Inhibition of miR-449a Promotes Cartilage Regeneration and Prevents Progression of Osteoarthritis in In Vivo Rat Models

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INTRODUCTION

Traumatic and degenerative lesions of articular cartilage usually progress to osteoarthritis (OA), a leading cause of disability in humans. MicroRNAs (miRNAs) can regulate the differentiation of human bone marrow-derived mesenchymal stem cells (hBMSCs) and play important roles in the expression of genes related to OA. However, their functional roles in OA remain poorly understood. Here, we have examined miR-449a, which targets sirtuin 1 (SIRT1) and lymphoid enhancer-binding factor-1 (LEF-1), and observed its effects on damaged cartilage.

The levels of chondrogenic markers and miR-449a target genes increased during chondrogenesis in anti-miR-449a-transfected hBMSCs. A locked nucleic acid (LNA)-anti-miR-449a increased cartilage regeneration and expression of type II collagen and aggregan on the regenerated cartilage surface in acute defect and OA models. Furthermore, intra-articular injection of LNA-anti-miR-449a prevented disease progression in the OA model. Our study indicates that miR-449a may be a novel potential therapeutic target for age-related joint diseases like OA.

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression through post-transcriptional or post-translational regulation in various biological processes and diseases. The miRNAs have many benefits that have mature sequences as short and completely conserved targeting multiple vertebrate species. Therefore, gene modulation therapy using miRNAs has emerged.

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miRNAs as gene modulators during cartilage regeneration and OA prevention remain largely unknown.

Our group has previously demonstrated that miR-449a regulates hBMSC chondrogenesis by targeting lymphoid enhancer-binding factor 1 (LEF1). 35 We also revealed that miR-449a regulates expression of sirtuin 1 (SIRT1) during IL-1β-induced cartilage destruction, which promotes OA. 36 Another group reported that SIRT1 has an anabolic effect in chondrocytes and promotes the chondrogenic differentiation of hBMSCs. 37 Taken together, these reports indicate that miR-449a regulates the chondrogenesis of hBMSCs and may be a useful regulator and therapeutic agent for the treatment of damaged cartilage.

To examine in vivo miR-449a function, we utilized a locked nucleic acid (LNA) to stably deliver the miRNA and enhance its function. The LNA is a modified RNA nucleotide with an extra bridge linking the 2’ oxygen and 4’ carbon, which confers improved stability against endonucleases and exonucleases in vivo and effective coherence with the complementary strand. 38 LNA-modified miRNAs have been developed for potential applications in tumor diagnosis and treatment. 39-41 However, there are few studies on cartilage regeneration using LNA-modified miRNA-based gene therapies. 34

In this study, we have investigated the function of miR-449a in the regulation of hMSC chondrogenic differentiation during cartilage regeneration using an LNA-modified miRNA as a delivery system in rat acute defect and OA models. We demonstrate that miR-449a enhances cartilage regeneration in an acute defect model and prevents cartilage degeneration in an OA model by targeting LEF1 and SIRT1.
Collectively, these results indicate that anti-miR-449a is a promising and novel therapeutic target for OA and general cartilage damage.

**RESULTS**

**Effect of miR-449a on hBMSC Chondrogenesis**

To investigate the effects of miR-449a, we observed the expression of miR-449a by miR-specific real-time qPCR at various time points during chondrogenic differentiation of hBMSCs microan culture. The results showed that the miR-449a expression was continuously expressed and remained upregulated after induction of chondrogenic differentiation (Figure 1A). Additionally, the expression levels of chondrogenic marker type II collagen (COL2A1) were also upregulated (Figure 1B). Next, we analyzed the mRNA levels of chondrogenic differentiation marker genes and miR-449a target genes, such as SRY-related high mobility group-box gene 9 (SOX9), aggrecan (ACAN), SIRT1, and LEFI by real-time qPCR. The expression levels of chondrogenic differentiation marker genes were significantly decreased in miR-449a-transfected hBMSCs compared with miR-sc (control)-transfected hBMSCs. Conversely, the expression of these genes was significantly increased in anti-miR-449a-transfected hBMSCs compared with anti-miR-sc-transfected hBMSCs (Figures 1C–1F). Furthermore, the expression levels of the miRNA-449a target genes SIRT1 and LEFI were consistently and significantly decreased in miR-449a-transfected hBMSCs, and significantly increased in anti-miR-449a-transfected hBMSCs (Figures 1C–1F). The protein levels showed consistent trends (Figure 1G).

Finally, we confirmed the effects of miR-449a on chondrogenesis by histological analysis using Alcian blue and safranin O staining and immunohistochemistry (IHC). With Alcian blue, anti-miR-449a-transfected hBMSCs were the most positively stained (Figure 2A). With safranin O staining, the synthesis of proteoglycans was mostly increased in anti-miR-449a-transfected hBMSCs (Figure 2A). Moreover, the mass was larger in anti-miR-449a-transfected hBMSCs than in anti-miR-sc-transfected and control hBMSCs (Figure 2A). By IHC analysis, COL2A1 and ACAN expression levels were decreased in miR-449a-transfected hBMSCs (Figure 2B). However, anti-miR-449a-transfected hBMSCs displayed increased COL2A1 and ACAN expression (Figure 2B). Taken together, these results indicate that miR-449a negatively regulates the chondrogenic differentiation of hBMSCs.

**Establishment of Optimal LNA-Anti-miR-449a Concentration**

To determine the effects of miR-449a on hBMSC chondrogenic differentiation during cartilage regeneration, we used LNA-anti-miR-449a, both as a delivery system and to improve the efficiency of miR-449a functional inhibition. We synthesized LNA-anti-miR-449a, with antisense oligonucleotides complementary to miR-449a (Figure 3A), and first investigated whether the synthesized LNA was cytotoxic in hBMSCs by 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide (MTT) assay. LNA-anti-miR-sc and LNA-anti-miR-449a had no effect on cell viability at all tested concentrations (Figure 3B). To establish the optimal concentration of LNA-anti-miR-449a in terms of improved target gene expression and inhibition of miR-449a expression, we treated hBMSCs with LNA-anti-miR-scramble control (LNA-anti-miR-sc) or LNA-anti-miR-449a in a dose-dependent manner and analyzed miRNA expression levels using miR-specific real-time qPCR. When hBMSCs were treated with 10–500 nM LNA-anti-miR-449a, miR-449a expression was inhibited by 50–100 nM LNA-anti-miR-449a, compared with hBMSCs treated with LNA-anti-miR-sc (Figure 3C). Next, we examined the expression of miR-449a target genes such as LEFI and SIRT1. When hBMSCs were treated with LNA-anti-miR-449a, the mRNA expression increased in a dose-dependent manner (Figure 3D), and protein expression was enhanced at concentrations ≥50 nM (Figure 3E). For these reasons, we determined that the optimal LNA-anti-miR-449a concentration for in vivo treatment was 100 nM.

**LNA-Anti-miR-449a Promotes Cartilage Regeneration in an Acute Defect Model**

To examine whether LNA-anti-miR-449a affected cartilage regeneration in an acute defect model, we created osteochondral defects in rats, and the defect sites were either left untreated or filled with...
hBMSCs, hBMSCs with LNA-anti-miR-sc, or hBMSCs with LNA-anti-miR-449a (Figure 4A). Histological analysis of regenerated articular cartilage was performed 4 and 8 weeks after surgery. After 4 weeks, the regenerated cartilage in the hBMSCs with LNA-anti-miR-449a group was smooth and resembled normal cartilage tissue (Figure 4B). However, the hBMSCs and hBMSCs with LNA-anti-miR-sc groups showed comparatively insufficient cartilage regeneration. Histological features were examined by H&E and Masson’s trichrome (MT) staining, and well-differentiated chondrocytes resembling those of normal cartilage were observed in the hBMSCs with LNA-anti-miR-449a group (Figure 4C). However, the other groups showed incompletely differentiated chondrocytes and severe disruption of normal cartilage surface. There was increased safranin O staining throughout the matrix, with abundant glycosaminoglycans (GAGs) and intact surface integrity in the hBMSCs with LNA-anti-miR-449a group (Figures 4D and 4E), whereas the other groups showed reduced safranin O staining. We evaluated the histological findings using a modified O’Driscoll score system (Figure 4F). The hBMSCs with LNA-anti-miR-449a group showed significantly higher scores (16.13 ± 2.77; p = 0.0001; Figure 4F) than the defect (7.65 ± 1.46), hBMSCs the imperfect restoration resulting from the implantation of hBMSCs alone.

LNA-Anti-miR-449a Increases Cartilage Regeneration in an OA Model

To determine whether LNA-anti-miR-449a has regenerative effects on damaged cartilage in an OA model, we induced OA surgically by destabilization of the medial meniscus (DMM) in 12-week-old rats. We induced OA for 8 weeks to establish the model, because OA was not definitively induced 6 weeks after DMM (data not shown). Eight weeks after DMM, intra-articular injections of PBS, LNA-anti-miR-sc, and LNA-anti-miR-449a were performed twice a week for up to 8 weeks (Figure 5A). In histological analysis, the LNA-anti-miR-449a-treated group showed intact surfaces resembling normal, well-differentiated chondrocytes, with reduced cartilage degradation and proteoglycan loss by safranin O and MT staining (Figure 5B). The PBS and LNA-anti-miR-sc-treated groups showed severe cartilage degradation and irregular chondrocyte morphology, severe fibrillation, and loss of proteoglycan, similar to the defect group (Figure 5B). Based on histological analysis, the LNA-anti-miR-449a-treated group had significantly lower
modified Mankin and Osteoarthritis Research Society International (OARSI) scores (4.00 ± 0.88 and 1.88 ± 0.83; Figure 5C) compared with the other groups (20 weeks defect group: 6.05 ± 0.63 and 2.77 ± 0.83; 28 weeks defect group: 10.22 ± 0.69 and 6.22 ± 0.50; PBS group: 10.00 ± 0.66 and 5.55 ± 0.50; and LNA-anti-miR-sc group: 10.78 ± 1.54 and 5.88 ± 0.83), indicating that LNA-anti-miR-449a had a regenerative effect on damaged cartilage. We also performed IHC analysis using primary antibodies against COL2A1 and ACAN. The expression of COL2A1 and ACAN was increased on regenerated tissue in the LNA-anti-miR-449a-treated group (Figure 5D). Additionally, the expression of inflammatory marker protein cyclooxygenase-2 (COX-2) and cartilage degradation marker protein MMP-13 were significantly upregulated in surgically induced OA, PBS, and LNA-anti-miR-sc-treated groups. However, the LNA-anti-miR-449a-treated group showed a significant decrease in their expression compared with the other groups. Therefore, we confirm the inflammatory state after injection of LNA-modified miRNA (Figure S2). Together, these findings suggest that the injection of LNA-anti-miR-449a can regenerate damaged cartilage in a rat OA model.

LNA-Anti-miR-449a Prevents Cartilage Destruction in an OA Model

To determine whether LNA-anti-miR-449a can prevent OA progression, we induced OA by DMM in 12-week-old rats. Starting immediately after surgery, intra-articular injections of PBS, LNA-anti-miR-sc, and LNA-anti-miR-449a were performed twice a week for up to 12 weeks (Figure 6A). We performed histological analysis using safranin O and MT staining. The LNA-anti-miR-449a-treated group maintained a relatively intact cartilage structure, with well-differentiated chondrocytes surrounding the matrix, and intact proteoglycan (Figure 6B). However, nearly complete cartilage destruction, including severe fibration, proteoglycan loss, chondrocyte clusters, and hypertrophic chondrocytes, was observed in the defect, PBS, and LNA-anti-miR-sc-treated groups (Figure 6B). The LNA-anti-miR-449a-treated group had distinctively lower modified Mankin and OARSI scores (2.22 ± 1.26 and 1.11 ± 0.83; Figure 6C) than the other groups (24 weeks defect group: 8.44 ± 0.69 and 4.96 ± 0.75; PBS group: 7.88 ± 0.83 and 5.66 ± 0.33; and LNA-anti-miR-sc group: 11.67 ± 1.76 and 6.74 ± 0.35). By IHC, the expression of COL2A1 and ACAN was the most abundant in the LNA-anti-miR-449a-treated group (Figure 6D). These results demonstrate that the intra-articular injection of LNA-anti-miR-449a slows down the progression of cartilage degradation in a rat OA model.

DISCUSSION

Our study is the first to report the role of anti-miR-449a in vivo using acute defect and OA models, and confirmed positive effect that can regenerate the damaged cartilage and slow down aggressive OA progression targeting LEF1 and SIRT1.

Previous reports have demonstrated that the intra-articular injection of hBMSCs causes no risk of immune rejection in a rat model.42,43 Additionally, hBMSC treatment has been used extensively in research and clinical trials because of the stability of hBMSCs and their potential to replace damaged tissue.14,44 However, studies have revealed lower regenerative capacities for hBMSCs because of the influence of culture protocols and application methods.16,47 This observation is in agreement with our study, because only the hBMSCs group showed less regeneration compared with the hBMSCs with LNA-anti-miR-449a group, which had increased cartilage regeneration in the acute defect model. This may indicate synergistic effects between hBMSCs and LNA-anti-miR-449a in cartilage restoration.

Only a few studies have examined the regulation of chondrogenesis by miRNAs. They have revealed that inhibition of miR-101 prevents cartilage degeneration by targeting SOX9 in a monoiodoacetate-induced rat model of OA,48 and that miR-34a regulates SIRT1 expression in human osteoarthritis chondrocytes.39 Additionally, inhibition of miR-34a promotes aggressive disease progression in a rat OA model.49 These groups all used adeno viral vectors for successful miRNA delivery. Viral vectors such as lentiviruses and adenoviruses have been widely used as miRNA delivery systems; however, limitations remain, including safety issues and loss of miRNA efficiency.19 Poly (lactide-co-glycolide) (PLGA), a polymer, has also been widely used as an miRNA delivery system because of its low toxicity;50,51 however, its use is limited by low loading efficiency.52 Liposomes consisting of lipid layers have been developed for miRNA delivery;53 however, these also have limitations, including toxicity and the induction of type I and type II interferons by positively charged lipids.54 Therefore, more effective miRNAs delivery systems are required. In contrast, we used LNA-modified oligonucleotides to not only successfully deliver miRNAs, but also enhance miR-449a inhibition. The LNA-modified miRNA delivery system is one of the most advanced in vivo delivery systems. LNA can interact with complementary miRNAs with high affinity, neutralizing the targeted miRNA with no cytotoxicity.55,56 We have demonstrated that LNA-anti-miR-449a is successfully delivered to defect sites, where it serves dual positive roles, regenerating damaged cartilage and preventing OA.

Figure 4. Increased Cartilage Regeneration by LNA-Anti-miR-449a in an Acute Defect Model

(A) Experimental design for cartilage regeneration induced by acute osteochondral defects. (B) Gross morphology of osteochondral defects after 4 weeks. (C) Histological analysis of osteochondral defects observed using H&E and MT staining. Scale bars, 500 μm (original magnification ×4). (D) Histological analysis of osteochondral defects observed using safranin O staining. Scale bars, 500 μm (original magnification ×4) and 100 μm (original magnification ×20). (E) Quantification of safranin O staining. (F) Quantification of histology by O’Driscoll score based on safranin O staining. (G) Left: IHC analysis of COL2A1 (PE; red fluorescence) and ACAN (FITC; green fluorescence) in an acute defect model. Scale bar, 50 μm (original magnification ×40). Right: quantification of IHC analysis. Data are defined as mean ± SD. p values were calculated compared with the defect group: *p < 0.05; **p < 0.001 (n = 7 for each group).
A

B

C

D

(legend on next page)
progression by targeting LEF1 and SIRT1. Hence, our results provide insights on the utility of LNAs as in vivo miRNA delivery systems.

In a previous report, we showed that miR-449a negatively regulates LEF1 expression during hBMSC chondrogenesis. This result suggested that the inhibition of miR-449a would promote the chondrogenic differentiation of hBMSCs by increasing LEF1 expression. Here, our in vivo study confirmed that miR-449a inhibition causes the regeneration of damaged cartilage in an acute defect model and OA model.

SIRT1 plays an inhibitory role in IL-1β-induced cartilage destruction associated with OA. In a previous report, we showed that miR-449a regulates the expression of SIRT1, which has anti-inflammatory effects on OA chondrocytes. We confirmed that the inhibition of miR-449a has a protective effect, inhibiting the expression of catabolic genes and restoring the expression of anabolic genes by targeting SIRT1 during IL-1β-induced cartilage degradation. Combined with these previous results, the in vivo results in this study suggest that miR-449a may be a promising novel therapeutic target for the prevention of cartilage degeneration.

Overall, our observations strongly indicate dual positive effects of silencing miR-449a, regenerating damaged cartilage and preventing OA progression by targeting LEF1 and SIRT1. These results suggest that miR-449a could be a promising therapeutic target for OA and other cartilage degenerative disorders.

MATERIALS AND METHODS

Culture of hBMSCs
Bone marrow aspirates were obtained from the posterior iliac crests of 10 healthy adult donors with a mean age of 47.5 years (range: 31–65 years), with approval from the Institutional Review Board of Yonsei University College of Medicine (IRB No. 4-2017-0232), and all participants agreed to participation. Cells were cultured and selected by adherence on a plastic culture plate surface, and their validations were confirmed by flow cytometry as in a previous study, then cultured for 7 days in DMEM-low glucose (GIBCO, USA) with 10% fetal bovine serum (FBS; GIBCO, USA) and 1% antibiotic-antimycotic solution (GIBCO, USA). Cells were subcultured for up to three passages, and all experiments were carried out in triplicate.

Chondrogenic Differentiation of hBMSCs
DMEM-high glucose (DMEM-HG; GIBCO, USA) with 1× antibiotic-antimycotic solution, 1× insulin transferrin selenium-A (ITS; Invitrogen, Carlsbad, CA, USA), 50 μg/mL ascorbic acid (Sigma, St. Louis, MO, USA), and 10 ng/mL transforming growth factor (TGF)-β3 (R&D Systems; Minneapolis, MN, USA) was used for the chondrogenic differentiation of hBMSCs. For micromass culture, 1 × 10⁵ hBMSCs were dropped in the center of each well of a 24-well plate. After 2 hr, chondrogenic medium was added. The medium was replaced every 3 days.

Cytotoxicity Assay
A cytotoxicity assay was performed using the MTT assay in 48-well plates. EZ-Cytox cell viability reagents (Daeil Lab, Seoul, Korea) and 300 μL of fresh medium were added, and the samples were incubated at 37°C for 4 hr. The absorbance was measured at 540 nm.

Transfection of miRNA
Cells were transfected with miR-scramble control (miR-sc; Genolution, Seoul, Korea), miR-449a mimic (Genolution, Seoul, Korea), anti-miR-sc mimic (ST Pharm, Seoul, Korea), and anti-miR-449a mimic (ST Pharm, Seoul, Korea) at 100 nM using Lipofectamine LTX & Plus Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. LNA-modified oligonucleotides were used as a delivery system into cartilage joints, and LNA was synthesized as unconjugated and fully phosphorothiolated oligonucleotides (Exiqon, Vedbaek, Denmark). LNA-anti-miR-sc and LNA-anti-miR-449a were directly applied to hBMSCs. After 48 hr, cells were harvested, and chondrogenic differentiation was induced by micromass culture. A list of all miRNA sequences used is provided in Table S1.

Real-Time qPCR
Total RNA was extracted from harvested cells using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized for real-time qPCR (Applied Biosystems, Foster City, CA, USA). MiR-specific real-time qPCR was performed according to guidelines of a manufacturer, and U6 small nuclear RNA (snRNA) was used as a control to quantify miRNAs (Clontech, Palo Alto, CA, USA). A list of all primer sequences used is provided in Table S2.

Western Blot Analysis
For protein extraction, cells were harvested and lysed in lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1% tergitol-type NP-40 [NP-40], and 0.1% SDS) and processed as previously described. The primary antibodies used targeted LEF1, SIRT1, SOX9, and GAPDH (all from Santa Cruz Biotechnology, USA).

In Vivo Surgical Induction of an Acute Defect Model in Rats
The Committee on the Ethics of Animal Experiments of Yonsei University College of Medicine approved all animal experiments and protocols (permit no. 2015-0168). Male Sprague-Dawley rats (n = 7 per group) at 12 weeks of age were anaesthetized with an intra-peritoneal...
injection of Zoletil (30 mg/kg) and Rompun (10 mg/kg). To expose the rat knee joint, we made a para-patellar incision using a surgical blade. After lateral dislocation of the patella, we created an osteochondral defect (2.0 mm in diameter and 1.5 mm in depth) on the patellar groove of the distal femur using a trephine burr. Next, scaffolds were inserted into the osteochondral defect sites, and we randomly allocated the animals into four groups as follows: (1) untreated defect, (2) hBMSCs only, (3) hBMSCs with LNA-anti-miR-sc, and (4) hBMSCs with LNA-anti-miR-449a. Fibrin gel (Greenplast Kit; Green Cross, Seoul, Korea) was used as a scaffold according to the manufacturer’s instructions. Finally, the incision site was closed with Vicryl, and Metacam (1 mg/kg) was used as an analgesic. Rats were sacrificed 4 and 8 weeks after surgery, and the knee joints were harvested.

**In Vivo DMM in Rats and Intra-articular Injection of LNA-Anti-miR-449a**

To establish a rat model of OA, we prepared the right knees of 12-week-old male Sprague-Dawley rats (n = 7 per group) for DMM surgery according to a previous study. In brief, the medial meniscus (MM) and medial menisco-tibial ligament (MMTL) were opened following the dissection of the fat pad. Then, the menisci were devalized due to transection of the MMTL with no. 11 blade, and the wound was closed with 3-0 Vicryl. Animals were randomly allocated into four groups as follows: DMM without treatment (defect), DMM with PBS, DMM with LNA-anti-miR-sc, and DMM with LNA-anti-miR-449a. Intra-articular injections of 50 μL of PBS, LNA-anti-miR-sc (100 nM), and LNA-anti-miR-449a (100 nM) were performed 8 weeks after DMM surgery twice a week and immediately following DMM surgery twice a week for 12 weeks. At 8, 12, and 16 weeks post-DMM, the rats were sacrificed under anesthesia and knee joints were harvested.

**Histological and IHC Analysis**

Knee joints were fixed with 3.7% formaldehyde, decalciﬁed with Calci-Clear Rapid (National Diagnostics, Atlanta, GA, USA), embedded in paraffin, and cut into 100-μm sections. For histological analysis, the sections were deparaffinized in xylene and serially rehydrated in ethanol. The sections were sequentially stained with MT, embedded in paraffin, and cut into 100-μm sections. For histological analysis, the sections were deparaffinized in xylene and serially rehydrated in ethanol. The sections were sequentially stained with MT, H&E, Alcian blue and safranin O staining according to conventional protocols. To evaluate the histological assessment of the acute defect model, we used the O’Driscoll scoring system and as demonstrated in Table S4; to evaluate the histological assessment of the OA models, we used modified Mankin and OARSI score systems for cartilage regeneration following as demonstrated in Table S4. For IHC, primary antibodies against ACAN, COL2A1, MMP13 (all from Santa Cruz Biotechnology, USA), and COX2 (BD Biosciences, USA) were used. All histological results were evaluated fairly by independent blind assessment. The numbers of positively stained cells were calculated using ImageJ 1.51f software (NIH).

**Statistical Analysis**

For each experiment, samples were analyzed in triplicate. For comparisons of over three groups, we used one-way ANOVA and post hoc comparison with the Tukey correction. Two-tailed independent t tests were used for comparisons between two groups. GraphPad Prism software (version 6.0) was used for statistical analysis. p values less than 0.05 were considered statistically significant. All data are presented as mean ± SD.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes two ﬁgures, four tables, and Supplemental Materials and Methods and can be found with this article online at https://doi.org/10.1016/j.omtn.2018.09.015.

**AUTHOR CONTRIBUTIONS**

D.B. wrote the manuscript. D.B. and K.W.P. performed all in vitro and in vivo experiments together. K.-M.L. discussed the data. J.W.S. devised the animal surgery protocols and suggested methodology for the animal studies. S.M.C. performed the in vivo experiments. K.H.P. analyzed the data and kindly recommended experiments. J.W.L. and S.-H.K. supervised the preparation of this manuscript and provided important intellectual contributions to the final draft.

**CONFLICTS OF INTEREST**

There authors have no conflicts of interest.

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