BMJ open

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Journal:	*BMJ Open*
Manuscript ID:	bmjopen-2013-003200
Article Type:	Research
Date Submitted by the Author:	16-May-2013
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Primary Subject Heading:	Neurology
Secondary Subject Heading:	Geriatric medicine
Keywords:	Dementia < NEUROLOGY, Adult neurology < NEUROLOGY, GENETICS

Estrogen receptor polymorphisms are an associated risk factor for mild cognitive impairment and Alzheimer disease in women APOE ε4 carriers

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ABSTRACT

Objetives: Examine the role of the single nucleotide polymorphisms (SNPs) in the estrogen receptor genes: rs9340799, rs2234693, rs2228480 (in the ESR1 gene) and rs4986938 (in the ESR2 gene) as a risk factor for amnesic mild cognitive impairment (MCI) and Alzheimer's disease (AD) and its possible association with APOE gene

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

Setting: Subjects were prospectively recruited from the Neurology Departments of several Basque Country hospitals.

Participants: This study comprised 816 Caucasian subjects that were aged 50 years and older: 204 MCIa, 350 sporadic AD patients 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 MCI and AD was established using Logistic Regression Models.

Results: Neither alleles nor genotypes of SNPs rs9340799, rs2234693, rs2228480 and rs4986938 of estrogen 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 1.28-8.54, p=0.014) and AD (OR= 5.16, 95% CI 2.19-12.14, p<0.001) in women APOE*ε4 allele carriers.

Conclusions: The less represented alleles of SNPs studied are associated with DCLa y AD in subjects APOE*E4 carriers. Particularly, 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.

ARTICLE SUMMARY

Article focus:

- Alzheimer's disease´s aetiology is complex and multifactorial
- Estrogen receptors have several polymorphisms that seem to be related with the effect of the main risk factor to Alzheimer disease (AD), the APOE gene.
- The aim of the study is to examine the role of the single nucleotide polymorphisms (SNPs): rs9340799, rs2234693, rs2228480 and rs4986938 as a risk factor for mild cognitive impairment (MCI) and AD and its possible association with APOE gene

Key message

- APOE*ε4 allele is an independent risk factor for the AD population, and this risk is highest for women
- rs9340799, rs2234693, rs2228480 and rs4986938 are not independently associated with the risk of MCI and AD

- The less represented alleles of SNPs studied are associated with MCI and AD in subjects APOE*E4 carriers

Strengths and limitations of this study

- It was one of the first studies to investigate and association between polymorphisms of ER and cognitive function not only in AD patients, but also in MCI.
- 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 estrogen replacement therapy were unknown.

INTRODUCTION:

Alzheimer´s disease (AD) is the most common form of dementia, currently affecting over 9 million americans and europeans, its ethiology 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 [1].

The apolipoprotein E gene (*APOE*) is a genetic factor closely related to late onset AD disease, and constitutes and strong independent risk factor for sporadic AD [2]. Women have a slightly higher risk of AD compared to men [3]. 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 last years genetic research has focused on identifying common population polymorphism loci, not only *APOE*, but also other genes such as *CLU*, *CR1 PICALM* and *EXOC3L2* have been associated with an increased risk for developing AD [4-7]. 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*.

Estrogens are pleiotropic hormones having an influence not only on reproductive system but also in central nervous system (CNS). These hormones are synthesized by ovaries and by glia in CNS having a wide spectrum of effects such as neuroprotective and antiapoptotic [8-10]. Synaptogenic effects of estradiol-17-Beta have been demonstrated in the adult mammalian brain, 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 [11, 12]. Among other positive effects of estrogens[13], estradiol-17-Beta has an effect on 1) the maintenance and increase of the neurotransmitter systems, 2) the APP processing, Abeta levels and factors that alter its clearance and aggregation [14], 3) mechanisms of oxidative damage [15], Low endogenous estrogen levels have been broadly related with the increased risk of Alzheimer's in postmenopausal women. However, despite the initial data [16-19], there is disagreement regarding hormonal replacement therapy [20, 21].

Furthermore, it has also described an interaction with apolipoprotein E (ApoE). Estradiol increased ApoE levels and neurite outgrowth. ApoE2 isoform increased neurite length more than ApoE3 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 ApoE4, the isoform that is associated with increased risk of neurological disease, was exogenously supplied [22]. These data support the hypothesis that *APOE* gene plays an integral role in the neurotrophic effects of estradiol-17-Beta, and the presence of a probable synergism between ApoE subtype expression and the effects of estrogens.

The mechanism through which estrogens exert its neuroprotective and anti-neurodegenerative effects in the CNS is poorly understood overall are mediated by two estrogen receptors (ERs), ERalpha and ERbeta

(coded by *ESR1* and *ESR2* genes). ERs are located through around the brain, especially in hippocampus and amygdale [23, 24], regions involved in memory and learning process. Thus, genetic variants in ER genes have been studied in relation to EA. There are several polymorphic loci in intron 1 of *ESR1* gen, highlighting the PvuII and XbaI locus [25]. These loci may influence the expression of *ESR1* gen; xp haplotype has higher expression than the XP one, but with no significant differences [26]. Several studies, [27-29] but not all [26] have found an increased frequency of the PvuII and Xbal *ESR1* polymorphisms in AD patients.

Other interesting SNP is rs2228480, this polymorphism is the coding synonymous variant at 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 [30]. In addition, this SNP has been associated with neurodegenerative disorders and the mechanism of this association may involve alternative gene regulation and transcript processing [31].

Other studies have shown an association between several polymorphism of *ESR2* gene and late onset AD, and they found that variations in this gene could modify disease susceptibility [32]. The polymorphism located in 3'UTR of *ESR2* gene, rs4986938, has been associated with the onset of Parkinson disease [33] and the susceptibility for VaD in an Israeli cohort, but not with AD [34]. In the study of Dresener-Pollack et al. (2009), VAD is differentiated from AD by clinical criteria, but in the absence of imaging data, the potential for misclassification is high. Thus, results should be confirmed.

To date no studies have been conducted in the prodromal stages of EA such as mild cognitive impairment of amnestic type (MCIa). Such studies could provide information on the beginning of the disease process, helping to ensure that suitable therapeutic measures are 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 *APOE* gene; and whether such 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 ε4 status carriers. We have studied this association in AD patients and in MCIa patients, the latter condition possibly representing a prodrome for dementia of AD type [35].

With the purpose of examining the association of the *ESR1* and *ESR2* genes involved in estrogen 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 *APOE* genotype.

METHODS:

This study comprised 816 caucasian subjects, included in 3 groups: MCIa patients (n=204), AD patients (n=350) and healthy controls (n=262). Subjects were prospectively recruited from the Neurology Departments of several hospitals. Participants were aged 50 years and older. For AD and MCIa patients, evaluation also included routine blood tests: haematology, biochemistry, thyroid-stimulating hormone, vitamin B12 levels, syphilis serology and neuroimaging test (CT scan or MRI).

The subjects were evaluated using a broad battery of neuropsychological tests: Minimental State Examination, Clinical Dementia Rating scale, CERAD protocol, Stroop test, unilateral and bilateral motor praxis, 7-minute test, trial making part A and B; and Neuropsychiatric Inventory (NPI).

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

The diagnosis of MCIa patients was based on Petersen's criteria [35, 56]. 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 CDR scale was required to be 0.5, and performance in relation to other cognitive functions and daily living activities were required to be normal. The diagnosis

of AD was based on the DSM IV [57] and NINCDS-ADRDA [58] criteria for probable and possible AD. Patients with a total score of less than 3 on CDR scale (mild to moderate dementia) were included.

Healthy control subjects 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 ischemic 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:

At the first visit, peripherical blood samples were collected at EDTA vacuum tubes from all individuals. Genomic DNA was extracted by proteolytic lysis from white blood cells using standard phenol/chloroform extraction method.

APOE gene was amplified by PCR with the primers 112F and 158R, under the PCR conditions described by Wilton and Lim [59]. Digestion of the amplified product was carried out with Hae II and Afl III, as described by Álvarez-Álvarez et al. (2003)[60].

Three single nucleotide polymorphisms (SNPs) in the ESR1 gene (rs9340799, rs2234693 and rs2228480) and one SNP in the ESR2 gene (rs4986938) were evaluated. 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 characterized 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 polymorphism rs2228480; G>A (SNP1) of ESR1 gen and polymorphism rs4986938; G>A (SNP2) of ESR2 gen.

SNP genotypes of candidate genes (ESR1 and ESR2) and 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 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 version 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 [61]. The G test was also used to check the differences between demographic and clinical variables, allele frequencies and genotype frequencies.

Statistical analysis was also performed using the SPSS® package, version 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 effect of X, P and SNP1-A alleles of ESR1 gen and SNP2-A allele of ESR2 gen in the total sample and in the absence of APOE*ε4 allele. The effect of 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 polymorphism of ESR1 and ESR2 genes and the APOE*ε4 allele, based on the hypothesis that the effect of estrogens might exists only in APOE*ε4 allele carriers.

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

RESULTS:

We have investigated the independent and combined association of X, P and SNP1-A alleles of ESR1 gen and SNP2-A allele of ESR2 gen and APOE*ε4 allele by using a case-control design.

In the present study we analysed a sample of 204 MCIa patients, 350 AD patients, 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).

Table 1. Baseline Demographic

Group	n	Age[a]	Women (%)[b]	MMSE[c]
MCIa	204	70,25 ± 8,6	61,3	26.38 ± 2.05
AD	350	72,17± 8,3	71,1	19.68 ± 4.60
CONTROLS	262	74,00 ± 9,6	59,5	28.45 ± 1.63

[a] Years, mean ± Standard Desviation (S.D.). [b] % of Women in group. [c] MMSE score, mean ± S.D.

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

Table 2. Allelic and genotypic frequency.

ESR1

	XbaI	MCIa (N = 204)	AD (N =350)	CONTROLS (N = 262)
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[a]	**p-Value**	0.197	0.376	1.000

PvuII

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[a]	**p-Value**	0.575	0.110	1.000
	SNP1			
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[a]	**p-Value**	0.818	0.861	1.000
ESR2				
	SNP2			
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[a]	**p-Value**	0.245	0.325	0.591
APOE				
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[a]	**p-Value**	0.217	0.814	0.102

Genetic Profile			
XPAA(+)	0.709	0.708	0.674
XPAA(-)	0.291	0.292	0.326

a Hardy-Weinberg probability test.

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

Table 3. Exact G test

XbaI [a]			**XbaI** [b]		
	P-value	**S.E.** [c]		**P-value**	**S.E.** [c]
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

PvuII [a]			**PvuII** [b]		
	P-value	**S.E.** [c]		**P-value**	**S.E.** [c]
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

SNP1 [a]			**SNP1** [b]		
	P-value	**S.E.** [c]		**P-value**	**S.E.** [c]
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

SNP2 [a]			**SNP2** [b]		
	P-value	**S.E.** [c]		**P-value**	**S.E.** [c]
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

APOE [a]			APOE [b]		
	P-value	S.E. [c]		P-value	S.E. [c]
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

a Allelic frecuency. b Genotypic frecuency. c Standard Error

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 MCIa, AD and control individuals with the presence of at least one of these alleles. None of them had a significant effect (data not shown).

In the total sample, APOE*ε4 allele is a risk factor for cognitive impairment; the odds ratios (ORs) of developing MCIa and AD were 2.44 (95%CI 1.61-3.69, p<0.001) and 4.23 (95%CI 2.93-6.12, p<0.001), respectively (Table 4). The higher risk conferred by APOE*ε4 allele was observed even when the samples were subgrouped by sex, but in AD women the risk was higher than in men, 4.85 (95%CI 3.04-7.73, p<0.001) versus 3.19 (95%CI 1.73-5.88, p<0.001).

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

	MCI		AD	
Global Effects	**OR CI95%**	**p**	**OR CI95%**	**p**
X (+)[a]	1.39 (0.93-2.06)	0.104	1.18 (0.85-1.67)	0.324
P (+)[b]	1.25 (0.82-1.90)	0.293	1.26 (0.88-1.23)	0.205
SNP1-A[c]	1.14 (0.76-1.71)	0.506	1.13 (0.78-1.62)	0.510
SNP2-A[d]	1.05 (0.71-1.54)	0.304	1.08 (0.77-1.51)	0.649
E4 (+)[e]	2.44 (1.61–3.69)	<0.001	4.23 (2.93–6.12)	<0.001
Women	1.07 (0.73-1.56)	0.705	1.67 (1.19-2.35)	0.003
E4 (+)*Women[f]	2.27 (1.32-3.87)	0.003	4.85 (3.04-7.73)	<0.001
E4 (+)*Men[g]	2.74 (1.43-5.23)	0.002	3.19 (1.73-5.88)	<0.001
Independent Effects				
X (+) E4(-)[h]	1.04 (0.65-1.66)	0.863	1.18 (0.76-1.81)	0.452
P (+) E4(-)[h]	0.86 (0.52-1.40)	0.545	1.19 (0.754-1.90)	0.444
SNP1-A(+)*E4(-)[h]	1.19 (0.74-1.92)	0.469	1.13 (0.73-1.76)	0.568
SNP2-A(+)*E4(-)[h]	1.03 (0.65-1.66)	0.879	1.07 (0.70-1.64	0.758
ESR1				

Combined Effects				
E4(+)*X[i]	3.17 (1.80-5.59)	<0.001	5.07 (3.00-8.55)	<0.001
E4(+)*P[i]	2.74 (1.55-4.85)	0.001	5.35 (3.11-9.17)	<0.001
E4(+)*SNP1-A[i]	2.53 (1.31-4.90)	<0.001	4.44 (2.48-7.93)	<0.001
ESR2				
Combined Effects				
E4(+)*SNP2-A[i]	2.77 (1.55-4.93)	0.001	4.87 (2.91-8.17)	<0.001
Genetic Profile (XPAA)				
Independent Effects				
XPAA*E4(-)[j]	1.31 (0.48-3.54)	0.590	1.19 (0.49-2.91)	0.696
XPAA(-)*E4(+)[k]	2.53 (1.61-3.93)	<0.001	4.32 (2.91-6.40)	<0.001
Combined Effects				
XPAA*E4(+)[l]	3.30 (1.28-8.54)	0.014	5.16 (2.19-12.14)	<0.001
XPAA*E4(+)*Women[m]	3.84 (1.09-13.57)	0.036	8.04 (2.60-24.80)	<0.001
XPAA*E4(+)*Men[m]	3.20 (0.73-14.11)	0.124	3.57 (0.88-14.47)	0.075

*[a] Sample selected by at least one X of RFLP XbaI. [b] Sample selected by at least one P of RFLP PvuII. [c] Sample selected by at least one A allele of rs2228480.[d] Sample selected by at least one A allele of rs4986938. [e] Sample selected by at least one E4 allele of APOE gene.[f] Women selected by at least one E4 allele of APOE gene. [g] Men selected by at least one E4 allele of APOE gene. [h] Sample selected by at least one allele that is indicated and the absence of E4 allele of APOE gene [i] Sample selected by at least one E4 allele of APOE gene and one of the alleles that is indicated. Reference category was sample control. [j] Sample selected by absence of E4 allele of APOE gene and the presence of XPAA. [k] Sample selected by absence of XPAA and the presence by at least one E4 allele of APOE gene. Sample selected by at least one E4 allele of APOE gene and the presence XPAA.[l] Sample selected by at least one E4 allele of APOE gene and the presence of XPAA. [m] Women or Men selected by at least one E4 allele of APOE gene and the presence of XPAA. * In all models reference category was sample control*

Aiming to avoid the combined effect of the less represented alleles of SNPs in candidates genes and APOE*ε4 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*ε4 allele. We did not found 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 candidates genes and APOE*ε4 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*ε4 allele. A slight increase in nominal risk of MCI and AD was observed.

In order to analyse the combined effect between estrogen polymorphisms, we created a genetic profile with the less represented alleles of these SNPs, expressed as XPAA. We did not found a significant risk in the absence of one APOE*ε4 allele, but analysing the combined effect of XPAA with APOE*ε4 allele, ORs were as follows: MCIa, OR= 3.30 (95%CI 1.28-8.54, p=0.014) and AD, OR= 5.16 (95%CI 2.19-

12.14, p<0.001), these ORs were even greater than the independent effect of APOE*ε4 allele with XPAA(-) (absence of this genetic profile). However when the samples were subgrouped by sex, MCIa and AD women showed an increased OR, 3.84 (95%CI 1.09-13.57, p<0.036) and 8.04 (95%CI 2.60-24.80, p<0.001) respectively, comparing with men.

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 absence of APOE*ε4. Furthermore, the genetic profile created with the less represented alleles of SNPs in candidates genes were associated with an increased risk for MCIa and AD in women APOE*ε4 allele carriers.

In our series, APOE*ε4 allele seems to be an independent risk factor for the AD population, and this risk is highest for women. The APOE*ε4 allele also constitutes a risk factor for MCIa patients.

On evaluating the combined effect of the APOE*ε4 allele in the presence of alleles or genotypes of *ESR1* and *ESR2* SNPs the risk for AD remains significant; though 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 found a significant risk in the absence of one APOE*ε4 allele. However, the presence of XPAA and at least one APOE*ε4 allele increases the risk in MCIa and AD women.

Nowadays the most well-known polymorphism of *ESR1* gene related with AD are SNPs rs9340799 (A>G; XbaI) and rs2234693 (PvuII; T>C). Regarding the association between XbaI with AD, several studies show that *ESR1* XbaI polymorphism is an additional risk factor [27, 36-38]. However, other studies have not found this association [29, 39-42]. These results and several meta-analysis [1, 43] suggested that *ESR1* gene polymorphisms might be related to the individual susceptibility to AD, especially in the females.

Concerning the association between *ESR1* PvuII polymorphism with AD, several published studies have shown a great heterogeneity. In some of them no association has been found [26, 39, 40, 42, 44]. Other studies claimed a protective role of P allele of *ESR1* PvuII polymorphism [29, 37, 38], whereas others found an opposite effect [27, 28, 36, 45-47]. Some studies [36] have established an association between *ESR1* PP and XX genotypes with an increased risk for AD only in males (OR = 3.6, 95% CI = 1.2-10.9) and conferred a relevant additional risk of AD to subjects also carrying APOE*ε4 allele, and in AD women. In this last study *ESR1* PP and XX genotypes were also associated with lower MMSE values (p = 0.0007). This data suggests 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 APOE * ε4 allele.

In our knowledge, this is the first study to show evidences in support of the association of SNP rs2228480 with MCI and AD patients APOE*ε4 allele carriers. Previously, this SNP only has been linked to the alternative regulation and transcript processing of *ESR1* gene [31, 48]. To date had not been provided other information in relation to neurodegenerative disorders.

Regarding polymorphisms of *ESR2* gen, several studies have been published with conflicting results: susceptibility for vascular dementia (VaD) but not for sporadic AD in elderly Jewish women was found in *ESR2* rs4986938 polymorphism [34]. Pirskanen et al. (2005)[32] found that some gene variants of *ESR2* gen are associated with increased risk of AD in women (rs1271573 T/T genotype and rs1256043 T/T genotype) while others not (IVS31842, rs4986938). Lambert et al. (2001) [40] found no independent association of these polymorphisms with the risk of developing AD. One study suggests the *ESR2* allele 5 seems to be a protective factor [49]. Meta-analyses have not been performed on the following polymorphisms of *ESR2* gen since they lack published genotype data or the published genotype data was

not eligible for inclusion. Other studies [50] 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 *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 APOE*ε4 allele confers an increased risk in women, whereas it does not contribute to the disease susceptibility in men. According to our results, some variations in the ER genes in synergy with APOE*ε4 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 both *ESR1* and *ESR2* genes have several polymorphisms, which might be related to their expression and biological activities. The variations in the ERs genes may involve alternative gene regulation and transcript processing in the brain [31]. *APOE* gene expression can be differentially regulated depending on activation of ER subtypes. A recent study [15] demonstrated that activation of *ESR1* gene up-regulated APOE*ε4 mRNA and protein expression in hippocampus. In contrast, activation of *ESR2* gene down-regulated the mRNA and protein expression of *APOE*. Thus, it is expected lower regulation in postmenopausal women [51], conferring less protection against the effect of APOE*ε4 allele.

Estrogens have been shown to affect amyloid precursor protein metabolism, by increasing the secretory metabolism of amyloid protein precursor (*APP*). Estrogens are also a potent factor that not only prevents vascular disease but also improves blood flow, including blood flow in regions on the brain affected by AD [52]. Synaptic sprouting by estradiol in a model of AD may operate via an APOE*ε4-dependent mechanism [53]. Cholinergic neurons that are implicated in cognitive functions may be regulated by estrogens. The distribution of ERs corresponds to that of cholinergic system [54]. The important decrease in endogenous estrogen levels after menopause may contribute to the development of AD [55]. Despite the protective effect of estrogens upon AD, this effect might to be modified by ERs polymorphisms, particularly in APOE*ε4 allele carriers. Thus, the current state of knowledge of the role of estrogens for preventing dementia in postmenopausal women should be reviewed.

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

Some limitations to 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 test used in our sample might ensure a highest homogeneity.

CONCLUSIONS

In our study, APOE*ε4 allele is an independent risk factor for MCIa and AD patients. 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; although this association does not confer a relevant additional risk of AD and MCIa. Furthermore, the genetic profile with the less represented alleles of *ESR1* and *ESR2* gene polymorphisms, expressed as XPAA, did not increased 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 increases the risk in MCIa and AD women.

OTHER INFORMATION:

Competing interests:

None.

Funding:

This work was sponsored by grants from the Federación de Asociaciones de Familiares de enfermos de Alzheimer de Euskadi, Fondo de Investigación Sanitaria del Instituto Carlos III (Madrid), Pfizer Foundation and Ayudas a la Investigación de la Obra Social de la Caja Vital Kutxa.

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STROBE Statement—Checklist of items that should be included in reports of ***case-control studies***

	Item No	Recommendation
Title and abstract	1	(a) Indicate the study's design with a commonly used term in the title or the abstract Page 2
		(a) Provide in the abstract an informative and balanced summary of what was done and what was found Page 2
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported Page 3
Objectives	3	State specific objectives, including any prespecified hypotheses Page 4
Methods		
Study design	4	Present key elements of study design early in the paper Page 4
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection Page 4-5
Participants	6	*(a)* Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls Page 4-5
		(b) For matched studies, give matching criteria and the number of controls per case
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable Page 4-6
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group Page 4
Bias	9	Describe any efforts to address potential sources of bias Page 6
Study size	10	Explain how the study size was arrived at
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why
Statistical methods	12	*(a)* Describe all statistical methods, including those used to control for confounding Page 5-6
		(*b*) Describe any methods used to examine subgroups and interactions
		(*c*) Explain how missing data were addressed
		(*d*) If applicable, explain how matching of cases and controls was addressed

		(*e*) Describe any sensitivity analyses
Results		
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed Page 4-5
		(b) Give reasons for non-participation at each stage
		(c) Consider use of a flow diagram
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders Page 4-6
		(b) Indicate number of participants with missing data for each variable of interest Page 4
Outcome data	15*	Report numbers in each exposure category, or summary measures of exposure Page 6-9
Main results	16	*(a)* Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included Page 9-10
		(*b*) Report category boundaries when continuous variables were categorized
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period

Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
Discussion		
Key results	18	Summarise key results with reference to study objectives Page 11
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias Page 12
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence Page 11-12
Generalisability	21	Discuss the generalisability (external validity) of the study results Page 12 (multicenter nature)
Other information		
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based Page 13

*Give information separately for cases and controls.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at http://www.strobe-statement.org.

BMJ open

Estrogen receptor polymorphisms are an associated risk factor for mild cognitive impairment and Alzheimer disease in women APOE ε4 carriers

Journal:	*BMJ Open*
Manuscript ID:	bmjopen-2013-003200.R1
Article Type:	Research
Date Submitted by the Author:	30-Jul-2013
Complete List of Authors:	Fernández-Martínez, Manuel; Hospital Universitario Cruces, Neurology Elcoroaristizabal, Xabier; University of Basque Country UPV/EHU, Blanco, Elisa; Hospital Universitario Cruces, Neurology Galdós, Luis; Hospital Universitario Txagorritxu,, Neurology Ugarriza-Serrano, Iratxe; Hospital Universitario Cruces, Neurology Gómez-Busto, Fernando; Vitoria-Gasteiz city Council, San Prudencio Comprehensive Care Center for elderly Álvarez-Álvarez, Maite; University of Basque Country UPV/EHU, Molano, Ana; Hospital Universitario Cruces, Neurology Bereincua, Rocío; Hospital Universitario Cruces, Neurology Inglés, Sandra; Hospital Universitario Txagorritxu, Neurology Uterga, Juan; Hospital Universitario Basurto, Neurology Indakoetxea, Begoña; Hospital Universitario Donostia, Neurology Gómez-Beldarraín, María; Hospital Galdakao, Neurology Moraza, Josefa; Hospital Santiago Apóstol, Neurology Barandiarán, Myriam; Hospital Universitario Donostia, Neurology Martinez-De Pancorbo, Marian; University of Basque Country UPV/EHU,
Primary Subject Heading:	Neurology
Secondary Subject Heading:	Geriatric medicine, Genetics and genomics
Keywords:	Dementia < NEUROLOGY, Adult neurology < NEUROLOGY, GENETICS

Estrogen receptor polymorphisms are an associated risk factor for mild cognitive impairment and Alzheimer disease in women APOE ε4 carriers

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ABSTRACT

Objetives: Examine the role of single nucleotide polymorphisms (SNPs) in the estrogen receptor 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 *APOE* gene

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

Setting: Subjects were prospectively recruited from Neurology Departments of several Basque Country hospitals.

Participants: This study comprised 816 Caucasian subjects that were aged 50 years and older: 204 MCIa, 350 sporadic AD patients 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 MCI and AD was established using Logistic Regression Models.

Results: Neither alleles nor genotypes of SNPs rs9340799, rs2234693, rs2228480 and rs4986938 of estrogen 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 1.28-8.54, p=0.014) and AD (OR= 5.16, 95% CI 2.19-12.14, 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*E4 carriers. Particularly, 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.

ARTICLE SUMMARY

Article focus:

- Alzheimer's disease´s etiology is complex and multifactorial
- Estrogen receptors have several polymorphisms that seem to be related to the effect of the main risk factor to Alzheimer disease (AD), the *APOE* gene.
- The aim of the study is to examine the role of the single nucleotide polymorphisms (SNPs): rs9340799, rs2234693, rs2228480 and rs4986938 as a risk factor for mild cognitive impairment (MCI) and AD and its possible association with *APOE* gene

Key message

- APOE*ε4 allele is an independent risk factor for the AD population, and this risk is higher in women

- rs9340799, rs2234693, rs2228480 and rs4986938 are not independently associated with the risk of MCI and AD
- The less represented alleles of SNPs studied are associated with MCI and AD in APOE*E4 carriers

Strengths and limitations of this study

- It was one of the first studies to investigate and association between polymorphisms of ER genes and cognitive function not only in AD patients, but also in 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 estrogen replacement therapy were unknown.

INTRODUCTION:

Alzheimer´s disease (AD) is the most common form of dementia, currently affecting over 9 million americans and europeans, its etiology 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 [1].

The apolipoprotein E gene (*APOE*) is a genetic factor closely related to late onset AD disease, and constitutes an strong independent risk factor for sporadic AD [2]. 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 last years genetic research has focused on identifying common population polymorphism loci, not only *APOE*, but also other genes such as *CLU*, *CR1, PICALM* and *EXOC3L2* have been associated with an increased risk for developing AD [3-6]. These genes are implicated in chaperone action, positive regulation immune response, 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*.

Estrogens are pleiotropic hormones having an influence not only on reproductive system but also in central nervous system (CNS). These hormones are synthesized by ovaries and are also produced in smaller amounts by other tissues such as glia in CNS, having a wide spectrum of effects such as neuroprotective and antiapoptotic [7-9]. 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 [10 11]. Among other positive effects of estrogens [12], estradiol-17-Beta has an effect on 1) the maintenance and increase of the neurotransmitter systems, 2) the *APP* processing, Abeta levels and factors that alter its clearance and aggregation [13], 3) mechanisms of oxidative damage. Multiple lines of evidence suggest that loss of estrogens in the aging brain of both women and men may play a role in the cognitive declines associated with AD [14] but whether female sex is also a risk factor is controversial although some past and a recent study show higher rates of cognitive decline for women and apolipoprotein E4 carriers (APOE*ε4) [15 16], and mouse AD-transgenic mice studies generally show great amyloid and neurodegeneration in females

[17] [18]. However, despite the initial data [19-22], there is disagreement regarding hormonal replacement therapy in women [14] [23-25].

Furthermore, it has also described an interaction With ApoE. Estradiol increased ApoE levels and neurite outgrowth. APOE*ε2 isoform increased neurite length more than APOE*ε3 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*ε4, the isoform that is associated with increased risk of neurological disease, was exogenously supplied[26]. These data support the hypothesis that *APOE* gene plays an integral role in the neurotrophic effects of estradiol-17-Beta, and the presence of a probable synergism between ApoE subtype expression and the effects of estrogens.

The mechanism through estrogens exert its neuroprotective and anti-neurodegenerative effects in the CNS is poorly understood overall are mediated by two estrogen receptors (ERs), ERalpha and ERbeta (coded by *ESR1* and *ESR2* gene),expressed in neurons and glia throughout the brain, especially in hippocampus and amygdale [27] [28], regions involved in memory and learning process. Thus, genetic variants in 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 [29]. 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. Subjects were described as pp or xx homozygotes, Pp or Xx heterozygotes, or PP or XX homozygotes [30].The xp haplotype has higher expression than the XP one, but with no significant differences [31]. Several studies, [32-34] but not all [31] have found an increased frequency of the PvuII and XbaI *ESR1* polymorphisms in AD patients.

Other interesting SNP is rs2228480, this polymorphism is the coding synonymous variant at 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 [35]. In addition, this SNP has been associated with schizophrenia and the mechanism of this association may involve alternative gene regulation and transcript processing[36].

Other studies have shown an association between several polymorphism of *ESR2* gene and late onset AD, and they found that variations in this gene could modify disease susceptibility [37]. The polymorphism located in 3'UTR of *ESR2* gene, rs4986938, has been associated with the onset of Parkinson disease [38] and the susceptibility for vascular dementia (VaD) in an Israeli cohort, but not with AD [39]. In the study of Dresener-Pollack et al. (2009), VaD is differentiated from AD by clinical criteria, but in the absence of imaging data, the potential misclassification is high. Thus, results should be confirmed.

To date no studies have been conducted in the prodromal stages of AD such as mild cognitive impairment of amnestic 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 *APOE* gene; and whether such 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 APOE*ε4 carriers. We have studied this association in AD patients and in MCIa patients, the latter condition possibly representing a prodrome for AD type dementia. [40].

With the purpose of examining the association of the *ESR1* and *ESR2* genes involved in estrogen 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 *APOE* genotype.

METHODS:

This study comprised 816 caucasian subjects, included in 3 groups: MCIa patients (n=204), AD patients (n=350) and healthy controls (CTL) (n=262). Subjects were prospectively recruited from Neurology Departments of several hospitals. Participants were aged 50 years and older. For AD and MCIa patients, evaluation also included routine blood tests: haematology, biochemistry, thyroid-stimulating hormone, vitamin B12 levels, syphilis serology and neuroimaging test: CT (Computerized Tomography) scan or MRI (Magnetic Resonance Imaging).

The subjects were evaluated using a broad battery of neuropsychological tests: Minimental State Examination (MMSE), Clinical Dementia Rating scale, CERAD protocol, Stroop test, unilateral and bilateral motor praxis, 7-minute test, trial making part A and B; and Neuropsychiatric Inventory (NPI).

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

The diagnosis of MCIa patients was based on Petersen's criteria [40]. 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 CDR scale was required to be 0.5, and performance in relation to other cognitive functions and daily living activities were 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 3 on CDR scale (mild to moderate dementia) were included.

Healthy control subjects 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 ischemic 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, peripherical blood samples were collected in EDTA vacuum tubes from all individuals. Genomic DNA was extracted by proteolytic lysis from white blood cells using standard phenol/chloroform extraction method.

APOE gene was amplified by PCR with 112F and 158R primers, under the PCR conditions described by Wilton and Lim [41]. Digestion of the amplified product was carried out with Hae II and Afl III, as described by Álvarez-Álvarez et al. (2003) [42].

Three single nucleotide polymorphisms (SNPs) in the *ESR1* gene (rs9340799, rs2234693 and rs2228480) and one SNP in the *ESR2* gene (rs4986938) were evaluated. 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 characterized 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 polymorphism rs2228480; G>A (SNP1) of *ESR1* gen and polymorphism rs4986938; G>A (SNP2) of *ESR2* gen.

SNP genotypes of candidate genes (*ESR1* and *ESR2*) and *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 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 version 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 [43]. The G test was also used to check the differences between demographic and clinical variables, allele frequencies and genotype frequencies.

Statistical analysis was also performed using the SPSS ® package, version 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 effect of X, P and SNP1-A alleles of *ESR1* gen and SNP2-A allele of *ESR2* gen in the total sample and in the absence of APOE*ε4 allele. The effect of 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 polymorphism of *ESR1* and *ESR2* genes and the APOE*ε4 allele, based on the hypothesis that the effect of estrogens might exists only in APOE*ε4 allele carriers.

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

RESULTS:

We have investigated the independent and combined association of X, P and SNP1-A alleles of *ESR1* gen and SNP2-A allele of *ESR2* gen and APOE*ε4 allele by using a case-control design.

In the present study we analysed a sample of 204 MCIa patients, 350 AD patients, 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 1. Baseline Demographic

Group	n	Age[a]	Women (%)[b]	MMSE[c]	Education [d]
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

[a] Years, mean ± Standard Desviation (S.D.). [b] % of Women in group. [c] MMSE score, mean ± S.D. [d] Years of education

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

Table 2. Allelic and genotypic frequency.

ESR1				
	XbaI	**MCIa (N = 204)**	**AD (N =350)**	**CONTROLS (N = 262)**
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[a]	**p-Value**	0.197	0.376	1.000
	PvuII			
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[a]	**p-Value**	0.575	0.110	1.000
	SNP1			
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[a]	**p-Value**	0.818	0.861	1.000
ESR2				
	SNP2			
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[a]	**p-Value**	0.245	0.325	0.591

APOE				
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[a]	**p-Value**	0.217	0.814	0.102
Genetic Profile				
	XPAA(+)	0.709	0.708	0.674
	XPAA(-)	0.291	0.292	0.326

a Hardy-Weinberg probability test.

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

Table 3. Exact G test

XbaI [a]			**XbaI** [b]		
	P-value	**S.E.**[c]		**P-value**	**S.E.**[c]
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

PvuII[a]			**PvuII**[b]		
	P-value	**S.E.**[c]		**P-value**	**S.E.**[c]
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

SNP1[a]			**SNP1** [b]		
	P-value	**S.E.**[c]		**P-value**	**S.E.** [c]
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

SNP2[a]			**SNP2**[b]		
	P-value	**S.E.**[c]		**P-value**	**S.E.** [c]
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

APOE [a]			**APOE** [b]		
	P-value	**S.E.** [c]		**P-value**	**S.E.** [c]
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

a Allelic frecuency. b Genotypic frecuency. c Standard Error

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 MCIa, AD and control individuals with the presence of at least one of these alleles. None of them had a significant effect (data not shown).

In the total sample, APOE*ε4 allele is a risk factor for cognitive impairment; the odds ratios (ORs) of developing MCIa and AD were 2.44 (95%CI 1.61-3.69, p<0.001) and 4.23 (95%CI 2.93-6.12, p<0.001), respectively (Table 4). The higher risk conferred by APOE*ε4 allele was observed even when the samples were subgrouped by sex, but in AD women the risk was higher than in men, 4.85 (95%CI 3.04-7.73, p<0.001) versus 3.19 (95%CI 1.73-5.88, p<0.001).

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

	MCI		**AD**	
Global Effects	**OR CI95%**	**p**	**OR CI95%**	**p**
X (+)[a]	1.39 (0.93-2.06)	0.104	1.18 (0.85-1.67)	0.324

P (+)[b]	1.25 (0.82-1.90)	0.293	1.26 (0.88-1.23)	0.205
SNP1-A[c]	1.14 (0.76-1.71)	0.506	1.13 (0.78-1.62)	0.510
SNP2-A[d]	1.05 (0.71-1.54)	0.304	1.08 (0.77-1.51)	0.649
E4 (+)[e]	2.44 (1.61–3.69)	<0.001	4.23 (2.93–6.12)	<0.001
Women	1.07 (0.73-1.56)	0.705	1.67 (1.19-2.35)	0.003
E4 (+)*Women[f]	2.27 (1.32-3.87)	0.003	4.85 (3.04-7.73)	<0.001
E4 (+)*Men[g]	2.74 (1.43-5.23)	0.002	3.19 (1.73-5.88)	<0.001
Independent Effects				
X (+) E4(-)[h]	1.04 (0.65-1.66)	0.863	1.18 (0.76-1.81)	0.452
P (+) E4(-)[h]	0.86 (0.52-1.40)	0.545	1.19 (0.754-1.90)	0.444
SNP1-A(+)*E4(-)[h]	1.19 (0.74-1.92)	0.469	1.13 (0.73-1.76)	0.568
SNP2-A(+)*E4(-)[h]	1.03 (0.65-1.66)	0.879	1.07 (0.70-1.64	0.758
ESR1				
Combined Effects				
E4(+)*X[i]	3.17 (1.80-5.59)	<0.001	5.07 (3.00-8.55)	<0.001
E4(+)*P[i]	2.74 (1.55-4.85)	0.001	5.35 (3.11-9.17)	<0.001
E4(+)*SNP1-A[i]	2.53 (1.31-4.90)	<0.001	4.44 (2.48-7.93)	<0.001
ESR2				
Combined Effects				
E4(+)*SNP2-A[i]	2.77 (1.55-4.93)	0.001	4.87 (2.91-8.17)	<0.001
Genetic Profile (XPAA)				
Independent Effects				
XPAA*E4(-)[j]	1.31 (0.48-3.54)	0.590	1.19 (0.49-2.91)	0.696
XPAA(-)*E4(+)[k]	2.53 (1.61-3.93)	<0.001	4.32 (2.91-6.40)	<0.001
Combined Effects				
XPAA*E4(+)[l]	3.30 (1.28-8.54)	0.014	5.16 (2.19-12.14)	<0.001
XPAA*E4(+)*Women[m]	3.84 (1.09-13.57)	0.036	8.04 (2.60-24.80)	<0.001
XPAA*E4(+)*Men[m]	3.20 (0.73-14.11)	0.124	3.57 (0.88-14.47)	0.075

[a]Effect of sample with at least one X of RFLP XbaI. [b]Effect of sample with at least one P of RFLP PvuII.
[c] Effect of sample with at least one A allele of rs2228480.[d] Effect of sample with at least one A allele of rs4986938. [e] Effect of sample with at least one E4 allele of APOE gene. [f] Women selected by at least one E4 allele of APOE gene. [g] Men selected by at least one E4 allele of APOE gene. [h] Sample selected by at least one allele that is indicated and the absence of E4 allele of APOE gene. [i] Sample selected by at least one E4 allele of APOE gene and one of the alleles that is indicated. Reference category was sample control. [j] Sample selected by absence of E4 allele of APOE gene and the presence of XPAA. [k] Sample selected by absence of

XPAA and the presence by at least one E4 allele of APOE gene. Sample selected by at least one E4 allele of APOE gene and the presence XPAA.[l] Sample selected by at least one E4 allele of APOE gene and the presence of XPAA.[m] Women or Men selected by at least one E4 allele of APOE gene and the presence of XPAA. * In all models reference category was sample control considering the age and sex (as appropriate)

Aiming to avoid the combined effect of the less represented alleles of SNPs in candidates genes and APOE*ε4 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*ε4 allele. We did not found 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 candidates genes and APOE*ε4 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*ε4 allele. A slight increase in nominal risk of MCI and AD was observed. The statistical analyzes were also conducted according to the gender (Supplementary table 1)

In order to analyse the combined effect between estrogen polymorphisms, we created a genetic profile with the less represented alleles of these SNPs, expressed as XPAA. We did not found a significant risk in the absence of one APOE*ε4 allele, but analysing the combined effect of XPAA with APOE*ε4 allele, ORs were the following: MCIa, OR= 3.30 (95%CI 1.28-8.54, p=0.014) and AD, OR= 5.16 (95%CI 2.19-12.14, p<0.001), these ORs were even greater than the independent effect of APOE*ε4 allele with XPAA(-) (absence of this genetic profile). Although it was expected to obtain a greater effect in MCI men and AD women, according to the results showed in table 3, when the samples were subgrouped by sex taking into account the genetic profile, MCIa and AD women showed an increased OR, 3.84 (95%CI 1.09-13.57, p<0.036) and 8.04 (95%CI 2.60-24.80, p<0.001) respectively, comparing 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 absence of APOE*ε4. Furthermore, the genetic profile created with the less represented alleles of SNPs in candidates genes were associated with an increased risk for MCIa and AD in women APOE*ε4 allele carriers.

In our serie, APOE*ε4 allele seems to be an independent risk factor for the AD population, and this risk is highest for women. The APOE*ε4 allele also constitutes a risk factor for MCIa patients.

On evaluating the combined effect of the APOE*ε4 allele in the presence of alleles or genotypes of *ESR1* and *ESR2* SNPs the risk for AD remains significant; though 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 found a significant risk in the absence of one APOE*ε4 allele. However, the presence of XPAA and at least one APOE*ε4 allele increases the risk in MCIa and AD women.

Nowadays the most well-known polymorphism of *ESR1* gene related to AD are SNPs rs9340799 (A>G; XbaI) and rs2234693 (PvuII; T>C). Regarding to the association between XbaI with AD, several studies show that *ESR1* XbaI polymorphism is an additional risk factor [32 44-46]. However, other studies have not

found this association [34 47-50]. These results and several meta-analysis [1 51] suggested that *ESR1* gene polymorphisms might be related to the individual susceptibility to AD, especially in the females.

Concerning to the association between *ESR1* PvuII polymorphism and AD, several published studies have shown a great heterogeneity. In some of them no association has been found [31 47 48 50 52]. Other studies claimed a protective role of P allele of *ESR1* PvuII polymorphism [34 45 46], whereas others found an opposite effect [32 33 44 53-55]. Some studies [44] have established an association between *ESR1* PP and XX genotypes with an increased risk for AD only in males (OR = 3.6, 95% CI = 1.2-10.9) and conferred a relevant additional risk of AD to subjects also carrying APOE*ε4 allele, and in AD women. In this last study *ESR1* PP and XX genotypes were also associated with lower MMSE values (p = 0.0007). This data suggests 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 to the increased risk of MCI and AD observed in patients with APOE*ε4 allele.

In our knowledge, this is the first study to show evidences in support of the association of SNP rs2228480 with MCI and AD patients APOE*ε4 allele carriers. Previously, this SNP only has been linked to the alternative regulation and transcript processing of *ESR1* gene [36 56]. To date have not been provided other information in relation to neurodegenerative disorders.

Regarding polymorphisms of *ESR2* gen, several studies have been published with conflicting results: susceptibility for VaD but not for sporadic AD in elderly Jewish women was found in *ESR2* rs4986938 polymorphism [39]. Pirskanen et al. (2005)[37] found that some gene variants of *ESR2* gen are associated with increased risk of AD in women (rs1271573 T/T genotype and rs1256043 T/T genotype) while others not (IVS31842, rs4986938). Lambert et al. (2001) [48] found no independent association of these polymorphisms with the risk of developing AD. One study suggests the *ESR2* allele 5 seems to be a protective factor [57]. Meta-analyses have not been performed on the following polymorphisms of *ESR2* gen since they lack published genotype data or the published genotype data was not eligible for inclusion. Other studies [58] 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 *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 APOE*ε4 allele confers an increased risk in women, whereas 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 expected results differ slightly from those expected. The combined effect observed between X , P, SNP1-A and SNP2-A alleles and at least one APOE*ε4 allele seemed to point to an increased risk in MCIa men and AD women. Our case-control study is relative medium size there are a small number samples carrying the genetic profile (<8% in MCI and AD patients, and <2% in controls) and APOE*ε4 allele that may affects negatively the power. Nevertheless, according to our results, some variations in the ER genes in synergy with APOE*ε4 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 both *ESR1* and *ESR2* genes have several polymorphisms, which might be related to their expression and biological activities. The variations in the ERs genes may involve alternative gene regulation and transcript processing in the brain[36]. *APOE* gene expression can be differentially regulated depending on activation of ER subtypes. A recent study [59] demonstrated that activation of *ESR1* gene up-regulated APOE*ε4 mRNA and protein expression in hippocampus. In contrast, activation of *ESR2* gene down-regulated the mRNA and protein expression of *APOE* gene. Thus, it is expected lower regulation in postmenopausal women [60], conferring less protection against the effect of APOE*ε4 allele.

Relatively few studies have examined the epistatic effects between estrogen-related pathway genes and APOE*ε4 allele. Postmenopausal women with down syndrome showed an increased risk of AD and elevated sex hormone binding globulin in those carrying *CYP17* and *CYP19* variants and APOE*ε4 allele[61]. Both genes are involved in the production of neurosteroids (estrogens and testosterone). In addition,

estrogens have been shown to affect amyloid precursor protein metabolism, by increasing the secretory metabolism of amyloid protein precursor (*APP*). Estrogens are also a potent factor that not only prevents vascular disease but also improves blood flow, including blood flow in regions on the brain affected by AD[62]. Synaptic sprouting by estradiol in a model of AD may operate via an APOE*ε4-dependent mechanism [63]. Cholinergic neurons that are implicated in cognitive functions may be regulated by estrogens. The distribution of ERs corresponds to that of cholinergic system[64]. The important decrease in endogenous estrogen levels after menopause may contribute to the development of AD [65]. Despite the protective effect of estrogens upon AD, this effect might to be modified by ERs polymorphisms, particularly in APOE*ε4 allele carriers. Thus, the current state of knowledge of the role of estrogens for preventing dementia in postmenopausal women should be reviewed.

Although the prevalence and incidence of AD are higher in women, men also may have the same effect due to SNPs in ER genes. It has been observed that while androgens have specific receptors to exert its neuroprotective action, they may also exert their actions indirectly via *CYP17* by aromatization of testosterone to estradiol[66] or directly through *ESR2* binding capacity of the metabolite dihydrotestosterone[67]. To date, it is unclear whether SNPs in ER genes would increase the risk of AD or MCIa in men. Our partial data trend 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 AD patients, healthy controls, and MCIa patients. In our knowledge, ours is the first study to investigate an association between polymorphisms of ER (rs9340799, rs2234693, rs2228480 and rs4986938) and cognitive function not only in AD patients, but also in 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 test used in our sample might ensure a highest homogeneity.

CONCLUSIONS

In our study, APOE*ε4 allele is an independent risk factor for MCIa and AD patients. 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 *ESR1* and *ESR2* gene polymorphisms, expressed as XPAA, did not increased 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 MCIa and AD women.

OTHER INFORMATION:

Competing interests:

None.

Funding:

This work was sponsored by grants from the Federación de Asociaciones de Familiares de enfermos de Alzheimer de Euskadi, Fondo de Investigación Sanitaria del Instituto Carlos III (Madrid), Pfizer Foundation and Ayudas a la Investigación de la Obra Social de la Caja Vital Kutxa.

Contributorship

MFM: main investigator, conceived of the study, and participated in its design and coordination, and drafted the manuscript.
XEM: co-investigator; participated in its design and coordination, and drafted the manuscript
EBM: Participated in the drafting of the manuscript.
IUS: Participated in the drafting of the manuscript.
LGA: co-investigator; participated in its design and coordination, and drafted the manuscript.
FGB: co-investigator; participated in its design and coordination, and drafted the manuscript.
MAA: co-investigator; participated in its design and coordination, and drafted the manuscript.
AMS: performed the battery of neuropsychological tests.
RBG: performed the battery of neuropsychological tests.
SIB: performed the battery of neuropsychological tests.
JMUV: co-investigator; participated in its design and coordination. BIJ: co-investigator; participated in its design and coordination.
MAGB: co-investigator; participated in its design and coordination. JML: co-investigator; participated in its design and coordination.
NO: performed the battery of neuropsychological tests.
MBA: performed the battery of neuropsychological tests.
MCZ: performed the battery of neuropsychological tests.
MMP: co-investigator; participated in its design and coordination, and drafted the manuscript.

All authors read and approved the final manuscript.

Data sharing

No additional data available.

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STROBE Statement—Checklist of items that should be included in reports of ***case-control studies***

	Item No	Recommendation
Title and abstract	1	(a) Indicate the study's design with a commonly used term in the title or the abstract Page 2
		(a) Provide in the abstract an informative and balanced summary of what was done and what was found Page 2
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported Page 3
Objectives	3	State specific objectives, including any prespecified hypotheses Page 4
Methods		
Study design	4	Present key elements of study design early in the paper Page 4
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection Page 4-5
Participants	6	*(a)* Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls Page 4-5
		(b) For matched studies, give matching criteria and the number of controls per case
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable Page 4-6
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group Page 4
Bias	9	Describe any efforts to address potential sources of bias Page 6
Study size	10	Explain how the study size was arrived at
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why
Statistical methods	12	*(a)* Describe all statistical methods, including those used to control for confounding Page 5-6
		(*b*) Describe any methods used to examine subgroups and interactions
		(*c*) Explain how missing data were addressed
		(*d*) If applicable, explain how matching of cases and controls was addressed

		(*e*) Describe any sensitivity analyses
Results		
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed Page 4-5
		(b) Give reasons for non-participation at each stage
		(c) Consider use of a flow diagram
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders Page 4-6
		(b) Indicate number of participants with missing data for each variable of interest Page 4
Outcome data	15*	Report numbers in each exposure category, or summary measures of exposure Page 6-9
Main results	16	*(a)* Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included Page 9-10
		(*b*) Report category boundaries when continuous variables were categorized
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period

Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
Discussion		
Key results	18	Summarise key results with reference to study objectives Page 11
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias Page 12
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence Page 11-12
Generalisability	21	Discuss the generalisability (external validity) of the study results Page 12 (multicenter nature)
Other information		
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based Page 13

*Give information separately for cases and controls.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at http://www.strobe-statement.org.

Estrogen receptor polymorphisms are an associated risk factor for mild cognitive impairment and Alzheimer disease in women APOE ε4 carriers

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ABSTRACT

Objetives: Examine the role of ~~the~~ single nucleotide polymorphisms (SNPs) in the estrogen receptor 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 *APOE* gene

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

Setting: Subjects were prospectively recruited from ~~the~~ Neurology Departments of several Basque Country hospitals.

Participants: This study comprised 816 Caucasian subjects that were aged 50 years and older: 204 MCIa, 350 sporadic AD patients 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 MCI and AD was established using Logistic Regression Models.

Results: Neither alleles nor genotypes of SNPs rs9340799, rs2234693, rs2228480 and rs4986938 of estrogen 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 1.28-8.54, p=0.014) and AD (OR= 5.16, 95% CI 2.19-12.14, p<0.001) in women APOE*ε4 allele carriers.

Conclusions: The less represented alleles of SNPs studied are associated ~~with DCIa yto~~with MCIa and AD in ~~subjects~~ APOE*E4 carriers. Particularly, 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.

ARTICLE SUMMARY

Article focus:

- Alzheimer's disease´s ~~aetiology~~etiology is complex and multifactorial
- Estrogen receptors have several polymorphisms that seem to be related ~~with~~to the effect of the main risk factor to Alzheimer disease (AD), the *APOE* gene.
- The aim of the study is to examine the role of the single nucleotide polymorphisms (SNPs): rs9340799, rs2234693, rs2228480 and rs4986938 as a risk factor for mild cognitive impairment (MCI) and AD and its possible association with *APOE* gene

Key message

- APOE*ε4 allele is an independent risk factor for the AD population, and this risk is ~~highest for~~higher in women

- rs9340799, rs2234693, rs2228480 and rs4986938 are not independently associated with the risk of MCI and AD
- The less represented alleles of SNPs studied are associated with MCI and AD in ~~subjects~~ APOE*E4 carriers

Strengths and limitations of this study

- It was one of the first studies to investigate and association between polymorphisms of ER genes and cognitive function not only in AD patients, but also in ~~MCI~~ 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 estrogen replacement therapy were unknown.

INTRODUCTION:

Alzheimer´s disease (AD) is the most common form of dementia, currently affecting over 9 million americans and europeans, its ~~ethiology 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 [1].~~etiology 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 [1]

~~The apolipoprotein E gene (*APOE*) is a genetic factor closely related to late onset AD disease, and constitutes and strong independent risk factor for sporadic AD [2]. Women have a slightly higher risk of AD compared to men [3]. 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.~~

The apolipoprotein E gene (*APOE*) is a genetic factor closely related to late onset AD disease, and constitutes an strong independent risk factor for sporadic AD [2]. 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 last years genetic research has focused on identifying common population polymorphism loci, not only *APOE*, but also other genes such as *CLU*, *CR1*, *PICALM* and *EXOC3L2* have been associated with an increased risk for developing AD ~~[4-7].~~[3-6]. These genes are implicated in chaperone action, positive regulation immune response, 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*.

Estrogens are pleiotropic hormones having an influence not only on reproductive system but also in central nervous system (CNS). These hormones are synthesized by ovaries and ~~by~~ are also produced in smaller amounts by other tissues such as glia in CNS, having a wide spectrum of effects such as neuroprotective and antiapoptotic ~~[8-10].~~[7-9]. 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 ~~[11, 12]~~[10, 11].

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~~Among other positive effects of estrogens[13], estradiol-17-Beta has an effect on 1) the maintenance and increase of the neurotransmitter systems, 2) the APP processing, Abeta levels and factors that alter its clearance and aggregation [14], 3) mechanisms of oxidative damage [15], Low endogenous estrogen levels have been broadly related with the increased risk of Alzheimer's in postmenopausal women. However, despite the initial data~~ . Among other positive effects of estrogens [12], estradiol-17-Beta has an effect on 1) the maintenance and increase of the neurotransmitter systems, 2) the *APP* processing, Abeta levels and factors that alter its clearance and aggregation [13], 3) mechanisms of oxidative damage. Multiple lines of evidence suggest that loss of estrogens in the aging brain of both women and men may play a role in the cognitive declines associated with AD [14] but whether female sex is also a risk factor is controversial although some past and a recent study ~~says~~show ~~that there is~~ higher rates of cognitive decline for women and apolipoprotein E4 carriers (APOE*ε4) ~~[16-19]~~[15, 16], ~~there is disagreement regarding hormonal replacement therapy~~ , and mouse AD-transgenic mice studies generally show great amyloid and neurodegeneration in females ~~[20, 21]~~[17,18] . However, despite the initial data [19-22], there is disagreement regarding hormonal replacement therapy in women [14,23-25]

Furthermore, it has also described an interaction ~~with apolipoprotein E (ApoE).~~With ApoE, Estradiol increased ApoE levels and neurite outgrowth. ~~ApoE2~~APOE*ε2 isoform increased neurite length more than ~~ApoE3~~APOE*ε3 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 ~~ApoE4~~APOE*ε4, the isoform that is associated with increased risk of neurological disease, was exogenously supplied ~~[22].~~[26] These data support the hypothesis that *APOE* gene plays an integral role in the neurotrophic effects of estradiol-17-Beta, and the presence of a probable synergism between ApoE subtype expression and the effects of estrogens.

The mechanism through ~~which~~ estrogens exert its neuroprotective and anti-neurodegenerative effects in the CNS is poorly understood overall are mediated by two estrogen receptors (ERs), ERalpha and ERbeta (coded by *ESR1* and *ESR2* ~~genes). ERs are located through around~~gene),expressed in neurons and glia throughout the brain, especially in hippocampus and amygdale ~~[23, 24]~~[27,28] ~~, regions involved in memory and learning process. Thus, genetic variants in ER genes have been studied in relation to EA. There are several polymorphic loci in intron 1 of *ESR1* gen, highlighting the PvuII and XbaI locus [25]. These loci may influence the expression of *ESR1* gen; xp haplotype has higher expression than the XP one, but with no significant differences [26]. Several studies, [27-29] but not all [26] have found an increased frequency of the PvuII and XbaI *ESR1* polymorphisms in AD patients.~~

, regions involved in memory and learning process. Thus, genetic variants in ER genes have been studied in relation to ~~E~~AD. There are several polymorphic loci in intron 1 of *ESR1* gen, highlighting the PvuII and XbaI locus [29]. 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. Subjects were described as pp or xx homozygotes, Pp or Xx heterozygotes, or PP or XX homozygotes [30].The xp haplotype has higher expression than the XP one, but with no significant differences [31]. Several studies, [32-34] but not all [31] have found an increased frequency of the PvuII and XbaI *ESR1* polymorphisms in AD patients.

Other interesting SNP is rs2228480, this polymorphism is the coding synonymous variant at 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 ~~[30]. In addition, this SNP has been associated with neurodegenerative disorders~~[35]. In addition, this SNP has been associated with schizophrenia and the mechanism of this association may involve alternative gene regulation and transcript processing ~~[31].~~[36]

Other studies have shown an association between several polymorphism of *ESR2* gene and late onset AD, and they found that variations in this gene could modify disease susceptibility ~~[32].~~[37] The polymorphism located in 3'UTR of *ESR2* gene, rs4986938, has been associated with the onset of Parkinson disease ~~[33]~~[38] and the susceptibility for vascular dementia (VaD) in an Israeli cohort, but not with AD ~~[34].~~[39] In the study of Dresener-Pollack et al. (2009), ~~VAD~~VaD is differentiated from AD by clinical criteria, but in the absence of imaging data, the potential ~~for~~ misclassification is high. Thus, results should be confirmed.

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To date no studies have been conducted in the prodromal stages of ~~EA~~AD such as mild cognitive impairment of amnestic type (MCIa). Such studies could provide information ~~on~~about the beginning of the disease process, helping to ensure that suitable therapeutic measures ~~are~~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 *APOE* gene; and whether such 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 APOE*ε4 ~~status~~ carriers. We have studied this association in AD patients and in MCIa patients, the latter condition possibly representing a prodrome for ~~dementia of AD type [35].~~AD type dementia. [40]

With the purpose of examining the association of the *ESR1* and *ESR2* genes involved in estrogen 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 *APOE* genotype.

METHODS:

This study comprised 816 caucasian subjects, included in 3 groups: MCIa patients (n=204), AD patients (n=350) and healthy controls (CTL) (n=262). Subjects were prospectively recruited from ~~the~~ Neurology Departments of several hospitals. Participants were aged 50 years and older. For AD and MCIa patients, evaluation also included routine blood tests: haematology, biochemistry, thyroid-stimulating hormone, vitamin B12 levels, syphilis serology and neuroimaging test ~~(~~: CT (Computerized Tomography) scan or MRI~~)~~. (Magnetic Resonance Imaging).

The subjects were evaluated using a broad battery of neuropsychological tests: Minimental State Examination~~,~~ (MMSE), Clinical Dementia Rating scale, CERAD protocol, Stroop test, unilateral and bilateral motor praxis, 7-minute test, trial making part A and B; and Neuropsychiatric Inventory (NPI).

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

The diagnosis of MCIa patients was based on Petersen's criteria ~~[35, 56].~~[40] 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 CDR scale was required to be 0.5, and performance in relation to other cognitive functions and daily living activities were required to be normal. The diagnosis of AD was based on the DSM IV ~~[57]~~ and NINCDS-ADRDA ~~[58]~~ criteria for probable and possible AD. Patients with a total score of less than 3 on CDR scale (mild to moderate dementia) were included.

Healthy control subjects 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 ischemic 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

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consent to undergo the examination. The study was conducted in accordance with the Declaration of Helsinki concerning medical research in human subjects.

Genetic analysis:

~~At~~On the first visit, peripherical blood samples were collected ~~at~~in EDTA vacuum tubes from all individuals. Genomic DNA was extracted by proteolytic lysis from white blood cells using standard phenol/chloroform extraction method.

APOE gene was amplified by PCR with ~~the primers 112F and 158R, under the PCR conditions described by Wilton and Lim [59].~~112F and 158R primers, under the PCR conditions described by Wilton and Lim [41]. Digestion of the amplified product was carried out with Hae II and Afl III, as described by Álvarez-Álvarez et al. ~~(2003)[60].~~(2003) [42].

Three single nucleotide polymorphisms (SNPs) in the *ESR1* gene (rs9340799, rs2234693 and rs2228480) and one SNP in the *ESR2* gene (rs4986938) were evaluated. 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 characterized 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 polymorphism rs2228480; G>A (SNP1) of *ESR1* gen and polymorphism rs4986938; G>A (SNP2) of *ESR2* gen.

SNP genotypes of candidate genes (*ESR1* and *ESR2*) and *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 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 version 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 ~~[61].~~ [43]. The G test was also used to check the differences between demographic and clinical variables, allele frequencies and genotype frequencies.

Statistical analysis was also performed using the SPSS® package, version 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 effect of X, P and SNP1-A alleles of *ESR1* gen and SNP2-A allele of *ESR2* gen in the total sample and in the absence of APOE*ε4 allele. The effect of 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 polymorphism of *ESR1* and *ESR2* genes and the APOE*ε4 allele, based on the hypothesis that the effect of estrogens might exists only in APOE*ε4 allele carriers.

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

RESULTS:

We have investigated the independent and combined association of X, P and SNP1-A alleles of *ESR1* gen and SNP2-A allele of *ESR2* gen and APOE*ε4 allele by using a case-control design.

In the present study we analysed a sample of 204 MCIa patients, 350 AD patients, 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).~~ (Table 1). Years of education were not significantly different between groups (p=0,148).

Table 1. Baseline Demographic

Group	n	Age[a]	Women (%)[b]	MMSE[c]	Education[d]
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

[a] Years, mean ± Standard Desviation (S.D.). [b] % of Women in group. [c] MMSE score, mean ± S.D. [d] Years of education

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

Table 2. Allelic and genotypic frequency.

ESR1				
	XbaI	**MCIa (N = 204)**	**AD (N =350)**	**CONTROLS (N = 262)**
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[a]	**p-Value**	0.197	0.376	1.000
	PvuII			
Allele	**P**	0.488	0.480	0.462
	p	0.512	0.520	0.538
Genotype	**PP**	0.225	0.209	0.214

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	Pp	0.525	0.543	0.496
	pp	0.250	0.249	0.290
H-W[a]	**p-Value**	0.575	0.110	1.000
	SNP1			
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[a]	**p-Value**	0.818	0.861	1.000
ESR2				
	SNP2			
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[a]	**p-Value**	0.245	0.325	0.591
APOE				
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[a]	**p-Value**	0.217	0.814	0.102
Genetic Profile				
	XPAA(+)	0.709	0.708	0.674

XPAA(-)	0.291	0.292	0.326

a Hardy-Weinberg probability test.

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

Table 3. Exact G test

XbaI [a]			**XbaI** [b]		
	P-value	**S.E.** [c]		**P-value**	**S.E.** [c]
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
PvuII [a]			**PvuII** [b]		
	P-value	**S.E.** [c]		**P-value**	**S.E.** [c]
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
SNP1 [a]			**SNP1** [b]		
	P-value	**S.E.** [c]		**P-value**	**S.E.** [c]
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
SNP2 [a]			**SNP2** [b]		
	P-value	**S.E.** [c]		**P-value**	**S.E.** [c]
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
APOE [a]			**APOE** [b]		
	P-value	**S.E.** [c]		**P-value**	**S.E.** [c]

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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

a Allelic frecuency. b Genotypic frecuency. c Standard Error

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 MCIa, AD and control individuals with the presence of at least one of these alleles. None of them had a significant effect (data not shown).

In the total sample, APOE*ε4 allele is a risk factor for cognitive impairment; the odds ratios (ORs) of developing MCIa and AD were 2.44 (95%CI 1.61-3.69, p<0.001) and 4.23 (95%CI 2.93-6.12, p<0.001), respectively (Table 4). The higher risk conferred by APOE*ε4 allele was observed even when the samples were subgrouped by sex, but in AD women the risk was higher than in men, 4.85 (95%CI 3.04-7.73, p<0.001) versus 3.19 (95%CI 1.73-5.88, p<0.001).

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

	MCI		AD	
Global Effects	**OR CI95%**	**p**	**OR CI95%**	**p**
X (+)[a]	1.39 (0.93-2.06)	0.104	1.18 (0.85-1.67)	0.324
P (+)[b]	1.25 (0.82-1.90)	0.293	1.26 (0.88-1.23)	0.205
SNP1-A[c]	1.14 (0.76-1.71)	0.506	1.13 (0.78-1.62)	0.510
SNP2-A[d]	1.05 (0.71-1.54)	0.304	1.08 (0.77-1.51)	0.649
E4 (+)[e]	2.44 (1.61–3.69)	<0.001	4.23 (2.93–6.12)	<0.001
Women	1.07 (0.73-1.56)	0.705	1.67 (1.19-2.35)	0.003
E4 (+)*Women[f]	2.27 (1.32-3.87)	0.003	4.85 (3.04-7.73)	<0.001
E4 (+)*Men[g]	2.74 (1.43-5.23)	0.002	3.19 (1.73-5.88)	<0.001
Independent Effects				
X (+) E4(-)[h]	1.04 (0.65-1.66)	0.863	1.18 (0.76-1.81)	0.452
P (+) E4(-)[h]	0.86 (0.52-1.40)	0.545	1.19 (0.754-1.90)	0.444
SNP1-A(+)*E4(-)[h]	1.19 (0.74-1.92)	0.469	1.13 (0.73-1.76)	0.568
SNP2-A(+)*E4(-)[h]	1.03 (0.65-1.66)	0.879	1.07 (0.70-1.64	0.758
ESR1				
Combined Effects				
E4(+)*X[i]	3.17 (1.80-5.59)	<0.001	5.07 (3.00-8.55)	<0.001
E4(+)*P[i]	2.74 (1.55-4.85)	0.001	5.35 (3.11-9.17)	<0.001

E4(+)*SNP1-A[i]	2.53 (1.31-4.90)	<0.001	4.44 (2.48-7.93)	<0.001
ESR2				
Combined Effects				
E4(+)*SNP2-A[i]	2.77 (1.55-4.93)	0.001	4.87 (2.91-8.17)	<0.001
Genetic Profile (XPAA)				
Independent Effects				
XPAA*E4(-)[j]	1.31 (0.48-3.54)	0.590	1.19 (0.49-2.91)	0.696
XPAA(-)*E4(+)[k]	2.53 (1.61-3.93)	<0.001	4.32 (2.91-6.40)	<0.001
Combined Effects				
XPAA*E4(+)[l]	3.30 (1.28-8.54)	0.014	5.16 (2.19-12.14)	<0.001
XPAA*E4(+)*Women[m]	3.84 (1.09-13.57)	0.036	8.04 (2.60-24.80)	<0.001
XPAA*E4(+)*Men[m]	3.20 (0.73-14.11)	0.124	3.57 (0.88-14.47)	0.075

[a]Effect of sample with at least one X of RFLP XbaI. [b]Effect of sample with at least one P of RFLP PvuII. [c] Effect of sample with at least one A allele of rs2228480.[d] Effect of sample with at least one A allele of rs4986938. [e] Effect of sample with at least one E4 allele of APOE gene. [f] Women selected by at least one E4 allele of APOE gene. [g] Men selected by at least one E4 allele of APOE gene. [h] Sample selected by at least one allele that is indicated and the absence of E4 allele of APOE gene. [i] Sample selected by at least one E4 allele of APOE gene and one of the alleles that is indicated. Reference category was sample control. [j] Sample selected by absence of E4 allele of APOE gene and the presence of XPAA. [k] Sample selected by absence of XPAA and the presence by at least one E4 allele of APOE gene. Sample selected by at least one E4 allele of APOE gene and the presence XPAA.[l] Sample selected by at least one E4 allele of APOE gene and the presence of XPAA. [m] Women or Men selected by at least one E4 allele of APOE gene and the presence of XPAA. * In all models reference category was sample control considering the age and sex (as appropriate) ~~[a] Sample selected by at least one X of RFLP XbaI. [b] Sample selected by at least one P of RFLP PvuII. [c] Sample selected by at least one A allele of rs2228480.[d] Sample selected by at least one A allele of rs4986938. [e] Sample selected by at least one E4 allele of APOE gene.[f] Women selected by at least one E4 allele of APOE gene. [g] Men selected by at least one E4 allele of APOE gene. [h] Sample selected by at least one allele that is indicated and the absence of E4 allele of APOE gene [i] Sample selected by at least one E4 allele of APOE gene and one of the alleles that is indicated. Reference category was sample control. [j] Sample selected by absence of E4 allele of APOE gene and the presence of XPAA. [k] Sample selected by absence of XPAA and the presence by at least one E4 allele of APOE gene. Sample selected by at least one E4 allele of APOE gene and the presence XPAA.[l] Sample selected by at least one E4 allele of APOE gene and the presence of XPAA. [m] Women or Men selected by at least one E4 allele of APOE gene and the presence of XPAA. * In all models reference category was sample control~~

Aiming to avoid the combined effect of the less represented alleles of SNPs in candidates genes and APOE*ε4 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*ε4 allele. We did not found a significant effect, even when the samples were subgrouped by sex (data not shown).

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We further evaluated a possible synergistic effect between the less represented alleles of SNP in candidates genes and APOE*ε4 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*ε4 allele. A slight increase in nominal risk of MCI and AD was observed. The statistical analyzes were also conducted according to the gender (Supplementary table 1)

Supplementary Table 1: Risk factors for combined effects in MCI and AD from Logistic Regresion Models

ESR1	MCI		AD	
Combined Effects	**OR CI95%**	**p**	**OR CI95%**	**p**
E4 (+)*X*Women[a1]	4.32 (1.80-10.39)	0.001	7.46 (3.46-16.10)	<0.001
E4 (+)*X*Men[a2]	5.02 (1.95-12.89)	0.001	3.84 (1.60-9.21)	0.003
E4 (+)*P*Women[b1]	3.62 (1.51-8.67)	0.004	9.71 (4.20-22.43)	<0.001
E4 (+)*P*Men[b2]	3.87 (1.52-9.82)	0.004	4.67 (1.86-11.71)	0.004
E4 (+) *A*Women[c1]	1.51 (0.93-3.63)	0.348	4.45 (2.18-9.08)	<0.001
E4 (+) *A*Men[c2]	5.05 (1.77-14.42)	0.002	3.87 (1.39-10.76)	0.010
ESR-2				
Combined Effects				
E4 (+)*A* Women[d1]	2.14 (1.03-4.49)	0.041	4.71 (2.49-8.90)	0.001
E4 (+)*A* Men[d2]	4.20 (1.62-10.87)	0.003	4.74 (1.94-11.56)	0.001

[a1] Women selected by at least one E4 allele of APOE gene and at least on X allele of XbaI. Reference category was sample control. [a2] Men selected by at least one E4 of APOE gene and at least one X allele of XbaI. Reference catecogry was sample control. [b1] Women selected by at least one E4 allele of APOE gene an at least one P allele of P vull. Reference category was sample control. [b2] Men slected by at least one E4 an APOE gene and at least one P allele of Pvull. Reference category was sample control . [c1] Women selected by at least one Er allele of AOE an at least one A allele of rs2228480. Reference category was sample control. [c2] Men selected by at least one E4 allele of AOE an at least: One A allele of rs2228480. Reference category was sample control. [d1] Women selected by at leas one E4 allele of AOE an at least one A allele of rs4986938. Reference category was sample control. [d2] Men selected by at least on EA allele of APOE and at least one A allele of rs4986938. Reference category was sample control.

A significant increased OR was found between the X, P, SNP1-A and SNP2-A alleles tested and MCI men, but it has not been clear observed in women. The opposite effect was observed in the AD group, women showed a greater OR than men. Supplementary table 2 shows the size of samples that carry the genetic characteristic considered in the input of combined models in all groups. Overall, significant differences between the control frequencies and patient's frequencies provided enough power to address this question for a minimum detectable OR between 2.0 and 5.

Supplementary table 2. Samples size for each group considered in combined calculations.

	MCI (N=204)				AD (N=350)				CTL (N=262)			
	E4(+)		E4(-)		E4(+)		E4(-)		E4(+)		E4(-)	
	Women	Men	Women	Men	Women	Men	Women	Men	Women	Men	Women	Men
Alleles	N(%)	N(%)	N(%)	N(%)	N(%)	N(%)	N(%)	N(%)	N(%)	N(%)	N(%)	N(%)
XbaI X(+)	34 (16.67)	27 (13.24)	47 (23.04)	34 (16.67)	88 (25.14)	31 (8.86)	76 (21.71)	37 (10.57)	19 (7.25)	13 (4.96)	79 (30.15)	55 (20.99)

	X(-)	11 (5.39)	6 (2.94)	33 (16.18)	12 (5.88)	48 (13.71)	15 (4.29)	37 (10.57)	18 (5.14)	12 (4.58)	9 (3.44)	46 (17.56)	29 (11.07)
	P(+)	38 (18.63)	29 (14.22)	52 (25.49)	34 (16.67)	102 (29.14)	35 (10.00)	83 (23.71)	43 (12.29)	21 (8.02)	15 (5.73)	90 (34.35)	60 (22.90)
PvuII	P(-)	7 (3.43)	4 (1.96)	28 (13.73)	12 (5.88)	34 (9.71)	11 (3.14)	30 (8.57)	12 (3.43)	10 (3.82)	7 (2.67)	35 (13.36)	24 (9.16)
	A(+)	12 (5.88)	14 (6.86)	28 (13.73)	16 (7.84)	46 (13.14)	15 (4.29)	45 (12.86)	13 (3.71)	12 (4.58)	6 (2.29)	46 (17.56)	19 (7.25)
ESR1													
SNP1	A(-)	33 (16.18)	19 (9.31)	52 (25.49)	30 (14.71)	90 (25.71)	31 (8.86)	68 (19.43)	42 (12.00)	19 (7.25)	16 (6.11)	79 (30.15)	65 (24.81)
	A(+)	29 (14.22)	21 (10.29)	51 (25.00)	31 (15.20)	88 (25.14)	30 (8.57)	73 (20.86)	36 (10.29)	21 (8.02)	11 (4.20)	80 (30.53)	51 (19.47)
ESR2													
SNP2	A(-)	16 (7.84)	12 (5.88)	29 (14.22)	15 (7.35)	48 (13.71)	16 (4.57)	40 (11.43)	19 (5.43)	10 (3.82)	11 (4.20)	45 (17.18)	33 (12.60)
	XPAA(+)	8 (2,29)	5 (1,43)	14 (4,00)	7 (2,00)	21 (6,00)	7 (2,00)	17 (4,86)	6 (1,71)	4 (1,14)	3 (0,86)	13 (3,71)	9 (2,57)
Genetic													
profile	XPAA(-)	37 (10,57)	28 (8,00)	66 (18,86)	39 (11,14)	115 (32,86)	39 (11,14)	96 (27,43)	49 (14,00)	27 (7,71)	19 (5,43)	112 (32,00)	75 (21,43)

The percentages are calculated over the total size of each group. (+) presence of the allele, (-) Absence of the allele.

In order to analyse the combined effect between estrogen polymorphisms, we created a genetic profile with the less represented alleles of these SNPs, expressed as XPAA. We did not found a significant risk in the absence of one APOE*ε4 allele, but analysing the combined effect of XPAA with APOE*ε4 allele, ORs were ~~as follows~~the following: MCIa, OR= 3.30 (95%CI 1.28-8.54, p=0.014) and AD, OR= 5.16 (95%CI 2.19-12.14, p<0.001), these ORs were even greater than the independent effect of APOE*ε4 allele with XPAA(-) (absence of this genetic profile). ~~However~~Although it was expected to obtain a greater effect in MCI men and AD women, according to the results showed in table 3, when the samples were subgrouped by sex taking into account the genetic profile, MCIa and AD women showed an increased OR, 3.84 (95%CI 1.09-13.57, p<0.036) and 8.04 (95%CI 2.60-24.80, p<0.001) respectively, comparing ~~with~~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 absence of APOE*ε4. Furthermore, the genetic profile created with the less represented alleles of SNPs in candidates genes were associated with an increased risk for MCIa and AD in women APOE*ε4 allele carriers.

In our ~~series~~serie, APOE*ε4 allele seems to be an independent risk factor for the AD population, and this risk is highest for women. The APOE*ε4 allele also constitutes a risk factor for MCIa patients.

On evaluating the combined effect of the APOE*ε4 allele in the presence of alleles or genotypes of *ESR1* and *ESR2* SNPs the risk for AD remains significant; though 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 found a significant risk in the absence of one APOE*ε4 allele. However, the presence of XPAA and at least one APOE*ε4 allele increases the risk in MCIa and AD women.

Nowadays the most well-known polymorphism of *ESR1* gene related ~~with~~ to AD are SNPs rs9340799 (A>G; XbaI) and rs2234693 (PvuII; T>C). Regarding to the association between XbaI with AD, several studies show that *ESR1* XbaI polymorphism is an additional risk factor [~~27, 36-38~~]^[32,44-46]. However, other

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studies have not found this association [29, 39-42][34 47-50]. These results and several meta-analysis [1, 43][51] suggested that *ESR1* gene polymorphisms might be related to the individual susceptibility to AD, especially in the females.

Concerning to the association between *ESR1* PvuII polymorphism withand AD, several published studies have shown a great heterogeneity. In some of them no association has been found [26, 39, 40, 42, 44][31 47 48 50 52]. Other studies claimed a protective role of P allele of *ESR1* PvuII polymorphism [29, 37, 38][34 45 46], whereas others found an opposite effect [27, 28, 36, 45-47][32 33 44 53-55]. Some studies [36]. Some studies [44] have established an association between *ESR1* PP and XX genotypes with an increased risk for AD only in males (OR = 3.6, 95% CI = 1.2-10.9) and conferred a relevant additional risk of AD to subjects also carrying APOE*ε4 allele, and in AD women. In this last study *ESR1* PP and XX genotypes were also associated with lower MMSE values (p = 0.0007). This data suggests 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 withto the increased risk of MCI and AD observed in patients with APOE**ε4 allele.

In our knowledge, this is the first study to show evidences in support of the association of SNP rs2228480 with MCI and AD patients APOE*ε4 allele carriers. Previously, this SNP only has been linked to the alternative regulation and transcript processing of *ESR1* gene [31, 48][36 56]. To date hadhave not been provided other information in relation to neurodegenerative disorders.

~~Regarding polymorphisms of *ESR2* gen, several studies have been published with conflicting results: susceptibility for vascular dementia (VaD) but not for sporadic AD in elderly Jewish women was found in *ESR2* rs4986938 polymorphism [34]. Pirskanen et al. (2005)[32] found that some gene variants of *ESR2* gen are associated with increased risk of AD in women (rs1271573 T/T genotype and rs1256043 T/T genotype) while others not (IVS31842, rs4986938). Lambert et al. (2001) [40] found no independent association of these polymorphisms with the risk of developing AD. One study suggests the *ESR2* allele 5 seems to be a protective factor [49]. Meta-analyses have not been performed on the following polymorphisms of *ESR2* gen since they lack published genotype data or the published genotype data was not eligible for inclusion. Other studies [50] have not detected a significant gene-gene interaction between ESR1, ESR2 SNPs and APOE status but the analysis was performed in late onset AD.~~

Regarding polymorphisms of *ESR2* gen, several studies have been published with conflicting results: susceptibility for ~~vascular dementia (~~VaD~~)~~ but not for sporadic AD in elderly Jewish women was found in *ESR2* rs4986938 polymorphism [39]. Pirskanen et al. (2005)[37] found that some gene variants of *ESR2* gen are associated with increased risk of AD in women (rs1271573 T/T genotype and rs1256043 T/T genotype) while others not (IVS31842, rs4986938). Lambert et al. (2001) [48] found no independent association of these polymorphisms with the risk of developing AD. One study suggests the *ESR2* allele 5 seems to be a protective factor [57]. Meta-analyses have not been performed on the following polymorphisms of *ESR2* gen since they lack published genotype data or the published genotype data was not eligible for inclusion. Other studies [58] 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 *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 APOE*ε4 allele confers an increased risk in women, whereas it does not contribute to the disease susceptibility in men. ~~According~~Analysis of haplotypes offers more power to detect associations than does simply focusing on a single variant, but in our case the expected results differ slightly from those expected. The combined effect observed between X , P, SNP1-A and SNP2-A alleles and at least one APOE*ε4 allele seemed to point to an increased risk in MCIa men and AD women. Our case-control study is relative medium size there are a small number samples carrying the genetic profile (<8% in MCI and AD patients, and <2% in controls) and APOE*ε4 allele that may affects negatively the power. Nevertheless, according to our results, some variations in the ER genes in synergy with APOE*ε4 allele may be associated with an increased risk of MCIa and AD in women.

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Our results may suggest that the risk for MCIa and AD may be modulated only when both *ESR1* and *ESR2* genes have several polymorphisms, which might be related to their expression and biological activities. The variations in the ERs genes may involve alternative gene regulation and transcript processing in the brain ~~[31].~~[36] *APOE* gene expression can be differentially regulated depending on activation of ER subtypes. ~~A recent study [15]~~A recent study [59] demonstrated that activation of *ESR1* gene up-regulated APOE*ε4 mRNA and protein expression in hippocampus. In contrast, activation of *ESR2* gene down-regulated the mRNA and protein expression of *APOE* gene. Thus, it is expected lower regulation in postmenopausal women ~~[51],~~[60] conferring less protection against the effect of APOE*ε4 allele.

~~Estrogens have been shown to affect amyloid precursor protein metabolism, by increasing the secretory metabolism of amyloid protein precursor (*APP*). Estrogens are also a potent factor that not only prevents vascular disease but also improves blood flow, including blood flow in regions on the brain affected by AD [52]. Synaptic sprouting by estradiol in a model of AD may operate via an APOE*ε4-dependent mechanism [53]. Cholinergic neurons that are implicated in cognitive functions may be regulated by estrogens. The distribution of ERs corresponds to that of cholinergic system [54]. The important decrease in endogenous estrogen levels after menopause may contribute to the development of AD [55]. Despite the protective effect of estrogens upon AD, this effect might to be modified by ERs polymorphisms, particularly in APOE*ε4 allele carriers. Thus, the current state of knowledge of the role of estrogens for preventing dementia in postmenopausal women should be reviewed.~~

Relatively few studies have examined the epistatic effects between estrogen-related pathway genes and APOE*ε4 allele. Postmenopausal women with down syndrome showed an increased risk of AD and elevated sex hormone binding globulin in those carrying *CYP17* and *CYP19* variants and APOE*ε4 allele[61]. Both genes are involved in the production of neurosteroids (estrogens and testosterone). In addition, estrogens have been shown to affect amyloid precursor protein metabolism, by increasing the secretory metabolism of amyloid protein precursor (*APP*). Estrogens are also a potent factor that not only prevents vascular disease but also improves blood flow, including blood flow in regions on the brain affected by AD[62]. Synaptic sprouting by estradiol in a model of AD may operate via an APOE*ε4-dependent mechanism [63]. Cholinergic neurons that are implicated in cognitive functions may be regulated by estrogens. The distribution of ERs corresponds to that of cholinergic system[64]. The important decrease in endogenous estrogen levels after menopause may contribute to the development of AD [65]. Despite the protective effect of estrogens upon AD, this effect might to be modified by ERs polymorphisms, particularly in APOE*ε4 allele carriers. Thus, the current state of knowledge of the role of estrogens for preventing dementia in postmenopausal women should be reviewed.

Although the prevalence and incidence of AD are higher in women, men also may have the same effect due to SNPs in ER genes. It has been observed that while androgens have specific receptors to exert its neuroprotective action, they~~also~~ ~~they~~ may ~~exert~~also exert their actions indirectly via *CYP17* by aromatization of testosterone to estradiol[66] or directly through *ESR2* binding capacity ~~of~~of the metabolite dihydrotestosterone[67]. To date, it is unclear whether SNPs in ER genes would increase the risk of AD or MCIa in men. Our partial data trend to increase the risk of MCIa in men~~, although the data seems to indicate otherwise~~. 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 AD patients, healthy controls, and MCIa patients. In our knowledge, ours is the first study to investigate ~~and~~an association between polymorphisms of ER (rs9340799, rs2234693, rs2228480 and rs4986938) and cognitive function not only in AD patients, but also in MCIa. Moreover, the patient sample is not small, allowing gender stratification.

Some limitations ~~to~~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 test used in our sample might ensure a highest homogeneity.

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CONCLUSIONS

In our study, APOE*ε4 allele is an independent risk factor for MCIa and AD patients. 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; ~~although~~ this association ~~does not~~ confers a relevant additional risk of AD and MCIa ~~Furthermore~~ , in women and men respectively. Nevertheless, the genetic profile with the less represented alleles of *ESR1* and *ESR2* gene polymorphisms, expressed as XPAA, did not increased 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 MCIa and AD women.

OTHER INFORMATION:

Competing interests:

None.

Funding:

This work was sponsored by grants from the Federación de Asociaciones de Familiares de enfermos de Alzheimer de Euskadi, Fondo de Investigación Sanitaria del Instituto Carlos III (Madrid), Pfizer Foundation and Ayudas a la Investigación de la Obra Social de la Caja Vital Kutxa.

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Supplementary Table 1: Risk factors for combined effects in MCI and AD from Logistic Regresion Models

ESR1	MCI		AD	
Combined Effects	**OR CI95%**	**p**	**OR CI95%**	**p**
E4 (+)*X*Women[a1]	4.32 (1.80-10.39)	0.001	7.46 (3.46-16.10)	<0.001
E4 (+)*X*Men[a2]	5.02 (1.95-12.89)	0.001	3.84 (1.60-9.21)	0.003
E4 (+)*P*Women[b1]	3.62 (1.51-8.67)	0.004	9.71 (4.20-22.43)	<0.001
E4 (+)*P*Men[b2]	3.87 (1.52-9.82)	0.004	4.67 (1.86-11.71)	0.004
E4 (+) *A*Women[c1]	1.51 (0.93-3.63)	0.348	4.45 (2.18-9.08)	<0.001
E4 (+) *A*Men[c2]	5.05 (1.77-14.42)	0.002	3.87 (1.39-10.76)	0.010
ESR-2				
Combined Effects				
E4 (+)*A* Women[d1]	2.14 (1.03-4.49)	0.041	4.71 (2.49-8.90)	0.001
E4 (+)*A* Men[d2]	4.20 (1.62-10.87)	0.003	4.74 (1.94-11.56)	0.001

[a1] Women selected by at least one E4 allele of APOE gene and at least on X allele of Xbal. Reference category was sample control. [a2] Men selected by at least one E4 of APOE gene and at least one X allele of Xbal. Reference catecogry was sample control. [b1] Women selected by at least one E4 allele of APOE gene an at least one P allele of P vull. Reference category was sample control. [b2] Men slected by at least one E4 an APOE gene and at least one P allele of Pvull. Reference category was sample control . [c1] Women selected by at least one Er allele of AOE an at least one A allele of rs2228480. Reference category was sample control. [c2] Men selected by at least one E4 allele of AOE an at least: One A allele of rs2228480. Reference category was sample control. [d1] Women selected by at leas one E4 allele of AOE an at least one A allele of rs4986938. Reference category was sample control. [d2] Men selected by at least on EA allele of APOE and at least one A allele of rs4986938. Reference category was sample control.

A significant increased OR was found between the X, P, SNP1-A and SNP2-A alleles tested and MCI men, but it has not been clear observed in women. The opposite effect was observed in the AD group, women showed a greater OR than men. Supplementary table 2 shows the size of samples that carry the genetic characteristic considered in the input of combined models in all groups. Overall, significant differences between the control frequencies and patient's frequencies provided enough power to address this question for a minimum detectable OR between 2.0 and 5.

Supplementary table 2. Samples size for each group considered in combined calculations.

		MCI (N=204)				AD (N=350)				CTL (N=262)			
		E4(+)		E4(-)		E4(+)		E4(-)		E4(+)		E4(-)	
		Women	Men	Women	Men	Women	Men	Women	Men	Women	Men	Women	Men
	Alleles	N(%)	N(%)	N(%)	N(%)	N(%)	N(%)	N(%)	N(%)	N(%)	N(%)	N(%)	N(%)
Xbal	X(+)	34 (16.67)	27 (13.24)	47 (23.04)	34 (16.67)	88 (25.14)	31 (8.86)	76 (21.71)	37 (10.57)	19 (7.25)	13 (4.96)	79 (30.15)	55 (20.99)
	X(-)	11 (5.39)	6 (2.94)	33 (16.18)	12 (5.88)	48 (13.71)	15 (4.29)	37 (10.57)	18 (5.14)	12 (4.58)	9 (3.44)	46 (17.56)	29 (11.07)
Pvull	P(+)	38 (18.63)	29 (14.22)	52 (25.49)	34 (16.67)	102 (29.14)	35 (10.00)	83 (23.71)	43 (12.29)	21 (8.02)	15 (5.73)	90 (34.35)	60 (22.90)

	P(-)	7 (3.43)	4 (1.96)	28 (13.73)	12 (5.88)	34 (9.71)	11 (3.14)	30 (8.57)	12 (3.43)	10 (3.82)	7 (2.67)	35 (13.36)	24 (9.16)
	A(+)	12 (5.88)	14 (6.86)	28 (13.73)	16 (7.84)	46 (13.14)	15 (4.29)	45 (12.86)	13 (3.71)	12 (4.58)	6 (2.29)	46 (17.56)	19 (7.25)
ESR1 SNP1	A(-)	33 (16.18)	19 (9.31)	52 (25.49)	30 (14.71)	90 (25.71)	31 (8.86)	68 (19.43)	42 (12.00)	19 (7.25)	16 (6.11)	79 (30.15)	65 (24.81)
	A(+)	29 (14.22)	21 (10.29)	51 (25.00)	31 (15.20)	88 (25.14)	30 (8.57)	73 (20.86)	36 (10.29)	21 (8.02)	11 (4.20)	80 (30.53)	51 (19.47)
ESR2 SNP2	A(-)	16 (7.84)	12 (5.88)	29 (14.22)	15 (7.35)	48 (13.71)	16 (4.57)	40 (11.43)	19 (5.43)	10 (3.82)	11 (4.20)	45 (17.18)	33 (12.60)
	XPAA(+)	8 (2,29)	5 (1,43)	14 (4,00)	7 (2,00)	21 (6,00)	7 (2,00)	17 (4,86)	6 (1,71)	4 (1,14)	3 (0,86)	13 (3,71)	9 (2,57)
Genetic profile	XPAA(-)	37 (10,57)	28 (8,00)	66 (18,86)	39 (11,14)	115 (32,86)	39 (11,14)	96 (27,43)	49 (14,00)	27 (7,71)	19 (5,43)	112 (32,00)	75 (21,43)

The percentages are calculated over the total size of each group. (+) presence of the allele, (-) Absence of the allele.