Variants Affecting the C-Terminal of CSF1R Cause Congenital Vertebral Malformation Through a Gain-of-Function Mechanism

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CSF1R encodes the colony-stimulating factor 1 receptor which regulates the proliferation, differentiation, and biological activity of monocyte/macrophage lineages. Pathogenic variants in CSF1R could lead to autosomal dominant adult-onset leukoencephalopathy with axonal spheroids and pigmented glia or autosomal recessive skeletal dysplasia. In this study, we identified three heterozygous deleterious rare variants in CSF1R from a congenital vertebral malformation (CVM) cohort. All of the three variants are located within the carboxy-terminal region of CSF1R protein and could lead to an increased stability of the protein. Therefore, we established a zebrafish model overexpressing CSF1R. The zebrafish model exhibits CVM phenotypes such as hemivertebral and vertebral fusion. Furthermore, overexpression of the mutated CSF1R mRNA depleted of the carboxy-terminus led to a higher proportion of zebrafish with vertebral malformations than wild-type CSF1R mRNA did (p = 0.03452), implicating a gain-of-function effect of the C-terminal variant. In conclusion, variants affecting the C-terminal of CSF1R could cause CVM though a potential gain-of-function mechanism.

Keywords: colony stimulating factor 1 receptor, congenital vertebral malformation, C-terminal variant, gain-of-function mechanism, zebrafish model

INTRODUCTION

Colony-stimulating factor 1 receptor (CSF1R) regulates the proliferation, differentiation and biological activity of monocyte/macrophage (Mφ) lineages (Stanley et al., 1997). CSF1R is also expressed in Paneth cells, renal proximal tubule epithelial cells, and placental trophoblasts, indicating a pleiotropic role of CSF1R protein in embryonic development (Arceci et al., 1989, 1992; Pixley and Stanley, 2004; Huynh et al., 2009; Menke et al., 2009). The CSF1R protein includes an extracellular ligand-binding domain and an intracellular tyrosine kinase domain (PTK domain) (Pixley and Stanley, 2004). The extracellular domain of CSF1R binds to ligands such as CSF1 and IL34, which further induces autophosphorylation and protein dimerization (Wang et al., 2012; Stanley and Chitu, 2014). Downstream molecules interact with the intracellular part of CSF1R and are phosphorylated by the PTK domain. The phosphorylated signal molecules...
could activate the downstream signaling pathway, promoting the proliferation and differentiation of hematopoietic precursor cells, especially mononuclear phagocytes. Heterozygous deleterious variants in CSF1R have been reported to cause adult-onset leukoencephalopathy with axonal spheroids and pigmented glia (ALS-P, MIM: 221820) (Oosterhof et al., 2019), a neurological disease characterized by executive dysfunction, memory decline, personality changes, motor impairments, and seizures (Svensson et al., 2011; Rodriguez-Tornos et al., 2016; Makrythanasis et al., 2018). Notably, most of the reported ALS-P-related heterozygous pathogenic variants were located at the intracellular PTK domain

More recently, homozygous variants in CSF1R have been linked to brain abnormalities, neurodegeneration, and dysosteosclerosis (MIM: 618476), which is characterized by progressive neurologic deterioration and sclerotic bone dysplasia (Guo et al., 2019; Oosterhof et al., 2019). Csf1r−/− mouse model resembled the skeletal phenotypes of the patients carrying bi-allelic CSF1R variants, implicating the important role of CSF1R in bone development (Erblich et al., 2011). Furthermore, an abnormal vertebral arch compared to normal individuals was observed in the zebrafish model with bi-allelic loss-of-function mutations in csf1ra and csf1rb, indicating the potential effect of CSF1R variants on vertebral morphology (Oosterhof et al., 2019).

In this study, we analyzed variants in CSF1R in a cohort of congenital vertebral malformation (CVM), which has been partially attributed to genetic defects previously (Wu et al., 2015, 2019; Chen et al., 2016, 2020; Liu et al., 2019; Yang et al., 2019, 2020; Lin et al., 2020; Ren et al., 2020). In vitro and in vivo functional experiments were then performed to explore the effect of these variants on protein expression and vertebral morphology.

METHODS

Human Subjects

Five hundred and eighty-three patients diagnosed with CVM were consecutively enrolled and collected in the cohort between 2009 and 2018 at Peking Union Medical College Hospital, as a part of the Deciphering Disorders Involving Scoliosis and CoMorbidities (DISCO) study (http://www.discostudy.org/). Detailed phenotypic data was recorded. X-ray, computed tomography (CT), and magnetic resonance imaging (MRI) were also taken. Exome sequencing and bioinformatic analysis were conducted as described previously (Zhao et al., 2021). Variant interpretation was then performed based on the genome Aggregation Database (gnomAD, http://gnomad.broadinstitute.org/). Rare variants in CSF1R were extracted and filtered with the following criteria: (1) truncating (non-sense, frameshift, splice acceptor/donor) and minor allele frequency (MAF) ≤0.001 or (2) missense variants absent from public databases in the general population.

Sanger Sequencing

Candidate variants of CSF1R identified in our cohort were validated by Sanger sequencing. The variant-encoding amplicon was amplified by PCR from genomic DNA obtained from patients and purified using an Axygen AP-GX-50 kit (lot no. 05915KE1). Sanger sequencing was then performed on an ABI3730XL instrument.

Plasmid Construction

The pEGFP-C1-based vector was used for the construction of wild-type CSF1R and mutated CSF1R (NM_005211.3: c.2906_2909dupATCA, c.2797G>T, c.2749_2758delGACAGGAGAG) plasmids. All plasmids were verified by DNA sequencing.

Cell Culture, Transfection, and Western Blotting Analysis

Cos-7 cells were maintained in Dulbecco’s Modified Eagle’s medium (DMEM, Invitrogen) supplemented with fetal bovine serum (Gibco), penicillin (50 U/ml), and streptomycin (50 µg/ml) in six-well plates. The full-length wild-type or mutant CSF1R constructs (plasmid pEGFP-C1 1 µg) was transfected into the cells, respectively. After 48 h, western blotting was used to assess the protein expression level in the cell lysates with the following antibodies: rabbit anti-human CSF1R monoclonal antibody (1:1,000, Abcam, ab229188) and mouse anti-GAPDH monoclonal antibody (1:1,000, ZSJB Co., Ltd.).

Zebrafish Husbandry and Fertilization

Tg (Ola.Sp7:nlsGFP) transgenic zebrafish, where GFP expression was driven by an Sp7 promoter in osteoblasts, was utilized for animal model establishment. The zebrafish were kept at 28°C and fed with brine shrimp twice a day. From the 2–4-cell stage to 3 days post fertilization (dpf), zebrafish were exposed to methylene blue which inhibited fungal contamination.

In vitro mRNA Transcription

A human wild-type CSF1R DNA sequence was cloned into the PCS2+ plasmid, constructing a human wild-type CSF1R plasmid. Then, an indel variant (c.2749_2758delGACAGGAGAG) was constructed to generate a human-mutated CSF1R plasmid containing a mutated gene sequence. After the linearization of plasmids, transcription was performed with mMESSAGE mACHINE™ SP6 Ultra Transcription Kit (Ambion) to obtain corresponding mRNA.

In vivo mRNA Overexpression Experiment

Human CSF1R mRNA (50 pg for each), i.e., the wild-type mRNA or the mutated mRNA, was dissolved into distilled water (2 nl for each) and injected into embryos at the 1–2-cell stage, respectively. An equal amount of water was injected into the embryos, which were fertilized as the control group. All the individuals in the experimental groups and control group were maintained for phenotype evaluation.

Fluorescence Imaging and Phenotype Evaluation

Fluorescence images were collected at 14 dpf by fluorescent microscopy. The vertebral morphology was observed and recorded for phenotypic evaluation and statistical analysis.
Genetic Analysis of CSF1R-Associated Genes

Twenty genes which are biologically linked to CSF1R (IL34, CSF1, SOS, GRB2, INPP5D, INPPL1, PIK3R1, PIK3R2, SOCS1, SOCS3, CBL, FYN, GRAP2, LYN, RASA1, SHC11, THOC5, TES1, CBL, PLCG2, SLA2) were selected for analysis according to public databases including Pubmed (https://pubmed.ncbi.nlm.nih.gov/) and Online Mendelian inheritance in Man (OMIM, https://www.omim.org/). Further, variant filtration of those genes was performed according to the same interpretation method as we did for CSF1R.

Statistics

Statistical differences between different experiment groups and the control group were evaluated with the Chi-square test. All statistical procedures were carried out using GraphPad Prism 8. *, **, *** and **** denote $p < 0.05$, $< 0.01$, $< 0.001$, and $< 0.0001$, respectively.

RESULTS

Pathogenic Variants Identified in CSF1R

From three patients with CVM, three deleterious heterozygous variants in CSF1R were identified, including two predicted truncating variants and one novel missense variant (Table 1). DISCO-CSS170368 was a 14-year-old girl admitted for spinal surgery (Figure 1A). The spinal plain radiograph showed that the coronal Cobb angle of the main curve (T2–L1) was 114°. The CT scan and three-dimensional reconstruction showed segmentation defects of T5–T9, dysplasia of T3–9, L4 hemivertebra, and left 6th and 7th rib abnormality (Figure 1B). MRI indicated mild diastematomyelia and syringomyelia. The girl showed no dyspnea, numbness or weakness of the limbs, backache or extremity pain, or marfanoid symptoms during the course of the disease. The clinical diagnoses of this patient were severe congenital scoliosis, pulmonary dysfunction, and diastematomyelia along with syringomyelia. WES identified a heterozygous truncating CSF1R variant (NM_005211.3: c.2749_2758delGACAGGAGAG), which was then confirmed by Sanger sequencing (Figure 1A). This variant is absent from public databases and our in-housed database encompassing 849 exome data (Table 1). Ten nucleotides were deleted between positions 2749 and 2758 in exon 21, resulting in a frameshift mutation in CSF1R. The mRNA harboring this mutation is predicted to escape from non-sense-mediated mRNA decay (NMD) by the NMD Esc Predictor (Figure 2A) (Coban-Akdemir et al., 2018). Hence, this frameshift mutation could consequently generate a truncated protein product (p.Asp917SerfsTer32).

DISCO-CSS180319 was a 10-year-old female patient with CVM. Imaging examination revealed a 56° Cobb angle of the main curve (T3–T7). Besides, a structure disorder of T3–T7 was also observed in CT scan and three-dimensional reconstruction (Figure 1B). A missense variant (NM_005211.3: c.2797G>T) was identified in this girl (Figure 1A) and was also absent from the public and in-house databases. This variant is predicted to be deleterious by Polyphen-2 (Polyphen2 HDIV score: 0.981).

| Patient | Ref transcript | Variant nomenclature | Mutation type | gnomAD allele frequency | gnomAD allele count | In-house allele frequency | In-house count | CADD | Polyphen2 HDIV score |
|---------|----------------|----------------------|--------------|-------------------------|-------------------|--------------------------|----------------|------|---------------------|
| DISCO-CSS170368 | NM_005211.3 | c.2749_2758delGACAGGAGAG | Frameshift variant | 0 | 0 | 0 | 0 | NA | NA |
| DISCO-CSS180319 | NM_005211.3 | c.2797G>T | Missense variant | 0 | 0 | 0 | 0 | 11.22 | 0.981 |
| DISCO-CSS170278 | NM_005211.3 | c.2906_2909dupATCA | Frameshift variant | 0.00003254 | 10 | 0 | 0 | 0.002594 | 0.002594 |

TABLE 1 | Deleterious and rare CSF1R variants identified in patients with congenital vertebral malformation.

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DISCO-CSS170278 was a 16-year-old boy with thoracic and lumbar scoliosis. The spinal plain radiograph revealed the presence of T10 hemivertebra (Figure 1B). WES analysis revealed a deleterious variant (NM_005211.3: c.2906_2909dupATCA) in CSF1R, which has been reported in the East Asian population with low frequency (0.0005) (Figure 1A). This deleterious variant could lead to a reading-frame shift. As a result, the last two amino acids of CSF1R were deleted and six extra amino acids were added to the C-terminus of the protein.

Interestingly, all of the three rare CSF1R variants identified in our cohort were located within or near the carboxy-terminal region downstream of the PTK domain (Figure 2B), which has been reported to be responsible for protein degradation mediated through the ubiquitination–proteasome pathway (Pawson, 1995; Schlessinger, 2000). No pathogenic variant in this region has ever been reported to be related to human diseases. Hence, those newly identified deleterious CSF1R variants might contribute to CVM through a distinct mechanism, which remains to be further investigated.

**Variants Affecting the C-Terminal of CSF1R Increased the Stability of Protein and Could Induce Vertebral Malformation in Zebrafish**

To determine the functional consequence of the C-terminal CSF1R variants in vitro, we transfected wild-type and mutant CSF1R constructs into cos-7 cells and examined the cellular levels of protein products with Western blotting. As a result, all of the three mutants accumulated at greater levels than the wild type, suggesting increased stability of mutant CSF1R proteins (Figures 3A,B).

Therefore, we proposed that the heterozygous deleterious CSF1R variants might exert a gain-of-function effect on CSF1R biological function through disturbing the carboxy-terminal region of the protein. We then overexpressed the mutated CSF1R alleles (NM_005211.3: c.2749_2758delGACAGGAGAG) which is depleted of all the carboxy-terminal regions downstream of the PTK domain in the zebrafish model. This mutated mRNA-deleted carboxy-terminal region is referred to as CSF1RΔC mRNA in the following text.

The human CSF1R and CSF1RΔC mRNAs were injected into Tg (Ola.Sp7:nlsGFP) zebrafish embryos, respectively. Fluorescence images of the spine were collected at 21 dpf by fluorescent microscopy. The zebrafish of the CSF1R mRNA overexpression group exhibited vertebral malformations including vertebral fusion, hemivertebra, and fused vertebral arches (Figure 3C), which recapitulated the human CVM phenotype. These phenotypes indicate that the dose effect of CSF1R expression might be related to vertebral malformation. Intriguingly, injection of human CSF1RΔC mRNA led to a higher proportion of zebrafish with vertebral malformations than the wild-type mRNA did (p = 0.03452) (Figure 3D).

**FIGURE 1** | Variant information and clinical phenotype of CSF1R carboxy-terminal variants in three patients with CVM. (A) Results of Sanger sequencing of the rare CSF1R carboxy-terminal variants. (B) Spinal plain radiograph and computed tomography (CT) scan three-dimensional reconstruction of those CVM patients. CVM, congenital vertebral malformation.
These results revealed that the carboxy-terminus-mutated CSF1R alleles might exert gain-of-function effects and thereby cause vertebral malformations.

**Mutational Spectrum in Genes Interacting With CSF1R**

As an essential signaling transducer, CSF1R participates in several biological processes in individual development and interact with many other genes. To explore whether the variants in CSF1R-related genes may also be related with clinical phenotypes like vertebral abnormalities, we identified 21 genes of which the protein products interacted with CSF1R (IL34, CSF1, SOS, GRB2, INPP5D, INPPL1, PIK3R1, PIK3R2, SOCS1, SOCS3, CBL, FYN, GRAP2, LYN, RASA1, SHC11, THOC5, TES1, CBL, PLCG2, SLA2) and performed a genetic spectrum analysis. Twelve potential deleterious rare variants were identified in different patients, including four truncating variants and eight missense variants (Table 2). Among those CSF1R-related genes, CBL encodes an adaptor protein c-Cbl for CSF1R. It positively regulates CSF1R ubiquitination in a manner dependent on its variant SH2 and RING finger domains, then marking active CSF1R for degradation (Pawson, 1995; Schlessinger, 2000). One of the binding sites of c-Cbl on CSF1R is 969aa, which lies in the carboxy-terminal region of CSF1R where all three pathogenic variants identified in CSF1R were located (Wilhelmsen et al., 2002). We prioritized two heterozygous CBL variants, including a rare truncating variant and a novel missense variant, from two patients in our CVM cohort. Those deleterious variants of CBL may affect the interaction between CSF1R and c-Cbl and lead to CVM by a similar mechanism as carboxy-terminal region variants of CSF1R do.

**DISCUSSION**

In this study, we used an exome-level genomic approach to identify potentially pathogenic variants of CSF1R genes in a CVM cohort. We identified three rare deleterious heterozygous C-terminal CSF1R variants, which were proved to increase the stability of protein in vitro. The CVM patients carrying those CSF1R variants exhibited vertebral malformations and early onset of scoliosis clinically. The zebrafish overexpression model resembled those vertebral phenotypes of CVM patients, indicating a gain-of-function mechanism.

In previous studies, CSF1R has been related to ALSP, which was inherited in an autosomal dominant pattern (Rademakers et al., 2011). ALSP is a fatal neurological disease characterized by progressive cognitive and motor impairment and seizures. The majority of the reported variants related with ALSP are missense or in-frame indels within the PTK domain (Guo...
et al., 2019). Recent studies indicated that bi-allelic CSF1R variants contribute to skeletal abnormalities in an autosomal recessive pattern (Guo et al., 2019; Oosterhof et al., 2019). The skeletal phenotypes of affected patients were referred to as the DOS-Pyle disease spectrum, which is characterized by sclerotic skull and flat and diffusely dense vertebral bodies (Guo et al., 2019). The pleiotropic effects of CSF1R could be attributed to the different inheritance modes and the variety of variants. Unlike the reported pathogenic CSF1R mutations, the variants identified in our cohort were located within the carboxyl-terminal region of protein. This terminal region contains two autophosphorylation sites (923aa and 969aa), which offer binding sites for c-Cbl and participate in the degradation of CSF1R (Wilhelmsen et al., 2002; Ho et al., 2020). We proposed that the biological function of the protein could be affected when the carboxyl-terminal region is...
Apart from the variety of variants and different pathogenic mechanisms, the age of onset is another factor which potentially affects the clinical symptoms of CSF1R variant carriers. The average age of symptom onset for ALSP is about 40 years (Sundal and Wszolek, 1993; Adams et al., 2018), while the average age of the patients with CSF1R deleterious variants identified in our cohort is around 10 years. The CVM patients may be too young to exhibit neurological phenotypes, which makes vertebral malformation-related scoliosis the only clinical symptom of those patients.

As an indispensable regulator of the monocyte/macrophage lineage, CSF1R has already been studied in variable animal models with the assistance of the transgenic technique. For instance, bi-allelic Csf1r deficiency in mouse was reported to cause brain development abnormalities and sclerosing skeletal dysplasia which leads to death within 6 weeks (Dai et al., 2004). Besides, the zebrafish model with biallelic loss-of-function mutations in csf1ra and csf1rb was also established in recent studies. This csf1r<sup>−/−</sup> (csf1ra<sup>−/−</sup>, csf1rb<sup>−/−</sup>) zebrafish also exhibited neurological abnormalities primarily, along with skeletal phenotypes like osteopetrosis (Oosterhof et al., 2019). Considering the gain-of-function effect of the newly identified heterozygous CSF1R variants, the overexpression experiment could be used to prove the pathogenicity of the CSF1R mono-allelic mutations identified in patients with spine abnormalities like CVM.

Furthermore, we focused on the detailed structural and biological effects of the variants and hypothesized how these variants affected the biological function of CSF1R. One of the CSF1R variants (NM_005211.3: c.2749_2758delGACAGGAGAG) identified in our cohort was related to higher protein expression level and led to more severe clinical phenotypes as compared with that of the other two deleterious variants. This variant (NM_005211.3: c.2749_2758delGACAGGAGAG) deletes the entire carboxy-terminus region sequence (p.Asp917SerfsTer32), which is important for the binding of c-Cbl and thus the ubiquitination-mediated protein degradation. However, the other two CSF1R variants only lead to minor structural disturbance of the protein carboxy terminus and consequently confer milder effects on the protein expression and human orthopedic manifestations. This phenomenon indicates a genotype-phenotype correlation in CSF1R-related CVM.

Correspondingly, considering the potential role c-Cbl plays in the etiology of CSF1R-related CVM, we also focused on this protein and identified two deleterious rare CBL variants in our cohort. It is demonstrated that c-Cbl binds to activated CSF1R and mediates its degradation through the tyrosine-kinase binding (TKB) domain (Wilhelmsen et al., 2002). Interestingly, the novel deleterious CBL missense variant (NM_005188.3: c.640C>T) identified in our CVM patient is located within the TKB domain exactly, which potentially interrupts the interaction between c-Cbl and CSF1R, further affecting CSF1R degradation. Besides, another variant (NM_005188.3: c.2435-2A>G) of CBL is predicted to affect the splicing process of c-Cbl and create mutated, which contributes to different clinical phenotypes like vertebral malformation.

Table 2: Deleterious and rare variants in genes interacting with CSF1R.

| Gene symbol | Ref transcript | Variant nomenclature | Mutation type | In-house allele count | In-house frequency | gnomAD allele count | gnomAD allele frequency | CADD score |
|-------------|----------------|----------------------|---------------|----------------------|-------------------|---------------------|------------------------|--------------|
| CBL         | NM_005188.3    | c.2435-2A>G          | Splice acceptor variant | 17 | 0.00000122 | 3 | 0.000004103 | NA |
| SLA2        | NM_005211.3    | c.640C>T             | Missense variant | 0 | 0 | 0 | 0 | 17 |
| GAB2        | NM_005274.3    | c.2749_2758delGACAGGAGAG | Frameshift variant | 0 | 0 | 0 | 0 | NA |
| IL34        | NM_001172772.1 | c.279T>A             | Missense variant | 0 | 0 | 0 | 0 | 24.3 |
| NM_001172772.1 | c.352T>A   | p.Tyr118Asn | Missense variant | 0 | 0 | 0 | 0 | 16.25 |
| NM_153047.3 | c.779C>T     | p.Ala260Val | Missense variant | 0 | 0 | 0 | 0 | 19.28 |
| FYN         | NM_001567.3    | c.1294A>C             | Missense variant | 0 | 0 | 0 | 0 | 19.28 |
| INPPL1      | NM_001002877.1 | c.655A>G             | Missense variant | 0 | 0 | 0 | 0 | 29.9 |
| THOC5       | NM_001002877.1 | c.235G>A             | Missense variant | 0 | 0 | 0 | 0 | 26.3 |
| SOCS1       | NM_003745.1    | c.77C>T              | Missense variant | 0 | 0 | 0 | 0 | 17.6 |

TABLE 2: Deleterious and rare variants in genes interacting with CSF1R.
a critical structure disorder, which could also inhibit c-Cbl biological function. All those CBL deleterious variants were identified in CVM patients, who exhibited similar clinical phenotypes as CSF1R carboxy-terminus variant carriers did. This phenomenon indicates that the CSF1R ubiquitination and degradation might be one of the important biological processes related to CVM. The inhibition of CSF1R degradation may contribute to the enhancement of CSF1R biological function, further affecting bone metabolism and vertebral development, leading to vertebral malformation.

Besides, the variants in our cohort may also affect the biological function of CSF1R through generating structural disorders potentially. For instance, the missense variant (NM_005211.3: c.2797G>T) leads to amino acid change (p.Gly933Cys), for which glycine was replaced by cystine. Glycine is a relatively stable amino acid with low molecule weight. The amino acid is replaced by cystine, which is much more active and easily oxidizable. The oxidized cystidine tends to form a disulfide bond with each other, potentially promoting the formation and stabilization of the CSF1R homodimer. The biological activity of protein could be enhanced by such structure disorder, which contributes to vertebral malformation. All the hypothesized mechanisms mentioned before remains to be explored further.

In conclusion, our study reveals the pathogenicity of heterozygous carboxy-terminal variants of CSF1R in CVM. The variants affect the biological function of the CSF1R product through potential gain-of-function effects.

DATA AVAILABILITY STATEMENT

The datasets generated during the current study are available in the Mendeley repository, https://data.mendeley.com/datasets/wzjxyz99st/1.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by ethics committee at Peking Union Medical College Hospital (JS-098). Written informed consent was obtained from the individual(s), and minor(s)’ legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

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AUTHOR CONTRIBUTIONS

NW, ZW, TZ, BL, SZ, and GQ conceived of the project and designed the study. NW, BL, SZ, ZY, SW, and YN recruited the patients and collected and interpreted the data. BL and SZ conducted the statistical analysis and bioinformatic analyses. BL and JL performed the in vivo experiments. BL, LZ, and XL performed the in vitro experiments. BL, SZ, and NW wrote the first draft of the manuscript and critically revised the work for important intellectual content. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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