



## APOE-TOMM40 in the Pharmacogenomics of Dementia

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### Abstract

Polymorphic variants present in the APOE-TOMM40 region (19q13.2) are implicated in Alzheimer's disease (AD) and have been shown to modify disease risk, age at onset of symptoms, and the therapeutic response to conventional drugs. We have investigated the structure of the APOE-TOMM40 region and the influence of APOE and TOMM40 poly T variants (rs10524523) in the therapeutic response to a multifactorial treatment in 920 Spanish patients with AD. The frequencies of TOMM40 poly T genotypes were: 18.37% S/S, 7.83% S/L, 38.80% S/VL, 1.52% L/L, 7.17% L/VL, and 26.31% VL/VL. The most frequent APOE-TOMM40 associations were as follows: 82% APOE-3/3 with S/S, 63% with S/VL, and 40% with VL/VL; 90% APOE-3/4 with S/L, 57% with L/VL, and 43% with VL/VL; and 100% APOE-4/4 with L/L. Globally, the response rate was about 59%, with no difference between females and males. APOE-4 carriers were found to be the worst responders (45-56%) whereas APOE-3/3 carriers were the best responders (70%) for a transient period of cognitive improvement (<12 m). Among TOMM40 variants, S/S carriers were the best responders (70%), followed by S/VL (61%), VL/VL (57%), and L/VL carriers (51%). The worst responders were those patients harboring the L/L genotype (35%). Therefore, specific APOE-TOMM40 poly T variants exert a powerful influence on the therapeutic response to conventional treatments in AD.

**Keywords:** Alzheimer's disease; APOE; Multifactorial treatment; Pharmacogenomics; Therapeutic Response Rate; TOMM40

**Abbreviations:** A2BP1: Ataxin-2 Binding Protein 1; ABAT: 4-aminobutyrate aminotransferase; ABCA7: ATP-binding cassette, sub-family A (ABC1), member 7; ABCB1: ATP-binding cassette, sub-family B (MDR/TAP), member 1; ABCs: ATP-binding cassette family; ABO: ABO blood group (transferase A, alpha 1-3-N-acetylgalactosaminyltransferase; transferase B, alpha 1-3-galactosyltransferase); ACE: Angiotensin I Converting Enzyme; AD: Alzheimer's Disease; AGT: Angiotensinogen; APOC1: Apolipoprotein C-I; APOC2: Apolipoprotein C-II; APOC4: Apolipoprotein C-IV; APOE: Apolipoprotein E; APOs: Apolipoproteins; APP: Amyloid beta (A4) Precursor Protein; BIN1: Bridging Integrator 1; CD2AP: CD2-associated protein; CDH2: Cadherin 2, type 1; CLU: Clusterin; CNVs: Copy Number Variations; CR1: Complement Component (3b/4b) Receptor 1; CRMP1: Collapsin Response Mediator Protein 1; CRP: C-reactive protein; CVD: Cardiovascular Disease; CYP2D6: Cytochrome P450, family 2, subfamily D, polypeptide 6; CYPs: Cytochrome P450 family; DMRT1: Doublesex and mab-3 related transcription factor 1; EO-FAD: Early-onset Familial Alzheimer's Disease; EPHA5: EPH receptor A5; EPHA6: EPH receptor A6; ERMP1: Endoplasmic Reticulum metalloproteinase 1; GWAS: Genome-wide Association Studies; HMGCR: 3-hydroxy-3-methylglutaryl-CoA reductase; ILs: Interleukins; L: Long; LOAD: Late Onset Alzheimer's Disease; MAPT: Microtubule-associated protein tau; MS4A4E: Membrane-spanning 4-domains, subfamily A, member 4E; MS4A6A: Membrane-spanning 4-domains, subfamily A, member 6A; MTHFR: Methylene tetrahydrofolate Reductase (NAD(P)H); NATs: N-acetyltransferase family; NFT: Neurofibrillary Tangles; NOS: Nitric Oxide Synthase; PICALM: Phosphatidylinositol binding clathrin assembly protein; PRNP: Prion protein; PSEN1: Presenilin 1; PSEN2: Presenilin 2; S: Short; SLCs: Solute Carrier Family; SNPs: Single-nucleotide Polymorphisms; TOMM40: Translocase of Outer Mitochondrial Membrane 40 homolog (yeast); UGTs: UDP glucuronosyltransferase family; VL: Very Long; VLDLR: Very Low Density Lipoprotein Receptor

### Introduction

The genes involved in the pharmacogenomic response to drugs in Alzheimer's disease (AD) fall into five major categories: (i) genes associated with AD pathogenesis and neurodegeneration (*APP*, *PSEN1*, *PSEN2*, *MAPT*, *PRNP*, *APOE* and others); (ii) genes associated with the mechanism of action of drugs (enzymes, receptors, transmitters, messengers); (iii) genes associated with drug metabolism (phase I (*CYPs*) and phase II reactions (*UGTs*, *NATs*); (iv) genes associated with drug transporters (*ABCs*, *SLCs*); and (v) pleiotropic genes involved in multifaceted cascades and metabolic reactions (*APOs*, *ILs*, *MTHFR*, *ACE*, *AGT*, *NOS*, etc) [1-4]. The genetic and epigenetic defects identified so far in AD include Mendelian mutations, susceptibility single-nucleotide polymorphisms (SNPs), mitochondrial DNA (mtDNA) mutations, and epigenetic changes. Mendelian mutations affect genes directly linked to AD, including mutations in the amyloid beta precursor protein (*APP*) gene (21q21)(*AD1*), mutations in the presenilin 1 (*PSEN1*) gene (14q24.3)(*AD3*), and mutations in the presenilin 2 (*PSEN2*) gene (1q31-q42) (*AD4*) [5-10]. *PSEN1* and *PSEN2* are important determinants of  $\gamma$ -secretase activity responsible for proteolytic cleavage of *APP* and *NOTCH* receptor proteins. Mendelian mutations are very rare in AD (1:1000). Mutations in exons 16 and 17 of the *APP* gene appear with a frequency of 0.30% and 0.78%, respectively, in AD patients. Likewise, *PSEN1*, *PSEN2*, and

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microtubule-associated protein Tau (*MAPT*) (17q21.1) mutations are present in less than 2% of the cases. In the Alzgene database [11] there are over 600 genes potentially associated with AD, of which the top ten are *APOE* (19q13.2), *BIN1* (2q14), *CLU* (8p21-p12), *ABCA7* (19p13.3), *CRI* (1q32), *PICALM* (11q14), *MS4A6A* (11q12.1), *CD33* (19q13.3), *MS4A4E* (11q12.2), and *CD2AP* (6p12) [6]. Potentially defective genes associated with AD represent about 1.39% (35,252.69 Kb) of the human genome, which is integrated by 36,505 genes (3,095,677.41 Kb). The highest number of AD-related defective genes concentrate on chromosomes 10 (5.41%; 7337.83 Kb), 21 (4.76%; 2,289,15 Kb), 7 (1.62%; 2,584.26 Kb), 2 (1.56%; 3,799.67 Kb), 19 (1.45%; 854.54 Kb), 9 (1.42%; 2,010.62 Kb), 15 (1.23%; 1,264.4 Kb), 17 (1.19%; 970.16 Kb), 12 (1.17%; 1,559.9 Kb), and 6 (1.15%; 1,968.22 Kb) [3]. Ten novel private pathogenic copy number variations (CNVs) in 10 early-onset familial Alzheimer's disease (EO-FAD) families overlapping a set of genes (*A2BP1*, *ABAT*, *CDH2*, *CRMP1*, *DMRT1*, *EPHA5*, *EPHA6*, *ERMP1*, *EVC*, *EVC2*, *FLJ35024* and *VLDLR*) have also been identified [3].

Multiple polymorphic risk variants can increase neuronal vulnerability to premature death. Among these susceptibility genes, the apolipoprotein E (*APOE*) gene (19q13.2) (*AD2*) is the most prevalent as a risk factor for AD, especially in those subjects harboring the *APOE-4* allele [12,13], whereas carriers of the *APOE-2* allele are prone to longevity [14] and might be protected against dementia [15-17].

*APOE* is the prototypical paradigm of a pleiotropic gene with multifaceted activities in physiological and pathological conditions [5,18]. *APOE* is consistently associated with the amyloid plaque marker for AD. *APOE-4* may influence AD pathology by interacting with APP metabolism and A $\beta$  accumulation, enhancing hyperphosphorylation of tau protein and neurofibrillary tangles (NFT) formation, reducing choline acetyltransferase activity, increasing oxidative processes, modifying inflammation-related neuroimmunotrophic activity and glial activation, altering lipid metabolism, lipid transport and membrane biosynthesis in sprouting and synaptic remodeling, and inducing neuronal apoptosis [5,18-21]. In addition, multiple studies over the past two decades have demonstrated that *APOE* variants may affect the therapeutic response to anti-dementia drugs [20-30].

The TOMM40 locus is located adjacent to and in linkage disequilibrium with *APOE* on 19q13.2. A poly T repeat in an intronic polymorphism (rs10524523) (intron 6) in the TOMM40 gene, which encodes an outer mitochondrial membrane translocase involved in the transport of amyloid- $\beta$  and other proteins into mitochondria, has been implicated in AD [31-44], and *APOE-TOMM40* genotypes have been shown to modify disease risk and age at onset of symptoms [32-37,45], although the latter assumption needs replication due to contradictory results [36,46-49]. A fixed-effect meta-analysis approach showed that rs4420638 at the TOMM40/*APOE*/*APOC1* gene locus is associated with longevity [50,51]. Two independent associations with cognitive decline were found among European-Americans in the 19q13.32 region (rs769449, *APOE* intron; and rs115881343, TOMM40 intron); rs769449 was also associated with cognitive decline among African-Americans, but rs115881343 was not [52]. The *APOE-TOMM40* genomic region is associated with cognitive aging [53] and with pathological cognitive decline [54].

Linnertz et al. [55] investigated the genomic region spanning the TOMM40 and *APOE* genes, to determine whether intronic poly T (rs10524523) within this region affects expression of the *APOE* and TOMM40 genes in the brain of patients with late-onset Alzheimer's disease (LOAD). The expression of both genes was significantly increased with disease. Mean expression of *APOE* and TOMM40 mRNA

levels was higher in VL homozygotes compared with S homozygotes in the temporal and occipital cortex from normal and LOAD cases. The 523 VL poly T resulted in significantly higher expression than the S poly T. These results suggest that the 523 locus may contribute to LOAD susceptibility by modulating the expression of TOMM40 and/or *APOE* transcription [55]. Recent studies also suggest that the TOMM40 gene rs10524523 ("523") variable length poly T repeat polymorphism is associated to a certain extent with similar AD phenotypes as those reported for *APOE*, such as brain white matter changes [56,57] or different biomarkers [58-61]. In addition, the TOMM40 rs2075650 G allele may be a risk factor for the development of depression [62] and sporadic inclusion body myositis [63]. Different markers at the 19q13-q13.2 chromosomal region, including the rs2075650 and rs157590 (TOMM40), rs1064725 (*APOC1*), and rs429358 and rs7412 (*APOE*) SNPs also show association with primary progressive aphasia and the behavioural variant frontotemporal dementia [64].

The TOMM40/*APOE*/*APOC1* loci have been associated with C-reactive protein (CRP), a heritable biomarker of systemic inflammation and a predictor of cardiovascular disease (CVD) [65]. Genome-wide association studies (GWAS) have identified LDL-cholesterol-associated loci near HMGCR, ABO and TOMM40 [66], and also an association of TOMM40 with blood lipid levels [67,68] and body mass index [69]. Genetic variants in TOMM40/*APOE*-C1-C2-C4 genes have also been found to be associated with multiple cardiovascular-related traits [70-72].

In the present study we have investigated the structure of the *APOE-TOMM40* region in Spanish patients with dementia, and the influence of polymorphic variants in this genomic segment on the therapeutic response to a multifactorial treatment adapted to the pathogenic profile of the patients. The main aims of the study were: (i) structural analysis of the *APOE-TOMM40* region (distribution and frequency of major genotypes, with special emphasis on TOMM40 poly T variants) in the Spanish population with dementia; and (ii) *APOE*- and TOMM40 poly T1/T2-related therapeutic response to a multifactorial therapy in AD.

## Materials and Methods

### Patients and treatment

In this retrospective study, selected Spanish patients with AD (DSM-IV/NINCDS-ADRDA criteria), treated at the EuroEspes Biomedical Research Center (period 1992-2011)(N=920, age: 71.11  $\pm$  9.09 years, range: 41-96 years; 556 females, age: 71.14  $\pm$  9.07 years, range: 43-96 years; and 364 males, age: 71.06  $\pm$  9.13 years, range: 41-94 years), received for one year a multifactorial therapy integrated by CDP-choline (500 mg/day, p.o.) (choline donor and intermediate metabolite in DNA synthesis and repair), Piracetam (1600 mg/day, p.o.) (nootropic drug), Sardilipin (E-SAR-94010) (250 mg, t.i.d.) (nutraceutical with lipid-lowering effects and anti-atherosclerotic properties, Patent ID: P9602566) [3,24,73], and Animon Complex<sup>®</sup> (2 capsules/day) (a nutraceutical compound integrated by a purified extract of *Chenopodium quinoa* (250 mg), ferrous sulphate (38.1 mg equivalent to 14 mg of iron), folic acid (200  $\mu$ g), and vitamin B<sub>12</sub> (1  $\mu$ g) per capsule (RGS: 26.06671/C)). Patients with chronic deficiency of iron (<35  $\mu$ g/mL) (4.45%), folic acid (<3 ng/mL) (5.43%) or vitamin B<sub>12</sub> (<170 pg/mL) (4.13%) received an additional supplementation of iron (80 mg/day), folic acid (5 mg/day) and B complex vitamins (B<sub>1</sub>, 15 mg/day; B<sub>2</sub>, 15 mg/day; B<sub>6</sub>, 10 mg/day; B<sub>12</sub>, 10  $\mu$ g/day; nicotinamide, 50 mg/day), respectively, to maintain stable levels of serum iron (50-150  $\mu$ g/mL), folic acid (5-20 ng/mL) and vitamin B<sub>12</sub> levels (500-1000 pg/mL) in order to avoid the negative influence of these metabolic

factors on cognition [27,28]. Patients with hypertension (>150/85 mmHg) (28.04%) received enalapril (5-20 mg/day, p.o.); patients with hypercholesterolemia (>220 mg/dL) (43.48%) received atorvastatin (10-20 mg/day); patients with diabetes (glucose >105 mg/dL) (24.24%) received metformin (850-1700 mg/day, p.o.); and patients (<3%) with other ailments (e.g. hypothyroidism, hyperuricemia, etc.) received the appropriate treatment according to their medical condition. Psychotropic drugs (antidepressants, neuroleptics, hypnotics, sedatives) were avoided, and less than 5% of the patients required a transient treatment with benzodiazepines for short periods of

time. Blood pressure, psychometric assessment (Mini-Mental State Examination, MMSE; Alzheimer's Disease Assessment Scale, ADAS; Hamilton Rating Scale-Depression, HAM-D; Hamilton Rating Scale-Anxiety, HAM-A), and blood parameters Table 1 were evaluated prior to treatment (baseline) and after 1, 3, 6, 9, and 12 months of treatment. All patients and/or their legal representatives gave informed consent for genotyping and clinical assessment.

### APOE genotyping

Genomic DNA was extracted from EDTA-treated blood samples

Parameter (normal range)	Total	Females	Males	Differences
N	920	556	364	
Age (years) Range	71.11 ± 9.09 41-96	71.14 ± 9.07 43-96	71.06 ± 9.13 41-94	P=0.74
Systolic Blood Pressure (SBP) (mm Hg) (120-160)	140.61 ± 20.64 <120: 122 (13.26%) >160: 188 (20.43%)	140.10 ± 20.66 74 (13.31%) 105 (18.88%)	141.38 ± 20.62 48 (13.18%) 83 (22.80%)	P=0.41 P(χ <sup>2</sup> )=0.92 P(χ <sup>2</sup> )=0.15
Diastolic Blood Pressure (DBP) (mm Hg) (70-85)	79.49 ± 11.89 <70: 71 (7.72%) >85: 258 (28.04%)	79.52 ± 11.70 40 (7.19%) 142 (25.54%)	79.64 ± 11.62 31 (8.51%) 116 (31.87%)	P=0.71 P(χ <sup>2</sup> )=0.52 P(χ <sup>2</sup> )=0.04
Pulse (bpm) (60-100)	67.93 ± 12.23 <60: 224 (24.34%) >100: 15 (1.63%)	68.74 ± 11.65 117 (21.04%) 8 (1.44%)	66.69 ± 12.96 107 (29.39%) 7 (1.92%)	P=0.001 P(χ <sup>2</sup> )=0.005 P(χ <sup>2</sup> )=0.60
Weight (Kg)	69.70 ± 12.93	65.39 ± 11.76	76.54 ± 11.71	P<0.001
Height (m)	1.58 ± 0.09	1.53 ± 0.06	1.66 ± 0.06	P<0.001
Body mass index (BMI) (Kg/m <sup>2</sup> )	27.88 ± 4.45	28.01 ± 4.95	27.68 ± 3.49	P=0.72
Underweight (15-18.5)	10 (1.08%)	8 (1.44%)	2 (0.55%)	P(χ <sup>2</sup> )=0.33
Normal weight (18.5-25)	309 (33.59%)	185 (33.27%)	124 (34.07%)	P(χ <sup>2</sup> )=0.83
Overweight (25-30)	382 (41.52%)	213 (38.31%)	169 (46.43%)	P(χ <sup>2</sup> )=0.01
Obese Class I (moderate)(30-35)	162 (17.61%)	102 (18.35%)	60 (16.48%)	P(χ <sup>2</sup> )=0.48
Obese Class II (severe)(35-40)	49 (5.33%)	41 (7.37%)	8 (2.20%)	P(χ <sup>2</sup> )=0.001
Obese Class III (very severe)(>40)	8 (0.87%)	7 (1.26%)	1 (0.27%)	P(χ <sup>2</sup> )=0.15
Glucose (Glc) (mg/dL) (70-105)	101.77 ± 27.36 <70: 6 (0.65%) >105: 223 (24.24%)	98.74 ± 25.03 3 (0.54%) 103 (18.52%)	106.37 ± 30.02 3 (0.82%) 120 (32.97%)	P<0.001 P(χ <sup>2</sup> )=0.68 P(χ <sup>2</sup> )=0.0005
Cholesterol (Cho) (mg/dL) (140-220)	221.17 ± 47.75 <140: 31 (3.37%) >220: 400 (43.48%)	231.69 ± 46.28 9 (1.62%) 336 (60.43%)	205.12 ± 45.48 22 (6.04%) 64 (17.58%)	P<0.001 P(χ <sup>2</sup> )=0.0005 P(χ <sup>2</sup> )=0.0005
HDL-Cholesterol (mg/dL) (35-75)	53.88 ± 14.43 <35: 59 (6.41%) >75: 86 (9.35%)	58.17 ± 14.45 15 (2.67%) 72 (12.95%)	47.39 ± 11.76 44 (12.09%) 14 (3.85%)	P<0.001 P(χ <sup>2</sup> )=0.0005 P(χ <sup>2</sup> )=0.0005
LDL-Cholesterol (mg/dL) (80-160)	144.37 ± 41.62 <80: 44 (4.78%) >160: 318 (34.56%)	150.47 ± 41.49 16 (2.88%) 219 (39.39%)	135.16 ± 40.16 28 (7.69%) 99 (27.20%)	P<0.001 P(χ <sup>2</sup> )=0.001 P(χ <sup>2</sup> )=0.0005
Triglycerides (TG) (mg/dL) (50-150)	113.51 ± 65.36 <50: 49 (5.33%) >150: 180 (19.56%)	111.56 ± 64.65 32 (5.75%) 97 (17.45%)	116.50 ± 66.41 17 (4.67%) 83 (22.80%)	P=0.38 P(χ <sup>2</sup> )=0.54 P(χ <sup>2</sup> )=0.05
Urea (BUN) (mg/dL) (15-30)	43.93 ± 13.52 >30: 825 (89.67%)	42.33 ± 12.53 466 (83.81%)	46.39 ± 14.60 359 (98.62%)	P<0.001 P(χ <sup>2</sup> )=0.0005
Creatinine (Cr) (mg/dL) (0.70-1.40)	0.92 ± 0.47 <0.70: 129(14.02%) >1.40: 56 (6.08%)	0.81 ± 0.17 123 (22.12%) 18 (3.24%)	1.08 ± 0.68 6 (0.82%) 38 (10.44%)	P<0.001 P(χ <sup>2</sup> )=0.0005 P(χ <sup>2</sup> )=0.0005
Uric Acid (UA) (mg/dL) (3.4-7.0)	4.27 ± 1.43 <3.4: 260 (28.26%) >7.0: 45 (4.89%)	3.81 ± 1.29 226 (40.65%) 13 (2.34%)	4.97 ± 1.35 34 (9.34%) 32 (8.79%)	P<0.001 P(χ <sup>2</sup> )=0.0005 P(χ <sup>2</sup> )=0.0005
Total Protein (T-Pro)(g/dL) (6.5-8.0)	6.92 ± 0.43 <6.5: 123 (13.37%) >8.0: 8 (0.87%)	6.94 ± 0.43 68 (12.23%) 5 (0.90%)	6.89 ± 0.43 55 (15.11%) 3 (0.82%)	P=0.07 P(χ <sup>2</sup> )=0.23 P(χ <sup>2</sup> )=0.80
Albumin (Alb) (g/dL) (3.5-5.0)	4.28 ± 0.29 <3.5: 7 (0.76%) >5.0: 7 (0.76%)	4.28 ± 0.29 3 (0.54%) 3 (0.54%)	4.28 ± 0.29 4 (1.10%) 4 (1.10%)	P=0.92 P(χ <sup>2</sup> )=0.44 P(χ <sup>2</sup> )=0.44
Calcium (Ca) (mg/dL) (8.1-10.4)	9.21 ± 0.44 <8.1: 1 (0.11%) >10.4: 14 (1.52%)	9.21 ± 0.45 0 (0%) 8 (0.87%)	9.18 ± 0.42 1 (0.27%) 6 (1.65%)	P=0.48 P(χ <sup>2</sup> )=0.39 P(χ <sup>2</sup> )=0.80
Phosphorus (P) (mg/dL) (2.5-5.0)	3.37 ± 0.54 <2.5: 29 (3.15%) >5.0: 7 (0.76%)	3.45 ± 0.52 14 (2.52%) 2 (0.36%)	3.25 ± 0.54 15 (4.12%) 5 (1.37%)	P<0.001 P(χ <sup>2</sup> )=0.18 P(χ <sup>2</sup> )=0.12

Aspartate Aminotransferase (GOT/ASAT) (IU/L) (10-40)	22.58 ± 20.14 <10: 2 (0.22%) >40: 40 (4.35%)	22.64 ± 23.24 2 (0.36%) 13 (2.34%)	22.49 ± 14.20 0 (%) 27 (7.42%)	P=0.30 P(χ <sup>2</sup> )=0.52 P(χ <sup>2</sup> )=0.0005
Alanine Aminotransferase (GPT/ALAT) (IU/L) (9-43)	23.80 ± 22.60 <9: 21 (2.28%) >43: 60 (6.52%)	22.07 ± 21.13 21 (3.77%) 24 (4.32%)	26.46 ± 24.47 0 (%) 36 (9.89%)	P<0.001 P(χ <sup>2</sup> )=0.0005 P(χ <sup>2</sup> )=0.001
Gamma-glutamyl transpeptidase (GGT) (IU/L) (11-50)	30.76 ± 41.97 <11: 65 (7.06%) >50: 110 (11.95%)	27.51 ± 41.01 57 (10.25%) 52 (9.35%)	35.69 ± 42.99 8 (2.20%) 58 (15.93%)	P<0.001 P(χ <sup>2</sup> )=0.0005 P(χ <sup>2</sup> )=0.003
Alkaline Phosphatase (ALP) (IU/L) (37-111)	78.34 ± 30.51 <37: 12 (1.30%) >111: 130 (14.13%)	79.93 ± 31.71 8 (1.44%) 79 (14.21%)	75.90 ± 28.46 4 (1.10%) 51 (14.01%)	P=0.01 P(χ <sup>2</sup> )=0.77 P(χ <sup>2</sup> )=0.93
Bilirubin (BIL) (mg/dL) (0.20-1.00)	0.74 ± 0.35 <0.20: 3 (0.37%) >1.00: 158(17.17%)	0.69 ± 0.30 2 (0.36%) 68 (12.23%)	0.82 ± 0.42 1 (0.27%) 90 (24.72%)	P<0.001 P(χ <sup>2</sup> )=0.82 P(χ <sup>2</sup> )=0.0005
Creatine Phosphokinase (CPK)(IU/L) (38-174)	85.41 ± 59.51 <38: 96 (10.43%) >174: 75 (8.15%)	82.51 ± 57.92 53 (9.53%) 38 (6.83%)	89.80 ± 61.67 43 (11.81%) 37 (10.16%)	P=0.20 P(χ <sup>2</sup> )=0.27 P(χ <sup>2</sup> )=0.08
Lactate Dehydrogenase (LDH) (IU/L) (200-480)	304.11 ± 71.75 <200: 16 (1.74%) >480: 30 (3.26%)	316.16 ± 68.13 5 (0.90%) 9 (1.62%)	285.74 ± 73.34 11 (3.02%) 21 (5.77%)	P<0.001 P(χ <sup>2</sup> )=0.02 P(χ <sup>2</sup> )=0.001
Na <sup>+</sup> (mEq/L) (135-148)	142.18 ± 2.47 <135: 7 (0.76%) >148: 15 (1.63%)	142.35 ± 2.36 3 (0.54%) 7 (1.26%)	141.91 ± 2.61 4 (1.10%) 8 (2.20%)	P=0.005 P(χ <sup>2</sup> )=0.44 P(χ <sup>2</sup> )=0.29
K <sup>+</sup> (mEq/L) (3.5-5.3)	4.35 ± 0.37 <3.5: 9 (0.98%) >5.3: 45 (4.89%)	4.29 ± 0.36 8 (1.44%) 0 (0%)	4.44 ± 0.37 1 (0.27%) 45 (12.36%)	P<0.001 P(χ <sup>2</sup> )=0.09 P(χ <sup>2</sup> )=0.0005
Cl <sup>-</sup> (mEq/L) (98-107)	104.18 ± 2.54 <98: 13 (1.41%) >107: 60 (6.52%)	104.36 ± 2.48 5 (0.90%) 42 (7.55%)	103.90 ± 2.62 8 (2.20%) 18 (4.94%)	P=0.01 P(χ <sup>2</sup> )=0.15 P(χ <sup>2</sup> )=0.13
Fe <sup>2+</sup> (μg/dL) (35-160)	84.65 ± 33.88 <35: 41 (4.45%) >160: 15 (1.63%)	80.04 ± 31.75 28 (5.03%) 8 (1.44%)	91.09 ± 36.12 13 (3.57%) 7 (1.92%)	P<0.001 P(χ <sup>2</sup> )=0.33 P(χ <sup>2</sup> )=0.60
Ferritin (ng/mL) (F: 11-307) (M: 24-336)	118.57 ± 136.38 <11: 26 (2.83%) >307: 44 (4.78%)	88.21 ± 109.60 <11: 26 (4.67%) >307: 11 (55.21%)	162.53 ± 158.09 <24: 23 (6.32%) >336: 33 (9.06%)	P<0.001 P(χ <sup>2</sup> )=0.36 P(χ <sup>2</sup> )=0.0005
Folate (ng/mL) (>3.00)	7.01 ± 3.88 <3: 50 (5.43%)	7.12 ± 3.79 30 (5.39%)	6.84 ± 4.01 20 (5.49%)	P=0.06 P(χ <sup>2</sup> )=0.94
Vitamin B <sub>12</sub> (pg/mL) (170-1000)	509.21 ± 314.45 <170: 38 (4.13%)	507.48 ± 312.21 24 (4.32%)	511.84 ± 318.23 14 (3.84%)	P=0.92 P(χ <sup>2</sup> )=0.74
Thyroid-stimulating Hormone (TSH) (μIU/mL) (0.20-4.50)	1.49 ± 2.86 <0.20: 33 (3.58%) >4.50: 18 (1.95%)	1.62 ± 3.58 24 (4.32%) 13 (2.34%)	1.29 ± 1.06 9 (2.47%) 5 (1.37%)	P=0.04 P(χ <sup>2</sup> )=0.15 P(χ <sup>2</sup> )=0.34
Thyroxine (T4) (ng/mL) (0.54-1.40)	0.94 ± 0.35 <0.54: 9 (0.98%) >1.40: 67 (7.28%)	0.93 ± 0.23 8 (1.44%) 26 (4.67%)	0.94 ± 0.48 1 (0.27%) 41 (11.26%)	P=0.41 P(χ <sup>2</sup> )=0.09 P(χ <sup>2</sup> )=0.0005
Red Blood Cell Count (RBC) (x10 <sup>6</sup> /μL) (3.80-5.50)	4.59 ± 0.45 <3.80: 27 (2.93%) >5.50: 26 (2.82%)	4.47 ± 0.39 21 (3.77%) 3 (0.54%)	4.78 ± 0.48 6 (1.65%) 23 (6.32%)	P<0.001 P(χ <sup>2</sup> )=0.07 P(χ <sup>2</sup> )=0.0005
Hematocrit (HCT) (%) (40.0-50.0)	41.84 ± 5.01 <40.0: 308(33.48%) >50.0: 26 (2.82%)	40.34 ± 3.35 248 (44.60%) 1 (0.18%)	44.14 ± 6.13 60 (16.48%) 25 (6.87%)	P<0.001 P(χ <sup>2</sup> )=0.0005 P(χ <sup>2</sup> )=0.0005
Hemoglobin (Hb) (g/dL) (13.5-17.0)	13.98 ± 1.39 <13.5: 324(35.22%) >17.0: 26 (2.82%)	13.50 ± 1.14 259 (46.58%) 0 (0%)	14.72 ± 1.42 65 (17.86%) 26 (7.14%)	P<0.001 P(χ <sup>2</sup> )=0.0005 P(χ <sup>2</sup> )=0.0005
Mean Corpuscular Volume (MCV) (fL) (80-100)	90.94 ± 5.06 <80: 16 (1.74%) >100: 32 (3.48%)	90.41 ± 5.00 9 (1.62%) 10 (1.80%)	91.75 ± 5.05 7 (1.92%) 22 (6.04%)	P<0.001 P(χ <sup>2</sup> )=0.79 P(χ <sup>2</sup> )=0.001
Mean Corpuscular Hemoglobin (MCH) (pg) (27.0-33.0)	30.48 ± 1.93 <27.0: 31 (3.37%) >33.0: 67 (7.28%)	30.27 ± 1.94 21 (3.77%) 44 (7.91%)	30.80 ± 1.88 10 (2.74%) 23 (6.32%)	P<0.001 P(χ <sup>2</sup> )=0.45 P(χ <sup>2</sup> )=0.36
Mean Corpuscular Hemoglobin Concentration (MCHC) (g/dL) (31.0-35.0)	33.46 ± 1.25 <31.0: 3 (0.33%) >35.0: 29 (3.15%)	33.40 ± 1.50 3 (0.54%) 10 (1.80%)	33.56 ± 0.71 0 (0%) 19 (5.22%)	P=0.13 P(χ <sup>2</sup> )=0.28 P(χ <sup>2</sup> )=0.004
Red Blood Cell Distribution Width (RDW) (%) (11.0-15.0)	13.21 ± 1.54 <11.0: 5 (0.54%) >15.0: 61 (6.63%)	13.21 ± 1.64 4 (0.72%) 40 (7.19%)	13.21 ± 1.38 1 (0.27%) 21 (5.77%)	P=0.78 P(χ <sup>2</sup> )=0.65 P(χ <sup>2</sup> )=0.42
White Blood Cell Count (WBC) (x10 <sup>3</sup> /μL) (4.0-11.0)	6.37 ± 1.89 <4.0: 49 (5.33%) >11.0: 16 (1.74%)	6.14 ± 1.85 36 (6.47%) 8 (1.44%)	6.71 ± 1.90 13 (3.57%) 8 (2.20%)	P<0.001 P(χ <sup>2</sup> )=0.07 P(χ <sup>2</sup> )=0.44

% Neutrophils (45.0-70.0)	61.66 ± 9.28 <45.0: 33 (3.58%) >70.0: 173(18.80%)	61.39 ± 8.90 19 (3.42%) 100 (17.98%)	62.07 ± 9.84 14 (3.84%) 73 (20.05%)	P=0.14 P( $\chi^2$ )=0.85 P( $\chi^2$ )=0.43
% Lymphocytes (20.0-40.0)	28.80 ± 8.20 <20: 124 (13.48%) >40: 80 (8.69%)	29.52 ± 8.02 63 (11.33%) 56 (10.07%)	27.69 ± 8.37 61 (16.76%) 24 (6.59%)	P<0.001 P( $\chi^2$ )=0.02 P( $\chi^2$ )=0.07
% Monocytes (3.0-10.0)	7.19 ± 2.10 <3.0: 11 (1.19%) >10.0: 79 (8.58%)	7.00 ± 2.08 11 (1.98%) 38 (6.83%)	7.49 ± 2.10 0 (0%) 41 (11.26%)	P<0.001 P( $\chi^2$ )=0.009 P( $\chi^2$ )=0.02
% Eosinophils (1.0-5.0)	2.72 ± 2.64 <1.0: 48 (5.22%) >5.0: 60 (6.52%)	2.49 ± 1.57 33 (5.93%) 24 (4.32%)	3.05 ± 3.62 15 (4.12%) 36 (9.89%)	P=0.01 P( $\chi^2$ )=0.28 P( $\chi^2$ )=0.001
% Basophils (0.0-1.0)	0.57 ± 1.31 >1.0: 33 (3.58%)	0.48 ± 0.19 10 (1.80%)	0.69 ± 2.01 23 (6.32%)	P<0.001 P( $\chi^2$ )=0.0005
Platelet Count (PTL) ( $\times 10^3/\mu\text{L}$ ) (150-450)	227.42 ± 68.69 <150: 75 (8.15%) >450: 6 (0.65%)	238.21 ± 66.51 30 (5.39%) 4 (0.72%)	210.62 ± 68.92 45 (12.36%) 2 (0.55%)	P<0.001 P( $\chi^2$ )=0.0005 P( $\chi^2$ )=0.91
Mean Platelet Volume (MPV) (fL) (6.0-10.0)	8.31 ± 1.02 <6.0: 4 (0.43%) >10.0: 58 (6.30%)	8.28 ± 0.95 1 (0.18%) 29 (5.21%)	8.37 ± 1.11 3 (0.82%) 29 (7.97%)	P=0.44 P( $\chi^2$ )=0.30 P( $\chi^2$ )=0.09
BL-MMSE Score (30) <24/30 >24/30	19.63 ± 7.17 650 (70.65%) 270 (29.35%)	18.78 ± 7.11 393 (70.68%) 163 (29.32%)	20.92 ± 7.09 257 (70.60%) 107 (29.80%)	P<0.001 P( $\chi^2$ )=0.97 P( $\chi^2$ )=0.97
BL-ADAS-Cognitive	12.45 ± 10.48	12.71 ± 10.37	12.04 ± 10.65	P=0.11
BL-ADAS-Memory	13.59 ± 4.99	13.75 ± 5.09	13.35 ± 4.84	P=0.24
BL-ADAS-Cognitive Total	25.87 ± 14.74	26.30 ± 14.58	25.23 ± 14.99	P=0.14
BL-ADAS-Non Cognitive	5.93 ± 4.74	6.32 ± 4.87	5.32 ± 4.47	P<0.001
BL-ADAS-Total	31.77 ± 17.58	32.59 ± 17.51	30.53 ± 17.67	P=0.02

Data: mean ± SD

Table 1: Sample features.

using a QIAamp DNA Micro Kit (QIAGEN, Hilden, Germany). To determine the *APOE*  $\epsilon 2$ ,  $\epsilon 3$  and  $\epsilon 4$  alleles, SNPs rs7412 and rs429358 were analyzed by allelic discrimination using the TaqMan real-time PCR system. Validated SNP genotyping assays (ID number C\_3084793\_20 for rs429358 and C\_904973\_10 for rs7412) were purchased from Applied Biosystems (Applied Biosystems, CA, USA). Both SNP genotyping reactions were optimized in a total volume of 15  $\mu\text{L}$  using 20 ng of DNA, and fluorescence was detected in a StepOne Plus Real-Time PCR system (Applied Biosystems, CA, USA). Control samples with known *APOE* genotypes were included in each PCR run, which were analyzed by the alternative PCR-RFLP method.

### TOMM40 genotyping

The chr19:50094819+50094997 (NCBI36/hg18 human genome assembly) fragment containing rs10524523 was amplified from genomic DNA samples (10-20 ng) with 5'-fluorescently labeled forward primer ACCTCAAGCTGTCCTCTTGC and unlabeled reverse primer GAGGCTGAGAAGGGAGGATT (each at 0.5  $\mu\text{M}$  concentration). The PCR reaction was performed with FailSafe PCR kit (Epicentre Biotechnologies, Madison, WI) using buffer G from the kit. The PCR reaction was carried out in 20  $\mu\text{L}$  volumes under the following conditions: 3 min at 95°C, 40 s at 98°C, then 10 cycles with 30 s at 95°C, 90 s at 63°C, then 18 cycles with 30 s at 95°C, 1 min at 61°C, then final extension 7 min at 72°C. One microliter of each PCR product was diluted with 15  $\mu\text{L}$  of HiDi Formamide (Applied Biosystems, Foster city, CA) and supplemented with 0.3  $\mu\text{L}$  of 600 LIZ size standard (Applied Biosystems, Foster city, CA). The size analysis was performed by Cornell University Genomics Core Laboratory (Ithaca, NY) on an ABI 3730 DNA analyzer (Applied Biosystems, Foster city, CA). ABI 3730 data were analyzed using GeneMarker software (SoftGenetics, State College, PA). The presence of poly T stretch in the fragment causes "stuttering" during the PCR reaction [74]. Therefore, an additional procedure

was implemented in order to interpret complex peak patterns and to determine the genotypes with precision. Briefly, PCR products from several patients were subcloned and the length of the poly T stretch in each clone was determined by sequencing. A clone library that covered poly T lengths from 14 to 38 was generated. Plasmid DNA from each clone was diluted and PCR amplified under exactly the same conditions as described above. Peak patterns from PCR amplification of individual cloned DNA were compared to peak patterns obtained from each individual patient's DNA. The accuracy of the method was verified by determination of poly T lengths in coded samples of patients' DNA that were previously deep sequenced and coded by an independent group. Our method was found to be 100% accurate.

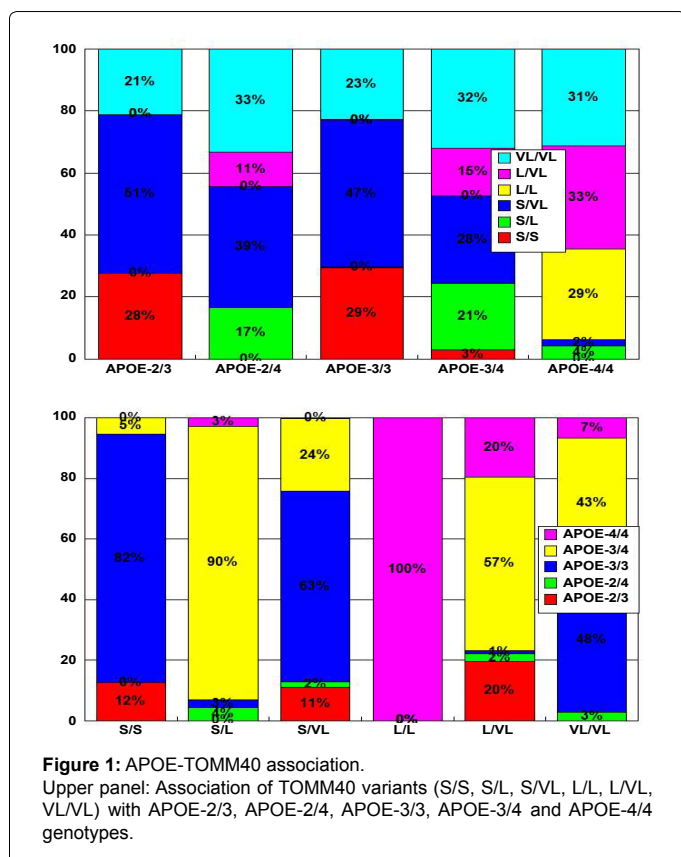
### Statistical analysis

Statistical analysis (paired t-test, Analysis of Variance,  $\chi^2$  and Fisher exact test, Mann-Whitney Rank Sum test, Linear and Non-linear Regression analysis, Durbin-Watson statistic, Pearson correlation, Spearman rank) were performed by using the IBM SPSS Statistics 20 and Sigma Stat 3.5 programs, where appropriate. Results are expressed as mean ± SD in the text and as mean ± SEM in Figs. 2, 3 and 5 for clarity. A 2-tailed  $p < 0.05$  (or  $p$  value ( $\chi^2$ )  $\leq 0.05$ ) was considered statistically significant.

### Results

#### Structural features of the APOE-TOMM40 region

The distribution and frequency of APOE genotypes was the following: APOE-2/3, 8.26%; APOE-2/4, 1.96%; APOE-3/3, 51.52%; APOE-3/4, 33.04%; and APOE-4/4, 5.22%. The distribution of 6 major TOMM40 poly T variants was: 18.37% S/S, 7.83% S/L, 38.80% S/VL, 1.52% L/L, 7.17% L/VL, and 26.31% VL/VL Figure 1. The APOE-2/3 genotype was found to be associated with S/S (27.63%), S/VL (51.32%), and L/VL (21.05%); APOE-2/4 was associated with S/L (16.67%), S/



VL (38.89%), L/VL (11.11%), and VL/VL (33.33%); APOE-3/3 was associated with S/S (29.32%), S/L (0.42%), S/VL (47.26%), L/VL (0.21%), and VL/VL (22.79%); APOE-3/4 was associated with S/S (2.96%), S/L (21.38%), S/VL (28.29%), L/VL (15.46%), and VL/VL (31.91%); and APOE-4/4 was associated with S/L (4.17%), S/VL (2.17%), L/L (29.17%), L/VL (33.33%), and VL/VL (31.25%). Likewise, the S/S genotype was associated with APOE-2/3 (27.63%), 3/3 (29.32%), and 3/4 (2.96%); S/L with APOE-2/4 (16.67%), 3/3 (0.42%), 3/4 (21.38%), and 4/4 (4.17%); S/VL with APOE-2/3 (51.32%), 2/4 (38.89%), 3/3 (47.26%), 3/4 (28.29%), and 4/4 (2.08%); L/L was exclusively associated with APOE-4/4 (100%); L/VL with APOE-2/4 (11.11%), 3/3 (0.21%), 3/4 (15.46%), and 4/4 (33.33%); and VL/VL with APOE-2/3 (21.05%), 2/4 (33.33%), 3/3 (22.79%), 3/4 (31.91%), and 4/4 (31.25%) Figure 1. S/VL and VL/VL are the only TOMM40 poly T genotypes which interact with all major APOE genotypes; in contrast, the APOE-4/4-TOMM40-L/L association is unique, representing approximately 30% of APOE-4/4 carriers.

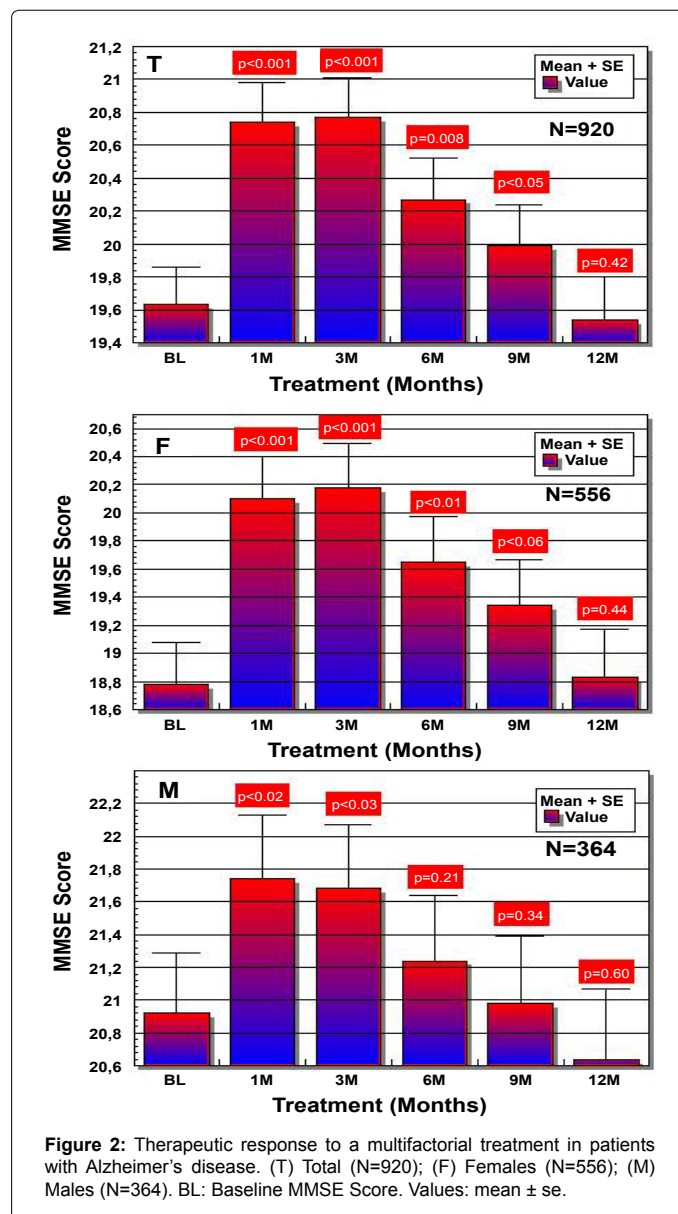
### Influence of APOE and TOMM40 poly T repeats on age at onset

APOE-4/4 carriers were the youngest at age of onset ( $69.95 \pm 7.10$  yrs), showing a significant difference as compared with APOE-3/4 carriers ( $72.45 \pm 8.54$  yrs;  $p=0.008$ ). APOE-3/3 carriers ( $70.28 \pm 9.52$  yrs,  $p<0.001$ ) were also younger than APOE-3/4 carriers; and no differences were found between the age at onset of APOE-2/3 ( $71.43 \pm 8.64$  yrs) or APOE-2/4 carriers ( $71.77 \pm 11.05$  yrs) and that of patients with other APOE genotypes. The age at onset of patients according to their TOMM40 poly T genotypes was as follows: S/S,  $69.42 \pm 10.30$  yrs; S/L,  $72.37 \pm 7.46$  yrs; S/VL,  $71.88 \pm 8.68$  yrs; L/L,  $69.14 \pm 7.36$  yrs; L/VL,

$72.51 \pm 9.42$  yrs; and VL/VL,  $70.51 \pm 9.07$  yrs. Significant differences were found between S/S and S/VL ( $p=0.02$ ), S/VL and VL/VL ( $p<0.05$ ), L/L and L/VL ( $p<0.05$ ); and L/VL vs S/S ( $p=0.03$ ), and L/VL vs VL/VL ( $p=0.04$ ).

### Sex-related therapeutic response

In the whole group Figure 2, there was a clear pattern in terms of cognitive performance, with a significant improvement in MMSE scores at 1 ( $p<0.001$ ), 3 ( $p<0.001$ ), 6 ( $p=0.008$ ), and 9 months ( $p<0.05$ ) of treatment. The peak effect was observed during the first 3 months of treatment with a gradual cognitive decline thereafter. This trend was almost identical in females and males Figure 2, with a response rate (% of cases with a MMSE score superior or equal to baseline values after 12 months of treatment) of 60% in females and 59% in males (59% responders (R) and 41% non-responders (NR) in the global sample), despite substantial gender-related differences in biochemical and hematological parameters Table 1; however, the degree of significance appeared to be more apparent in females than in males Figure 2.



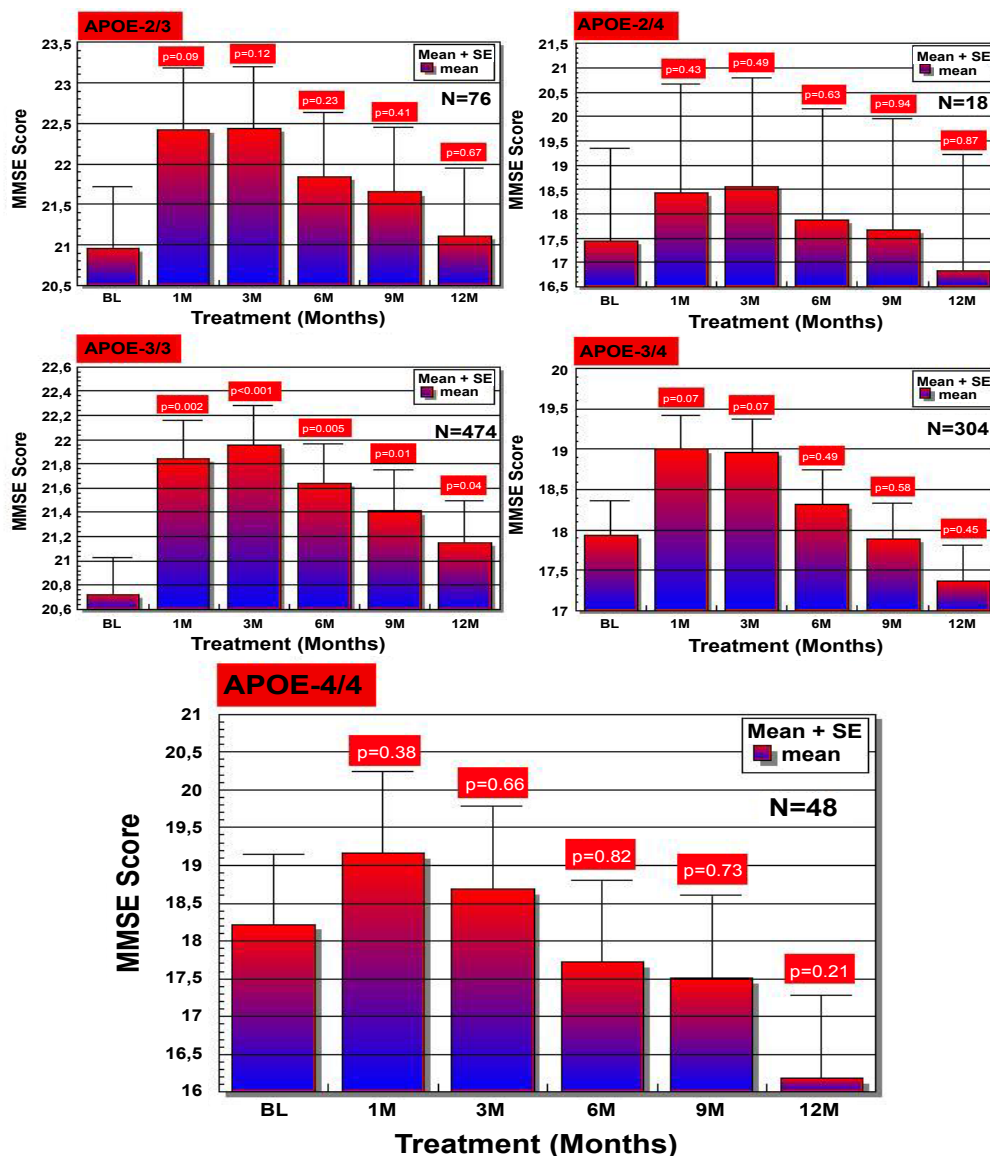


Figure 3: APOE-Related therapeutic response to a multifactorial treatment in patients with Alzheimer's disease. BL: Baseline MMSE Score. Values: mean  $\pm$  se.

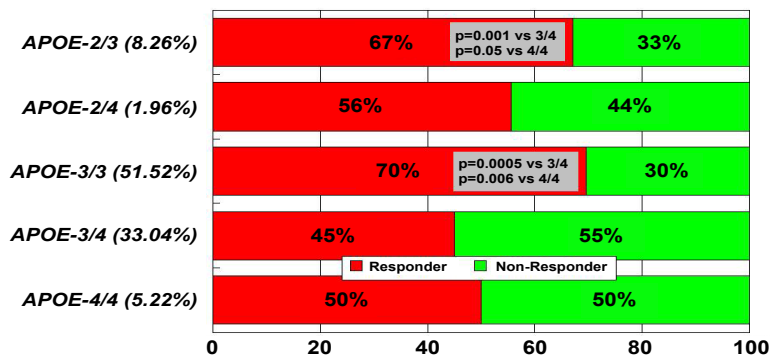


Figure 4: APOE-Related response rate (%) in patients with Alzheimer's disease treated with a multifactorial therapy for one year.

### APOE- and TOMM40 poly T-related therapeutic response

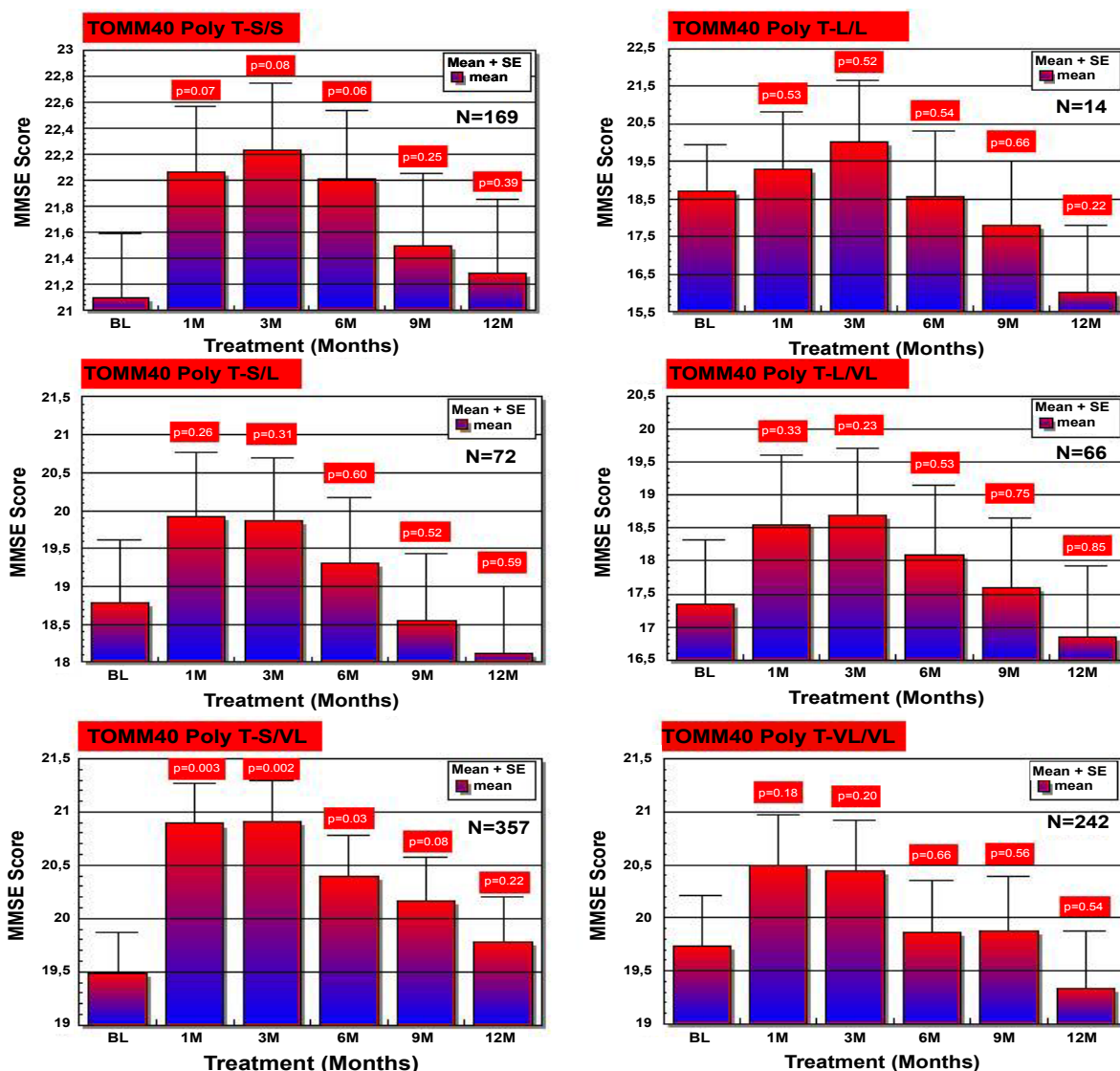
The same pattern of mental performance, with a transient profile of cognitive improvement for 6-12 months and maximum effect during the first 3 months of treatment, was observed in APOE-2/3, APOE-2/4, APOE-3/3, APOE-3/4, and APOE-4/4 carriers Figure 3, with significant effects in APOE-3/3 carriers for 12 months Figure 3. The response rate (RR) was 70% in APOE-3/3, 67% in APOE-2/3, 56% in APOE-2/4, 50% in APOE-4/4, and 45% in APOE-3/4 carriers Figure 4, with significant differences between APOE-2/3 and APOE-3/4 ( $p=0.001$ ), APOE-2/3 and APOE-4/4 ( $p=0.05$ ), APOE-3/3 and APOE-3/4 ( $p>0.0005$ ), and APOE-3/3 and APOE-4/4 ( $p(\chi^2) <0.006$ ) ( Figure 4.

The time-dependent profile of cognitive performance after treatment, according to the TOMM40 poly T genotype, was similar to that observed in the total group or in the APOE-related study, with an apparent improvement during the first 3-9 months of treatment;

however, significant effects were only observed in patients harboring the TOMM40 poly T-S/S and S/VL genotypes Figure 5. S/S carriers were the best responders (70%), followed by S/VL (61%), VL/VL (57%), and L/VL carriers (51%), and L/L (35%) and S/L carriers (45%) were the worst responders Figure 6 and 7.

### Discussion

The present results appear to indicate that the combination therapy used in this study, probably together with the lipid-lowering effects and anti-atherosclerotic properties of sardilipin [18,24,73] and the prometabolic activity of animon complex, exert a positive synergistic effect on brain function accompanied by a transient improvement in mental performance, lasting for about 9-12 months Figure 2. The effect of CDP-choline, either alone or in combination with donepezil, as well as the effects of sardilipin, are APOE-dependent [3,18-28]. In our study, the administration of cholinesterase inhibitors was avoided in



**Figure 5:** TOMM40-Related therapeutic response to a multifactorial treatment in patients with Alzheimer's disease. TOMM40 poly T repeat variants: S/S, S/L, S/VL, L/L, L/VL, VL/VL. BL: Baseline MMSE Score. Values: mean  $\pm$  se.



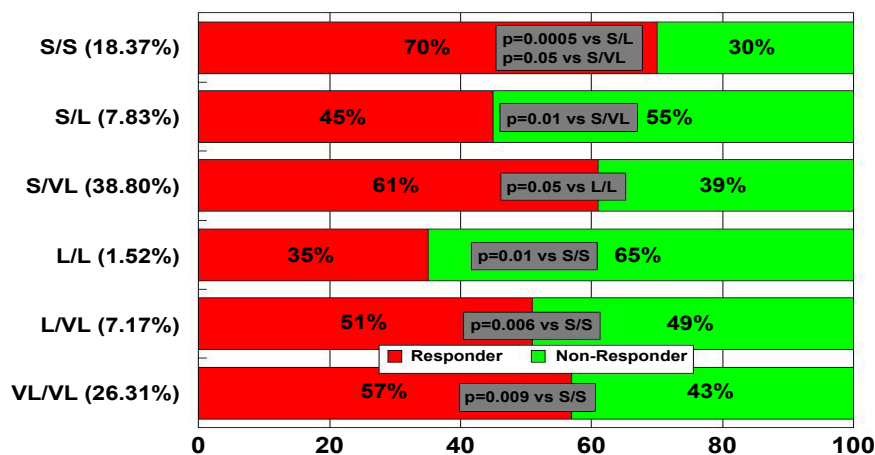


Figure 6: TOMM40-Related response rate (%) in patients with Alzheimer's disease treated with a multifactorial therapy for one year.

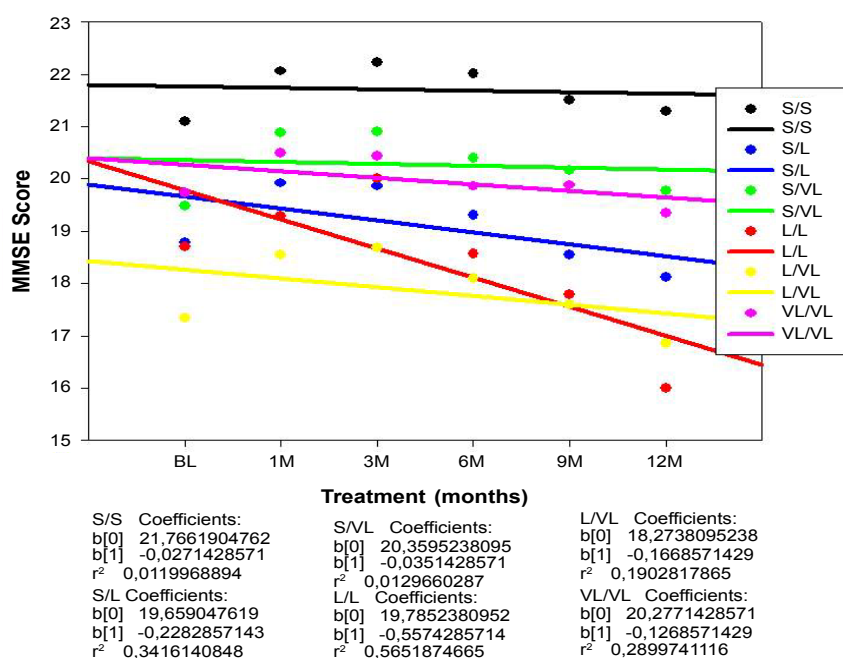


Figure 7: Comparative linear regression analysis of the cognitive response to a multifactorial treatment in patients with Alzheimer's disease according to their TOMM40 poly T variants. BL: Baseline MMSE Score. Each point depicts the mean value of the MMSE scores in Figure 5 at baseline (BL) and after 1, 3, 6, 9, and 12 months of treatment.

order to eliminate distorting variability and side-effects associated with CYP2D6-related factors [25,27,75].

Our data also illustrate that polymorphic variants in the APOE-TOMM40 region are determinant for the therapeutic response to conventional drugs in patients with AD Figure 3-7. The role of APOE in this endeavor seems to be fundamental due to the influence of the APOE gene on the AD phenotype [76]; however, the impact of the TOMM40 gene on AD pathogenesis and pharmacogenetics is a novelty [33-36]. From studies designed to define APOE-related AD phenotypes, several conclusions can be drawn: (i) the age-at-onset is 5-10 years earlier in approximately 80% of AD cases harboring the APOE-4/4 genotype;

(ii) the serum levels of ApoE are lowest in APOE-4/4, intermediate in APOE-3/3 and APOE-3/4, and highest in APOE-2/3 and APOE-2/4; (iii) serum cholesterol levels are higher in APOE-4/4 than in the other genotypes; (iv) HDL-cholesterol levels tend to be lower in APOE-3 homozygotes than in APOE-4 allele carriers; (v) LDL-cholesterol levels are systematically higher in APOE-4/4 than in any other genotype; (vi) triglyceride levels are significantly lower in APOE-4/4; (vii) nitric oxide levels are slightly lower in APOE-4/4; (viii) serum and CSF Aβ levels tend to differ between APOE-4/4 and the other most frequent genotypes (APOE-3/3, APOE-3/4); (ix) blood histamine levels are dramatically reduced in APOE-4/4 as compared with the other genotypes; (x)

brain atrophy and AD neuropathology is markedly increased in *APOE-4/4*>*APOE-3/4*>*APOE-3/3*; (xi) brain mapping activity shows a significant increase in slow wave activity in *APOE-4/4* from early stages of the disease; (xii) brain hemodynamics, as reflected by reduced brain blood flow velocity and increased pulsatility and resistance indices, is significantly worse in *APOE-4/4* (and in *APOE-4* carriers in general, as compared with *APOE-3* carriers); brain hypoperfusion and neocortical oxygenation is also more deficient in *APOE-4* carriers; (xiii) lymphocyte apoptosis is markedly enhanced in *APOE-4* carriers; (xiv) cognitive deterioration is faster in *APOE-4/4* patients than in carriers of any other *APOE* genotype; (xv) in approximately 3-8% of the AD cases, the presence of some dementia-related metabolic dysfunctions accumulates more in *APOE-4* carriers than in *APOE-3* carriers; (xvi) some behavioral disturbances, alterations in circadian rhythm patterns, and mood disorders are slightly more frequent in *APOE-4* carriers; (xvii) aortic and systemic atherosclerosis is also more frequent in *APOE-4* carriers; (xviii) liver metabolism and transaminase activity also differ in *APOE-4/4* with respect to other genotypes; (xix) hypertension and other cardiovascular risk factors also accumulate in *APOE-4*; and (xx) *APOE-4/4* carriers are the poorest responders to conventional drugs. These 20 major phenotypic features clearly illustrate the biological disadvantage of *APOE-4* homozygotes and the potential consequences that these patients may experience when they receive pharmacological treatment for AD and/or concomitant pathologies [1-5,18-30,77-79]. In our study, it is clear that *APOE-4* carriers are the worst responders to conventional treatments Figures 3 and 4.

Linnertz et al. [39] defined 3 allele groups for rs10524523 ('523'), based on the number of 'T'-residues: 'Short' (S, T ≤ 19), 'Long' (L, 20 ≤ T ≤ 29) and 'Very Long' (VL, T ≥ 30). Roses et al. [33-36] reported that longer lengths of rs10524523 are associated with a higher risk for LOAD; for *APOE-3/4* patients who developed LOAD after 60 years of age, individuals with long poly T repeats (19-39 nucleotides) linked to *APOE-3* develop LOAD on an average of 7 years earlier than individuals with shorter poly T repeats (11-16 nucleotides) linked to *APOE-3* [33,34,37]. Apparently, these results could not be replicated by other authors [46-49]. In our case, we clearly found that patients harboring the *APOE-4/4-L/L* cluster developed dementia at an earlier age (<70 yrs) than their counterparts with other genotypes. In fact, L/L carriers were the youngest at age of onset, followed by S/S carriers. In addition, virtually 100% of L/L carriers were exclusively associated with *APOE-4/4* Figure 1, representing the worst responders to our combination therapy Figure 6. The *APOE-3/3-VL/VL* cluster, with an earlier age at onset (mean age ~70 yrs), was present in approximately a quarter of *APOE-3/3* carriers Figure 1.

Bernardi et al. [45] studied the association between TOMM40 rs10524523, age of onset, and memory performance in patients with the PSEN1 M146L mutation in a large familial AD Calabrian kindred, and found that *APOE33/TOMM40VL/VL* patients showed a tendency for an earlier age at onset compared to those with *APOE33/TOMM40VL/S* and *APOE33/TOMM40S/S*. TOMM40VL/VL patients had better memory performance, when compared to TOMM40S/S but not to TOMM40VL/S patients. For Li et al. [46], TOMM40 intron 6 poly T length may explain some of the variation in age at onset in PSEN2 familial AD and may be associated with AD neuropathology in persons with *APOE-3/3*.

The allele distribution of TOMM40 poly T repeats in the Spanish population reflects a high proportion of heterozygous S/VL (39%), followed by homozygous VL (27%) and homozygous S (19%). Homozygous L/L represents 1.52% of the Spanish population, and

both S/VL and L/VL genotypes conform a group of about 7-8% of the population Figure 6. Potential dissimilarities with other White and Hispanic populations [39], might be due to the ancestral admixture of different cultures in the Iberian peninsula. The linkage pattern between TOMM40-'523' and *APOE* alleles in Whites and Hispanics reflects that the L is primarily linked to *APOE-4*, while the majority of the VL and S are linked to *APOE-3*. In African-Americans, Ghanaians and Japanese, there is an increased frequency of the '523'S-*APOE-4* haplotype [39].

Several reports suggest that both *APOE* and TOMM40 influence memory performance in normal [53] and pathological conditions [54,80]. For some authors, both TOMM40 and *APOE* significantly influence age-related memory performance, but they appear to do so independently of each other [80]. Others suggest important *APOE*-independent associations between the TOMM40 '523' polymorphism and specific cognitive domains of memory and executive control that are preferentially affected in early-stage AD, with S homozygotes performing better than the S/L-S/VL and the VL/L-L/VL-VL/VL genotype groups on measures associated with memory and executive function [54]. According to our data, the best mental performance (and response rate to treatment) is observed in patients harboring the *APOE-3/3-S/S* haplotype (R~70%), followed by those with the *APOE-3/3-S/VL* haplotype (R~60%)(Figs. 3-6). In general, S/S carriers are the best responders > S/VL (61%) > VL/VL (57%) > L/VL (51%) > S/L (45%) > L/L (35%) Figure 6. The presence of the L allele appears to contribute to a poor therapeutic outcome, and when the L/L genotype associates with the *APOE-4/4* genotype, carriers of the *APOE-4/4-S/S* haplotype (30% of *APOE-4/4* carriers) are converted into the worst responders.

Although a retrospective study such as this, designed to assess the potential influence of the *APOE-TOMM40* region on cognitive performance in response to a heterogeneous multifactorial therapy in a natural setting, has its limitations, interpretation of the obtained results appears to indicate that the *APOE-TOMM40* cluster is important in both the clinical course of the disease and the biological conditions of AD patients in their response to the different drugs necessary to protect brain function, transiently halting disease progression. The relevance of the *APOE-TOMM40* locus in the complex phenotype of dementia might be due to the pleiotropic properties of these genes. Although a large number of pharmacogenetic studies in AD used *APOE* as a reference gene with plural results [3,76], more specific studies exploring the influence of *APOE*, TOMM40, CYP, and ABCB1 variants on the pharmacogenetic outcome are needed to optimize therapeutics in AD [3,35,81].

## Conclusions

1. A multifactorial treatment with conventional neuroprotectants and other drugs or metabolic factors to treat concomitant pathologies or metabolic deficiencies, respectively, seems to be useful for patients with dementia in approximately 50% of the cases, stabilizing or improving cognitive deterioration for a transient period of time (<12 months).
2. *APOE-4* carriers are the worst responders and *APOE-3* carriers are the best responders to conventional treatments.
3. TOMM40 poly T-S/S carriers are the best responders, VL/VL and S/VL carriers are intermediate responders, and L/L carriers are the worst responders to treatment.
4. Patients harboring a large (L) number of poly T repeats in intron 6 of the TOMM40 gene (L/L or S/L genotypes) in haplotypes associated with *APOE-4* are the worst responders to treatment.

5. Patients with short (S) TOMM40 poly T variants (S/S genotype), and to a lesser extent S/VL and VL/VL carriers, in haplotypes with APOE-3 are the best responders to treatment.
6. In 100% of the cases, the L/L genotype is exclusively associated with the APOE-4/4 genotype, and this haplotype (4/4-L/L) is probably responsible for early onset of the disease, a faster cognitive decline, and a poor response to different treatments.

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