$P = 0.28$	$P = 0.63$
TNFR2 R	$P = 0.71$	$P = 0.43$	$P = 0.63$

[a] *P*-values not corrected for multiple comparisons

Mean age of onset was compared between affected patients with an M/M genotype and patients with one or two copies of the TNFR2 R allele using a t-test from SAS Release 8.0 [SAS, 1999]. Age of onset was not significantly different (t-test *P*-value = 0.59) in the 191 affected siblings with the M/M genotype (mean = 68.1 years) when compared to the 131 affected siblings with the M/R or R/R genotypes (mean = 68.8 years).

Flanking markers D1S507 and D1S455 at 12.7 and 32.1 cM, respectively, were typed at chromosome 1p36 to further narrow the broad region that was associated with AD in the full and age of onset over 70 datasets. The results from the analyses of these markers and from the updated multipoint analyses of this region are shown in Table 7 and Figure 16.

SIBPAL was used to examine the sib-pair difference in age of onset as a quantitative trait in these AD families to help localize an AD gene. As it is well known

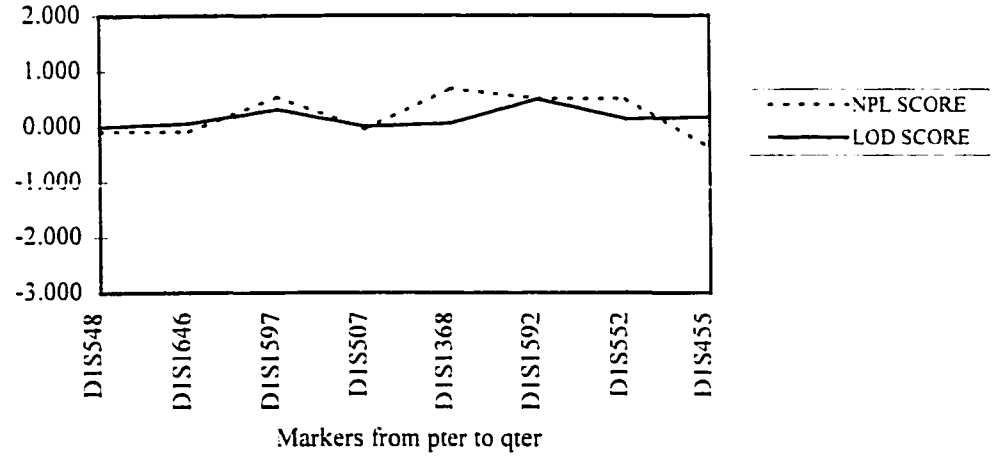
that ApoE ε4/ε4 homozygotes have a significantly earlier age of onset [Blacker et al., 1997], ApoE ε4/ε4 typings were used as a positive control for these analyses ($P = 0.006$). Typings from 15 markers on chromosome 14 were used as a negative control ($0.24 < P < 0.99$), as these markers had previously been shown to be unassociated with AD in our families. The results from analyses of chromosome 1p36 and 6p21 are listed in Tables 8 and 9, respectively. No markers in chromosome 6p21 ε4/ε4 subset were positive, however, in the full dataset of families at 6p21, markers D6S1017 and D6S271 were positive. At chromosome 1p36, markers D1S507 and D1S552 were positive in the whole dataset, and D1S552 was also slightly positive in the AO > 70 subset.

Table 7. 1p36 Region Single-Point Linkage Analyses.

			SIBPAL		GENEHUNTER		FASTLINK	
Marker	Distance	Stratum (# fams)	Mean	*P*-Value	NPL score	*P*-Value	lod score	θ
[a]D1S548	4.2 cM	TOTAL (266)	0.51	0.35	0.162	0.43	0.00	0.45
[a]D1S548	4.2 cM	AO >70 (145)	0.53	0.04	1.015	0.15	0.14	0.25
D1S1646	4.4 cM	TOTAL (266)	0.51	0.17	0.518	0.30	0.06	0.30
D1S1646	4.4 cM	AO >70 (145)	0.53	0.07	0.971	0.17	0.15	0.25
D1S1597	9.1 cM	TOTAL (266)	0.52	0.03	1.036	0.15	0.31	0.25
D1S1597	9.1 cM	AO >70 (145)	0.52	0.04	1.184	0.12	0.19	0.20
[a]D1S507	12.7 cM	TOTAL (266)	0.50	0.57	-0.486	0.69	0.00	0.40
[a]D1S507	12.7 cM	AO >70 (145)	0.51	0.28	0.043	0.33	0.03	0.35
D1S1368	~15 cM	TOTAL (266)	0.52	0.14	1.022	0.15	0.06	0.35
D1S1368	~15 cM	AO >70 (145)	0.54	0.04	1.429	0.08	0.23	0.20
[a]D1S1592	16.8 cM	TOTAL (266)	0.51	0.16	0.840	0.20	0.49	0.20
[a]D1S1592	16.8 cM	AO >70 (145)	0.52	0.12	0.940	0.17	0.35	0.20
D1S552	22.6 cM	TOTAL (266)	0.50	0.40	0.406	0.34	0.13	0.30
D1S552	22.6 cM	AO >70 (145)	0.52	0.16	1.227	0.11	0.14	0.25
[a]D1S455	32.1 cM	TOTAL (266)	0.51	0.21	-0.250	0.60	0.17	0.30
[a]D1S455	32.1 cM	AO >70 (145)	0.50	0.59	-0.265	0.60	0.04	0.30

[a]indicates flanking marker

A. Chromosome 1 results for all 266 families



B. Chromosome 1 results for the 145 families with an age of onset over 70

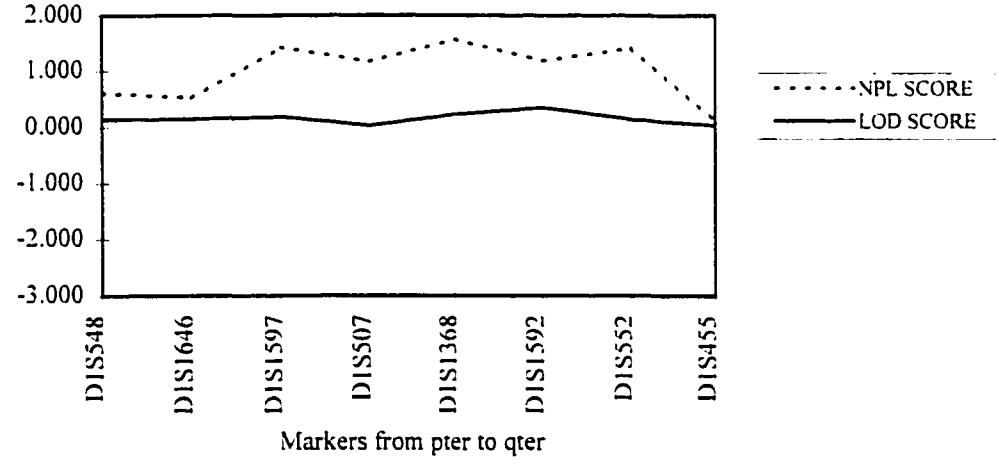


Fig. 16. Chromosome 1p36 multipoint results in the total subset (A). and in the 145 families with an age of onset over 70 (B).

Table 8. SIBPAL Analyses of Age of Onset as a Quantitative Trait at 1p36.

Marker	Distance	Stratum (# fams)	*P*-Value
[a]D1S548	4.2 cM	TOTAL (266)	0.04
[a]D1S548	4.2 cM	AO >70 (145)	0.09
D1S1646	4.4 cM	TOTAL (266)	0.06
D1S1646	4.4 cM	AO >70 (145)	0.26
D1S1597	9.1 cM	TOTAL (266)	0.03
D1S1597	9.1 cM	AO >70 (145)	0.09
[a]D1S507	12.7 cM	TOTAL (266)	0.007
[a]D1S507	12.7 cM	AO >70 (145)	0.09
D1S1368	~15 cM	TOTAL (266)	0.18
D1S1368	~15 cM	AO >70 (145)	0.27
[a]D1S1592	16.8 cM	TOTAL (266)	0.80
[a]D1S1592	16.8 cM	AO >70 (145)	0.81
D1S552	22.6 cM	TOTAL (266)	0.017
D1S552	22.6 cM	AO >70 (145)	0.04
[a]D1S455	32.1 cM	TOTAL (266)	0.99
[a]D1S455	32.1 cM	AO >70 (145)	0.61

[a]indicates flanking marker

Table 9. SIBPAL Analyses of Age of Onset as a Quantitative Trait at 6p21.

MARKER	Distance	Stratum (# fams)	*P*-Value
D6S105	31.9 cM	ALL (266)	0.25
D6S105	31.9 cM	ε4/ε4 (84)	0.29
D6S1051	35.2 cM	ALL (266)	0.49
D6S1051	35.2 cM	ε4/ε4 (84)	0.63
[a]D6SMIB	37.7 cM	ALL (266)	0.53
[a]D6SMIB	37.7 cM	ε4/ε4 (84)	0.26
[a]TNFa	37.8 cM	ALL (266)	0.31
[a]TNFa	37.8 cM	ε4/ε4 (84)	0.09
[a]D6S9N3	38.0 cM	ALL (266)	0.56
[a]D6S9N3	38.0 cM	ε4/ε4 (84)	0.15
[a]D6S1017	42.3 cM	ALL (266)	0.013
[a]D6S1017	42.3 cM	ε4/ε4 (84)	0.15
D6S271	42.9 cM	ALL (266)	0.04
D6S271	42.9 cM	ε4/ε4 (84)	0.11
D6S1280	44.8 cM	ALL (266)	0.07
D6S1280	44.8 cM	ε4/ε4 (84)	0.53

[a]indicates flanking marker

DISCUSSION

The TNF promoter polymorphisms, -308 and –238, were not found to be individually associated with AD using family-based association testing. Their genotype frequencies were also not found to be significantly different, when comparing affected and unaffected siblings. This could be due to the low level of heterozygousity at these markers, which could decrease the number of informative families in the analyses. Due to this, these polymorphisms were combined with TNFa to form a haplotype, 2-1-2, which was then found to be associated with AD.

The TNF–308 promoter polymorphism TNF2 (G→A) allele, part of the AD associated haplotype, has been shown to have an increased frequency in autoimmune and inflammatory diseases [Wilson et al., 1995] and is associated with stronger transcriptional activation than the TNF1 allele [Wilson et al., 1997]. The TNFA allele of the TNF–238 promoter polymorphism has no effect on TNF production [Poicot et al., 1995], and our associated haplotype includes the more common –238 TNFG allele. The TNF microsatellite TNFa 2 allele (99 basepairs) has been previously associated with higher TNF secretion [Poicot et al., 1993] and susceptibility to rheumatoid arthritis [Mulcahy et al., 1996, Field et al., 1997].

Thus, two of the TNF alleles comprising this AD haplotype are associated with increased TNF production, which could lead to the chronic inflammatory state and free radical damage hypothesized to be involved in AD pathogenesis [Wood, 1995; McGeer

et al., 1996]. This could potentially lead to a lower age of onset for individuals carrying this haplotype. Although the mean age of onset for affecteds carrying the haplotype is 1.5 years lower than the mean age of onset for affecteds not carrying the haplotype, this finding is not significant.

When flanking markers D1S455 and D1S507 were typed in the chromosome 1p36 region, they were not found to be individually associated with AD using parametric and non-parametric linkage analyses. They actually lowered the multi-point score at marker D1S1368, from 1.58 to 1.56, in the group of families with an age of onset over 70. However, part of this could be explained by the uncertain location of marker D1S1368. If, in fact, it is closer to D1S1597 (on the other side of D1S507), our multipoint results could improve.

The TNFR2 locus in the 1p36 region with an exon 6 T→G polymorphism did not have significantly different genotype frequencies when compared to affected and unaffected siblings. It was also not found to be individually associated with AD using family-based association testing, however, this could be secondary to the lack of heterozygousity at this marker. Mean ages of onset were also not significantly different when comparing patients with different genotypes.

We analyzed age of onset as a quantitative trait using SIBPAL in our total dataset and subsets. Here, the age of onset difference between two affected siblings was squared and regressed on the mean IBD. We used ApoE as a positive control, and markers on chromosome 14 as a negative control. It was interesting to note that, when using the squared difference in age of onset between siblings as a quantitative trait, ApoE was significantly associated with a *P*-value of 0.006. However, when ApoE was run using

status as a binary trait, there was no association. This agrees with the literature that ApoE acts more as a modifier of age of onset [Blacker et al., 1997].

The results of the age of onset analyses identified the same regions previously linked to AD in our families, but with some differences. For example, the chromosome 6p21 ε4/ε4 subset was not positive, although this could be because it had the smallest number of families to analyze and, therefore, lower power. However, in the whole set of families at 6p21, markers D6S1017 and D6S271 were positive. At chromosome 1p36, D1S507 was closely linked ($P = 0.007$), which was interesting because it is not positive in our previous analyses, although it is in the linked region. Once again at chromosome 1p36, the whole dataset is linked, but the subset only has one positive marker, D1S552. This could also be secondary to a decreased number of families and decreased power. However, as we have seen with ApoE, some AD genes may just influence the age of onset.

In a recent study by Daw et al. [1999], they also analyzed AD using age of onset as a quantitative trait. They believe that they found another region on chromosome 14 that is linked to AD, while in our study chromosome 14 was completely negative. They also excluded our 1p region, which is opposite of our results. However, their quantitative model was parametric, and ours was nonparametric. It must be remembered that the traits analyzed were different. Daw et al. [1999] used age of onset of disease, and our trait is defined as the squared pair difference between affected siblings. Thus, our positive linkage results may be identifying genes that make onsets similar; for example, the ApoE results.

TNF has been found in the brain lesions of AD along with other inflammatory cytokines such as IL-1, IL-6, and IL-12 [Yen, 1995; Fiala et al., 1998]. It is of interest to note that recently polymorphisms in both IL-1A and IL-1B have been associated with increased risk of AD and a signicantly eariler age of onset [Tanzi et al., 2000]. This chronic inflammatory state could lead to subsequent neuronal damage [Tarkowski et al., 1999] and memory loss [Hauss-Wegrzyniak et al., 1998].

Previous studies have shown that NSAIDs protect against, or slow the progression of, AD [McGeer et al., 1996; Makenzie and Munoz, 1998], with the level of protection directly related to the level of NSAID use [in't Veld et al., 1998]. This protection may be due to the inhibition of cyclo-oxygenase-2 (COX-2), which then reduces the generation of reactive oxygen species harmful to the CNS. COX-2 expression has been shown to be higher in AD patients [Pasinetti, 1998], especially within neurofibrillary tangles [Oka and Takashima, 1997], and TNF has been shown to upregulate COX-2 expression [Geng et al., 1995]. In addition, TNF secretion can be induced by Aβ [Fiala et al., 1998; Klegeris et al., 1997], which upregulates microglia, releasing TNF and free oxygen radicals, [Schubert et al., 1998] which can then oxidize neuronal proteins [Yatin et al., 1999] and overstimulate the immune system [Behl, 1997; Kaltschmidt et al., 1997].

The three genes which have been found to cause early-onset AD, APP, PS1, and PS2, are proposed to cause AD by increasing the production of Aβ42 [Citron et al., 1997; Selkoe, 1996; Scheuner et al., 1996], which aggregates [Jarrett and Lansbury, 1993] to form neurotoxic AD NPs [Yankner et al., 1989]. Free radicals produced during normal brain metabolism oxidize Aβ and make it aggregate more easily [Dyrks et al., 1992] into this neurotoxic form. We hypothesize that the known early-onset AD mutations

upregulate TNF and other cytokines by increasing Aβ production, leading to increased free radical production and senile plaque formation, which eventually leads to neuronal lysis.

The A2M gene in late-onset AD may also be related to TNF. A2M is an acute phase protein and AD plaque component [Rebeck et al., 1995; van Gool et al., 1993] that binds to [Hughes et al., 1998] and degrades Aβ [Qiu et al., 1996]. Additionally, A2M binds TNF [Webb and Gonias, 1998] and may be regulated by the release of TNF and other cytokines [Lyoumi et al., 1998]. This A2M deletion may potentially affect Aβ and TNF binding sites, leading to less degradation, additional plaque formation, and immune stimulation.

Confirmation is still needed to determine if the TNF locus is the primary AD associated gene in this region; however, there is further evidence that this region is implicated in late-onset AD families. Pericak-Vance et al. [1997], in a 54 family late-onset AD genomic screen, found a peak lod score of 1.37 at marker D6S1019 [Garcia et al., 1999], which maps very close to the TNF gene. Also, Kehoe et al. [1999] found a lod score of 1.4 near the HLA region in a genome screen of 230 families with late-onset AD, which were derived from the same pool of families collected by the NIMH AD Genetics Initiative.

The reconstruction of parental genotypes and haplotypes for these analyses by GENEHUNTER may introduce bias by increasing the type one error rate, especially in families of particular heterozygous parental mating types [Curtis, 1997; Knapp, 1999; Clayton, 1999]. This procedure may also introduce bias by restricting the analysis to families for which a haplotype assignment can be made [Clayton, 1999]. In individual

TNF marker analyses using S-TDT and SDT, parental genotypes were not reconstructed, but the use of these programs for haplotype analysis may introduce bias, as haplotypes were constructed from sibship genotypes. However, the results from the SIBASSOC test are valid and do not incur the false-positive bias when conditioning on reconstructed haplotypes [Curtis, 1997]. Our dataset consists of 151 sibships, a mean sibship size of 3.7, and a median sibship size of three, which increases the power of the S-TDT and SDT, and keeps the true type one error rate close to the expected [Knapp, 1999]. Therefore, the increased average sibship size and typing of unaffected siblings allows more accurate reconstruction of parental genotypes [Curtis, 1997; Knapp, 1999] and haplotypes [Clayton, 1999]. Furthermore, only 5% of the siblings (11 affected and 17 unaffected) for whom no haplotype could be assigned were dropped from the analysis.

In this study, we chose to examine a broad region associated with AD because it has been established that peaks harboring disease genes are longer than false-positive peaks [Terwilliger et al., 1997], even though the individual screening markers may not meet the stringent criteria discussed by Lander and Kruglyak [1995]. It has been estimated that four additional loci may play a role in late-onset AD [Warwick et al., 2000]. Therefore, individual gene contributions may be difficult to elucidate. We realize that, with the use of subsets as well as nonparametric, parametric, and association analyses, the level of significance of our results may be questioned. However, because of the implication of this region by others and the hypothesized role of TNF in neuroinflammation and free radical damage, these results indicate that TNF may play a crucial role in the development of AD.

CONCLUSION

In conclusion, we found that the TNF haplotype 2-1-2, whose alleles are associated with inflammatory diseases and heightened TNF levels, was significantly associated with AD. This, along with the evidence that TNF levels are affected by other known AD mutations and that increased TNF production can lead to an exacerbation of the inflammatory state and free radical generation, allows us to hypothesize that increased TNF production can lead to an increased severity of symptoms or decreased onset age in AD patients, for which NSAIDs and antioxidants could be protective. Therefore, our results implicating a TNF haplotype lend further support for the possible role of inflammatory cytokines and free radicals in the pathogenic process of AD.

Further studies to explore this effect of TNF expression could be done in transgenic mice overexpressing human TNF and APP, in which neuropathological examination could determine whether plaque development is affected. In addition, TNF expression could also be compared between samples of AD affected and unaffected brain tissue using expression arrays on chips.

Although the results from the TNFR2 polymorphism and initial flanking marker analyses were not significant, there does seem to be evidence that the region may be harboring a candidate gene or modifier. This region had been previously implicated in our nonparametric analyses, and marker D1S507 had a *P*-value of 0.007 when analyzing 1p36 with the squared pair difference in age of onset as a quantitative trait, which is close

to the *P*-value of 0.006 for the ApoE gene. This candidate region needs to be explored further, and other candidate genes in the area, such as C1q, which binds to Aβ and is involved in inflammation, should be considered.

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APPENDIX

INSTITUTIONAL REVIEW BOARD APPROVAL FORM

OMB No. 0990-0263
Approved for use through 7/31/94

Protection of Human Subjects
Assurance Identification/Certification/Declaration
(Common Federal Rule)

POLICY: Research activities involving human subjects may not be conducted or supported by the Components and Agencies adopting the Common Rule (56 FR 28002, June 18, 1991) unless the activities are exempt from or approved in accordance with the common rule. See Section 101(b) the common rule for exemptions. Institutions submitting applications or proposals for support must submit certification of appropriate Institutional Review Board (IRB) review and approval to the Department or Agency in accordance with the common rule.

Institutions with an assurance of compliance that covers the research to be conducted on file with the Department, Agency, or the Department of Health and Human Services (HHS) should submit certification of IRB review and approval with each application or proposal unless otherwise advised by the Department or Agency. Institutions which do not have such an assurance must submit an assurance and certification of IRB review and approval within 30 days of a written request from the Department or Agency.

1. Request Type	2. Type of Mechanism	3. Application or Proposal Identification No. (if known)
☐ ORIGINAL	☐ GRANT ☐ CONTRACT ☐ FELLOWSHIP	
☐ FOLLOWUP	☐ COOPERATIVE AGREEMENT	
☐ EXEMPTION	☐ OTHER: _____	

4. Title of Application or Activity	5. Name of Principal Investigator, Program Director, Fellow, or Other
A Search for Alzheimer's Disease Genes in the NIMH Sibling Dataset	Rodney C.P. Go

6. Assurance Status of this Project (Respond to one of the following)

☐ This Assurance, on file with the Department of Health and Human Services, covers this activity

Assurance identification no. M- 1149 IRB identification no. 01MR

☐ This Assurance, on file with (Agency/Grantee) _____ covers this activity

Assurance identification no. _____ IRB identification no. _____ (if applicable)

☐ No assurance has been filed for this project. This institution declares that it will provide an Assurance and Certification of IRB review and approval upon request.

☐ Exemption Status: Human subjects are involved, but this activity qualifies for exemption under Section 101 (b), paragraph _____

7. Certification of IRB Review (Respond to one of the following if you have an Assurance on file)

☒ This activity has been reviewed and approved by the IRB in accordance with the common rule and any other governing regulations or submits on (date) 08-04-98 by: ☐ Full IRB Review or ☒ Expedited Review

☐ This activity contains multiple projects, some of which have not been reviewed. The IRB has granted approval on condition that all projects covered by the common rule will be reviewed and approved before they are initiated and that appropriate further certification will be submitted.

8. Comments

9. The official signing below certifies that the information provided above is correct and that, as required, future reviews will be performed and certification will be provided.	10. Name and Address of Institution
	The University of Alabama at Birmingham 1120A Administration Building 701 South 20th Street Birmingham, AL 35294-0111
11. Phone No. (with area code): (205)934-3789	12. Fax No. (with area code): (205)975-5977
13. Name of Official: Marilyn Doss, M.A.	14. Title: Vice Chair-IRB
15. Signature: [signature]	16. Date: 8-4-98

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OPTIONAL FORM 310 (9-92)
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GRADUATE SCHOOL
UNIVERSITY OF ALABAMA AT BIRMINGHAM
DISSERTATION APPROVAL FORM
DOCTOR OF PHILOSOPHY

Name of Candidate Julianne Shea Collins

Graduate Program Medical Genetics

Title of Dissertation A Search for Genes Influencing Late-Onset Alzheimer's Disease on Chromosomes One and Six

I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this student are such that she may be recommended for the degree of Doctor of Philosophy.

Dissertation Committee:

Name		Signature
Rodney C. P. Go	**, Chair**	Rodney C.P. Go
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Gail V. Johnson		
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Date 1/4/01