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ANALYSIS OF GWAS-IDENTIFIED VARIANTS ASSOCIATED WITH ALZHEIMER'S DISEASE RISK IN CASS4, TREM2, CD2AP, AND MS4A4E GENES

ANÁLISE DE VARIANTES IDENTIFICADAS POR GWAS ASSOCIADAS AO RISCO DE DOENÇA DE ALZHEIMER NOS GENES *CASS4*, *TREM2*, *CD2AP* E *MS4A4E*

ANÁLISIS DE VARIANTES IDENTIFICADAS EN GWAS ASOCIADAS AL RIESGO DE LA ENFERMEDAD DE ALZHEIMER EN LOS GENES CASS4, TREM2, CD2AP Y MS4A4E

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ABSTRACT

Understanding genetic and biochemical aspects of complex diseases like Alzheimer's disease (AD) is crucial for developing new treatments. Genome-wide association studies (GWAS) identify genetic risk factors, but validating these variants is necessary to confirm their role in disease susceptibility. This observational analytical case-control genetic association study included 221 unrelated participants, comprising 82 patients with late-onset Alzheimer's disease (LOAD) and 139 older adults without dementia. Four GWAS-identified variants associated with LOAD were analyzed: rs911159 in CASS4, rs75932628 in TREM2, rs9349407 in CD2AP, and rs670139 in MS4A4E, using real-time PCR and PCR-RFLP. Logistic regression showed an association between rs911159 in CASS4 and LOAD (OR = 0.187; 95% CI 0.059–0.59; p = 0.005), suggesting a protective effect in our sample. However, no association was found for rs9349407 in CD2AP and rs670139 in MS4A4E. The rs75932628-T variant was not detected in our sample. These findings contribute to the understanding of the genetic basis of LOAD in an admixed Brazilian population. However, these results should be interpreted with caution due to the limited sample size, and further studies with larger cohorts are needed to confirm these associations.

KEYWORDS: *Late-onset Alzheimer's disease. GWAS-validation. Association study. CASS4 gene. Genetic risk factors*

RESUMO

Compreender os aspectos genéticos e bioquímicos de doenças complexas, como a doença de Alzheimer (DA), é fundamental para o desenvolvimento de novos tratamentos. Estudos de associação genômica ampla (GWAS) identificam fatores genéticos de risco, mas a validação dessas variantes é necessária para confirmar seu papel na suscetibilidade à doença. Este estudo observacional analítico de associação genética do tipo caso-controle incluiu 221 participantes não aparentados, sendo 82 pacientes com doença de Alzheimer de início tardio (DAIT) e 139 idosos sem demência. Foram analisadas quatro variantes identificadas por GWAS associadas à DAIT: rs911159 em *CASS4*, rs75932628 em *TREM2*, rs9349407 em *CD2AP* e rs670139 em *MS4A4E*, utilizando PCR em tempo real e PCR-RFLP.

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A regressão logística demonstrou associação entre rs911159 em *CASS4* e DAIT (OR = 0,187; IC 95% 0,059–0,59; p = 0,005), sugerindo efeito protetor em nossa amostra. Entretanto, não foi observada associação para rs9349407 em *CD2AP* e rs670139 em *MS4A4E*. A variante rs75932628-T não foi detectada em nossa amostra. Esses achados contribuem para a compreensão da base genética da DAIT em uma população brasileira miscigenada. Contudo, os resultados devem ser interpretados com cautela devido ao tamanho amostral limitado, e estudos futuros com coortes maiores são necessários para confirmar essas associações.

PALAVRAS-CHAVE: Doença de Alzheimer de início tardio. Validação de GWAS. Estudo de Associação. Gene *CASS4*. Fatores de risco genéticos.

RESUMEN
Comprender los aspectos genéticos y bioquímicos de enfermedades complejas, como la enfermedad de Alzheimer (EA), es fundamental para el desarrollo de nuevos tratamientos. Los estudios de asociación del genoma completo (GWAS) identifican factores genéticos de riesgo, pero la validación de estas variantes es necesaria para confirmar su papel en la susceptibilidad a la enfermedad. Este estudio observacional analítico de asociación genética de tipo caso-control incluyó a 221 participantes no emparentados, compuestos por 82 pacientes con enfermedad de Alzheimer de inicio tardío (EAIT) y 139 adultos mayores sin demencia. Se analizaron cuatro variantes identificadas por GWAS asociadas con la EAIT: rs911159 en CASS4, rs75932628 en TREM2, rs9349407 en CD2AP y rs670139 en MS4A4E, utilizando PCR en tiempo real y PCR-RFLP. La regresión logística mostró una asociación entre rs911159 en CASS4 y la EAIT (OR = 0,187; IC del 95%: 0,059–0,59; p = 0,005), lo que sugiere un efecto protector en nuestra muestra. Sin embargo, no se observó asociación para rs9349407 en CD2AP ni para rs670139 en MS4A4E. La variante rs75932628-T no fue detectada en nuestra muestra. Estos hallazgos contribuyen a la comprensión de la base genética de la EAIT en una población brasileña mestiza. No obstante, los resultados deben interpretarse con cautela debido al tamaño muestral limitado, y se necesitan estudios futuros con cohortes más grandes para confirmar estas asociaciones.

PALABRAS CLAVE: *Enfermedad de Alzheimer de início tardio. Validación de GWAS. Estudio de Asociación. Gen CASS4. Factores de riesgo genéticos.*

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder and the most common type of dementia in older people, affecting an estimated 55 million people worldwide, with nearly 10 million new cases diagnosed each year (Teixeira *et al.* 2024). Pathological changes occur within the brains of individuals with AD. Neurofibrillary tangles are formed inside neurons due to the hyperphosphorylation of tau protein, whereas amyloid plaques, formed by Aβ42 peptides generated through abnormal processing of amyloid precursor protein (APP), accumulate outside neurons (Rostagno, 2023). Both changes are the main features of AD and support the amyloid cascade hypothesis (Uddin *et al.* 2020; Gin *et al.* 2024).

Most AD cases are late-onset AD (LOAD), which has a multifactorial etiology and occurs after 65 years of age (Yu *et al.* 2014; Valdez-Gaxiola *et al.* 2024). The ε4 allele in the *Apolipoprotein E* (*APOE*) gene is considered a risk factor for LOAD worldwide (Lambert and Amouyel 2011; Ismail

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et al. 2024). Genome-wide association studies (GWAS) have identified several genes associated with LOAD, such as *Cas scaffold protein family member 4* (*CASS4*)*, Triggering receptor expressed on myeloid cells 2* (*TREM2), CD2-associated protein* (*CD2AP*), and *Membrane-spanning 4-domain subfamily A4E* (*MS4A4E*) (Hollingworth *et al.* 2011; Hou *et al.* 2021; Gunter *et al.* 2024; Wang *et al.* 2024). These genes are involved in cellular processes, such as cytoskeletal function, axonal transport, and inflammatory, endocytic, and immunological pathways (Hu *et al.* 2013; Lambert *et al.* 2013; Karch and Goate 2015; Ubelmann *et al.* 2017).

Because AD is a complex disorder with an important genetic component, replication studies are essential to determine whether GWAS-identified variants are consistently associated with disease susceptibility across different populations. The polymorphisms investigated in this study were selected based on two criteria. First, previous GWAS and genetic association studies identified these loci as potentially associated with LOAD. Second, their corresponding genes participate in biological pathways relevant to AD pathogenesis, including cytoskeletal organization, axonal transport, endocytosis, immune response, microglial function, and inflammatory mechanisms. However, evidence regarding these variants remains limited in admixed Brazilian populations, which may present distinct allele frequencies and genetic risk profiles.

Therefore, this study tested the hypothesis that GWAS-identified variants in *CASS4*, *TREM2*, *CD2AP*, and *MS4A4E* are associated with susceptibility to LOAD in an admixed Brazilian sample. The general objective was to evaluate the association of rs911159 in *CASS4*, rs75932628 in *TREM2*, rs9349407 in *CD2AP*, and rs670139 in *MS4A4E* with LOAD. Specifically, the study aimed to analyze the distribution of these polymorphisms in patients with AD and controls, investigate their association with disease susceptibility, and contribute to the understanding of the molecular pathophysiology of AD in the Brazilian population.

MATERIALS AND METHODS

Sample

This was an observational analytical case-control genetic association study conducted in 2022. Laboratory procedures were performed at the Human and Molecular Genetics Center of the Federal University of Espírito Santo, Brazil.

The study included 221 unrelated participants, comprising 139 older adults without dementia (control group) and 82 patients with probable LOAD, matched for age and sex. Participants were diagnosed with probable LOAD, according to the criteria of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA). Moreover, they were diagnosed by a comprehensive diagnostic evaluation for dementia and fulfilled other criteria, such as the Mini-

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Mental State Examination (MMSE), with scores ranging from 4 to 14, and the Clinical Dementia Rating (CDR), with a score of 2. The score of older adults without dementia was >25 on the MMSE.

Patients were included in the AD group when they had a clinical diagnosis of probable LOAD, age compatible with late-onset disease, available clinical and demographic information, and biological samples suitable for genetic analysis. Controls were included when they were older adults without dementia, had MMSE scores above 25, and had no clinical diagnosis of dementia at the time of evaluation. Individuals with insufficient clinical information, unavailable biological samples, or inconclusive genotyping results for a given polymorphism were excluded from the respective analysis.

All participants were from Grande Vitória, a metropolitan region in the state of Espírito Santo, southeastern Brazil. This study was Approved by the Human Research Ethics Committee of the Health Sciences Center of the Federal University of Espírito Santo. Participants were assisted and diagnosed by a geriatrician at the Geriatric Unit of the Hospital Santa Casa de Misericórdia de Vitória (HSCMV) and at the *Centro de Atendimento ao Idoso* (CRAI – Center for Senior Care). Participants or their relatives provided a written informed consent before their participation in the study, as well as information regarding age, sex, ethnicity/skin color, and schooling level. This study was conducted in accordance with the Declaration of Helsinki and Approved by the Ethics Committee (CAAE: 13994419.5.0000.5060).

Blood sampling and genotyping

Peripheral blood was collected in 5 mL EDTA tubes at the Geriatric Unit of the HSCMV and at the CRAI and stored at 4°C before analysis. Genomic DNA was extracted according to Miller *et al.* (1988).

The rs75932628 polymorphism in the *TREM2* gene (C>T RefSeq NM_018965.4) was assessed by real-time quantitative polymerase chain reaction (qPCR). Genomic DNA (30 ng/µL) was used for qPCR, according to the manufacturer's instructions (Applied Biosystems TaqMan SNP Genotyping Assay, Carlsbad, California, USA), on the 7500 Fast Real-Time PCR System. Genotypes were analyzed using SDS version 2.0.5.

The rs670139 polymorphism in the *MS4A4E* gene was assessed by conventional polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), using the primers described by Mao *et al.* (2015). PCR products underwent digestion at 55°C for 16 hours with the enzyme BSL-1 (Thermo Fisher Scientific). Digestion products were electrophoresed on a 7% polyacrylamide gel and stained with silver nitrate. The rs911159 polymorphism in the *CASS4* gene was assessed by PCR-RFLP using the forward primer (5'-TGGGATTGGAGTAGCAGTCA-3') and the reverse primer (5'-AGCAAACACTTCCACCAACC-3') designed by Primer3 version 0.4.0. After amplification, PCR products underwent digestion at 37°C for 15 minutes with the

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enzyme *RsaI*. The rs9349407 polymorphism in the *CD2AP* gene was assessed by PCR-RFLP using degenerate primers. The forward primer (5'-AATCTATAGTAGTGTATACTAAG-3') was designed by dCAPS Finder 2.0 with a G nucleotide introduced at the 3' end to form the restriction site (GGWCC) for the enzyme AvaII (Thermo Fisher Scientific). The reverse primer (5'-TGTAGGCAACTGTAACACAATGG-3'), which was designed by Primer3 version 0.4.0, was not degenerate. After amplification, PCR products underwent digestion at 37°C for two hours with AvaII. The digestion products of rs670139 in *MS4A4E*, rs911159 in *CASS4*, and rs9349407 in *CD2AP* were electrophoresed on a 7% polyacrylamide gel stained with silver nitrate.

For some polymorphisms, the number of valid genotypes differed from the total sample size because samples with insufficient DNA quality, unsuccessful amplification, inconclusive electrophoretic patterns, or missing genotyping data were excluded from the respective gene-specific analysis. Therefore, the denominator varied among polymorphisms according to the number of successfully genotyped individuals.

Statistical analysis

Statistical analysis was performed using SPSS (IBM), version 23.0 for Windows. P-values lower than 0.05 were considered statistically significant. Chi-square tests were used, and odds ratios (ORs) with 95% confidence intervals (95% CIs) were calculated to assess the association between AD and the analyzed polymorphisms. Hardy-Weinberg equilibrium (HWE) was assessed with 1 degree of freedom. Logistic regression was used to evaluate the association between AD and the analyzed polymorphisms, adjusting for *APOE* ε4 status, ethnicity/skin color, age, sex, and schooling level. *APOE* status was included in the analysis based on previous evidence of its association with AD (Camporez *et al.* 2021).

Differences in sex, schooling level, *APOE* status, and ethnicity/skin color among participants with AD and without dementia were evaluated using the chi-square test (χ^2). Regarding schooling level, participants were classified as literate or illiterate. *APOE* status with at least one ε4 allele was considered as ε4+ whereas those without ε4 alleles were considered as ε4−. Regarding ethnicity/skin color, participants were classified as European descendants or African descendants. The Mann-Whitney test was performed to compare age among participants with AD and without dementia.

RESULTS AND DISCUSSION

Results

We found no significant difference regarding schooling level, age, sex, and ethnicity/skin color among participants with AD and without dementia (Table 1). However, as expected, we

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observed a significant difference in *APOE*-ε4 allele frequencies. The genotype distributions of rs911159 in *CASS4*, rs9349407 in *CD2AP*, and rs670139 in *MS4A4E* were in Hardy-Weinberg equilibrium. We could not test rs75932628 in *TREM2* for Hardy-Weinberg equilibrium due to the absence of the T allele. All individuals in our sample had the CC genotype for rs75932628 in *TREM2.*

Table 2 presents the genotype frequencies of rs911159 in *CASS4,* rs75932628 in *TREM2,* rs9349407 in *CD2AP*, and rs670139 in *MS4A4E* in the sample and the logistic regression analysis. The number of valid genotypes varied among polymorphisms. Complete genotyping data were available for 221 participants for *MS4A4E* and *TREM2*, 143 participants for *CASS4*, and 161 participants for *CD2AP*. These differences were due to unsuccessful amplification, inconclusive genotyping patterns, or insufficient DNA quality in specific assays. The genotype frequency of rs911159 in *CASS4* was significantly different between the control group and participants with AD, even after adjusting for schooling level, ethnicity/skin color, age, sex, and *APOE* status. However, we found no significant difference for rs670139 in *MS4A4E* or rs9349407 in *CD2AP*. No association analysis could be performed for rs75932628 in *TREM2* because the T allele was not detected in the sample.

Table 1. Characteristics of sample

Variable	**AD Patients 82 (100%)**	**Controls 139 (100%)**	***p*-value**
Gender			
Man	28 (34.1%)	36 (25.9%)	0.192 [a]
Woman	54 (65.9%)	103 (74.1%)	
Schooling			
Literate	44 (53.7%)	89 (64.0%)	
Illiterate	29 (35.4%)	43 (30.9%)	0.154 [a]
No identification	9 (11.0%)	7 (5.0%)	
Ethnic background			
European descendants	47 (57.3%)	82 (59.0%)	
African descendants	30 (36.6%)	55 (39.6%)	0.159 [a]
No identification	5 (6.1%)	2 (1.4%)	
APOE status			
ε4 +	47 (57.3%)	43 (30.9%)	<0.001[a]
ε4 -	35 (42.7%)	96 (69.1%)	
Age (mean ± SD)	81 ± 7	80 ± 8	0.186 [b]

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MMSE (mean ±SD)	13±6	25±5	<0.001
CDR	2	–	

AD Patients = Alzheimer's disease patients; ε4 += ε4 carriers; ε4 - = ε4 non-carriers; SD= standard deviation; [a] = AD patient versus control group by x^2 test; [b]= p-value of AD patient versus control group by Mann-Whitney test; p-value ≤ 0.05 considered significant. MMSE = Mini-Mental State Examination (value considering the schooling level and the average evolution time of the disease in AD patients); CDR = Dementia Rating Scale.

Table 2. Genotypes frequencies and logistic regression analyses

Gene/ Polymorphism		AD patients	Controls	*p*-value [a]	OR (95%CI) [b]	*p*-value [c]
MS4A4E						
	CC	38 (46.3%)	56 (40.3%)		1 (reference)	-
	CA	33 (40.2%)	63 (45.3%)	0.675	0.77 (0.42 – 1.39)	0.261
	AA	11 (13.4%)	20 (14.4%)		0.81 (0.34 – 1.88)	0.489
	Total	82 (100%)	139 (100%)			
CASS4						
	GG	70 (94.6%)	51 (73.9%)		1 (reference)	-
	GA	4 (5.4%)	18 (26.1%)	0.001	0.187 (0.059 – 0.59)	0.005
	AA	0	0			
	Total	74 (100%)	69 (100%)			
CD2AP						
	GG	27 (45.0 %)	47 (46.5%)		1 (reference)	-
	GC	30 (50.0%)	46 (45.5%)	0.723	1.13 (0.58 – 2.19)	0.610
	CC	3 (5.0%)	8 (7.9%)		0.65 (0.16 – 2.67)	0.629
	Total	60 (100%)	101 (100%)			
TREM2						
	CC	82 (100%)	139 (100%)	-	-	-
	CT	0	0	-	-	-
	TT	0	0	-	-	-
	Total	82 (100%)	139 (100%)			

The numbers with percentages in parentheses show the proportions of genotypes in AD patients and controls; AD Patients = Alzheimer's disease patients; *p*-value ≤0.05 was considered significant; [a] =AD patient versus control group by x^2 test; OR = Odds ratio; CI = Confidence inter-val; [b] = Logistic regression; [c] = *p-value* adjusted by the variables age, gender, educational attainment and ethnic background.

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Discussion

GWAS identified several genes associated with LOAD, including *CASS4*, *TREM2*, *CD2AP*, and *MS4A4E.* However, the replication of GWAS variants in different studies is essential to validate these results. Although the presence of ε4 is the major genetic risk factor for AD worldwide, this *APOE* allele represents less than 20% of the genetic risk of Alzheimer's disease, which intensifies the interest in the validation of new genetic factors (Beck *et al.* 2014). The results of this study show that *CASS4* might play a role in the susceptibility of LOAD. However, no association was observed for the analyzed variants in *CD2AP* and *MS4A4E* in our sample. For rs75932628 in *TREM2*, association analysis could not be performed because the T allele was not detected in the sample.

The *CASS4* gene is located on 20q13.31 and encodes the CASS4 protein. This protein belongs to the CAS scaffold protein family involved in integrin-dependent signaling processes, which are essential for cell proliferation, survival, migration, and motility (Lambert *et al.* 2013; Deneka *et al.* 2015; Wong *et al.* 2023). Evidence shows that *CASS4* plays a role in the pathogenesis of AD through its involvement in cytoskeletal function, axonal transport, and *APP* and tau metabolism (Lambert *et al.* 2013; Karch and Goate 2015). Functional studies show that this protein can modulate tau toxicity (Dourlen *et al.* 2017). *CASS4* contributes to microtubule integrity in neurons. In addition, specific single nucleotide polymorphisms (SNPs) in the *CASS4* gene may influence protein expression levels and, consequently, protein function.

Beecham *et al.* (2014) support the relationship between *CASS4* and AD, as they assessed the association of this protein in response to brain injury in a clinicopathological GWAS with autopsy data. Their study showed that the rs7274581 variant in *CASS4* acts as a protective genetic factor for LOAD. Our study found an association between rs911159 in *CASS4* and LOAD. In our sample, the variant rs911159 acted as a protective factor. Lin *et al.* (2017) analyzed this polymorphism in a gene-lifestyle interaction study in Taiwan and found an association between this variant and increased cognitive decline, acting as a risk factor for AD. Our data show that the rs911159 variant in *CASS4* is associated with LOAD, but more studies are necessary to clarify the influence of different variants in *CASS4* on LOAD.

The *CD2AP* gene is located on 6p12 and encodes the CD2AP protein, which regulates endocytosis, cytoskeletal structure, cell adhesion, and intracellular trafficking (Tao *et al.* 2017; Tao *et al.* 2019). Several studies show the role of this protein in LOAD by affecting the endocytosis of *APP* in neurons and impairing the control of *APP* degradation in dendrites in the brain of affected individuals (Tao *et al.* 2017; Kong *et al.* 2024; Maninger *et al.* 2024). The C allele of rs9349407 in *CD2AP* was considered a risk factor for LOAD in GWAS (Hollingworth *et al.* 2011; Kong *et al.* 2024). This SNP was correlated with increased neuritic plaques and tau proteins in a functional study and, consequently, neuronal loss and cognitive decline (Shulman *et al.* 2013; Ramos de

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Matos *et al.* 2018). However, no association was observed between rs9349407 in *CD2AP* and AD in our sample.

Studies in China found a positive association between the rs9349407 variant and LOAD (Jiao *et al.* 2015; Xiao *et al.* 2015). Studies with white individuals in Canada (n = 2,864) and the United States and Europe (n = 6,835) did not find a significant association between this variant and LOAD, even with large samples (Carrasquillo *et al.* 2011; Omoumi *et al.* 2014). A meta-analysis by Chen *et al.* (2015) showed that rs9349407 was a risk factor for LOAD. These authors stated that this association was probably due to the large sample and the variety of ethnicities. However, most studies that showed a positive association with LOAD resulted from GWAS with white individuals.

These differences may stem from distinct risk profiles in different ethnic groups. According to Lins *et al.* (2010) and Pena *et al.* (2011), the Brazilian population is primarily composed of White, Black, and mixed-race individuals, which was reflected in our sample. Since the allele frequency of each genetic marker varies among populations, the contribution of different risk factors due to distinct ethnicities may also vary. In our sample, no association was observed between rs9349407 and LOAD. However, this result should be carefully interpreted due to the limited sample size.

The *MS4A4E* gene, which is located on 11q12.2 and expressed on microglial cells, is part of the *MS4A* gene family of cell surface proteins (Liang *et al.* 2001; Mehdizadeh *et al.* 2019). The MS4A4E protein is involved in immune modulation and the regulation of calcium homeostasis inside cells (LaFerla 2002; Karch and Goate 2015; Villegas-Llerena *et al.* 2016; Mehdizadeh *et al.* 2019). High levels of intracellular calcium may facilitate the formation of amyloid plaques and tau hyperphosphorylation (LaFerla 2002; Guan *et al.* 2021). Therefore, among the two possible roles of this protein in the association with AD, one is related to an immunological pathway and another to an amyloidogenic mechanism.

Hollingworth *et al.* (2011), in a GWAS with white individuals, showed that the A allele in the rs670139 variant in *MS4A4E* is a risk factor for LOAD. Karch *et al.* (2012) also obtained this result by analyzing parietal lobes of autopsied brains with AD using data from the Clinical Dementia Rating (CDR), the increased Braak tangle score and the Braak plaque score of European American patients. Tan *et al.* (2013) and Wang *et al.* (2016) found a positive association between this SNP and AD in China. Mao *et al.* (2015) and Miyashita *et al.* (2013) performed association studies in East Asia and Japan, respectively, and showed no association with LOAD. Mao *et al.* (2015) obtained the same results in a meta-analysis of the rs670139 variant in *MS4A4E* in an Asian population. Our results showed no association between rs670139 and AD, thus, the effect of this variant on LOAD is probably small and only a larger sample may detect its overall influence.

The *TREM2* gene, which is located on 6p21.1, encodes a receptor of the TREM family involved in innate immune responses (Hu *et al.* 2013; Mehdizadeh *et al.* 2019). The TREM2 protein

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is related to AD and other neurodegenerative diseases through inflammatory and immune pathways (Hu *et al.* 2013; Liu *et al.* 2024). Wang *et al.* (2018) and Jonsson *et al.* (2013) showed that the rare rs75932628-T (p.R47H) polymorphism increases susceptibility to AD through inflammatory processes. This protein is correlated with the production of pro-inflammatory cytokines and the stimulation of CD4+ T cells in microglia in the brain (Zheng *et al.* 2024). Moreover, the expression of *TREM2* was related to the formation of amyloid plaques in *APP* transgenic mouse models, suggesting a role in the development of AD (Melchior *et al.* 2010; Jonsson *et al.* 2013; Delizannis *et al.* 2021).

Studies in the United States, Germany, the Netherlands, Norway, Spain, and Colombia also found the association between rs75932628-T and AD (Benitez *et al.* 2013; Arboleda-Bustos *et al.* 2018). However, no statistically significant association was found in Iran, even after the detection of a significant frequency of the T allele (Mehrjoo *et al.* 2015). All participants in our study had the CC genotype; therefore, association analysis for rs75932628 in *TREM2* could not be performed in our sample. Similarly, studies by Wang *et al.* (2018) and Ma *et al.* (2014) in China also reported only the CC genotype. Despite the frequency of this rare genetic variant in specific populations, several studies support that the rs75932628-T polymorphism in *TREM2* is associated with an increased risk of LOAD.

Our study shows that rs911159 in *CASS4* is associated with LOAD. No association was observed for rs9349407 in *CD2AP* or rs670139 in *MS4A4E* in our sample. For rs75932628 in *TREM2*, association analysis could not be performed because the T allele was not detected in the sample, which may reflect its low frequency in the population analyzed. These results should be carefully interpreted due to the limited sample size. The replication of GWAS variants in different studies is essential to support the role of genetic risk factors in complex diseases. Our findings contribute to the investigation of GWAS-identified variants associated with LOAD, particularly rs911159 in *CASS4*, in an admixed Brazilian population.

Population stratification is an important methodological challenge in genetic association studies conducted in admixed populations, such as the Brazilian population. In the present study, self-reported ethnicity/skin color was considered in the adjusted analysis; however, genetic ancestry markers were not available. Therefore, future studies should include larger samples and ancestry-informative markers to better evaluate the contribution of GWAS-identified variants to LOAD susceptibility in Brazilian and Latin American populations.

CONCLUSION

Our findings support an association between rs911159 in *CASS4* and late-onset Alzheimer's disease in the analyzed sample, suggesting a possible protective effect of this variant

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in an admixed Brazilian population. No association was observed for rs9349407 in *CD2AP* or rs670139 in *MS4A4E*, and association analysis for rs75932628 in *TREM2* could not be performed because the T allele was not detected. These results reinforce the relevance of validating GWAS-identified variants in underrepresented populations and suggest that *CASS4* may be a candidate gene for further investigation in Brazilian studies on AD genetics. However, the findings should not be broadly generalized due to the limited sample size, the absence of genetic ancestry markers, and the limited ability to evaluate rare variants in the present sample. Further studies with larger cohorts, ancestry-informed analyses, and integrative approaches are needed to confirm these findings.

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