Astaxanthin (AST) is a red carotenoid pigment and a natural product of microalgae Haematococcus pluvialis with unique structural and chemical characteristics. AST is a powerful scavenger capable particularly chemoresistance contributes to breast cancer (BC) recurrence post-surgery followed by secondary tumor formation and distant metastasis. Natural compounds that can help chemo-sensitization of tumors without raising unwanted side effects have drawn attention for some time.

Introduction
Induction of cell death pathways constitutes the basis of cancer chemotherapy. Drug resistance

Background: We investigated molecular mechanisms behind astaxanthin-mediated induction of apoptosis in breast cancer cell lines toward combination therapy against cancer drug resistance.

Methods: Breast cancer cell lines were treated with serial concentrations of astaxanthin to determine its IC50. We used drug-design software to predict interactions between astaxanthin and receptor tyrosine kinases or other key gene products involved in intracellular signaling pathways. Changes in gene expression were examined using RT-PCR. The effect of astaxanthin-nanocarbons combinations on cancer cells was also evaluated.

Results: Astaxanthin induced cell death in all three breast cancer cell lines was examined so that its IC50 in two HER2-amplifying lines SKBR3 and BT-474 stood, respectively, at 36 and 37 µM; however, this figure for MCF-7 was significantly lowered to 23 µM (P<0.05). Astaxanthin-treated SKBR3 cells showed apoptotic death upon co-staining. Our in silico examinations showed that some growth-promoting molecules are strongly bound by astaxanthin via their specific amino acid residues with their binding energy standing below -6 Kca/Mol. Next, astaxanthin was combined with either graphene oxide or carboxylated multi-walled carbon nanotube, with the latter affecting SKBR cell survival more extensively than the former (P<0.05). Finally, astaxanthin co-induced tumor suppressors p53 and PTEN but downregulated the expression of growth-inducing genes in treated cells.

Conclusions: These findings indicate astaxanthin carries' multitarget antitumorigenic capacities and introduce the compound as a suitable candidate for combination therapy regimens against cancer growth and drug resistance. Development of animal models to elucidate interactions between the compound and tumor microenvironment could be a major step forward towards the inclusion of astaxanthin in cancer therapy trials.
of degrading free radicals in aqueous solvents. It is a free oxygen inhibitor and an effective inhibitor of lipid peroxidation.

Cancer incidence is rare among North American tribes who traditionally consume salmon fish (a rich source of AST). AST induces reduction of mouse tumor cells and synthesis of DNA. The compound also reduces mouse mammary tumor cells by 40%. Among eight carotenoids tested, AST most effectively inhibited the invasion of hepatoma cells. AST inhibits growth among human cancer cell lines too. For example, two human colon cancer cell lines incubated with AST for 4 days lost their growth significantly compared to their controls. The inhibiting effect of AST on human prostate cancer cells has also been reported. Finally, AST can prevent UV damage on DNA of skin fibroblasts, melanocytes and intestine cells.

The anti-oxidant properties of AST restore superoxide dismutase (SOD) and catalase (CAT), thereby reducing intracellular O\(^2\)- production and reversing lipopolysaccharide-induced toxicity and reactive oxygen species production in cancer cells. Such properties can also arrest cell cycle progression, inhibit cell proliferation and induce apoptosis by activating Nrf2-mediated anti-oxidant defense system. Unlike other carotenoids, there exists no report on the pro-oxidative properties of AST.

The anti-proliferative effects of AST on cancer cells of breast, liver and lung have been shown in parallel with a stronger growth inhibitory effect of AST compared to other carotenoids. Also, AST differentially affects cancer cells without harming normal ones. AST indeed protects wild-type cells against oxidative stress, ultraviolet, neurotoxins, and cell-tissue inflammation via inhibiting apoptosis. In contrast, it forces cancer cells to commit apoptosis. AST-treated cancer cells show a significant peak of hypodiploid, indicative of apoptosis with typical characteristics including modified mitochondrial morphology, transmembrane potential and respiratory chain and regulated levels of Bcl-2, Bax, Bad, Bcl-xL.

In mouse models of cancer, the potential of AST in inducing apoptosis among tumor cells has been demonstrated. As an example, the compound inhibits tumorigenesis in rat colon via inducing apoptosis. AST can do this by controlling ERK-2, AKT, MMPs-2/9, COX-2 and NFKB genes. In another study, AST inhibited colon carcinogenesis by regulating inflammatory cytokines. In hamster model of mouth cancer, dietary AST inhibited NFKB and Wnt/β-catenin by inhibiting Erk/MAPK and PI3K/AKT, eventually leading to apoptosis. Based on its anti-inflammatory and anti-oxidant properties, AST was able to reduce proliferation and migration of BC cells compared to control normal breast epithelial cells. The anti-invasive and anti-metastatic potential of AST has been shown in increasing metastasis-specific miRNAs and suppressing the expression of MYC transcription factor.

In this study, we examined the effect of AST on the fate of BC cell lines and the changes it causes in expression levels of some key genes involved in BC cell tumorigenesis. We tested AST potential in binding to key growth signaling molecules including receptor tyrosine kinases, examined its impact on gene expression, and used nano particles to potentiate anti-tumorigenic effects of the carotenoid.

**Methods**

**Cell culture**

Three BC cell lines namely MCF-7, BT-474 and SKBR3 plus MCF-10, a breast epithelial line as a control, were obtained from the cell bank in NIGEB and fed with Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS and incubated in 37 °C and 5% CO\(_2\).

**Cell treatment**

To make a stock solution of AST, 53.1 mg of its powder (Santa Cruz Biotechnology Inc., USA) was dissolved in 9 mL DMEM and 1 mL DMSO to obtain a 10 mM solution. This solution was filtered and stored at -20 °C until use. The cells were seeded in 96-well plates with 5000-7000 cells per well in 100-150 µL medium and incubated for 24 hrs before treatment began. Serial dilutions of AST were added to the cells followed by cell incubation for 5 hrs. We used AST doses of 8, 16, 24, 36, 40, 50, 65, 80 µM and then tested cell viability in each cell line using the MTT Viability assay. Each dose was tested in triplicate wells for each cell line and the whole experiment was repeated three times. Due to the lethal properties of DMSO as a solvent, and to determine AST independent effect, the cells were also treated with DMSO for the equivalent volumes in parallel.

**Cell death analyses**

We used MTT assay to measure cell viability as a percentage of untreated WT control cells. Briefly, a 5-mg/mL stock of MTT solution in PBS was formed from which 20 µL was added to 80 µL cell medium in each well of 96-well plates and incubated for 4 hrs. The medium was then replaced with 100 µL DMSO for another 30-min shaking-incubation at dark before reading absorbance in 580 nM (excitation) and 620 nM (emission) using an ELISA reader. An average of the produced figures in various doses of AST was used to determine AST effective dose.

**Cell co-staining and live cell count**

Since acridine orange (AO) co-applied with ethidium bromide (EB) stains nuclear DNA green in live cells and orange in dead cells, we followed the standard co-staining method to measure the percentage of viable SKBR3 cells. Cell groups were
suspended at a normal counting concentration from which 50 µL was mixed with 50 µL of the stain in 96-well plates and subjected to counting under fluorescent light. Images were captured using a fluorescence microscope coupled with a Nikon digital camera. Six random microscopic fields per well were selected to count an average of 120 cells counted for each cell group in triplicates (three wells per group). Green cells and orange cells were considered, respectively, as living and dead cells. The data collected from three independent experiments were then expressed as the percentage of living cells by dividing the number of living cells by the number of total cells counted (living + dead).

**Bioinformatic analysis of astaxanthin interactions with target molecules**

Three classes of luminal B, HER2⁺ and basal like/triple negative are considered as the main subtypes in BC. First, we used KEGG (Kyoto Encyclopedia of Genes and Genomes) database to identify molecular pathways involved in BC tumorigenesis. In this database, diseases are considered as induced molecular network systems. The aim of using this database was to reach a common signaling pathway among BC subtypes and, therefore, to reach therapeutic aims and target proteins. The next step was to determine the structure of selected proteins from Protein Data Bank (www.rcsb.org).

In order to prepare protein structures, we used the AutoDockTools software (http://autodock.scripps.edu/resources/adt). We omitted ligands and water molecules from the structure of each protein without deleting cofactors. The AST structure was captured from Chemspider (http://www.chemspider.com/) and ATB site developed by the University of Queensland, Australia (https://atb.uq.edu.au) was utilized to design and optimize the AST molecular structure. We ultimately examined molecular docking using AutoDockTools. In this procedure, we first converted pdb files of ligands and proteins to pdbqt. Then, we applied the AutoGrid program in order to obtain a map for docking simulation. To do this, after defining proteins and ligands, Grid box in the active site of each protein was adjusted. In the next step, AutoDock was used for simulation using genetic algorithm as the searching parameter. In order to obtain dock scores, the docking protocol used in the study consisted of 200 independent runs along with population size=150 and the Lamarckian genetic algorithm was applied for all docking runs reported in Kcal/mol. Finally, the figures of interactions between AST and the selected proteins were portrayed through Discovery Studio Visualization software.

**Reverse transcription (RT) PCR**

RT-PCR was carried out as reported. Briefly, total RNA was extracted, DNase treated, and used (2µg) for cDNA synthesis. To amplify target DNA fragments, the cDNA samples were first denatured at 95 °C for 2 min before amplification. Table 1 shows the primer pairs we used for each gene candidate. PCR reactions were carried out for 30 cycles consisting of denaturation at 95 °C for 45”, annealing at various temperatures. Gel electrophoresis and band intensity measurements were duplicated as reported.

**Table 1. Primer sequences for RT-PCR**

| Name | Primer Sequence | Size (bp) | Anneal. Temp | Accession # |
|------|----------------|-----------|--------------|-------------|
| EGFR | F TCTTGGAAAACCTGCAGA R TGCCTCCAGGTCAGCA | 188 | 52.2°C × 20" | NM_005228.5 |
| PTEN | F CAATTCAAGGACACCACAGCAG R CCTCTGTCCTGTAGGAAGA | 173 | 53.2°C × 20" | NM_000314.7 |
| IGF1R | F CTTGTAAGGTCCTTGCCC R GAGCCCACTGAAGCTCCA | 225 | 53°C × 20" | NM_000875.5 |
| AKT1 | F AGGATGTCGAGAAGCTGAG R GTCCGGTCGATGACAGG | 133 | 54.2°C × 20" | NM_051632.3 |
| AKT2 | F CATCAGAGGACGCCTGAGG R ACCGAGAAGTGTAGGG | 153 | 53.8°C × 60" | NM_001626.6 |
| ERK1 | F ATCATGCTGCAACTAC R ACAAGGTGTCGCTTCACAGG | 172 | 52°C × 60" | NM_002746.3 |
| ERK2 | F ACATTCAAGGCTGACAGG R TCAGACCTGAGGTGGCTCG | 254 | 53.4°C × 60" | NM_001626.6 |
| P53 | F CCCCTCCCTGGCCCTGTACATTC R GCAGCAGCCTTACAACCTCCGCTCAT | 265 | 54.8°C × 60" | NM_00112618.1 |
| GAPDH | F GTCTCTGTGACTTCAACAGG R GTCTCTGTGACTTCAACAGG | 130 | 56°C × 60" | NM_002046 |
Combination of astaxanthin and carbon nanomaterials

Two carbon nanomaterials were used to combine with AST: graphene oxide (GO) and carboxylated multi-walled carbon nanotube (MWCNT-COOH). Their characteristics were as follows:
1. MWCNT-COOH: %95 purity, OD: 10-30 nm, Length: 0.5-2 μm, Neutrino Co., Ltd.
2. GO nanoplatelets (99%, Thickness 3.4-7 nm with 6-10 Layers).

The concentration of AST was performed by Unico UV-2100 Model variable-wavelength UV-Vis spectrophotometry. Field emission scanning electron microscope (FESEM) was taken using an MIRA3\TESCAN-XMU model. Fourier transform infrared spectroscopy (FT-IR) was recorded using KBr tablets on a Thermo Nicolet Nexus 870 FTIR spectrometer.

In order to load AST on nanotubes, 30 mg of GO or MWCNT-COOH was dispersed into 15 mL ethanol containing 100 mg/L of AST for 4 h. The amounts of absorbed AST were calculated as the difference between the initial and final concentrations when the equilibrium was reached. After 4h, the AST concentration in the aqueous solutions was determined by UV-Vis spectroscopy. The adsorption (%) was obtained as follows:

\[
\text{% Adsorption} = \frac{C_0 - C_e}{C_0} \times 100
\]

where C0 and Ce are, respectively, the initial and final concentrations (mg/L) of AST in the aqueous solution.

Statistical analyses

Data in the figures are represented as the mean ± standard error of the mean (SEM) of three or more separate experiments. Student’s T-test was used to analyze differences between two groups. Differences among three or more groups were analyzed by one-way analysis of variance (ANOVA), followed by a post hoc Duncan Multiple-comparisons Test (P<0.05, statistically significant; P<0.01, highly significant).

Results

Death induction in breast cancer cell lines by astaxanthin

We measured viability of AST-treated BC cell lines and found that the compound kills the cells at its lethal dose within 5 hours of treatment. Our results showed that IC50s for BT-474, SKBR3 and MCF-7 are, respectively, 36, 37 and 23 µM with no significant effect on normal controls MCF-10 (Figure 1). We also found that the cell death is dose dependent and accelerated beyond a range of the drug dose (Fig. 1B-D).

Morphological changes towards apoptosis post-treatment with astaxanthin

Cell co-staining using acridine orange and ethidium bromide reveals altered morphologies typical of apoptosis among cell samples (Figure 2). Over 29% of SKBR3 cells treated with 36 µM AST

Figure 1. The effect of astaxanthin on survival of breast cancer cell lines. Each column represents an average of three independent experiment carries out in triplicates. Symbols * and ** indicate statistical differences between each column with its counterpart in control, whereas symbols # and ## show differences between each column of AST-treated cells and its control treated with DMSO.
The KEGG database encompasses data of genomics, biological pathways, human diseases, drugs and chemicals (Figure 3.1). The KEGG pathway analysis introduced PI3K/AKT and MAPK/ERK as the common signaling pathways that are actively present in all BC subtypes, hence their targeting can be translated to control all BC subgroups. To perform the docking studies, the following protein structures were captured from Protein Data Bank (PDB): AKT1 (1UNQ) and AKT2 (1O6L) as key proteins involved in the PI3K/AKT pathway, IGFR (5FXS) and EGFR (2RGP) as common receptors involved in cancer development, and ERK1 (4QTB) and ERK2 (3SA0) as principal proteins included in the MAPK/ERK pathway (Figure 3.2).

Following the preparation of ligands and proteins, docking was carried out in order to simulate drug-

Strong binding between astaxanthin and key intracellular molecules

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Following the preparation of ligands and proteins, docking was carried out in order to simulate drug-

underwent apoptosis compared to their untreated controls (P<0.05), whereas this rate increased to 46% in the presence of 40 µM AST (P<0.01).

Figure 3.1. The KEGG database representing molecular pathways involved in breast cancer tumorigenesis.

Figure 2. Apoptotic death amongst SKBR3 cells treated with two astaxanthin doses. Each column represents an average of three independent experiments carried out in triplicates by random counting of microscopic fields as detailed in Methods. Symbols * and ** indicate statistical differences between each column with its control counterpart.
receptors bindings. Table 2 shows the information related to five conformers with the least binding energy for each protein. These results indicate that AST can strongly bind to each of these proteins via their specific amino acid residues, leading to their inhibition.

Simulation by docking showed that the lowest binding energies of AST with AKT1 and AKT2 were -8.49 and -10.69 between, respectively, two amino acids THR160, PHE161 in AKT1 and three amino acids GLY235, TYR231 and ASN233 within AKT2. IGF1R with -11.37 and EGFR with -9.48 binding energies form an H bound with AST in, respectively, amino acids VAL1132 and MET766. Finally, AST interaction with ERK1/2 shows binding energies of -10.38 and -11 involving, respectively, amino acids TRY81, ARG84 in ERK1 and THR160, ARG67 in ERK2 (Figure 3). We also tested AST binding to various sites in random within the CD44 receptor protein but did not find favorable binding sites in the receptor structure.

Altered expression of growth signaling genes in astaxanthin-treated SKBR3 cell line

| Protein | Lowest Binding Energy (KCal/mol) |
|---------|----------------------------------|
| AKT1    | -8.49                            |
| AKT2    | -10.69                           |
| ERK1    | -10.38                           |
| ERK2    | -11.00                           |
| IGF1R   | -11.37                           |
| EGFR    | -9.48                            |

Table 2. Binding energy for 5 initial conformers of target proteins.

Changes in mRNA expression of genes involved in growth pathways and tumor suppressor gene PTEN were monitored in SKBR3 cells. As shown in Figure 4, expression of RTKs, EGRF and IGF1R, and other cell growth-promoting genes AKT1, AKT2, ERK1 and ERK2 was reduced upon AST treatment of the cells and most of these changes were statistically significant compared to untreated controls (Fig. 4; P<0.01). In contrast, the expression of tumor suppressors p53 and PTEN that promote apoptosis against cell growth, significantly increased upon AST treatment (P<0.01).

Figure 4. Altered expression of key genes under astaxanthin treatment. The gel image shows RT-PCR products. The intensity of the gel bands was measured as outlined in Methods to produce the graph. Each column in the graph represents two independent RT-PCR experiments. Symbols * and ** show statistical differences between each column and its control counterpart.
After producing our nano-drugs, cancer cell lines were treated with nano-astaxanthin compounds. As shown in Fig. (5C), the percentage of the cells killed by GO-AST was comparable to the percentage of those killed by AST alone. However, the combination of AST+MWCNT-COOH accelerated cell death in both lines so that the difference with those under AST alone became significant (Figure 5C; P<0.05). Also, MCF-7 cells suffered more death than SKBR3 lines after treatment with AST+MWCNT-COOH (P<0.05).

### Discussion

In this study, we showed that AST significantly reduces viability and induces apoptosis in three different BC cell lines. Based on the levels of HER2 we have previously detected in these cell lines, it is conceivable from our current data that AST impact on cell survival might conversely correlate with HER2 content.

According to the KEGG database, several key molecules are involved in the cells’ survival and growth; therefore, among them, we selected EGFR, IGF1R, AKT, ERK1/2, and PTEN for analysis. Our data in silico indicated strong binding of AST to these molecules. These observations primarily suggest that although HER2 is a determinant receptor in cancer cell growth, AST binding to alternative molecules on the row may eventually overcome HER2 pro-growth function by providing a bypassing route toward disruption of growth signals. This might explain the reduced levels of viability among HER2-amplifying BC cell lines upon receiving higher doses of AST.

The strong binding of AST to key RTKs and downstream molecules also indicates the unexplored anti-neoplastic capacity of the photochemical compound. Overexpression or dysfunction of EGFR

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**Figure 5.** Acceleration of BC cell death treated with nano-astaxanthin compounds. The two images on the left show electron microscopy and adsorption spectroscopy, respectively. The right graph shows survival of treated BC cells. Each column represents an average of three independent experiments carried out in triplicates by random counting of microscopic fields as detailed in Methods. Symbols * and ** indicate statistical differences between each column with its control counterpart, whereas symbol ‡ shows difference between nano-AST and AST alone and symbol # shows differences between nano-AST and nano particle control.
(HER1 in humans) due to mutations plays a role in tumorigenesis.\textsuperscript{30} Ligand binding can activate EGFR molecules to homo- or hetero-dimerize with HER2\textsuperscript{31} or another EGFR member and these changes induce EGFR auto-phosphorylation in several of its tyrosine residues. These changes initiate downstream signaling pathways including MAPK, AKT and JNK, leading to DNA synthesis and cell proliferation plus cell migration. Conversely, inhibition of EGFR signaling may inhibit tumor formation or growth. Inhibitors of the EGFR oncogene have been developed against various cancer types that include several small molecules and antibodies e.g. gefitinib. However, the use of these therapeutics is likely to cause resistance in cancer cells through increasing the activity of key molecules such as STAT3.\textsuperscript{33}

We showed that beside EGFR, AST also strongly binds the insulin-like growth factor 1 (IGF-1) receptor (IGF1R). This receptor promotes cell growth by interacting IGF-1 and IGF-2, and IGF1R amplification and/or overexpression has implications in chemo- and radio-resistance and metastasis in several cancer types including BC.\textsuperscript{34,35} Indeed, triple-negative BC (TNBC) cells overexpress IGF1R at considerable levels, leading to elevated proliferation, aggressive subtypes, and survival of all TNBCs.\textsuperscript{36} Cross-talks between EGFR and IGF1R prevent EGFR inhibitors from activity and so allow EGFR signaling to resume in the presence of these inhibitors. Our analysis in silico indicated that AST can strongly bind to a number of amino acid residues in these molecules. Simultaneous inhibition of EGFR and IGF1R by AST indicates the potential of the compound in blocking EGFR-IGF1R cross-talks and bypassing drug resistance caused upon utilizing inhibitors. Therefore, the anti-tumorigenic potency of the compound can be explored along with chemical inhibitors or chemotherapy drugs against BC.

In addition to inhibitory binding to key molecules of the common cell growth pathways, AST treatment also induced mRNA expression of p53 in our SKBR3 cells. Similar to hepatoma and colon cancer,\textsuperscript{37,38} AST-mediated dose-dependent induction of phospho-p53 leading to induced cell cycle arrest and enhanced apoptosis has been shown in BC lines MCF-7 with wild-type p53 and MDA-MB-231 that carries mutant p53.\textsuperscript{39} P53 can act as both an oncogene and a tumor suppressor so that its mutations are largely translated to tumor aggression, whereas its restoration leads to tumor regression. AST-mediated induction of p53 expression adds to the multitarget potential of the carotenoid against tumorigenesis, with its therapeutic benefits exceeding those of the anti-cancer dexamethasone.\textsuperscript{40} An extension of our study would examine how changes in p53 mRNA levels correlate with alterations in protein levels and activity.

Astaxanthin further downregulated the expression of Phosphatase and Tensin Homolog (PTEN). The PTEN tumor suppressor gene, via its phosphatase activity, plays a role in cell cycle regulation and prevents cells from overgrowth and rapid division.\textsuperscript{41} The phosphatase activity of PTEN preferentially dephosphorylates PIP3 and so results in the inhibition of the AKT/PKB signaling pathway that regulates cell growth, survival, and migration.\textsuperscript{42,43} Conversely, PTEN mutations contribute to the development of many cancers, with loss of PTEN tumor suppressor commonly occurring in human cancer particularly in prostate cancer\textsuperscript{44} and reduction of its expression in BC.\textsuperscript{45} These add to the importance of PTEN induction by AST as an anti-tumor measure.

AST interacts with p53 to regulate EMT and cell migration.\textsuperscript{46} Our study on SKBR3 cells showed that AST induces p53 expression and, in parallel, restores lost levels of PTEN. In vivo studies using patient-derived xenograft HER2 models of BC similar to what we have developed before\textsuperscript{20} could provide deeper insights into the ultimate impact of PTEN-p53 co-induction by AST on HER2\textsuperscript{2} BC tumor growth and outcome.

In fact, combined versus individual disruption of p53 and PTEN induces TNBC growth with clear activating impact on PI3K/AKT signaling, changes that lead to distinct mesenchymal features and poor clinical outcome.\textsuperscript{47} Compared to the wild-type p53 expressed in MCF-7,\textsuperscript{48} MDA-MB-231 carries mutant p53, which confers survival advantage to the TNBC cell line by suppressing apoptosis.\textsuperscript{49} Due to these differences, higher doses of AST are needed to kill MDA-MB-231 cells.\textsuperscript{50} If applied in TNBC line MDA-MB-231 and therapy-resistant TNBC tumor models carrying either wild-type or mutant p53, our \textit{in silico} strategy might shed more light on the impact of PTEN-p53 interactions, upon AST treatment, on cancer cell growth and ultimately on tumor fate.

In conclusion, our \textit{in silico} experiments showed that AST promotes induction of apoptotic cell death by interactions with key genes of tumorigenesis. When combined with nanotubes, the cell death rate amongst cancer cells increases significantly, an important development in breaking drug resistance in BC tumors. These anti-tumorigenic effects of AST provide a glimpse of a global picture of the effect of phytochemicals on BC therapy and/or prevention. Animal studies focusing on AST and nano-AST-mediated changes in expression of tumor suppressors, RTKs and growth pathway molecules as well as AST interactions with anti-metastatic miRNAs will consolidate our findings in the actual tumor cells and tumor microenvironment and assess AST utilities against tumor chemoresistance and metastasis in combined modes of clinical BC therapy.

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**Conflict of Interest**
The authors declare no conflict of interest.

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