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Original article

Suppression of nectin-2 induced early apoptosis in MDA-MB-468 triple-negative breast cancer cell line

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Abstract

Background: Breast cancer, the most common cancer in women, caused 670,000 deaths worldwide in 2022. Triple-negative breast cancer (TNBC), lacking estrogen (ER), progesterone (PR), and human epidermal growth factor (HER2) receptors, are especially aggressive with poor outcomes. Nectin-2 is overexpressed in breast cancer tissues and TNBC cell lines, suggesting a potential role in tumor progression and making it a candidate for further investigation.

Objective: To evaluate the role of nectin-2 in breast cancer progression, focusing on cell proliferation, apoptosis, and migration in the MDA-MB-468 TNBC cell line.

Methods: A stable *NECTIN2*-knockdown (KD) MDA-MB-468 cell line was generated using lentiviral transduction. Knockdown efficiency was confirmed by RT-qPCR and Western blot analysis. Cell proliferation was evaluated using the MTT assay, apoptosis was detected through Annexin-V/7-AAD staining, and migration was assessed via the transwell migration assay.

Results: Knockdown of *NECTIN2* resulted in an 86% reduction at the gene level, with a corresponding protein reduction. The MTT assay showed that the knockdown of NECTIN2 had no significant effect on cell proliferation, and the transwell migration assay showed no notable difference in cell movement. However, there was a significant increase in early apoptotic cells to 17.26% in the NECTIN2 KD group.

Conclusion: Nectin-2 knockdown had no significant effect on proliferation and migration but slightly increased early apoptosis in MDA-MB-468 cells, suggesting a limited role in this model with moderate expression. Further studies across TNBC subtypes with varying nectin-2 levels are needed to clarify its role in tumor progression and potential as a therapeutic target.

Keywords: Early apoptosis, nectin-2, MDA-MB-468, TNBC.

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Breast cancer is the most common cancer among females, with approximately 2.3 million women diagnosed and 670,000 deaths reported globally in 2022. Treatment options for breast cancer vary based on its molecular subtype and may include radiation therapy, hormonal therapies, chemotherapies and biologically targeted therapies. [1] The classification of breast cancer primarily relies on the expression patterns of hormonal receptors, including estrogen (ER), progesterone (PR), and human epidermal growth factor (HER2) receptors. The four main molecular subtypes include luminal A (ER+, PR+/-, HER2-), luminal B (ER+, PR+/-, HER2+/-), HER2-positive (ER+/-, PR+/-, HER2+) and triple-negative breast cancer (TNBC) (ER-, PR- and HER2-). [2, 3] Among these, luminal A demonstrates a better prognosis and responds well to hormonal therapy. In contrast, TNBC is particularly aggressive, associated with poor prognosis and low responsiveness to endocrine therapy due to the lack of hormonal receptors, [4] and is characterized by high rates of metastasis to other organs such as brain, liver and lung. [5] Therefore, it is crucial to evaluate novel therapeutic approaches for TNBC.

Nectin-2 (CD112 or PVRL2) is a $Ca^{2}z$ - independent immunoglobulin superfamily cell adhesion molecule. This type I single-pass transmembrane protein facilitates cell-cell adhesion among epithelial cells through adherens junctions. It forms adhesion complexes through homophilic interaction, where the same nectin molecule binds between adjacent cells, or heterophilic interactions with other members of the nectin family, including nectin-1, nectin-3, and nectin-4. Additionally, the interaction of nectin-2 with the scaffolding protein afadin links the nectin complex to actin cytoskeleton and regulate cytoskeleton dynamics. [6] Additionally, nectin-2 plays a crucial role in regulating the functions of $CD8^{+}$ T-cells and NK cells by interacting with the inhibitory receptor PVRIG, which is expressed on the surface of the immune cells. [7] Notably, several types of cancer exhibit high levels of nectin-2 expression compared to normal cells. Its overexpression is linked to the epithelial-to-mesenchymal transition (EMT) pathway, which is associated with cancer metastasis. [8] Nectin-2 is overexpressed in human esophageal squamous cell carcinoma (ESCC) tissues and is associated with cancer progression. Silencing nectin-2 using siRNA has been shown to suppress cell migration and invasion. [9] In lung adenocarcinoma (LUAD), elevated levels of nectin-2 expression have been observed in clinical tissues. Nectin-2 depletion in LUAD promoted cell apoptosis and reduced migration. [10] These findings highlight the potential of nectin-2 as a therapeutic target for cancer treatment.

Overexpression of nectin-2 has been reported in clinical breast and ovarian cancers at the nectin-2 mRNA expression level and the nectin-2 protein in cancer tissues. Among the breast cancer cell lines, MDA-MB-468 had moderate nectin-2 protein expression. Furthermore, targeting nectin-2 using Y-443 monoclonal antibody suppressed TNBC tumor growth *in vivo*. [11] Elevated nectin-2 mRNA levels have been detected in the serum of patients with grade 3 breast carcinoma patient, [12] suggesting a potential association between nectin-2 expression and breast cancer progression. However, the specific role of nectin-2 in breast cancer, particularly in TNBC, an aggressive and hard-to-treat subtype, remains unexplored.

In this study, the MDA-MB-468 cell line was selected as a model for the TNBC subtype. Nectin-2 suppression was performed to assess its role in cancer progression. The data revealed that nectin-2 plays a crucial role in regulating apoptosis but may not be a major factor in cancer cell proliferation and migration in the MDA-MB-468 cell line. This study aims to explore the roles of nectin-2 in MDA-MB-468 breast cancer cell line, focusing on its involvement in cancer cell progression, including cell proliferation, apoptosis and migration.

Materials and methods

Generation of NECTIN2 gene knockdown stable cell line and validation

To study *NECTIN2* gene function, stable *NECTIN2*-knockdown (KD) MDA-MB-468 cell line was generated. HEK293FT cells were utilized as the lentiviral production host. Viral packaging was achieved by co-transfecting pMD2.G, psPAX2 and *NECTIN2* shRNA containing a puromycin resistance gene or scramble (SCR) shRNA with EGFP into overnight cultured of 5×10^{6} HEK293FT cells in a 10 cm petri-dish using Lipofectamine™ 3000 Transfection Reagent (Thermo Fisher Scientific, USA). At 48 hours post-transfection, viral particles were collected, filtered through a 0.2 μm filter, and concentrated. Approximately 4×10^{5} MDA-MB-468 cells were seeded in a 6-well plate and incubated overnight. The whole viral particles, mixed with high-

glucose DMEM medium containing 2% FBS and 8 µg/ml polybrene, were then added to the cells *NECTIN2*-KD cell lines were selected by treating the cells with 1 µg/ml puromycin. [13] Nectin-2 expression levels were assessed using reverse transcription quantitative polymerase chain reaction (RT-qPCR) and Western blot analysis.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis

Levels of *NECTIN2* gene were analyzed using RT-qPCR. A total of 1×10^6 cells were collected, and total RNA was extracted using Trizol reagent (Thermo Fisher Scientific, USA), following the manufacturer's instructions. Next, 250 ng of RNA was reverse transcribed into cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) with oligo dT primers, as per the manufacturer's guidelines. The RT-qPCR reaction was carried out using iTaq Universal SYBR Green Supermix (Bio-Rad) and analyzed on a CFX-96 real-time PCR instrument (Bio-Rad). The relative expression of *NECTIN2* was normalized to β-actin and calculated using the comparative $2^{-\Delta\Delta CT}$ method.

Western blot analysis

Protein extraction was performed on MDA-MB-468 cells, including control, scramble (SCR), and *NECTIN2*-KD cells, at a cell density of $1x10^6$, using RIPA buffer containing protease inhibitors. The protein concentration was quantified using Bradford assay (Bio-Rad protein assay dye). A total of 20 µg of protein samples were denatured and separated by SDS-PAGE using 10% separating gel and 4% stacking gel in 1x running buffer. Wet blotting system was used in transferring the proteins to a nitrocellulose membrane. The blocking and antibody preparation were based on 5% skim milk in TBST buffer. A concentration of 1:1000 dilution of nectin-2/CD112 (D8D3F) XP® Rabbit mAb (Cell signaling, USA) and 1:2000 dilution of goat anti-rabbit antibody conjugated with HRP were used, all washing steps was done by 1x TBST buffer. The membranes were visualized by using ECL detection reagents (Cytiva, USA) and exposed to X-ray film. Image J was used to quantify protein band density and the relative expression of nectin-2 to β-actin was calculated.

Cell proliferation assay

Cell proliferation was assessed using MTT assay. All the experimental groups including control, SCR and *NECTIN2*-KD of MDA-MB-468 cells were synchronized by culturing in serum-free media for one day prior to cell seeding. Each group was seeded at a density of 5×10^3 cells per well in a 96-well plate and incubated overnight. Following this, a final concentration of 0.5 mg/ml MTT solution was added and incubated for an hour allowing for formazan crystal development. The media was replaced with DMSO to dissolve the crystal and continued incubation for an hour. The optical density (OD) measured at 570 nm using a spectrophotometer to quantify cell viability. This process was repeated daily for four days to monitor the proliferation rate. Cell viability was calculated as a fold-change by comparing the OD values from each subsequent day to that of day one.

Transwell migration assay

Cell migration was assessed using the transwell assay. A total of 5×10^4 cells/well in serum-free DMEM of the three experimental groups, including control, SCR, and *NECTIN2*-KD cell lines of MDA-MB-468, were seeded into the 6.5 mm upper chamber, supported by an 8 µm PC membrane of a 24-well plate under serum-deprived conditions. To induce chemotaxis, the lower chamber was filled with DMEM containing 10% FBS, creating a serum gradient that stimulates directional cell migration. After incubation, the migrated cells were fixed using 4% paraformaldehyde and stained with crystal violet. The number of migrated cells was counted under a light microscope at 100x magnification, with at least five random fields per well.

Cell apoptosis detection

The impact of *NECTIN2*-KD on cell apoptosis was analyzed using the PE-conjugated annexin-V/7-AAD Apoptosis detection kit (BD biosciences, USA). MDA-MB-468 cells were collected at a density of 1×10^5 cells for the three experimental groups including control, SCR, and *NECTIN2*-KD. Cells were processed according to the manufacturer's instructions. Apoptosis was assessed using a BD Accuri™ C6 Plus flow cytometer (BD Biosciences, USA). Each sample was analyzed by dividing the data into 4 quadrants to evaluate apoptosis stages including live (Annexin-V^-/7-AAD^-), early (Annexin V^+/7-AAD^-), and late apoptosis (Annexin V^+/7-AAD^+), based on the percentage of cells in each quadrant.

Statistical analysis

All experiments were performed with three independent replicates. Data are presented as the mean ± standard deviation (SD). Statistical comparisons between the three experimental groups were conducted using one-way analysis of variance (ANOVA) for RT-qPCR, Western blot analysis, transwell migration assay, and apoptosis assay. Two-way ANOVA was used for the MTT assay. *, **, *** and **** denote *P*-value < 0.05, < 0.01, < 0.001, and < 0.0001, respectively, indicating statistical significance. A *P*-value greater than 0.05 indicates no significant difference. Statistical analysis was conducted using GraphPad Prism software (version 10.4.1, GraphPad Software, San Diego, CA). Each bar represents the mean value of three biological replicates.

Results

Reduction of nectin-2 *expression in NECTIN2-KD MDA-MB-468 cell line*

After viral transduction, MDA-MB-468 cells: control, scramble (SCR), and *NECTIN2*-KD were then assessed at both the gene and protein levels. RT-qPCR analysis revealed a significant *NECTIN2* gene reduction at 86% in *NECTIN2*-KD MDA-MB-468 compared to that of the control group **(Figure 1A)**. These findings were further confirmed by Western blot analysis, which showed no detectable nectin-2 protein **(Figures 1B and 1C)**.

NECTIN2-KD did not affect cell proliferation but induced cell apoptosis

Cell proliferation in MDA-MB-468 cells was assessed using the MTT assay. The fold change in OD was calculated relative to day 1. Knockdown of NECTIN2 did not significantly affect cell proliferation compared to the control group on days 2 to 4, with *P*-values of 0.9738, 0.2008, and 0.2274, respectively. Similarly, no significant differences were observed when compared to the SCR group, with *P*-values of 0.8251, 0.3020, and 0.9923 on days 2, 3 and 4, respectively **(Figure 2A)**. Apoptosis analysis of the control, SCR, and *NECTIN2*-KD groups revealed that live cells comprised 87%, 88%, and 73%, respectively. Early apoptotic cells were significantly increased in the *NECTIN2*-KD cells (15.9%) compared to the control and SCR cells (7.7% and 4.8%, respectively). There was no significant difference in the proportion of late apoptotic cells among the three groups **(Figures 2B and 2C)**.

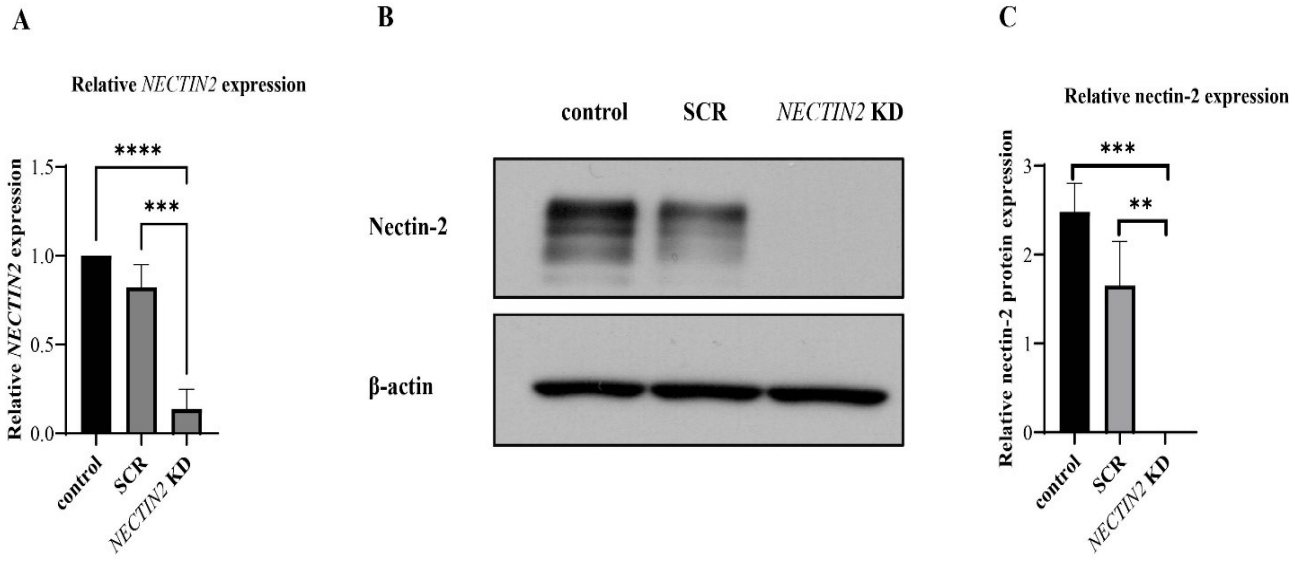

Figure 1. shRNA-mediated reduction of *NECTIN2* in the MDA-MB-468 cell line. The experimental group included control, scramble (SCR), and *NECTIN2*-KD. **(A)** The relative *NECTIN2* gene expression was analyzed by RT-qPCR and normalized to the *ACTB* gene. **(B)** Western blot analysis result demonstrates nectin-2 protein expression. **(C)** The quantitative levels of nectin-2 protein from Western blot analysis.
, * and **** denote $P < 0.01$, < 0.001, and < 0.0001, respectively.

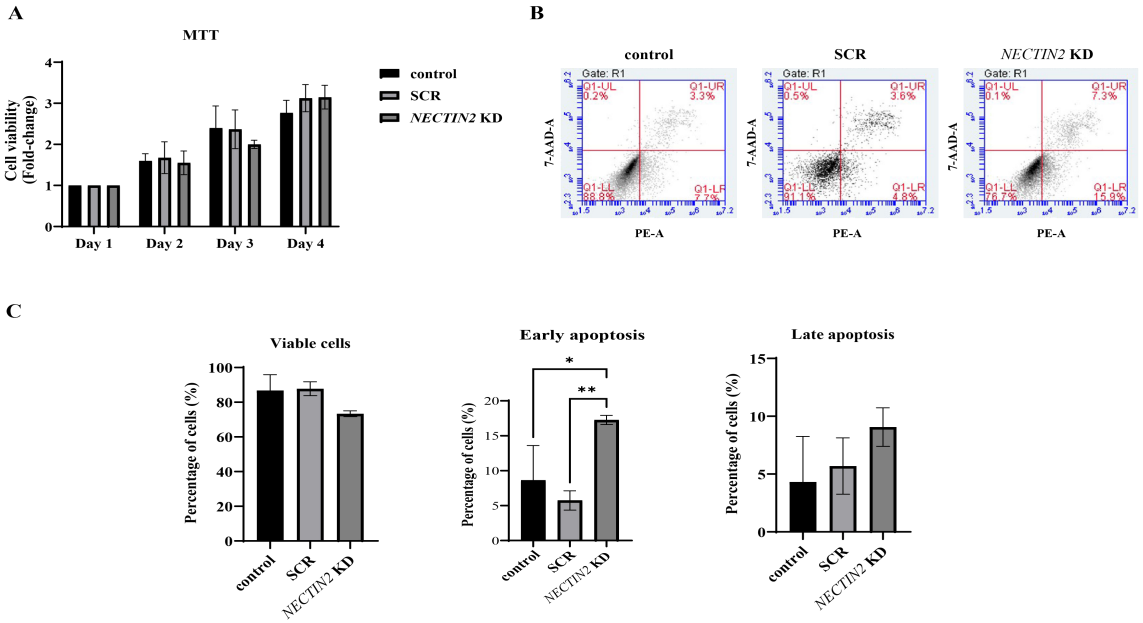

Figure 2. Reduction of *NECTIN2* minimally affected cell apoptosis. **(A)** Cell proliferation was assessed using the MTT assay. Cell viability was measured as OD at 570 nm on day 1 to 4. OD values of each subsequent day were calculated as a fold change relative to day 1. **(B)** Apoptosis results of PE-Annexin V/7-AAD staining, with cell categorized into four quadrants representing viable, early apoptotic, and late apoptotic populations. **(C)** Bar graph summarizing the apoptosis results, including the proportions of viable cells, early apoptosis and late apoptosis.
* and ** denote $P < 0.05$ and < 0.01, respectively.

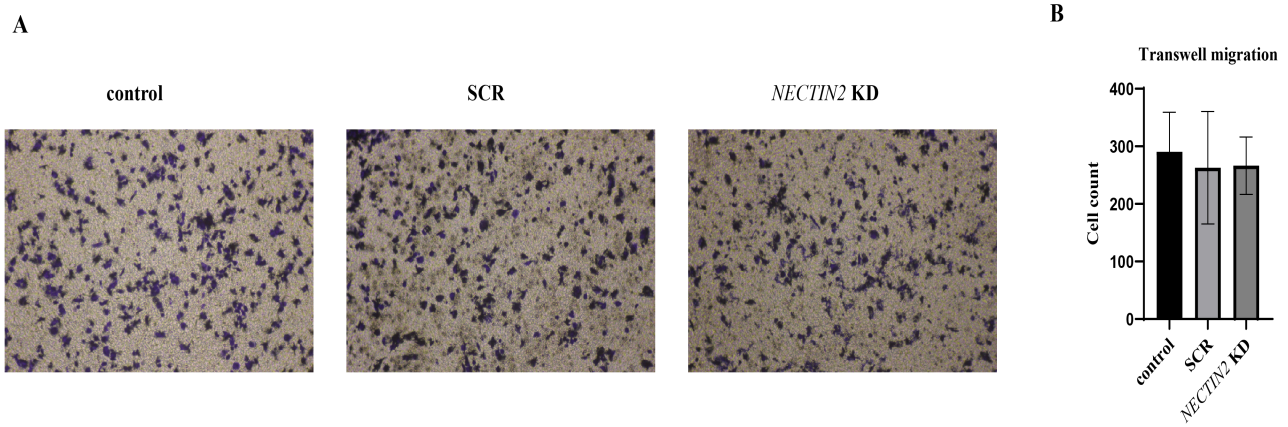

Figure 3. Reduction of *NECTIN2* did not affect MDA-MB-468 cell migration. **(A)** Migrated cells on the transwell membrane, captured at 100x magnification**. (B)** The bar graph illustrated the quantitative analysis of the migrated cell number.

Nectin2-KD did not affect cell migration in transwell migration assay

Cell migration was assessed using a transwell migration assay in three MDA-MB-468 cell groups; control, SCR, and *NECTIN2*-KD. The results demonstrated that suppression of nectin-2 did not affect the migratory ability of MDA-MB-468 cells. The number of migrated cells was quantified as 290 ± 63, 227 ± 24, and 267 ± 39 cells, respectively **(Figures 3)**. Statistical analysis showed no significant difference between the NECTIN2-KD group and either the control or SCR groups, with *P*-values of 0.9066 and 0.9982, respectively.

Discussion

In this study, the knockdown of *NECTIN2* in MDA-MB-468 breast cancer cells was confirmed at both the gene and protein levels. Cancer cell progression, including cell proliferation, was assessed through the MTT assay, which revealed no significant effect on cell viability at any time point, suggesting that nectin-2 possibly not a major factor in MDA-MB-468 breast cancer cell proliferation. However, previous research has reported that knockout of *NECTIN2* decreased LUAD cell proliferation, while its overexpression had a slight effect on cell proliferation, [10] indicating that the functional roles of

nectin-2 may vary across the cancer cell types. Despite the lack of impact on cell proliferation, flow cytometry results showed potential minimally induces the cell into early apoptosis stage following *NECTIN2* reduction. Although nectin-2 may not directly regulate cell proliferation, it might be involved in modulating cell survival mechanisms. Cell migration was assessed using the transwell migration assay, where cells were induced to migrate by gradient chemotaxis from FBS-supplemented DMEM in the bottom chamber through the supporting membrane. The results showed no significant changes in *NECTIN2*-KD MDA-MB-468 cells compared to control and SCR. However, previous studies have confirmed the role of nectin-2 as a migration factor in human esophageal squamous cell carcinoma (ESCC) [9] and LUAD cells. [10]

One of the limitations of this study is that its reliance on a single TNBC cell line may not fully address the role of nectin-2 across the TNBC subtypes. The variation of nectin-2 expression across TNBC cell lines may not be generalizable to all forms of TNBC by the results of MDA-MB-468 alone. These findings suggest the need for further investigations, the use of additional breast cancer models, especially those with higher nectin-2 expression such as MDA-MB-231 TNBC, [11] could provide further insights into functional roles in nectin-2 in TNBC. Furthermore, including invasion assays and molecular pathway analyses may help elucidate the underlying mechanism following the nectin-2 knockdown.

The minimal phenotypic changes observed following the nectin-2 knockdown in MDA-MB-468 may be due to compensatory mechanisms by other members of the nectin family. The nectin proteins are known to interact both homophilically and heterophilically. [6] It is possible that the functions of nectin-2 were maintained through compensation by these related protein, [14] potentially maintaining cell progression even the absence of nectin-2. This redundancy highlights the need of further investigation into the nectin-2 signaling network in breast cancer cells.

In conclusion, suppression of nectin-2 in MDA-MB-468 does not significantly affect cell proliferation and migration. However, it slightly induces early apoptosis. These findings suggested that nectin-2 did not play a major role in migration for MDA-MB-468 and may have cell line specific functions. To gain deeper insight, further studies are needed to be investigating the effects of nectin-2 knockdown groups across breast cancer models through pathway analysis of upstream and downstream genes or proteins, using transcriptomics and proteomics could help identify molecular changes associated with varying levels of nectin-2 expression. These further studies could enhance our understanding of cancer cell progression mechanisms and reveal potential compensatory pathways. Additionally, exploring the role of nectin-2 in breast cancer progression may offer novel therapeutic insights, as nectin-2 acts as both a cell adhesion molecule and an immune checkpoint. Combination strategies, such as small-molecule inhibitors targeting nectin-2 and genetically engineered chimeric antigen receptors, could offer beneficial precision medicine approaches as a novel therapeutic option.

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Conflict of interest statement

All authors have completed and submitted the International Committee of Medical Journal Editors Uniform Disclosure Form for Potential Conflicts of Interest. None of the authors disclose any conflict of interest.

Data sharing statement

All data generated or analyzed during the present study are included in this published article. Further details are available for non-commercial purposes from the corresponding author on reasonable request.

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