

# Oestrogen receptor polymorphisms are an associated risk factor for mild cognitive impairment and Alzheimer disease in women APOE ε4 carriers: a case-control study

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## ABSTRACT

**Objectives:** Examine the role of single nucleotide polymorphisms (SNPs) in the oestrogen receptor (ER) genes: rs9340799, rs2234693, rs2228480 (in the *ESR1* gene) and rs4986938 (in the *ESR2* gene) as a risk factor for amnesic mild cognitive impairment (MCIa) and Alzheimer's disease (AD) and its possible association with the apolipoprotein E (*APOE*) gene.

**Design:** We have investigated the independent and combined association of different alleles of the oestrogen receptor genes and APOE\*ε4 allele with cognitive impairment using a case-control design.

**Setting:** Participants were prospectively recruited from the neurology departments of several Basque Country hospitals.

**Participants:** This study comprised 816 Caucasian participants who were aged 50 years and older: 204 MCIa, 350 sporadic patients with AD and 262 healthy controls.

### Primary and secondary outcome measures:

Clinical criteria and neuropsychological tests were used to establish the diagnostic groups (MCIa, AD and healthy controls). A dichotomous variable was used for each allele and genotype and the association with MCIa and AD was established using Logistic Regression Models.

**Results:** Neither alleles nor genotypes of SNPs rs9340799, rs2234693, rs2228480 and rs4986938 of oestrogen receptor genes (*ESR1* and *ESR2*) are independently associated with the risk of MCIa or AD. However, the genetic profile created with the combination of the less represented alleles of these SNPs (expressed as XPAA) was associated with an increased risk for MCIa (OR=3.30, 95% CI 1.28 to 8.54, p=0.014) and AD (OR=5.16, 95% CI 2.19 to 12.14, p<0.001) in women APOE\*ε4 allele carriers.

**Conclusions:** The less represented alleles of SNPs studied are associated with MCIa and AD in APOE\*ε4

## ARTICLE SUMMARY

### Strengths and limitations of this study

- It was one of the first studies to investigate the association between polymorphisms of the ER genes and cognitive function not only in patients with AD, but also in patients with MCIa.
- It is a multicenter study with a patient sample that allows gender stratification.
- The study population comes from the hospital setting. A community-based study could provide more information.
- The levels of estradiol and the previous oestrogen replacement therapy were unknown.

carriers. In particular, the genetic profile created with the less represented alleles of *ESR1* and *ESR2* SNPs are associated with an increased risk for MCIa and AD in women APOEε4 allele carriers.

## INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia, currently affecting over 9 million Americans and Europeans; its aetiology is complex and multifactorial. Several genes associated with sporadic and familial AD have been identified, but it is estimated that probably more than 50% of genetic risk remains unidentified.<sup>1</sup>

The apolipoprotein E gene (*APOE*) is a genetic factor closely related to the late onset AD disease, and constitutes a strong independent risk factor for sporadic AD.<sup>2</sup> However, the *APOE* gene explains only a fraction of the

genetic risk associated with AD, and it is possible that other genes or metabolic factors may modify the *APOE* effect to initiate the pathogenesis of AD.

In the past, genetic research had focused on identifying common population polymorphism loci, such as *APOE*, as well as other genes such as *CLU*, *CRI*, *PICALM* and *EXOC3L2*, which have been associated with an increased risk for developing AD.<sup>3–6</sup> These genes are implicated in chaperone action, positive regulation immune response and regulation of receptor-mediated endocytosis. Strikingly, although these genes have a significant effect on the risk of AD, risks differ by more than two orders of magnitude lower than *APOE*.

Oestrogens are pleiotropic hormones having an influence not only on the reproductive system but also on the central nervous system (CNS). These hormones are synthesised by ovaries and are also produced in smaller amounts by other tissues such as glia in CNS, having a wide spectrum of neuroprotective and antiapoptotic effects.<sup>7–9</sup> Synaptogenic effects of estradiol-17- $\beta$  have been demonstrated in the adult mammalian brain (rodent and monkey models); low levels of estradiol are correlated with lower synapse density, while high estradiol levels are correlated with a higher density of synapses in the hippocampal region and dendritic spine density in CA1 pyramidal cells.<sup>10–11</sup> Among the other positive effects of oestrogens,<sup>12</sup> estradiol-17- $\beta$  has an effect on (1) the maintenance and increase of the neurotransmitter systems, (2) the *APP* processing, A $\beta$  levels and factors that alter its clearance and aggregation<sup>13</sup> and (3) mechanisms of oxidative damage. Multiple lines of evidence suggest that loss of oestrogens in the ageing brain of women and men may play a role in the cognitive declines associated with AD,<sup>14</sup> but whether the female sex is also a risk factor is controversial, although some past studies and a recent study show higher rates of cognitive decline for women and apolipoprotein E4 carriers (*APOE*\* $\epsilon$ 4),<sup>15–16</sup> and mouse AD-transgenic mice studies generally show great amyloid and neurodegeneration in females.<sup>17–18</sup> However, despite the initial data,<sup>19–22</sup> there is disagreement regarding hormonal replacement therapy in women.<sup>14–23–25</sup>

Furthermore, it has also described an interaction with ApoE. Estradiol increased ApoE levels and neurite outgrowth. The *APOE*\* $\epsilon$ 2 isoform increased the neurite length more than did the *APOE*\* $\epsilon$ 3 isoform in the presence of estradiol-17- $\beta$ . The hormone had no effect on neurite outgrowth from mice lacking the *APOE* gene or when only *APOE*\* $\epsilon$ 4, the isoform that is associated with increased risk of neurological disease, was exogenously supplied.<sup>26</sup> These data support the hypothesis that the *APOE* gene plays an integral role in the neurotrophic effects of estradiol-17- $\beta$  and in the presence of a probable synergism between the ApoE subtype expression and the effects of oestrogens.

The mechanism through oestrogens exerts its neuroprotective and antineurodegenerative effects in the CNS is poorly understood and is mediated by two oestrogen

receptors (ER), ER $\alpha$  and ER $\beta$  (coded by the *ESR1* and *ESR2* genes), expressed in neurons and glia throughout the brain, especially in the hippocampus and amygdala<sup>27–28</sup> regions involved in the memory and learning process. Thus, genetic variants in the ER genes have been studied in relation to AD. There are several polymorphic loci in intron 1 of *ESR1* gen, highlighting the PvuII and XbaI locus.<sup>29</sup> The polymorphisms of PvuII were coded as P or p and the polymorphisms of XbaI as X or x, in which the capital letter signifies the absence of the restriction site and the lower case letter signifies its presence. Participants were described as pp or xx homozygotes, Pp or Xx heterozygotes or PP or XX homozygotes.<sup>30</sup> The xp haplotype has a higher expression than the XP one, but with no significant differences.<sup>31</sup> Several studies,<sup>32–34</sup> but not all,<sup>31</sup> have found an increased frequency of the PvuII and XbaI *ESR1* polymorphisms in patients with AD.

Another interesting SNP is rs2228480; this polymorphism is the coding synonymous variant to codon 594 (rs2228480) within the last exon of the gene *ESR1* gen. This variant is thought to play a role in distinguishing between the receptor agonist or antagonists binding to the receptor molecule.<sup>35</sup> In addition, this SNP has been associated with schizophrenia and the mechanism of this association may involve alternative gene regulation and transcript processing.<sup>36</sup>

Other studies have shown an association between several polymorphisms of the *ESR2* gene and the late onset AD, and they found that variations in this gene could modify disease susceptibility.<sup>37</sup> The polymorphism located in the 3'UTR of the *ESR2* gene, rs4986938, has been associated with the onset of Parkinson disease<sup>38</sup> and the susceptibility for vascular dementia (VaD) in an Israeli cohort, but not with AD.<sup>39</sup> In the study of Dresener-Pollack *et al*,<sup>39</sup> VaD is differentiated from AD by clinical criteria, but in the absence of imaging data, the potential misclassification is high. Thus, the results should be confirmed.

Until now, no studies have been conducted in the prodromal stages of AD such as mild cognitive impairment of amnesic type (MCIa). Such studies could provide information about the beginning of the disease process, helping to ensure that suitable therapeutic measures would be implemented at an early stage.

According to the above, the aim of the present study was to determine whether the *ESR1* and *ESR2* genes are linked to the risk of MCIa; whether there is an interaction with the *APOE* gene; and whether such an interaction could influence the risk of AD and MCIa. Our hypothesis is that the association of the *ESR1* and *ESR2* genes with cognitive impairment may exist only in the *APOE*\* $\epsilon$ 4 carriers. We have studied this association in patients with AD and in patients with MCIa, the latter condition possibly representing a prodrome for AD-type dementia.<sup>40</sup>

With the purpose of examining the association of the *ESR1* and *ESR2* genes involved in oestrogen metabolism, as a genetic risk factor for cognitive impairment, we

conducted a study on a sample of patients with MCIa, AD and a control group. All subjects were analysed for the *ESR1* (rs9340799, rs2234693 and rs2228480) and *ESR2* (rs4986938) polymorphisms and the *APOE* genotype.

## METHODS

This study comprised 816 Caucasian participants, included in three groups: patients with MCIa (n=204), patients with AD (n=350) and healthy controls (CTL) (n=262). Participants were prospectively recruited from the neurology departments of several hospitals. Participants were aged 50 years and older. For patients with AD and MCIa, evaluation also included routine blood tests: haematology, biochemistry, thyroid-stimulating hormone, vitamin B<sub>12</sub> levels, syphilis serology and neuroimaging test: CT scan or MRI.

The participants were evaluated using a broad battery of neuropsychological tests: Mini-Mental State Examination (MMSE), Clinical Dementia Rating scale, CERAD protocol, Stroop test, unilateral and bilateral motor praxis, 7 min test, Trail Making Test (TMT) Parts A & B; and neuropsychiatric inventory (NPI).

Based on the results of these evaluations, the participants were classified into the following groups: patients with MCIa, patients with AD and healthy control subjects.

The diagnosis of patients with MCIa was based on Petersen's criteria.<sup>40</sup> Patients had memory complaints corroborated by an informant, representing a decline from a previous level of functioning given their age and educational level. The score in the CDR scale was required to be 0.5, and performance in relation to other cognitive functions and daily living activities was required to be normal. The diagnosis of AD was based on the DSM IV and NINCDS-ADRDA criteria for probable and possible AD. Patients with a total score of less than three on the CDR scale (mild to moderate dementia) were included.

Healthy control subjects were scored within the normal ranges for age and educational level in psychometric testing, with a CDR score of 0.

The exclusion criteria included: severe comorbidities making adequate follow-up unlikely, acute psychiatric diseases, previous cerebrovascular diseases (transient ischaemic attacks, stroke or intracranial haemorrhage), other neurodegenerative diseases and the absence of a reliable informant.

A specific database was designed and declared to the Spanish Data Protection Agency. The study was approved by the Ethics Committee of Cruces Hospital (Barakaldo, Spain). All patients signed informed consent to undergo the examination. The study was conducted in accordance with the Declaration of Helsinki concerning medical research in human subjects.

## Genetic analysis

On the first visit, peripheral blood samples were collected in EDTA vacuum tubes from all individuals.

Genomic DNA was extracted by proteolytic lysis from white blood cells using the standard phenol/chloroform extraction method.

The *APOE* gene was amplified by PCR with 112F and 158R primers, under the PCR conditions described by Wilton and Lim.<sup>41</sup> Digestion of the amplified product was carried out with Hae II and Afl III, as described by Álvarez-Álvarez *et al.*<sup>42</sup>

Three single nucleotide polymorphisms (SNPs) in the *ESR1* gene (rs9340799, rs2234693 and rs2228480) and one SNP in the *ESR2* gene (rs4986938) were evaluated. The first two SNPs in *ESR1* (rs9340799 and rs2234693) are in intron 1 and are separated by only 46 base pairs. The rs9340799 polymorphism marks an A→G transition 351 nucleotides upstream in intron 1 (also known as c.454-351A>G). Those with the G allele have an absent XbaI site, which has previously been called X in the literature, with the A allele denoted by x. The rs2234693 polymorphism is characterised by a T→C transition 397 nucleotides upstream in the intron (also known as c.454-497T>C) that obliterates the PvuII restriction site. The T allele has previously been called the p allele, while the C allele has been called the P allele, denoting the absence of the PvuII restriction site. Subjects were described as XX, xx, PP, pp, homozygotes; and Xx or Pp heterozygotes.

Taqman SNP Genotyping Assays were used to analyse the polymorphism rs2228480; G>A (SNP1) of *ESR1* gene and polymorphism rs4986938; G>A (SNP2) of *ESR2* gene.

SNP genotypes of candidate genes (*ESR1* and *ESR2*) and the *APOE* gene were analysed blinded to clinical diagnosis.

The less frequent alleles of each SNP were evaluated, such as a combined genotype (XPAA). Therefore, with the name of XPAA, we are referring to all haplotypes with at least one X allele (rs9340799), one P allele (rs2234693), one A allele (rs2228480) and one A allele (rs4986938).

## Statistical analyses

Genepop V.4.0 was used to test the goodness of the fit to the Hardy-Weinberg equilibrium by means of the Guo-Thompson exact test for all three groups studied.<sup>43</sup> The G test was also used to check the differences between the demographic and clinical variables, as well as between the allele frequencies and genotype frequencies.

Statistical analysis was also performed using the SPSS package, V.15.0. A dichotomous variable was used for each polymorphism: 'yes' or 'no' for 'carrier' or 'non carrier' of the *APOE*\*ε4 allele and for different alleles and genotypes of the SNPs in candidate genes (*ESR1* and *ESR2* genes).

Several multinomial regression models were created in order to determine the independent effects of X, P and SNP1-A alleles of *ESR1* gene and SNP2-A allele of *ESR2* gene in the total sample and in the absence of the *APOE*\*ε4 allele. The effect of the *APOE*\*ε4 allele in the

total sample and in the different diagnostic groups was also calculated. Another model was created to assess the combined effect of different polymorphisms of *ESR1* and *ESR2* genes and the APOE\* $\epsilon$ 4 allele, based on the hypothesis that the effect of oestrogens might exist only in APOE\* $\epsilon$ 4 allele carriers.

Because age and gender could be associated with the frequency of some polymorphisms, we adjusted our analysis for these covariates in the total sample. *p* Values of less than 0.05 were considered statistically significant.

## RESULTS

We have investigated the independent and combined associations of X, P and SNP1-A alleles of *ESR1* gen and SNP2-A allele of *ESR2* gen and the APOE\* $\epsilon$ 4 allele by using a case-control design.

In the present study, we analysed a sample of 204 patients with MCIa, 350 patients with AD and 262 healthy control subjects without significant differences in terms of age ( $p>0.05$ ). There was, however, a significant difference in the MMSE score between groups ( $p<0.05$ ) (table 1). Years of education were not significantly different between groups ( $p=0.148$ ).

Table 2 shows the allele and genotype frequencies of *ESR1* and *ESR2* polymorphisms and the *APOE* gene in MCIa, AD and controls. In all studied groups, frequencies were in the Hardy-Weinberg equilibrium ( $p>0.05$ ).

There were no significant differences in the allele and genotype frequencies in MCIa and AD compared to controls for *ESR1* and *ESR2* gene polymorphisms, while the differences proved to be significant for the *APOE* gene (table 3).

In order to determine whether the less represented alleles of SNPs in candidate genes (*ESR1* and *ESR2* genes) were an independent risk factor for MCIa and AD, we selected a subgroup of individuals with MCIa and AD and controls with the presence of at least one of these alleles. None of them had a significant effect (data not shown).

In the total sample, the APOE\* $\epsilon$ 4 allele is a risk factor for cognitive impairment; the ORs of developing MCIa and AD were 2.44 (95% CI 1.61 to 3.69,  $p<0.001$ ) and 4.23 (95% CI 2.93 to 6.12,  $p<0.001$ ), respectively (table 4). The higher risk conferred by the APOE\* $\epsilon$ 4 allele was observed even when the samples were subgrouped by sex, but in the AD women the risk was higher than in men,

4.85 (95% CI 3.04 to 7.73,  $p<0.001$ ) vs. 3.19 (95% CI 1.73 to 5.88,  $p<0.001$ ).

Aiming to avoid the combined effect of the less represented alleles of SNPs in candidate genes and the APOE\* $\epsilon$ 4 allele, we analysed the risk of MCIa and AD according to the presence of X, P, SNP1-A and SNP2-A alleles and the absence of one APOE\* $\epsilon$ 4 allele. We did not find a significant effect, even when the samples were subgrouped by sex (data not shown).

We further evaluated a possible synergistic effect between the less represented alleles of SNP in candidate genes and the APOE\* $\epsilon$ 4 allele by using a multivariate logistic regression model. To analyse this effect, we subgrouped the subjects according to the presence of X, P, SNP1-A and SNP2-A alleles and at least one APOE\* $\epsilon$ 4 allele. A slight increase in the nominal risk of MCI and AD was observed. The statistical analyses were also conducted according to gender (see supplementary tables S1 and S2).

In order to analyse the combined effect between oestrogen polymorphisms, we created a genetic profile with the less represented alleles of these SNPs, expressed as XPAA. We did not find a significant risk in the absence of one APOE\* $\epsilon$ 4 allele, but analysing the combined effect of XPAA with the APOE\* $\epsilon$ 4 allele, ORs were the following: MCIa, OR=3.30 (95% CI 1.28 to 8.54,  $p=0.014$ ) and AD, OR=5.16 (95% CI 2.19 to 12.14,  $p<0.001$ ); these ORs were even greater than the independent effect of the APOE\* $\epsilon$ 4 allele with XPAA(–) (absence of this genetic profile). Although it was expected to obtain a greater effect in men with MCI and women with AD, according to the results shown in table 3, when the samples were subgrouped by sex taking into account the genetic profile, women with MCIa and AD showed an increased OR, 3.84 (95% CI 1.09 to 13.57,  $p<0.036$ ) and 8.04 (95% CI 2.60 to 24.80,  $p<0.001$ ), respectively, compared to men (table 4).

## DISCUSSION

Our study shows that neither alleles nor genotypes of SNPs rs9340799 (A>G; XbaI), rs2234693 (PvuII; C>T) and rs2228480 (A>G) (*ESR1* gene) and SNP rs4986938 (A>G) (*ESR2* gene) are independently associated with the risk of MCIa or AD. The less represented alleles of SNPs in candidate genes (*ESR1* and *ESR2* genes) were not an independent risk factor for MCIa and AD in the

**Table 1** Baseline demographic

Group	n	Age*	Women (%)†	MMSE‡	Education§
MCIa	204	70.25±8.6	61.3	26.38±2.05	8.08±4.36
AD	350	72.17±8.3	71.1	19.68±4.60	8.41±7.90
CONTROLS	262	74.00±9.6	59.5	28.45±1.63	9.51±4.80

\*Years, mean±SD.

†Per cent of women in group.

‡MMSE score, mean±SD.

§Years of education.



**Table 2** Allelic and genotypic frequency

<b>ESR1</b>			
<b>XbaI</b>	<b>MCIa (N=204)</b>	<b>AD (N=350)</b>	<b>CONTROLS (N=262)</b>
Allele			
X	0.426	0.409	0.395
x	0.574	0.591	0.605
Genotype			
XX	0.157	0.154	0.156
Xx	0.539	0.509	0.477
xx	0.304	0.337	0.366
H-W*			
p Value	0.197	0.376	1.000
<b>Pvull</b>			
Allele			
P	0.488	0.480	0.462
p	0.512	0.520	0.538
Genotype			
PP	0.225	0.209	0.214
Pp	0.525	0.543	0.496
pp	0.250	0.249	0.290
H-W*			
p Value	0.575	0.110	1.000
<b>SNP1</b>			
Allele			
A	0.191	0.189	0.174
G	0.809	0.811	0.826
Genotype			
AA	0.039	0.037	0.030
AG	0.304	0.303	0.286
GG	0.657	0.660	0.684
H-W*			
p Value	0.818	0.861	1.000
<b>ESR2</b>			
<b>SNP2</b>			
Allele			
A	0.424	0.419	0.378
G	0.576	0.581	0.622
Genotype			
AA	0.201	0.189	0.133
AG	0.446	0.460	0.489
GG	0.353	0.351	0.378
H-W*			
p Value	0.245	0.325	0.591
<b>APOE</b>			
Allele			
2	0.027	0.034	0.057
3	0.743	0.665	0.842
4	0.230	0.301	0.101
Genotype			
2,2	0.000	0.000	0.008
2,3	0.044	0.046	0.092
2,4	0.010	0.017	0.008
3,3	0.574	0.434	0.698
3,4	0.294	0.420	0.195
4,4	0.078	0.083	0.000
H-W*			
p Value	0.217	0.814	0.102
<b>Genetic profile</b>			
XPAA(+)	0.709	0.708	0.674
XPAA(-)	0.291	0.292	0.326

\*Hardy-Weinberg probability test

absence of APOE\* $\epsilon$ 4. Furthermore, the genetic profile created with the less represented alleles of SNPs in candidate genes were associated with an increased risk for MCIa and AD in women and APOE\* $\epsilon$ 4 allele carriers.

In our series, the APOE\* $\epsilon$ 4 allele seems to be an independent risk factor for the population with AD, and this risk is highest for women. The APOE\* $\epsilon$ 4 allele also constitutes a risk factor for patients with MCIa.

On evaluating the combined effect of the APOE\* $\epsilon$ 4 allele in the presence of alleles or genotypes of *ESR1* and *ESR2* SNPs the risk for AD remains significant, although this association did not confer a relevant additional risk of MCIa and AD.

When we created a genetic profile with the less represented alleles of *ESR1* and *ESR2* SNPs, expressed as XPAA, we did not find a significant risk in the absence of one APOE\* $\epsilon$ 4 allele. However, the presence of XPAA and at least one APOE\* $\epsilon$ 4 allele increases the risk in women with MCIa and AD.

Nowadays, the most well-known polymorphism of the *ESR1* gene related to AD are SNPs rs9340799 (A>G; XbaI) and rs2234693 (PvuII; T>C). As regards the association between XbaI with AD, several studies show that the *ESR1* XbaI polymorphism is an additional risk factor.<sup>32 44-46</sup> However, other studies have not found this association.<sup>34 47-50</sup> These results and several meta-analyses<sup>51</sup> suggested that the *ESR1* gene polymorphisms might be related to the individual susceptibility to AD, especially in women.

Several published studies have shown a great heterogeneity concerning the association between the *ESR1* PvuII polymorphism and AD. In some of them, no association has been found.<sup>31 47 48 50 52</sup> Some studies claimed a protective role for the P allele of the *ESR1* PvuII polymorphism,<sup>34 45 46</sup> whereas others found an opposite effect.<sup>32 33 44 53-55</sup> Some studies<sup>44</sup> have established an association between *ESR1* PP and XX genotypes with an increased risk for AD only in men (OR=3.6, 95% CI 1.2 to 10.9) and conferred a relevant additional risk of AD on subjects also carrying the APOE\* $\epsilon$ 4 allele and in women with AD. In this last study, the *ESR1* PP and XX genotypes were also associated with lower MMSE values (p=0.0007). These data suggest that the involvement of *ESR1* polymorphisms (XbaI and PvuII) in AD onset is mediated by the regulation of *APOE* expression. Our data support this hypothesis, in accordance with the increased risk of MCI and AD observed in patients with the APOE\* $\epsilon$ 4 allele.

To our knowledge, this is the first study to show evidence in support of the association of SNP rs2228480 with patients with MCI and AD and the APOE\* $\epsilon$ 4 allele carriers. Previously, this SNP had only been linked to the alternative regulation and transcript processing of the *ESR1* gene.<sup>36 56</sup> Until now additional information has not been provided with regard to neurodegenerative disorders.

Regarding polymorphisms of the *ESR2* gen, several studies have been published with conflicting results: susceptibility for VaD but not for sporadic AD in elderly

Table 3 Exact G test

	p Value	SE		p Value	SE
<b>Xbal*</b>			<b>Xbal†</b>		
MCI vs CTL	0.339	0.006	MCI vs CTL	0.336	0.004
MCI vs AD	0.571	0.005	MCI vs AD	0.564	0.004
AD vs CTL	0.638	0.005	AD vs CTL	0.635	0.004
<b>Pvull*</b>			<b>Pvull†</b>		
MCI vs CTL	0.479	0.006	MCI vs CTL	0.464	0.004
MCI vs AD	0.853	0.002	MCI vs AD	0.846	0.002
AD vs CTL	0.562	0.006	AD vs CTL	0.548	0.005
<b>SNP1*</b>			<b>SNP1†</b>		
MCI vs CTL	0.483	0.005	MCI vs CTL	0.491	0.004
MCI vs AD	0.935	0.002	MCI vs AD	0.935	0.001
AD vs CTL	0.532	0.011	AD vs CTL	0.552	0.007
<b>SNP2*</b>			<b>SNP2†</b>		
MCI vs CTL	0.180	0.009	MCI vs CTL	0.153	0.005
MCI vs AD	0.896	0.003	MCI vs AD	0.904	0.003
AD vs CTL	0.139	0.007	AD vs CTL	0.173	0.009
<b>APOE*</b>			<b>APOE†</b>		
MCI vs CTL	0.000	<0.001	MCI vs CTL	0.000	<0.001
MCI vs AD	0.033	0.002	MCI vs AD	0.033	0.002
AD vs CTL	0.000	<0.001	AD vs CTL	0.000	<0.001

\*Allelic frequency.

†Genotypic frequency.

Jewish women was found in the *ESR2* rs4986938 polymorphism.<sup>39</sup> Pirskanen *et al*<sup>37</sup> found that some gene variants of the *ESR2* gene are associated with increased risk of AD in women (rs1271573 T/T genotype and rs1256043 T/T genotype) while others are not (IVS31842, rs4986938). Lambert *et al*<sup>48</sup> found no independent association of these polymorphisms with the risk of developing AD. One study suggests that the *ESR2* allele 5 seems to be a protective factor.<sup>57</sup> Meta-analyses have not been performed on the following polymorphisms of the *ESR2* gene since they lack published genotype data or the published genotype data were not eligible for inclusion. Other studies<sup>58</sup> have not detected a significant gene-gene interaction between *ESR1*, *ESR2* SNPs and *APOE* status, but the analysis was performed in late onset AD.

In contrast with previous studies, we have analysed the genetic profile of the less represented alleles of the *ESR1* and *ESR2* gene polymorphisms, XPAA; when considering the XPAA isolatedly, the genetic profile was not an independent risk factor for MCIa and AD, but the combined effect with the *APOE*\* $\epsilon$ 4 allele confers an increased risk in women, while it does not contribute to the disease susceptibility in men. Analysis of haplotypes offers more power to detect associations than does simply focusing on a single variant, but in our case the actual results differed slightly from those expected. The combined effect observed between X, P, SNP1-A and SNP2-A alleles and at least one *APOE*\* $\epsilon$ 4 allele seemed to point to an increased risk in men with MCIa and women with AD. Our case-control study is relatively

medium sized with a small number of samples carrying the genetic profile (<8% in patients with MCI and AD and <2% in controls) and the *APOE*\* $\epsilon$ 4 allele that may affect the power negatively. Nevertheless, according to our results, some variations in the ER genes in synergy with the *APOE*\* $\epsilon$ 4 allele may be associated with an increased risk of MCIa and AD in women.

Our results may suggest that the risk for MCIa and AD may be modulated only when the *ESR1* and *ESR2* genes have several polymorphisms, which might be related to their expression and biological activities. The variations in the ER genes may involve alternative gene regulation and transcript processing in the brain.<sup>36</sup> The *APOE* gene expression can be differentially regulated depending on the activation of ER subtypes. A recent study<sup>59</sup> demonstrated that activation of the *ESR1* gene upregulated the *APOE*\* $\epsilon$ 4 mRNA and protein expression in the hippocampus. In contrast, an activation of the *ESR2* gene downregulated the mRNA and protein expression of the *APOE* gene. Thus, it is expected to lower regulation in postmenopausal women<sup>60</sup> conferring less protection against the effect of the *APOE*\* $\epsilon$ 4 allele.

Relatively few studies have examined the epistatic effects between the oestrogen-related pathway genes and the *APOE*\* $\epsilon$ 4 allele. Postmenopausal women with Down syndrome showed an increased risk of AD and an elevated sex hormone binding globulin in those carrying *CYP17* and *CYP19* variants and the *APOE*\* $\epsilon$ 4 allele.<sup>61</sup> Both genes are involved in the production of neurosteroids (oestrogens and testosterone). In addition, oestrogens have been shown to affect amyloid precursor

**Table 4** Risk Factors for MCI and AD from Logistic Regression Models

	MCI		AD	
	OR CI 95%	p Value	OR CI 95%	p Value
<b>Global effects</b>				
X (+)†	1.39 (0.93 to 2.06)	0.104	1.18 (0.85 to 1.67)	0.324
P (+)‡	1.25 (0.82 to 1.90)	0.293	1.26 (0.88 to 1.23)	0.205
SNP1-A§	1.14 (0.76 to 1.71)	0.506	1.13 (0.78 to 1.62)	0.510
SNP2-A¶	1.05 (0.71 to 1.54)	0.304	1.08 (0.77 to 1.51)	0.649
E4 (+)**	2.44 (1.61 to 3.69)	<0.001	4.23 (2.93 to 6.12)	<0.001
Women	1.07 (0.73 to 1.56)	0.705	1.67 (1.19 to 2.35)	0.003
E4 (+)*Women††	2.27 (1.32 to 3.87)	0.003	4.85 (3.04 to 7.73)	<0.001
E4 (+)*Men‡‡	2.74 (1.43 to 5.23)	0.002	3.19 (1.73 to 5.88)	<0.001
<b>Independent effects</b>				
X (+) E4(-)§§	1.04 (0.65 to 1.66)	0.863	1.18 (0.76 to 1.81)	0.452
P (+) E4(-)§§	0.86 (0.52 to 1.40)	0.545	1.19 (0.754 to 1.90)	0.444
SNP1-A(+)*E4(-)§§	1.19 (0.74 to 1.92)	0.469	1.13 (0.73 to 1.76)	0.568
SNP2-A(+)*E4(-)§§	1.03 (0.65 to 1.66)	0.879	1.07 (0.70 to 1.64)	0.758
<b>ESR1</b>				
<b>Combined effects</b>				
E4(+)*X¶¶	3.17 (1.80 to 5.59)	<0.001	5.07 (3.00 to 8.55)	<0.001
E4(+)*P¶¶	2.74 (1.55 to 4.85)	0.001	5.35 (3.11 to 9.17)	<0.001
E4(+)*SNP1-A¶¶	2.53 (1.31 to 4.90)	<0.001	4.44 (2.48 to 7.93)	<0.001
<b>ESR2</b>				
<b>Combined effects</b>				
E4(+)*SNP2-A¶¶	2.77 (1.55 to 4.93)	0.001	4.87 (2.91 to 8.17)	<0.001
<b>Genetic profile (XPAA)</b>				
<b>Independent effects</b>				
XPAA*E4(-)***	1.31 (0.48 to 3.54)	0.590	1.19 (0.49 to 2.91)	0.696
XPAA(-)*E4(+)*†††	2.53 (1.61 to 3.93)	<0.001	4.32 (2.91 to 6.40)	<0.001
<b>Combined effects</b>				
XPAA*E4(+)*§§§	3.30 (1.28 to 8.54)	0.014	5.16 (2.19 to 12.14)	<0.001
XPAA*E4(+)*Women§§§	3.84 (1.09 to 13.57)	0.036	8.04 (2.60 to 24.80)	<0.001
XPAA*E4(+)*Men§§§	3.20 (0.73 to 14.11)	0.124	3.57 (0.88 to 14.47)	0.075

†Effect of sample with at least one X of RFLP XbaI.

‡Effect of sample with at least one P of RFLP PvuII.

§Effect of sample with at least one A allele of rs2228480.

¶Effect of sample with at least one A allele of rs4986938.

\*\*Effect of sample with at least one E4 allele of the APOE gene.

††Women selected by at least one E4 allele of the APOE gene.

‡‡Men selected by at least one E4 allele of the APOE gene.

§§Sample selected by at least one allele that is indicated and the absence of the E4 allele of the APOE gene.

¶¶Sample selected by at least one E4 allele of the APOE gene and one of the alleles that is indicated. The reference category was sample control.

\*\*\*Sample selected by absence of the E4 allele of the APOE gene and the presence of XPAA.

†††Sample selected by absence of XPAA and the presence by at least one E4 allele of the APOE gene. Sample selected by at least one E4 allele of the APOE gene and the presence XPAA.

‡‡‡Sample selected by at least one E4 allele of the APOE gene and the presence of XPAA.

§§§Women or men selected by at least one E4 allele of the APOE gene and the presence of XPAA. \* In all models, the reference category was sample control considering the age and sex (as appropriate).

protein metabolism, by increasing the secretory metabolism of amyloid protein precursor (App). Oestrogens are also a potent factor that not only prevents vascular disease but also improves blood flow, including blood flow in regions of the brain affected by AD.<sup>62</sup> Synaptic sprouting by estradiol in a model of AD may operate via an APOE\*ε4-dependent mechanism.<sup>63</sup> Cholinergic neurons that are implicated in cognitive functions may be regulated by oestrogens. The distribution of ERs corresponds to that of the cholinergic system.<sup>64</sup> The important decrease in endogenous oestrogen levels after

menopause may contribute to the development of AD.<sup>65</sup> Despite the protective effect of oestrogens on AD, this effect might be modified by ER polymorphisms, particularly in the APOE\*ε4 allele carriers. Thus, the current state of knowledge of the role of oestrogens in preventing dementia in postmenopausal women should be reviewed.

Although the prevalence and incidence of AD are higher in women, men may also have the same effect due to SNPs in ER genes. It has been observed that while androgens have specific receptors to exert their neuroprotective action, they may also exert their actions

indirectly via *CYP17* by aromatisation of testosterone to estradiol<sup>66</sup> or directly through the binding of the metabolite dihydrotestosterone to *ESR2*.<sup>67</sup> Until now, it is unclear whether SNPs in ER genes would increase the risk of AD or MCIa in men. Our partial data show a tendency to increase the risk of MCIa in men. Future studies should elucidate whether there is a relationship between ER genes and MCIa in men.

The strengths of our study are its multicenter nature including patients with AD, healthy controls and patients with MCIa. To our knowledge, our study is the first to investigate an association between polymorphisms of ER (rs9340799, rs2234693, rs2228480 and rs4986938) and cognitive function not only in patients with AD, but also in patients with MCIa. Moreover, the patient sample is not small, allowing gender stratification.

Some limitations in our study must be addressed. The study population comes from the hospital setting. A community-based study could provide more information. The serum levels of estradiol have not been measured, and we do not know whether the patients received ERT in the last years. We also include a sample of patients with MCIa; this stage is probably a heterogeneous clinical entity, but the broad battery of neuropsychological tests used in our sample might ensure the highest homogeneity.

## CONCLUSIONS

In our study, the APOE\*ε4 allele is an independent risk factor for patients with MCIa and AD. The combined effect of the APOE\*ε4 allele and the less represented alleles of *ESR1* and *ESR2* SNPs remains the risk for MCIa and AD; this association confers a relevant additional risk of AD and MCIa in women and men, respectively. Nevertheless, the genetic profile with the less represented alleles of the *ESR1* and *ESR2* gene polymorphisms, expressed as XPAA, did not increase the risk of cognitive impairment in the absence of one APOE\*ε4 allele, but the presence of XPAA and at least one APOE\*ε4 allele only increases the risk in women with MCIa and AD.

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study design and coordination and helped to draft the manuscript, EBM, IUS and MAA were involved in the drafting of the manuscript. LGA and FGB were involved in the study design and coordination and helped to draft the manuscript. AMS, RGB, SIB, NO, MBA and MCZ performed the battery of neuropsychological tests. JMUUV, BIJ, MAGB and JML participated in the design of the study and coordination of the manuscript. MMP participated in the study design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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