GENETIC SUSCEPTIBILITY IN ALZHEIMER'S DISEASE AND THE ROLE OF LIPID METABOLISM

by

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Genetic Susceptibility in Alzheimer's Disease
and the Role of Lipid Metabolism

Abstract

by

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With significant evidence supporting the role of lipid transport and/or lipid metabolism in late onset Alzheimer's Disease (LOAD) susceptibility, we hypothesized that polymorphisms in genes coding for proteins involved in lipid pathways would make good candidates in an investigation of genetic risk and LOAD. We selected six genes associated both with LOAD and either directly or indirectly with lipid pathways to test in three case-control populations: Brain-derived Neurotrophic Factor (BDNF), Transthyretin (TTR), Tumor Necrosis Factor alpha (TNFa), Low-density Lipoprotein Receptor-related Protein (LRP1), Apolipoprotein L-3 (APOL3), and Sterol O-acyltransferase 1 (SOAT1). As an additional, exploratory analysis, we tested the hypothesis that dietary intake of lipids may modify the effect of these genes. Our results demonstrate a potentially significant role of LRP1 in protection against LOAD in the younger LOAD case-control population. In the elderly LOAD case-control population, we observed an association of borderline significance for TNFa, APOL3, and BDNF. Additional results of interest include observed but statistically non-significant effect modification of TTR by dietary fish intake.

1. Background

Alzheimer Disease (AD) is a complex neurodegenerative disease characterized by progressive and irreversible dementia. There are two forms of Alzheimer disease: early-onset, where disease development is before age 60, and late onset, with development after age 60, with the latter being the focus of our investigation. Late-onset Alzheimer Disease (LOAD) is the more common form of disease, affecting an estimated 4.5 million people in the United States currently. It is anticipated that the prevalence of LOAD will increase to between 11 to 16 million in the next 40 to 50 years[1-3] as life expectancy increases and as the 76 million Americans born during the post-World War II baby boom become elderly.

1.1 Disease Symptoms and Pathology

1.1.2 Symptoms

When symptoms of AD develop before age 60 (or 65 depending on clinician judgment or study criteria), the disease is classified as early-onset AD. Less than 5 to 10 percent of all AD cases are this early-onset form [4] which is normally diagnosed in the age ranges of 45-60[4]. The more common, late-onset form of disease is defined by symptoms which present after the age of 65. Because the early- and late-onset forms of disease are

pathologically and clinically indistinguishable except by age at onset, misclassification of disease type can occur when onset of symptoms cannot be clearly dated.

The symptoms of Alzheimer's disease are variable in expression and include memory loss, impairments in attention, language, visual-spatial abilities, praxis (purposeful movement), calculations, visual, auditory, and/or olfactory perception, problem solving ability, and judgment. The first observed symptom is often memory loss and many AD patients exhibit depression, personality changes, apathy, or irritability. A person with AD will live an average of eight years and as many as 20 years or more from the onset of symptoms as estimated by relatives[5]. From the time of diagnosis, people with AD survive about half as long as those of similar age without dementia. Average survival time is affected by age at diagnosis and severity of other medical conditions[6]. Some research suggests that people with early-onset AD decline at a faster rate than do those with late-onset AD[7].

Many of the symptoms which characterize the dementia associated with AD are not exclusive to AD. Dementia is an umbrella term for acquired impairment of intellect and memory, of which AD is the most common cause. Other causes can include vascular dementia or Lewy body-type dementia. To clinically diagnose AD, a combination of tests are used which include cognitive screening tests, physical examination to exclude alternative causes of dementia which can include neuro-imaging and blood tests, and a recording of family history for AD (see **defining phenotype**, section 2.1.3). Only

examination of brain tissue at autopsy can provide a definitive pathological diagnosis of AD pathology.

1.1.2 Pathology

There are two pathological signatures of AD, amyloid plaques and neurofibrillary tangles, and the presence of both is required for definitive diagnosis of AD. Regions of the brain that are important in memory are those most affected by AD and include the hippocampus and entorhinal and cerebral cortexes [8; 9] . Together these plaques and tangles contribute to the loss of function and eventual cell death associated with progression of the disease.

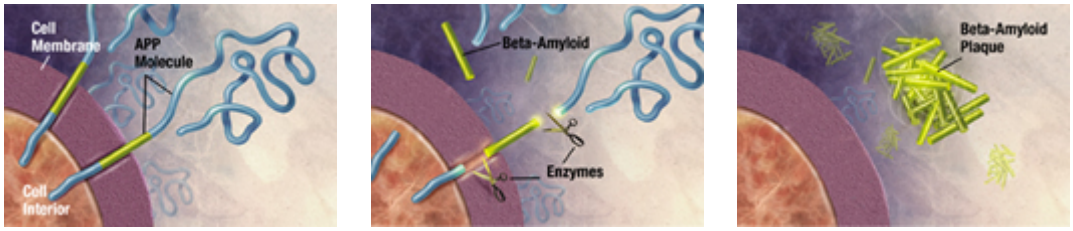
1.1.2.a Plaques

Amyloid plaques are formed in the extracellular spaces and are composed primarily of beta-amyloid (Aβ), also known as amyloid beta or Abeta. Aβ is a protein fragment of 39 to 43 amino acids in length snipped from a larger protein called the amyloid precursor protein (APP)[8; 9]. APP has several isoforms generated by alternative splicing of the 19-exon APP gene. The predominant isoform found in neuronal tissue is APP695 and includes translated sequences from exons 1-6 and 9-18. Exons 16 and 17 of the APP gene code for the portion of the APP protein from which, after cleavage, Aβ is derived.

Non-pathogenic processing of APP by alpha secretases releases soluble N-terminal APP fragments into the extracellular space. These fragments are not amyloidgenic (do not aggregate) and do not contribute to plaque formation. However, when APP is cleaved by the beta and gamma secretase, the amyloid β peptide fragment (Aβ) is produced, which is

the foundation of pathogenic amyloid plaques in AD [10]. Generation of Aβ is caused by sequential cleavage of the APP protein at β and γ sites through two biochemical reactions (Figure 1). The first reaction involves a β-site APP cleaving enzyme (BACE1) and a protein complex (γ-secretase). BACE1 is the rate-limiting enzyme for Aβ biogenesis.

Figure 1: Amyloidgenic processing of the APP molecule leads to formation of Beta-Amyloid (Aβ) and subsequently amyloid plaques



Couresy of ADEAR, Alzheimer's Disease Education and Referral Center, a service of the National Institute on Aging

The resulting Aβ fragment contains the amino-acid residues 29–42 that are normally embedded in the membrane-spanning region of the precursor APP. This pathway is known as the amyloidogenic pathway of APP processing. There are two forms of Aβ, one with 40 amino acids and the other with 42 amino acids, the latter of which is more hydrophobic and, hence, aggregates more readily into plaques.

The pathogenic properties of these plaques are not completely understood. It has been suggested that presence of these plaques in affected brain regions increase oxidative stress to the neurons [11] and trigger an inflammatory response. However, these pathogenic mechanisms may not represent the full scope of effects. (see **the relationship between plaques and tangles**, section 1.1.2.c).

1.1.2.b Tangles

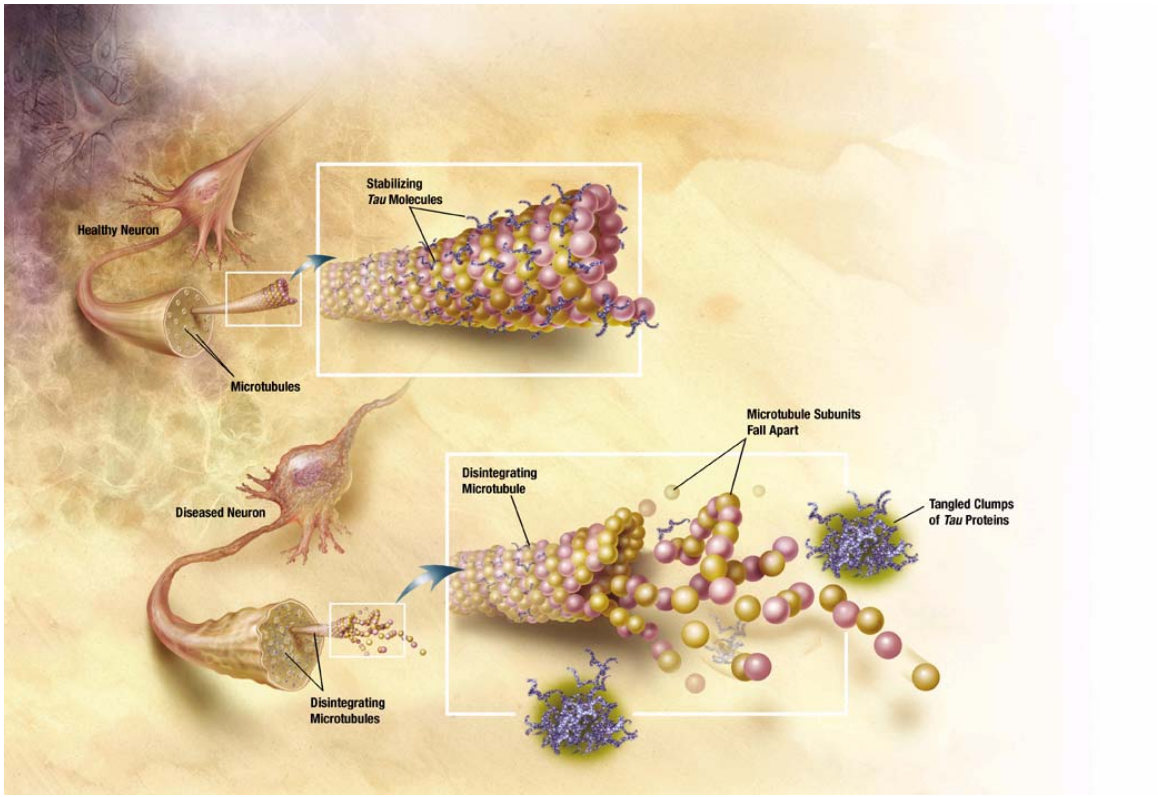
Healthy neurons have an internal support structure of microtubules which are stabilized by the protein, tau. In addition to providing structural support, these microtubules act as a transport and communication system for the cell. In AD, this stabilizing tau protein becomes hyperphosphorylated, which results in a disruption of the ability of this protein to bind. As a result, the role of this protein as the stabilizing factor for microtubles is hindered (Figure 2). Phosphorylation also increases aggregation of the protein and thus fosters tangle formation. Because microtubles are key players in structural support, cell communication and transport, collapse of this system is directly related to a collapse of cell function. This loss of cell function is a central event in the pathogenesis of AD and is a precedent to cell death.

1.1.2.c The relationship of plaques and tangles

Recent findings have indicated that the effects of amyloid deposition and tangle development are linked.[12]. Aβ has been shown to induce mitogen activated protein kinase (MAPK) which leads to tau phosphorylation and subsequent tangle development [13]. MAPK activity normally declines with aging of the immune system, but MAPK pathways are increased in AD. There has been some suggestion that the formation of neurofibrillary tangles is the primary agent in the cognitive impairment characteristic of clinical AD and that this relationship is mediated by the amount of amyloid plaques

present[14]. However, there is

Figure 2: Destabilization of microtubules and subsequent tangle formation



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evidence that Aβ fibrils form pores in neurons leading to calcium influx and the neuron death associated with AD which may indicate pathogenesis independent of tangles [15].

Of particular interest when considering the relationship between plaques and tangles in AD is a new theory of synergistic plaque and tangle effect [16; 17] which provides protection against cell damage. In summary, this theory advances that plaques and tangles are initially formed as a survival response to pre-clinical disease-caused oxidative stress. The theory is based on the premise that unchecked oxidative cell stress will cause almost immediate cell death. For those cells that are able to survive in chronic conditions

of oxidative damage such as AD, unique features of these cells must play a role in their continued survival. A recent article comparing the strikingly similar pathological features of AD and a degenerative age-dependent muscle disease called sporatic inclusion-body myosintis (s-IBM) [17] advances that the development of plaques and tangles in both diseases must be the unique cell features that allow continued survival in chonic stress conditions.. A homeostasis is achieved in which plaques and tangles are formed to manage oxidative stress and the cell manages the plaques and tangles by removing excess production. It is only after disease progression drives free radical activity increasingly high, that plaque and tangle formation overwhelms cell efforts at maintaining a functioning homeostasis. When formation of plaques and tangles in response to oxidative stress is too rapid for effective removal from the cell, plaque and tangles become pathogenic.

The appeal of this theory is that it addresses inconsistency issues in AD. For example, individual variability in the neuron's ability to maintain homeostasis could explain the variability in clinical age of onset. As well, non-demented individuals who are found to have AD pathology upon death could have plaques and tangles as evidence of the early, protective role in AD pathology.

1.2 Disease Genetics

1.2.1 Heritability and variability

Measures of heritability of LOAD are high, with the estimated genetic influence on disease development between 60 and 80%[18; 19]. Studies of LOAD families, however, have not demonstrated Mendelian inheritance. Additionally, disease presentation is variable, with a wide range of values observed for age of onset and rate of decline[19-22]. The cause of this observed variability, both in incidence and severity, is not completely understood, but is likely the result of both genetic and environmental factors acting in concert.

Studies contrasting monozygotic and dizygotic twins are particularly useful in determining the relevant influences of genes and environment because they compare those with shared environments but with potentially differing genetic profiles (dizygotic twins) to those with both shared environments and shared genetic profiles (monozygotic twins). If a trait such as age of onset were, for example, solely the consequence of genetic influences, one would expect complete phenotypic concordance among monozygotic twins as well as genetic concordance at the causative locus or loci. Monozygotic twins are expected to be identical by decent at all alleles. Dizygotic twin pairs, however, are only identical by decent, on average, at 50% of their alleles. Expected concordance for a trait solely genetic in cause among dizygotic twin pairs would be half, with dizygotic twins sharing the identical by decent causative allele on

average 50% of the time. Such studies investigating age of onset have, in fact, suggested that the variability between twin pairs is the result of both environmental and genetic factors but the exact factors involved are still not known. [23; 24].

1.2.2 Known genetic factors

1.2.2.a Early onset disease

There are three known genetic factors that lead to familial early-onset AD: the APP gene on chromosome 21 (see **plaques**, section 1.1.2.a) and the genes coding for the two presenilin proteins on chromosome 14 (PSEN1) and chromosome 1 (PSEN2). Mutations in these genes follow autosomal dominant inheritance and are nearly 100% penetrant. However, approximately 40% of early-onset cases do not follow an autosomal dominant model [25]. Even in families where early onset disease aggregates, many show no mutations in any of the three known genes despite extensive sequencing of open reading frames and adjacent intronic regions [4].

The role of the APP gene in AD was revealed from investigations involving those affected with Down syndrome. Down syndrome patients typically develop AD if they live beyond age 30. Because Down syndrome is caused by chromosome 21 trisomy, genes on this chromosome came under scrutiny as disease-causing candidates. The APP gene, located at 21q21, contains 18 exons, the alternate splicing of which results in eight isoforms. Mutations in this gene can cause an increase in Aβ and/or an increase in the more hydrophobic, amyloidgenic Aβ42 isoform (see **plaques**, section 1.1.2.a). Mutations

in the APP gene are estimated to account for up to 5% of familial AD[26], and are associated with an average disease age of onset of 51.5 years [4].

The most common cause of familial early-onset AD is mutation of the genes encoding presenilins 1 and 2, which alter gamma-secretase activity to increase the production of the amyloidgenic Aβ42 fragment. Mutations in the presenilins, PSEN1 and PSEN2, are thought to cause up to 80% of familial early-onset AD cases [26], and are associated with an average age of onset at 44.1 years and 57.1 years respectively[4].

Although APP and the presenilins are associated with early-onset disease, which is not the focus of this study, identification of these genes was crucial in elucidating the pathology of AD. Studying the biological consequences of the disease-causing mutations established key pathogenic pathways such as APP cleavage to Aβ. Since the early- and late- onset diseases are pathologically identical, such information is relevant to both forms of disease.

1.2.2.b Late onset

Currently, the sole established susceptibility gene in LOAD is the gene coding Apolipoprotein E protein, APOE. This gene has 3 common alleles, E2, E3, and E4 with E3 as the most common allele with an estimated frequency in the general population of 78%. E2 is a relatively rare allele (7%) and may provide some protection against AD. E3 appears to play a neutral role in AD whereas E4 (15%) has been shown to confer an increased risk of development of disease [27-31] [32]. The risk for those with one copy

of the E4 allele has been estimated at 3 times that of a non-carrier and, for those with two copies of the E4 allele, 9 times that of a non-carrier[33]. The E4 allele has also been associated with an earlier age of onset of LOAD [34] and an increased risk of advancement from cognitive impairment to AD[33]. However, APOE4 is neither necessary nor sufficient for development of disease, and only 40% of all LOAD patients carry at least one copy of the allele [8].

The increased risk associated with the E4 allele may be due to isoform-specific differences in binding affinity of APOE. Extracellular cholesterol, such as that released from degenerating nerve terminals, forms lipoprotein complexes with APOE[35]. Altered ability of APOE4 to bind this cholesterol and serve as a ligand to receptors, which remove this potentially toxic extracellular cholesterol build-up, is one theoretical mechanism behind the increased risk for LOAD from the APOE4 allele [36; 37]. Additionally, altered lipid binding of E4 limits APOE's protective role in neuronal repair. Lipids form the foundation of the neuron cell membrane and it is APOE-lipid complexes that serve as the supply for maintenance and remodeling of synapses and dendrites [38]. Furthermore, in vitro evidence has demonstrated APOE-allelic differences in stimulation of neuronal growth in the presence of lipids, with the E3 allele demonstrating protective stimulation but E4 showing no such response [39; 40]. These allelic differences in protective effects may be particularly critical in the early stages of AD.

Differences in binding capacity between APOE alleles may also affect plaque and tangle formation. Alleles E2 and E3 can augment the microtubule binding domains of tau,

which may stabilize its interaction with beta tubulin and inhibit tangle formation [32; 41]. Isoform specific differences are also evident in binding with Aβ. Creation of APOE-Aβ complexes may promote plaque formation by stabilizing the aggregating Aβ and in vitro evidence has shown increased affinity of E4 for Aβ [42]. Furthermore, a dose effect has been shown between E4 and amyloid plaque density [43] with those homozygous for the E4 allele exhibiting the greatest deposition of plaques.

It has been suggested that familial risk for LOAD is linked to APOE status. Recent study results show an effect of APOE E4 status on susceptibility for LOAD independent of familial history, yet find an effect of familial history on development of LOAD only among those with an APOE E4 allele.[44]. Given the complex nature of LOAD, it is likely that additional genetic susceptibility loci are causal in disease development and progression.

1.3 Non-genetic risk factors – the role of lipids

Non-genetic risk factors that have been associated with LOAD include age, sex, education, cardiovascular disease and stroke, diabetes mellitus, smoking, depressive illness, traumatic head injury[45], and a number of dietary factors such as antioxidant intake, alcohol consumption[46], and lipid intake[46-49] The role of lipids in LOAD is a particularly promising avenue of study for several reasons.

First, lipids are an essential part of the structure of neurons. The phospholipid bilayer forms the foundation of the cell membrane, and, because of the extensive surface area of central nervous system neurons, this lipid bilayer represents the primary component of brain tissue. Furthermore, myelin, which insulates the neurons, is particularly lipid enriched, containing 76% lipid as compared to the 43% lipid composition of red blood cell membranes[50]. Cholesterol molecules within this membrane are also vital and serve to increase viscosity and strength while also increasing membrane permeability to non-polar solutes[51]. Secondly, pathways involving lipids are associated with neuronal health and disease[27; 35; 51-58]. Extracellular cholesterol, such as that released from degenerating nerve terminals, forms lipoprotein complexes with APOE[35]. Although the role of APOE in LOAD is not completely understood, impaired ability of APOE4 to bind this cholesterol and serve as a ligand to receptors, which remove this potentially toxic extracellular cholesterol build-up, is one theoretical mechanism behind the increased risk for LOAD from the APOE4 allele [59; 60]. As important however, is the role of lipid transport by APOE in neuronal repair. As age increases so does demand for maintenance and remodeling of synapses and dendrites [38]. APOE's role in transporting lipids to a site of injury is vital to this process, and the impaired ability of the E4 isoform to bind lipids is a critical hindrance [27]. Furthermore, it has been found that, in the presence of lipids, the E3 isoform will stimulate neuronal growth whereas the E4 isoform will not [61; 62].

Cholesterol itself can influence amyloid plaque formation through an effect on amyloid precursor protein (APP) splicing and, through this mechanism, may also be associated

with neurofibrillary tangles. Non-pathogenic processing of APP by alpha secretases releases soluble N-terminal APP fragments into the extracellular space. These fragments are not amyloidgenic and do not contribute to plaque formation. However, when APP is cleaved by the beta and gamma secretase, the amyloid β peptide fragment (Aβ) is produced, which is the foundation of pathogenic amyloid plaques in LOAD [63]. The formation of cholesterol enriched lipid rafts in the cell membrane can favor the co-aggregation of beta and gamma secretases with their necessary substrates, increasing potential for amyloidogenic splicing of Aβ[38; 54; 55; 57; 58]. Experimental work with mouse models showed that an increase in the amyloidogenic Aβ can increase hyperphosphorylation of tau protein.[13] Hyperphosphorylation is the mechanism behind tau's disassociation as stabilizer of neurofilaments and the cause of neurofibrillary tangles. The protein, flotillin 1, has also been found to be associated with cholesterol-rich lipid rafts. Flotillin1 has been found to be over-expressed in neurons containing neurofibrillary tangles and may point to an additional mechanism by which cholesterol increases the formation of neurofibrillary tangles[64].

Observational studies link vascular risk factors to an increased risk for LOAD and include increased age[38; 65; 66], diet[21; 67-72], cardiovascular disease and stroke[8; 54; 66; 73-80], diabetes mellitus[8; 81-87], smoking, and traumatic head injury[8; 88-90]. These factors point to a possible common risk mechanism of compromised circulation and the associated hypoxia and hypoglycemia. The brain is incapable of storing or synthesizing its own glucose and is, as such, particularly vulnerable to this impaired delivery mechanism. Low levels of oxygen and glucose weaken all aspects of brain

function, particularly those in the hippocampal region, which is a region associated with memory [91]. Seriously compromised circulation can lead to direct brain injury through mechanisms such as ischemia. Conversely, brain injuries from stroke or traumatic events may have a component of vascular injury, which further limits oxygen and glucose delivery to surrounding healthy tissue.

Further studies indicate diets high in polyunsaturated fats such as fish to be protective against LOAD[67; 69; 92-102]. Associations between diets rich in fatty fish and protection against LOAD are thought to result from the omega 3 fatty acid content of fish. Because omega 3s cannot be synthesized by the body, a dietary source is necessary and fish is the most common source. These fatty acids, most crucially docashexaenoic acid (DHA), are a fundamental element of neuronal synapses and are essential in their maintenance and repair[92]. Demands for such repair mechanisms increase with age, and are particularly imperative in those with preclinical or diagnosed LOAD. An additional association between high fish intake and LOAD may be through its protection against the pathogenic effects of oxidative stress. Oxidation of DHA renders it useless in synaptic maintenance[103] and, in an environment of severe and sustained oxidative stress, a significant intake of DHA is critical to override the oxidative depletion and maintain the supply needed for synaptic repair. Gene-environment interaction has been implicated between DHAs and APOE in animal models where, among other factors including male gender, a lack of APOE increased oxidation of DHA by 81% [104].

It has been shown that diets rich in polyunsaturated fats increase circulating high density lipoproteins (HDLs) [105], which further protects against LOAD through its effect on circulating cholesterol. Excess intake of saturated fat and cholesterol has been associated both with high circulating cholesterol and with LOAD[35; 54; 66; 68; 106; 107]. HDLs bind with circulating cholesterol and remove it to the liver where it is metabolized. Reduction in arterial plaque-causing cholesterol from circulation helps protect vascular health and its effect on the brain as discussed above. A direct link between circulating cholesterol and pathogenic processes in the brain is yet unknown. However, it has been hypothesized that, although transport of cholesterol from circulation into the brain is usually prevented by the blood brain barrier (BBB), vascular injury, which can be caused by excess cholesterol, may compromise the BBB[35]. A compromised BBB may allow increased influx of cholesterol from circulation into the brain and may confer risk through an increase in neuronal cell membrane concentrations of cholesterol in the lipid rafts, as described above. A summary of some of the published findings regarding lipid intake as a non-genetic risk factor are enumerated in Table 1 below.

Table 1: Summary of some recent findings regarding fat intake and risk for AD

Reference	Environmental factor	Direction of Association	Magnitude of Association
[48]	1 serving of fish/week (v. rarely/never ate fish)	Protective	RR 0.4, [0.2–0.9]
[108]	Fish consumption	Protective	RR = 0.3 [0.1-0.9]
[109]	High monounsaturated fatty acid (MUFA) intake	Protective	OR 0.7 [1.0-4.5]
[49]	upper 5th of saturated-fat intake (v. lower 5th)	Detrimental	RR 2.2, [1.1-4.7]
	upper 5th of trans-unsaturated fat intake (v. lower 5th)	Detrimental	RR 2.4; [1.1-5.3]
[108]	total fat saturated fat cholesterol	Detrimental	RR = 2.4 [1.1-5.2] RR = 1.9 [0.9-4.0] RR = 1.7 [0.9-3.2]
[49]	total fat, animal fat, and dietary cholesterol		Not associated
[47]	High intake of total, saturated, trans fat, and cholesterol and low intake of MUFA, polyunsaturated fatty acids (PUFA), n-6 PUFA, and n-3 PUFA		Not associated

This evidence suggests genes involved in lipid transport and metabolism are promising candidates in an investigation of LOAD susceptibility. We chose six genes as candidates for investigation based on their potential association with neuronal health and their link to pathways involving lipids: Brain-derived Neurotrophic Factor (BDNF), Transthyretin (TTR), Tumor Necrosis Factor alpha (TNFa), Low-density Lipoprotein Receptor-related Protein (LRP1), Apolipoprotein L-3 (APOL3), and Sterol O-acyltransferase 1 (SOAT1). Secondary considerations were distribution in the genome and novelty.

1.4 Candidate Genes

1.4.1 BDNF

Brain-derived neurotrophic factor (BDNF) is a cytokine, a protein that acts on other cells to control cell growth and differentiation as well as regulate immune and inflammatory responses. Neurotrophic factors and their pathways are diverse and, in many cases, can be restricted to particular populations of neurons within the brain[110]. Experimental results suggest that BDNF is active in areas of the brain related to learning and memory. Genetic variants of the BDNF gene have been implicated in differences in cognitive functions[111]. The mechanism behind this effect may be found in BDNF's role supporting colinergic neurons. BDNF supports neuron growth and survival particularly in the hippocampal, cortical and basal forebrain cholinergic neurons[8]. These neurons, which use acetylcholine, are critical to memory and learning. Located in the nucleus basalis and septal region of the brain, they provide connections to areas of cognitive function such as the cortex and hippocampus. It is primarily cholinergic neurons that are affected by AD. The decrease in cholinergic function correlates closely with onset and disease progression in patients with AD [42]. BDNF-supported neuroplasticity, the ability of these neurons to adjust to or compensate for injury, could be critical in AD. Areas of the brain compromised by AD pathogenesis could potentially continue to function if aided by the pathways of growth and pro-survival induced by BDNF.

So, too, the process of memory acquisition and memory consolidation itself may promote expression of BDNF. Learning is associated with an increase of BDNF in synapses [110] and it is possible that epidemiologic associations found between education and LOAD, and between cognitive activity and LOAD, could be through this biochemical pathway. There is evidence that those affected with AD have reduced levels of BDNF in the synapses of the hippocampus and cerebral cortex[112]. In brain tissue affected by AD it has been found that the level of BDNF mRNA is decreased three- to four-fold compared with tissues from those unaffected by the disease. However, the biochemical pathway causing this down-regulation of BDNF in AD is not yet known [113].

In an additional protective pathways, BDNF has been shown in mouse cell cultures to inhibit phosphorylation of tau [114]. Since tau phosphorylation initiates the cascade of tangle formation, inhibition of this process by BDNF will diminish the level and severity of one of two key pathological features of AD.

BDNF expression has been extensively studied in relation to diet with inconclusive results. Although in animal studies, BDNF was found to be up-regulated in key brain regions such as the hippocampus and cerebral cortex under dietary restriction[110; 115], these results have been disputed [116; 117]. Of particular interest to this project, it was recently found that polyunsaturated fatty acids can effect BDNF expression [118]. After mild head injury with subsequent reduced BDNF expression was induced in rats, dietary supplementation with omega 3 fatty acids returned BDNF levels to normal.

1.4.2 TTR

Transthyretin (TTR) is a protein that influences amyloid deposition. As amyloid deposition and plaque formation are key factors involved in AD, the action of TTR on Aβ makes it of interest in a study of AD pathogenesis. TTR has been shown in vitro to inhibit amyloid fibril formation, a precursor of amyloid plaques, by sequestering the Aβ protein[119]. The concentration of TTR in the brains of AD mouse models was significantly lower than in age-matched, cognitively normal control mice[120]. Additional work with human participants has found similar impaired TTR expression in the cerebral spinal fluid of those affected with AD [121]. Experimental work with mice has shown that when Aβ levels are abnormally high in the brain, TTR is up-regulated [120]. This up-regulation may point to a protective mechanism employed by the organism to prevent detrimental plaque formation through TTR's sequestering activity. As further evidence of this, in those mice genetically engineered to produce high levels of Aβ, a lack of both plaque formation and neuronal loss was seen in conjunction with up-regulation of TTR [120].

Also of interest is the relationship between polyunsaturated fatty acids and TTR. Polyunsaturated fatty acids are critical to neuronal function. In infants they are required for growth and functional development of the brain, with deficiency resulting in cognitive disorder. In adults, polyunsaturated fatty acids are essential for maintenance of normal brain function [122; 123]. Epidemiological investigation has linked the intake of polyunsaturated fatty acids, particularly through the medium of fish oil, to cognitive

function. In those who consume significant amounts of fish, a lower incidence of mental illness has been observed [123].

Intake of polyunsaturated fatty acids and cognitive function may be linked through up-regulation of TTR. In studies using rats it was found that as much as a 10 fold increase in TTR expression occurred when the diets were enriched with polyunsaturated fatty acids [124]. Pregnant mice fed a diet supplemented with fish oil had offspring with improved cognitive performance [125]. This mechanism may also be central in AD pathogenesis. Reduced brain levels of polyunsaturated fatty acids were observed in those affected with AD [124] and in those who ate fish at least once a week it was found that risk for development of LOAD was reduced by 60% [126]. Additionally, the intake of polyunsaturated fatty acids has been associated with protection against vascular disease, insulin resistance, schizophrenia and inflammatory disease [126-128], all diseases that have been linked to AD. Polyunsaturated fatty acid intake may contribute to a common metabolic pathway which protects against LOAD either by preventing risk factors such as vascular disease or by contributing to LOAD protection directly.

1.4.3 TNF

Tumor Necrosis Factor alpha (TNF) is a cytokine that plays a major role in the inflammatory process. As inflammation is considered a potential partner in the pathogenesis of AD, proteins involved in this process are of interest. It has been

demonstrated that TNF is up-regulated in AD tissues, with TNF levels found to be 25 times higher in those affected with AD than in those unaffected [129]. Furthermore, it has been established that this TNF is produced locally by the affected brain tissue rather than from a systemic source [129].

Activated microglia may be the source of the inflammatory pathway leading to TNF production and neuronal degeneration. Microglia are macrophages of the central nervous system. Normally inactive, they become activated in response to introduction of an antigen. Once activated, they proliferate and migrate to the site of injury, where they bind to the antigens and produce the toxic cytokins IL-1 and TNF. It has been suggested that Aβ may activate microglial cells, leading to TNF production. Tan et al. [130] demonstrated that increased TNF-alpha production and induction of neuronal injury occurred when Aβ-stimulated microglia were treated with CD40 ligand (a member of the tumor necrosis factor ligand superfamily that reacts with the CD40 receptor to mediate a broad variety of immune and inflammatory responses). Further results from the same study showed microglia from Tg APPsw mice (mice that produce high levels of Aβ) deficient for CD40 ligand had less abnormal tau phosphorylation, suggesting that the CD40-CD40 ligand interaction and the resulting TNF alpha increase is an early event in AD pathogenesis [130].

In addition to its role in the inflammatory process, TNF has been linked to a processes involving APP that may contribute to disease. TNF can upregulate the amyloidogenic pathway of APP processing (see **plaques**, section 1.1.2.a) through stimulation of the

gamma secretase cleavage of APP [131]. TNF, in combination with interferon g, has also been shown to increase the production of Aβ in vitro and inhibits the secretion of neuroprotective soluble APP[132].

A recent study correlated a disease-induced increase in TNF levels with a reduction in insulin-like growth factor-I (IGF-I) [133]. IGF-I is a neurotrophic factor and, like BDNF, supports the ability of these neurons to adjust to or compensate for injury. If increased TNF levels compromise IGF-I availability to the neuron, areas of the brain compromised by AD loose a critical compensatory mechanism.

Conversely, TNF alpha may be expressed in AD affected tissue as a protective mechanism. TNF has been associated both with exciting an inflammatory response and with reining in such a response once begun [134]. This inflammation control may be critical in managing injury to the brain caused by the accumulation of plaques and tangles. In rats, TNF has also been shown to protect against injury in the hippocampus and cortex – areas particularly susceptible to AD. Evidence has also pointed to TNF expression as protective against Aβ induced toxicity [129]. Incubation of human neurons with TNF produced bcl-2, a substance known to down regulate apoptosis [129]. Finally, TNF has been associated with synaptic plasticity and strength [135].

Investigators have looked at various TNF-alpha polymorphisms in relation to AD with mixed results [132; 136-140]. Associations have been found in some populations but not others. Intriguingly, TNF-alpha has been associated with conferring protection from AD

in the form of increased age at onset [141]. Some gene-gene interaction has been observed between TNF and APOE with a TNF polymorphism increasing the risk conferred by the APOE E4 allele but only in some populations [137]. This interaction may be executed through TNF's effect on nuclear factor-kappa B (NFkB) protein, which activates both the release of APOE and more TNF-a [132].

Of particular interest to this project, a recent study in mouse models found dietary enrichment of both cholesterol and polyunsaturated fatty acid down-regulated the expression of TNF-a [124]. Reduced levels of TNF early in AD pathogenesis may be beneficial by limiting TNF induced pathways to cellular damage.

1.4.4 LRP1

The low-density lipoprotein receptor-related protein LRP1 is a multifunctional receptor that works on the intake and degradation of lipoproteins and protease/protease inhibitor complexes [142]. As an alpha-2-macroglobulin (A2M) receptor, LRP1 mediates degradation and production of Aβ [142]. LPR1 also serves as a ligand to APOE and APP, both of which have established roles in AD pathogenesis. The multifunctionality of LRP1, considered with its wide tissue distribution, implicate LRP1 as having a central role in cellular maintenance [143].

Polymorphisms in the LRP1 gene have been shown in vitro to directly affect LRP1 expression [143]. Differential expression of LRP1 may be related to disease mechanisms

in AD. For example, LRP1 expression in those affected with AD is lower than that of age matched unaffected controls, and a later age of onset was observed in those with higher levels of LRP expression who went on to develop disease. It was further shown that the normal age-related decline of LRP expression is more pronounced in APOE E4-negative controls [142].

The effect of LRP1 expression on disease development is uncertain but may be accomplished through one or more pathways which LRP1 shares with other substances associated with AD. Of particular interest to this project is the role LRP1 plays with APOE in cholesterol transport for neuronal repair. APOE-cholesterol complexes bind to LRP1 receptors, which internalize the complex for use in neuronal membrane maintenance [35]. This process serves both to aid cell repair and to clear excess cholesterol from the extracellular space, where its presence may lead to an increase in lipid raft-facilitated amyloidgenic APP splicing. LRP1 has been shown in vitro to mediate clearance of Aβ complexes, the products of amyloidgenic APP splicing and precursors to plaques, via the α2-macroglobulin-LRP1 pathway [142; 144]. It has been observed that reduced LRP expression is correlated with higher soluble Aβ levels and amyloid deposition [142].

1.4.5 APOL3

Apolipoprotein L-3 is a member of the apolipoprotein L gene family. It codes for a high density lipoprotein found in the cytoplasm, where it may affect the movement of lipids or

allow the binding of lipids to organelles. APOL has not received much attention as a potential factor in the pathology of AD (a search of the literature in August 2006 revealed no published investigations of APOL3 and Alzheimer's disease) but has been investigated with regard to its role in atherosclerosis. APOL, as a high density lipoprotein (HDL), is associated with a reduced risk for cardiovascular disease. HDLs take cholesterol from peripheral tissues back to the liver and help lower total serum cholesterol. As the cholesterol content of membranes is important in cellular processes such as gene transcription and signal transduction both in the adult brain and during neurodevelopment [145], the role of APOL in cholesterol transport may be a mechanism for cognitive health and is, thus, of interest in an investigation of AD pathology. Of particular note, APOL expression is up-regulated by tumor necrosis factor-alpha (TNF), described above as another potential agent in AD pathology [146]. The interaction between these two substances may point toward a shared mechanism or pathway for disease.

1.4.6 SOAT1

The gene sterol O-acyltransferase 1 (SOAT1) codes for the protein acyl-CoA:cholesterol acyltransferase 1 (ACAT1). Of the two isoforms ACAT1 and ACAT2, ACAT1 is the protein distributed most uniformly among tissues, including the brain (ACAT2 is expressed in the liver and intestine only). ACAT is an endoplasmic reticulum resident membrane protein that generates cholesterol esters from free cholesterol and fatty acids. ACAT plays a central role in the regulation of cholesterol homeostasis and distribution in

multiple tissues. These include uptake of dietary cholesterol to assembly of very low-density lipoprotein (VLDL) complexes in the liver to control macrophage ability to synthesize and accumulate cholesterol esters. ACAT inhibitors reduce cholesterol absorption from the diet, VLDL/LDL levels in the blood, and the formation of atherosclerotic plaques.

Although ACAT may impact development of AD through its effect on vascular health, ACAT's role in cholesterol transport in the brain is of particular interest for this project. Esterification by ACAT is a critical step in the brain's synthesis and storage of cholesterol. Since cholesterol is a basic component of the neuronal membrane, this ability to synthesize and amass cholesterol is essential to cellular maintenance and repair [35]. ACAT has also been shown to play a role in Aβ generation. A mutant form of ACAT in *Drosophila melanogaster* resulted in a decrease of both cholesterol esters and processing APP[147]. In humans, a polymorphism in SOAT1 is associated with low amyloid load in the brain, low cerebrospinal fluid levels of cholesterol, and reduced risk for AD in ethnically distinct populations[148]. Finally, ACAT inhibitors have recently been proposed for the treatment of AD[149].

2. Methods

2.1 Populations

Evidence exists to suggest genetic risks in LOAD may differ between those in the younger age range of late onset disease (60s to 70s) and those who are more elderly (70s to 80s). For example, the susceptibility for LOAD conferred by the APOE4 allele is associated with earlier onset for disease. After age 73, E4 allele homozygotes do not appear to be at increased risk for LOAD (after age 87 for E4 heterozygotes) [150]. In our investigation, we constructed three case-control populations, one "Younger" (median current age of 74), one "Elderly" (median current age of 87), and one which pools the two previous populations ("Pooled") from three study sets: the National Institute of Mental Health (NIMH), the Oregon Brain Aging Study (OBAS), and the Case Western Reserve University Memory and Aging Study (CWRU) (Table 3).

From the NIMH study set with a sample size (n) of 250 affected siblings, one randomly selected sibling from each affected sib pair (n=97) was chosen for the cases in our Elderly LOAD population. Controls were selected from the OBAS study set of 131 participants above age 85 who, upon ascertainment, met strict criteria for neurological health. Individuals who developed symptoms of dementia during the course of the OBAS study were excluded from our analysis (n=41), with the remaining (n=90) chosen as the Elderly LOAD population controls. The Younger LOAD case-control population was constructed from the CWRU study set (n=427) and consists of 105 cases aged 65 –

75 years old and 322 age frequency matched controls recruited from neighbors, friends, or associates of the cases, and determined to be free of dementia at the time of recruitment. By pooling the Elderly and Younger populations, we created an additional, larger and more heterogeneous case-control population (Pooled) group (n=614). It was thought that with this larger sample size, we may have more power to detect genetic effect despite the heterogeneity.

In all of our case-control populations, cases and controls were assessed in the same way to determine neurological, psychiatric, or medical diseases affecting cognition. Cases had either a possible or probable diagnosis of AD, reached by consensus conference using NINCDS/ADRDA criteria. Controls were excluded from analysis if they received a borderline score suggestive of cognitive impairment. All participants in our case-control populations were Caucasian.

2.1.1 Case ascertainment and recruitment of controls

2.1.1.a Younger

Cases in the Younger population were recruited from clinical settings, the community, and enrollees in the Research Registry of the University Alzheimer Center, University Hospitals of Cleveland. Surrogate respondents for cases were asked to identify friends, neighbors, and organizations to which the case members belonged. From this pool, control participants were recruited for the study. Controls were frequency-matched on age

and gender with cases. The frequency matching method ensures the frequency of ages and genders within the control group match that of the case group, as opposed to matching individual cases to individual controls. Controls who were recruited from friends or neighbors were compared with those recruited from organizations in which cases were members and no differences were found with regard to demographic variables, cognitive performance, or personality.[151]

Of note, in this study additional information was gathered on lifestyle factors such as diet. Because the nature of Alzheimer disease affects recall, surrogate respondents were used for cases enrolled in the study. The surrogates, as a group, comprised 62% spouses, 28% children, and 10% siblings or friends. All cases had surrogates available who had known the case for at least the last 10 years, and who had a close personal relationship with the case [151].

2.1.1.b Elderly

Cases in the Elderly population were recruited as a part of the National Institute of Mental Health (NIMH) Genetics Initiative [152]. Study families were recruited from local memory disorder clinics, nursing homes, and the community surrounding the University of Alabama at Birmingham, Johns Hopkins University, and Massachusetts General Hospital, Harvard Medical School. For inclusion in the study, at the time of ascertainment each family had to include at least two living blood relatives with memory problems. One of the two affected family members was required to meet National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease

and Related Disorders Association (NINCDS/ADRDA) criteria for probable Alzheimer's disease. Although unaffected relatives were recruited as controls when available and willing to participate, no unaffected participants from the NIMH study have been used in our analysis since the sample size was small (n=24).

Controls in the Elderly population were recruited as part of the Oregon Brain and Aging Study (OBAS) from active and healthy, cognitively normal volunteers in the Portland, Oregon community [153]. Recruitment efforts included advertising, contact with senior groups, and mass mailings. Inclusion criteria required participants to be independent in daily activities and unaffected by chronic medical conditions such as hypertension, diabetes, or heart disease. Participants were followed for an average of 6 years, and were assessed every 6 months for cognitive impairment.

2.1.2 Inclusion/exclusion criteria

Cases and controls were examined by PhD- or MD-level clinicians using NINCDS/ADRDA criteria. All were determined to be free of neurological, psychiatric, or medical diseases affecting cognition other than AD in cases. Controls in both the Younger and Elderly population were excluded if they received a score that suggested any cognitive impairment. Spouses were excluded from all control groups to avoid overmatching [151; 154].

2.1.2.a Younger

In the Younger population, those with a history of illness that could affect or impair cognition, such as alcoholism, drug abuse, major head trauma, cancer, stroke, significant medical surgical, or psychiatric illnesses and insulin-dependent diabetes, were excluded from both case and control groups. Furthermore, in the Younger population, cases were excluded if their onset of symptoms were not within 5 years of evaluation at the University Alzheimer Center (to minimize the contribution of pre-morbid features).

2.1.2.b Elderly

OBAS participants, from which Elderly controls were drawn, were excluded from the study if they were found to have chronic medical conditions such as hypertension, diabetes, or heart disease. Documentation of the ascertainment process for the NIMH Genetics Initiative from which Elderly cases were drawn does not indicate if exclusion criteria, such as a history of illness, were imposed after inclusion criteria were met although NINCDS/ADRDA criteria, which was used to assess all NIMH participants includes screening for alternate forms of dementia and risk factors which may lead to vascular dementia rather than AD.

2.1.3 Defining phenotype

2.1.3.a Younger

Among the Younger cases, participants had a probable (79%) or a possible (21%) AD diagnosis that was reached by consensus conference using NINCDS-ADRDA. Probable AD was defined by this criterium as deficits in two or more cognitive areas, progressive worsening of memory and other cognitive functions, no disturbance in consciousness, onset between 40 and 90 years of age, and an absence of systemic disorders or other brain diseases that could account for the progressive deficits in memory and cognition. This diagnosis was supported by progressive deficits in language (aphasia), motor skills (apraxia), and perception (agnosia), impaired activities of daily living and altered patterns of behavior, family history of similar disorders, and consistent laboratory or radiologic results (e.g., cerebral atrophy on computed tomography). Possible AD was defined similarly with regard to cognitive function but with less stringency. Although deficits in two or more cognitive areas as defined above remained a requirement, exceptions could be made in two areas while still being classified as possible AD: (1) symptoms may not have been completely typical of AD or (2) another disease may not have been ruled out as responsible for the dementia [151].

2.1.3.b Elderly

Among Elderly cases, using NINCDS/ADRDA criteria as detailed above, 89.8% were found to have a clinically probable AD diagnosis and 9.8% met criteria for possible AD. An additional 2 participants with insufficient documentation were included, having been found to meet research neuropathologic criteria for definite AD on autopsy. Diagnosis was made by either an MD- or PhD- level clinician at the originating site, based on

clinical evaluation and review of medical records. Consensus conference involving clinicians from all three sites is in progress.

2.1.3 Pathological confirmation of AD diagnosis

Of the 70 Younger cases for whom an autopsy was performed, 57 (81%) were confirmed using neuropathologic criteria as having had AD. Ten of the 70 had suspected dementia but not definitive AD and three were found to have some other form of neurological disorder. Finally, one case was found to be normal. Of note, one control was autopsied and found to have had the pathological signatures of AD. One hundred and twelve (112) of 113 cases (99.1%) autopsied in the NIMH study set, from which the Elderly cases were drawn, were confirmed pathologically as having AD [152]. In the Elderly controls, from the OBAS study, autopsies indicated some participants who died clinically non-demented had significant AD neuropathology.

2.1.4 Crossovers

Controls in the Younger population were assessed at baseline to be free of dementia but were not followed over time to determine if they remained AD free. It is unknown in the Younger population if some controls crossed over to case status post-assessment. Controls in the Elderly group were followed an average of 6 years (until death, withdraw from the study, or study conclusion). Any Elderly controls who showed cognitive impairment were excluded from analysis. If defining AD by clinical criteria, cross over

of controls to cases should not be a problem in the Elderly group. However, as noted above, some controls who died clinically non-demented were found upon autopsy to have significant AD neuropathology.

Case and control status in our analyses was based on clinical symptoms since these data were available for all participants. However, misclassification by clinical assessment or by clinical onset after evaluation may lead to bias toward the null. This bias is due to phenotypic heterogeneity. For example, if controls are actually pre-clinical AD cases, they may have the same causative alleles as the cases. This similarity between cases and controls at the causative locus (loci) will minimize any observed difference between the two groups, despite causality.

2.2 Measurement of lipid exposure

Dietary intake data was available in a subset of the Younger population (n=188) . Measurements of lipid exposure were compiled using responses to detailed dietary questions adapted from the Block Health Habits and History questionnaire. Cases (through surrogate respondents) and controls were queried on the 98 most frequently consumed foods and were asked to recall intake level at each of three age categories in their life: (a) 20s & 30s (b) 40s & 50s (c) 60s and above. They were further asked to provide an estimate of the numbers of servings eaten (per day, per week, per month, or rarely/never eaten). Servings were assumed to be medium in size [154]. Lipid exposure was then calculated from this intake information using software from the National Cancer

Institute (HHHQ-DIETSYS) which provides measurements for 33 nutrients, including total fat, total saturated fat, oleic and linoleic acids, and total cholesterol. Fat intake was standardized for gender, body size, and activity differences[154].

Fish intake, of interest in its approximation of polyunsaturated fatty acid intake through fish oil, was measured from 4 categories: (1) fried fish/fish sandwiches (2) tuna fish, tuna salad, tuna casserole (3) shell fish, and (4) other fish broiled or baked. Servings for these sources were summed to create a single, quantitative variable. Several factors were considered in creating this variable such as common cooking methods and availability of items like pre-packaged fish-fillets at the time these participants were in mid-life. The most limiting factor however was sample size. Only 24 cases had additional dietary data and among these 24, fish intake was low. A larger sample size may have allowed for a more complex assessment of fish intake, however, use of a sum measure helped insure no potentially significant source of fish is eliminated from analysis.

Deriving data from food frequency questionnaires can pose challenges. Issues such as recall bias can be a problem under the best of circumstances and, in the case of AD research, can be a major hurdle. Because the very nature of AD affects recall, surrogate respondents were used for cases enrolled in the study. These surrogates were the source for all environmental data, such as food intake, for cases. All cases had surrogates available who had known the case for at least the last 10 years, and who had a close personal relationship with the case member.

A number of issues surround the use of surrogates in data gathering. There may be concern that surrogates cannot provide accurate information, raising the issue of validity. Even when the surrogate's responses are close to the true responses, their use may introduce systematic bias from consistent over- or under-estimation of environmental exposures. This systematic bias can affect the measure of association. As surrogates were used in this study for data gathering in the case group but not in the control group, further issues of differential misclassification are a concern. To test the validity of surrogate responses in this study population, surrogates were used for healthy controls and the agreement of responses analyzed. Differences between measurements derived from surrogate responses and those derived from the healthy controls themselves were not found to be significant [155].

One strength of the method used in this study, which queries for three age categories, is that it captures information that can be used to reflect early and mid life habits, not just those that occur in the few years immediately preceding disease onset. A particular weakness, however, is that our study relies heavily on recall to gather data on early and mid life habits. Efforts were made to enhance recall, such as reminding respondents that food supply and behaviors may have been different in the time period being queried (for example, respondents were reminded that items such as low fat yogurt or margarine may not have been available at the time). However, the questionnaire itself is among the best of such tools and has recently been validated [156].

Although recall bias may be less of a concern when querying recent food history, limiting the use of food intake data to those years immediately preceding the study may be

problematic. It is not yet understood at what point environmental exposures contribute to disease progression. Some theorize [35] that early and mid-life exposures are those most related to AD development later in life. Studies that rely on measurements of dietary habits of later life must assume that either dietary habits do not change from early to mid life or that it is these later life intakes that are the effect of interest in AD.

The first assumption, that dietary habits remain stable, has been investigated, showing that a change in dietary habits is associated with pre-morbid AD [157]. Significant differences that are found between the food intake of cases and controls when using later life dietary data may simply be an artifact of these pre-morbid changes and not a causative factor in disease development. As well, the second assumption, that it is these later intakes that are the causative factors, is premature at this time since much is not yet known about the time dependency of these exposures. As a result, we have used mid-life values for dietary intake. Use of mid-life values avoids incorporating the biases due to disease-induced changes in dietary habits among cases that may be reflected in the later-life measurements. As well, mid-life values may be more reliably measured by recall methods as compared to reported early-life dietary habits, since they are more recent and may reduce the effect of recall bias on our measurements.

Other methods to determine lipids include the use of plasma or serum total cholesterol as measurements of exposure. These measurements may assess more than one contributing factor, for example, a high cholesterol level may represent a measure of both dietary intake of fats and a genetic predisposition to high serum cholesterol. An advantage of

dietary measures is that the point of intervention is clear – diet – whereas circulating lipids may require multiple intervention points to address both diet and other contributing factors. As well, a problem with circulating lipid measures is that they only query a single time point – the time of the blood draw, and may be highly dependent on individual consumption in the period preceding blood draw. However, these blood-based measures also provide an excellent characterization of lipid exposure experienced by the body, which may be a more useful tool when investigating biological pathways. Future work with our study population could include further processing of our samples to assess the relationship of these measures to the food intake data as an independent measure of exposure.

2.3 SNP Selection

Single nucleotide polymorphisms (SNPs) were used for genotyping. These markers are abundant in the genome, with a SNP that has a minor allele frequency greater than 1%. occurring about once every 300 base pairs of sequence. Numerous on-line resources exist that provide information related to SNPs and aid in selection. These convenient and extensive information sources, in combination with the cost-effective laboratory methods used to genotype SNPs, determined our choice of markers.

Our SNP selection process was developed with the intent of both maximizing the potential for identifying causative SNPs (Table 2) and sufficiently representing the diversity of a gene. This process is enumerated below.

(1) no less than 3 SNPs per gene
(2) investigation of SNP and AD or potential pathway to AD in previous study
(3) informative SNPs
(4) validated SNPs
(5) bioplausibility
(6) gene characterization

(1) No less than 3 SNPs per gene

Rationale: Because genotyping issues, such as failure to amplify the PCR product, or monomorphism in our population, will necessitate excluding some SNPs from analysis, we genotyped at least three SNPs per gene. Even with loss to exclusion of one third, we can expect to have at least two SNPs on each gene to characterize, create haplotypes, and to use in association analysis. Although identification of a causative SNP would be ideal, it is not necessary to find association between the gene in which a causative SNP exists and disease if we genotype SNPs that are in Linkage Disequilibrium (LD) (see section 2.6.2 for a detailed description of LD) with a causative SNP. More than three SNPs were selected when the gene was larger and thus more difficult to characterize with only a few SNPs, or when the gene was of particular interest for biological reasons.

(2) Association implicated in AD literature between SNP and AD or between SNP and potential pathway to AD

Rationale: Although some of the genes in our study have been implicated in AD pathogenesis, there is hardly a consensus regarding true effect. Associations found in some populations are not replicated in others and further study is necessary. Of particular interest with regards to lipid metabolism would be differences in effect from previous studies. A review of the current literature was conducted to identify SNPs in our

candidate genes that had been previously investigated in relation to AD. If such SNPs exist, they were included for genotyping. .

(3) Informative SNPs (heterozygosity greater than 1%)

Rationale: SNPs with exceedingly low heterozygosity may have such rare minor alleles that our sample may be monomorphic at that locus. With a sample size such as ours, even if very rare minor alleles were to appear, they would be in such a small number as to be statistically insignificant. Hence, an association study to test for differences between cases and controls at that locus (see **association studies**, section 2.6.1) is ill advised. Because AD is not a rare disease in our population, affecting 25% of people 75 and older and 50% of all people aged 85 and older, we would also expect causative mutations to be less rare. Assuming that the hypothesis of common disease – common mutation (see **haplotypes,** section 2.6.3) is correct, rarer SNPs are less likely to be causative and thus less desirable as genotyping targets.

(4) Validated SNPs

Rationale: We chose to use validated SNPs with established allele frequencies. Unvalidated SNPs may not be true polymorphisms and may have appeared as such due to sequencing error. Choice of validated SNPs provides more insurance that these are true polymorphisms and that we can expect variation in our population. As well, a SNP of unknown frequency may be uninformative in our population and thus not allow for analysis, as stated above. As our genotyping resources were limited, the risk of uninformative, unvalidated SNPs was too costly.

(5) Bioplausibility

Rationale: For a SNP to be causative, it must have an effect on the gene product. SNPs in a position in the coding sequence to cause changes in the amino acid chain, and possibly in the conformation of the resultant protein, were selected when available. By selecting SNPs with such functionality, the potential exists for identifying the causative mutation itself, not just the area in LD with the mutation.

Bioplausibility was assessed based on (1) location in the DNA sequence, such as in a promoter region, a stop codon, or splice site, (2) functionality of the region, such as intronic or exonic SNPs, and (3) identified as causing amino acid changes. The following resources provided the information to aid in assessing function. Polyphen, an on-line reference which identifies potential effects of SNPs, provided evaluations of SNP function such as "possibly damaging," "probably damaging," and "benign." Not all SNPs that appeared in other databases such as Locus Link and Ensembl appeared in Polyphen. All SNPs investigated, however, had neighboring sequence information. These sequences were tested in the Human Gene Mutation Database (HGMD) which identifies functional properties of DNA sequences. Although some SNPs selected based on previous studies were in areas of interest such as promoter regions, we found limited success in identifying other SNPs with significant properties in terms of sequence. Functionality with regard to region was assessed using Ensembl, an online gene database. Exonic SNPs, which will be translated, were chosen above intronic SNPs that are removed from the final coding sequence by the normal process of splicing. By matching

SNP position with start and stop sites of introns and exons, it was possible to identify SNPs that occurred on splice sites or on the cusp of such sites. Mutations at these sites may alter splicing and potentially alter the amino acid sequence. Finally, as reported in both Ensembl and Locus Link, amino acid changes resulting from a nucleotide change at the SNP site were noted.

Validated, informative SNPs that were indicated as having biological implications in the above assessment were selected for genotyping.

(6) Gene characterization (SNP location)

Because genotyping resources were limited, it was desirable to consider how SNPs were distributed over the gene. SNPs in close proximity to each other were less likely to be chosen than those farther apart. Exon and intron numbers were assigned so that SNPs would not be selected from the same coding region. Identification of haplotype blocks was attempted so that haplotype-tagging SNPs could be utilized.

The theory behind haplotype tagging SNPs is that areas in tight LD will be inherited as blocks together over generations (see **linkage disequilibrium**, section 2.6.2). Since these areas are inherited together, SNPs that occur in the same block are redundant in the information they give. For example, even if one SNP is closer to the causative mutation than the other, if both are within a block of tight LD, both should show association with disease and, thus, only one of the two SNPs is necessary to identify the area. Using only one SNP per block can reduce the number of SNPs needed when genotyping while

maintaining power to detect association. Haplotype blocks were compiled using software on the HapMap site which graphically shows pairwise LD values between SNPs. For each gene, users can manipulate haploytpe blocks to maximize the amount of LD between SNPs within each block but with parsimony of blocks. The result is a gene divided into blocks of LD which are conserved over meioses and between which recombination is more likely. Not all SNPs found in Ensembl or Locus Link were available on HapMap, and so not all SNPs could be assigned definitively to a block. Blocks were used to reduce redundancy in SNP selection.

A summary of the selection procedure for each SNP is detailed in Table 2. The selection criterion "informativity" includes both expected heterozygosity and SNP validation. For SNPs selected based on previously reported associations with AD, reference to the relevant literature is provided in the References and notes column. At the time of SNP selection, the number of validated SNPs with sufficient expected variability was not large within some of our genes and finer criteria for selection such as shared LD with another selected SNP was not a concern. In the few cases where one SNP was selected above another so as to reduce redundancy and characterize a separate LD block, that block membership is noted in the References and notes column.

Table 2: Genotyped SNPs and Criteria for Selection

Gene	Size (kb)	SNP ID	Selection Criteria	Frequency [Heterozygosity]	Functionality	Ref or notes
BDNF	66.72	rs6265 Val66Met	Implicated in prev study	0.675 / 0.325; 0.73 / 0.27 (Multi nat)	Non-syn AA change Assoc. w/ risk for AD	[158], [112], [159; 160]
		C270T	In prev study		Assoc. w/ risk for AD	[161-165]
		rs1048220	Informativity Bioplausible	0.98 / 0.02 (Multi nat); Some populations homozygous for c allele	Polyphen: probably damaging non-syn AA change	
TTR	7.16	rs3764478	Informativity Location	0.218 / 0.782 (E. Asia)		
		rs723744	Informativity Location	0.43 / 0.57		
		rs1080093	Informativity Location	0.54 / 0.46 (CEPH); 0.625 / 0.375 (E Asia) 0.25 / 0.75; 0.643 / 0.357 (N.Amer)		
		rs3764476	Informativity Location	0.774 / 0.226 (N.Amer)		
		rs3794884	Informativity			
		rs1804118	Informativity Bioplausible Location	0.771 / 0.229 (N. Amer)	polyphen: probably damaging non-syn AA change	
TNF	2.96	C-850T	In prev study		risk of AD w/ APOE4	[137; 139]
		-308/A	In prev study		increase promoter activity; effects age at onset	[132; 140; 141; 166; 167]
		-238/G	In prev study		associated w/ decreased transcriptional activity	[132; 141; 166; 167]
		rs3093661	Informativity	0.93 / 0.07 (Multi-nat); 0.96 / 0.04 (S. Amer); 0.913 / 0.087 (E. Asia); 0.979 / 0.021 (Africa)		
		rs3093664	Informativity	0.854 / 0.146 (PGA, Africa, N. Amer) 0.935 / 0.065 (Multi nat)		
LRP1	85.04	rs1799986	In prev study	[0.128]	Assoc. w/ risk for AD	[80]
		rs2228186	Informativity Location	[0.270]		
		rs2229278	Informativity Bioplausible Location	[0.293]	non-syn AA change	
		rs1800139	Informativity Location	[0.471]		
		rs7397167	Bioplausible Location		splice site, non-syn AA change	
		rs1800156	Informativity Location	[0.424]		
APOL3	26.05	rs132618	Informativity Location	0.5 / 0.5 (Multi nat); 0.360 / 0.640 (E Asia)		Hapblock 1
		rs132622	Informativity Location	0.370 / 0.630 (E Asia)		Hapblock 3
		rs132638	Informativity Location	[0.42]		Hapblock 5
SOAT1	110.2	rs1543876	Implicated in previous study			[148]
		rs2152318	Implicated in previous study	[0.427]		[148]
		rs1044925	Implicated in previous study		splice site, assoc w/ low amyloid	[148]

2.4 Genotyping (Laboratory) methods

Genotyping was performed using the TaqMan SNP genotyping assays. Both ready-to-use and custom assays were utilized. In brief, the TaqMan process uses multiplex polymerase chain reaction (PCR) with florescent tagged probes to identify SNPs. What is unique about the TaqMan probes is that they are labeled with a reporter florescence at the 5' end and a florescence quencher at the 3'end. When the reporter and quencher are held together by the probe (just 20 to 30 base pairs apart), the florescence of the reporter is absorbed by the quencher and no signal can be observed. As the Taq polymerase replaces the probe with bases, the 5' florescent label is released into the substrate, moving away from the 3' quencher, and producing a signal. As this is a multiplex process, each allele has a distinct florescence. Heterozygotes emit two florescent signals and homozygotes emit only one. Because both strands of DNA are copied during PCR, there is both an exponential amplification of the gene and of the florescent signal(s). A threshold is set for the florescence above which the alleles are called. In the event of a mismatch between probe and allele, incomplete annealing of the probe to the DNA strand will prevent the Taq polymerase from replacing the probe base by base and freeing the reporter from the quencher.

There are a number of issues that can arise in this process impacting the genotyping data. Stringency of primers is essential to avoid non-specific amplification (where primers are complementary to and anneal to areas of the genome other than the target sequence, initiating amplification of DNA that does not include the SNP). Primer sequences can be

checked in BLAST, NCBI's sequence database, to ensure primers are specific to the sequence of interest. Contamination can also be an issue when DNA from a person who is not the study participant is introduced into the sample from sources such as saliva. This can result in inaccurate allele calling but can be controlled through good laboratory practices. Volume issues such as poor DNA concentration or evaporation of the wells can cause poor or failed amplification, resulting in an inability to call an allele. Evaporation can be prevented with laboratory techniques and devices. Repeating the TaqMan procedure for poorly amplified wells can often resolve the ambiguous or missing signals. A genotyping issue which is unrelated to process is monomorphism in the population. If, at a locus, we find our population is uninformative (monomorphic), this locus will be discarded. The pre-made, validated assays included C_11592758 and CR014434 in BDNF, C_1278231 and C_11735433 in TTR, C_11918223 and 2215707 in TNF, C_1955081 in LRP1, C_1088426, C_11476856, and C_15851 in APOL3, and C_1805922 in SOAT. The custom assays are detailed in Table 3.

Despite excellent laboratory methods to minimize genotyping error and SNP selection criteria which included consideration of infomativity, we had a loss to genotyping error/monomorphism of 38.5%. For the remaining SNPs, pair-wise Linkage Disequilibrium (LD) was estimated between SNPs using the Haploview program to calculate D' and R^2 (see figures 5-14 for LD figures). SNP density as a representation of genetic diversity is enumerated in Table 4.

Table 3: Custom assays for polymorphisms analyzed in our study
*Position given reflects reported positions in Ensembl Human GeneSNPview in September 2004

Gene Name (Symbol)	Chr. Loc	Position*	RS #	Alleles	Forward Primer Sequence	Reverse Primer Sequence	Probe Sequences
Transthyretin (TTR)	18q12.1	27422470	rs3764478	A/C	5'-agtgctccaaaccggacttg-3'	5'-agacaaacatgtagaaaacatagagaagacaa-3'	VIC-tcttggcttgtatttg FAM-tcttggcttttatttg
		27425667	rs1080093	C/G	5'-ccaggctaatcccacgatcac-3'	5'-tgaactggctaatgaaggtgagaag-3'	VIC-cactcttggacatgaa FAM-cactcttgcacatgaa
		27428447	rs3764476	G/T	5'-ctacccctcagccagcag-3'	5'-aacagtctcaggtaggcagatctat-3'	VIC-cagcccctaccctcga FAM-agcccctaacctcga
Low-density Lipoprotein Receptor-related Protein (LRP1)	12q13-q14	55854029	rs2228186	C/T	5'-ggcgacggctcagatgag-3'	5'-ccaccctccctacccttctc-3'	VIC-ctcaccgcagagct FAM-cctcaccacagag
		55871411	rs1800139	C/T	5'-tccacaggtgatcctaaagtcaga-3'	5'-acccagtcagtccagaaaatgtg-3'	VIC-catacacggccagcc FAM-catacacagccagcc

Table 4: Measures of SNP density after genotyping

Gene	Chr. Loc	Gene Size (Kb)	Exons per gene	Position	Distance to previous SNP	LD to previous SNP**
BDNF	11p13	66.72	2	27644225		
BDNF	11p13	66.72	2	promoter	3273	64
TTR	18q12.1	7.16	4	27422470		
TTR	18q12.1	7.16	4	27424463	1993	
TTR	18q12.1	7.16	4	27425667	1204	98
TTR	18q12.1	7.16	4	27428447	2780	99
TTR	18q12.1	7.16	4	27428958	511	99
TNF	6p21.3	2.96	4			
TNF	6p21.3	2.96	4		data not available	81
LRP1	12q13-q14	85.04	89	55821533		
LRP1	12q13-q14	85.04	89	55854029	32496	29
LRP1	12q13-q14	85.04	89	55871411	17382	92
APOL3	22q13.1	26.05	3	34809313		
APOL3	22q13.1	26.05	3	34812281	2968	99
APOL3	22q13.1	26.05	3	34815534	3253	20
SOAT1	1q25	110.2	18	176519190		

**Linkage Disequilibrium (LD) was measured using D' and R^2 with the results from the D' calculation shown here. The D' scale is of 0 to 100: 100 = strongest non-random association between two loci, 0 = complete independence.

2.5 Hardy Weinberg Proportions/Equilibrium (HWE)

Departure from Hardy-Weinberg proportions was evaluated for each case-control group using Pearson's chi-squared test which compares observed genotype frequencies with

expected Hardy-Weinberg values as applied in the Genetics package of R. Cases and controls were each tested separately as well as the case-control population as a whole.

Statistically significant deviation from HWE in the data set may result from violations of HWE assumptions, which include random mating, infinite population size, with no selection, mutation or migration. Violation of these assumptions can have varying effects on expected genotype frequency, such as excess homozygosity due to non-random mating or selection or deviation from expected proportion due to genetic drift in a small population size. Departure from the assumptions of HWE may not be the only source of HWE deviation. Genotyping error and linkage to a disease susceptibility locus can also contribute to deviations from expected proportions. Assessment of conformance with HWE in the data can help identify loci which require added scrutiny.

Loci that displayed goodness of fit deviations from HWE received added attention to determine the manner in which they departed from HWE. Deviations at loci which appeared to have resulted from laboratory error were considered for exclusion from analysis. Because added scrutiny of loci which diverge from HWE may also help identify a susceptibility locus, careful consideration was given to the potential for HWE divergence due to underlying biology. A polymorphism associated with disease risk may or may not be in HWE among cases but there is more power to detect association between a locus and disease if a departure from HWE is found at that locus among cases[168; 169]. Departure from HWE due to underlying biology can also be found in controls, as well [170], and so HWE was assessed in the population as a whole as well as

in cases and controls separately. Because these multiple tests of HWE conformity can result in a high type I error rate, the significance level for the tests of HWE was adjusted using a Bonferroni correction.

2.6 Assessment of Association

2.6.1 Association Studies

A case control study is a fitting design to investigate genetic determinants of AD. Because age of onset in AD is quite late, study designs using family based methods pose distinct challenges. Most affected offspring have long out-lived their parents, and so a sampling design such as parent offspring trios is rarely practical. Even sib-based methods face difficulties. Competing causes of death are common in the age group most affected by LOAD and siblings may have died before AD status could be ascertained. Even under the best circumstances, recruitment in family based studies is more labor intensive (read: costly). Because the use of unrelated individuals allows one to cast a wider sampling net, case control studies can allow for a larger sample size. Finally, although recall bias is a primary concern in case control study designs, since genotypes are fixed, their assessment is not subject to the vagaries of memory and is free of this most often referenced source of bias.

Linkage methods, often used with family based designs, are unlikely to identify the causative locus in a complex disease. As linkage methods are based on observed

recombinations, at a certain level (about 1 centiMorgan), the probability of observing a recombination between two markers is quite small even within large pedigrees[171]. Narrowing a linkage region to an actual locus using family based designs is further hampered by incomplete penetrance, genetic heterogeneity, and the presence of phenocopies [172]. Additionally, the limited contribution of any one locus to a complex disease decreases the power to detect the loci using linkage methods as compared to association studies [173].

Association studies are based on the simple logic that if the cause of a disease is observable, then you should expect to observe this causal factor more often in those with the disease than those without the disease. Such studies measure the extent to which a factor is observed more often in those with disease and employs a statistical test to determine what amount of risk this factor confers. Association studies can test any number of measures, such as allele frequencies, behaviors or dietary values, for these significant differences between cases and controls. When an allele is found significantly more often in those affected with disease than among those unaffected, one can say the allele is associated with the disease. This association, however, is not necessarily causative. When investigating genes, the associated allele can be the causative mutation, tightly linked to the causative locus (i.e., linkage disequilibrium), or correlated with the causative locus for other reasons.

To test association between single loci and disease in our populations, we utilized two approaches: assessment of a main, unadjusted SNP effect and assessment of SNP effect

after adjustment for covariates. To test main SNP effects, contingency tables were constructed and chi square tests executed for each case-control group to assess the unadjusted association between genotype and dichotomous disease status. To evaluate adjusted SNP effect, logistic regression models, as applied in SAS 9.1 software, were used to assess and adjust for the covariates age, gender, and APOE genotype**.** Both main effects and interactions with genotype were examined for each covariate. Age was defined uniformly for cases and controls as current age, which represented either age at last examination or age at death. Age at disease onset was examined in a separate case-only analysis. Both main effects and interactions were examined for each covariate. Mode of inheritance was modeled initially as additive, with later tests executed to investigate dominant models.

2.6.2 Allelic Association and Linkage Disequilibrium

Allelic association occurs when alleles at two distinct loci appear together more frequently than expected by chance alone given the known allele frequencies. For example, at equilibrium, for two loci with alleles 'A' and 'a' at the first locus, and alleles 'B' and 'b' at the second locus, the frequency of the haplotype AB should be equal to the product of the allele frequencies for A and B. Deviation from this expected value indicates allelic association. This non-random association between two loci can relate to physical proximity on the chromosome (i.e., linkage disequilibrium [LD]) or can be due to chance, genetic drift (the stochastic process of allele frequencies drifting to 0 or 1, reducing heterozygosity), founder effect (genetic drift due to isolation of a small mating

population), mutation (where high mutation rates at a SNP will show little or no LD with nearby markers), recombination (where high recombination rates will disturb evidence of linkage even with nearby markers) selection (SNPs neighboring the preferred variant will increase in frequency), or population stratification (see **population stratification**, section 2.6.5).

Allelic association can be measured using a number of methods, most commonly with the correlation coefficients R (or Δ) and R^2, Lewontin's D', population attributable risk δ, Yule's *Q*, or Kaplan and Weir's proportional difference *d* [174]. All 5 measures are based on calculating differences between observed haplotypes and expected haplotypes under independence (See box below).

$R^2 = (p_{AB}p_{ab}-p_{Ab}p_{aB})^2/(p_{A.}*p_{a.}*p_{.B}*p_{.b})$

$\delta = (p_{AB}p_{ab}-p_{Ab}p_{aB})/(p_{\_B}*p_{ab})$

$d = (p_{AB}p_{ab}-p_{Ab}p_{aB})/(p_{\_B}*p_{\_b})$

$D' = (p_{AB}p_{ab}-p_{Ab}p_{aB})/ Dmax$

Where

$Dmax = \min[p_{.b}p_{A.}-p_{.B}p_{.a}]$ if $D>0$,

$\min[p_{A.}p_{.B.}-p_{a.}p_{.b}]$ if $D<0$

$Q = (p_{AB}p_{ab}-p_{Ab}p_{aB})/ (p_{AA}*p_{bb} + p_{aB}*p_{Ab})$

Formulas based on two biallelic loci A,a and B,b with haplotype frequencies, p.

Both D' and R^2 were calculated, as applied in Haploview software [175] between all SNPs in each gene using the Pooled dataset. The measures of D' and R^2 provide somewhat different assessments of SNP association. D' incorporates in its calculation a measure of expected allele frequencies under independence whereas R^2 is a more direct measure of colinearity. D'is a better measure of LD than R^2 when minor allele frequencies vary across the marker loci [174] and D' is scaled so that regardless of minor

allele frequency, the value always ranges from 1 to -1. However, D' can be upwardly biased in small samples [176]. R^2 describes the capability of a "tag" SNP to predict the variability of a comparison SNP. However, when one of the two SNPs has a low minor allele frequency, it is uninformative for the other SNP, even if the two SNPs are closely associated. In this situation, R^2 may be quite low and not reflective of the amount of LD between the two markers.

When multiple SNPs on the same gene are found to be associated with disease, pairwise tests that result in large D or R^2 values would indicate strong LD between SNPs and provide further evidence of a causative mutation in that area of the genome. When only a single SNP in a gene shows association with disease, low pairwise measurements of LD with other SNPs on that gene would explain the singular association.

2.6.3 Haplotypes

One assumption underlying our analyses is that genomic regions near a disease-causing locus are shared more closely among those affected with disease than of those unaffected. These genomic regions may take the form of haplotypes. The common disease/common allele hypothesis posits alleles with high frequency (greater than 1%) and low penetrance are those that confer susceptibility to common disease [177]. Under this hypothesis, if the disease is common then the disease-causing allele must also be fairly common and the mutation must be relatively ancient (as the proliferation of the allele is a function of both the number of offspring and the number of generations since the original mutation).

Seemingly unrelated individuals with the same disease may have inherited a common genetic component over many generations from the same ancestor in whom the sporadic mutation first developed.

Because LD degenerates with each recombination and since recombination is a function of meiosis, if the mutation is quite old (the number of meioses that have occurred is large), then the ancestral portion of the genome in LD with the causative locus will be quite small. To capture an association in this small region, ideally a high density of SNPs should be genotyped. However, when this has not been the case, a haplotype analysis may provide some power to assess association across the region.

Haplotyping can be an important step in the analysis of genetic data. An advantage of testing haplotypes for disease association, in addition to single SNPs, is the potential of additional power to detect association. Simultaneous consideration of multiple SNPs across a genomic region, as done with a haplotype analysis, adds information and may ease demonstration of association with disease. Take, for example, the event in which the true disease locus has not been genotyped but two flanking SNPs have. The power to detect association of this genomic region with disease will be greater when evaluating the two-locus haplotype than if testing the two SNPs separately, especially if LD between the flanking SNPs and the true locus is not very high. Conversely, if the true disease locus has been genotyped, a haplotype-based analysis incorporates superfluous information which may obscure the observed effect (by causing "noise"). However, by assessing each SNP individually before conducting a haplotype analysis, weakening the observed

effect of a true disease locus is not a concern. Any individual SNP effect will be captured in this first analysis.

Several methods may be used to construct haplotypes. Molecular methods, such as long-range PCR that sequence the DNA for each individual at the gene of interest, are thorough but prohibitively expensive and time-consuming methods in most situations. Pedigree analysis can also be used to determine haplotypes but can only be used when sufficient family data are available. Haplotypes for unphased data can be estimated using algorithms, one of which, the EM algorithm, we will employ with these data. Other methods include Clark's algorithm, the Markov chain Monte Carlo and psuedo-Gibbs sampler approaches proposed by Stevens and Donnelly, [178; 179] and a Bayesian inference algorithm[180]. Estimates derived from Clark's algorithm are not as accurate or unique as those generated by the EM algorithm and the algorithm cannot start when there are no homozygotes or single-site heterozygotes in the population [181]. Baysian approaches give as equally good estimates as the EM algorithm if there is low intermarker LD, and slightly better estimates if there is high intermarker LD. The main advantage of the Baysian methods is the ability to haplotype larger numbers of markers, which is not a concern here.

Our choice in haplotyping methods was driven by our choice of analysis method to evaluate allelic association, namely the regression approach implemented in the Haplo.Stat package [175]. Haplo.Stat employs the EM algorithm to determine haplotypes in a "progressive insertion" variation. Essentially, instead of considering all

possible haploypes before iterating the algorithm, Haplo.Stat progressively inserts loci onto increasingly longer haplotypes with a trimming function. If a haplotype probability falls below a pre-determined cut off (trimming value), then it will be eliminated from the algorithm even if all loci have not been added to it. If a trimming value of zero is entered, the computation of the haplotypes follows the classic EM algorithm.

2.6.3.a EM algorithm to estimate haplotype frequencies

The EM Algorithm constructs haplotypes using maximum likelihood estimation in a two-step iterative process of first expectation (the E-step) and then maximization (the M step). The essential problem in unphased data is that there may be more than one pair of haplotypes consistent with the observed genotype. The EM algorithm first assigns haplotypes to the ambiguous individuals by calculating the frequency of the known haplotypes and then makes an estimate of the ambiguous ones. This is done in the E step by computing the conditional expectation of the complete data log likelihood (where the complete data comprise the combination of both known, assigned haplotypes and estimated, ambiguous haplotypes) given the observed data and assigned parameters. This function is then maximized in the M-step. The goodness of fit between the expectation estimate and the maximum likelihood of this estimate based on the observed data is then tested. The process is reiterated until convergence (when the difference between the estimate and the maximum likelihood of the estimate is negligible). The EM algorithm assumes HWE but has been shown to be robust to departures from HWE [181]. Although this algorithm is dependent on the initial parameter values chosen, starting the

algorithm from divergent points can help ensure convergence at a true global, rather than local, maximum likelihood [175]. A disadvantage of the EM algorithm is that it can become computationally intense with many loci. Given the number of markers in our sample, however, this is not a problem with our data.

2.6.3.b Use of Haplotypes in a Generalized Linear Model

Haplotype frequencies were calculated for cases and controls in each population and association with disease evaluated using score tests, as executed in the Haplo.Score program of the Haplo.Stats software [175]. For each haplotype, a score statistic, based on a generalized linear model (GLM) is calculated under the null hypothesis of no association of the trait with genotype. Given a simple logistic GLM where Y is the trait, X_n are the haplotypes, and β_n are the haplotype specific log-odds, the model can be writen as

$E[f(Y)] = \alpha + X_1\beta_1 + X_2\beta_{2...} + X_n\beta_n$. Haplo.stats uses a GLM to expresses the probability of the subject's trait for each individual. The product of all individual probability functions for the sample forms the likelihood function. The score statistic, $S = U'V^{-1}U$, is determined by taking the log of the likelihood and computing partial derivatives. U, the score function, is a vector gradient of partial derivatives of the log-likelihood function and U' is the transpose of that vector. The variance (V) is equal to the Fisher's Information Matrix which is the negative of the second derivative of the log-likelihood (the first derivative of the score function). This score statistic computes a value for the comparison of the score function evaluated under the null hypothesis to the maximum

likelihood (which is equal to a vector of zeros). These values are distributed as a chi square random variable with degrees of freedom equal to the number of parameters. Although this model becomes considerably more complicated when ambiguous haplotypes are considered, Haplo.score adjusts the variance matrix accordingly [175]. This GLM framework has the additional advantage of assessing confounding by covariates.

2.6.4 Age of onset analysis

The alternative, continuous disease measure of age at onset was examined in a separate case-only analysis. Using a one-way ANOVA test with two degrees of freedom as applied in SAS 9.1, significant differences in age at onset were assessed at each locus between three groups – those homozygous either for the minor or major allele at the locus and those heterozygous at the locus. For the purpose of the test, the three groups were coded as independent genotypes. An F statistic was used as a measure if variation in average age at onset between the three groups is significantly different and so indicate a particular genotype as associated with either an earlier or later age at onset of disease.

2.6.5 Population stratification

In a case-control study of unrelated individuals, when association is found between a locus and disease, a consideration of population stratification must be made. Population stratification, which can occur when two or more sub-populations of separate genetic descent are present in the population or when separate sub-populations have recently

mixed, can confound a causal association between the susceptibility locus and disease. Although a locus may appear causal to disease, it may, instead, only indicate membership in a sub-population. If this sub-population has a higher incidence of disease for whatever reason (environmental or genetic) any allele that is informative for this subgroup will appear associated with disease regardless of causation. A classic example of this kind of confounding is detailed in Knowler's work with the Pima Indians [182]. Assessing and controlling for the potential confounder of population stratification should be considered when using case-control populations of unrelated individuals.

Assessment of population stratification can be done using a variety of methods. An intuitive method is to match cases and controls on group membership such as ethnicity. Ethnicity has been found to be a good surrogate for group membership[183; 184]. Ardlie demonstrated in 2002 that controlling for participant and parental birthplace can significantly reduce a population stratification effect [185] with this conclusion repeated in subsequent studies [186; 187].

Subgroups may not, however, be easily recognized [188]. A method that may be effective when sub-populations are not easily categorized is Genomic Control. Advanced by Devlin and Roeder in 1999 [189], this method is based on the premise that type-I error rate inflation due to population substructure, and/or cryptic relatedness is fairly uniform across the genome and can be approximated by $\lambda\chi^2$ with 1 degree of freedom. The measure, λ, is a constant which represents the uniform inflation due to population structure can be estimated from the empirical distribution of tests statistics at unlinked

loci by calculating the median value of the test statistics and dividing by a constant such as 0.675 [189] or 0.456 [190]. To correct for this inflation, the test statistics at the experimental markers are divided by λ (when λ is greater than 1) and then p-values are calculated from the chi square distribution based on these adjusted test statistics.

Another method that can assess and adjust for population substructure is Structured Association proposed by Pritchard, et al in 2000. This method is based on the theory that if sub-populations are present, null alleles in this subpopulation will, simply by virtue of being informative for the sub-population, show association to the trait [191]. Pritchard's method first tests for population stratification using unlinked markers and then infers population structure and ancestry. These values for structure and shared ancestry are incorporated into the model that calculates a likelihood ratio statistic thereby controlling for ancestry and increasing power. The result is improved detection of true association to disease.

Computer programs such as ADMIXMAP and Structure [191] with its companion program STRAT provide an automated approach to infer population and control for population stratification based on the principles of Structured Association. Structure and ADMIXMAP use a model-based clustering method to determine population membership using unlinked marker genotypes. Both require an a priori estimation of the number of sub-populations and ancestry proportions, with the use of allele frequencies from ancestral populations.

Based on factors that included assumption violations, dataset size, and homogeneity, the decision was made not to assess/control for population structure in our data sample.

A central assumption of Genomic Control and Structured Association is that test alleles are random, unlinked, and unlikely to be associated with disease. Because SNP selection in our data set was premised on potential association with disease, meeting the assumption of marker independence from disease causation may be problematic. In addition, the requirement of unlinked markers is difficult. Although extensions to Stucture and ADMIXMAP have been developed to manage linked markers, the method by which they do so (treating haplotypes as the unlinked units) effectively reduces the number of loci which can inform ancestry. Our small dataset has genotype information at only 16 markers in 600 individuals. After consideration of LD structure (where a haplotype is defined conservatively as LD between markers at 80% or greater), the effective marker number in our entire data set is 10 with all considered a priori as associated with disease. As well, our focus on potentially causative markers did not include genotyping of ancestry informative markers (AIMs) which are required for optimal use of Stucture and ADMIXMAP. The combination of limitations regarding independence of markers and lack of AIMs makes our dataset ill suited for Genomic Control and Structured Association.

An additional justification for the decision to forgo controlling for population structure was that all participants analyzed in our dataset are U.S.-based Caucasians. Much attention has been given to the fact that Caucasian samples can contain sub-populations

[186; 188]. However, when stratification has been demonstrated in these populations, standard analytical methods such as Genomic Control or use of AIMs were not able to identify the subpopulations and only techniques that utilized detailed information such as extensive genealogical data [188] or specific nationality data [186; 187] were able to reveal the substructure. Information on nationality of origin is available only in a small subset of the Younger population and cannot be assessed across the dataset. Because cases and controls in the Elderly dataset are drawn from geographically separate areas in the US (Boston and Portland, respectively), the potential for substructure in this group exists but cannot be assessed accurately at this time.

A similar concern to population substructure is that of cryptic relatedness among cases. Cases that share a disease with a genetic component, in theory, have inherited the same genetic susceptibilities from a distant common ancestor which may supersede an ethnic or national ancestry. This shared ancestry is referred to as cryptic relatedness. It follows that controls are more likely to be independent than cases. Genomic Control was developed to address this cryptic relatedness but it has since been shown that in real (as opposed to simulated) outbred populations, the effect of cryptic relatedness is negligible [192].

Ultimately, the question for our purposes is not does substructure exist or can substructure be defined, but instead does substructure contribute to false associations with disease in our dataset. Positive association due to population stratification rather than underlying biology is often cited as the cause of lack of reproducibility in genetic

association studies. However, this explanation has been criticized [193] and, in fact, lack of replication also plagues linkage studies in which population stratification should not play a role [194]. The phenomenon of irreproducibility may be simply the result of complex disease mechanisms where significant environmental, polygenic and epistatic factors which may be unknown or difficult to measure contribute in varying degrees to the effect of genes in different samples. As well, sample size may differentially impact power to detect gene effect. Inconsistent phenotype definition from study to study may lead to inconsistent measurement of gene contribution. Finally, naïve use of methods to detect genetic effect can alter results.

Because of the limitations delineated above, assessment of the contribution of substructure to our dataset cannot be accurately measured. However, given our Caucasian population and a priori expectation of causation, population substructure is unlikely to be a major contributor to false positive associations with disease.

2.6.6 Correction for Multiple Testing

Similar to population stratification, tests of multiple hypotheses may lead to falsely positive association of genetic variants with disease. As the number of hypotheses tested increases, there is a concomitant increase that an unlikely result will occur due to chance alone. Lack of reproducibility in genetic association studies has been attributed to this phenomenon and must be considered to avoid reporting falsely positive associations.

Methods exist to control for this inflated type-I error due to multiple testing by adjusting the threshold for a significant result.

The most stringent method of controlling for multiple tests is the Bonferroni correction which divides the desired type-one error rate (typically 0.05) by the number of hypotheses being tested. Although computationally straightforward, this adjustment has been criticized as having potential for an excessively low threshold p-value which may lead to a loss of detection of true associations [195; 196]. An additional limit of this method is that it assumes independent tests. Many of our loci are strongly correlated and do not comply with an assumption of independence.

Methods such as Benjamini and Hochberg's [195] False Discovery Rate (FDR) have been developed to provide a less strict control for multiple testing. In summary, FDR uses ordered p-values to calculate a new error rate and estimate a rejection region. P-values from each hypothesis tested are ordered from smallest to largest and assigned a corresponding rank. This rank is multiplied by the desired type-one error rate (typically 0.05) and then divided by the total number of tests. If the p-value at that rank is less than or equal to the calculated value, it is considered significant after correction for the FDR. Like Bonferroni, this method assumes independent tests, which may not be appropriate for our dataset.

Extensions have been made to both the Bonferroni and FDR methods to better account for correlated data. For example, a simple adjustment which has been proposed for

Bonferroni is to use principle components analysis or LD structure to form pseudoindependant signals [197] for correction with multiple testing. A supplement for FDR in the case of dependant data was proposed by Storey and Tibshirani in 2001 with additional extensions having been proposed to more accurately address definition of the significance threshold [196]. However, correlated data may not be the primary reason for which these methods could be inappropriate for our dataset.

A central component of type-I error is the prior probability of association with disease at the locus being tested. There is a qualitative difference between our candidate gene study and genome scans with regard to type-I error. Our study applied a systematic approach to locus selection which incorporated evidence across disciplines to support our hypothesis of association of lipid-related genes to AD. We conducted a review of epidemiological evidence on lipid intake and neuronal health, in vitro research on protein function, and previous studies investigating genes and AD to select our candidate genes. This systematic approach contributes to a greater prior probability of association with disease than that of a genome scan, and, as follows, a potentially smaller chance of false positives. Yet much of the discussion which is directed toward the issue of multiple testing refers to large scale scans and expression data. Methods developed to address the issue of multiple testing in these large scale studies, where there is no a priori assumption of association with disease at any one locus, may be too conservative for candidate gene studies like ours which have a different hypothesis building process.

Recent work has focused on the unique issues that arise when seeking to correct for multiple testing in candidate gene studies. Two related approaches have been published using omnibus tests which incorporate both haplotype-based tests and SNP-based tests into an overall statistic. The first, termed a resampling-based multiple hypothesis testing procedure, was recently published in Genetic Epidemiology [198]. This paper describes the omnibus tests statistic which incorporates a global test for control of multiplicity of SNP association tests and a minimum p-value control for haplotypes tests multiplicity into a single statistic. The work published in Statistics in Medicine [199], proposes a two step procedure. In the first step gene effect is summarized with a single p-value estimated by assessing SNP and haplotype effect and controlling for multiple testing within the gene using an omnibus statistic. The second step adjusts for multiple testing across genes using FDR. These two methods are advantageous in that they explicitly address concerns specific to multiple testing in candidate gene studies. Like all methods which adjust for multiple testing, however, they cannot avoid the fact that control of type-I error is inexorably linked to an increase in type-II error.

A correction that assigns a low threshold for p-value significance may, in its stringency, deem null a true association with disease. It is not known with certainty which risk is greater – that of falsely reported positive associations or that of missed true associations. Scientists have spoken out on both sides of the multiple testing debate. Notable epidemiologist Kenneth Rothman has expressed concern that correction for multiple testing may lead to errors in interpretation, both by restricting the evidence available for observation and by discounting non-random natural principles [200]. A summary of the

argument was provided by clinician Giusppe Bianchi: “the probability models of frequentist statistics, based on randomness, trivialize the ordered complexity of biological systems based on natural laws.”[201] For some diseases, such as cancer, consortiums have been formed that pool data and evaluate results using meta-analyses and consensus approaches [202]. When done skillfully, this approach, which allows judgment by replication, may be the ideal method to balance type-I and type-II error rates in assessment of genetic effect. A naïve meta-analysis, however, may seek to characterize gene function on insufficient information. A recent meta-analysis of the role of LRP1 in AD reviewed only studies which had investigated a single SNP in this large (85 Kb) gene [203] but nonetheless drew conclusions about the overall genetic effect of LRP1 in AD. This case demonstrates one flaw of type-I error rate control using judgment based on replication - it depends on who is doing the judging.

Although there is some uncertainty as to the best approach for addressing inflation of type-I error due to multiple testing, the FDR approach was applied to our association results to gain an understanding of how this control measure may adjust measures of significance. We have also reported uncorrected p-values in the interest of providing all evidence with regard to potential gene effect.

3. Results

3.1 Populations

Association between genes and disease was tested in three case-control populations which differed by design in current age, defined as age of last examination or age at death (Table 5). The younger population (Younger), with a median age of 74, had a greater proportion of male participants. The older population (Elderly), with a median age of 87, had fewer men as one would expect with a population-based sample of older US Caucasians. These two case-control populations also differed in frequency of the APOE4 allele, with a higher prevalence of the E4 allele, at 18.1%, in the Younger group as compared to 11% in the Elderly group. Distribution of the E4 allele between cases and controls also differed between the Younger and Elderly groups with E4 prevalence in the Elderly cases at 6 times that of Elderly controls as compared to a 3 and a half fold difference between cases and controls in the Younger population. By pooling the Elderly and Younger populations, we created an additional, larger and more heterogeneous case-control population which we refer to as the "Pooled" group (n=613). The defining characteristics of this population fall between the Younger and Elderly group but do not represent and average of the two since the Younger population is more than twice the size of the Elderly group.

Table 5: Population characteristics

Population	Size	% Male	Median Age	Age Range	APOE4 Frequency (%)
Young LOAD	426	45.5	74	64-84	18.1
Cases	104	50.5	78	69-84	43.3
Controls	322	43.9	74	64-84	12.1
Elderly LOAD	187	31.0	87	68-103	11.0
Cases	97	26.8	83	68-103	28.0
Controls	90	35.2	89	86-99	4.5
Pooled	613	41.0	78	64-103	15.2
Cases	201	38.8	80	68-103	27.0
Controls	412	42.1	76	64-99	10.8

In a subset of participants for whom dietary intake and genotype data were available, we examined gene-diet interaction for fish and cholesterol intake. This sub-population consisted of 23 cases and 166 controls from the Younger population and was found to be representative of the Younger group in terms of age, sex, and APOE frequency as demonstrated in Table 6 below.

Table 6: Characteristics of sub-group for whom gene and diet information was available

Population	Size	% Male	Median Age	Age Range	APOE4 Frequency (%)
Younger sub-set with diet info	189	49.2	74	65 - 84	18
Cases	23	47.8	78	72 - 82	48
Controls	166	49.4	73	65 - 84	14

It is important to note that the fish intake variable used in analysis was a composite of four separate measurements of fish consumption based on mid-life intake values. It was possible that differences in the type of fish consumed (fried instead of broiled, for example) between cases and controls may contribute to AD pathology. Differences in

type of fish consumed was tested and found to be statistically insignificant at the 0.05 level between cases and controls as assessed by t tests.

3.2 Divergence from expected Hardy Weinberg Proportions (HWP)

Conformance with Hardy Weinberg Proportions (HWP) was tested in all three populations at each locus, with p-values for the chi square goodness of fit tests enumerated in Table 7. After correction for multiple testing by the Bonferroni method which placed significance at the 0.001 level, deviation from expected proportions was observed at the C270T locus in the BDNF gene in all sub-groups in the Younger population (p-value 0.0001) and among cases in the Elderly population (p-value 0.0001). We anticipated all tests to conform to HWP. Controls in the Elderly population conform to the expected HWP (p-value 0.6425). The deviation at this locus was the only observed deviation at the Bonferroni-adjusted significance level across datasets in cases and controls. Because the deviation could be due to linkage with a disease susceptibility locus [170], we included the SNP in our analysis but with an understanding that any observed significant associations with disease would be treated with caution.

As discussed in section 2.5, divergence from HWP can be due to underlying biology. A polymorphism associated with disease risk may or may not be in HWP among cases but there is more power to detect association between a locus and disease if a departure from HWE is found at that locus among cases. It is important to note that deviation from HWP was also observed in Younger cases at the rs2228186 locus in the LRP1 gene. This SNP

was found to be significantly associated with disease at the 0.005 level in univariate analysis.

Deviations at both the BDNF C270T and LRP1 rs2228186 loci were due to excess homozygosity. Excess homozygosity can result from a number of influences including genetic drift, selection and inbreeding. Of most concern, however, is excess homozygosity resulting from population sub-structure (the Wahlund effect). Because population substructure may contribute to spurious associations, associations at these two loci must be considered carefully. No association was observed at the BDNF locus and was therefore not investigated further. Since deviation from HWP at the LRP1 locus was observed in a relatively homogeneous group of US-based Caucasians all from the Cleveland area and observed among cases only, this deviation is unlikely to be due to population substructure.

Table 7: P-Values for lack of fit to HWP

		Young			Elder			Pooled		
Gene	**SNP**	**Case**	**Control**	**All**	**Case**	**Control**	**All**	**Case**	**Control**	**All**
BDNF	rs6265	0.017	0.251	0.227	1.000	0.540	0.668	0.109	0.551	0.629
	C270T	<.001	<.001	<.001	<.001	0.643	0.001	1.000	<.001	<.001
TTR	rs3764478	0.632	0.233	<.001	1.000	1.000	0.634	1.000	0.615	0.717
	rs723744	0.509	0.533	0.383	0.652	0.410	0.234	0.353	0.310	0.141
	rs1080093	0.138	0.808	0.348	0.829	0.315	0.517	0.450	0.447	0.250
	rs3764476	0.505	1.000	0.659	0.368	0.165	0.164	0.261	0.485	0.217
	rs3794884	0.266	1.000	0.591	0.514	0.416	0.292	0.342	0.567	0.269
TNFa	C850T	0.056	0.758	0.405	0.680	1.000	0.482	0.304	0.549	1.000
	-238G	0.631	0.223	<.001	1.000	1.000	0.624	1.000	0.609	0.713
LRP1	rs1799986	0.123	0.202	0.713	0.581	0.674	1.000	0.649	0.459	0.764
	rs2228186	<.001	0.102	0.001	0.230	0.335	0.175	0.038	0.291	0.028
	rs1800139	0.006	0.027	0.002	0.344	0.644	0.319	0.194	0.069	0.033
APOL	rs132618	0.118	0.821	0.564	0.309	0.133	0.100	0.071	0.686	0.175
	rs132622	0.027	0.531	0.595	0.253	0.827	0.331	0.347	0.516	1.000
	rs132638	0.469	0.216	0.227	0.645	0.219	0.499	0.867	0.077	0.117
SOAT	rs1044925	0.540	0.055	0.205	0.804	0.775	1.000	0.632	0.079	0.272

3.3 Unadjusted association of SNPs with disease outcome

Asymptotic p-values were calculated from chi square tests to assess association of individual SNPs with disease outcome. Mode of inheritance was modeled both as additive and dominant with the additive results shown in Table 8 (full results shown in Appendix 1). Association with disease was observed in the LRP1 gene at locus rs2228186 in the Younger population (p-value 0.004) **(**Table 8) with a more significant association observed under the dominant model (p-value 0.0009) which remained significant after correction for multiple testing with the Bonferroni method which set cut off for significance at 0.001.

Table 8: Unadjusted association of SNPs with disease outcome

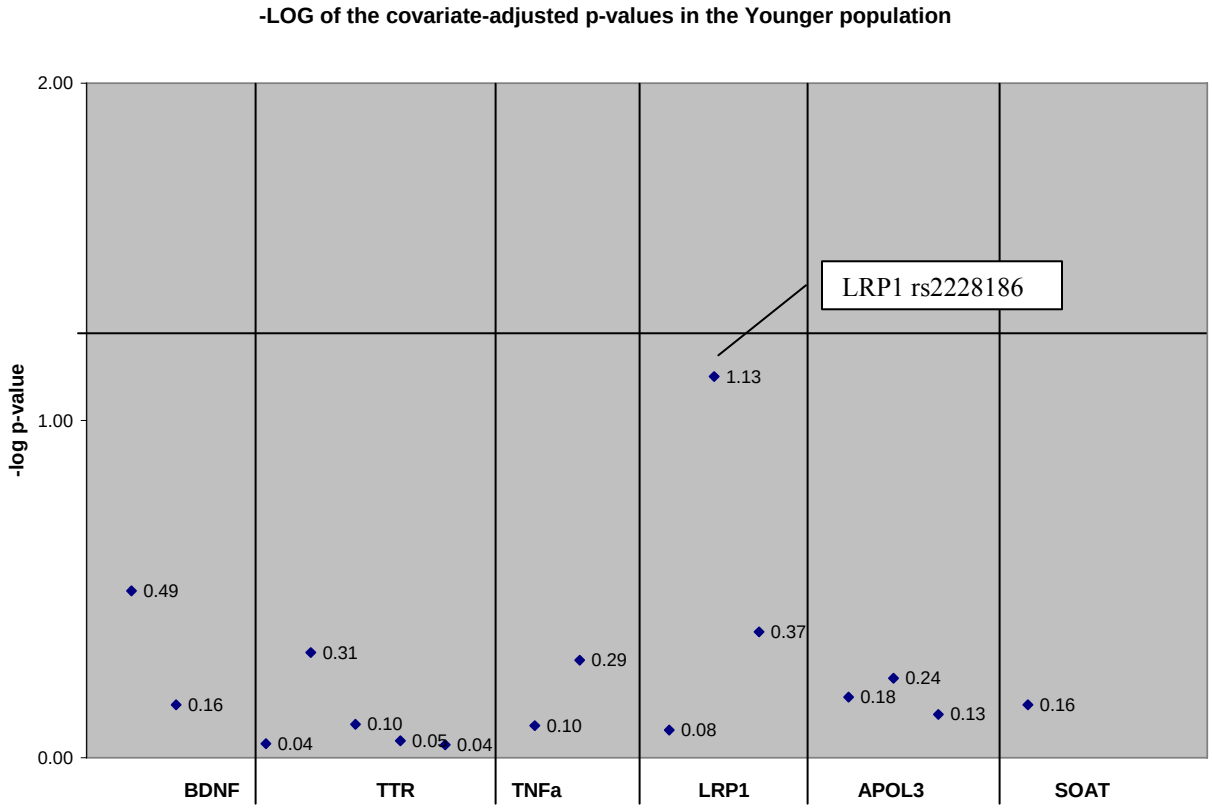
	Younger					Elderly					Pooled				
Gene & SNP ID	**MAF**	**Pval**	**OR**	**CI Low**	**CI Hi**	**MAF**	**Pval**	**OR**	**CI Low**	**CI Hi**	**MAF**	**Pval**	**OR**	**CI Low**	**CI Hi**
BDNF															
rs6265	0.22	**0.031**	1.58	0.999	2.499	0.21	0.548	1.061	0.579	1.945	0.22	0.25	1.339	0.941	1.906
C270T	0.09	0.692	0.564	0.123	2.595	0.07	**0.024**	0.25	0.078	0.8	0.07	0.348	0.483	0.194	1.204
TTR															
rs3764478	0.1	0.934	0.967	0.524	1.783	0.11	0.299	1.363	0.652	2.847	0.1	0.592	1.177	0.755	1.835
rs723744	0.34	0.794	0.898	0.57	1.415	0.31	0.112	1.502	0.828	2.724	0.33	0.805	1.033	0.732	1.458
rs1080093	0.37	0.631	0.794	0.505	1.249	0.35	0.248	1.488	0.828	2.674	0.36	0.836	0.995	0.707	1.401
rs3764476	0.33	0.663	0.868	0.551	1.368	0.3	0.241	1.443	0.788	2.642	0.32	0.721	0.98	0.693	1.387
rs3794884	0.33	0.058	0.852	0.542	1.339	0.3	0.155	1.502	0.828	2.724	0.33	0.601	0.999	0.709	1.409
TNF															
C850T	0.1	0.169	0.733	0.389	1.381	0.3	0.065	2.228	1.042	4.761	0.1	0.202	1.201	0.776	1.858
-238G	0.06	0.483	1.352	0.736	2.483	0	0.342	1.567	0.617	3.98	0.06	0.346	1.367	0.84	2.226
LRP1															
rs1799986	0.16	0.079	1.33	0.813	2.177	0.11	0.084	1.396	0.745	2.619	0.17	0.341	1.412	0.973	2.048
rs2228186	**0.33**	**0.004**	0.633	0.381	1.051	0.31	0.699	1.03	0.578	1.834	0.32	0.229	0.923	0.647	1.317
rs1800139	0.32	0.118	1.228	0.89	1.696	0.32	0.781	0.896	0.569	1.41	0.31	0.58	1.108	0.861	1.425
APOL3															
rs132618	0.49	0.128	1.384	0.884	2.165	0.49	0.797	0.918	0.513	1.643	0.5	0.296	1.238	0.88	1.74
rs132622	0.35	0.071	0.972	0.695	1.36	0.36	0.196	0.857	0.565	1.3	0.37	0.495	0.932	0.723	1.202
rs132638	0.34	0.112	0.78	0.496	1.225	0.31	0.451	0.718	0.402	1.282	0.3	0.5	0.755	0.535	1.063
SOAT1															
rs1044925	0.4	0.34	1.008	0.734	1.384	0.33	0.473	1.364	0.821	2.265	0.34	0.058	1.017	0.788	1.313

MAF = minor allele frequency

3.4 Adjusted association of SNPs with disease outcome

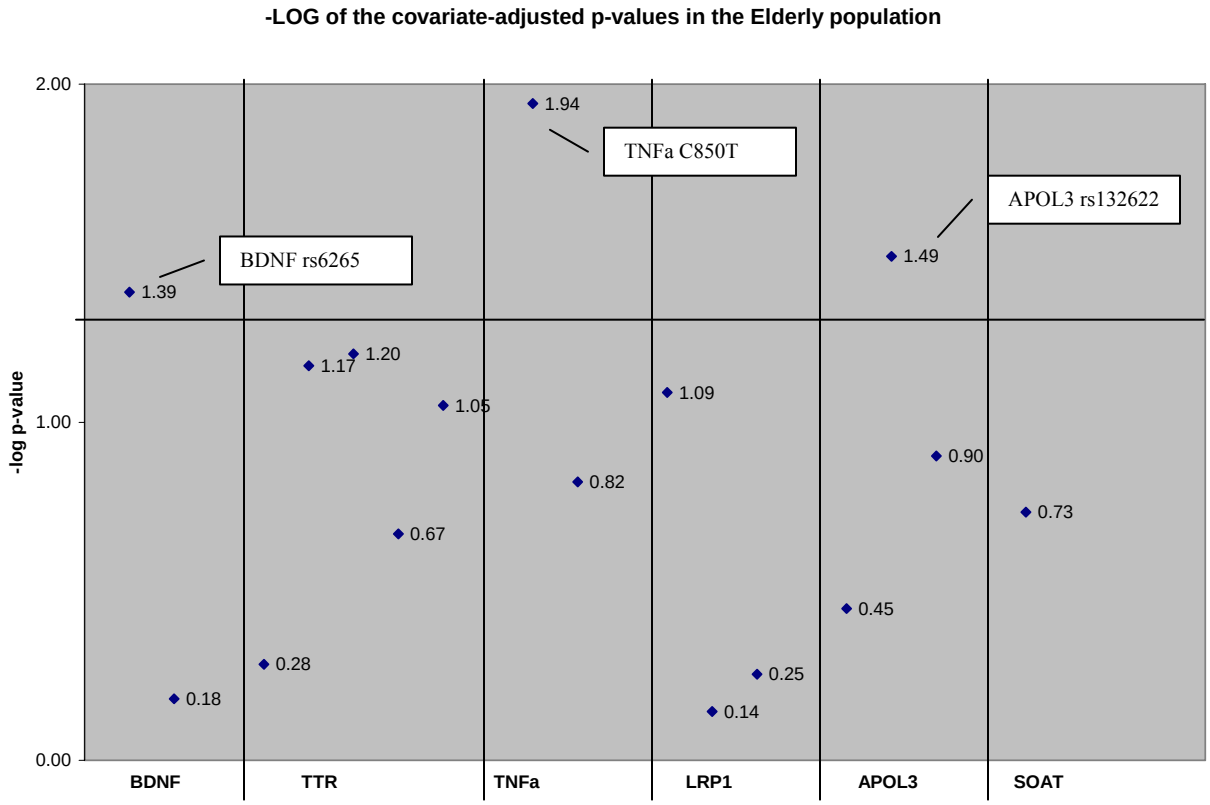
Figures 3 and 4 graphically display the negative log p-values for tests of association between SNPs and disease in each population when controlling for the potential confounders age, gender, and APOE genotype using logistic regression methods. This graphical presentation better demonstrates the relative significance of p-values among those observed in the dataset. Table 9 shows p-values with corresponding odds ratios and confidence intervals. The major effect observed in the Younger population at SNP rs2228186 in the LRP1 gene remained only marginally significant after adjustment for covariates (p-value of 0.074). Conversely, where no effect had been observed in the Elderly population in unadjusted analysis, controlling for covariates revealed three genetic effects of varying significance below the 0.05 level: TNFa C850T (p-value of 0.011), at APOL3 rs132622 (p-value 0.0323) and at BDNF rs6265 (p-value 0.41). No associations were found significant at the 0.05 level in the pooled population when adjusting for covariates. Including a covariate for Younger or Elderly group membership in the logistic analyses for the Pooled population did not reveal any genetic effects. No interactions were found to be significant at the 0.05 level when assessed in the logistic model. After adjustment for multiple testing, none of the results were considered significant at the FDR-adjusted level.

Figure 3: Relative significance of p-values calculated from tests of adjusted association with disease in the Younger population



P-value significance level of 0.05 is denoted by the line horizontal line at the y axis point of 1.30.

Figure 4: Relative significance of p-values calculated from tests of adjusted association with disease in the Elderly population



P-value significance level of 0.05 is denoted by the line horizontal line at the y axis point of 1.30.

Table 9: Results for association between SNPs and disease adjusted for age, sex, and APOE genotype

		Young				Elder				Pooled			
Gene	**SNP**	**Pvalue**	**OR**	**CI low**	**CI High**	**Pvalue**	**OR**	**CI low**	**CI High**	**Pvalue**	**OR**	**CI low**	**CI High**
BDNF	rs6265	0.32	1.703	0.973	2.982	**0.041**	1.315	0.624	2.773	0.352	1.423	0.965	2.098
	C270T	0.697	0.398	0.052	3.059	0.658	0.118	0.027	0.522	0.516	0.46	0.176	1.203
TTR	rs3764478	0.909	1.009	0.477	2.134	0.52	2.03	0.815	5.06	0.573	1.177	0.721	1.923
	rs723744	0.488	0.832	0.48	1.44	0.068	1.689	0.82	3.479	0.241	0.999	0.685	1.457
	rs1080093	0.797	0.616	0.354	1.07	0.063	1.783	0.876	3.629	0.272	0.917	0.631	1.334
	rs3764476	0.891	0.839	0.485	1.454	0.214	1.646	0.787	3.443	0.361	0.967	0.661	1.415
	rs3794884	0.916	0.765	0.442	1.325	0.089	1.725	0.837	3.554	0.45	0.961	0.66	1.401
TNF	C850T	0.803	0.607	0.281	1.31	**0.011**	2.749	1.119	6.758	0.972	1.022	0.631	1.656
	-238G	0.514	1.191	0.56	2.531	0.15	2.207	0.751	6.487	0.208	1.358	0.782	2.358
LRP1	rs1799986	0.828	1.346	0.742	2.444	0.082	1.773	0.829	3.788	0.723	1.308	0.872	1.963
	rs2228186	**0.074**	0.69	0.375	1.269	0.717	1.243	0.623	2.481	0.135	0.93	0.629	1.373
	rs1800139	0.423	1.205	0.812	1.787	0.556	0.852	0.501	1.451	0.692	1.093	0.829	1.442
APOL	rs132618	0.661	1.361	0.791	2.341	0.356	0.862	0.432	1.723	0.491	1.224	0.843	1.777
	rs132622	0.581	0.979	0.654	1.465	**0.032**	0.896	0.54	1.487	0.52	0.966	0.734	1.272
	rs132638	0.745	0.627	0.361	1.089	0.126	0.542	0.269	1.091	0.815	0.718	0.494	1.045
SOAT	rs1044925	0.697	1.101	0.752	1.613	0.185	1.595	0.8	3.178	0.926	1.023	0.775	1.352

3.5 Haplotype-based Tests of Association with Disease

Significant association was observed at the 0.05 level between the LRP1 gene and disease in our haplotype-based tests of association with disease. In the Younger population, the global test of differences in haplotype frequencies between cases and controls was significant for the LRP1 gen,e with a p-value of 0.002. After adjustment for APOE genotype, the difference was even more significant, with a p-value of less than 0.001 (Table 10).

Table 10: P-values for global tests of differences in haplotype frequencies between cases and controls after adjustment for APOE

Gene	Younger	Elderly	Pooled
BDNF	0.790	0.534	0.377
TTR	0.952	0.251	0.881
TNFa	0.631	0.118	0.769
LRP1	< 0.001	0.343	0.057
APOL3	0.382	0.771	0.584

Individual haplotypes for the LRP1 gene were also seen to be significant at the 0.005 level or below in the Younger population when adjusting for age, sex or APOE (Table 11). The individual haplotype "CGG" (where the three bases correspond to the three polymorphisms, rs1799986, rs2228186, and rs1800139 respectively) was most strongly association with disease.

Table 11: Frequencies and measures with disease of association for individual LRP1 haplotypes in the Younger population when adjusting for APOE (significant values are in bold)

Haplotype	Freq cases	Freq controls	Overall freq	p-values
T A G	0.471	0.552	0.533	0.103
T G A	0.286	0.265	0.267	0.405
C A G	0.109	0.132	0.128	0.186
C G A	0.016	0.026	0.023	0.926
T G G	0.049	0.011	0.021	**0.005**
T A A	0.044	0.016	0.023	0.114
C G G	0.025	0	0.006	**0**

3.6 Assessment of the role of dietary intake of lipids

In the Younger population, a sub-group logistic regression analysis investigated the potential for confounding and effect modification by the dietary factors of fish intake and cholesterol consumption. No main associations were observed between SNPs and disease when adjusting for fish intake or cholesterol intake assessed at mid-life. However, when interaction between fish intake and each locus was assessed in the logistic models, this interaction term was found to be significant at the 0.05 level at three SNPs: TTR rs3764478, TNFa rs1799986 and LRP1 C_1955081. Similarly, using a logistic model to assess interaction between cholesterol intake and each locus found the interaction term to be significant at the 0.05 level at two SNPs: TTR rs3764478, and LRP1 rs2228186.

Effect modification was assessed with stratification by low and high consumption of fish or cholesterol (Table 12). Although not statistically significant (confidence intervals cross 1), among those who had higher fish intake, the protective effect was increased two fold in TTR and 50 percent in LRP1. When adjusting for cholesterol intake, a four fold

increase in protective effect was observed in the TTR gene for those with high cholesterol intake, but no effect modification was observed in the LRP1 gene.

Table 12: Assessment of effect modification by dietary intake

FISH		Low Intake		High Intake	
Gene	**SNP**	**OR**	**CI**	**OR**	**CI**
TTR	rs3764478	0.935	0.528-1.654	0.429	0.052-3.504
TNFa	-238G	0.693	0.353-1.36	0.899	0.182-4.442
LRP1	rs1799986	1.09	0.675-1.763	0.384	0.089-1.652
CHOLESTEROL		**Low Intake**		**High Intake**	
Gene	**SNP**	**OR**	**CI**	**OR**	**CI**
TTR	rs3764478	1.157	0.644-2.078	0.25	0.032-1.938
LRP1	rs2228186	1.345	0.943-1.917	1.442	0.695-2.991

The interaction between TTR and LRP1 and the dietary measures may be due, in part, to interaction between the genes themselves. Allelic association between SNPs in the TTR gene and LRP1 in the Younger population was assessed using Haploview with D' as the measure used for strength of association. Low D' values indicate a weak association between SNPs, with the value 0 indicating no association and the value 100 indicating complete association. For the full population, association between the LRP1 locus rs1799986 and the TTR locus rs3764478 was measured as D'=16 and the LRP1 locus rs2228186 and the TTR locus rs3764478 with a D'=1. In cases only, association between rs1799986 and rs3764478 showed D'=10 and rs2228186/rs3764478 showed D'=8. With such low D' measures, allelic association between these two genes in the Younger dataset is unlikely.

3.7 Age of onset analysis

The alternative outcome of age at onset was assessed at each locus in the three populations (Table 13). Borderline significance at the 0.05 level was found at LRP1 locus rs1800139 in the Pooled population (p-value 0.055) and at TNFa locus -238G in the Elderly population (p-value 0.039).

Table 13: Test statistics and p-values for case-only analysis of differences in age at onset by allele at each locus

		Young		**Elder**		**Pooled**	
GENE	**SNP**	F Value	Pr > F	F value	Pr > F	F Value	Pr>F
BDNF	rs6265	0.8	0.496	0.69	0.503	1.36	0.256
	C270T	1.04	0.376	0.92	0.432	1.24	0.296
TTR	rs3764478	1.1	0.352	0.52	0.667	0.8	0.493
	rs723744	0.02	0.997	2.09	0.129	0.97	0.406
	rs1080093	0.05	0.984	1.9	0.136	1.92	0.128
	rs3764476	0.2	0.896	2.1	0.106	1.62	0.185
	rs3794884	0.04	0.988	2.55	0.084	1.48	0.220
TNFa	C850T	0.68	0.569	1.69	0.191	0.84	0.472
	-238G	0.4	0.672	**4.38**	**0.039**	0.89	0.412
LRP1	rs1799986	0.07	0.930	0.12	0.884	0.26	0.854
	rs2228186	0.32	0.809	1.25	0.296	2.21	0.088
	rs1800139	1.42	0.240	1.25	0.292	**2.58**	**0.055**
APOL	rs132618	0.31	0.817	0.76	0.518	0.41	0.748
	rs132622	1.03	0.382	0.32	0.808	0.44	0.726
	rs132638	1.62	0.189	0.88	0.419	1.36	0.257
SOAT	rs1044925	0.53	0.589	0.31	0.738	0.33	0.716

3.8 Assessment of Linkage Disequilibrium

Linkage disequilibrium as measured by D' or R^2 was calculated between all SNPs in each gene in the Pooled population (Figures 5-14). When multiple SNPs on the same gene are found to be associated with disease, pairwise tests that result in large D' or R^2 values indicate strong LD between these SNPs and provide further evidence of a causative mutation in that area of the genome. When a single SNP in a gene shows association with disease, low pairwise measurements of LD with other SNPs on that gene explains the singular association.

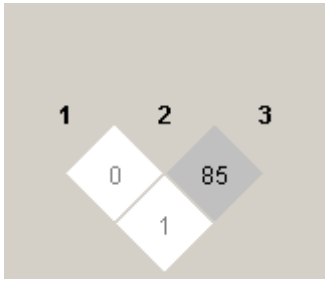
It is therefore surprising to see strong LD by both D' and R^2 measurements (Figures 5 and 6) between the disease-associated SNP in LRP1, rs2228186, and the neighboring polymorphism, rs1800139, which showed no significant association with disease. One explanation is that although there is a disparity between the observed main effects at the two SNPs, after adjustment for covariates this disparity is reduced. Adjusted associations may best represent the true effect at these two SNPs and so, as one would expect based on the LD structure, the adjusted effects are similar at the two SNPs.

For the associations observed in the BDNF and TNFa genes, the singular associations are not such a concern. Both polymorphisms in BDNF and TNFa have very low variability and, as such, provide little power to assess LD accurately (Figures 7-8 and 9-10 respectively). The significant SNP, rs132622, in the APOL3 gene has better overall variability but the variability differs from neighboring SNPs (Figures 13 and 14). This discrepancy may lead to low informativity to assess LD. As a result, R^2 and D' measurement of LD in all three genes are quite different and portray a disparate picture of

the amount of LD. R^2 may be artificially low because of its reliance on allele frequency. However, D' may be upwardly biased in this situation since it represents the maximum potential for LD when an allele does not have sufficient variability to be truly informative.

Figures 5-14: Linkage Disequilibrium (D'& R^2) between SNPs (with Haploview displaying the absolute value of D' multiplied by 100)

Figure 5: LRP1 LD structure assessed using R^2



LRP1 SNP IDs and corresponding numbers	Minor allele frequency
rs1799986 - 1	0.166
rs2228186 - 2	0.316
rs1800139 - 3	0.315

Figure 6: LRP1 LD structure assessed using D'

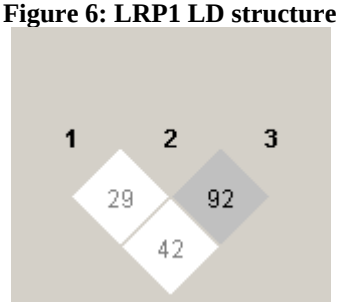
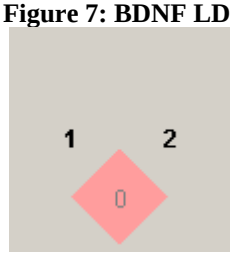


Figure 7: BDNF LD structure assessed using R^2



BDNF SNP IDs and corresponding numbers	Minor allele frequency
rs6265 - 1	0.214
C270T - 2	0.067

Figure 8: BDNF LD structure assessed using D'

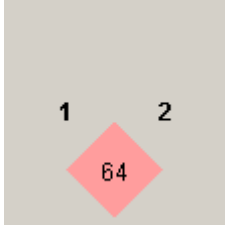
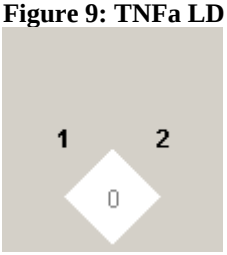


Figure 9: TNFa LD structure assessed using R^2



TNFa SNP IDs and corresponding numbers	Minor allele frequency	D'
C850T - 1	0.099	
-238G - 2	0.058	

Figure 10: TNFa LD structure assessed using

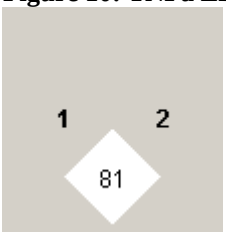
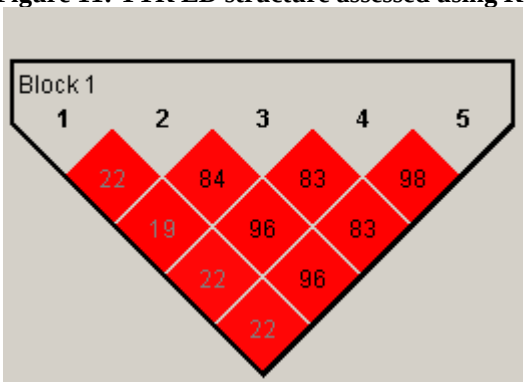


Figure 11: TTR LD structure assessed using R^2



TTR SNP IDs and corresponding numbers	Minor allele frequency
rs3764478 - 1	0.099
rs723744 - 2	0.326
rs1080093 - 3	0.358
rs3764476 - 4	0.317
rs3794884 - 5	0.321

Figure 12: TTR LD structure assessed using D'

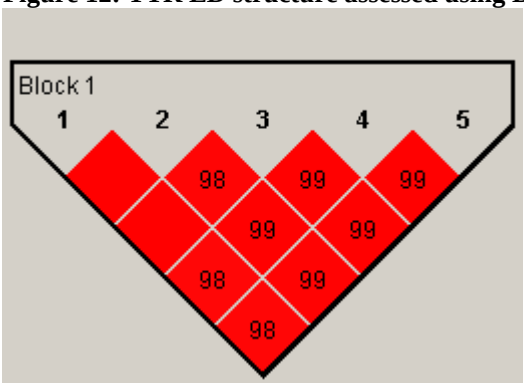
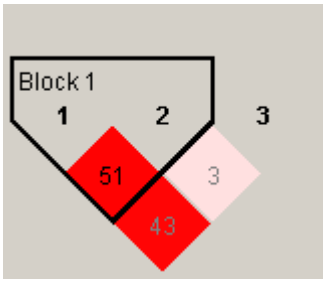
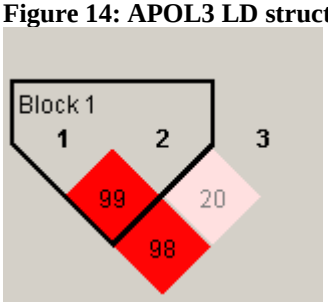


Figure 13: APOL3 LD structure assessed using R^2



APOL3 SNP IDs and corresponding numbers	Minor allele frequency
rs132618 - 1	0.479
rs132622 - 2	0.355
rs132638 - 3	0.328

Figure 14: APOL3 LD structure assessed using D'



4. Discussion

Our study presents evidence for a role of *LRP1* in the pathogenesis of LOAD in the Younger population, with a significant main effect observed at the novel locus rs2228186 (p-value 0.001), and a highly significant global test of association for inferred haplotypes after adjustment for APOE genotype (p-value 0.0001). Additionally, our study has added support to the reported association in the TNF gene between the rs17799724 SNP and AD in the Elderly population, and has provided evidence in support of a possible role among the oldest-old of the novel gene, *APOL3*, in AD.

All observed associations were confined to either one population or the other. Although by design these two populations differed by current age, they also differed by geography, ascertainment method, and *APOE* allele frequency. Additional analyses in our dataset further support the role of age as a key factor in the observed differences of association in each population (see section 4.1). However, because Elderly cases were ascertained only when at least two siblings were affected with disease, genetic heterogeneity between the familial group (Elderly) and the sporadic group (Younger) could be the cause of associations which differed between the two groups. As well, because cases and controls in the Elderly dataset are drawn from geographically separate areas in the US (Boston and Portland, respectively), the potential for substructure in this group exists but cannot be assessed accurately at this time (see section 2.6.5). Regardless of the cause of genetic heterogeneity, the observed associations were significant at the 0.05 level or below and were supported by considerable biological evidence linking the genes to LOAD.

4.1 The role of LRP1

Our study presents evidence for a role of LRP1 in the pathogenesis of AD in the Younger population, with a marginally significant main effect evaluated at the Bonferroni-corrected value of 0.001 observed at the novel locus rs228186 (p-value 0.0009 for the dominant model). The effect was less significant after adjustment for covariates (p-value 0.074). A highly significant global test of association (p-value 0.0001) for inferred haplotypes was observed after adjustment for APOE genotype. Previous studies reporting no association between LRP1 and AD [203] may have been premature to dismiss this candidate.

Located in an established linkage region on chromosome 12 [204], LRP1 is a multifunctional receptor with at least 25 ligands identified to date [205]. LRP1 ligands include AD associated lipoproteins such as APOE and alpha-2-macroglobulin. LRP1 is central to the pathways involving transport of cholesterol out of the extracellular space in the brain, where its presence may encourage the formation of cholesterol-rich lipid rafts in the cell membrane and, in turn, facilitate the amyloidgenic splicing activity of beta and gamma secretase. As well, through the endocytotic function of LRP1, cholesterol is made available inside the neuron for uses such as repair of the cellular membrane [35]. Aside from its function in cholesterol transport, LRP1 has been shown in vitro to mediate clearance of Aβ through the alpha-2-macroglobulin-LRP-pathway and reduced LRP expression has been correlated with higher soluble Aβ levels and amyloid

deposition[142]. Expression studies have shown disease-specific differences in LRP1 levels in the brain [143].

Despite this evidence, genetic associations between the LRP1 gene and AD have been inconsistent. Two meta-analyses have investigated the associations and found conflicting results[203; 206]. This can be explained, however, by a number of factors such as differences in loci studied, difference in sample size and power, differences in population, and difference in study participant selection criteria. The present study is a good example of how differences in selection criteria can affect observed associations. LRP1 associations were observed exclusively in the Younger population, with separate associations observed in the Elderly population, although both were US-based Caucasians. We recognize that genetic risk in AD may be age-dependent, as evidenced by the activity of the APOE gene, and believe this may have been an additional factor in the reported inconsistencies regarding LRP1.

An age-dependant role for LRP1 is further supported by results from our case-only analysis using age at disease onset as an alternative outcome. In the pooled population, which combined the Younger and Elderly study groups, the difference between mean age at onset between alleles was found to be of borderline significance at the rs1800139 locus of LRP1 with a p-value of 0.055. As well, when we stratified by age in the Younger population, the greatest effect at the rs2228186 SNP was seen in the youngest of the Younger population (Table 14) with diminishing effect correlated with greater age.

LRP1 is a relatively large gene containing 89 exons, and many studies have focused on a single locus, a silent C -T polymorphism in exon 3, in their investigation of this gene as it relates to AD. This single polymorphism may not be the causative locus nor even linked with the causative locus in such a large gene. Reliance on studies investigating a single polymorphism in LRP1 to assess the role of this gene in AD may not be sufficiently powered if the single SNP is not an adequate surrogate for all of the variability in the gene. Our study of a novel SNP in the LRP1 established that a more comprehensive treatment of LRP1, either by assessment of new polymorphisms or inclusion of an analysis of inferred haplotypes, may provide more evidence that is in agreement with the biological plausibility of this gene.

Of particular concern in our dataset, however, is the observed reduction in significance at the LRP1 rs2228186 locus in our Younger population after adjustment for the covariates sex, age, and APOE genotype. Although studies have found that the effect of APOE is modified both by gender and by age [207], when assessed singularly, it is the effects of age and APOE which contribute most to the reduction in effect observed at LRP1 rs2228186 under the full model. One explanation of this phenomenon may simply be that APOE and LRP1 share a causal pathway. Since APOE has been linked to age in AD pathogenesis, it follows that all three may belong to a common mechanism. A shared mechanism would be expected since APOE is a primary ligand of LRP1 and it is this relationship which is often invoked when linking LRP1 to AD.

This mechanism was supported by a further stratified analysis. APOE4 negative participants in the Younger population had a lower odds ratio which was also of borderline significance (OR = 0.634, p-value = 0.08) (Table 7) whereas, among those with one or more APOE4 alleles, the odds ratio was higher and not significant (OR = 0.823, p-value = 0.41) (Table 14).

Table 14: Age- and APOE-stratified analysis of association of LRP1 rs2228186 with AD

LRP1 rs2228186 association with disease stratified by Age

Age Strata	strata size (case/control)	p-value	OR	CI
LE 71	100 (6/94)	0.0348	0.301	0.097, 0.938
LE 75 and GT 71	102 (16/86)	0.495	0.774	0.374, 1.603
LE 79 and GT 75	89 (28/61)	0.3815	0.749	0.393, 1.427
LE 85 and GT 79	24 (10/14)	0.2092	1.842	0.680, 4.991

LRP1 rs2228186 association with disease stratified by *APOE*

APOE Strata	strata size (case/control)	p-value	OR	CI
No APOE4	258 (31/227)	0.0854	0.634	0.380, 1.059
At least one APOE4	127 (62/65)	0.4088	0.823	0.518, 1.308

In the Younger population, using a dominant genetic model with an effect size of 0.4, assuming a disease prevalence of 3% and a sample size equivalent to that available for this study, we had a power of 87% to detect this locus at a significance level of 0.05. Power in the Elderly population however, was less. Using the same genetic model but with a disease prevalence of 50%, power to detect an effect assuming a significance level of 0,05 was only 67%. This discrepancy in power may also explain why an effect was observed in the Younger but not Elderly population.

Although there is considerable evidence to support the role of LRP1 in AD and our results provide some indication that an association exists, the true effect of LRP1 in our Younger population remains uncertain. Inconsistencies such as lack of association at markers in LD with the significant locus rs2228186 as well as the undetermined role of APOE in the observed effect may call into question the validity of our results. However, given the strong biological plausibility of LRP1, and our evidence associating LRP1 with disease in unadjusted analysis, haplotype analysis, and age at onset analysis, further study of LRP1 is certainly justified.

4.2 The role of TNFa

Our study also confirmed previous reports of association of TNFa with AD as evidenced by the covariate-adjusted association with disease at locus C850T (p-value of 0.011). TNFa plays a central role in lipid metabolism and, through this mechanism, can affect the level of circulating lipids as well as other circulating metabolites associated with AD such as glucose. Adipose is not simply a storage tissue but is an active participant in regulation of systemic energy levels. Adipose tissue has been shown to produce TNFa which, in turn, inhibits the uptake of circulating free fatty acids and lipoproteins and increases lipolysis by adipocytes. Lipolysis results in release of glycerol, free fatty acids, and other metabolites into circulation with a net increase in circulating lipids [208].

Although its role in lipid metabolism represents one potential risk-conferring mechanism linking TNFa to AD, this cytokine also plays a major role in inflammation. Inflammation is considered central to the pathogenesis of AD, and proteins involved in this process have been an area of strong interest. Expression of TNFa has been shown to be up-regulated in AD affected tissues and is produced locally by the affected brain[129]. Conversely, TNFa is also associated with protection against AD through its secondary role in limiting the inflammatory response after it has begun[134]. This inflammation control may be critical in management of further brain injury caused by an un-checked inflammatory response to plaques and tangles.

An additional role of TNFa may be in modification of age at onset. The TNFa locus -238G was found to contribute significantly at the 0.05 level to an earlier age at onset in the Elderly population (p-value 0.039). This locus was not significant at the 0.05 level in the analyses that assessed main or adjusted gene effect, although this may be due to low variability and a resultant lack of power (see table 15). Precedence exists to support a role of TNFa in age at disease onset. A similar effect was reported when locus -238G was included in a haplotype with APOE4 [141]. Evidence for age of onset modification by TNFa has also been reported at the C850T locus in two previous studies [138; 166].

Elucidating the pathogenic mechanism between TNFa and AD has been difficult with studies indicating both potential for protective and detrimental effects. A protective effect of TNFa was demonstrated by Beattie in 2002 showing glia-produced TNFa enhanced synaptic efficacy by increasing expression of AMPA receptors [135].

However, TNFa has also been implicated in gamma secretase stimulation and, thus, up-regulation of the pathway leading to amyloidgenic splicing of APP [131]. Not surprisingly, researchers have investigated TNFa and AD with mixed results. Although our sample size is not large, the variability at the TNF locus is good in the Elderly population with a minor allele frequency of 0.3. Using the genetic models described in table 4, and assuming a disease prevalence of 50%, and a sample size equivalent to that available for this study, we had a power of 55% to detect this locus assuming a significance level of 0.05. As such, our study provides further evidence that this cytokine is involved in AD.

4.3 The novel gene APOL3 and AD

Our study also points to a potential role for APOL3 in AD as evidenced by the observed association at locus rs132622 (OR 0.717, p-value 0.032). APOL3 has not received much attention as a potential factor in the pathology of AD and has mostly been investigated with regard to its role in atherosclerosis (see **APOL3**, section 1.4.5). It is this role, however, that is of most interest to us as we investigate the intersection between lipid metabolism and transport and AD. APOL3 is a member of the apolipoprotein L gene family which codes for a high density lipoprotein found in the cytoplasm where it may affect the movement of lipids. Of particular note, APOL has been shown to be up-regulated by TNFa [146]. Although our investigation of the gene was limited by SNP density and sample size, with a power of only 39% to detect this locus assuming a

significance level of 0.05, an additional analysis with affected siblings replicated the association we observed.

This supplementary family-based analysis paired our Elderly cases with their affected siblings from the NIMH dataset in a model-free linkage analysis that investigated single point marker allele sharing identity by decent (IBD) as executed in the **S.A.G.E.** v5.3.0 SIBPAL program. The proportion of sibling pairs sharing zero alleles IBD and two alleles IBD at the rs132622 locus was highly significant with p-values of 7.65×10^{-5} and 9.37×10^{-4} respectively. Mean allele sharing was also highly significant with a p-value of 276×10^{-4}. Although low SNP density and small sample size limited our investigation of the APOL3 gene, the observed associations at the rs132622 locus using case-control and family-based methods are intriguing and deserving of further research.

4.4 BDNF and the val66met polymorphism

BDNF has received a great deal of attention as a candidate in brain disorders because of its role in stimulating and facilitating neuroplasticity, particularly in cholinergic regions of the brain which can be particularly susceptible to AD pathologies. The val66met polymorphism (SNP id rs6265) represents a functional amino acid change in the promoter region which was found to correlate with poor performance in memory tests [209]. Because memory is a central deficit in those affected by AD, this association may point to the biological cause of the role of BDNF in AD. Additional work has shown association between the val66met polymorphism in the BDNF gene (rs6265) and AD in

several populations [160; 210]. These results, however, have not been consistently reproduced [111; 112; 211-213].

In our Elderly population, with a minor allele frequency of 0.22 and sample size of 426 participants (104 controls), we have a power of approximately 31% to detect the BDNF val66met locus (rs6265) assuming a significance level of 0.05. Despite this power limitation, we observed an association of borderline significance (p-value 0.041) in our Elderly population after adjustment for the covariates of age, sex, and APOE genotype. If considering inflated type-I error due to multiple testing or population substructure, our finding may not support the val66 met polymorphism as a causal factor in AD risk. However, given the strong biological plausibility of BDNF and the low power of our sample, our study does not conclusively eliminate the val66met SNP from consideration in further studies.

4.5 Gene-Environment interactions between TTR and LRP1 and dietary lipids

Intriguing results were seen when we stratified by dietary intake of fish and cholesterol and then assessed association between our candidate genes and AD. Although not statistically significant owing possibly to limits in sample size (only 23 cases with dietary data were available for genotyping), there was a dramatic correlation between level of fish intake and effect of TTR and LRP1.

With a high level of fish intake, the protective effect of TTR was twofold. This result confirms in humans what has been observed in rodent models. Pregnant mice fed a diet supplemented with fish oil had offspring with superior cognitive performance [125]. Rats fed a diet enriched with polyunsaturated fatty acids like fish oil were found to have as much as a ten-fold increase in TTR expression. TTR has been shown to inhibit amyloid accumulation in the brain by sequestering Aβ [119], and it is this protective mechanism that may indirectly be driven by high fish intake. The link between fish intake and LRP1 function is not so clearly supported by previous research but deserves further investigation.

The increased protective effect of TTR with high cholesterol intake is more puzzling. An analysis of dietary patterns found that some participants in this sub-group with high fish intake also have a high cholesterol intake. The protective effect observed for those with high cholesterol intake my simply be due to concordant fish intake. A potential explanation may also lie in the role of TTR in repair mechanisms. As mentioned above, TTR has been shown to sequester Aβ [119]. When levels of Aβ are abnormally high in the brain, it has been shown that TTR is up-regulated [120]. It is possible that those with a high cholesterol intake are producing excess Aβ, but not sufficiently to cause disease symptoms. It is possible that the up-regulation of TTR may show an initial protective effect but that over time this high intake of cholesterol may, through its own risk-conferring pathways, overwhelm this protective effect and prove detrimental.

4.6 Negative Results

Some polymorphisms previously reported as associated with AD were tested in our populations but were not found to be significant at the 0.05 level. Power limitations of our study, however, call into question the confidence of our negative results at these loci. Power to detect a moderate gene effect with an odds ratio of 1.5 is well below 50% in both our populations at all but one locus previously associated with disease in our sample (Table15).

If, however, an expectation of greater gene effect in warranted, power to detect genetic effect is close to 80% or above in both populations at LRP1 rs1799986, and SOAT rs1044925. In the Younger population, sufficient power exists to detect a gene effect with an odds ratio of 2.0 or above at the C270T locus in BDNF as well. In previous studies, the magnitude of effect reported at LRP1 rs1799986 ranged from odds ratios of1.3 to 2.4[142; 203]. The effect at SOAT rs1044925 was reported as an odds ratio of 0.6 [148].

These previously reported associations may support the premise of a larger gene effect. Under the assumption of a two-fold or greater genetic effect, we can determine with certainty that the polymorphisms rs1799986 in LRP1 and rs1044925 in SOAT do not significantly contribute to or protect from AD risk in our populations and that the C270T locus in BDNF does not contribute to AD risk in the Younger group.

Given the complex nature of AD, however, the concern remains that a strong genetic risk of 2.0 may not be a valid assumption. With lack of certainty regarding gene effect taken with consideration of the biological plausibility of these genes and previous association with disease, I would not eliminate these SNPs from further study based on our findings.

Table 15: Power to detect gene effect at SNPs associated with AD in previous studies but not found to be significant at the 0.05 level in our study.

		Young	Power		Elder	Power	
			effect size	effect size		effect size	effect size
Gene	**SNPs**	**MAF**	**1.5**	**2.0**	**MAF**	**1.5**	**2.0**
BDNF	C270T	0.09	.344	.776	0.07	.141	.312
TNF	-238G	0.06	.207	.516	0	0	0
LRP1	rs1799986	0.16	.453	.891	0.19	.362	.769
SOAT1	rs1044925	0.4	.685	.985	0.33	.470	.88

4.6 False associations due to multiple testing

A criticism of our results may be that our reported associations are not truly significant but instead an artifact of a liberal type-one error rate which has not been adjusted to account for the multiple hypotheses tested. Methods such as Benjamini and Hochberg's False Discovery Rate (FDR) [195] have been developed to provide a control for multiple testing. When applied to our data set, associations formerly significant at the 0.05 level were no longer significant at the FDR-adjusted level (Table 16).

Table 16: SNPs in the Elderly population significant at the 0.05 level after adjustment for covariates and the FDR adjusted cutoff for significance.

Gene and SNP	P-value for assoc. w/ disease	FDR cut off for significant assoc.
TNFa C850T	0.0114	0.003125
APOL3 rs132622	0.0323	0.00625
BDNF rs6265	0.0412	0.009

It is important to note, however, that FDR may remain too conservative for our dataset. Unlike genome scans, for which FDR was developed, that have an a priori expectation of no association with disease at a given SNP, our study selected genes based on a deliberate and systematic approach incorporating biological plausibility and potential for causation. This hypothesis building process was used specifically to increase the prior probability of association with disease at a given SNP and therefore may justify a less stringent type-I error threshold (see **correction for multiple testing**, section 2.6.6).

More importantly, however, current knowledge of AD pathogenesis is still limited and elucidation of disease pathways remains a priority. The risk of inflated type-two error, or that of missing a true genetic effect, may outweigh the risk of false positives at this point in our understanding of disease and further supports the reporting of observed risk without an overly conservative adjustment for multiple testing.

5. Limitations of the current study and future work

Approaches to assess genetic contribution to disease in case-control studies are numerous. Two common approaches are the candidate gene study and the genome scan. Our decision to pursue a candidate gene analysis as opposed to a genome scan was based largely on availability of data and budget constraints. Genome scans, which can provide unprecedented identification of novel loci in association with disease, are also expensive, using sometimes thousands of markers spread across the human genome. The usefulness of this type of data for fine mapping may be limited, however, if the density of the scan is inadequate. Density of markers genotyped is related both to cost as well as to characterization of the genome and potential for association with disease (i.e. the denser the polymorphism coverage, the greater the likelihood of identifying a causative locus). When budgets are limited, using a priori knowledge of regions of interest in disease can help narrow the search for disease causing loci. Use of this type of knowledge in identifying gene variants associated with disease often takes the form of candidate gene studies. These types of studies have the advantage of building on previous findings from divergent disciplines to assess in advance the plausibility of genomic regions for genotyping.

Like genome scans, however, candidate gene studies can also fail to sufficiently characterize genes of interest and miss potential associations with disease if genotyping density is poor. Although every attempt was made in our study to maximize the potential for causality at every SNP, gene coverage in our study would not be considered ample,

especially in genes such as SOAT where loss to genotyping error and monomorphism reduced coverage to a single SNP and eliminated the option of haplotype analysis. Future work with our dataset could be to pursue additional genotyping in the candidate genes most suggestive of a causative role in AD such as LRP1, TNFa, APOL3 and BDNF. Additional considerations should include cost-saving measures such as utilization of pre-made, validated assays.

One limitation of our study is that it was unable to eliminate certain polymorphisms from consideration in future work because the test of difference between the two samples was insufficiently powered due in part to small sample size. With a larger sample size, an additional test of equivalence could also be performed at those loci where a significant difference was not found. Equivalence testing would be a method which could allow for more certainty in rejecting SNPs from further consideration and focusing future work on more promising loci. Our failure to detect significant differences between genotypes of cases and controls does that mean they are equivalent and specific statistical methods have been designed to prove similarity [214; 215]. Similarity between cases and controls may be a more meaningful hypothesis to test at loci with borderline association to disease because the statistical test provides proof of no association. Used in clinical trials, equivalence testing assesses the primary hypothesis that the difference between groups falls at or below a pre-determined, trivial level, with an alternative hypothesis that the differences is above that level. Further work using this method in our study would include identification a value for the level of similarity acceptable for our purposes.

Additional analyses may have also revealed more information about disease process. For example, further investigation into the individuals who have both high cholesterol intake and high fish intake in the sub-sample data may reveal additional similarities between these two groups, beyond dietary habits, that might better explain the seeming protective effect of cholesterol intake. At this time, lifestyle data is available for fewer than 25 genotyped cases.

Future work could include gaining additional consent and gathering supplementary lifestyle data from genotyped participants.

In this study, we investigated two outcomes: disease status and age at onset. Rate of decline in AD, however, is an additional measurement of disease, or phenotype, that may be of keen interest to clinicians and caregivers. At this time, there are no published studies that investigate the role of our candidate genes with regard to rate of decline in AD. Exploration of rate of decline as an alternative outcome in our study could provide additional information as to the contribution of our candidate genes to this aspect of disease

Investigation of rate of decline, however, is not a simple matter. Unlike the universally recognized NINCDS-ADRDA method used to determine AD status, rate of decline does not have a standardized clinical measure. Furthermore, our dataset provides only a limited measure of cognitive decline, defined as time to death or last evaluation from age at onset. This simplistic measure does not differentiate between death attributed to AD and competing causes of death, nor does it incorporate any measure of cognitive or

functional decline. A measure of cognitive/functional decline may better represent disease progression and severity and, as such, may be a better surrogate for disease pathogenesis.

Inaccurate definition of phenotype can cause spurious results when tested for association with genetic factors and is a concern. Considerable work would have to be invested to research rate of decline phenotype definition and the ability to define this phenotype within our dataset or with supplementary work. Future work which sought to assess this outcome might include gaining consent to examine complementary materials like medical records which could provide a better characterization of cognitive/functional decline.

Supplementary data gathering in future work could also include a measure of early-life cognition level and of disease severity. Again, considerable work would have to be invested to research phenotype definition and the ability to gather this information with supplementary work. Like rate of decline, though, these two alternative phenoptype measures could be of great importance to clinicians, caregivers and policy makers. As well, the measures early-life cognition level and of disease severity could be used in conjunction with the measures age at onset and rate of decline to investigate relationships between the four phenotypes and the effect our genes may have on the relationship.

Additional analyses may have also revealed more information about genetic mechanism. Significant preliminary work assessed the main effect at each SNP across multiple

populations using differing models of inheritance (Appendix 1). In a number of cases, SNPs used for the primary analysis not found to be significantly associated with disease in our populations were found to be significant in other populations. Many of these were significant at the 0.05 level, which, after consideration of multiple testing, may not justify further investigation. Of particular note, however, in a population similar to the Elderly population, four of the five SNPs in the TTR gene were significant at or below the 0.01 level under a dominant model, with all SNPs in the gene significant at or below the 0.05 level.

Investigation of this effect in the alternate Elderly population may establish an important relationship between transthyretin and disease in the oldest-old. Like the Elderly population, this alternative group was also composed from the NIMH study set (n=250), using the randomly selected sibling from each affected sib pair, which was not chosen for the cases in the Elderly LOAD population. Controls were the same as those in the Elderly population. It is surprising that two populations that have identical control participants and whose cases are first-degree relatives, would at all five markers show a difference in association with disease of a full magnitude or greater. Future work to explore the TTR mechanism would include a comparison of the two elderly groups to identify differences between the two with the potential to explain the disparity in observed effect of TTR. Further analysis could also include a more through treatment of the genetic effect such as haplotype-based tests and tests of gene-gene interaction.

Gene-gene interaction is a rich area for potential research among our candidate genes. Because all genes selected for our study are related both to lipid metabolism and/or transport and to AD pathologies, gene-gene interaction due to common mechanisms would be expected. An exploratory assessment of gene-gene interaction using SNPs in a logistic model to assess interaction terms was executed in this study but with results that failed to reject the null hypothesis. This result is not surprising since excessive dimensionality is a common issue with the use of logistic regression since, with each main effect added to the model, the number of interaction terms grows exponentially [216]. Another simple approach to assessing gene-gene-interaction may be by estimating Hardy-Weinberg Equilibrium patterns where unlinked loci, which deviate in the same way from HWE in cases but not controls, might indicate an epistatic pattern [216].

Many more sophisticated methods exist to evaluate gene-gene interaction in case control studies and utilization of these methods with this or an expanded data set may prove informative. Multifactor Dimensionality Reduction (MDR), which has been shown to have good power in smaller sample sizes [217], has been developed specifically to address excessive dimensionality. MDR as a method has the added advantage of a companion open-access software package that automates much of the test algorithm. The Combinatorial Searching Method (CSM) has been developed for genome scans but is also appropriate, with modification, for candidate gene studies and may provide an effective tool to investigate in our dataset gene-gene interaction for an alternative continuous outcome such as age at onset or rate of decline. A novel method recently

published, described as a double penalized log-likelihood [218], has proposed evaluating both gene-gene and gene-environment interactions simultaneously.

Pattern recognition is another area of analysis that may lead to identification of disease-relevant relationships in the data. This type of machine learning is a computationally intense approach which can operates on little a priori information about a dataset. Methods of this kind, such as classification tree models, sequentially define tree-like clusters of data which may correlate with biologically germane patterns.

Like all statistical tests, however, choice of analytical method is not trivial. Although data mining with methods such as pattern recognition or MDR may be a promising area of research, I am as yet unfamiliar with the implementation of such methods. Future work in this area will require additional research into the advantages and disadvantages of each approach as applied to the particular dataset.

With additional exploration of relationships in the dataset come additional concerns for inflation of type-I error due to multiple testing. Data mining techniques in particular, have high potential for type-I error inflation. Balancing multiple testing concerns with what information a dataset may divulge upon further analyses may be less a strictly statistical problem and more an evaluation based on researcher judgment. In this dataset where observed associations have been only marginally significant at the 0.05 level and not significant after correction for multiple testing, additional analyses seem unlikely to reveal an effect of consequence.

6. Conclusions:

This study provides evidence to support the hypothesis that genes involved in lipid transport and metabolism can affect risk for Alzheimer's disease. Further investigation is necessary to elucidate these pathways, particularly in regard to the genes Low-density Lipoprotein Receptor-related Protein (LRP1), Tumor Necrosis Factor alpha (TNFa), Apolipoprotein L-3 (APOL3), Brain-derived Neurotrophic Factor (BDNF), and Transthyretin (TTR). Our approach was unique in that it selected genes based on a common mechanism linked to disease susceptibility (lipid metabolism/transport). Testing these genes for association with disease in populations that differed in current age allowed for assessment of age-dependent genetic effects that may not contribute to, or be assessed by, an age at onset analysis. Novel results included the observed association at locus rs2228186 in LRP1 in the Younger population, the association between APOL3 and disease in the Elderly population, and the observed but statistically non-significant effect modification of TTR by fish intake. Although aspects of our study may be limited, our results confirm that this avenue of research holds particular promise, both for developing a greater understanding of disease process and in the future identification of interventions or treatments.

Appendix 1: P-Values and genotype frequencies for various populations

Key: Populations:

NIMH1 =nimh group1 + nimh controls
OHSU = ohsu cases and controls
CWRU = cwru cases and controls
NIOH1 = nimh grp 1 + ohsu controls
NIOH1 = nimh grp 2 + ohsu controls
NIOHCW = NIOH1 + CWRU
NIOHCC = NIOH1 + OHSU
ALLDAT = NIOHCC + CWRU

Models:

ADD = additive model (0=aa, 1=ab, 2=bb)
DOM1 = binary, or dominant model: 0=aa and 1=ab, bb
DOM2 = dominant model: 0=aa, ab and 1=bb

GENE	ASSAY ID	P-VALUE for associations w/in Populations							
		NIMH1 N=121 Cas=97 Con=24	**OHSU** N=131 Cas=41 Con=90	**CWRU** N=427 Cas=105 Con=322	**NIOH1** N=187 Cas=97 Con=90	**NIOH2** N=186 Cas=96 Con=90	**NIOHCW** N=614 Cas=202 Con=412	**NIOHCC** N=252 Cas=138 Con=114	**ALLDAT** N=679 Cas=436 Con=243
BDNF	RS6265	Add .723	Add .597	Add .**0311**	Add .548	Add .621	Add .2495	Add . 639	Add .2510
		Dom1 .307	Dom1 .333	Dom1 .**0473**	Dom1 .298	Dom1 .454	Dom1 .279	Dom1 .375	Dom1 .169
		Dom2 .674	Dom2 .636	Dom2 .2388	Dom2 .551	Dom2 .747	Dom2 .226	Dom2 .604	Dom2 .3348
	Allele freq (%) 0=gg,1=ga,2=aa	0=3.36 1=29.41 2=67.23	0=1.45 1=33.08 2=65.38	0=4.44 1=34.35 2=61.21	0=3.76 1=34.41 2=61.83	0=3.26 1=31.52 2=65.21	0=4.27 1=34.32 2=61.41	0=2.79 1=33.47 2=63.75	0=3.86 1=33.98 2=62.17
BDNF	C270T	Add .538	Add .451	Add .6922	Add .**0235**	Add .157	Add .3475	Add .0893	Add .4045
		Dom1 .4758	Dom1 .947	Dom1 .441	Dom1 .**0530**	Dom1 .176	Dom1 .868	Dom1 .**0685**	Dom1 .6375
		Dom2 .287	Dom2 .420	Dom2 .270	Dom2 .169	Dom2 .486	Dom2 .1746	Dom2 .3344	Dom2 .2671
	Allele freq (%) 0=cc, 1=ct, 2=tt	0=1.67 1=8.33 2=90	0=1.54 1=13.08 2=85.38	0=4.52 1=3.72 2=91.76	0=2.2 1=9.34 2=88.46	0=1.1 1=12.02 2=86.89	0=3.97 1=5.42 2=90.60	0=1.61 1=8.9 2=89.47	0=3.55 1=5.66 2=90.78
TTR	rs3764478	Add .375	Add .294	Add .934	Add .299	Add .0925	Add .592	Add .3231	Add .5982
		Dom1 .476	Dom1 .141	Dom1 .811	Dom1 .0945	Dom1 .173	Dom1 .3033	Dom1 .**0683**	Dom1 .2166
		Dom2 .184	Dom2 .479	Dom2 .987	Dom2 .187	Dom2 **.0365**	Dom2 .262	Dom2 .4029	Dom2 .4602
	Allele freq (%) 0=aa, 1=ac, 2=cc	0=1.67 1=25.83 2=72.5	0=0.775 1=14.73 2=84.5	0=1.18 1=16.04 2=82.78	0=1.63 1=19.57 2=78.8	0=1.09 1=22.83 2=76.08	0=1.33 1=17.24 2=81.43	0=1.61 1=17.67 2=80.72	0=1.35 1=16.77 2=81.89
TTR	RS723744	Add .786	Add .363	Add .7943	Add .112	Add .**0073**	Add .805	Add .1936	Add .8394
		Dom1 1.0	Dom1 .571	Dom1 .839	Dom1 .251	Dom1 .442	Dom1 .4272	Dom1 .327	Dom1 .4606
		Dom2 .505	Dom2 .155	Dom2 .821	Dom2 .**0405**	Dom2 **.0081**	Dom2 .442	Dom2 .**0764**	Dom2 .4487
	Allele freq (%) 0=aa, 1=ac, 2=cc	0=12.59 1=51.67 2=35.83	0=10 1=36.92 2=53.08	0=12.26 1=42.22 2=45.52	0=11.83 1=38.71 2=49.46	0=10.18 1=43.78 2=45.41	0=12.07 1=41.49 2=46.45	0=11.95 1=40.24 2=47.81	0=12.09 1=41.79 2=46.12
TTR	rs1080093	Add .788	Add .332	Add .6314	Add .248	Add .**0181**	Add .836	Add .2971	Add .7758

		Dom1 .794	Dom1 .584	Dom1 .481	Dom1 .498	Dom1 .498	Dom1 .413	Dom1 .4719	Dom1 .4021
		Dom2 .492	Dom2 .137	Dom2 .685	Dom2 .**0736**	Dom2 .**0048**	Dom2 .542	Dom2 .0922	Dom2 .4562
	Allele freq (%)	0=14.17	0=12.31	0=14.32	0=12.97	0=12.97	0=13.86	0=13.2	0=13.86
	0=cc, 1=cg, 2=gg	1=54.15 2=31.67	1=41.54 2=46.15	1=44.13 2=41.55	1=43.24 2=43.78	1=47.03 2=40.00	1=43.89 2=42.24	1=44.8 2=42	1=44.41 2=41.73
TTR	rs3764476	Add .839	Add .4413	Add .6629	Add .241	Add .**0145**	Add .7212	Add .359	Add .7465
		Dom1 1.0	Dom1 .586	Dom1 .553	Dom1 .317	Dom1 .458	Dom1 .2739	Dom1 .3933	Dom1 .2925
		Dom2 .570	Dom2 .136	Dom2 .896	Dom2 .**0734**	Dom2 .**0022**	Dom2 .516	Dom2 .1168	Dom2 .4970
	Allele freq (%)	0=12.5	0=10.08	0=11.14	0=11.54	0=10.87	0=11.19	0=11.74	0=11.14
	0=tt, 1=tg 2=gg	1=50.83 2=36.67	1=36.43 2=53.49	1=42.42 2=46.45	1=37.36 2=51.1	1=42.83 2=46.2	1=41.23 2=47.58	1=39.27 2=48.99	1=41.57 2=47.14
TTR	RS3794884	Add .839	Add .340	Add .**05773**	Add .155	Add .**0114**	Add .6012	Add .2525	Add .6418
		Dom1 1.0	Dom1 .888	Dom1 .440	Dom1 .342	Dom1 .442	Dom1 .255	Dom1 .518	Dom1 .3514
		Dom2 .570	Dom2 .155	Dom2 .921	Dom2 .**0564**	Dom2 .**0028**	Dom2 .432	Dom2 .0975	Dom2 .4292
	Allele freq (%)	0=12.5	0=9.23	0=11.48	0=11.29	0=10.81	0=11.35	0=11.16	0=11.29
	0=aa, 1=ac, 2=cc	1=50.83 2=36.67	1=37.69 2=53.08	1=42.39 2=46.14	1=38.71 2=50	1=43.24 2=45.95	1=41.61 2=47.04	1=40.64 2=48.21	1=42.05 2=46.66
TNFa	C850T	Add .474	Add .**014**	Add .1689	Add .**0647**	Add .**0329**	Add .2016	Add .0714	Add .0849
		Dom1 mono (all 1)	Dom1 .140	Dom1 .**0656**	Dom1 .337	Dom1 mono (all 1)	Dom1 .**0798**	Dom1 .199	Dom1 .**0502**
		Dom2 .474	Dom2 .**0062**	Dom2 .652	Dom2 .**0244**	Dom2 .**0329**	Dom2 .221	Dom2 .**0370**	Dom2 .0961
	Allele freq (%)	0=0	0=7.7	0=1.18	0=.54	0=0	0=1	0=.8	0=1.05
	0=cc, 1=ct, 2=tt	1=27.5 2=72.5	1=19.23 2=80	1=17.06 2=81.75	1=19.89 2=79.57	1=20 2=80	1=17.74 2=81.26	1=23.11 2=76.10	1=19.16 2=79.79
TNFa	-238G	Add .873	Add .888	Add .4827	Add .342	Add .579	Add .346	Add .3775	Add .3498
		Dom1 .873	Dom1 .888	Dom1. 345	Dom1 .342	Dom1 .688	Dom1 .185	Dom1 .378	Dom1 .2230
		Dom2 mono (all 0)	Dom2 mono (all 0)	Dom2 .730	Dom2 mono (all 0)	Dom2 mono (all 0)	Dom2 .994	Dom2 mono (all 0)	Dom2 .9232
	Allele freq (%)	0=92.44 1=8.4 2=0	0=90.77 1=9.23 2=0	0=89.28 1=10.26 2=.47	0=88.71 1=11.29 2=0	0=91.35 1=8.65 2=0	0=89.01 1=10.49 2=0.49	0=89.24 1=10.76 2=0	0=89.19 1=10.37 2=0.44
LRP1	RS1799986	Add .565	Add .151	Add .0794	Add .0841	Add .327	Add .3414	Add .0539	Add .4213
		Dom1 .309	Dom1 .057	Dom1 .**0550**	Dom1 .**0700**	Dom1 .202	Dom1 .976	Dom1 .0231	Dom1 .4999
		Dom2 .564	Dom2 .890	Dom2 .708	Dom2 .0823	Dom2 .2854	Dom2 .0819	Dom2 .1478	Dom2 .1107
	Allele freq (%)	0=33.3	0=3.08	0=2.57	0=3.76	0=2.70	0=2.96	0=3.98	0=3.12
	0=cc, 1=ct, 2=tt	1=30.83 2=65.83	1=25.38 2=71.54	1=26.17 2=71.26	1=30.65 2=65.59	1=29.19 2=68.11	1=27.26 2=69.77	1=29.08 2=66.93	1=27 2=69.88

LRP1	rs2228186	Add .227	Add .1824	Add .**0010**	Add .699	Add .704	Add .229	Add .376	Add .5879
		Dom1 .155	Dom1 .414	Dom1 .**0009**	Dom1 .482	Dom1 .673	Dom1 .**0886**	Dom1 .1656	Dom1 .3080
		Dom2 .577	Dom2 .**0691**	Dom2 .585	Dom2 .783	Dom2 .559	Dom2 .532	Dom2 .3351	Dom2 .9854
	Allele freq (%)	0=9.17	0=7.69	0=14.46	0=7.57	0=8.11	0=11.9	0=8	0=11.64
	0=cc, 1=ct,	1=50	1=43.08	1=35.29	1=47.57	1=50.27	1=39.12	1=44.4	1=38.74
	2=tt	2=40.83	2=49.23	2=50.25	2=44.86	2=41.62	2=48.98	2=47.6	2=49.62
LRP1	rs1800139	Add .512	Add .191	Add .1177	Add .781	Add .580	Add .795	Add .5168	Add .9908
		Dom1 .577	Dom1 .**069**	Dom1 .469	Dom1 .833	Dom1 .559	Dom1 .407	Dom1 .3639	Dom1 .8333
		Dom2 .410	Dom2 .609	Dom2 .**0585**	Dom2 .482	Dom2 .496	Dom2 .513	Dom2 .3471	Dom2 .7687
	Allele freq (%)	0=40.83	0=49.23	0=51	0=44.62	0=41.62	0=49.06	0=47.44	0=49.69
	0=cc, 1=ct,	1=50.83	1=41.54	1=35.32	1=46.77	1=46.77	1=39.11	1=43.82	1=38.73
	2=tt	2=8.33	2=9.23	2=13.68	2=8.60	2=8.60	2=11.84	2=8.76	2=11.57
APOL3	RS132618	Add .546	Add .863	Add .1283	Add .797	Add .296	Add .486	Add .7968	Add .5193
		Dom1 .598	Dom1 .591	Dom1 .971	Dom1 .919	Dom1 .349	Dom1 .985	Dom1 .766	Dom1 .8887
		Dom2 .47	Dom2 .947	Dom2 .0835	Dom2 .811	Dom2 .376	Dom2 .148	Dom2 .817	Dom2 .145
	Allele freq (%)	0=25	0=21.54	0=22.66	0=20.54	0=23.24	0=21.55	0=21.2	0=21.69
	0=aa, 1=at,	1=48.33	1=56.15	1=51.17	1=56.22	1=51.35	1=52.8	1=56	1=53.05
	2=tt	2=26.67	2=22.31	2=26.17	2=23.24	2=25.41	2=25.65	2=22.8	2=25.26
APOL3	RS132622	Add .710	Add .970	Add .0707	Add .196	Add .495	Add .635	Add .267	Add .7085
		Dom1 .617	Dom1 .817	Dom1 .388	Dom1 .346	Dom1 .271	Dom1 .996	Dom1 .433	Dom1 .9899
		Dom2 .404	Dom2 .997	Dom2 .**0652**	Dom2 .904	Dom2 .403	Dom2 .216	Dom2 .848	Dom2 .2745
	Allele freq (%)	0=45.83	0=40	0=41.81	0=42.86	0=43.48	0=41.64	0=42.51	0=41.63
	0=cc, 1=ct,	1=41.67	1=45.38	1=46.71	1=42.86	1=44.02	1=45.82	1=42.91	1=45.55
	2=tt	2=11.67	2=14.62	2=11.40	2=14.29	2=12.5	2=12.54	2=14.57	2=12.82
APOL3	RS132638	Add .509	Add .090	Add .1119	Add .451	Add .4999	Add .324	Add .0884	Add .**0678**
		Dom1 .281	Dom1 .904	Dom1 .250	Dom1 .386	Dom1 .256	Dom1 .598	Dom1 .348	Dom1 .5979
		Dom2 .553	Dom2 .**038**	Dom2 .092	Dom2 .527	Dom2 .956	Dom2 .**0622**	Dom2 .1002	Dom2 .**0079**
	Allele freq (%)	0=10.08	0=6.92	0=9.69	0=8.60	0=9.24	0=9.44	0=7.97	0=9.12
	0=cc, 1=cg,	1=47.06	1=43.08	1=46.81	1=45.16	1=52.17	1=46.69	1=43.82	1=46.04
	2=gg	2=42.86	2=50	2=43.50	2=46.24	2=44.02	2=43.87	2=48.21	2=44.84
SOAT1	RS1044925	Add .522	Add .606	Add .	Add .473	Add .**0577**	Add .3864	Add .9047	Add .5011
		Dom1 .601	Dom1 .718	Dom1 .495	Dom1 .326	Dom1 **.0198**	Dom1 .4718	Dom1 .7941	Dom1 .8666
		Dom2 .411	Dom2 .443	Dom2 .331	Dom2 .319	Dom2 .248	Dom2 .394	Dom2 .6674	Dom2 .3033
	Allele freq (%)	0=31.78	0=50.52		0=44.76	0=38.56	0=40.15	0=45.36	0=40.74
	0=aa, 1=ac,	1=53.27	1=40.21		1=44.76	1=50.33	1=44.57	1=42.78	1=43.94
	2=cc	2=14.95	2=9.28		2=10.49	2=11.11	2=15.29	2=11.86	2=15.32

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