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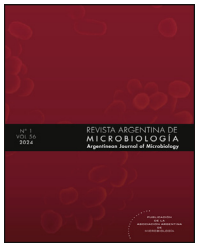

ORIGINAL ARTICLE

Molecular epidemiology and antifungal susceptibility patterns of *Aspergillus* and *Candida* species isolated from Argentinian patients with COVID-19-associated pulmonary aspergillosis and candidemia

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KEYWORDS
COVID-19;
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Abstract COVID-19-associated pulmonary aspergillosis (CAPA) and candidemia (CAC) have emerged as serious complications with poor prognoses among critically ill patients with SARS-CoV-2 pneumonia and acute respiratory distress syndrome at intensive care units (ICU). This study aimed to provide an overview of the diversity of *Aspergillus* and *Candida* species in critically ill patients with CAPA and CAC, as well as to analyze their antifungal susceptibility profiles. Ninety-six *Aspergillus* isolates were recovered from respiratory specimens of 78 COVID-19 patients with probable CAPA, and 52 *Candida* isolates were recovered from blood stream specimens of 50 COVID-19 patients with proven CAC. All patients were admitted to ICUs in different medical centers across from Argentina, between August 2020 and August 2021. *Aspergillus* species were identified by morphology and DNA sequencing, while *Candida* species

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were identified using matrix assisted laser desorption/ionization time-of-flight mass spectrometry. Antifungal susceptibility testing was performed by microdilution. The *Aspergillus* isolates were identified as members of the sections *Fumigati* (n = 51), *Flavi* (n = 23), *Nigri* (n = 17), *Terrei* (n = 4), and *Nidulantes* (n = 1). The *Candida* isolates belonged to the following clades or genera: *Lodderomyces* (n = 41), *Nakaseomyces* (n = 4), *Meyerozyma* (n = 2), *Metschnikowia* (n = 2), *Yarrowia* (n = 1), and members of the family *Wickerhamomycetaceae* (n = 2). All *Aspergillus* isolates were susceptible to the tested antifungal agents, whereas 26.9% of *Candida* isolates exhibited a non-wild-type or resistant phenotype to at least one antifungal drug. Our results provide insights into the diversity of *Aspergillus* and *Candida* species, as well as their antifungal susceptibility profiles, in critically ill COVID-19 patients.

PALABRAS CLAVE
COVID-19;
CAPA;
CAC;
Epidemiología molecular;
Ensayos de sensibilidad

Epidemiología molecular y patrones de sensibilidad a antifúngicos de especies de *Aspergillus* y *Candida* aisladas de pacientes argentinos con aspergilosis pulmonar y candidemia asociadas a COVID-19

Resumen La aspergilosis pulmonar (CAPA) y la candidemia asociadas a COVID-19 (CAC) son complicaciones graves con mal pronóstico en pacientes críticos con SARS-CoV-2 y síndrome de distrés respiratorio agudo en unidades de cuidados intensivos (UCI). Este estudio tuvo como objetivo analizar la diversidad de especies de *Aspergillus* y *Candida* en pacientes críticos con CAPA y CAC, y sus perfiles de sensibilidad a antifúngicos. Se aislaron 96 cepas de *Aspergillus* de muestras respiratorias de 78 pacientes con CAPA y 52 cepas de *Candida* de hemocultivos de 50 pacientes con CAC. Todos los pacientes fueron internados en UCI de distintos centros médicos de Argentina, entre agosto de 2020 y agosto de 2021. Las especies de *Aspergillus* fueron identificadas por morfología y secuenciación de ADN, mientras que las de *Candida* por espectrometría de masas por desorción/ionización láser asistida por matriz con análisis de tiempo de vuelo. Los aislamientos de *Aspergillus* pertenecieron a las secciones *Fumigati* (n = 51), *Flavi* (n = 23), *Nigri* (n = 17), *Terrei* (n = 4) y *Nidulantes* (n = 1). Los aislamientos de *Candida* pertenecieron a los siguientes clados o géneros: *Lodderomyces* (n = 41), *Nakaseomyces* (n = 4), *Meyerozyma* (n = 2), *Metschnikowia* (n = 2) y *Yarrowia* (n = 1); también se encontraron miembros de la familia *Wickerhamomycetaceae* (n = 2). Todos los aislamientos de *Aspergillus* fueron sensibles o presentaron un fenotipo salvaje a los antifúngicos evaluados, mientras que el 26,9% de los aislamientos de *Candida* mostraron un fenotipo no silvestre o resistente a al menos uno. Nuestros resultados aportan información sobre la diversidad de especies de *Aspergillus* y *Candida*, y sus perfiles de sensibilidad en pacientes críticos con COVID-19.

Introduction

Coronavirus disease-2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARSCoV-2), has become a major threat to human health and has triggered a global public health crisis.

Patients with severe COVID-19 pneumonia are particularly susceptible to developing acute respiratory distress syndrome (ARDS), a condition that often requires mechanical ventilation[32].

Critically ill COVID-19 patients are also prone to invasive fungal infections, particularly during the middle and late stages of the disease[8,18]. Among the most common fungal co-infecting agents in these patients are *Aspergillus* and *Candida* species, which cause COVID-19-associated pulmonary aspergillosis (CAPA) and COVID-19-associated candidemia (CAC), respectively[25,39]. Both CAPA and CAC are linked to poor clinical outcomes, including high morbidity and mortality rates as well as prolonged hospital stays[25,39]. Diagnosis is frequently delayed due to their nonspecific clinical presentations and the limitations of conventional diagnostic methods[4].

Several pathophysiological factors, including damage to the respiratory epithelium, impaired mucociliary clearance, and compromised local immunity, contribute to the development of CAPA and other bacterial or fungal superinfections[30]. Furthermore, immunosuppressive treatments particularly corticosteroids in severe COVID-19 cases exacerbate immunosuppression, increasing susceptibility to opportunistic fungal infections[34].

Additional risk factors for CAPA and CAC in this population include the use of broad-spectrum antibiotics, central venous catheters, parenteral nutrition, prolonged intensive care unit (ICU) stays, mechanical ventilation, and underlying conditions such as malignancies, immunodeficiencies, or recent organ transplants[8,33].

Some studies have shown that the cumulative incidence of CAPA in the ICU ranges from 1.0% to 39.1% with a mortality rate of 52.2%[31], while the incidence of CAC in the ICU ranges from 5.1% to 10.6%, with a mortality rate between 51% and 87.1%[21,22]. Strikingly, most of these patients (52.7% of CAPA patients and 58.7% of CAC patients) received antifungal therapy[22,31].

Adding to the challenges posed by CAPA and CAC, there is a rising concern regarding the emergence of antifungal resistance in both *Aspergillus* and *Candida* isolates, which makes the treatment of infections caused by these resistant strains even more challenging and associated with poorer outcomes[3].

Data regarding the microbiological and molecular characteristics of these infections in COVID-19 patients are scarce.

This study aimed to provide an overview of the diversity of *Aspergillus* and *Candida* isolates recovered from critically ill patients with CAPA and CAC, as well as to analyze their antifungal susceptibility profiles with their molecular resistant mechanisms.

Materials and methods

Samples, culture and identification of strains

A total of 148 clinical isolates were studied, which included 96 *Aspergillus* isolates recovered from respiratory specimens (bronchoalveolar lavage and tracheal aspirate) of 78 COVID-19 patients with probable CAPA, and 52 *Candida* isolates recovered from bloodstream specimens of 50 COVID-19 patients with proven CAC or candidemia. All patients were admitted to ICUs at various medical centers across Buenos Aires, Salta and Santa Fe provinces, Argentina, between August 2020 and August 2021. In all cases, the diagnosis of CAPA was based on the ECMM/ISHAM consensus criteria[23].

Organisms were first identified at the medical institutions using the routine methods available in each laboratory. These isolates were immediately submitted to the Research and Development Laboratory in Mycology of the Institute of Microbiology and Medical Parasitology of the University of Buenos Aires-CONICET, Buenos Aires, Argentina.

The isolates were subcultured and maintained onto Sabouraud dextrose agar (SDA) supplemented with 500 mg/l chloramphenicol and/or potato dextrose agar at 37 °C for further taxonomical identification and susceptibility testing. Species identification of all yeast isolates was performed by using matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF VITEK®MS).

Aspergillus isolates were identified at the species level by PCR amplification and sequencing of a portion of the β-tubulin gene[19]. For this purpose, genomic DNA was first extracted from pure fungal cultures in GYEP broth (2% glucose, 0.5% yeast extract, 1% peptone, Merck KGaA) and incubated 24–48 h at 37 °C. Then, genomic DNA was isolated by the phenol–chloroform–isoamyl alcohol extraction procedure[35] and PCR amplification reactions were performed as previously described[6]. PCR products were purified using the PCR and DNA fragment purification kit™ (Dongsheng Biotech, Guangzhou, Guangdong, China) and bidirectionally sequenced using the ABI BigDye Terminator v.3.1 kit (Macrogen Inc., Seoul, South Korea).

The resulting nucleotide sequences were compared with curated sequences published in the NCBI GenBank database obtained from Houbraken et al.[20].

Antifungal susceptibility testing

Antifungal susceptibility testing for *Aspergillus* isolates was performed against amphotericin B (AMB), itraconazole (ITR), voriconazole (VRC), posaconazole (POS) and isavuconazole (ISA) following the CLSI M38 3rd ed. Document[9].

Antifungal susceptibility testing for *Candida* species was performed against AMB, ITR, VRC, POS, fluconazole (FLC), micafungin (MCF), caspofungin (CAS) and anidulafungin (AND) following the CLSI document M27 4th ed[10].

Drugs were obtained as standard powders from their manufacturers or from Merck-Sigma-Aldrich (Argentina). The final drug concentration ranges were 0.015–8 μg/ml for all antifungal agents, except when testing FLC against *Candidozyma haemuli* (*Candida haemulonii*), *Meyerozyma guilliermondii* (*Candida guilliermondii*) and *Nakaseomyces glabratus* (*Candida glabrata*), where the range was 0.25–128 μg/ml.

Antifungal susceptibilities were interpreted by using the available epidemiological cut off values (ECV) and/or clinical breakpoints published in the CLSI documents M57S-Ed4, M38M51S-Ed3, and M27M44S[11–13]. For POS and *Aspergillus fumigatus*, the ECV used were those published by Espinel-Ingroff et al. (≤0.25 μg/ml)[15]. Susceptibility tests of non-WT isolates were performed on different days by triplicate.

A. fumigatus ATCC 204305, *Aspergillus flavus* ATCC 204304, *Candida parapsilosis* ATCC 22019, and *Pichia kudriavzevii* (*Candida krusei*) ATCC 6258 were used as quality control strains for susceptibility testing.

PCR amplification and sequencing of the FKS1 and ERG11 genes of *Candida albicans* and *C. parapsilosis*

The FKS1 gene, which encodes 1,3-beta-glucan synthase, the echinocandin drug target, and the ERG11 gene, which encodes lanosterol 14-α demethylase, the azole drug target, were amplified and sequenced for all *C. albicans* and *C. parapsilosis* isolates exhibiting a non-wild-type or resistant phenotype to at least one antifungal drug, using the previously described PCR conditions[17,24,37].

To reduce errors during amplification, the high-fidelity *Pfu* DNA polymerase (Thermo Fischer Scientific) was used. Sequences were analyzed using the Muscle algorithm contained in the Unipro UGENE v34.0 software[28]. The DNA sequences were compared to *C. parapsilosis* reference sequences (GenBank accession no. EU221325 for the FKS1 gene and CPAR2_303740 for the ERG11 gene), and to *C. albicans* reference sequences (GenBank accession no. D88815

for the FKS1 gene, and GenBank accession no. X13296 for the ERG11 gene).

Results

Species distribution

From August 2020 to August 2021, 148 clinical isolates of *Aspergillus* spp. and *Candida* spp. isolated from the respiratory secretions of patients with probable CAPA and from the bloodstream of patients with CAC, were retrospectively analyzed.

Among the 96 *Aspergillus* isolates analyzed, five species complexes were found: 53.1% belonged to section *Fumigati* (51 *A. fumigatus*), 23.9% to section *Flavi* (20 *A. flavus*, 2 *A. parasiticus* and 1 *A. tamarii*), 17.7% to section *Nigri* (16 *A. niger*, and 1 *A. tubigensis*), 4.2% to section *Terrei* (2 *A. terreus* and 2 *A. alabamensis*) and 1.1% to section *Nidulantes* (1 *A. nidulans*).

The 52 *Candida* isolates analyzed belonged to the following five clades and genera: 78.8% to the *Lodderomyces* clade (19 *C. parapsilosis*, 14 *C. albicans*, 6 *C. tropicalis* and 2 *C. orthopsilosis*), 7.7% to the *Nakaseomyces* clade (4 *N. glabratus* —*C. glabrata*—), 3.8% to the genus *Meyerozyma* (2 *M. guilliermondii* —*C. guilliermondii*—), 3.8% to the genus *Clavispora* within the *Metschnikowia* clade (1 *C. haemulid* —*C. haemulonii*— and 1 *Clavispora lusitaneae* —*Candida lusitaniae*—), 1.9% to the genus *Yarrowia* (1 *Yarrowia lipolytica* —*Candida lipolytica*—), and 3.8% to the family *Wickerhamomycetaceae* (2 *Wickerhamomyces anomalus* —*Candida pelliculosa*—).

Antifungal susceptibilities

All *Aspergillus* species analyzed were susceptible or wild-type to azole antifungals and AMB (Tables S1–S5), while 26.9% (n = 14) of *Candida* isolates exhibited a non-wild-type or resistant phenotype to at least one antifungal drug (Table 1 and Tables S6–S13). Of these, three isolates (21.4%) exhibited a resistant phenotype to FLC, and 11 isolates (78.6%) exhibited a resistant phenotype to at least one echinocandin. One *C. albicans* isolate was resistant to FLC and VRC; one *C. parapsilosis* isolate was resistant to FLC and AND; one *M. guilliermondii* (*C. guillermondii*) isolate was resistant to FLC; one *C. tropicalis* isolate was resistant to CAS; five *C. parapsilosis* isolates were resistant to AND; one *C. parapsilosis* isolate was resistant to CAS and intermediate to AND; and three *C. albicans* isolates were resistant to CAS and AND. Of these, two 2 *C. albicans* isolates were additionally intermediate to MCF. These results showed that 50% (1 out of 2) *M. guilliermondii* (*C. guilliermondii*) isolates, 36.8% (7 out of 19) of *C. parapsilosis* isolates, 28.6% (4 out of 14) of *C. albicans* isolates, and 16.7% (1 out of 6) of *C. tropicalis* isolates recovered from the bloodstream of CAC patients were resistant to at least one antifungal drug.

The *C. haemuli* (*C. haemulonii*) isolate exhibited a non-wild-type phenotype to VRC and POS and a very high MIC value to FLC. Lastly, 12 *Candida* isolates exhibited an intermediate phenotype to AND and/or CAS: one *C. tropicalis* and one *N. glabratus* (*C. glabrata*) isolates were intermediate to CAS while 9 *C. parapsilosis* and 1 *M. guilliermondii* (*C. guilliermondii*) isolates were intermediate to AND.

Although no ECV value has been described for *A. parasiticus*, it should be noted that one out of two isolates exhibited a MIC value $\geq 8\,\mu g/ml$.

Sequence analysis of the FKS1 and ERG11 genes of *C. albicans* and *C. parapsilosis*

The sequencing of the gene encoding the azole drug target, ERG11, revealed the presence of the amino acid substitutions K128R + D223E + E260G in the FLC-resistant *C. parapsilosis* strain, and the amino acid substitutions Y132F + E266D in the FLC- and VRC-resistant *C. albicans* strain (Table 1). The sequencing of the FKS1 gene in *C. albicans* and *C. parapsilosis* strains that exhibited resistance to at least one echinocandin drug revealed the presence of the S593L and F593L amino acid substitutions in the hotspot 1 region, respectively (Table 1). Additionally, another *C. parapsilosis* strain harbored the S575STOP, L601S, and S634L substitutions in the same region (Table 1). In the remaining seven isolates resistant to at least one echinocandin drug, no mutations in the FKS1 gene were found (Table 1).

The nucleotide sequences were deposited in GenBank under accession numbers PV867447 to PV867457.

Discussion

The COVID-19 pandemic led to a significant increase in the incidence of invasive fungal diseases, particularly CAPA and CAC, among hospitalized patients in ICUs[18].

A. fumigatus, the most frequently recovered mold from the respiratory tract of patients with CAPA, is a saprophytic fungus, ubiquitous in the environment and organic matter, and responsible for causing a wide spectrum of diseases, some of which are severe invasive infections, such as CAPA, which was a major cause of morbidity and mortality among ICU COVID-19 patients[18,26].

Due to its clinical importance, the World Health Organization has designated *A. fumigatus* as one of the four critical fungal pathogens[38].

A. flavus, which is the second most common pathogenic species of *Aspergillus* in humans, is also of great importance due to its ability to cause disease in animals and crops and to produce carcinogenic mycotoxins[36].

Candida species are typically harmless commensals in mucosal surfaces, the gastrointestinal tract, respiratory tract, genitourinary tract and skin[1]. *C. albicans* is one of the predominant representatives and affects around 6–10% of all ICU patients[2]. Nevertheless, non-*albicans Candida* (NAC) infections are increasing, with multidrug resistant *N. glabratus* (*C. glabrata*) and *C. auris* being the most prominent[1,2].

The high morbidity and mortality associated with CAPA and CAC, along with the increasing burden of triazole- and/or echinocandin-resistant isolates and the emergence of COVID-19 as a new risk factor for these fungal diseases, have raised growing awareness of the need to better understand the dynamics of these fungi. Understanding the epidemiology of CAPA and CAC isolates provides better insight into the interactions between these fungi and their novel susceptible host: the critically ill COVID-19 patient.

Table 1 Antifungal susceptibility values of non-wild type or resistant *C. albicans* and *C. parapsilosis* isolates and their molecular features.

Isolate ID	Species	Antifungal MIC (μg/ml) of:					ERG11 mutations	FKS1 mutations	GenBank accession no.
		FLC	VRC	AND	MCF	CAS			
CpL8_FKS1	*C. parapsilosis*	0.5	0.015	≥8	0.5	0.5	N.S.	Wild-type	PV867447
CpL32_FKS1	*C. parapsilosis*	1	0.03	≥8	0.5	0.5	N.S.	Wild-type	PV867448
CpL41_FKS1	*C. parapsilosis*	0.5	0.125	≥8	1	0.5	N.S.	Wild-type	PV867449
CpL44_FKS1	*C. parapsilosis*	≥8	0.125	≥8	0.5	0.5	K128R + D223E + E260G	Wild-type	PV867450*
CpL31_FKS1	*C. parapsilosis*	2	0.06	≥8	0.5	2	N.S.	F593L	PV867451
CpL3_FKS1	*C. parapsilosis*	0.5	0.06	4	0.5	≥8	N.S.	S575Stop + L601S + S634L	PV867452
CaL9_FKS1	*C. albicans*	0.125	0.03	4	0.25	2	N.S.	Wild-type	PV867453
CaL15_FKS1	*C. albicans*	0.5	0.06	4	0.5	2	N.S.	Wild-type	PV867454
CaL13_FKS1	*C. albicans*	0.5	0.015	4	0.125	2	N.S.	S593L	PV867455
CpL44_ERG11	*C. parapsilosis*	≥8	0.125	≥8	0.5	0.5	K128R + D223E + E260G	Wild-type	PV867456
CaL25_ERG11	*C. albicans*	≥8	1	0.03	0.06	0.125	Y132F + E266D	N.S.	PV867457*

N.S.: not sequenced.
* PV867450 and PV867457 correspond to strain CpL44, as they represent the same isolate.

In the present study, conducted in Argentina, *A. fumigatus*, *A. flavus* and *A. niger* ranked first (53.1%), second (20.8%) and third (10.4%) among the *Aspergillus* species recovered from the airways of patients with probable CAPA, respectively. Similarly, *C. parapsilosis*, *C. albicans* and *C. tropicalis* ranked first (36.5%), second (26.9%) and third (11.5%) among the *Candida* species recovered from the bloodstream specimens of patients with proven CAC, respectively. These results are similar to those described in Italy, where *C. parapsilosis* was the most frequently isolated yeast (49.8% of the cases), followed by *C. albicans* and *N. glabratus* (*C. glabrata*) (35% and 10% respectively)[29].

Notably, a predominance of NAC species was observed (73.1% *vs* 26.9%). In contrast, non-*fumigatus* species were less frequent than *fumigatus* species (46.9% *vs* 53.1%).

All *Aspergillus* species analyzed were susceptible or wild-type to azole antifungals and AMB. One isolate of *A. parasiticus* exhibited a high MIC value to AMB (≥8 μg/ml), while another *A. parasiticus* isolate displayed a MIC value of 1 μg/ml against AMB.

With regard to *Candida* spp., 26.9% of the isolates exhibited a non-wild-type or resistant phenotype to at least one antifungal drug. Of these, three isolates (1 *C. albicans*, 1 *C. parapsilosis* and 1 *M. guilliermondii* (*C. guilliermondii*)) were resistant to FLC, while 11 isolates (7 *C. parapsilosis*, 3 *C. albicans* and 1 *C. tropicalis*) were resistant to at least one echinocandin. *C. haemuli* (*C. haemulonii*), a multidrug-resistant species closely related to the emerging multidrug-resistant *C. auris*, exhibited a non-wildtype phenotype to VRC and POS and a high MIC value against FLC (≥128 μg/ml). According to CLSI document M57S-Ed4, the FLC ECV for *C. haemuli* (*C. haemulonii*) is very high (128 μg/ml), which might suggest intrinsic resistance or limited susceptibility to this agent[11,13].

Among the ten *C. albicans* and *C. parapsilosis* isolates showing elevated MICs to at least one echinocandin, seven exhibited a wild-type FKS1 genotype. This discrepancy may reflect alternative resistance mechanisms (e.g., FKS2 mutations), or could result from technical variability, particularly in cases where only one echinocandin showed an elevated MIC. The FLC- and VRC-resistant *C. albicans* strain exhibited amino acid changes Y132F + E266D, which are associated with azole resistance[27], while the amino acid changes K128R + D223E + E260G in Erg11 of *C. parapsilosis* have not been described before. However, given the high MIC value for FLC in this isolate, these alterations most likely have an impact on azole susceptibility. Further studies are needed to confirm their role. In addition to ERG11 mutations, other molecular mechanisms may also contribute to azole resistance in *C. parapsilosis*, such as gain-of-function mutations in transcriptional regulators such as UPC2, TAC1, and MRR1, or increased azole resistance through high copy number variations[7]. These alternative mechanisms should be considered in future investigations.

Although no ECV value has been described for *A. parasiticus* and none of the cryptic species in section *Flavi* are known to carry natural resistance to antifungal drugs[16], it should be noted that one out of two isolates of *A. parasiticus* exhibited a MIC value ≥8 μg/ml. Previous reports have described MIC values of 2 μg/ml[14] and 8 μg/ml[5] for this cryptic species.

This study has some limitations due to the lack of data, particularly concerning the patients from whom the isolates were recovered, and the non-generalizability of the results given the local fungal epidemiology at the study centers. Lastly, we were unable to assess whether any of the isolates developed antifungal drug resistance during treatment, as no sequential isolates were collected. Although we did not have access to full clinical records, information regarding previous antifungal exposure may be available and could be analyzed in future studies to better understand the emergence of resistance.

Conclusions

This study shows that among *Aspergillus* species, *A. fumigatus*, *A. flavus* and *A. niger* had a high frequency in patients with probable CAPA from Argentina. Similarly, among *Candida* species, *C. parapsilosis*, *C. albicans* and *C. tropicalis*

had a high frequency in patients with proven CAC from Argentina. The high frequency of resistant or non-wild-type *Candida* isolates to azoles and/or echinocandins was also reported, with the presence of the multidrug resistant *C. haemuli* (*C. haemulonii*) isolate, a species closely related to the emerging multidrug-resistant *C. auris*. Continuous clinical surveillance is required to detect antifungal resistant isolates.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors do not use any generative AI and/or AI-assisted technology in the writing process.

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Conflicts of interest

The authors declare that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version available at https://doi.org/10.1016/j.ram.2026.100712.

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