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Estimation of phagocytic activity by normal human peripheral blood mononuclear cells on various oral isolates of *Candida* species: an *in-vitro* study

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ABSTRACT

Polymorphonuclear neutrophils (PMN) and mononuclear phagocytes represent an important first line and effector function in controlling *Candida* infections. The study aims to determine the *in-vitro* phagocytic activity of human peripheral blood mononuclear cells against oral isolates of *Candida* species and its antifungal susceptibility. The study also evaluates the degree of respiratory burst activity of PBMCs. Phagocytic and lytic indices by PBMCs were determined for *Candida spp*. The respiratory burst activity was evaluated using a nitroblue tetrazolium test. Antifungal disc diffusion susceptibility testing was performed. 100 *Candida* were isolated, belonging to *C. albicans*, *C. tropicalis*, *C. krusei* and *C. auris*. The phagocytic and lytic indices of *C. albicans* were significant compared to the standard strain of *C. albicans*. *C. tropicalis* and *C. krusei* phagocytic index were significant, while the lytic index was insignificant compared to the standard strain. The inter-species comparison of both indices was not significant for the clinical isolates of *Candida*. A significant reduction in phagocytic activity was observed for clinical isolates of *Candida spp.* However, lytic activity was variable compared to the standard strain of *C. albicans*.

Introduction

There are around 150 different species in the *Candida* genus, most of which grow as single-celled yeasts. However, some species, like *Candida albicans*, can also exhibit variant forms, such as pseudohyphae and hyphae. *Candida species* exist in various environments, but only a few are

directly linked to human disease and colonisation [1]. *Candida* species, classified as opportunistic pathogens, contribute substantially to increased morbidity and mortality on a global scale, thereby posing a severe threat to public health. Furthermore, *Candida* species can lead to vaginitis, oral candidiasis, cutaneous candidiasis, candidemia, and systemic infections [2]. Within the *Candida* genus, *C. albicans* is the most significant human fungal pathogen, accounting for approximately 50% of candidemia cases [3]. Additionally, various other species such as *C. glabrata*, *C. tropicalis, C. krusei* (currently renamed *Pichia kudriavzevii)*, *C. auris*, and *C. dubliniensis* have been linked to infections, either independently or in conjunction with *C. albicans* [4]. The Nosocomial Infections Surveillance System (NNISS) reports *Candida* species as the fourth most common nosocomial bloodstream pathogen [5].

Host defence mechanisms against candidiasis involve the activation of an acute inflammatory response by innate immunity, followed by a specific T cell (cell-mediated immunity) or B cell (humoral immunity)-mediated immune response [6]. Through innate immunity, polymorphonuclear leucocytes (PMNL) and macrophages protect against invasive *Candida* infections. Cell-mediated immunity (CMI), on the other hand, controls mucosal infections. Antibody-mediated immunity's (AMI) role in candidiasis remains controversial [7]. Among the various mechanisms responsible for the PMN-mediated killing of *Candida spp*., the 'oxidative burst' mechanism appears to be widely accepted. Oxidative burst is the process of rapid formation of reactive oxygen intermediates. The NADPH oxidase enzyme complex must first be assembled in the cytoplasmic or phagosomal membrane to release superoxide [8, 9]. This study evaluates the *in-vitro* phagocytic activity of human peripheral blood mononuclear cells (PBMCs) against oral isolates of *Candida spp.* Additionally, the study aims to determine the degree of respiratory burst activity of PBMCs and the antifungal susceptibility of these *Candida* isolates.

Materials and methods

The present cross-sectional study was conducted at the School of Medical Education (SME), Kottayam, Kerala, India, between January 2023 and September 2023. One hundred isolates of *Candida spp.* from known non-diabetic patients with oral candidiasis were collected from diagnostic microbiology laboratories in central Kerala, India. *Candida albicans* MTCC 227, procured from the Institute of Microbial Technology (IMTECH), Chandigarh, India, was used as a standard control for phagocytic assay and antifungal susceptibility testing. The isolates were reconfirmed by subculturing onto chromogenic media – HiCrome™ Candida differential agar followed by Gram staining, and the colonies were confirmed to be Gram-positive. Tests like germ tube and chlamydospore production provided further identification. The VITEK II system confirmed the identification of all candida species. All reagents, culture media, and antifungal discs were procured from HiMedia Laboratories in Mumbai, India.

Blood for the phagocytic assay was collected from adult males and females (20–25 years) with no known systemic illness or prior antimicrobial therapy in the past three months. Individuals with a history of past Candida infection were excluded from the study. Blood samples from healthy individuals exhibiting a respiratory burst above 50% by Nitroblue tetrazolium test were employed for the phagocytic assay.

Isolation of PBMCs

Per the manufacturer's guidelines, the peripheral blood mononuclear cells were isolated using HiSep™ LSM 1077. 2.5 mL of HiSep™ LSM 1077 was aseptically transferred to a clean centrifuge tube (15 mL), overlaid with 7.5 mL diluted blood (1:2 dilution of whole blood in isotonic phosphate buffered saline) and centrifuged at 2000 rpm for 30 minutes at room temperature. Centrifugation sediments erythrocytes and polynuclear leukocytes, and bands mononuclear lymphocytes above HiSep™ LSM 1077. The supernatant above the interface band, rich in plasma and platelets, was discarded by aspiration. The mononuclear cells were carefully aspirated and transferred to a clean centrifuge tube using a pipette. 10 mL of PBS was added to the layer of mononuclear cells in the centrifuge tube and mixed by gentle aspiration. The cells were centrifuged at 1200 rpm for 10 minutes at room temperature. This washing with isotonic

phosphate buffered saline removes HiSep™ LSM and reduces the number of platelets. The washed cells were rinsed further with isotonic PBS and resuspended in Hank's balanced salt solution.

Assessment of phagocytic function

Candida phagocytosis test

Candida phagocytic test was performed according to Shanmugam et al. [10]. *Candida* was heat killed at 56^{0}C for 30 minutes, suspended in PBS, adjusted to 1 × 10^8 cells/mL and stored at -20°C until used. 0.1 mL of HBSS, 0.1 mL of pooled serum, and 0.1 mL of heat-killed Candida were added to 0.2 mL of 2 × 10^6 PBMC from a buffy coat and centrifuged at 1000 rpm for 5 min. The supernatant was removed, 10 µl sediment was smeared to a microscopic glass slide, then fixed with methanol, stained with Leishman's stain and viewed under a light microscope at 100X. The PBMCs with engulfed *Candida* were considered positive after counting the first 100 cells. *Candida* inside the mononuclear cells were also counted. Phagocytic and Lytic indices were calculated using the following formulae:

$$\text{Phagocytic index} = \frac{Number\ of\ positive\ cells}{100\ cells}$$

$$\text{Lytic index (Avidity index)} = \frac{Total\ number\ of\ phagocytosed\ Candida}{100\ cells}$$

Nitroblue tetrazolium (NBT) test

Shanmugam et al. also performed a nitroblue tetrazolium test [10]. 0.5 mL of heparinised blood was taken on a clean glass slide and incubated at 37°C for 30 min. It was gently washed with cold saline, tapped gently, and excess saline was removed. NBT medium was added before the smear dried, a coverslip was placed, and the slide was incubated at 37°C for 30 minutes. Then, the smear was washed with cold saline, air dried, fixed with methanol for 3 min, and washed with distilled water. Furthermore, the slide was air-dried, stained with safranin (0.77%) for 7 minutes, and washed with distilled water. The number of formazon-positive cells (blue crystals around the cells) was counted under 100X magnification under a light microscope.

Antifungal susceptibility

Antifungal disc diffusion susceptibility testing was performed as prescribed by CLSI M44-A22 [11]. Mueller-Hinton agar supplemented with 2% glucose and 0.5 µg/ml methylene blue was used for sensitivity testing. Antifungals used were fluconazole (25 µg), voriconazole (1 µg) and clotrimazole (10 µg). Per the manufacturers' instructions, CLSI M44-A22 [11] prescribed interpretive criteria for fluconazole, voriconazole, and clotrimazole.

The Institutional Ethical Committee (IEC) at the School of Medical Education approved this study (IEC/25/MICRO/SME-GNR/2022).

Statistical analysis

The results of the phagocytic and lytic indices were analysed using a one-sample t-test and ANOVA. *P*-value <0.05 was considered as significant. The SPSS 17 software was used for statistical analysis.

Results

A total of 100 clinical isolates of *Candida* obtained from various diagnostic laboratories were identified as belonging to four species: *C. albicans* (n = 49), *C. tropicalis* (n = 32), *C. krusei* (n = 18) and *C. auris* (n = 1). On HiCrome Candida differential agar, *C. albicans* produced light green colonies, *C. tropicalis* produced blue colonies, *C. krusei* produced purple colonies, and *C. auris* produced cream colonies, as shown in **Figure 1**.

Phagocytic Index

Engulfed yeast cells were enumerated, and the phagocytic index was calculated, as shown in **Figure 2**. The phagocytic index of *C. albicans* iso-

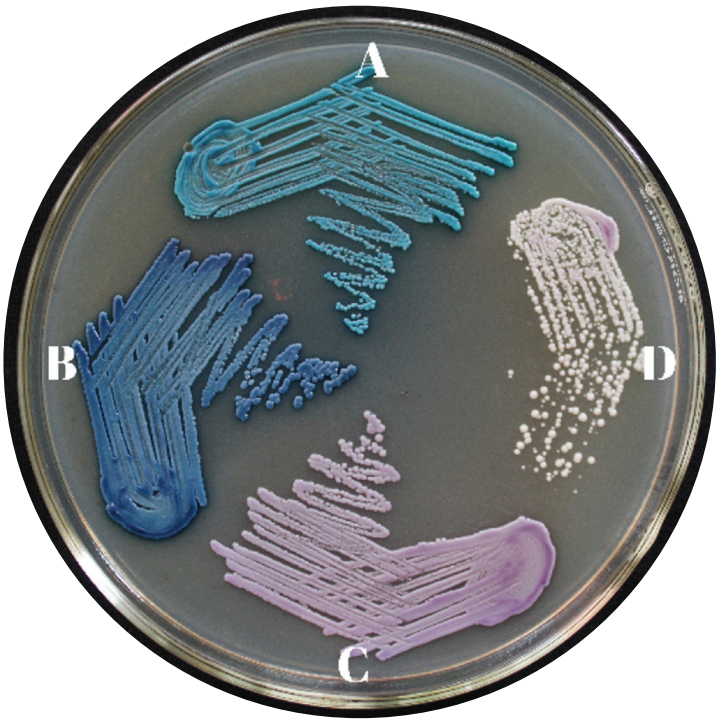

Figure 1. HiCrome Candida differential agar exhibiting growth of A: *C. albicans*, B: *C. tropicalis*, C: *C. krusei*, D: *C. auris*.

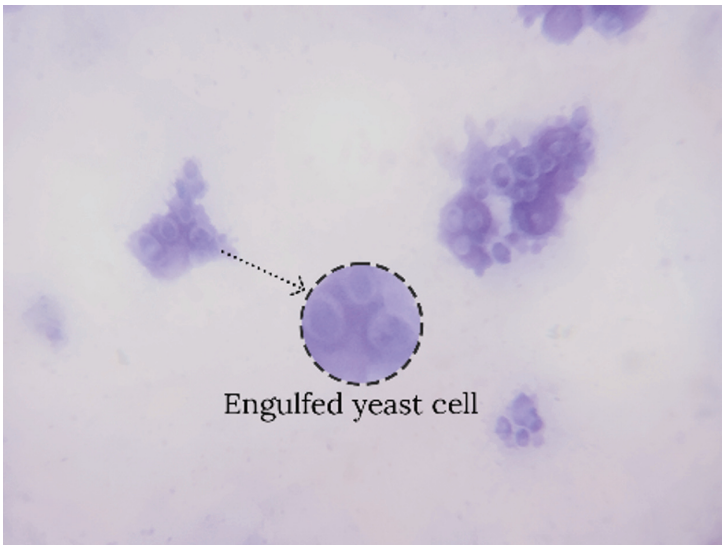

Figure 2. *Candida* phagocytic test – Engulfed yeast cell by PBMCs.

Table 1. Comparison of phagocytic index of *Candida spp.* with *C. albicans* MTCC 227 using One-Sample t-test.

	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
Phagocytic Index for *C. albicans*	-3.92	48	0	-0.05	-0.08	-0.03
Phagocytic Index for *C. tropicalis*	-2.63	31	0.01	-0.04	-0.06	-0.01
Phagocytic Index for *C. krusei*	-2.12	17	0.04	-0.04	-0.09	-0.0001

95% Confidence Interval of the Difference
C. albicans MTCC 227 test value = 0.15

lates (p = 0) was compared to that of the standard strain of *C. albicans* (*Candida albicans* MTCC 227). As the p-value is <0.05, the phagocytic index of *C. albicans* isolates and *C. albicans* MTCC 227 was statistically significant. The phagocytic index for *C. tropicalis* was 0.01 and was found to be statistically significant as it is <0.05. The p-value for the phagocytic index of *C. krusei* was 0.04 and was also statistically significant (**Table 1**).

Lytic index

The lytic index of *C. albicans* MTCC 227 was compared to that of *Candida* isolates. Lytic index for *C. albicans* was 0.001 and is <0.05. So, the lytic indices of *C. albicans* and *C. albicans* MTCC227 are statistically significant. The p-value for the lytic index of *C. tropicalis* was 0.43 and is not statistically significant. The lytic index for *C. krusei* was 0.35, not less than 0.05. Therefore, the lytic indices of *C. krusei* and *C. tropicalis* are not statistically significant (**Table 2**).

Intergroup phagocytic index comparison

The variations in phagocytic index among the three groups were derived using the ANOVA test. Even though differences in the mean between the three groups were observed, there was no

Table 2. Comparison of lytic index of *Candida spp.* with *C. albicans* MTCC 227 using a one-sample t-test.

	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
Lytic index for *C. albicans*	-3.66	48	0.001	-0.13	-0.21	-0.06
Lytic index for *C. tropicalis*	0.79	31	0.43	0.32	-0.49	1.13
Lytic Index for *C. krusei*	-0.96	17	0.35	-0.07	-0.21	0.08

95% Confidence Interval of the Difference
C. albicans MTCC 227 test value = 0.39

Table 3. Intergroup Phagocytic index comparison using ANOVA.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.01	2	0.003	0.40	0.67
Within Groups	0.78	96	0.008		
Total	0.79	98			

Table 4. Intergroup Lytic index comparison using ANOVA.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.15	2	2.06	1.23	0.29
Within Groups	162.29	96	1.69		
Total	166.44	98			

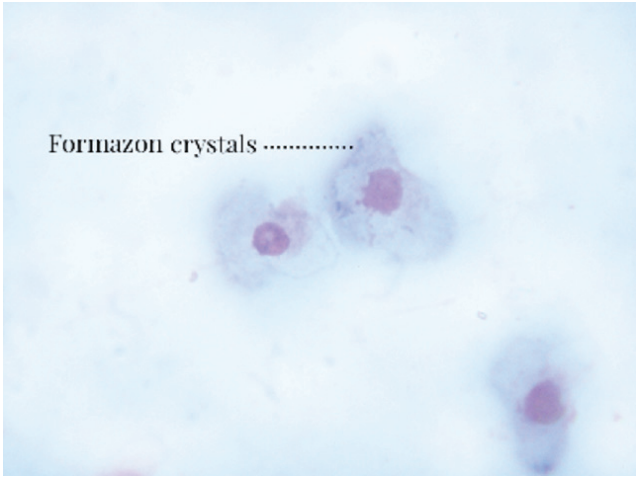

Figure 3. NBT test- showing blue coloured formazon positive cells.

significant difference in the intergroup phagocytic index, with an F value of 0.40 and p-value of 0.67 (**Table 3**) [Supplementary data Table 1 and Figure 1].

Intergroup lytic index comparison

The ANOVA test determined the differences in the lytic indices of the three groups. Even though differences in the mean between the three groups were observed, there was no significant difference in the intergroup lytic index with an F-value of 1.23 and p-value of 0.29 (**Table 4**) [Supplementary data Table 2 and Figure 2].

Statistical analysis did not include *C. auris*, as only one isolate was identified. The phagocytic index and lytic index for *C. auris* were 0.01.

Nitroblue tetrazolium test

The degree of respiratory burst activity of PBMCs was ascertained using the Nitroblue tetrazolium test. NBT freely enters the cells, and intracellular reduction of the dye by phagosomes converts it to an insoluble blue crystalline form (formazon crystals), as shown in **Figure 3**. The percentage of formazon-positive cells varied among the different blood samples studied. The average respiratory burst activity of the blood samples was 73.57%, with a range variation of 54–92%.

Antifungal susceptibility testing

C. albicans exhibited 100% susceptibility to fluconazole and voriconazole. 93.8% of isolates were susceptible to clotrimazole, while 6.12% displayed intermediate susceptibility. Of the isolates of *C. tropicalis*, 96.8% were susceptible to fluconazole, and 3.12% were Susceptible Dose-Dependent (SDD). *C. tropicalis* exhibited 96.8% susceptibility and 3.12% resistance to voriconazole. 81.25% of isolates were sensitive to clotrimazole, 9.31% exhibited intermediate susceptibility, and 9.37% were resistant.

C. krusei are intrinsically resistant to fluconazole. Susceptibility to voriconazole was at 100% in the case of *C. krusei*. *C. krusei* exhibited 83.3% susceptibility, 11.11% intermediate susceptibility and 5.55% resistance to clotrimazole. *C. auris* turned out to be sensitive to Voriconazole and resistant to Fluconazole and Clotrimazole (**Figure 4**).

Discussion

Candida is a commensal organism residing in the mucosal surfaces of the gastrointestinal tract, skin, and female genital tract in most humans. When perturbations in immunity or microbial

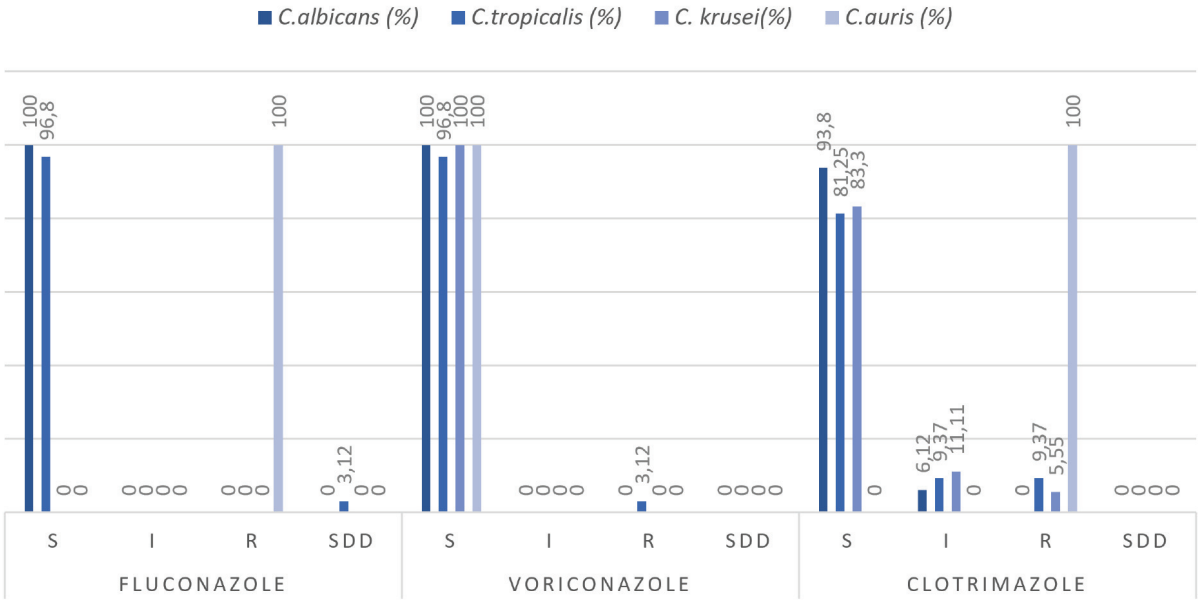

Figure 4. Antifungal susceptibility of *Candida spp*.

flora occur, *Candida* can convert to an opportunistic pathogen and cause mucosal or systemic infections [12.] *Candida spp.* is associated with non-life-threatening, mucosal diseases like vaginal and oral candidiasis to systemic infection, the most common deep-seated invasive mycosis in the developed world and the third leading cause of nosocomial bloodstream infection in modern intensive care units [13]. Experimental models evaluating host defence against *Candida spp.* have shown that innate resistance and acquired cell-mediated immunity are involved in anti-*Candida* responses [14]. Essential components of both arms of the immune defence against infections by *Candida spp*. are phagocytic cells, i.e., polymorphonuclear neutrophils (PMN) and mononuclear phagocytes [15].

Lehrer et al. reported that neutrophils are important in defence against candidiasis [16]. Many studies have evaluated the phagocytic activity of *C. albicans* by PBMCs. Our study compared the phagocytic activity of PBMCs against different *Candida spp* and evaluated this activity compared to a standard strain, *C. albicans* MTCC227. When comparing the phagocytic and lytic indices of the clinical isolates of

C. albicans with *C. albicans* MTCC227, both indices were found to be statistically significant. The clinical isolates are less phagocytosed and lysed by PBMCs. For isolates of *C. tropicalis* and *C. krusei*, the phagocytic indices were statistically significant compared to *C. albicans* MTCC227, but the lytic indices were insignificant. Clinical isolates are less phagocytosed and more lysed by PBMCs, suggesting that non-*C. albicans spp*. are better lysed by PBMCs. Moran G P [17] and Priest S J et al. reported that this difference in lysing capacity could be a possible reason for the increased pathogenicity of *C. albicans* compared to non-albicans isolates. [18]. The clinical isolates of *C. albicans*, *C. tropicalis* and *C. krusei* did not exhibit significant differences in their phagocytic and lytic indices. However, all three species significantly varied from *C. albicans* MTCC227.

The reduction of the yellow dye NBT to blue formazon indicates that the phagocytes produce oxygen radicals that play an important microbicidal role in pathogen destruction [19,20]. The blood sample from healthy volunteers showed a substantial respiratory burst activity of 73.57% compared to blood from diabetic individuals, who exhibited a lesser respiratory burst activity of 40.8% (our unpublished data).

The most common antifungals for candidiasis are azoles, polyenes and echinocandins. In the present study, two species of clinical isolates (*C. albicans* and *C. tropicalis*) showed high levels of sensitivity to the tested agents viz fluconazole, voriconazole and clotrimazole, and *C. krusei* exhibited high-level sensitivity to Voriconazole and clotrimazole which is by the study of Kuriyama et al. [21]. The single isolate of *C. auris* was only sensitive to voriconazole. Studies by Nihal Bandara et al. [22], Pfaller MA et al. [23], and Lockhart SR et al. [24] have reported variable resistance of 12.67%, 28% and 54%, respectively, to the drug. All *C. albicans* isolates were sensitive to fluconazole and voriconazole but the susceptibility to clotrimazole was 93.8%.*C.tropicalis* showed 96.8% sensitivity to fluconazole and voriconazole and 81.25% sensitivity to clotrimazole. All the *C. krusei* isolates were sensitive to voriconazole, and 83.3% were susceptible to clotrimazole.

The present study's possible limitation includes extrapolating in vitro laboratory findings of phagocytic and lytic function to the in vivo relationship between PBMCs. This study utilised isolates of three species of *Candida, C. albicans, C. tropicalis, and C. krusei,* from oral candidiasis and tested them with PBMCs from healthy donors. An expansive study involving more *Candida* species from different forms of candidiasis will certainly lead to a broader understanding of phagocytic and lytic functions.

In conclusion, PBMCs, which include polymorphonuclear neutrophils, monocyte/macrophages and dendritic cells, which are the first line of defence for professional phagocytes and also critical players during candidial infections, showed significant reduction in phagocytic activity against clinically isolated *Candida spp.* when compared to the standard strain of *C. albicans*. The lytic activity of phagocytic cells was significantly less towards *C. albicans* when compared to the standard strain of *C. albicans*. The lytic action against clinically isolated non-albicans species proved higher than the standard strain of *C.albicans*, which may result from its polymorphic nature and other surface adhesins. The inter-species comparison of the clinical isolates of *Candida spp*. did not reveal any significant differences in phagocytic or lytic activity.

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Conflict of interest statement
The authors declare no conflict of interest.

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