

Original Research Article

The effect of cynaropicrin, a sesquiterpene lactone, on the migratory properties of triple-negative breast cancer cells and the underlying mechanisms

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Abstract

Objective: Triple-negative breast cancer (TNBC) is the most metastatic type of breast cancer. Cynaropicrin, a sesquiterpene lactone, shows potential anticancer effects. This study evaluated cynaropicrin's impact on metastasis and angiogenesis in TNBC cells.

Materials and Methods: MDA-MB-231 and MDA-MB-468 cell lines were exposed to incrementing concentrations of cynaropicrin. The proliferation of the cell lines was assayed using the MTT method. A wound scratch technique was chosen to appraise the migratory properties of cells following cynaropicrin treatment. The transcript levels of epithelialmesenchymal transition (EMT) and pro-angiogenic factors were quantified via quantitative polymerase chain reaction. The western blotting technique estimated the amount of E-cadherin, N-cadherin, Fibronectin, Vimentin, and VEGFA.

Results: The proliferation of MDA-MB-231 and MDA-MB-468 cells was significantly lowered due to cynaropicrin in a concentration-associated way. Results of the wound healing method uncovered that cynaropicrin could mitigate the migration of breast-derived MDA-MB-231 and MDA-MB-468 cells. Cynaropicrin also upregulated E-cadherin and hindered the protein expression of N-cadherin, Vimentin, Fibronectin 1, and VEGFA in breast-derived MDA-MB-468 and MDA-MB-231 cells.

Conclusion: The present findings indicated the anti-metastatic capacity of cynaropicrin against TNBC by a mechanism that implicated the inhibition of the EMT and pro-angiogenic factor VEGFA. These outcomes suggest cynaropicrin as an anti-metastatic and anti-angiogenic sesquiterpene lactone against TNBC.

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Introduction

Breast cancer is the most prevalent type of cancer throughout the globe (Wilkinson and Gathani 2022). According to recent estimates, patients with breast tumors may augment to more than 3 million cases with a mortality rate of 1 million per year by 2040 (Arnold et al. 2022). Among a variety of breast cancers, triple-negative breast cancer (TNBC) is a particular histologic type with highly aggressive features, poor clinical outcomes, aggressive high metastatic capability, high recurrence, and poor prognosis (Medina et al. 2020). TNBC tumors generally show resistance to therapeutic agents due to the loss of three important receptors, including estrogen receptor (ER), progesterone receptor (PR), or human epidermal growth factor receptor 2 (HER2) (Kamalabadi-Farahani et al. 2025; Yin et al. 2020). Classical methods to cure breast tumor patients are surgery, hormone therapy, radiotherapy, and chemotherapy. Patients with TNBC tumors are recommended to be treated with agents chemotherapeutic to enhance therapy of the disease due to the absence of necessary receptors (Kumar and Aggarwal 2016). However, breast surgery with radiotherapy or without it, or breast keeping with radiotherapy is another wellestablished therapy for TNBC patients (Kumar and Aggarwal 2016). Recently, several specific targeted therapies have been created for the suppression of different types of breast tumors (Masoud and Pagès 2017). The Food and Drug Administration (FDA) has verified three molecular targeted therapeutics that include olaparib and talazoparib for patients bearing a germline breast cancer gene (BRCA) mutation and combination of atezolizumab and nabpaclitaxel for subjects who are positive for programmed death-ligand 1 (PD-Ll+)(Lyons 2019).

Metastasis is a process with several stages that is initiated by the invasion of the metastatic cancer cell into the adjacent tissue followed by several steps such as intravasation, circulation in the blood or lymph, extravasation, and colonization within the distant organ (Hosseini et al. 2015; Rajabi et al. 2023). The first phase in the course of metastasis is indicated by the epithelial-mesenchymal transition (EMT), which is a process by which the cancer cell obtains metastatic characteristics (Hajimehdipoor et al. 2023). Angiogenesis is another important mechanism by which the tumors can easily metastasize into distant organs (Rajabi et al. 2019). This process is a fundamental step in the promotion and advancement of metastasis.

Sesquiterpene lactones are sesquiterpenoids known for their diverse pharmacological properties (Forouzanfar et al. 2020; Noori et al. 2014; Rajabi et al. 2024). Cynaropicrin, as a sesquiterpene lactone derived from Cynara cardunculus, has several biological activities against aging, hyperlipidemia, parasites, inflammation, and cancer (Elsebai et al. 2016; Fuhr et al. 2022). Its anti-cancer effects have been numerously mentioned in the literature (Choi et al. 2005; Muhammad et al. 2003; Yang et al. 2008). However, no previous investigation has studied the influence of cynaropicrin on invasion, migration, and metastasis in TNBC cells. Thus, this current investigation was conducted for assessing the anti-metastatic and anti-angiogenic impacts of cynaropicrin on TNBC cell lines, including MDA-MB-231 and MDA-MB-468, and underlying mechanisms.

Materials and Methods Cell culture and materials

Human TNBC cell lines, MDA-MB-468 and MDA-MB-231, were bought from Sigma Company, USA. The TNBC cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium at 37°C with 98% humidity and 5% CO₂ in a standard incubator. Fetal bovine serum, penicillin/streptomycin, and trypsin for cell culture were sourced from Gibco Co., Germany. Cynaropicrin was obtained from Sigma Company, USA. The solvent for the preparation of cynaropicrin solution to treat the cells was dimethyl sulfoxide (DMSO; Merck, Germany).

Cell viability

Both MDA-MB-231 and MDA-MB-468 cells were cultivated at an amount of 10^4 cells in each well in 96-well cell culture plates. Afterward, both cell lines received augmenting concentrations of cynaropicrin (2, 4, 6, 8, 10, 15, 20, and 30 µM) for 24, 48, and 72 hr. DMEM was administered to the control wells with sham wells receiving DMSO solution. Subsequently, the MTT viability assay was performed to estimate the proliferation percent of the cells. Briefly, the MTT reagent was applied at an amount of 5 mg/ml followed by the incubation with both cancer cell lines for 4 hr. Then, the MTT solution was discarded and 200 µl of DMSO solution was poured into each well. Finally, the formazan solution's absorbance was read at 570 and using 630 nm a plate reader spectrophotometer (Epoch, USA).

Wound scratch technique

Using *in-vitro* wound scratch technique, the possible influence of cynaropicrin treatment on the invasion and migration of MDA-MB-468 and MDA-MB-231 breastderived cell lines was examined. Briefly, two TNBC cell lines were cultivated in 6well cell culture plates at an amount of 2×10^5 cells per well. The cultured cell lines were permitted to stick and proliferate as 50–60%. Then, three horizontal scratches were made in each well using 200 µl yellow pipette tips. To remove cell debris, washing of each well was done twice using Phosphate Buffered saline (PBS) buffer. Subsequently, the treatment wells containing MDA-MB-231 cells received cynaropicrin at doses of 4 (IC25) and 8 (IC50) μ M and the wells that contained MDA-MB-468 cells received 5 (IC25) and 10 (IC50) μ M of cynaropicrin. The control wells were administered with a standard DMEM culture medium. Afterward, the pictures of all cynaropicrin-treated and control wells were grabbed at 0, 12, 24, 48, and 72 hr.

Gene transcript expression examination

The amount of gene markers related to EMT (Cadherin 1: *CDH1*, Cadherin 2: *CDH2*, Vimentin: *VIM*, and fibronectin 1: *FN1*) and angiogenic marker Vascular Endothelial Growth Factor A (*VEGFA*) in MDA-MB-468 and MDA-MB-231 breastderived cell lines was measured using quantitative real-time PCR method.

RNA molecules were isolated by utilizing an RNeasy mini kit (Qiagen, Hilden, Germany) following the protocols of the manufacturer. RNA of samples was reversed to cDNA by utilizing a standard kit for cDNA synthesis (Qiagen, Hilden. Germany). Amplification cDNA of molecules was carried out by SYBR-green master mix (Ampliqon, Denmark) on ABI PRISM 7900HT (Applied Biosystems, USA). Melting curves were analyzed for each product to confirm the specificity of primers and products. The expression level of the transcripts of the GAPDH gene was chosen as the housekeeping gene and the expression of all transcripts was estimated by using the $2^{-\Delta\Delta Ct}$ method. Table 1 lists the primer sequences used in this study for gene amplification.

Table 1. Primer sequences used in the present study

Gene name	Forward primer	Reverse primer
CDH1	5'-GGGGTCTGTCATGGAAGGTG-3'	5'-CGACGTTAGCCTCGTTCTCA-3'
CDH2	5'-GCGTCTGTAGAGGCTTCTGG-3'	5'-GCCACTTGCCACTTTTCCTG-3'
FN1	5'-ACAAGCATGTCTCTCTGCCAA-3'	5'-TCAGGAAACTCCCAGGGTGA-3'
VIM	5'-TCCGCACATTCGAGCAAAGA-3'	5'-ATTCAAGTCTCAGCGGGCTC-3'
VEGFA	5'-GAGCAAGACAAGAAAATCCC-3'	5'-CCTCGGCTTGTCACATCTG-3'
GAPDH	5'-ACCCACTCCTCCACCTTTGA-3'	5'-CT GTTGCTGTAGCCAAATTCGT-3'

Protein expression analysis

The influence of cynaropicrin treatment amount EMT the of protein on concentrations, which include E-cadherin, N-cadherin, Vimentin, and Fibronectin 1 together with an angiogenic factor VEGFA, was estimated in MDA-MB-468 and MDA-MB-231 breast-derived cell lines by utilizing western blotting. Briefly, the cells trypsinized and subjected were to centrifugation (2500 RPM) for 7 min. Then, both cell lines were washed with PBS and incubated with radioimmunoprecipitation assay buffer (RIPA) lysis buffer (Thomas Scientific Inc., USA). RIPA buffer lysed the harvested cancer cells, and the resulting lysate was centrifuged. The supernatants underwent western blotting, with total protein content measured by the Bradford method. Next, 40 µg of total protein was mounted onto 10% SDS-PAGE and transferred to a standard PVDF membrane. Upon the blockage of the membrane by utilizing 5% non-fat milk, primary antibodies namely E-cadherin, N-cadherin, Vimentin, Fibronectin 1, VEGFA, and GAPDH (Santa Cruz Biotech, Santa Cruz, USA) were added onto the membrane. The membrane was incubated overnight at 4°C, followed by the addition of horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour in the dark. Finally, the immunoblots were observable by applying an enhanced chemiluminescent standard kit (Bio-Rad, CA, USA) and exposed to autoradiography film.

Statistical assessments

Data were analyzed using one-way ANOVA followed by Tukey's *post hoc* test. Results from triplicate experiments are presented as mean \pm standard deviation (SD). Statistical analyses were conducted with GraphPad PRISM software version 8, and p-values less than 0.05 were deemed statistically significant.

Results

Impact of cynaropicrin compound on the viability rates of MDA-MB-231 cell line

Cynaropicrin treatment for 24 hr declined the proliferation of the MDA-MB-231 cells at doses that are greater than $30 \,\mu M$ but lower concentrations had no significant effect on their proliferation rate (p=0.063) (Figure 1A). Treatment of these cells for 48 hr significantly inhibited their proliferation at doses that are greater than $6 \,\mu M$ with the inhibitory concentration 50 (IC50) value of 8.05 µM (p=0.04) (Figure 1B). Cynaropicrin treatment for 72 hr also suppressed MDA-MB-231 cell proliferation at doses that are greater than 6 µM (p=0.032). The IC50 value of cynaropicrin hindering impact on these cancer cells for 72 hr was 7.96 µM (Figure 1C).





Figure 1. Anti-proliferative effect of cynaropicrin on the MDA-MB-231 cell line. Different concentrations of cynaropicrin (2, 4, 6, 8, 10, 15, 20, and 30 μ M) were used to treat MDA-MB-231 cells for 24 (A), 48 (B), and 72 (C) hr. The MTT method was used to measure the viability of the cells. The data are expressed as mean \pm SD of three independent experiments. *p<0.05 as compared to the control was considered statistically significant.

Impact of Cynaropicrin compound on the viability rates of MDA-MB-468 cell line

Treatment of the MDA-MB-468 cell incrementing line with doses of cynaropicrin (2-30 µM) for 24, 48, and 72 hr is illustrated in Figures 2A, B, and C. The obtained data suggested that cynaropicrin prohibiting impact on the proliferation of these breast tumor cells in a dose-associated way. In detail, cynaropicrin administration to the MDA-MB-468 cell line for 24 hr had no marked effects on their proliferation (p=0.07) (Figure 2A). However, exposing of these cancer cells to augmenting doses of cynaropicrin for 48 (p=0.042) and 72 hr (p=0.04)markedly hindered their proliferation with IC50 values of 9.90 and 9.95, respectively.



Figure 2. Anti-proliferative effects of cynaropicrin on the MDA-MB-468 cell line. The cells were treated with different doses of cynaropicrin (2, 4, 6, 8, 10, 15, 20, and 30 μ M) for 24 (A), 48 (B), and 72 (C) hr. The cell viability was determined using MTT assay. The data are expressed as mean ± SD of three independent experiments. *p<0.05 as compared to the control was considered statistically significant.

Effects of cynaropicrin on the propagation of MDA-MB-231 and MDA-MB-468 cell lines

To examine influence the of cynaropicrin on the invasiveness and migratory capacity of MDA-MB-231 and MDA-MB-468 cell lines, these malignant cell lines were exposed to IC25 and IC50 concentrations of this phytochemical drug anti-migration activity of and the compound was evaluated using wound healing assay. The outcomes revealed that cynaropicrin at a concentration of 8 µM (IC50) considerably inhibited the migration of the MDA-MB-231 cell line even after 48 hr in comparison to untreated control cells (Figure 3A). However, treating MDA-MBcells with cynaropicrin 231 at a concentration of 4 µM (IC25) elicited no marked impact on the migratory properties of these cells compared to the untreated cells. Figure 3B indicates that cynaropicrin treatment prohibited the invasion and migration of MDA-MB-468 cells at all amounts of this natural material. However, the cells treated with IC50 concentration of cynaropicrin more considerably hampered the invasiveness of these TNBC cells in comparison to the cells treated with IC25 concentration of the drug.



Figure 3. (A) Anti-invasion effect of cynaropicrin on MDA-MB-231 cell line at the concentrations of 4 (IC₂₅) and 8 (IC₅₀) μ M for 48 hr. (B) Anti-invasion effect of cynaropicrin on MDA-MB-468 cell line at the concentrations of 5 (IC₂₅) and 10 (IC₅₀) μ M for 48 hr. The scratch assay was performed in triplicate with three horizontal scratches within each well.

Influence of cynaropicrin on gene expression in MDA-MB-231 and MDA-MB-468 cell lines

The effects of cynaropicrin use (48 hr) on the gene expression of EMT markers such as *CDH1*, *CDH2*, *VIM*, and *FN1* along with the angiogenesis activator gene *VEGFA* were measured both in MDA-MB-468 and MDA-MB-231 breast-derived cell lines. According to the data shown in Figure 4A, cynaropicrin, at amounts of 4 and 8 μ M, meaningfully increased the levels of the *CDH1* gene (p=0.046) along with no influence on the levels of the *FN1* gene (p=0.08) in the MDA-MB-231 cell line compared with the control. It also downregulated the mRNA expression of CDH2 (p=0.045) at the amount of 4 μ M and lowered the levels of VIM (p=0.038) and VEGFA (p=0.043) genes at doses of 4 and 8 µM in MDA-MB-231 cells. In the MDA-MB-468 cell line, cynaropicrin treatment (5 and 10 µM) markedly elevated the transcript level of the CDH1 (p=0.031) gene and decreased the expression levels of VIM (p=0.037) and VEGFA (p=0.033) genes when comparing the control cells (Figure 4B). This sesquiterpene lactone also hindered the expression of CDH2 (p=0.032) and FN1 (p=0.04) mRNA levels at a dose of 5 µM in the MDA-MB-468 cells when comparing with the control cells.



Figure 4. (A) Effect of cynaropicrin treatment at the concentrations of 4 (IC₂₅) and 10 (IC₅₀) μ M for 48 hr on the expression of *CDH1*, *CDH2*, *VIM*, *FN1*, and *VEGFA* genes in the MDA-MB-231 cell line. (B) Effect of cynaropicrin ate doses of 5 (IC₂₅) and 10 (IC₅₀) μ M for 48 hr on the mRNA expression of *CDH1*, *CDH2*, *VIM*, *FN1*, and *VEGFA* genes in the MDA-MB-468 cell line. The experiments were performed in triplicate. The results are representative of means ± SD. *p<0.05 as compared to the control was considered statistically significant.

Effects of cynaropicrin on protein expression in MDA-MB-231 and MDA-MB-468 cell lines

The protein expression of EMT and angiogenic markers in cynaropicrin-treated MDA-MB-231 and MDA-MB-468 cell lines was measured using western blotting. The results of western blotting illustrated that treatment of MDA-MB-231 cells with cynaropicrin (4 and 8 μ M for 48 hr) markedly heightened the protein expression of E-cadherin and lowered VEGFA and vimentin compared with untreated MDA-MB-231 cells (Figure 5A). Cynaropicrin, at a dose of 8 μ M, diminished the

A

concentration of N-cadherin protein in these cells. However, no remarkable effect was shown in the amount of fibronectin 1 protein in cynaropicrin-treated MDA-MB-231 cells. As depicted in Figure 5B, the protein expression of E-cadherin was increased and the protein level of Ncadherin, vimentin, and VEGFA was decreased in MDA-MB-468 cells treated with cynaropicrin (5 and 10 μ M) as compared with the control. In addition, cynaropicrin at a dose of 10 μ M reduced the amount of fibronectin 1 protein in MDA-MB-468 cells in comparison to untreated cells.



B

Figure 5. (A) Effect of cynaropicrin treatment on the expression of E-cadherin, N-cadherin, vimentin, fibronectin 1, and VEGFA at protein levels in the MDA-MB-231 cell line. (B) Effect of cynaropicrin on the protein expression of E-cadherin, N-cadherin, vimentin, fibronectin 1, and VEGFA in the MDA-MB-468 cell line. The experiments were performed in triplicate.

Discussion

In this current investigation, the results of the MTT technique uncovered that cynaropicrin treatment for 48 and 72 hr diminished the augmentation of the MDA-MB-231 cells with the IC₅₀ values of 8.05 μ M and 7.96 μ M, respectively. Also, treatment of MDA-MB-468 cells with incrementing amounts of cynaropicrin for 48 and 72 hr meaningfully blocked their proliferation with IC₅₀ values of 9.90 μ M and 9.95 μ M, respectively. Accordingly, the other experiments in the present study were performed by treating MDA-MB-468 and MDA-MB-231 cells with IC50 and IC25 concentrations of cynaropicrin after 48 hr treatment period. In the second step, a wound scratch assay was performed to assess the influence of cynaropicrin on the invasive and migratory capability of MDA-MB-231 and MDA-MB-468 cell lines. The outcomes of scratch wound assay on MDA-MB-231 unraveled that cynaropicrin (8 µM) markedly reduced the migration of the MDA-MB-231 cell line. Moreover. cynaropicrin treatment (5 and 10 μ M) inhibited the invasion and migration of MDA-MB-468 cells. In the final step, the effect of cynaropicrin on the amount of EMT indexes (E-cadherin, N-cadherin, and Fibronectin and Vimentin, 1) angiogenic factor VEGFA was investigated at mRNA and protein levels to uncover the mechanism of anti-metastatic activity of cynaropicrin in both TNBC cell lines. Looking at the results of western blotting technique and real-time PCR, cynaropicrintreated MDA-MB-468 and MDA-MB-231 cells showed augmented expression of Ecadherin and declined levels of N-cadherin. Vimentin, Fibronectin 1, and VEGFA when compared with untreated control cells.

The anticancer activity of cynaropicrin has been proven in several previous studies in the literature (Boulos et al. 2023; Lepore et al. 2019; Li et al. 2021; Yang et al. 2022). In a study on the *in-vitro* and *in-vivo* models of colorectal cancer (CRC), cynaropicrin suppressed the proliferation of CRC cells and initiated apoptosis by a mechanism that induced the activation of the JAK/STAT signaling pathway (Zheng et al. 2020a). In another study on a human CRC cell line, cynaropicrin administration HCT-116, considerably triggered apoptosis in these cancer cells by acting on the intrinsic pathway of apoptosis (Wang et al. 2023). Rotondo et al. unraveled that cynaropicrinexposed human glioblastoma cell line, U-87MG, underwent cell death in a doseassociated manner. They also unraveled that apoptosis was the prominent cell death mechanism by which this cancer cell line died following cynaropicrin treatment (Rotondo et al. 2022). To the best of our knowledge, only three studies have evaluated the anti-metastatic or antiinvasion properties of cynaropicrin on cancer cells. For example, De Cicco et al. investigated to examine pro-apoptotic and anti-metastatic roles of cynaropicrin in human melanoma cell line A375. They indicated that cynaropicrin significantly hampered the proliferation and augmented apoptosis of A375 cells. It also prohibited the migration, invasion, and colony

formation of these cancer cells by blocking mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF- κ B) pathways (De Cicco et al. 2021). In another study, the migration potential of three human CRC cell lines was estimated using a transwell assay. The results of that study unraveled that cynaropicrin markedly prevented the cell migration of CRC cells compared with the control group (Zheng et al. 2020b). The outcomes of the third study suggested that cynaropicrin may act as an anti-metastasis agent in leukocyte cancer cells (Cho et al. 2004).

To date, no prior investigation has explored the anti-metastatic capability of cynaropicrin in breast cancer cells. Therefore, the current research is the first report of the anti-metastasis function of cynaropicrin in TNBC cells as the most invasive and highly metastatic subtype of breast cancer. Our findings provided invitro evidence to highlight the inhibitory effect of this sesquiterpene lactone on the invasion, migration, metastatic or propagation of two distinct TNBC cell lines. The present data also suggested a potential mechanism by which cynaropicrin hindered the migratory activity of TNBC cells. This mechanism may acted by the blockage of the EMT process. This process is the first step in the promotion of metastasis by which the cancer cells gain the ability to invade the adjacent tissue (Rajabi et al. 2020; Ribatti et al. 2020). Metastatic cancer cells undergo the EMT process by downregulating E-cadherin and upregulating N-cadherin, Vimentin, and Fibronectin 1 to obtain mesenchymal characteristics for migration to distant (Francou and Anderson 2020). organs Angiogenesis is another critical process that is pivotal for the metastatic propagation of cancer cells (Liu et al. 2023). In addition, angiogenesis provides oxygen, nutrients, and growth factors for growing tumors (Al-Ostoot et al. 2021). The process of angiogenesis is a dynamic pathway modulated by different pro- and antiangiogenic factors (Larionova et al. 2021). Vascular endothelial growth factor (VEGF) family members are key inducers of angiogenesis for the stimulation of endothelial cells for neovascularization (Melincovici et al. 2018). Decreased expression of VEGFA in the present investigation may underscore the antiangiogenic and anti-metastatic activity of cynaropicrin against TNBC invasiveness.

The strengths of the present investigation is the simultaneous study of influence of phytochemical the cynaropicrin on two types of TNBC cell lines with some different features. The other strength of the current study is uncovering the probable mechanism of cynaropicrin anti-metastatic action on TNBC cells. The weakness of the study may arise from budget limitations that inhibited a comprehensive examination of some other key metastatic factors. Due to some budget limitations, we could not assess extracellular matrix factors associated with metastatic cascade of breast cancer cells. Therefore, exaltation of these factors might be incorporated into future work.

The present study provided evidence to unravel the anti-metastatic or antimigratory influences of cynaropicrin on two different TNBC cell lines MDA-MB-231 and MDA-MB-468. Our data also highlighted a novel mechanism by which cynaropicrin hindered the migration and invasion of these TNBC cells. This mechanism involved the blockage of the EMT process to hamper the migratory properties of both TNBC cell lines. Further experiments showed that cynaropicrin downregulated the levels of VEGFA as a key inducer of angiogenesis, which is pivotal for cancer cell metastasis. This study is the first to evaluate the antimetastatic activity of cynaropicrin in breast cancer cells, specifically in TNBC, which is known for its invasiveness and high metastatic potential. Our in vitro results demonstrate that this sesquiterpene lactone effectively inhibits invasion, migration, and metastatic spread in two different TNBC

cell lines. Taken together, the present results may propose cynaropicrin as a novel and effective drug for blocking metastasis in TNBC.

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Conflicts of interest

The authors have no conflict of interest to declare.

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