

1 Article

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

6

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

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

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

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

58

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

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62

63     **Introduction**

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

76     The study of genetic traits is important to investigate the early stages of complex diseases  
77     such as AD. In fact, the ApoE- $\epsilon$ 4 variant has been demonstrated to be the major genetic risk factor for  
78     AD in the general population [17]. The apolipoprotein E (APOE) has three isoforms ApoE- $\epsilon$ 2,  
79     ApoE- $\epsilon$ 3, and ApoE- $\epsilon$ 4 with direct genetic correspondence to the  $\epsilon$ 2,  $\epsilon$ 3, and  $\epsilon$ 4 alleles. Besides the  
80     allelic variant, increased plasmatic apolipoprotein E levels have been examined in relation to AD  
81     risk [18]. However, reduced plasma apolipoprotein E levels have been considered a marker of  
82     progression of cognitive impairment independently of the APOE genotype [19,20]. Moreover,  
83     subjects with different dementia types and with one or two copies of the  $\epsilon$ 4 allele of the APOE gene

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

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

93 Although the expression of APOE and its differential methylation levels in LOAD has been  
94 explored, there are no studies in subjects with MCI in LA describing the relationship between APOE  
95 methylation levels and apolipoprotein E differential expression. Therefore, we conducted this study  
96 to estimate the DNA methylation levels for APOE gene (Chr19; exon four; from 44,909,188 to  
97 44,909,373) and plasma apolipoprotein E levels in a sample of Colombian patients with MCI;  
98 furthermore, we explore the relationship between APOE genotype, the DNA methylation of the  
99 APOE gene and the risk of MCI.

100

## 101 **Methods**

### 102 **Study design and population sample**

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

### 114 **Mild cognitive impairment**

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

### 121 **DNA Extraction and Bisulfite Treatment**

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

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

## 129 **Bisulfite sequencing PCR (BSP)**

130 The APOE-CGI primers sequences from APOE-F-(5'-TGGAGAAGGTGAGGTT-3') and  
131 APOE-R-(5'-TTATTAAACTAAAATCCACCCC-3') was designed following the parameters  
132 proposed by Tusnady et al. [38]. Each amplification reaction contained 200 ng of DNA, 20 pmol of  
133 each primer, 10% dimethyl sulfoxide, two mM dNTP, 0.125 U of Taq DNA. Both primers were used  
134 in a final concentration of 200 nmol/L. Specificity of the assay for converted DNA was verified with  
135 the inclusion of unconverted genomic DNA as a control (non-converted DNA Human Methylated &  
136 Non-methylated DNA set D5014 Zymo Research) [39]. Methylation percentage for each sample was  
137 calculated by analysis with ESME (Epigenetic Sequencing Methylation analysis Software) [40].  
138 Conditions in the PCR stage of the BSP assay were 95 °C for 5 minutes, 40 cycles 95 °C for 30 seconds,  
139 58.2 °C for 30 seconds, and 72 °C for 45 seconds and standardized in a thermocycler C1000 touch  
140 from BIORAD then the products were purified and sequencing in a ABI PRISM 3500 (Applied  
141 Biosystem).

## 142 **Apolipoprotein plasma levels**

143 Genotyping for APOE  $\epsilon$ 2,  $\epsilon$ 3 and  $\epsilon$ 4 allelic variants was determined [40] and ApoE plasma levels  
144 [26] were measured [41] using ELISA technique (Thermofisher-Invitrogen Human Apo E (AD2)  
145 ELISA Kit).

## 146 **Statistical Analysis**

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

## 161 **Results**

### 162 **Baseline characteristics**

163 Of the total 100 participants evaluated, 41 had MCI and 59 were the control subjects. (Table 1). The  
164 mean age of the whole sample selected was  $68.9 \pm 9.5$  yrs, and 71% (n=71) were women with an age  
165 range of 43-91 years old. The distribution of ApoE- $\epsilon$ 4 was similar between MCI and controls.

### 166 **Plasma ApoE levels and APOE methylation**

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

170 (P=0.010) and 227 (P<0.001) were lower in MCI participants (CpGs = 118, 165, 190, and 198) and only  
171 one was reversed (CpG-227). Comparisons between non-APOE- $\epsilon$ 4 carriers and APOE- $\epsilon$ 4 carriers  
172 (**Table 3**) showed that only CpG-148 was differently distributed (P=0.003) being higher among  
173 APOE- $\epsilon$ 4 carriers; the remaining CpG islands were similar distributed (P>0.05).

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

175 Logistic regression models adjusted by age and sex (**Table 4**) demonstrated that the increment on  
176 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;  
177 P=0.045), and CpG-190 (OR=1.52; 95% CI=1.06-2.19; P=0.042) are risk factors for MCI. Higher  
178 CpG-227 methylation (OR=0.49; 95% CI=0.31-0.78; P=0.002) correlated with lower risk for MCI.

179 **Correlation between Plasma ApoE levels and APOE methylation levels**

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

184

185 **Discussion**

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

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

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

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

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

### 231 **Limitations**

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

### 239 **Conclusion**

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

250

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259 **Declarations**

260 **Ethics approval and consent to participate**

261 This study was approved by the ethics committee of the National University of Colombia Act  
262 011-107-15. All participants, or their closest relatives, gave a written informed consent before  
263 participating in this study.

264

265 **Competing interests**

266 The authors declare that they have no competing interests.

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270 **Authors' contributions**

271 Conceptualization, G.C.R O.M, R.P and H.A; Methodology, O.M.P.K.E, J.M and E.G; Validation,  
272 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,  
273 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 –  
274 Original Draft Preparation, O.M, K.E, J.M, D.S, R.P and H.A.; Writing – Review & Editing, O.M, H.A,  
275 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  
276 Administration, H.A; Funding Acquisition, GCR, O.M.P and H.A.

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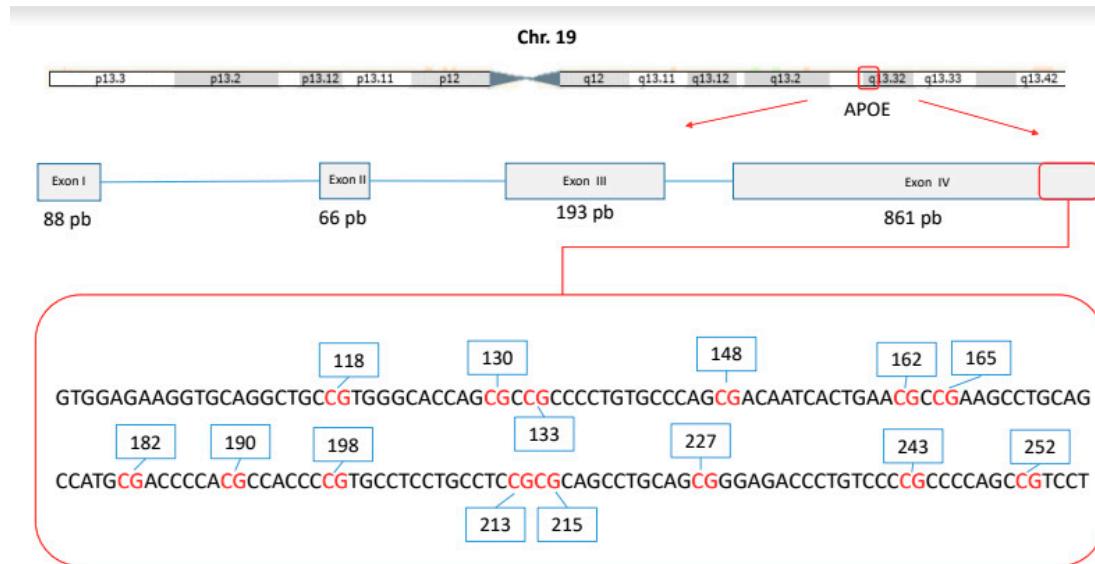
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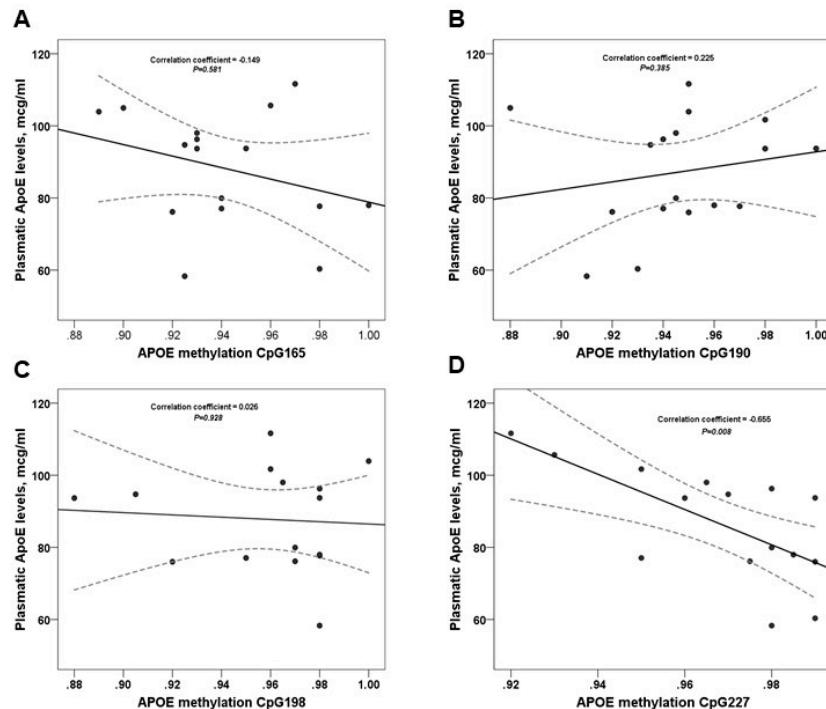
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291 **Figure 1. Schematic map of the human APOE gene.** The APOE gene contains four exons, in the final  
 292 region of the Exon IV is a region of new 15 CpG. For CpGs 118, 130, 133, 148, 162, 165, 182, 190,  
 293 198, 213, 215, 227, 243 and 252, the genomic positions were chr19: 44,909 plus 208, 220, 223, 238, 252, 254,  
 294 272, 280, 303, 305, 317, 333, and 342, respectively.



295

296 **Figure 2. Correlation between Plasmatic ApoE levels and APOE Methylation CpGs.** The  
 297 correlation coefficient and p values for figures A, B and C were calculated using Pearson's test  
 298 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	MCI	Control	P value*
	(n = 100)	(n = 41)	(n = 59)	
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	MCI	Control	P value*
		(n = 100)	(n = 41)	(n = 59)	
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

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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307

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

310

Variables	Non-APOE- <b>ε4</b> Carriers	APOE- <b>ε4</b> Carriers	<i>P</i> value*
	(n = 70)	(n = 25)	
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

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.

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

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Variables	Risk for Mild Cognitive Impairment		
	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

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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