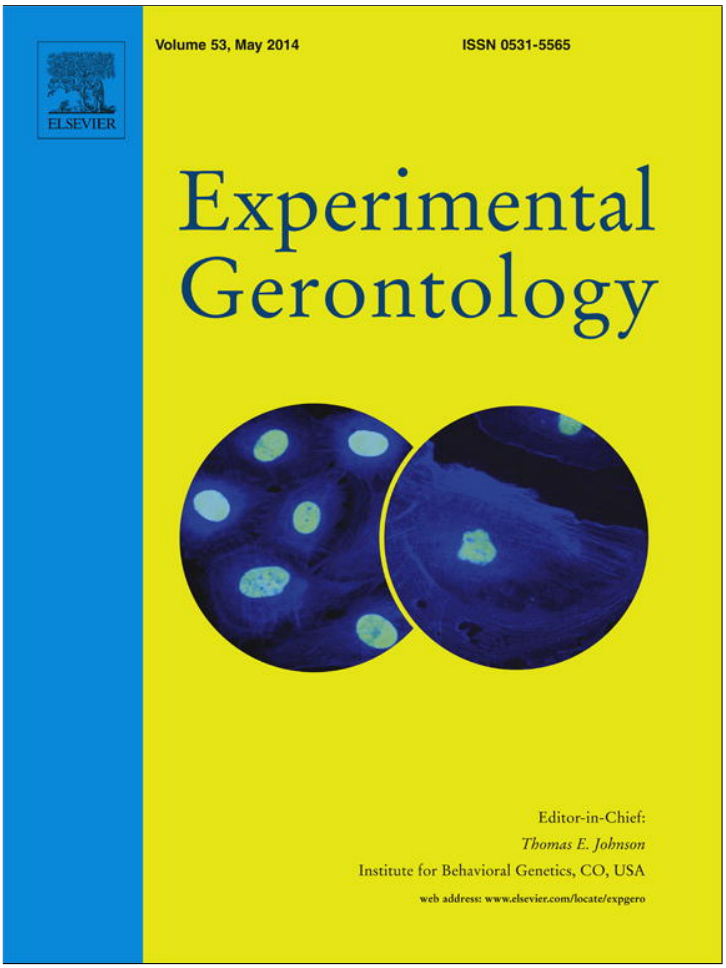

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ApoE gene and exceptional longevity: Insights from three independent cohorts

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ABSTRACT

The *ApoE* gene is associated with the risk of Alzheimer or cardiovascular disease but its influence on exceptional longevity (EL) is uncertain. Our primary purpose was to determine, using a case–control design, if the *ApoE* gene is associated with EL. We compared *ApoE* allele/genotype frequencies among the following cohorts: cases (centenarians, most with 1 + major disease condition; n = 163, 100–111 years) and healthy controls (n = 1039, 20–85 years) from Spain; disease-free cases (centenarians; n = 79, 100–104 years) and healthy controls (n = 597, age 27–81 years) from Italy; and cases (centenarians and semi-supercentenarians, most with 1 + major disease condition; n = 729, 100–116 years) and healthy controls (n = 498, 23–59 years) from Japan. Our main findings were twofold. First, the ε4-allele was negatively associated with EL in the three cohorts, with the following odds ratio (OR) values (adjusted by sex) having been found: 0.55 (95% confidence interval (CI): 0.33, 0.94), $P = 0.030$ (Spain); 0.41 (95%CI: 0.18, 0.99), $P = 0.05$ (Italy); and 0.35 (95%CI: 0.26, 0.57), $P < 0.001$ (Japan). Second, although no association was found in the Spanish cohort (OR = 1.42 (95%CI: 0.89, 2.26), $P = 0.145$), the ε2-allele was positively associated with EL in the Italian (OR = 2.14 (95%CI: 1.18, 3.45), $P = 0.01$) and Japanese subjects (OR = 1.81 (95%CI: 1.25, 2.63), $P = 0.002$). Notwithstanding the limitations of case–control designs, our data suggest that the *ApoE* might be a candidate to influence EL. The ε4-allele appears to decrease the likelihood of reaching EL among individuals of different ethnic/geographic origins. An additional, novel finding of our study was that the ε2-allele might favor EL, at least in the Italian and Japanese cohorts.

1. Introduction

Apolipoprotein E (ApoE) is a major cholesterol carrier that supports lipid transport and injury repair in the brain. The *ApoE* gene is polymorphic, and the vast majority of population studies have focused on the three most common alleles (ε2, ε3 and ε4) that result in six possible genotypes (ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4 and ε4/ε4). The *ApoE* is associated with the risk of having several age-related conditions, such as cardiovascular disease (CVD, including coronary heart disease and cerebrovascular disease) (Kumar et al., 2012) and Alzheimer's disease (AD) (Liu et al., 2013) including also late-onset AD (Sadigh-Eteghad et al., 2012), with the ε4-allele being the unfavorable allele. Thus, the latter variant increases mortality risk (Christensen et al., 2006). Recent data also support an association of *ApoE* and mortality in long-lived individuals. A genome-wide linkage analysis with nonagenarians identified *ApoE* as a *longevity gene* (Beekman et al., 2013), which is in agreement with the results of a recent case–control genome-wide association study comparing long-lived individuals (mean age: 99.7 years) and

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younger controls from Germany (Nebel et al., 2011). However, whether *ApoE* influences exceptional longevity (EL), i.e., reaching 100+ years of age, is more controversial.

The majority of reports indicate a lower frequency of the *ApoE* ε4-allele in centenarians compared with younger controls, i.e., in French (Blanche et al., 2001; Schachter et al., 1994), Finnish (Louhija et al., 1994), Southern Italian (Panza et al., 1999), or Chinese cohorts (Feng et al., 2011). However, others found no association of this variant with EL in Japanese (Asada et al., 1996), Finnish (Louhija et al., 2001) or Southern-Italian centenarians (Capurso et al., 2004). Concurrently, the *ApoE* ε2-allele has been reported to be more frequent in centenarians (Blanche et al., 2001; Louhija et al., 1994; Schachter et al., 1994; Seripa et al., 2006) than in younger people, but others found no differences (Asada et al., 1996; Feng et al., 2011; Louhija et al., 2001; Panza et al., 1999). A meta-analysis reported a point estimate of 1.50 [95% confidence intervals (CI): 1.27, 1.78] and 0.49 (95%CI: 0.41, 0.58) for the frequency of ε2 and ε4-alleles, respectively, in centenarians vs. younger controls, but only studies published before 2004 were included in the review (Lewis and Brunner, 2004).

Our purpose was to determine if *ApoE* is associated with EL. To this end, we compared *ApoE* allele/genotype frequencies in centenarians (cases) and disease-free controls of the same ethnic and geographic origin among three independent cohorts from Spain, Italy and Japan. We also performed subgroup comparisons (i.e., oldest vs. the rest) within each centenarians' group. Based on previous research, we hypothesized that the *ApoE* gene may be associated with EL, with the ε2 and ε4-alleles playing favorable and unfavorable roles, respectively.

2. Material and methods

2.1. Participants

Written consent was obtained from each participant. The study protocol was approved by the corresponding institutional ethics committees [European University of Madrid and *Instituto de Salud Carlos III* (Spanish cohort), University of Pavia (Italian cohort), and National Institute of Health and Nutrition, Medical Research Institute and Keio University (Japanese cohort)] and was in accordance with the Declaration of Helsinki for Human Research of 1974 (last modified in 2008).

2.1.1. Spanish cohort

Two groups of Spanish subjects (most born and living in the central area of Spain, *Meseta Castellana*) were investigated: (i) 175 cases (centenarians, age range 100–111 years, 144 women, 31 men); and (ii) 1081 healthy controls (20–85 years, 622 women, 459 men). All the Spanish participants were of the same Caucasian (Spanish) descent for ≥3 generations and all spoke Spanish as mother language.

Centenarians were living mainly in nursing residencies of the Spanish central area, where they were recruited during years 2009–2012 after we had access to the ages' lists of the people living in the residencies. The participants' ages were ascertained by the dates of birth as stated on identity cards. Their DNA was obtained from saliva samples (Pinos et al., in press). This group included the oldest European individual (111 years) alive in June 2012 (http://www.grg.org/Adams/E.HTM) and ~7% of the cohort was aged ≥105 years. The most prevalent diseases were osteoarthritis (66%), hypertension (57%), dementia (51%) and cardiovascular disease (29%). Only two centenarians were free of any diagnosed disease.

The DNA of 387 younger controls (20–50 years, 170 women, 217 men) was collected from saliva samples during 2008–2009 in the *European University* (Madrid, Spain). This was a convenient sample composed of students and staff from this institution; all of them were free of any major disease (including CVD or AD) and had no known family history of high longevity (90+ years), as reported in a questionnaire. The DNA of the remaining part of the control group (n = 794, 68–85 years, 452 women, 242 men) was obtained between October 2011 and April 2013 in the *Fundación CIEN* (Madrid, Spain) from blood samples of a cohort of the *Vallecas Project*. The latter is a longitudinal study of older subjects with either no or mild cognitive impairment aimed at the early detection of AD. The history of past and current disease was collected by personal interview with the participants. Exclusion criteria were having stroke or traumatic brain injury with severe physical or cognitive impairment, clinically evident cancer, major psychiatric disorders or severe systemic diseases (e.g., chronic renal or liver failure). None of the subjects had criteria of cognitive impairment after being submitted to an extensive neuropsychological battery that included: Mini-mental-State Examination (MMSE), Free and Cued Selective Reminding Test (FCSRT), Verbal Fluency Task (P, M & R), Rey-Osterrieth Complex Figure (ROCF), Drawing Clock Test, Global Deterioration Scale (GDS)-15 of Reisberg and Global Clinical Dementia Rating (CDR). All subjects with CDR > 0 were excluded from the present study.

2.1.2. Italian cohort

Two groups of Italian subjects born and living in Northern Italy were studied: (i) 79 cases (healthy centenarians, 100–104 years, 40 women, 39 men); and (ii) 597 healthy controls (27–81 years, 315 women, 282 men). The participants' ages were defined by the dates of birth as stated on identity cards. All patients and controls were Caucasian whites ascertained to be of Italian descent. The criterion of 'Italian descent' was met when all the parents and grandparents of an individual originated from Italy.

The Italian centenarians were ascertained mainly via general practitioners in the community. These centenarians represent a convenience sample that has been previously described (Emanuele et al., 2010). The history of past and current diseases was accurately collected, checking the centenarians' medical documentation and the current drug therapy. Accordingly, all the Italian centenarians were free of major age-related diseases, i.e., severe cognitive impairment, clinically evident cancer, CVD, renal insufficiency, or severe physical impairment. Only part of this group had decreased visual or auditory acuity. Thus, all of the Italian centenarians were in good health relative to their very advanced age.

Controls were in apparent good physical health, with exclusion criteria being: presence of major CVD or cerebrovascular disease, cancer, dementia, chronic autoimmune or inflammatory disorders, renal or hepatic failure, and major psychiatric disorders.

2.1.3. Japanese cohort

Two groups of Japanese subjects [of the same Asian (Japanese) descent] were studied: (i) 742 cases (centenarians, 100–116 years, 623 women, 119 men); and (ii) 499 healthy controls (23–59 years, 356 women, 143 men). The group of cases was gathered from two prospective cohorts: the Tokyo Centenarians Study (TCS) and the Semi-Supercentenarians Study in Japan (SSC-J). A detailed description of population-based recruitments for the TCS has been previously reported (Gondo et al., 2006). The TCS cohort included 304 centenarians (65 men, 239 women) aged 100–108 years.

The SSC-J is a nationwide longitudinal survey consisting mainly of individuals aged 105 years or older, which started in 2002 (with n = 135 SSC). After 2002, the recruitment strategy has relied on responses to local governments and nursing homes in the whole country, and direct inquiries to our research team. Consequently, a total of 450 centenarians (58 men, 392 women) were enrolled in the SSC-J by the end of November 2011. The phenotype and disease characteristics of the Japanese centenarians are described elsewhere (Takayama et al., 2007), with a prevalence of hypertension, CAD and dementia of 63.6%, 28.8% and 59.4%, respectively.

Inclusion criteria for the control group, which was recruited during the years 2008–2012 by advertising were being men or women aged <60 years, and free of diagnosed stroke, cardiac disease, and chronic renal failure (as reported in a questionnaire).

2.2. Genotype assessment

2.2.1. Spanish cohort

In the cases and younger healthy controls, genomic DNA was extracted from buccal cells according to standard phenol/chloroform procedures followed by alcohol precipitation. *ApoE* genotyping was performed with pre-designed Life Technologies TaqMan® SNP Genotyping Assays on demand considering two SNPs: Cys112Arg (rs429358; ID: C_3084793_20) and Arg158Cys (rs7412; ID: C_904973_10). Individuals ε2 were carriers of 112Cys and 158Cys alleles, individuals ε3 were carriers of 112Cys and 158Arg alleles, and individuals ε4 were carriers of 112Arg and 158Arg alleles. Polymerase chain reaction (PCR) amplification was performed by using a StepOne™ Real-Time PCR System (Life Technologies, Foster City, CA) with a denaturation stage at 95 °C for 10 min, 50 cycles of denaturation at 92 °C for 15 s, annealing/extension at 60 °C for 1 min, and a final extension stage of 30 s at 60 °C.

For the controls gathered from the *Vallecas* Project, total DNA was isolated from peripheral blood following standard procedures. Genotyping of *ApoE* polymorphisms (rs429358 and rs7412) was determined by Real-Time PCR (Calero et al., 2009).

2.2.2. Italian cohort

ApoE genotyping was performed from venous blood samples in all subjects (cases and controls) using PCR restriction fragment length polymorphism (PCR-RFLP) as described previously by Hixson and Vernier (Hixson and Vernier, 1990). In brief, a 244-bp *ApoE* fragment was amplified by PCR (35 cycles; 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min) with the primer pairs: 5′-GATCAAGCTTCCAATCACAGGCAGGA AG-3′ and 5′ GATCCGGCCGCACACGTCCTCCATG-3′. The amplified fragment was digested by using *HhaI* (Promega, Madison, WI) and the products were visualized on polyacrylamide gels. Each genotype gives a specific combination of *HhaI* fragment sizes, i.e., ε2/2: 91 and 83 bp; ε3/3: 91 and 48 bp; ε4/4: 72 and 48 bp and a mixed genotype; ε2/3: 91, 83, and 48 bp; ε3/4: 91, 72 and 48 bp; ε2/4: 91, 83, 72 and 48 bp.

2.2.3. Japanese cohort

Samples were genotyped in the genomics laboratory of the Tokyo Metropolitan Institute of Gerontology (Tokyo, Japan). Total DNA was isolated from venous blood by the use of QIAamp DNA Blood Maxi Kit (QIAGEN, Hilden, Germany). Genotyping was performed during Fall 2013 by using custom designed Taqman® SNP genotyping assays [IDs: C___3084793_20 for Cys112Arg (rs429358) and C____904973_10 for Arg158Cys (rs7412)] (Applied Biosystems, Foster City, CA, USA). All samples were run in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). A total of 5 ml genotyping mixture contained 2.5 ml GTXpress™ master mix, 0.125 ml assay mix (40×) and 1.375 ml distilled water to mix with 1 ml genomic DNA (10 ng/ml) in each reaction. One or two negative controls were included on each plate. TaqMan® SNP Genotyping Assays for genotype calls were analyzed by using StepOne™ Software v2.1 (Applied Biosystems, Foster City, CA, USA).

2.3. Statistical analysis

ApoE allele frequencies were calculated from genotypes by using the gene-counting method. Hardy–Weinberg equilibrium (HWE) was tested with the χ^2 test. Genotype/allele frequencies were compared between cases and controls in each of the three cohorts by using the Fisher exact test. We compared each genotype and allele with all other genotypes and alleles. We also conducted binary logistic regression adjusted by sex to examine the likelihood of reaching EL if carrying a given allele/genotype. The same statistical approach was used to perform specific subgroup comparisons among centenarians in each cohort, i.e., oldest quartile vs. the rest of centenarians. A *P*-value ≤ 0.05 was considered statistically significant. All statistical analyses were performed by using the PASW (v. 18.0 for WINDOWS, Chicago), except for statistical power, which we calculated with the StatMate software, version 2.0 (GraphPad, San Diego, CA, USA).

3. Results

3.1. Spanish cohort

Failure rate of genotyping was 6.9% in cases and 3.9% in controls. The distribution of *ApoE* genotypes was consistent with the HWE in both groups (cases, $P = 0.918$; controls, $P = 0.910$). The results of genotype/allele frequency distributions as well as of binary logistic regression are shown in Table 1 and summarized below. The frequency of the ε2 or ε3-allele did not differ between cases and controls ($P > 0.05$). The ε4-allele was less frequent in Spanish cases than in controls ($P = 0.027$), but no difference was found for the ε4/ε4 genotype ($P = 0.608$). The ε4-allele was negatively associated with EL [odds ratio (OR) adjusted by sex = 0.55 (95%CI: 0.33–0.94, $P = 0.030$]. Table 2 shows the results of the subgroup analyses within the centenarians' group, with no significant *P*-value having been found.

Based on the observed prevalence of the ε4-allele in the control group, the Spanish cohort's sample size had a 95% power to detect a relative likelihood of 2.22 for being a centenarian between ε4-allele non-carriers and carriers with a significance level (alpha) of 0.05 (two-tailed).

3.2. Italian cohort

Rate failure of genotyping was 0%. The distribution of the *ApoE* genotypes met HWE in the two groups (cases, $P = 0.513$; controls, $P = 0.056$). The results of genotype/allele frequency distributions

Table 1
Genotype/allele distributions of the apolipoprotein E (*ApoE*) gene and results of binary logistic regression in the Spanish cohort.

	Cases (centenarians)		Controls		Cases vs. controls Fisher's test *P*-value	Cases vs. controls Binary logistic regression	
	n	%	n	%		OR (95%CI)	*P-value*
Genotype							
ε2/ε2	1	0.6	4	0.4	0.518	0.31 (0.14, 12.18)	0.810
ε2/ε3	21	12.9	93	8.9	0.114	1.57 (0.94, 2.64)	0.086
ε2/ε4	1	0.6	10	1.0	1.000	0.62 (0.35, 1.09)	0.095
ε3/ε3	125	76.7	774	74.5	0.628	1.11 (0.75, 1.64)	0.617
ε3/ε4	15	9.2	150	14.4	0.086	0.55 (0.07, 4.41)	0.574
ε4/ε4	0	0.0	8	0.8	0.608	–	–
Allele							
ε2	24	7.4	111	5.3	0.153	1.42 (0.89, 2.26)	0.145
ε3	286	87.7	1791	86.2	0.487	1.15 (0.80, 1.64)	0.454
ε4	16	4.9	176	8.5	**0.027**	**0.55 (0.33, 0.94)**	**0.030**

Significant *P*-values and associations are in bold. Abbreviations; 95% CI, 95% confidence interval; OR, odds ratio (adjusted by sex).

Table 2
Subgroup comparisons of genotype/allele distributions of the apolipoprotein E (*ApoE*) gene in Spanish centenarians.

		Oldest centenarians (oldest quartile)		Rest of centenarians		Oldest vs. the rest Fisher's test *P*-value	Oldest vs. the rest Binary logistic regression	
		n	%	n	%		OR (95%CI)	*P-value*
Genotype								
	ε2/ε2	0	0.0	1	0.8	1.000	–	–
	ε2/ε3	4	9.8	17	13.9	0.598	0.67 (0.21, 2.12)	0.497
	ε2/ε4	0	0.0	1	0.8	1.000	–	–
	ε3/ε3	34	82.9	91	74.6	0.393	1.65 (0.66, 4.10)	0.282
	ε3/ε4	3	7.3	12	9.8	0.763	0.73 (0.19, 2.72)	0.636
	ε4/ε4	0	0.0	0	0.0	1.000	–	–
Allele								
	ε2	4	4.9	20	8.2	0.464	0.57 (0.19, 1.73)	0.326
	ε3	75	91.5	211	86.5	0.330	1.67 (0.71, 3.94)	0.240
	ε4	3	3.7	13	5.3	0.769	0.68 (0.19, 2.44)	0.551

Abbreviations: 95% CI, 95% confidence interval; OR, odds ratio (adjusted by sex).

as well as the data of binary logistic regression are shown in Table 3 and summarized below. The frequency of the ε3-allele did not differ between cases and controls ($P > 0.05$). The ε4-allele was less frequent in cases than in controls ($P = 0.046$), whereas the ε2-allele was more frequent in the former ($P = 0.013$). The ε4-allele was negatively associated with healthy EL [OR adjusted by sex = 0.41 (95%CI: 0.18, 0.99), $P = 0.05$], whereas a positive association was found for the ε2-allele [OR adjusted by sex = 2.14 (95%CI: 1.18, 3.45), $P = 0.01$]. Table 4 shows the results of the subgroup analyses within the centenarians' group, which are summarized below. The ε2/ε3 and ε3/ε3 genotypes were more frequent in the oldest centenarians compared with the rest of cases ($P = 0.001$ and $P = 0.002$, respectively). The frequency of the ε2- and ε3-allele was higher and lower, respectively, in the oldest quartile compared with the rest of centenarians ($P = 0.001$ and $P = 0.005$).

Based on the observed prevalence of the ε4-allele in controls, the Italian cohort's sample size had a 95% power to detect a relative likelihood of 3.82 for being a centenarian between ε4-allele carriers and non-carriers with $\alpha = 0.05$ (two-tailed).

3.3. Japanese cohort

Failure rate of genotyping was 1.75% in cases and 0.20% in controls. The distribution of *ApoE* genotypes was consistent with the HWE in both groups (cases, $P = 0.111$; controls, $P = 0.293$). The results of genotype/allele frequency distributions as well as of binary logistic regression are shown in Table 5 and summarized below. The frequency of ε2/ε3 and ε3/ε3 genotypes was higher in cases than in controls ($P < 0.01$), whereas the frequency of ε3/ε4 and ε4/ε4 genotypes was lower in the former ($P < 0.05$). The frequency of the ε2 and ε3-allele was higher in cases than in controls and ε4-allele frequency was lower in the former (all $P < 0.01$). The ε4-allele was negatively associated with healthy EL [OR adjusted by sex = 0.35 (95%CI: 0.26, 0.57), $P < 0.001$], whereas a positive association was found for the ε2-allele [OR adjusted by sex = 1.81 (95%CI: 1.25, 2.63), $P = 0.002$] and ε3-allele [OR adjusted by sex = 1.43 (1.13, 1.81), $P = 0.003$].

Table 6 shows the results of the subgroup analyses within the centenarians' group, with no significant *P*-value having been found.

Based on the observed prevalence of the *ApoE* ε4 in controls, the Japanese cohort's sample size had a 95% power to detect a relative likelihood of 1.64 for being a centenarian between ε4-allele non-carriers and carriers with a significance level (alpha) of 0.05 (two-tailed).

3.4. Analysis of the three cohorts combined

When studying the three cohorts combined, genotype distributions were: ε2/ε2 = 0.4%; ε2/ε3 = 13.9%; ε2/ε4 = 0.4%; ε3/ε3 = 76.9%; ε3/ε4 = 8.0%; and ε4/ε4 = 0.3% (cases) and ε2/ε2 = 0.4%; ε2/ε3 = 8.5%; ε2/ε4 = 0.9%; ε3/ε3 = 74.2%; ε3/ε4 = 14.9%; and ε4/ε4 = 1.1% (controls). The frequency of ε3/ε4 and ε4/ε4 genotypes was lower in cases than in controls ($P < 0.001$ and $P = 0.032$, respectively) whereas the ε2/ε3 genotype was more frequent in the former ($P < 0.001$). Allele distributions were: ε2 = 7.6%: ε3 = 87.9%; and ε4 = 4.5% (cases) and ε2 = 5.1%: ε3 = 85.9%; and ε4 = 9.0% (controls). The ε2 and ε3-allele were more frequent in centenarians than in controls ($P = 0.003$ and $P = 0.037$, respectively), whereas the ε4-allele was less frequent in the former ($P < 0.001$).

Table 3
Genotype/allele distributions of the apolipoprotein E (*ApoE*) gene and results of binary logistic regression in the Italian cohort.

		Cases (centenarians)		Controls		Cases vs. controls Fisher's test *P*-value	Cases vs. controls Binary logistic regression	
		n	%	n	%		OR (95%CI)	*P-value*
Genotype								
	ε2/ε2	2	2.5	4	0.3	0.148	1.68 (0.12, 29.52)	0.64
	ε2/ε3	12	15.2	50	9.5	0.060	**1.94 (1.01, 3.77)**	**0.05**
	ε2/ε4	1	1.2	7	0.8	1.000	0.88 (0.09, 7.27	0.91
	ε3/ε3	60	76.0	460	75.9	0.887	0.96 (0.49, 1.71)	0.84
	ε3/ε4	4	5.1	70	13.0	0.084	0.41 (0.17, 1.17)	0.09
	ε4/ε4	0	0.0	6	0.5	1.000	0.59 (0.04, 10.25)	0.73
Allele								
	ε2	17	10.7	65	5.4	**0.013**	**2.14 (1.18, 3.45)**	**0.01**
	ε3	136	86.1	1040	87.1	0.706	0.94 (0.51, 1.33)	0.68
	ε4	5	3.2	89	7.5	**0.046**	**0.41 (0.18, 0.99)**	**0.05**

Significant *P*-values and associations are in bold. Abbreviations: 95% CI, 95% confidence interval; OR, odds ratio (adjusted by sex).

Table 4
Subgroup comparisons of genotype/allele distributions of the apolipoprotein E (*ApoE*) gene and results of binary logistic regression in Italian centenarians.

		Oldest centenarians (oldest quartile)		Rest of centenarians		Oldest vs. the rest Fisher's test *P*-value	Oldest vs. the rest Binary logistic regression	
		n	%	n	%		OR (95%CI)	*P-value*
Genotype								
	ε2/ε2	1	5.2	1	1.6	0.175	3.22 (0.18, 54.12)	0.43
	ε2/ε3	8	42.1	4	6.7	**0.001**	**10.99 (2.39, 36.74)**	**0.001**
	ε2/ε4	0	0	1	1.6	1.000	1.05 (0.04, 25.21)	0.94
	ε3/ε3	9	47.5	51	85	**0.002**	**0.17 (0.08, 0.51)**	**0.009**
	ε3/ε4	1	5.2	3	5.1	1.000	1.09 (0.17, 9.77)	0.98
	ε4/ε4	0	0	0	0	–	1.00 (0.02, 255.12)	1.00
Allele								
	ε2	10	26.3	7	5.8	**0.001**	**5.64 (2.04, 16.27)**	**0.003**
	ε3	27	71.1	109	90.8	**0.005**	**0.24 (0.11, 0.71)**	**0.008**
	ε4	1	2.6	4	3.4	1.000	0.79 (0.12, 7.24)	0.88

Significant *P*-values and associations are in bold. Abbreviations: 95% CI, 95% confidence interval; OR, odds ratio (adjusted by sex).

4. Discussion

Our results indicate that the ε4-allele was negatively associated with the likelihood of becoming a centenarian in the three cohorts. A novel finding of our study was that the ε2-allele seems to specifically favor EL in the Italian and Japanese population, although this result was not corroborated in the Spanish cohort. Of note, the ε2-allele might also specifically favor *successful* or *healthy* EL in the Italian cohort we studied. This allele was indeed more frequent in Italian centenarians, who were free of dementia or any other major age-related condition compared with their young controls, and was also more frequent in the oldest Italian centenarians than in the rest of this group. More research is however needed in other cohorts of disease-free centenarians to corroborate the potential benefit of the ε2-allele on *healthy* EL.

There are large differences in *ApoE* allele frequencies among geographical areas and populations (Hallman et al., 1991; Singh et al., 2006), e.g., with high relative frequencies of *ApoE* ε4 in Northern Europe (15–21%), intermediate frequencies in Central Europe (11–13%), and low frequencies in Southern Europe (4–6%) (Lewis and Brunner, 2004). The ε4-allele frequency in our Spanish (10%) and Italian (8%) young controls was relatively higher than that previously reported by Lewis and Brunner (2004) in Southern Europe. Whereas, the *ApoE* ε4-allele frequency in Spanish and Italian centenarians was 4.9 and 3.2% respectively, which is within the range of previously published frequencies (1–10%) for European centenarians (Blanche et al., 2001; Capurso et al., 2004; Castro et al., 1999; Gerdes et al., 2000; Louhija et al., 1994; Maruszak et al., 2012; Panza et al., 1999; Panza et al., 2003; Schachter et al., 1994; Seripa et al., 2006). On the other hand, allele frequencies in the Japanese centenarians studied here (ε2 = 7.3%; ε3 = 88.1%; ε4 = 4.6%) are comparable to those previously reported in centenarians of the same country, e.g., ε2 = 4.5%; ε3 = 90.9%; ε4 = 4.5% (Asada et al. 1996).

Our results indicate that the ε4-allele is negatively associated with EL, a finding which was corroborated in our three independent cohorts. Some debate exists, with some authors traditionally viewing *ApoE* more as a 'frailty' or 'disease-risk' gene than as a longevity gene (Christensen et al., 2006; Fried et al., 2001; Gerdes et al., 2000), with its effects on death theoretically decreasing at very old ages (Christensen et al., 2006). This view is in line with the 'heterogeneity hypothesis', according to which mortality of individuals carrying the 'frail' genotype (e.g., one or more ε4-alleles) in a population will approach that of non-carriers with advancing age because of a selection pressure on carriers; that is, surviving carriers might possess other factors compensating for the 'frail' genotype, and thus heterogeneity becomes a more important factor at old ages than carriage of a given risk-allele (Christensen et al., 2006). However, our results seem to be in support of previous data on Scandinavian twins showing an increasing mortality risk associated with the *ApoE* ε4-allele with advancing age (Hjelmborg et al., 2006) or of those showing lower frequency of this allele in centenarians compared with younger controls of French (Blanche et al., 2001; Schachter et al., 1994), Finnish (Louhija et al., 1994), Southern Italian (Panza et al., 1999) or Chinese origin (Feng et al., 2011). Thus, our data seem in agreement with an another hypothesis, the so-called 'multifactorial threshold model', according to which many single factors (e.g., carriage of a frail genotype such as one or more ε4-alleles) each with small yet significant effect add up and contribute to a disorder/condition (or death) to happen with age (Jacobsen et al., 2010).

There are several biological mechanisms to postulate *ApoE*, and particularly the ε4-allele, as a candidate to influence longevity. The

Table 5
Genotype/allele distributions of the apolipoprotein E (*ApoE*) gene and results of binary logistic regression in the Japanese cohort.

		Cases (centenarians)		Controls		Cases vs. controls Fisher's test *P*-value	Cases vs. controls Binary logistic regression	
		n	%	n	%		OR (95%CI)	*P-value*
Genotype								
	ε2/ε2	1	0.1	0	0.0	1.000	–	–
	ε2/ε3	102	14.0	39	7.8	**0.001**	**1.86 (1.26, 2.75)**	**0.002**
	ε2/ε4	2	0.3	2	0.4	1.000	0.68 (0.09, 5.00)	0.709
	ε3/ε3	562	77.1	350	70.3	**0.008**	**1.34 (1.04, 1.74)**	**0.024**
	ε3/ε4	59	8.1	98	19.7	**<0.001**	**0.34 (0.24, 0.89)**	**<0.001**
	ε4/ε4	3	0.4	9	1.8	**0.018**	**0.22 (0.60, 0.83)**	**0.025**
Allele								
	ε2	106	7.3	41	4.1	**0.002**	**1.81 (1.25, 2.63)**	**0.002**
	ε3	1285	88.1	837	84.0	**0.004**	**1.43 (1.13, 1.81)**	**0.003**
	ε4	67	4.6	118	11.9	**<0.001**	**0.35 (0.26, 0.57)**	**<0.001**

Significant *P*-values and associations are in bold. 95% CI, 95% confidence interval; OR, odds ratio (adjusted by sex).

Table 6
Subgroup comparisons of genotype/allele distributions of the apolipoprotein E (*ApoE*) gene and results of binary logistic regression in Japanese centenarians.

		Oldest centenarian (oldest quartile)		Rest of centenarians		Oldest vs. the rest Fisher's test *P*-value	Oldest vs. the rest	
		n	%	n	%		OR (95%CI)	*P-value*
Genotype								
	ε2/ε2	1	0.7	0	0.0	0.210	–	–
	ε2/ε3	23	15.0	79	13.7	0.694	1.17 (0.70, 1.94)	0.546
	ε2/ε4	0	0.0	2	0.3	1.000	–	–
	ε3/ε3	121	79.1	441	76.6	0.588	1.28 (0.83, 1.98)	2.58
	ε3/ε4	7	4.6	52	9.0	0.094	0.45 (0.20, 1.02)	0.056
	ε4/ε4	1	0.7	2	0.3	0.507	1.73 (0.16, 19.22)	0.655
Allele								
	ε2	25	8.2	81	7.0	0.535	1.21 (0.75, 1.93)	0.433
	ε3	272	88.9	1013	87.9	0.692	1.12 (0.75, 1.66)	0.593
	ε4	9	2.9	58	5.0	0.127	0.53 (0.26, 1.09)	0.083

Abbreviations: 95% CI, 95% confidence interval; OR, odds ratio (adjusted by sex).

ε4-allele is a well-established risk factor for age-related conditions such as mainly CVD and AD. A meta-analysis including 121 studies showed that this allele is associated with higher CVD risk, coupled with higher plasma cholesterol levels (Bennet et al., 2007). The association with CVD may be the consequence of higher LDL cholesterol and triglyceride levels in the plasma of ε4-carriers (Dallongeville et al., 1992). A recent meta-analysis of 21 studies on *ApoE* polymorphisms and AD concluded that the ε4-allele is strongly associated with a significantly higher risk of sporadic late onset AD (Sadigh-Eteghad et al., 2012). *ApoE*-lipoproteins bind to several cell-surface receptors to deliver lipids, and also to hydrophobic amyloid-β (Aβ) peptide, which is thought to initiate toxic events that lead to synaptic dysfunction and neurodegeneration in AD (Liu et al., 2013). *ApoE* isoforms differentially regulate Aβ aggregation and clearance in the brain, and have important functions in regulating brain lipid transport, glucose metabolism, neuronal signaling, neuroinflammation, and mitochondrial function (Liu et al., 2013), with the ε4-allele being associated with higher Aβ cellular uptake, leading to more Aβ aggregation compared to ε3 (Liu et al., 2013; Smith 2002; Verghese et al., 2013).

The explanation for our novel finding, i.e., that the ε2-allele could benefit EL in the Italian and Japanese population, and specifically favor *healthy* attainment of EL in the Italian cohort, is less apparent and warrants further research. The *ApoE* ε2-allele has previously been reported to be more frequent in centenarians (Blanche et al., 2001; Louhija et al., 1994; Schachter et al., 1994; Seripa et al., 2006) than in younger people, but others found no differences (Asada et al., 1996; Capurso et al., 2004; Feng et al., 2011; Louhija et al., 2001; Panza et al., 1999). A meta-analysis considering only studies of centenarians published before 2004 gave a point estimate of 1.50 (95%CI, 1.27–1.78) for ε2-allele frequency in centenarians vs. younger controls (Lewis and Brunner, 2004). However, scant data are available on the disease status of the centenarians' cohorts of the above-mentioned reports. Capurso et al. (2004) studied Southern-Italian centenarians without dementia, or other chronic neurological or cerebrovascular disease, but found no differences in *ApoE* genotype frequencies between this group and younger controls. To date, much more data are available on the deleterious role of the ε4-allele, particularly with regards to AD risk, than on a potential protective effect of the ε2-allele. Inheritance of two copies of the ε4-allele is indeed associated with a >10-fold increased risk for developing AD compared to the most common ε3/ε3 genotype (Corder et al., 1993; Strittmatter et al., 1993). There is strong evidence that *ApoE* ε4 facilitates Aβ deposition, with senile plaque density being significantly higher in the brains of AD ε4/ε4 patients compared with those with the ε3/ε3 genotype (Gomez-Isla et al., 1996; Rebeck et al., 1993). Although Aβ deposition is the pathological hallmark of AD, oligomeric, soluble forms of Aβ have been implicated as the synaptotoxic component, in which higher levels of Aβ oligomers are increasing the loss of dendritic spines and accelerating memory impairments in AD (Hashimoto et al., 2012; Wu et al., 2010). With regards to this, a recent study showed that the levels of Aβ oligomers in AD patients were lowest in those carrying the ε2-allele (Hashimoto et al., 2012). The ε2-allele also seems to play a protective role against CVD (Bennet et al., 2007; Gerdes et al., 2000; Kolovou et al., 2002), though the evidence is probably weaker than for negative effect of the ε4-allele (Drenos and Kirkwood, 2010). In addition, previous large population-based longitudinal studies, i.e., the Rotterdam study (Slooter et al., 2001) and the Danish 1905 birth cohort (Lindahl-Jacobsen et al., 2013) reported no favorable effect of ε2-allele on mortality in the elderly, despite protecting against cognitive decline in the oldest old (≥93 years) (Lindahl-Jacobsen et al., 2013).

Besides the rationale for postulating the *ApoE* as a candidate to influence EL, a major strength of our study was the clear definition we used for the criterion of EL (with all cases being centenarians). A novelty with regards to previous research on EL and *ApoE* stems from the fact that (i) the analyses were conducted in three independent cohorts (with one of them, the Japanese cohort, including a very large sample of centenarians) and (ii) we studied both 'normal' and *super healthy* (Italian) centenarians. Nonetheless, it must be kept in mind that the cross-sectional nature of our design precludes comments on causality and we only concentrated on EL as a categorical trait (i.e., to be centenarian or not) rather than on the continuous life-span, which runs the risk of losing some information. On the other hand, some caution is needed when attempting to extrapolate our findings to the whole Italian, Spanish or Japanese population because we used mostly convenience samples, which raises the risk of bias due to population stratification. Another limitation from our study stems from the relatively high rate of genotyping failure in the Spanish cohort, particularly in cases. The main reason might lie on the fact that DNA extraction from saliva in frail individuals such as centenarians might result in small amounts of DNA available for analyses (we did not collect blood samples based on ethical constraints). Thus, blood sampling is likely preferable in this population segment. Also, two different methodologies were used in the Spanish cohort, i.e., TaqMan® and real-time PCR. With regard to the two aforementioned limitations, we believe the fact that enotype distributions met HWE in the two Spanish groups (cases and controls) and allele/genotype frequencies were similar to those reported in other Southern European cohorts (Blanche et al., 2001; Capurso et al., 2004) support the validity of our data. Finally, interpretation of case–control studies comparing allele/genotype frequencies among centenarians and younger counterparts may be flawed by differences in date of birth, e.g., 20+ year difference in our cohorts (with centenarians and controls been born in the early 1900s and after 1930 respectively). Indeed, mortality risk depends on the interaction of genotype with environmental or lifestyle risk factors and the pattern of exposure to such risk factors is related to year of birth (Lewis and Brunner, 2004).

5. Conclusions

In summary, our data corroborate that *ApoE* genotypes are associated with EL. The ε4-allele appears to decrease the likelihood of reaching EL among individuals of different ethnic/geographic backgrounds. An additional finding of our report was that the ε2-allele seems to favor EL in the Italians and Japanese cohorts we studied. Moreover, the ε2-allele seems to specifically favor *healthy* EL in the Italian cohort we assessed, although this finding remains to be corroborated in other cohorts of disease-free centenarians from the same country and from other parts of the world, and especially in longitudinal studies.

Conflict of interests

The authors declare that none of them has any conflict of interest related to this manuscript.

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