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Article

Differential Methylation in *APOE* (Chr19; exon four; from 44,909,188 to 44,909,373) and Increased Apolipoprotein E Plasmatic Levels in Subjects with Mild Cognitive Impairment

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Abstract: Background: Biomarkers are essential for identification of individuals at high risk of mild cognitive impairment (MCI) for potential prevention of dementia. We investigated DNA methylation in the *ApoE* gene and plasmatic apolipoprotein E (ApoE) levels as MCI biomarkers in Colombian subjects with MCI and controls.

Methods: 100 participants were included (71% women, average age, 70 yrs., range 43-91). MCI was diagnosed by neuropsychological testing, medical and social history, activities of daily living, cognitive symptoms and neuroimaging. Multivariate logistic regression models adjusted by age and gender were performed to examine the risk association of MCI with plasma ApoE and *APOE* methylation

Results: MCI was diagnosed in 41 subjects (average age, 66.5±9.6 yrs.) and compared with 59 controls. Elevated plasma ApoE and *APOE* methylation of CpGs 165, 190, and 198 were risk factors for MCI ($P<0.05$). Higher CpG-227 methylation correlated with lower risk for MCI ($P=0.002$). Only CpG-227 was significantly correlated with plasmatic ApoE levels (correlation coefficient=-0.665; $P=0.008$).

Conclusion: Differential *APOE* methylation and increased plasma ApoE levels were correlated with MCI. These epigenetic patterns can be used as potential biomarkers to identify early stages of MCI.

Keywords: ■APOE gene; ■Apolipoprotein E; ■DNA methylation; ■Mild cognitive impairment; ■Hispanics.

Introduction

Mild Cognitive Impairment (MCI) affects between 3-20% of adults over 65 years old and varies by geographic regions [1-4]. Approximately 20% of elderly individuals would develop dementia after the diagnosed of MCI [5,6]. Interestingly, although Hispanics from Latin America (LA) have almost two-fold higher risk of developing Late-Onset Alzheimer's disease (LOAD) than white North Americans [7,8], the rates of MCI reported in individuals from the United States (US) [9,10] are particularly higher than Hispanics in LA (20% vs. <10%) [11]. This could be attributed to the fact that LA regions, as part of developing countries, fail to diagnose individuals with MCI and/or to identify individuals at high risk for dementia. Additionally, Hispanics have higher rates of a number of vascular risk factors associated with both MCI and dementia, such as hypertension, diabetes mellitus, smoking, sedentary lifestyle, hyperhomocysteinemia, obesity and dyslipidemia[12-16]. Thus, it is critical to implement new strategies to identify subjects at high risk for MCI in order to prevent and/or delay the development of dementia in this highly susceptible population.

The study of genetic traits is important to investigate the early stages of complex diseases such as AD. In fact, the ApoE-ε4 variant has been demonstrated to be the major genetic risk factor for AD in the general population [17]. The apolipoprotein E (APOE) has three isoforms ApoE-ε2, ApoE-ε3, and ApoE-ε4 with direct genetic correspondence to the ε2, ε3, and ε4 alleles. Besides the allelic variant, increased plasmatic apolipoprotein E levels have been examined in relation to AD risk [18]. However, reduced plasma apolipoprotein E levels have been considered a marker of progression of cognitive impairment independently of the APOE genotype [19,20]. Moreover, subjects with different dementia types and with one or two copies of the ε4 allele of the APOE gene

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exhibit decreased expression levels of serum apolipoprotein E with regard to both earlier onset of symptoms and deposits of beta-amyloid plaques [21-23].

Epigenetic modifications influence the protein expression levels such as DNA methylation at CpG sites within the genome [24]. Hypermethylated promoters are primarily associated with gene expression inhibition [25]; however, in some instances hypermethylation has been associated with enhanced expression of some genes as TREM2 in LOAD [26]. The APOE gene has a bimodal methylation structure, with a hypomethylated CpG promoter and comparatively hypermethylated CpG sites located in the APOE exon 4 to 3′ UTR region. In AD brains, the APOE CpG sites are differentially methylated both in a tissue-specific and an APOE genotype-specific manner [27].

Although the expression of APOE and its differential methylation levels in LOAD has been explored, there are no studies in subjects with MCI in LA describing the relationship between APOE methylation levels and apolipoprotein E differential expression. Therefore, we conducted this study to estimate the DNA methylation levels for APOE gene (Chr19; exon four; from 44,909,188 to 44,909,373) and plasma apolipoprotein E levels in a sample of Colombian patients with MCI; furthermore, we explore the relationship between APOE genotype, the DNA methylation of the APOE gene and the risk of MCI.

Methods

Study design and population sample

Participants from a cohort of Colombian patients enrolled at the Dementia Clinic of the National University of Colombia agreed to participate in this research [28]. Inclusion criteria: (i) subjects free dementia at baseline and (ii) available data of plasmatic ApoE or APOE methylation levels. Exclusion criteria: history of schizophrenia, maniac-depressive disorders, schizoaffective disorder, drug/dependence abuse, severe brain trauma or significant disability or unstable medical conditions (i.e., chronic renal failure, chronic hepatic disease, or severe pulmonary disease). From a total of 100 participants, 41 had plasma ApoE levels and 59 had APOE methylation data available (only 18 participants had both genetic phenotypes). All participants were assessed at the Dementia Clinic of the National University of Colombia in Bogotà. Informed consent was obtained from both the participants and their closest relatives. This study was approved by the ethics committee of the School of Medicine at the National University of Colombia, Act 011-107-15,

Mild cognitive impairment

MCI was diagnosed by consensus of a multidisciplinary group that included neurologists, neuropsychologists and neuroscientists, according to the criteria of Petersen et al [4]. Differential diagnostic of other related cognitive disorders included information of neuropsychological testing, medical and social history, activities of daily living, reported cognitive symptoms, and neuroimaging findings. Global cognitive functioning was assessed with the Neuronorma-Colombia diagnostic neuropsychological battery [28-34] and other functional scales [35,36].

DNA Extraction and Bisulfite Treatment

Genomic DNA was isolated from blood from patients and controls using the kit ReliaPrep Blood gDNA Miniprep System™(A5082-PROMEGA) following the protocol recommended by the company. DNA was Quantified in a spectrophotometer NanoDrop2000c ThermoScientific and afterward saved at 4°C. Subsequently, the isolated DNA was bisulfite-converted using the EZ DN Methylation-Direct Kit (D5021– ZymmoResearch). We then evaluated the methylation status of the

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APOE-CGI (APOE-ExonVI-CpG118 to CpG252) located in Chr19:44,909,188-44,909,373 by analysis of the sequence from the RT- PCR products [37].

Bisulfite sequencing PCR (BSP)

The APOE-CGI primers sequences from APOE-F-(5′-TGGAGAAGGTGTAGGTT-3′) and APOE-R-(5′-TTATTAAACTAAAATCCACCCC-3′) was designed following the parameters proposed by Tusnady et al. [38]. Each amplification reaction contained 200 ng of DNA, 20 pmol of each primer, 10% dimethyl sulfoxide, two mM dNTP, 0.125 U of Taq DNA. Both primers were used in a final concentration of 200 nmol/L. Specificity of the assay for converted DNA was verified with the inclusion of unconverted genomic DNA as a control (non-converted DNA Human Methylated & Non-methylated DNA set D5014 Zymo Research) [39]. Methylation percentage for each sample was calculated by analysis with ESME (Epigenetic Sequencing Methylation analysis Software) [40]. Conditions in the PCR stage of the BSP assay were 95° C for 5 minutes, 40 cycles 95 °C for 30 seconds, 58.2° C for 30 seconds, and 72 °C for 45 seconds and standardized in a thermocycler C1000 touch from BIORAD then the products were purified and sequencing in a ABI PRISM 3500 (Applied Biosystem).

Apolipoprotein plasma levels

Genotyping for APOE ε2, ε/3 and /ε4 allelic variants was determined [40] and ApoE plasma levels [26] were measured [41] using ELISA technique (Thermofisher-Invitrogen Human Apo E (AD2) ELISA Kit).

Statistical Analysis

The continuous variables were shown as mean and standard deviation (±) meanwhile categorical ones were summarized as frequencies and percentages (%). Global methylation level was calculated averaging each of the CpG islands. We compared the ApoE plasmatic and APOE methylation levels between participants with MCI and control group. Those APOE methylation CpGs following a non-parametric distribution were analyzed using the U-Man Whitney test for determining statistically significant differences; for the remaining traits, we used t-student test. For categorical variables, we used Chi-square test. The ApoE plasmatic and APOE methylations levels were compared according to the APOE allelic variants. To determine the risk association of plasmatic ApoE levels and APOE methylation with MCI, we performed multivariate adjusted models accounting by age and sex. Regression models were performed in those genetic phenotypes with significant average differences. Finally, CpG regions found as risk factors for MCI were correlated with plasmatic ApoE levels using Pearson's correlation or Spearman's rank tests when appropriated. Data management and statistical analysis were performed using SPSS version 23 (statistical package for social science). Statistical significance was accepted at $p<0.05$ for two-tailed tests.

Results

Baseline characteristics

Of the total 100 participants evaluated, 41 had MCI and 59 were the control subjects. (**Table 1**). The mean age of the whole sample selected was 68.9±9.5 yrs, and 71% (n=71) were women with an age range of 43-91 years old. The distribution of ApoE-ε4 was similar between MCI and controls.

Plasma ApoE levels and APOE methylation

Table 2 shows the genomic position of each CpG island and compares the genotype traits between individuals with MCI and the normal control group. The plasma ApoE levels were higher among those with MCI (P<0.001). APOE methylation of CpGs 118 (P=0.009), 165 (P=0.040), 190 (P=0.045), 198

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(P=0.010) and 227 (P<0.001) were lower in MCI participants (CpGs = 118, 165, 190, and 198) and only one was reversed (CpG-227). Comparisons between non-APOE-ε4 carriers and APOE-ε4 carriers (**Table 3**) showed that only CpG-148 was differently distributed (P=0.003) being higher among APOE-ε4 carriers; the remaining CpG islands were similar distributed (P>0.05).

Plasma ApoE levels and APOE methylation levels as risk factors for MCI

Logistic regression models adjusted by age and sex (**Table 4**) demonstrated that the increment on plasma ApoE levels (OR=1.07; 95% CI=1.02-1.13; P=0.003), CpG-165 (OR=1.20; 95% CI=1.01-1.43; P=0.045), and CpG-190 (OR=1.52; 95% CI=1.06-2.19; P=0.042) are risk factors for MCI. Higher CpG-227 methylation (OR=0.49; 95% CI=0.31-0.78; P=0.002) correlated with lower risk for MCI.

Correlation between Plasma ApoE levels and APOE methylation levels

The linear comparisons of plasma ApoE levels and APOE methylation are shown in **Figure 2**. Although CpG-165 and CpG-190 demonstrated a tendency, the association with plasma ApoE levels was not significant (P>0.05). For instance, there was a negative significant association between plasma ApoE levels and CpG-227 (P=0.008).

Discussion

In the present study, we examined the plasma ApoE levels and APOE methylation in 14 CpGs in Chr19; exon four; from 44,909,188 to 44,909,373 between participants with MCI and control subjects from Bogotá, Colombia, South America. Our key findings were: (i) individuals with MCI had increased plasma ApoE levels in contrast with normal controls; (ii) rather than considering global methylation levels, we found that diverse APOE CpGs were differentially methylated when comparing participants with MCI and control subjects; (iii) after adjustment by age and sex, increments in ApoE plasma levels and CpG-165, CpG-190 and CpG-198 were found to be associated with increased risk of MCI, whereas lower CpG-227 methylation was related with lower risk; (iv) only CpG-227 showed a significant correlation with plasma ApoE levels. The assessment of MCI by considering plasma ApoE levels and APOE methylation levels can be important to clinically identify individuals at high risk to develop dementia. [41]

Although previous studies have shown that decreased serum ApoE levels [41] and hypomethylation in the CpG-252 [26] are risk factors for the development of dementia, we found an inverse relationship in which higher plasma levels of ApoE were associated as a risk factor for MCI group. Our findings might be due to differences in methylation patterns between cell types, with neurons holding higher global levels of DNA methylation [27] and with methylation variations in peripheral blood mononuclear cells related with shortening telomere length [42]. On the other hand, it should be noted that our study examined the possible pathophysiological process involved in a pre-dementia phase and thus our findings may suggest that high concentrations of ApoE would generate a more significant burden of amyloid B deposits that need to be removed. This is supported to the fact that ApoE protein has amyloid beta removal effect [43]. However, this hypothesis cannot be verified with this model of study.

This study reports that the serum ApoE and CpG regions are differentially methylated in MCI patients in contrast with control group. We found both decreased and increased DNA methylation associated with MCI. Whether the increased DNA methylation of the APOE CpG-165, CpG-190 and CpG-198 is a cause or a consequence of cognitive decline remains to be studied. Foraker et al [27] suggest an enhancer role of CGI which can be altered by DNA methylation and can modulate gene expression of both APOE and TOMM40 with possible implications in apolipoprotein

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E expression and mitochondrial function. Additionally, these alterations in DNA methylation within genes that are essential for mitochondrial function could contribute to structural changes in protein and mRNA instability [44]. Our findings support this view, as we found that CpG-227 was correlated with plasma ApoE levels. In spite of no significant association, CpG-165 and CpG-190 showed a tendency in relation to ApoE plasma levels.

Liu et al. [45] suggested that hypermethylation levels at multiple CpGs in the APOE genomic region are associated with delayed recall during cognitive aging. A previous study of our group [40] found an absence of differences in global LINE-1 DNA methylation in LOAD subjects; however, this does not imply lack of alterations in DNA methylation for specific loci and their contribution to exonization events and lately in the epigenetic modifications of the landscape. [44] Consequently, global APOE DNA methylation can be useful to complement locus-specific subanalysis. In the same way, the ability to detect DNA methylation in patients with MCI could be enhanced by new approaches focused on specific cell-analysis, such as distinct cerebral cortex layers [27] and correlation with in vivo brain flow biomarkers [47-51]. Developing countries do not usually have advanced diagnostic methods that can be implemented to identify patient at high risk for MCI. Thus, the study of peripheral blood DNA methylation promises to be a useful pre-clinical biomarker of MCI.

Limitations

The present study must be interpreted within the context of its potential limitations. First, the population sample presents a risk of selection bias because analyzed individuals attended a specialized care center for patients with memory complaints. It was controlled by local radio and television campaigns to capture subjects from other regions of the country. Second, the small sample size limits the generalization of the findings. Third, although unlikely, it is possible that peripheral cellular populations with normal DNA methylation levels could mask the detection of more substantial methylation changes [52].

Conclusion

Dependently of the CpG region, decreased or increased DNA methylation levels were found to be a risk factor for MCI, as well as increased plasma ApoE levels. The current findings might have implications for clinical practice as these genetic phenotypes, detected from peripheral blood samples, can potentially be used as early biomarkers to MCI. Moreover, if a high-risk profile for vascular cognitive impairment is identified, [14] clinical intervention strategies to treat and control modifiable risk factors [16] associated with MCI progression can be intensively implemented in order to prevent or delay the development of dementia.[14,16] Further studies are needed to confirm these findings and to clarify the risk-association of DNA methylation from different tissues with MCI and to determine whether the clinical intervention of controlling modifiable risk factors found in dementia can modify the DNA methylation pattern and reduce the risk for MCI progression.

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Declarations

Ethics approval and consent to participate

This study was approved by the ethics committee of the National University of Colombia Act 011-107-15. All participants, or their closest relatives, gave a written informed consent before participating in this study.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conceptualization, G.C.R O.M, R.P and H.A; Methodology, O.M.PK.E, J.M and E.G; Validation, H.A, O.M and RP,; Formal Analysis, M.M, E.G, J.O, D.S and J.M.; Investigation, O.M, R.P, K.B, K.E, F.C and H.A.; Resources, H.A.; Data Curation, O.M, K.B, F.C, K.E, E.V, H.A and N.S.; Writing – Original Draft Preparation, O.M, K.E, J.M, D.S, R.P and H.A.; Writing – Review & Editing, O.M, H.A, K.E, K.B, D.S, G.C.R, and R.P.; Visualization, X.X.; Supervision, G.C.R O.M, R.P and H.A; Project Administration, H.A; Funding Acquisition, GCR, O.M.P and H.A.

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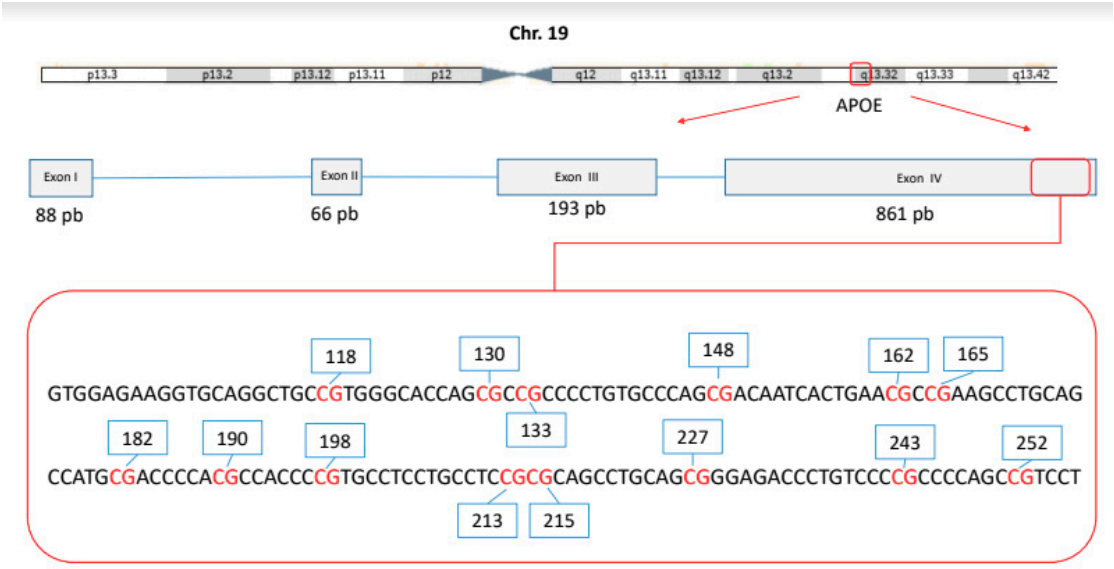

Figure 1. Schematic map of the human *APOE* gene. The *APOE* gene contains four exons, in the final region of the Exon IV is a region of new 15 CpG. For CpGs 118, 130, 133, 148, 162, 165, 182, 190, 198, 213, 215, 227, 243 and 252, the genomic positions were chr19: 44,909 plus 208, 220, 223, 238, 252, 254, 272, 280, 303, 305, 317, 333, and 342, respectively.

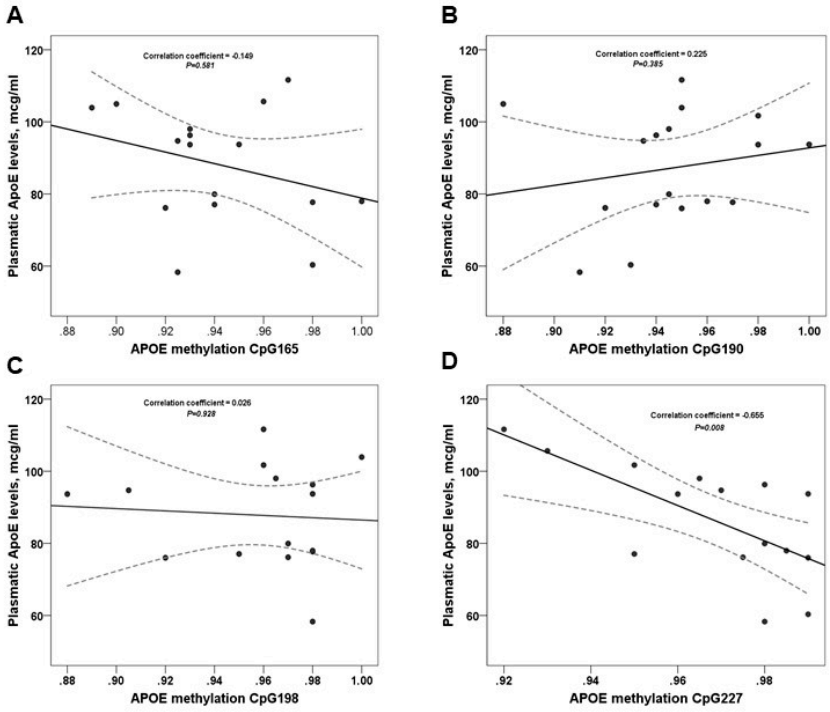

Figure 3. Correlation between Plasmatic ApoE levels and APOE Methylation CpGs. The correlation coefficient and p values for figures A, B and C were calculated using Pearson's test meanwhile for figure D was used the Spearman's rank correlation.

Figure 2. Correlation between Plasmatic ApoE levels and APOE Methylation CpGs. The correlation coefficient and p values for figures A, B and C were calculated using Pearson's test meanwhile for figure D was used the Spearman's rank correlation.

299

300 **Table 1. Baseline Characteristics of the Selected Sample and Divided by**
301 **Individuals with Mild Cognitive**
302

Baseline characteristics	Whole sample (n = 100)	MCI (n = 41)	Control (n = 59)	*P* value*
Demographic data				
Age, years				
Average	68.9±9.5	66.5±9.6	70.5±9.1	0.029
Range	43-91	43-91	50-88	
Gender				0.008
Women, n (%)	71 (71.0)	35 (85.4)	36 (61.0)	
Men, n (%)	29 (29.0)	6 (14.6)	23 (39.0)	
Genetic traits				
APOE-ε4	25 (25.0)	10 (24.4)	15 (25.4)	0.999

MCI=mild cognitive impairment. **P* value of comparison between controls and individuals with MCI. The t-student test implemented for calculating differences for age average meanwhile chi-square test for gender and APOE-ε4.

303

304 **Table 2. Description of ApoE Plasmatic Levels and APOE Methylation of CpGs Islands and**
305 **Comparison between Individuals with Mild Cognitive Impairment and Normal Control Group**

Variables	Genomic position	Whole sample (n = 100)	MCI (n = 41)	Control (n = 59)	*P* value*
ApoE plasmatic Levels, mcg/ml	**-**	**103.2±26.5**	**113.8±26.4**	**86.0±15.7**	**<0.0001†**
Global methylation	**-**	**91.9±3.0**	**92.8±2.6**	**91.6±3.1**	**0.154†**
Methylation by CpG Islands					
CpG118	**chr19:44,909,208**	**85.6±5.1**	**89.6±4.1**	**84.7±4.9**	**0.009†**
CpG130	**chr19:44,909,220**	**88.9±7.1**	**89.0±3.9**	**88.9±7.6**	**0.484**
CpG133	**chr19:44,909,223**	**87.9±10.1**	**85.7±13.3**	**88.4±9.3**	**0.620**

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CpG148	chr19:44,909,238	94.2±4.2	94.9±3.4	93.9±4.4	0.255
CpG162	chr19:44,909,252	89.7±6.1	90.9±3.9	89.3±6.7	0.361
CpG165	chr19:44,909,254	92±5.2	94.5±2.3	91.2±5.6	0.040†
CpG182	chr19:44,909,272	95.2±8.2	93.3±11.7	95.9±6.6	0.324
CpG190	chr19:44,909,280	93.8±5.8	96.4±2.3	93.0±6.3	0.045†
CpG198	chr19:44,909,288	90.8±7.9	96.2±3.1	89.3±8.2	0.01†
CpG213	chr19:44,909,303	95.1±6.7	96.6±6.8	94.7±6.7	0.212
CpG215	chr19:44,909,305	90.7±11.5	91.3±12.9	90.5±11.2	0.272
CpG227	chr19:44,909,317	97.7±2.3	95.6±2.5	98.4±1.8	<0.0001
CpG243	chr19:44,909,333	91.9±10.4	92.5±11.9	91.6±9.9	0.157
CpG252	chr19:44,909,342	90.4±6.2	90.0±6.1	90.6±6.4	0.833

MCI=mild cognitive impairment. **P* value of comparison between controls and individuals with MCI. †The t-student test was implemented for calculating differences. The remaining quantitative variables were analyzed by using U-Mann Whitney as they followed a non-parametric distribution.

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308 **Table 3. ApoE Plasmatic Levels and APOE Methylation of CpGs Islands according to APOE**
309 **Genotype**

310

	Non-APOE-ε4 Carriers	APOE-ε4 Carriers	
Variables	(n = 70)	(n = 25)	*P* value*
ApoE plasmatic Levels, mcg/ml	106.0±31.3	103.0±26.2	0.738†
Global methylation	92.2±2.2	91.6±3.3	0.557†
Methylation by CpG Islands			

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CpG118	**84.5±6.0**	**85.8±4.5**	**0.428†**
CpG130	**90.9±6.5**	**88.0±7.3**	**0.079**
CpG133	**84.8±11.9**	**89.9±7.0**	**0.087**
CpG148	**96.2±2.9**	**93.3±4.4**	**0.003**
CpG162	**90.8±3.1**	**89.4±7.0**	**0.447**
CpG165	**89.7±4.3**	**92.6±5.4**	**0.076†**
CpG182	**94.2±10.1**	**95.4±7.7**	**0.474**
CpG190	**95.0±2.0**	**94.1±3.1**	**0.316†**
CpG198	**91.1±4.6**	**91.4±5.6**	**0.860†**
CpG213	**95.6±5.2**	**94.8±7.4**	**0.872**
CpG215	**93.0±8.1**	**89.6±12.7**	**0.650**
CpG227	**97.5±2.4**	**98.0±2.1**	**0.482**
CpG243	**95.0±3.6**	**90.0±12.3**	**0.363**
CpG252	**89.9±3.5**	**90.5±7.3**	**0.200**

***P value of comparison between controls and individuals with MCI. †The t-student test was implemented for calculating differences. The remaining quantitative variables were analyzed by using U-Mann Whitney as they followed a non-parametric distribution.**

311

312 **Table 4. Adjusted Logistic Regression Models to Examine the Association of Plasmatic ApoE**
313 **levels and APOE Methylation with Mild Cognitive Impairment**
314

	Risk for Mild Cognitive Impairment		
Variables	**Odds ratios**	**95% Confident Interval**	***P* value**
Plasmatic ApoE levels	1.07	1.02-1.13	0.003
APOE methylation		-	
cpg118	1.25	0.96-1.62	0.092

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cpg165	1.20	1.01-1.43	0.045
cpg190	1.52	1.06-2.19	0.023
cpg198	1.30	1.01-1.67	0.042
cpg227*	10.05	1.50-67.30	0.017

Models were adjusted by age and sex. *As CpG227 followed a non-parametric distribution, we divided it into 4 quartiles and <percentile 25th was considered as the risk reference.

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