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EFFECT OF PEMETREXED AND METHOTREXATE ON FLUCONAZOLE SUSCEPTIBILITY IN YEAST

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Abstract: Previous research has confirmed that *Candida* spp. incubated with the folic acid pathway inhibitor methotrexate can develop multidrug resistance to azoles. However, it remains unclear whether this phenomenon also occurs with other antifoliate agents. The aim of this study was to assess whether a different antifolate —pemetrexed— can induce resistance to azoles among endogenous yeast strains. For this purpose, 15 strains of *Candida* and 3 strains of *Nakaseomyces* were stimulated twice with either methotrexate or pemetrexed. Subsequently, minimum inhibitory concentration for fluconazole for each strain was determined before and after stimulation. Susceptibility to fluconazole increased in 7 strains, decreased in 10 strains and did not change in case of 1 strain after exposure to pemetrexed. After exposure to methotrexate susceptibility to fluconazole increased in 7 strains, decreased in 5 strains and did not change in case of 6 strains. One strain of *Candida albicans* became resistant to fluconazole after stimulation with either methotrexate or pemetrexed.

1. Introduction. 2. Materials and Methods. 3. Results. 4. Discussion. 5. Conclusions.

Keywords: antifolates; cross-resistance; drug-induced resistance; fluconazole; yeast

1. Introduction

Fungal infections, commonly caused by *Candida* species, remain a significant problem in patients suffering from various types of neoplasms (Tang et al. 2014). Risk factors for candidal infection include among others chemotherapy, immunosuppression and long term, broad spectrum antibiotic therapy (Al-Dossary and Al-Shamahy 2018; Yapar 2014). Illness-related immune dysfunction and the effects of antineoplastic drugs used in oncological and hematological patients make them particularly vulnerable to serious fungal infections, such as candidemia and invasive candidiasis, in which the selection of an effective, empirical antimycotic may determine the prognosis.

Antifolates, such as methotrexate (MTX) and pemetrexed (PMX), are commonly used in the treatment of both solid tumors and hematological neoplasms. Their main mechanism of action is based on the inhibition of tetrahydrofolate dehydrogenase, which results in impaired metabolism of folic acid (Hanoodi and Mittal 2023). Unlike MTX, PMX is an inhibitor of at least two other enzymes involved in synthesis of purines and pyrimidines: glycinamide ribonucleotide formyltransferase and thymidylate synthase. Numerous studies have demonstrated that MTX can induce cross-resistance to azoles among *Candida* species. Even though MTX-induced cross-resistance is consistently observed *in vitro*, and some studies seem to suggest its clinical presence as well, evidence for its occurrence *in vivo* remains limited (Żyrek et al. 2021). To the authors' knowledge, no studies have been conducted so far on the potential of PMX to induce cross-resistance to azoles. Given the similar mechanism of action and comparable chemical structure, it is highly probable that this substance may also exhibit the same phenomenon. Therefore, the aim of our study was to assess whether PMX similarly to MTX can induce cross-resistance to fluconazole (FLU) among clinical *Candida* and *Nakaseomyces* strains.

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2. Materials and Methods

16 clinical strains of yeast were collected, isolated and identified from the oral cavity of azole-naive patients of the Rheumatology Outpatient Clinic and the Rheumatology Day Care Unit of Wrocław Teaching Hospital. The analyzed strains were stored in deep freezers (−80 °C) in a liquid tryptic soy broth (TSB; Biomaxima, Lublin, Poland) medium with 15% glycerol and cultured in Sabouraud agar (Biomaxima, Lublin, Poland) at 37 °C for 48 h under aerobic conditions. Clinical strains (12 strains of *C. albicans*, 3 strains of *C. tropicalis*, three strains of *Nakaseomyces glabratus* - formerly known as *Candida glabrata*) and two reference strains (*C. albicans* ATCC 90028 and *N. glabratus* ATCC 15126) were then stimulated twice with either PMX or MTX.

Stimulation procedure with each of the mentioned above antifolates was based on the MTX stimulation protocol proposed by Karuga et al., with minor modifications introduced to increase the selective pressure exerted by the antifolate on fungal cells (Karuga et al. 2021; Góralska et al. 2024). For the stimulation procedure, pemetrexed disodium heptahydrate (PMX; ≥98% purity by HPLC; Sigma-Aldrich, USA) and methotrexate disodium (MTX; 50 mg/ml; Metex®, medac GmbH, Germany) were used. Suspensions of the tested strains (0.5-2.5 × 10^3 CFU per mL) were applied to 96-well flat-bottom polystyrene plates with previously prepared serial dilutions of MTX or PMX in the range of 0.18 to 375 µg/ml (instead of 0.0088–45 µg/ml as used in protocol of Karuga et al.). The plates were incubated for 48 hours (rather than 24 hours described by Karuga et al.) at 37 °C and assessed visually. Fungal cells incubated with the highest non-inhibitory concentration of the tested drug were collected, cultured and used in a second identical stimulation procedure.

Minimum inhibitory concentrations (MIC) for FLU for all strains were determined in a quadruplicate before and after stimulation with PMX or MTX, using the microdilution method in RPMI 1640 liquid medium based on the guidelines of the CLSI (Clinical and Laboratory Standards Institute). Suspensions of the tested strains (0.5-2.5 × 10^3 CFU per mL) were applied to 96-well flat-bottom polystyrene plates with previously prepared serial dilutions of FLU concentrations ranging from 0.125 to 256 µg/mL. The plates were incubated for 48 hours at 37 °C. All experiments included a strain growth control (positive control; K+) and a negative control (K-), which served as a medium sterility test. Following CLSI recommendations, when testing susceptibility to azoles among *Candida* isolates, MIC is defined as a prominent decrease in turbidity (2/4 in numerical score), which corresponds to 50% inhibition of growth assessed spectrophotometrically. Therefore, to determine MICs, we modified the CLSI method by measuring optical density (OD) spectrophotometrically (BiochromAsys UVM 340) at a wavelength of 530 nm after 24 and (in case of insufficient 24-hour growth in K+ wells) 48 hours of incubation. The MIC was considered as the concentration of FLU at which the growth inhibition of at least 50% of microorganisms was detected, using the equation:

$$\left(\frac{OD_{well}\text{-}OD_{K\text{-}}}{OD_{K+}\text{-}OD_{K\text{-}}}\right) \times 100\%.$$

In instances where the MIC values differed across the four repetitions, the median was accepted as the final MIC result.

The data were interpreted based on the clinical breakpoints recommended by the CLSI. For each isolate, the percentage change in MIC following antifolate stimulation (%ΔMIC$_i$) was calculated using the following formula:

$$\%\Delta MIC_i = \frac{MIC_{i,\ after\ stimulation} - MIC_{i,before\ stimulation}}{MIC_{i,before\ stimulation}} \times 100\%$$

where $MIC_{i,\ before\ stimulation}$ and $MIC_{i,\ after\ stimulation}$ denote the MIC values of isolate *i* before and after antifolate stimulation, respectively. For groups of isolates stratified by species or by their behavior following exposure to a specific antifolate, the mean percentage change in MIC ($\%\Delta MIC_{group}$) was calculated according to the formula:

$$\%\Delta MIC_{group} = \frac{1}{m}\sum_{i\in G}\left(\frac{MIC_{i,after\ stimulation} - MIC_{i,before\ stimulation}}{MIC_{i,before\ stimulation}}\right) \times 100\%$$

where $m=|G|$ is the number of isolates in the group.

The Shapiro-Wilk test was used to establish whether the data has a distribution similar to a normal distribution. The Wilcoxon signed-rank test was performed to assess the differences between corresponding MIC values before and after stimulation procedure. The results of the research were statistically processed using TIBCO Statistica v13.3 software (TIBCO Software Inc., Palo Alto, USA). The result was considered statistically significant if p ≤ 0.05.

Minimal inhibitory concentration to Fluconazole [μg/mL]					
		All strains n= 18	***C. albicans*** n= 12	***C. tropicalis*** n= 3	***N. glabratus*** n= 3
Before stimulation	Range	0.13 - 16.00	0.13 - 1.00	0.25 - 1.00	1.00 - 16.00
	Median (Q1-Q3)	0.25 (0.25-0.88)	0.25 (0.22-0.25)	0.50 (0.38-0.75)	1.00 (1.00-8.50)
	Geometric mean MIC	0.40	0.24	0.50	2.52
After MTX	Range	0.13 - 16.00	0.13 - 12.00	0.25 - 1.00	0.38 - 16.00
	Median (Q1-Q3)	0.28 (0.25-0.84)	0.25 (0.13-0.31)	0.25 (0.25-0.63)	16.00 (8.19-16.00)
	Geometric mean MIC	0.55	0.35	0.40	4.58
	Average MIC change [%]	191%	171%	-17%	479%
	Maximal MIC change [%]	1500%	1100%	-50%	1500%
p value		0.75	0.86	0.16*	0.67*
After PMX	Range	0.13 - 16.00	0.13 - 8.00	0.13 - 4.00	0.25 - 16.00
	Median (Q1-Q3)	0.19 (0.13-0.45)	0.16 (0.13-0.22)	0.25 (0.19-2.13)	6.00 (3.13-11.00)
	Geometric mean MIC	0.41	0.24	0.50	2.88
	Average MIC change [%]	75%	61%	67%	142%
	Maximal MIC change [%]	700%	700%	300%	500%
p value		0.48	0.44	0.50*	0.67*

Table 1. Summary characteristics of minimum inhibitory concentration (MIC) values for fluconazole among the tested strains before and after exposure to methotrexate (MTX) or pemetrexed (PMX). The p-value in the first column reflects the outcome of the one-sided Wilcoxon test for paired samples. * In the case of *C. tropicalis* and *N. glabratus*, the small sample size (n=3) confers very limited statistical power, and therefore the test results should be interpreted with caution. Q1, first quartile; Q3, third quartile.

3. Results

The summary characteristics of MIC values for FLU among the tested strains before and after exposure to MTX or PMX are presented in Table 1. Although the mean MIC values following stimulation with either MTX or PMX were higher than those observed before stimulation, the changes were not statistically significant—neither across all tested strains nor within species-specific groups analyzed collectively.

The change in the MIC value for FLU before and after the stimulation procedure for each strain is presented in Table 2. In most of the strains, the MIC value for FLU changed after double stimulation with an antifolate. After MTX stimulation, the MIC value increased in 7 of the tested strains (6/12 *C. albicans* strains and 1/3 *N. glabratus* strains), decreased in 5 of the tested strains (3/12 *C. albicans* strains, 1/3 *C. tropicalis* strains and 1/3 *N. glabratus* strains), and did not change in 6 of them (3/12 *C. albicans* strains, 2/3 *C. tropicalis* strains and 1/3 *N. glabratus* strains).

After PMX stimulation, 7 strains had a higher MIC value for FLU than before stimulation (5/12 *C. albicans* strains, 1/3 *C. tropicalis* strains and 1/3 *N. glabratus* strains); in 10 of the tested strains the MIC value decreased (7/12 *C. albicans* strains, 2/3 *C. tropicalis* strains and 1/3 *N. glabratus* strains), and in 1 strain (*N. glabratus*) the MIC value did not change. Five out of the seven strains whose MIC increased after PMX stimulation also responded with an MIC increase after MTX stimulation (4 strains of *C. albicans* and 1 strain of *N. glabratus*). One *C. albicans* strain and one *C. tropicalis* strain responded with an increase in MIC only after stimulation with PMX, but not with MTX. Two other *C. albicans* strains responded with an increase in the MIC value only after stimulation with MTX, whereas PMX exposure led to a slight increase in their susceptibility to FLU.

Changes in MIC among strains that responded to stimulation with a shift in MIC values are presented in Table 3. Among the strains that showed an increase in

Species	Strain number	MIC before stimulation [µg/mL]	MIC after MTX exposure (% of MIC change)	MIC after PMX exposure (% of MIC change)
C. albicans	1	0.13	0.13 (0%)	0.19 (50%)
	2	0.25	0.31 (25%)	0.13 (-50%)
	3	0.25	0.13 (-50%)	0.13 (-50%)
	4	1.00	12.00 (1100%)	8.00 (700%)
	5	0.25	0.13 (-50%)	0.13 (-50%)
	6	0.25	0.13 (-50%)	0.13 (-50%)
	7	0.25	0.25 (0%)	0.19 (-25%)
	8	0.25	0.25 (0%)	0.13 (-50%)
	9	0.25	2.25 (800%)	0.50 (100%)
	10	0.25	0.31 (25%)	0.13 (-50%)
	11	0.13	0.25 (100%)	0.19 (50%)
	12*	0.13	0.31 (150%)	0.31 (150%)
C. tropicalis	13	0.50	0.25 (-50%)	0.25 (-50%)
	14	0.25	0.25 (0%)	0.13 (-50%)
	15	1.00	1.00 (0%)	4.00 (300%)
N. glabratus	16	1.00	16.00 (1500%)	6.00 (500%)
	17	16.00	16.00 (0%)	16.00 (0%)
	18**	1.00	0.38 (-63%)	0.25 (-75%)

Table 2. Change in the minimum inhibitory concentration (MIC) value for Fluconazole before and after exposure to methotrexate (MTX) or pemetrexed (PMX) for each of the tested strains. The color of the cell reflects changes in MIC for FLU after antifolate stimulation: red indicates an increase, green a decrease and white- no MIC change. * *C. albicans* reference strain ATCC 90028; ***N. glabratus* reference strain ATCC 15126

the MIC value after MTX or PMX stimulation, the average change in relation to the initial MIC was 529% (367% for *C. albicans* strains and 1500% for *N. glabratus* strain) and 264% (210% for *C. albicans* strains, 300% for *C. tropicalis* strain and 500% for *N. glabratus* strain), respectively. The shift in the MIC values among all strains exhibiting an MIC increase was statistically significant after both PMX ($p<0.01$) and MTX stimulation ($p<0.01$). Considering only the *C. albicans* subgroup, the observed increase in the MIC values after either MTX or PMX stimulation also reached statistical significance ($p=0.02$ and $p=0.03$, respectively).

The decrease in the MIC value following the antifolate stimulation procedure was always moderate and never exceeded twofold the initial MIC. The mean change in the MIC among all the strains that showed a decrease in MIC was -53% (-50% for *C. albicans*, -50% for *C. tropicalis*, and -63% for *N. glabratus*) for MTX and -50% (-46% for *C. albicans*, -50% for *C. tropicalis*, and -75% for *N. glabratus* strain) for PMX. The shift in the MIC values among all strains exhibiting an MIC decrease was statistically significant for both MTX ($p=0.03$) and PMX ($p<0.01$), whereas considering only the *C. albicans* subgroup the observed reduction in MIC values reached statistical significance after PMX stimulation but not after MTX stimulation ($p<0.01$ and $p=0.13$, respectively).

Prior to stimulation with MTX or PMX, all tested strains demonstrated sensitivity to FLU as defined by the CLSI breakpoints. One strain of C. *albicans* became resistant to FLU after stimulation with either MTX or PMX, with the MIC being higher after MTX stimulation (12 µg/mL) than after PMX stimulation (8 µg/mL). One of the *C. tropicalis* strains, after PMX

Change of minimal inhibitory concentration to Fluconazole [µg/mL] after stimulation with an antifolate					
		All strains that showed an increase in MIC	***C. albicans* strains that showed an increase in MIC**	**All strains that showed a decrease in MIC**	***C. albicans* strains that showed a decrease in MIC**
After MTX	Minimal MIC change [%]	25%	25%	-50%	-50%
	Maximal MIC change [%]	1500%	1100%	-63%	-50%
	Average MIC change [%] ±SD	529% ± 602%	367% ± 464%	-53% ± 6%	-50% ± 0%
	Number of strains	7	6	5	3
p value		<0.01	0.02	0.03	0.13*
After PMX	Minimal MIC change [%]	50%	50%	-25%	-25%
	Maximal MIC change [%]	700%	700%	-75%	-50%
	Average MIC change [%] ±SD	264% ± 251%	210% ± 277%	-50% ± 12%	-46% ± 9%
	Number of strains	7	5	10	7
p value		<0.01	0.03	<0.01	<0.01

Table 3. Change of minimum inhibitory concentration (MIC) values for fluconazole among the tested strains of which MIC changed after exposure to methotrexate (MTX) or pemetrexed (PMX). The p-value in the first column reflects the outcome of the one- sided Wilcoxon test for paired samples. * In the case of *C. albicans* strains that showed a decrease in MIC after stimulation with MTX, the small sample size (n=3) confers very limited statistical power, and therefore the test results should be interpreted with caution. SD- standard deviation

stimulation (but not after MTX stimulation), reduced its sensitivity to FLU, reaching the SDD (susceptible dose-dependent) category threshold according to the CLSI breakpoints.

4. Discussion

The study is the first to demonstrate that PMX, similarly to MTX, can increase the level of resistance to FLU among clinical yeast strains previously unexposed to azoles, although this effect is not universal and appears to be strain-specific. These findings indicate that individual strains within the same species may exhibit distinct and reproducible responses to antifolate stimulation, including increased MIC, decreased MIC, or no measurable change in susceptibility to FLU. This phenomenon was observed not only in respect to *C. albicans* but also in *C. tropicalis* and *N. glabratus* strains. While only one of the tested *C. albicans* isolates met the CLSI clinical breakpoint for FLU resistance, it should be remembered that the stimulation process was carried out only twice, and the time during which the tested strains were exposed to the antifolate was relatively short. In clinical settings, we encounter long-term and repetitive exposure to high, antineoplastic doses of MTX or PMX. If the change in the sensitivity to azoles is proportional to the selective pressure exerted by an antifolate on fungal cells, then the higher the drug concentration and the longer the exposure, the greater the chance of developing drug resistance. It can be assumed that after multiple repetitions of the stimulation procedure, some other strains tested in this study could eventually also become completely resistant to FLU. Moreover, considering the varying susceptibility of the tested isolates to stimulation, we can hypothesize that in the general population we also find strains in which the increase in MIC, even after a single exposure to an antifolate, will be sufficient to exceed the resistance threshold.

If the process of acquiring azole resistance occurs in patients receiving PMX (*in vivo*), this must be taken into account when selecting empirical antifungal therapy. In such cases, non-azole antimycotics may be considered as drugs of choice. Given that oncological patients treated with antifolates are at high risk for neutropenic fever and fungal sepsis, the immedi-

ate implementation of an effective antifungal agent is particularly important, since the administration of an inadequate antimycotic will certainly contribute to an increased mortality. For this reason, we believe that it is necessary to determine whether the phenomenon of induction of azole resistance upon exposure to PMX also occurs in clinical conditions.

Among *Candida albicans*, induction of azole resistance occurs by several mechanisms, the most important being the one dependent on the increased *CaMDR1* expression after MTX exposure. The *CaMDR1* gene encodes an efflux pump that can actively transport xenobiotics, including MTX and FLU, out of the cell, hindering their effective action (Kohli et al. 2001; Hiller et al. 2006). Other mechanisms contributing to azole resistance among *Candida* isolates include biofilm formation and mutations in the genes of the enzymes involved in ergosterol synthesis, such as *ERG11* and *ERG3* (Ruiz-Baca et al. 2021 Nov 17; Rojas et al. 2023). Azoles exert their antifungal effect mainly by disrupting the function of lanosterol 14-α demethylase encoded by *ERG11*, leading to accumulation of toxic 14α-methylated sterols. Overexpression of *ERG11* or a mutation that changes the structure of the 14-α demethylase can, therefore, significantly reduce sensitivity to these antimycotics. *ERG3* gene encodes sterol C5,6-desaturase, an enzyme necessary for the conversion of nontoxic intermediate metabolites of the ergosterol synthesis pathway, formed under the effect of azoles, to their toxic 14α-methylated derivatives. By preventing the formation of 14α-methylated sterols, its inactivation modifies ergosterol metabolism, thereby contributing to azole cross-resistance, promoting aggregation and biofilm formation, yet concurrently diminishing the virulence of *Candida* isolates (Chau et al. 2005; Hirayama et al. 2021).

Genes homologous to the *CaMDR1* gene, as well as alternative genes encoding efflux pumps (*CDR1*, *CDR2*), have been observed among various yeast species, including *C. parapsilosis* and *C. tropicalis*, but only a few studies demonstrated that species other than *C. albicans* (*Candida parapsilosis* and *Meyerozyma guilliermondii*) can develop azole resistance after exposure to MTX (Wirsching et al. 2001; Grossman et al. 2015; Karuga et al. 2021; Rojas et al. 2023; Góralska et al. 2024). A study from 2024 showed that stimulation of *C. albicans* with MTX in some isolates leads to the reduced sensitivity to voriconazole, which was associated with the increased expression of the *ERG11*. Interestingly, the researchers observed that after exposure to MTX most of the isolates showed reduced expression of *ERG11*, although in such cases overexpression of *MDR1* or *CDR1* was frequently present simultaneously (Góralska et al. 2024). No studies directly investigating the influence of MTX exposure on *ERG3* expression have been conducted so far. However, in our opinion, examining this relationship may be an interesting subject for further research.

Due to their similarity in the chemical structures and modes of action, the mechanisms leading to decreased susceptibility to FLU after exposure to PMX may resemble those developed by yeast after exposure to MTX. This hypothesis is further supported by the fact that in the experiment most of the strains in which MIC to FLU increased after MTX stimulation were also susceptible to stimulation with PMX. Nevertheless, understanding how PMX exposure influences antifungal susceptibility in yeast strains will require additional research, including molecular and genetic investigations focused on gene expression patterns similar to those linked to resistance following MTX treatment.

The decrease in the MIC value to FLU after exposure to both compounds was not expected by us and requires an adequate explanation. As previously mentioned, this effect was strain-specific, manifested consistently, and occurred in most strains whose MIC for FLU was initially low. However, the change in MIC never exceeded one order of magnitude (twofold FLU concentration). It has been demonstrated that MTX at concentrations resembling the ones achieved *in vivo* during chemotherapy has an intrinsic fungistatic effect (Warnock et al. 1989). Furthermore, synergy of antimicrobial action of MTX and azoles was observed in laboratory conditions (Yang et al. 2019). Despite all preventive efforts, it is possible that during the transfer of biological material in the subsequent stages of the study (from a 96-well titration plate to a plate with Sabouraud Agar and then to another titration plate containing successive dilutions of FLU) some of the medium containing residual amounts of MTX or PMX got into the RPMI in which the sensitivity to FLU was tested following the resistance stimulation procedure. The lower MIC values would then be the result of additive or synergistic antimicrobial activity of both compounds against the tested strains. Such an explanation seems unlikely for at least two reasons. Firstly, we attempted to collect yeast cells without medium, and the amounts of supposedly contaminated biological material were very small in relation to the utilized medium volume. Secondly, the fungistatic effect of antifolates is

pH-dependent and does not occur in the pH-neutral environment provided by RPMI, in which the sensitivity of all the stimulated strains was tested (DeJarnette et al. 2020).

The authors wish to emphasize that the primary objective of this study was to demonstrate the potential of PMX to alter fungal susceptibility to fluconazole, rather than to explore the underlying molecular mechanisms responsible for this phenomenon. Another limitation of the study is that fluconazole was the only antifungal agent tested, despite existing evidence that MTX-induced resistance in yeasts is often accompanied by cross-resistance to multiple azole antifungals. Whether PMX exposure elicits a similar cross-resistance pattern remains to be determined. In our opinion, the direction of changes in azole sensitivity following antifolate exposure depends on the individual metabolic profile of the strain. Determining why some isolates consistently responded with an increase, while others demonstrated a decrease in sensitivity to FLU after exposure to MTX or PMX may significantly expand our knowledge of fungal cross-resistance mechanisms and requires proper explanation in further research.

5. Conclusions

In most isolates the MIC value changed after the exposure to an antifolate. In some strains of *C. albicans*, *C. tropicalis* and *N. glabratus* prior exposure to either MTX or PMX significantly decreased their susceptibility to FLU. One tested strain of C. *albicans* became resistant to FLU after double stimulation with either MTX or PMX. In contrast, some of the tested *Candida* and *Nakaseomyces* strains demonstrated lower susceptibility to FLU after previous exposure to MTX or PMX. It is necessary to conduct additional research to establish whether the phenomenon of PMX-induced cross resistance to azoles occurs in clinical conditions.

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Institutional Review Board Statement
The study was conducted in accordance with the Declaration of Helsinki, and approved by the Bioethical Committee at Opole University, Poland (approval No. UO/0025/KB/2023 provided on 26.10.2023).

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Conflict of interest
The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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