Effect of Methylenetetrahydrofolate Reductase (MTHFR) gene mutations and oxidative stress in silent brain infarction

Pınar Aslan Koşar
Suleyman Demirel Universitesi Tip Fakultesi
Muhammet Yusuf Tepebaşı (gultepe74@windowslive.com)
Süleyman Demirel University https://orcid.org/0000-0002-1087-4874
Nihat Şengeze
Suleyman Demirel Universitesi Tip Fakultesi
İlter İlhan
Suleyman Demirel Universitesi Tip Fakultesi
Halil İbrahim Büyükbayram
Suleyman Demirel Universitesi Tip Fakultesi
Süleyman Kutluhan
Suleyman Demirel Universitesi Tip Fakultesi

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Abstract

Ischemic infarctions occur under the influence of genetic and environmental factors. In our study, the role of ischemia-modified albumin and thiol balance, which are new markers in determining oxidative damage together with MTHFR gene mutations and homocysteine levels, in the development of SBI was investigated. White matter lesions in the magnetic resonance imaging (MRI) results of the patients were evaluated according to the Fazekas scale and divided into groups (Grade 0, 1, 2, and 3). Homocysteine, folate, B12, IMA, total thiol, and native thiol were measured by biochemical methods. The mutations in MTHFR genes were investigated by the RT-PCR method. According to our results, a significant difference was found between the groups in age, homocysteine, folate, IMA, total thiol, and native thiol parameters ($p<0.05$). When we compared the groups in terms of genotypes of the C677T gene, we found a significant difference in TT genotype between grades 0/3 and 1/3 ($p<0.05$). We determined that homocysteine and IMA levels increased and folate levels decreased in CC/TT and CT/TT genotypes in the C677T gene ($p<0.05$). Considering our results, the observation of homocysteine and IMA changes at the genotype level of the MTHFR C677T gene and between the groups, and the deterioration of thiol balance between the groups suggested that these markers can be used in the diagnosis of silent brain infarction.

Introduction

Silent brain infarcts (SBIs) have been defined as ischemic lesions that are common in patients without a history of stroke, especially in the elderly and some other populations at risk [1, 2]. SBI is widely used to identify cerebral infarctions seen on magnetic resonance imaging (MRI) without neurological signs or a history of transient ischemic attacks [3]. Studies have shown that the prevalence of silent brain infarctions varies from 8–28% depending on age, and this rate increases to 38% in patients with stroke [4, 5]. It has been determined that risk factors such as age, ethnic factor, metabolic syndrome, hypertension, and diabetes are risk factors and increase the frequency of silent brain infarctions [6, 7]. Patients with silent brain infarction may have cognitive deficits and the risk of developing dementia is doubled. Also, it was determined that the risk of recurrent stroke in elderly patients with lesions due to free brain infarction increased three times [2, 8].

Stroke is a polygenic multifactorial disease that occurs as a result of occlusion in the vessels with the effect of genetic and environmental factors [9, 10]. There are studies on different prothrombotic gene mutations that are thought to be effective in the etiopathogenesis of cerebrovascular diseases. These studies focus on the methylenetetrahydrofolate reductase (MTHFR) C677T and A1298C mutation [11–14]. The human MTHFR gene is localized on chromosome 1p36.3 and encodes the MTHFR enzyme consisting of 656 amino acids. Mutation in MTHFR genes decreases this enzyme activity. Plasma homocysteine level increases as a result of the decrease in MTHFR enzyme activity [15–17]. In severe MTHFR deficiency in which hyperhomocysteinemia and homocystinuria occur, clinical features such as peripheral neuropathy, growth retardation, hypotonia, stroke, and thrombosis are observed. Also, cases of mild MTHFR deficiency are quite common in the population, and it is claimed to be a risk factor especially in the formation of arterial diseases [18, 12, 19].

Enzymes involved in homocysteine metabolism use B12 and folic acid as cofactors. Deficiencies of these vitamins together with MTHFR gene mutations constitute the most common cause of hyperhomocysteinemia [20, 21]. Studies conducted to explain the relationship between hyperhomocysteinemia and thrombosis and stroke hypothesize that homocysteine is a highly reactive amino acid and enhances oxidation, which is toxic to endothelial cells and can cause thrombosis [22]. Hyperhomocysteinemia makes this effect by showing a cytotoxic effect on the endothelium through oxidative damage. This oxidative effect occurs when homocysteine is oxidized in plasma and transforms into hydrogen peroxide and superoxide [23].

Ischemia-modified albümin (IMA) is the altered form of albumin as a result of oxidative stress and is defined as a new marker used in determining ischemic events [24]. Although IMA was first defined as a myocardial ischemic marker, studies have shown that it increases in non-cardiac oxidative stress and ischemia states, such as cerebrovascular diseases, inflammatory diseases, pulmonary embolism, and chronic liver diseases [24–28]. Besides, the thiol/disulfide balance developed by Erel et al. is used to evaluate the oxidant/antioxidant balance in oxidative damage [29]. Thiol balances oxidative stress by reducing the formation of reactive oxygen species or accelerating inactivation [30]. In oxidative stress conditions, while native thiol and total thiol values are expected to decrease, disulfide/thiol, disulfide / total thiol, and native thiol/ total thiol indices are expected to increase [29].

In the present study, we investigated the relationship between mutations in MTHFR genes, homocysteine levels, and oxidative stress in patients with SBI.

Material And Methods

Patient collection

103 patients with suspected SBI who were hospitalized in the Department of Neurology, Süleyman Demirel University Faculty of Medicine, were included in our study. Demographic information about the age and gender of the patients and cranial MRI results were recorded for evaluation. The blood of the patients was taken into EDTA and biochemistry tubes and removed to -80ºC for DNA isolation, folate, B12, total thiol, native thiol, and IMA.

Cranial images were obtained using 1.5 Tesla MR (MAGNETOM Avanto, Siemens Healthcare, Erlangen, Germany). SBI was defined as 3 mm and larger lesions using T2 weighted and FLAIR images. Patients with SBI were evaluated by two clinicians.

The Fazekas scale was used to define the load and location of the lesions. It was used to measure the burden of white matter T2 hyperintense lesions, mostly attributed to chronic small vessel ischemia. A grade was given depending on the size and confluence of the lesions. Lesions were rated as grade 0 = absence, grade 1 = “caps” or pencil-thin lining, grade 2 = smooth “halo,” or grade 3 = irregular white matter hyperintensity extending into the deep white matter [31, 32].

DNA isolation was done according to the manufacturer's protocol (High pure PCR template preparation, Roche Diagnostics, USA).
The DNAs obtained were removed to -80 ºC.

Real-time Pcr

MTHFR C677T, MTHFR A1298C gene mutation analysis was performed on Rotor-Gene Q Qiagen (Hilden, Germany) instrument according to the manufacturer's protocol as follows. The kit includes Primer Probe Mix, Master Mix (MTHFR677, MTHFR1298), Heterozygous PC, and Homozygous PC. For analysis, 2x Real-Time Master Mix 10µl, 5x Primer Probe Mix 4µl, Water (ddH2O) 3µl mixtures were prepared. 3µl of DNA sample was placed on a total of 17µl master mix and placed in the Rotor-Gene Q device. Program terms; 15 minutes 1 cycle at 95C, 40 cycles for 15 seconds at 95ºC, and 40 cycles for 45 seconds at 58ºC. Subsequently, the results were analyzed and wild type, heterozygous and homozygous mutations were detected.

Ischemia Modified Albumin (ima)

For serum IMA measurement, 200 µl of patient serum was mixed with 50 µl cobalt chloride and incubated for 10 minutes. After the incubation step, 50 µl of dithiothreitol (DTT) was added to the measuring cuvette to determine cobalt that did not bind to albumin, and mixed, allowing dithiothreitol to form a colored complex with cobalt not bound to albumin. After 2 minutes, 1 ml of saline was added to stop the reaction. The colored complex formed was measured spectrophotometrically at 470 nm wavelength. Again with the same method, the results obtained after the sample without DTT was zeroed against its blank was given as absorbance unit (ABSU).

Homocysteine

Total homocysteine level in serum samples was measured by using Axis-Shield Liquid Stable (LS) 2- Part Homocysteine kit with the spectrophotometric method in Beckman Coulter AU 5800 instrument. Results were reported in µmol/L.

B12 And Folate

Vitamin B12 and Folic acid levels in serum samples were studied with the Roche Cobas e601 hormone autoanalyzer instrument using the electrochemiluminescence immunoassay (ECLIA) method. Results are reported in B12 pg/mL and folate ng/mL.

Total Thiol And Native Thiol

Total Thiol and Native Thiol parameters were measured by the spectrophotometric method using Rel Assay Diagnostics Commercial Kits with Beckman Coulter AU5800 autoanalyzer instrument to evaluate serum disulfide and thiol amounts. Serum native thiol (-SH), total thiol (-SH + -S-S-) levels were determined and disulfide (-S-S-) was calculated.

Statistical analysis

The genotype frequency of the A1298C and C677T genes was performed using Arlequin v352 software [32]. The significance of genotype differences between groups formed according to the Fazekas scale of these genes was compared with the chi-square test with Yates

correction and Fisher's exact tests. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated as a measure of the relationship between genotypes of groups and genes.

The normality distribution of the data was calculated by the Kolmogorov-Smirnov Test. Statistical significance in numerical parameters was evaluated by the Kruskal Wallis-H Test in all groups.

Comparison between groups was made according to their number and normal distribution using Mann-Whitney U Test.
The chi-square test was used to compare nominal parameters. Pearson's or Spearman's analyzes were used in the correlation analysis. The strength of correlation was classified as weak (0.20-0.39), moderate (0.40-0.59), strong (0.60-0.79), or very strong (0.80-1.00). Statistical analyzes were performed at a 95% confidence interval using IBM SPSS 22.0 and $P < 0.05$ was considered significant.

Results

In our study, groups (Grade 0, 1, 2, and 3) were formed according to the Fazekas scale by evaluating the white matter T2 hyperintense lesions in the MR results of 103 patients (Table 1). There were 19 patients in grade 0, 58 patients in grade 1, 19 patients in grade 2, and 7 patients in grade 3. Gender distribution in the patient groups was determined as 12 males and 7 females in grade 0, 19 males and 39 females in grade 1, 6 males and 13 females in grade 2, and 3 males and 4 females in grade 3. There was no statistically significant difference between the groups in terms of gender (p = 0.107).

Table 1
Mean and statistical analysis of parameters in all groups.

Groups	Total	Age	Homocysteine	Folate	B12	İMA	Total Thiol	Native thiol	Disulfide
		Mean ± SD							
Grade 0 (n = 19)	103	35.3 ± 12.9	8.6 ± 2.8	8.8 ± 3.9	349 ± 152	0.303 ± 0,025	540 ± 115	428 ± 99	56 ± 15
Grade 1 (n = 58)		44.3 ± 10.6	9.9 ± 3.6	7.9 ± 3.1	341 ± 193	0.313 ± 0.018	476 ± 91	368 ± 71	54 ± 15
Grade 2 (n = 19)		49.0 ± 10.5	11.5 ± 2.0	6.7 ± 3.2	328 ± 124	0.320 ± 0.013	464 ± 90	358 ± 79	53 ± 13
Grade 3 (n = 7)		53.1 ± 7.2	15.7 ± 3.4	5.6 ± 1.5	270 ± 119	0.329 ± 0.010	413 ± 31	307 ± 39	53 ± 16
P value		0.001*	0.000*	0.017*	0.552	0.006*	0.015*	0.008*	0.855
SD: Standard deviation, * p-value statistically significant									

Statistical analyzes were performed by determining the age, homocysteine, folate, B12, IMA, total thiol, native thiol, and disulfide ratios in all groups. According to these results, a statistically significant difference was found in age, homocysteine, folate, IMA, total thiol, and native thiol parameters (p = 0.001, p = 0.000, p = 0.017, p = 0.006, p = 0.015, and p = 0.008, respectively) There was no significant difference in B12 and disulfide (p = 0.552 and p = 0.855) (Table 1). As a result of the statistical analysis for age between the groups, a significant difference was found between grade 0/1, grade 0/2, grade 0/3, and grade 1/3 (p = 0.006, p = 0.002, p = 0.003, and p = 0.039, respectively). There was no significant difference between grade1/2 and grade 2/3 (p = 0.129 and p = 0.395, respectively). When homocysteine was evaluated statistically between the groups, a significant difference was found between grade 0/2, grade 0/3, grade 1/2, and grade 1/3 (p = 0.000, p = 0.000, p = 0.008, and p = 0.001, respectively). There was no significant difference for grade 0/1 (p = 0.136). As a result of the statistical analysis of folate between the groups, it was determined that there was a significant difference between grade 0/2, grade 0/3, grade 1/2, and grade 1/3 (p = 0.025, p = 0.022, p = 0.038, and p = 0.023, respectively). There was no significant difference for grade 0/1 and grade 2/3 (p = 0.531 and p = 0.534, respectively). As a result of the statistical analysis of IMA between the groups, it was determined that there is a significant difference between grades 0/2, 0/3, and 1/3 (p = 0.018, p = 0.007, and p = 0.015, respectively). There was no significant difference in grade 0/1, grade 1/2, grade 2/3 groups (p = 0.091, p = 0.089 and p = 0.120, respectively). As a result of the statistical analysis of total thiol between the groups, it was determined that there is a significant difference between grades 0/1, 0/3, and 1/3 (p = 0.029, p = 0.006, and p = 0.026, respectively). There was no significant difference in grade 0/2, grade 1/2, grade 2/3 groups (p = 0.061, p = 0.671, and p = 0.209, respectively). As a result of the statistical analysis of native thiol between the groups, it was determined that there was a significant difference between grades 0/1, 0/3, and 1/3 (p = 0.023 p = 0.002 and p = 0.019, respectively). There was no significant difference in grade 0/2, grade 1/2, grade 2/3 groups (p = 0.050, p = 0.684, and p = 0.094, respectively).

In groups, normal, heterozygous, and homozygous allele frequencies of A1298C and C677T genes were determined and statistical analysis was made between groups. When the groups were compared in terms of A1298C gene mutation, no statistically significant difference was found. When the C677T gene mutation was evaluated in the groups, it was determined that there was a statistically significant difference in the TT genotype between 0/3, 1/2, and 1/3 groups (p = 0.04, p = 0.02, and p = 0.01, respectively) (Table 2).

Table 2
Distribution and statistical analysis of genotypes of A1298C and C677T genes between groups.

Genes	Groups	Genotypes Frequency			Association analyses	Genetic model [OR (95% CI)] / p-value		
A1298C		AA	AC	CC		AA	AC	CC
(n = 103)	0 (n = 19)	7 (36.8%)	11 (57.9%)	1 (5.3%)	Grade 0/1	(0.40–3.53) 0.78	(0.39–3.18) 1.0	(0.04–3.52) 0.67
					Grade 0/2	(0.17–2.37) 0.74	(0.8–11.2) 0.19	(0.02–2.36) 0.34
	1 (n = 58)	19 (32.8%)	32 (55.1%)	7 (12.1%)	Grade 1/2	(0.19–1.55) 0.28	(0.89–7.99) 0.11	(0.13–1.99) 0.45
					Grade 1/3	(0.33–26.03) 0.42	(0.19–4.50) 1	(0.05–2.11) 0.25
	2 (n = 19)	9 (47.4%)	6 (31.5%)	4 (21.1%)	Grade 2/3	(0.54–53.9) 0.19	(0.05–2.06) 0.37	(0.09–4.81) 1.0
	3 (n = 7)	1 (14.3%)	4 (57.1%)	2 (28.6%)	Grade 0/3	(0.35–35.4) 0.37	(0.18–5.9) 1.0	(0.01–1.9) 0.17
C677T		CC	CT	TT		CC	CT	TT
(n = 103)	0 (n = 19)	7 (36.8%)	11 (57.9%)	1 (5.3%)	Grade 0/1	(0.28–2.40) 0.79	(0.69–1.70) 0.79	(0.09–10.4) 1.0
					Grade 0/2	(0.40–6.51) 0.73	(0.42–5.49) 0.75	(0.02–1.49) 0.18
	1 (n = 58)	24 (41.4%)	31 (53.4%)	3 (5.2%)	Grade 1/2	(0.63–6.22) 0.28	(0.45–3.6) 0.79	(0.03–0.71) 0.02*
					Grade 1/3	(0.31–9.86) 0.69	(0.51–16.01) 0.26	(0.01–0.48) 0.01*
	2 (n = 19)	5 (26.3%)	9 (47.4%)	5 (26.3%)	Grade 2/3	(0.12–6.16) 1.0	(0.34–14.6) 0.65	(0.08–2.91) 0.63
	3 (n = 7)	2 (28.6%)	2 (28.6%)	3 (42.8%)	Grade 0/3	(0.22–9.61) 1.0	(0.52–22.4) 0.39	(0.01–0.91) 0.04*

OR: odds ratio, Cl: Confidence interval, * p-value statistically significant

The effects of normal (AA), heterozygous (AC), and homozygous (CC) genotypes of A1298C and C677T genes on homocysteine, folate, B12, IMA, total thiol, native thiol, and disulfide were investigated. When the CC genotype in the A1298G gene was compared to the AA and AC genotype, it was found that the TT genotype significantly increased homocysteine level (p = 0.025 and p = 0.004, respectively). Also, a significant difference was found in total thiol in terms of AC/CC (p = 0.024). When the TT genotype in the C677T gene was compared with CC and CT genotypes, a significant difference was found in homocysteine folate and IMA levels According to these results, the statistical significance level was found CC/CT (p = 0.001) and CT/TT (p = 0.001) for homocysteine, CC/CT (p = 0.002) and CT/TT (p = 0.001) for folate, CC/TT (p = 0.018) ve CT/TT (p = 0.003) for IMA (Table 3).

Table 3
The effect of A1298C and C677T gene mutations on homocysteine, folate, B12, IMA, total thiol native thiol and disulfide.

	A1298C						C677T					
	AA (n = 36)	AC (n = 53)	CC (n = 14)	AA/AC	AA/CC	AC/CC	CC (n = 38)	CT (n = 53)	TT (n = 12)	CC/CT	CC/TT	CT/TT
	Mean ± SD			P value			Mean ± SD			P value		
Homocysteine	10.3 ± 4.02	9.8 ± 2.9	12.9 ± 3.8	0.735	0.025*	0.004*	9.76 ± 3.14	10.1 ± 3.51	13.9 ± 3.4	0.527	0.001*	0.001*
Folate	7.9 ± 3.1	7.8 ± 3.2	12.9 ± 3.8	0.757	0.157	0.139	7.8 ± 3.2	8.2 ± 3.4	5.2 ± 1.4	0.426	0.002*	0.001*
B12	343 ± 108	344 ± 215	278 ± 76	0.288	0.053	0.209	347 ± 95	339 ± 219	281 ± 95	0.070	0.069	0.483
IMA	0.311 ± 0.02	0.315 ± 0.02	0.317 ± 0.01	0.364	0.304	0.883	0.314 ± 0.02	0.310 ± 0.02	0.327 ± 0.01	0.649	0.018*	0.003*
Total Thiol	488 ± 105	490 ± 96	434 ± 73	0.828	0.076	0.024*	477 ± 99	482 ± 89	497 ± 132	0.664	0.785	0.987
Native thiol	377 ± 81	381 ± 85	334 ± 63	0.963	0.092	0.058	370 ± 79	374 ± 78	376 ± 106	0.837	0.794	0.933
Disulfide	55 ± 17	55 ± 13	50 ± 16	0.682	0.218	0.287	53 ± 17	54 ± 11	60 ± 19	0.607	0.229	0.310

SD: Standard deviation, * p-value statistically significant

Correlation analysis was performed between parameters. According to the results of this analysis, a positive significant correlation was found between homocysteine-age and homocysteine-IMA levels (p = 0.012 r = 0.247, p = 0.000 r = 0.492, respectively). In addition, a significant negative correlation was found between homocysteine-folate, homocysteine-total thiol and homocysteine-native thiol (p = 0.000 r=-0.478, p = 0.002 r=-0.302 and p = 0.003 r=-0.291, respectively).

Discussion

Silent brain infarcts are ischemic lesions detected by MRI [33]. It is thought that endothelial dysfunction, chronic cerebral hypoperfusion, blood-brain barrier destruction, and impairment of auto-regulatory dysfunction of small vessels may be effective in the formation of these lesions [34]. SBI clinically lacks stroke-like symptoms but more than doubles the risk of subsequent stroke and dementia [8]. Silent brain infarctions develop multifactorial due to factors such as age, genetics, oxidative stress, and environmental factors [9].

As a result of the statistical analysis we performed with silent brain infarction patients, we found that age, homocysteine, and IMA increased, but folate, total thiol, and native thiol were decreased. Also, we could not find a significant difference in B12 and disulfide levels (Table 1). Previous studies have demonstrated that silent brain infarctions increase with age [4, 5]. There have also been studies showing that homocysteine levels increase with age [35]. Also, studies have shown that there is an increase in homocysteine in SBI patients [36]. In another study, it was shown that there is an increase in homocysteine in patients with ischemic stroke [37]. In a study conducted by Gunduz et al. in 2008, it was found that IMA levels were significantly higher in patients with cerebral infarction, brain hemorrhage, and subarachnoid hemorrhage [27]. Also, in the study conducted by Menon et al. in 2018, the IMA level was found to be significantly higher in patients with acute ischemic stroke [26]. In another study, when patients with ischemic stroke and hemorrhagic stroke were compared in terms of IMA levels, it was found to be higher in patients with ischemic stroke [38]. Similar to the results of our study, in the study conducted by Bektaş et al. in 2016, while total thiol and native thiol were found to be significantly lower in patients with acute ischemic stroke, they did not find any change in disulfide balance [39].

Also, in our study, we investigated the MTHFR genes that we think may affect the development of SBI and the effects of genotypes of these genes on homocysteine, IMA, folate, B12, total thiol, native thiol, and disulfide.

When we evaluated the MTHR A1298C gene among the groups in terms of genotype, we could not find a statistically significant difference. Also, when we compared the genotypes of the C677T gene between the groups, we found that there was a significant difference in the TT genotype between grade 0/3 and grade 1/3 (p = 0.04 and p = 0.01, respectively) (Table 2). Similarly, Nan et al. study in young ischemic

cerebrovascular patients did not find a significant difference in genotype distribution and allele frequency of the A1298C gene, while there was a significant difference in genotype distribution and allele frequency of the C677T gene ($p > 0.05$ and $p < 0.01$, respectively) [40]. Also, a significant difference was found in the genotypes of MTHFR A1298C (AC + CC) and C677T (CT + TT) genes in the study conducted by Fekih-Mrissa et al. in 2006 on patients with ischemic stroke ($p < 0.01$) [37]. Sazci et al. in their study conducted in patients with ischemic and hemorrhagic stroke in 2006, found a significant difference in the A1298C gene in both patient groups, while the C677T gene was significant only in patients with hemorrhagic stroke (CC genotype $p = 0.004$ $p = 0.001$ and TT genotype $p = 0.014$) [12]. In a case report published in 2014, the C677T mutation was detected in a dalmatian woman with silent brain infarction [10]. In the meta-analysis results published in 2019 and conducted in China in the elderly population with ischemic stroke, it was stated that the T allele of the C677T gene increased the ischemic stroke [41]. In the publication published in 2018 and investigating the relationship between white matter lesions and the genotypes of the MTHFR C677T gene, it was stated that the severity of white matter lesions was not related to the MTHFR C677T gene polymorphism [42].

In our study, when we compared CC/AC and AA/CC genotypes in terms of homocysteine levels in the A1298C gene, we found that it was significantly higher in homozygous mutations. Also, when we compared the AC/CC genotypes, the folate level was found to be significantly lower in homozygous mutation. We could not find any significant difference in other parameters in terms of genotype. We found that the homocysteine and IMA levels in the genotypes of the C677T gene were significantly higher in homozygous mutations. On the contrary, we found the folate level significantly lower. There was no significant difference in other parameters in terms of genotype (Table 3). Some studies have stated that while variants of the A1298C gene do not affect homocysteine levels, some studies have been found [19, 37, 43–46]. In studies, it has been stated that variants of the C677T gene increase the homocysteine level [45, 19, 36, 37, 47]. In similar studies, it was stated that there was no significant difference in the B12 level, where folate level decreased especially in C677T gene mutations. Since IMA, total thiol, native thiol, and disulfide are new oxidative parameters, there were no studies on the effects of variants of these genes on these parameters.

Conclusions

We determined that mutations in the MTHFR C677T gene were effective in silent brain infarctions and that IMA increased due to the increase in homocysteine as a result of enzyme metabolism. Also, we found that the thiol balance was impaired in these patient groups. Considering these results, it suggested that IMA and thiol balance parameters are important in determining the development of silent brain infarction.

Declarations

Author contributions

All of the authors contributed to the design of the study, collection of samples, and analysis and interpretation of data.

Compliance with ethical standards

Conflict of interest The authors have no conflict of interest in this study.

Ethical approval This study was performed in accordance with Declaration of Helsinki ethics guidelines, and informed consent was obtained from each of its participants. Protocol and informed consent forms was approved by Isparta Süleyman Demirel University Medical Faculty Ethics Committee (Dated:27 February 2020, No: 42423).

Informed consent Written informed consent was obtained from all patients prior to their participation in the study.

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