

Do *MTHFR* C677T Genetic Polymorphism Influence in the Pathogenesis of Amyotrophic Lateral Sclerosis?

Rômulo Moraes Azevedo

Universidade Federal de Goiás

Kamilla de Faria Santos

Universidade Federal de Goiás

Rayana Pereira Dantas de Oliveira

Universidade Federal de Goiás

Júlia Costa Pereira

Universidade Federal de Goiás

Dhiogo da Cruz Pereira Bento

Universidade Federal de Goiás

Angela Adamski da Silva Reis

Universidade Federal de Goiás

Rodrigo da Silva Santos (✉ rdssantos@ufg.br)

Universidade Federal de Goiás <https://orcid.org/0000-0002-9480-4362>

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Abstract

Amyotrophic Lateral Sclerosis (ALS) is a progressive and lethal neurodegenerative disease without a definitive diagnostic test and effective treatment. A plethora of studies suggest that genetic factors play an important role in ALS development, and potentially link folate pathway dysregulation to disease pathogenesis. This study aims to evaluate folate dysregulation due to *MTHFR* C677T polymorphism and other factors such as sociodemographic and clinical, to better elucidate the involvement of these factors in ALS pathogenesis, and to investigate possible biomarkers for use as disease diagnostics or prognostics. This hospital-based case-control study analyzed 101 patients diagnosed with ALS and 119 considered healthy, with no suspicion or diagnosis of neurodegenerative disease. Blood samples were collected, stored, and underwent DNA extraction. Clinical and sociodemographic data from patients were collected through a questionnaire, as well as consultation of medical records. Genotypic analyses were performed using PCR-RFLP, and statistical analysis of clinical and genotypic data was conducted with SPSS software, version 23. The results show a higher presence of the mutant genotype ($p = 0.02$) in the case group, and suggest that mutant allele (T) is a risk factor for ALS susceptibility (OR = 1.54; 95% CI = 1.05–2.29; $p = 0.03$). Mutant genotype (T/T) interacts with both demographics (White $p = 0.005$ / Brown $p = 0.001$) and clinical factors (Physical activity $p = 0.006$) as risk factors for ALS. Also, a significant difference in alcohol consumption ($p = 0.001$) between the case and control group was observed. Moreover, a statistical trend towards faster disease progression and death was observed for patients with the mutant allele (T) ($p = 0.06$). Thus, the results of this study suggest that folate deficiency due to *MTHFR* C677T polymorphism is implicated in ALS through pathogenic mechanisms and interaction with other risk factors, resulting in faster disease progression and early death.

Introduction

Amyotrophic Lateral Sclerosis (ALS) is a lethal and progressive neurodegenerative disease that selectively affects the upper and lower motor neurons. It is characterized by symptoms such as cramps, fasciculations, muscle weakness and atrophy, dysphagia, dysarthria, and respiratory failure, which is the leading cause of death [1–3]. The disease has an annual incidence of about 1.55–1.96 / 100,000 inhabitants and a prevalence of 5.2–6.2 / 100,000 inhabitants, predominantly affecting individuals aged 55 to 75 years, Caucasian populations, and males [4, 5].

The causes of disease development are still undefined. ALS is thought to be a multifactorial pathology, associated with smoking, alcoholism, prolonged physical activities, and genetic factors [6, 7]. There is ample evidence that genetic factors contribute to ALS susceptibility, and several genes have already been implicated in the neurodegenerative process, such as *SOD1*, *VAPB*, *C9orf72*, *FUS*, *NEK1*, *TRPM7*, and others [8, 9]. Despite the continued discovery of an increasing number of genetic biomarkers in ALS, over 50% of patients do not have a genetic diagnosis [10].

Without a specific diagnostic test for the disease, ALS diagnosis is currently performed through clinical evaluation aided by neuroimaging and electroneuromyography tests [11]. The average time for the

diagnosis is estimated to be nine months to one year, from the symptom onset [12, 13]. Riluzole, a glutamate antagonist, is currently the only medicine with proven efficacy in improving ALS survival, but it only increases life expectancy by two to three months. It is known that early diagnosis associated with drug therapy using riluzole may enhance its efficacy in clinical improvement [14, 15].

Variation in genes involved in the folate pathway has been suggested as a possible factor implicated in ALS since this vitamin is essential to DNA synthesis and homocysteine (Hcy) metabolism, the dysregulation folate metabolism unbalances the cellular homeostasis, and could contribute to neurodegenerative processes in motor neurons [16, 17]. The *Methylenetetrahydrofolate reductase* (*MTHFR*) gene stands out as a potential target, considering that it is responsible for encoding the MTHFR enzyme, which plays a crucial role in intracellular folate metabolism [18]. MTHFR enzyme regulates the intracellular methylation pathway and influences DNA synthesis by converting 5,10-methylenetetrahydrofolate (5,10-MTHF) into 5-methyltetrahydrofolate (5-MTHF), the most abundant circulating form of folic acid in the body [18–22].

It is known that polymorphisms in the *MTHFR* gene result in the production of a defective enzyme, which deregulates the entire intracellular biochemical process of folate. Among the main polymorphisms studied in the gene, the C677T (rs1801133) mutation codes for a thermolabile enzyme with reduced activity by up to 65% at 35 °C, resulting in increased plasma levels of Hcy [23, 24]. The mutation has been identified as a risk factor for several diseases, such as cardiovascular diseases [25, 26], cancers [27, 28], diabetes [29], hypertension [30], and congenital malformations [31].

In the nervous system, dysregulation of the folate cycle directly impacts neuronal physiology by causing hyperhomocysteinemia (HHcy). Homocysteine is a neurotoxic factor and is associated with several neuronal death mechanisms [32, 33]. Thus, increasing numbers of studies have hypothesized the folate metabolic dysfunctions in ALS pathogenesis, especially after the detection of HHcy in ALS patients affected by the disease and the discovery of its effect on the motor neuron [34, 35].

Therefore, this study aims to evaluate the MTHFR C677T polymorphism, socio-demographic and clinical factors in ALS patients in the population of central Brazil, to understand how genetic causes of intracellular folate dysregulation, as well the influences of environmental and clinical factor affect disease susceptibility and progression.

Material And Methods

Population study (Patients)

This hospital-based case-control study consists of 101 patients diagnosed with ALS (case group) who underwent medical follow-up at the referral hospital for neuromuscular diseases in Goiânia, GO, Brazil, Dr. Henrique Santillo Rehabilitation and Readaptation Center (CRER). The control group consists of 119 individuals not diagnosed with ALS or other neurodegenerative diseases, collected from the Laboratory of Clinical Analysis and Health Education of the Federal University of Goiás, Brazil (LACES-UFG).

We selected patients with a minimum age of 18 years and a maximum of 90 years, regardless of the stage of disease progression, who followed the protocol of laboratory and imaging tests defined for the disease diagnosis, standardized by the CRER. Patients unable to give venous blood samples were excluded.

Patients who agreed to participate signed the Informed Consent Form (ICF) and completed a questionnaire concerning age, gender, ethnicity, physical activity, occupation, smoking, and alcohol consumption. The groups were matched for gender and age according to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) and STrengthening the REporting of Genetic Association studies (STREGA) guidelines [36].

Regarding the lifestyle questions, patients who reported smoking for more than one year were considered smokers. Likewise, patients who reported regularly consuming alcohol for at least one year before their ALS diagnosis were considered consumers of alcohol. Also, in the case group, medical records were consulted to obtain clinical information, such as disease classifications, symptoms, treatments, and outcomes.

Ethical Principles

The study was conducted with the assent of the Research Ethical Committee from the Federal University of Goiás, Brazil (CEP/UFG) under protocol number CAAE 79593117.7.0000.5083, and also followed the Ethical Principles for Medical Research Involving Humans of the Declaration of the World Medical Association of Helsinki.

Sample Collection and Analysis for *MTHFR* C677T Polymorphism

The peripheral blood samples from the patients were collected using a venous puncture technique in 4 ml tubes containing an anticoagulant (EDTA) identified and processed at 5000 rpm for ten minutes. Then, the samples were subjected to DNA extraction and purification with a commercial kit (Invitrogen®) using the protocol suggested by the manufacturer. The extracted samples were identified and stored at -80 °C, and subsequently subjected to genomic material quantification evaluation using the NanoDrop™ ND-1000 spectrophotometer (ThermoFisher®, USA).

Genotypic analyses for the *MTHFR* C677T polymorphism were performed at the Laboratory of Molecular Pathology, Institute of Biological Sciences (ICB), Federal University of Goiás, Goiânia, GO, Brazil. Polymerase Chain Reaction and Restriction Fragment Length Polymorphism (PCR-RFLP) analysis were employed. Thermocycling protocols were set as suggested [37]. Forward primer: 5'-AGGCTGACCTGAAGCACTTGAA-3', Reverse primer: 3'-CTCAAAGAAAAGCTGCGTGATGA-5'.

PCR conditions were 94 °C for 5 min of denaturation and 36 cycles of 61 °C for 1 min, 72 °C for 1 min 30 seconds, and 4 °C at the end. The *HinfI* enzyme was used for digestion, and the resulting restriction fragments were visualized on a polyacrylamide gel (15%), stained in 4g / L silver nitrate solution, allowing

for the identification of wildtype genotypes (C/C) 198bp fragment, heterozygotes (C/T) 198bp, 175bp, and 23bp fragments, and mutants (T/T) 175bp and 23bp fragments.

Statistical analysis

Statistical analysis was performed using the SPSS statistical package, version 23. The general characterization of the case group and control group individuals was performed with descriptive statistics, with absolute (n) and relative (%) frequencies reported, while mean and standard deviation were applied to continuous variables.

For genotypic analysis, PostHoc Chi-square tests and Pearson's test (MacDonald and Gardner, 2000) were performed. To study survival (time between ALS diagnosis and death) according to genotype, Kaplan-Meier curves were created. For all results, a significance level of 5% was used.

Results

Sociodemographic and clinical data

Sociodemographic data were evaluated for ALS (101 individuals) and control (119 individuals) groups. Mean ages were 57.3 ± 12.8 years for the case group and 58.0 ± 10.3 years for the control group (Table 1).

The case group had 44.6% of female patients (45 individuals) and 55.4% of male patients (56 individuals). In the control group, 57.1% were female (68 individuals), and 42.9% were male (51 individuals). Age ($p = 0.64$) and gender ($p=0.08$) showed no statistically significant associations with the disease.

In the analysis of risk factors, smoking was not different between the case and control groups ($p = 0.28$). However, alcohol consumption was higher in the case group than in the control group ($p = 0.01$), therefore, it is a possible risk condition for the disease among the studied groups (Table 1).

Table 1 - Comparison of clinical and sociodemographic variables between case and control group.

	Samples n (%)		Total	<i>p</i>
	Control (54.1)	119 Case (45.9)	101	
Age (Mean ± SD)	58.0 ± 10.3	57.3 ± 12.8	57.7 ± 11.5	0.64**
Gender				
Female	68 (57.1)	45 (44.6)	113 (51.4)	0.08*
Male	51 (42.9)	56 (55.4)	107 (48.6)	
Alcohol Intake				
No	84 (70.6)	55 (54.5)	139 (63.2)	0.01*†
Yes	35 (29.4)	46 (45.5)	81 (36.8)	
Smoking				
No	68 (57.1)	65 (64.4)	133 (60.5)	0.28*
Yes	51 (42.9)	36 (35.6)	87 (39.5)	

*Pearson's Chi-square test, ** Student t-test, † $p < 0,05$.

n= absolute frequency, % = relative frequency

SD = standard deviation, ALS- Amyotrophic Lateral Sclerosis,

In the ALS group, 48.5% identified as white and 44.6% identified as brown. When asked about physical activity, 46.5% declared that they did not engage in any type of physical activity before the diagnosis of the disease, while 53.5% reported physical activity. The reported physical activities included walking, soccer, water aerobics, workout, pilates, and others. Most cases reported work in the following categories: general service category (36.6%), housework (13.9%), administrative services (12.9%), commerce (9.9%), teachers (7.9%), health professional (5.9%), chef cook (1%), student (1%), and others (5%). However, 5.9% chose not to inform.

The patients in the case group were classified according to the El Escorial World Federation of Neurology, as reported in their medical records. 5.9% of patients reported a recurrence of ALS in the family (fALS), and 94.1% were considered sporadic cases (sALS). Moreover, 72.3% fit the cALS category, 23.8% were bALS, and 4% were juvenile ALS. Previous pathologies were cited by 46.5% in the case group, and 40.6% reported a history of another neurological disease in the family.

Regarding treatment with riluzole, 75.2% of the case group said they followed the treatment, and 24.8% reported not using it. During the research period, 10.9% of the ALS patients in the case group died. Therefore, death was considered as a disease outcome in this study.

Genotypic data and association with risk factors

According to the genotypic analysis, the mutant genotype (T/T) was more frequent in patients diagnosed with ALS than in the control group, representing 9.9% and 2.5% of the groups, respectively ($p = 0.02$).

Allelic evaluation corroborates the genotypic analysis, showing that the frequency of the T allele is a higher risk for the development of the disease than the C allele (OR= 1.54, 95% CI= 1.05 - 2.29) ($p = 0.03$), shown in Table 2.

Table 2 - Association of *MTHFR* C677T genotype and allele frequency with ALS.

<i>MTHFR</i> C677T	Samples n (%)		Total	Odds Ratio (95% CI)	<i>p</i>
	Control 119 (54.1)	Case 101 (45.9)			
Genotypic Frequency					
Wild (C/C)	61 (51.3)	41 (40.6)	102 (46.4)	Reference	0.11**
Heterozygous (C/T)	55 (46.2)	50 (49.5)	105 (47.7)	0.85 (0.51-1.41)	0.63**
Mutant (T/T)	3 (2.5)	10 (9.9)	13 (5.9)	1.83 (1.27-2.65)	0.02**†
Allele Frequency					
C	219 (74.5)	132 (65.3)	351 (70.8)	Reference	0.03*†
T	75 (25.5)	70 (34.7)	145 (29.2)	1.54 (1.05-2.29)	

* Pearson's Chi-square test, ** *Post hoc* Chi-square, † $p < 0,05$.

n = absolute frequency, % = relative frequency

ALS - Amyotrophic Lateral Sclerosis.

These results suggest a possible association of *MTHFR* C677T polymorphism with ALS, which could be due to dysregulation of the folate metabolism, leading to increased plasma Hcy levels, alterations in DNA synthesis, RNA and protein hypomethylation, oxidative stress, neuroinflammation, and ultimately the death of motor neurons (Fig. 1).

When analyzing associations of clinical data with the results of the genotypic analysis of ALS patients, the patients' gender ($p = 0.85$), alcohol intake ($p = 0.16$), smoking ($p = 0.42$), disease classification ($p = 0.57$), neurological disease in the family ($p = 0.99$), and previous pathologies ($p = 0.90$) did not present a statistically significant association with genotype in disease development (Table 3). However, ethnicity, when compared with genotypic profile, was significantly associated with (T/T) genotype patients, specifically for self-identified white ($p = 0.005$) and brown ($p = 0.001$) individuals. Engaging in physical activity was also associated with the (T/T) genotype ($p = 0.006$), shown in Table 3.

Table 3 - Association between *MTHFR* C677T polymorphism genotype with the demographic and clinical profile of ALS patients.

	<i>MTHFR</i> n (%)			<i>p</i> *
	Wild	Heterozygous	Mutant	
Gender				
Female	19 (46.3)	21 (42.0)	5 (50.0)	0.85
Male	22 (53.7)	29 (58.0)	5 (50.0)	
Ethnicity				
White	20 (48.8)	28 (56.0)	1 (10.0)†	0.005
Black	6 (14.6)	1 (2.0)	0 (0.0)	0.23
Brown	15 (36.6)	21 (42.0)	9 (90.0)†	0.001
Physical Activity				
No	23 (56.1)	24 (48.0)	0 (0.0)	0.006
Yes	18 (43.9)	26 (52.0)	10 (100.0)†	
Alcohol Intake				
No	27 (65.9)	23 (46.0)	5 (50.0)	0.16
Yes	14 (34.1)	27 (54.0)	5 (50.0)	
Smoking				
No	29 (70.7)	29 (58.0)	7 (70.0)	0.42
Yes	12 (29.3)	21 (42.0)	3 (30.0)	
Classification				
Sporadic	39 (95.1)	46 (92.0)	10 (100.0)	0.57
Familial	2 (4.9)	4 (8.0)	0 (0.0)	
Neurological disease in the Family				
No	24 (58.5)	30 (60.0)	6 (60.0)	0.99
Yes	17 (41.5)	20 (40.0)	4 (40.0)	
Previous pathologies				
No	23 (56.1)	26 (52.0)	5 (50.0)	0.90
Yes	18 (43.9)	24 (48.0)	5 (50.0)	

* *Post hoc* Chi-square, † *p* < 0,05, n = absolute frequency, % = relative frequency.

Association of genotypic profile and ALS outcome

Survival analysis was performed with the onset of symptoms as the starting point and death as the outcome according to the *MTHFR* C667T polymorphism genotypes. This analysis demonstrated a statistical trend towards more significant progression and earlier death of patients with the heterozygous genotype (C/T) when compared to (C/C) patients ($p = 0.06$), presented in Fig. 2.

Discussion

ALS is a heterogeneous disease with ethnic and sociodemographic differences. In this study, the ALS group has an average age of 57.3 ± 12.8 years, younger patients represent 4% of the group, and 55.4% are males. ALS disease affects mainly men, with a male-to-female ratio between 1 and 2, and the initial symptoms usually manifest between 55–75 years [2, 7, 38]. Despite the prevalence in the elderly, young people with an average age of 20 years are also affected, representing 1–10% of all cases, classified as juvenile ALS [39]. In this study, the average age and gender proportions in the case group correspond to the literature [40]. However, age and gender are not significantly different between groups (Table 1).

The reason for the difference in occurrence between the genders is not yet known. Some studies associate a lower prevalence of ALS in women with a hormonal protective effect or lower exposure to risk factors [41, 42]. Recent studies suggest that estrogen acts as a protective factor in neurodegenerative processes by countering mechanisms of oxidative stress and excitotoxicity [43–45]. Despite the lower incidence of women among ALS patients in general, they are more associated with a disease-specific classification, the FTD-ALS cases, which has been justified by genetic factors [46]. However, further studies are needed to elucidate gender biases in ALS.

Looking at the sociodemographic make-up of the case group, white people comprised the most substantial proportion, representing 48.5% of the individuals, and 44.6% were brown. These results corroborate other studies that report a higher incidence of the disease in white populations, which has been associated with genetic factors [5, 47]. Besides, the white population has more easy access to health programs than the black population, traditionally considered a minority in a society that often neglects social rights [48].

Among the modifiable variables analyzed, alcohol consumption as a risk factor for ALS is still controversial. Some studies show the consumption of alcohol as a protective factor for the disease [49, 50]. However, in the present study, alcohol consumption was more reported in the case group; thus, it is a possible risk condition for the disease ($p = 0.01$). Such a hypothesis does have a potential mechanistic explanation. Excessive alcohol consumption increases the sensitivity of glutamate receptors and increases the concentration of glutamate in the central nervous system, leading to excitotoxicity and neuronal death [51]. Additionally, it is known that alcohol consumption can activate astrocytes through alterations in proteins expressed in microglia, implying in neuroinflammatory mechanisms [52].

Smoking as a risk factor for ALS is also controversial. Some studies relate smoking to an increased risk for the disease, possibly linked to the toxic effects of cigarette substances [49, 53]. Smoking results in neuronal death since nicotine interacts with acetylcholine receptors in the central nervous system, causing the dysregulation of Ca^{2+} , Na^+ , and K^+ ion concentrations, increased cell permeability, oxidative stress, and neuroinflammation [54, 55]. Nevertheless, there was no statistically significant difference in identification as a smoker between the groups in this study ($p = 0.28$).

Other factors were investigated in the case group, such as physical activity and occupation before the diagnosis of ALS. Some studies indicate that physical activity could contribute to the risk of disease development, as it results in increased metabolic rates in the body, which is related to the production of reactive oxygen species and oxidative stress [56, 57]. In this sense, the hypermetabolic condition was reported as a characteristic of ALS pathogenesis, and it has been discussed as a determining factor in neurodegenerative processes [58, 59]. Besides, physical activity has been associated with neuronal death, due to its relation to the increase of pro-inflammatory cytokine expression resulting in neuroinflammation processes. Additionally, it is known that physical injuries during such practices may contribute to the genesis of the pathological process [57, 60]. In the present study, physical activity was reported by most ALS individuals, representing 53.5% in the case group (Table 3). Similar results were found in studies by [57] and [61].

In the professional occupation analysis of the ALS group, most individuals reported working in professions that require physical exertion or repetitive activities, like general services (36.6%), housework (13.9%), administrative services (12.9%), commerce (9.9%), teachers (7.9%), and health professionals (5.9%). In general, activities in which professionals are exposed to chemicals and require significant physical performance are considered risk factors for ALS [62, 63]. The present study corroborates the literature regarding occupational exposure, in which professions such as health professionals, teachers, scientists, and athletes are most affected by the disease [64, 65]. However, further research is needed on the association of occupational factors and clinical factors with disease susceptibility.

ALS patients were also classified into major disease variations according to the recommendations of the El Escorial World Federation of Neurology and information contained in medical records. We observed a higher percentage of sALS (94.7%) and fALS (5.9%). Moreover, 72.3% fit into the cALS category, 23.8% were bALS, and 4% were juvenile cases. These data corroborate the prevalence of the classifications described in the literature, where sALS represents ~ 90 to 95% of all ALS cases, and ~ 5 to 10% are fALS. The other classifications also follow the same pattern, in which the classical form of the disease is most prevalent ~ 50 to 75%, the bulbar form affects ~ 20 to 25% of all cases, and juveniles vary from ~ 1 to 10% [3, 39].

Also, in the case group, individuals were asked about their past medical history and cases of neurodegenerative diseases in the family. Research on patients' previous diseases and the occurrence of neurodegenerative diseases in the families of ALS patients has grown considerably in recent years due to the discovery of the common etiopathogenesis among some diseases, which aroused scientists'

attention to a better understanding of the mechanisms involved in ALS [66, 67]. The present study showed that 46.5% of the patients in the case group reported some type of disease before the ALS diagnosis, and 40.6% reported cases of neurodegenerative diseases in the family. Significant results of neurological disease history in family and ALS were found in the studies by [66], [68] and [69].

Regarding drug treatment, 75.2% of ALS patients reported taking riluzole, and 24.8% reported not having followed the treatment. Riluzole is the only drug approved for ALS treatment in Brazil. Nevertheless, studies report no significant increase in patient survival attributed to this drug treatment – only about two to three months [14, 70]. Other medications are currently being studied for ALS treatment, such as Edaravone (Radicut), Masitinib [14], Tirasemtiv [71], and others. However, the progressive character of the disease and the lack of effective medication contribute to the short life expectancy after the diagnosis, which varies between three and five years.

Concerning the genotyping analyses, this study demonstrates a higher prevalence of genotype (T/T) in ALS patients, making it a possible risk factor for the disease (OR = 1,83; 95% CI = 1.27–2.65) ($p = 0.02$). Similarly, the allele frequency analysis showed that the T allele confers risk for disease susceptibility (OR = 1.54; 95% CI = 1.05–2.29) ($p = 0.03$) (Table 2). The same results were found in studies by [72] and [73].

The hypothesis that *MTHFR* C677T polymorphism may contribute to ALS pathogenesis is supported by evidence showing that this polymorphism causes an imbalance of the intracellular folate pathway [74], which is associated with several pathogenic mechanisms, such as hypomethylation, increased Hcy levels in the organism, and altered nucleotide synthesis. These mechanisms have been implicated in central nervous system malformation, cognitive impairment, depression, epilepsy, Down syndrome, and the development of neurodegenerative diseases [75, 76].

Intracellular folate dysregulation could be driven by several factors, especially alterations in genes acting on its biochemical pathway [23]. Remarkably, the *MTHFR* gene stands out due to its coding of the enzyme MTHFR, which plays a crucial role in folate intracellular metabolism by converting 5,10-MTHF into 5-MTHF, the main circulating form of folic acid and most abundant form in the body [77]. The enzyme MTHFR directly regulates the intracellular methylation cycle, since the 5-MTHF reduced by the enzyme is an intermediate necessary for the methylation of Hcy to produce methionine. Such a process is essential to cellular homeostasis, and vital to the synthesis of SAM, the main active molecule in folic acid intracellular methylation processes [24]. Also, the enzyme MTHFR indirectly influences purine synthesis via the Methylene tetrahydrofolate dehydrogenase (MTHFD) pathway through the use of the 5,10-MTHF molecule, which can be used in both pathways [21].

The *MTHFR* gene is more expressed in the brain, muscle, placenta, and stomach, possibly due to the greater need for folate homeostasis in these tissues [78]. More than 20 genetic variations in the gene are known to result in the coding of non-functional enzymes. Among these, the C677T single nucleotide polymorphism – SNP is the main alteration studied. Cytosine is replaced with thymine at position 677 in exon 4, causing the change in coding for the amino acid alanine to valine. This change results in the production of a thermolabile enzyme with activities reduced by 35% in the case of heterozygous (C/T)

and 65% in homozygous (T/T) at 35 °C [22, 24]. Various diseases are associated with this production, such as cardiovascular disease [25, 26], diabetes [29], hypertension [30], congenital anomalies [31], cancers [27, 28], and neurodegenerative diseases such as Alzheimer [79, 80] and Parkinson [81, 82].

The C677T polymorphism directly interferes with the available concentration of 5-MTHF, a molecule that is used in the bioconversion of Hcy to methionine and is essential in the production of SAM [24]. Low SAM synthesis is a limiting factor in the methylation cycle, which is crucial for DNA interactions, chromatin structure, and transcription rates, all of which are involved in epigenetic mechanisms that are essential for neuronal development and differentiation [22, 83].

Epigenetic changes during embryonic development are known to result in life-long functional changes, predisposing individuals to various neurological disorders [22]. Although not well understood, some studies report that folate cycle dysregulation and epigenetic changes are associated with alterations in stem cell differentiation and proliferation rates of neurons, the formation of neuromuscular junctions, neural cell specialization, and decreased cells in cortical regions [74, 75, 84]. Thus, the importance of epigenetic changes for motor neurons should be considered, as they are highly differentiated with large prolongations in the central nervous system and responsible for specialized functions in the contraction of effector muscles in the periphery [85].

Furthermore, hypomethylation resulting from the *MTHFR* C677T polymorphism could be associated with ALS pathogenesis since it may affect proteins that compound the neuronal cytoskeleton such as tubulin, β -actin [86], myelin, and cell membrane phospholipids, thus supporting the neurodegenerative process through structural changes and demyelination of the nervous system [83, 87]. Moreover, disturbances in the methylation process are associated with lower synthesis of inhibitory neurotransmitters such as gamma-aminobutyric acid (GABA) and serotonin, which causes neuroplasticity dysregulation and exposes motor neurons to glutamate excitotoxicity in ALS [88–90].

Additionally, HHcy due to the *MTHFR* C677T polymorphism is known to affect cellular redox states, associated with higher production of reactive oxygen species and oxidative stress [35] (Fig. 1). HHcy also affects ion channels, inducing intracellular calcium influx associated with mitochondrial dysregulation, DNA damage, and cell death [91–93]. Studies on ALS have reported HHcy in patients plasma and cerebrospinal fluids [94, 95], discussed as a factor in the predisposition of motor neurons to the process of excitotoxicity and oxidative stress, due to interactions in glutamatergic receptors, which causes higher sensitivity in the release process of glutamate neurotransmitters [94, 96].

Moreover, the increased Hcy level in the bloodstream could contribute to muscle denervation and neurodegeneration, given that the concentration of this amino acid is ten times higher in the bloodstream of ALS patients than in their central nervous system [95]. This suggests that Hcy might act at neuromuscular junctions and skeletal muscles [35].

Of the main process arising from the *MTHFR* gene polymorphism linked to HHcy, oxidative stress presents itself as a mechanism implicated in many intracellular alterations in ALS neurodegeneration [97]. In the

transsulfuration pathway, Hcy is converted to cystathionine and later to cysteine, the precursor molecule in protein and glutathione (GSH) synthesis. Glutathione is an essential antioxidant compound that acts against oxidative imbalance and cell damage by capturing reactive oxygen species (ROS) [98, 99]. Intracellular folate deficiency due to *MTHFR* gene polymorphism compromises glutathione metabolism since SAM allosterically regulates GSH production [100, 101] (Fig. 1). In brain tissue, the conversion of cystathionine to cysteine does not occur, and the process of GSH synthesis is dependent on cysteine transporters [102]. In this way, studies report low levels of glutathione in brain tissue, causing greater susceptibility of neurons to oxidative stress and development of ALS [103, 104] (Fig. 1). On the other hand, folate deficiency and HHcy contribute to oxidative stress, once the Hcy interacts with cysteine transporters preventing GSH synthesis [105].

As previously mentioned, the *MTHFR* C677T polymorphism can also affect DNA synthesis via the thymidylate pathway by MTHFD and TMS enzymes [19]. In this pathway, the 5,10-MTHF is reduced and oxidized to 10-Formiltetrahydrofolate, which donates a methyl group for the conversion of deoxyuridine-5-monophosphate (dUMP) to dTMP [106, 107] (Fig. 2). Although not well understood, deficiency in the *MTHFR* enzyme is known to result in the accumulation of the 10-Formiltetrahydrofolate molecule due to the non-conversion of 5,10-MTHFR to 5-MTHF, stimulating dTMP synthesis [108]. Thus, the synthesis of new thymidylate is an alternative for intracellular folate reactions in cases of *MTHFR* enzyme deficiency, possibly increasing the dTMP concentration in plasma.

The increased production of thymidylate contributes to oxidative stress, as its entire synthesis process is performed by oxidation-reduction reactions that depend on NADPH [20, 21]. NADPH is a major ROS generator that contributes to the development of neurodegenerative diseases, including ALS, due to the release of free radicals in the brain and the vulnerability of this tissue to oxidizing agents [109]. Increased plasma thymidine levels have been reported in neurogastrointestinal mitochondrial encephalomyopathy disease (MNGIE), discussed as a cause in mitochondrial DNA imbalance associated with symptoms such as mental regression, ophthalmoplegia, and fatal gastrointestinal complications [110]. Moreover, increased thymidylate was reported in carcinoma cells as an angiogenic factor associated with oxidative stress, stimulation of pro-inflammatory interleukin secretion, and vascular endothelial growth factor secretion (VEGF) [111].

In addition to analyzing the *MTHFR* C677T polymorphism as a possible risk factor for ALS, this study also identified associations between the genotypic profile and sociodemographic and clinical factors reported by the individuals in the case group. Interactions between genotype (T/T) and factors such as ethnicity in white ($p = 0.005$) and brown ($p = 0.001$) individuals, as well as physical activity ($p = 0.006$) were observed (Table 3). Other important variables, such as gender, alcohol consumption, smoking, ALS classification, neurological disease in the family, and previous pathologies showed no interactions with the genotypic profile presented in the case group.

The association of ethnicity with the *MTHFR* C677T polymorphism is consistent with the higher prevalence of this genetic variable in white people such as Hispanic, Italian, Californian, and others.

Conversely, the lowest incidence was reported in Black people, including North America, Brazil, and sub-Saharan Africa [24]. Since studies suggest that genetic influences can explain the higher frequency in white populations [5], it is possible that the variation in the *MTHFR* gene is a factor, in agreement with the results presented in our study.

Physical activity is considered a risk factor for ALS due to the increase in energy expenditure associated with oxidative stress (discussed earlier in this study). Moreover, the relationship between physical activity and genetic contributions in ALS susceptibility has been discussed [61]. In this context, the results obtained in this study show an interaction between physical activity and *MTHFR* C677T polymorphism in disease susceptibility. This interaction perhaps results from the possibility that both of these risk factors contribute to oxidative stress.

ALS is a rapidly progressing disease and has a median survival time of three to five years. However, there are discrepant cases in which survival exceeds ten years, as was the case of the famous physicist Stephen Hawking [112, 113]. The precise causes of these longer survival cases are still unknown; however, genetic and environmental factors are possible causes [114].

In this study, we performed a comparative analysis between the C677T *MTHFR* polymorphism and the death, which showed a statistical trend towards more significant ALS progression and early death in patients with the heterozygous (C/T) genotype compared to the wild genotype (C/C) ($p = 0.06$) (Fig. 2). Until the data collection period, there were no cases of death with the genotype (T/T). A study by Tsang et al. [115] reports that the heterozygous genotype (C/T) exhibited the most significant effect of *MTHFR* C677T polymorphism on decreasing blood folate, which surprisingly corroborates our data. This result suggests a higher pathogenic risk and earlier death in heterozygous (C/T) than mutant homozygous (T/T) genotypes.

Conclusion

There is ample evidence that folate deficiency and *MTHFR* C677T polymorphism are associated with the development of various disorders, including neurodegenerative diseases. This polymorphism is known to result in hypomethylation, HHcy, aberrant stimulation of nucleotide synthesis, oxidative stress, and cell death, which may be implicated in the pathogenesis and progression of ALS neurodegeneration.

We have found that the *MTHFR* C677T polymorphism is possibly implicated in the pathogenesis of ALS and is a risk factor for the development and progression of the disease. Furthermore, we found a risk interaction of the mutant genotype with sociodemographic and clinical factors, which increases susceptibility to the disease. Moreover, within the modifiable factors, alcohol consumption was a possible risk factor, independently of genotype.

Since ALS is a fast-progressing disease without a definitive diagnosis and effective treatment, the results of this study are highly relevant in the search for a possible biomarker for its diagnosis and prognosis. Such a biomarker could serve as a complementary tool in early diagnosis and improve the effectiveness

of riluzole treatment. This study also highlights the possible relationship between folate cycle dysregulation to the pathogenesis of the disease; however, further studies are needed to elucidate how these factors contribute to ALS pathogenesis.

Declarations

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Author contributions

Conceptualization: Rômulo Morais Azevedo, Angela Adamski da Silva Reis and Rodrigo da Silva Santos, Methodology and Validation: Rômulo Morais Azevedo, Kamilla de Faria Santos, Rayana Pereira Dantas de Oliveira, Júllia Costa Pereira, Dhiogo da Cruz Pereira Bento, Angela Adamski da Silva Reis and Rodrigo da Silva Santos, Formal analysis: Rômulo Morais Azevedo, Kamilla de Faria Santos, Rayana Pereira Dantas de Oliveira and Júllia Costa Pereira, Investigation: Rômulo Morais Azevedo, Kamilla de Faria Santos, Rayana Pereira Dantas de Oliveira, Júllia Costa Pereira, Dhiogo da Cruz Pereira Bento, Angela Adamski da Silva Reis and Rodrigo da Silva Santos, Resources: Rômulo Morais Azevedo, Angela Adamski da Silva Reis and Rodrigo da Silva Santos, Data curation: Rômulo Morais Azevedo, Kamilla de Faria Santos, Rayana Pereira Dantas de Oliveira, Júllia Costa Pereira, Angela Adamski da Silva Reis and Rodrigo da Silva Santos, Writing: Rômulo Morais Azevedo, Angela Adamski da Silva Reis and Rodrigo da Silva Santos, Writing-review and editing: Rômulo Morais Azevedo, Kamilla de Faria Santos, Júllia Costa Pereira, Angela Adamski da Silva Reis and Rodrigo da Silva Santos, Visualization: Rômulo Morais Azevedo, Kamilla de Faria Santos, Rayana Pereira Dantas de Oliveira, Júllia Costa Pereira, Angela Adamski da Silva Reis and Rodrigo da Silva Santos, Supervision and Project administration: Angela Adamski da Silva Reis and Rodrigo da Silva Santos, Funding acquisition: Angela Adamski da Silva Reis and Rodrigo da Silva Santos.

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Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflict of interest or no financial conflict of interest to disclose.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by the Ethical Committee of Federal University of Goiás (protocol: CAAE 79593117.7.0000.5083).

Informed consent: Patients signed their written informed consent, which was approved by the Ethical Committee of Federal University of Goiás (protocol: CAAE 79593117.7.0000.5083). The informed consent was obtained from the patient(s) and/or legally authorized representative (LAR) signed informed consent regarding publishing their data.

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Figures

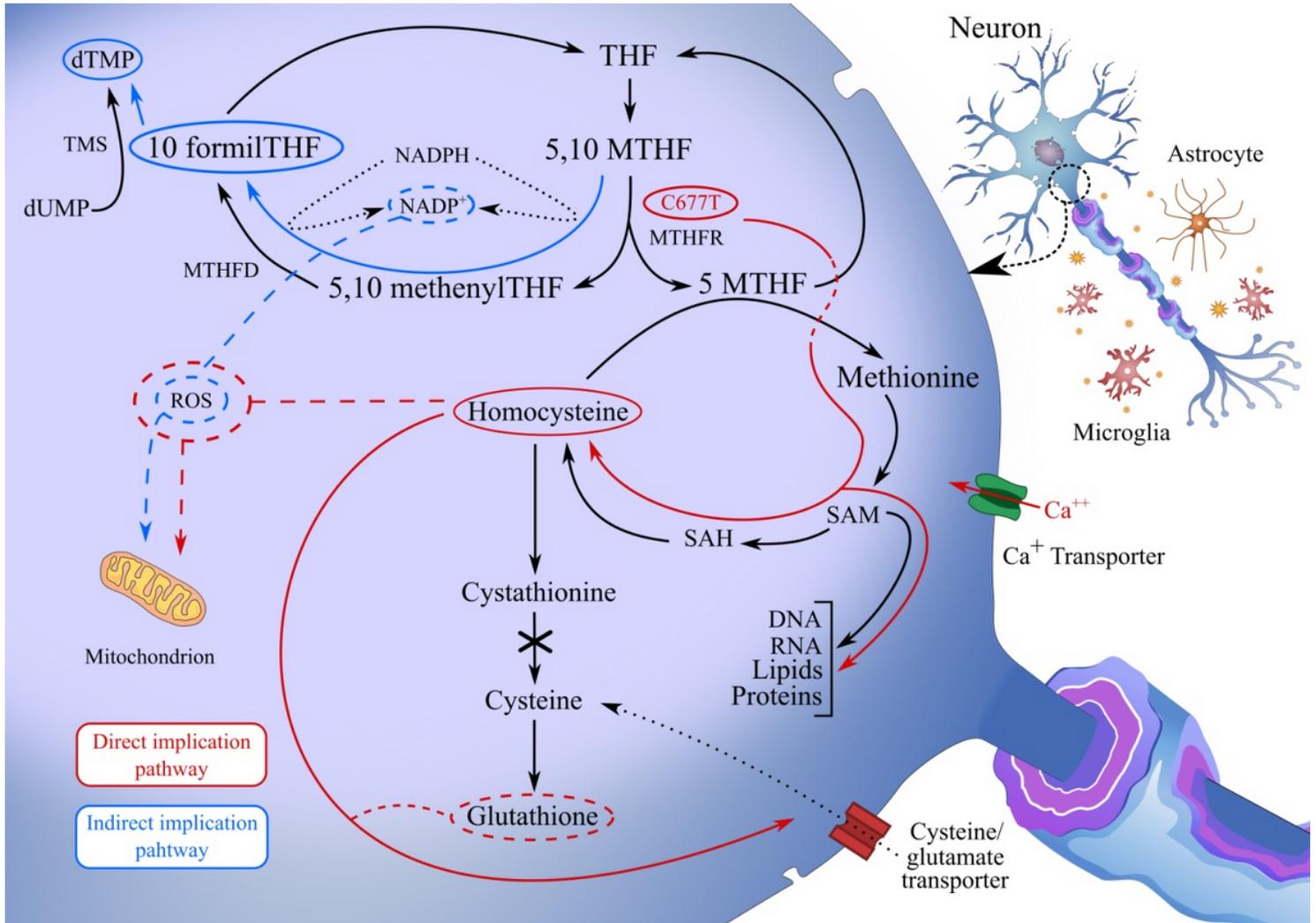


Figure 1

Hypothetical Model: MTHFR C677T polymorphism and possible folate cycle implication pathways.

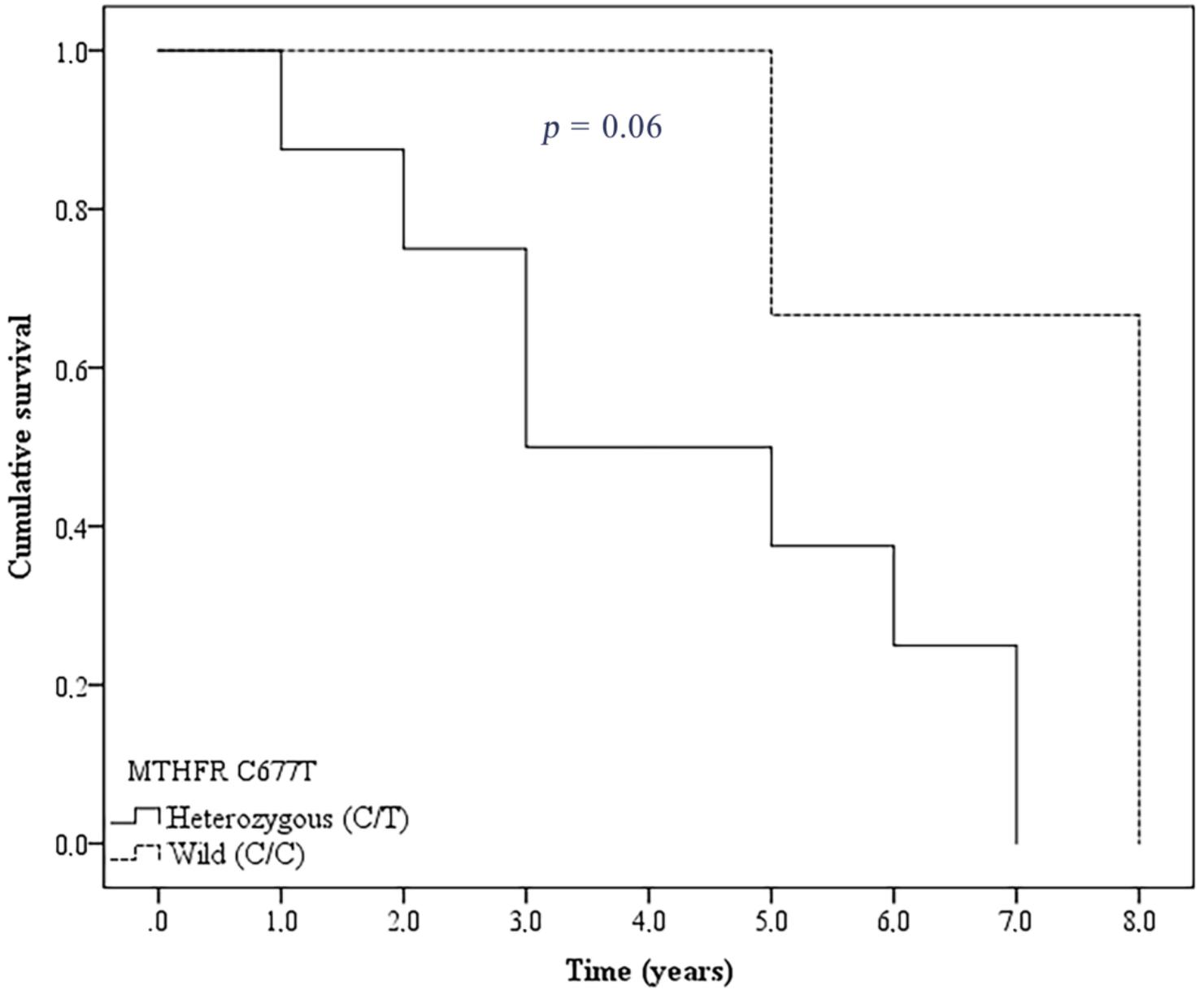


Figure 2

Kaplan-Meier curve analyzing survival at the onset of symptoms to outcome according to (C/C) and (C/T) MTHFR C667T polymorphism.