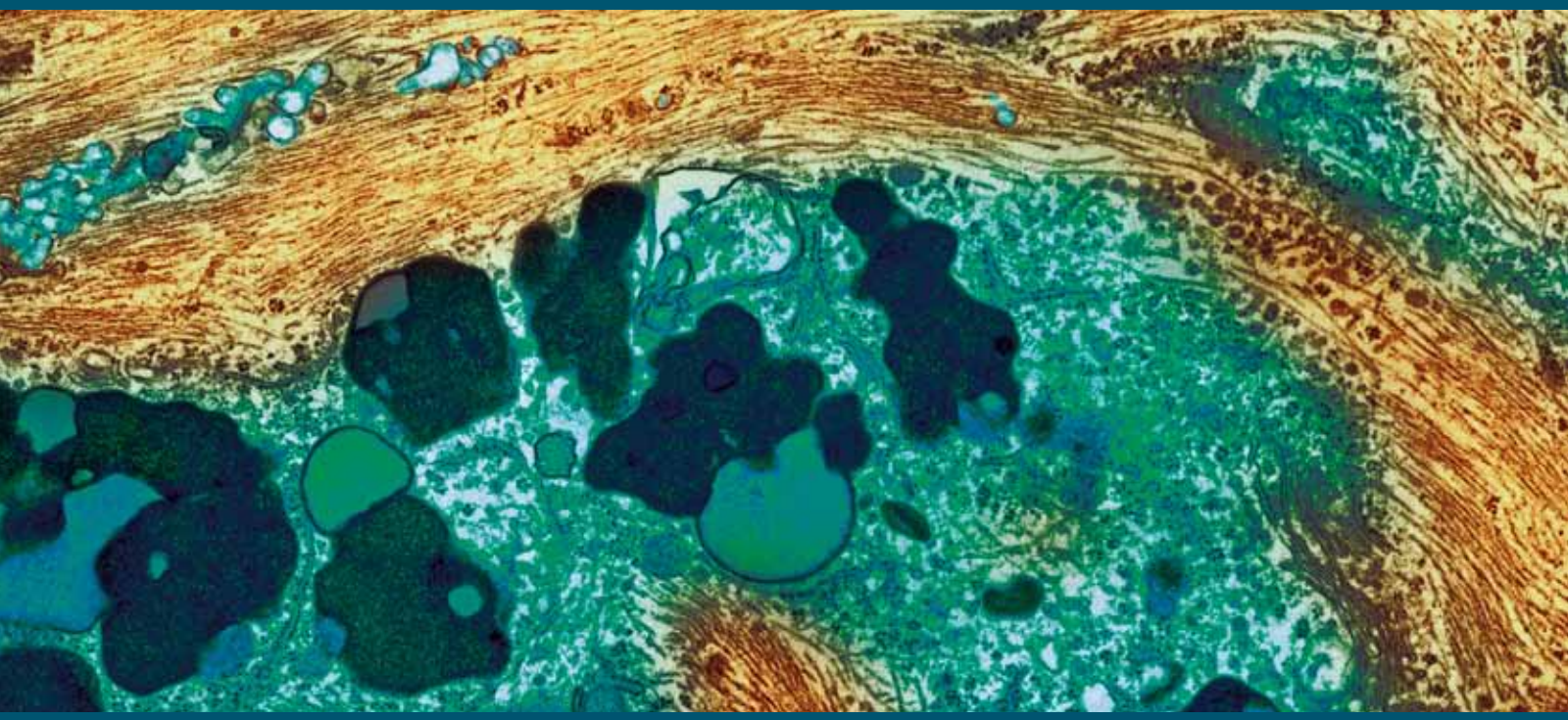
International Journal of Alzheimer's Disease

# Genetic Risk Factors: Their Function and Comorbidities in Alzheimer's Disease

Guest Editors: Mikko Hiltunen, Lars Bertram, and Aleister J. Saunders



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*Editorial*

# Genetic Risk Factors: Their Function and Comorbidities in Alzheimer's Disease

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Alzheimer's disease (AD) is an epidemiologically complex disorder, in which both genetic and environmental factors play important roles contributing to disease susceptibility. Identification of these risk factors is crucial as they may provide new avenues for the identification of novel disease biomarkers as well as for the design of intervention approaches. Several novel AD susceptibility genes with small risk effects have been recently identified by employing genetic association analyses, in particular by those using a genome-wide approach. For most of the newly identified AD risk factors, however, the biological mechanisms driving these associations remain elusive, emphasizing the need for comprehensive functional characterization of these genes and for determining their relevance for AD pathogenesis. In addition, epidemiological and clinical studies have revealed that certain comorbidities often precede or cooccur with AD. These are often correlated with modifiable life-style factors potentially providing promising alternative routes to be exploited in treatment studies. For this special issue of the *International Journal of Alzheimer's Disease*, we invited investigators to contribute original research and review articles that stimulate efforts to identify novel molecular targets involved in AD pathogenesis. Eventually, we selected to include eight articles on the topic, which we believe to be of particular interest to the readers of the journal.

The first set of four studies in this special issue elucidates a number of different genetic aspects of AD. The study by G. Hamilton et al. assessed the impact of recently identified AD genome-wide association signals on cognitive functioning in two birth cohorts from Scotland. Their strongest results implicate a haplotype at the *TRAPPC6A* locus in individuals lacking the *APOE*$\varepsilon$4 allele. Less-pronounced effects on cognition are also observed for genetic variants in *APP* and *BIN1*. The study by L. Polito et al. investigated the potential role of *SLC6A4*, a serotonin transporter highly expressed in the brain, in contributing to AD risk. While they found nominally significant risk effects in their own case-control sample from Italy, combining these data with those from other groups yields a more ambiguous answer. The study by R. Dominici et al. followed a similar approach investigating the potential effects of DNA sequence variants in G protein-coupled receptor 3 (GPR3) in AD cases and controls from Italy. In agreement with recent genome-wide association studies, they found no evidence that *GPR3* is involved in AD epidemiology. Finally,the paper by E. Blom et al. chose a more functionally orientated approach to AD genetics. The authors performed a genome-wide gene expression study in transgenic mouse models of AD, which suggested differences in expression patterns in genes of the Wnt pathway. Validation experiments in human brain samples confirm these findings and suggest that *TCF7L2* and *MYC* show the largest expression differences in AD versus control subjects.

The second set of studies focus on comorbidities and life-style factors that are associated with AD. In the paper by V. Leinonen et al., investigators assessed the well-known AD-related biomarkers, such as A$\beta$42, tau protein, and inflammatory components from the cerebrospinal fluid samples obtained from the patients of idiopathic normal pressure hydrocephalus (iNPH). As AD is the most important differential diagnosis for iNPH, brain biopsy samples obtained from NPH patients provide valuable information

on pathogenic events taking place in early phase of AD. The paper by G. Pasinetti et al. summarizes the effects of caloric intake, dietary life-style and macronutrient composition on the risk of AD. More specifically, the investigators discuss how certain cardiovascular and diabetic conditions can induce an increased susceptibility for AD and provide potentialmechanisms through which this may occur. The paper by E. Tuminello et al. provides a comprehensive review related to the hypothesis of apolipoprotein E (protein: apoE; gene: *APOE*) antagonistic pleiotropy. The leading hypothesis is that the *APOEε*4 allele may be beneficial in earlier ages, while it leads to cognitive decline later in life. Finally, the last paper by V. Leduc et al. highlights the pleiotropic roles and the structure-function relationship of apoE particularly in the lipid homeostasis-related events in AD and cardiovascular diseases.

Taken together, we believe that the articles included in this special issue of the *International Journal of Alzheimer's Disease* shed new light on a number of different aspects of AD pathogenesis. In concert with the work from hundreds of other AD laboratories worldwide, these exciting new data will hopefully translate into the identification of novel biomarkers and the development of new therapeutic strategies to efficiently diagnose and treat this devastating disorder.

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*Research Article*

# Alzheimer's Disease Genes Are Associated with Measures of Cognitive Ageing in the Lothian Birth Cohorts of 1921 and 1936

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Alzheimer's disease patients have deficits in specific cognitive domains, and susceptibility genes for this disease may influence human cognition in nondemented individuals. To evaluate the role of Alzheimer's disease-linked genetic variation on cognition and normal cognitive ageing, we investigated two Scottish cohorts for which assessments in major cognitive domains are available: the Lothian Birth Cohort of 1921 and the Lothian Birth Cohort of 1936, consisting of 505 and 998 individuals, respectively. 158 SNPs from eleven genes were evaluated. Single SNP analyses did not reveal any statistical association after correction for multiple testing. One haplotype from *TRAPPC6A* was associated with nonverbal reasoning in both cohorts and combined data sets. This haplotype explains a small proportion of the phenotypic variability (1.8%). These findings warrant further investigation as biological modifiers of cognitive ageing.

## 1. Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease, and it is predicted to affect over a million people in the UK by 2025 (Dementia UK 2007 report). AD is characterised initially by impaired episodic memory [1] and, as the disease progresses, other cognitive deficits appear, particularly in attention and executive functions, semantic memory, language, and spatial orientation [2, 3].

AD is a genetically heterogeneous disease. Mutations in three genes (the amyloid precursor protein, *APP*; presenilin 1, *PS1*; presenilin 2, *PS2*) are known to cause a rare early-onset form of AD [4–6]. The most common form of AD occurs sporadically and with a late age at onset. Until recently, the only well-replicated risk factor for this form of AD was the $\varepsilon 4$ allele of the apolipoprotein E (*APOE*) gene [7]. However, three recent genome-wide association studies (GWASs) have identified four new candidate genes for sporadic AD—*BIN1, CLU, CR1,* and *PICALM*—and one new genomic region near *BLOC1S3/EXOC3L2/MARK4* [8–10]. Associations with *CLU, CR1,* and *PICALM* have been replicated [11–13].

Nonpathological age-related cognitive decline is a major and growing concern in developed societies [14]. General cognitive ability is an important predictor of life outcomes, including in old age. The determinants of normal cognitive ageing are not fully understood, but are likely to include both genetic and environmental influences [14]. Genetic influences on cognitive ability increase from about 30% in childhood to as much as 80% in later adulthood, and these decrease slightly in very old-age when, probably, stochastic effects become relatively more important [15]. As is still

Table 1: Details of the Lothian Birth Cohorts of 1921 and 1936.

| | LBC1921 | LBC1936 |
|---|---|---|
| Total | 505 | 998 |
| Females (%) | 296 (58.7) | 494 (49.5) |
| Males (%) | 209 (41.3) | 504 (50.5) |
| Mean age in years[1] (± s.d) | 10.9 ± 0.28 | 10.9 ± 0.28 |
| Mean age in years[2] (± s.d) | 79.11 ± 0.57 | 69.58 ± 0.83 |
| ≥1 *APOE ε*4 allele | 135 | 287 |
| no *APOE ε*4 allele | 370 | 672 |

[1]Mean age at original test date, [2]Mean age when revisited.

true for many complex phenotypes, there are few replicated genotype-phenotype associations with cognitive ageing [15, 16]. There is suggestive evidence for genes such as *BDNF* and *COMT* but, to date, *APOE* is the only gene that has been consistently shown to have a significant, but small, influence on age-related cognitive decline [17]. We hypothesise that other genes involved in AD may play a role in normal cognitive ageing. Indeed, a recent study has described the association of variants in the *CLU* and *PICALM* genes with cognitive function [18].

Here, we examine genetic variants from the *APP*, *PS1*, *PS2, BIN1, CLU, CR1, PICALM* genes, and the region surrounding the *BLOC1S3/EXOC3L2/MARK4* genes on chromosome 19 in two large, phenotypically well-defined cohorts, the Lothian Birth Cohorts of 1921 and 1936 [19, 20]. The individuals in these cohorts took a general mental ability test in childhood and then took a range of mental tests in old age. They are, therefore, unusually useful in understanding the genetic contributions to cognitive change across most of the human life course. The *APOE* gene has previously been investigated in these cohorts and shown to explain a small percentage (0.005–0.01) of the variance associated with the general cognitive factor, two nonverbal tests, and choice reaction time variability [21–24].

## 2. Materials and Methods

*2.1. Sample.* The samples examined were the Lothian Birth Cohort of 1921 (LBC1921) and the Lothian Birth Cohort of 1936 (LBC1936). They were born in 1921 and 1936, respectively and, at a mean age of 11 years, they were tested on general cognitive ability by means of the Scottish Mental Survey of 1932 (SMS1932) or the Scottish Mental Survey of 1947 (SMS1947) (each cohort has a mean age = 10.9 ± 0.28 years). Since 1999 for LBC1921 and 2004 for LBC1936, a number of the original Surveys' participants who were living in the Edinburgh area of Scotland have been revisited. Participants from LBC1921 were tested for a variety of cognitive phenotypes at approximately 79 years of age (mean age = 79.11 ± 0.57 years), whereas participants from the LBC1936 were tested at approximately 70 years of age (mean age = 69.58 ± 0.83 years) (Table 1) [19, 20].

Individuals were excluded from this study if there was a personal history of dementia, if they had an MMSE score of less than 24, or if they did not have GWAS data. Four individuals were removed from the LBC1921 due to a family history of dementia, and eight were removed due to MMSE < 24. Seven individuals were removed from the LBC1936 with MMSE < 24. The total number of participants included from the LBC1921 was 505 (41.3% male: 58.7% female), and the total number of participants from the LBC1936 was 998 (50.5% male: 49.5% female) (Table 1).

The LBC1936 was used as the discovery cohort. Significant results meeting the chosen statistical criteria were carried forward and investigated using the LBC1921.

*2.2. Cognitive Tests.* Individuals from the LBC1936 were tested on the Moray House Test (MHT) no. 12 at age 11 (10.9 ± 0.28 years) and subsequently at age 70 (69.58 ± 0.83 years) [19]. At age 70, they were also tested for a variety of cognitive phenotypes, with the ones of interest to this study being verbal fluency (a test of executive function using the letters C, F, and L) [25], matrix reasoning (a subtest from the Wechsler Adult Intelligence Scale-III$^{UK}$ used to assess nonverbal reasoning) [26], and logical memory (a test of immediate and delayed verbal declarative memory from the Wechsler Memory Scale-III$^{UK}$) [27].

Individuals from the LBC1921 were tested on the MHT no. 12 at age 11 (10.9 ± 0.28 years) and subsequently at age 79 (79.11 ± 0.57 years) [20]. This cohort's participants were tested for three cognitive phenotypes; verbal fluency (exactly as applied in the LBC1936), Raven's Standard Progressive Matrices (a test of non-verbal reasoning) [28], and logical memory (a test of immediate and delayed verbal declarative memory from the Wechsler Memory Scale-Revised [29].

From this point forward, age 11 for both cohorts indicates 10.9 ± 0.28; age 70 for the LBC1936 cohort indicates 69.58 ± 0.83 years; age 79 for the LBC1921 cohort indicates 79.11 ± 0.57 years.

*2.3. Genotyping.* Genomic DNA from the LBC1936 cohort was isolated from whole blood by standard procedures at the Wellcome Trust Clinical Research Facility (WTCRF), Genetics Core, Western General Hospital, Edinburgh. Genomic DNA from the LBC1921 cohort was isolated from whole blood by standard procedures at Medical Research Council (MRC) Technology, Western General Hospital, Edinburgh. All samples were genotyped at the WTCRF Genetics Core with the Illumina Human 610-Quadv1 chip as part of a larger study [30]. SNPs were included in the analyses if they met the following conditions: call rate ≥ 0.98, minor allele frequency ≥ 0.01, and Hardy-Weinberg Equilibrium test with $P \geq .001$ [30]. For this study, specific SNPs were selected from the GWAS data set. Genomic regions approximately 5 kb upstream to 5 kb downstream of each candidate gene were identified using positional information from the Santa Cruz Genome Browser, March 2006 Assembly (NCBI36) (http://genome.ucsc.edu/) [31]. All SNPs with available genotype data from each region were used in this study. A further five SNPs that showed association with sporadic AD were included: four that were outside the above genomic regions and one that was within the genomic region but that had not been genotyped. This SNP (rs6656401)

was imputed using the HapMap phase II CEU data (NCBI build 36 (UCSC hg18)) as the reference sample and MACH software. The imputation quality score for this SNP was high ($r^2 = 0.92$). A total of 158 SNPs were selected; *66* from APP, 9 from *PS1*, 6 from *PS2,* 17 from *BIN1*, 6 from *CLU,* 9 from *CR1,* 29 from *PICALM*, and 16 from the *BLOC1S3/EXOC3L2/MARK4* region, which included three SNPs from the 5′ end of *TRAPPC6A* gene (Table S1). *APOE* haplotype data were available for all samples.

### 2.4. Statistical Analysis

*2.4.1. Significance Threshold.* To determine the correct level of significance for regression and haplotype analyses of the LBC1936 cohort, a spectral decomposition program, SNPSpD, was used [9]. SNPSpD calculates an approximate estimate of the effective number of independent SNPs using a previously described method [32]. A Bonferroni calculation using this number of SNPs was used to determine the appropriate level of significance for regression and haplotype analysis. A significance level for pairwise interaction analyses of the LBC1936 cohort was determined using $\alpha = 0.05/x$, where $x = n(n-1)/2$ ($n$ = effective number of independent SNPs) [33].

*2.4.2. Cognitive Phenotypes.* Standardized residual scores were calculated for each cognitive phenotype to incorporate age at time of testing and gender, using linear regression in SPSS, v14.0.

*2.4.3. Association Analysis.* Unless otherwise noted, all statistical analyses were carried out using PLINK v1.07 (http://pngu.mgh.harvard.edu/purcell/plink) [34]. Three approaches to association analysis were used.

The first approach examined all SNPs in relation to the selected cognitive phenotypes and applied a stringent Bonferonni threshold to the $P$ values. Linear regression analysis was performed under an additive model in PLINK. Additional analyses included two covariates; (i) the presence or absence of an *APOE ε4* allele and (ii) general cognitive ability at age 11 (MHT score adjusted for age) to adjust for prior cognitive ability. Using general cognitive ability at age 11 as a covariate enables the role of each SNP in cognitive ageing to be explored. Two stratified data sets, with or without the *APOE ε4* allele, were analysed similarly. Adaptive permutation analysis was carried out on all linear regression analyses.

The second approach was haplotype analysis. Each gene was examined for association with cognitive phenotypes using a sliding window of three SNPs, shifting one SNP at a time. Two stratified data sets, with or without the *APOE ε4* allele, were analysed similarly. In the haplotype analysis, the presence or absence of an *APOE ε4* allele and general cognitive ability at age 11 (MHT score adjusted for age) were not used as covariates. SNP regions meeting the significance threshold were analysed using max(T), a label swapping-based permutation method.

The third and final approach used pairwise interaction analysis to determine any effect of gene-gene interaction on the association with cognitive phenotypes. The full data set and two stratified data sets, with or without the *APOE ε4* allele, were analysed similarly. In the pairwise interaction analysis, the presence or absence of an *APOE ε4* allele and general cognitive ability at age 11 (MHT score adjusted for age) were not used as covariates. The results file was controlled so that only associations having $P \leq .0001$ were reported. Additionally, only where SNPs were located in different genes are the pairwise interactions described here. Significant interactions were analysed using a one-way ANOVA in SPSS v14.0. To examine each interaction, both the cognitive mean of each genotype (aabb, aaBB, aaBb, AAbb, Aabb, AABB, AABb, AaBB, AaBb) and the cognitive mean of the groups representing the presence or absence of each minor allele (aabb, aaB-, A-bb, A-B-) were compared (where a and b represent the minor allele of each SNP).

*2.4.4. Linkage Disequilibrium Analysis.* Linkage disequilibrium (LD) values were generated and visualised using Haploview [35].

## 3. Results

*3.1. Significance Threshold.* 158 SNPs in total were selected for analysis in this study (Table S1 in Supplementary Material available online at doi:10.4061/2011/505984). The LBC1936 cohort was used as a discovery sample and the LBC1921 cohort as a replication cohort. Different significance thresholds were applied to each cohort. To determine an appropriate threshold for analyses of the discovery cohort, two methods were used. Spectral decomposition analysis calculated that the approximate estimate of the effective number of independent SNPs was 89.24. Therefore, in our regression and haplotype analyses, only where $P \leq .00056$ ($\alpha = 0.05/89.24$), were results considered significant associations. For pairwise interaction analysis, only where $P \leq .000013$ ($\alpha = 0.05/x$, x = [89.24 (89.24-1)]/2) were results considered significant associations. max(T) permutation analysis was carried out on significant haplotype results, and a significance threshold of $P \leq .05$ was applied to the results. Results with $P \leq .05$ were considered significant in our replication cohort.

*3.2. Association of AD SNPs with Cognitive Phenotypes.* No individual SNP in the LBC1936 was associated with any cognitive phenotype in the overall or *APOE* stratified sample at $P \leq .00056$ (Table S2, Table S3).

*3.3. Association of AD Gene Blocks with Cognitive Phenotypes.* Tables S4, S5, and S6 detail the effect of each 3-SNP window on each cognitive phenotype in the complete LBC1936 data set and in the LBC1936 data sets stratified for presence or absence of the *APOE ε*4 allele.

Two 3-SNP windows, comprising four adjacent SNPs from *BIN1*, reached our corrected $P$ value level ($P \leq .00056$) with general cognitive ability at age 11 (MHT adjusted) in

the overall LBC1936 sample (Table 2). These results were not replicated in the LBC1921 and were nonsignificant following permutation analysis of both the LBC1936 and the combined data set.

Two separate 3-SNP windows from the *APP* locus reached significance with logical memory in the *APOE* $\varepsilon 4$ positive subgroup (Table 2). These SNP windows were not significant postpermutation analysis of the LBC1936. Further, this result was not replicated in the LBC1921 or following permutation analysis of the combined sample.

One 3-SNP window from the *TRAPPC6A* locus reached significance with matrix reasoning in the *APOE* $\varepsilon 4$ negative subgroup (Table 2). Though not significant postpermutation analysis in the LBC1936, this finding was replicated in the LBC1921 and in post permutation analysis of the combined cohort.

*3.4. Gene-Gene Interaction Analysis.* Tables S7 11 detail the results obtained in the pairwise interaction analyses with each cognitive phenotype in the LBC1936. Data were extracted for interactions if $P \leq .0001$. Results were considered significant if $P \leq .000013$.

One SNP-SNP interaction from the chromosome 19 locus (*MARK*4, rs344807) and *APP* (rs12482753) was significantly associated with general cognitive ability at age 70 (MHT adjusted for age) in the *APOE* $\varepsilon 4$ negative LBC1936 subset (Figure 1; Table 3). However, analysis of the cognitive means for each genotype group indicated that the association was due to the low score of a single individual who expressed the aaBb genotype. Analysis of the cognitive means of the four groups representing the presence or absence of the minor alleles showed no significant difference, and following the removal of the aaBb individual the genotype result was no longer significant (results not shown). This interaction was not replicated in the LBC1921.

A single SNP-SNP interaction from *PS1* (rs214260) and *APP* (rs440666) was significantly associated with verbal fluency in the *APOE* $\varepsilon 4$ negative LBC1936 subset (Figure 1; Table 3). Analysis of the cognitive means for each genotype group indicated that the association was due to the lower verbal fluency scores of the group expressing the Aabb genotype; however, analysis of the cognitive means of the four groups representing the presence or absence of the minor alleles showed no significant difference (results not shown). This interaction was not replicated in the LBC1921.

One SNP-SNP interaction from *BIN1* (rs10200967) and *APP* (rs2830036) was significantly associated with verbal declarative memory in the *APOE* $\varepsilon 4$ positive LBC1936 subset (Figure 1; Table 3). Analysis of the cognitive means for each genotype and for the four groups representing the presence or absence of the minor alleles indicated that this association was due to the low logical memory scores of two individuals homozygous for each minor allele (Figure 2). Although not a direct replication of the result observed in the LBC1936 cohort, two *BIN1-APP* interactions approached significance in the LBC1921 cohort. The associations were observed with the *BIN1* SNP (rs10200967) that was associated in the LBC1936 *APOE* $\varepsilon 4$ positive sample set, but with two different *APP* SNPs: rs396969 and rs383700 (Table 3). The two *APP* SNPS were in complete LD (Figure 1). Both interactions were associated with higher logical memory scores, with the opposite of that observed in the LBC1936. Analysis of the cognitive means for each genotype indicated that both associations were due to the high logical memory score of one individual homozygous for each minor allele. Following the removal of this individual, this result was no longer significant (results not shown). No *BIN1-APP* SNP interactions were observed in the *APOE* $\varepsilon 4$ positive samples in LBC1921, and there was no significant interaction when the samples were combined.

A single SNP-SNP interaction from *PS2* (rs1150895) and *PICALM* (rs3851179) was significantly associated with verbal declarative memory in the *APOE* $\varepsilon 4$ negative LBC1936 subset (Figure 1; Table 3). Analysis of the cognitive means for each genotype group indicated that the association was due to the higher logical memory scores of the groups expressing either the AAbb or aaBB genotype compared to the AABB genotype. Further analysis of the cognitive means of the four groups representing the presence or absence of the minor alleles showed that aaBB and aaBb individuals had higher logical memory scores than other allele groups (results not shown). This interaction was not replicated in the LBC1921.

## 4. Discussion

In this study, we have screened polymorphisms from three causal and five putative risk genes for Alzheimer's disease in two cohorts with extensive and unique cognitive phenotypes available. Evidence was found to suggest a role for variation in a gene at the chromosome 19 locus, *APP* and *BIN1* in cognitive ability.

Each gene will be discussed individually.

*4.1. Chromosome 19 Locus.* A genomic locus on chromosome 19 was recently implicated in a single LOAD-GWAS [10]. It identified a locus distal to and not in linkage disequilibrium with *APOE*. The SNPs chosen in this study span the 5′ end of the *TRAPPC6A* gene and cover *BLOC1S3*, *EXOC3L2, MARK4,* and the 3′ end of the *CKM* gene.

One 3-SNP window located at the 5′ end of the *BLOC1S3/EXOC3L2/MARK4* region was significantly associated with non-verbal reasoning in individuals lacking an *APOE* $\varepsilon 4$ gene in the LBC1936 data set. This SNP window consisted of the SNPs (rs7247764, rs28555639, rs12460041) located at the 5′ end of the *TRAPPC6A* gene. They span a genomic region of 1442 bp and are in complete LD ($D' = 1$). The genotype of this associated haplotype was TTT, and it was the most common haplotype ($f = 0.70$). This haplotype was associated with a small decrease in Wechsler matrix reasoning scores ($\beta = -0.21$) and explained 1.8% of the variation in the LBC1936. This was replicated in the LBC1921 cohort ($\beta = -0.18$), where it explained 1.3% of the variation in Raven's Standard Progressive Matrices scores. Permutation analysis of the combined data set confirmed this result.

Table 2: Significant haplotype results.

| Gene | dbSNP ID (rs) | Haplotype | Cohort | Sample | *N* | Frequency | Cognitive phenotype | Beta | $r^2$ | *P* | max(T) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *BIN1* | 3768857/17014873/2276575 | GAG | LBC1936 | Overall | 941 | 0.13 | GCA11 | −0.24 | 0.012 | .00048* | 0.21 |
| | | GAG | LBC1921 | Overall | 453 | 0.13 | GCA11 | −0.11 | 0.0031 | .24 | |
| | | GAG | Both | Overall | 1394 | 0.13 | GCA11 | −0.19 | 0.0091 | .00036 | 0.14 |
| | 17014873/2276575/13430599 | AGT | LBC1936 | Overall | 941 | 0.13 | GCA11 | −0.24 | 0.014 | .0003* | 0.16 |
| | | AGT | LBC1921 | Overall | 453 | 0.13 | GCA11 | −0.12 | 0.003 | .23 | |
| | | AGT | Both | Overall | 1394 | 0.13 | GCA11 | −0.2 | 0.0096 | .00025 | 0.1 |
| *APP* | 2829997/440666/2014146 | GTG | LBC1936 | *APOE* ε4 positive | 287 | 0.013 | LM | −1.3 | 0.043 | .0004* | 0.18 |
| | | GTG | LBC1921 | *APOE* ε4 positive | 134 | 0.011 | LM | −0.051 | 0.00004 | .94 | |
| | | GTG | Both | *APOE* ε4 positive | 421 | 0.012 | LM | −1 | 0.023 | .0017 | 0.49 |
| *APP* | 1783025/380417/1787438 | TTG | LBC1936 | *APOE* ε4 positive | 287 | 0.053 | LM | 0.72 | 0.048 | .00017* | 0.072 |
| | | TTG | LBC1921 | *APOE* ε4 positive | 134 | 0.053 | LM | 0.042 | 0.00016 | .88 | |
| | | TTG | Both | *APOE* ε4 positive | 421 | 0.053 | LM | 0.516 | 0.024 | .0014 | 0.43 |
| *TRAPPC6A* | 7247764/28555639/12460041 | TTT | LBC1936 | *APOE* ε4 negative | 669 | 0.7 | MR | −0.21 | 0.018 | .00043* | 0.24 |
| | | TTT | LBC1921 | *APOE* ε4 negative | 369 | 0.71 | MR | −0.18 | 0.013 | .024** | |
| | | TTT | Both | *APOE* ε4 negative | 1039 | 0.7 | MR | −0.2 | 0.016 | .000036 | 0.019*** |

A result is significant with the LBC1936 cohort if $P \leq .00056$ (*) and with the LBC1921 cohort if $P \leq .05$ (**). A result is significant postpermutation analysis if $P \leq .05$ (***). The following abbreviations are used: *N*, sample number; Beta, regression coefficient of the trait value; $r^2$, proportion of the variance explained; GCA11, general cognitive ability at age 11 (MHT adjusted for age); LM, logical memory; MR, matrix reasoning. max(T) *P* value is controlled for all SNPs tested.

Table 3: Significant pairwise interaction results.

| Cohort | Samples | Gene 1 | dbSNP ID (rs) | Gene 2 | dbSNP ID (rs) | Cognitive phenotype | Beta | *P* |
|---|---|---|---|---|---|---|---|---|
| LBC1936 | *APOE* ε4 negative | *MARK4* | 344807 | *APP* | 12482753 | GCA70 | −1.69 | .000012* |
| LBC1921 | *APOE* ε4 positive | intergenic chr 19 | 2627641 | *APP* | 2829984 | GCA70 | −1.21 | .000032 |
| | | intergenic chr 19 | 597668 | *APP* | 2829984 | GCA70 | −1.21 | .000032 |
| Both | *APOE* ε4 negative | *MARK4* | 344807 | *APP* | 12482753 | GCA70 | −1.27 | .000056 |
| LBC1936 | *APOE* ε4 negative | *PS1* | 214260 | *APP* | 440666 | VF | −0.5 | .000012* |
| LBC1936 | *APOE* ε4 negative | *PS2* | 1150895 | *PICALM* | 3851179 | LM | −0.43 | .0000048* |
| LBC1936 | *APOE* ε4 positive | *BIN1* | 10200967 | *APP* | 2830036 | LM | −0.67 | .000011* |
| LBC1921 | Overall | *BIN1* | 10200967 | *APP* | 396969 | LM | 0.62 | .00033 |
| | | *BIN1* | 10200967 | *APP* | 383700 | LM | 0.62 | .00032 |

A result is significant with the LBC1936 cohort if $P \leq .000013$ (*) and with the LBC1921 cohort if $P \leq .05$ (**). The following abbreviations are used: *N*, sample number; Beta, regression coefficient of the trait value; GCA70, general cognitive ability at age 70 (MHT adjusted for age); VF, verbal fluency; LM, logical memory.

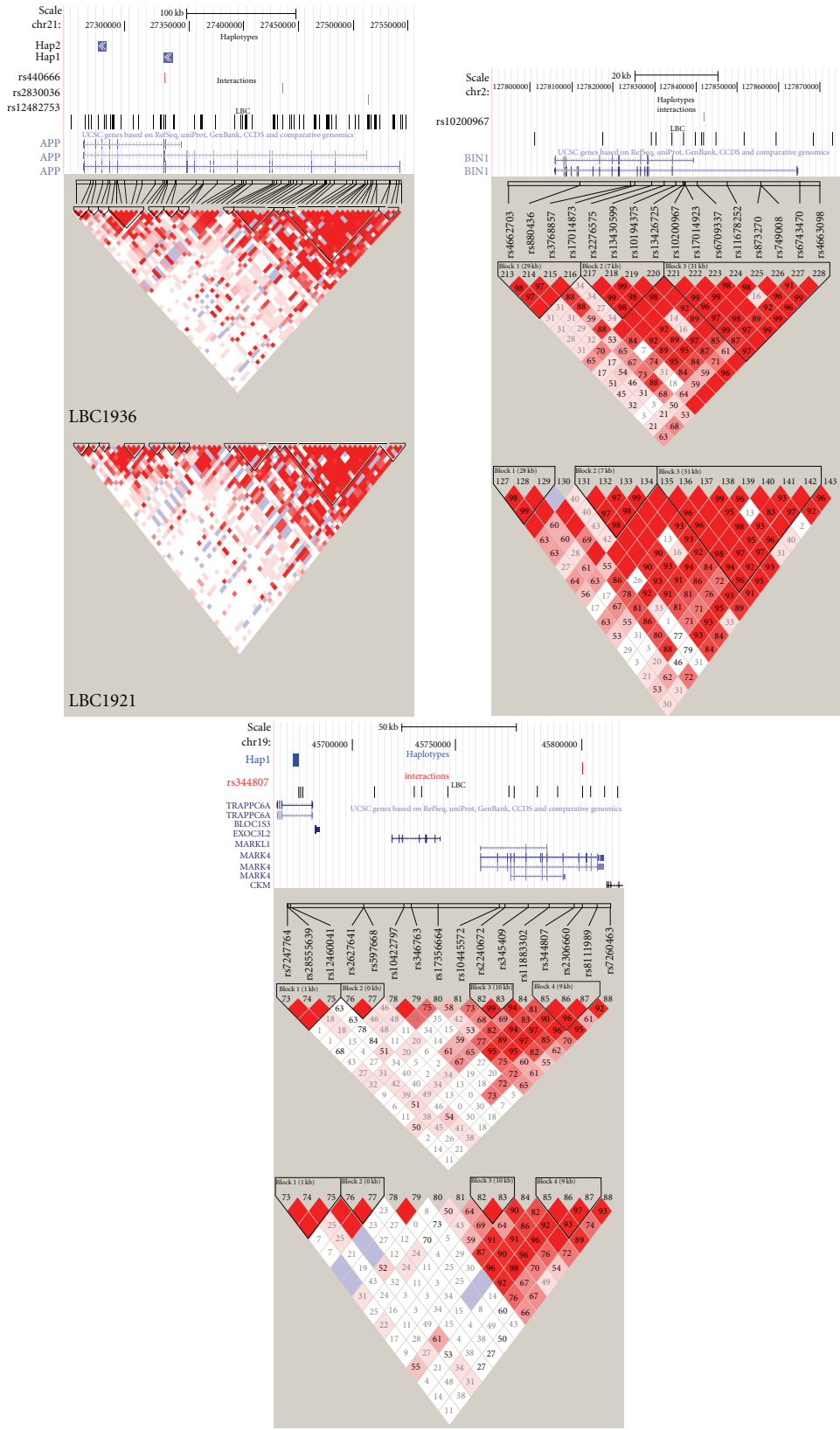
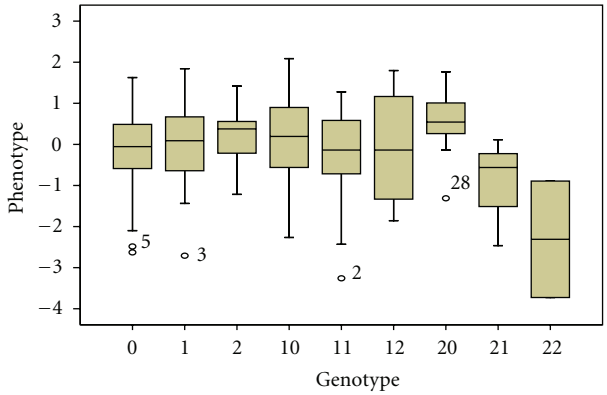


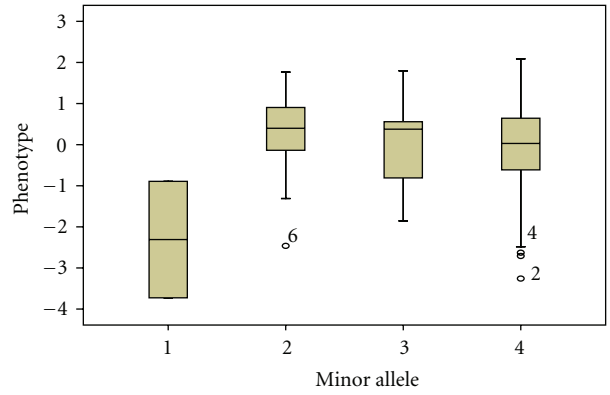

FIGURE 1: Genomic structure of positively associated genes. (a) Genomic structure of *APP*, *BIN1*, and chromosome 19. Highlighted are the location of each SNP genotyped and the location of positively associated haplotypes and gene-gene interactions. (b) LD structure of *APP*, *BIN1*, and chromosome 19 in the Lothian Birth Cohorts of 1936 (top) and 1921 (bottom). LD values used were $D'$.

| | Sum of squares | *df* | Mean square | *F* | Sig. |
|---|---|---|---|---|---|
| Between groups | 21.611 | 8 | 2.701 | 2.96 | .003 |
| Within groups | 252.824 | 277 | .913 | | |
| Total | 274.434 | 285 | | | |




(a)

| | Sum of squares | *df* | Mean square | *F* | Sig. |
|---|---|---|---|---|---|
| Between groups | 11.58 | 3 | 3.86 | 4.141 | .007 |
| Within groups | 262.854 | 282 | .932 | | |
| Total | 274.434 | 285 | | | |




(b)

Figure 2: The interaction of an SNP pair from *BIN1* and *APP* is likely to influence logical memory in the *APOE* ε4 positive subset of LBC1936. Analysis of both the (a) genotype cognitive means and (b) the allele specific means shows that the initial positive result is due to two individuals carrying both minor alleles, aabb. Genotype legend; 11 = AaBb, 10 = AaBB, 01 = AABb, 00 = AABB, 12 = Aabb, 01 = AAbb, 21 = aaBb, 20 = aaBB, 22 = aabb. Allele legend; 1 = aabb, 2 = aaB-, 3 = A-bb, 4 = A-B-.

The SNP associated with LOAD in the recent GWAS study [10], rs597668, is located in an intergenic region between *TRAPPC6A* and *EXOC3L2*. This SNP was included in our study although we did not observe an association with any cognitive phenotype. The *TRAPPC6A* haplotype is located 31573 bp from the GWAS SNP, and analysis of the LD in this region shows that SNPs from the haplotype were not in the same LD block as the GWAS SNP ($D' = 0.22$), so it is unclear whether our results are detecting the same effect. Replication of the *TRAPPC6A* haplotype is required in a larger cohort.

*4.2. APP.* *APP* was the first disease gene identified in familial AD [4]. It is a transmembrane protein, and sequential cleavage by $\beta$- and $\gamma$-secretase releases the $\beta$-amyloid peptide. Although the exact role of the *APP* protein is unknown, it is considered central to AD pathogenesis.

Two 3-SNP windows at the *APP* locus, each consisting of three SNPs, were associated with verbal declarative memory in individuals carrying at least one *APOE* ε4 allele in the LBC1936. These results correspond to two genomic regions located at the 3' end of the *APP* gene. The first region consisted of three SNPs, rs2829997, rs440666, and rs2014146, and spanned 8163 bp. These SNPs were in high LD ($D' > 0.7$) and constituted a haplotype block. The associated haplotype, with genotype GTG, was rare, with a frequency of 0.013. This haplotype, *APP* Hap1, was associated with a decrease in logical memory scores ($\beta = -1.312$) and explained 4.3% of the variation. The second genomic region spanned 7326 bp and consisted of three SNPs, rs1783025, rs380417, and rs1787438. These SNPs are located near known pathogenic AD mutations, in sites encoding the $\alpha$, $\beta$, and $\gamma$-secretase sites. The latter two SNPs were in complete LD ($D' = 1$); however, rs1783025 was not ($D'$ 0.48, 0.64 with rs380417, rs1787438, respectively). The associated genotype, TTG, was rare, with a frequency of 0.053. This genotype was associated with an increase in logical memory scores ($\beta = 0.72$) and explained 4.8% of the variation. These two genotypes explain a small, but important, amount of the variance, 4.3% and 4.8% respectively, especially considering that *APOE* ε4 contributes 0.5–1% to variance in cognitive traits. However, these results were not replicated following permutation analysis. Further, this effect was not observed in the LBC1921 or in the combined data set.

These results may not have been replicated in the LBC1921 cohort for a couple of reasons: the replication cohort contains fewer individuals and the logical memory test used with the LBC1921 cohort differed slightly from that used with the LBC1936 cohort. Nonetheless, the haplotype frequencies are consistent between cohorts and, although not significant, LBC1921 individuals with *APP* Hap1 (GTG) have lower logical memory scores while individuals with the second associated genotype (TTG) have higher logical memory scores in the LBC1921.

Further evidence of a role for *APP* in logical memory was obtained in our gene-gene interaction analysis. SNPs at the *APP* locus were observed to statistically interact with polymorphisms at the *BIN1* locus to influence verbal declarative memory.

*4.3. BIN1.* *BIN1* was identified as a putative risk factor for LOAD in a recent GWAS study [10]. It encodes several

Table 4: Comparison of significant findings between studies.

| Gene | Paper | Genotyping method | SNP (rs) | *P* value | OR | $\beta$ | Trait |
|---|---|---|---|---|---|---|---|
| *CLU* | Harold et al. [8] | Illumina 610 quad Illumina Human Hap550/300 | 11136000** | $8.5 \times 10^{-10}$ | 0.86 | | LOAD |
| | Lambert et al. [9] | Illumina 610 quad | 11136000 | $7.5 \times 10^{-9}$ | 0.86 | | LOAD |
| | Carrasquillo et al. [11] | Taqman | 11136000 | $8.6 \times 10^{-5}$ | 0.82 | | LOAD |
| | Corneveaux et al. [12] | Genome-wide Human SNP6.0 array, Affymetrix | 11136000 | 0.04 | 0.86 | | LOAD |
| | Kamboh et al. [13] | Taqman | 11136000 | $4.4 \times 10^{-16}$ | 0.86 | | LOAD |
| | Mengel-From et al. [18] | Taqman | 11136000 | 0.016 | | 0.5 | CCS |
| *PICALM* | Harold et al. [8] | Illumina 610 quad Illumina Human Hap550/300 | 3851179** | $1.3 \times 10^{-9}$ | 0.86 | | LOAD |
| | Carrasquillo et al. [11] | Taqman | 3851179 | $1.3 \times 10^{-5}$ | 0.8 | | LOAD |
| | Kamboh et al. [13] | Taqman | 3851179 | $3.4 \times 10^{-9}$ | 0.88 | | LOAD |
| | Mengel-From et al. [18] | Taqman | 3851179 | 0.024 | | 1.4 | CCS* |
| | Hamilton et al. 2011 | Illumina 610 quad v1.0 | 3851179 (interaction with *PS2*) | 0.0000048 | | −0.43 | LM |
| | Corneveaux et al. [12] | Genome-wide Human SNP6.0 array, Affymetrix | 541458** | 0.01 | 0.81 | | LOAD |
| | Kamboh et al. [13] | Taqman | 541458 | $3.5 \times 10^{-9}$ | 0.87 | | LOAD |
| *CR1* | Lambert et al. [9] | Illumina 610 quad | 6656401** | $3.7 \times 10^{-9}$ | 1.21 | | LOAD |
| | Corneveaux et al. [12] | Genome-wide Human SNP6.0 array, Affymetrix | 6656401 | 0.008 | 1.28 | | LOAD |
| | Kamboh et al. [13] | Taqman | 6656401 | $2.3 \times 10^{-9}$ | 1.17 | | LOAD |
| | Carrasquillo et al. [11] | Taqman | 3818361** | 0.014 | 1.15 | | LOAD |
| | Kamboh et al. [13] | Taqman | 3818361 | $5.2 \times 10^{-13}$ | 1.21 | | LOAD |
| *BIN1* | Seshadri et al. [10] | Illumina 610 quad 6.0 (amongst others) | 744373** | $1.6 \times 10^{-11}$ | 1.13 | | LOAD |
| *BIN1* | Hamilton et al. 2011 | Illumina 610 quad v1.0 | 10200967 (interaction with *APP*) | 0.000011 | | −0.67 | LM |
| chr19 | Seshadri et al. [10] | Illumina 610 quad 6.0 (amongst others) | 597668** | $6.4 \times 10^{-9}$ | 1.18 | | LOAD |
| | Hamilton et al. 2011 | Illumina 610 quad v1.0 | 7247764, 28555639, 12460041 | 0.000036 | | 0.016 | MR |
| | Hamilton et al. 2011 | Illumina 610 quad v1.0 | 344807 (interaction with *APP*) | 0.000012 | | | GCA70 |

Results are provided from recent GWAS for sporadic AD and compared to the results obtained in this study. The following abbreviations are used: OR, odds ratio; Beta, regression coefficient of the trait value; GCA70, general cognitive ability at age 70 (MHT adjusted for age); LM, logical memory; MR, matrix reasoning; LOAD, late-onset Alzheimer's disease; CCS, cognitive composite score; n/a, not applicable. *observed in males. **included in the LBC1921 and LBC1936 study.

isoforms that are expressed in the central nervous system and may be involved in synaptic vesicle endocytosis.

An interaction between rs10200967 (*BIN1*) and rs2830036 (*APP*) was significantly associated with verbal declarative memory in the *APOE* $\varepsilon 4$ positive LBC1936 subset. Further analysis showed that this was due to the low logical memory scores of two individuals expressing both minor alleles of rs10200967 (C, *BIN1)* and rs2830036 (T, *APP*) (Figure 2). This result was not replicated in the LBC1921 cohort or in the combined analysis.

However, these results are consistent with the association of *APP* Hap1, GTG, which is associated with a similar decrease in logical memory scores in the *APOE* $\varepsilon 4$ positive subset of LBC1936. The *APP* SNP involved in the *APP-BIN1* interaction (rs2830036) is located 5′ to *APP* haplotype 1 but there are low levels of LD between them ($D' = 0.34$). Indeed the two individuals contributing to the interaction association do not carry the *APP* Hap1 genotype associated with a decrease in logical memory scores (*APP* Hap1 genotype, GTG; individual genotypes, both AA-CC-AG).

Although the *BIN1-APP* interaction was not replicated in the LBC1921, an association approaching significance was observed with variants from *APP* and *BIN1* and verbal declarative memory in the overall LBC1921 cohort. This was due to the higher logical memory score of a single individual expressing both minor alleles of the two SNPs (*BIN1,* rs10200967; *APP,* rs396969 and rs383700), so may not hold up in a replication study. The two *APP* SNPs involved in this interaction were in LD ($D' = 0.98$) with the second *APP* region, genotype TTG, which was associated with higher logical memory scores in the *APOE* $\varepsilon 4$ positive subset of LBC1936. Again, the individual responsible for the interaction result did not carry the haplotype associated with increased logical memory scores (*APP* region 2 genotype, TTG; individual genotype, CT-TT-TT).

The two *BIN1* SNPs involved in the association of *APP-BIN1* with verbal declarative memory (rs10200967 and rs4663098) are located near to the 5′ end of the *BIN1* gene. There is high LD in this region of *BIN1*, and the SNP associated with LOAD in the recent GWAS, rs744373, is located 21580 bp 5′ of rs4663098 ($D' = 0.93$). There are no current reports of an *in vivo* interaction between *BIN1* and *APP.* However, *APP* is a transmembrane protein and is transported through the secretory pathway. It is possible that through its role in endocytosis, *BIN1* may interact with *APP.*

## 5. Conclusions

This study indicates that gene specific variation and gene-gene interactions may influence cognition. Our strongest results implicate a role for a haplotype at the *TRAPPC6A* locus in non-verbal reasoning in individuals lacking the *APOE* $\varepsilon 4$ allele. A less clear role for *APP* and *BIN1* in influencing verbal declarative memory in individuals carrying at least one *APOE* $\varepsilon 4$ allele is suggested.

The effect sizes we have observed in this study are small. Indeed, despite the comparability of genomic LD structure, the majority of these associations were not replicated in the LBC1921 cohort. However, it should be noted that the replication cohort ($n = 505$) is smaller than the discovery cohort ($n = 998$). Particularly, our main results were observed in the smaller *APOE* stratified groups. In addition, the individuals in each cohort were retested at different ages; the LBC1921 were re-tested at age 79, while the LBC1936 were re-tested at age 70, and not all cognitive tests used were all identical, although they were similar.

The results presented here were obtained with SNPs not previously associated with sporadic AD, suggesting that either allelic heterogeneity or a functional SNP is not yet identified (Table 4). Nonetheless, the results presented here identify interactions between recently identified and previously known AD genes and provide an interesting insight into potential molecular pathways underlying cognitive traits. They require further investigation in larger identically phenotyped cohorts.

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*Research Article*

# A Novel Study and Meta-Analysis of the Genetic Variation of the Serotonin Transporter Promoter in the Italian Population Do Not Support a Large Effect on Alzheimer's Disease Risk

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Alzheimer's disease (AD) is a neurodegenerative disorder whose clinical onset is mainly characterized by memory loss. During AD progression, behavioral and psychological symptoms of dementia (BPSD) frequently occur. In this paper we evaluated the association between AD and the short/long (S/L) functional polymorphism of the promoter region of the 5-hydroxytryptamine (5-HT) transporter gene (*SLC6A4*). The S-allele shows a 2-fold reduced transcriptional rate, causing an imbalance in 5-HT intracellular availability that might in turn trigger behavioral and cognitive alterations. We also genotyped the *SLC6A4* promoter functional variant *rs25531* ($A \rightarrow G$). By comparing the genotypic and allelic frequencies in an Italian population of 235 AD and 207 controls, we found an association between *5-HTTLPR* and AD (odds ratio for the L-allele versus the S-allele: 0.74, associated *P* value = .03), while no difference was found for the *rs25531*. A meta-analysis of studies in Italy assessing *5-HTTLPR* and AD risk gave an estimation of odds ratio for the L-allele versus the S-allele of 0.85 (associated *P* value = .08). Overall, our findings are not supportive of a large genetic effect of the explored polymorphisms on AD risk.

## 1. Introduction

Alzheimer's disease (AD) is a neurodegenerative process whose prevalence increases with age. The number of AD patients is expected to raise considerably in the next future [1]. More than 90% of AD cases are sporadic, and only a little percentage has a clear genetic cause [2–4]. The main clinical feature of AD at onset is memory loss, accompanied by behavioral and psychological symptoms of dementia (BPSD) encompassing agitation, aggression, sexual disinhibition, delusions, hallucinations, and sleeping or eating disorders that are an outstanding managing problem for the caregiver [5, 6].

Despite the fact that the most important neurochemical deficiency in AD is related to acetylcholine loss, a possible role for serotonin (5-HT) in AD was suggested by post-mortem assays showing reduced level of serotonin in AD brains [7]. 5-HT role in cognitive processes and memory has been recently suggested, both in animal models and in studies on human subjects [8, 9]. Consequently, 5-HT imbalance might contribute to AD pathological signs. The 5-HT transporter gene (*SLC6A4*, 17q11.1-q12) codes for a neuronal transmembrane protein that is devoted to 5-HT reuptake at presynaptic level, a key regulatory event for serotonergic transmission [10]. The promoter region of the *SLC6A4* gene bears a functional polymorphism,

named serotonin-transported linked-polymorphic region (*5-HTTLPR*), consisting of a 43-bp insertion or deletion (ind/del) leading to a hypofunctional short (S) or to a normal long (L) variant [11, 12]. This polymorphism has been investigated in association with AD risk (Table 1) [13–19], and a meta-analysis of the available data shows no significant effect [20]. A different *SLC6A4* promoter polymorphism, *rs25531* (A → G), is able to modulate *5-HTTLPR* transcriptional efficiency, as the presence of the *rs25531* G-variant in an L-allele carrier reduces the normal transcriptional rate to a level comparable to the S-allele [21]. The genome-wide association studies performed so far did not report significant effect for these two genetic variants in relation to AD susceptibility [22]. In the Italian population, the *5-HTTLPR* has been investigated as risk factor for AD with conflicting results, while to our knowledge no data are available for *rs25531*. To contribute in this field, we have made an association study in a population from Northern Italy.

## 2. Materials and Methods

*2.1. Patients Recruitment.* We recruited 235 independent AD subjects from two clinical centers: "Luigi Sacco" Hospital (Milan, Italy) and Ospedale Maggiore Policlinico (Milan, Italy). Probable AD was diagnosed according to the National Institute of Neurological and Communicative Disorders and Stroke criteria (NINCDS-ADRDA). A Hachinski Ischemic Score >4 was an exclusion criteria [23, 24]. Patients underwent physical and neurological examination, screening laboratory tests, cognitive evaluation, brain Magnetic Resonance Imaging (MRI), Computed Tomography (CT), or Positron Emission Computed Tomography (PET) when required. Dementia severity was assessed by the Minimental State Examination (MMSE) [25]. Controls ($n = 207$) were from the same clinical centers as above; they were mainly elderly outpatients coming to clinical attention for non-neurological illness or spouses of the cases. The absence of cognitive impairment in controls was measured by MMSE, at baseline and at least after one-year follow-up. All subjects (or their relatives) gave an informed consent to the participation in the study (approved by the local ethical committees) that followed the principles of the Declaration of Helsinki.

*2.2. Blood Samples Collection, 5-HTTLPR, rs25531, and Apolipoprotein E Genotyping.* About 5 mL of blood were collected by venipuncture, frozen at −20°C, and extracted to collect genomic DNA (gDNA) using a commercial kit according to the manufacturer's instructions (Promega, Madison, USA).

To assess *5-HTTLPR* genotype, 50 ng of gDNA were amplified by polymerase chain reaction (PCR) with the following primers: forward: 5′-ggcgttgccgctctgaatgc, reverse-5'-gagggactgagctggacaacca (size of the amplified bands: L-allele 529 bp; S-allele 486 bp). The *rs25531* genotype was assessed by allele-specific PCR using as primers: forward A-allele specific: 5′-acccctcgcggcatccccccctgcacccaca-3′; forward G-allele specific: 5′-acccctcgcggcatccccccctgcacccacg-3′; common reverse: 5′-tggagtccgcgcgggattctggtgccacct-3′. Finally, apolipoprotein E (*APOE*) genotype was assessed as previously published [26]. To avoid false genotyping, samples were assessed at least twice and only unambiguous results were considered.

*2.3. Meta-Analysis, Statistical Analysis, and Power Calculation.* The meta-analysis was performed based on association studies data shown in the public available database http://www.alzforum.org/ [20]. Calculations were done using the MetaEasy software v1.0.4 (http://www.jstatsoft.org/v30/i07/paper). Genotypic or allelic frequencies were compared using hypothesis-free $\chi^2$ test by a free available online resource [27]. The odds ratios (ORs) were calculated by $2 \times 2$ contingency table at 95% confidence interval (CI) by GraphPad Prism 5.0. Power analysis (performed by G∗Power 3.03). With the sample size of our novel study (a total of 417 subjects), we had 85% power to detect an increase of the S-allele of 7%, corresponding to a small-to-medium effect size of $w = 0.14$ (where $w$ stands for the effect size conventional index for chi-square test [28]), and a power of 57% to detect a small effect size of $w = 0.10$ (an increase of S-allele of 5%). As for the meta-analysis performed in the Italian population, the total number of subjects was 1178, and in this case we had a power of 77% to detect an increase of the S-allele of 5%. The statistical significance limit was set at $P = .05$.

## 3. Results

*3.1. 5-HTTLPR, rs25531 Genotyping, and APOE-ε4 Stratification.* AD subjects ($n = 235$) and controls (CNTR, $n = 207$) were screened to assess *5-HTTLPR* and *rs25531* genotype. Their demographic data are summarized in Table 2. AD cases and CNTR were people of Italian ancestry (self-reported, at least two generations before the patient were born and resident in Italy), balanced for age and sex proportion.

Genetic results are shown in Table 3. Of the available 235 AD and 207 CNTR, 220 AD (93.6%) and 197 CNTR (95.2%) were considered for subsequent analysis, with a genotyping efficacy of 99.5% and an accuracy of 94.8%. We checked at first Hardy-Weinberg equilibrium and found no deviation in cases and controls separately for *5-HTTLPR*, while for *rs25531* controls had a significant difference from the expected genotypic frequencies ($\chi^2$=8.29, $P = .003$), probably due to a slight overrepresentation of the rare G/G homozygous genotype in this group. The distribution of genotypic frequencies of *5-HTTLPR* and *rs25531* did not differ between AD and CNTR. However, by assuming that the presence of at least one S-allele was sufficient to modulate AD risk, we calculated the odds ratio OR (95% confidence interval (CI)) for the *5-HTTLPR* L/L versus (S/L+S/S) genotype that was 0.62[0.41–0.94], with associated $P$-value of $P = .02$. For *rs25531*, the comparison considering (G/A+G/G) genotypes versus A/A genotype gave an OR (95% (CI)) of 0.83 (0.5–1.5), with associated $P$ value of $P = .59$. As for allelic frequencies, the *5-HTTLPR* S-allele was significantly more frequent in AD than CNTR (47.5% versus 40.1%). We have also verified whether the *5-HTTLPR* allelic distribution was independent of the presence of the *APOE-ε4* allele (Table 4). The *APOE-ε4* allele by itself was a strong risk factor for AD (OR (95% CI) for carriers versus noncarriers: 5.4 (3.2–8.8), $P < .0001$). When we divided the *5-HTTLPR*

Table 1: Literature overview of the *5-HTTLPR* polymorphism in association with AD in the Caucasian population.

| Reference | Population | No. of cases | No. of controls | Main result |
|---|---|---|---|---|
| [13] | UK | 196 | 271 | No association |
| [14] | Germany | 84 | 118 | No association |
| [15] | Germany | 50 | 199 | Association of S-allele with AD (S-allele frequency in AD and controls: 51% and 41%, resp.) |
| [16] | Italy | 208 | 116 | No association |
| [17] | Italy | 105 | 114 | No association |
| [18] | Austria | 127 | 479 | No association |
| [19] | Italy | 164 | 54 | Association of S-allele with AD (S-allele frequency in AD and controls: 47% and 34%, resp.) |
| This study (no overlapping with the above-cited populations) | Italy | 220 | 197 | Association of S-allele with AD (S-allele frequency in AD and controls: 47.5% and 40.1%, resp.) |

AD: sporadic Alzheimer's disease.

Table 2: Demographics of the AD sample.

| Diagnosis | No. of subjects (male : female) | Age at sampling (years ± SD) | Age at onset (years ± SD) | Disease duration (years ± SD) | MMSE score at sampling (mean ± SD) |
|---|---|---|---|---|---|
| AD | 235 (74 : 161) | 78.6 ± 9.8 | 77.2 ± 8.0 | 4.1 ± 1.8 | 18.7 ± 5.8 |
| CNTR | 207 (69 : 138) | 77.0 ± 9.3 | NA | NA | 28.2 ± 2.6** |

AD: sporadic Alzheimer's disease;
CNTR: controls;
MMSE: Minimental State Examination;
SD: standard deviation;
NA: not applicable;
**$P < .001$, Student's $t$-test versus AD.

cases and CNTR according to *APOE-ε4* status, we did not find a difference in the genotypic or allelic distributions. No variation was found by comparing *5-HTTLPR* genotypic or allelic frequencies between AD *APOE-ε4* carriers and noncarriers.

We also performed a multivariate logistic regression considering variables: age, sex, *rs25531*, *5-HTTLPR*, and *APOE-ε4* status. The contribution to AD of *5-HTTLPR* was no longer significant (OR: 0.61, 95% CI: 0.38–1.05, $P = .10$). As for *rs25531*, no risk modulation was found ($P = .96$). We confirmed the strong influence of *APOE-ε4* allele (OR: 6.4, 95% CI: 3.5–11.8, $P < .00001$).

Finally, we have assessed whether *5-HTTLPR* or *rs25531* influenced other clinical parameters as age at onset. We found no association between the *5-HTTLPR* or *rs25531* genotype and dementia onset (data not shown).

*3.2. Meta-Analysis for 5-HTTLPR Studies.* Taking advantage from the public available database http://www.alzforum.org/, we have performed a meta-analysis of the Italian studies focused on *5-HTTLPR* and risk of AD, including our own data. We found a marginal effect, with an odds ratio (OR) and 95% confidence interval (95% CI) of the L-allele versus the S-allele of 0.85 (0.70–1.03) (Figure 1). We compared the Italian meta-analysis with a second meta-analysis based on thirteen studies (regardless of ethnicity) and a third including eight Caucasian studies only. In the general meta-analysis the OR (95% CI) was 0.97 (0.87–1.07), while for the Caucasian meta-analysis the OR (95% CI) was 0.90 (0.79–1.02) (Figure 1).

## 4. Discussion

5-HT imbalance might be the biochemical basis of the etiology of behavioral disturbances that are frequent features in late-onset AD [29, 30]. However, the genetic variability linked to the promoter region of the *SLC6A4* gene has been considered as predisposing factor for the development of AD dementia, too (Table 1). The increased frequency we have detected in *5-HTTLPR* S-allele corresponds to a small-to-medium effect size (odds ratio (OR) of 1.6), a magnitude far below the *APOE-ε4* allele, but that seems independent of the presence of this strong risk factor as suggested by our stratification analysis. However, this observation should suffer from reduced sample size in the groups analyzed, even though both in AD *APOE-ε4* carriers (+) and AD noncarriers (−) the *5-HTTLPR* S-allele frequency had a positive trend in comparison to controls. As for the significance of our

Table 3: *5-HTTLPR* and *rs25531* genotypic and allelic frequencies.

| | Genotype count (%) | | | Allele count (%) | | OR (95% CI) and *P* value for allelic distribution (L-allele versus S-allele) |
|---|---|---|---|---|---|---|
| | S/S | S/L | L/L | S | L | |
| *5-HTTLPR* | | | | | | |
| AD (220) | 51 (23.2) | 107 (48.6) | 62 (28.2) | 209 (47.5) | 231 (52.5) | |
| CNTR (197) | 37 (18.8) | 84 (42.6) | 76 (38.6)* | 158 (40.1) | 236 (59.9) | 0.74 (0.56–0.97) and 0.03 |
| | Genotype count (%) | | | Allele count (%) | | OR (95% CI) and *P* value for allelic distribution (G-allele versus A-allele) |
| | G/G | G/A | A/A | G | A | |
| *rs25531* | | | | | | |
| AD (220) | 0 (0.0) | 31 (13.9) | 189 (86.1) | 31 (7.0) | 409 (93.0) | |
| CNTR (197) | 5 (2.5) | 26 (13.2) | 166 (84.3)# | 36 (9.1) | 358 (90.9) | 0.75 (0.45–1.24) and 0.29 |

AD: sporadic Alzheimer's disease;
CNTR: controls;
*= 0.07; # = 0.06*, *P*-value calculated from $\chi^2$ test for AD versus CNTR as for genotypic distribution;
OR (95% CI): odds ratio and 95% confidence interval calculated from $2 \times 2$ contingency table.

Table 4: Stratification of *5-HTTLPR* genotype according to *APOE-ε4* status.

| | Genotype count (%) | | | Allele count (%) | | *P* value |
|---|---|---|---|---|---|---|
| | S/S | S/L | L/L | S | L | |
| *APOE-ε4 (−)* | | | | | | |
| AD (117) | 25 (21.6) | 57 (48.6) | 35 (29.8) | 107 (45.7) | 127 (54.3) | .35[a] |
| CNTR (171) | 31 (18.1) | 75 (43.8) | 65 (38.1)[a] | 137 (40.0) | 205 (60.0)[b] | .17[b] |
| | Genotype count (%) | | | Allele count (%) | | *P* value |
| | S/S | S/L | L/L | S | L | |
| *APOE-ε4 (+)* | | | | | | |
| AD (103) | 27 (26.3) | 50 (48.5) | 26 (25.2) | 104 (50.5) | 102 (49.5) | .57[c] |
| CNTR (26) | 5 (19.2) | 12 (46.1) | 9 (34.7)[c] | 22 (42.3) | 30 (57.7)[d] | .29[d] |

AD: sporadic Alzheimer's disease;
CNTR: controls;
*P* value:*P* calculated from $\chi^2$ test for AD versus CNTR;
[a,c] genotypic distribution;
[b,d] allelic distribution.

findings, the association found with unadjusted analysis was no longer significant after correction by logistic regression performed considering other variables, such as age, sex, and *APOE-ε4* status, even if the OR coming from the multivariate regression was similar to the uncorrected analysis, suggesting that the potential confounders age, sex, and *APOE-ε4* do not strongly influence the association result. A haplotypic study assessing *5-HTTLPR* and *rs25531* together might have been of interest, but our sample was too small to perform this analysis.

To our knowledge, none of the genome-wide association studies (GWASs) so far reported in the literature found an association signal for the *SLC6A4* promoter region and AD, while the case-control studies available to date addressing the same association are conflicting, even if they are mostly negative (Table 1). In the Caucasian population two studies linked *5-HTTLPR* and AD, while others did not reproduce this association (including two studies in the Italian population, the largest enrolling $n = 324$ subjects) [16, 17]. To this respect, our population ($n = 417$ subjects with successful genotyping) is a bigger independent sample, and the indication of an increased frequency of the *5-HTTLPR* S-allele in AD deserves further replication in Italy, also considering the recent data by Lorenzi et al. reporting an increased frequency of *5-HTTLPR* S-allele in sporadic demented subjects (AD and frontotemporal lobar dementia (FTLD)) from the same country [19]. Our data are also in agreement with the positive association between *5-HTTLPR* and AD from Hu et al. ($n = 249$ subjects, AD group $n = 50$) [15], but represent a more accurate replication due to the increased sample size, in particular for the AD group (AD group genotyped in our study $n = 220$). Due to the Italian population structure and the age of included patients, we do not envisage a possible bias in our analysis due to population stratification, even if the Italian ancestry was self-reported.

The meta-analysis performed by comparing studies with mixed ethnicity, Caucasian ancestry and Italian ethnicity have pointed out a marked heterogeneity of results as demonstrated by the Cochran's Q-test for heterogeneity (significant in all the meta-analyses that we made). It is worth to notice that a trend for an increased risk of

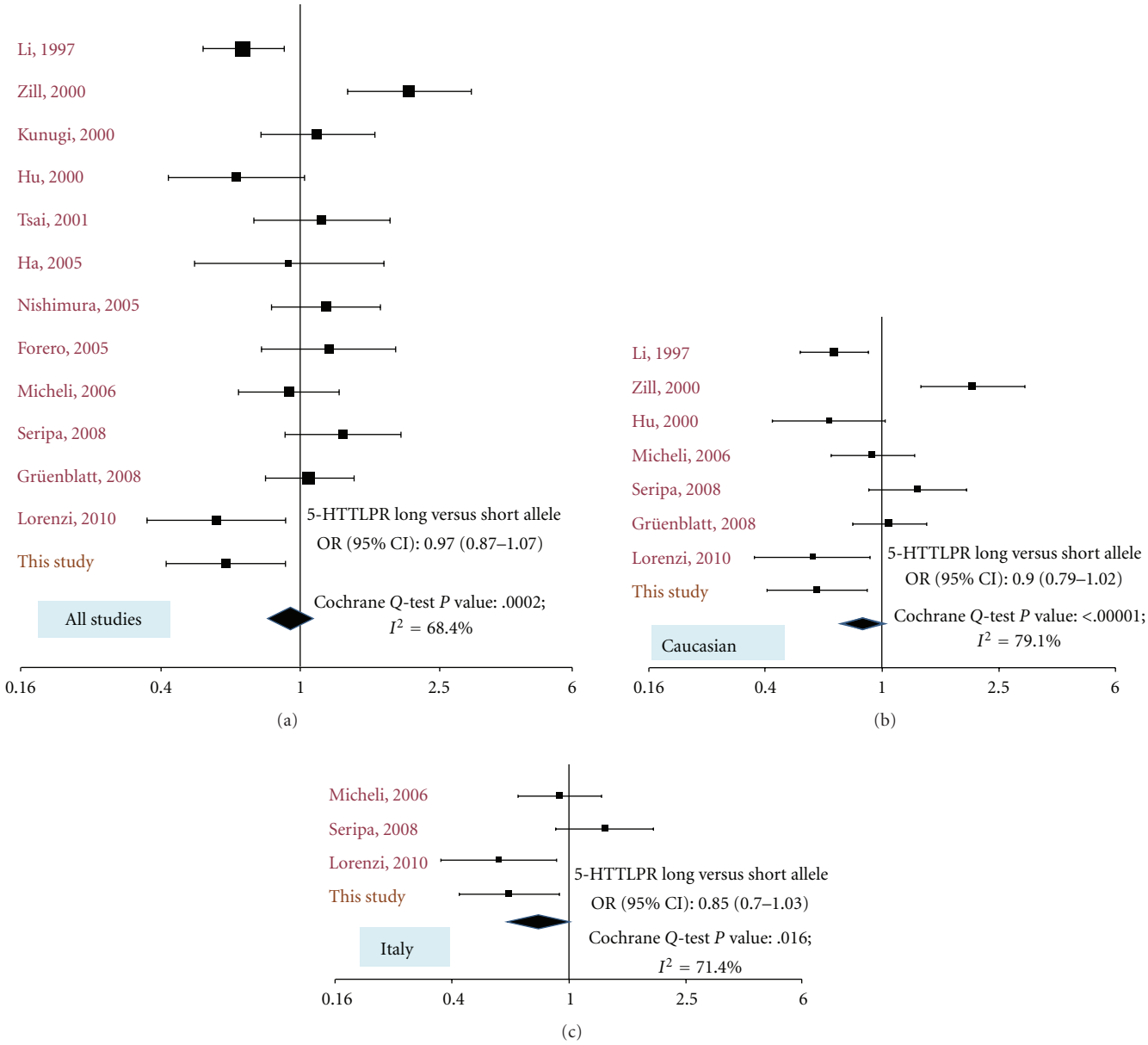


Figure 1: Meta-analysis of *5-HTTLPR*. We have included our own data in three datasets: (a) all nonoverlapping studies regardless of ethnicity available online (http://www.alzforum.org/ [20]); (b) Caucasian studies only; (c) Italian studies only. The odds ratio (OR) and 95% confidence interval (CI) are calculated for the long (L) versus short (S) allele. The diamond is the output of meta-analysis calculation, with its OR (95% (CI)).

the S-allele in comparison to the L-allele came to light, with the Italian population having the highest risk among the group considered, although not significant. We can speculate that *5-HTTLPR* might be an AD risk factor with a selective ethnicity effect.

Overall, we acknowledge that the result of our novel association study is most likely inflated by type-I error, while the meta-analysis results do not provide nominally significant evidence for association. However, before a robust conclusion can be drawn, larger studies would be needed to definitely assess the role of this marker in AD pathogenesis.

The analysis on *rs25531* had first of all the important limitation of a deviation from Hardy-Weinberg equilibrium (HWE) in controls. They revealed that *rs25531* frequency in our population considered as a whole was quite limited (G-allele frequency of 8.0% and homozygous G/G genotype frequency of 1.2%), so it might explain a small percentage of AD genetic risk. Moreover, due to the frequency of this SNP and the departure from HWE in controls, our negative data should be confirmed in larger datasets. As for a possible linkage disequilibrium (LD) between *rs25531* and *5-HTTLPR* we have already addressed this point with negative results [31].

## Conflict of Interests

The authors declare no conflict of interests regarding the present manuscript.

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*Research Article*

# Lack of Association between the GPR3 Gene and the Risk for Alzheimer's Disease

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Alzheimer's disease is the most frequent form of dementia and its incidence is rapidly increasing. Genetic factors are important determinants of the individual susceptibility to the disease and many efforts have been made to identify loci and markers involved. Recent finding describes the GPR3 gene as a modulator of $\beta$-amyloid production, suggesting that perturbation of its activity and function may contribute to the pathogenesis of AD. Furthermore, the gene is located at chromosome 1, in a region proposed as a susceptibility locus for the disease. We searched for nucleotide variations in the coding sequence and in the region 5 prime of it by dHPLC and analysed their distribution in a group of 104 AD patients and 109 age-matched controls. We identified 5 types of variation, two in the putative promoter region (g.27718954A>G and g.27719102A>T) and the others in exon 2 (c.51C>A, c.80C>G, and c.771C>T). All of them were equally represented in the two cohorts of the study, thus suggesting the absence of an association between GPR3 gene and AD in our population.

## 1. Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease and an important cause of illness and death in the industrialized world [1]. It often initiates with difficulties in remembering new information and gradually progresses to dementia and loss of normal life abilities. The major histopathological features are extracellular senile plaques and intracellular neurofibrillary tangles (NFTs), both related to the deposition of misfolded proteins; beta amyloid (A$\beta$) is the main component of plaques and hyperphosphorylated tau that of NFTs. A very small proportion of AD cases is directly linked to genetic variations in three genes coding for the amyloid precursor protein (APP) on chromosome 21, presenilin 1 (PSEN1) on chromosome 14, and presenilin2 (PSEN2) on chromosome 1. These genetic forms are collectively defined as early-onset familial AD (EOFAD), since the disease tends to start earlier than the age of 65, sometimes even before 30 years of age. Most cases of AD are sporadic, with less evident familial aggregation and later onset of the symptoms (late-onset AD, LOAD). The causes of sporadic AD are not defined yet, but current thinking suggests that multiple factors, including environment, epigenetics, and genetics concur in determining the individual risk. Advancing age is surely the prominent risk factor for LOAD, but genetics is estimated to determine up to 60–80% of the individual susceptibility [2]. Both candidate gene and genome wide association studies (GWASs) have identified different genes and loci potentially capable of modifying the predisposition to develop AD; the Apolipoprotein E gene (APOE) is by large the most important of these, with carriers of the e4 allele having a 4-fold increased risk [3], while those of the e2 allele benefit of a protective effect [4, 5].

Table 1: Demographic, clinical, and genetic characteristics of patients with AD and controls.

| | AD | CNT | *P* value |
|---|---|---|---|
| *n* | 104 | 109 | |
| Sex F/M | 71/33 | 74/35 | |
| Age[a,b], years | 78.4 ± 6.7 | 72.6 ± 7.9 | |
| MMSE score[a] | 19.2 ± 5.7 | 27–30 | |
| APOE ε4+ | 39.4% | 13.2% | <.001 |

[a] Data are given as mean ± SD.
[b] The age of onset of the disease was considered for AD patients.
AD: Alzheimer's disease patients; CNT: controls, MMSE: Mini Mental Scale Examination.

Table 2: Nucleotide variants in GPR3 promoter and coding regions and their allele frequencies in AD patients and controls.

| | | Allele frequencies | | |
|---|---|---|---|---|
| SNP | position | AD | CNT | *P* value |
| g.27718954A>G | Upstream 5′ end | 0.084 | 0.082 | 1.0 |
| g.27719102A>T | Upstream 5′ end | 0.084 | 0.082 | 1.0 |
| c.51C>A | Exon 2 | 0.026 | 0.023 | .84 |
| c.771C>T | Exon 2 | 0.011 | 0.010 | .89 |

The g.27718953A>G (rs2504785) and g.27719102A>T SNPs are in the genomic region (NC_000001.10) upstream of GPR3 exon1 and probably linked in a haplotype (AA or GT). The c.51C>A and c.771C>T (NM_05821.2) are synonymous SNPs and correspond to Rs11586015 and Rs2230880, respectively. SNP: single nucleotide polymorphism; AD: Alzheimer's disease patients; CNT: controls.

Recent findings have indicated the orphan G protein-coupled receptor 3 (GPR3) has a possible agent in the modulation of A$\beta$ metabolism [6]. Overexpression of GPR3 stimulated the production of the peptide, while its genetic ablation reduced it, both in transduced neural cells and in a mouse model of AD. This effect was exerted through the modulation of $\gamma$-secretase, one of the enzymes involved in APP proteolytic processing [7]; overexpression of GPR3 determined an increase in the amount of mature $\gamma$-secretase and of its localization at the cellular surface. Interestingly GPR3 is significantly expressed in the central nervous system [8], is elevated in the sporadic AD brain [6], and maps to chromosome 1 p36.1–p35 [9], which has been suggested as a potential AD linkage region in a high-resolution genome screen [10]. On the basis of these data we scanned the putative promoter and coding regions of GPR3 gene by DHPLC and evaluated frequencies and distribution of the identified sequence variations in cohorts of Italian AD patients and age-matched controls.

## 2. Materials and Methods

*2.1. Patients and Controls.* AD patients and controls were recruited by the Alzheimer Unit of Ospedale Maggiore in Milan (Italy). All patients (104) underwent a standard battery of examinations and dementia severity was assessed by the Clinical Dementia Rating (CDR) and the Mini Mental Scale Examination (MMSE). The diagnosis of probable AD was made according to NINCDS-ADRDA criteria [11]. The control group consisted of 109 nondemented subjects (MMSE ranging from 27 to 30). All individuals or caregivers provided an informed consent to participate in genetic studies. Table 1 shows the demographic and clinical characteristics of the population sample. Considering the age of onset of the disease, the AD population (78.4 ± 6.7) had similar age than the control one (72.6 ± 7.9) and no differences in gender distribution were present.

*2.2. Patients and Controls.* Genomic DNA was obtained from whole blood [12] and stored at 4°C until use. APOE genotyping was performed by DHPLC as previously described [13]. Table 1 shows number and percentage of APOE ε4 carriers among patients and controls. The GPR3 gene consists of two exons separated by a single intron. The coding sequence (NM_005281.2, from base 1 to base 1324) and about 500 bp upstream of exon 1 (hereafter referred to as "promoter" region) were analyzed by the Navigator software (Transgenomic Inc, Neb., USA) in order to evaluate the melting profiles in the sequence and optimize the design of primers for PCR amplification. The sequence of interest was subdivided in 5 different fragments, ranging from 280 to 540 bp. About 100 ng of genomic DNA were amplified by primers and settings described in the supplementary (Table 1SD, material available online at doi: 10.4061/2011/576143) DHPLC analysis was performed by a 3500A System (Transgenomic Inc, Neb., USA.); 5 $\mu$L of each PCR reaction were loaded on a SepA column and analysed at the temperatures described in Table 1SD. Different elution profiles were obtained and associated to specific genotypes by further DHPLC analysis and sequencing. Finally, homozygous samples always eluted with single-peak, symmetric profiles, with no evident differences in the retention time. In order to distinguish wild-type samples from those carrying mutation in homozygosis, each sample was mixed with a known wild-type one, denatured and reannealed, and further analysed by DHPLC; when the elution profile was no more symmetric, sequencing allowed to confirm the presence of a homozygous nucleotide variation.

*2.3. Patients and Controls.* Allelic and genotypic frequencies were obtained by direct counting. The Hardy-Weinberg equilibrium and differences in the allele frequency distribution between patients and controls were assessed by the $\chi$-squared test.

## 3. Results and Discussion

The analysis of GPR3 "promoter" and coding regions by dHPLC in about 400 chromosomes evidenced few nucleotide variations, most of which with low frequency (Table 2). We identified a possible haplotype in the "promoter" region, consisting of the SNPs g.27718954A>G (rs2504785) and the g.27719102A>T (NC_000001.10). All the subjects carrying the G variant in the most upstream position were also positive for the second variation. Given the short distance between the two SNPs (150 bp),

they are probably inherited as a haplotype, AA and GT being the two alternative genotypes. The overall allelic frequency was 8.3%; five individuals were homozygous and 19 heterozygous. No frequency is reported in the database (Entrez SNP, http://www.ncbi.nlm.nih.gov/) for rs2504785 and the comparison with our data is impossible. The g.27719102A>T seems to be unreported yet. The analysis of the possible biological effects of the two SNPs was performed by TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) and resulted in no difference in types and positions of putative transcription factor-binding sites among the ancestral sequence and the variant one.

The analysis of the coding sequence (NM_05821.2) revealed only three substitutions, two synonymous (Table 2), c.51C>A (G17G, Rs11586015) and c.771C>T (A257A, Rs2230880) and one nonsynonymous, c.80c>G (A27G), all at the heterozygous state. The frequency of the c51C>A SNP was 0.024, very close to that described in the database (0.042, pilot 1 CEU sample). That of c.771C>T was 0.010 (database = 0.033, MITOGPOP6 sample), while the third mutation was identified in a single subject.

When we compared the distribution of these variants in the cohort of AD patients versus that of age-matched controls we did not find any statistically significant difference, the frequency being virtually the same in patients and controls (Table 2).

Since we analysed each amplicon at different temperatures, we believe that our search is complete and that the other types of substitution in GPR3 open reading frame (rs34890664, rs34585631, and rs734852) that are described in the database are not present in our population. As a matter of fact their allelic frequency is either not reported or very low (rs734852 = 0.017, HapMapCEU).

## 4. Conclusions

The number of subjects enrolled in the study together with the low incidence of the nucleotide variations found in GPR3 gene sequence limit the statistical power of the study (between 40 and 50%, http://www.dssresearch.com/toolkit/default.asp); nonetheless the results let us infer that the SNPs identified do not determine modification of the individual risk to develop AD. The analysis of a larger population sample would be useful for the definitive confirmation of the results.

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*Research Article*

# Increased mRNA Levels of *TCF7L2* and *MYC* of the Wnt Pathway in Tg-ArcSwe Mice and Alzheimer's Disease Brain

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Several components in the Wnt pathway, including $\beta$-catenin and glycogen synthase kinase 3 beta, have been implied in AD pathogenesis. Here, mRNA brain levels from five-month-old tg-ArcSwe and nontransgenic mice were compared using Affymetrix microarray analysis. With surprisingly small overall changes, Wnt signaling was the most affected pathway with altered expression of nine genes in tg-ArcSwe mice. When analyzing mRNA levels of these genes in human brain, transcription factor 7-like 2 (*TCF7L2*) and v-myc myelocytomatosis viral oncogene homolog (*MYC*), were increased in Alzheimer's disease (AD) ($P < .05$). Furthermore, no clear differences in *TCF7L2* and *MYC* mRNA were found in brains with frontotemporal lobar degeneration, suggesting that altered regulation of these Wnt-related genes could be specific to AD. Finally, mRNA levels of three neurogenesis markers were analyzed. Increased mRNA levels of dihydropyrimidinase-like 3 were observed in AD brain, suggesting that altered Wnt pathway regulation may signify synaptic rearrangement or neurogenesis.

## 1. Introduction

Alzheimer's disease (AD) is the most common form of dementia. The most prominent histopathological features of the AD brain are extracellular plaques, mainly containing amyloid-$\beta$ (A$\beta$) peptides, and neurofibrillary tangles largely consisting of hyperphosphorylated tau. Studies on familial cases of AD have identified several disease-causing mutations in the genes for the amyloid-$\beta$ precursor protein (*APP*) [1] and for the presenilins (*PSEN1* and *PSEN2*) [2, 3]. In addition, the *ε4* allele of the gene for apolipoprotein E (*APOE*) lowers the age at onset of sporadic late-onset AD in a dose-dependent manner [4, 5]. All these genetic variants result in increased levels or in an altered aggregation of A$\beta$, indicating a central role of A$\beta$ in the disease process. However, it is still unclear which downstream pathways that are causing the synaptic dysfunction and cognitive failure seen in AD.

Gene expression studies have found a wide range of pathways that are altered in both human postmortem AD brains and transgenic mouse models of AD. Examples of such pathways include mitochondrial abnormalities, oxidative stress, inflammation, and calcium dyshomeostasis [6]. Moreover, the Wnt signaling pathway, consisting of a complex network of proteins, has been implicated in AD pathogenesis. For example, glycogen synthase kinase 3 beta (GSK3$\beta$) hyperphosphorylates tau [7] and increases levels of $\beta$-catenin in *PSEN1* transgenic mice [8]. During Wnt

Table 1: Mouse samples studied.

| Type | $n$ | Age (weeks ± SEM) | % female |
|---|---|---|---|
| Tg-ArcSwe | 6 | 20.5 ± 1.5 | 33 |
| Nontransgenic | 6 | 20.6 ± 1.4 | 67 |

Tg-ArcSwe: transgenic mice, Nontransgenic: nontransgenic littermates.

signaling, $\beta$-catenin translocates to the nucleus where it forms complexes with transcription factor LEF/TCF (lymphoid enhancer factor/T-cell factor) and activates target gene expression. Finally, the Wnt pathway is also involved in cell differentiation and cancer development [9].

Transgenic AD mice models have been of importance to the understanding of disease mechanisms. One of these models, the tg-ArcSwe mouse, overexpresses human APP with the Swedish and the Arctic mutation. Studies on tg-ArcSwe mice suggest that intraneuronal A$\beta$ aggregates, which appear at one month and increase with age before the onset of extracellular plaque deposition at six months [10, 11], may be responsible for the spatial learning deficits in these mice [12]. This mouse model would thus be suitable to study molecular changes related to early A$\beta$ aggregation.

In this study we wanted to analyze and compare gene expression patterns between young tg-ArcSwe and nontransgenic mice. In addition, we aimed at investigating whether differentially expressed genes in this model also were altered in brains from AD patients.

## 2. Materials and Methods

*2.1. Samples.* Six tg-ArcSwe and six nontransgenic mice (littermates) were sacrificed by cervical dislocation. The brain stem, cerebellum, and olfactory bulb were removed by dissection, and the remaining hemispheres were frozen separately on dry ice and stored at −80°C until processed. All mice were approximately five months old and were of the same genetic background, C57Bl/6J (Table 1). The use of mice had been approved by the local ethical committee for research on laboratory animals.

Human brain tissue from the anterior part of temporal neocortex of 13 AD cases and nine age-matched controls was obtained from the Alzheimer's Disease Research Center (ADRC) at Massachusetts General Hospital in Boston, USA (Table 2). In addition, a separate set of tissues from the anterior part of frontal neocortex from nine cases with frontotemporal lobar degeneration (FTLD) and nine age-matched controls, also from ADRC, were included (Table 2). The tissues were stored at −80°C until processed. This study was approved by the Regional Ethical Committees in Uppsala, Sweden, and Massachusetts General Hospital, Boston, USA.

*2.2. RNA Extraction and cDNA Synthesis.* Total RNA was extracted from both mouse and human brain tissues. The samples were homogenized in TRIzol (Invitrogen, Carlsbad, CA, USA), and total RNA was extracted according to the manufacturer's protocol. The total RNA was further purified using RNeasy Mini Kit (Qiagen, Valencia, CA, USA), following the manufacturer's protocol. Total RNA quantity was measured with NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and RNA integrity through the 28S : 18S rRNA ratio, as measured by the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). First-strand cDNA synthesis was carried out on 2 $\mu$g total RNA with Superscript II first-strand synthesis kit for RT-PCR (Invitrogen), according to the manufacturer's protocol. Finally, 2.5 ng of cDNA was used for quantitative PCR (qPCR).

*2.3. Microarray Analyses.* Individual total RNA samples from tg-ArcSwe and nontransgenic mice were hybridized to Affymetrix GeneChip Mouse Genome 430 2.0 arrays (Affymetrix, Santa Clara, CA, USA). Robust multichip average expression values were obtained from six arrays per group and further analyzed for differential expression. Using the Database for Annotation, Visualization and Integrated Discovery (DAVID) [13], differentially expressed genes with a $P$-value of less than .05 from the microarray analysis were classified into different molecular pathways according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) [14]

*2.4. Quantitative PCR.* Levels of mRNA from selected genes were measured in mouse and human brain samples with qPCR using the SYBR green chemistry on the MyiQ Single Color Real Time PCR Detection System (Bio-Rad, Richmond, CA, USA). The samples were prepared in a volume of 25 $\mu$L, containing 2.5 ng cDNA, 2x Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), and 200 nM of each primer (sequences available upon request). Next, the mixture was denatured in 95°C (10 min.), followed by 40 cycles at 95°C (15 sec.) and 60°C (1 min.) before a final incubation at 95°C (1 min.). All primers were designed to have an annealing temperature of 60°C.

All samples were run in triplicates, including a calibrator sample. Wells without template were included as negative controls. Gene expression was determined relative to *GAPDH* and normalized to the calibrator sample using the $2^{-\Delta\Delta Ct}$ method (Applied Biosystems). Primers were designed using Primer Express Software (Applied Biosystems) or obtained from online Real Timer PCR Primer and Probe Database [15]. Primers were evaluated in a qPCR efficiency test on a dilution series of template. Unpaired $t$-tests with Welch correction were performed using GraphPad InStat (GraphPad Software, San Diego, CA, USA).

*2.5. Western Blot on Human Brain Samples.* For all subjects, human brain tissue samples from the same region as used for qPCR were homogenized in RIPA buffer (50 mM Tris (pH 8.0), 1% NP-40, 0.5% sodium deoxycholate, and 150 mM NaCl, 0.1% SDS) supplemented with Complete Protease Inhibitor Cocktail Tablet (Roche Applied Science, Indianapolis, IN, USA), 1000 $\mu$L per 50 mg tissue. The homogenate was incubated at 4°C for two hours under constant agitation. In the following, all incubation steps were at room temperature.

Table 2: Human subjects studied.

| Diagnosis | Area | $n$ | Age (years ± SEM) | PMI (hours ± SEM) | % female |
|---|---|---|---|---|---|
| AD | Temporal ctx | 13 | 79.8 ± 1.5 | 14.8 ± 1.5 | 46 |
| C | Temporal ctx | 9 | 79.8 ± 1.8 | 19.9 ± 3.9 | 44 |
| FTLD | Frontal ctx | 9 | 71.6 ± 3.4 | 12.9 ± 2.3 | 44 |
| C | Frontal ctx | 9 | 82.4 ± 2.6 | 19.3 ± 4.2 | 56 |

PMI: postmortem interval, AD: Alzheimer's disease, C: control subjects, and FTLD: frontotemporal lobar degeneration.

Forty $\mu$g of total protein from each homogenate was mixed with sample buffer (1x Laemmli buffer, 0.1% bromophenol blue, and 2.5% mercaptoethanol) and boiled (five min.) before SDS-PAGE on a 10–20% Tris-Tricine gel (Bio-Rad). Next, the proteins were transferred (over night) to a nitrocellulose membrane (Bio-Rad) and blocked (1 h.) in TBS with 5% milk powder (BioRad). After washing in TBS with 0.1% Tween (TBS-T), the samples were incubated with primary antibody (1 h.) and washed in TBS-T before incubation with fluorophore-conjugated secondary antibody (30 min.). After washing in TBS-T and TBS, respectively, fluorescence was detected with the Odyssey imaging system (LI-COR/Westburg, Leusden, Netherlands).

In order to adjust for unequal sample loading, the protein levels were calculated as a ratio to GAPDH levels. Therefore, after detection, the membranes were incubated with a primary GAPDH antibody (30 min.). Next, they were washed in TBS-T, followed by incubation with fluorophore-conjugated secondary antibody (30 min.). After washing in TBS-T and TBS, respectively, fluorescence was measured with the Odyssey.

As primary antibodies, C-33 (1 : 250, Santa Cruz biotechnology, Santa Cruz, CA, USA) was used for detection of MYC, ab60727 (1 : 500, Abcam, Cambridge, MA, USA) and 6H5-3 (1 : 500, Upstate, Lake Placid, NY, USA) for TCF7L2 and G9545 (1 : 10000, Sigma-Aldrich, St. Louise, MO, USA) for GAPDH. As secondary antibodies, IRdye 800CW-conjugated anti-mouse antibody (1 : 5000, Rockland, Gilbertsville, PA, USA) was used for MYC and TCF7L2 whereas Alexa fluor 680-conjugated anti-rabbit antibody (1 : 5000, VWR, West Chester, PA, USA) was used for GAPDH. All antibodies were diluted in 50/50 Odyssey buffer/TBS-T 0.2%.

*2.6. Immunohistochemistry on Human Brains.* Ten $\mu$m brain sections from tissue blocks embedded in paraffin from temporal cortex of five of the AD and four of the control brains were included for immunohistochemistry. The tissues were deparaffinized in xylene for ten minutes and incubated in $H_2O_2$/methanol for 20 minutes followed by hydration in decreasing concentrations of ethanol and $H_2O$. Antigen retrieval was performed in citrate buffer (20 minutes, 10 mM, pH 6.0, 95°C). After a brief wash in PBS, the tissues were blocked in normal goat serum (NGS, 5%) followed by incubation overnight at 4°C with the primary antibody in 1.5% NGS (mouse anti-c-myc, 1 : 50, Santa Cruz Biotechnology; anti-TCF7L2 1 : 500, Millipore, Billerica, MA). Next day, the sections were washed in PBS followed by incubation with the secondary antibody in 1.5% NGS (biotinylated goat anti-mouse, 1 : 200). After additional washing in PBS, the Vectastain Elite ABC kit (Vector Labs, Burlingame, CA) and the DAB kit (Vector Labs) were used according to the manufacturer's instructions. Finally, sections were washed in PBS and dehydrated in increasing concentrations of EtOH and xylene before they were mounted with Permount.

## 3. Results

Altogether 530 genes were found to be differentially expressed ($P < .05$) when comparing mRNA levels between six tg-ArcSwe and six nontransgenic mice. The altered genes were either down- or upregulated, ranging from 88% to 112% of the reference levels in nontransgenic mice. Using DAVID, the genes were categorized according to their respective KEGG pathways. Thereby, three pathways emerged as being particularly altered in the tg-ArcSwe mice: the thyroid cancer pathway, the adipocytokine signaling pathway, and the Wnt signaling pathway. Four of the genes with altered expression were previously described to be involved in thyroid cancer development, six of the genes in adipocytokine signaling, and nine of the genes in the Wnt signaling pathway. Of the Wnt-related genes, mRNA levels of *TCF7L2*, *CCND3*, *MYC*, *NKD2,* and *PPP3CB* were increased whereas mRNA levels of *PLCB1*, *NFAT5*, *LEF1,* and *PPP2R2C* were decreased in transgenic as compared to nontransgenic mice (Table 3).

As the Wnt signaling pathway demonstrated the highest number of differentially expressed genes, we focused on this pathway for further analyses. First, we wanted to investigate whether the relatively small changes of the nine Wnt pathway genes could be detected with an independent technique. Therefore, we performed qPCR on cDNA preparations derived from the same RNA samples that were used for microarray analyses. However, after normalization to GAPDH, the small changes between transgenic and nontransgenic mice for the nine Wnt genes could not be verified with this method (Table 3).

When examining mRNA levels of the nine Wnt signaling pathway genes in human brains, two of the genes with increased mRNA levels in transgenic brains, *TCF7L2* and *MYC*, displayed significantly increased mRNA levels also in AD brain ($n = 13$) as compared to nondemented control brain ($n = 9$) (both $P < .05$) (Table 3, Figure 1). Moreover, the mRNA levels of these two genes were found to correlate with each other in the AD samples (Spearman $r = 0.896$, $P < .001$) (Figure 1(c)). Unlike in transgenic mouse brains, the mRNA levels of the other seven investigated genes did not

Table 3: Fold changes and *P* values when comparing tg-ArcSwe mice and nontransgenic littermates and when comparing AD patients and control subjects (C).

| Gene | Protein | Tg-ArcSwe versus nontransgenic | | | | AD versus C | |
|---|---|---|---|---|---|---|---|
| | | Microarray | | qPCR | | qPCR | |
| | | Fold change | *P* | Fold change | *P* | Fold change | *P* |
| *CCND3* | Cyclin D3 | 1.10 | <.05 | 0.88 | n.s. | 1.14 | n.s. |
| *LEF1* | Lymphoid enhancer-binding factor 1 | 0.92 | <.05 | 0.88 | n.s. | 0.90 | n.s. |
| *MYC* | V-myc myelocytomatosis viral oncogene homolog | 1.09 | <.05 | 0.91 | n.s. | 2.02 | <.05 |
| *NFAT5* | Nuclear factor of activated T-cells 5 | 0.91 | <.05 | 0.96 | n.s. | 1.21 | n.s. |
| *NKD2* | Naked cuticle homolog 2 (Drosophila) | 1.08 | <.05 | 1.05 | n.s. | 0.92 | n.s. |
| *PLCB1* | Phospholipase C, beta 1 | 0.90 | <.01 | 0.99 | n.s. | 0.82 | n.s. |
| *PPP2R2C* | Protein phosphatase 2, regulatory subunit B, gamma | 0.93 | <.05 | 1.21 | n.s. | 1.24 | n.s. |
| *PPP3CB* | Protein phosphatase 3, catalytic subunit | 1.08 | <.05 | 1.03 | n.s. | 0.79 | n.s. |
| *TCF7L2* | Transcription factor 7-like 2 | 1.10 | <.05 | 0.95 | n.s. | 1.43 | <.05 |

n.s.: not significant.

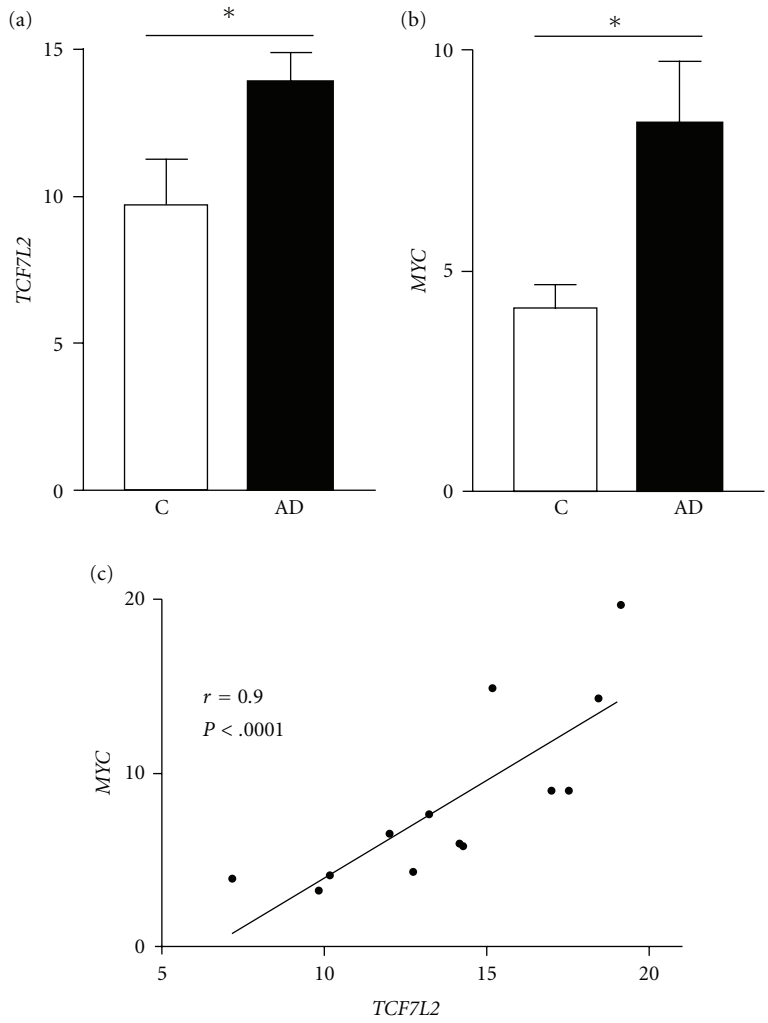


Figure 1: Relative mRNA levels of (a) *TCF7L2* and (b) *MYC* in control (C) ($n = 9$) and AD ($n = 13$) brain as measured by qPCR, normalized to *GAPDH* and to a calibrator sample (mean ± SEM, $P < .05$ for both). (c) Correlation of *TCF7L2* and *MYC* in the individual AD brains (Spearman $r = 0.9$, $P < .0001$).

show any altered levels in AD as compared to brains from healthy controls (Table 3).

In order to elucidate whether the observed alterations in mRNA levels of *TCF7L2* and *MYC* were AD-specific features or a result of the neurodegenerative process itself, the two genes were also analyzed in frontal neocortical tissue from a set of cases ($n = 9$) with frontotemporal lobar degeneration (FTLD) and compared to frontal cortex tissue from nondemented controls ($n = 9$). No significant differences were seen, although there was a trend for increased levels of *TCF7L2* mRNA in the FTLD group ($P = .08$) (data not shown).

As the Wnt signaling pathway is known to be upregulated in neurogenesis, we wanted to explore whether the increased mRNA levels of *TCF7L2* and *MYC* reflected neurogenesis in the AD brains. Thus, mRNA levels of two neurogenesis markers (neural cell adhesion molecule 1—*NCAM1* [16] doublecortin—*DCX* [17]) and one marker for neurons that are migrating or undergoing axonal growth (dihydropyrimidinase-like 3—*DPYSL3* [18]) were analyzed by qPCR. Only mRNA levels of *DPYSL3* were increased in AD brain ($P < .05$) whereas the *NCAM1* and *DCX* mRNA levels were unchanged as compared to control brains.

To investigate if the observed changes in mRNA levels of *TCF7L2* and *MYC* were paralleled by changes in the corresponding proteins (transcription factor 7-like 2 and v-myc myelocytomatosis viral oncogene homolog), western blot and immunohistochemical analyses were carried out on temporal neocortex from the same AD and control subjects. For western blot, we used human brain tissue homogenates from the same regions as that used for the mRNA analyses. For transcription factor 7-like 2, no signals could be seen in any of the brains using either of two antibodies. For v-myc myelocytomatosis viral oncogene homolog, clear signals were obtained but no significant change was detected in the AD cases (not shown). For immunohistochemistry on temporal cortical sections, we obtained signals only from transcription factor 7-like 2 but could not see any differences between AD and control brains (not shown). For v-myc myelocytomatosis viral oncogene homolog, no signals were seen on immunohistochemistry from any of the tissues investigated (not shown).

## 4. Discussion

The amyloid cascade hypothesis [19] is widely accepted, but it is still unclear which form of A$\beta$ that initiates the disease process or has the most neurotoxic properties. Studies have suggested that soluble A$\beta$ oligomers are particularly toxic [20] and effects on gene expression by such A$\beta$ species should therefore be of particular interest.

For this study, microarray analyses were performed on five-month old tg-ArcSwe mice with marked accumulation of intraneuronal A$\beta$ and increased levels of soluble A$\beta$ protofibrils in the brains [21]. For comparison, age-matched nontransgenic littermates were used. The fold changes in brain levels of various mRNAs from the transgenic mice were surprisingly small, ranging from 88% to 122% of that in nontransgenic littermates. These subtle changes could have been due to the fact that very young animals were used, when A$\beta$ possibly does not yet affect biological pathways to a great extent. The relatively small differences could also have been explained by the fact that whole hemispheres were used for extraction of RNA. If only areas displaying intracellular A$\beta$ had been used, the changes might have been more pronounced. Nevertheless, the genes found to be alternatively expressed in this microarray analysis may represent downstream molecular cascades triggered by the early formation of intracellular A$\beta$ seen in this mouse model.

Three biochemical pathways—the thyroid cancer pathway, the adipocytokine signaling pathway, and the Wnt signaling pathway—were overrepresented among the genes that had altered mRNA levels in the transgenic mice. Nine genes from the Wnt pathway were found to be upregulated in the mice brain. Although the fold changes were very small and despite that we could not verify the increase for all nine mRNA species by qPCR, we decided to further study the Wnt pathway in postmortem AD brains. We then found that two of the nine investigated genes, *TCF7L2* and *MYC*, had significantly increased mRNA levels also in AD brain. Both genes encode proteins that are part of the Wnt signaling pathway, in which the transcription factor 7-like 2 regulates transcription of the *MYC* gene [22]. This link in biological activity was also well illustrated by the correlation between the respective mRNA levels demonstrated in this study.

*MYC* is of particular interest as it has been illustrated on a transgenic mouse model that induction of this proto-oncogene can drive cell cycle re-entry and result in neuronal cell death, gliosis, and cognitive deficits [23]. Thus, the upregulation of MYC mRNA in human brain could possibly contribute to some of the neurodegenerative features seen in AD.

The observed increase in mRNA levels of *TCF7L2* and *MYC* indicates an increase in Wnt signaling in AD pathogenesis. Accordingly, recent evidence suggests that levels of nuclear calcineurin are increased in post mortem AD brains [24]. On the contrary, other studies have reported increased levels of GSK3$\beta$, suggesting a repression of the Wnt signaling pathway in AD [25]. However, in our study the mRNA levels of both *GSK3B* and $\beta$-catenin were unchanged, both in tg-ArcSwe mouse brain and in AD brain (data not shown). In addition, the mRNA levels of PPP2R2C were slightly decreased in tg-ArcSwe mouse brain whereas they were nonsignificantly increased in the AD brain (Table 3).

The observed increase in mRNA levels of *TCF7L2* and *MYC* between AD and control brains was not paralleled by increased levels of the respective proteins (transcription factor 7-like 2 and v-myc myelocytomatosis viral oncogene homolog). The discrepancy between mRNA and protein levels may be explained by a rapid turnover in brain of these signaling molecules.

Several lines of evidence suggest that both cell cycle activation and neurogenesis may be features in neurodegeneration. In the AD brain, cell cycle re-entry is believed to occur prior to the development of tangles and plaques (reviewed in [26]). Moreover, Wnt signaling has been demonstrated to be the principal regulator of adult hippocampal neurogenesis [27], which has been suggested to occur in the AD brain

[28]. We therefore reasoned that an upregulation of the Wnt signaling pathway may be explained by an increased neurogenesis in the investigated brains. One of the analyzed genes known to be upregulated in neurogenesis indeed displayed increased mRNA levels in our samples, suggesting that an upregulated Wnt signaling pathway may be related to increased neurogenesis in the AD brain. On the other hand, mRNA levels of the two other neurogenesis-related genes investigated were not found to be changed in AD, thus making the interpretation more difficult.

Interestingly, *TCF7L2* was shown to be associated with type II diabetes [29], and increased levels of *TCF7L2* mRNA in human islets have been found in type II diabetes [30]. There are commonalities between AD and diabetes with both disorders being associated with amyloidogenic deposits, and patients with diabetes have been reported to be more prone to develop AD [31]. Also, soluble A$\beta$ oligomers may impair insulin signaling in the brain through a reduction in neuronal surface insulin receptors [32]. Therefore, *TCF7L2* may represent a molecular link between AD and diabetes pathogenesis.

In conclusion, we have compared gene expression patterns using microarray analysis of tg-ArcSwe and nontransgenic mice, indicating only subtle changes in mRNA levels in young tg-ArcSwe. Interestingly, we found that the tg-ArcSwe mice displayed altered mRNA levels of several members of the Wnt signaling pathway as compared to nontransgenic mice. Moreover, we have investigated whether differentially expressed genes in this model also had altered mRNA levels in brains from AD patients. The mRNA levels of two of these genes, *TCF7L2* and *MYC*, were significantly increased also in AD brain ($P < .05$), whereas their levels were not significantly changed in frontal cortex of FTLD brains as compared to frontal cortex from control brains. Although the number of brains analyzed was limited, these results cautiously suggest that the observed differences may be AD specific, rather than a general feature of neurodegeneration. Finally, increased mRNA levels of a neuronal plasticity marker suggest that the upregulation of certain Wnt genes may give further support for ongoing neurogenesis in the AD brain.

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*Clinical Study*

# Cerebrospinal Fluid Biomarkers in Idiopathic Normal Pressure Hydrocephalus

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The diagnosis of idiopathic normal pressure hydrocephalus (iNPH) is still challenging. Alzheimer's disease (AD), along with vascular dementia, the most important differential diagnosis for iNPH, has several potential cerebrospinal fluid (CSF) biomarkers which might help in the selection of patients for shunt treatment. The aim of this study was to compare a battery of CSF biomarkers including well-known AD-related proteins with CSF from patients with suspected iNPH collected from the external lumbar drainage test (ELD). A total of 35 patients with suspected iNPH patients were evaluated with ELD. CSF was collected in the beginning of the test, and the concentrations of total tau, ptau$_{181}$, A$\beta_{42}$, NFL, TNF-$\alpha$, TGF$\beta$1, and VEGF were analysed by ELISA. Twenty-six patients had a positive ELD result—that is, their gait symptoms improved; 9 patients had negative ELD. The levels of all analyzed CSF biomarkers were similar between the groups and none of them predicted the ELD result in these patients. Contrary to expectations lumbar CSF TNF-$\alpha$ concentration was low in iNPH patients.

## 1. Introduction

Normal pressure hydrocephalus (NPH) is characterized by a clinical triad of symptoms including cognitive impairment, gait difficulty, and urinary incontinence along with ventricular enlargement in brain imaging [1]. NPH is considered as idiopathic (iNPH) when there are no known predisposing factors such as subarachnoid haemorrhage or brain trauma [1]. NPH can be treated by shunt [1] but the response rate is highly sensitive for selection of patients [2, 3]. Alzheimer's disease (AD) is along with vascular dementia (VaD) the most frequent differential diagnosis for iNPH [4].

Several supplementary diagnostic tests of cerebrospinal fluid (CSF) dynamics are used in the selection of patients to shunt surgery. The CSF tap test or external lumbar drainage test (ELD) can predict the shunt response with high specificity and are widely used [5]. Infusion tests where usually Ringer$^{R}$-solution is infused into CSF space with simultaneous CSF pressure monitoring to calculate outflow conductance or outflow resistance are also used [6]. In addition, intracranial pressure monitoring alone [7] or together with cortical brain biopsy to detect AD-related pathological findings [4] has been suggested.

CSF biomarkers reflecting ongoing pathophysiological processes might further help in the evaluation of patients with suspected iNPH. Previous reviews have pointed out several potential CSF biomarkers associated with NPH [8] but all of them still requiring further verification [9]. There are numerous experimental studies in both acute and chronic hydrocephalus that provide translational evidence for the role of metabolic changes and markers in parallel with hydrocephalus and disturbed CSF dynamics, for example, as reported by Kondziella et al. [10].

Several CSF proteins are potentially important in iNPH or Alzheimer's disease.

Tumour necrosis factor-$\alpha$ (TNF-$\alpha$), a proinflammatory cytokine, seems to be one of the most promising since up to 45-fold increased CSF concentrations compared with healthy controls have been observed in NPH prior to treatment along with normalization after shunt [11]. However, the patients with known aetiology that is secondary NPH were mixed

Table 1: Characteristics of the patients and CSF concentrations of the analyzed biomarkers.

| | Positive ELD | Negative ELD | $P$ |
|---|---|---|---|
| N. patients | 26 | 9 | |
| Median age at ELD (range) | 74 (65–88) | 77 (69–88) | .29 |
| Median age at onset (range) | 71.5 (62–86) | 75 (68–88) | .14 |
| Male/female | 13/13 | 5/4 | .77 ($X^2$) |
| Gait ($n$) | 25 | 9 | .55 ($X^2$) |
| Cognition ($n$) | 21 | 9 | .16 ($X^2$) |
| Incontinence ($n$) | 14 | 6 | .50 ($X^2$) |
| Major symptom gait/cogn/inc | 21/2/1 | 7/1/1 | .72 ($X^2$) |
| | 2 cases n.d.* | | |
| Shunt | 25 | 1 | |
| Shunt response fair/good/exc | 1/3/21 | 0/0/1 | |
| NFL pg/mL | 1940** ± 2662 (280–>10000) | 2046 ± 2886 (483–9306) | .92 |
| Total tau pg/mL | 274 ± 358 (44–1860) | 291 ± 283 (89–983) | .90 |
| A$\beta_{1–42}$ pg/mL | 250 ± 179 (31–765) | 209 ± 120 (102–486) | .52 |
| Total tau/A$\beta_{1–42}$ > 1.15 | 7 (27%) | 4 (44%) | .33 ($X^2$) |
| $p$-Tau$_{181}$ pg/mL | 55 ± 45 (16–234) | 53 ± 25 (24–95) | .88 |
| TNF-$\alpha$ pg/mL | 0.7 ± 1.1 (0–4.9) | 1.3 ± 1.4 (0–3.4) | .16 |
| TGF$\beta$-1 pg/mL | 61 ± 24 (29–146) | 71 ± 62 (20–228) | .52 |
| VEGF pg/mL | n.d.*** | n.d.*** | |

The values are given as mean±SD (range) unless otherwise stated.
*not able to determine one major symptom.
** two cases with concentration >10 000 pg/mL (10 000 used as a value and therefore the real mean value is expected to be higher.)
***not determined due to low concentration.
ELD: external lumbar drainage test, NFL: neurofilament light chain, $t$-tau: total tau, $p$-tau$_{181}$: phosphorylated tau$_{181}$, A$\beta_{42}$: amyloid-$\beta_{1–42}$, TNF-$\alpha$: tumour necrosis factor-$\alpha$, TGF$\beta$1: transforming growth factor $\beta$1, and VEGF: vascular endothelial growth factor.

with idiopathic cases and no further studies on this marker have been published in NPH.

Transforming growth factor-$\beta$ (TGF-$\beta$) is associated with brain response to injury and inflammation, and increased CSF TGF-$\beta$ concentrations are reported in AD patients [12]. Subarachnoid haemorrhage (SAH) increased TGF-$\beta$ concentrations and correlated with the risk of shunt-dependent hydrocephalus [13] but in iNPH the role of TGF-$\beta$ is somewhat controversial [14, 15].

Neurofilament protein is a marker of neurodegeneration especially axonal injury, and clearly increased NF light (NFL) concentrations have been detected both in iNPH and sNPH patients [16, 17].

Vascular endothelial growth factor (VEGF) is associated with cerebral ischemia and has been correlated negatively with neurofilament heavy chain (NFh) protein in iNPH patients and increased during ELD [18].

Increased total tau ($t$-tau) and phosphorylated-tau$_{181}$ ($p$-tau$_{181}$) in CSF are associated with neurodegeneration and AD [19]. In iNPH both normal [17] and increased [20] $t$-tau concentrations have been observed. Decreased amyloid-$\beta_{42}$ (A$\beta_{42}$) in CSF is associated with AD and indicates risk of AD in mild cognitive impairment [21] but may be normal in iNPH [22].

In the present study, we correlated the concentrations of seven biomarkers, NFL, $t$-tau, $p$-tau$_{181}$ A$\beta_{42}$, TNF-$\alpha$, TGF$\beta$1, and VEGF in the CSF of 35 patients with suspected iNPH with the result of the lumbar drainage test.

Table 2: Black Scale for assessment of shunt outcome [3].

| | |
|---|---|
| Excellent | Resumed preillness activity without deficit |
| Good | Resumed preillness activity with deficit, improved in two or more categories |
| Fair | Improved but did not return to previous work, improved in one category |
| Transient | Temporary major improvement |
| Poor | No change or worsening |
| Dead | Died within 6 wk of surgery or as a result of surgery |

## 2. Material and Methods

*2.1. Study Series.* This study includes 35 patients referred to Brigham and Woman's Hospital (BWH) Neurosurgery with suspected idiopathic normal pressure hydrocephalus (iNPH) according to clinical and radiological examination. Patient characteristics are presented in Table 1. Patients with known cause of NPH (sNPH) were excluded.

*2.2. External Lumbar Drainage Test (ELD).* All patients were evaluated by standard previously described ELD [23] between 2007 and 2010. Continuous drainage was applied with targeted CSF drainage rate of 5 to 10 mL/h. Neurological (including Folstein Mini-Mental Status Examination in patients with cognitive symptoms) and physical therapy

Table 3: CSF biomarkers.

| Protein | Abbreviation | Mean detection limit | Manufacturer |
|---|---|---|---|
| Neurofilament light chain | NFL | 31 pg/mL | Uman Diagnostics, Umeå, Sweden |
| Total tau | $t$-tau | 12 pg/mL | Invitrogen, Camarillo, Calif, USA |
| Phosphorylated $tau_{181}$ | $p$-$tau_{181}$ | 10 pg/mL | Invitrogen, Camarillo, Calif, USA |
| Amyloid-$\beta_{1–42}$ | A$\beta_{42}$ | 10 pg/mL | Invitrogen, Camarillo, Calif, USA |
| Tumour necrosis factor $\alpha$ | TNF-$\alpha$ | 0.106 pg/mL | Quantikine HS, R&D Systems, Minneapolis, Minn, USA |
| Transforming growth factor $\beta$1 | TGF$\beta$1 | 4.61 pg/mL | Quantikine HS, R&D Systems, Minneapolis, Minn, USA |
| Vascular endothelial growth factor-165 | VEGF | 5 pg/mL | Invitrogen, Camarillo, Calif, USA |

NFL: neurofilament light chain, $t$-tau: total tau, $p$-$tau_{181}$: phosphorylated $tau_{181}$, A$\beta_{42}$: amyloid-$\beta_{1–42}$, TNF-$\alpha$: tumour necrosis factor-$\alpha$, TGF$\beta$1: transforming growth factor $\beta$1, and VEGF: vascular endothelial growth factor.

(including Timed Up and Go test—the patient was timed while rising from a chair, walking 3 m, turning around, walking back to the chair, and sitting down) evaluations were completed prior to drainage and daily until the end of the test, which continued for a maximum of 5 days. If the patient demonstrated documented improvement before 5 days or if the patient experienced side effects of drainage refractory to conservative measures, the trial was considered complete and the drain was removed. Criteria for positive ELD were 20% improvement in objectively measured gait or cognition [23].

ELD test was negative in nine and positive in 26 patients.

*2.3. Response to Shunt.* Patients were shunted or not according to ELD result. One patient was shunted despite of primary negative response but later noted subjective improvement.

Clinical symptoms, history of any possible known cause of NPH, and other neurological disorders were recorded as well as clinically evaluated response for treatment in shunted patients. Shunt response was graded according to Black Scale (Table 2) as no response, fair, good, or excellent response after 3-month followup. All shunted patients responded to the treatment (Table 1).

*2.4. CSF Analysis for Biomarkers.* The cerebrospinal fluid (CSF) samples (4 mL) were collected in the beginning of the ELD immediately after the puncture, centrifuged, and stored in polypropylene tube at −80°C until analysis to ensure the stability of the CSF biomarker levels during the storage.

Measurements of CSF were performed in BWH Neurosurgery laboratory using commercially available solid phase sandwich enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's protocol and blinded to the ELD results (Table 3). All samples were analyzed as duplicates.

*2.5. Ethical Aspects.* The study was approved by the Partners Human Research Committee. Written informed consent was obtained from all patients.

*2.6. Statistical Analysis.* The CSF concentrations of the analyzed markers were compared between the positive and negative ELD groups by independent samples $t$-test. Dichotomized variables were compared by $X^2$-test. Pearson's correlation coefficients were calculated between the markers. Statistical analyzes were performed using SPSS 17.0.

## 3. Results

The biomarker concentrations in the CSF of 26 patients with a positive ELD result and nine patients with negative ELD result are presented in Table 1. Nine patients had initially negative ELD, and one of them was shunted with excellent response. The levels of all analyzed CSF biomarkers were similar between the groups, and none of them could predict the ELD result in these patients.

NFL concentrations were increased similarly both in patients with positive (range 280–>10000) and negative (range 438–9306) ELD (Table 1).

TNF-$\alpha$ concentration was equally low around 1 pg/mL in both groups (Table 1). In 10 cases the concentration was even under the mean detection limit (0.106 pg/mL).

TGF$\beta$1 concentrations were similar (mean 61 versus 71 pg/mL, range 20–228, $P = .52$) in both groups (Table 1). TGF$\beta$1 concentration had positive correlation with $t$-tau ($r = 0.413$, $P = .014$, Table 4).

Mean A$\beta_{42}$ concentrations did not differ significantly between the groups (250 versus 209 pg/mL) (Table 1).

Mean $t$-tau concentration was 274 pg/mL (range 44–1860) in positive and 291 (range 89–983) in negative ELD groups without significant difference ($P = .90$, Table 1) and increased with age ($r = 0.38$, $P = .023$, Table 4). Also $p$-$tau_{181}$ concentrations were equal between positive and negative ELD groups (55 versus 53 pg/mL, $P = .88$, Table 1).

VEGF concentrations were under the detection limit (5 pg/mL) in all except two cases and therefore were not included in the correlation analysis.

## 4. Discussion

The most important finding of this study is the unexpectedly low CSF TNF-$\alpha$ concentrations observed in iNPH patients and the inability of CSF biomarkers to predict the ELD result.

Using a standard commercial ELISA, the CSF TNF-$\alpha$ levels between 0 and 5.0 pg/mL—close to the standard

Table 4: Correlations between CSF biomarkers.

| | NFL | $t$-Tau | A$\beta_{1–42}$ | $p$-Tau$_{181}$ | TNF-$\alpha$ | TGF$\beta$1 |
|---|---|---|---|---|---|---|
| Age | −0.112 (0.52) | 0.383* (0.023) | 0.215 (0.22) | 0.010 (0.95) | 0.246 (0.15) | −0.009 (0.96) |
| NFL | | 0.030 (0.86) | −0.225 (0.19) | 0.063 (0.71) | 0.130 (0.46) | −0.013 (0.94) |
| $t$-Tau | 0.030 (0.86) | | −0.071 (0.69) | 0.667* (<0.001) | 0.143 (0.41) | 0.413* (0.014) |
| A$\beta_{1–42}$ | −0.225 (0.19) | −0.071 (0.69) | | −0.169 (0.33) | −0.024 (0.89) | −0.103 (0.56) |
| $p$-Tau$_{181}$ | 0.063 (0.71) | 0.667* (<0.001) | −0.169 (0.33) | | −0.138 (0.43) | 0.205 (0.24) |
| TNF-$\alpha$ | 0.130 (0.46) | 0.143 (0.41) | −0.024 (0.89) | −0.138 (0.43) | | 0.267 (0.12) |
| TGF$\beta$1 | −0.013 (0.94) | 0.413* (0.014) | −0.103 (0.56) | 0.205 (0.24) | 0.267 (0.12) | |

NFL: neurofilament light chain, $t$-tau: total tau, $p$-tau$_{181}$: phosphorylated tau$_{181}$, A$\beta_{42}$: amyloid-$\beta_{1–42}$, TNF-$\alpha$: tumour necrosis factor-$\alpha$, and TGF$\beta$1: transforming growth factor $\beta$1.

levels previously observed in serum (0.5–2.8 pg/mL)—were detected. Concentrations up to 700 pg/mL would have been expected according to one previous study [11]. Differences in sampling and analyzing processes (different ELISA was used in the previous study [11]) might have effect on the results although likely not crucial. The most probable explanation could be that in the previous study [11] the idiopathic cases were not separated from the secondary cases. This indicates that the inflammatory reaction would be associated with secondary NPH rather than idiopathic form.

Our study did not include healthy controls, and our negative result on TNF-$\alpha$ should be reproduced in study were NPH patients with possible known cause of the disease are separated from idiopathic cases and compared with healthy controls. It would also be very interesting to study the possible role of inflammation in the formation of secondary hydrocephalus for example after SAH or trauma. Experimental studies clearly indicate the increased production of TNF-$\alpha$ as well as other proinflammatory cytokines due to accession of blood products to CSF [24].

Notably increased TGF$\beta$1 levels after SAH indicated risk of persistent hydrocephalus [13]. We detected rather low TGF$\beta$1 levels (varied from 20 to 228 pg/mL) in iNPH patients contrary to a previous observation of increased concentrations [14] but supporting other studies with low concentrations [15]. Interestingly TGF$\beta$1 levels correlated with $t$-tau but not with the levels of other studied biomarkers. This is contrary to a previous study in AD patients and controls where TGF$\beta$1 levels correlated with A$\beta_{42}$ but not with tau [12]. This can be explained by different patient population and by rather small number of cases in both studies. The clear correlation between $p$-tau$_{181}$ and total tau is expected since phosphorylated tau is an isoform included into total tau. Interestingly only total tau correlated with age and TGF$\beta$1. The correlation analysis can be justified to indicate obvious associations between different biomarkers but has to be interpreted cautiously because of low statistical power due to rather small number of cases.

Equally increased CSF VEGF levels are detected both in AD and VaD patients also correlating with TGF$\beta$1 levels [25]. Decreased cerebral blood flow associated with chronic hydrocephalus potentially leads to hypoxia, which could induce increased VEGF formation also in iNPH. In an experimental study the increased CSF VEGF levels were seen only in a short-term model of chronic hydrocephalus but not in the long-term model [26]. Therefore low VEGF concentrations detected here are not very surprising.

Based on studies comparing AD patients and healthy subjects it could be expected that iNPH patients with low A$\beta_{42}$ and increased $t$-tau and/or $p$-tau might have concomitant early AD or could be in the risk of developing AD. CSF tau and A$\beta_{42}$ protein levels might help in the detection of the patients who have AD instead of NPH or may have significant risk for concomitant AD. The low A$\beta_{42}$ together with increased tau concentrations ($t$-tau/A$\beta_{42}$ ratio >1.15) seems to indicate increased risk of AD [19]. Thus total tau/A$\beta_{42}$ ratio over 1.15 could be a potential cut-off limit for increased risk of AD [19] and according to that one-third of the current iNPH patients diagnosed by LDT would be in increased risk of future AD. However, increased CSF tau and decreased A$\beta_{42}$ could be detected also in other neurodegenerative disorders than AD [27]. In any case, shunted iNPH patient with still progressing amnestic cognitive impairment and the profile of increased tau and decreased A$\beta_{42}$ should be noted and managed interdisciplinary with a thorough dementia workup.

Specific CSF biomarkers of iNPH indicating shunt response and the possible "point of no return" in the course of the disease would be valuable. Also indicators of the long-term prognosis especially those predicting risk of dementia despite of shunting would be useful. However, this would need a battery of biomarkers and long-term follow-up studies since causes of dementia in these patients are variable from AD to dementia with vascular origin and perhaps dementia due to iNPH itself. Markers indicating possibility of AD are already available but their predictive value of concomitant AD in iNPH patients still needs to be shown in follow-up studies.

Currently there seems to be no CSF biomarker available which could clearly predict the result of lumbar CSF drainage test. It should also be kept in mind that the sensitivity of ELD is not 100% and as also in this series there can be patients who benefit from shunt despite negative test result. Therefore we can not exclude the possibility that some of the markers analyzed here could somehow predict response for shunt treatment in case of negative ELD. In tau and NFL the current results are in line with the previous study where they did not correlate with outcome of shunt [28].

Further research is needed to evaluate the molecular biological basis of idiopathic NPH and to obtain CSF biomarkers for the clinical diagnosis of iNPH. New methods may detect novel proteins to clarify the pathophysiology of iNPH [29]. The CSF, blood, and even brain tissue samples which are possible to obtain without significant additional risk for the patient during diagnosis and treatment of NPH may be useful in differential diagnosis and further research [4]. Also they can be useful for validation of less invasive methods to detect for example A$\beta$ and other possible markers of neurodegeneration and help in the discovery of new surrogate markers. Neurosurgeons are encouraged to collect blood, CSF, and tissue samples for future biobanks with detailed clinical characterisation of patients with careful history taking and use of standardized outcome scales. It is also obligatory to present the results of the analyses separately in different entities of NPH since idiopathic and secondary forms of the disease have different molecular biological background. The secondary forms of NPH should also be separated depending on the specific aetiology.

In conclusion the CSF biomarkers analyzed here could not predict the ELD result. Contrary to the expectations lumbar CSF TNF-$\alpha$ concentrations were low in iNPH patients.

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*Review Article*

# Caloric Intake, Dietary Lifestyles, Macronutrient Composition, and Alzheimer' Disease Dementia

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Alzheimer's disease is a devastating neurodegenerative condition currently affecting over 5 million elderly individuals in the United States. There is much evidence suggesting that certain dietary lifestyles can help to prevent and possibly treat Alzheimer's disease. In this paper, we discuss how certain cardiovascular and diabetic conditions can induce an increased susceptibility for Alzheimer's disease and the mechanisms through which this occurs. We further discuss how the consumption of certain foods or food components can help to reduce one's risk for Alzheimer's disease and may possibly be developed as a therapeutic agent.

## 1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by a progressive decline in memory functions, which has become a growing public health concern. This condition of clinical dementia was first described by Alois Alzheimer in 1907, and ever since, the incidence of AD has increased exponentially. There are presently 5 million Americans affected with AD, and the estimated annual health care cost is almost 100 billion dollars. Further, due to the expected increase in the number of individuals 65 years or older, it has been estimated that the total incidence of AD will quadruple by the year 2050 [1].

As there is presently no cure for this devastating condition, there is an urgent need to find a means of preventing, delaying the onset, or reversing the course of AD. Recent research has provided evidence that certain dietary lifestyle choices can help to prevent Alzheimer's disease. This area of research has been quite exciting in light of the fact that delaying the onset of AD by just five years could cut its incidence in half.

In this paper, we first discuss the major pathological features of AD clinical dementia, followed by an examination of research on certain dietary factors that have been found to influence AD. These dietary factors include calorie, fat, and glucose/sugar intake, in addition to the inclusion of foods like fish, certain fruits and vegetables, plant extracts, spices and red wine, or polyphenol-rich foods in one's diet.

AD is pathologically characterized by the depositions of beta-amyloid aggregates in extracellular spaces and cerebral vasculatures as well as intracellular depositions of aggregated tau protein [2]. The "amyloid cascade hypothesis" is a popular model of AD pathogenesis and most of the autosomal dominant familial AD cases are caused by mutations in the amyloid precursor protein (APP), presenilin 1, or presenilin 2, which lead to increased generation of beta-amyloid (A$\beta$) peptides. However, the majority of the AD cases are sporadic. Cause(s) of sporadic AD are largely unknown. Multiple risk factors may contribute to the onset of sporadic AD. These risk factors include genetic risk factor such as the apolipoprotein E (APOE) genotype, as well as nongenetic and environmentally modifiable factors, including dietary, cardiovascular risk factors, physical and intellectual activities, and environment factors. As such, these factors have been generally considered as potential targets of intervention for AD prevention and therapy.

*1.1. Alzheimer 's Disease Neuropathology.* AD is characterized in the brain by accumulated extracellular $\beta$-amyloid (A$\beta$) plaques and intracellular neurofibrillary tangles composed of

abnormally hyperphosphorylated microtubular tau proteins. The manifestation of AD clinically is a progressive loss of cognitive abilities, including deficits in memory and planning. Neurotoxic A$\beta$ is known to exist in multiple assembly states, which often result in varying pathophysiological effects. Additionally, although A$\beta$ is classically understood to be deposited extracellularly, there is new evidence in mice and humans that A$\beta$ peptides can also accumulate intraneuronally [3]. A$\beta$ species are generated from the ubiquitously expressed amyloid precursor protein (APP) through sequential proteolysis by $\beta$- and $\gamma$-secretases [4–6]. Although the 40-amino acid form of A$\beta$ (A$\beta_{1–40}$) is considered to be the major secreted species in AD, the 42-amino acid form of A$\beta$ (A$\beta_{1–42}$), which contains two additional residues at its carboxyl terminus, is thought to initiate AD pathogenesis [7]. In addition, tau proteins in the brain (most particularly hyperphosphorylated tau), which aggregate into paired helical filaments and deposit as intracellular neurofibrillary tangles, [8, 9], are also considered to be a major pathology associated with Alzheimer's disease. Researchers posit that abnormal hyperphosphorylation of tau leads to the sequestration of normal and hyperphosphorylated tau in microtubules, leading to alternations in the healthy functioning of tau in the brain (including changes in axon transport and microtubule stability and polymerization [10, 11]). As A$\beta$ species and tau neurofibrillary tangles are the major hallmarks of AD neuropathology, research on therapies or preventions for AD are often geared toward attenuating or treating these neuropathologies.

In addition to A$\beta$ and tau pathologies, mitochondrial functions also play a major role in AD clinical dementia [12]. Mitochondria regulate energy metabolism in cells and contribute largely to cell life or death (apoptosis). In the presence of increased A$\beta$ content in the brain, mitochondria increase the generation of reactive oxygen species (ROS), which function as damaging agents and as signaling molecules. Highly reactive ROS, in fact, unleash a mechanism involving the liberation of cytochrome c, leading to neuronal apoptosis [13, 14]. In human AD patients, positron emission tomography (PET) imaging assessments have suggested that the AD brain is characterized by impaired mitochondrial glucose metabolism, leading to neuronal hyperglycemic conditions [15]. In light of this evidence, controlling mitochondrial glucose/energy metabolism in the brain has also been of high interest to AD researchers for the prevention and treatment of AD.

## 2. Obesity and the Metabolic Syndrome in AD

High-fat diets and sedentary lifestyles have become major concerns throughout the world. They have led to a growing incidence of obesity, dyslipidemia, high blood pressure, and hyperglycemic conditions, known collectively to be components of metabolic syndrome [16]. These health conditions are well known to develop along with, or be precursors of, atherosclerosis, cardiovascular disease, and diabetes. Recent studies have found that most of these disorders can also be linked to an increased risk of AD. Of note, accumulating evidence suggests a mechanistic link between cholesterol metabolism in the brain and the formation of amyloid plaques in AD development [17, 18].

Epidemiological studies have demonstrated that individuals with obesity and diabetes have a fourfold increased risk for AD. Health risks associated with obesity, including evidence that obesity may causally promote the AD degenerative process, are of high concern for public health. By the beginning of the twenty-first century, the fraction of Americans considered to be obese reached "epidemic" levels, according to a study published in [19]. This study, carried out between 1991 and 1998, observed a steady increase in weight in all 50 states, across genders, age groups, races, and educational levels, and occurring regardless of smoking status. This study found that obesity had increased from 12.0% in 1991 to 17.9% in 1998. Likewise, national survey data has shown that between 1976–1980 and 1988–1994, the age-adjusted prevalence of obesity increased by 8 percentage points, from 14.5% to 22.5%, in the US adult population ages 20–74 years [20].

Several major studies have been conducted in humans to explore the relationship between obesity and the brain. Recently, Pannacciulli and colleagues [21] explored the association between body fat and regional alterations in brain structure using voxel-based morphometry (VBM) imaging (based on high-definition 3D magnetic resonance imaging [MRI]). Compared to lean subjects, obese individuals were found to have significantly lower gray matter density in the postcentral gyrus, frontal operculum, putamen, and middle frontal gyrus, indicating differences in the brain regulation of taste, reward and behavioral control. Additionally, Whitmer and colleagues [22] evaluated the possible association between obesity (as measured by body mass index (BMI) and skinfold thickness) in middle age and risk of dementia in later life in a large scale, multiethnic population-based cohort. Findings revealed that obese individuals (BMI > 30) in middle age had a 35% higher risk for dementia compared to normal weight individuals (18.6 < BMI < 24.9), independent of other comorbid conditions. Additionally, Balakrishnan and colleagues [23] investigated the association between blood plasma A$\beta$ levels (which promote AD development), BMI, and fat mass (FM) in healthy adults and found significant correlations of BMI and FM with plasma A$\beta_{1–42}$ levels and also noted that the presence of certain proteins known to play a role in inflammation, cardiovascular disease, and type 2 diabetes strengthened these correlations.

Researchers have also investigated the role of leptin, a protein hormone secreted in fat cells associated with obesity (which regulates appetite and metabolism), in AD pathogenesis. In pathological conditions of aging, such as in AD, it has been demonstrated that the transport of leptin across the blood brain barrier (BBB) is significantly impaired, in particular, by the downregulation of megalin, a protein to which leptin must bind in order to enter the brain [24]. Leptin has also been shown to reduce $\beta$-secretase activity in neuronal cells, possibly by altering the lipid composition of membrane rafts, and thereby affecting A$\beta$ generation. In fact, chronic administration of leptin actually reduced A$\beta$ load in the brains of AD transgenic mice, suggesting the potential

of leptin as a treatment for AD [25] and providing further support for the hypothesized link between obesity and AD.

## 3. Calorie Intake and Caloric Restriction

Research has demonstrated that caloric intake (among other nongenetic factors) influences one's risk for AD, and, accordingly, that curbing obesity/calorie intake might play an important role in delaying the AD degenerative process. Clarifying the mechanisms through which caloric intake may ultimately influence AD neuropathology, and how caloric restriction (CR) may exert anti-$\beta$-amyloidogenic activities, may provide new avenues for designing preventive and/or therapeutic lifestyle strategies for AD and other neurodegenerative conditions.

The hypothesized preventive effects of CR on the development of mild cognitive impairment (MCI) or AD are supported by epidemiological evidence indicating that individuals who habitually consume fewer calories have a reduced incidence of AD [26, 27]. Additionally, studies have demonstrated that CR is one method by which one can mitigate the risk factor presented by elevated plasma homocysteine levels (which increase with age) for AD. Specifically, as high homocysteine levels render neurons to be more vulnerable by impairing DNA repair mechanisms (and thereby promoting cell death), lowering homocysteine levels through CR could potentially help to maintain the brain's neuroprotective abilities and help prevent against AD [28].

Halagappa and colleagues [29] tested the hypothesis that two different dietary energy restriction regimens—40% calorie restriction (CR) and intermittent fasting (IF)—could protect against cognitive decline in a transgenic mouse model of AD and found that both regimens ameliorated age-related cognitive impairments but could not directly link the observed effects to A$\beta$ and tau pathologies. Further, Wu and colleagues [30] investigated the effects of CR for 4 months on different AD phenotypes in another mouse model, finding that CR diets improved cognitive impairments in treated mice based on improved scores on assessments of novel object recognition and contextual fear conditioning memory. Further histological and biochemical analyses revealed that CR actually attenuated ventricle enlargement, caspace-3 activation and astrogliosis and reduced the induction of tau hyperphosphorylation. Importantly, DNA microarray analysis in this study also demonstrated that CR could increase the expression of neurogenesis-related genes and decrease the expression of inflammation-related genes in the hippocampus of the mice, indicating that CR could induce neuroprotective activity in the brain.

In accordance with this line of work, we initiated a series of studies to investigate whether AD pathogenesis can be prevented by reducing calorie intake to levels appropriate for cardiac health. At the beginning of this endeavor, although evidence had supported a possible neuroprotective role of CR in neurodegeneration, there had been no information regarding whether CR could attenuate AD neuropathology until recently.

We explored whether a clinically acceptable weight reduction/CR regimen, based on an approximately 30%-reduced carbohydrate intake, could attenuate AD neuropathology and possibly mitigate preexisting amyloid neuritic pathology (by a reduction in plaque size) resulting in the recovery of amyloid-associated neuritic dystrophy as a function of time in Tg2576 AD-type mice [31]. In this study [32], 3-month old Tg2576 mice, which developed AD-type amyloid neuropathology at 8–10 months of age, were fed with a daily low-carbohydrate (low-carb) diet for 9 months, resulting in 30% lower caloric intake compared to age- and gender-matched control mice fed *ad libitum* (AL) with a standard laboratory rodent diet (dietary content of protein, fat, cholesterol, vitamins, and minerals were identical across both mice groups). We found that the low-carb/CR diet in mice resulted in body weight stabilization, 3-fold lower ependymal fat pad weight, and improved glucose tolerance responses compared to the AL-fed mice at 9 months of age. This finding was consistent with clinical evidence indication that low-carb/CR diets considerably improve abnormal glucose control and obesity [33–35], which are risk factors for diabetes and AD [27, 36]. Moreover, when examined for AD-type neuropathology, we found that the CR intervention almost completely prevented cortical and hippocampal AD-type amyloid plaque (lower $A\beta_{1–40}$ and $A\beta_{1–42}$ concentrations) development relative to the AL-fed group. We next proceeded to explore APP processing and A$\beta$ peptide generation in the CR- and AL-fed mice, and, consistent with our previous evidence, we confirmed decreased levels of $A\beta_{1–40}$ and $A\beta_{1–42}$ in CR-fed compared to AL-fed mice. Further investigation revealed that $\gamma$-secretase activity had no involvement in the anti-amyloidogenic activity observed but that $\alpha$-secretase activity (which promotes the nonamyloidogenic processing of APP) likely played a role in the observed benefits of CR treatment in mice. As $\alpha$-secretase cleavage of APP is known to involve the release of a soluble and neuroprotective form of APP (sAPP$\alpha$), it is possible that CR may not only promote a non-amyloidogenic pathway in the brain, but also may promote brain repair activities as a result of sAPP$\alpha$ neurotrophic function [37].

Current therapeutic approaches to AD are aimed at preventing the generation of amyloidogenic A$\beta$ peptides, and for this reason, $\beta$- and $\gamma$-secretase activities required for the formation of A$\beta$ peptides are central targets in the development of therapeutic agents in AD [38]. However, it has been difficult for scientists to find safe and selective $\beta$- and $\gamma$-secretase inhibitors, as these activities are vital in the processing of other cellular substrates [38]. Our studies collectively revealed that CR dietary intervention benefitted AD mice by promoting $\alpha$-secretase activity, and thereby inhibiting the generation of high molecular weight A$\beta$ peptides.

*3.1. Sirtuins (SIRT1) in CR-Mediated AD Prevention.* Sirtuins are class III histone deacetylases (HDAC) known as silent information regulators, which serve to catalyze deacetylation reactions in an NAD(+)-dependent manner. Often called "longevity" genes, sirtuins regulate important cell functions by deacetylating histone and nonhistone targets. Activation of sirtuins is known to extend lifespan by promoting healthy

aging in a variety of species and by protecting crucial tissues in the body, including those in the heart and brain. In mammalian systems, sirtuin activators protect against axonal degeneration, polyglutamine toxicity, and microglia-mediated A$\beta$ toxicity, suggesting the potential therapeutic value of sirtuin activation in patients with AD [39]. SIRT1 has been found to protect against microglia-dependent A$\beta$ toxicity by inhibiting NF-$\kappa$B signaling in the brain [40].

In 2006, we reported for the first time that promotion of SIRT1-mediated deacetylase activity may be a mechanism through which CR influences AD-type amyloid neuropathology. CR by 30% reduced carbohydrate intake was found to prevent amyloid neuropathology in young-adult Tg2576 mice, which may have been mediated in part through mechanisms involving activation of mammalian SIRT1 [41]. Consistent with this evidence in Tg2576 mice, we confirmed this finding in a new animal model by showing that a similar 30% CR regimen in squirrel monkeys coincided with a significant reduction in A$\beta_{1–40}$ and A$\beta_{1–42}$ peptide content in the brain, which inversely correlated with the elevation of SIRT1 protein concentrations, relative to AL-fed monkeys [42]. In view of the fact that several studies in squirrel monkeys have been successfully used to provide important human physiological and biological information at organism, tissue, cellular, and molecular levels, these studies in squirrel monkeys strongly support our hypothesis that clinically applicable CR regimens in humans might be effective in preventing amyloid neuropathology and possibly MCI and AD.

Collectively, our studies on CR in AD mice and in squirrel monkeys revealed that certain experimental CR dietary regimens may promote, attenuate, or even partially reverse features of AD [32, 41, 42]. In our studies, we found that high caloric intake, in particular of saturated fat, promotes AD-type $\beta$-amyloidosis and that reducing carbohydrate intake may actually prevent it, possibly through SIRT1-mediated response mechanisms.

## 4. The Role of Insulin in AD

Insulin and insulin signaling have been suggested to play a role the pathophysiology of AD [43, 44]. In population-based studies, individuals with type 2 diabetes mellitus are at an increased risk for cognitive impairment, dementia, and neurodegeneration. Mechanisms through which diabetes presents a risk factor include glycemia, insulin resistance, oxidative stress, advanced glycation endproducts, inflammatory cytokines, and microvascular and macrovascular disease [45]. The principal defect in type 2 diabetes is insulin resistance, leading to insulin deficiency. The islet of Langerhans (in the pancreas) in type 2 diabetes is characterized by $\beta$-cell loss and islet amyloid derived from islet amyloid polypeptide (IAPP) [46–48], a protein co-expressed and se-creted with insulin by $\beta$-cells. As with A$\beta$ peptides, IAPP spontaneously forms into amyloid aggregates in an aqueous environment [49]. Additionally, as with AD, the incidence of type 2 diabetes strongly increases with age. Borderline diabetes is also associated with increased risks of dementia and AD, independent of whether one develops diabetes in later life, and may interact with severe systolic hypertension to multiply one's risk for Alzheimer's disease [50]. These findings implicate a close biological relationship between type 2 diabetes and AD.

In addition to complications affecting the eyes, kidneys, heart, blood vessels and nerves, diabetes mellitus is associated with damage to the central nervous system (CNS) and cognitive deficits [51, 52]. Impairments in learning and memory have been documented in both types 1 and 2 diabetes. CNS deficits range from moderate to severe, depending on the quality of glycemic control, and involve mainly verbal memory and complex information processing [53–55]. Furthermore, it has been shown that insulin affects several brain functions including cognition and memory, and several studies have established links between insulin resistance, diabetes mellitus and AD [56]. Recent evidence indicates that insulin regulates the metabolism of A$\beta$ and tau proteins [57–59]. It has also been suggested that desensitization of neuronal insulin receptors and certain signaling events in AD could lead to reduced acetylcholine levels and cerebral blood flow, resulting in chronic and increasing deficits in oxidative metabolism [60]. Additionally, insulin is known to facilitate the hepatic clearance of plasma A$\beta_{1–40}$ by intracellular translocation of low-density lipoprotein receptor-related protein 1 (LRP-1) to the plasma membrane in hepatocytes [61].

Alzheimer's disease (AD) is associated with major impairments in insulin and insulin-like growth factor (IGF) gene expression and signaling in the brain, which increase with severity of dementia and deficits in energy metabolism and acetylchoine homeostasis. This coexistence of insulin/IGF deficiency and resistance in the brain suggests that AD may represent a brain-specific form of diabetes (i.e., type 3 diabetes). This hypothesis is supported by findings from de la Monte and colleagues [62] in an experimental animal model in which intracerebral streptozotocin (ic-STZ) was used to deplete brain, and not pancreatic, insulin. The ic-STZ treatment produced brain-specific insulin depletion and insulin resistance and was associated with progressive neurodegeneration sharing many features in common with AD. They demonstrated that early treatment with peroxisome-proliferator activated receptor agonists can effectively prevent ic-STZ-induced neurodegeneration and its associated deficits in learning and memory and that the observed effects were mediated by increased binding to insulin receptors, reduced levels of oxidative stress and tau phosphorylation, and increased choline acetyltransferase expression in the brain, suggesting potential therapeutic efficacy of insulin sensitizing agents in AD.

*4.1. Diabetogenic Diets and AD Amyloid Pathology.* There is *in vitro* evidence that insulin itself may significantly promote the generation of extracellular amyloidogenic A$\beta$ peptides through mechanisms that involve accelerated APP/A$\beta$ trafficking from the trans-Golgi network (a major cellular site for A$\beta$ generation) to the plasma membrane [63]. While this evidence tentatively suggests that abnormal carbohydrate metabolism might play an important role in AD through mechanisms that involve A$\beta$ peptide generation, experimental studies also suggest that insulin resistance may promote AD amyloid neuropathology in Tg2576 mice, possibly by

limiting A$\beta$ degradation via competition with insulin for degradation by the insulin-degrading enzyme (IDE) [64], a zincmetallopeptidase that preferentially cleaves proteins with a propensity to form $\beta$-pleated sheet-rich amyloid fibrils, such as monomeric A$\beta$ peptides [64].

Recent evidence suggests a role for insulin even in normal memory function, thereby supporting the hypothesis that insulin by itself affects mechanisms related to neuronal activity and cognitive function. Of particular interest to our research group, chronic hyperinsulinemia and insulin resistance, or reduced insulin effectiveness, has been demonstrated to negatively influence memory [65, 66]. For example, Hoyer [60] proposed that low concentrations in circulating insulin in the CNS, along with reduced expression of insulin receptors and subsequent altered downstream signaling in AD, would ultimately lead to reduced levels of acetylcholine and a corresponding decrease in cerebral blood flow.

Based on this evidence, and the fact that type 2 diabetes appears to be associated with an increased relative risk for AD [60, 63, 65–67], we recently explored in our laboratory the role of experimental type 2 diabetes in a Tg2576 mouse model of AD amyloid neuropathology. We found that a diabetogenic diet, resulting in elevated circulating levels of insulin, promoted amyloidogenic A$\beta_{1-40}$ and A$\beta_{1-42}$ peptide generation and amyloid plaque burden in the brain of Tg2576 mice. This also corresponded with increased $\gamma$-secretase activities and decreased IDE activities. Moreover, the increased AD-type amyloid neuropathology also coincided with increased impairments in spatial memory function [68]. Further exploration of this interrelationship between insulin resistance and brain amyloidosis revealed a functional decrease in insulin receptor- (IR-) mediated signal transduction in the brain, as suggested by decreased IR $\beta$-subunit (IR-$\beta$ $Y^{1162/1163}$) autophosphorylation and reduced phosphatidylinositol 3 (Pl3)-kinase/$pS^{473}$-AKT/protein kinase- (PK-) B in these same brain samples [68]. This study collectively suggests that diet-induced insulin resistance in AD mice may significantly promote AD-type amyloidosis in the brain through mechanisms involving the elevation of $\gamma$-secretase activity as a result of impaired IR signaling and also that type 2 diabetes may contribute to AD amyloid pathology by attenuating the degradation of A$\beta$ peptide pathways associated with IDEs.

Interestingly, a later study by Li and colleagues [69] explored whether AD-type pathological changes in the brain occur in two experimental rat models which develop type 1 and type 2 diabetes. They found accumulations of $\beta$-amyloid and phosphor-tau in these mice and that these pathologies were associated with neurite degeneration and neuronal loss. Changes in the rat model of type 2 diabetes were more severe and appeared to be associated with insulin resistance and possibly hypercholesterolemia. Additionally, Huang and colleages [70] found that compared to normal mice, a mouse model of hyperglycemia was more vulnerable to $\beta$-amyloid oxidative stress. These findings further support the role of insulin and insulin resistance in AD neuropathology and provide evidence that preventing diabetic conditions may in turn help to prevent AD dementia.

## 5. Hypertension and AD

Several studies have demonstrated an association between high blood pressure and AD [71–73]. It has been suggested that hypertension can increase one's risk for AD by potentially causing cerebrovascular disease or changes in blood vessel walls (which could lead to hypoperfusion, ischemia, and hypoxia), among other conditions, which can potentially initiate the pathological degenerative AD process. Additionally, subclinical AD has also been suggested as a risk factor for high blood pressure; thus, similar biological mechanisms may be involved in the pathogenesis of these two conditions [74].

The relationship between cognitive function and antihypertensive drug therapy has been investigated in several studies of hypertensive elderly human patients. For example, Guo and colleagues [75] found that a combination of certain calcium channel and $\beta$-adrenergic blockers used as antihypertensive agents protected elderly individuals from developing AD. Similarly, other studies have demonstrated that certain calcium channel blockers, such as dihydropyricine [76] and nitrendipine [77], decreased the incidence of AD in hypertensive individuals. Further, it has also been found that antihypertensive drugs that are $K^+$-sparing diuretics, in particular, reduce the risk of AD in elderly individuals with hypertension [78]. Moreover, it was demonstrated that antihypertensive agents that cross the BBB and affect the renninangiotensin-aldosterone system (including perindopril or losartan), or brain calcium metabolism (like nitrendipine), provide additional protection against cognitive decline in addition to blood pressure control. All of these studies suggest a neuroprotective effect of certain antihypertensive agents.

In light of these findings, animal studies in our laboratory have shown that the application of certain antihypertensive drugs can improve cognition in animal models of AD. We conducted a high-throughput drug screening of 55 commercially available antihypertensive drugs and found 7 candidate agents that significantly reduced AD-type A$\beta$ accumulation in the brains of Tg2576 mice. Of these 7 drugs, we found that valsartan, an angiotensin receptor blocker, attenuated the oligomerization of A$\beta$ into high-molecular-weight (HMW) oligomeric peptides (known to be involved in cognitive deterioration) *in vitro* and reduced the content of soluble HMW oligomeric A$\beta$ in the brain in preventive studies. Additionally, we also found that valsartan, delivered at a dose 2-fold lower than the equivalent clinical dosage used in humans for hypertension, significantly attenuated the development of A$\beta$-mediated cognitive deterioration [79]. Another *in vitro* study in this line of investigation revealed that the antihypertensive drugs furosemide, nitrendipine, and candesartan cilexetil prevented A$\beta_{1-40}$ and A$\beta_{1-42}$ oligomerization and that furosemide in particular dissociated preaggregated A$\beta_{1-42}$ oligomers; follow-up studies revealed that short-term treatment with furosemide in Tg2576 mice resulted in reduced A$\beta$ content in the brain [80]. Most recently, we have investigated the potential beneficial effects of carvedilol, a nonselective $\alpha/\beta$-adrenergic receptor blocker used to treat hypertension, on AD pathogenesis and treatment in two AD

mouse models. We found that chronic oral treatment with carvedilol significantly attenuated brain contents of oligomeric A$\beta$ and cognitive deterioration in two mouse models of AD (the Tg2576 model of $\beta$-amyloidosis and TgCRND8 model of tauopathy), which coincided with improvements in neuronal transmission and the maintenance of less stable "learning" thin dendritic spines (associated with learning and memory functions) in the brains of these AD mice [81]. A related study in our laboratory investigated the benefits of carvedilol in AD on an electrophysiological parameter of learning and memory, long-term potentiation (LTP), as assessed in the TgCRND8 mouse model [82]. In this *ex vivo* study, hippocampal slices from carvedilol-treated TgCRND8 mice chronically treated with carvedilol showed improved basal neurotransmission and improved LTP, relative to slices from nontreated TgCRND8 mice, indicating that carvedilol improves neuroplasticity in this mouse model of AD.

Collectively, these studies suggest a clear link between hypertensive conditions and AD and, importantly, that antihypertensive agents may benefit AD. In turn, these studies also indicate that taking dietary precautions to prevent hypertension may, in turn, reduce one's risk for AD.

## 6. The Link between Dietary Choices and AD

A large area of research in the field of neurodegeneration has been focused on the role of specific foods and food components in the neurodegenerative process. Luchsinger et al. [65, 66] posited that taking nutritional supplements alone (e.g., carotenoids versus carrots) might not be as effective as whole foods in providing nutrients, perhaps because the *interaction* of nutrients within whole foods or certain dietary patterns might contribute largely to any food's benefit. As an example, one study [83] demonstrated that plant-based low-fat diets might be superior to low-fat diets containing little plant-based food intake (e.g., lower consumption of fruits, vegetables, nuts, etc.), even if the two diets have identical contents of fat, protein, carbohydrates, and cholesterol. The authors further noted that the beneficial effect of low-density lipoprotein (LDL) cholesterol in one's diet should not be underestimated.

One food of high interest in the AD prevention field has been fish. For example, researchers have investigated the benefits of certain omega-3 fatty acids found in fish and fish oils, specifically docosohexaenoic acid (DHA) and eicosapentaenoic acid, which have been shown to affect psychiatric and behavioral symptoms in AD, as demonstrated in animal studies and in human epidemiological studies [84, 85]. In this line of research, Lim and colleagues [86] demonstrated that DHA-enriched diets significantly reduced AD-type amyloid neuropathology by approximately 70%, including a decrease in A$\beta_{1-42}$ levels, compared to low-DHA or control diets, in a mouse model of AD. Moreover, Hashimoto and colleagues [87] studied the effects of DHA on AD-type pathology following 12 weeks of DHA administration and found that DHA treatment led to a decreased number of working memory errors in A$\beta$-infused rats in addition to an increase in corticohippocampal DHA levels and in the molar ratio of DHA/arachidonic acid, suggesting that DHA treatment attenuated impaired spatial cognition and learning abilities. They further demonstrated that DHA suppressed increases in levels of lipid peroxide and reactive oxygen species in the cerebral cortex and hippocampus of these A$\beta$-infused rats, which suggested that DHA may also increase antioxidative defenses. These findings collectively demonstrated DHA's potential as a therapeutic agent in AD.

Another area of interest to researchers has been the benefits of certain plant extracts and spices in AD. In traditional Asian medicine, various leaves, fruits, barks, roots, and so forth have been used as agents to improve memory functions. In Ayurvedic medicine (a traditional system of Indian medicine), for example, *Bacopa monnieri, Centella asiatica, Withania somnifera, Glycrrhiza glabra, Acorus calamus,* and *Emblica officinalis* have been considered to enhance one's memory. Based on this notion, various laboratories have tested some of these memory-enhancing compounds in mouse models of AD. Mulberry leaf, for example, has been shown to inhibit A$\beta_{1-42}$ fibril formation and protect hippocampal neurons from A$\beta_{1-42}$-induced cell death in a concentration-dependent manner [88]. Additionally, in a screening of 27 herbs for their ability to protect A$\beta_{1-42}$-induced neuronal death, *Curcuma aromatia* and *Zingiber officinale* (ginger) extracts were found to most effectively protect neurons. Several other herbs were also found to be neuroprotective (such as *Ginkgo biloba* (Ginkgo), *Polygonatum* sp. (King Solomon's seal), *Cinnamum cassia* (Chinese cinnamon), and *Rheum coreanum* (Korean rhubarb)), but did not exert as potent effects [89].

Ginkgo biloba extract in particular has been heavily investigated for its use as a preventive and therapeutic agent in AD. It has been shown to exhibit neuroprotective effects in several mouse models [90] and improve cognitive function in AD patients [91, 92]. Several studies have demonstrated the mechanisms by which Ginkgo biloba extract may benefit AD. For example, it has been shown to improve age-related memory deficits and A$\beta$-peptide burden, act as a nitric oxide scavenger [93, 94], and regulate APP metabolism toward the $\alpha$-secretase pathway [95]. Ginkgo biloba extract has also been shown to inhibit A$\beta$-induced free radical generation in a dose-dependent manner [96]. Further, Yao and colleagues [97] examined a specific Ginkgo biloba extract EGb761 in relation to cholesterol and amyloidogenesis and found that EGb761 treatment reduced APP and A$\beta$ generation coincidental with decreased levels of free circulating cholesterol in *in vivo* (in rats) and *in vitro* studies. Moreover, Lee and colleagues [98] investigated ginkgolides A and B for their effect on A$\beta$-modulated acetylcholine release from hippocampal brain slices and found that ginkgolide B may produce antiamnestic effects by mitigating A$\beta$ peptides' inhibitory effect on cholinergic transmission. These studies have provided evidence supporting further investigation of Ginkgo biloba extract in AD.

Another plant extract, curcumin, a polyphenolic yellow pigment in the turmeric spice used in Indian curries and in Indian herbal medicine, has been investigated for its potential use in AD prevention and therapy. Epidemiological studies demonstrated that the prevalence of AD in individuals 70–79 years of age is 4.4-fold less in the India compared

to the U.S. [99]. The curcumin compound (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6 heptadiene-2,5 dione) has been shown to be neuroprotective against A$\beta$ toxicity *in vitro* [100], antiamyloidogenic [101, 102], and capable of reducing brain amyloid load and plaque burden [103]. Spectrophotometric studies have suggested that curcumin binds to the more readily redox-reactive metals Cu and Fe, but does not bind to Zn, and, in turn, acts as an antioxidant by chelating the redox active metal ions in the body [104]. Lim and colleagues [105] found that dietary curcumin treatment in AD mice significantly lowered levels of oxidized proteins, interleukin-1 $\beta$ (a proinflammatory cytokine elevated in these mice), and insoluble and soluble A$\beta$ in the brain and reduced amyloid plaque burden by 43%–50%.

Several other spices have been investigated for their role in AD. For example, aged garlic extract has been shown *in vitro* to suppress the generation of reactive oxygen species, which are known to be involved in apoptosis as a result of A$\beta$-mediated neurotoxicity [106], suggesting that garlic compounds may enhance antioxidant defenses in the brain. Additional *in vitro* evidence demonstrated that garlic treatment inhibits caspase-3 in a dose-dependent manner, which indicates that garlic may inhibit apoptotic neuronal death in the brain [107]. Another spice of interest to researchers as been *Crocus sativus*, or saffron, due to its unusually polar carotenoid components. Notable, certain saffron extracts have been shown to inhibit A$\beta$ fibrillogenesis [108]. Further research on these extracts may illuminate precise mechanisms of action on AD neuropathology and their potential as preventive or therapeutic agents in AD.

*6.1. Fruit Juices and Wine.* Polyphenols, the most abundant dietary antioxidants, have been heavily investigated for their ability to provide neuroprotection against oxidative damage in the brain. One study that propagated research on polyphenols in AD, conducted by Dai and colleagues [109], revealed that long-term fruit juice consumption can reduce one's risk for AD. The investigators suggested that the neuroprotective effects of fruit juices can be enhanced by consuming a combination of juices that are rich in phenolic compounds, which include juices derived from purple grapes, grapefruit, cranberries, and apples.

Several studies have examined the effects of certain fruit juices and extracts on AD. For example, apple juice was shown to prevent A$\beta$-induced oxidative damage *in vitro* [110], and blueberry treatment has been found to reverse the effects of aging on motor behavior and neuronal signaling in animal models [111], possibly through mechanisms involving signal transduction, neuronal communication, and enhancement of hippocampal plasticity [112, 113]. Moreover, treatment with antioxidant-rich pomegranate juice has been shown to reduce $A\beta_{1-42}$ content and amyloid deposition in the hippocampus by approximately 50% in mice. A study conducted by Mullen and colleagues [114] examined 13 different fruit juices and reported that purple grape juice contained the highest number of individual phenolic compounds in addition to the highest concentration of total phenolics. The main components found in purple grape juice, accounting for 93% of the total phenolic conent, were flavan-3-ols, anthocyanins, and hydroxycinnamates. White grape juice, on the contrary, containing mainly hydroxycinnamates, had the lowest phenolic content of the juices examined.

Resveratrol is a naturally occurring polyphenol, found in the skin of grapes and red wine as a result of exposure to fungi or bacteria, which has been investigated for its ability to neuroprotect. Resveratrol has been demonstrated to maintain cell viability, exert antioxidant activity, exert proteasome-dependent antiamyloidogenic activity, and attenuate A$\beta$-induced cytotoxicity in PC12 cells *in vitro* [115–117]. Importantly, resveratrol is also understood to activate the expression of sirtuins, often referred to as the "longevity gene," in yeast [118] and in mammalian animal models of neurodegeneration [119, 120]—this resveratrol-induced sirtuin activation has been shown to promote neuroprotective activities against neuronal apoptosis. However, given recent evidence suggesting that resveratrol may not directly activate sirtuins [121], it is not quite clear if sirtuin activation plays a role in resveratrol's observed benefits in AD-type neuropathology. Further research will certainly illuminate resveratrol's bioactivity and the mechanisms through which it benefits AD.

Several studies have suggested that moderate red wine consumption reduces the incidence of AD clinical dementia [65, 66, 115, 122–124] and may even benefit the course of AD [125]. Derived from red grapes, red wine is rich in antioxidants and holds neuroprotective properties. Studies in our laboratory, using an AD mouse model, examined whether moderate consumption of the red wine cabernet sauvignon (the most polyphenol-rich red wine, likely due to increased inclusion of grape skins) reduces AD-type neuropathology and cognitive deterioration. We found that cabernet sauvignon treatment was capable of attenuating AD-type cognitive deterioration and A$\beta$ neuropathology by mechanisms involving nonamyloidogenic processing of APP, ultimately inhibiting A$\beta$ generation [126, 127].

*6.2. Grape Seed Polyphenolic Extract.* Another area of investigation of high interest in the AD field has been the potential beneficial role of grape seed polyphenolic extract (GSPE) in attenuating AD-type neuropathology and cognitive impairments. Studies in our laboratory have investigated a specific GSPE (MegaNatural), which is comprised primarily of catechin and epicatechin in monomeric, oligomeric, and polymeric forms, is readily absorbed through the intestinal mucosua due to modification of the constituent polyphenols in its preparation and has been demonstrated to be safe in animal models [128–131] and in humans with pre-hypertensive conditions [132]. In an initial investigation in a mouse model of AD [129], mice were treated for 5 months with 200 mg/kg/day GSPE in drinking water (equivalent to 1 g/day in humans, according to Food and Drug Administration criteria for converting drug dosages across species), after which *in vitro* and *in vitro* assessments were conducted at 6 (for behavior) and 10 (for neuropathology) months. *In vitro* studies revealed that GSPE prevented A$\beta$ peptides from aggregating into high molecular weight (HMW) oligomers, and *in vivo* studies showed that GSPE treatment significantly

reduced $A\beta_{1–40}$ and $A\beta_{1–42}$ peptide and HMW $A\beta$ oligomer levels and amyloid plaque burden in the brain, relative to age- and gender-matched water-treated mice. Moreover, GSPE-treated mice also performed significantly better tests of cognitive function compared to age- and gender-matched water treated mice. A follow-up mechanistic study [130] investigated *in vitro* GSPE's ability to alter the assembly of $A\beta_{1–40}$ and $A\beta_{1–42}$ oligomers and $A\beta$-induced cytotoxicity in $A\beta$-treated PC12 cells; these studies revealed that GSPE blocked $A\beta$ protofibril formation, preprotofibrillar oligomerization, and the structure transition from initial coil to $\alpha$-helix/$\beta$-sheet. Additionally, GSPE exerted protective activities in assays of $A\beta$-induced cytoxicity (prior to peptide assembly, following assembly, and just prior to peptide addition in cells). These studies collectively suggest a neuroprotective and possibly therapeutic role of GSPE in AD-type $A\beta$ neuropathology and cognitive deterioration.

To follow this line of work, we also investigated *in vitro* the potential beneficial role of GSPE on AD-type tau neuropathology [133], another major hallmark of AD. Using an *in vitro* model system, we found that GSPE treatment significantly inhibited the aggregation of tau peptides into filaments and was also capable of dissociating preformed tau aggregates. This finding suggests that GSPE treatment may attenuate deposits of tau aggregates in the AD brain.

In light of our evidence that GSPE was capable of attenuating $A\beta$ and tau pathology, we next explored GSPE bioavailability [131] to further assess its potential as an AD treatment. We found that acute oral administration of GSPE in Sprague Dawley rats led to detectable contents of catechin, epicatechin and their metabolites in the brain. Following repeated GSPE exposure, we detected accumulations of catechin, epicatechin, and gallic acid and their metabolites in the blood and similarly, catechin and epicatechin and their metabolites in the brain.

These studies, which demonstrate GSPE's ability to attenuate AD-type $A\beta$ pathology *in vivo* and *in vitro* and tau pathology *in vitro*, combined with its demonstrated safety and bioavailability, support the continued development of GSPE as a treatment for $A\beta$- and tau-mediated neurodegeneration and cognitive impairments.

## 7. Conclusions and Future Trends

Collectively, epidemiological and experimental research has demonstrated that dietary choices can play a key role in the prevention of AD and dementia. For example, much of the research described here suggest that preventing and managing conditions such as diabetes, hypertension, obesity, and heart disease may in turn prevent the onset of pathological aging and dementia. Research from our laboratory and others has demonstrated that reducing calorie intake can help prevent AD and similarly, given the demonstrated relationship between diabetogenic conditions and AD, that preventing diabetes (perhaps by limiting one's glucose intake) may also decrease one's risk for AD. Moreover, studies have demonstrated that consuming foods that are rich in polyphenols, such as blueberries or grapes, may also prevent AD and cognitive deterioration, perhaps through their antioxidant and antiamyloidogenic activities. Based on the evidence described, it seems possible that in the near future, we may be able to utilize our knowledge of AD prevention through dietary changes and work toward intensive dietary intervention(s) that might be capable of preventing or treating AD.

However, when reviewing this body of scientific literature, one must understand that this area of research is still in its infancy, and further research must be conducted before making any dietary recommendations to the general public for preventing neurodegenerative conditions of aging. For example, conditions such as AD are chronic and have a long latency period, and conducting clinical trials for dietary interventions under such circumstances, over long enough periods of time and on large enough samples to draw accurate and repeatable conclusions, would be a highly complex endeavor. Moreover, any dietary recommendations made toward this aim must always be incorporated into a general healthy diet. Future research on dietary lifestyles and their role in the prevention or treatment of dementia will certainly elucidate which diets and foods are capable of exerting neuroprotective activities, in what quantities, and by what mechanism(s) of action.

The current medical model for preventing and treating neurodegenerative conditions such as AD lacks a "whole organism" approach. For example, the onset of AD is likely a result of genomic and proteomic factors, but also psychosocial and lifestyle factors, such as nutrient intake or levels of stress. Today, Medicare and other insurers and individuals will pay billions of dollars for various surgical and medical procedures to treat chronic conditions (such as heart disease or diabetes), and yet they pay very little for integrative and preventive medicine approaches (such as alterations in diet) that can prevent or reverse many chronic conditions. By further investigating the role that dietary choices may play in AD and other dementias and diseases of aging, we will work toward the utilization of an integrative approach to medicine, taking into account all aspects of an individual's lifestyle when working toward the maintenance and curing of chronic diseases.

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*Review Article*

# The Apolipoprotein E Antagonistic Pleiotropy Hypothesis: Review and Recommendations

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Research on apolipoprotein E (APOE) has consistently revealed a relationship between the gene's $\varepsilon 4$ allele and risk for development of Alzheimer's disease (AD). However, research with younger populations of $\varepsilon 4$ carriers has suggested that the APOE $\varepsilon 4$ allele may in fact be beneficial in earlier ages and may only confer risk of cognitive decline later in life. Accordingly, we and others have proposed that APOE may represent an example of antagonistic pleiotropy. Antagonistic pleiotropy is an evolutionary biology concept that proposes certain genes or alleles that may differentially impact fitness during different life stages. We critically review this hypothesis in light of new research of the impact of APOE on cognition and neural integrity across the lifespan. We provide recommendations for the revision of the antagonistic pleiotropy hypothesis of APOE and suggest important avenues for future research in this area.

## 1. Introduction

Apolipoprotein E (APOE) is a protein coded by a gene located on chromosome 19 and plays an important role in cholesterol metabolism and synaptogenesis in the brain [1, 2]. The APOE gene has three variants, or alleles: $\varepsilon 2$, $\varepsilon 3$, and $\varepsilon 4$. The $\varepsilon 4$ allele has been consistently associated with a higher risk of developing late-onset or sporadic Alzheimer's disease (AD) and a faster rate of disease progression (e.g., [3]), and as such is perhaps the most frequently studied genetic risk factor for AD. Studies of nondemented older adults utilizing event-related fMRI paradigms have also suggested that $\varepsilon 4$ carriers may more strongly recruit task-related brain regions or may recruit additional brain regions beyond task-related areas in order to compensate for preclinical AD changes to achieve the same performance as noncarriers (e.g., [4]). Preferentially recruited areas beyond task-related regions have frequently been found to include frontal regions with a possible predilection for the right hemisphere [1]. While $\varepsilon 4$ carriers may initially maintain cognitive performance through these compensatory recruitment processes, once AD disease burden increases, compensatory recruitment processes cannot sustain premorbid cognitive performance levels and cognitive decline ensues [1].

Although APOE has been extensively studied in older populations, its impact on cognition in younger ages has been seldom considered. Interestingly, the few studies that have investigated APOE's impact in early life have frequently reported that $\varepsilon 4$ carriers may cognitively outperform noncarriers. For instance, Yu et al. [5] found young $\varepsilon 4$ carriers to have higher IQs than noncarriers, Alexander et al. [6] found $\varepsilon 4$ carriers as young as six to have higher verbal fluency scores than noncarriers, and Wright et al. [7] found $\varepsilon 4$ infants to produce higher scores on the 24-month Mental Development Index of the Bayley Scale.

As we proposed previously in our review on this topic [1], these seemingly contradictory patterns of results may be reconciled through the antagonistic pleiotropy concept. Antagonistic pleiotropy is a concept from evolutionary biology purporting that certain genes may impact fitness (i.e., survival and reproduction) differently during different life stages. Utilizing the concept of antagonistic pleiotropy,

we [1] suggested a model of the impact of APOE on cognition across the lifespan. According to this model, we have the following.

(a) Young ε4 carriers outperform noncarriers on tests of memory and other cognitive domains.

(b) They may also recruit additional right hemisphere frontal regions in order to achieve this advantage.

(c) By middle age, there are slight if any neurocognitive differences between ε4 carriers and noncarriers.

(d) In more advanced ages, ε4 carriers may begin to preferentially recruit right frontal brain regions in order to maintain cognitive performance comparable to noncarriers.

Finally, the model proposes that once AD disease burden sufficiently accrues, ε4 carriers' compensatory recruitment will begin to fail, marking the beginning of cognitive decline.

Much research investigating APOE across the lifespan has been conducted since the antagonistic pleiotropy hypothesis of APOE was proposed. We aim to review our model in light of this most recent research. We provide recommendations for revisions of this model to include recent findings and suggest important areas for future research to evaluate and expand the antagonistic pleiotropy hypothesis of APOE.

We utilized the PubMed database in searching the literature for this review. All searches were limited to those articles published in the last three years because of our intent to focus on research published following our proposal [1] of the antagonistic pleiotropy hypothesis of APOE in 2008. Search terms included "APOE and brain imaging," "APOE and cognition," and "APOE and neuropsychology." The abstracts of articles returned through these searches were examined and relevant articles were gathered. Articles were considered relevant if they examined APOE genotype in relation to cognitive performance or functional imaging in any age group. The reference lists of obtained articles were also searched by hand for additional relevant literature. The articles discussed in this review as well as their sample size, focus, and relevant findings are listed in Table 1.

## 2. APOE and Cognition in Early Life

Fortunately, research on the impact of APOE in early life has become more prevalent in recent years. However, this work has yielded inconsistent conclusions regarding the impact of APOE on cognition early in the lifespan. Consistent with the model of antagonistic pleiotropy, some studies have reported an ε4 advantage in early life. Bloss et al. [8] found that school-age children with the ε4 APOE allele performed better than children with the ε2 allele on the Rey Complex Figure Test (RCFT) copy trial. Children with the ε2 allele were also more likely than carriers of other alleles to be left-handed, a possible sign of abnormal neural development. Because of ε4 carriers' apparent benefits and ε2 carriers' deficits in this young sample, a reversal of typical findings in older age groups, the authors suggested that these results are consistent with antagonistic pleiotropy. Also in support, Mondadori [46] reported young adult ε4 carriers to outperform noncarriers on the delayed recall portion of an episodic memory task, although no other differences by genotype were found in working memory, visuospatial processing, or executive functioning. Marchant et al. [9] reported that 18 to 30-year-old APOE ε4 carriers showed an advantage over noncarriers on tests of frontal lobe functioning. Noé et al. [10] studied a sample with moderate to severe traumatic brain injury (TBI) and found that while ε4 carriers were more severe at baseline, they also exhibited a steeper rate of improvement in working memory compared to noncarriers. This work is consistent with our model [1], suggesting an advantage in cognition and head injury outcome for young ε4 carriers.

Nevertheless, studies reporting the opposite findings are equally as prevalent. Gozal et al. [47] found that the APOE ε4 allele was associated with cognitive impairment in 5- to 7-year-old children with obstructive sleep apnea. Dennis et al. [11] and Filbey et al. [12] both failed to find cognitive differences between ε4 carriers and noncarriers in their 20's and 30's and Liu et al. [48] failed to find differences by APOE genotype in those younger than age 50. Hiekkanen et al. [13] also reported that APOE genotype was unassociated with one-year outcome of mild TBI in adults aged 18 to 70. Luciano et al. [14], studying a sample of 70-year olds in the Lothian Birth Cohort, failed to find differences by APOE genotype in IQ measured at age 11. It is noteworthy that several studies finding reduced cognition in ε4 carriers only did so when carriers also had other risk factors for AD. For instance, Ruiz et al. [15] reported finding no differences in cognition by APOE status in a sample of adolescents. However, the authors discovered that ε4 carriers who also carried the MTHFR 677TT allele, a gene possibly detrimental to cognition, displayed significantly worse cognitive performance than adolescents with other allelic combinations. Bloss et al. [16] found that school-age children with the APOE ε4 allele as well as a family history of AD performed more poorly on standardized achievement tests of reading and language and the RCFT copy. Importantly, there was no main effect of APOE genotype; children with the ε4 allele were only found to exhibit depressed scores when they also had a family history of AD, suggesting that these two risk factors interact to influence cognition. Acevedo et al. [17] found that 7 to 10-year-old ε4 carriers were more likely to have been placed in an intensive care unit after birth, achieved lower spatial memory retention, and, among girls only, ε4 carriers achieved lower visual recall scores on the Family Pictures Test. The authors conceptualized the latter finding as representing an interaction between the potentially gendered risk of AD, which is more prevalent in women, with the APOE ε4 risk factor. It is important to note that while the authors indicated that participants' birth complications were unlikely to have impacted cognition, the presence of birth complications was not controlled for in analyses. Because ε4 carriers were more likely to experience birth complications, this fact could explain the association between ε4 status and cognition in this sample.

Table 1: Samples, methods, and relevant findings of reviewed articles.

| Article | Sample | Methods used | Relevant findings |
|---|---|---|---|
| Bloss et al. [8] | 147 youth, age 11–16 | Assessment of verbal cognition and visuospatial processing | APOE ε4 carriers performed better than ε2 carriers on a test of visuospatial processing |
| Marchant et al. [9] | 156 college students, age 18–30 | Assessment of spatial working memory, estimated IQ, immediate verbal memory, verbal fluency, sustained attention, and decision-making ability | APOE ε4 carriers showed an advantage over noncarriers on tests of verbal fluency and decision-making |
| Noé et al. [10] | 82 patients currently with post-traumatic amnesia (PTA; mean age = 31.5) and 107 patients without PTA (mean age = 29.5) | Assessment of PTA severity, verbal memory, and working memory | APOE ε4 carriers were more severe at baseline but exhibited a steeper rate of improvement in working memory over time than noncarriers |
| Dennis et al. [11] | 24 young adults (mean age = 21.3) | Functional MRI during the encoding portion of an object memory task followed by a recall session 24 hours later, and an assessment of memory, processing speed, attention, and executive functioning | APOE ε4 carriers and noncarriers performed similarly on all cognitivetests, while ε4 carriers showed more bilateral MTL activity and functional connectivity of MTL and posterior cingulate and perilimbic structures during memory encoding |
| Filbey et al. [12] | 36 healthy adults, age 50–75 and 16 adults, age 19–32 | Functional MRI during a visual working memory task and an assessment of global cognition | APOE ε4 carriers and noncarriers did not differ in cognitive performance, while ε4 carriers showed more medial frontal and MTL activity compared to noncarriers during the working memory task. Older ε4 carriers also showed decreased activation compared to noncarriers in several frontal, parietal, temporal, and cingulate cortices |
| Hiekkanen et al. [13] | 33 mild TBI patients (mean age = 44.2) | Structural MRI, assessment of PTA, and Glasgow Coma Scale ratings over a one year follow-up period | While MRI findings and PTA severity predicted TBI outcome after one year, APOE genotype was unassociated with TBI outcome |
| Luciano et al. [14] | 1,091 participants in the Lothian Birth Cohort | Assessment of IQ at age 11 and measures of global cognition, working memory, nonverbal reasoning, construction, verbal fluency, and processing speed at age 70 | APOE genotype was unrelated to IQ at age 11, yet the ε4 allele was associated with lower processing speed, nonverbal reasoning, and general cognition measured at age 70 |
| Ruiz et al. [15] | 412 participants, age 13–18 | Assessment of verbal and quantitative skills and problem solving ability | APOE genotype was not associated with cognition, but ε4 carriers who also had the MTHFR 677TT allele had lower quantitative and reasoning abilities |
| Bloss et al. [16] | 109 youth, age 11–16 | Assessment of verbal cognition and visuospatial processing | APOE genotype was not associated with cognition, but ε4 carriers who also had a family history of AD had lower verbal and visuospatial abilities |
| Acevedo et al. [17] | 50 youth, age 7–10 | Assessment of general intelligence, memory, attention, executive functioning, and visuospatial processing | APOE ε4 carriers were more likely to have been placed in intensive care after birth and had lower spatial memory abilities, especially among girls |
| Filippini et al. [18] | 36 adults, age 20–35 | Structural MRI, perfusion MRI at rest, and functional MRI at rest and during a memory encoding task | APOE ε4 carriers showed more connectivity among default-mode network regions and more hippocampal activation during the memory task than noncarriers |
| Kukolja et al. [4] | 18 healthy older adults (mean age = 60.5) | Functional MRI during a spatial contextual memory task | APOE ε4 carriers had poorer memory performance than noncarriers. ε4 carriers also more strongly activated prefrontal, temporal, and parietal regions during encoding than did noncarriers but showed less activation in prefrontal cortex during retrieval |
| Trivedi et al. [19] | 155 healthy adults, age 18–84 | Functional MRI during episodic encoding and metacognitive self-appraisal tasks | APOE ε4 carriers showed increasing hippocampal activation with age during the memory task, while noncarriers showed age-related reductions in hippocampal activity |
| Wierenga et al. [20] | 22 healthy older adults (mean age = 78.10) | Functional MRI during an object naming task | APOE genotype was unrelated to naming ability, yet ε4 carriers exhibited greater activity in the left fusiform, right perisylvian cortex, and bilateral medial prefrontal cortex than noncarriers |

Table 1: Continued.

| Article | Sample | Methods used | Relevant findings |
|---|---|---|---|
| Seidenberg et al. [21] | 69 healthy older adults, age 65–85 | Functional MRI during a semantic memory task | Those with an $\varepsilon 4$ allele and family history of AD showed greater activations in bilateral cingulate, temporoparietal, and prefrontal regions than those without risk factors. $\varepsilon 4$ carriers also showed greater recruitment of right middle frontal regions |
| Woodard et al. [22] | 57 older adults, age 65–85 with or without amnestic MCI | Functional MRI during a semantic memory task | Those with an $\varepsilon 4$ allele and family history of AD displayed increased activation in temporoparietal, hippocampal, and posterior cingulate regions than those without risk factors. MCI patients showed similar patterns along with enhanced frontal recruitment |
| Bartrés-Faz et al. [23] | 32 older adults with mild memory impairments (mean age = 66.83) | Functional MRI during a face-name learning task | APOE $\varepsilon 4$ carriers showed increased connectivity of the hippocampus with anterior cingulate, postcentral gyrus, and caudate nucleus during encoding compared to noncarriers |
| Borghesani et al. [24] | 14 healthy older adults | Functional MRI during a visuospatial memory task | APOE $\varepsilon 4$ carriers showed less MTL activation during encoding than noncarriers despite equal performance |
| Xu et al. [25] | 74 healthy adults, age 50–65 | Functional MRI during an episodic face recognition task | APOE $\varepsilon 4$ carriers showed reduced activation in posterior and anterior cingulate and precuneus than noncarriers during recall |
| Suthana et al. [26] | 32 healthy older adults (mean age = 61.1) | Functional MRI during a word memory task | APOE $\varepsilon 4$ carriers displayed reduced hippocampal activation during encoding than noncarriers |
| Welsh-Bohmer et al. [27] | 507 healthy older adults, age 66–103 | Assessment of object naming, verbal fluency, memory, construction, processing speed, and global cognition | APOE genotype was unrelated to all measures of cognition |
| Adamson et al. [28] | 50 healthy pilots, age 50–76 | Structural MRI and assessment of memory | APOE $\varepsilon 4$ carriers performed more poorly on visual paired associate recall than noncarriers but showed no structural brain differences |
| Debette et al. [29] | 717 healthy adult offspring from the Framingham cohort (mean age = 59) | Structural MRI, assessment of memory, abstract reasoning, and mental flexibility, and determination of parental dementia | APOE $\varepsilon 4$ carriers were more likely to have a parent with dementia. Among $\varepsilon 4$ carriers, parental dementia was associated with lower memory performance |
| Honea et al. [30] | 53 healthy older adults age, 60 and older | Structural MRI, diffusion tensor imaging, and an assessment of memory, language, executive functioning, and visuospatial ability | APOE $\varepsilon 4$ carriers performed more poorly on measures of memory and working memory and had smaller hippocampi and parahippocampal FA |
| Caselli et al. [31] | 815 healthy adults age, 21–97 | A longitudinal assessment of long-term memory, global cognition, verbal fluency, and visuospatial abilities | APOE $\varepsilon 4$ carriers were found to experience memory decline in their 50's, while noncarriers did not show decline until their 70's. A dose-dependent effect was found in which $\varepsilon 4$ homozygotes displayed earlier memory decline than heterozygotes. $\varepsilon 4$ carriers showed steeper decline than noncarriers in memory, global cognition, and visuospatial processing |
| De Blasi et al. [32] | 620 healthy older adults, age 65–85 | Assessment of memory and global cognition | While APOE $\varepsilon 4$ was associated with memory encoding and recall, it was unrelated to global cognition |
| Knopman et al. [33] | 1130 adults (mean age = 59) | Assessment of memory, verbal fluency, processing speed, and vascular risk factors | APOE $\varepsilon 4$ carriers exhibited increased decline in processing speed and memory compared to noncarriers |
| Mungas et al. [34] | 369 older adults (mean age = 74.3) | Assessment of object naming, verbal fluency, memory, and working memory | APOE $\varepsilon 4$ carriers exhibited lower episodic memory scores at baseline and increased decline in memory and executive functioning than noncarriers |
| Walhovd et al. [35] | 161 older adults, age 55–90 | Structural MRI, FDG-PET, and an assessment of memory | APOE $\varepsilon 4$ allele was associated with poorer recognition memory beyond imaging variables |

Table 1: Continued.

| Article | Sample | Methods used | Relevant findings |
|---|---|---|---|
| Hayden et al. [36] | 2957 older adults (mean age = 74) | Longitudinal assessment of global cognition and family history of dementia | APOE ε4 carriers had lower baseline global cognitionand steeper decline in cognition than noncarriers |
| Whitehair et al. [37] | 516 amnestic MCI patients, age 55–90 | Longitudinal assessment of global cognition, memory, processing speed, verbal fluency, working memory, naming, and functioning | APOE ε4 carriers had lower baseline scores on nearly every assessment and also showed steeper declines in nearly every domain |
| Yaffe et al. [38] | 2509 healthy older adults, age 70–79 | Longitudinal assessment of global cognition and health variables | APOE ε4 allele was associated with likelihood of cognitive decline |
| Thambisetty et al. [39] | 94 healthy older adults (mean age = 69.2) | Longitudinal structural MRI, PET imaging, and assessment of memory, verbal intelligence, verbal fluency, attention, working memory, and executive functioning | APOE ε4 carriers performed more poorly on category fluency and also had greater decline in regional cerebral blood flow, particularly in areas commonly implicated in AD |
| Raz et al. [40] | 189 healthy adults, age 18–82 | Assessment of fluid intelligence, memory, executive functioning, and processing speed | APOE ε4 carriers evidenced greater age-related interference effects in a Stroop task than noncarriers |
| Barabash et al. [41] | 89 amnestic MCI patients (mean age = 79) and 90 healthy adults (mean age = 76) | Longitudinal assessment of cognitive diagnosis | APOE ε4 allele was associated with higher risk of developing MCI, but not AD |
| Wang et al. [42] | 20 healthy older adults (mean age = 75) and 58 amnestic MCI patients (mean age = 76.6) | Longitudinal structural MRI and an assessment of global cognition, memory, attention, language, construction, and abstract thinking | APOE ε4 allele was unassociated with rate of decline in cognition or brain volumes |
| Heun et al. [43] | 200 healthy older adults (mean age = 80.3) | Longitudinal assessment of cognitive diagnosis, global cognition, memory, construction, attention, and language | APOE ε4 allele was unassociated with likelihood of conversion to MCI |
| Carrión-Baralt et al. [44] | 87 nonagenarians | Assessment of global cognition, memory, naming, verbal fluency, attention, and processing speed | APOE ε4 carriers displayed higher global cognition, attention, visuospatial processing, naming, praxis, and memory encoding than noncarriers |
| Kozauer et al. [45] | 659 adults (mean age = 58.4) | Longitudinal assessment of global cognition and memory | APOE ε4 carriers in the younger cohort, who were in their 50's, showed increased decline in memory and global cognition compared to noncarriers. However, older ε4 carriers in their 70's showed no cognitive differences from noncarriers |

While these studies do not unequivocally support our model of antagonistic pleiotropy and APOE [1], interpretation of the findings of studies in this area is complicated by the nature of the samples studied. When the potential benefit of holding an ε4 allele could be confined to a very narrow window early on in the lifespan, the importance of studying the impact of APOE in specific age ranges and of examining the interaction of age and APOE status in lifespan samples becomes apparent. Moreover, it is likely that APOE status interacts with other genetic and environmental risk factors (e.g., birth complications, MTHFR 677TT allele, family history of AD) to impact cognition [15–17], implying that participants' additional risk factors beyond APOE should also be considered. Taking such measures will greatly clarify the literature in this area and lead to more reliable findings regarding the impact of APOE on cognition in early life.

## 3. APOE and Compensatory Recruitment in Early Life

Although comparatively less research has examined neural activity than cognition early in the lifespan, fMRI research with children has produced results more consistently supporting our model of antagonistic pleiotropy and APOE. Dennis et al. [11] examined activity in medial temporal lobe (MTL) structures important for memory and functional connectivity between MTL regions and other brain regions using event-related fMRI. Young adult participants were asked to encode visually presented stimuli and were given a surprise recall session 24 hours later. Results revealed that ε4 carriers had more bilateral activity in MTL structures during memory encoding and had more functional connectivity of MTL structures with posterior cingulate and perilimbic

regions than noncarriers. However, ε4 carriers also showed a tendency towards decreased connectivity between anterior and posterior cortices. Dennis et al. [11] suggested that findings of increased functional connectivity between MTL regions and other regions known to be affected by AD (e.g., posterior cingulate) may suggest that APOE begins to be expressed in AD-associated brain regions long before cognitive decline and that these findings may also be consistent with antagonistic pleiotropy. Filbey et al. [12, 49] also found that young adult ε4 carriers showed more medial frontal [12, 49], cingulate [49], and MTL [12] activity compared to noncarriers in a working memory task. In a slightly older sample of 20-to-35-year-olds, Filippini et al. [18] reported ε4 carriers to have more default mode network (DMN) connectivity and more hippocampal activation during a memory encoding task than noncarriers. However, at least one study [46] reported that young adult ε4 carriers exhibited *less* neural activity in bilateral MTL and left frontal regions during the encoding and retrieval portions of an episodic memory task than performance-matched noncarriers. The authors attributed these findings to enhanced neural efficiency of memory networks in young adult ε4 carriers [46].

The majority of these studies appear to support the proposed model of antagonistic pleiotropy in that ε4 carriers tend to recruit task-related regions more intensely than do noncarriers. However, the above studies provide less support for the proposal that ε4 carriers will preferentially recruit more right hemisphere frontal regions during task performance. While it is unclear whether the ε4 carriers studied by Dennis et al. [11] also showed higher activations in frontal regions because the authors focused on activity in MTL structures, Filippini et al. [18] used voxel-based morphometry and did not reveal any involvement of frontal regions by APOE status. Additionally, while Filbey et al. [12] indicated that ε4 carriers recruited medial frontal regions more than noncarriers, this may be due to the nature of the task, as working memory tasks are more frontally-mediated than assessments of episodic or semantic memory.

## 4. APOE and Compensatory Recruitment in Old Age

As Trachtenberg et al. [50] note in their thorough review, research examining the blood-oxygen-level-dependent (BOLD) response using fMRI has produced inconsistent findings regarding the impact of APOE that are not easily understood even when considering differences in task, family history of AD, and age. While this observation was also borne out in our review of recent research with older adults, the majority of studies provided evidence of compensatory neural recruitment in older APOE ε4 carriers.

Of studies that found evidence of compensatory recruitment in nondemented older adults with the ε4 allele, many found an increase in activation of task-related regions, regions known to be affected by AD (e.g., posterior cingulate), and frontal regions. For instance, Bookheimer et al. [51] found that nondemented middle-aged and older adult ε4 carriers more strongly activated AD-associated brain regions, such as hippocampal, parietal, and prefrontal regions, than ε3 carriers during a paired-associate word learning task. Furthermore, the authors found that the magnitude and extent of neural activation at baseline was predictive of cognitive decline after two years, as would be expected according to the compensatory recruitment hypothesis [51]. Dickerson et al. [52] also reported findings suggestive of compensatory recruitment in a sample of cognitively intact older adults and patients with MCI or AD. Results revealed that APOE ε4 carriers, regardless of diagnosis, showed enhanced hippocampal activity during encoding in a face-name memory task [52]. Similarly, Bondi et al. [53] reported healthy older adult ε4 carriers display more intense and widespread activations in parietal, frontal, and right MTL regions than noncarriers during encoding in a picture learning task. Notably, ε4 carriers exhibited reduced activations in left hippocampal regions compared to ε3 carriers, supportive of our model's [1] prediction of a predilection for compensatory responses in the right hemisphere [53]. A more recent study by Kukolja et al. [4] found that nondemented older ε4 carriers more strongly activated right hippocampal and predominantly right prefrontal, temporal, and parietal regions during the encoding portion of a spatial context memory task than did noncarriers, as would be expected according to antagonistic pleiotropy. However, the authors also reported that ε4 carriers showed less activity in the prefrontal cortex bilaterally during retrieval. Interestingly, the authors noted that hippocampal activity was inversely associated with items remembered for ε4 carriers but positively associated with recall for noncarriers. The opposite relationship was found in bilateral inferior frontal gyrus, right middle frontal gyrus, and left parietal cortex, the activity of which was positively associated with recall among ε4 carriers. In light of these findings, Kukolja et al. [4] suggested that compensation may occur during encoding among ε4 carriers, coinciding with increased frontal activations during this portion of the task. However, they note that activity decreases during the retrieval portion of the task reveal the likelihood of approaching cognitive decline. Filbey et al. [12] also found nondemented older adult ε4 carriers to more strongly recruit medial frontal and temporal regions during a working memory task, although ε4 carriers showed less activity than noncarriers in several other frontal and temporal regions. Trivedi et al. [19] examined a large sample from age 18 to 84 using an episodic encoding task and found that ε4 carriers exhibited increases in hippocampal activity with age, while noncarriers showed age-related decreases in hippocampal activity. Wierenga et al. [20], studying cognitively intact older ε4 carriers with a confrontation naming task, found that ε4 carriers displayed increased activations compared to noncarriers in the left fusiform gyrus, bilateral medial prefrontal cortex, and right perisylvian cortex despite equal performance across the groups. Seidenberg et al. [21] utilized a semantic memory task designed to require low levels of effort and produce high accuracy rates in a cognitively normal sample of older adults. They found that those with the ε4 allele and a family history of AD preferentially recruited bilateral posterior cingulate/precuneus, bilateral

temporoparietal junction, and bilateral prefrontal cortex than participants without any AD risk factors during recall of familiar versus unfamiliar names. Moreover, in comparing participants with both the ε4 allele and family history of AD to those with just a family history, ε4 carriers exhibited more activation in right middle frontal and right supramarginal gyri. Seidenberg et al.'s [21] results suggest that the APOE ε4 allele was uniquely associated with preferential activation of right hemisphere frontal regions, as we have suggested [1]. Woodard et al. [22] also used a low-effort famous name discrimination task and found that participants with an ε4 allele and family history of AD displayed increased activation compared to those without risk factors in lateral temporoparietal regions, left hippocampus, and posterior cingulate/precuneus. Participants with MCI also showed similar increases in activations, but they did not show enhanced recruitment of the hippocampus and instead showed increased activations in frontal regions, suggesting that compensatory recruitment processes may evolve with changes in cognition and neuropathology burden [22]. Finally, Bartrés-Faz et al. [23] studied hippocampal connectivity in older adults with mild memory impairments. They reported that ε4 carriers exhibited increased connectivity of the hippocampus with the anterior cingulate, inferior parietal/postcentral gyrus, and the caudate nucleus during the encoding portion of a face-name memory task. The authors suggested that this may indicate increased cortical-subcortical connections in the episodic memory networks in ε4 carriers.

Overall, these studies uniformly support the presence of compensatory neural recruitment processes in ε4 carriers. Yet, while some studies support our proposal [1] that ε4 carriers would have a predilection for recruiting right hemisphere regions [4, 20, 21], this was not true for all studies reviewed [12, 19, 22, 23]. This could be a factor of type of task, as Kukolja et al. [4], who found the most evidence for a right hemisphere dominant compensatory response, used a spatial context memory task, which may be expected to preferentially activate right hemisphere regions due to the involvement of visuospatial processing. In contrast, several studies that found a predominantly left hemisphere or bilateral compensatory response (e.g., [12, 20–22]) used tasks (e.g., confrontation naming, working memory, and famous name discrimination) that may be more expected to activate left rather than right hemisphere regions due to the emphasis on language.

There were also studies reviewed which did not support the existence of a compensatory recruitment process in older cognitively intact ε4 carriers. Johnson et al. [54] found that APOE genotype alone did not impact neural activity during a self-appraisal task in middle-age adults, although APOE status did interact with a family history of AD to produce greater frontal and posterior cingulate activations in ε4 carriers without a family history of AD. Johnson et al. [55] reported similar results in an earlier study of middle-age adults using an episodic memory task. While APOE ε4 carriers with no family history of AD did show the predicted enhanced recruitment of MTL structures, ε4 carriers with a family history of AD actually showed the lowest levels of MTL recruitment of all participant groups [55]. As noted by the authors, [54, 55], such results suggest that the impact of APOE on brain activity may be moderated by genetic factors underlying a familial history of AD. Lind et al. [56] found that healthy middle aged and older adult ε4 carriers displayed reduced neural activity in left inferior parietal and anterior cingulate cortices than noncarriers during a semantic categorization task. Borghesani et al. [24], studying MTL activation in nondemented older adults with a visuospatial memory task, also found that ε4 carriers exhibited lower MTL activation during encoding. Filbey et al. [12], while simultaneously finding regions more activated in ε4 carriers (as discussed above), also found that ε4 carriers exhibited less activity than noncarriers in lateral frontal, basal ganglia, cingulate, parietal, and temporal regions during a working memory task. Reporting similar findings, Xu et al. [25] found that middle aged ε4 carriers showed reduced activation in left dorsal posterior cingulate, precuneus, and anterior cingulate than noncarriers during the recall portion of an episodic face recognition task. A study by Suthana et al. [26] reported reduced hippocampal activation in nondemented older ε4 carriers compared to noncarriers during the encoding portion of a word memory task. The authors proposed that this decrease in hippocampal activation may in fact allow for compensatory processes in other regions, which would explain findings of decreased activation in MTL regions in ε4 carriers and may also explain Kukolja et al.'s [4] finding of reduced memory retrieval with increasing hippocampal activation among those with the ε4 allele.

While many of these studies support the compensatory recruitment aspect of our model [1] of antagonistic pleiotropy and APOE, some studies did not find evidence of compensatory recruitment processes in ε4 carriers, instead finding lower levels of activation in ε4 carriers than noncarriers. A notable trend is that those studies finding decreased levels of activity in ε4 carriers frequently did so in regions commonly associated with AD (e.g., posterior cingulate, precuneus, and hippocampus), while studies finding increased activity in ε4 carriers commonly did so in regions beyond those typically involved in early AD (e.g., prefrontal cortex). As several authors have pointed out (e.g., [12]), ε4 carriers may exhibit decrements in activity in regions initially impacted in AD due to increased pathology burden in these areas while continuing to compensate for these decrements with increased activity in regions not initially affected by AD pathological processes. Another possible explanation for discrepancies among studies could be due to differences in vascular risk factors present in the samples. Age-related vascular changes have been found to attenuate neurovascular coupling upon which the BOLD response in fMRI is based (e.g., [57, 58]). Such alterations can complicate the interpretation of the BOLD response and lead to erroneous conclusions if vascular changes are not taken into account [57]. This is particularly relevant as the APOE ε4 allele has been associated with vascular risk factors (e.g., [59, 60]). Therefore, careful consideration of vascular changes and clinically silent or prodromal forms of dementia

are required to clarify the existence of compensatory neural recruitment in older adult ε4 carriers.

## 5. APOE and Declining Cognition in Old Age

Much recent research has considered the implications of APOE genotype for declining cognition in old age, and the majority of this research has supported our proposal [1] of exaggerated cognitive decline in ε4 carriers compared to noncarriers. While not universally the case [12, 21, 27], most studies reporting cross-sectional differences by APOE genotype found that ε4 carriers performed more poorly in tasks of verbal and visual episodic memory [4, 28–35, 59]. Other reported discrepancies were in working memory [30], general cognition [2, 4, 30, 36–38], category fluency [39], the Stroop task [40], matrix reasoning [14], and symbol search [14]. Many studies also considered cognition longitudinally and found ε4 carriers to display an increased risk of developing mild cognitive impairment (MCI) (e.g., [41]), AD (e.g., [3]), or vascular dementia [59], although other studies have failed to find a relationship between the ε4 allele and risk of developing MCI [42, 43]. Furthermore, although less consistent in the literature, some reported finding steeper rates of cognitive decline in ε4 carriers compared to noncarriers [3, 31, 36, 37], with a number reporting evidence of a dose-dependent response [3, 31, 59]. One interesting study by Casselli et al. [31] longitudinally followed patients between 21 and 97 years of age. Growth curve modeling predicted that APOE ε4 carriers begin to decline in their memory around their 50's, while participants without an ε4 allele only begin to experience memory decline in their 70's. Memory decline also evidenced a dose-dependent relationship, so that participants with two ε4 alleles were predicted to experience memory decline before those with only one ε4 allele. Moreover, ε4 carriers had an overall steeper rate of decline in memory, on the MMSE, and on Judgment of Line Orientation than noncarriers. Importantly, the study's longitudinal design allowed authors to remove from analysis those participants who eventually developed MCI or dementia. The authors therefore suggest that APOE detrimentally affects cognition in older adults independent of the influence of dementia. Liu et al. [48] also examined the impact of the APOE ε4 allele on cognition in a lifespan sample and reported that ε4 carriers' cognitive performance was only reduced compared to noncarriers in those 50 and older; APOE ε4 had no effect on cognition in younger age groups.

Carrión-Baralt et al. [44] reported intriguing results with a sample of Puerto Rican oldest-old. They found ε4 carriers in this sample of nonagenarians to exhibit *higher* performance than noncarriers in overall cognition as well as measures of visuospatial abilities, naming, and attention. The authors suggest that this unexpected finding may have two sources. It may be that those who survive into their 90's have genetic protective factors that confer benefit regardless of APOE genotype. Because of the higher risk of dementia and eventual mortality found with ε4 carriers, nonagenarian ε4 carriers who survived beyond the prime ages for AD development may be more likely to have this protective factor and may therefore perform better than noncarriers. A second possibility noted by Carrión-Baralt et al. [44] is that APOE may exhibit antagonistic pleiotropic effects, being detrimental for cognition and survival in young-old age, but being beneficial in old-old age. Reporting similar findings, Kozauer et al. [45] studied a large sample of two age cohorts after a 22-year followup. Differences in cognition by APOE status were only found in the younger cohort, which was in their 50's, in which ε4 carriers displayed worse performance than noncarriers. These differences were not found at other waves of data collection, implying that they developed upon reaching middle and older ages. Interestingly, cognitive differences by APOE status were not observed in the older cohort, which had a mean age in the mid-70's. This also supports the contention that the APOE ε4 allele may not be detrimental to those who survive into later ages without developing dementia. Similarly, Welsh-Bohmer et al. [27], who also studied APOE in oldest-old, found no relationship between APOE and cognition. While these latter findings of cognitive similarity by APOE status in the oldest-old may not be as persuasive as those of Carrión-Baralt et al. [44], they still suggest that the very common finding of cognitive decline in older ε4 carriers may not hold true for the oldest-old and may fail to do so because of antagonistic pleiotropy. This argues for an extension of our model [1] into old-old age, where APOE ε4 may again impart beneficial cognitive effects to its carriers.

As suggested in our [1] model of APOE as an example of antagonistic pleiotropy, much research has found that the APOE ε4 allele is associated with disproportionate cognitive decline in old age [3, 4, 14, 28–41, 59], although it may be that the ε4 allele confers an advantage on its carriers who reach old-old age without developing dementia, suggesting an extension to our model [1, 27, 45, 48]. The association of APOE ε4 with cognitive decline follows from findings suggesting a close relationship of the ε4 allele with amyloid neuropathology, a hallmark of AD [61–65]. Indeed, many individual studies and reviews have reported APOE to be strongly associated with A$\beta$42, the most insoluble form of the A$\beta$ peptide, which aggregates to form senile plaques in AD (e.g., [61, 64, 65]). Some studies have also demonstrated a dose-dependent relationship between the APOE ε4 allele and amyloid pathology, in which ε4 homozygotes exhibit more amyloid pathology than those with only one ε4 allele (e.g., [64, 65]). However, the demonstrated relationship between the APOE ε4 allele and AD-related amyloid pathology might argue against the ability of APOE to confer protective effects to its old-old nondemented carriers. With respect to this possibility, it is important to note that studies have found that amyloid pathology, while being strongly associated with the development of AD, does not account for all of the cognitive decline found in these patients (e.g., [61, 65, 66]). Other biomarkers, particularly brain atrophy and tau pathology, commonly account for a large portion of variance in cognitive decline found in AD (e.g., [61, 65, 66]). The fact that the APOE ε4 allele has not been as strongly associated with these other AD biomarkers [61, 65, 66] may explain why old-old APOE ε4 carriers may still exhibit cognitive benefits

despite the strong association of the ε4 allele with amyloid pathology.

## 6. Conclusions and Recommendations

Our model [1] of antagonistic pleiotropy in APOE was critically re-evaluated in light of recent research. Some aspects of the model were strongly supported. Research suggests that younger ε4 carriers do indeed exhibit compensatory neural recruitment, as proposed by our model [1]. However, our prediction of a predilection for right hemisphere frontal regions in this compensatory process was not clearly corroborated. It instead seems that young ε4 carriers invoke compensatory processes in task-related regions. The cognitive decline in older ε4 carriers predicted by the model was also supported in recent literature, revealing a strong effect on memory as well as other cognitive domains. Yet inconsistent evidence was found to support other aspects of the model. For instance, the presence of compensatory neural recruitment in older ε4 carriers was not unanimously supported. Studies with older ε4 carriers also only partially supported our prediction [1] of a higher likelihood of recruitment of right frontal regions. Differences in compensatory regions may be linked to type of task, as those thought to invoke more of a right hemisphere response also found evidence for right hemisphere compensatory processes, while left hemisphere compensatory processes were occasionally observed for tasks emphasizing language that would be thought to rely more heavily on left hemisphere regions. It is also unclear whether young ε4 carriers show better cognitive performance than noncarriers. As discussed, these inconsistent findings may be explained by the need to consider APOE and cognition in narrow age ranges or to statistically test the interaction between APOE genotype and age in predicting cognition for lifespan samples. It also appears important to control for detrimental health comorbidities that may be more prevalent in ε4 carriers in such analyses. However, if it is determined that APOE ε4 carriers do not show a cognitive benefit in early life, this strongly argues against APOE as displaying antagonistic pleiotropic effects. In fact, findings of compensatory recruitment in young ε4 carriers in the presence of similar, instead of enhanced, cognitive performance implies that the ε4 allele may be detrimental even in young ages, as young carriers have to activate more brain regions to produce the same level of performance as noncarriers. Research in this area is vital to further evaluate the antagonistic pleiotropy hypothesis of APOE.

In light of the reviewed research, we suggest certain revisions to our model [1] of antagonistic pleiotropy and APOE. In the absence of conclusive contrary findings, we maintain that APOE ε4 carriers exhibit cognitive benefits early in life. We also propose that compensatory neural recruitment processes occur in task-related regions in young ε4 carriers but transfer to frontal regions among older adults, once task-related regions become overburdened with AD pathology. While compensatory regions may be in the right hemisphere, lateralization of the compensatory response does not seem to be a consistent trend. Second, we propose that cognitive decline will begin much earlier in ε4 carriers and will progress more rapidly. In light of new research [27, 44, 45], we also predict that the APOE ε4 allele again becomes beneficial in the oldest-old, who survive into old-old age without developing dementia. Additional research is needed to test this notion, as functional imaging to examine compensatory recruitment in nonagenarian populations of ε4 carriers would be particularly elucidating.

The finding of cognitive superiority in nondemented ε4 nonagenarians suggests a second hypothesis of gene-gene interaction that may explain the apparent APOE antagonistic pleiotropic effects as well as the inconsistencies in the literature. It may be that the APOE ε4 allele is not antagonistically pleiotropic but instead interacts with other AD risk factors to differentially influence cognition depending on a person's profile of risk. Indeed, APOE has been found by many studies to interact with a family history of AD to influence cognition (e.g., [25, 29]) and also to interact with other risk genes such as APOCI AA, BCHE, and CHRNA4 [59, 67, 68]. Therefore, it may be that among people without AD risk factors, APOE ε4 does not adversely affect cognition and could perhaps be beneficial to its carriers. Some have gone so far as to suggest that APOE has no influence on cognition beyond increasing risk for AD [69]. These authors suggest that APOE, while putting one at risk for prodromal dementia, does not create a specific cognitive phenotype of its own [69]. APOE's interaction with other genetic risk factors to influence cognition would explain why only some young ε4 carriers show enhanced cognition over noncarriers, why only some ε4 carriers show disproportionate cognitive declines with age, and why nondemented oldest-old ε4 carriers show cognitive enhancements. Perhaps those children with APOE ε4 and other AD risk factors are those that show no difference from noncarriers, due to compensatory recruitment processes, or actually show reductions in cognitive abilities compared to noncarriers. Perhaps those children with APOE ε4 and other AD risk factors are the ones to show disproportionate cognitive decline in old age compared to noncarriers, whereas ε4 carriers without other risk factors do not show more cognitive decline than would be expected. This hypothesis of the interaction of APOE with AD-specific risk factors is further supported by the lack of associations between APOE and cognition in other diseases such as multiple sclerosis [70]. Future research in genetics and epigenetics is needed which carefully considers participants' AD risk profiles. Careful, comprehensive studies conducted in this way will help the field determine whether APOE displays antagonistic pleiotropy or gene-gene interactions with other AD risk factors.

This paper suggests several avenues for future research as well as other important factors when considering APOE as an example of antagonistic pleiotropy. Beyond considering neurovascular decoupling with age and participants' AD risk profiles, future research examining the impact of APOE ε4 allele on cognition and neural activity across the lifespan is needed. Continued evaluation of the antagonistic pleiotropy and gene-gene interaction hypotheses of APOE will move the field closer to realizing effective treatments and preventative measures for AD.

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*Review Article*

# Function and Comorbidities of Apolipoprotein E in Alzheimer's Disease

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Alzheimer's disease (AD)—the most common type of dementia among the elderly—represents one of the most challenging and urgent medical mysteries affecting our aging population. Although dominant inherited mutation in genes involved in the amyloid metabolism can elicit familial AD, the overwhelming majority of AD cases, dubbed sporadic AD, do not display this Mendelian inheritance pattern. Apolipoprotein E (APOE), the main lipid carrier protein in the central nervous system, is the only gene that has been robustly and consistently associated with AD risk. The purpose of the current paper is thus to highlight the pleiotropic roles and the structure-function relationship of APOE to stimulate both the functional characterization and the identification of novel lipid homeostasis-related molecular targets involved in AD.

## 1. Introduction

Cardiovascular diseases (CVDs), a group of disorders involving the heart and blood vessels, are currently the world's leading cause of death and the top ranking therapeutic category in terms of prescription drug spending. However, given the alarming aging of the population and the increase longevity of humans, Alzheimer's disease (AD) and other related dementias are set to become the next great health crusade of the coming decades [1]. Interestingly, these progressive degenerative disorders share common etiological grounds: advancing age, apolipoprotein (apo) E4 inheritance, cigarette smoking, high blood pressure, diabetes, obesity, oxidative stress, and abnormal blood cholesterol levels all concur to increase one's liability to develop CVD [2, 3] and dementias [4]. While the connection between vascular factors and cognition remains obscure, converging evidence associates the deficiency of APOE with impaired cognition (see Section 4). Importantly, APOE is the only locus known to significantly contribute to the risk of developing the late-onset form of AD, with the E4 and E2 alleles, respectively, increasing and decreasing the risk level [5–7]. Given its pleiotropic function (see Sections 2 and 3), the mechanisms by which APOE may exert its effects remain unclear. Yet, APOE primarily functions as a major lipid transporter in the periphery and as the main one in the central nervous system (CNS), putting lipid homeostasis center stage for the maintenance of cognitive function and the promotion of AD (see Section 3). Accordingly, alterations in lipid homeostasis are known to severely impair neuronal function and elicit progressive disorders such as Farber's, Gaucher and Niemann-Pick type C diseases [8–10]. In addition to its association with AD, APOE genotype also correlates with a wide range of other dementias and neurodegenerative disorders (see Section 5). These converging findings strongly support the implication of cholesterol/lipid metabolism as a key factor in neurodegenerative disorder etiology, a concept still under-studied in the field of AD. To stimulate interest in identifying novel, lipid homeostasis-related molecular targets involved in AD pathogenesis, this paper reviews the latest advances and concepts associated with APOE functions.

## 2. Structural Organization and Toxicological Properties of the APOE Protein

*2.1. Association between APOE and AD: Two Antipode Isoforms.* The APOE gene is mapped onto chromosome 19 and mainly exists in humans as three possible isoforms differing from each other by single amino acid substitutions at positions 112 and 158. APOE isoforms are unevenly distributed in the general population as 77% of people carry the E3 allele, 15% the E4 allele, and 8% the E2 allele [5, 11]. Second only to aging, APOE is now recognized as the most important risk factor for the late-onset form of AD. Indeed, in addition to numerous case control studies [5, 6, 12] several independent genome-wide association studies (GWASs) have been performed in homogeneous and heterogeneous population of AD and age-matched control cases in North America, Europe, and Asia [13–17]. Using genome-wide statistical criterion, the APOE4 allele was found to be associated with AD in all these independent studies. Accounting for as much as 50% of the genetic variation in liability to develop AD [18], carriers of the APOE4 allele who develop AD do so at an earlier age at onset and exhibit higher levels of soluble beta-amyloid (A$\beta$) peptide, increased senile plaque (SP) [19], and neurofibrillary tangle (NFT) accumulation, as well as more extensive cholinergic deficits [20–22].

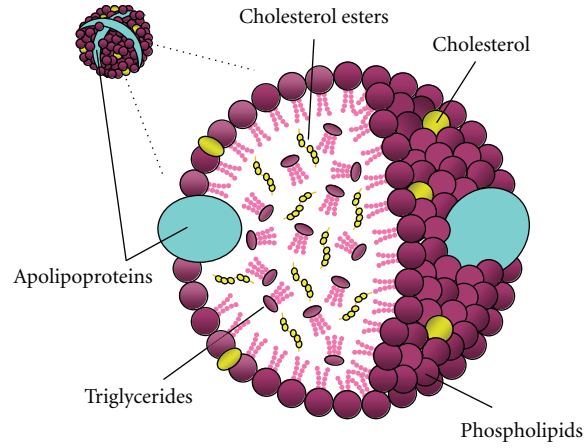
In contrast, the APOE2 variant is associated with a marked risk reduction of AD [7]. Indeed, carriers of the APOE2 allele have less AD pathological changes than APOE3 carriers, that is, less pathological A$\beta$, SP, and NFT levels [23–26], as well as larger regional cortical thicknesses and volumes indicative of greater brain reserve against cognitive decline [27]. These diametrically opposite effects of APOE4 and APOE2, which only differ by subtle amino acid substitutions at positions 112 and 158, sparked a large interest in understanding how these proteins differ at the molecular level.

*2.2. APOE Basic Structural Arrangement.* APOE is a major protein constituent of plasma and CNS lipoproteins (see Box 2.2.1) and functions primarily as a lipid transporter in the human body. Through its binding to members of the low-density lipoprotein receptor (LDLR) family present on the plasma membrane, APOE effectively mediates the uptake of lipoproteins by cells [28, 29] or activate signalling pathways that modulate lipid homeostasis [30]. At physiological levels, a substantial amount of APOE is bound to lipoprotein, whereas a significant fraction of APOE could be associated with cell surface proteoglycans in a lipid-free state [31]. As will be discussed in more detail below, these lipid-bound and lipid-free conformational states likely affect the biological functions of APOE.

In the lipid-free state, the APOE protein (299 residues) is organized as two independently folded domains linked by a protease-sensitive loop (Figure 1): an N-terminal (NT) domain (residues 1–191) comprised of a four-helix bundle [32, 33] and a C-terminal (CT) domain (residues 210–299) whose structural organization has still not been elucidated despite the crystallization of a proteolytic fragment comprising residues 223–272 [34]. The NT domain was shown to bear the LDLR binding site [28, 35–37], whereas residues within the lipid-binding CT domain mediate the lipoprotein binding [38–40] and the APOE self-association sites [31, 41, 42] (Table 1). Indeed, at physiological concentrations (micromolar), APOE exists predominantly as a tetramer [43]. Latest results indicate that, in a lipid-free state, the CT domain of APOE forms dimer, which then dimerizes further to form a tetramer [31]. However, APOE is likely to bind to lipid from its monomeric rather than tetrameric state. The transition from lipid-free to lipid-bound APOE may thus involve the formation of multiple intermediate conformational states [44].

As suggested by the initial discovery that only lipid-bound APOE binds to LDLR with high affinity [45], the structural organization of the APOE protein significantly changes when bound to lipids or lipoproteins [44, 46, 47]. Importantly, APOE adopts many lipid-bound conformations that depend on lipoprotein size [48], lipid composition [49] and on the presence of other apolipoproteins [50]. Interestingly, recent evidence indicates, however, that dipalmitoylphosphatidylcholine-(DPPC-) bound APOE adopts an alpha-helical hairpin conformation in the shape of a horseshoe, with the CT domain oriented toward the lipoprotein surface [44, 51, 52] (Figure 1). This hairpin conformation puts all the known elements of the LDLR binding site into a structural apex, potentially explaining why only lipid-bound APOE binds to LDLR with high affinity [44, 51].

*2.2.1. Box 1*




Lipids are hydrophobic molecules that use lipoproteins to move through aqueous environments. These lipoprotein particles comprise a nonpolar core of triglycerides (TGs) and cholesterol esters surrounded by an outer shell of phospholipids, cholesterol, and apolipoproteins that confer water solubility on the lipid constituents. In the periphery, lipoproteins are classified into four major classes on the basis of their associated apolipoproteins and their lipid content: chylomicrons, very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein

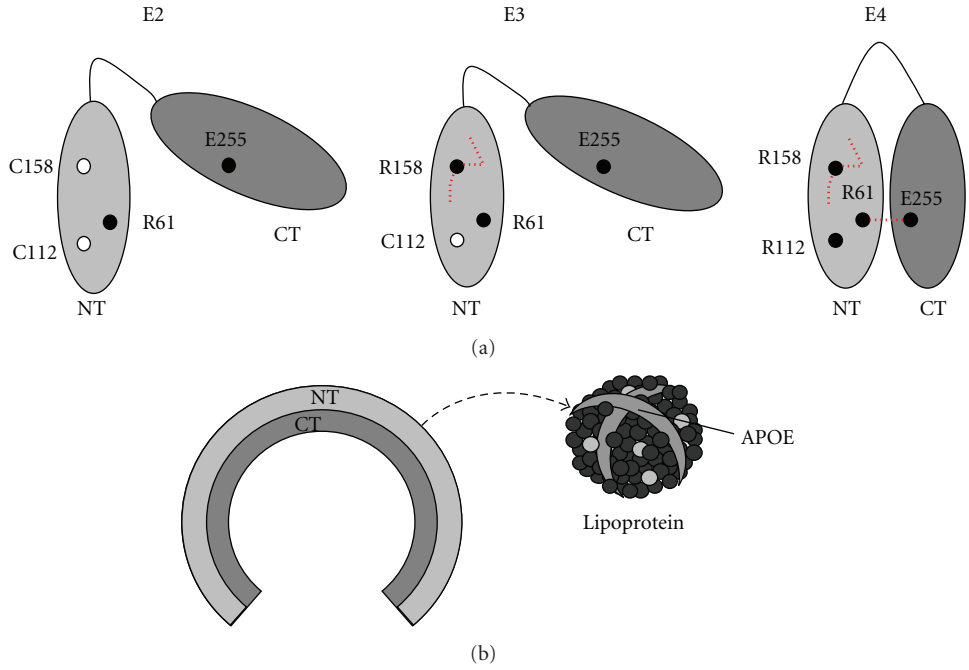



Figure 1: Illustration of the lipid-free (a) and DPPC-bound (b) conformational states of APOE isoforms. (a) In a lipid-free state, the NT and CT domains of APOE interact with each other. (b) The tridimensional conformation of APOE significantly changes when bound to lipid and adopts the shape of a horseshoe, with the CT domain oriented toward the lipoprotein surface. APOE or E: apolipoprotein E; the red dots line represents salt bridges; C: cysteine; R: arginine; E: glutamate; DPPC: dipalmitoylphosphatidylcholine; NT: N-terminal domain containing the low-density lipoprotein binding sites; CT: C-terminal domain comprising the lipoprotein binding and APOE self-association sites.

Table 1: Key structural and functional differences between the three main human APOE isoforms.

| Description | E2 | E3 | E4 |
|---|---|---|---|
| Primary sequence difference | C112 | C112 | **R112** |
| | **C158** | R158 | R158 |
| Structure particularity | Disruption of salt bridge network in the NT domain | | Domain interaction (creation of salt bridges R112-E109 and **R61-E255**) |
| LDLR binding | <2% normal receptor binding activity | High | High |
| Lipoprotein binding | HDL | HDL | VLDL/LDL |
| Protein stability | +++ | ++ | + |
| Molten-like-globule propensity | + | ++ | +++ |

E: glutamate; C: cysteine; R: arginine; LDLR: low-density lipoprotein receptor; HDL: high-density lipoprotein; VLDL: very-low-density lipoprotein; +/++/+++, respectively, low/medium/high.

(HDL) [53]. Chylomicrons are rich in APOB and represent the larger lipoproteins. Their function is that they carry exogenous (dietary) fatty acids from the intestine to the liver, skeletal muscles, and adipose tissues. VLDLs are assembled in the liver with TG, APOC, B, and E and ensure transport of endogenous lipids from the liver to adipose tissues. Once in the circulation, VLDLs undergo important TG hydrolysis, and their apolipoproteins (except APOB) are progressively eliminated. When cholesterol content becomes greater than the content of TG, VLDLs are converted into LDL with APOB as their main apolipoproteins. LDLs are taken up by the liver and other tissues through LDL receptor-(LDLR-) mediated endocytosis. Finally, HDLs are the smallest but the densest lipoprotein particles because they associate with the highest proportion of apolipoproteins, mainly APOA-I. Contrary to the other lipoproteins, HDLs mediate the reverse cholesterol transport as they extract cholesterol from peripheral tissues and transport it to the liver for excretion. In the CNS, only HDL-like lipoproteins composed primarily of APOE, APOA-I, and APOJ are present [54, 55].

*2.3. Structural Arrangement and Toxicological Properties of the APOE Isoforms: Understanding the Antipodes.* The APOE

protein is polymorphic, with three common isoforms bearing identical CT domain primary structure, but distinct NT domain sequence: at positions 112 and 158, E2 has cysteines, E4 has arginines, and E3 has a cysteine and an arginine, respectively [32, 56, 57] (Figure 1, Table 1). These particularities profoundly affect the structural and functional properties of APOE (reviewed in [44]).

For instance, while APOE3 and APOE4 bind to LDLR with similar affinities, the APOE2 isoform has less than 2% of the LDLR binding activity of APOE3, at least in peripheral cells [58, 59]. This inhibition of the LDLR binding activity is mediated not only by the arginine-to-cysteine substitution at position 158, which disrupts the salt bridge network between residues R92, E96, R103, R150, D151, and D154 [33], but also by part of the CT domain [59] (Figure 1, Table 1). Consequently, a double dose of APOE2 is associated with type III dyslipidemia, a disorder characterized by increased plasma levels of cholesterol and triglycerides as well as premature CVD resulting from a defective clearance in chylomicron remnants [29, 60].

For its part, the cysteine-to-arginine substitution at position 112 in the APOE4 NT domain induces the formation of a salt bridge between arg112 and glu109, modifying the orientation of the side chain of arg61, which subsequently forms a salt bridge with glu255 in the CT domain [39] (Figure 1, Table 1). This *domain interaction* apparently influences the binding kinetics of lipid-free APOE to lipids, thereby contributing to APOE4 preferences for VLDL and LDL particles, whereas APOE3 and APOE2 isoforms preferentially bind HDL particles [38, 39, 50, 61] (Figure 1, Table 1).

As aforementioned, APOE undergoes as extensive conformational changes upon binding its ligand (lipid or lipoprotein), and the transition from lipid-free to lipid-bound APOE can be facilitated thermodynamically by the formation of intermediate, partially unfolded APOE conformational states [44]. These partially unfolded structures, also called *molten-globule-like conformations*, are believed to be crucial for lipid binding by numerous apos, including APOE, apoAI [62], and apoAII [63]. Yet, molten-globule-like conformations are more prone to proteolysis, more vulnerable to degradation pathways and have been implicated in several diseases [44, 64]. Accordingly, the AD-associated APOE4 isoform possesses the highest propensity to form molten globule-like conformations, followed by APOE3 and finally, APOE2 [65] (Table 1). Conversely, APOE2 possesses the highest resistance to thermal and chemical denaturation, followed by APOE3 and APOE4 [66–68] (Table 1). In accordance with their disparate protein stability, APOE2 is associated with the highest levels of APOE lipoprotein, whereas APOE4 is associated with the lowest in both the blood and brain [69–72].

The lower stability of APOE4, its increased susceptibility for proteolysis as demonstrated by turnover studies in humans [73] and APOE human knockin mice [74] and its higher propensity to form molten-globule-like intermediates that actively bind to phospholipids and membranes could, in concert with fibrillar A$\beta$, promote lysosome leakage and apoptosis through lysosomal membrane disruption [75, 76]. The suboptimal features of APOE4 might also promote neurotoxicity and neuroinflammation through the proteolysis of APOE4 into putative neurotoxic NT and CT fragments, a process postulated to occur solely in neurons and not in astrocytes [77]. Furthermore, this proteolytic processing of APOE is proposed to occur only in the secretory pathway, and not in the internalization pathway of neurons (i.e., there is no fragmentation of the astrocyte-derived APOE acquired by neurons following internalization of APOE-lipoproteins) [77]. However, synthesis of APOE by neurons remains to be clarified (see Section 3) through additional studies both, in model systems and in humans.

In sum, studies on the conformational structure of APOE have yielded valuable insights into the relationships that exist between the structure and function of APOE. A noteworthy finding from such studies that resulted in our further understanding of APOE4's toxicological properties is that of apoE4's domain interaction, which promotes the formation of molten-globule-like conformations. Although progress has been made toward understanding how the structural differences of the three APOE isoforms relate to phenotype and disease, much work remains to be done.

## 3. Biological Functions of APOE and Interaction with Its Associated Partners

*3.1. An Evolutionary Perspective.* APOE3 allele appears to have spread during the later stages of human evolution after originating from the ancestral APOE4 allele. According to DNA sequences representing four distinct ethnic groups, APOE3 is estimated to have spread some 225,000 years ago. The depth of the tree is estimated at 311,000 years ago (range 0.176–0.579) [78]. Although these sequences analyses do not inform us of when APOE3 originated as a mutation, they imply that APOE3 arose before anatomically modern Homo sapiens first migrated from Africa about 100,000 years ago. Thus, APOE3 was present in Neanderthals (from 300,000 years ago) and in earlier African or European Homo from which Homo sapiens is thought to have diverged. Only one APOE genotype has been reported in chimpanzees and other primate species, which closely resembles human APOE4 with arginines at positions homologous to amino acids 112 and 158 (Table 2) [79–81]. Many other mammals, including rats, mice, pigs and cows, also have arginines at these positions [80]. Given the depth of human APOE genealogy tree and the similarities between human APOE4 and mammal APOEs, the APOE4 is considered as the ancestral allele in primates [79, 80].

*3.2. APOE in Cholesterol and Phospholipid Transport.* APOE is the major apolipoprotein in the CNS and plays a central role in lipid transport in the nervous system [82]. The dependence of brain cells toward APOE as their chief lipid carrier and provider is emphasized by the complete absence of synthesis of other key plasma apolipoprotein such as apoA1 and apoB in the CNS [83]. In the brain, APOE is produced mainly by astrocytes [83–86] and to a lesser extent by microglia [87]. Initial studies investigating the site of APOE production in the brain suggested that only astrocytes,

Table 2: APOE polymorphisms in humans and species differences.

| ApoE residue (+signal peptide) | Population prevalence (%) | 112 (130) | 158 (176) |
|---|---|---|---|
| Human | | | |
| APOE2 | 8 | C | C |
| APOE3 | 77 | C | R |
| **APOE4** | **15** | **R** | **R** |
| Chimp | 100 | R | R |
| Gorilla | 100 | R | R |
| Orangutan | 100 | R | R |
| Mice | 100 | R | R |
| Rats | 100 | R | R |

APOE: apolipoprotein E; C: cysteine; R: arginine.

oligodendrocytes, and ependymal cells synthesized APOE [86, 88]. Under diverse physiological and pathological conditions, neurons have also been reported to express APOE, albeit at a much lower level than astrocytes, in humans [89], neuronal cell lines [90–92], mice [93], and APOE transgenic mice [94]. Surprisingly, a significant number of studies failed to observe the *synthesis* of APOE in neurons both in rodent and human brains [20, 86, 95–99]. Clearly, additional studies are needed to clarify the issue of APOE synthesis within neurons, especially since numerous studies found no evidence of APOE mRNA expression within neurons [86, 95, 100, 101].

Cholesterol homeostasis in the CNS is regulated independently from the periphery due to the presence of the blood-brain barrier (BBB) which prevents the plasma cholesterol from crossing into the CNS [102]. Maintenance of the cholesterol pool in the brain is based on the regulation of three important steps: synthesis, transport (recycling), and excretion [103]. The first step, synthesis, is provided by *de novo* anabolism that converts acetyl-CoA to cholesterol through a series of 20 complex reactions in which 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) is the rate limiting enzyme [104, 105]. While neurons of the mature brain can synthesize cholesterol, they mainly rely on astrocytes to meet their cholesterol requirements for neuronal maintenance, growth, repair and dendritic reorganization [102, 106, 107].

Recycling of lipids reflects an energy-efficient system when compared to the biosynthesis of cholesterol because the latter involves a complex pathway requiring over 20 reactions [103, 107, 108]. Cholesterol homeostasis is maintained through a series of interdependent pathways including that of cholesterol transport in which APOE is of central significance. APOE is the primary apolipoprotein in the CNS followed by APOJ (also known as Clusterin) [109], which has also been identified as a genetic risk factor in AD by genome-wide association approaches [17, 106, 110]. In the brain, APOE is produced and secreted by astrocytes and microglia and is subsequently lipidated by the ATP-binding cassette transporter A1 (ABCA1) to form lipoprotein particles (Figure 2) [71, 102]. The role of ABCA1 is to regulate the efflux of cholesterol and phospholipids from the cell onto HDL in plasma [111]. It has been proposed that ABCA1 catalyses the initial transfer of cholesterol onto lipid-poor APOE and that ATP-binding cassette transporter G1 (ABCG1) finalizes the full lipidation of the apolipoprotein (Figure 2) [112, 113]. Although many lines of evidence support a role of ABCG1 in the regulation of cholesterol efflux, its function remains elusive. Tangier disease provides supporting evidence for the central role of ABCA1 in cholesterol homeostasis as this disease is caused by mutations in the ABCA1 gene and is characterized by HDL deficiency and cholesterol accumulation in macrophages and hepatocytes [114, 115]. Consistent with this phenomenon, ABCA1 mouse knockouts exhibit poorly lipidated APOE which in turn has been shown to influence A$\beta$ metabolism [111, 116]. The pivotal role of ABCA1 in cholesterol homeostasis makes it of potent interest as a target for AD treatment.

Following lipidation, the APOE-HDL-like lipoparticles are endocytosed by specific members of the LDLR family (including LDLR, LDLR-related protein (LRP), APOER2, and the VLDLR) present on both neuronal and nonneuronal cells (Figure 2) [102, 117]. APOE endocytosis provides cholesterol to the neuron that can subsequently be used for synthesis of plasma membranes, synaptogenesis, and dendritic proliferation [118]. The functions of APOJ in lipid homeostasis and A$\beta$ metabolism are very similar to those of APOE in that they are both carriers of cholesterol in the CNS and they both modulate amyloid fibrillogenesis and clearance [109, 119, 120]. Albeit their similar functions, APOE and APOJ are present on different HDL-like lipoprotein particles which differ in composition: APOJ-lipoprotein particles are lipid poor and have a higher phospholipid-to-cholesterol ratio compared to APOE-lipoprotein particles [85, 119, 121]. As well, there is some debate as to whether the cholesterol transport pathway used by APOE and APOJ differs since the receptor for APOJ, megalin/LRP-2, is not expressed by neurons and APOJ levels which are significantly increased in AD brain [122, 123] are unaltered in ABCA1 knockout mice suggesting that APOJ does not use ABCA1 for lipidation [111, 119, 124].

Excess cholesterol cannot be degraded in the brain due to its sterol ring. The predominant pathway to excrete cholesterol from the CNS is therefore to convert it into a more lipophilic 24(S)-hydroxycholesterol which can cross the BBB [112, 125]. This metabolite, only produced in neurons, is then directed to the liver where it can be excreted in the form of bile acids [126]. Other pathways account for about 36% of the excretion; however, the mechanisms remain unclear and controversial [125, 127].

APOE variants continue to receive great attention today and account for more genetic variance (25%) in cholesterol metabolism than any other gene [128]. APOE4/4 versus APOE3/3 carriers have 3 to 15% higher LDL and total cholesterol, depending on the population, diet, and exercise. APOE alleles show marked effects on blood lipids during dietary shifts. For example, in humans on a low-fat baseline diet, adding 300 mg cholesterol/day (2 egg yolks) caused serum total cholesterol to increase fourfold more in APOE4/4

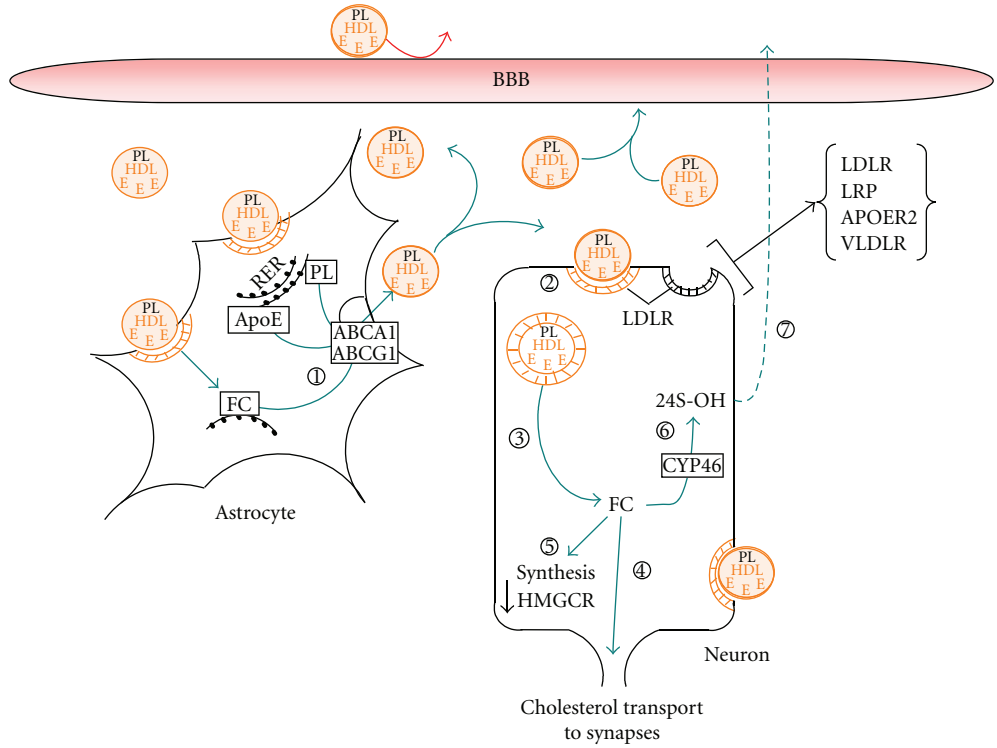



Figure 2: Cholesterol transport in the CNS. APOE is synthesized by astrocytes and assembles free cholesterol (FC) and phospholipids (PLs) to form HDL-like particles. (1) Lipidation of these lipoparticles is facilitated via the mobilization and distribution of lipids to the cell surface by ABCA1/G1. Once secreted in the extracellular space, these HDL-like particles are directed either toward the circulation through the BBB and/or to neurons requiring lipids. (2) These APOE-HDL-like particles are recognized and endocytosed by members of the cell surface LDLR family (LDLR, LRP, APOER2, VLDLR), and (3) the FC is released within neurons and can be used for neurite elongation and/or synaptogenesis (4). As a result of lipid internalization, the endogenous synthesis of cholesterol within neurons (via the HMGCR pathway) is repressed (5). Excess cholesterol will be removed from neurons through its conversion into 24S-hydroxycholesterol (24S-OH) which is mediated by cholesterol 24S-hydroxylase (CYP46) (6). This sterol can now freely cross lipophilic membranes of the BBB and exit the brain for elimination (7). APOE or E: apolipoprotein E; BBB: blood-brain barrier; RER: rough endoplasmic reticulum; HMGCR: 3-hydroxy-3-methylglutaryl coenzyme A reductase; HDL: high-density lipoprotein; LDLR: low-density lipoprotein receptor.

carriers than in APOE3/3 and even greater relative increases of LDL cholesterol [129]. As aforementioned, APOE4 preferentially binds triglyceride-rich lipoproteins (LDL and VLDL), whereas APOE3 binds preferentially to HDL [38, 39, 50, 61]. These differences in lipoprotein binding by APOE3 and APOE4 greatly influence lipoprotein clearance and the LDL/HDL ratios, which are risk factors in cardiovascular diseases. APOE4 has smaller effects on the risk of cardiovascular disease than on AD, in the range of 10% to 50%, with effects peaking during middle age [130]. In contrast, APOE4 is the most common AD risk factor throughout the world, with a 10- to 20-fold higher risk in Caucasian homozygous E4 carriers. The impact of the APOE4 variant varies widely across different populations/ethnicities, which can likely be explained, at least partly, by an interaction between genetic and environmental factors. For example, Yorubans in Nigeria showed 70% less dementia than African Americans [131], an incidence difference which may be related to their lifelong low-fat diet.

*3.3. APOE-Mediated Beta Amyloid Transport.* Because the possible roles of APOE in amyloid metabolism have been extensively reviewed recently by Kim and colleagues [106], only the potential role of APOE lipoproteins as scavengers of soluble beta amyloid (A$\beta$) peptides will be discussed in this section [21]. Indeed, evidence suggests that APOE binds avidly to soluble nonaggregated A$\beta$ fragments [6, 132]. As it was demonstrated in rat primary neuronal cell cultures, the APOE lipoproteins containing A$\beta$ may then be internalized via the APOE receptor internalization pathway [133, 134]. Following internalization, these A$\beta$ fragments could be released and degraded via the endosomal/lysosomal pathway [20, 21]. The observation that A$\beta$ reaches high intracellular concentration without affecting neuronal survival strengthens the proposed compartmentalization of internalized A$\beta$ in endosomes/lysosomes [133, 134]. Interestingly, APOE binding affinity for A$\beta$ was shown to follow an E2 > E3 > E4 gradient [135]. This provides an additional mechanism explaining, at least in part, the marked discrepancy that exists between the APOE2/E4 variants and the risk to develop AD. Indeed, the protective APOE2 variant binds A$\beta$ more avidly than the deleterious APOE4 variant and might therefore be more efficient than its APOE4 counterpart at clearing A$\beta$ fragments from the extracellular space [21].

*3.4. APOE and Neuroinflammation.* In addition to mediating the endocytosis of cholesterol and phospholipids into neurons, APOE have been associated with antioxidant properties in both *in vitro* and *in vivo* models [136, 137]. Additionally, in the periphery, APOE- and APOB-enriched lipoproteins are known to transport vitamin E and other lipid soluble antioxidant species [138]. Irrespective of APOE genotype, a plethora of oxidative reaction products has been found increased in the brains of AD and mild cognitive impairment subjects [139–141]. However, markers of oxidative damage are more intense in individuals who carry one or two copies of the APOE4 allele [137, 142].

As in many neurodegenerative diseases including Parkinson's disease [143], neuroinflammation caused by an abnormal activation of astrocytes and microglia is featured prominently as a pathological characteristic of AD [144–146]. This neuroinflammatory response is primarily driven locally by neuronal cell loss and extracellular A$\beta$ deposits evidenced by the colocalization of numerous inflammation-related proteins (i.e., cytokines, complement receptors and acute-phase proteins), activated microglia clusters, and amyloid plaques [147, 148]. *In vitro* studies affirm that A$\beta$ peptides can trigger an inflammatory response as measured by increases in standard neuroinflammatory proteins (i.e., cytokines and nitric oxide synthase) as well as nitric oxide release [149]. Neuroinflammation, especially when prolonged, is of concern for AD due to the accumulation of inflammatory molecules that are proven toxic to neurons resulting in neuronal dysfunction or death [146, 150].

As previously discussed, APOE is involved in numerous pathways influencing AD onset and progression, including A$\beta$ production, clearance, and degradation as well as its role in cholesterol homeostasis. Since the initial finding of decreased inflammation from glial A$\beta$-induced APOE production [145, 151], numerous studies have reported that APOE has an anti-inflammatory function and that its stimulation by A$\beta$ acts as a negative feedback system [145, 151, 152]. In support of this, APOE-deficient mice have a greater neuroinflammatory response relative to control mice [145]. *In vitro* studies also confirm that exogenously administered human APOE has the ability to attenuate A$\beta$-induced astrocyte activation as measured by a decrease in cyclo-oxygenase 2 (COX2) and inducible nitric oxide synthase [153] levels [145, 151]. However, the anti-inflammatory effect of APOE is reversed in the absence of an A$\beta$-induced inflammatory response as assessed by expression of interleukin-1b, a proinflammatory cytokine, following exogenously administered APOE [151]. Taken together these findings suggest a dual role for APOE in which it attenuates A$\beta$-induced neuroinflammation but also overproduction of APOE by this same activated glia can lead to an exacerbation of the inflammation [151, 154]. Although the mechanisms by which APOE influences the inflammatory response remain elusive, the primary candidate is the modulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-$\kappa$B) transcription and signalling pathway [147, 155, 156].

Also investigated is whether the role of APOE in neuroinflammation is affected in an isoform-specific manner. *In vivo* studies using human *APOE* knockin mice have shown that following lipopolysaccharide (LPS) administration, APOE4 mice have a greater neuroinflammatory response relative to the APOE3 mice, suggesting that APOE4 has a less effective anti-inflammatory effect [157]. In addition to the decreased anti-inflammatory function of APOE4, cell culture studies investigating the proinflammatory function of APOE in the absence of A$\beta$ have demonstrated that astrocytes and microglia show a greater inflammatory response when exogenous APOE4 is added compared with the APOE3 isoform [151].

Collectively, these findings imply that APOE4 has a reduced anti-inflammatory effect as well as a more vigorous proinflammatory function. Although these results infer an isoform-specific immunomodulatory effect, the efficiency of APOE2 requires further investigation.

Neuroinflammation is a prominent characteristic of AD; however, it remains uncertain whether it is promoting AD progression or merely a byproduct of the disease [148]. Numerous studies have investigated the effects of nonsteroidal anti-inflammatory drugs (NSAIDs) on AD risk and treatment [158–161]. While they remain controversial, a trend toward a decrease in AD risk is observed in population-based studies with long-term use of NSAIDs, suggesting an early role of inflammation in AD [160, 162]. Recently, the NSAID risk reduction benefits have been shown to be restricted to the apoE4 carrier subgroup in the population-based Cardiovascular Health Cognition Study [163], further highlighting the interactions linking apoE metabolism to the immune system.

## 4. The Neurophysiological and Functional Correlates of the APOE4 Aging Brain

*4.1. Overview of APOE Functions.* Acting as the main cholesterol transporter of the CNS [83], APOE plays a determinant role in neuronal maintenance, growth, repair, and reorganization [164, 165]. While CNS neurons produce just enough cholesterol to survive and grow inefficient synapses [166], a glia-derived factor consisting of cholesterol complexed to APOE-containing lipoproteins strongly promotes synapse formation [107, 167]. Indeed, the formation of multiple, highly efficient synapses in the developing CNS and in the injured adult brain was shown to be highly dependent on additional cholesterol supplies provided by the glial system [107]. It is therefore understood that the plastic properties of the brain through terminal remodeling and synaptogenesis are highly dependent on CNS cholesterol bioavailability. Needless to say that ineffective cholesterol transport associated with APOE deficiencies or deficiency in one of its most important receptors, the LDL receptor (LDLR), should invariably alter plasticity-dependent cognitive refinement and synaptic remodeling.

*4.2. APOE-Deficient Mouse: A Model of Impaired Cognition.* Consistent with the human APOE4/AD observations indicating the APOE levels are tightly regulated by its polymorphisms, APOE-deficient mice which express no APOE in the brain display: (a) progressive age-related

memory loss [168–174], (b) progressive loss of cortical and hippocampal synapses that reaches nearly 70% by 18 months of age [170, 173, 175], (c) a marked reduction of cholinergic activity [176], (d) impaired injury-mediated synaptic remodeling [171], (e) impaired reinnervation processes [177, 178], and (f) deterioration of phospholipids metabolism during maturation [179]. Moreover, APOE deficiency also potentiates the detrimental effects of oxidative stress on learning/memory through the expression of inflammatory proteins in the cerebral vasculature [180]. Taken together, these APOE-deficient mouse model findings, whether through oxidative stress, altered cholinergic system function, or compromised synaptic plasticity mechanisms, all corroborate the particular susceptibility of the hippocampus to APOE deficiency in mice. Strikingly, this increased vulnerability of hippocampus-related functions to APOE deficiency is consistent with disproportionate hippocampal volume atrophy in the early stages of AD [181], particularly so in carriers of the APOE4 allele [182, 183].

Similarly, in absence of LDLR, the main receptor for APOE, synaptic remodelling, and plasticity is gravely impaired in LDLR knockout (KO) mice [184]. These mice exhibit progressive age-related memory loss and significant synaptic loss in the CA1 area (−35% at 11 months) of the hippocampus [184]. Interestingly, adult LDLR KO mice expressing no brain LDLR have a 50% reduction in APOE synthesis relative to wild-type mice [185], a feature not that dissimilar from the finding that APOE4/4 AD subjects express only 50% of the normal brain APOE levels of APOE3/3 individuals [72, 186]. Relative to wild-type mice, basal hippocampal A$\beta$1-42 are found to be significantly higher in both the LDLR KO and dual APOE/LDLR KO mice, corroborating the potential role of APOE lipoproteins as scavengers of soluble A$\beta$ peptides (see Section 3). Furthermore, the fact that the APOE4 allele and LDLR genetic variations synergistically enhance the risk of developing AD by 11-fold [187] highlights the importance of both APOE synthesis and internalization in AD. Interestingly, it was shown a few years ago that cultured astrocytes from APOE4 and APOE3 human knock-in mice synthesize and release APOE to the same extent [188]. This suggests that the reported lower levels of brain APOE protein associated with the APOE4 allele might also be mediated by the internalization of APOE within brain cells. Whether the recycling and/or degradation of the APOE4–LDLR complex is differentially regulated when compared to its APOE3 counterpart warrants further studies.

Interestingly, the introduction of human APOE3 or APOE4 in the APOE KO mice completely prevents the cognitive deficit typical of these APOE-deficient mice [189, 190]. However, it should be noted that human APOE4 expression in targeted replacement mice leads over time to (a) a marked reduction of APOE levels in the brain [191], (b) compromised synaptic plasticity [192], and (c), defective cognitive performance as well as impaired long-term potentiation (LTP) [190, 193, 194]. More interestingly, cross-breeding of the humanized APOE4 or APOE3 mice with APP717 amyloid overexpressing transgenic mice almost completely prevented the characteristic accumulation of A$\beta$ deposits reported in hippocampal and cortical areas [195]. These findings are entirely consistent with the proposed notion that brain APOE acts as a local active scavenger of extracellular A$\beta$ [132, 134].

*4.3. APOE4 and Impaired Cognition in Nondemented Individuals.* In humans, APOE4 is known to increase the risk of both familial and sporadic AD [5, 6] and to precipitate conversion to AD among mild-cognitive impairment (MCI) patients [196, 197]. MCI refers to a condition in which memory or, less commonly, another cognitive function is below normal but does not interfere with daily functioning. MCI is considered a transitional state between normal forgetfulness and AD. Moreover, converging evidence indicates that, over time, APOE4 increases the likelihood of cognitive impairments in clinically normal 50+ years old individuals [198]. Indeed, APOE4 carriers under the age of 60 years exhibited greater acceleration of age-related memory decline relative to noncarriers, despite ongoing normal clinical status [199]. This memory decline occurring prior to MCI diagnosis was previously found to be relatively specific as no differences were found in the domains of language, spatial skills, or executive function [200].

Among dominant views on what underlies this APOE4 and AD association, one is based on the observation that APOE4 proteins are the least effective in facilitating the metabolism of pathogenic A$\beta$ forms, which indirectly augments A$\beta$ burden [201]. Alternatively, another potent explanation for this increased risk of AD in APOE4 carriers is the deleterious effects of reduced protein expression on cholesterol homeostasis. Indeed, the APOE4 gene was shown to encode significantly less APOE proteins than E3/E2 counterparts, thereby providing insufficient levels of functional APOE4 to maintain CNS cholesterol homeostasis and neuronal health [134, 191, 193]. This notion finds compelling support in the neuroprotective properties of the APOE2 allele against late-life development of sporadic AD [24], as this APOE polymorphism is associated with a tenfold increase in APOE protein levels compared to both APOE3 and APOE4 [193]. Equally important is the demonstration that treatment of hypercholesterolemia with HMGCR inhibitors (statins), a family of lipids-lowering agents [27], in middle-aged individuals confers neuroprotection against late-life development of sporadic AD [202–204]. Furthermore, treatment by statins resulted in selective improvement of AD-prone hippocampal and frontal-related cognitive functions in APOE4 carriers, but without affecting CSF A$\beta$42 or total tau levels [205]. Further studies are clearly needed to assess the impact of different statin therapy regimens on histopathological hallmarks of AD-like A$\beta$ metabolism and tau deposition.

*4.4. APOE4 Effects on Hippocampal/Entorhinal Cortex Imaging and Volume Measurements.* Alongside the association between APOE4 and cognitive alterations mostly in the sphere of memory among nondemented individuals, neuroimaging studies have provided significant structural as well as functional evidence of medial temporal lobe alterations

in APOE4 carriers. While structural neuroimaging studies have yielded mixed results over the past decade, recent years have witnessed significant advances in our ability to image structural atrophy using techniques such as diffusion tensor imaging (DTI) and voxel-based morphometry (VBM). A VBM study that compared gray matter density between cognitively intact APOE4/E3 carriers and APOE3 homozygotes of all ages (age 19 to 80) showed reduced gray matter density in carriers of the APOE4 allele in right medial temporal and bilateral frontotemporal regions [206]. Another study demonstrated that the presence of an APOE4 allele in non-demented older adults was also associated with decreases in cognition joint with white/gray matter changes in the medial temporal cortex using VBM and DTI [207]. A similar VBM study more recently demonstrated that late-onset AD patients displayed a selective pattern of parahippocampal white matter loss, while early-onset AD patients experienced a more widespread pattern of posterior white matter atrophy. Among both AD groups, APOE4 positivity was associated with a greater parahippocampal white matter loss, supporting the contention that the APOE4 effect is restricted to parahippocampal white matter regions and not related to age of onset [208]. In MCI patients, left hippocampus grey matter atrophy was found to exert a stronger effect than the right hippocampus or bilateral basal forebrain in the prediction of amnestic MCI occurrence, and this left hippocampal atrophy was accentuated in APOE4 carriers relative to noncarriers [209]. This hippocampus-specific pattern of cerebral atrophy was also found in mild AD APOE4 carriers as opposed to APOE4 noncarriers who tended to exhibit greater frontoparietal atrophy [210]. In parallel, an emerging AD Neuroimaging Initiative (ADNI) study showed that APOE4 positive amnestic MCI patients with more brain atrophy were at greatest risk of functional degradation [211], highlighting the value of genetic and volumetric MRI information as predictors of disease conversion to AD.

Recent extensions to these volumetric studies described the implication of the APOE gene on brain atrophy in relation with AD cerebrospinal fluid (CSF) biomarkers levels at different disease stages. This emerging line of research finds compelling support in the recent ADNI-derived demonstration that the APOE gene reached genome-wide significance for association with CSF levels of both A$\beta$ (1–42) and tau [212]. Interestingly, another ADNI study looking to define the genetic backgrounds to normal cognition, MCI (AD disease stages), and AD in relation to CSF levels found lower CSF A$\beta$ (1–42) levels with APOE4 gene dose in each disease stage. Moreover, AD patients who were APOE4 homozygotes exhibited elevated total-tau (t-tau) and phosphorylated-tau (p-tau) 181 levels [213]. This is consistent with previous findings of a significant age $*$ APOE4 genotype interaction for p-tau231, isoprostane, and t-tau CSF concentrations increased with age [214]. In keeping with this notion, cognitively intact older adults with reduced CSF A$\beta$ (1–42) levels were more likely to be APOE4 positive (48% versus 11% in high A$\beta$ (1–42) levels older adults), to exhibit increased whole brain loss, increased ventricular expansion, and faster hippocampal atrophy rates [215]. Similarly, APOE4-related decreased CSF A$\beta$ (1–42) and increased tau concentrations were associated with significantly higher rates of brain tissue loss that were both regional as well as disease stage specific [216]. Conversely, APOE2 carriers had slower rates of hippocampal atrophy concomitant with decreased preclinical AD pathology (i.e., higher CSF levels of A$\beta$ (1–42), lower CSF p-tau and t-tau concentrations) [217]. It therefore seems that along with genetics and volumetric MRI, CSF biomarkers of AD provide valuable quantitative measurements for early detection/disease progression across disease stages.

Functional neuroimaging findings in APOE4 carriers have also abounded in the last decade. A recent study conducted with non-demented older adults found an association between APOE4 and decline in regional cerebral blood flow (rCBF) over time in brain regions especially susceptible to pathological changes in AD [218]. Accelerated rates of decline in brain functions of APOE4 carriers were suggested to contribute to an increased risk of AD and a younger age at onset [218]. These findings are based on a previous experiment conducted with a group of healthy elderly subjects among whom APOE4 carriers exhibited significantly different patterns of brain activation during a nonverbal memory task. Interestingly, these differences in brain activation were not thought to reflect task difficulty, but were rather interpreted as memory-related alterations of cognitive processing that may result from subclinical incipient AD pathology and/or APOE-related neurophysiologic heterogeneity [219]. Other evidence suggests that baseline metabolic reductions in the entorhinal cortex (EC) accurately predicted the conversion from normal aging to MCI. At follow-up, those who declined showed memory impairment and hypometabolism in temporal lobe neocortex and hippocampus particularly in APOE4 carriers [198]. These convergent cognitive and neuroanatomic findings support the notion that APOE genotype modulates the clinical phenotype of AD through influence on selective brain networks [210] and highlights the influence of genetic variance on imaging, cognitive measures, and risk for AD.

In sum, findings on the role of APOE on cognition have converged to highlight its manifest involvement in AD-prone memory and learning functions. Indeed, these APOE-related cognitive alterations were found to be concomitant with reduced cerebral metabolism, impoverished neuronal interconnections as well as damaged cerebral vasculature particularly exacerbated in medial temporal brain structures. Owing to substantial technical advances made over recent years, prevention of AD could greatly benefit from our acquired ability to relate genetic variances with abnormal brain neurophysiology patterns in cognitively intact individuals.

## 5. APOE and Other Neurodegenerative Diseases

*5.1. Other Dementias.* Next to AD, one of the leading causes of neurodegenerative dementia is Lewy body dementia (LBD). As its name clearly points on, the central pathological hallmark is the cortical Lewy bodies, as opposed to the

classical Lewy bodies described in Parkinson's disease [143], which are intracytoplasmic (ubiquitin-positive) aggregates of $\alpha$-synuclein that accumulates in the substancia nigra. While they contain less NFT, the majority of LBD brains contain as much SP as in AD brains [220]. Moreover, APOE4 is consistently found associated with LBD [221–223].

Frontotemporal dementia (FTD) represents a heterogeneous group of neurodegenerative disorders characterized pathologically by frontal and/or temporal lobes atrophy and their tau isoforms pattern. Indeed, while specific FTD tauopathies typically result from the pathological aggregation and phosphorylation of one or two tau protein isoforms, all 6 tau isoforms are hyperphosphorylated in AD (for a review of FTDs classification see [224]). The best known FTD-associated diseases that will be reviewed here for their associations with APOE are Pick's disease, corticobasal degeneration (CBD), and progressive supranuclear palsy (PSP).

Pick's disease (PiD) is characterized by ballooned neurons named Pick cells that are swollen due to the presence of cytoplasmic inclusions containing tau proteins (Pick bodies). APOE associations with PiD risk or age at onset are not consistently found [225, 226]. Of note, Farrer et al. reported that the APOE4 frequency is higher in PiD than in controls (but lower than in AD) and that the number of *APOE4* allele copies is inversely proportional to the age at onset [227]. Consistent with results from Singleton et al. study in LBD [223], Gustafson et al. also found that the APOE4 allele frequency was higher in PiD than in controls, but with APOE4/4 and APOE2 frequencies being, respectively, lower and higher than those reported for AD [228].

The next two FTDs, CBD and PSP, are also referred to as Parkinson-plus diseases due to parkinsonism symptoms. Differential diagnostic between AD, CBD, PSP, and PD is clinically difficult. However, CBD and PSP pathologies manifest themselves in tau-positive astroglial and neuronal inclusions. CBD and PSP differ both by the form of their tau inclusions—namely, "doughnut-shaped" in CBD as opposed to "tuft-shaped" in PSP—and by their intracerebral distribution [229]. Two studies illustrate particularly well the controversy about APOE4 association with CBD. On one hand, Pickering-Brown et al. did not find any association between APOE4 and clinical expression of CBD [226]. On the other hand, Schneider et al. reported a higher APOE4 allele frequency in 11 CBD cases [230]. To date, evidence of an association between PSP and APOE genotype is scarce, mostly due to small sample size [226, 231–235]. However, a higher frequency of the APOE2 allele (but not the APOE4 allele) in PSP relative to controls was found in a Japanese population [236].

Parkinson's disease, 2nd age-related neurodegenerative disorder in importance after AD and 1st extrapyramidal disorder, is also associated with dementia. Parkinson's disease dementia (PDD) accounts for 0.2 to 0.5% of dementia cases in the general population over the age of 65 and affects 24% to 31% of PD patients [237]. The risk of dementia is 4 to 6 times higher in PD patients than in controls [238]. More than one-third of PD patients meet criteria for AD and the differential diagnostic between PDD with and without AD is difficult [239, 240]. These results conducted to the rise of associations studies between APOE polymorphism and PD. However, these studies have yielded mixed results. While APOE polymorphism was not associated with PD in several studies [143, 241–248], others did find associations but differed in the terms of this association. Indeed, lower E4 allele frequency and higher E4 allele frequency were, respectively, associated in sporadic and familial PD with cognitive decline [249]. Dementia in PD was repetitively associated with the E4 allele [250–253] and also with the E2 allele [254]. Age at onset appeared to be modulated by APOE genotype (earlier onset E4 > E3 > E2) [251, 252, 255, 256] and by sex [257]. Moreover, two meta-analyses sought to further corroborate the association between APOE and the risk of PD and dementia in PD. The first one [258] confirmed previous results indicating that APOE2, which is protective in AD, increases the risk of PD [254, 259]. The more recent one [260] acknowledged that APOE4 is significantly associated with an increased risk of dementia in PD but the authors warn that publication bias and heterogeneous source of data could have confounded this result.

As regard to Huntington's disease [261], another well-known neurodegenerative extrapyramidal movement disorder with neuronal intranuclear inclusion and late dementia, the APOE4 allele has been associated with a later age at onset [262], whereas the E2 and E3 alleles appear to require other factors in order to modulate age at onset [263, 264]. Finally, multiple system atrophy [265], a rare Parkinson-plus extrapyramidal disorder characterized mostly by brainstem glial Lewy body-like inclusions and subsidiary tau inclusions, has not been associated with any APOE allele [231, 232, 266].

*5.2. Other Neurodegenerative Disorders.* Amyotrophic lateral sclerosis (ALS), the most common motor neuron disease, also presents $\alpha$-synuclein-positive inclusions, and, notably, 5% of patients will develop an FTD [267]. The association between APOE and ALS risk is controversial [268–272]. Most striking results are the association with age at onset, with the APOE4 and APOE2 alleles, respectively, decreasing [270, 272] and increasing the age [270, 273], as well as the finding that APOE plasma levels (but not APOE genotype) were correlated with a faster rate of deterioration and shortened survival time [274]. Multiple sclerosis (MS) has also been tested for its association with APOE genotype given the importance of inflammation for the disease process and the affliction of the myelin, which is vulnerable to lipid deficiency. There is a relative agreement on the negative effect of the APOE4 allele on disease severity and progression rate [275–279], but a single cross-sectional study showed that homozygosis for this allele increases both the risk for and the rate of progression of MS [280]. Contradictory results to these reported findings have also been published [241, 265, 278, 281–284].

The last neurodegenerative disease investigated in this paper section is age-related macular degeneration (ARMD). ARMD is the leading cause of vision loss in the elderly in developed countries. ARMD is associated with druse, an extracellular deposit primarily composed of activated

Table 3: Summary of the most common neurodegenerative diseases of the CNS, some distinctive features, and their associations with the human APOE isoforms.

| Neurodegenerative disease | Pathological and clinical characteristics | | APOE isoform association | | | |
|---|---|---|---|---|---|---|
| | Protein deposition | Dementia | Risk[1] | Age at onset[1] | Dementia | Severity/progression rate |
| AD | Extracellular amyloid-$\beta$ ("SP") NCI tau ("NFT") | ++ | $\varepsilon4\nearrow$ (dose effect) | $\varepsilon4\searrow$ $\varepsilon2\nearrow$ (dose effect) | | |
| PiD | NCI tau ("Pick Body") | ++ | $\varepsilon4\nearrow$(4/4<, 2/2>/-AD) | $\varepsilon4\searrow$ (dose effect) | | |
| CBD | GCI (+NCI) tau ("doughnut") | ++ | $\varepsilon4\nearrow$ | | | |
| PSP | GCI (+NCI) tau ("tuft") | + | $\varepsilon2\nearrow$ | | | |
| LBD | NCI $\alpha$-synuclein ("non- classical LB") (+ SP, + NFT) | ++ | $\varepsilon4\nearrow$(4/4<, 2/2>/-AD) | | | $\varepsilon4\searrow$ survival |
| MSA | GCI (+NCI) $\alpha$-synuclein ("Papp-Lantos Body") (GCI + NCI tau) | (+) | | $\varepsilon4\nearrow$ | | |
| PD | NCI $\alpha$-synuclein (classical LB) | + | ($\varepsilon4\nearrow$) $\varepsilon2\nearrow$ | $\varepsilon4\searrow$ (> $\varepsilon3$> $\varepsilon2$) | $\varepsilon4\nearrow$ | |
| HD | Neuronal intranuclear huntingtin | + | | $\varepsilon4\nearrow$ | | |
| ALS | (NCI TDP-43) | 5% FTD | $\varepsilon4\nearrow$ | $\varepsilon4\searrow$ $\varepsilon2\nearrow$ | | |
| MS | | | 4/4$\nearrow$ | | | $\varepsilon4\nearrow$ severity |
| ARMD | Extracellular amyloid-$\beta$ ("drusen") | | $\varepsilon4\searrow$ $\varepsilon2\nearrow$ | $\varepsilon4\nearrow$ $\varepsilon2\searrow$ | | |

AD: Alzheimer's disease; PiD: Pick's disease; CBD: Corticobasal dementia; PSP: Progressive supranuclear palsy; LBD: Lewy body dementia; MSA, multiple system atrophy; PD: Parkinson's disease; HD: Huntington's disease; ALS: Amyotrophic lateral sclerosis; MS: Multiple sclerosis; ARMD: Age-related macular degeneration; NCI: neuronal cytoplasmic inclusion; GCI: glial cytoplasmic inclusion; SP: senile plaque; NFT: neurofibrillary tangle; LB: Lewy body; ++ indicates very present, + moderately present, (+) present in a subset; [1]the parentheses indicate that the association is less consistently found.

complement components, A$\beta$ peptide, APOE, and ubiquitin. It is noteworthy that the molecular composition of the drusen is highly similar to that of the SP found in AD. Interestingly, AD and ARMD also share some cardiovascular risk factors. These evidence prompted association studies between APOE genotype and ARMD. Two associations were reproductively observed: the *APOE4* allele is less frequent among ARMD patients [285] and reduces the risk of developing the disease by up to 40%, whereas the *APOE2* allele is more frequent and increases the risk by up to 20% [286]. Kovács et al. stressed the opposite frequencies between *APOE4/APOE2* and ARMD/AD and highlighted the rare occurrence of ARMD among AD patients [287]. As for Baird et al., they pointed that APOE is the most consistently associated gene with ARMD. They showed that *APOE4* is protective against ARMD and/or increases its age at onset, whereas *APOE2* decreases the age at onset of ARMD [288]. Interestingly, Malek et al. presented a mouse model of ARMD in which aged human APOE transgenic mice were fed a high-fat cholesterol-rich diet [289]. They found that the mice displayed APOE isoform-dependent pathologies of different severity. Mice expressing the human APOE4 were the most severely affected ones; they developed changes that mimicked ARMD pathology, but that could not be attributed solely to age or high-fat cholesterol-rich factors.

In sum, neurodegenerative diseases, with or without dementia, encompass a large spectrum of disorders. The more we learn about these pathologies, the more similarities and differences are found, which result in a constant reclassification of these diseases. One common pathological hallmark is the deposition of misfolded protein (amyloid-$\beta$, tau, $\alpha$-synuclein, etc.). Through the modulation of disease risk, age at onset, and/or rate of progression, APOE is involved in an isoform-dependent manner in all the diseases reviewed here (Table 3). With the noticeable exception of ARMD, the APOE4 allele is predominantly deleterious, whereas the APOE2 is beneficial. This evidence suggests that APOE polymorphism confers a risk susceptibility not specific to AD, but to neurodegenerative disease in general.

## 6. Conclusion

We have reviewed the postulated roles of APOE4 in the development of different forms of dementia and particularly, in sporadic AD. While age remains a key determinant that modulates the onset and expression of AD pathology, genetic risk factors such as APOE4 appear to play a central role in the pathophysiology of this disease, years, if not decades, before clinical diagnosis. The combined use of genetic profiling and gene targeting will allow scientists to better target the biochemical mechanisms regulating the loss of synapses and the accumulation of amyloid deposits in the aging and diseased brain. The discovery that compounds such as estrogens, probucol, indomethacin, and even rosiglitazone can significantly induce APOE synthesis and secretion both *in vitro* and *in vivo*, and enhance cognitive performances [290–294] in small clinical trials certainly suggested a potential therapeutic role for APOE modulators in AD. The surprising convergence of these biochemical, pharmacogenomic, and clinical observations raises exciting new possibilities and certainly interesting new therapeutic avenues for the treatment and prevention of a genetically-defined, sizeable subset of Alzheimer's disease subjects.

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