A Common Variant in the MC1R Gene (p.V92M) is associated with Alzheimer’s Disease Risk

Gemma Tell-Martí, Joan Anton Puig-Butillle, Miriam Potrony, Estel Plana, Celia Badenas, Anna Antonell, Raquel Sanchez-Valle, José L. Molinuevo, Alberto Lleó, Daniel Alcolea, Juan Forteá, Rubén Fernández-Santiago, Jordi Clarimón, Albert Lladó, and Susana Puig

Dermatology Department, Melanoma Unit, Hospital Clinic & IDIBAPS (Institut d’Investigacions Biomèdiques August Pi i Sunyer), Barcelona, Spain
Centro Investigación Biomédica en Enfermedades Raras (CIBERER), ISCIII, Barcelona, Spain
Biochemical and Molecular Genetics Service, Hospital Clinic & IDIBAPS (Institut d’Investigacions Biomèdiques August Pi i Sunyer), Barcelona, Spain
RTI Health Solutions, Travesera de Gracia 56 Atic 1era, Barcelona, Spain
Alzheimer’s Disease and Other Cognitive Disorders Unit, Neurology Service, Hospital Clínic, Institut d’Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain
Memory Unit, Neurology Department, Hospital de Sant Pau (Sant Pau Biomedical Research Institute), Universitat Autònoma de Barcelona, Barcelona, Spain
CIBERNED, Center of Networker Biomedical Research into Neurodegenerative Diseases, Madrid, Spain
Laboratory of Neurodegenerative Disorders, Institut d’Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS)-Hospital Clínic de Barcelona-Centro de Investigación sobre Enfermedades Neurodegenerativas (CIBERNED), ISCIII, Barcelona, Spain
Medicine Department, Universitat de Barcelona, Barcelona, Spain

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Abstract. Despite the recent identification of some novel risk genes for Alzheimer’s disease (AD), the genetic etiology of late-onset Alzheimer’s disease (LOAD) remains largely unknown. The inclusion of these novel risk genes to the risk attributable to the APOE gene accounts for roughly half of the total genetic variance in LOAD. The evidence indicates that undiscovered genetic factors may contribute to AD susceptibility. In the present study, we sequenced the MC1R gene in 525 Spanish LOAD patients and in 160 controls. We observed that a common MC1R variant p.V92M (rs2228479), not related to pigmentation traits, was present in 72 (14%) patients and 15 (9%) controls and confers increased risk of developing LOAD (OR: 1.99, 95% CI: 1.08–3.64, p = 0.026), especially in those patients whose genetic risk could not be explained by APOE genotype. This association remains and even increased in the subset of 69 patients with typical AD cerebrospinal fluid profile (OR: 3.40 95% CI: 1.40–8.27, p = 0.007). We did not find an association between p.V92M and age of onset of AD. Further studies are necessary to elucidate the role of MC1R in brain cells through the different MC1R pathways.

Keywords: Cerebrospinal fluid biomarkers, common variant, late-onset Alzheimer’s disease, melanocortin 1 receptor (MC1R) gene, p.V92M, risk

Correspondence to: Susana Puig, MD, PhD, Chief Dermatology Service, Melanoma Unit, Dermatology Department, Hospital Clinic Barcelona, Villarroel 170. 08036, Barcelona, Spain. Tel.: +34 93 2275400/Ext. 2422; Fax: +34 93 2275438; E-mail: susipuig@gmail.com.
INTRODUCTION

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder characterized clinically by memory and cognitive dysfunction and represents the most common form of dementia in the elderly [1]. The prevalence of the disease increases after the age of 65 years and the disease onset is usually after the age of 70 years [2]. However, familial AD (FAD) patients carry autosomal dominant mutations in high-risk Alzheimer susceptibility genes (APP, PSEN1, and PSEN2) and present an early age of onset (<65 years). These genes do not play an important role in either Sporadic AD cases or late onset cases (>65 years) indicating that other genomic variants may be involved in the common forms of the disease [3]. To date, the ε4 allele polymorphism in the Apolipoprotein E (APOE) gene has been well established as a risk factor for developing late-onset Alzheimer’s disease (LOAD) [3]. The disease-attributable risk in LOAD patients related to the ε4 allele in APOE is less than 50% [4]. Previous genome-wide association studies (GWAS) have identified low-risk variants associated with LOAD [5–10], which account for a small proportion of risk. The inclusion of these novel risk genes to the risk attributable to the APOE gene accounts for roughly half of the total genetic variance [11], indicating that additional undiscovered genetic factors may contribute to AD susceptibility.

The main pathological hallmarks of AD are extracellular amyloid plaques, intracellular neurofibrillary tangles, and loss of neurons and synapses, resulting in brain atrophy [12]. Moreover, an elevated level of oxidative damage products has been observed in areas of degeneration in AD brains, suggesting that oxidative stress and consequent protein oxidation may be potential mechanisms of neuronal death in AD [13]. The accumulation of intracellular damage determined by reactive oxygen species might produce the progressive loss of control over biological homeostasis and the functional impairment typical of damaged brain cells in AD [14].

The Melanocortin 1 receptor (MC1R) gene encodes for a G protein-coupled seven transmembrane receptor for melanocortin peptides (α-MSH, ACTH) and mediates its effects mainly by activating a cAMP-dependent signaling pathway [15]. MC1R expression is observed in several types of neuronal cells suggesting that it may be a key regulator in brain cell functions and survival [16]. The MC1R activation has anti-inflammatory and immunomodulatory effects in brain cells [16] and promotes pigmentation synthesis in melanocytes [17]. It has been established that several MC1R polymorphisms constitute a risk factor to develop skin cancer (melanoma and non-melanoma skin cancer) [18, 19], in part, by promoting an increased oxidative stress in skin cells [20]. Notably, co-occurrence of Parkinson’s disease (PD) and cutaneous melanoma (CM) has been reported in epidemiological studies [21] and previous evidence indicates that MC1R is involved in the bidirectional link between both diseases [22, 23]. Thus, we hypothesized that certain MC1R variants may increase the oxidative damage and/or deregulate inflammatory processes in brain cells, which consequently, increase the susceptibility of developing other neurodegenerative disorders beyond PD. In the present study, we analyzed the role of the MC1R gene as a putative genetic risk factor in LOAD patients, and we observed that a common MC1R variant, not related to pigmentation traits, confers risk of developing LOAD in a Spanish population.

MATERIAL AND METHODS

We performed a case-control study of 525 unrelated LOAD patients (Mean age ± SD, 76.35 ± 5.61 years (the age of onset was after 65 years in all patients studied; male 29.7%/female 70.3%) and 160 controls (Mean age ± SD, 73.81 ± 5.87 years; male 36.3%/female 63.7%). All patients studied were recruited from two hospital-based series from the same geographical area: the Alzheimer’s Disease and Other Cognitive Disorders Unit at Clinic Hospital of Barcelona and from the Memory Unit at Sant Pau Hospital of Barcelona. All AD patients were diagnosed using the NINCDS-ADRDA criteria [24]. Furthermore, 69/525 had a CSF biomarker profile typical of AD (Aβ42/p-tau ratio <6.43) [25]. The control group included healthy individuals without signs of neurodegenerative or psychiatric disorders obtained from three independent control series from Spain: Hospital Clinic of Barcelona (N = 13), Sant Pau Hospital of Barcelona (N = 85), and the Spanish National Bank of DNA (N = 62) (Table 1).

All individuals included in the study gave their written informed consent according to the Declaration of Helsinki. The Ethical Committee of Clinical Investigation at the Hospital Clinic of Barcelona approved the study.

APOE genotype analysis

DNA was isolated from blood samples using the Wizard® Genomic DNA Purification Kit (Promega,
Fitchburg, Wisconsin, USA). APOE genotype was determined through the analysis of rs429358 and rs7412 using TaqMan (Applied Biosystems) genotyping technologies.

CSF biomarkers determination

69 subjects underwent a spinal tap during the morning. The samples were centrifuged and stored in polypropylene tubes at −80°C within 2 h. Levels of Aβ42, t-tau, and p-tau were measured by experienced laboratory personnel using commercial sandwich ELISA kits (Innogenetics, Ghent, Belgium) [26]. We are participants of the QC program, and Aβ42, t-tau, and p-tau levels obtained in our lab for the Alzheimer’s Association QC samples were within mean ± 2 SD.

MC1R molecular screening

The MC1R gene, which consists of one single exon encoding a 317 amino-acid protein (ENST00000555147), was sequenced using 50–100 ng of total DNA per sample. PCR amplification was carried out as previously described [27] using an initial denaturing step at 95°C 5 min, followed by 35 PCR cycles (94°C 1 min, 55°C 1 min, 72°C 3 min), and a final extension at 72°C 10 min. PCR products were purified using Multiscreen Filter plates (Millipore). We sequenced the entire coding region of MC1R (a 1,107 bp fragment) using the following internal primers (TM-F: 5’AACCTGCACTCACCATGTA3’ and TM-R: 5’TTTAAGGCCAAAGCCCTGT3’) and the BigDye Terminator v3.1 Cycle Sequencing kit, according to manufacturer’s instructions (Applied Biosystems, Foster City, CA). Sequences were run on an ABI3100 automatic sequencer (Applied Biosystems) and analyzed using the SeqPilot 4.0.1 software (JSI Medical Systems). The entire coding region was sequenced in 110 LOAD patients and in all controls. In 415 LOAD patients, the MC1R gene was exclusively sequenced using TM-R primer. This strategy allows us to detect all MC1R variants, except for the presence of p.D294H and p.T314T variants.

Statistical analysis

We focused the analysis on the non-synonymous MC1R variants with an observed minor allele frequency (MAF) in at least 1% of cases. Synonymous variants were considered as wild-type MC1R alleles. Public databases such as dbSNP (http://www.ncbi.nlm.nih.gov/), MelGene DB (http://www.melgene.org/), and Ensembl genome browser (http://www.ensembl.org/) were used to determine whether the detected non-synonymous variants have been previously described. In-silico analysis of each rare non-synonymous variant was carried out using software Polyphen2 (http://genetics.bwh.harvard.edu/pph2/) [28].

The genotypic association analysis was performed using multiple logistic regression models (co-dominant, dominant, recessive, over-dominant, and log-additive) in the whole set of patients (N = 525) and in the subset of patients with CSF biomarkers data (N = 69). The selection of the most suitable model of inheritance was performed based on both Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC). All reported odds ratios (ORs), 95% confidence intervals (CI), and p-values were adjusted for age, gender, and APOE genotype.

The goodness-of-fit of our logistic regression model was evaluated by the Hosmer-Lemeshow test. In the genotypic association analysis with the whole set of samples (525 patients and 160 controls), we obtained the best fit of our model by categorical transformation of the age. We converted the numerical
<table>
<thead>
<tr>
<th>MC1R variants</th>
<th>Subjects</th>
<th>LOAD (N = 525)</th>
<th>Controls (N = 160)</th>
<th>Polyphen</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs146544450</td>
<td>0.2151/0.0969/0.1761</td>
<td>p.Q233Q (c.699G&gt;A)</td>
<td>Het 4 (0.7)</td>
<td>–</td>
</tr>
<tr>
<td>rs181269865</td>
<td>0.1064/0.0712/0.0947</td>
<td>p.I264I (c.792C&gt;T)</td>
<td>Het 2 (0.4)</td>
<td>–</td>
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<tr>
<td>UN</td>
<td>UN</td>
<td>p.L309L (c.927G&gt;A)</td>
<td>Het 1 (0.2)</td>
<td>–</td>
</tr>
<tr>
<td>rs2228478</td>
<td>10.79%/60.86%/20.81%</td>
<td>p.T314T (c.942G&gt;A)</td>
<td>Het 21 (19)</td>
<td>24 (15)</td>
</tr>
<tr>
<td>Non-synonymous</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs1805005</td>
<td>13.34%/2.26%/9.60%</td>
<td>p.V60L (c.178T&gt;G)</td>
<td>Het 137 (26)</td>
<td>40 (25)</td>
</tr>
<tr>
<td>rs372590533</td>
<td>0.0116/0.0/0.0078</td>
<td>p.R67Q (c.200G&gt;A)</td>
<td>Het – 3 (2)</td>
<td>–</td>
</tr>
<tr>
<td>rs221192930</td>
<td>0.0116/0.0228/0.0154</td>
<td>p.V122M (c.366G&gt;A)</td>
<td>Het 1 (0.2)</td>
<td>–</td>
</tr>
<tr>
<td>rs374235260</td>
<td>0.0116/0.0/0.0077</td>
<td>p.M128T (c.385G&gt;C)</td>
<td>Het – 1 (0.6)</td>
<td>–</td>
</tr>
<tr>
<td>rs11547464</td>
<td>0.0102/0.0182/0.0725</td>
<td>p.V92M (c.274G&gt;A)</td>
<td>Het 72 (14)</td>
<td>15 (9)</td>
</tr>
<tr>
<td>rs1805007</td>
<td>7.61%/1.70%/5.62%</td>
<td>p.R151C (c.451C&gt;T)</td>
<td>Het 26 (5)</td>
<td>9 (6)</td>
</tr>
<tr>
<td>rs201326893</td>
<td>0.0349/0.0/0.0231</td>
<td>p.Y298H (c.892T&gt;C)</td>
<td>Het – 2 (1.2)</td>
<td>1.000</td>
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<tr>
<td>rs1110400</td>
<td>1.04%68/0.29%/0.79%</td>
<td>p.V174I (c.520G&gt;A)</td>
<td>Het – 1 (0.6)</td>
<td>0.002</td>
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<tr>
<td>rs1805008</td>
<td>7.74%7/1.34%5/2.56%</td>
<td>p.R160W (c.478C&gt;T)</td>
<td>Het 18 (3)</td>
<td>4 (2)</td>
</tr>
<tr>
<td>rs885479</td>
<td>4.82%7/1.52%6/3.71%</td>
<td>p.R163Q (c.488G&gt;A)</td>
<td>Het 24 (5)</td>
<td>3 (2)</td>
</tr>
<tr>
<td>rs200000734</td>
<td>0.0594/0.0239/0.0476</td>
<td>p.R121W (c.367C&gt;T)</td>
<td>Het 1 (0.2)</td>
<td>–</td>
</tr>
<tr>
<td>rs1805009</td>
<td>2.07%6/0.68%5/1.61%</td>
<td>p.D294H (c.880G&gt;C)</td>
<td>Het 5 (4)</td>
<td>6 (4)</td>
</tr>
<tr>
<td>UN</td>
<td>UN</td>
<td>p.V298H (c.892T&gt;C)</td>
<td>Het – 2 (1.2)</td>
<td>1.000</td>
</tr>
</tbody>
</table>

*The minor-allele frequency in percent listed in the order of European American (EA), African American (AA) and all populations (All) (delimited by /). Variants in bold have not been reported before. UN = Unknown. Het: variant in heterozygosis. Homo: variant in homozygosis. MC1R common variants. Score Polyphen2: predicts possible impact of an amino acid substitution on the structure and function of a human protein (scores close to 0.0, indicate a benign mutation; score close to 1.0, damaging mutation) a MC1R variants evaluated in 110 LOAD patients and 160 controls.

variable (age) into a categorical variable, dividing the age into different categories. In this analysis, the logistic regression model was adjusted for age (as categorical variable), gender, and APOE genotype. Otherwise, in the genotypic association analysis with the subset of patients with typical AD CSF biomarkers (69 patients and 160 controls), we obtained the best fit of our model, including the age as a numerical variable. In this second analysis, the logistic regression model was adjusted for age (as numerical variable), gender, and APOE genotype.

We evaluated whether MC1R and APOE genotypes modify age of onset (AOO) among the Spanish LOAD group using the Student’s t-test. We evaluated whether interaction between MC1R variants and APOE alleles exists using the two-tail χ² test.

*p-values less than 0.05 were considered statistically significant. All tests were two sided, and Bonferroni correction for multiple comparisons was applied to all p-values <0.05. We performed all the analyses using STATA v.11 software.

RESULTS

MC1R genotyping was carried out in 525 LOAD patients and in 160 control subjects. Non-significant difference in the gender distribution was detected between patients and controls (p = 0.118). The study identified 22 MC1R variants (4 synonymous and 18 non-synonymous), four rare variants (p.V156E, p.V174E, p.Y298H, and p.L309L) had not been previously identified (Table 2). Five recurrent non-synonymous variants showed a frequency ≥1% in LOAD patients: p.V60L (16%), p.V92M (7%), p.R151C (2%), p.R160W (2%), and p.R163Q (2%). All variants were in Hardy-Weinberg equilibrium within both the control population and LOAD
patients (allelic and genotypic frequencies are listed in Table 3). Moreover, frequencies of \textit{MC1R} variants observed in control subjects were compared with those observed in other set of controls (N = 736) free from neurodegenerative disorders used in other study [29]. No statistical significant differences were observed between both groups.

The \textit{APOE} genotype was obtained for all cases and controls. The frequency of heterozygous \textit{APOE} e4 carriers was 41.5\% (218/525) in LOAD patients and 20\% (32/160) in controls. The \textit{APOE} e4/e4 genotype was only detected in 4.6\% (24/525) of LOAD patients. The carriers of at least one \textit{APOE} e4 allele had a higher than three-fold increased risk of developing LOAD (OR: 3.47, 95\% CI: 2.24–5.39, \(p < 0.0001\)).

We evaluated the 5 most common \textit{MC1R} variants detected in the study with the risk of developing LOAD (Table 3). We detected that variant p.V92M was enriched in LOAD patients compared to controls, being associated with an increased risk of developing LOAD under the log-additive genetic model (OR: 1.99, 95\% CI: 1.08–3.64, \(p = 0.026\)), after Bonferroni correction this association did not reach statistical significance (Bonferroni corrected \(p = 0.13\)).

We re-evaluated the association between \textit{MC1R} variant p.V92M and LOAD risk within the subset of 69 patients with typical AD CSF profile (Table 4) and we found it was statistically significant and we evidenced an even higher OR (OR: 3.40, 95\% CI: 1.40–8.27, \(p = 0.007\)). We did not detect statistically significant differences in the different CSF biomarkers levels between p.V92M carriers and non-carriers (data not shown).

In order to identify whether the p.V92M association with LOAD risk was modulated by the presence of the \textit{APOE} e4 allele, we evaluated the interaction between both alleles within the whole set of LOAD patients (Table 5). The frequency of variant p.V92M in the \textit{MC1R} gene was significantly lower (\(p = 0.041\)) in carriers of at least one \textit{APOE} e4 allele compared to non-carriers (35.1\% and 64.9\%, respectively). This result suggests an inverse correlation between both alleles.

Finally, we observed a significantly lower AOO associated with \textit{APOE} e4 allele (\(p < 0.0001\)), in contrast, no significant effect on AOO was observed for \textit{MC1R} variant p.V92M (Table 6).

### DISCUSSION

LOAD form accounts for more than 90\% of AD cases [30]. To date, the \textit{APOE} gene is the major genetic factor in LOAD susceptibility [31], while other genetic factors related with LOAD susceptibility remain largely unknown. Thus, identification of novel genetic factors may be crucial to detect individuals with an inherited AD risk.

In this study, we report a novel association between the p.V92M variant in the \textit{MC1R} gene and the risk of developing LOAD. After Bonferroni correction, the association detected in the overall set of patients did not reach statistical significance. However, the
association in the subset of biochemically confirmed AD patients was even stronger than in the overall set of patients, suggesting that the MC1R variant plays a role in the etiology of AD. This gene is highly polymorphic, and p.V92M and other variants are common in the Caucasian population. During the last few years, several GWAS have been conducted to identify common LOAD risk variants [5–10], and none of these studies reported an association between MC1R and the disease. This could be caused by methodological issues (coverage level of MC1R in SNP-array platforms or conservative statistical correction procedures) or by the molecular or clinical heterogeneity of patients included. Interestingly, we observed an inverse tendency between the APOE genotype and p.V92M suggesting that the presence of the MC1R variant could contribute to AD susceptibility, especially in those patients whose genetic risk could be not attributable to the APOE genotype. Further studies restricted to MC1R variants and APOE alleles should be conducted using previous GWAS data to elucidate such an inverse correlation.

Clinicopathological studies have shown that the sensitivity and specificity of the classical clinical criteria for AD diagnosis are roughly 85% and 70%, respectively [32]. CSF studies measuring Ab42 and tau protein levels in AD patients with confirmed pathology have demonstrated that abnormal levels of both biomarkers constitute a specific signature of the underlying AD-pathology (senile plaques and neurofibrillary tangles, respectively). Furthermore, multiple studies have shown that the sensitivity and specificity with the inclusion of specific CSF biomarkers profiles are roughly 90% and 85%, respectively [33, 34]. In accordance with this evidence, a recent study has found after inclusion of CSF results, 90% of amnestic and 82% of the non-amnestic AD presentation could be categorized as “high probability of AD etiology”, while 3% of AD patients fit into the category “dementia probably not due to AD” [35]. Thus, we included a subset of patients with typical CSF AD biomarkers, demonstrating evidence of AD pathophysiological process and increased probability of AD etiology as a cause of symptomatology of the patient according to NIA-AA criteria [36]. This fact is relevant, because different studies have demonstrated neuropathological changes that sometimes do not correlate with clinical diagnosis [37]. Therefore, the role of the MC1R variant p.V92M in the AD risk was re-analyzed in these well-characterized AD patients. Notably, in spite of the sample size reduction, the association remains statistically significant and the LOAD risk in p.V92M carriers increased more than three fold. Thereby, the risk of p.V92M is more evident within more accurately diagnosed AD patients.

In this study, decreasing age of onset was restricted to the APOE e4 allele. Thus, the p.V92M variant should be considered like those prior variants which increase the risk of developing AD but do not modulate AOO [38].

The MC1R gene encodes a membrane receptor, which is expressed, in neurons of the periaqueductal gray matter, astrocytes, and Schwann cells activated by melanocortin peptides [16, 39]. This receptor may have an important role in the anti-inflammatory brain response [16] and in female specific mediation mechanisms of analgesia [40]. MC1R is also expressed in melanocytes, a cell type with a common embryonic origin with brain cells [41], which determine hair and skin color [42], and certain variants increase the risk for skin cancer (melanoma and non-melanoma skin cancer) [18, 19]. Functional studies of MC1R variants conducted in melanocytes, reveal that certain variants reduce cell surface protein expression and diminished capacity to stimulate cAMP, resulting in the red hair color phenotype [17]. Interestingly, two MC1R variants related to red hair color phenotype modulate the risk to develop PD [22, 29]. These findings partially explain the previous epidemiological evidence

### Table 5

<table>
<thead>
<tr>
<th>APOE genotype</th>
<th>0</th>
<th>p.V92M</th>
<th>≥1</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>APOE e4 (–)</td>
<td>235</td>
<td>52.1</td>
<td>48</td>
<td>64.9</td>
</tr>
<tr>
<td>APOE e4 (+)</td>
<td>216</td>
<td>47.9</td>
<td>26</td>
<td>35.1</td>
</tr>
</tbody>
</table>


### Table 6

<table>
<thead>
<tr>
<th>APOE genotype</th>
<th>Age of onset</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>^APOE genotype</td>
<td>N</td>
<td>Mean ± SD*</td>
</tr>
<tr>
<td>APOE e4 –</td>
<td>283</td>
<td>77.56 ± 5.33</td>
</tr>
<tr>
<td>APOE e4 +</td>
<td>242</td>
<td>75.17 ± 5.70</td>
</tr>
<tr>
<td>MC1R genotype</td>
<td>p.V92M controls</td>
<td>451</td>
</tr>
<tr>
<td>p.V92M carriers</td>
<td>(+)</td>
<td>74</td>
</tr>
</tbody>
</table>

describing a bidirectional link between PD and CM [21]. In contrast to PD, an increased incidence of CM among AD patients and overrepresentation of individuals with natural red hair within AD patients compared to control population has not been reported. This can be explained as the p.V92M variant does not confer a risk to develop CM [18] and by the fact that variant p.V92M promotes a decrease in the affinity of the receptor for its ligand α-MSH, but showed normal cell surface expression and normal capacity to stimulate cAMP, consequently it does not impact on the phenotype [17, 39]. Notably, a functional deficiency of α-MSH in the brain cells of LOAD patients had been previously reported, suggesting that α-MSH may be critical in the development of LOAD [43].

To date, functional evaluation of MC1R variants in other cell types such as the nervous system cells is limited. However, there is evidence that certain variants may also impact physiological conditions beyond skin and hair pigmentation, such as risk of depression disorders [44], pain response [40], and anesthetic requirement [45]. Interestingly, a case-control study indicates that variant p.V92M is associated with the response of desipramine treatment in depression disorder [44].

Our study, although exploratory, has some limitations. The major one being sample size, which is not large enough to provide reliable evidence for a genetic AD risk factor, especially if we focus on the controls size (N = 160) which is small. However, our work provides positive results from our hypothesis and highlights a putative role of the MC1R gene in the genetic susceptibility to developing neurodegenerative diseases, which is in the same line as previously published works [22, 23].

Another minor limitation is the exclusion of the p.D294H MC1R variant from the genetic association analysis. As we explained in the Material and Methods section, the entire coding region was sequenced in 110 LOAD patients, but the other 415 LOAD patients were exclusively sequenced using TM-R primer. This strategy allowed us to detect all MC1R variants except one common non-synonymous variant (p.D294H). However, we compared the frequency of the p.D294H variant observed in 110 LOAD patients (minor allele frequency was 3%) with the frequency observed in 160 controls (minor allele frequency was 2%), and we did not detect any statistically significant difference.

In conclusion, the present study suggests that MC1R variant p.V92M may increase the risk to develop LOAD. Although, the molecular mechanisms underlying the increased risk of LOAD associated with p.V92M variant are not known, this variant may have biological relevance through non-pigmentation pathways involved in inflammatory or immunomodulatory processes.

Larger genetic studies are necessary to confirm the association of p.V92M with AD. Additionally, further functional studies should be carried out to elucidate the role of the MC1R receptor in brain cells.

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