

# Individual and Combined Effects of *ApoE* and *MTHFR* 677C/T Polymorphisms on Cognitive Performance in Spanish Adolescents: The AVENA Study

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**Objective** To examine the individual and combined associations of *ApoE* and *MTHFR* 677C/T polymorphisms with cognitive performance in adolescents.

**Study design** The study comprised 412 Spanish adolescents (13 to 18.5 years of age). Cognitive performance (verbal, numeric and reasoning abilities, and an overall score) was measured by the Spanish-version of the SRA-Test of Educational-Ability.

**Results** We observed no differences in the cognitive performance study variables in adolescents carrying or not carrying the *ApoE*  $\epsilon 4$  variant. Adolescents without the *MTHFR* 677TT genotype had significantly better cognitive performance than their TT peers. The analysis of the combined effect of these polymorphisms revealed that those individuals carrying both the *ApoE*  $\epsilon 4$  variant and the *MTHFR* 677TT genotype had significantly worse cognitive performance than their peers with other genotype combinations. These findings were independent of sex, age pubertal status, socioeconomic status, physical activity, and skipping breakfast.

**Conclusions** The results of the present study suggest that the *ApoE*  $\epsilon 4$  alone is not associated with cognitive performance in adolescents. Individuals with the *MTHFR* 677TT genotype had slightly impaired cognitive performance, whereas we observed a combined effect of both the *ApoE*  $\epsilon 4$  variant and the *MTHFR* 677TT genotype on cognitive performance. More research is needed in larger population samples to corroborate our findings. (*J Pediatr* 2010;156:978-84).

**A**polipoprotein E (*ApoE*) and its receptors (also known as LDL-receptors) play a pivotal role in neural development, synaptic plasticity, and neuroprotection.<sup>1</sup> The *ApoE* gene is polymorphic, and its 3 common *ApoE* alleles ( $\epsilon 2$ ,  $\epsilon 3$ , and  $\epsilon 4$ ) have been associated with risks for several diseases.<sup>2,3</sup> The presence of the  $\epsilon 4$  variant is a strong risk factor for Alzheimer disease.<sup>4,5</sup> Adults who are  $\epsilon 4$  homozygotes have up to 15 times higher risk of developing Alzheimer disease compared with non-carriers,<sup>6</sup> and their median age of Alzheimer disease onset is 68 years, compared with 84 years in non- $\epsilon 4$  carriers.<sup>7</sup> The presence of *ApoE*  $\epsilon 4$  acts in synergy with several lifestyle factors (eg, fat and alcohol intake, smoking, and physical inactivity) during adult life.<sup>8</sup>

The *ApoE*  $\epsilon 4$  variant is also associated with normal age-related cognitive decline.<sup>9,10</sup> Whether the *ApoE*  $\epsilon 4$  influences cognition during youth is, however, a subject of debate. Two studies did not find an association between *ApoE* polymorphisms and cognitive ability in children.<sup>9,10</sup> In contrast, other studies involving healthy young individuals reported a positive association between *ApoE*  $\epsilon 4$  and intelligence quotient (IQ),<sup>11</sup> predisposition to reach higher levels of education,<sup>12</sup> better performance in some neuropsychological measures after brain injury,<sup>13</sup> improved memory and neural efficiency,<sup>13</sup> or verbal fluency.<sup>14</sup>

Elevated total plasma homocysteine is another strong, independent predictor for the development of vascular dementia and Alzheimer disease later in life.<sup>15</sup> High homocysteine levels are also associated with decreased cognitive function

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Supported by the Spanish Ministry of Health (FIS No. 00/0015), grants from the Spanish Ministry of Education (EX-2007-1124; EX-2008-0641), grants from Panrico S.A., Madaus S.A., and Procter and Gamble S.A., the Swedish Council for Working Life and Social Research (FAS), the ALPHA study, a European Union-funded study, in the framework of the Public Health Programme (Ref: 2006120) the Spanish Ministry of Health: Maternal, Child Health and Development Network (No. RD08/0072). The authors declare no conflicts of interest.

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AVENA	[Alimentación y Valoración del Estado Nutricional de los Adolescentes Españoles (Food and Assessment of the Nutritional Status of Spanish Adolescents)] Study
ANCOVA	One-way analysis of covariance
<i>ApoE</i>	Apolipoprotein E
IQ	Intelligence quotient
<i>MTHFR</i>	5,10-Methylenetetrahydrofolate reductase
PCR	Polymerase chain reaction
TEA	Test of Educational Ability

in the normal aging population.<sup>16-18</sup> Among other mechanisms, elevated homocysteine may negatively affect brain vasculature, resulting in low delivery of nutrients, and could interfere with neurotransmitter formation and DNA repair mechanisms.<sup>19</sup> Plasma homocysteine levels are in turn influenced by the 5,10-methylenetetrahydrofolate reductase (*MTHFR*) 677C/T polymorphism,<sup>20</sup> with the T allele being associated with higher homocysteine levels even in young people.<sup>20,21</sup> Several studies showed no association between the *MTHFR* 677C/T polymorphism and cognitive performance in aging populations,<sup>22-25</sup> and only one report is available in younger cohorts, showing no differences in the distribution of *MTHFR* 677C/T genotypes in children with high IQ compared with peers with average IQ.<sup>26</sup> On the other hand, the possible combined effect of deleterious *ApoE* and *MTHFR* 677C/T polymorphisms on cognitive function in adolescents remains to be elucidated.<sup>27</sup>

The aim of the present study was to examine the association of *ApoE* and *MTHFR* 677C/T polymorphisms with cognitive performance (including verbal, numeric, and reasoning abilities) in Spanish (Caucasian) adolescents. We also examined the combined effects of both polymorphisms on cognitive performance.

## Methods

Participants were recruited from the AVENA [*Alimentación y Valoración del Estado Nutricional de los Adolescentes Españoles* (Food and Assessment of the Nutritional Status of Spanish Adolescents)] Study. This is a cross-sectional study that was primarily designed to assess the nutritional status of a sample of urban Spanish adolescents ages 13 to 18.5 years. Data collection took place from 2000 to 2002 in 5 Spanish cities (Madrid, Murcia, Granada, Santander, and Zaragoza). The complete methodology of the study is detailed elsewhere.<sup>28-30</sup> The number of adolescents included in the AVENA Study was 2859. Blood samples were randomly obtained from 581 participants. The subgroup from which blood samples were obtained was similar to the remaining subjects with regard to the variable selected to calculate the number of participants to be included in the study, for example, body mass index<sup>29</sup> and age and sex proportions (all  $P > .2$ ). The present study comprised 412 adolescents (204 boys and 210 girls) for whom we obtained complete data on cognitive performance and *ApoE* and *MTHFR* 677C/T genotypes.

A comprehensive verbal description of the nature and purpose of the study was given to the parents, school supervisors, and adolescents. Written consent to participate was requested from both parents and adolescents. Adolescents with personal history of cardiovascular disease, cognitive dysfunction, on medication at the time of the study, or those who were pregnant, were excluded. The study protocol was performed in accordance with the ethical standards laid down in the 1961 Declaration of Helsinki (as revised in 2000) and approved by the Review Committee for Research Involving Human Subjects of the Hospital Universitario Marqués de Valdecilla (Santander, Spain).

We assessed cognitive performance with the Spanish version of the SRA Test of Educational Ability (TEA).<sup>31</sup> The TEA measures the subject's ability to learn, by evaluating 3 areas: (1) verbal, command of language; (2) numeric, speed and precision in performing operations with numbers and quantitative concepts; and (3) reasoning, the ability to find logical ordination criteria in sets of figures, numbers, or letters. Direct scores were obtained for each of these parameters. We also computed an overall cognitive performance score by summing up the individual scores of the 3 items.

Genomic DNA for polymorphism analysis was extracted from EDTA-collected peripheral blood using the Quiagen procedure.<sup>32</sup> The *ApoE* (rs7412 and rs429358) genotypes were determined by polymerase chain reaction (PCR) and allele-specific restriction digestion of the amplified products with the restriction enzyme *HhaI*.<sup>33</sup> Genotyping of the 677C/T variant in the *MTHFR* gene (rs1801133) was performed with PCR and allele-specific restriction digestion of the amplified products with the restriction enzyme *HinfI* (GE Healthcare, Madrid, Spain).<sup>20</sup> More detailed information about the *ApoE*<sup>30</sup> and *MTHFR* genotyping procedures is available.<sup>34</sup> The genotypes were in Hardy-Weinberg equilibrium ( $P > .1$ ).

## Potential Confounding Factors

Before any testing was performed, the parents completed a questionnaire that addressed the adolescents' previous and current health status and socioeconomic status, as defined by the educational level and occupation of the father. According to this information, and following the recommendation of the Spanish Society for Epidemiology,<sup>35</sup> the adolescents were classified into 5 categories: (I) low, (II) medium-low, (III) medium, (IV) medium-high, and (V) high socioeconomic status. We also obtained information regarding maternal education level (primary, secondary or university).

We obtained information about family structure through the aforementioned questionnaire. Family structure was defined as living with both mother and father or any other arrangement (only mother, only father, grandparents, others).

We assessed leisure physical activity by means of a questionnaire in which the adolescents answered the following question: "Do you practice any type of physical activity outside school time?" The possible answers were 0 (no) or 1 (yes).

We assessed whether the adolescents skipped breakfast by means of a questionnaire in which they answered the following question: "Do you have breakfast?" The possible answers were either 0 (no) or 1 (yes).

We assessed pubertal development according to Tanner and Whitehouse.<sup>36</sup>

Anthropometric measurements were obtained.<sup>37</sup> Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared ( $\text{kg}/\text{m}^2$ ). Skinfold thickness was measured at the biceps, triceps, subscapular, suprailiac, thigh, and calf on the left side of the body to the nearest 0.2 mm using a Holtain skinfold caliper. All measurements were taken twice and in rotation, and the mean value was calculated. The sum of 6 skinfold, as well as BMI, was used as marker of body fat.

We assessed cardiorespiratory fitness with the 20-meter shuttle run test.<sup>38</sup> It was based on the number of stages completed (precision of 0.5 stage).

### Statistical Analysis

We compared the genotype frequencies between adolescent boys and girls with the  $\chi^2$  test. We analysed the differences in cognitive performance between groups of *ApoE* and *MTHFR 677C/T* polymorphisms by 1-way analysis of covariance (ANCOVA) after adjusting for age, pubertal status, socioeconomic status, and physical activity. For analyses, *ApoE* polymorphism was dichotomized as  $\epsilon 2 + \epsilon 3$  and  $\epsilon 4$ ,<sup>39</sup> and *MTHFR 677C/T* polymorphism was analysed in an additive model (0 = CC, 1 = CT, 2 = TT), and as recessive (0 = CC + CT, 1 = TT) and dominant traits (0 = CC, 1 = CT + TT).

We examined the potential interaction between both polymorphisms in determining cognitive performance by ANCOVA after adjusting for the above-mentioned covariates. We also performed ANCOVA to examine the combined effects of both polymorphisms on cognitive performance. For the combined analyses, we grouped individuals into 3 categories: (I) those having 0 theoretically deleterious genotypes (ie, *ApoE*  $\epsilon 2 + \epsilon 3$  and *MTHFR 677CC + CT*); (II) those with one theoretically deleterious genotype (ie, either carrying the *ApoE*  $\epsilon 4$  variant or the *MTHFR 677TT* genotype); and (III) those having the 2 theoretically deleterious genotypes (ie, carrying both the *ApoE*  $\epsilon 4$  variant and the *MTHFR 677TT* genotype).

Because we did not observe an interaction effect between sex polymorphisms and cognitive performance (all  $P > .1$ ), all the analyses were performed with boys and girls together, and sex was included in the analyses as a covariate. Multiple comparisons were adjusted for mass significance.<sup>40</sup>

Analyses were performed using the Statistical Package for Social Sciences (SPSS, v. 16.0 for Windows; SPSS Inc., Chicago, Illinois), and the level of significance was set to .05.

## Results

Genotype distributions of the *ApoE* and *MTHFR 677C/T* polymorphisms were similar in boys and in girls (Table I). There were no adolescent homozygotes for the  $\epsilon 2$  or  $\epsilon 4$  genotype. Mean (SD) estimates of cognitive performance by *ApoE* genotypes are shown in Table II. We did not observe any difference in the cognitive performance study variables between adolescents carrying or not carrying the  $\epsilon 4$  variant.

Mean (SD) estimates of cognitive performance by *MTHFR 677C/T* genotypes are shown in Table III. We observed that the minor T allele was negatively associated with overall cognitive performance ( $-3.14$  points per risk allele,  $P = .017$ ), verbal ability ( $-1.15$  point per risk allele,  $P = .045$ ), and numeric ability ( $-1.14$  points per risk allele,  $P = .009$ ), whereas it was not associated with reasoning ability ( $-0.84$  points per risk allele,  $P = .105$ ). Adolescents without the TT genotype had significantly better cognitive performance than those with the TT genotype (recessive model) after adjusting for sex, age, pubertal status, socioeconomic status, and physical

**Table I.** Genotype frequencies (percentage) of *ApoE* (rs7412 and rs429358) and *MTHFR 677C/T* (rs1801133) polymorphisms by sex in Spanish (Caucasian) adolescents

	<i>ApoE</i>			$\chi^2$ (P value)
	$\epsilon 2/\epsilon 3$	$\epsilon 3/\epsilon 3$	$\epsilon 3/\epsilon 4$	
Boys	19 (9.4)	144 (71.3)	39 (19.3)	0.449 (.799)
Girls	17 (8.1)	156 (74.3)	37 (17.6)	
	<i>MTHFR 677 C/T</i>			$\chi^2$ (P value)
	CC	CT	TT	
Boys	72 (35.6)	106 (52.5)	24 (11.8)	2.132 (.344)
Girls	75 (35.7)	100 (47.6)	35 (16.7)	

activity. Further adjustments for cardiorespiratory fitness, BMI, skinfold thickness, and skipping breakfast did not materially alter the results (data not shown). When maternal educational status was used instead of socioeconomic status, the findings remained unaltered (data not shown).

We also examined whether the association between the *MTHFR 677C/T* polymorphism and cognitive performance-related traits was modified by socioeconomic status, leisure time physical activity, cardiorespiratory fitness (dichotomized into above and below the median), or fatness (thirds of BMI or skinfold thickness), but we did not find evidence for a significant interaction (all  $P > .2$ ). Likewise, there was no significant interaction between the *ApoE* polymorphism and any of the above mentioned variables (all  $P > .2$ ).

We observed a significant interaction between both polymorphisms in determining overall cognitive performance ( $P = .019$ ), numeric ( $P = .012$ ), and reasoning ( $P = .024$ ) but not in verbal performance ( $P = .150$ ). The analysis on the combined effects of *ApoE* and *MTHFR 677C/T* polymorphisms on cognitive performance are depicted in the Figure. Adolescents with 2 deleterious genotypes (ie, carrying both the *ApoE*  $\epsilon 4$  variant and the *MTHFR 677TT* genotype) had worse scores compared with their peers having either 0 or 1 deleterious genotypes.

## Discussion

We studied the effect of candidate genes on cognitive performance during adolescence, a period of life when the brain has profound plasticity, and thus there are high possibilities of

**Table II.** Mean (SD) estimates of cognitive performance by *ApoE* (rs7412 and rs429358) genotypes in Spanish (Caucasian) adolescents

	$\epsilon 2 + \epsilon 3$ (n = 336)	$\epsilon 4$ (n = 76)	P
Overall cognitive performance (0-99)	53.9 (15.4)	53.0 (15.1)	.661
Verbal ability (0-33)	21.6 (6.1)	20.8 (6.8)	.433
Numeric ability (0-33)	14.5 (4.6)	13.8 (5.3)	.357
Reasoning ability (0-33)	18.0 (6.1)	18.4 (6.0)	.665

Sex, age, and pubertal status plus socioeconomic status and physical activity were entered as covariates in the model.

**Table III.** Mean (SD) estimates of cognitive performance by *MTHFR* 677C/T genotypes (rs1801133) in Spanish (Caucasian) adolescents.

	CC (n = 147)	CT (n = 206)	TT (n = 59)	P Add	P Recess	P Dom
Overall cognitive performance (0-99)	55.3 (15.3)	54.3 (15.2)	48.4 (14.9)	.017	.009	.202
Verbal ability (0-33)	22.1 (6.7)	21.6 (6.7)	19.5 (6.8)	.045	.026	.244
Numeric ability (0-33)	15.0 (5.0)	14.4 (5.0)	12.6 (5.1)	.009	.022	.092
Reasoning ability (0-33)	18.2 (6.1)	18.3 (6.3)	16.3 (6.1)	.105	.041	.601

Sex, age, and pubertal status plus socioeconomic status and physical activity were entered as covariates in the model. Add indicates additive (0 = CC, 1 = CT, 2 = TT); recess, recessive (0 = CC + CT, 1=TT); dom, dominant (0 = CC, 1 = CT + TT).

stimulating cognitive function.<sup>27</sup> A main finding of the present study is that the *MTHFR* 677C/T polymorphism was associated with cognitive performance in Spanish adolescents, with those carrying the TT genotype having worse/lower cognitive performance. For the *ApoE*  $\epsilon$ 4 variant, we did not observe a deleterious effect on cognitive performance. A key finding was the combined effect of these polymorphisms, revealing that those individuals carrying both the *ApoE*  $\epsilon$ 4 variant and the *MTHFR* 677TT genotype had significantly worse cognitive performance than their peers with other genotype combinations.

While keeping in mind our small sample size (especially for the group of 9 children with 2 deleterious *ApoE*  $\epsilon$ 4 and *MTHFR* 677TT genotypes) and the need for an additional population cohort to replicate our findings, the data shown here are informative and provide insights regarding the association of *ApoE* and *MTHFR* 677C/T polymorphisms with cognitive performance in Spanish (Caucasian) adolescents. The fact that the findings were independent of sex, age, pubertal status, socioeconomic status, and physical activity and that further adjustments for other potential confounders such as maternal education, cardiorespiratory fitness, fatness, or skipping breakfast did not materially modify the results are strengths of this study. It should be also noted that there might be other genetic variants that influence cognitive function individually and that could exert complex interactions with candidate genes as those studied here. Further, the important role that epigenetic mechanisms (ie, environment-gene interactions during critical phases of development) play on gene expression and thus on complex phenotypes might be at least as important as the genetic endowment.

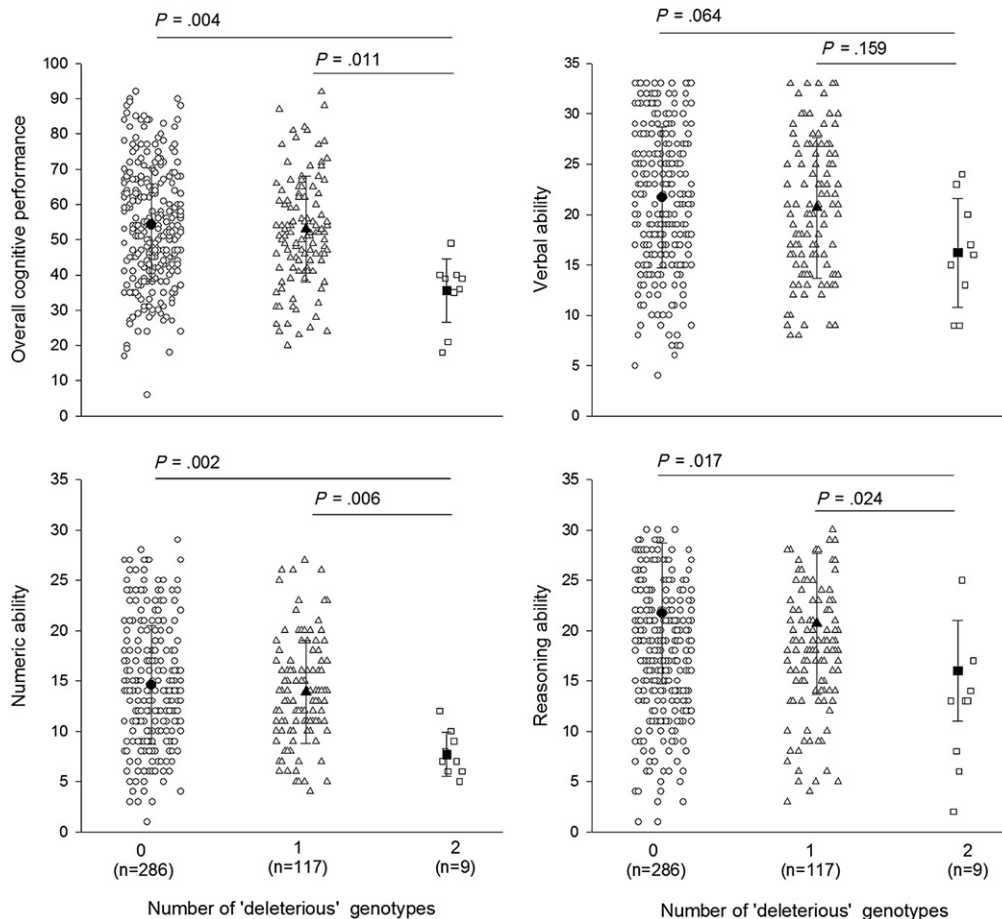
Our observation that *MTHFR* 677C/T polymorphism is associated with cognitive performance in adolescents is the first direct evidence that a functional gene variant of an enzyme involved in the homocysteine metabolism may play a role in cognitive function from the first decades of life. This finding is intriguing and warrants further investigation. Although the association between homocysteine and cognitive performance is consistent,<sup>15-18</sup> it is remarkable that studies in adults failed to find an association between polymorphisms involved in homocysteine metabolism and cognitive performance.<sup>22-25</sup> This raised some concern about the role of homocysteine in cognitive decline, and it was suggested that elevated homocysteine levels are an epiphenomenon rather than a true causal factor.<sup>41</sup> The magnitude of the effect we observed for the *MTHFR* 677C/T polymorphism

was relatively low, which might be explained by the fact that the effect of individual genes are likely to be small.<sup>42,43</sup>

Our findings do not concur with those reported by Barbaux et al<sup>26</sup> in a smaller sample of children, in which they compared the genotype frequencies of several functional polymorphisms involved in homocysteine metabolism between children with high (n = 101) or average IQ (n = 101). They showed that the frequency of the *MTHFR* 677TT variant was 13% and 17% for children with high and average IQ, respectively. They did not report information on the IQ tests and age of participants, which makes comparisons between studies difficult. Our finding that the genotype distribution of the *MTHFR* 677C/T polymorphism was similar in boys and in girls concurs with Anderson et al.<sup>44</sup>

The lack of association between *ApoE* polymorphism and cognitive performance in our cohort is in agreement with earlier studies.<sup>9,10</sup> One study showed no association of *ApoE* variants with general cognitive ability (as assessed by several IQ tests) in a case control study with 2 groups of children (n = 101 each) with high and average cognitive ability.<sup>10</sup> Deary et al<sup>9</sup> observed no differences among *ApoE* genotypes in the score of a general intelligence test performed by 11-year-old children. In contrast, Yu et al<sup>11</sup> observed that young females with the  $\epsilon$ 4 variant had higher IQ scores compared with their counterparts who were non- $\epsilon$ 4 carriers. Similarly, Wright et al<sup>45</sup> reported that infants with the  $\epsilon$ 4 variant of *ApoE* gene had higher scores in the 24-month Mental Development Index of the Bayley Scale compared with non- $\epsilon$ 4 carriers. Discrepancies between studies can arise from factors related to differences in the cognitive parameters being analyzed and assessment methods, sample sizes, or ethnic background and obviously the influence of epigenetic mechanisms.

*ApoE* is the major apolipoprotein constituent of the central nervous system, where it plays an important role in injured and aging brain by mediating neuronal remodeling.<sup>46</sup> The harmful effects of *ApoE*  $\epsilon$ 4 are well known and include increased risk for Alzheimer disease<sup>4,5</sup> as well as cognitive decline in nondemented persons.<sup>47</sup> However, several studies indicated that young *ApoE*  $\epsilon$ 4 carriers may have an improved cognitive performance.<sup>11,45</sup> These apparently contradictory findings suggest the existence of an antagonistic pleiotropic effect of *ApoE* at the extremes of lifespan.<sup>47,48</sup> Williams<sup>49</sup> developed the theory of antagonistic pleiotropy based on the fact that certain types of gene expression were beneficial for increasing fitness in early life, supposedly when natural selection is strong, but had the opposite effects at later ages, when



**Figure.** Cognitive performance in individuals having 0 deleterious genotype (ie, *ApoE*  $\epsilon 2 + \epsilon 3$  and *MTHFR* 677CC + CT), those having at least 1 deleterious genotype in any of the studied polymorphisms (ie, either carrying the *ApoE*  $\epsilon 4$  variant or the *MTHFR* 677TT genotype), and those having 2 deleterious genotypes (ie, carrying both the *ApoE*  $\epsilon 4$  variant and the *MTHFR* 677TT genotype). Dark points are means (standard deviation).

selection is weak. To note, human studies showing polymorphisms with antagonistic pleiotropic effects are scarce and not convincing.<sup>50</sup> Findings from longitudinal studies may provide useful insights for interventions before significant clinical decline occur. In this regard, several follow-up studies showed greater cognitive decline among people with the *ApoE*  $\epsilon 4$  genotype.<sup>51-53</sup>

We also examined whether the associations of *ApoE* and *MTHFR* 677C/T polymorphisms with cognitive performance-related phenotypes were modified by sex, physical activity, socioeconomic status, cardiorespiratory fitness or fatness, and skipping breakfast. There was no evidence that these factors modified the effects of the *ApoE* or *MTHFR* 677C/T polymorphism in our cohort. We did not assess diet-related factors (particularly plasma homocysteine and folate levels), and therefore whether they modulate the association between the study genetic polymorphisms and cognitive function in adolescents remains to be elucidated.

Interestingly, we observed a large influence of having both *ApoE*  $\epsilon 4$  and *MTHFR* 677TT genotypes on overall cognitive

performance in Spanish adolescents. Adolescents having the 2 theoretically deleterious genotypes had an ~33% lower score compared with those with either 0 or 1 deleterious genotype. Our findings highlight the combined effect of having 2 deleterious genotypes in 2 different genes on cognitive performance; yet, the low prevalence of adolescents with both the *ApoE*  $\epsilon 4$  variant and the *MTHFR* 677TT genotype ( $n = 9$ , 2%) precludes drawing any firm conclusions. The lack of studies analyzing the combined effect of these polymorphisms in young and old people prevents the possibility of comparing our results with others.

The *MTHFR* 677C/T polymorphism is associated with cognitive performance in Spanish adolescents, yet the effect is relatively small. Though we did not find an association between the *ApoE* polymorphism and cognitive performance in adolescents, we observed that individuals carrying both the *ApoE*  $\epsilon 4$  variant and the *MTHFR* 677TT genotype had significantly worse cognitive performance than their peers with different genotype combinations. More research is warranted examining both the individual and the combined effect of

these and other candidate genes on cognitive performance in young people. Longitudinal studies are needed to explore the impact on cognitive function later in life of having either the *ApoE*  $\epsilon 4$  variant, the *MTHFR* 677TT genotype, or both in childhood and adolescence. ■

Submitted for publication Jul 17, 2009; last revision received Sep 25, 2009; accepted Dec 9, 2009.

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## Appendix

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