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
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; in
98 addition, we explore the relationship between APOE genotype, the DNA methylation of the
99 APOE gene and the risk of MCI.

100 Methods

101 Study design and population sample

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

113 Mild cognitive impairment

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

120 DNA Extraction and Bisulfite Treatment

121 Genomic DNA was isolated from blood from patients and controls using the kit ReliaPrep Blood
122 gDNA Miniprep System™(A5082-PROMEGA) following the protocol recommended by the
123 company. DNA was Quantified in a spectrophotometer NanoDrop2000c ThermoScientific and
124 afterward saved at 4°C. Subsequently, the isolated DNA was bisulfite-converted using the EZ DN
125 Methylation-Direct Kit (D5021– ZymmoResearch). We then evaluated the methylation status of the
Apolipoprotein (ApoE) was genotyped using a human Apo E (AD2) ELISA kit (Thermofisher-Invitrogen). The presence of ApoE alleles was determined using ELISA technique.

**Statistical Analysis**

The continuous variables were shown as mean and standard deviation (±) meanwhile categorical ones were summarized as frequencies and percentages (%). The data was analyzed using the U-Mann 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 methylation 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.

**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
(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
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,
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

Depending on 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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Regional Committee (LARC) short research training grant.
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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.

**Funding**

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**Authors’ contributions**

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, 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.
Table 1. Baseline Characteristics of the Selected Sample and Divided by Individuals with Mild Cognitive Impairment

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

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.

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

<table>
<thead>
<tr>
<th>Variables</th>
<th>Genomic position</th>
<th>Whole sample (n = 100)</th>
<th>MCI (n = 41)</th>
<th>Control (n = 59)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoE plasmatic Levels, mcg/ml</td>
<td>-</td>
<td>103.2±26.5</td>
<td>113.8±26.4</td>
<td>86.0±15.7</td>
<td>&lt;0.0001†</td>
</tr>
<tr>
<td>Global methylation</td>
<td>-</td>
<td>91.9±3.0</td>
<td>92.8±2.6</td>
<td>91.6±3.1</td>
<td>0.154†</td>
</tr>
<tr>
<td>Methylation by CpG Islands</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CpG118</td>
<td>chr19:44,909,208</td>
<td>85.6±5.1</td>
<td>89.6±4.1</td>
<td>84.7±4.9</td>
<td>0.009†</td>
</tr>
<tr>
<td>CpG130</td>
<td>chr19:44,909,220</td>
<td>88.9±7.1</td>
<td>89.0±3.9</td>
<td>88.9±7.6</td>
<td>0.484</td>
</tr>
<tr>
<td>CpG133</td>
<td>chr19:44,909,223</td>
<td>87.9±10.1</td>
<td>85.7±13.3</td>
<td>88.4±9.3</td>
<td>0.620</td>
</tr>
</tbody>
</table>
Table 3. ApoE Plasmatic Levels and APOE Methylation of CpGs Islands according to APOE Genotype

| CpG     | 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.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Non-APOE-ε4 Carriers (n=70)</th>
<th>APOE-ε4 Carriers (n=25)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoE plasmatic Levels, mcg/ml</td>
<td>106.0±31.3</td>
<td>103.0±26.2</td>
<td>0.738†</td>
</tr>
<tr>
<td>Global methylation</td>
<td>92.2±2.2</td>
<td>91.6±3.3</td>
<td>0.557†</td>
</tr>
</tbody>
</table>

Methylation by CpG Islands
<table>
<thead>
<tr>
<th>Variable</th>
<th>Odds ratios</th>
<th>95% Confident Interval</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmatic ApoE levels</td>
<td>1.07</td>
<td>1.02-1.13</td>
<td>0.003</td>
</tr>
<tr>
<td>APOE methylation</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>cpG118</td>
<td>1.25</td>
<td>0.96-1.62</td>
<td>0.092</td>
</tr>
</tbody>
</table>

*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.
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.

References


