Protective effect of miR-33-5p on the M1/M2 polarization of microglia and the underlying mechanism

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ABSTRACT
This study was aimed to investigate the influence of miR-33-5p on the M1/M2 polarization of microglia and the underlying mechanism. Transcriptome sequencing was performed using microglia from miR-33-5p mimic and control groups. In total, 507 differentially expressed genes, including 314 upregulated genes and 193 downregulated genes, were identified. The subnetwork of module A, which was extracted from the protein–protein interaction networks, mainly contained the downregulated genes. Cdk1, Ccnb1, and Cdc20, the members of module-A networks with the highest degrees, possess the potential of being biomarkers of ischemic stroke due to their function in the cell cycle. NFY, a transcription factor, was predicted to have the regulatory relation with nine downregulated genes. Overall, our findings will provide a valuable foundation for genetic mechanisms and treatment studies of ischemic stroke.

Highlights
- PCA analysis showed significant differences between HAPI-mimic and blank control groups.
- Cell cycle-related genes, such as Cdk1, Ccnb1, and Cdc20, were identified based on modularized genes.
- The transcription factor NFY regulated nine downregulated genes.

Introduction
Cardiovascular and cerebrovascular diseases are common and serious threats to humans worldwide [1]. Approximately 80 million people have experi-
encealed stroke, and more than 50 million survivors suffer from some form of permanent disability. Cerebral apoplexy is divided into ischemic stroke and hemorrhagic stroke, among which ischemic stroke is the most common [2]. The morbidity, mortality, and recurrence rate of ischemic stroke are extremely high [3]. The pathophysiological basis of ischemic stroke includes cell apoptosis, imbalance in body oxidation and antioxidation, toxicity effects of excitatory amino acids, and cell inflammation [4]. In many neurodegenerative diseases, the inflammatory response is closely related to the activation and polarization of microglia [5], a group of inflammatory cells [6].

Microglia are the smallest cells in the central nervous system, with small nuclei and little cytoplasm [7]. Microglia are mainly concentrated in the telencephalon, basal ganglia, olfactory bulb, and hippocampus, and are the brain’s inherent immune effector cells, participating in dynamic balance and host defense against pathogens and central nervous system diseases [8]. Microglia are activated under pathological conditions, which are named polarization of microglia [9]. Microglial activation is divided into two major phenotypes: classical activation (also known as M1 phenotype) and substitution activation (M2 phenotype) [10]. M1-type microglia is associated with cytotoxicity, superoxide production, and cytokine secretion [11]. The factors released by M1 microglial cells can inhibit tissue repair, destroy the blood-brain barrier, and participate in neuronal degeneration [12]. In contrast to the M1 phenotype, the M2 microglial phenotype exerts anti-inflammatory effects and promotes wound healing and tissue repair. M2-type microglia can also promote the expression of neuroprotective factors and participate in tissue repair and remodeling by changing gene expression [13]. Therefore, it is of great value to inhibit common markers on the surface of M1 microglia to reduce the cytotoxic effect and enhance the beneficial effect of M2 microglia [14]. However, the mechanism of M1/M2 polarization in microglia remains unclear.

MiR-33-5p has been shown to play a crucial role in the inflammatory response [15], macrophage lipid accumulation [16], and cell proliferation [17]. Zeng et al. demonstrated that miR-33-5p may be a potential biomarker for acute ischemic stroke [18]. Direct intracerebral delivery of miR-33 also changed gene expression [19]. Nevertheless, whether miR-33 is associated with the M1/M2 polarization of microglia and thus indirectly participates in the occurrence of ischemic stroke is still unknown.

As a result, this study was aimed to investigate the influence of miR-33-5p on the M1/M2 polarization of microglia and the underlying mechanism. The changes in gene expression after miR-33-5p overexpression were analyzed by RNA sequencing and bioinformatics methods. Western blotting was used to verify the results.

Materials and methods

Cell culture and transfection

Rat microglial HAPI cells were purchased from BNCC (Art. No. BNCC340723, Beijing, China). Briefly, HAPI cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium with 10% fetal bovine serum at 37°C and 5% CO2 in an incubator.

HAPI cells, at ~80% confluence, were harvested using a trypsin detachment solution and inoculated into a 6-well plate at a density of 5 × 105 cells/well. Cells were transfected with miR-33-5p mimics according to the manufacturer’s instructions (GenePharma Co., Ltd, Shanghai, China). After 48 h of transfection, the cell precipitate was collected and lysed with 1 mL TRIzol for qPCR detection.

Real-time PCR

Real-time PCR was performed as described previously [20]. Briefly, the reverse transcription system contained 4 μL 5× primeScript RT Master MIX (perfect Real Time), 1 μg RNA, and 15 μL RNase Free water (up to 20 μL). RT-PCR was performed using a quantitative PCR (ABI 7500, Thermo Fisher Scientific, MA, USA) in the presence of a fluorescent dye (SYBR Green I; Takara, NJ, USA). The primers used in this study are shown in Table 1.
dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to a PVDF membrane. After transfer, the membranes were incubated with 5% skim milk. Then, the blots were washed thrice with 1× PBS-T (1000 mL 1× PBS + 1 mL Tween-20) for 5–10 min. The primary antibody diluted with 5% skim milk was incubated overnight at 4°C. After washing the membrane six times, secondary antibody was added and transferred to a table concentrator at 37°C for 2 h. Finally, bands were detected using the Millipore ECL system. Tanon Image Software was used for grayscale analysis. P < 0.05 was the screening criterion for significant difference.

**cDNA library construction and transcriptome sequencing**

The sequencing experiment was performed using the Illumina TruseqTM RNA sample prep Kit method for library construction. Briefly, total RNA was extracted using TRIzol reagent (Invitrogen) and its concentration and purity were detected using Nanodrop 2000. After reverse transcription, jointing adaptor, and PCR amplification, a cDNA library was constructed. The library was sequenced using an Illumina HiSeq™ 2000 sequencer (Illumina, San Diego, CA, USA).

**Raw reads filtering**

To ensure the accuracy of the subsequent analysis, the original sequencing data were filtered by removing joint sequences, low-quality read segments, and high N (N represents uncertain base information) rate sequence. SeqPrep [21] and Sickle [22] were used to remove the joint sequence from reads, sequences of less than 50 bp, and low-quality sequences.

**Mapping and differential expression analysis**

Based on the clean data, TopHat2 [23] was used to perform a sequence alignment analysis. Based on the existing reference genome, the mapped reads were assembled and spliced to obtain differentially expressed genes (DEGs) and new transcripts using Cufflinks [24] and StringTie [25]. The screening

### Western blotting

After lysis with RIPA lysis buffer, proteins were extracted from the fully lysed sample. Proteins from each sample were separated by sodium

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**Table 1. The primers used in this study.**

| Primers | Sequences (5'-3') |
|---------|------------------|
| rat-mIrr-33-Sp-F | AGCTCGTGCAAGTGTAAACATTC |
| rat-mIrr-33-Sp-R | GTGCAGCTTGTTCTGCAG |
| rat-U6-F | GCCTTGCAAGGTGAGTGTAAACATTC |
| rat-U6-R | CGCTTCAGCAGTTGGGTCAT |
| β-actin-rat-F | ATTGCTGACCAGGAGCAAGAA |
| β-actin-rat-R | TAGAGCACACCAATCCACAG |
| rat-CCL2-F | ACCACGACGAGGTGTCAT |
| rat-CCL2-R | TGGCTTGTTCTGCAG |
| rat-IL-1β-F | CAGGATGAGGAGCCTCATCCT |
| rat-IL-1β-R | GTGCAGCTTGTTCTGCAG |
| rat-TNF-a-F | GCCCTCTTCATCCCTGTCAG |
| rat-TNF-a-R | TCCGCTTTGTTTCCTGAC |
| rat-Ym-1-F | TGGAGGCTGGAAGTTGGAAT |
| rat-Ym-1-R | GCATATGCTGCTGTCAG |
| rat-CD206-F | GTGCTCTCTGTGCTGCTG |
| rat-CD206-R | TCCACGATCCACATCAAG |
| rat-Ang-1-F | GAGAAAAGTCCCGGAGCAAT |
| rat-Ang-1-R | CAGACCGTGGGATCTCCACAA |
| rat-Slc7a5-F | TGGAGGCTGCCATCAAGGT |
| rat-Slc7a5-R | GAGACCGCTGCCAGGGAAGAG |
| rat-Rhod-F | AGCTTGGAAGCCTCATCCT |
| rat-Rhod-R | CGACGTCGATCTGCTG |
| rat-Smad1-F | CAGGCTGTGTTGGATGAGT |
| rat-Smad1-R | TCACTGAGGCACTCCGAGAT |
| rat-Rhod-F | GGAGCAAGATTTGGGAAT |
| rat-Rhod-R | TGAGACGTCGATCTGCTG |
| rat-Myb1-2-F | TTGTGATGAGATGAGGAAGAG |
| rat-Myb1-2-R | CAGACGCGACATCCGTCAGT |
| rat-GAPDH-F | AGAGACCGCGAGATCCGTCAGT |
| rat-GAPDH-R | CAGACCGCGAGATCCGTCAGT |

**Table 2. Pathways and BP terms (top 5) enriched by upregulated DEGs.**

| Category | Term | Count | PValue |
|----------|------|-------|--------|
| PATHWAY  | mro04010:MAPK signaling pathway | 12 | 5.16E-03 |
| PATHWAY  | mro05202:Transcriptional misregulation in cancer | 9 | 9.38E-03 |
| PATHWAY  | mro05000:Starch and sucrose metabolism | 4 | 1.25E-02 |
| PATHWAY  | mro04213:Longevity regulating pathway – multiple species | 5 | 1.63E-02 |
| PATHWAY  | mro05135:Yersinia infection | 7 | 1.72E-02 |
| GO_BP    | GO:0045944~ positive regulation of transcription from RNA polymerase II promoter | 42 | 7.89E-07 |
| GO_BP    | GO:006357~ regulation of transcription from RNA polymerase II promoter | 44 | 6.59E-06 |
| GO_BP    | GO:0048704~ embryonic skeletal system morphogenesis | 8 | 2.44E-05 |
| GO_BP    | GO:0007399~ nervous system development | 14 | 4.30E-05 |
| GO_BP    | GO:0086010~ membrane depolarization during action potential | 5 | 6.75E-05 |
criteria for DEGs were $|\log(FC)| > 1$ and p-value $< 0.05$.

**Functional enrichment analysis of differentially expressed genes (DEGs)**

The DEGs were subjected to Gene Ontology (biological process; GO BP) and Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation using the common enrichment analysis tool DAVID [26] (version 6.8). The thresholds were count ≥ 2 and p-value < 0.05.

**Construction of a protein–protein interaction (PPI) network of DEGs**

The interaction relationship between DEG-coding proteins was predicted and analyzed using the STRING [27] (version 10.0) database (PPI score: 0.15). Cytoscape plugin MCODE (version 1.4.2) was used to analyze the module in the PPI network (score > 5).

Additionally, the module genes were mapped using GO BP and KEGG databases for functional annotation. DAVID [26] (version 6.8) was used to perform the function analyses, with thresholds of count ≥ 2 and p-value < 0.05.

**Transcription Factor (TF)-target and miRNA-target regulatory network prediction**

Based on the significant module genes, the Overrepresentation Enrichment Analysis (ORA) method in WebGestalt [28] was used to predict the TF-target and miRNA-target regulatory relation for network construction.

**Statistical analysis**

All experiments were repeated three times. Data are shown as mean ± standard deviation. GraphPad Prism 5 (San Diego, CA, USA) was used to analyze the data from this study. One-way analysis of variance was used for comparisons among groups, followed by Newman-Keuls multiple comparison test. Statistical significance was considered for p-values less than 0.05.

**Results**

**Expression of miR-33-5p and M1/M2 biomarkers**

The expression level of miR-33-5p was detected by RT-PCR. As shown in Figure 1(a), the expression of miR-33-5p in the mimic group was significantly higher than that in the blank control (BC) and negative control (NC) groups ($p < 0.01$). The biomarkers of M1 microglia (CCL2, IL-1, and TNF-α) and biomarkers of M2 microglia (Ym-1, CD206, and Arg1) were detected. The expression levels of the three biomarker genes of M1 in the mimic group were significantly increased compared with those in the BC and NC groups, while M2 in the mimic group were significantly reduced compared with those in the BC and NC groups (Figure 1(b)).

**Genes differentially expressed upon miR-33-5p overexpression**

To investigate the action mechanism of miR-33-5p in the M1/M2 polarization of microglia, DEGs between the groups with or without miR-33-5p mimic treatment were identified. In total, 507 DEGs were found, which included 314 upregulated genes and 193 downregulated genes. The heatmap and volcano plot of DEGs are shown in Figure 2(a-b).

**Functional enrichment analysis of differentially expressed genes (DEGs)**

The upregulated DEGs were significantly enriched in 84 BP terms, such as positive regulation of transcription from RNA polymerase II promoter, and regulation of transcription from RNA polymerase II promoter, and 6 KEGG pathways, such as MAPK signaling pathway and transcriptional misregulation in cancer. The downregulated DEGs were significantly enriched in 59 BP terms, such as, mitotic DNA replication initiation, and DNA unwinding involved in DNA replication, and 8 KEGG pathways, such as cell cycle, and DNA replication. The top 5 terms for the enrichment results are shown in Tables 2 and 3.
Protein–protein interaction (PPI) network and module analysis

To obtain more interactions, PPI networks were constructed using STRING. As shown in Figure 3, 407 nodes and 1347 edges were included in the networks. The top ten nodes, with higher degrees, were Cdk1, Ccnb1, Cdc20, Mad2l1, Ccna2, Ube2c, Mcm3, Mcm4, Kif2c, and Kif23. Due to the large number of nodes in the network, we further selected the key module from the network. Two modules were finally obtained with the threshold of score > 5, as shown in Table 4 and Figure 4. Module A (score: 23.33) contained 25 nodes and 280 edges. All of the genes in module A were downregulated, and the top five were Cdk1, Ccnb1, Cdc20, Mad2l1, and Ccna2. Module B (score: 5.24) contained 22 nodes and 55 edges (Figure 3). Most genes in this module were upregulated except for Colla1 and Cyr61.

Function analysis of module genes

Genes in module A were significantly enriched in six KEGG pathways, including cell cycle, DNA replication, oocyte meiosis, progesterone-mediated oocyte maturation, and foxo signaling pathway. For GO BP, microtubule-based movement, mitotic cell cycle, cell

Figure 1. The expression of miR-33-5p (a) and biomarkers of M1/M2 microglia (b). *p < 0.05 and ** p < 0.01.
division, and DNA unwinding involved in DNA replication terms were significantly enriched. The top five BP terms of module B were anterior/posterior pattern specification, embryonic skeletal system morphogenesis, embryonic skeletal system development, positive regulation of transcription from RNA polymerase II promoter, and cellular response to hormone stimulus.

**Transcription factor (TF)-target and miRNA-target networks**

In total, 6 TFs were predicted for the module genes, involving 58 pairs of TF-target regulatory relationships. As shown in Figure 5(a), the six TFs were NFY, NFAT, GFI1, PAX4, HNF1, and GER1. NFY had the highest degree, which regulated the most target genes, such as the downregulated genes of Ncapd2, Ube2c, Pola1, Ccna2, Cdk1, Mcm4, etc., and upregulated genes of Rhoq, Stat3, Pbx2, etc. GFI1, NFAT, PAX4, and EGR1 regulated eight target genes, respectively. HNF1 regulated seven target genes. The downregulated gene of Col1a1 was regulated by four TFs, including NFY, NFAT, PAX4, and HNF1. Based on the module genes, 17 miRNAs were predicted, such as MIR106A, MIR106B, MIR20B, and MIR519D. The miRNA-target network was conducted, which included 17 miRNAs and 18 genes (five downregulated and 13 upregulated), involving 99 regulatory relation pairs (Figure 5(b)). Among the 18 genes, Pbx1, Pbx2, and Col1a1 were the center nodes with degrees greater than 10. In addition, Arhgef12, Rhoc, Kif23, and Stat3 also showed high connectivity degrees with the miRNAs.

**Table 3. Pathways and BP terms (top 5) enriched by downregulated DEGs.**

| Category | Term | Count | PValue          |
|----------|------|-------|----------------|
| PATHWAY  | rno04110:Cell cycle | 11 | 4.31E-07 |
| PATHWAY  | rno03030:DNA replication | 6 | 2.16E-05 |
| PATHWAY  | rno04914:Progestosterone-mediated oocyte maturation | 6 | 2.11E-03 |
| PATHWAY  | rno04114:Oocyte meiosis | 6 | 6.39E-03 |
| PATHWAY  | rno03008:Ribosome biogenesis in eukaryotes | 5 | 1.07E-02 |
| GO_BP    | GO:1902975- mitotic DNA replication initiation | 4 | 1.10E-05 |
| GO_BP    | GO:0006268- DNA unwinding involved in DNA replication | 5 | 2.96E-05 |
| GO_BP    | GO:0045944- positive regulation of transcription from RNA polymerase II promoter | 26 | 3.90E-05 |
| GO_BP    | GO:0000727- double-strand break repair via break-induced replication | 4 | 1.16E-04 |
| GO_BP    | GO:0045893- positive regulation of transcription, DNA-templated | 17 | 3.32E-04 |
Verification of differentially expressed genes (DEGs) by qPCR and western blotting

Efferocytosis-related genes, Slc7a5, Rhob, Smad1, Rhog, and Mybl2, were selected from DEGs and their expression levels were verified by qPCR and western blotting. As shown in Figure 6(a), the mRNA expression levels of Slc7a5, Rhog, and Smad1 were significantly different between the two groups. After that, the protein levels of Slc7a5 and Rhog were detected by western blotting. As shown in Figure 6(b), Slc7a5 was significantly downregulated, while Rhog was significantly upregulated in the miR-33-5p mimic group.

Discussion

In this study, gene expression data were analyzed to identify genes involved in microglia upon overexpression of miR-33-5p. Compared with the control groups, 507 DEGs were identified in groups with mimics. Cdk1, Ccnb1, and Cdc20 had higher degrees in the PPI module. TFs of NFY, NFAT,
Table 4. Genes in module-A and module-B.

| Genes | Description | Degree | Genes | Description | Degree |
|-------|-------------|--------|-------|-------------|--------|
| Cdk1  | down        | 55     | Stat3 | up          | 24     |
| Ccnb1 | down        | 49     | Egr1  | up          | 18     |
| Cdc20 | down        | 45     | Rhoc  | up          | 14     |
| Mad21 | down        | 41     | Pbx1  | up          | 12     |
| Ccnb2 | down        | 37     | Col1a1| down        | 12     |
| Ube2c | down        | 36     | Pbx2  | up          | 11     |
| Mcm3  | down        | 35     | Rhog  | up          | 11     |
| Kif23 | down        | 34     | Rhob  | up          | 10     |
| Mmc4  | down        | 34     | Hoxc9 | up          | 9      |
| Kif2c | down        | 34     | Plocl | up          | 8      |
| Top2a | down        | 33     | Rhoq  | up          | 8      |
| Mcm2  | down        | 33     | Cyr61 | down        | 8      |
| Rfc4  | down        | 33     | Hoxb6 | up          | 8      |
| Incenp| down        | 32     | Hoxb5 | up          | 8      |
| Ncapd2| down        | 32     | Hoxb3 | up          | 7      |
| Rad51 | down        | 30     | Plocl | up          | 7      |
| Ect2  | down        | 29     | Hoxb4 | up          | 7      |
| Pola1 | down        | 29     | P4ha2 | up          | 6      |
| Kif4a | down        | 28     | Fgd1  | up          | 6      |
| Pold1 | down        | 27     | Col4a5| up          | 6      |
| Pbk2  | down        | 27     | Arhgef12| up        | 6      |
| Dsc2  | down        | 26     | Col25a1| up          | 5      |
| Plk3  | down        | 25     |       |             |        |
| Plk2  | down        | 25     |       |             |        |
| Cks2  | down        | 23     |       |             |        |

GF11, PAX4, HNF1, and GER1 had regulatory relationships with the DEGs.

The differential expression of Slc7a5 and Rhog and the proteins encoded by them was verified by RT-PCR and western blotting, respectively. Slc7a5 plays a critical role in cell growth and proliferation [29]. To our knowledge, this is the first report to demonstrate the regulatory relationship between miR-33-5p and Slc7a5. There is convincing evidence that Slc7a5 is deeply involved in the occurrence of ischemic stroke [30]. Therefore, more detailed studies are needed to prove the regulatory relationship between Slc7a5 and miR-33-5p. Rhog is a member of the Rho family, which plays an important role in regulating cytoskeletal reorganization in physiological and pathophysiological situations [31]. To our best knowledge, there was no report about the associations between Rhog and miR-33-5p or ischemic stroke; therefore, we hypothesized that Rhog might participate in M1/M2 polarization based on our results.

All the genes in module A were downregulated. Among the 25 genes in module A, Cdk1, Ccnb1, and Cdc20 possessed the most interactions with other genes. Cyclin-dependent kinases (CdkS) have already been reported to mediate the death of ischemic neuronal cells. Zhang et al. proved that the expression of Cdk1 was induced when primary cortical neuron cultures were exposed to oxygen-glucose deprivation (OGD) for 4 h [32]. Cdk1 also showed partial resistance to OGD-induced neuronal cell death [33]. Moreover, Cdk1 has also been shown to play a critical role in neuronal death and has been reported to contribute to the pathogenesis of neurodegenerative diseases [34]. Currently, it

Figure 4. Subnetworks (a, module-A; b, module-B) of PPI network. Yellow circles indicate upregulated genes, and green squares indicate downregulated genes. The size of a node is based on the degree value, with higher degree values indicated by larger nodes.
is generally accepted that $Cdk1$ regulates the cell cycle. Importantly, miR-33 has been demonstrated to play a crucial role in cell proliferation and cell cycle progression by modulating the expression of $Cdk1$ [35,36]. Our results were consistent with the abovementioned studies, indicating that the interactions between miR-33 and $Cdk1$ may affect the development of ischemic stroke.

Cyclin B1 ($Ccnb1$), an important regulator of the cell cycle machinery, is essential for mouse embryonic development [37]. Several studies have shown that $Ccnb1$ is involved in central nervous system regeneration driven by microglia [38]. However, there was no evidence to prove the direct regulation between $Ccnb1$ and miR-33-5p. Cdc20 is an important cell-cycle regulator for the completion of mitosis in organisms [39]. Lloyd et al. found that Cdc20 could promote the proliferation of microglia through its population replacement process [40]. Elevated Cdc20 increased extensive mitotic errors, leading to chromosome
mis-segregation [41]. Based on the existing literature, we speculated that miR-33-5p may regulate the expression of genes involving in caryomitosis and cell cycle, such as Cdk1, Ccnb1, and Cdc20.

Interestingly, we found that collagen type I alpha I (Col1a1) appeared in module-B, TF-target, and miRNA-target networks. It has been reported that Col1a1 is highly related to osteoporotic fracture [42], bone mineral density, and osteoporotic fracture [43]. The only research that associated Col1a1 with ischemic stroke was completed by Choi et al., who investigated the changes in gene expression after ischemic stroke [44]. In our results, the TF of nuclear factor Y (NFY) showed a wide range of interactions with nine downregulated genes. NFY was proved to be associated with the sterol regulation of human fatty acid synthase promoter I [45]. However, no studies have identified the direct relationship between NFY and microglia or ischemic stroke. We hypothesize that NFY may be involved in microglial polarization by indirectly regulating other genes.

Conclusions
In conclusion, our result for the first time demonstrated that miR-33-5p plays a crucial role in the M1/M2 polarization of microglia. Overexpression of miR-33-5p induced a significant change in the expression of Slc7a5 and Rhog. Genes that regulate neuron cell cycle and death, such as Cdk1, Ccnb1, and Cdc20, attracted our attention due to their high potential for M1/M2 polarization.

Authors’ contributions
Bo Yu, Dingying Ma, and Wenhua Chen carried out the conception and design of the research and obtained the funding. Ran Sun, Fei He and Lihua Chen participated in data acquisition. Song Chai, Yilan Sheng, Fei He and Ran Sun participated in the data analysis, and interpretation. Yilan Sheng, Jieshi He and Lihua Chen performed the statistical analysis. Song Chai, Yilan Sheng, Bo Yu and Ran Sun drafted this paper. Bo Yu and Dingying Ma participated in the revision of the manuscript for important intellectual content. All authors have read and approved the final manuscript.

Availability of data and materials
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Disclosure statement
No potential conflict of interest was reported by the author(s).

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