

FOXO1 locus and acetylcholinesterase inhibitors in elderly patients with Alzheimer's disease

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Objective: Acetylcholinesterase inhibitors (AChEIs) may reduce the oxidative stress in brain of Alzheimer's disease (AD) patients. Forkhead box O1 (*FOXO1*) protein has been reported as the link between oxidative stress and AD. We evaluated a potential association between *FOXO1* gene locus and the response to AChEI treatment in patients with sporadic AD.

Methods: In this prospective study, 109 Caucasian AD patients were treated with standard doses of donepezil, galantamine, or rivastigmine for 6 months. Functional and cognitive status were evaluated at baseline and after treatment. Response to therapy was defined according to the National Institute for Health and Clinical Excellence criteria. Genotype analyses, including the *APOE* polymorphism, were made in blinded fashion.

Results: A significantly higher frequency of *FOXO1* rs7981045 G/G genotype was observed in nonresponders compared with responders (17.14% versus 2.70%, $P=0.010$). Age, sex, and *APOE*-adjusted logistic regression analysis confirmed that patients with the G/G genotype had a significantly higher risk of poor response to AChEI treatment (odds ratio = 10.310; 95% confidence interval, 1.510–70.362). Haplotype analysis revealed significant differences in haplotype frequency distribution between these groups.

Conclusion: *FOXO1* may influence the clinical response to AChEIs in AD patients.

Keywords: forkhead box O1, acetylcholinesterase inhibitors, response to treatment

Introduction

The global increases in population size and life expectancy have led sporadic Alzheimer's disease (AD) to become a main health problem. Sporadic AD occurs in later life with a prevalence of 20% after 75 years increasing to 30% after 85 years. About 5% of people aged 65 years or older have AD, and with about 3 to 4 million people affected and about 350,000 new cases per year, AD is the most frequent cause of dementia in Western countries. Accordingly, it has been estimated that AD prevalence will nearly double every 20 years, rising to 65.7 million in 2030 and 115.4 million in 2050.^{1–3}

Despite the progress of modern pharmacology in developing drugs against AD, for most of these drugs, positive clinical outcomes are missing.^{4,5} Thus, acetylcholinesterase inhibitors (AChEIs), and in particular donepezil, rivastigmine, and galantamine still remain the main drugs currently used for the symptomatic treatment of AD.^{6,7}

Recent studies reported strong evidence toward a key role of functional variants in gene encoding drug-metabolizing enzymes on the efficacy of AChEIs, such as donepezil.^{8–10} However, unrelated drug-metabolism pathways that conversely underlie the pathogenesis of AD may contribute to the response to AChEIs. Oxidative stress, recently related to the pathogenesis of AD, may be one of these pathways. An overproduction of reactive oxygen species may activate microglia and astrocytes, inducing neurotoxicity through deposition of amyloid β peptides.^{11,12} Recent data

suggested that AChEIs may act directly on this process, enhancing antioxidant activity and attenuating oxidative stress.¹³

Many genes have been described for their role in the physiological response to oxidative stress. In particular, proteins of the forkhead box family, class O (FoxO) has been suggested as a link among pathophysiology of AD and the response to oxidative stress. Recent data strongly suggested that FoxO protein 1 (*FOXO1*), the most important protein of the FoxO family, plays this role.¹⁴ *FOXO1* is encoded by the *FOXO1* gene at locus 13q14.1. We also analyzed all samples for *APOE* genotype, which is being used clinically to provide additional information regarding patients with dementia and indicates whether there is an increased risk of AD, although not specifically diagnostic of AD.

The aim of the present study was to investigate the role of the *FOXO1* gene as a potential background factor influencing the response to AChEIs in older patients with AD.

Materials and methods

Patient recruitment

This was a prospective cohort study fulfilling the Declaration of Helsinki,¹⁵ the guidelines for Good Clinical Practice,¹⁶ the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines,¹⁷ and the National Institute for Health and Clinical Excellence (NICE) requirements.¹⁸ Approval of the study for experiments using human subjects was obtained from the local ethics committees on human experimentation. Written informed consent for research was obtained from each patient or from a relative or legal guardian in the case of critically disabled demented patients, prior to participation in the study. All patients included in this study were Caucasians, with most individuals from families that have lived in Central and Southern Italy for at least two generations. All patients included in this study were selected from patients consecutively attending from July 1, 2009, through July 31, 2011, the Geriatric Unit of the Istituto di Ricovero e Cura a Carattere Scientifico Casa Sollevio della Sofferenza in San Giovanni Rotondo, Italy.

Inclusion/exclusion criteria

Inclusion criteria were 1) age ≥ 65 years; 2) diagnosis of mild or moderate AD; 3) informed consent for research. Exclusion criteria were 1) diagnosis of vascular dementia (VaD), mixed dementia, or mild cognitive impairment; 2) presence of serious comorbidity, tumors, other diseases, or physiological status (ascertained blood infections, vitamin B₁₂ deficiency, anemia, disorders of the thyroid, kidneys, or liver) that could

be causally related to cognitive impairment; 3) history of alcohol or drug abuse; 4) head trauma; 5) current or previous use of psychoactive substances; or 6) diabetes mellitus.

Data collection

Baseline demographic and clinical characteristics were collected by a structured interview, clinical evaluation, and review of records from patients' general practitioners. All patients included in the study were initially treated for 1 month with an AChEI, that is, donepezil 5 mg/daily; or rivastigmine 1.5 mg $\times 2$ /daily (pill) or 4.6 mg/daily (transdermal patch); or galantamine 8 mg/daily. Thereafter, patients who had followed the treatment with a satisfactory or good compliance without clinically relevant drug-related adverse events increased the dosage of donepezil to 10 mg/daily; rivastigmine to 3 mg $\times 2$ /daily (pill) or to 9.5 mg/daily (transdermal patch) for the following 5 months; or galantamine to 16 mg/daily for a further 1 month, which was increased to 24 mg/daily for the following 4 months. Patients who needed a coadministration of memantine were excluded from the study. At 6-month follow-up, the clinical assessment, including the evaluation of cognitive and functional status, compliance, and drug-related adverse events, was repeated.

Cognitive-functional evaluation and diagnosis of AD

In all patients, cognitive status was screened by means of the mini-mental state examination (MMSE),¹⁹ the Alzheimer's Disease Assessment Scale-cognitive subscale (ADAS-cog),²⁰ and the Clinical Dementia Rating scale (CDR).^{21,22} Dementia (CDR 1+) was confirmed and diagnosed by the *Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DMS-IV)*, whereas diagnosis of questionable dementia (QD) was made according to a CDR value of 0.5+.²³ Diagnosis of mild cognitive impairment was made according to the Petersen criteria²⁴ in subjects with CDR 0.5+ and an MMSE value from 24 to 27. Diagnosis of QD or MCI caused the exclusion from the study. Diagnosis of possible/probable AD was made according to the criteria of the National Institute of Neurological and Communicative Disorders and Stroke – Alzheimer's Disease and Related Disorders Association Work Group (NINCDS-ADRDA).²⁵ Diagnosis of possible/probable vascular dementia (VaD) was made according to the criteria of the National Institute of Neurological Disorders and Stroke – Association Internationale pour la Recherche et l'Enseignement en Neurosciences Work Group (NINDS-AIREN).²⁶ Differential diagnosis between AD and VaD was also based on the Hachinski Ischemic Score to address

unclear AD/VaD diagnoses.²⁷ In particular, scores ≤ 4 were considered as probable AD, scores ≥ 7 were included in the VaD group that was excluded from the study. Scores between 5 and 6 were diagnosed as mixed dementia and were also excluded from the study. Diagnosis of AD or VaD was always supported by neuroimaging evidences (computed tomography scan and/or nuclear magnetic resonance). In particular, the presence of multiple cortical/subcortical infarcts or an infarct in a strategic area such as the thalamus or temporal lobe and/or lesions of the white matter indicated probable VaD. The absence of the above-mentioned cerebrovascular lesions indicated AD. Functional status was evaluated using the Activities of Daily Living (ADL) index²⁸ and the Instrumental Activities of Daily Living (IADL) scale.²⁹

Responder/nonresponder assessment criteria

According to the NICE requirements,¹⁸ a responder to AChEI treatment was defined as a patient who showed improvement or no deterioration in cognition, as evaluated by means of ADAS-cog and MMSE, and an improvement in functional status, as evaluated by means of ADL or IADL indexes.

Genetic analysis

Genomic deoxyribonucleic acid (DNA) was purified from fresh/frozen blood samples following a salting-out method.³⁰ On the basis of their level of heterozygosity ($>5\%$ in Caucasians),³¹ we selected the three single-nucleotide polymorphisms (SNPs) rs2721069 (41,143,720 bases from pter) ($C^{102,015} \rightarrow T$), rs4943794 (41,173,408 bases from pter) ($T^{72,327} \rightarrow G$), and rs7981045 (41,209,236 bases from pter) ($C^{36,499} \rightarrow T$) spanning a 65 kb block at the *FOXO1* locus (13q14.1). All SNPs were investigated in a blinded fashion by means of the allele discrimination assay. The analysis was made using TaqMan technology on an ABI PRISM 7700 Sequence Detector system (Life Technologies Corporation, Carlsbad, CA, USA) with assay c_15926664_10 (rs2721069), c_30366093_20 (rs4943794), and c_30886685_10 (rs7981045) according to manufacturer's instructions. The *APOE* genotypes were determined as previously described.³²

Statistical analyses

Patients' baseline characteristics and *FOXO1* genotypes were reported as frequencies and percentage, mean \pm standard deviation (SD), and comparisons between groups were performed with Pearson's chi-square test and Mann-Whitney *U*-test for categorical and continuous variables, respectively.

Differences between clinical evaluation at baseline and after 6 months of follow-up were assessed using a Student's *t*-test for paired samples. Age, sex, *APOE*, and AChEI administration were evaluated as potential predictors of responsiveness to AChEI treatment at the 6-month follow-up. Results were reported as odds ratios (ORs) along with their 95% of confidence intervals (CIs). Furthermore, univariate and multivariate logistic regression models were assessed in order to test the association between the genotypes and the responsiveness to AChEIs under free, dominant, recessive, and additive genetic models of inheritance. The Hardy-Weinberg equilibrium (HWE) was verified for all the investigated polymorphisms using the exact test proposed by Wigginton et al.³³ The HaploView 4.2 genetic software package³⁴ was used to estimate values of linkage disequilibrium (LD) coefficient r^2 as well as to estimate haplotype frequency and to compare it among the study groups. A *P*-value of <0.05 was considered for statistical significance. All statistical analyses were performed using SAS Release 9.1 (SAS Institute, Cary, NC, USA).

Results

Characteristics of patients at baseline according to sex are summarized in Table 1. No difference was observed in age distribution between men and women. Conversely, the educational level was higher in men (6.10 ± 4.49 years versus 3.60 ± 2.22 years; $P < 0.001$). Men also had a higher MMSE score than women (17.57 ± 4.41 versus 15.14 ± 3.64 ; $P = 0.003$). No differences were observed in mean values of ADL and IADL scores. Men had a higher ADAS-cog score than women (40.39 ± 10.27 versus 35.27 ± 8.47 ; $P = 0.003$). A significant difference was also observed in mean value of CDR score, which was lower in men than in women (1.51 ± 0.65 versus 1.76 ± 0.57 ; $P = 0.041$). Donepezil was administered less frequently in men than in women (35.14% versus 43.06%; $P < 0.001$). Conversely, rivastigmine was administered more frequently in men (62.16% versus 55.56%; $P < 0.001$). Accordingly, pill administration was more frequent in men than in women (24.32% versus 19.44%; $P = 0.002$). Similarly, transdermal patch administration was more frequent in men than in women (37.84% versus 36.11%; $P < 0.001$, respectively). No difference in frequency of galantamine treatment was observed ($P = 0.999$). *APOE* genotype and estimated allele frequencies according to sex were evaluated. No significant differences were observed in the distribution of the *APOE* genotypes and alleles between males and females.

The clinical evaluation of responder/nonresponder phenotype to AChEI treatment at the 6-month follow-up

Table 1 Characteristics of patients at baseline according to sex

	All (n=109)	Men (n=37)	Women (n=72)	P-value
Age (years)	78.72±6.49	78.25±6.26	78.97±6.64	0.591
Educational level (years)	4.46±3.39	6.10±4.49	3.60±2.22	<0.001
MMSE	15.96±4.06	17.57±4.41	15.14±3.64	0.003
ADL	3.84±1.93	4.30±1.69	3.61±2.02	0.193
IADL	1.84±2.33	2.50±2.59	1.51±2.15	0.120
ADAS-cog	37.19±9.47	40.93±10.27	35.27±8.47	0.003
CDR	1.68±0.61	1.51±0.65	1.76±0.57	0.041
Donepezil	44 (40.37%)	13 (35.14%)	31 (43.06%)	0.536
Rivastigmine (pill)	23 (21.10%)	9 (24.32%)	14 (19.44%)	0.622
Rivastigmine (transdermal patch)	40 (36.7%)	14 (37.84%)	26 (36.11%)	0.999
Rivastigmine (all)	63 (57.79%)	23 (62.16%)	40 (55.56%)	0.544
Galantamine	2 (1.83%)	1 (2.70%)	1 (1.39%)	0.999

Notes: Data are reported as means ± standard deviation and frequency and percentages for continuous and categorical variables, respectively. P-values are from Mann–Whitney U-tests or Pearson chi-square tests for continuous and categorical variables, respectively.

Abbreviations: ADAS-cog, Alzheimer's Disease Assessment Scale-cognitive subscale; ADL, Activities of Daily Living; CDR, Clinical Dementia Rating; IADL, Instrumental Activities of Daily Living; MMSE, mini-mental state examination.

is summarized in Table 2. At follow-up, 68% of patients were responders, and 32% were nonresponders to AChEI treatment. As compared with baseline, according to the NICE criteria,¹⁸ responder patients had higher mean MMSE (18.61±4.86 versus 15.78±4.14; $P<0.001$), ADL (4.42±2.66 versus 3.89±2.00; $P=0.001$), IADL (2.40±2.68 versus 2.09±2.58; $P=0.003$), and ADAS-cog (43.36±11.33 versus 36.77±9.64) scores at follow-up. No difference in CDR scores were observed. Conversely, nonresponder patients had lower mean MMSE (12.73±3.90 versus 16.35±3.93; $P<0.001$), IADL (0.47±0.61 versus 1.18±1.33; $P=0.016$) and ADAS-cog (29.68±9.10 versus 38.10±9.15; $P<0.001$) scores at follow-up, whereas no difference in mean ADL

score was observed. In these patients, a higher CDR score (2.06±0.77 versus 1.74±0.56; $P=0.005$) at follow-up was also observed. No differences in age and sex distribution were observed between responder and nonresponder patients.

Genotype distribution at *FOXO1* locus according to the response to AChEI treatment is summarized in Table 3. Two multivariate analyses were performed: the first one included MMSE score at baseline, AChEI treatment, and age and sex as confounding factors; whereas the second one also included *APOE* genotypes. No associations were observed for rs2721069 and rs4943794. Conversely, a significant association was observed for rs7981045. Genotype G/G was

Table 2 Clinical evaluation of responders/nonresponders to AChEI treatment at the 6-month follow-up

Variable	Category	Responders (n=74)	P-value	Nonresponders (n=35)	P-value
Sex (M/F)		26/48		11/24	0.829
Age		78.31±6.92		79.60±5.47	0.333
MMSE	Baseline	15.78±4.14	<0.001	16.35±3.93	<0.001
	Follow-up	18.61±4.86		12.73±3.90	
ADL	Baseline	3.89±2.00	<0.001	3.71±1.79	0.079
	Follow-up	4.42±1.66		3.20±1.47	
IADL	Baseline	2.09±2.58	0.003	1.18±1.33	0.012
	Follow-up	2.40±2.68		0.47±0.61	
ADAS-cog	Baseline	36.77±9.64	<0.001	38.10±9.15	<0.001
	Follow-up	43.36±11.33		29.68±9.10	
CDR	Baseline	1.65±0.63	0.111	1.74±0.56	0.005
	Follow-up	1.51±0.76		2.06±0.77	

Notes: Data are reported as mean ± standard deviation. P-values are from paired Student's t-tests.

Abbreviations: AChEIs, acetylcholinesterase inhibitors; ADAS-cog, Alzheimer's Disease Assessment Scale-cognitive subscale; ADL, Activities of Daily Living; CDR, Clinical Dementia Rating; IADL, Instrumental Activities of Daily Living; MMSE, mini-mental state examination.

Table 3 Genotype distribution at *FOXO1* locus according to the response to AChEI treatment and results from univariate and multivariate logistic regressions

	Responders		Nonresponders		Univariate analysis			Multivariate analyses [§]					
	(n=74)		(n=35)		P-value	OR	(95% CI)	Age/sex			Age/sex/APOE		
	N	%	N	%				P-value	OR	(95% CI)	P-value	OR	(95% CI)
rs2721069*													
C/C	44	59.46	20	57.14	–	1.00	–	–	1.00	–	–	1.00	–
C/T	22	29.73	10	28.57	1.000	1.00	0.400–2.498	0.895	1.07	0.412–2.756	0.955	0.971	0.356–2.650
T/T	8	10.81	5	14.29	0.614	1.38	0.399–4.733	0.566	1.46	0.404–5.235	0.383	1.825	0.472–7.056
rs4943794 [#]													
G/G	53	71.62	28	80	–	1.00	–	–	1.00	–	–	1.00	–
G/C	19	25.68	6	17.14	0.325	0.60	0.214–1.667	0.311	0.58	0.203–1.662	0.415	0.636	0.214–1.890
C/C	2	2.7	1	2.86	0.965	0.95	0.082–10.899	0.990	0.94	0.087–13.889	0.935	0.899	0.069–11.698
rs7981045 [†]													
A/A	44	59.46	14	40	–	1.00	–	–	1.00	–	–	1.00	–
A/G	28	37.84	15	42.86	0.240	1.68	0.706–4.014	0.198	1.73	0.734–4.454	0.182	1.895	0.741–4.845
G/G	2	2.7	6	17.14	0.010	9.43	1.706–52.108	0.017	9.19	1.481–57.086	0.017	10.31	1.510–70.362

Notes: [§]Both analyses were also adjusted for the MMSE score at baseline and AChEI. Hardy-Weinberg equilibrium: * $P=0.067$ (responders) and $P=0.095$ (nonresponders); [#] $P=0.679$ (responders) and $P=0.362$ (nonresponders); [†] $P=0.447$ (responders) and $P=0.720$ (nonresponders).

Abbreviations: AChEI, acetylcholinesterase inhibitor; CI, confidence interval; OR, odds ratio; MMSE, mini-mental state examination.

overrepresented in nonresponder as compared with responder patients (17.14% versus 2.70%; $P=0.010$). This association was present also when age and sex or age, sex, and *APOE* genotypes were considered as confounding factors ($P=0.017$ in both cases). Thus, the G/G genotype may be at risk for a poor response to AChEI treatment in the crude analysis (OR =9.429, 95% CI 1.706–52.108) as well as in the adjusted analysis (OR =9.194, 95% CI 1.481–57.086 and OR =10.308, 95% CI 1.510–70.362, respectively).

An evaluation of the relationships between *FOXO1* genotypes and the response to AChEI treatment assuming different genetic models of inheritance is summarized in Supplementary Table S1. Assuming a dominant, recessive, or additive model of inheritance, in both univariate and multivariate analyses, the SNPs rs2721069 and rs4943794 did not show significant associations. Conversely, for the SNP rs7981045, assuming a dominant model of inheritance, a significant association was observed when the analysis was adjusted for age and sex ($P=0.042$) or age, sex, and *APOE* genotypes ($P=0.038$). Thus, rs7981045 may be at risk for a poor response to AChEIs in this model when the analysis was adjusted for age and sex (OR =2.398, 95% CI 1.033–5.566)

or when the analysis was adjusted for age, sex, and *APOE* genotypes (OR =2.452, 95% CI 1.055–6.122). Assuming a recessive model of inheritance a significant association was observed in both crude ($P=0.018$) and adjusted analyses ($P=0.027$ and $P=0.028$, respectively), suggesting that rs7981045 may be at risk for a poor response to AChEI in the univariate analysis (OR =7.448, 95% CI 1.420–39.069) as well as in the multivariate analysis considering age and sex (OR =7.675, 95% CI 1.256–48.885) or age, sex, and *APOE* (OR =8.511, 95% CI 1.265–57.258) as confounding factors. Similarly, assuming an additive model of inheritance, this association was confirmed in both univariate ($P=0.011$) and multivariate analyses ($P=0.012$ and $P=0.011$, respectively), suggesting that rs7981045 may be at risk for a poor response to AChEIs in the crude analysis (OR =2.342, 95% CI 1.217–4.508) as well as in the age- and sex-adjusted analysis (OR =2.367, 95% CI 1.208–4.638) and in the age-, sex- and *APOE*-adjusted analysis (OR =2.488, 95% CI 1.231–5.028).

Estimated haplotype frequencies at *FOXO1* locus according to the response to AChEI treatment are summarized in Table 4. In both responders and nonresponders, CGA

Table 4 Estimated haplotype frequencies at *FOXO1* locus according to the response to AChEI treatment

rs2721069	rs4943794	rs7981045	Responders	Nonresponders	χ^2	P-value	All
C	G	A	0.346	0.531	6.51	0.01	0.47
C	G	G	0.368	0.212	5.96	0.014	0.26
T	C	A	0.114	0.155	0.659	0.417	0.14
T	G	A	0.154	0.098	1.477	0.224	0.12

Note: Only haplotypes with and estimated frequencies >1% are reported.

Abbreviation: AChEI, acetylcholinesterase inhibitor.

and CGG haplotypes were the most frequent, followed by haplotypes TCA and TGA. Notably, the most frequent C- haplotypes (CGA + CGG) accounted for about 70% of the overall haplotype frequencies estimated in both responder and nonresponder patients. Haplotype CGA was significantly overrepresented in nonresponder than in responder patients (0.346 versus 0.531; $P=0.010$). Conversely, CGG haplotype was underrepresented in nonresponder than in responder patients (0.212 versus 0.368; $P=0.014$). No significant differences were observed for haplotypes TCA and TGA. In both responder and nonresponder patients, a high value of LD coefficient r^2 at *FOXO1* locus (according to the response to AChEI treatment) was observed between SNPs rs2721069 and rs4943794 ($r^2=0.53$ and $r^2=0.32$, respectively). Conversely, a high value of LD coefficient between SNPs rs2721069 and rs7981045 was observed in nonresponder but not in responder patients ($r^2=0.17$ versus $r^2=0.09$, respectively) as shown in Supplementary Figure S1.

Discussion

The influence of unbalances in biological mechanisms underlying AD pathogenesis, such as oxidative stress, may add an important contribution in considering the overall response to AChEI treatment. Indeed, recent data suggested that AChEIs might act directly on oxidative stress by enhancing antioxidant activity.¹³ Thus, imbalances in oxidative stress/antioxidant activities may reduce the overall response to AChEIs by two ways. First of all, they may reduce the overall health of cholinergic neurons,^{35,36} thus limiting their capability to respond to the nervous signal. Second, they may directly contrast the efficacy of AChEI treatment by inducing an increased production of reactive oxygen species.

Personalized medicine is a medical model that proposes the customization of health care – with medical decisions, practices, and/or products being tailored to the individual patient. A method of targeting medication for an individual based on genetic characteristics would enable doctors to prescribe more effectively. Progress in this direction has been much slower than what the initial excitement suggested. A great deal of this delay relates to the fact that an individual's response to drugs is multifactorial, resulting from multiple gene and environmental interactions.³⁷ Scientists also recognize that even as the knowledge base continues to expand, the clinical translation of that knowledge still requires empirical evidence, generated for a particular disease and drug combination, before treatment can be customized to a patient's genotype.

In the present study we investigated the *FOXO1* gene locus, encoding *FOXO1* protein, as a potential genetic factor reducing the overall therapeutic response to AChEIs in patients with sporadic AD.

FOXO1 is one of the fox proteins playing pivotal roles in several human intracellular pathways. These proteins are transcription factors acting as nuclear regulator of transcriptional activity of a number of metabolic processes such as the cellular response to oxidative stress.³⁸ Indeed, it has been recently proposed that *FOXO1* protein response to oxidative stress may be linked to AD by means of insulin brain resistance.¹⁴ For this reason, in the present study, we enrolled only highly selected AD patients free from any form of insulin metabolism imbalance.

In the present study, we selected three SNPs showing a sufficient level of heterozygosity spanning 65 kb, ie, 60% of the full length of *FOXO1* gene, thus suitable for a genetic analysis of *FOXO1* locus. In the analysis, we observed a significant association of rs7981045 G/G with a poor response to AChEI treatment and important differences in LD coefficient values among these SNPs producing significant differences in haplotype distribution between responder and nonresponder patients. The correct recruitment of our sample is also warranted by the HWE that was correctly checked for each SNP in both responder and nonresponder groups. Notably, in the evaluation of the *APOE* polymorphism, we observed a deviation from the HWE in responder patients. This may be a limitation of the study. However the correct frequencies of the *APOE* polymorphism observed in our AD sample do not differ from those observed in AD Caucasians. Moreover, the association of rs7981045 G/G genotype with a poor response to AChEIs treatment was observed in both crude and *APOE*-adjusted analyses. In the estimation of the LD coefficients across 65 kb spanning *FOXO1*, we observed important differences in values of the LD coefficient between the study groups. As expected, these different LD values produced significant differences in the estimated haplotype frequency distribution between responders and nonresponders. Indeed, we observed a haplotype that may be at risk for a poor response to AChEI treatment as well as a haplotype that may be protective against a poor response to AChEI treatment. This condition is expected from a classical haplotype analysis showing both at risk and protective associations, thus confirming the quality of our analysis. We can speculate that the contribution of *FOXO1* to the overall response to AChEI treatment may be due to a different genetic background at *FOXO1* locus.

Current data on the clinical response to AChEIs in AD patients are not homogeneous, mainly due to the inclusion of patients with different degrees of disease severity, duration of treatments, and criteria to identify responder or nonresponder patients. For these reasons, in this study, we enrolled only highly selected patients with mild to moderate AD who were treated for 6 months, and responders to treatment were defined conservatively according to the NICE criteria as patients who showed improvement or no deterioration in cognition and improvement in functional status.¹⁸ After 6 months of treatment with AChEIs, we showed that responder patients reached significant improvements of the clinical assessment, in particular better values of ADL, IADL, and ADAS-cog.

At 6 months we observed response to AChEI treatment in more than 60% of patients. This rate is in agreement with meta-analyses of randomized clinical trials of AChEIs donepezil, rivastigmine, and galantamine reporting 30% to 68% of patients responding to treatment after 6 months of follow-up.^{6,39} Moreover, these patients showed the typical AD-related *APOE* genotype distribution. Recent studies suggested that the $\epsilon 4$ allele of the *APOE* polymorphism seems to improve the responsiveness to specific AChEIs, such as donepezil.^{40,41} Indeed, we observed significant differences in the distribution of *APOE* $\epsilon 2/\epsilon 4$ and $\epsilon 3/\epsilon 4$ genotypes between the study groups. However, these differences may be due to the non-HWE distribution of the *APOE* genotype frequencies in responder patients. In fact, these differences did not produce differences in the *APOE* allele distribution. Thus, in agreement with other authors,^{8,42,43} our study failed to find a significant role of the $\epsilon 4$ allele in improving the clinical response to AChEIs. Multivariate analyses demonstrated that the significant role of *FOXO1* polymorphism in influencing the clinical response to donepezil was independent from the age, sex and MMSE score at baseline as well as the *APOE* polymorphism. In particular, multivariate analysis did not show a significant role of *APOE* polymorphism in improving clinical responsiveness to donepezil, even after adjustment for sex, age, MMSE at baseline, and *FOXO1* genotypes. All these findings suggest that the *APOE* gene is unrelated to the AChEI metabolism and do not support the hypothesis of a direct interaction between *APOE* and *FOXO1* polymorphisms.

The genotype frequencies of the SNP rs7981045 in the study cohort were comparable to the *FOXO1* genotype distribution reported in Caucasians.³¹ Moreover, the observed genotype frequencies at the *FOXO1* and *APOE* loci did not differ from the expected HWE frequencies nor did they differ

after dividing patients according to sex. These conditions minimize the risk of a genetic bias in patient enrollment.

A limitation of this study is the potential lack of generalizability of our findings, since our AD patients were selected according to strict inclusion criteria. Moreover, the large confidence interval associated with the G/G genotype in both crude (range from 1.706 to 52.108) and adjusted analysis (range from 2.510 to 70.362) could reflect imprecise OR values. However, given the high OR values associated with the G/G genotype (9.429 and 10.308 for the crude and the adjusted analysis, respectively), it is difficult to draw negative conclusions.

Clearly, *FOXO1* is a minor actor in the theater of the events underlying the response to AChEI treatment since it is not an AChEI-metabolizing enzyme such as cytochrome P450 (CYP) 2D6, which has been demonstrated to play a major role in such response.^{8–10} Nevertheless, our results, if confirmed on large samples of highly selected patients, may suggest the presence of factors playing a background role in AChEI efficacy, which must be considered in the prediction of the overall clinical response to drug treatments. Further studies are needed to evaluate the role of *FOXO1* in the response to AChEI treatment in AD patients with different CYP metabolizer phenotypes.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

Table S1 Association of *FOXO1* genotypes with the response to AChEI treatment assuming different genetic models of inheritance

+θ	Genotype/ allele	Univariate model			Multivariate model*					
		P-value	OR	95% CI	Age/sex			Age/sex/APOE		
					P-value	OR	95% CI	P-value	OR	95% CI
rs2721069										
0.0	C/C	–	1.00	–	–	1.00	–	–	1.00	–
	C/T + T/T	0.819	1.10	0.487–2.484	0.715	1.17	0.502–2.727	0.721	1.174	0.486–2.841
0.5	C/C + C/T	–	1.00	–	–	1.00	–	–	1.00	–
	T/T	0.602	1.375	0.415–4.555	0.576	1.424	0.412–4.917	0.364	1.841	0.493–6.869
1.0	C	–	1.00	–	–	1.00	–	–	1.00	–
	T	0.686	1.125	0.636–1.987	0.617	1.162	0.645–2.092	0.487	1.245	0.671–2.308
rs4943794										
0.0	G/G	–	1.00	–	–	1.00	–	–	1.00	–
	G/C + C/C	0.352	0.631	0.239–1.665	0.352	0.622	0.229–1.690	0.436	0.662	0.235–1.866
0.5	G/G + G/C	–	1.00	–	–	1.00	–	–	1.00	–
	C/C	0.963	1.059	0.093–12.086	0.852	1.27	0.102–15.796	0.993	1.011	0.079–12.943
1.0	G	–	1.00	–	–	1.00	–	–	1.00	–
	C	0.433	0.714	0.308–1.656	0.605	1.168	0.648–2.107	0.508	0.741	0.305–1.800
rs7981045										
0.0	A/A	–	1.00	–	–	1.00	–	–	1.00	–
	A/G + G/G	0.06	2.2	0.969–4.996	0.042	2.398	1.033–5.566	0.038	2.452	1.055–6.122
0.5	A/A + A/G	–	1.00	–	–	1.00	–	–	1.00	–
	G/G	0.018	7.448	1.420–39.069	0.027	7.675	1.256–46.885	0.028	8.511	1.265–57.258
1.0	A	–	1.00	–	–	1.00	–	–	1.00	–
	G	0.011	2.342	1.217–4.508	0.012	2.367	1.208–4.638	0.011	2.488	1.231–5.028

Notes: *Both Student's t-test analyses were also adjusted for the MMSE score at baseline and AChEIs. Where θ=0.0, dominant model; θ=0.5, recessive model; θ=1.0, additive model.

Abbreviations: AChEI, acetylcholinesterase inhibitor; CI, confidence interval; MMSE, mini-mental state examination; OR, odds ratio.

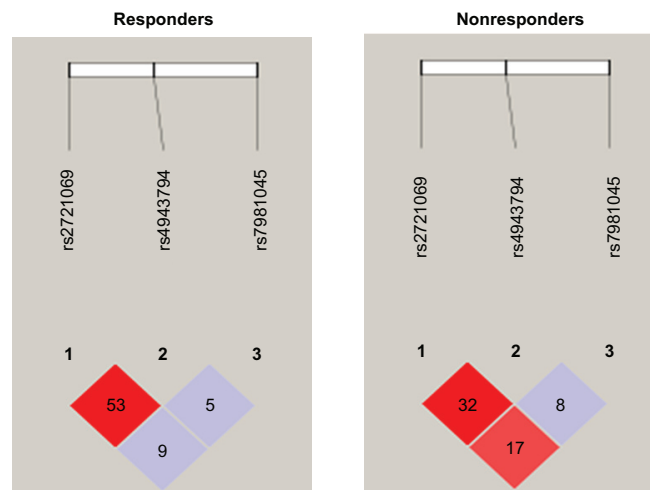


Figure S1 Estimated values of linkage disequilibrium coefficient r^2 at *FOXO1* locus according to the response to AChEI treatment.

Abbreviation: AChEI, acetylcholinesterase inhibitor.

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