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MTHFR Polymorphisms and Plasma Homocysteine in Early-Onset Alzheimer's Disease: A Case-Control Study

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Early-onset Alzheimer's disease (EOAD) constitutes 1-2% of all Alzheimer's cases, presenting with poorer prognosis, progressive symptoms, and reduced life expectancy compared to late-onset Alzheimer's, thereby increasing socioeconomic burden. Elevated plasma homocysteine levels due to MTHFR gene polymorphisms are implicated in Alzheimer's etiology. The present study aims to explore the association between MTHFR gene polymorphisms in Sudanese population. Seventy-three EOAD patients were assessed for MTHFR C677T and A1298C polymorphisms, alongside plasma homocysteine levels. Significant associations were observed between CT and TT alleles, elevated plasma homocysteine levels, and EOAD. MTHFR C677T polymorphism was associated in EOAD in Sudanese population. Elevated plasma homocysteine levels might frame this association and potentially contribute to the disease onset before the age of 65.

Keywords: A1298C, C677T; Allele; Alzheimer's disease, Early-onset; Heterozygous; Homozygous; MTHFR, Polymorphism.

Alzheimer's disease (AD) is an age-accelerated progressive neurodegenerative disorder characterized by a decline in both behavioural and cognitive functions[1,2]. The disease is the most common type of dementia in individuals aged 65 years or above[2,3], accounting for 50-70% of all cases[4]. Globally, dementia accounted for 3.9 % in people aged 65 years or above[5]. Regionally, the lowest prevalence of dementia was reported in Africa (1.6%), followed by China and Western Pacific regions (4.0%). In Latin America, it accounted for 4.6% of cases, The highest prevalence was reported in North America (6-4%) and Western Europe (5.4%)[5].

Alzheimer's disease is classified as early-onset Alzheimer's disease (EOAD) and late-onset Alzheimer's disease (LOAD); based to the onset of the disease under or over 65 years old[6], respectively. About 10-30% of individuals aged above 65 years develop late-onset Alzheimer's disease[7]. Early-onset Alzheimer's disease, on the other side, represented 5-10% of all cases of AD[8].

In other studies, however, the prevalence was as low as 3%[9]. The mean age for AD patients varied among different populations. In one study, the mean age was 72 ± 5 years for LOAD and 60 ± 4 years for EOAD[10]. In another study, the mean age for LOAD and EOAD was 74 ± 6 years and 56 ± 5 years, respectively[11].

Literature has already established the correlation between gender and AD, with highest rates detected in women than men[12]. This gender-related variation was attributed to hormonal and psychological factors[12]. In an attempt to find an explanation, some studies have suggested a protective role of the female sex hormone oestrogen against neuronal degeneration, and hence postmenopausal women have a greater risk of AD[13].

Clinically, Alzheimer's disease is characterized by a progressive decline in episodic memory and cognitive function parameters such as language, attention, reasoning, memory, comprehension, judgement, execution, and visuospatial abilities[14]. The disease has three stages which take place in a chronological manner. The first stage is called the preclinical or asymptomatic stage, owing to the asymptomatic nature of the stage. The second stage is the cognitive impairment stage, which is followed by the third stage of dementia[2]. It is worth mentioning that the clinical presentation of early-onset and late-onset disease is almost the same[14]. A notable difference is that patients with the early-onset disease tend to preserve the episodic memory function rather than the cognitive function[14]. Additionally, patients of the early-onset disease tended to show a more aggressive course with more frequent pathological patterns and a relatively shorter life span[14].

Generally, AD is diagnosed on clinical bases as the disease pathology can be seen in otherwise asymptomatic individuals. Moreover, disease biomarkers like plasma and cerebrospinal fluid markers[2] were unable to detect all abnormalities in amyloid peptide and tau protein[15]. The clinical criteria for the diagnosis of dementia and Alzheimer's disease were both implemented in the diagnosis of AD[16].

The aetiology of Alzheimer's disease is not fully understood[16] and is thought to have a multifactorial nature[17]. This is applied, in particular; to cases of LOAD[18]. The main pathological abnormalities in AD included the extracellular accumulation of amyloid-beta (Aâ) plaques and the intraneuronal deposition of tau proteins (p-tau)[19]. Other abnormalities included the synaptic and neuronal loss[20], the vascular dysfunction[9], and the brain atrophy[21]. Furthermore, infection[22], genetic susceptibility[23], and mutations[23] were all incriminated in the disease pathology. Other factors included abnormal acetylcholine in the brain, insulin resistance, vascular dysfunction, oxidative stress, mitochondrial dysfunction and inflammation[9]. In addition, several risk factors for the disease were identified, including advanced age[18], ethnicity[24], diabetes[9], Down syndrome[9], cardiovascular and cerebral diseases[9], environmental factors[9], smoking[25] and alcohol consumption[25].

Genetically, the å4 allele of the APOE gene is the most common identified mutation associated with the late onset disease[8]. For the early-onset disease, almost all cases have familial nature[9], with 30-60% of cases have at least one affected first-degree relative[26], and 10% have autosomal dominant pattern of inheritance[8]. The familial pattern of the disease was attributed to mutations affecting Aâ precursor protein (APP) gene, Presenilin 1 (PSEN1) gene, and Presenilin 2 (PSEN2) gene[9]. Those genes are affected by over 400 known mutations[8].

Pathologically, the mutations are thought to increase not only the production of â-amyloid proteins[23] but also its deposition[8]. Other suggested mutations include those affecting APOE, BIN1, SORL1, CLU, TREM2[9], and ABCA7[27] genes. The methylene tetrahydrofolate reductase (MTHFR) gene has three polymorphisms associated with AD: C677T, A1298C, and A1793G[28]. MTHFR C677T was reported as the most significant of these mutations[28]. Regarding the other two polymorphisms, it was hypothesized that they protect against the development of AD[28]. Furthermore, MTHFR C677T was proposed as a risk factor for AD in a study on Asians[29], and in a meta-analysis study[30].

Both the heterozygous (CT) and homozygous alleles (TT) of MTHFR C677T polymorphism were associated with an increased risk for AD[31]. Interestingly, a study on Chinese population concluded that both MTHFR C677T and A1298C polymorphisms were associated with the late onset disease[28]. This conclusion

was supported by a case study which detected both C677T and A1298C polymorphisms in a patient diagnosed with early-onset Alzheimer's disease[32]. Moreover, another study found that A1298C polymorphism, but not MTHFR C677T; was associated with the disease[33]. Among the theories that explained the role of MTHFR C677T polymorphism in the development of AD were the white matter signal abnormalities caused by hyperhomocysteinemia[34], the impaired function and deposition of the neuronal amyloid-â protein precursor (AâPP) caused by the polymorphism-induced phosphorylation of amyloid- â protein precursor[35], and the impaired cognitive function caused by the low folate levels secondary to MTHFR C677T polymorphism[36].

Methylene tetrahydrofolate reductase (MTHFR) is an enzyme that catalyses the biochemical conversion of 5,10-methylenetetrahydrofolate to 5-methyltrahydrofolate[37]. The 5-methyltetrahydrofolate is a coenzyme in the formation of methionine from homocysteine[37]. The gene encoding the enzyme is located on the short arm of chromosome 1 (1p36.3)[37]. More than 40 single nucleotide polymorphisms in MTHFR gene were described[38], the most common being C677T and A1298C[39]. MTHFR C677T polymorphism results in the formation of a thermolabile enzyme[40]. Accordingly, enzyme activity will be diminished, resulting in elevated levels of homocysteine, a condition known as hyperhomocysteinemia[41]. MTHFR C677T polymorphism and the resulting hyperhomocysteinemia were significantly associated with the development of several diseases and syndromes including subacute combined degeneration of the spinal cord[42], cardiac syndrome X[43], hypertrophic cardiomyopathy[44], cleft lip/palate[45], â-thalassemia[46], preeclampsia[47], diabetes mellitus[48], rectal cancer[49], stroke[50], neural tube defects[51], psoriasis[52], late-onset Alzheimer's[53], and bipolar disorders[54]. Literature has reported a global frequency of MTHFR 677CT of about 25%. Regionally, the highest frequency was 47%, reported among Hispanics. The frequency dropped to 36% in Europeans, 30% in East Asians, 12% in South Asians, and finally 9% among Africans[55]. Additionally, the frequency of the homozygous and heterozygous alleles in the general population was 8.5% and 2.5%, respectively[56].

Currently, there is no treatment available for AD[19]. The life expectancy of the disease depends on age, gender, and genetics[19] and is relatively shorter in EOAD than LOAD[14]. Furthermore, the early-onset disease has a poorer prognosis than the late-onset disease as its course is more progressive[14]. Accordingly, EOAD has a greater socioeconomic burden[14] given that the associated responsibilities like raising children and working[53]. Moreover, EOAD is associated with a considerable delay in diagnosis[54], reflected as higher rates of premature mortality[6].

MATERIALS AND METHODS

Study type and population

This case-control study was conducted among Alzheimer's patients in Khartoum, Sudan, during the period from May 2021-May 2022. Participants were recruited from different neuropsychiatric and healthcare facilities in Khartoum. The sample size was calculated based on a 95% confidence interval, a 5% margin of error, an estimated 5% population proportion, and a population size of 6000 citizens, assuming a normal sample distribution[57]. The population proportion was taken as 5% based on previous well-designed review study[8]. The following formula was used to calculate the sample size[58]:

$$n = \frac{t\alpha 2 \; x \; p \; x \; q \; x \; N}{(N-1) \; X \; e2 + t\alpha 2 \; x \; p \; x \; q}$$

whereas:
n = sample size
N = population size
p = expected percentage of the variable
q = 1-p
e = accepted margin of error
$t\alpha$ = 1.96 for 95% confidence interval.

Accordingly, a total of 73 patients; clinically diagnosed with EOAD, were enrolled using simple random sampling. All patients diagnosed by expert neurologists and met the clinical criteria for diagnosis of dementia[15], and AD clinical stages[15] during the study period were included in the study. The control group included 73 healthy subjects recruited from individuals attending the same facilities for other reasons.

Data collection

Sociodemographic data and medical data related to Alzheimer's disease were collected from

the hospital's medical records after obtaining an ethical approval.

DNA extraction and PCR

Whole blood was collected, and DNA was extracted using Qiagen kits (FlexiGene DNA Kit)[59]. DNA was amplified by polymerase chain reaction using GeneAmp PCR kit (PerkinElmer Cetus)[60]. The 198 bp amplified fragments were digested with HinfI endonuclease which identifies C-T substitution. Accordingly, fragments were cut into 175 and 23 bp fragments. Fragments were then electrophoresed in polyacrylamide gel and stained with ethidium bromide[43]. The wildtype (CC) allele appeared as a 198 bp band whereas the homozygous (TT) allele appeared as a 175 bp band. The heterozygous (CT) allele appeared as two bands (198 and 175 bp)[43]. For A1298C polymorphism, the amplified 163 bp was digested with MboII. The AA genotype produced 5 fragments of 56, 31, 30, 28 and 18 bp. The homozygous (CC) allele gives 4 fragments of 84, 31, 30 and 18 bp whereas the heterozygous (AC) allele produced 6 fragments of 84, 56, 31, 30, 28 and 18 bp[43].

Plasma homocysteine

Plasma homocysteine level was measured using Human Homocysteine (HCY) ELISA Kit[61] . EDTA tube was used to collect blood. sample was immediately Centrifuged for 15 minutes at 1000 x g, 2-8°C. Plasma homocysteine levels over 15 micromole/l were considered as hyperhomocysteinemia[62].

Data presentation and analysis

Descriptive data were presented as means and standard deviations for quantitative variables and frequencies for qualitative variables. Results were statistically analysed using the SPSS (18th version). Chi-Square test was used to compare gender and the distribution of MTHFR C677T genotypes between EOAD patients and controls. The two-tailed t-test was used to compare means of plasma homocysteine between AD cases and control subjects. Analysis of variance (ANOVA) test was used to correlate plasma homocysteine with the distribution of MTHFR C677T genotypes. The p-value was considered significant when d" 0.05.

Ethical approval

The study was ethically approved by the local ethical committee at Faculty of Medicine, El-Neelain University, and the Ministry of Health, Sudan.

Table 1. Mean age of EAOD patients

Count, n	73
Sum, Σx	4221
Mean, x	57.821917808219
Variance, s2	9.9539573820396
Standard deviation	3.1549892839817
Standard error of mean (SEM)	0.36926356518872
Confidence of interval	95%, 1.960sx
Margin of error (confidence interval 95%)	57.8219 ±0.724 (±1.25%)

Table 2. Sex distribution among EOAD cases and controls

		Males	Females	Raw totals	
Cases	Observed	20 (27.4%)	53 (72.6%)	73	df = 1
	Expected	33	40		p-value = 0.000
	Chi Square contribution	5.1212	4.225		Chi Square = 18.6924
Controls	Observed	46 ()	27	73	
	Expected	33	40		
	Chi Square contribution	5.1212	4.225		
Column totals		66	80	146	

RESULTS

Demographic Data

The current study recruited 73 Sudanese patients with early-onset Alzheimer's and other 73 control subjects. The mean age of participants was 57.9 years (table 1). With regards to gender, males represented 27.3% and 53 females represented 72.4% (table 2). The difference between cases and control subjects was significant (p=0.000).

Plasma Homocysteine

Table 3 shows that the mean plasma homocysteine level was 15.5 micromole/l among cases and 13.01 micromole/l among control subjects. The difference in the mean plasma homocysteine level between cases and control subjects was significant (p=0.0001).

MTHFR C677T Genotypes

Regarding MTHFR C677T genotype distribution among cases of EOAD, we found that CC, CT, and TT frequencies were 24.7%, 27.4% and 47.9% respectively. Genotype frequencies among control subjects were 78%, 12.3%, and 9.6%, respectively (table 4). The difference in genotype frequencies between cases and control subjects was statistically significant (p=0.0001). Table 5 shows the mean age among different MTHFR C677T genotypes of EOAD patients. Statistical analysis was significant (p= 0.000). The gender distribution of MTHFR C677T genotypes among cases of EOAD was shown in table 6. The difference among MTHFR C677T genotypes was significant (p= 0.000). Moreover, we compared the plasma homocysteine levels among MTHFR

Table 3. Mean plasma homocysteine levels among EOAD and controls

AD	Count, N:	73	df = 144
	Sum, Óx:	1129	Standard error of
	Mean, x:	15.465753424658	difference = 0.172
	Variance, s2:	1.8356164383562	t = 14.221F
	Standard deviation	1.3548492308579	two-tailed
	Standard error of mean (SEM)	0.1585731082574	p value = 0.000195%
	Confidence of interval	95%, 1.960sx	CI = 2.1112572999529675
	Margin of error	15.4658 ±0.311 (±2.01%)	-2.7928522890890335.
Control	Count, N:	73	
	Sum, Óx:	950	
	Mean, x:	13.013698630137	
	Variance, s2:	0.33453196347032	
	Standard deviation	0.57838738183878	
	Standard error of mean (SEM)	0.067695122694177	
	Confidence of interval	95%, 1.960sx	
	Margin of error	13.0137 ±0.133 (±1.02%)	

Table 4. MTHFR C677T genotype distribution among EOAD cases and controls

		CT	TT	CC	Raw Totals	
Cases	Observed	20 (27.4%)	35 (47.9%)	18 (24.7%)	73	df = 2
	Expected	14.5	21	37.5		p-value = 0.0001
	Chi Square contribution	2.0862		9.3333	10.14	Chi Square = 43.1191
Controls	Observed	9 (12.3%)	7 (9.6%)	57 (78%)	73	
	Expected	14.5	21	37.5		
	Chi Square contribution	2.0862	9.3333	10.14		
Column Totals		29	42	75	146	

C677T genotypes (table 7). Statistical analysis found the difference between genotypes to be significant (p= 0.000).

MTHFR A1298C genotypes

Regarding MTHFR 1298C, we reported frequencies of 38.4%, 27.4%, and 34.2% for AA, AC, and CC genotypes, respectively. Genotype frequencies among control subjects were 35.6%, 32.9%, and 31.5%, respectively (table 8). There was no statistical difference between cases and control subjects (p= 0.6228).

DISCUSSION

In the current study, we explored the relationship between MTHFR C677T and A1298C polymorphisms and early (young)-onset Alzheimer's disease among Sudanese population. We recruited seventy-three subjects clinically diagnosed with EOAD who were attending or admitted to different neuropsychiatric healthcare facilities in Khartoum, Sudan. EOAD is defined as dementia that occurs before the age 65 years[6].

Table 5. Mean age among MTHFR C677T genotypes of cases

Group	N	Mean age	SD	
CT	20	57.95	0.6863	
TT	35	57.5143	0.7425	
CC	18	59.0556	0.8726	
	df	SS	F-statistic	*p*-value
Variation among samples	2	28	24.4160	0.000
Variation within samples	70	42		
Total	72	69		

Table 6. Gender distribution of MTHFR C677T genotypes among cases

		CT	TT	CC	Raw Totals	
Males	Observed	2 (10%)	5 (25%)	13 (65%)	20	df = 2
	Expected	5.4795	9.5890	4.9315		p-value = 0.000
	Chi Square contribution	2.2095	2.1962	13.2010		Chi Square = 25.2506
Females	Observed	18 (33.9%)	13 (24.5%)	5 (9.4%)	53	
	Expected	14.5205	25.4110	13.0685		
	Chi Square contribution	0.8338	0.8287	4.9815		
	Column Totals	20	35	18	73	

Table 7. Mean plasma homocysteine levels among MTHFR C677T genotypes

	N	MEAN	95% CI	
CT	20	15.1	(14.8565, 15.3435)	
TT	35	15.5714	(15.3874, 15.7555)	
CC	18	13.8333	(13.5767, 14.09)	
	df	SS	F-statistic	p-value
Variation among samples	2	36	60.5622	0.000
Variation within samples	70	21		
Total	72	57		

Table 8. MTHFR A1298C genotype distribution among EOAD cases and controls

		AC	CC	AA	Raw totals	
Cases	Observed	20 (27.4%)	25 (34.2%)	28 (38.4%)	73	df = 3
	Expected	22	24	27		p-value = 0.6228
	Chi Square contribution	0.1818	0.0417	0.037		Chi Square = 1.764
Controls	Observed	24 (32.9%)	23 (31.5%)	26 (35.6%)	73	
	Expected	22	24	27		
	Chi Square contribution	0.1818	0.0417	0.037		
	Column totals	44	48	54	146	

This entity of AD is of particular clinical and socioeconomic importance owing to its poor prognosis, early presentation, and tremendous consequences on life quality[14]. Clinically, EOAD differs from LOAD in some respects. Firstly, EOAD tends to preserve the episodic memory function but not the cognitive impairment[14]. Secondly, EOAD tends to have more aggressive course and relatively shorter life span than LOAD[14]. Thirdly, the disease is often underestimated and misdiagnosed[54], which was reflected as relatively higher mortality rates[6]. The global frequency of EOAD was 24.2 in 100000 (0.2%)[6]. Despite the low frequency, EOAD is considered was considered as the most common dementia before the age 65 years[6]. Because the study belonged to the case-control entity, we were not able to compute the prevalence of EOAD in Sudanese population.

The mean age of EOAD patients in our study was 57.9 ±3 years, which was within the range given in the definition of EOAD[6]. The literature review in this regard suggested a variation in the mean age of EOAD even within the same population. For an instance, the mean age in one Dutch study was 60 ± 4 years[10]. In another Dutch study, the mean age was 56 ± 5 years[11]. This variation in our opinion, depended on the period after which an established diagnosis of EOAD was given. We usually expect a relatively later onset for the disease in developing countries due to the lack of modern diagnostic tools. However, the mean age for EOAD in our study did not vary much with literature in this context.

Our findings regarding gender suggested that females were more affected than males (72.4% vs. 27.6%). Our findings agreed with the general assumption that Alzheimer's disease occurs primarily in women as suggested by Mary[12] , despite the fact that some studies did not discriminate between males and females[12]. In her study, Mary has attributed this variation in gender to hormonal and psychological factors and pregnancy-related morbidities[12]. It was established that the female hormones oestrogen and progesterone increased the risk for dementia[12]. Mental disorders related to pregnancy such as psychosis and post-partum depression were also known to aggravate the cognitive impairment in AD[12].

When we looked into the mean age and gender together, we could see that our patients were mainly post-menopausal women. Therefore, our results supported the study by Geeske[13] who suggested that oestrogen may protect against neuronal degeneration, and hence postmenopausal women have a greater risk of AD than perimenopausal women or women taking hormone replacement therapy. From our point of view, the lack of oestrogen in our patients might have played a role in the acceleration of the neuronal degeneration seen in AD patients. Our findings confirmed that age and gender have altered the correlation between MTHFR C677T polymorphism and EOAD to some degree.

The frequency of MTHFR C677T in the current study was 39.7% for the heterozygous allele and 57.5% for the homozygous allele. Both frequencies were within the proposed range for the *t* alle prevalence of 12-57%[28]. The current study concluded that both the homozygous (TT) and the heterozygous alleles (CT) of MTHFR C677T polymorphism were associated with EOAD in Sudanese population. Because the mutation

was associated with the early onset disease, our findings supported the conclusion drawn by Yaling[28] that MTHFR C677T polymorphism might advance the onset of the disease. The current study, however, did not find a significant association between the disease and A1298C polymorphism, supporting the fact that MTHFR C677T is the most clinically significant polymorphism associated with AD among the three polymorphisms[28]. Our study, however, could not finalize whether this polymorphism might be considered as a risk factor for AD or not[29,30]. Our results also confirmed the findings of the study by Ye Hu[31] who concluded that both the heterozygous and homozygous alleles of MTHFR C677T were associated with AD.

Considering A1298C polymorphisms, we could not totally agree with the case study by Leila[32] who detected both C677T and A1298C polymorphisms in a patient with EOAD[32]. Furthermore, we could not support or deny whether A1298C polymorphism has a causative[33], or a protective[28] role in the pathogenesis of AD[28]. Because EOAD is basically multifactorial, we could not conclude that C677T polymorphism was the sole etiology behind AD[4].

The current study has confirmed a significant elevation in plasma homocysteine levels among cases, suggesting a link between MTHFR 677T mutation and AD. It was already proven that MTHFR C677T polymorphism causes hyperhomocysteinemia[41], which is associated with AD[53]. Furthermore, it was already established that elevated homocysteine levels might induce white matter signal abnormalities[34] that can be responsible for the impaired cognitive function in patients with AD[34]. This assumption, however, was not conclusive because of the multifactorial nature of AD and the fact that both genetic and environmental factors together decide who will get the disease[4]. The low folate probably due to MTHFR C677T polymorphism was thought to ameliorate the cognitive impairment seen in patients with AD[36]. It was suggested that hyperhomocysteinemia might be caused by factors other than MTHFR C677T polymorphism. These factors included low folate and vitamin B_{12} levels, aging, smoking and renal impairment[34]. Because we did not measure folate and B_{12} levels in our participants, we could not confirm whether low folate level has contributed to the cognitive impairment or not. Moreover, we could not exclude low folate as a cause of hyperhomocysteinemia. Interestingly, our findings clashed with one study by Luchsinger[40] who found no significant association between high plasma homocysteine levels and AD. We concluded that individuals with elevated plasma homocysteine levels were more likely to develop EOAD than individuals with normal homocysteine levels.

We have also looked into the means of plasma homocysteine levels within patients having CC, CT, and TT genotypes. Our results suggested that homocysteine levels were significantly elevated in the group with the homozygous allele, followed by the heterozygous allele, whereas levels were within the normal range in the group having the wildtype allele. This finding supported the conclusion drawn by Munshi[38] who reported higher plasma homocysteine levels in the TT genotype, followed by the CT then the CC genotype.

CONCLUSION

In conclusion, the frequency of EOAD among Sudanese population was as low as 0.0012%. The mean age of EOAD patients was 57.9 ±3 years. Females were more commonly affected than males due to hormonal and psychological variation. The frequency of MTHFR C677T in the current study was 39.7% for the heterozygous allele and 57.5% for the homozygous allele. Both the homozygous and heterozygous alleles were associated with EOAD. It appears that the mutation might advance the onset of the disease and the elevated plasma homocysteine levels might be partially responsible for the neurodegenerative abnormalities seen in those patients. Our results suggested that homocysteine levels were significantly increased in the group with the homozygous allele, followed by the heterozygous allele, whereas levels were within the normal range in the group having the wildtype allele. MTHFR A1298C polymorphism was not associated with EOAD in the study. Individuals with family history of Alzheimer's disease may need to be screened for this mutation as early as possible, so that proper preventive and therapeutic measures could be implemented once the polymorphism is detected. MTHFR C677T-positive individuals should be considered at-risk for progression to Alzheimer's disease.

The study was without limitations. We could not exclude all factors that might elevate plasma homocysteine levels like diet, low folate and B_{12} levels. Additionally, we were unable to establish a direct effect or causation as a case-control study. Moreover, the study might be less useful for examining the risk factors for such a rare disease as in cohort studies.

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Conflict of Interest

The authors do not have any conflict of interest

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