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DNA methylation status of interspersed repetitive sequences in patients with migraine

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Abstract

Objective: To analyse the methylation status of the Long Interspersed Nuclear Element-1 (LINE-1) and Short Interspersed Nuclear Element Alu (Alu) of peripheral blood mononuclear cells (PBMCs) from patients with migraine compared with healthy control subjects.

Methods: This case–control study recruited patients with migraine without aura and age-matched healthy control subjects. PBMCs were purified from peripheral blood samples. Methylation levels and patterns of LINE-1 and Alu sequences were evaluated using combined bisulfite restriction analysis-interspersed repetitive sequences polymerase chain reaction.

Results: A total of 84 patients with migraine and 82 age-matched healthy controls were enrolled in the study. High levels of unmethylated cytosines in both the LINE-1 and Alu repetitive elements were observed in the migraine group compared with the control subjects. In addition, a significant difference was detected in the methylation level of LINE-1 between TT and CC genotype groups of the methylenetetrahydrofolate reductase gene.

Conclusions: These results suggest that analysis of epigenetic biomarkers in PBMCs may help to identify patients at a higher risk of migraine development.

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Introduction

Migraine is a common neurovascular disorder characterized by recurrent headache attacks of moderate to severe intensity that may last up to 72 h. Migraine is subclassified into migraine with aura and migraine without aura.[1] In approximately 25% of patients, headache attacks are accompanied by a migraine aura, consisting of focal neurological symptoms such as visual disturbances, sensory and motors deficits, and language impairment.[2] The exact mechanism for migraine is not completely understood. The vascular theory proposed that there was vasoconstriction of the intracerebral arterial vessels, followed by extracranial vasodilation and then the pain associated with the migraine.[3,4] There are several risk factors associated with migraine occurrence, including genetics, sex and age. For example, numerous genes have been implicated in the pathogeneses of migraine, including genes involved in the regulation the vascular system making the vascular class of genes a priority and critical for migraine studies.[5] Environmental factors, such as alcohol, smoking, nutrition, stress, environmental changes, exercise and menstrual cycles in women, have been reported to play some role in causing migraine.[6] In addition, there is growing evidence for the contributions of epigenetic mechanisms to the pathogenesis of migraine. For example, methylenetetrahydrofolate reductase (*MTHFR*) variants, which are associated with migraine susceptibility, have direct links to epigenetic alterations.[7,8] The *MTHFR* gene encodes one of the main regulatory enzymes of homocysteine (Hcy) metabolism that catalyses the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the methyl donor for the remethylation of Hcy to methionine.[9] A common C677T transition (rs1801133) in the *MTHFR* gene is a well-established genetic determinant of hyperhomocysteinaemia and results in a thermolabile protein with decreased enzyme activity.[10] Thus, regarding the interaction between environment and genome, epigenetic mechanisms and more specifically DNA methylation seem to be of great importance in development of various conditions.[11,12] DNA methylation plays a role in brain development and the diverse functions of the brain including neuronal activity, learning and memory, degeneration and substance addiction.[13,14]

Most of the DNA methylation present in mammalian genomes is associated with interspersed repetitive sequences (IRSs) of DNA such as long interspersed elements and short interspersed elements.[15,16] Hypomethylation of IRSs is frequently observed in malignant tissues and proposed to be a useful biomarker for the prediction and prognosis of cancers and various inflammatory conditions.[12,17,18]

This current study analysed the methylation status of the Long Interspersed Nuclear Element-1 (LINE-1) and Short Interspersed Nuclear Element Alu (Alu) mobile elements of peripheral blood mononuclear cells (PBMCs) in individuals with migraine using combined bisulfite restriction analysis (COBRA)-IRS polymerase chain reaction (PCR), which is a highly

accurate quantitative methylation measurement method to describe DNA methylation patterns. The study also evaluated the impact of the *MTHFR* rs1801133 variant on the LINE-1 and Alu methylation levels in patients with migraine.

Patients and methods

Study population

This case–control study recruited consecutive patients with migraine at the Department of Neurology, Tbilisi Institute of Medicine, Tbilisi, Georgia between October 2018 and May 2021. The inclusion criteria for the patients were as follows: (i) aged >20 years; (ii) male or female; (iii) episodic migraine without aura that was diagnosed by a neurological examination and based on The International Classification of Headache Disorders, 3rd edition (ICHD-III) determined by the International Headache Society;[1] (iv) not using pain management medications for at least 7 days prior to sample collection. Age-matched control subjects without migraine were recruited from the patients' relatives and friends as well as employees of the Tbilisi Institute of Medicine and the Tbilisi State Medical University (Tbilisi, Georgia). Healthy control subjects were only allowed to participate if they did not suffer from any relevant headache. Detailed information on the medical history from all study participants was recorded including demographic characteristics and headache features if appropriate (pain duration, frequency and accompanying symptoms during attacks).

The study protocol was approved by the Tbilisi State Medical University Biomedical Research Ethics Committee, Tbilisi, Georgia (approval number: N5-2017/65; December 13, 2017). All patient details were de-identified. Written informed consent was obtained from all patients and controls. The reporting of this study conforms to STROBE guidelines.[19]

DNA preparation and bisulfite modification

Blood samples (5 ml) were collected into 0.369 M ethylenediaminetetra-acetic acid BD Vacutainer® blood collection tubes. Blood was taken in the period between migraine episodes in the patients and at enrolment in the control subjects. Blood samples were processed immediately. PBMCs were isolated from whole blood using Ficoll-Paque™ (Sigma-Aldrich, St Louis, MO, USA) gradient centrifugation at 400 ***g*** for 30–40 min at 18–20°C (LMC-56 Laboratory Centrifuge; Biosan, Riga, Latvia). DNA from PBMCs was obtained using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). A 500-ng sample of DNA was treated with bisulfite using an EpiTect Bisulfite Kit (QIAGEN) according to the manufacturer's instructions. The bisulfite-treated DNA samples were stored at −20°C until further analysis.

MTHFR genotyping

Single nucleotide polymorphism (SNP) genotyping of the *MTHFR* rs1801133 variant was undertaken using a TaqMan™ SNP Genotyping Assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Each TaqMan™ SNP Genotyping Assay contained sequence-specific forward and reverse primers to amplify the polymorphic sequence of interest and two TaqMan minor groove binder probes with nonfluorescent quenchers: one VIC dye-labelled probe to detect the Allele 1 sequence; and one FAM dye-labelled probe to detect the Allele 2 sequence. The final PCR reaction volume was 25 µl: DNA polymerase, forward and reverse primers at a final concentration 200 nM, probes at a final concentration

250 nM and 20 ng of genomic DNA. The PCR cycling programme involved preliminary denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 60 s and extension at 60°C for 60 s. The post-PCR read and analysis was undertaken using a real-time PCR instrument (QuantStudio™ 3 Real-Time PCR System; Thermo Scientific) with software that plots the results of the allelic discrimination (AD) data as a plot of Allele 1 (VIC dye) versus Allele 2 (FAM dye). The AD plot represents each sample well as an individual point on the plot. A typical AD plot shows homozygote clusters, a heterozygote cluster and the no-template controls. The points in each cluster are grouped closely together and each cluster is located well away from the other clusters.

COBRA of LINE-1 and Alu nuclear elements

The bisulfite-treated DNA samples were subjected to 40 PCR cycles with LINE-1 forward 5′-CGTAAGGGGTTAGGGAG TTTTT-3′ and reverse 5′-RTAAAACCC TCCRAACCAAATATAAA-3′ or Alu forward 5′-GGYGYGGTGGTTTAYGTTTG TAA-3′ and reverse 5′-CTAACTTTTTAT ATTTTTAATAAAAACRAAATTTCAC CA-3′ primers at an annealing temperature of 50°C for LINE-1 and 57°C for Alu.[20] After PCR amplification, the LINE-1 and Alu amplicons (160 base pairs [bp] and 133 bp, respectively) were digested with TaqI and TasI (LINE-1) and TaqI (Alu) in CutSmart® Buffer (New England Biolabs, Ipswich, MA, USA). Digested products were then electrophoresed on a 3% agarose gel. Intensity of the bands was detected using gel analyser 19.1 software.[21]

The COBRA LINE-1 amplicons generated four bands based on the methylation status of the two CpG dinucleotides as follows: mCuC, 160 bp; uCuC, 98 bp; 1 methylated CpG (mC, 80 bp); and 1 unmethylated CpG (uC, 62 bp) (Figure 1). The LINE-1 methylation level of each pattern was calculated according to a previously described method to determine the precise percentage of CpG dinucleotides.[20] The intensity of each band was separated by the length (bp) of the double-stranded DNA as follows: %160/160 = A, %98/94 = B, %80/78 = C and %62/62 = D. Then the LINE-1 methylation levels were computed using the following formulas: percentage of overall methylated loci (% mC) = 100 × (C + A)/(C + 2A + B + D), % mCmC = 100 × ((C−D + B)/2)/(((C−D + B)/2) + D + A), %uCuC = 100 × B/(((C−D + B)/2) + A + D), %mCuC = 100 × (A)/(((C−D + B)/2) + A + D) and %uCmC = 100 × (D−B)/((C−D + B)/2) + A + D).

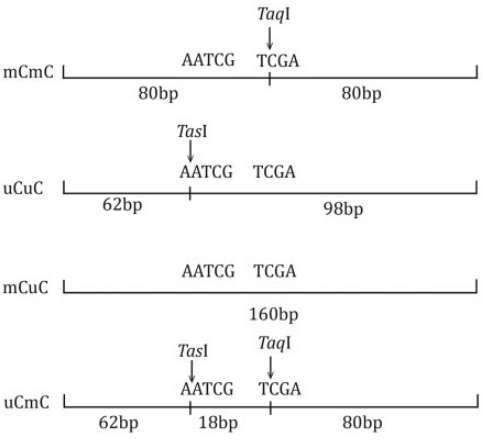

Figure 1. Methylation levels and patterns of the Long Interspersed Nuclear Element-1 (LINE-1) mobile elements of peripheral blood mononuclear cells. Combined bisulfite restriction analysis LINE-1 possesses four possible methylation patterns: hypermethylated loci (mCmC), hypomethylated loci (uCuC) and two partially methylated loci (mCuC and uCmC). TaqI is a restriction enzyme that is specific to methylated cytosine, whereas TasI is specific to unmethylated cytosine. The various methylation patterns of the digested LINE-1 polymerase chain reaction products yielded four differently sized digested products of 160 base pairs (bp), 98 bp, 80 bp and 62 bp.

Enzymatic digestion of 133 bp COBRA Alu products with the TaqI enzyme generated bands of 133, 90, 75, 58, 43 and 32 bp, with different intensities based on the methylation status of the two CpG dinucleotides (Figure 2). The number of CpG dinucleotides was calculated by dividing the intensity of each band by the number of double-stranded bp of DNA sequence as follows: %133/131 = A; %58/56 = B %75/73 = C; %90/88 = D; 43/41 = E; %32/30 = F. Alu methylation level percentage was calculated as follows: $\%\mathrm{mCmC} = 100 \times F/(A+C+D+F)$, $\%\mathrm{uCmC} = 100 \times C/(A+C+D+F)$, $\%\mathrm{mCuC} = 100 \times D/(A+C+D+F)$, $\%\mathrm{uCuC} = 100 \times A/(A+C+D+F)$, the percentage of methylated loci, and % methylation $(\mathrm{mC}) = 100 \times (2F+D+C)/(2A+2C+2D+2F)$.

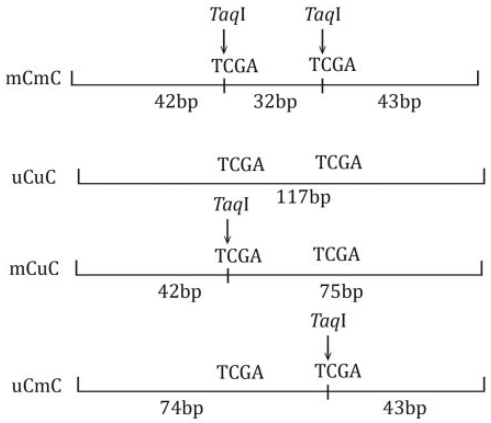

Figure 2. Methylation levels and patterns of the Short Interspersed Nuclear Element Alu (Alu) family mobile elements of peripheral blood mononuclear cells. Combined bisulfite restriction analysis Alu possesses four possible methylation patterns: hypermethylated loci (mCmC), hypomethylated loci (uCuC) and two partially methylated loci (mCuC and uCmC). TaqI is a restriction enzyme that is specific to methylated cytosine. The various methylation patterns of the Alu polymerase chain reaction products yielded four differently sized digested products of 117 base pairs (bp), 74/75 bp, 42/43 bp and 32 bp.

Statistical analyses

All statistical analyses were performed using IBM SPSS Statistics for Windows, Version 21.0 (IBM Corp., Armonk, NY, USA). Data are presented as the mean ± SD. Independent sample *t*-tests (two tailed) were used to compare LINE-1 and Alu methylation patterns between groups. The genotype frequencies were assessed to determine whether they were in Hardy–Weinberg equilibrium. Multivariate regression analysis was carried out between the *MTHFR rs1801133* variant and independent variables such as age, sex, attack frequency and attack duration; and the Wald test was used to calculate the *P*-value. A *P*-value ≤ 0.05 was considered statistically significant.

Results

This case–control study enrolled 84 patients with migraine and 82 age-matched healthy controls. The clinical and demographic characteristics of the patients are shown in Table 1. The frequency of attacks in the migraine group ranged from 2–10 attacks/month and disease duration ranged from 1–20 years. The majority of patients ($n = 63$) had no history of prophylaxis treatment and the remaining 21 patients had not taken any prophylactic medications for at least 2 months prior to sampling. Both categories of patient were receiving current acute treatments so they were asked to find a gap between migraine episodes when they were not using pain management medications for at least 7 days prior to sample collection. All patients in the study were diagnosed as migraine (episodic) without aura. There were no significant differences in sex distribution and age between the migraine and control groups.

The COBRA LINE-1 analysis demonstrated different levels and patterns of LINE-1 methylation in the PBMCs from

Table 1. Demographic and clinical characteristics of the patients with migraine ($n = 84$) and age-matched healthy control subjects ($n = 82$) that participated in a study of the methylation status of the Long Interspersed Nuclear Element-1 (LINE-1) and Short Interspersed Nuclear Element Alu (Alu) mobile elements of peripheral blood mononuclear cells.

Characteristics	Migraine group ($n = 84$)	Healthy group ($n = 82$)
Age, years	41.9 ± 8.7	36.3 ± 9.8
Sex		
Male	14	20
Female	70	62
Disease duration, years	10.3 ± 9.4	–
Attack frequency/month	6.0 ± 4.5	–
Attack duration, h	36 ± 14	–
Current acute treatment		
NSAIDs only (including in combinations of NSAIDs)	46 (55%)	–
Triptans	22 (26%)	–
Other (including combinations of NSAIDs and triptans)	16 (19%)	–

Data presented as mean ± SD or *n* of patients (%).
NSAID, nonsteroidal anti-inflammatory drug.

Table 2. Methylation levels and patterns of the Long Interspersed Nuclear Element-1 (LINE-1) mobile elements of peripheral blood mononuclear cells from patients with migraine ($n = 84$) and age-matched healthy control subjects ($n = 82$).

	LINE-1 methylation levels, %				
Sample groups	mC	mCmC	uCmC	mCuC	uCuC
Control group ($n = 82$)	44.15 ± 4.37	25.32 ± 4.22	17.02 ± 6.43	20.29 ± 2.42	35.14 ± 4.01
Migraine group ($n = 84$)	41.46 ± 4.12	19.29 ± 5.67	14.43 ± 6.02	21.17 ± 1.15	46.11 ± 5.30
Statistical analysis[a]	$P = 0.014$	$P = 0.010$	$P = 0.020$	NS	$P = 0.001$

Data presented as mean ± SD.
[a]Independent sample *t*-test (two tailed) was used to compare LINE-1 methylation patterns between the two groups.
mC, overall methylation level of cytosine; mCmC, hypermethylated cytosine; uCmC, semi methylated cytosine; mCuC, semi methylated cytosine; uCuC, unmethylated cytosine; NS, no significant between-group difference ($P > 0.05$).

patients with migraine ($n = 84$) and healthy controls ($n = 82$). When each pattern was analysed, the number of hypermethylated loci at both positions (mCmC) of LINE-1 from the migraine patients was significantly lower (19%) than the healthy controls (25%) ($P = 0.01$) (Table 2). Moreover, the number of unmethylated loci at both positions (uCuC) for LINE-1 from the migraine patients was significantly higher (46%) than the healthy controls (35%) ($P = 0.001$). The level of methylation of uCmC in the PBMCs from patients with migraine compared with control PBMCs was 14% versus 17% ($P = 0.02$), respectively. There were no significant differences in the percentages for the mCuC pattern for LINE-1 between the patients and the healthy controls.

A similar methylation pattern was observed in the Alu repetitive elements. The overall methylation level (mC) of Alu was significantly lower in the PBMCs from migraine patients (46%) compared with the healthy controls (53%) ($P = 0.021$), while

unmethylated cytosine levels (uCuC) were significantly higher in the patient group compared with the healthy controls (24% and 16%, respectively) ($P = 0.01$) (Table 3). There were no significant differences in the percentages for the semi-methylation patterns of uCmC and mCuC.

The interaction between the *MTHFR* polymorphism rs1801133 (known as C677T) and the global DNA methylation status in migraine patients was examined. Patients were stratified according to their *MTHFR* genotypes (CC, CT and TT). The *MTHFR* C677T genotypes were in Hardy–Weinberg equilibrium and there were no significant differences observed in age, sex, attack frequency or attack duration between the three genotypes (Table 4). For the *MTHFR* TT genotype group, a statistically significant difference was observed in the methylation level of LINE-1 compared with the CC genotype (44% versus 38%; $P = 0.015$) (Table 5).

Discussion

Methylation of the Alu and LINE-1 sequences are interspersed throughout genomic DNA and represents up to 50% of global genomic methylation.[22] Usually LINE-1 and Alu elements are heavily methylated in normal human tissues.[16] Hypomethylation of these retrotransposable elements has been associated with genomic instability and is therefore biologically significant.[16] More than 90% of all

Table 3. Methylation levels and patterns of the Short Interspersed Nuclear Element Alu (Alu) mobile elements of peripheral blood mononuclear cells from patients with migraine ($n = 84$) and age-matched healthy control subjects ($n = 82$).

	Alu methylation levels, %				
Sample groups	mC	mCmC	uCmC	mCuC	uCuC
Control group ($n = 82$)	52.76 ± 1.43	28.11 ± 1.31	22.86 ± 2.34	27.63 ± 2.32	16.46 ± 1.18
Migraine group ($n = 84$)	46.48 ± 1.36	24.51 ± 2.82	21.52 ± 1.34	28.94 ± 2.76	23.52 ± 1.23
Statistical analysis[a]	$P = 0.021$	$P = 0.017$	NS	NS	$P = 0.010$

Data presented as mean ± SD.

[a]Independent sample *t*-test (two tailed) was used to compare Alu methylation patterns between the two groups.

mC, overall methylation level of cytosine; mCmC, hypermethylated cytosine; uCmC, semi methylated cytosine; mCuC, semi methylated cytosine; uCuC, unmethylated cytosine; NS, no significant between-group difference ($P > 0.05$).

Table 4. Multivariate regression analysis of the demographic and clinical characteristics of the patients with migraine ($n = 84$) stratified according to the methylenetetrahydrofolate reductase (*MTHFR*) genotype.

	MTHFR genotype		
Characteristic	CC	TT	CT
Age, years	39.7 ± 5.4	42.8 ± 4.6	41.0 ± 6.6
Male	5	3	6
Female	38	8	24
Attack frequency/month	6.0 ± 2.5	7.0 ± 1.5	7.0 ± 1.5
Attack duration, h	35 ± 11	32 ± 15	34 ± 12

Data presented as mean ± SD or *n* of patients.

No significant between-group differences ($P > 0.05$).

Table 5. The methylation status of the Long Interspersed Nuclear Element-1 (LINE-1) and Short Interspersed Nuclear Element Alu (Alu) mobile elements of peripheral blood mononuclear cells of patients with migraine ($n = 84$) stratified according to the methylenetetrahydrofolate reductase (*MTHFR*) genotype.

	MTHFR genotype		
	CC	CT	TT
LINE-1 methylation, %	44.0 ± 2.58	43.0 ± 2.82	38.0 ± 3.35*
Alu methylation, %	52.0 ± 3.45	50.0 ± 1.98	48.0 ± 2.15

Data presented as mean ± SD.
*$P = 0.05$, TT versus CC genotype for LINE-1 methylation; low methylation levels of the Alu element were found in individuals with TT and CT genotypes compared with CC, but this was not statistically significant; Wald test.

genomic differentially methylated CpGs are located in CpG-rich sequences of transposable repetitive elements, including LINE-1 and Alu sequences.[17,23]

Several studies have suggested that hypomethylation of LINE-1 and Alu are the causes for global DNA hypomethylation and genomic instability in many malignancies and inflammatory diseases, including chronic inflammatory pain.[24–26] A genome-wide analysis of DNA methylation in migraine patients identified 62 non-overlapping differentially methylated regions in relation to CpG islands.[27] In addition, a recent study suggested a strong association between DNA methylation of migraine-related genes in migraine.[28] Research showed a different methylation pattern in two CpG sites at the proximal promoter region of the calcitonin related polypeptide alpha (*CALCA*) gene, which encodes calcitonin gene-related peptide (CGRP) in patients with episodic migraine.[29] In addition, DNA methylation levels at different CpG sites in the *CALCA* gene promoter was correlated with several clinical characteristics of migraine.[29] In agreement with this study,[29] a low methylation level was observed at the promoter region of the receptor activity modifying protein 1 gene, which encodes a key receptor subunit of CGRP in patients with migraine compared with controls.[30]

To the best of our knowledge, there are no or limited data on the methylation status of IRS in patients with migraine. The present study analysed methylation in the LINE-1 and Alu repetitive elements in PBMCs from 84 patients with migraine without aura and 82 healthy control subjects. Due to the dynamic nature of DNA methylation, the current study selected a homogeneous patient population with episodic migraine without aura. Migraine treatment, especially prophylaxis therapy, includes medications that can modulate DNA methylation. In this regard, a small number of patients with a history of prophylaxis therapy had not taken any prophylactic medications for at least 2 months prior to sampling. All patients received acute drugs, but PBMCs were obtained during a drug-free period of at least 7 days between migraine episodes.

This current study found high levels of unmethylated cytosine in both the LINE-1 and Alu repetitive elements in patients with migraine without aura compared with healthy control subjects. The current study also examined the interaction between the *MTHFR* gene C677T polymorphism and DNA methylation status of IRS in migraine

patients. MTHFR is one of the main regulatory enzymes that is involved in Hcy metabolism and it catalyses the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the methyl donor for the remethylation of Hcy to methionine.[9] Research suggests that *MTHFR* polymorphisms are associated with global DNA methylation levels.[9] There was a significant difference detected in the methylation level of LINE-1 between the *MTHFR* TT and CC genotype groups. However, the low methylation levels of the Alu element in individuals with the *MTHFR* TT and CT genotypes were not significant compared with the CC genotype group.

This current study had several limitations. First, it had a relatively small sample size. Secondly, the study participants may have had different dietary habits and other environmental factors that might influence DNA methylation levels in PBMCs. In this current study, folate, vitamin B_6, vitamin B_{12} and Hcy plasma levels were not determined, so it was not possible to investigate the possible correlation between their levels and the methylation of the LINE-1 and Alu repetitive elements.

In conclusion, this current study demonstrated that there is an increased frequency of unmethylated (uCuC) LINE-1 and Alu sequences in PBMCs from migraine patients compared with healthy control subjects. The study also provided evidence that a particular SNP in the *MTHFR* gene might be a potential risk factor for disease development associated with DNA methylation status. These current results suggest that methylation analysis in IRE might help to identify patients at a higher risk of migraine development. To our best knowledge, methylation levels and patterns of the LINE-1 and Alu repetitive elements have not been previously analysed and further studies are needed that focus on the effect of folate status, vitamin B_{12}, vitamin B_6, plasma Hcy levels and other environmental factors on global DNA methylation patterns.

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Author contributions

Conceptualization: EK, MZ and EA; methodology: NK, MK and SS; formal analysis: EK and NK; data curation: MK, EA and TG; laboratory analysis: NK, MZ and SS; writing – original draft preparation: EK and NK; writing – review and editing: all authors. All authors have read and agreed to the published version of the manuscript.

Declaration of conflicting interests

The authors declare that there are no conflicts of interest.

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