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## LOW DENSITY LIPOPROTEIN RECEPTOR AND ALZHEIMERS DISEASE

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ABSTRACT OF DISSERTATION

Rangaraj K. Gopalraj

The Graduate School  
University of Kentucky  
2008

LOW DENSITY LIPOPROTEIN RECEPTOR AND ALZHEIMERS DISEASE

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ABSTRACT OF DISSERTATION

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A thesis submitted in partial fulfillment of the  
requirements for the degree of Doctor of Philosophy in the  
College of Medicine Department of Physiology  
at the University of Kentucky

By

Rangaraj K. Gopalraj

Lexington, Kentucky

Director: Dr. Steven Estus, Professor of Physiology

Lexington, Kentucky

2008

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## ABSTRACT OF DISSERTATION

### LOW DENSITY LIPOPROTEIN RECEPTOR AND ALZHEIMERS DISEASE

Since apoE allele status is the predominant Alzheimers disease (AD) genetic risk factor, functional single nucleotide polymorphisms (SNPs) in brain apoE receptors represent excellent candidates for association with AD. Therefore, three low density lipoprotein receptor (LDLR) SNPs were evaluated by TaqMan allelic discrimination assays for association with AD and I found that certain haplotypes alter the odds of AD. A SNP within LDLR exon 12, rs688, was identified *in silico* as neutralizing a putative exon splicing enhancer (ESE). Since LDLR is a major apoE receptor in the brain, I hypothesized that rs688 modulates LDLR splicing in neural tissues and associates with AD. To evaluate this hypothesis, I analyzed splicing patterns in human hippocampus samples and established that this SNP was associated with significantly decreased LDLR exon 12 splicing efficiency when the minor allele T is present *in vivo*. Lastly, I evaluated whether rs688 associates with AD by genotyping DNA from the Religious Orders Study (ROS) series. The rs688T/T genotype was associated with increased AD odds in males, but not in females, in a dataset consisting of 1,457 men and 2,055 women drawn from three case-control series. The rs688T/T genotype was associated with increased AD odds in males (recessive model, odds ratio (OR) of 1.49, 95% confidence interval (CI) of 1.13-1.97, uncorrected  $p=0.005$ ), but not in females. In summary, these studies identify a functional apoE receptor SNP that is associated with AD in a sex-dependent fashion.

KEYWORDS: Alzheimers disease, cholesterol, LDLR, polymorphism, SNP

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December 02, 2008

LOW DENSITY LIPOPROTEIN RECEPTOR AND ALZHEIMERS DISEASE

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DISSERTATION

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I dedicate this work to my wonderful teachers and my loving family

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## **Chapter One: Introduction and Background**

### **Alzheimers Disease**

#### ***Impact***

Alzheimers Disease (AD) is a devastating disorder. It is not considered a normal part of aging even though increasing age is an inescapable and pervasive risk factor (Giannakopoulos, Bouras et al. 2008). One in 10 individuals over 65 and nearly half of those over 85 are affected (Evans, Funkenstein et al. 1989). The number of Americans with AD has more than doubled since 1980 to an estimated 4.5 million and will continue to grow as the proportion of elderly in the population increases. It could be as high as 16 million by 2050 (Hebert, Scherr et al. 2003). The impact on individuals, families and the health care system makes the disease one of the nation's greatest medical, social and fiscal challenges especially in view of the burgeoning proportion of the elderly. Average survival time is affected by age at diagnosis and severity of other medical conditions (Larson, Shadlen et al. 2004). According to estimates used by the Alzheimer's Association and the National Institute on Aging national direct and indirect annual costs of caring for individuals with AD are at least \$100 billion (Ernst and Hay 1994). The implications of interventions or therapies that could delay onset of the disease could have profound effects prevalence. So much so a five year delay could decrease the number of individuals with AD by nearly fifty percent after fifty years (Brookmeyer, Gray et al. 1998). The critical breakthroughs have been achieved to a large extent through advances in the understanding of the biochemical pathology and genetics as explained below.

#### ***Pathology***

The description rendered by the German neuropsychiatrist Alois Alzheimer in 1906 is relevant even after almost a century. Grossly, there is atrophy of the brain and dilatation of the ventricles. Microscopically, the numbers of neuritic plaques and neurofibrillary tangles are vastly in excess of those seen in older persons without dementia. They are abundantly present in the amygdala, hippocampus and neocortex in AD. Indeed, the current pathologic criteria for AD are based on the demonstration of sufficient numbers

of these hallmark lesions during autopsy. Experienced physicians, following NINCDS-ADRDA (National Institute of Neurological and Communicative Diseases and Stroke/Alzheimer's Disease and Related Disorders Association) criteria, can diagnose possible or probable AD with up to 90% accuracy in live patients (McKhann, Drachman et al. 1984).

Plaques are extracellular deposits of proteinaceous amyloid core surrounded by dystrophic neurites. The amyloid beta ( $A\beta$ ) is a result of proteolytic cleavage of the larger APP. But whether the deposition of amyloid initiates or triggers AD or is just a relic marking neuronal degeneration is still debated (Mattson, Carney et al. 1995). Also  $A\beta$  oligomers may be equally toxic and deleterious (Haass and Selkoe 2007; Walsh and Selkoe 2007). Clinical trials of experimental drugs targeting amyloid that are under way should help clarify the role of plaques and may lend further credence to the 'amyloid hypothesis'.

Neurofibrillary tangles (NFTs) are intracellular accumulations of the cytoskeletal protein tau that is abnormally phosphorylated and arranged in the form of paired helical filaments (Arnaud, Robakis et al. 2006). The limbic and association cortices of AD brains demonstrate innumerable NFTs. Subcortical nuclei that project to these regions are also affected. These nuclei include the cholinergic basal forebrain complex, the locus ceruleus and the median raphe nuclei (Arnold, Hyman et al. 1991; Arriagada, Growdon et al. 1992). Though this protein does not find a prominent role in the 'amyloid hypothesis', it is still an active area of intense investigation (Haroutunian, Davies et al. 2007).

Although the decline in cholinergic system markers such as choline acetyltransferase and acetylcholinesterase has been correlated with both the degree of dementia and the number of neuritic plaques, cholinergic loss should not be considered the preeminent neurotransmitter alteration in AD because the neuronal loss is not restricted to cholinergic neurons.

### ***Risk factors***

AD is a progressive neurological disorder with complex etiology that encompasses both environmental and genetic risk factors.

The existence of **environmental risk factors** is suggested by twin studies. Though monozygotic twins have a high degree of concordance for AD, it is still less than 40%. Further, even when both identical twins develop the disease, the presentation and clinical course is not identical (Breitner and Murphy 1992; Small, Leuchter et al. 1993; Raiha, Kaprio et al. 1996). Anti-inflammatory drug use constitutes one “environmental” factor that lowers AD risk, as individuals receiving high doses, e.g., arthritic individuals, are at a lower AD risk (reviewed in (McGeer, Schulzer et al. 1996)). A history of head trauma and certain polymorphisms in the inflammatory mediator interleukin-2 (see below) have been associated with AD, providing further support for inflammation playing a role in AD (Guo, Cupples et al. 2000; Mrazek and Griffin 2000). A role for estrogen as a neuroprotectant has also been suggested (reviewed in (Behl 2002)). Life style choices such as controlling blood pressure, weight and cholesterol levels; exercising both body and mind; and staying socially active may not only promote successful healthy aging but it may also help reduce the risk of developing AD.

As seen in other chronic diseases of the elderly, first-degree relatives seem to have a greater risk of disease. Several robust **genetic risk factors** have been identified for AD over the past several years. Three genes have been implicated in familial AD (**FAD**), including APP, presenilin-1 and presenilin-2; mutations in these genes produce AD marked by autosomal dominance, early disease onset (can strike individuals as early as their 30s and 40s (Bird, Sumi et al. 1989)), high penetrance, and relative rarity, on the order of a few hundred families worldwide (reviewed in (Pericak-Vance, Grubber et al. 2000; Selkoe 2001)). APP is located in chromosome 21 and it was known widely that nearly all Down’s syndrome (Trisomy 21) patients developed AD. Because each of the mutated forms of these genes enhance the production of A $\beta$  or of A $\beta$  that is longer, and hence more likely to form toxic oligomeric aggregates, the discovery of these genes has suggested that genes that impact on A $\beta$  production or clearance could be critical to the

disease (Goate, Chartier-Harlin et al. 1991; Citron, Oltersdorf et al. 1992; Cai, Golde et al. 1993; Roher, Lowenson et al. 1993; Suzuki, Cheung et al. 1994; 1995; Levy-Lahad, Wijsman et al. 1995; Duff, Eckman et al. 1996).

This is the basic tenet for the ‘amyloid hypothesis’ which assigns a central causative role to abnormal amyloid processing. Amyloid precursor protein (APP) undergoes proteolytic cleavage by several different secretases. The bulk of the APP circumvents generation of A $\beta$  when processed by  $\alpha$ -secretase releasing the neurotrophic ectodomain of APP into the CSF (Sisodia 1992; Haass and Selkoe 1993). On the other hand  $\beta$ - and  $\gamma$ -secretases working in tandem can generate the A $\beta$  peptide (Sisodia 1992; Haass and Selkoe 1993). Due to slippage in the site of cleavage by  $\gamma$ -secretase the C terminal of amyloid is variable resulting in different species upto 43 amino acids long. The major species is 40 amino acids long, but of special interest is 42 which is ~10% of the amyloid peptides but aggregates with alacrity and could be a nidus for plaque formation and help recruit more amyloid (Iwatsubo, Odaka et al. 1994).

Several genes have been hypothesized to contribute to the much more common “sporadic”, late onset, AD (**LOAD**) which affects individuals over age 65. The premier example of a genetic risk factor for LOAD is apoE4, which surfaced on a biochemical screen of CSF proteins that can bind A $\beta$  (Strittmatter, Saunders et al. 1993). This is remarkable in that (i) multiple groups have confirmed that even a single copy of apoE4 increases susceptibility to AD, (ii) apoE4 has been associated with earlier disease onset, and (iii) apoE4 is associated with increased brain A $\beta$  in affected individuals ((Roses 1994); reviewed in (Pericak-Vance, Grubber et al. 2000)). These effects of apoE on A $\beta$  burden can be seen in mice as well, with studies in mice deficient for apoE or transgenic for human apoE supporting a role for apoE in A $\beta$  fibrillogenesis and neuritic plaque formation (Holtzman, Bales et al. 1999; Holtzman, Bales et al. 2000; Holtzman, Fagan et al. 2000). In summary, apoE4 (i) provides a point of reference for evaluating additional LOAD risk factors and (ii) must be considered when evaluating additional genetic polymorphisms to ensure that an underlying apoE4 does not dictate an apparent association between these additional SNPs and AD.

Overall, apoE4 and the rare FAD mutations appear to account for approximately 50% of the genetic risk of AD (Pericak-Vance, Grubber et al. 2000). One recent simulation analysis suggests the existence of at least four additional genes with an impact similar to ApoE4 on age of AD onset (Warwick Daw, Payami et al. 2000).

## **Cholesterol**

### ***Physiology***

Cholesterol is a vital component of all mammalian cell membranes. It confers important properties such as membrane fluidity and permeability. Apart from its structural function cholesterol is also the precursor of cellular signaling molecules such as oxysterol in the brain (Simons and Ikonen 2000), steroid hormones in endocrine tissue, Vitamin D in skin cells (Ohvo-Rekila, Ramstedt et al. 2002). It is also necessary for bile acid synthesis in the liver. Cholesterol biosynthesis is known to occur in the endoplasmic reticulum, and also in the peroxisomes (Reinhart, Billheimer et al. 1987) of most eukaryotic cells.

At the cellular level there is a fine balance between biosynthesis and uptake of lipoprotein cholesterol into cells and esterification. The process by which peripheral cholesterol is returned to the liver for catabolism and excretion is known as reverse cholesterol transport (RCT) (Fielding and Fielding 1995). This is important in order to achieve sterol homeostasis because extrahepatic tissue cannot catabolize cholesterol.

### ***CNS Cholesterol***

The brain and spinal cord harbors 25 percent of all unesterified cholesterol in an individual while accounting for only 2% of the body mass. Apart from the plasma membrane cholesterol is also a critical component of myelin and is deposited early in development (Spady and Dietschy 1983). The daily turnover rate of cholesterol in human brain is believed to be only 0.02% while it is a 100 times more rapid in the whole body (Wilson 1970). It is estimated that cholesterol has a half life of nearly six months in the brain (Andersson, Elmberger et al. 1990).

### ***Blood Brain Barrier***

The blood–brain barrier (BBB) is impermeable to the plasma lipoproteins and secludes the central nervous system (CNS) from the peripheral cholesterol pool (Danik, Champagne et al. 1999). Local tissue demand for cholesterol is supplied by synthesis within the brain (Morell and Jurevics 1996). Even though the BBB is a formidable barrier that preserves the cellular milieu in the brain, the component cerebral microvasculature could be damaged in the presence of disease such as stroke (Hulthe, Wikstrand et al. 1997) and possibly AD (Skoog, Wallin et al. 1998). And this process occurs early in the process well before the manifestation of the disease.

Only a miniscule amount of cholesterol is transported from the brain to the CSF via an ApoE-dependent mechanism. The majority of brain cholesterol is converted to 24-hydroxycholesterol by the enzyme 24-hydroxylase which is expressed predominantly in the brain. This metabolite readily crosses the BBB, and helps sustain brain cholesterol homeostasis. This oxysterol could potentially serve as a biomarker for cholesterol turnover in the brain (Lund, Guileyardo et al. 1999).

### **Animal models of hypercholesterolemia and AD pathology**

A high cholesterol diet in animal models has been shown to increase A $\beta$  formation and deposition. Cholesterol-fed rabbits are prone to accumulate A $\beta$  in the brain and this deposition can be reversed by withholding the dietary cholesterol (Sparks, Scheff et al. 1994; Sparks 1996). A double transgenic mouse model over expressing mutant APP and presenilin-1 manifest elevated numbers and size of A $\beta$  deposits in the brains in response to cholesterol (Refolo, Malester et al. 2000). Cholesterol-lowering drug treatment of these mice decreased not only plasma cholesterol, but also brain A $\beta$  generation and deposition (Refolo, Pappolla et al. 2001). The reversible nature of the pathology gives hope to the notion that AD is treatable if appropriate therapeutic modalities are availed of at an early stage in the disease process.

Cholesterol could influence the pathological processes in AD as evidenced by: (1) the higher risk of AD when midlife plasma cholesterol is elevated; (2) a decrease in the prevalence of AD by cholesterol-lowering drugs; and (3) the role of intracellular

cholesterol in regulating the processing of APP and hence generation of A $\beta$  peptides that accumulate as amyloid plaques in the brains and in cerebral vasculature of AD patients. Thus genes involved in cholesterol metabolism could influence AD.

### **ApolipoproteinE**

ApolipoproteinE (ApoE), a ~34 kDa secreted protein product of the gene located in chromosome 19q13.3, is abundantly produced in the brain, and is also an essential constituent of various lipoproteins (Wilson, Mau et al. 1994). Transplantation studies indicate that CNS apoE is synthesized locally (Linton, Gish et al. 1991) by astrocytes and microglia (Pitas, Boyles et al. 1987; Ladu, Reardon et al. 2000). Freshly synthesized glial derived apoE bearing particles are discoidal but starts maturing in shape and lipid content so much so when it reaches the CSF the particles are 15–20 nm spheres bearing additional apoA1 and resemble HDL particles (LaDu, Gilligan et al. 1998; Demeester, Castro et al. 2000; Koch, Donarski et al. 2001).

### ***ApoE variants***

There are three alleles of which ApoE3 is the most prevalent and has following amino acids at the indicated positions (Cys112, Arg158). The other two variants differ only by a single amino acid substitution: APOE2 (Cys112, Cys158), and APOE4 (Arg112, Arg158) (Mahley 1988). ApoE4 was identified to be a genetic risk factor of AD in 1993 (Corder, Saunders et al. 1993), a finding that has now been well replicated in a multitude of studies. So much so, the genetic association of apoE4 with AD can be tested as an internal positive control in association studies of novel candidate genes (Finckh 2003).

In the early days following this discovery, it was a puzzle as to how apoE was mechanistically linked to AD and even now after more than a decade of research the picture is far from complete. Cholesterol redistribution brought about by apoE is thought to facilitate the repair and maintenance of neuronal membranes and myelin, and this is especially helpful in the stressful setting of CNS injury or disease which can further induce synthesis of apoE (Ignatius, Gebicke-Harter et al. 1986; Ignatius, Gebicke-Harter et al. 1987). Thus allele specific outcomes of disease status could be attributed at least

partly to this mechanism. For instance apoE4 is associated with poor outcomes in different types of neuronal damage including head trauma, cerebral hemorrhage, and stroke (MO, Muir et al. 1998; Buttini, Orth et al. 1999; Liaquat, Dunn et al. 2002).

More specifically in the setting of AD apoE could directly interact with APP (Hass, Weidemann et al. 2001), or A $\beta$  [reviewed and discussed in (Tomiyama, Corder et al. 1999)] or indirectly influence these proteins by binding to its receptors and competing them away and/or altering signaling.

### ***Allele specific effects***

In the periphery apoE genotype can be correlated with plasma LDL cholesterol (LDL-C), where apoE4 is associated with the highest LDL-C levels, apoE2 with lowest levels, and apoE3 with intermediate measurements (Menzel, Kladetzky et al. 1983). ApoE4 is also a significant risk factor for coronary artery disease, which is probably on account of differential clearance of VLDL and chylomicron (Knouff, Hinsdale et al. 1999; Welty, Lichtenstein et al. 2000; Lahoz, Schaefer et al. 2001). Therapeutic response to statin therapy can be correlated with apoE genotype as well. Efficacy in LDL reduction with pravastatin treatment is greatest in apoE2, smallest in apoE4, and intermediate with apoE3 reflecting binding affinities of these particles with their receptors (Ordovas, Lopez-Miranda et al. 1995).

The age of onset for AD has been associated with apoE variants, such that apoE4 homozygotes have a mean age of onset of <70 years which is about 8 years earlier than apoE3 homozygotes (Corder, Saunders et al. 1993; Strittmatter and Roses 1995). This differential effect of the isoforms on the pathologic course of AD is mirrored by the differential binding affinity of the various isoforms with their receptors (apoE4 > apoE3 > apoE2). For e.g. apoE2 which binds very poorly to the receptors, may be protective in AD (Saunders 2000).

## **Low density lipoprotein receptor:**

### ***Gene Structure***

The low density lipoprotein receptor (LDLR) gene (GeneID: 3949) consists of 18 exons, which correspond closely to the structural units of the protein. LDLR is a striking example of a mosaic protein encoded by a gene that was assembled by exon shuffling. LDLR transcription is regulated efficiently by sterols. The constitutive transcription factor, Sp1 can bind to two repeat elements in the promoter region. The sterol-responsive element-1 (SRE-1) is the third site on the promoter, and this is recognized by SRE-1 binding proteins (SREBPs) which can then ramp up the transcription rate. The LDLR is expressed in all tissues of the body at variable levels, including the brain. Mutations in this gene cause the autosomal dominant disorder, familial hypercholesterolemia (FH). The disorder leads to markedly elevated plasma levels of LDL-cholesterol, the deposition of cholesterol on blood-vessel walls, premature atherosclerosis and coronary artery disease (CAD).

### ***Homologues***

This gene family arose with the evolution of multicellular lifeforms underscoring its importance in moving cargo and communication between cells, and is remarkably conserved with time. Absence of functional LRP1 in *C. elegans* results in improper molting and failure to thrive (Yochem, Tuck et al. 1999). Six *Drosophila melanogaster* genes belong to the LDLR family. Mutants identified include (1.) *yolkless*, which leads to arrested embryonic development due to inability to accumulate yolk in the egg (Schonbaum, Lee et al. 1995) and (2.) *arrow*, the orthologue of mammalian LRP5 and LRP6, which is required for Wg/Wnt signaling (Wehrli, Dougan et al. 2000).

The mammalian LDLR gene family consists of seven core members, including LDLR, the LDLR-related protein (LRP, a.k.a. LRP1), LRP1b, megalin (a.k.a. gp330 or LRP2), the VLDL receptor (VLDLR), Apolipoprotein E receptor-2 (ApoER2, a.k.a. LRP8), and a multiple epidermal growth factor (EGF) repeat containing protein, MEGF7 (reviewed in (Herz and Bock 2002)).

### ***Distinguishing features of ‘core members’***

By popular consensus ‘core members’ are expected to display all of the following five distinct domains or sequence motifs: (a) cysteine-rich ligand binding domain, (b) epidermal growth factor type cysteine-rich repeats, (c) YWTD domains that form a fold of  $\beta$ -pleated sheets resembling a propeller structure (Springer 1998; Jeon, Meng et al. 2001), (d) a single membrane spanning segment, and (e) a cytoplasmic tail containing one or more 'NPxY' (Asp-Pro-any amino acid-Tyr) tetraamino acid motifs (Krieger and Herz 1994; Willnow 1999; Herz, Gotthardt et al. 2000). The only other feature that is allowable is the optional O-linked sugar domain.

Some three other proteins LRP5, LRP6, and LR11 (a.k.a. SORLA) are still considered close relatives of the core family members despite not meeting all the structural requirements. All LDLR family members share an ability to bind the receptor-associated protein (RAP), which acts as a molecular chaperone in the endoplasmic reticulum (ER) where it facilitates proper folding (Bu, Geuze et al. 1995; Bu, Rennke et al. 1997; Obermoeller-McCormick, Li et al. 2001) as well as preventing the precocious interaction with ligands that might be traveling in the same compartment along with the receptors (Willnow, Armstrong et al. 1995; Willnow, Rohlmann et al. 1996).

### ***Receptor mediated endocytosis***

LDLR belongs to a gene family of cell surface proteins involved in receptor-mediated endocytosis of specific ligands. Low density lipoprotein (LDL) is normally bound at the cell membrane by the transmembrane LDLR and internalized into the cell. The LDL dissociates from its receptors in the acidic environment of the endosome. The LDLR protein is then returned to the plasma membrane. Regardless of ligand binding status, LDLR typically makes one round trip into the cell and back to the plasma membrane every 10 minutes, making a total of several hundred trips in its 20-hour life-span.

By contrast the cargo i.e. LDL particle is broken down upon fusion with lysosomes. The apo-B protein is degraded to amino acids and the cholesterol esters are hydrolyzed to fatty acids and cholesterol. The free cholesterol is made available for repression of

microsomal enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, the rate-limiting step in cholesterol synthesis. Simultaneously, a reciprocal stimulation of cholesterol ester synthesis favors storage and utilization of excess cholesterol. Thus abundant imported cholesterol inhibits synthesis by the cell of both cholesterol and LDL receptor protein.

### ***Critical domains involved in ligand binding and release***

LDLR can bind to the apolipoprotein B-100 bearing LDL as well as the apolipoprotein E studded beta-VLDL. Lipid association can change the conformation of apoE and improve the affinity of apoE for its receptor. Out of the seven LDLR class A repeats, numbers 4 and 5 is sufficient to bind apoE-containing ligands, as revealed by LDLR-derived "minireceptor" studies (Fisher, Abdul-Aziz et al. 2004).

The propeller could well emerge as an intramolecular mechanism for displacing lipoprotein particles. The extracellular domain of LDLR maintains an extended linear shape at neutral pH when it is amenable to ligand binding, but assumes a more compact shape at acidic pH as found in endosomes. The R1–R4 fragment normally does not associate with the rest of the extracellular domain at neutral pH, but it has been shown to do so at an acidic pH and this could be a mechanism whereby the ligand is displaced competitively (Rudenko, Henry et al. 2002).

### **LDLR variants result in Familial hypercholesterolemia**

The ground breaking work by Brown and Goldstein (Brown and Goldstein 1986) demonstrated that FH can be caused by mutations in the *LDLR* gene. FH is an autosomal dominant genetic disorder where there is a deficiency of LDLR activity. The absence of functional LDLR in target organs especially in the liver results in the characteristic clinical phenotype. Homozygotes have a qualitative loss of functional protein and are severely affected and suffer from markedly elevated plasma levels of LDL-cholesterol, the deposition of cholesterol in blood vessels, tendinous xanthomata and premature heart attacks. Heterozygotes survive longer especially with treatment because they have functional protein although at a reduced level.

The frequency of heterozygous FH in Caucasian populations is reported to be 1/500 (0.2 percent) (Goldstein, Schrott et al. 1973). More than 800 LDLR variants have been identified in FH subjects, and these genetic variants have been extensively reviewed (Hobbs, Brown et al. 1992; Day, Haddad et al. 1997) and compiled online at two websites: <http://www.ucl.ac.uk/fh/> (Heath, Gahan et al. 2001) and [www.umd.necker.fr/LDLR/research.html](http://www.umd.necker.fr/LDLR/research.html) (Villegier, Abifadel et al. 2002). Interestingly not all of these variants are known to be functional.

### ***Classification of functional LDLR mutations***

There are five classes of functional LDLR mutations (Hobbs, Brown et al. 1992) (Hobbs, Russell et al. 1990). Class 1 mutations result in no protein synthesis (null alleles) due to disruptions of the promoter sequence, nonsense, frameshift, or splicing mutations. Class 2 mutations disrupt transport of the LDL receptor from the endoplasmic reticulum to the Golgi apparatus due to mutations in the ligand-binding and epidermal growth factor precursor regions. Class 3 mutations are also primarily found in the ligand-binding and epidermal growth factor precursor regions but interfere with cell surface binding of the receptor to LDL. Class 4 mutations in the cytoplasmic and membrane-spanning domains inhibit the clustering of LDL receptors on the cell surface, so that the bound LDL particle is not internalized. Class 5 mutations in the epidermal growth factor precursor region prevent the proper release of LDL particles in the endosome and, as a result, the LDL receptor is not recycled to the cell surface.

### ***The spectrum of hypercholesterolemia***

Hypercholesterolemia refers to a blood cholesterol measurement  $>200$  mg/dl. It is estimated that more than 100 million Americans have total blood cholesterol above the desirable levels (i.e.  $> 200$  mg/dL). A cholesterol level of 200 to 239 mg/dl is considered borderline high, and a level of  $\geq 240$  mg/dl is considered to be a high cholesterol measurement (<http://www.nhlbi.nih.gov/health/public/heart/chol/wyntk.htm>).

Hypercholesterolemia (a.k.a. hypercholesteremia, hypercholesterinemia or type II familial hyperlipoproteinemia) can be related to many factors, such as: a diet rich in saturated fat, trans fatty acids (trans fats), and cholesterol; obesity; sedentary lifestyle; increasing age; postmenopausal state; and, familial hyperlipidemias. The National

Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III) lays down the following guidelines which consider the following values as desirable. Total cholesterol level less than 200 mg/dL; LDL cholesterol < 100 mg/dL; HDL levels the higher the better, 60 mg/dL or above is deemed to be protective (see website <http://www.nhlbi.nih.gov/guidelines/cholesterol/atglance.htm>).

### ***Clinical management***

The clinical management of hypercholesterolemia is based on both cholesterol measurements and the presence of cardiovascular risk factors. Nonpharmacologic modalities are the first line of therapy. Options include low fat dietary modifications that limit cholesterol (to less than 200 mg/day), and saturated fat (<7% of total calories). Life style changes include regular aerobic exercise, cessation of smoking and counseling on CAD risk factors. Chronic drug treatment is additionally employed in severe cases where the goals are not reached by the above measures, or the values are considered high relative to the existing CAD risk factors. For instance, patients with LDL >160 mg/dl; LDL 130 to 159 mg/dl with two or more risk factors for CAD; LDL >100 mg/dl with known disease such as CAD, vascular disease or diabetes mellitus are all candidates for drug therapy. The physician can choose from cholesterol absorption inhibitors (ezetimibe); Bile acid sequestrants; Niacin; HMG-CoA reductase inhibitors (Statins); and, Fibrates. The drugs can be tailored to suit the individual lipid profile, lifestyle so as to decrease the side effects and maximize compliance.

### **LDLR variants could be aberrantly spliced**

Mutations could alter splicing. This is to be expected when the base change occurs in known splice site sequences. It is more difficult to appreciate the functional consequences when the mutation affects cis-acting elements in the exons. The polymorphisms could alter exon splicing by affecting either enhancers or silencers. In a recent study of FH in Spain 39 novel mutations were identified, of which ~15% involved splicing (Mozas, Castillo et al. 2004). Intronic mutations when present in regulatory regions could result in splice variants and could lead to truncated proteins (Nissen, Hansen et al. 1997).

### ***SR proteins are a part of the spliceosome***

The splicing apparatus for GU-AG introns are made of small nuclear ribonucleic acids (snRNAs) such as U1, U2, U4, U5 and U6. These are less than 200 nucleotides in length and associate with proteins to become small nuclear ribonucleoproteins (snRNPs). The snRNPs, together with other accessory proteins, bind to the transcript and ultimately results in the spliceosome, the complex structure within which the actual splicing reactions occur (Smith and Valcarcel 2000).

Splice-site selection is still a mystery, but it has become increasingly clear that a set of splicing factors called SR proteins are important in this process of determining the correct splice sites so that exons are not lost during splicing, and cryptic sites are ignored. The C-terminal domains of SR proteins contain an abundant number of serine (abbreviation S) and arginine (R) amino acids. They are components of the spliceosome and are implicated in the formation of a connection between bound U1-snRNP and bound U2AF in the commitment complex (Valcarcel and Green 1996). This is believed to be important for splice-site selection especially in conjunction with ESEs.

### ***Exon Splicing Enhancer mutations can affect splicing and phenotype***

Exon splicing enhancer (ESE) refers to purine-rich sequences located in exons that can enhance splicing (Blencowe 2000). Inactivation of ESEs might be the underlying cause of many human genetic diseases linked to mutations found in exons (Blencowe 2000). Nonsense, missense and silent mutations can inactivate genes by inducing skipping of the mutant exons.

For instance, some cases of C5 deficiency in the immune system (Pfarr, Prawitt et al. 2005), spinal muscular atrophy, Becker muscular dystrophy, cystic fibrosis transmembrane conductance regulator gene mutations (Aznarez, Chan et al. 2003), and Tau exon 10 inclusion in frontotemporal dementia with Parkinsonism-chromosome 17 type (FTDP-17) could be due to disruption of functional ESEs (D'Souza and Schellenberg 2000). Thus even innocuous looking coding-region SNPs that are predicted to be translationally silent could turn out to be functionally malicious by virtue of

affecting the splicing machinery. This could affect the phenotype by bringing about exon skipping (Cartegni, Chew et al. 2002).

### **LDLR a good candidate for AD association study**

The 18 exon, ~42,000 base pair LDLR gene is a strong candidate for AD association studies for several reasons. First, the LDLR gene is located on chromosome 19p13.3, within a recently reported linkage peak in late onset, familial AD (Wijsman, Daw et al. 2004). Second, LDLR is a receptor for apoE (Herz and Bock 2002); since apoE variants are associated with AD, LDLR variants may also be associated with AD. Third, LDLR is intimately linked with cholesterol homeostasis, which itself may emerge as an AD modulator. The link between LDLR and cholesterol is supported by the facts that LDLR mutations cause familial hypercholesterolemia (Pullinger, Kane et al. 2003) while LDLR haplotypes appear associated with cholesterol levels and variance (Knoblauch, Bauerfeind et al. 2002; Knoblauch, Bauerfeind et al. 2004). While high cholesterol adversely affects both the cardiovascular system and the nervous system (Marx 2001), recent observations have linked cholesterol and AD more specifically, including that increased cholesterol alters APP processing to increase A $\beta$  levels in vitro and possibly in vivo (reviewed in (Puglielli, Tanzi et al. 2003)), and that cholesterol lowering drugs, statins, are associated with a lower AD prevalence in some observational studies ((Jick, Zornberg et al. 2000; Wolozin, Kellman et al. 2000), reviewed in (Miller and Chacko 2004)). In summary, since LDLR is a strong candidate gene for AD association, the goal of this study was to evaluate LDLR SNPs and haplotypes for their association with AD.

## **Chapter Two: LDLr variants based on three single nucleotide polymorphisms**

### **Rationale**

The location of the LDLR gene, its function as an apoE receptor and in cholesterol transport makes it a strong candidate gene for AD association studies as explained in the introduction chapter. The goal of this study was to evaluate LDLR SNPs and haplotypes for their association with AD.

### **Hypothesis**

LDLR gene variants will associate with AD.

### **Specific Aims**

In order to test the hypothesis we decided to answer the following specific questions.

Aim 1: To evaluate specific SNPs in the LDLR gene for association with AD in the UKY Series.

Aim 2: To evaluate specific haplotypes constructed from the SNPs for association with AD in the UKY Series.

Aim 3: To evaluate if any association that is found is reproducible in a second study series (the ROS series).

### **Single Nucleotide Polymorphisms**

Sequence variations within the genome are responsible for the phenotypic differences observed between individuals that make them unique. Some of the very same variations could also account for differential susceptibility to diseases. SNPs are one particular type of variation, where there are single base nucleotide substitutions. This could be a substitution involving potentially any of the four nucleotides A, T, G, or C but usually only two alleles are present. It occurs as often as 1 kb in the genome making it the

most abundant of genetic variations. They are about 1.4 million SNPs throughout the genome with about 6% or a hundred thousand of them resulting in restriction fragment length polymorphisms (RFLPs). Unlike RFLPs which could be laborious to analyze, all SNPs are amenable to high throughput analysis using modern genotyping methodologies.

### ***Genotyping methodology***

Genomic DNA was extracted from peripheral blood leukocytes or autopsied brain using routine methods. Samples were genotyped for apoE status by using a restriction fragment length polymorphism approach (Hixson and Vernier 1990). Samples were genotyped for LDLR SNPs by using unlabeled PCR primers and TaqMan MGB probes (FAM and VIC dye labeled) obtained via the 'Assays-by-Design' service from Applied Biosystems (Foster City, CA, USA).

For each individual SNP, the sequences of the forward primer, reverse primer, major allele probe and minor allele probe are as follows: Exon10:

5'ACGGCGTCTCTTCCTATGACA3', 5'CAGCCAGCCCGTCGG3':

6FAMAGCAGGGACATCCMGBNFQ, VICAGCAGAGACATCCAGMGBNFQ; Exon

13: 5'AACTTGTTGGCTGAAAACCTACTGT3', 5'GCTGACCCACCCTTACCTCTT3':

VICTGTGGAAGAGAACCATMGBNFQ, 6FAMAAGAGGACCATATCCMGBNFQ;

Exon15: 5'TCCACAGCCGTAAGGACACA3', 5'CAGCCGGGAGGTGTCG3':

6FAMCACCCGGCCTGTTMGBNFQ, VICACCACCCGACCTGTMGBNFQ.

### ***Statistical methodology***

The Estimated Haplotype (EH) program was used to test for linkage disequilibrium between alleles and between overall haplotypes and AD (Terwilliger J 1994); as reported elsewhere, the LDLR SNPs were in significant linkage disequilibrium (Knoblauch, Bauerfeind et al. 2002). The indicated p values are nominal and are considered significant if less than 0.05. Statistical Analysis System (SAS®) software (SAS Institute, Inc., Cary, NC) was used for (i) chi-square ( $\chi^2$ ) tests of allelic and genotypic frequencies in persons with and without AD, (ii) determining odds ratios (OR), 95% confidence intervals (CI) and corresponding p values, and (iii) logistic regression to

evaluate the influence of apoE4 (one degree of freedom (df)), gender, age (one df), education (two df) and LDLR haplotype (two df).

### **Description and characterization of the study subjects from two distinct series:**

A hypothesis-generating series was derived from a case-control study at the University of Kentucky (UKY series) and a hypothesis-testing series was derived from a cohort of the Religious Orders Study at the Rush University Medical Center (ROS series).

#### ***UKY Series***

In the UKY series, DNA for the non-demented, control individuals, i.e., non-AD individuals, was obtained from the Biologically Resilient Adults in Neurological Studies (BRAiNS) program (Schmitt, Wetherby et al. 2001). To be included within this study, community volunteers were initially screened for alcohol or drug abuse, or psychiatric illness, or dementia; they were then evaluated longitudinally through bi-annual physical and neuropsychological dementia testing (Schmitt, Wetherby et al. 2001). AD individuals within the UKY series were identified through the UKY Alzheimers Center Memory Disorders Clinic; some of these individuals were initially within the BRAiNS program and converted to a diagnosis of AD over time (see below). The UKY series included 133 non-ADs (51 males, average age at last visit  $79.0 \pm 7.0$  years; 82 females,  $78.4 \pm 8.6$  years), 97 persons with probable AD, and 21 with possible AD (32 males, average age at diagnosis  $73.5 \pm 6.6$  years; 86 females,  $74.6 \pm 8.4$  years).

#### ***ROS Series***

The ROS cohort was comprised of older Catholic nuns, priests, and brothers who underwent annual neurological and cognitive performance testing as well as evaluation by a physician with expertise in the evaluation of older persons for dementia. The members of the ROS series included 69 non-AD individuals (36 males, average age  $80.0 \pm 7.1$  years; 33 females,  $84.0 \pm 5.4$  years), 67 deceased persons with probable AD, and 19 with possible AD (37 males, average age at death  $86.5 \pm 6.6$  years; 49 females,  $88.8 \pm 5.7$  years).

Thus within each series, the ages of AD and non-AD individuals were well matched. In this study, the datasets presented were comprised entirely of Caucasians. For both series, each subject was recruited following institutional review board-approved guidelines and appropriate informed consent was obtained. In both the UKY and ROS series, control individuals, i.e., non-AD individuals, were cognitively intact during the entire study period. In both series, individuals diagnosed with AD represented a pool of persons with a diagnosis of probable AD, i.e., met clinical criteria for AD without another condition possibly contributing to dementia, and possible AD, i.e., met clinical criteria for AD and had another condition, e.g., stroke, that was thought by the examining clinician to be contributing to dementia. These clinical criteria and diagnoses were made following the recommendations of the joint working group of the National Institute of Neurologic and Communicative Disorders and Stroke and the Alzheimers Disease and Related Disorders Association (NINCDS/ADRDA) (McKhann, Drachman et al. 1984).

### **ApoE association with AD serves as an internal positive control**

To characterize the study subjects initially, we examined whether apoE4 was associated with altered AD odds in the UKY and ROS series. Table 2-1 shows the distribution of apoE4 according to disease status in each series. ApoE4 was significantly associated with AD in both groups, confirming the reports of many others (reviewed in (Rocchi, Pellegrini et al. 2003)) and suggesting that apoE should be evaluated as a potential confounding factor. Thus after ascertaining that the study series are representative we proceeded to test aim one.

### **Characterization of SNPs chosen for this study**

Three SNPs in LDLR were chosen for this association study (see Table 2-2 and Figure 2-1). They are all common SNPs i.e.  $> 0.10$  in minor allele frequency. All the three SNPs are in the coding region of the gene and are synonymous i.e. do not result in any amino acid changes. They were chosen because they have been previously examined and shown to be useful in constructing haplotypes (Knoblauch, Bauerfeind et al. 2002).

## **Results from UKY Series - Hypothesis Formulation:**

### ***Single locus analysis for association with AD***

In order to test specific aim 1 we evaluated three exonic SNPs from LDLR (Table 2-2) in our hypothesis generating sample, i.e., the UKY series. Table 2-3 shows genotypic and allelic frequencies. After ascertaining that both the non-AD and AD series were in Hardy-Weinberg equilibrium, we tested the null hypothesis that there is no association between a SNP and AD, the alternate being that there is an association. None of the SNPs associated significantly with AD (Table 2-3). The exon 13 SNP has been evaluated previously with similar results (Lendon, Talbot et al. 1997). The LDLR SNPs were also not significantly associated with AD after parsing for apoE4 status.

### ***Multi locus or haplotypic analysis for association with AD***

Since a haplotypic analysis can provide insights regarding additional SNPs that have not been evaluated but are in linkage disequilibrium with the studied SNPs, we next evaluated whether LDLR haplotypes were associated with AD. As part of testing specific aim 2 unambiguous haplotypes could be assigned to 126 subjects who were either homozygous for each the three polymorphisms or heterozygous at only one of the three polymorphisms (Table 2-4). The resulting haplotypic frequencies were similar to those published elsewhere (Knoblauch, Bauerfeind et al. 2002). Inspection of the results suggested that the 211 haplotype was associated with AD, and, indeed, the 211 haplotype was associated with a lower odds of AD (OR=0.48, CI=0.24-0.95, p= 0.03) (nominal p value of 0.03,  $\chi^2$  analysis of 211 against all other haplotypes). Hence, although corrections for multiple testing would render this result non-significant, we interpret these data as suggesting the hypothesis that the 211 LDLR haplotype is associated with lower odds of AD.

## **Results from ROS Series - Hypothesis Testing:**

### ***Haplotypic analysis reveals association of 211 with AD***

We then proceeded to evaluate this hypothesis per se in the ROS series, thus addressing specific aim 3. The allele and genotype frequencies for the LDLR SNPs (Table 2-5) were determined along with the resulting unambiguous 211 and non-211

LDLR haplotypes (Table 2-6). Interestingly, although we were not formally testing individual SNPs and odds of AD, we noted that the exon 10 SNP alone was associated with odds of AD in this cohort. The 211 haplotype was significantly associated with decreased odds of AD (OR=0.40, CI=0.19-0.84,  $p=0.01$ ). This association remained significant when controlled for apoE status, age, gender and education by logistic regression analyses ( $p=0.03$ ).

Lastly, when we combined the ROS and UKY data sets, the significance of the association between the 211 LDLR haplotype and the odds of AD increased, as expected (OR=0.44, CI=0.26-0.73,  $p=0.001$ , Table 2-7). We also analyzed the dose dependency of the 211 LDLR haplotype and odds of AD (Table 2-8), which suggested that increasing copy number was associated with a reduced odds of AD (OR = 0.46, CI= 0.26-0.84,  $p=0.01$ ). Hence, the 211 LDLR haplotype appears associated with lower odds of AD.

## **Discussion**

The primary finding of this study was that the 211 LDLR haplotype associates with a lower likelihood of AD in two different AD/non-AD series. These results are significant for two primary reasons. First, an association between LDLR haplotypes and AD suggests a new genetic link between AD, apoE, and cholesterol because the LDLR is an apoE receptor and intimately involved in cholesterol homeostasis (reviewed in (Herz and Bock 2002; Pullinger, Kane et al. 2003)). Second, these studies are generally significant in that identifying genetic AD risk factors will aid in early diagnosis, and facilitate drug discovery by identifying patients at high risk for AD prior to symptomology. Moreover, these studies may lead to the discovery of novel molecular mechanisms underlying AD, and thereby suggest new therapeutic approaches.

Within this study, we used the UKY subjects to generate a hypothesis that was then tested in ROS. In doing so, we minimized concerns about multiple testing issues. However, we noted additional findings that merit discussion. First, among the LDLR haplotypes evaluated by Knoblauch et al., the 211 haplotype was associated with cholesterol values near the mean (Knoblauch, Bauerfeind et al. 2002). Although the relationship between

cholesterol in the periphery and CNS is unclear, this association may be consistent with the possibility that “normal” cholesterol (reviewed in (Puglielli, Tanzi et al. 2003; Miller and Chacko 2004)) and the 211 LDLR haplotype appear associated with reduced AD odds.

The second finding that merits comment is that the UKY and ROS series differed in that the exon 10 SNP was associated with AD in the ROS but not the UKY series (Table 2-3, 2-5). We have identified two possible explanations for this difference. First, we recognized that the average age at diagnosis of the UKY series was some 14 years earlier than the average age at death of the ROS AD subjects. Therefore, we parsed the UKY series to generate a subset that matched the AD age in the ROS series, i.e., by using only UKY AD individuals diagnosed at the age of 77 years or greater, or UKY non-AD individuals last observed at the age of 77 years or greater, we generated post hoc a series that contained 81 non-AD individuals (average age at last visit  $83.9 \pm 4.9$  years), and 48 persons with AD (average age at diagnosis  $81.2 \pm 4.0$  years); given that the average duration from diagnosis to death is approximately 5-8 years, an average age of diagnosis of 81 may be roughly comparable to an average age at death of 88 years. When the exon 10 SNP was analyzed within this subset, the exon 10 SNP frequency was 42% in the non-AD and 27% in the AD group, respectively (nominal p value 0.03), a result which mimics that of the ROS series. Hence, one possible explanation for the UKY/ROS discrepancy is that the AD ages of the two series are different. A second explanation for this difference may be attributed to the case-control versus cohort nature of the two series. The exon 10 SNP frequency in the AD individuals in the ROS and UKY series was similar, i.e., 31.9% and 34.6%, respectively. Moreover, the non-AD exon 10 SNP frequency in the ROS population, 44.9%, is similar to that reported for a European population, 43.5% (Knoblauch, Bauerfeind et al. 2002). However, the exon 10 SNP frequency in the UKY non-AD subjects was 37.2%. If the exon 10 SNP frequency in the UKY AD subjects was compared with the European or ROS non-AD frequency, the UKY AD series would approach significance. In summary, we are unclear as to why these two series differ with respect to the exon 10 SNP association with AD but speculate

that this association may be most discernible in individuals in their 8th decade, and/or in prospective, cohort-based series.

The third issue that merits comment is that an EH analysis, which is based on predicted, or ambiguous haplotypes did not reach significance in either the ROS or UKY series (data not shown). In considering the interpretation of this analysis versus the unambiguous haplotype analysis, we recognized that the primary difference was that the unambiguous haplotype analysis did not include individuals that were heterozygous at more than one position, e.g., 211/121 heterozygotes. Examination of the complete data from UKY and ROS series (Table 2-9) suggests that the 211 haplotype has its most robust effect when the second haplotype within the individual is 21X. Hence, we speculate that the odds associated with the 211 LDLR haplotype are attenuated in the EH, predicted haplotype analysis because this analysis includes individuals that are likely 211/12X heterozygotes, and the 12X haplotype carries a polymorphism that blunts the effects of the 211 haplotype. This speculation requires experimental evaluation.

Since the LDLR SNPs did not alter the amino acid coding sequence, the mechanistic underpinnings of this association study are unclear. However, the link between AD, cholesterol and genetic variants is derived from multiple angles, including (i) the apoE4 polymorphism has been associated with increased cholesterol and increased AD risk (reviewed in (Puglielli, Tanzi et al. 2003); (ii) SNPs in other members of the LDLR family, including oxidized-LDLR and LDLR-related protein (LRP), as well as SNPs in enzymes involved with cholesterol homeostasis, including cholesterol 24-hydroxylase (CYP46) and acyl-coenzyme A, have been implicated in AD risk in at least some series (Clatworthy, Gomez-Isla et al. 1997; Kang, Saitoh et al. 1997; Bertram, Blacker et al. 2000; Desai, DeKosky et al. 2002; Kolsch, Lutjohann et al. 2002; Lambert, Luedeking-Zimmer et al. 2003; Papassotiropoulos, Streffer et al. 2003; Wollmer, Streffer et al. 2003). Although the LDLR-associated risk observed here is independent of apoE4 status, we speculate that possible compensation among LDLR family members may confound association studies evaluating individual family members; (iii) increased cholesterol is associated with increased levels of A $\beta$ , a protein that appears critical to AD (reviewed in

(Puglielli, Tanzi et al. 2003)); (iv) statin use lowers cholesterol levels and may decrease AD risk (reviewed in (Miller and Chacko 2004)).

**Table 2-1: ApoE4 is associated with increased odds of AD in UKY and ROS subjects**

Series ApoE4 copy#	AD n (%)	Non-AD n (%)
UKY	111 (100.0)	133 (100.0)
0	49 (44.1)	93 (69.9)
1	48 (43.2)	38 (28.6)
2	14 (12.6)	2 (1.5)
ROS	87 (100.0)	69 (100.0)
0	51 (58.6)	56 (81.2)
1	32 (36.8)	13 (18.8)
2	4 (4.6)	0 (0.0)

These data show the numbers and frequencies of individuals carrying 0, 1 (heterozygous), or 2 (homozygous) copies of apoE4 in the AD and non-AD subjects. These odds ratios (OR) were similar to those reported in other studies (Saunders, Strittmatter et al. 1993; Roses 1994), e.g., the presence of apoE4 was significantly associated with AD in each series (UKY: OR = 2.91, CI= 1.73-4.92, p= 0.00005, ROS: OR=2.97, CI=1.43-6.15, p=0.003).

**Table 2-2: LDLR SNPs selected for association study**

SNP Position	NCBI SNP Cluster ID	Synonym	Minor Allele Frequency	Nucleotide exchange 1/2	Amino acid exchange
Exon 10	rs5930	WIAF-10989	0.358	G/A	R→R
Exon 13	rs5925	WIAF-10994	0.476	T/C	V→V
Exon 15	rs5927	WIAF-10996	0.221	G/A	R→R

**Table 2-3: LDLR Genotypic and Allelic Counts and Frequencies for the UKY series**

SNPs	AD n (%)	Non-AD n (%)	$\chi^2$ (p value)
<b>Exon 10</b>			
Genotype	114 (100.0)	133 (100.0)	0.40 (0.82)
1/1	47 (41.2)	51 (38.3)	
1/2	55 (48.2)	65 (48.9)	
2/2	12 (10.5)	17 (12.8)	
Allele	228 (100.0)	266 (100.0)	0.35 (0.55)
1	149 (65.4)	167 (62.8)	
2	79 (34.6)	99 (37.2)	
<b>Exon 13</b>			
Genotype	115 (100.0)	129 (100.0)	1.81 (0.40)
1/1	27 (23.5)	40 (31.0)	
1/2	63 (54.8)	62 (48.1)	
2/2	25 (21.7)	27 (20.9)	
Allele	230 (100.0)	258 (100.0)	0.85 (0.36)
1	117 (50.9)	142 (55.0)	
2	113 (49.1)	116 (45.0)	
<b>Exon 15</b>			
Genotype	115 (100.0)	131 (100.0)	2.10 (0.35)
1/1	74 (64.3)	75 (57.3)	
1/2	34 (29.6)	50 (38.2)	
2/2	7 (6.1)	6 (4.6)	
Allele	230 (100.0)	262 (100.0)	0.55 (0.46)
1	182 (79.1)	200 (76.3)	
2	48 (20.9)	62 (23.7)	

This table refers to the SNPs in exon 10, 13 and 15 with 1 denoting the major allele and 2 the minor allele. The actual number and frequency (%) are displayed.

**Table 2-4: Unambiguous LDLR haplotypes in the UKY series**

Haplotypes	AD n (%)	Non-AD n %)	Total n (%)
111	24 (20.0)	20 (15.2)	44 (17.5)
112	3 (2.5)	6 (4.5)	9 (3.6)
121	61 (50.8)	59 (44.7)	120 (47.6)
122	3 (2.5)	3 (2.3)	6 (2.4)
211	14 (11.7)	29 (22.0)	43 (17.1)
212	15 (12.5)	15 (11.4)	30 (11.9)
Total chromosomes	120 (100.0)	132 (100.0)	252 (100.0)

The three positions in the haplotype refer to the SNPs in exons 10, 13 and 15, respectively, with 1 denoting the major allele and 2 the minor allele. Unambiguous haplotypes were discerned for 126 subjects, i.e., 50% of the total. Chromosomal number and frequency (%) for AD and non-AD subjects are displayed. Although the 211 haplotype was not significantly associated with gender or apoeE4 status ( $p>0.05$ ), the 211 haplotype was associated with a lower odds of AD (OR=0.48, CI=0.24-0.95,  $p=0.03$ ).

**Table 2-5: LDLR Genotypic and Allelic Counts and Frequencies for the ROS series.**

SNPs	AD n (%)	Non-AD n (%)	$\chi^2$ (p value)
<b>Exon 10</b>			
Genotype	83 (100.0)	68 (100.0)	9.07 (0.01)
1/1	34 (41.0)	21 (30.9)	
1/2	45 (54.2)	33 (48.5)	
2/2	4 (4.8)	14 (20.6)	
Allele	166 (100.0)	136 (100.0)	5.31 (0.02)
1	113 (68.1)	75 (55.1)	
2	53 (31.9)	61 (44.9)	
<b>Exon 13</b>			
Genotype	83 (100.0)	69 (100.0)	5.52 (0.06)
1/1	19 (22.9)	28 (40.6)	
1/2	48 (57.8)	31 (44.9)	
2/2	16 (19.3)	10 (14.5)	
Allele	166 (100.0)	138 (100.0)	3.88 (0.05)
1	86 (51.8)	87 (63.0)	
2	80 (48.2)	51 (37.0)	
<b>Exon 15</b>			
Genotype	81 (100.0)	67 (100.0)	2.09 (0.35)
1/1	50 (61.7)	38 (56.7)	
1/2	29 (35.8)	24 (35.8)	
2/2	2 (2.5)	5 (7.5)	
Allele	162 (100.0)	134 (100.0)	1.05 (0.31)
1	129 (79.6)	100 (74.6)	
2	33 (20.4)	34 (25.4)	

This table refers to the SNPs in exon 10, 13 and 15 in the ROS series. The number of individuals and frequencies (%) are displayed. Although we were not formally testing the statistical significance of these results, we have included  $\chi^2$  and p values for the sake of completeness.

**Table 2-6: Unambiguous 211 and non-211 LDLR haplotypes in ROS series.**

Haplotypes	AD	Non-ADs	Total
211	12 (14.6)	27 (30.7)	39 (22.9)
Non-211	70 (85.4)	61 (69.3)	131 (77.1)
Total	82 (100.0)	88 (100.0)	170 (100.0)

This table presents the unambiguous 211 and non-211 LDLR haplotypes for the AD and non-AD individuals within the ROS series. The 211 haplotype was associated with a lower odds of AD (OR=0.40, CI=0.19-0.84, p= 0.01).

**Table 2-7: Unambiguous LDLR haplotypes in the combined UKY and ROS series**

Haplotypes	AD n (%)	Non-AD n (%)	Total n (%)
211	26 (12.9)	56 (25.5)	82 (19.4)
Non-211	176 (87.1)	164 (74.5)	340 (80.6)
Total	202 (100.0)	220 (100.0)	422 (100.0)

The 211 haplotype was associated with a lower odds of AD (OR=0.44, CI=0.26-0.73, p=0.001).

**Table 2-8: 211 LDLR haplotype copy number in the combined UKY and ROS series**

# 211 haplotype	AD n (%)	Non-AD n (%)
0	78 (77.2)	67 (60.9)
1	20 (19.8)	30 (27.3)
2	3 (3.0)	13 (11.8)
Total	101 (100.0)	110 (100.0)

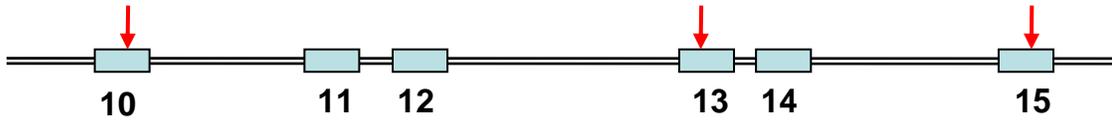
These data show the frequencies of individuals carrying 0, 1 (heterozygous), or 2 (homozygous) copies of the 211 LDLR haplotype in the combined AD and non-AD series. The presence of the 211 LDLR haplotype was significantly associated with AD (OR = 0.46, CI= 0.26-0.84, p= 0.01).

**Table 2-9: Complete data from UKY and ROS series**

Exon 10	Exon 13	Exon 15	Haplotypes	UKY		ROS	
				AD n (%)	Non-AD n (%)	AD n (%)	Non-AD n (%)
rs5930 G(1)/A(2)	rs5925 T(1)/C(2)	rs5927 G(1)/A(2)					
1/1	1/1	1/1	111/111	1 (0.9)	3 (2.4)	2 (2.5)	0 (0.0)
		1/2	111/112	1 (0.9)	0 (0.0)	0 (0.0)	0 (0.0)
		2/2	112/112	0 (0.0)	1 (0.8)	0 (0.0)	0 (0.0)
	1/2	1/1	111/121	16 (14.2)	8 (6.3)	11 (13.9)	9 (13.4)
		1/2	Ambiguous	5 (4.4)	7 (5.5)	4 (5.1)	2 (3.0)
		2/2	112/122	--	--	--	--
	2/2	1/1	121/121	21 (18.6)	24 (18.9)	13 (16.5)	9 (13.4)
		1/2	121/122	3 (2.7)	3 (2.4)	2 (2.5)	1 (1.5)
		2/2	122/122	--	--	--	--
1/2	1/1	1/1	111/211	5 (4.4)	6 (4.7)	9 (11.4)	8 (11.9)
		1/2	Ambiguous	6 (5.3)	8 (6.3)	4 (5.1)	1 (1.5)
		2/2	112/212	2 (1.8)	4 (3.1)	0 (0.0)	5 (7.5)
	1/2	1/1	Ambiguous	26 (23.0)	25 (19.7)	12 (15.2)	5 (7.5)
		1/2	Ambiguous	16 (14.2)	20 (15.7)	17 (21.5)	14 (20.9)
		2/2	Ambiguous	0 (0.0)	1 (0.8)	1 (1.3)	0 (0.0)
	2/2	1/1	121/221	--	--	--	--
		1/2	Ambiguous	--	--	--	--
		2/2	122/222	--	--	--	--
2/2	1/1	1/1	211/211	3 (2.7)	6 (4.7)	0 (0.0)	7 (10.4)
		1/2	211/212	3 (2.7)	11 (8.7)	2 (2.5)	5 (7.5)
		2/2	212/212	5 (4.4)	0 (0.0)	1 (1.3)	0 (0.0)
	1/2	1/1	211/221	--	--	1 (1.3)	0 (0.0)
		1/2	Ambiguous	--	--	0 (0.0)	1 (1.5)
		2/2	212/222	--	--	--	--
	2/2	1/1	221/221	--	--	--	--
		1/2	221/222	--	--	--	--
		2/2	222/222	--	--	--	--
			Total	113 (100.0)	127 (100.0)	79 (100.0)	67 (100.0)

These data reflect the overall results for this study and are included to facilitate eventual meta-analyses.

**Figure 2-1: Schematic of the three SNPs along the LDLR pre mRNA**



The diagram is not drawn to scale with regard to the length of the introns or exons. It is displayed so that the reader can appreciate the location of the three different SNPs.

## Chapter Three: The hunt for the functional SNP

### Rationale

This chapter focuses on understanding the mechanism whereby a genetic variation within the LDLR modulates LDLR function. The primary finding in our earlier study was that the 211 LDLR haplotype associates with a lower likelihood of AD in two different AD/non-AD series. Conversely the non 211 haplotypes are associated with increased odds of AD. Since the LDLR SNPs did not alter the amino acid coding sequence, the mechanistic underpinnings of this association study are unclear. Nearly fifty percent of the unambiguous haplotypes are formed by 121. It is possible that the 121 haplotype carries a polymorphism that blunts the effects of the 211 haplotype. This speculation requires experimental evaluation and that is exactly what we propose to do in this study. This chapter seeks to address the question as to what is the mechanism whereby a genetic variation within the LDLR genomic sequence modulates LDLR function.

### Hypothesis

The haplotypes harbour one or more functional SNP/SNPs

### Global Specific Aim

To identify linked SNP(s) within the haplotypes with physiologic function

**Specific Aim1:** To identify additional SNP(s) within the predetermined haplotypes by sequencing individuals displaying these haplotypes.

**Specific Aim2:** To perform theoretical evaluation of any identified SNP(s) for potential functional capacity using predictive modeling tools and to choose promising candidates

**Specific Aim3:** To test if any predicted effects actually occur in invitro/biologic models- a qualitative study

**Specific Aim4:** To test if the observed physiologic effect is associated with a specific haplotype/SNP- a quantitative study

### **Sequencing DNA to identify potentially functional SNP(s)**

The goal of this study was to unearth other functional SNPs that may be driving the LDLR haplotypic association with AD. We identified two individuals homozygous for haplotypes 211 or 121 from our previous study and selected these individuals for sequencing the exons and surrounding introns. We focused on the region between exon 9 and exon 14 using two complementary strategies. Our approach was to rapidly identify variants in exon-intron boundaries.

#### ***Methods:***

Genomic DNA of two haplotype 211 individuals and two haplotype 121 individuals was utilized in this study. Using standard molecular biology techniques primer pairs were designed that would amplify specific exons along with ~100 bp of the adjoining intron. Distinct bands were visualized by running on a polyacrylamide gel electrophoresis (PAGE) apparatus and the individual bands sliced out of the gel and purified using QIAEX II gel extraction kit (Qiagen) in isolation, taking care to avoid contamination. About 100 ng of the purified PCR product was added to 3  $\mu$ M of the respective sense or antisense primer in separate reactions. The volumes were made up to be 16  $\mu$ l and sequenced at an off-site facility, Davis Sequencing (Davis, CA).

#### ***Results:***

We chose to concentrate our work focusing on the region of exon 9 through 14 because the first two positions in our original haplotype appear to be the most robust and are defined by the SNPs in exons 10 and 13 and we felt that this was the most logical next step in our quest. In the course of sequencing the LDLR exons and adjoining introns in genomic DNA from two individuals homozygous for having 211 and comparing them with two individuals homozygous for 121 (the predominant non 211 haplotype), we identified one additional SNP in an exon. This SNP was in exon 12 (rs688, T for C) and the minor allele 'T' was found to label the 121 haplotype. There were several other SNPs identified in introns. One in intron 9 and three in intron 11. The SNP position and minor allele frequency for all of them are shown in Table 3-1. All of the SNPs have been

previously identified and readily available at the NCBI website online. Intron 11 is 646 bp long and rs2738447 is the closest to exon 12 at 54 base pairs 5' to exon 12. These SNPs appear to be differentiating the originally defined haplotypes. For instance the 211 haplotype is found to have 2---212---1 pattern in intron9--- intron11---exon12, which can be better appreciated visually in Figure 3-1.

### **Theoretical evaluation of identified SNP(s) for potential functional capacity**

#### ***Splicing of exon 12 is predicted to be affected by rs688***

rs688 is a SNP located within exon 12, at a position 68 nucleotides from the 5' end, at amino acid 591. This SNP has been previously identified and can be located online at NCBI a publicly available database ([http://www.ncbi.nlm.nih.gov/SNP/snp\\_ref.cgi?rs=688](http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=688)). This SNP has C as major allele and T as minor allele and is fairly common with a minor allele frequency of about 0.33. This abundant SNP has no prior clinical associations reported. The location of this SNP is at codon position 3 and does not alter the amino acid (Asn) at number 591. As such, this synonymous SNP is not predicted to effect the three dimensional structure of the protein (Wang, Address et al. 2007) or its interaction with its neighboring amino acid (Asn) in position 570 in three dimensions (<http://www.ncbi.nlm.nih.gov/SNP/snp3D.cgi?rsnum=688>). While searching for other tools to evaluate this SNP, we came across 'RESCUE-ESE Web Server' an online tool for identifying candidate ESEs in vertebrate exons (<http://genes.mit.edu/burgelab/rescue-ese/>). In brief ESEs are specific short oligo nucleotide sequences located in exons that enhance pre-mRNA splicing. The authors of this tool were able to identify 238 hexamers as candidate ESEs using a large database of human genes of known exon-intron structure containing over 30,000 non-redundant exons (<http://genes.mit.edu/burgelab/rescue-ese/ESE.txt>). One of these hexamers is TCAACG. Interestingly, rs688 alters this conserved DNA sequence termed an ESE element from the wild-type TCAACG to the polymorphic TCAATG. This subtle shift from the wild-type sequence is predicted to neutralize the ESE element such that the polymorphic sequence is not predicted to enhance splicing as shown in Figure 3-2 ((Fairbrother, Yeh et al. 2002; Fairbrother, Holste et al. 2004; Fairbrother, Yeo et al. 2004) on web at

<http://genes.mit.edu/burgelab/rescue-ese/>). Similar results were found using ESEfinder (<http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home>) (Cartegni, Wang et al. 2003; Smith, Zhang et al. 2006) as shown in Figure 3-3, 3-4). Thus when the SNP is present, the predicted splicing enhancer function is neutralized. This could result in an entire exon not being included in the transcript which has the potential to dramatically influence the structure of the protein (Fairbrother, Holste et al. 2004).

### ***Predicted effect of loss of exon 12***

The amino acids in proteins are encoded by codons in the mRNA with a unit length of three nucleotides. Exon 12 is 140 base pairs long which is not divisible by three, the unit length of a codon, and its loss results not only in the loss of the amino acids encoded by that exon but will also change the protein sequence encoded by the remaining exons following it. The exclusion of this exon 12 is predicted to cause a frameshift in exon 13, resulting in novel, carboxyl terminal sequences followed by a termination codon; the resulting LDLR protein would retain the LDL-binding domain encoded by exons 1-7 but lack the transmembrane domain encoded by exons 16-17 and hence in theory will be a truncated, secreted form of LDLR that retains the ability to bind apoE-containing lipoproteins. Similar secreted proteins are expected when the both exon 11 and 12 are spliced out. Both proteins will have identical sequence from the amino-terminus until the end of exon 10 but will have varying lengths of unique carboxyl termini. Due to the aberrant splicing and the frame shift premature stop codons occur well before the transmembrane domain encoding exons 16-17 and result in a truncated, secreted form of LDLR.

### **A Qualitative study to examine alternative splicing of LDLR exon 12**

The above mentioned effects are exciting. Do they actually happen in a physiologically relevant model? Does this alternative splicing actually happen in the brain? In order to address specific aim 3, we decided to set up the following experiment. We selected human hippocampal brain samples that were generously provided by the Sanders-Brown Alzheimers Disease Center Neuropathology Core. We used PCR to amplify the LDLR region of exons 10 through 14 from pooled human hippocampal cDNA samples, separated the DNA with polyacrylamide gel electrophoresis (PAGE),

stained with SYBR Gold and visualize the DNA on a fluorescent imager. The identity of the PCR products was determined by direct sequencing.

### ***PCR amplification Strategy***

Human hippocampal brain samples that were generously provided by the Sanders-Brown Alzheimers Disease Center Neuropathology Core. The samples were from deceased individuals and characterized previously. There were 25 samples in all and pooled human hippocampal cDNA preparation was generated by taking 1 microliter of each sample and mixing them together. The primer sequences are as follows, exon 10 sense 5' CATCGTGGTGGATCCTGTTC 3'; exon 14 antisense 5' GGTGGTCCTCTCACACCAGT 3'. PCR was performed on pooled human hippocampal cDNA preparation PCR profiles consisted of pre-incubation at 94°C for 60 sec, followed by 30 cycles of 94°C for 30s, 60°C for 45s, and 72°C for 90s (Perkin Elmer 9600). PCR products were separated by polyacrylamide gel electrophoresis (PAGE) and visualized by SYBR-gold fluorescence on a fluorescence imager (Fuji FLA-2000).

### ***Sequencing Methodology***

PCR product identities were determined by gel purification and direct sequencing (Davis Sequencing). Distinct bands were visualized by running on a polyacrylamide gel electrophoresis (PAGE) apparatus and the individual bands sliced out of the gel and purified using QIAEX II gel extraction kit (Qiagen) in isolation, taking care to avoid contamination. About 100 ng of the purified pcr product was added to 3 uM of the respective sense or antisense primer in separate reactions. The volumes were made up to be 16 µl and sequenced at an off-site facility, Davis Sequencing (Davis, CA).

### ***Results***

The PCR products upon separation by PAGE revealed multiple bands on analysis. The above mentioned approach revealed a 451 bp pcr product that is the predominant band comprising of exons 10 through 14 (which is the expected constitutive splicing product which could lead to full length LDLR). Other PCR products that were detected included a 311 bp band which could be due to a template lacking exon 12 and a 192 bp band which could be due to a template lacking exons 11 and 12. Primers were designed to anneal specifically to exon 10 and 14 of LDLR. This is ideal for studying the splicing

pattern of exon 12 and its neighbors because the bands will be well resolved and easily distinguishable from one another if indeed there is alternative splicing.

The identity of each band was determined definitively by direct sequencing, and shown to represent the alternative splicing patterns depicted in Figure 3-5. On close examination of the results the predicted model is confirmed to be true. The sequencing yielded excellent reads and were identical from either direction in the region of overlap. The exon boundaries were not violated and the exons 11 and/or 12 were included or omitted in a well defined manner.

### **Quantitative Splicing analyses: Compare splicing pattern of exon 12 in humans with different genotypes for rs688**

Having established that exon 12 can be alternatively spliced in the human brain tissue from the hippocampus, I proceeded to address the question as to how much of this alternative splicing occurs and if it associates with rs688. The goal was to identify individuals homozygous for the rs688 alleles, generate human hippocampus cDNA samples if needed and then analyze the splicing characteristics.

#### ***Methodology***

The choice was made to work with select autopsy specimens generously provided by the Sanders-Brown Alzheimers Disease Center Neuropathology Core. The cDNA or genomic DNA from 25 individuals were genotyped for rs688, by using unlabeled PCR primers and TaqMan MGB probes (FAM and VIC dye labeled) obtained via the 'Assays-by-Design' service from Applied Biosystems (Foster City, CA, USA) on an ABI-7000 or ABI-7900 (Applied Biosystems).

#### ***cDNA preparation:***

Total RNA was isolated from hippocampal autopsy samples from homozygous CC and homozygous TT individuals. This RNA was then quantified by UV spectroscopy to ascertain concentration and diluted to 1 ug per 5 ul to form a stock solution. It was then converted to cDNA (SuperScript II, Invitrogen) by reverse transcription by standard molecular techniques.

*PCR amplification and quantification:*

The region from exon 10 to exon 14 was PCR-amplified (Platinum Taq, Invitrogen) by using the primers specified earlier (refer section above), and the PCR products were separated by PAGE and visualized by SYBR-gold staining on a fluorescent imager (Fuji FLA-3000). To compare differences among RNA samples differential for the rs688 SNP, PCR signal intensities were quantified with the fluorescent imager. In an effort to improve the confidence in this assay background subtraction was performed within each lane, and additional normalization was performed wherein the length of the various bands were compared relative to the length of the full length exon 10-14 PCR product to obtain accurate stoichiometry. The tissues were processed simultaneously and splicing efficiency data obtained without regard to sex of the donor. For statistical analyses of splicing efficiency, data from CC and TT homozygous individuals were separated and analyzed by Students t test (StatSoft).

***Results: rs688T is associated with decreased splicing efficiency in the brain***

Out of the 25 samples we were able to identify 14 that were homozygous for rs688. To investigate whether rs688 is associated with exon 12 splicing efficiency in the human brain *in vivo*, we analyzed these 14 cDNA samples that were prepared from the human hippocampus, a brain area that is affected in AD. Eight of these individuals were homozygous for the major allele ‘C’ and six were homozygous for the minor allele ‘T’. We focused on how much constitutive splicing occurs in the vicinity of exon 12 by using appropriate PCR primers that anneal to exons 10 and 14. This approach revealed cDNA corresponding to the expected full length LDLR, i.e., containing exons 10, 11, 12, 13, and 14 (due to constitutive splicing), as well as LDLR sequences that lacked exon 12, or 11 and 12 (due to alternative splicing). A representative image of these experiments is shown in Figure 3-5. To compare the differences among RNA samples differential for the rs688 SNP, PCR signal intensities were quantified with the fluorescent imager, and normalized relative to the length of the full length exon 10-14 PCR product to obtain accurate stoichiometry. The resulting percentages of full-length LDLR across exons 10-14 as a function of rs688, the exon 12 SNP, are presented in Table 3-2.

This assay revealed that rs688T associated with a significantly lower proportion of exon 12 containing full length LDLR mRNA in the brain ( $p < 0.005$ , as shown in Table 3-2). The apparent difference in splicing was about 13%. In summary, rs688 associates with LDLR exon 12 splicing efficiency in the brain.

### **Compare the splicing pattern in human hippocampus vs cerebellum to check for regional variability**

The differences seen in the previous experiment could be attributed to either phenotypic or genotypic differences. Phenotypic differences could be due to cell population differences. AD for instance can damage the neurons in the hippocampus and alter the ratio of neurons to astrocytes. Neurons are believed to express LDLR and hence any decrease in the quantum of the receptor mRNA could be explained by presence of fewer neurons that can synthesize LDLR. And if this is the case then if we compare individuals with similar ratios of neuronal versus supporting cells in the hippocampus, the full length mRNA levels will be the same. And by extension if we compare another region of the brain that is relatively healthy the RNA levels will show no difference.

On the other hand it will be due to genotypic differences if we notice a persistence of the difference in the splicing pattern when we analyze a different region of the brain that will have a similar proportion of neurons versus supporting cells in all individuals. This could be quickly checked by performing the same study using a different region of the brain that is less vulnerable to the ravages of the neurodegenerative disorder. In this case I chose to examine tissue from the cerebellum which is a region that is not affected in AD. The exact methodology that was used to study the alternative splicing in the hippocampus was now employed to analyze the cerebellum derived cDNA.

### ***Results:***

Twenty individual samples were located and cDNA from the cerebellum was analyzed and compared to values obtained from the hippocampus. The results are provided in Figure 3-6. The positive slope of the line and the fair degree of correlation between the samples from the hippocampus and the cerebellum suggests that the differences are

unlikely to be due to regional variability. Thus genotypic differences seem to be the driving force for the splicing patterns observed.

**Summary:**

In this chapter we have discovered that the LDLR haplotypes of interest harbored several additional SNPs. Out of these SNPs we narrowed our focus on rs688. We identified that rs688 had the theoretical possibility for affecting splicing based on prediction models. Then we showed that the splicing occurs *in vivo*, in human hippocampus, and it varied based on the rs688 allele. The ESE prediction appears accurate as analyzing LDLR splicing patterns in human hippocampal RNA shows that individuals homozygous for rs688T have a significantly lower splicing efficiency.

**Table 3-1: LDLR SNPs identified by sequencing shown in context**

SNP Position	NCBI SNP Cluster ID	Minor Allele Frequency	Nucleotide exchange	Amino acid exchange
Intron 9	rs1003723	0.40	T/C	----
Exon 10	rs5930	0.36	G/A	R→R
Intron 11	rs2738445	0.43	T/C	----
Intron 11	rs2738446	0.29	C/G	----
Intron 11	rs2738447	0.28	C/A	----
Exon 12	Rs688	0.33	C/T	N→N
Exon 13	rs5925	0.48	T/C	V→V
Exon 15	rs5927	0.221	G/A	R→R

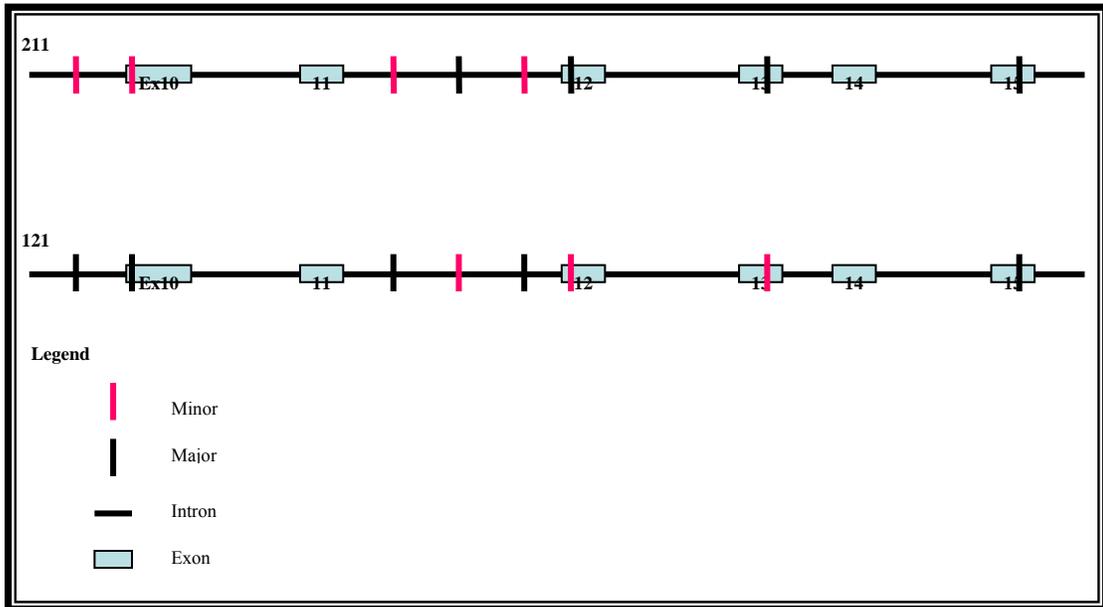
The SNPs were identified by sequencing genomic DNA from two 211 homozygous individuals and comparing them with two individuals homozygous for haplotype 121. The identified SNPs are shown in the table along with the SNPs in exon 10, exon 13 and exon 15 (highlighted in light grey) used to develop the original haplotypes.

**Table 3-2: rs688T is associated with decreased Full-Length LDLR mRNA**

Rs688	FL LDLR from exon 10 –14 Mean±SE (n)
C/C	74.1 ± 4.3 (8)
T/T	61.0 ± 1.9 (6)

This table shows the association between the exon 12 SNP rs688 and the amount of full length LDLR mRNA across exons 10-14. It shows the splicing efficiency in homozygous C/C and T/T individuals along with the standard error and the actual number of samples.

**Figure 3-1: Schematic of various SNPs along the pre mRNA:**



The diagram is not drawn to scale with regard to the length of the introns or exons. It is displayed so that the reader can appreciate the location of the different SNPs in table 3-1. It shows that several additional SNPs appear to be different among the originally defined haplotypes.

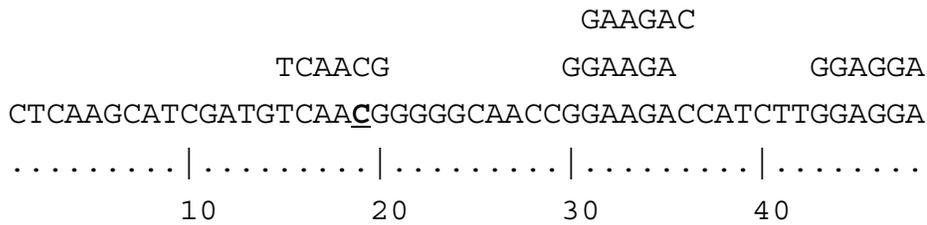
**Figure 3-2: Results from RescueESE program comparing influence of SNP rs688**

SPECIES: human

sequence length 48 bp, fragment of LDLR exon 12, rs688 with C

total matches 4

unique matches 4

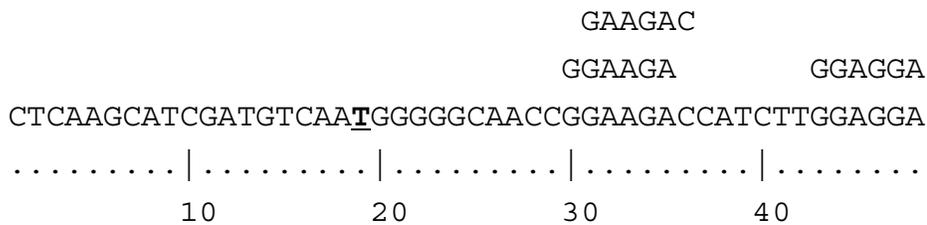


SPECIES: human LDLR

sequence length 48 bp, fragment of LDLR exon 12, rs688 with T

total matches 3

unique matches 3



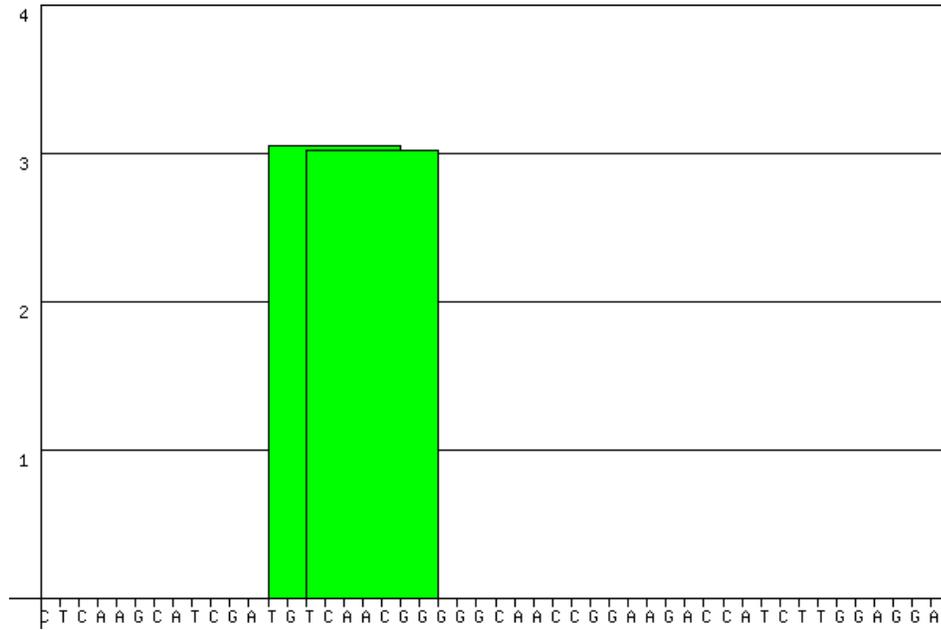
The SNP rs688 is at position 19 (in bold face and underlined) and this figure shows that when the sequence is analyzed with the RescueESE webtool as explained in the text, the T allele can alter the ESE sequence and hence potentially affect splicing.

**Figure 3-3: Results from ESEfinder program comparing influence of SNP rs688**

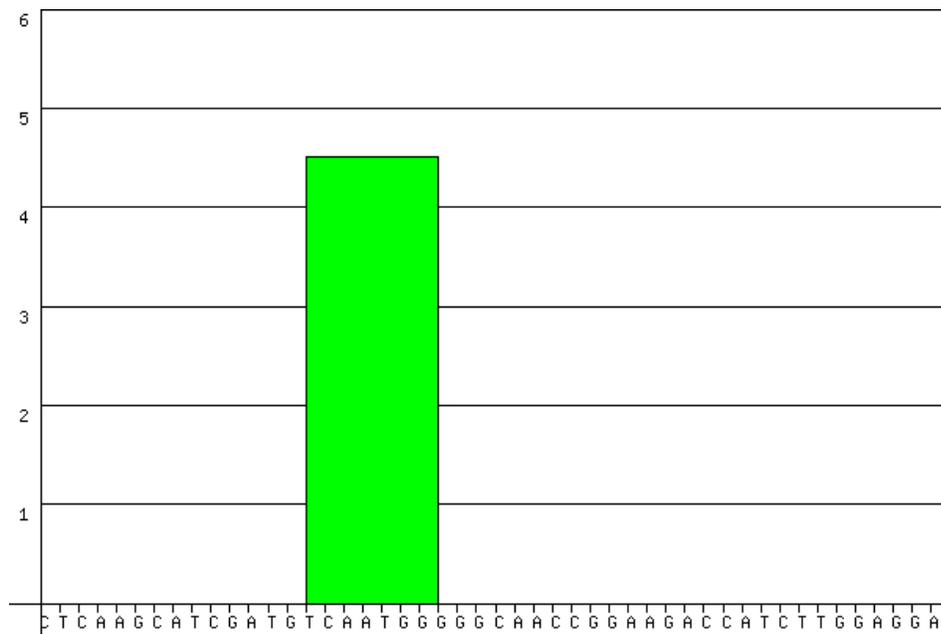
Seq	Motif	Position	Site	Score
rs688C	SRp40	13	TGTCAAC	3.04512
rs688C	SRp40	15	TCAACGG	3.01840
rs688T	SRp40	15	TCAATGG	4.50965

The SNP rs688 is at position 19 and its effects are shown in tabular form. This shows that the T allele can alter the ESE sequence resulting in lower threshold and loss of one of the SRp40 binding motif and hence may affect splicing.

**Figure 3-4a: Graphical results for rs688C and SRp40 binding thresholds**



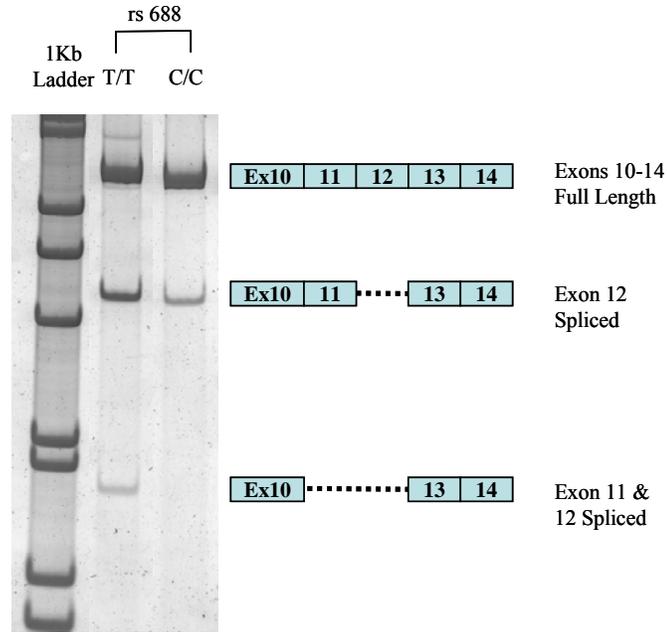
**Figure 3-4b: Graphical results for rs688T and SRp40 binding thresholds**



The SNP rs688 is at position 19 and its effects are shown in graph form from ESEfinder. The x axis represents the DNA sequences and the y axis represents the scores for the SRp40 protein binding for rs688C (Figure 3-4a) and rs688T (Figure 3-4b). The threshold

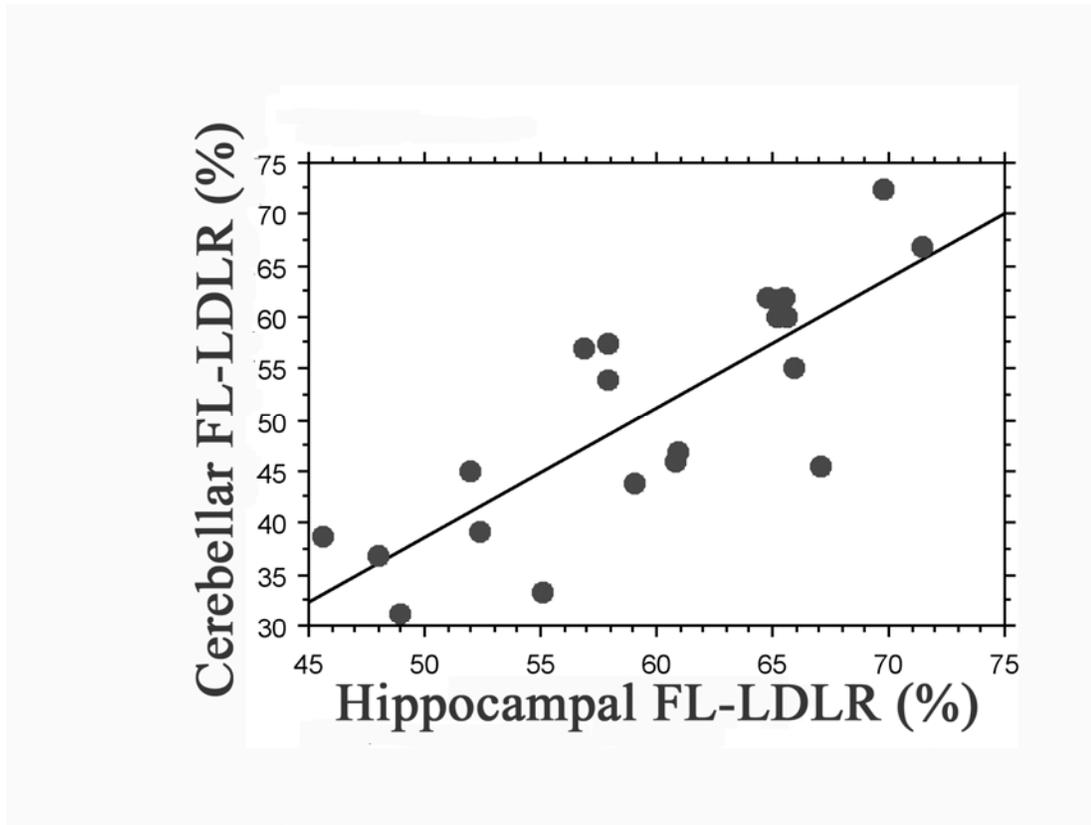
for binding is 2.67. Figure 3-4b predicts that the T allele can alter the binding sites for this protein involved in splicing.

**Figure 3-5: An exon 12 SNP rs688 associates with alternative LDLR Splicing**



Primers were designed to anneal specifically to exon 10 and 14 of LDLR. The PCR products upon separation by PAGE revealed multiple bands on analysis. The above mentioned approach revealed a 451 bp pcr product that is the predominant band comprising of exons 10 through 14 (which is the expected constitutive splicing product which could lead to full length LDLR). Other PCR products that were detected included a 311 bp band which lacks exon 12 and a 192 bp band which lacks exons 11 and 12.

**Figure 3-6: RNA splicing and regional variability**



Each data point indicates an individual. The x and y axis show the splicing efficiency of full length 10-14mRNA in the Hippocampus and Cerebellum respectively.

## Chapter Four: SNP rs688 and AD

### Rationale

This chapter focuses on exploring the association of rs688 with AD. In the previous chapter it was revealed that the LDLR haplotypes of interest harbored several additional SNPs. Out of these SNPs I narrowed my focus on rs688. I identified that rs688 had the theoretical possibility for affecting splicing based on prediction models. Then it was shown that the splicing occurs *in vivo*, in human hippocampus, and it varied based on the rs688 allele. In additional experiments with other members in our lab we evaluated the association of rs688 allele with exon 12 splicing efficiency *in vivo* further by quantifying LDLR splicing in human anterior cingulate tissue obtained at autopsy and found that the rs688T allele is associated with decreased LDLR exon 12 splicing efficiency in aged males, but not females (Zou, Gopalraj et al. 2008). Thus rs688 can modulate splicing in a sex dependant manner in the brain. This chapter will explore the association of rs688 with AD. The body of work in this chapter will also seek to find out if there is a sex dependant association in AD.

### Hypothesis

The SNP rs688 will associate with AD in a gender specific reproducible manner.

### Global Specific Aim

To test the SNP rs688 for association with AD and identify any gender specific effects that could influence this interaction

**Specific Aim1:** To test the SNP rs688 for genetic association with AD in the ROS series.

**Specific Aim2:** To test the effect of sex (male vs female) on this association in the ROS series.

**Specific Aim3:** To test if the sex and disease specific associations are consistently reflected in a larger population series

In order to address the specific aims I began by genotyping and analyzing data from the ROS series and then along with our collaborators study a large number of individuals across three population series for genetic association of rs688 with AD. The methodology for this chapter in its entirety is as described below.

## **Methodology for Genetic Studies**

### ***Study Population***

Three different series (JS, RS-AUT, and ROS) were analyzed (as described in (Zou, Gopalraj et al. 2008)). The JS series was collected through the Mayo Clinic Jacksonville memory disorders clinic (Ertekin-Taner, Ronald et al. 2005). This study included 215 AD men (mean age  $\pm$  standard deviation:  $79 \pm 8$ ) and 227 non-AD men (age:  $78 \pm 7$ ) as well as 362 women with AD (age:  $78 \pm 6$ ) and 339 non-AD women (age:  $78 \pm 8$ ). The RS series was collected through the prospective, community-based Mayo Clinic AD registry, as well as the Mayo Clinic Rochester memory disorders clinic (Ertekin-Taner, Ronald et al. 2005). For this cohort, we obtained data for 188 men with AD (age at diagnosis:  $81 \pm 8$ , and 519 non-AD men (age:  $78 \pm 6$ ), as well as 330 women with AD (age:  $81 \pm 8$ ), and 607 non-AD women (age:  $79 \pm 6$ ). The AD cases in the AUT series, which were combined with the RS series, were obtained from brains with neuropathologically confirmed AD that were collected at autopsy from Caucasians in Jacksonville, FL, Rochester, MN and many additional sites, and included 235 men (age at death:  $81 \pm 6$ ) as well as 337 women (age:  $85 \pm 7$ ). The ROS series represents the deceased members of the larger, prospective, community-based ROS series and have been described elsewhere (Gopalraj, Zhu et al. 2005). For this project, we used DNA from 37 men with AD (age:  $86 \pm 7$ ), and 36 non-AD men (age:  $80 \pm 7$ ), as well as 47 women with AD (age:  $89 \pm 6$ ), and 33 non-AD women (age:  $83 \pm 6$ ). Clinical AD diagnoses on all series were made with NINCDS-ADRDA criteria (McKhann, Drachman et al. 1984).

### ***Genotyping Method***

Genomic DNA was extracted from peripheral blood leukocytes or autopsied tissue by using routine methods (Zou, Gopalraj et al. 2008). Samples were genotyped for rs688 and/or rs5925, a surrogate which is in near perfect linkage disequilibrium with rs688, by using unlabeled PCR primers and TaqMan MGB probes (FAM and VIC dye labeled)

obtained via the ‘Assays-by-Design’ service from Applied Biosystems (Foster City, CA, USA) on an ABI-7000 or ABI-7900 (Applied Biosystems) (Zou, Gopalraj et al. 2008).

### ***Statistical Analyses***

The association of AD with rs688 was assessed by using logistic regression as adjusted for series, age, sex and presence of apoE4 (Zou, Gopalraj et al. 2008). Models were fit separately for males and females after strong evidence of an interaction of sex with the effect of rs688 was found in a single model (Zou, Gopalraj et al. 2008). Tests of interaction of rs688 by series, apoE4, and age were also conducted to evaluate whether there was evidence of heterogeneity across series (Zou, Gopalraj et al. 2008). For apoE and LDLR, the genotype frequencies in males and females from each series were consistent with Hardy Weinberg equilibrium (Zou, Gopalraj et al. 2008).

### **Results**

The data generated for all individuals across the three study series were analyzed together as laid out in the methods section. But for the purposes of this chapter I will initially present the findings from the ROS series in depth and then proceed to show the cumulative findings of all three population series.

#### ***rs688T associates with AD in the ROS population***

To test specific aim one the exon 12 SNP, rs688 was evaluated in the ROS series. Table 4-1 depicts the genotypic and allelic counts and frequencies. I tested the null hypothesis that there is no association between rs688 alleles and AD, the alternate being that there is an association. The minor allele ‘T’ is more abundantly present in the AD vs non-AD series, 48.2% vs 36.2% respectively. The SNP rs688 alleles associated significantly with AD ( $p = 0.04$  as shown in Table 4-1). This surprise finding though unexpected is heartening, because based on the findings in our lab I know that sex can influence the splicing efficiency in the brain. And so I was not expecting to find a robust difference in a mixed population comprising both sexes.

#### ***rs688T associates with AD in males***

The incidence and prevalence of AD varies by sex, with most studies drawing the conclusion that females generally appear to be at higher risk than males (McDowell

2001). Our lab has shown that rs688T allele is associated with decreased LDLR exon 12 splicing efficiency in human anterior cingulate tissue in aged males, but not females (Zou, Gopalraj et al. 2008).

To address specific aim two the data from ROS series are presented in detail to show rs688 and the genetic association with AD in men versus women. Table 4-2 depicts the genotypic and allelic counts and frequencies for the males in the ROS series. It was ascertained that both the non-AD and AD series were in Hardy-Weinberg equilibrium. Then we tested the null hypothesis that there is no association between rs688 alleles and AD, the alternate being that there is an association. The alleles for the SNP rs688 associated with AD significantly ( $p = 0.01$  as shown in Table 4-2). The minor allele 'T' is more abundantly present in the AD vs non-AD series, 54.1% vs 33.3% respectively.

Table 4-3 depicts the genotypic and allelic counts and frequencies for the females in the ROS series. It was ascertained that both the non-AD and AD series were in Hardy-Weinberg equilibrium. Then I tested the null hypothesis that there is no association between rs688 alleles and AD, the alternate being that there is an association. The alleles for the SNP rs688 did not show any association with AD ( $p = 0.59$  as shown in Table 4-3). The minor allele 'T' is still more abundantly present in the AD vs non-AD series, 48.2% vs 37.0% respectively.

#### ***rs688T associates with AD in males in three population series***

I was able to work with our collaborators both here at the University of Kentucky and outside and analyze whether rs688 associates with AD in a sex-dependent manner. In all DNA was genotyped from 1,457 men and 2,055 women drawn from three case control series. As described in the methods section these series were derived from clinically diagnosed subjects at the Mayo Clinic Jacksonville, FL (JS) and the Religious Orders Studies (ROS), as well as clinically diagnosed subjects at the Mayo Clinic Rochester, MN, which were combined with a series of AD autopsies (RS-AUT) (Ertekin-Taner, Ronald et al. 2005; Rogaeva, Meng et al. 2007; Zhu, Taylor et al. 2008). Analysis of the association of AD with rs688 (recessive model) using logistic regression adjusted for series, presence of apoE4, age and sex was performed (Zou, Gopalraj et al. 2008). The

interaction terms for rs688 with series, and apoE4, was also considered and it was found that there was strong evidence of an interaction of rs688 with sex ( $p=0.001$ ), but no evidence of heterogeneity in rs688 effects across series ( $p=0.46$ ) or of interactions with age ( $p=0.93$ ) or the presence of apoE4 ( $p=0.45$ ). Separate logistic regression models were then used to analyze male and female data further (Zou, Gopalraj et al. 2008). Males with genotype rs688T/T exhibited increased odds of AD (OR= 1.49,  $p=0.005$ , Figure 4-1) (Zou, Gopalraj et al. 2008). However, in females, the estimated odds ratio was 0.79 ( $p=0.056$ ), consistent with the sex-dependent splicing in the brain (Zou, Gopalraj et al. 2008). There was no significant difference in odds ratios among the three series for either males or females, but the RS-AUT and ROS populations displayed a more robust association in males than the JS series (Zou, Gopalraj et al. 2008). The statistical significance of this finding in the male series overall was maintained even after correction for multiple testing, for instance if the conservative Bonferroni correction is utilized for testing allelic, recessive and dominant models it would adjust the overall p-value to 0.015 (Zou, Gopalraj et al. 2008). Hence, the rs688T allele that is associated with decreased splicing efficiency in tissue samples from males is also found to be associated with increased odds of AD in males in the homozygous condition. In women the level of significance was only marginal, especially after correcting for multiple testing. Therefore I would cautiously interpret the results in women as suggestive evidence that rs688T may be associated with reduced AD risk in women (Zou, Gopalraj et al. 2008). In summary, rs688T/T is associated with increased odds of AD in males but not in females.

## **Discussion**

The primary findings of this study is that rs688 is a functional SNP that associates with LDLR exon 12 splicing efficiency in the human hippocampus and also associates with significantly increased odds of AD in men. Hence, the overall significance of this study is two-fold. First, these results specifically support the hypothesis that rs688 which is a functional SNP in LDLR, a brain apoE receptor, is associated with AD in a sex dependent manner. Second, the unusual sex-dependent association of rs688 with AD may be relevant to other genetic variants or candidate genes in general (Zou, Gopalraj et al.

2008). Other AD genetic studies may benefit by parsing the data based on sex and analyzing men and women separately.

rs688 is a common SNP located within LDLR. By virtue of its location in an excellent AD candidate gene, several prior studies have evaluated the association between AD and rs688 or linked SNPs (Retz, Thome et al. 2001; Cheng, Huang et al. 2005; Gopalraj, Zhu et al. 2005; Bertram, Hsiao et al. 2007). When these studies were undertaken, there was no awareness of any functional significance of rs688 or the association with sex. Therefore the investigators did not parse their series by sex. For instance even in this particular study if one were to analyze rs688 in males and females combined together by logistic regression, after adjusting for series, presence of apoE4 and sex, one will find no association (Zou, Gopalraj et al. 2008). The overall OR for rs688 in the recessive model was 1.04 (95% CI of 0.86-1.25,  $p=0.69$ ). The lack of association in women obscured the association resulting in an erroneous conclusion. Hence, stratifying for sex makes a huge difference in detecting the association described here. The use of a recessive model helped modestly in the detection of the association, but was not critical. For instance even an allelic dosage model showed that while the odds of AD for rs688C/T male individuals was equivalent to that of rs688C/C males (OR=1.00, 95% CI of 0.78-1.27), rs688T/T males had an increased odds of AD (OR= 1.49, 95% of CI-1.09-2.04,  $p=0.013$ ) (Zou, Gopalraj et al. 2008). In summary, I would suggest that additional case control series must be analyzed to confirm this finding that rs688T/T associates with increased odds of AD in male but not female subjects. I am confident that further studies that are well designed and of sufficient size will confirm these findings. And this expectation is also based on the demonstration of alteration in biological function such as altered splicing efficiency.

**Table 4-1: LDLR rs688 Genotypic and Allelic Counts and Frequencies for the ROS series.**

SNPs	AD n (%)	Non-AD n (%)	$\chi^2$ (p value)
<b>rs688</b>			
Genotype	84 (100.0)	69 (100.0)	5.14 (0.08)
1/1	21 (25.0)	29 (42.0)	
1/2	45 (53.6)	30 (43.5)	
2/2	18 (21.4)	10 (14.5)	
Allele	168 (100.0)	138 (100.0)	4.44 (0.04)
1	87 (51.8)	88 (63.8)	
2	81 (48.2)	50 (36.2)	

This table refers to the distribution pattern of SNP rs688 in the ROS series in AD and non-AD individuals. The number of individuals and frequencies (%) are displayed along with the  $\chi^2$  and p values.

**Table 4-2: LDLR rs688 Genotypic and Allelic Counts and Frequencies for the ROS series - Males.**

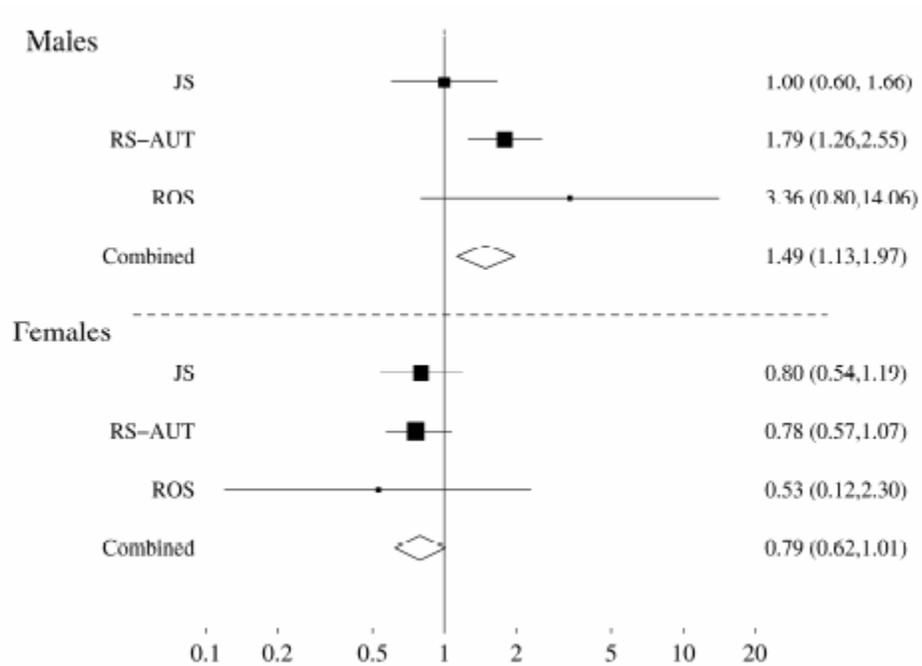
SNPs	AD n (%)	Non-AD n (%)	$\chi^2$ (p value)
<b>rs688</b>			
Genotype	37 (100.0)	36 (100.0)	5.98 (0.05)
1/1	08 (21.6)	17 (47.2)	
1/2	18 (48.6)	14 (38.9)	
2/2	11 (29.7)	05 (13.9)	
Allele	74 (100.0)	72 (100.0)	6.36 (0.01)
1	34 (45.9)	48 (66.7)	
2	40 (54.1)	24 (33.3)	

This table refers to the distribution pattern of SNP rs688 in the ROS series in AD and non-AD males. The number of individuals and frequencies (%) are displayed along with the  $\chi^2$  and p values.

**Table 4-3: LDLR rs688 Genotypic and Allelic Counts and Frequencies for the ROS series - Females.**

SNPs	AD n (%)	Non-AD n (%)	$\chi^2$ (p value)
<b>rs688</b>			
Genotype	47 (100.0)	33 (100.0)	0.76 (0.68)
1/1	13 (22.7)	12 (36.4)	
1/2	27 (57.4)	16 (48.5)	
2/2	07 (14.9)	05 (15.2)	
Allele	166 (100.0)	138 (100.0)	0.28 (0.59)
1	86 (51.8)	87 (63.0)	
2	80 (48.2)	51 (37.0)	

This table refers to the distribution pattern of SNP rs688 in the ROS series in AD and non-AD females. The number of individuals and frequencies (%) are displayed along with the  $\chi^2$  and p values.



**Figure 4-1: Rs688 is associated with AD in men.** This Forest plot depicts the OR and 95% CI for the association of rs688 with AD in each series, separately for men and women. The bar width reflects the 95% CI while the symbol size reflects the relative size of the series. The open diamonds represent the overall OR combining all three series with the diamond width corresponding to 95% CI. The sample sizes for each genotype for males were: rs688C/C (476), rs688C/T (704) and rs688T/T (277) while those for females were rs688C/C (656), rs688C/T (1021) and rs688T/T (278).

## **Chapter Five: Conclusion and Future Directions**

I am excited by the progression of our studies. I started with a promising candidate gene, then identified an interesting haplotype, then I found a SNP that seemed to label the haplotype, then I was able to predict the function by computational methods, then confirmed the findings in several different models with help from members of the Estus lab, and finally to associate the variant with disease. This final chapter will build on these studies and explain our model in the context of other research in the field.

### **Primary findings**

The primary findings of this study are four fold. First, the 211 LDLR haplotype associates with a lower likelihood of AD in two different AD/ non-AD population series. Second, the 211 haplotype does not contain the rs688T allele. Third, rs688T is associated with reduced splicing efficiency of exon 12 in human hippocampus, an area of the brain involved in AD. Fourth, this functional SNP rs688 associates with increased odds of AD in men.

### **Overall significance**

The overall significance of these studies are three-fold. First, these results specifically support the hypothesis that a functional SNP in LDLR, a brain apoE receptor, is associated with AD in a sex-dependent manner. Second, the unusual sex-dependent association of rs688 with AD may be relevant to other genetic variants in general, suggesting that AD genetic studies may benefit by analyzing men and women separately. Third, these studies are generally significant in that identifying genetic AD risk factors will aid in early diagnosis, and facilitate drug discovery by identifying patients at high risk for AD prior to symptomology. Moreover, these studies may lead to the discovery of novel molecular mechanisms underlying AD, and thereby suggest new therapeutic approaches.

LDLR has many different roles, including cholesterol metabolism, apoE sequestration and endocytosis. Other functions are emerging more slowly as more information becomes

available, such as its ability to influence signaling through other family members and competition for common ligands.

Although apoE and LDLR SNPs associate with both cholesterol and AD, we do not interpret our results as supporting a positive correlation between peripheral cholesterol and AD risk generally. ApoE4 and rs688T both associate with increased LDL-cholesterol and increased AD risk but these associations have clear differences in their sex dependence. The presence of an apoE4 allele in women is associated with a 5 mg/dl increase in LDL-cholesterol (Schaefer, Lamon-Fava et al. 1994) and an increased AD risk with an OR of 5.15 (95% CI of 4.23-6.27, data from this study). Males are similar in that apoE4 is associated with a 3 mg/dl increase in LDL-cholesterol (Schaefer, Lamon-Fava et al. 1994) and a 3.81 AD OR (95% CI 3.05-4.75). In contrast, rs688T is associated with a 6 mg/dl increase in LDL cholesterol in women (Zhu, Tucker et al. 2007) but an AD odds ratio of 0.79 (95% CI 0.62-1.01). Moreover, rs688T in men was associated with an insignificant trend towards decreased cholesterol (Zhu, Tucker et al. 2007) but had an odds ratio of 1.49 (95% CI 1.13-1.97) for AD risk. Thus the effects of apoE4 and rs688T on peripheral cholesterol do not correlate with their effects on AD risk.

## **Model**

For this reason, we interpret our data as suggesting that rs688 modulates LDLR function with respect to cholesterol in the periphery and possibly with apoE metabolism in the CNS. In the former, the relationship between LDLR and cholesterol has been well-substantiated; individuals with impaired LDLR function have less LDL-cholesterol removed from the extracellular space, resulting in increased intracellular cholesterol synthesis, further exacerbating plasma cholesterol levels (reviewed in (Hobbs, Brown et al. 1992)). Hence, LDLR in the periphery is intimately linked with cholesterol homeostasis per se. In contrast, LDLR deficiency in the brain does not alter brain cholesterol, at least as modeled by the LDLR deficient murine brain (Fryer, Demattos et al. 2005). Rather, LDLR deficiency increases murine brain apoE levels, as well as levels of apoE3 and apoE4 in mice expressing these human alleles (Fryer, Demattos et al. 2005) (Cao, Fukuchi et al. 2006). Since increased apoE expression associates with increased A $\beta$

pathology in the mouse (Holtzman, Fagan et al. 2000), the most parsimonious interpretation of our data in men is a model wherein rs688T decreases exon 12 splicing efficiency, leading to reduced functional LDLR and reduced apoE clearance, and thereby, increased amyloid pathology and AD risk.

This model is supported by several additional lines of reasoning. First, the rs688T allele increases the proportion of LDLR that lacks exon 12, which shifts the LDLR reading frame, leading to a premature stop codon in exon 13 and a LDLR isoform lacking the transmembrane domain encoded by exons 16-17; consistent with this possibility, a similarly truncated LDLR produced by a nonsense mutation in exon 13 is retained within the endoplasmic reticulum and causes familial hypercholesterolemia (Lehrman, Schneider et al. 1987). Second, LDLR deficiency in murine models of amyloidogenesis may exacerbate amyloid accumulation and memory deficits (Fryer, Demattos et al. 2005; Cao, Fukuchi et al. 2006). Third, the association between rs688 and increased AD risk was consistent with a recessive model; one can interpret this result as suggesting a threshold effect, i.e., LDLR expression must be reduced below a critical threshold for AD risk to be increased. Hence, while a partial reduction in LDLR due to a single rs688T allele is insufficient to increase AD risk, a homozygous rs688T/T genotype is sufficient to increase risk, consistent with the rs688T allele acting in a loss-of-function fashion. In summary, the parsimonious interpretation of these results is a recessive model wherein LDLR protein encoded by the exon 12-deficient LDLR isoform is not functional, representing a loss of apoE receptor, reduced apoE clearance and increased risk for AD in males (see model in Figure 5-1).

It is still unclear as to what are the underlying mechanism(s) restricting rs688 effects on splicing to male brain tissue and female liver tissue (Zhu, Tucker et al. 2007). This restriction may be more quantitative than qualitative as close inspection of the opposite sex data, i.e., splicing efficiency in female brain tissue and male liver tissue, reveals similar, albeit very modest trends. Further work in our lab has identified two related, estrogen centric hypotheses that may account for these sex-dependent rs688 effects. First, since (i) rs688T is predicted to neutralize an SRp40 binding site, (ii) SRp40 may be

estrogen regulated (Tyson-Capper, Bailey et al. 2005), and (iii) estrogen levels in the aged male are higher than those in the aged female due to aromatase acting upon testosterone (Vermeulen, Kaufman et al. 2002), one can speculate that at least in the brain, estrogen modulated SRp40 may account for rs688 sex-dependent actions. The second model proposes that since estrogen acts upon the estrogen-response element in the LDLR promoter to enhance LDLR expression (Li, Briggs et al. 2001), estrogen may also modulate LDLR splicing by concurrent recruiting of splicing factors such as CAPERalpha, as has been reported for estrogen actions on the transcription and splicing of other genes (Auboeuf, Honig et al. 2002; Dowhan, Hong et al. 2005). In summary, one can hypothesize that rs688 association with splicing in vivo in a sex-dependent fashion reflects estrogen actions on SRp40 activity and /or splicing factor recruitment concurrent with transcription.

### **Future Directions**

These studies have advanced the understanding of the role of LDLR in AD. My efforts have answered some questions, but while doing so it has raised more questions that need to be answered. These additional studies can help elucidate the role of rs688 in specific and LDLR in general in AD. The following are all valid questions to study and answers are needed. Whether SRp40 plays a role in regulating the splicing efficiency of LDLR? If so how? Does it recruit other cellular machinery directly by attracting them, or indirectly by negating the suppressive influences of other regulatory elements in the vicinity? Which splicing factors are involved in exon 12 alternative splicing? How important is the promoter region in LDLR and is it differential with rs688T bearing haplotypes?

One can design experiments to show that SRp40 is critical for alternative splicing of exon 12. SRp40 is supposed to bind in the region around rs688 as explained earlier in chapter three. One can use site directed mutagenesis to change the nucleotides around rs688 to show that when the footprint of the SR protein binding site is affected even the rs688C allele results in decreased splicing efficiency. With better prediction models becoming available with time one could also analyze the surrounding sequence for any silencer

elements that could negatively affect constitutive splicing. This will be interest because it will lend itself to further delineating the mechanism for SRp40s action in this instance, as to whether it directly recruits other helpful splicing machinery to the site or releases the repressive effects of a neighboring protein bound to a silencer element (exon splicing silencers) in the gene. And one can employ the same technique of site directed mutagenesis to change the nucleotides of the silencer element and study the effects on splicing efficiency. If there are no predicted elements one may even identify new regulatory elements if any over short stretches of DNA such as exon 12 by this approach.

One could also test if these effects are dependent on the RS domain which is a very important part of the SR proteins by using truncated proteins that lack this domain. One could study the effects of overexpressing or adding exogenous SRp40 or dominant negative proteins on splicing efficiency in these models. SRp40 and its interactions specifically with splicing factor U2AF35 and/or the snRNP protein U1-70K and splicing co-activator Srm160 can also be studied. I believe this targeted approach can be very fruitful in identifying the relevant players in this phenomenon. This could also help delineate whether these effects are RS domain dependent or independent (Cartegni, Chew et al. 2002).

It is known that there is a sterol-responsive element in the LDLR promoter that when activated will result in activating the SCAP/SREBP pathway (Duriez 2003; Dou, Fan et al. 2008; Morin, Nichols et al. 2008). One could sequence the promoter region from individuals with high vs low splicing efficiency and try to identify any linked SNPs and in functional assays one could investigate the effects of exogenous estrogen other steroids and drugs on the promoter activity and splicing efficiency.

So far I have been talking about events that happen upstream that can influence the levels of mRNA expression. But if the absolute amount of functional LDLR protein is a determinant in AD one also would need to be concerned about downstream events. We will need to look at the actual expression levels of LDLR protein. Some mechanisms that can alter gene expression levels can have no apparent change in protein levels, but this

again can be due to increased mRNA degradation or upregulation of other pathways that can downregulate the cell surface protein levels, such as the serine protease proprotein convertase subtilisin/kexin 9 (PCSK9). This will be instrumental in understanding the specific effects of rs688 alleles and will also open the door to the study of other genes and pathways that can influence the LDLR levels in general in association with AD. For instance PCSK9 has been shown to have a role in regulating LDLR protein levels in rat livers and this effect could be modulated by hormonal and dietary modalities (Persson, Galman et al. 2008). Lipid lowering medications could also affect the PCSK9 effects on LDLR (Gouni-Berthold, Berthold et al. 2008). It would be fascinating to study these effects in brain tissue and the interplay of rs688 and other LDLR SNPs. This line of investigation would advance the hypothesis that the amount of functional surface LDLR plays a pivotal role in AD by possibly altering the apoE and amyloid beta clearance and degradation, consistent with a recessive model for the biologic effects of rs688T influenced truncated soluble LDLR in the brain.

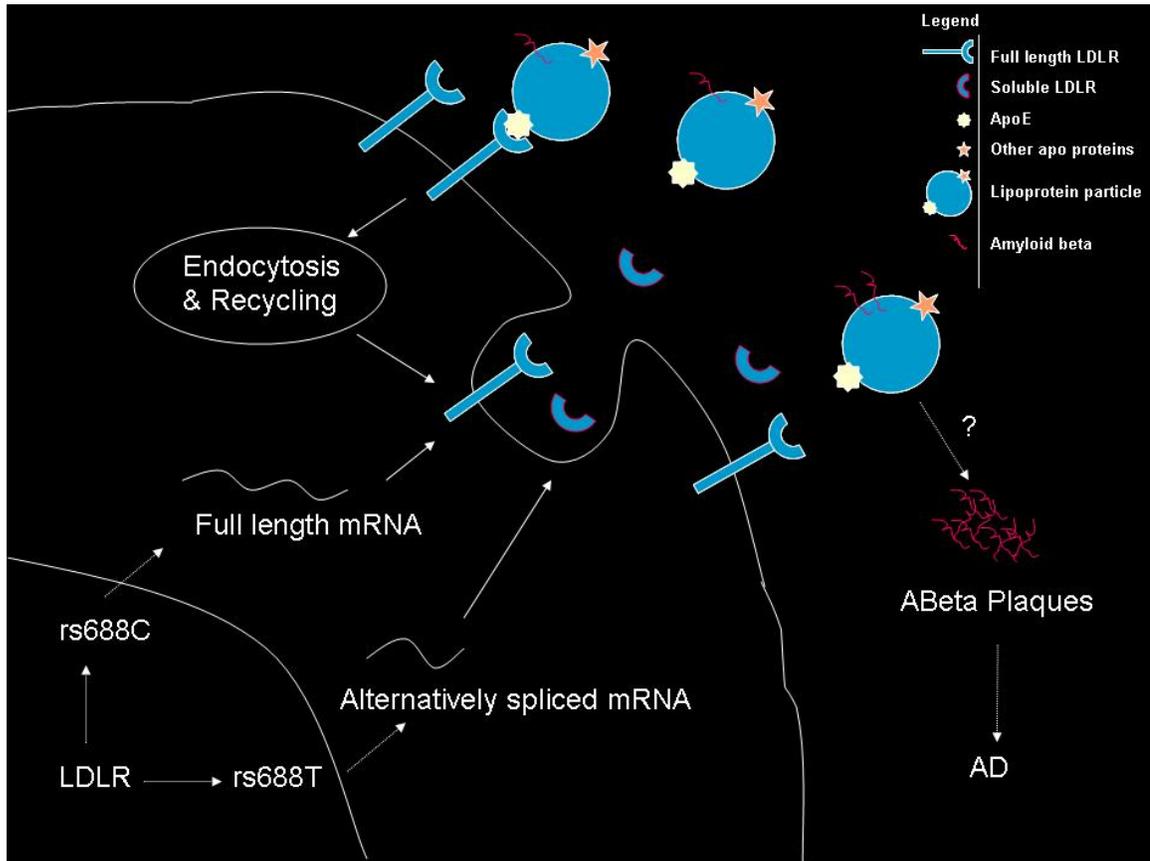
Once the accuracy of this model is established, it will pave the way to investigate drugs that increase LDLR and might be beneficial in AD. For instance statins can upregulate the expression of the LDLR. The findings from these additional experiments might also be applicable in lipid metabolism and cardiovascular disease. Also it will be interesting to look at new emerging research and how that may affect the model that has been developed from this study. For instance some studies suggest that gonadotrophins may be important in the brain signaling. So much so in some instances it is not just the absence of estrogens but the presence of gonadotrophins that can orchestrate multiple effects (Webber, Casadesus et al. 2005). Also it is tempting to speculate that the soluble LDLR can escape the ravages of PCSK9 and this might be deleterious especially in the periphery where soluble LDLR could act in a dominant fashion.

## **Conclusion**

In conclusion, rs688 is a common and functional LDLR SNP that modulates LDLR exon splicing efficiency in vitro in neural cells and in vivo in the brain. Moreover, rs688 associates significantly with increased AD risk in men. Since LDLR expression in the

CNS could be manipulated, e.g., by statins that penetrate the blood-brain barrier, these results suggest that LDLR modulation may represent a therapeutic target in at-risk populations. Overall, I anticipate these studies may prove useful for understanding the role of sex and apoE receptors in AD. Moreover, these studies provide a model for identifying AD susceptibility alleles by focusing on well-characterized functional SNPs in genes likely to influence risk for AD.

**Figure 5-1: Model of rs688 effect on AD**



This figure depicts the working model as to how rs688T could increase the odds of AD in men. rs688T decreases exon 12 splicing efficiency, leading to reduced functional LDLR and reduced apoE clearance, and thereby, increased amyloid pathology and AD risk.

# APPENDICES

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**Author:** Rangaraj K. Gopalraj, Haiyan Zhu, Jeremiah F. Kelly, Marta Mendiondo, Joseph F. Pulliam, David A. Bennett and Steven Estus  
**Publication:** Neurobiology of Aging  
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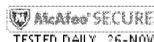
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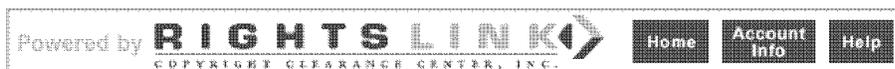
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- 2000-2008     **PhD** completed December 2, 2008, University of Kentucky, Department of Physiology. Dissertation: "LDL Receptor Polymorphisms and Alzheimers Disease", Mentor - Dr. Steven Estus, Lexington, KY
- 1993-1999     **M.B.B.S.** (Bachelor of Medicine and Bachelor of Surgery), Kilpauk Medical College and Hospital (KMCH), The T.N. Dr. M.G.R. Medical University, Madras (Chennai), India.

**Employment**

- 2005-2008     Resident, University of Kentucky Family Medicine Program at Hazard, KY
- 2005-2008     Resident Physician, East Kentucky Veteran Center at Hazard, KY
- 04/00-07/00     Duty Medical Officer, Voluntary Health Services, Adyar, India.
- 01/00-06/00     Special Trainee, Department of General Surgery, Stanley Medical College & Hospital, India
- 1998-1999     Compulsory Rotatory Resident Internship (CRRI), KMCH and Govt. Royapettah Hospital, India

**Awards/Support**

- 2002-2003     Kentucky Opportunity Fellowship, University of Kentucky, Lexington, KY.
- 2001-2002     Scholarship from Research Challenge Trust Fund, University of Kentucky, Lexington, KY.
- 2000-2001     Target Opportunity Research Assistantship, University of Kentucky, Lexington, KY.
- June, 2001     Excellence in Research Award for best research plan, 13<sup>th</sup> Annual Research Day, Department of Physical Medicine & Rehabilitation, University of Kentucky, Lexington, KY.
- Fall, 2000     Best overall performance among all students in the Integrated Biomedical Science Courses.

**Abstracts**

- R.K. Gopalraj**, F. Koura, R. Narayan and M. Dignan. Pulmonary Function Changes Among Alpha-1 Antitrypsin deficient and Chronic Obstructive Pulmonary Disease Patients and Effect of Augmentation Therapy. **American Thoracic Society, Toronto International Conference**, May 19, 2008.

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- F. Zou, **R. K. Gopalraj**, J. Lok, H. Zhu, I-F. Ling, J. F. Simpson, H. M. Tucker, J. F. Kelly, S. G. Younkin, D. W. Dickson, R. C. Petersen, N. R. Graff-Radford, D. A. Bennett, J. E. Crook, S. G. Younkin and S. Estus. Sex-dependent Association of a Common Low Density Lipoprotein Receptor Polymorphism with RNA Splicing Efficiency in the Brain and Alzheimers Disease. **Hum Mol Genet.** 2008 Apr 1;17(7):929-35. Epub 2007 Dec 8.
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