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ORIGINAL RESEARCH

Methylation of the *RIN3* Promoter is Associated with Transient Ischemic Stroke/Mild Ischemic Stroke with Early Cognitive Impairment

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Background: Early cognitive impairment after transient ischemic stroke (TIA)/mild ischemic stroke (MIS) is common but easily overlooked. It has been demonstrated that DNA methylation plays a significant role in cognitive impairment and ischemic stroke. Furthermore, it has been reported that the *RIN3* gene influences transportation of the amyloid β-protein. However, to our knowledge, there has been no research related to correlations between *RIN3* methylation and early-onset cognitive impairment after TIA/MIS. Therefore, this study aimed to investigate this relationship in TIA/MIS patients.

Methods: This study include 28 control subjects and 84 patients with TIA/MIS who were evaluated within 7 days of TIA/MIS onset using four single-domain cognitive scales. In addition, DNA methylation of whole blood was tested. *RIN3* methylation was compared between TIA/MIS and control groups and between TIA/MIS patients with early cognitive impairment and those without early cognitive impairment. Clinical variables and *RIN3* methylation sites with statistical differences were then used to construct a predictive model.

Results: Hypomethylation of the *RIN3* gene was observed in the whole blood of TIA/MIS patients relative to healthy controls. Furthermore, patients with early cognitive impairment after TIA/MIS had hypomethylation of *RIN3* relative to those without early cognitive impairment.

Conclusion: *RIN3* methylation is strongly associated with TIA/MIS and TIA/MIS with early cognitive impairment. It is possible to influence the disease process by methylation via appropriate lifestyle and clinical interventions, and methylation of *RIN3* gene sites may predict the occurrence of TIA/MIS with early cognitive impairment.

Keywords: amyloid B-protein, cognitive impairment, hypomethylation, ischemic stroke, transportation

Introduction

Ischemic stroke, one of the most common cerebral vascular diseases, can lead to neurological and cognitive impairment and can also accelerate cognitive disorders. Cognitive impairment can also occur in the early phase after a transient ischemic stroke (TIA) or a mild ischemic stroke (MIS). Since the dyskinetic symptoms of TIA/MIS are mild and short-lived, cognitive impairment due to TIA/MIS is easily overlooked, and the cognitive level of patients after onset of TIA/MIS is not routinely assessed in clinical practice. Currently, the specific pathogenesis of early cognitive impairment after TIA/MIS is unclear and requires further research.

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It has been demonstrated that the incidence of early cognitive impairment after TIA/MIS can be as high as 32%–70%.[1–4] Cognitive impairment is common in the acute phase of TIA/MIS and is more common within 7 days after TIA/MIS. Of note, the recovery of cognition in these patients does not parallel the resolution of somatic motor symptoms. Also, the acute phase of cognitive impairment is highly correlated with persistent future cognitive decline.[2]

DNA methylation, an important form of epigenetics, plays a significant role in several neurodegenerative pathologies, including Alzheimer's disease (AD) and parkinsonism. Abnormal methylation of several genes has also been observed in ischemic cerebrovascular disease. The *RIN3* gene, located on chromosome 14, acts as a stimulating factor to stabilise the transportation of GTP-RAB5 to endosomes at the plasma membrane. This process is associated with cellular endocytosis and has a negative effect on endocytosis of the amyloid β-protein (Aβ),[5,6] which plays a role in the onset of both AD and vascular cognitive impairment (VCI). Studies have shown that the *RIN3* gene is highly expressed in AD[7] and that this gene is hypomethylated in the whole blood of patients with early-onset AD.[8]

Based on these previous studies, we hypothesised that abnormal expression of the *RIN3* gene would impact the occurrence of cognitive impairment. Furthermore, we hypothesized that methylation, the most important manifestation of epigenetics, would affect expression of the *RIN3* gene. To our knowledge, there is no existing research on the correlation between *RIN3* gene methylation and early-onset cognitive impairment after TIA/MIS. Therefore, this study aimed to determine if hypomethylation of the *RIN3* gene was associated with TIA/MIS with early cognitive impairment.

In this study, we examined whole-blood methylation levels in three groups, including TIA/MIS patients with early cognitive impairment, TIA/MIS patients without cognitive impairment, and non-infarcted control subjects, in order to investigate the correlation between whole-blood *RIN3* methylation levels and TIA/MIS with early cognitive impairment. In addition, we aimed to identify significant predictive factors for construction of predictive models and for development of interventions.

Materials and Methods

Participants

A total of 84 patients (age < 75 years) with TIA/MIS were prospectively recruited from the Department of Neurology at the Qilu Hospital of Shandong University in Qingdao, China between June 2019 and July 2020. Mild stroke was defined as an acute cerebral infarction with a National Institutes of Health Stroke Scale (NIHSS) score of less than 5, and TIA was defined as a sudden focal neurological deficit in the brain, spinal cord, or retina lasting less than 24 h, which results from atherosclerotic vascular causes and is not associated with an acute cerebral infarction. All TIA/MISs were recorded using the Trial of Org 10172 in acute stroke treatment (TOAST) classification system and confirmed using cranial computed tomography (CT) and/or magnetic resonance imaging (MRI). Patients were evaluated within 7 days of TIA/MIS onset using four single-domain cognitive scales:[9–16] the Boston Naming Test (BNT) for language (abnormality: ≤21.5 points; adjusted value: ≤19.5 points if the education period was ≤9 years and ≤21.5 points if the education period was >9 years), the Auditory Verbal Learning Test (AVLT) for memory (abnormality: <5 points after 5 min), and the Trail Making Test (TMT)-A for visuospatial function (abnormality: ≥98.5 s; adjusted value: ≥80.5 s for patients aged ≤64 years, ≥90.5 s for those aged 64−74 years, and ≥90.5 s for those aged ≥75 years, with a maximum time of 150 s), and TMT-B for executive function (abnormality: ≥188.5 s; adjusted value: ≥150.5 s for patients aged ≤64 years, ≥165.5 s for those aged 64−74 years, and ≥199.5 s for those aged ≥75 years, with a maximum time of 300 s). Cognitive impairment was determined by more than one (≥1) scale that has abnormal results. All cognitive screening were performed by two neurologists with at least 5 years of experience. Exclusion criteria included (1) stroke mimics; (2) age >75 years; (3) cerebral haemorrhage, degenerative disease, tumour, or severe hepatic or renal insufficiency; and (4) inability to complete cognitive scales cooperatively because of dysphasia or hearing or visual impairment. The control group (28 patients) included healthy volunteers or patients without TIA/cerebral infarction suffering from dizziness or headaches recruited during the same period; all accepted cranial MRI/CT images and cerebrovascular screenings were used to exclude TIA/MIS. This study was approved by the Ethics Committee of the Qilu Hospital of Shandong University in Qingdao, China. All patients provided written informed consent for participation in this study.

DNA Extraction and Quality Control

Fasting blood samples were drawn using EDTA tubes, and whole-blood DNA was extracted using kits (Tiangen Biotech, Beijing, China). The quality of the DNA was detected by a NanoDrop 2000 (NanoDrop technologies,

Wilmington, DE, USA), which required a concentration of ≥20 ng/μL and a total sample purity of ≥400 ng.

CpG Island Selection

CpG islands located in the proximal promoter of the *RIN3* gene were selected for measurement according to the following criteria: (1) 200-bp minimum length; (2) 50% or higher GC content; and (3) 0.60 or higher ratio of observed/expected dinucleotide CpG. Two regions from CpG islands of the *RIN3* gene were selected and sequenced.

Bisulfite Conversion and Multiplex Amplification

DNA methylation levels were analysed using MethylTarget® (Genesky Biotechnologies Inc., Shanghai, China), an NGS-based multiple targeted CpG methylation analysis method. Specifically, the genomic regions of interest were analysed and transformed into bisulfite-converted sequences using GeneCpG software. Polymerase chain reaction (PCR) primer sets were designed using Methylation Primer software from bisulfate-converted DNA. The PCR primers for *RIN3_26* were as follows: forward, 5′-GTATATTTGTTAGGA ATGTGGAGGAG-3′; reverse, 5′-AAAAAAAAATC TTCCACTTAACTTAAAACC-3′. The PCR primers for *RIN3_27* were as follows: forward, 5′-TTAGTGTTTGGGTAGGGTTTAGG-3′; reverse, 5′-AAACCCAACCCCRAACAA-3′. Genomic DNA (400 ng) was subjected to sodium bisulfite treatment using the EZ DNA Methylation™-GOLD Kit (ZYMO, CA, USA), according to the manufacturer's protocols. Multiplex PCR was performed using optimised primer set combinations. A 20-μL PCR reaction mixture was prepared for each reaction, which included 10x reaction buffer (TaKaRa, Dalian, China), 25 mM of Mg^{2+}, 2.5 mM of dNTP, 1 μM of each primer, 5U of HotStarTaq polymerase (TaKaRa, Dalian, China), and 1 μL of template DNA. The cycling program included 95°C for 2 min; 11 cycles of 95°C for 20 s, 62°C for 40 s with a decreasing temperature step of 0.5°C per cycle, and 72°C for 1 min; and 24 cycles of 95°C for 20 s, 62°C for 30 s, 72°C for 1 min, and 72°C for 1 min.

Index PCR

PCR amplicons were diluted and amplified using indexed primers. Specifically, a 20-μL mixture was prepared for each reaction comprised of 5x reaction buffer (TaKaRa, Dalian, China), 2.5 mM of dNTP, 10 μM of F primer, 4 μM of index primer, 0.2μL of Herculase® II Fusion DNA polymerase (Agilent Technologies, CA, USA), 2 μL of diluted template, and ddH2O. The cycling program included 95°C for 2 min followed by 11 cycles of 95°C for 20 s, 65°C for 30 s, 72°C for 30 s, and 72°C for 3 min. PCR amplicons (170 bp–270 bp) were separated by agarose electrophoresis and purified using the TIANGEN Gel Extraction kit (TIANGEN, Beijing, China).

Sequencing

Libraries from different samples were quantified and pooled together followed by sequencing on the Illumina HiSeq platform according to the manufacturer's protocols. Sequencing was performed using a 2 × 150-bp paired-end mode.

Data Analysis

Fast Length Adjustment of SHort reads (FLASH) is an accurate and fast tool used to merge paired-end reads.[17] FASTQ to FASTA format was then processed using the Fastx toolkit (http://hannonlab.cshl.edu/fastx_toolkit/index.html). Reads in FASTA format were mapped to the targeted bisulfite genome (hg19) by Blast.[18] Unmapped reads were filtered, and mapped reads with a coverage greater than 90% and an identity greater than 90% were considered effective reads and used for the following statistics. The sequencing depth for each amplicon per sample was calculated by blasting the effective reads against the targeted genomic region. Reads less than 10-fold were removed, and the overall sequencing depth for each sample was evaluated. Methylation and haplotypes were analysed using Perl script.

Statistical Methods

R software was used for statistical analysis. Continuous data are presented as mean ± standard deviation ($\bar{x} \pm s$), and categorical data are expressed as numbers of cases and percentages (n [%]). Student's *t*-test was used for continuous variables with normal distribution and homogeneous variance to compare observations between both study groups, and the Wilcoxon test was used otherwise. The test of proportions, including the K-square test and Cochran-Armitage trend test, was applied to determine if there was a statistically significant difference in attribute percentages between data sets. The K-square test was used for nominal categorical variables. The Cochran-Armitage trend test was used for ordinal categorical variables. Single-factor logistic

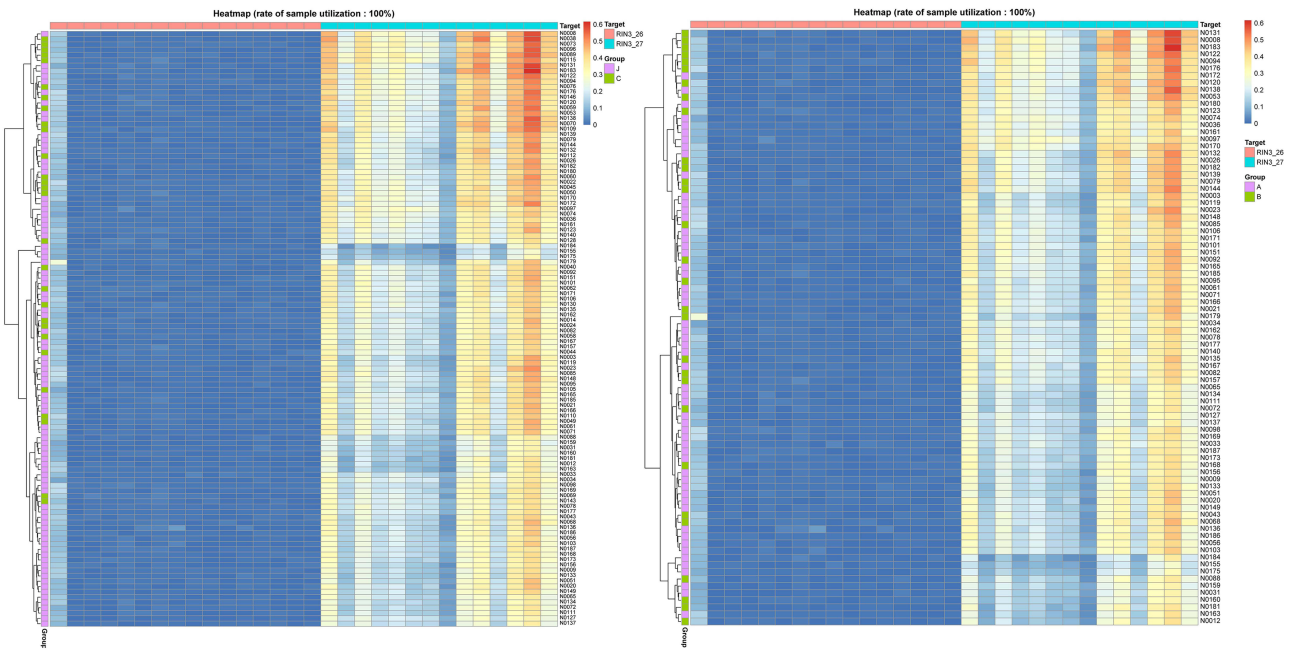

Figure 1 Shows a heatmap based on the methylation levels of CpG sites in all samples in groups J, C, A, and B. Each row is a sample, and each column is a CpG site. Each cell represents the relative methylation level at CpG sites in the corresponding row of samples. The colour gradient reflects the change in methylation level, with a tendency toward blue indicating a lower methylation level and a tendency toward red indicating a higher methylation level. The similarities between samples' methylation levels are indicated by the order of the rows, with adjacent rows indicating more similar overall methylation levels in the represented samples.

regression and multi-factor logistic regression analyses were performed to identify independent variables for TIS/MIS with early cognitive impairment. We screened variables using a stepwise regression analysis to construct predictive models and plot ROC curves. A p-value <0.05 was considered statistically significant.

Results

In this study, we tested whole-blood *RIN3* methylation in 112 subjects (Figure 1), including 84 patients with TIA/MIS in the case group (group J); 55 patients had early cognitive impairment (group A), whereas 29 participants did not (group B). Moreover, there were 28 participants in the control group (group C). Basic patient clinical information are shown in Tables 1 and 2.

Differences Between Patients with TIA/MIS and Healthy Controls in Terms of Total *RIN3* Methylation Level

By sequencing methylation in the target regions (Table 3) in the 84 TIA/MIS and 28 controls, we found that the overall methylation level of the *RIN3* gene was significantly lower in the TIA/MIS group than in the control group (Groupdiff=−0.02, P <0.001) (Figure 2), even after adjusting for age and gender (Adj. P <0.001) (Table 4).

Total *RIN3* Methylation Levels in Patients with TIA/MIS with and without Cognitive Impairment

The overall methylation level of the *RIN3* gene was significantly lower in the cognitive impairment group (n=55) than in the non-cognitive impairment group (n=29) (Groupdiff=−0.013, P=0.01) (Figure 3), even after adjusting for age, gender, education, CHD, and DWM (Adj. P=0.044) (Table 5).

Differences in Differentially Methylated Sites Between Patients with Transient Ischemic Stroke/Mild Ischemic Stroke and Healthy Controls

Whole blood *RIN3* gene methylation levels were significantly different between patients with TIA/MIS and controls. We evaluated differences in methylation levels at different sites between both groups and found statistically significant differences at several sites (S26-146,

Table 1 Clinical Information on the Transient Ischemic Stroke/ Mild Ischemic Stroke and Control Groups

	TIA/MIS Group (n=84)	Control Group (n=28)	*P* value
Age (years) ($\bar{x}$±s)	61.94 ± 8.03	57.64 ± 9.00	0.016
Females (n [%])	26 (31.0)	15 (53.6)	0.054
Education (years) ≤ 6 ≤ 12 > 12	 22 51 11	 1 19 8	0.004
Diabetes mellitus (n [%])	34 (40.5)	8 (28.6)	0.575
Hypertension (n [%])	62 (73.8)	18 (64.3)	0.622
Hyperlipemia (n [%])	22 (26.2)	12 (42.9)	0.035
Coronary heart disease (n [%])	16 (19.0)	6 (21.4)	0.425
Alcohol (n [%])	33 (39.3)	6 (21.4)	0.137
Smoking (n [%])	35 (41.7)	9 (32.1)	0.503
Abnormal scale BNT (n [%]) AVLT (n [%]) TMT-A (n [%]) TMT-B (n [%])	 16 (19.0) 43 (51.2) 39 (46.4) 38 (45.2)		
Fazekas PV 0 (n [%]) 1 (n [%]) 2 (n [%]) 3 (n [%])	 1 (1.2) 43 (51.2) 27 (32.1) 13 (15.5)	 10 (35.7) 17 (60.7) 1 (3.6) 0 (0)	<0.001
Fazekas DWM 0 (n [%]) 1 (n [%]) 2 (n [%]) 3 (n [%])	 7 (8.3) 44 (52.4) 26 (31.00) 7 (8.3)	 12 (42.9) 14 (50.00) 2 (7.1) 0 (0)	<0.001
LDL (mmol/L)	2.81 ± 1.00	3.12 ± 1.02	0.088
HDL (mmol/L)	1.10 ± 0.24	1.20 ± 0.31	0.074
HCY (μmol/L)	14.15 ± 7.19	12.45 ± 6.01	0.247
UA (μmol/L)	310.93 ± 84.17	339.37 ± 97.59	0.457
Vitamin B12 (pg/mL)	411.61 ± 251.58	476.30 ± 191.32	0.057

Notes: In the TIA/MIS group, 81 patients underwent low-density lipoprotein (LDL) testing, 80 underwent high-density lipoprotein (HDL) testing, 75 underwent homocysteine (HCY) testing, 72 underwent uric acid (UA) testing, and 63 underwent vitamin B12 testing. In the control group, 27 participants underwent LDL and HDL testing, 25 underwent HCY testing, 27 underwent UA testing, and 20 underwent vitamin B12 testing.
Abbreviations: PV, periventricular; DWM, deep white matter.

S27-25, 29, 36, 38, 40, 58, 61, 96, 111, 116, 127, 144, 146, and 152, P <0.05), even after adjustment for age and gender. All of these sites showed hypomethylation

Table 2 Clinical Information on the Cognitive-Impairment and Con-Cognitive-Impairment Groups

	Cognitive Impairment Group (n=55)	Non-Cognitive Impairment Group n=29)	*P* value
Age (years) ($\bar{x}$±s)	63.47 ± 6.89	59.03 ± 9.16	0.029
Female (n [%])	20 (36.4)	6 (20.7)	0.219
Education (years) ≤ 6 ≤ 12 > 12	 20 31 4	 2 20 7	0.001
Diabetes mellitus (n [%])	21 (38.2)	13 (44.8)	0.722
Hypertension (n [%])	38 (69.1)	24 (82.8)	0.145
Hyperlipemia (n [%])	14 (25.5)	8 (27.6)	1
Coronary heart disease (n [%])	14 (25.5)	2 (6.9)	0.077
TOAST			0.077
Large aryery atheroscierosis (n [%])	26 (47.3)	7 (24.1)	
Small artery occulusion (n [%])	29 (52.7)	22 (75.9)	
Alcohol (n [%])	22 (40.0)	11 (37.9)	1
Smoking (n [%])	24 (43.6)	11 (37.9)	0.786
Abnormal scale BNT (n [%]) AVLT (n [%]) TMT-A (n [%]) TMT-B (n [%])	 16 (29.1) 43 (78.2) 39 (70.9) 38 (69.1)		
NIHSS 0 (n [%]) 1 (n [%]) 2 (n [%]) 3 (n [%]) 4 (n [%])	 26 (47.3) 14 (25.5) 10 (18.2) 4 (7.3) 1 (1.8)	 14 (48.3) 7 (24.1) 5 (17.2) 2 (6.9) 1 (3.4)	0.935
Fazekas PV 0 (n [%]) 1 (n [%]) 2 (n [%]) 3 (n [%])	 1 (1.8) 26 (47.3) 19 (34.5) 9 (16.4)	 0 (0) 17 (58.6) 8 (27.6) 4 (13.8)	0.661
Fazekas DWM 0 (n [%]) 1 (n [%]) 2 (n [%]) 3 (n [%])	 3 (5.5) 26 (47.3) 20 (36.4) 6 (10.9)	 4 (13.8) 18 (62.1) 6 (20.7) 1 (3.4)	0.063
LDL (mmol/L)	2.78 ± 1.08	2.87 ± 0.82	0.483
HDL (mmol/L)	1.10 ± 0.23	1.05 ± 0.26	0.189

(*Continued*)

Table 2 (Continued).

	Cognitive Impairment Group (n=55)	Non-Cognitive Impairment Group n=29)	*P* value
HCY (umol/L)	14.15 ± 7.92	12.35 ± 5.26	0.320
UA (umol/L)	310.93 ± 78.53	347.35 ± 89.34	0.079
Vitamin B12 (pg/mL)	411.61 ± 263.40	389.32 ± 227.22	0.784

Notes: In the cognitive-impairment group, 54 patients underwent low-density lipoprotein (LDL) testing, 53 patients underwent high-density lipoprotein (HDL) testing, 50 underwent homocysteine (HCY) testing, 54 underwent uric acid (UA) testing, and 41 underwent vitamin B12 testing. In the non-cognitive-impairment group, 27 patients underwent LDL and HDL testing, 25 underwent HCY testing, 28 underwent UA testing, and 22 underwent vitamin B12 testing.
Abbreviations: PV, periventricular; DWM, deep white matter; NIHSS, National Institutes of Health Stroke Scale; TOAST, Trial of Org 10172 in acute stroke treatment.

(Groupdiff <0) in patients with TIA/MIS (Table 6 and Figure 4).

Differences in Differentially Methylated Sites in Patients with Transient Ischemic Stroke/Mild Ischemic Stroke with and without Cognitive Impairment

RIN3 gene methylation levels were significantly different between patients with TIA/MIS with and without cognitive impairment. We examined methylation levels at different sites between both groups and found statistically significant differences (P <0.05) at several sites (S26-113, 165, S27-25, 36, 111, 116, 127, 144, 146, and 152). All of these sites, except site S26-165 (Groupdiff=0.0019, P =0.03), showed hypomethylation in the cognitive impairment group after adjusting for gender, age, education, CHD, and DWM. There groups were not significantly different following adjustment at sites S26-113, 165, S27-36, and 127 (Table 7 and Figure 5).

Differences in Differentially Methylated Segments Between Patients with Transient Ischemic Stroke/Mild Ischemic Stroke and Healthy Controls

We investigated whether there were differences in the methylation levels of different fragments between patients with TIA/MIS and healthy controls. By calculating the mean methylation levels of all CpGs of the *RIN3* gene, we found that there were statistically significant differences in *RIN3-26* (P=0.03) and *RIN3-27* (P <0.001) between both groups. The overall methylation levels of *RIN3-26* (Groupdiff = −0.0011) and *RIN3-27* (Groupdiff =−0.0417) were significantly lower in the TIA/MIS group than in the control group. However, after adjusting for gender and age, there was only a statistically significant difference in *RIN3-27* between both groups (Adj. P <0.001) (Table 8).

Differences in Differentially Methylated Segments Between Patients with TIA/MIS

We investigated whether there were differences in the methylation levels of different fragments between the cognitive impairment and non-cognitive impairment TIA/MIS groups. By calculating the mean methylation levels of all CpGs of the *RIN3* gene, we found that there was a statistically significant difference in *RIN3-27* between both groups (P=0.01), even after adjusting for age, gender, education, CHD, and DWM (Adj. P=0.046) (Table 9). The methylation level of *RIN3-27* was significantly lower (Groupdiff=−0.0268) in the cognitive-impairment group than in the non-cognitive-impairment group.

Predictive Model for Acute-Phase TIA/MIS with Cognitive Impairment

After analysing clinical data from the cognitive-impairment and non-cognitive-impairment groups (Table 2) and

Table 3 Information on Target DNA Methylation Sequencing

Target	Chr	Gene	mRNA	mRNA Strand	TSS	TES	Start	End	Length	Target Strand	Distance2TSS
RIN3-26	14	*RIN3*	NM_024832	+	9298,012	93155339	92979633	92979850	218	+	−491
RIN3-27	14	*RIN3*	NM_024832	+	92980124	93155339	92980930	92981104	175	+	806

Abbreviations: Target, target segment name; Chr, chromosome; Gene, gene name; mRNA, mRNA closer to the product; mRNA strand, mRNA direction; TSS, transcription start site of mRNA; TES, transcriptional end site of mRNA; Start, starting position of the product on reference genomes; End, ending position of the product on reference genomes; Length, length of the product; Target strand, direction of the product; Distance2TSS, relative distance between product; TSS, a negative sign indicates that the site is upwards TSS.

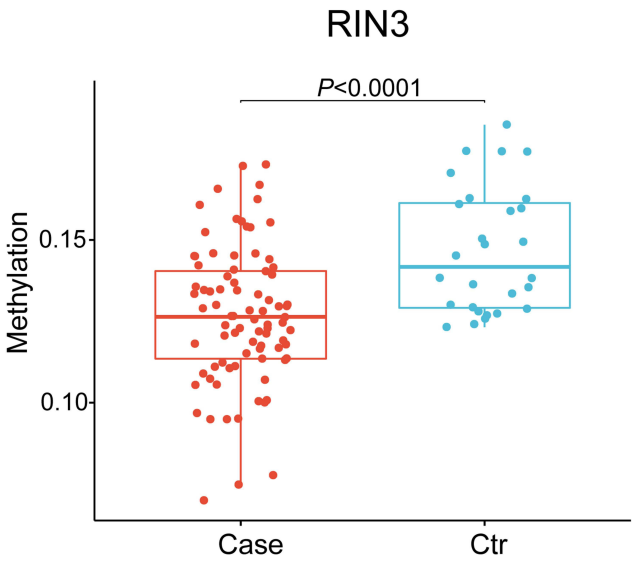

Figure 2 Shows the difference in *RIN3* methylation levels between the case group (group J) and the control group (group C). The *RIN3* gene was relatively hypomethylated in group J. Students *t*-test was used to compare the groups.

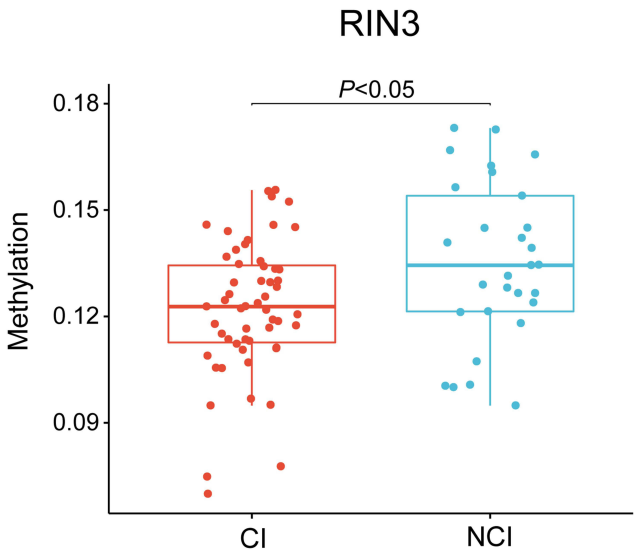

Figure 3 Compares the *RIN3* methylation levels between the group with cognitive impairment after TIA/MIS (group A) and the group without cognitive impairment after TIA/MIS (group B). The *RIN3* gene was hypomethylated in the former. Students *t*-test was used to compare the groups.
Abbreviations: Ctr, control; CI, cognitive impairment; NCI, non-cognitive impairment.

combining this information with statistically significant differences in *RIN3* methylation sites, we constructed a predictive model and plotted ROC curves. We found that the combination of three risk factors, including education level, presence of coronary heart disease, and S27-146 site methylation, showed the highest sensitivity/specificity for predicting early cognitive impairment after TIA/MIS. Thus, this model was able to scientifically forecast the occurrence of early cognitive impairment after TIA/MIS, with an area under the curve of 0.808 (95% CI: 0.7119–0.9044) (Figure 6).

Discussion

This study demonstrated that hypomethylation of the *RIN3* gene was associated with TIA/MIS and with early cognitive impairment after its onset. Furthermore, a combination of clinical indicators and methylation sites could predict the onset of early cognitive impairment after TIA/MIS. At present, there are few studies related to the relationship between gene methylation and early cognitive impairment after TIA/MIS, prompting this study.

Currently, there is no standardised scale for measuring vascular cognitive impairment. In this study, we used four scales to detect cognitive impairment in individual domains and found that the incidence of cognitive impairment after TIA/MIS was 65.5% (55/84), which is consistent with results reported in other studies.[1,3,4] Thus, the results obtained from these scales reflected the cognitive status of patients at that time. We investigated the correlation between *RIN3* methylation and TIA/MIS and between *RIN3* methylation and TIA/MIS with early cognitive impairment. Our results showed that the *RIN3* gene was hypomethylated in the whole blood of patients with TIA/MIS relative to a healthy control group. In addition, there was hypomethylation of the *RIN3* gene in TIA/MIS

Table 4 Differences in RIN3 Methylation Levels Between Transient Ischemic Stroke/Mild Ischemic Stroke Patients and Healthy Controls

Gene	*P value*	Groupdiff	Adj. *P value*
RIN3	<0.001	−0.02	<0.001

Note: Students *t*-test was used to compare the groups.
Abbreviations: Gene, gene name; Groupdiff, difference in mean methylation between the case and control groups; Adj. *P*-value, *P*-value after adjusting for age and gender.

Table 5 Differences in RIN3 Methylation Levels Between Patients with Transient Ischemic Stroke/Mild Ischemic Stroke with and without Cognitive Impairment

Gene	*P value*	Groupdiff	Adj.*P value*
RIN3	0.01	−0.013	0.044

Note: Students *t*-test was used to compare the groups.
Abbreviations: Gene, gene name; Groupdiff, difference in mean methylation levels between the cognitive impairment and non-cognitive impairment groups; Adj. *P*-value, *P*-value after adjusting for age, gender, education, CHD, and DWM; Edu, education; CHD, coronary heart disease; DWM, deep white matter.

Table 6 Differences in Methylation Sites Between Patients with Transient Ischemic Stroke/Mild Ischemic Stroke and Healthy Controls

Target	POS	Type	Groudiff	*P value*	Adj.*P value*
S26	*146*	*CG*	−0.0024	0.008	0.007
S27	*25*	*CG*	−0.0517	<0.001	<0.001
S27	*29*	*CG*	−0.0392	<0.001	<0.001
S27	*36*	*CG*	−0.0488	<0.001	<0.001
S27	*38*	*CG*	−0.0418	<0.001	<0.001
S27	*40*	*CG*	−0.0509	<0.001	<0.001
S27	*58*	*CG*	−0.0370	<0.001	<0.001
S27	*61*	*CG*	−0.0356	<0.001	<0.001
S27	*96*	*CG*	−0.0164	0.001	0.002
S27	*111*	*CG*	−0.0459	<0.001	0.003
S27	*116*	*CG*	−0.0525	<0.001	0.001
S27	*127*	*CG*	−0.0373	<0.001	<0.001
S27	*144*	*CG*	−0.0374	0.001	0.008
S27	*146*	*CG*	−0.0430	<0.001	0.008
S27	*152*	*CG*	−0.0465	<0.001	<0.001

Note: Students *t*-test was used to compare the groups.
Abbreviations: Target, site name; POS, position of the methylated site on the fragment; Type, methylation type; Groupdiff, difference in mean methylation between the case and control groups; *P*-value, *P*-value after adjusting for age and gender.

patients with acute-phase cognitive impairment in comparison to TIA/MIS patients without acute-phase cognitive impairment. Shen et al have shown that increased expression levels of *RIN3* can affect its transport function, which may lead to an increase in the αβ protein within neurons. Boden found that patients with early-onset AD showed hypomethylation of the *RIN3* gene in whole blood, which led to higher expression of the *RIN3* gene.[8] Another study confirmed that αβ plays a role in vascular cognitive impairment.[19] Therefore, we hypothesized that patients with TIA/MIS would also show abnormal methylation of the *RIN3* gene.

Cognitive impairment is common within 7 days after onset of TIA/MIS and may persist even after recovery of motor symptoms, leading to a greater risk of cognitive impairment and a reduced cognitive reserve in the distant future. Despite the rapid recovery of motor symptoms in TIA/MIS patients, cognitive impairment can easily be overlooked in these patients.[2] The occurrence of early cognitive impairment after TIA/MIS is influenced by multiple factors, and the current study found that some imaging changes may serve as predictors. Suda et al found that temporal horn atrophy on MRI education and smoking are independent risk factors for cognitive impairment.[1] Takahashi also confirmed that moderate temporal lobe atrophy on MRA source images combined with less years of education were predictors of cognitive impairment in the acute phase of ischemic stroke.[20] Finally, Zamboni found that white-matter brain damage on MRI was associated with TIA/MIS with early cognitive impairment damage.[21] These factors are, to some extent, nonmodifiable. Furthermore, it can be difficult to standardise their quantitative detection criteria. To date, there has been a lack of identification of modifiable biomolecular indicators related to disease onset, as well as a lack of quantifiable blood molecular indicators. This study, however, found that the *RIN3* gene and abnormal methylation of sites on this gene are associated with early cognitive impairment after TIA/MIS. Since methylation levels can be affected by environmental factors and can be measured quantitatively, they may be useful for the design of potential interventions and predictive models.

There is an increasing number of studies related to the correlation between methylation and ischemic stroke.[22–28] For example, methylation of the *ABCG1* and *APOE* genes has been correlated with cerebral infarction and atherosclerosis,[23] while *MMP*-2, *LINE3* and *TP53* methylation has been correlated with ischemic stroke,[24–26] and CDKN2B has been correlated with arterial calcification in ischemic stroke.[27,28] Furthermore, methylation has been strongly correlated with the pathogenesis of ischemic stroke. However, these studies have mainly focused on the relationship between gene methylation and ischemic stroke or atherosclerosis rather than specifically focusing on TIA/MIS. Moreover, there have been few studies related to the relationship between methylation and early cognitive impairment after stroke.

Some studies have investigated molecular indicators in the cerebrospinal fluid of patients with vascular dementia (VD) or VCI and have found that Aβ42 are significantly decreased in the cerebrospinal fluid of patients with VCI compared to controls.[29–33] Aβ concentration in red blood cell of VD patients is higher than that of controls.[34] Elevated Aβ levels in blood may aggravate vascular amyloidosis and thus affect cognition.[35] However, whether Aβ in blood can predict VCI still requires verification. NF-κB and VEGF levels have also been shown to be elevated in the cerebrospinal fluid of patients with VD, which may affect the β-amyloid protein.[36,37] Abnormal expression of the Aβ protein has been proposed to be significantly associated with vascular-related cognitive impairment.

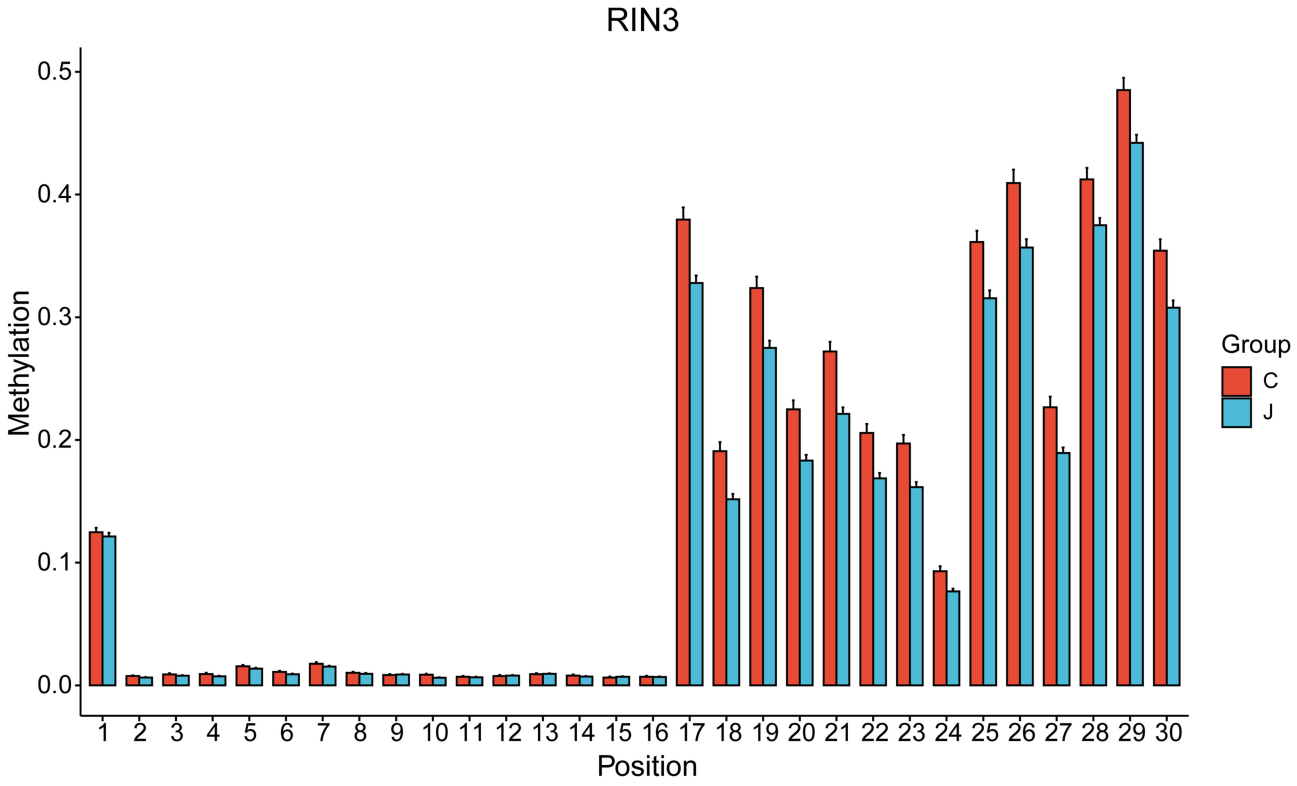

Figure 4 Compares methylation levels at measured sites in the case group (group J) and control group (group C), with the X-axis indicating the detected site and the Y-axis indicating the methylation level at each site. Students *t*-test was used to compare the groups.

The *RIN3* gene located on chromosome 14, however, has been less well studied. This gene has, however, been found to affect endocytosis of axonemes by interacting with BIN1, which influences the transport and metabolism of the Aβ protein, with high expression of the *RIN3* gene decreasing Aβ protein metabolism.[5,6] It has also been shown that abnormal *RIN3* gene expression is associated with AD[7] and that whole-blood *RIN3* methylation levels are low in AD patients.[8] *RIN3* affects cognitive function mainly by influencing Aβ protein transport. It is therefore likely that the appearance of early cognitive impairment in TIA/MIS patients is also associated with abnormal *RIN3* gene expression and with altered Aβ protein levels. The methylation level is an important factor affecting gene expression. This study confirmed that abnormal *RIN3* gene methylation was associated with early cognitive impairment in TIA/MIS.

Table 7 Different Methylation Sites in Patients with Transient Ischemic Stroke/Mild Ischemic Stroke with and without Cognitive Impairment

Target	POS	Type	Groudiff	*P value*	Adj.*P value*
S26	*113*	*CG*	−0.0033	0.02	0.16
S26	*165*	*CG*	0.0019	0.03	0.09
S27	*25*	*CG*	−0.0369	0.006	0.03
S27	*36*	*CG*	−0.0282	0.03	0.08
S27	111	*CG*	−0.0433	0.002	0.01
S27	116	*CG*	−0.0468	0.002	0.01
S27	127	*CG*	−0.0206	0.03	0.08
S27	144	*CG*	−0.0380	0.003	0.02
S27	146	*CG*	−0.0476	0.001	0.007
S27	152	*CG*	−0.0370	0.005	0.02

Note: Students *t*-test was used to compare the groups.
Abbreviations: Target, site name; POS, position of the methylated site on the fragment; Type, methylation type; Groupdiff, difference in mean methylation between both groups (patients with and without cognitive impairment); Adj. *P*-value, *P*-value after correction for age, gender, education, CHD, and DWM; Edu, education; CHD, coronary heart disease; DWM, deep white matter.

This study examined *RIN3* methylation levels in whole blood and found that the *RIN3* gene was hypomethylated in the TIA/MIS group compared to the control group and that *RIN3* methylation levels were lower in TIA/MIS patients with early cognitive impairment than in those without cognitive impairment. We hypothesize that *RIN3* hypomethylation occurs during the acute phase of TIA and mild stroke, which, in turn, leads to an abnormal accumulation of the Aβ protein in the blood. Indeed, this study did find a correlation between abnormal *RIN3* methylation in

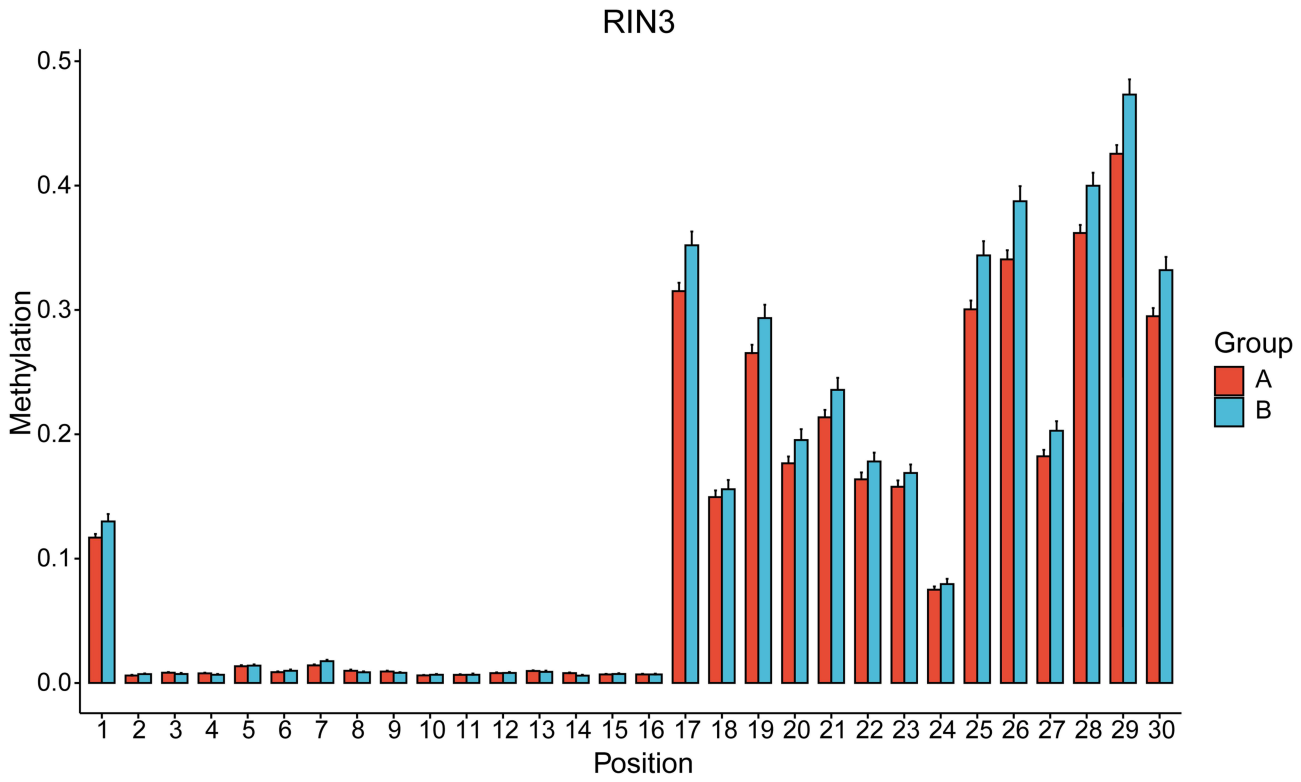

Figure 5 Compares methylation levels at measured sites between the group with cognitive impairment after TIA/MIS (group A) and the group without cognitive impairment after TIA/MIS (group B), with the X-axis indicating the detected site and the Y-axis indicating the methylation level. Students *t*-test was used to compare the groups.

blood and TIA/MIS with early cognitive impairment; however, the causal relationship between the two remains unclear. Based on our study, it is not possible to determine whether *RIN3* gene hypomethylation in the blood led to an accumulation of the Aβ protein, which resulted in the appearance or exacerbation of symptoms, or whether the disease itself first affected the level of *RIN3* methylation, which in turn affected the Aβ protein level in blood. These issues require further research.

This study had some limitations. First, the number of cases in this study was small and not fully matched by age nor sex. However, we corrected for age and sex to minimise the influence of these factors on our statistical analyses. Second, this study was designed to explore biomarkers in whole blood, so we examined DNA methylation in whole blood. However, we cannot confirm whether methylation levels in whole blood are reflected in methylation levels in brain tissue. However, we chose to test whole blood because the underlying cause of cerebrovascular disease involves changes to the blood vessels and the blood itself, which, in turn, leads to brain-tissue damage.

Conclusion

This study confirmed that there was hypomethylation of the *RIN3* gene in the whole blood of TIA/MIS patients relative to controls. Furthermore, there was hypomethylation of the

Table 8 Differences in Differentially Methylated Segments Between Patients with Transient Ischemic Stroke/Mild Ischemic Stroke and Healthy Controls

Target	Groupdiff	*P value*	Adj.*P value*
RIN3-26	−0.0011	0.03	0.17
RIN3-27	−0.0417	<0.001	<0.001

Note: Students *t*-test was used to compare the groups.
Abbreviations: Target, segment name; Groupdiff, difference in mean methylation between the case and control groups; Adj. *P*-value, *P*-value after adjusting for age and gender.

Table 9 Differences in Differentially Methylated Segments Between Patients with Transient Ischemic Stroke/Mild Ischemic Stroke with and without Cognitive Impairment

Target	Groupdiff	*P value*	Adj.*P value*
RIN3-27	−0.0268	0.01	0.046

Note: Students *t*-test was used to compare the groups.
Abbreviations: Target, segment name; Groupdiff, difference in mean methylation between the cognitive impairment and non-cognitive impairment groups; Adj. *P*-value, *P*-value after adjusting for age, gender, education, CHD, and DWM; Edu, education; CHD, coronary heart disease; DWM, deep white matter.

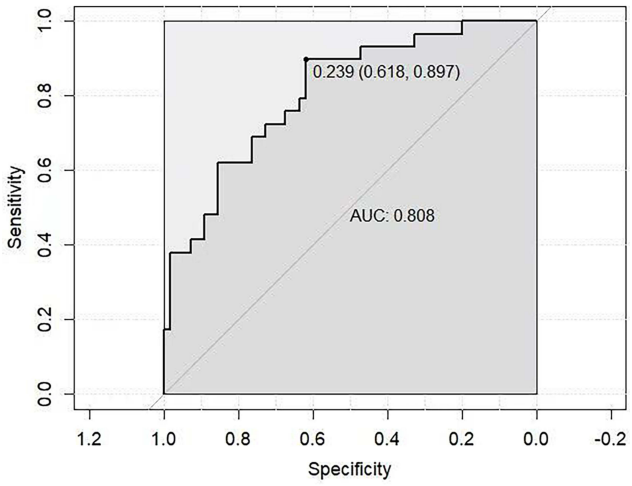

Figure 6 Showed the sensitivity/specificity for predicting early cognitive impairment after TIA/MIS.

RIN3 gene in the whole blood of patients with early cognitive impairment after TIA/MIS relative to patients without early cognitive impairment after TIA/MIS. Therefore, epigenetic modification of the *RIN3* gene was strongly associated with TIA/MIS and with TIA/MIS with early cognitive impairment. Based on the modifiable nature of methylation, our study suggests that it may be possible to influence this disease process by methylation via appropriate lifestyle and/or clinical interventions. Furthermore, since methylation can be quantified and easily analysed, the methylation levels of gene sites combined with clinical information can predict the occurrence of TIA/MIS with early cognitive impairment.

Data Sharing Statement

Datasets and codes of methylation used in the analyses are stored by the first author and will be provided upon request.

Ethics

This study was approved by the Ethics Committee of the Qilu Hospital of Shandong University (Qingdao). All patients provided written informed consent. The study complies with the Declaration of Helsinki.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors declare that there is no conflicts of interest or financial relationships that could be construed as a potential conflict of interest in this work.

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