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Yeast Nanometric Scale Oscillations Highlights Fibronectin Induced Changes in *C. albicans*

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Abstract: Yeast resistance to antifungal drugs is a major public health issue. Fungal adhesion onto the host mucosal surface is still a partially unknown phenomenon that is modulated by several actors among which fibronectin plays an important role. Targeting the yeast adhesion onto the mucosal surface could lead to potentially highly efficient treatments. In this work, we explored the effect of fibronectin on the nanomotion pattern of different *Candida albicans* strains by atomic force microscopy (AFM)-based nanomotion detection and correlated the cellular oscillations to the yeast adhesion onto epithelial cells. Preliminary results demonstrate that strongly adhering strains reduce their nanomotion activity upon fibronectin exposure whereas low adhering Candida remain unaffected. These results open novel avenues to explore cellular reactions upon exposure to stimulating agents and possibly to monitor in a rapid and simple manner adhesive properties of *C. albicans.*

Keywords: *Candida albicans*; adhesion; fibronectin; nanomotion; atomic force microscope (AFM)

1. Introduction

Yeast biotechnology is a recent field where nanotechniques are used to manipulate and analyze yeast cells and cell constituents at the nanoscale [1]. Among the nanotechniques, AFM-related approaches played a major role in unveiling morphological, mechanical and biochemical properties of yeast [2–4]. Recently, our team demonstrated that living cells attached onto a soft cantilever induce nanometric scale oscillations (referred to as nanomotion) that stop as soon as the organism dies [5]. Commercially available atomic force microscopes (AFM) or dedicated devices easily detect these oscillations. Nanomotion detection has been applied to numerous biological samples such as proteins, single organelles, and a plethora of living cells such as prokaryotes (bacteria) and eukaryotes (fungal, vegetal and mammalian cells) [6]. The most straightforward application of the technique is the ultra-rapid antibiotic sensitivity test (AST). AST can be performed within an hour as compared to long-lasting traditional AST methods, which depend on the replication rate of the bacteria [7–9]. The test consists in attaching the organism of interest onto an AFM cantilever and monitoring its oscillations as a function of time upon addition of antibiotics in the analysis chamber. It is worth noting

that the nanometric scale oscillations do not only reflect the living or death state of the organisms but also its activity [5,10].

Fungal infections are a major public health issue nowadays; it is estimated that every year fungi infect about 1.2 billion people [11]. *C. albicans* is a common fungal pathogen that belongs to the human microbiome of healthy individuals [12]. This commensal relationship is a complex interplay of candidial and human factors. However, impairment of the host immunity or the normal host microbiota can lead to *C. albicans* infection (candidiasis) [13]. *C. albicans* is the predominant cause of virtually all types of candidiasis [14]. The first step of the infection is the adhesion of *C. albicans* onto the host. This step is an essential determinant of pathogenesis, as it allows *C. albicans* to attach to host cells and to form biofilms or to disseminate in the host blood vessels. The biofilm increases yeast cell resistance to antifungal therapeutics and protects it from the host immune system [15]. *C. albicans* has developed multiple ways to colonize and infect host cells and tissues. One such mechanism is the specific ligand–receptor interaction through a whole range of adhesins displayed on the yeast cell wall [16–18]. These cell wall proteins are capable of recognizing protein ligands [16], glycolipids [19–22] and carbohydrates [23–29] on the host cells. Fibronectin is an important protein ligand of the host extracellular matrix (ECM) that plays an essential role in *C. albicans* adhesion [30]. Furthermore, targeting fibronectin has shown to alter *C. albicans* biofilm formation [31]. Therefore, a better understanding of the yeast–fibronectin interaction could lead to novel therapeutic options to fight candidiasis.

In this work, we applied nanomotion analysis to monitor the oscillatory activity of *C. albicans* upon exposure to fibronectin. We used an AFM-based nanomotion detector to follow the evolution of cellular oscillations in the absence and the presence of fibronectin on strongly and poorly adherent *C. albicans* cells. Interestingly, these two isolates reacted very differently to the interaction with fibronectin. These preliminary results demonstrate the potential of nanomotion analysis to monitor ligand–receptor interactions in a label free manner.

2. Materials and Methods

2.1. Yeast Strains

The *C. albicans* isolate 101 and CEC 3675 were kindly provided by Salomé Leibundgut and Christophe D'Enfert laboratories [32], respectively. The yeasts were cultured in yeast-extracted peptone-dextrose (YPD) medium (1% m/v yeast extract (Difco Laboratories, Fisher Scientific, Hampton, NH, USA), 2% m/v peptone (Difco Laboratories, Fisher Scientific, Hampton, NH, USA) and 2% m/v glucose (Sigma, St. Louis, MI, USA)) overnight at 30 °C with shaking (160 rpm).

2.2. Experimental Procedures

Rectangular tipless cantilevers (qp-CONT, NanoandMore GmbH, Wetzlar, Germany), with a nominal spring constant of 0.1 N/m and an average resonant peak in liquids of 8 kHz, were coated with 2 mg/mL of concanavalin A (Con A) (Sigma, St. Louis, MI, USA) for 30 min at room temperature. After removing the excess of Con A, the yeast cells were placed in contact with the cantilever for 1 h at room temperature to allow them to attach to its surface. Poorly attached *C. albicans* cells were removed by washing gently with YPD medium. Finally, the *C. albicans* covered cantilever was inserted into the analysis chamber containing 2 mL of filtered (0.2 µm syringe filter, Merck Millipore, Burlington, MA, USA) YPD medium. The measurements were performed at room temperature in YPD medium and in YPD medium containing 25 µg/mL of fibronectin (Sigma, USA). Fibronectin was directly added inside the chip reservoir. For the experiments performed with antifungals, caspofungin (Sigma, USA) was diluted in the YPD present in the analysis chamber to reach a final concentration of 100 µg/mL.

2.3. Nanomotion Detector

The cantilever oscillations were collected in real time using an in-house developed nanomotion detection device. The system relies on a laser-based signal transduction as typically used in commercial AFMs. A typical experiment lasted for 2 h. The control experiments were carried out for at least 4 h.

2.4. Software and Nanomotion Analysis

The cantilever oscillations were recorded and saved at 20 kHz using a USB-4431 DAQ card (National Instruments, Austin, TX, USA). The data acquisition program was developed in LabView. A dedicated Python program was used to process the recorded data and to display the deflection of the cantilever as a function of time. The software first removes the low frequency cantilever displacement signal by calculating a first order fit of the raw signal (deflection of the cantilever) by taking 20 seconds-long window frames. The obtained fit is then subtracted from the raw signal to remove thermally induced cantilever deflection. The thermal drift essentially occurs at the beginning of the experiment and during the fluid exchange procedures. The thermal drift free signal is further processed to obtain its variance in 10 seconds-window frames.

2.5. Viability Assay

Cells were placed inside a commercially available microfluidic chip (Ibidi, Planegg, Germany), and stained with calcofluor white (Sigma, USA), according to the manufacturer's instructions. To detect dead cells, propidium iodide (PI, Sigma, USA) was added to the YPD medium and the fluorescence of the yeast cells was recorded using an Axiovert microscope (Zeiss, Oberkochen, Germany).

2.6. Adhesion Assay

Adherence of *C. albicans* to TR146 cells was measured using the protocols previously described [33,34] with slight modifications (Figure S1). TR146 cells grown as monolayers in 6-well plates were incubated with 100 *C. albicans* cells for 20 min at 37 °C. The supernatant was carefully removed and spread on YPD agar plates to determine the number of non-adherent fungal cells. The adherent fungal cells that were left behind in the 6-well plates were rinsed with PBS and were overlaid with melted Wort agar at 40 °C. The plates were incubated at 30 °C for 36 h to count the colonies. Adherence was determined as the ratio of the number of colonies grown on Wort agar to the number of colonies grown on Wort agar and the number of colonies grown from the culture supernatant.

2.7. Statistical Analysis

Statistical analysis of nanomotion experiments were performed with the Python package Scipy. We performed the non-parametric Mann–Whitney U test for the three independent replicates. We used standard student t-test to process the adhesion assay on three independent replicates using the Graphpad Prism software.

3. Results

To assess a putative differential reaction of strongly and weakly interacting *C. albicans* to fibronectin, we quantified the adhesion of two different isolates, 101 and CEC3675, on oral keratinocytes (TR 146). As shown in Figure 1 isolate 101 was measured to have a significantly higher adhesion compared to isolate CEC3675.

To investigate the *C. albicans*–fibronectin interaction we used an in house nanomotion detector depicted in Figure 2A. The set up consists in an analysis chamber filled with liquid (in our case YPD) containing the cantilever to which yeast cells are attached (Figure 2B). The cantilever oscillations were recorded (Figure 2C) and processed to display the signal variance as a function of time (Figure 2D).

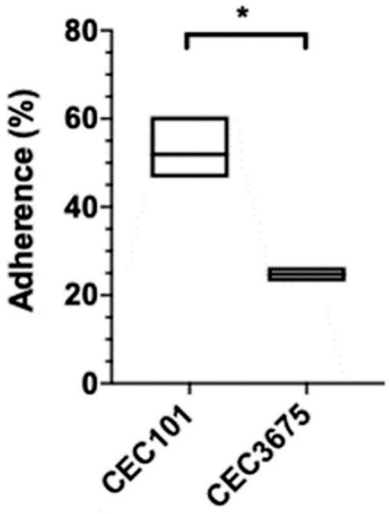

Figure 1. *C. albicans* isolates 101 and CEC3675 adhere differently to oral keratinocytes. Percentage of adherence of both isolates. Statistical analysis (n = 3) was done using standard t-test. The asterisk represents $p < 0.05$.

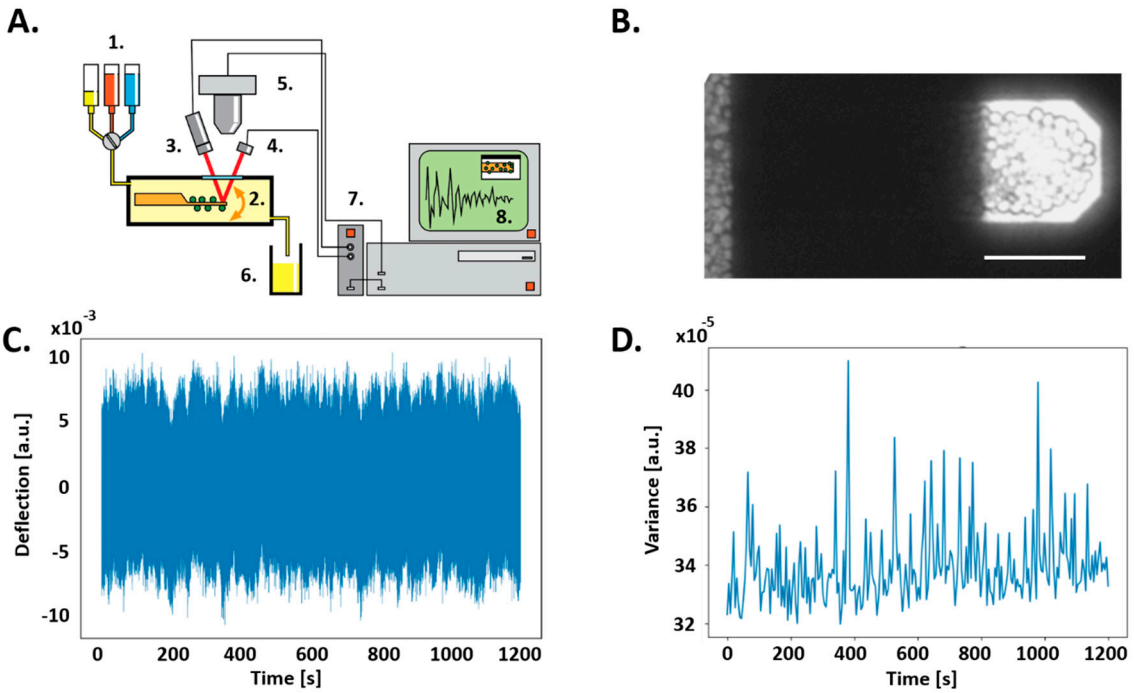

Figure 2. Nano-mechanical sensor system. (**A**) Representative image of a cantilever with attached *C albicans* cells. Scale bar 40 µm. (**B**) Schematic of the experimental system and data collection. (1) Liquids to be injected into the analysis chamber. In our case YPD, YPD containing fibronectin, and YPD containing caspofungin. (2) Analysis chamber with the AFM cantilever and *C. albicans* attached onto its surface (green circles). (3) Super luminescent diode. (4) Four-segment photodiode. (5) Optical microscopy with camera. (6) Liquid waste. (7) In-house dedicated electronics and National Instruments data acquisition card. (8) Desktop computer. (**C**). The collected raw data are processed; and (**D**). analyzed using the variance of the signal.

Using this system, we monitored the nanomotion pattern of *C. albicans* isolates 101 and CEC3675 in the absence and presence of fibronectin (Figure 3). Before addition of fibronectin, both isolates behaved similarly (Figure 3B). However, in the presence of fibronectin, nanomotion activity (variance) of isolate 101 drastically decreased (from 0.9 ± 0.5 to 0.3 ± 0.1) (Figure 3). In contrast, isolate CEC3675

did not present a significant decrease. To confirm that the drop of signal was not due to a change in the temperature, nor convective currents that can appear upon addition of a liquid in the analysis chamber, we performed control experiments, simultaneously, with another nanomotion detector. These experiments consisted in injecting the same quantity of medium, instead of fibronectin, into the analysis chamber. The obtained results showed no significant difference in the nanomotion pattern, for both isolates, upon addition of YPD media (Figure S2). Additionally, we assessed the number of cells present on the cantilever before and after the experiment to determine if the reduced signal was caused by cells being detached from the cantilever. The analysis of the images taken by the optical microscope located above the nanomotion detector (as depicted in the schematic in Figure 2A) confirmed that no cells detached from the cantilever throughout the experiments (Figure S3).

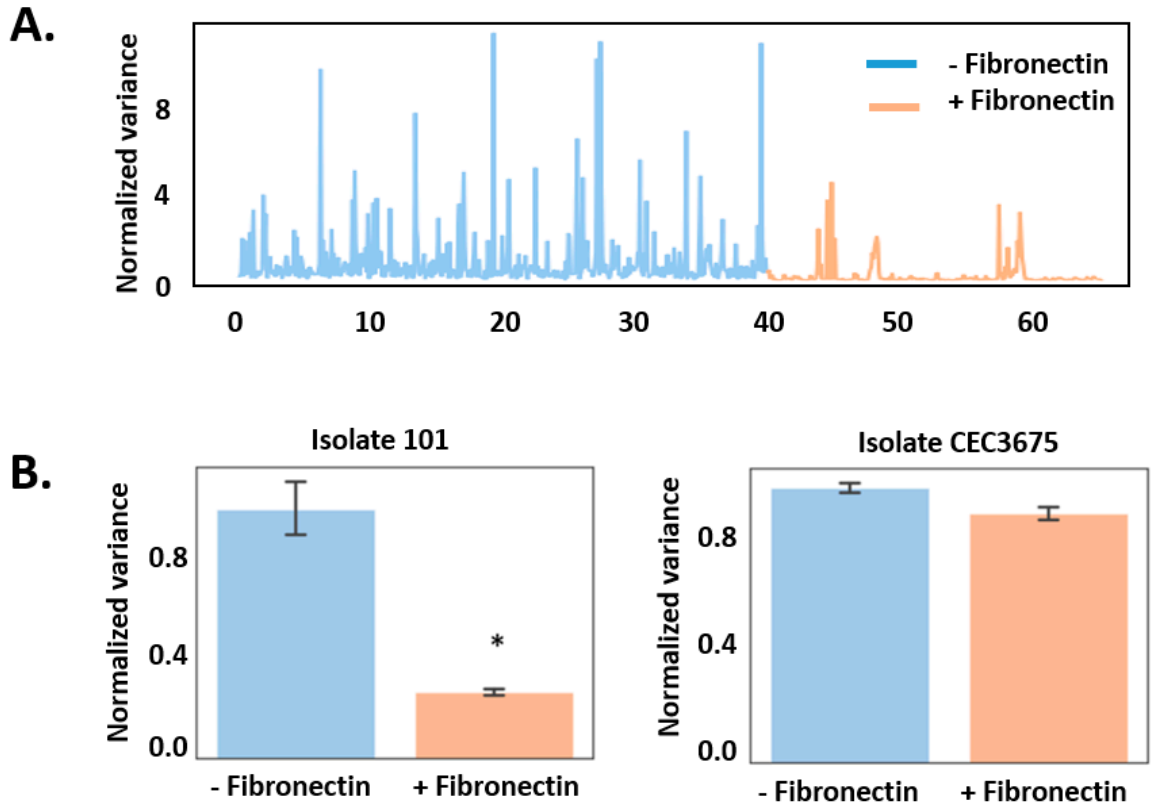

Figure 3. *C. albicans* isolate 101 and CEC 3675 react differently to fibronectin. (**A**). Representative graph of the normalized variance of isolate 101 in YPD (blue) and in YPD with fibronectin (orange). The decrease of the normalized variance is clearly visible between the two conditions. (**B**). The mean of the normalized variance (experiment in triplicate) represented as a bar plot for isolate 101 compared to isolate CEC 3675. Error bars are the confidence of intervals. Statistical analyses were done using Mann–Whitney U test, the asterisk represents $p < 0.05$.

To further exclude another cause of the decrease of the nanomotion signal for isolate 101, such as premature cell death, we monitored *C. albicans* viability by nanomotion and fluorescence microscopy in the absence and presence of fibronectin. Eventually the cells were killed by the antifungal caspofungin. As shown in Figure 3A, the variance of the nanomotion signal drastically dropped after the drug injection. The fluorescent viability test did not show any effect of fibronectin on the cellular viability as it can be noticed in Figure 4B. Similarly, fibronectin also did not have any effect on the viability of isolate CEC3675 (Figure S4).

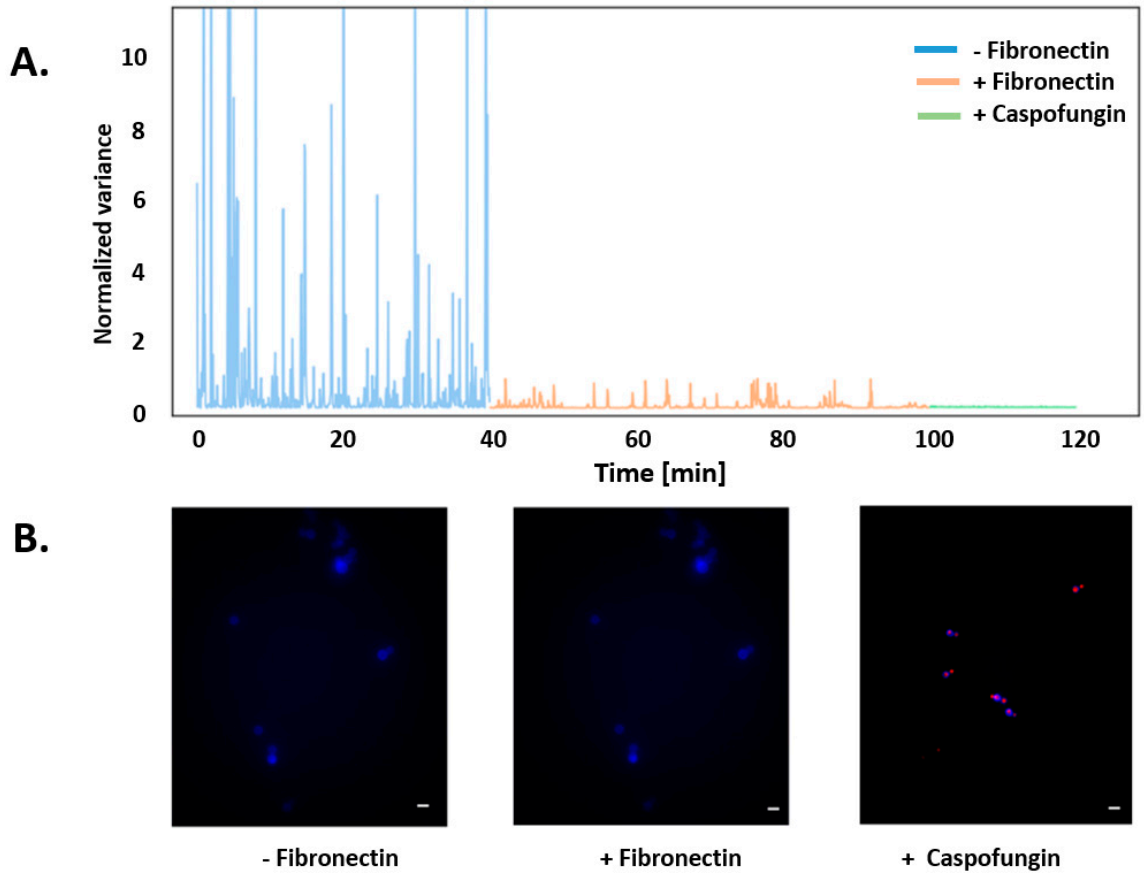

Figure 4. Viability assay of *C. albicans*. (**A**). Nanomotion signal of *C. albicans* isolate 101 in the absence (blue curve) and presence of fibronectin (orange curve), and after killing the cells by the antifungal caspofungin (green curve). (**B**). Representative fluorescence images of *C. albicans* isolate 101 in the absence (left panel) and presence of fibronectin (middle panel), and after killing (right panel). Scale bar 5 µm.

4. Discussion

C. albicans infection is a multistep process, consisting in the binding of *C. albicans* on epithelial cells. In a first adhesion step, the *C. albicans* adhesins of the agglutinin-like sequence (Als) family bind to ECM proteins of the host such as fibronectin [35,36], laminin and collagen. The attachment of the yeast cell to the host is followed by the penetration and transmigration of hypha into host cells, which then leads to vascular dissemination as soon the hypha reaches blood vessels. In this study we only explored the interaction of fibronectin with the yeast form. Adhesins playing a role in the planktonic *C. albicans* adhesion are the Als family members Als1 [27] and Als5 [37], Eap1 [38–40], Csh1 (cell surface hydrophobicity) [41,42], Ihd1 [43,44] and members of the SAP family [45–47]. It has been shown that Als1, Als5, and Csh1 interacts with fibronectin; Sap9 and Sap10 can interact with the ECM proteins collagen and vimentin. It has not yet been demonstrated that fibronectin is a ligand for Sap9/10, Eap1 and Ihd1.

Here, we used nanomotion detection to monitor the oscillation pattern of planktonic *C. albicans* cells upon exposure to fibronectin. Two different clinical isolates that showed a different adhesive phenotype, were used. The isolate 101 adhered significantly stronger to the host epithelial cells compared to isolate CEC3675. Nanomotion experiments showed that fibronectin affects isolate 101 significantly more than CEC3675. This drop of the nanomotion signal indicates a modification of the cellular activity upon fibronectin—*C. albicans* interaction. These results suggest that the initiation of adhesion related signaling in the yeast cell upon fibronectin attachment is mediated by the interaction with adhesins. Potential adhesion candidates that have been shown to interact with fibronectin are

Als1, Als3 and Csh1. The complete elucidation of the molecular mechanisms involved in the process are still unclear and deserve further research. We plan to investigate which specific adhesin(s) is (are) involved in the observed activity reduction. Additionally, the effect of other ligands such as laminin, collagen IV, fibrinogen and gelatin [28,48–50] should also be investigated.

This work demonstrated the ability of nanomotion detection to monitor in real time and in a label-free manner cellular activity changes induced by interacting ligands. Activity changes induced by increasing glucose concentration were observed for *Escherichia coli* in a previous study [5]. This technique opens novel avenues to detect cellular activation or inhibition induced by ligand–receptor interactions.

Supplementary Materials: The following are available online at http://www.mdpi.com/2311-5637/6/1/28/s1, Figure S1: Schematic representation of the adhesion assay protocol, Figure S2: Effect of the injection of YPD medium in the analysis chamber, Figure S3: Density of yeast cells on the cantilever, Figure S4: Viability assay of isolate CEC3675.

Author Contributions: Conceptualization, A.-C.K.; methodology, A.-C.K. and L.V.; software, A.-C.K.; validation, A.-C.K., L.V. and A.K.; formal analysis A.-C.K., investigation, A.-C.K., L.V., A.K.; resources, S.K., writing—original draft preparation, S.K. and A.-C.K.; writing—review and editing all the authors; visualization, A.-C.K., A.K.; supervision, A.-C.K., S.K.; project administration, S.K., G.D.; funding acquisition, S.K., G.D., D.S., R.W. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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