



Original article

Prevalence, antimicrobial resistance, and genetic lineages of nasal *Staphylococcus aureus* among medical students at a Spanish University: detection of the MSSA-CC398-IEC-type-C subclade

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ABSTRACT

Medical students could be a potential source of *Staphylococcus aureus* transmission to patients. This cross-sectional study involved samples collected from both nasal nostrils. Samples were processed for *S. aureus* recovery; the antimicrobial resistance (AMR) phenotype was determined by disc diffusion assays and the *spa* types and AMR genotypes by PCR/sequencing. A structured questionnaire was administered to students to collate data related to potential risk factors of nasal colonization. Ninety-eight students were included, 50 % were colonized by *S. aureus* and 12.2 % by MRSA. The *mecA* gene was detected in all MRSA isolates. The MSSA-CC398-IEC-type C lineage was found among 16.3 % of nasal carriers, of which t571 was the predominant *spa*-type. MRSA isolates were ascribed to *spa* types t2226 (CC5, 12 isolates) and t3444 (new *spa* type, 1 isolate). All MRSA were multi-drug resistant and MSSA were predominantly resistant to erythromycin-clindamycin (inducible-type, mediated by *ermT* gene). High rates of *S. aureus* and MRSA nasal carriage were observed in this study. The predominance of the CC398 lineage among MSSA (emergent invasive lineage) represent a relevant finding of public health concern. The role of medical students as potential source of MRSA and MSSA-CC398 transmissions in hospital and community needs to be elucidated in detail.

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1. Introduction

Staphylococcus aureus (*S. aureus*) is one of the main pathogens that cause nosocomial and community-acquired infections in humans [1], responsible for high morbidity and mortality [2].

S. aureus can colonize multiple sites like the skin and mucosal surfaces, but the main ecological niche in humans is the nostrils. It is usually found in healthy people without causing infection, but this represents a risk factor for acquiring staphylococcal infections. When the mucosal and cutaneous barriers are broken, *S. aureus* can produce several infections, which range from mild skin infections to severe diseases, such as bacteraemia, endocarditis, osteomyelitis and sepsis, among others [3].

In addition, the excessive use of antimicrobial agents has led to increasing resistance to some of them. Since the early 1960s, there has been an increase in the level of drug resistance and particularly methicillin-resistant *S. aureus* (MRSA) has emerged in hospitals and the community [4]. The resistance of MRSA strains to methicillin is mainly due to the acquisition of the *mecA* gene, transmitted on staphylococcal cassette chromosome *mec* (SCC*mec*), which codes for a modified PBP2a protein and implies resistance to almost all beta-lactam antibiotics (except some few cephalosporins). In 2011, a variant of *mecA* gene, *mecALGA251* (later named *mecC*), which produces an altered transpeptidase (PBP2c), also with a low affinity to beta-lactam agents, was initially detected in humans and animals in Scotland [5].

In the beginning, MRSA infections were confined to the hospital environment, and they caused infections with certain peculiarities due to their different molecular characteristics. On the other hand, community-acquired MRSA (CA-MRSA) often cause less severe infections and have less resistance to antibiotics than hospital-

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acquired MRSA. However, nowadays these more virulent and resistant strains can be found both in the community and in medical centres [4]. The livestock-associated MRSA (LA-MRSA) of lineage CC398 has been strongly associated with pigs and with persons in contact with them, and it has been detected a close correlation between the frequency of detection of MRSA-CC398 isolates and the pig-farming densities of the surrounding areas [6]. The pig-associated MRSA-CC398 isolates are mostly of *spa*-type t011. Nevertheless, recently it has been reported the emergence of the subclade MSSA-CC398, mostly associated with *spa*-type t571, that seems to be livestock-independent and is implicated in invasive human infections [7].

According to different publications, medical students could be a potential source of transmission of *S. aureus* to patients [8–11]. Nasal colonization with *S. aureus* among them, ranges from 20.3 % [11] to 46.25 % [10], and MRSA prevalence ranges from 0 % [8] to 29 % [10].

This study sought to determine the nasal carriage rate of *S. aureus*, their antimicrobial resistance (AMR) and genetic lineages among third-year medical students at the University of Las Palmas de Gran Canaria (ULPGC), Spain.

2. Material and method

2.1. Study design, subjects and sampling technique

A cross-sectional study of *S. aureus* nasal colonization in third-year medical students at the ULPGC was carried out. Samples from the right and left nostrils of 98 students were taken with sterile cotton swabs. Each sample came along with a survey, asking about age, gender, highest year matriculated, nose piercing, smoking habits, contact with animals, household member working in a healthcare centre, clinical practice, work in a healthcare centre, antibiotic treatment, previous hospitalization, skin and soft tissue infections, sinusitis, asthma, rhinitis, immunotherapy and corticoid treatment. The research was reviewed and approved by the FUN-CANIS' ethics committee (Code CEI/CEIm: 2019-203-1). Informed consent was obtained from all participating individuals included in the study.

2.2. Isolation and identification of *S. aureus*

Samples were cultured in Mannitol Salt Agar (MSA, Becton, Dickinson and Company (BD), MD, USA) for 24–48 h at 35–37 °C and on MRSA select chromogenic agar (BBL™ CHROMagar MRSA II) for 24–48 h at 35–37 °C. Due to the fermentation of mannitol, *S. aureus*-positive colonies appeared yellow and turn medium yellow. MRSA strains grew as purple-pink colonies on the chromogenic medium. All *S. aureus* and MRSA suspected strains were isolated in MSA and then identified by standard microbiological techniques such as Gram stain, microscopy morphology observation and the catalase test. If the isolates were Gram-positive cocci and had the catalase enzyme, two agglutination identification tests were performed: Pastorex Staph-Plus (BioRad) for Clumping Factor, Protein A and capsular polysaccharides 5 and 8; and Staph-Plus (BioMerieux) for Clumping Factor, Protein A and Glycopolysaccharide Antigen 18. If there was a positive reaction in at least one of these tests, the bacteria were considered *S. aureus*.

2.3. Antimicrobial resistance phenotypes and genotypes

The antimicrobial susceptibility of the isolates was evaluated by Agar Diffusion test (Kirby–Bauer test) using Müller-Hinton agar (Difco, Mo, USA) following the recommendations of CLSI 2020 [12]. The antimicrobial agents tested were as follows: ceftioxin (FOX),

quinupristin-dalfopristin (SYN), gentamicin (GM), vancomycin (VA), teicoplanin (TEC), fosfomicin (FOS), linezolid (LZD), levofloxacin (LEV), ciprofloxacin (CIP), trimethoprim-sulfamethoxazole (SXT), rifampicin (RA), clindamycin (CLI), erythromycin (ERY), tetracycline (TET) and chloramphenicol (C). The presence of inducible resistance to clindamycin in proximity to erythromycin (CLI^{ind}) was determined by D-test [13]. The ceftioxin disc (30 µg) was used to determine methicillin resistance (12)

The presence of *ermA*, *ermB*, *ermC*, and *ermT* genes was investigated by PCR in the erythromycin-resistant isolates [7,14], and the 16S rDNA (*Staphylococcus* spp. amplification control) [15] gene was also investigated by PCR. All MRSA isolates were subjected to specific PCR for *mecA* gene detection [15]. Positive and negative controls were used in all PCRs.

2.4. Slime production

Qualitative assessment of slime production was performed using a variation of Christensen's method, which detects the formation of bacterial populations that adhere to inert surfaces by staining the slime [16]. Bacteria were cultured in 2 ml of Brain Heart Infusion (BHI, BD, USA) for 24h at 37 °C. After incubation, the medium was drained, methyl violet solution was added to the tube to stain the adherent bacteria, and then washed with water. The test was considered positive when a halo of stained bacterial growth was observed in the tube.

2.5. Molecular typing and virulence genes of *S. aureus* isolates

S. aureus isolates obtained in the right and in the left nostril of the students were studied, unless they presented the same antimicrobial resistance profile. In this case, only one was characterized for its virulence factors and genetic lineage.

All *S. aureus* isolates were screened by PCR for the presence of the *arcA* gene (encoding the virulence factor Arginine Catabolic Mobile Element – ACME), as well as for the genes *lukF/S-PV* (encoding Panton-Valentine leukocidin) [17,18].

All *S. aureus* isolates (one per student or two if the isolates showed different phenotypes of AMR) were subjected to specific PCR screening for CC398 lineage [19]. Moreover, the *S. aureus* CC398 as well as the MRSA isolates were characterized by amplicon-sequencing of the hyper-variable region of the staphylococcal protein A (*spa*) gene specific for *S. aureus* [18]. The CC398 isolates were tested for the presence of the staphylococcal complement inhibitor (*scn*) gene, and all *scn*-positive isolates were typed by PCR for Immune Evasion Cluster (IEC) based on the combination of the IEC genes (*scn*, *sak*, *sep*, *sea* and *chp*) [20]. In addition, the presence of the gene *LukED* was screened by PCR in MRSA isolates [18]. The MSSA-CC398 isolates exhibiting a CLI^{ind} phenotype were tested for the presence of the *ermT* gene [18].

2.6. Statistical analysis

Categorical variables were expressed as frequencies and percentages and were compared, as appropriate, using the Chi-square (χ^2) test or the exact Fisher test. Statistical significance was set at $p < 0.05$. Data were analyzed using the R package, version 3.6.1 [21] and IBM SPSS statistic 26.00 (SPSS Inc., Chicago, Illinois, USA).

3. Results

A total of 98 medical students participated in the survey. Of them, 72.4 % (71/98) were females and the average age was 20.8 years (range 19–38). Most of the participants were in the third academic year (89/98, 90.8 %).

Seventy-three *S. aureus* isolates were obtained, of which 56 were from MSA and 17 from CHROMagar MRSA II. Two of the isolates from MSA and eleven of the ones obtained from a chromogenic medium were methicillin-resistant upon cefoxitin disk screening.

Fifty per cent (49/98) of students were nasal carriers of *S. aureus*, of which 24.5 % (12/49) were MRSA carriers. The final prevalence of MRSA nasal carriage among all students was 12.2 % (12/98). The nasal carriage rate of *S. aureus* was 46.5 % (33/71) in females and 59.3 % (16/27) in males, but this difference was not statistically significant. Half of the colonized students had no history of antibiotic use (25/49, 51 %), more than half had no taken corticoid treatment (34/49, 69.4 %) and none of them had received immunotherapy. Only 14.3 % (7/49) of the carriers were smokers. Fifty students had pets and 22 of them were *S. aureus* carriers. The most frequent pets were dogs (37.8 %). More than half of colonized students had suffered from sinusitis (28/49, 57 %), asthma (25/49, 51 %) or rhinitis (28/49, 57 %). However, none of these variables had a statistically significant association with *S. aureus* or MRSA carriage ($p > 0.05$).

Of the 73 *S. aureus* isolates obtained, 34.3 % (25/73) produced slime, 38.3 % (23/60) among methicillin-susceptible *S. aureus* (MSSA) and 15.4 % (11/13) among MRSA.

3.1. Antimicrobial resistance phenotypes and genotypes

Percentages of resistance to different antimicrobials are summarized in Table 1. All isolates were susceptible to quinupristin-dalfopristin, gentamicin, vancomycin, teicoplanin, linezolid, trimethoprim-sulfamethoxazole, rifampicin and tetracycline. The D-test was positive in 12 (16.4 %) of the erythromycin-resistant strains but none of them was MRSA (Table 1).

One isolate was considered Multidrug-resistant (MDR) when it showed resistance to 3 or more families of antimicrobial agents. All MRSA were MDR (Table 2) and 4.1 % (2/49) of the MSSA were also MDR.

All 13 MRSA isolates were selected for molecular typing. The *mecA* and the *lukED* genes were detected in all of them (Table 2). Most were typed as MRSA-CC5- *spa* type t2226 ($n = 12$), and only one as t13444 (new *spa* type). The *ermC* gene was found in 9 MRSA isolates that showed resistance for erythromycin and clindamycin.

Forty-nine MSSA isolates (one per student or two if they showed different phenotypes of antibiotic resistance) were selected for CC398-typing. Eight of the 49 MSSA isolates were typed as lineage

CC398 representing 16.3 % of total MSSA. Of the 8 students carrying CC398-MSSA (8 % of total tested, 8/98), four had dogs and one had a cat. The CC398-MSSA isolates were of *spa*-types t571 ($n = 4$), t1446 ($n = 1$), t1451 ($n = 1$), t3397 ($n = 1$) and t6606 ($n = 1$) (Table 3). All the MSSA-CC398 isolates showed the phenotype ERY-CLI^{Ind} and carried the *ermT* gene; moreover, six MSSA-CC398 isolates carried the IEC type-C system.

The *arcA* gene was detected in 6.5 % (4/62) of the isolates. The gene of PVL was not found in this collection of isolates included in this study.

4. Discussion

It is considered that healthcare providers are important reservoirs and vehicles for transmission of nosocomial pathogens. Medical students could also be a potential source of infections, particularly those who have close contact with patients [22–24].

The prevalence of *S. aureus* and MRSA nasal carriage among medical students at ULPGC was 50 % and 12.2 %, respectively. The *S. aureus* colonization rate in this study is comparable with a study from the Complutense University of Madrid (46.25 %) [10]. In other Spanish studies among medical students, colonization rates of 34.6 % in Navarra [24] and 39.3 % in Madrid were found [9]. Similar studies in other European countries among medical students from the Czech Republic [25], Ireland [26] and Portugal [27] have shown colonization rates of 20.9 %, 30.9 % and 37.1 %, respectively.

Different factors have been associated with carrier status such as sinusitis, antimicrobial treatments, pets, smoking and contact with health services by work or by health reasons [10,28,29]. However, in this study, there was no statistically significant association between nasal carriage of *S. aureus* or MRSA and the risk factors studied. Similar findings were reported by other previous studies [11,30–32].

As in previous reports, *S. aureus* isolated in our study showed high percentages of susceptibility to gentamicin, rifampicin, chloramphenicol, vancomycin and teicoplanin [10,11,23,33–36].

Remarkable high rate of erythromycin resistance was observed in our study (46.6 %), being consistent with previous literature [10,33,36], although higher percentage (95.24 %) of erythromycin resistance was identified among Brazilian medical students [37]. Perhaps these differences could be due to differences in use of macrolides-lincosamide-streptogramins-B (MLS_B). In 2015, the number of defined daily doses per 1000 inhabitants per day of total

Table 1
Frequencies of antimicrobial resistance among *S. aureus* isolates.

Antimicrobial agent	<i>S. aureus</i> isolates								
	MSSA (n = 60)			MRSA (n = 13)			SA (n = 73)		
	S	I	R	S	I	R	S	I	R
Cefoxitin	60 (100 %)	0	0	0	0	13 (100 %)	60 (82.2 %)	0	13 (17.8 %)
Quinupristin-Dalfopristin	59 (98.3 %)	1 (1.7 %)	0	13 (100 %)	0	0	72 (98.6 %)	1 (1.4 %)	0
Gentamicin	60 (100 %)	0	0	13 (100 %)	0	0	73 (100 %)	0	0
Vancomycin	60 (100 %)	0	0	13 (100 %)	0	0	73 (100 %)	0	0
Teicoplanin	60 (100 %)	0	0	13 (100 %)	0	0	73 (100 %)	0	0
Fosfomicin	47 (78.3 %)	0	13 (21.7 %)	12 (92.3 %)	0	1 (7.7 %)	59 (80.8 %)	0	14 (19.2 %)
Linezolid	60 (100 %)	0	0	13 (100 %)	0	0	73 (100 %)	0	0
Levofloxacin	59 (98.3 %)	1 (1.7 %)	0	0	1 (7.7 %)	12 (92.3 %)	59 (80.8 %)	2 (2.7 %)	12 (16.4 %)
Ciprofloxacin	59 (98.3 %)	0	1 (1.7 %)	0	0	13 (100 %)	59 (80.8 %)	0	14 (19.2 %)
Trimethoprim-Sulfamethoxazole	60 (100 %)	0	0	13 (100 %)	0	0	73 (100 %)	0	0
Rifampicin	60 (100 %)	0	0	13 (100 %)	0	0	73 (100 %)	0	0
Clindamycin	58 (96.7 %)	2 (3.3 %)	0	1 (7.7 %)	0	12 (92.3 %)	59 (80.8 %)	2 (2.7 %)	12 (16.4 %)
Erythromycin	37 (61.7 %)	7 (11.7 %)	16 (26.7 %)	1 (7.7 %)	0	12 (92.3 %)	38 (52.1 %)	7 (9.6 %)	28 (38.4 %)
Tetracycline	60 (100 %)	0	0	13 (100 %)	0	0	73 (100 %)	0	0
Chloramphenicol	59 (98.3 %)	1 (1.7 %)	0	13 (100 %)	0	0	72 (98.6 %)	1 (1.4 %)	0

Table 2
Genetic characteristics of the MRSA isolates.

Number of strains	Antimicrobial resistance phenotypes	Antimicrobial resistance genes detected	<i>spa</i> -type/CC	Virulence factors detected
9	FOX-ERY-CLI-CIP-LEV	<i>mecA</i> , <i>ermC</i>	t2226/CC5	<i>lukED</i>
3	FOX-ERY-CLI-CIP-LEV	<i>mecA</i>	t2226/CC5	<i>lukED</i>
1	FOX-CIP-LEV-FOS	<i>mecA</i>	t13444	<i>lukED</i>

FOX: cefoxitin; ERY: erythromycin; CLI: clindamycin; CIP: ciprofloxacin; LEV: levofloxacin; FOS: fosfomicin.

Table 3
Genetic characteristics of the 8 MSSA-CC398 isolates.

No. of strain	Antimicrobial resistance	Genotype	<i>spa</i> -type/CC	IEC type
1	FOS- ERY- CLI ^{ind}	<i>ermT</i>	t571/CC398	C
2	ERY- CLI ^{ind}	<i>ermT</i>	t571/CC398	C
1	ERY- CLI ^{ind}	<i>ermT</i>	t571/CC398	–
1	ERY- CLI ^{ind}	<i>ermT</i>	t1446/CC398	C
1	ERY- CLI ^{ind}	<i>ermT</i>	t6606/CC398	C
1	ERY- CLI ^{ind}	<i>ermT</i>	t1451/CC398	–
1	ERY- CLI ^{ind}	<i>ermT</i>	t3307/CC398	C

FOS: fosfomicin; ERY: erythromycin; CLI^{ind}: inducible resistance to clindamycin in proximity to erythromycin.

antibiotics (DDD) of the macrolides-lincosamide-streptogramin in Spain was 2.26 (12.6 %) in the European countries, and in Brazil 3.69 (16.2 %) in the Regions of the Americans [38]. In Spain in 2020, the use of MLS_B (ATC group J01F) in the community, expressed as DDD per 1000 inhabitants per day was 2.39 [39].

In contrast to other previous studies [33,35,36], the findings from our study showed that all the *S. aureus* isolates were susceptible to trimethoprim-sulfamethoxazole. This agrees with the ones of Treeririchod et al. [11] and Rodríguez-Avial et al. [10]. In this current study, all the MRSA isolates were resistant to fluoroquinolones. This result is consistent with other studies which indicated a high frequency of resistance to fluoroquinolones in MRSA strains [35,36].

The production of slime facilitates bacterial adherence, constituting a virulence factor. In our study, slime production was found in 34.3 % of *S. aureus* isolates. Similar findings have been found by Satorres and Alcaráz [40] among healthcare workers (22.2 %) and Herrera-Rodríguez et al. [41] among veterinary students (45.45 %). Although some studies have shown that slime-producing strains are highly resistant to antimicrobial agents [42,43], the slime-producing MRSA isolates were 15.4 % against 34.3 % of the MSSA and only in three of the multiresistant strains versus twenty-two of no multiresistant strains. Hence, no association between MDR and slime production in our isolates was found in our study.

We detected *arc* gene in 6.5 % of our isolates. This percentage was higher than the one reported by Budri et al. [26] in preclinical medical students (0.7 %) but lower than the one described by Herrera-Rodríguez et al. [41] among veterinary students from ULPGC (18.18 %).

We analyzed the presence of the clonal complex CC398 of *S. aureus*, as this lineage is increasingly detected in various human populations. This CC398 lineage has acquired great interest in public health and two subclades have been found, one MRSA-CC398 subclade, especially associated with pigs and to the *spa* type t011 (among others) and the animal-independent MSSA-CC398 subclade, generally associated to the *spa* type t571 (and t1451). In this study, we have detected 8 MSSA-CC398 isolates, that could correspond to the human subclade, and 4 of them were t571 and one t1451 (other 3 *spa* types were identified). Interestingly, most of them carried the ERY- CLI^{ind} resistance phenotype with the *ermT* gene and the IEC-type C, characteristics of the human subclade [7].

The presence of MSSA-CC398 nasal carriage had been reported by other studies in different healthy populations [44–46], including medical students [47]. We found a higher percentage (16.3 %) of MSSA-CC398 carriers than those found by Sakar et al. [47] among Saudi Arabian medical students (3.2 %), or Sarrau et al. [44] in a Greek healthy population (7.1 %). However, our finding on the detection rate of MSSA-CC398 was similar to those found by Valour et al. [48] in patients awaiting surgery (18.6 %) in France, but lower than those found in healthy population in France (24.7 %) [45]. These differences in the detection rate of MSSA-CC398 could be due to multifactorial reasons which may include the contact rate of subjects with sick people prior to sample collection, the year in which the study was conducted, and the methodology employed for isolation and characterization of *S. aureus*.

Classically, the MSSA-CC398 subclade is often of the IEC type C and carry the *ermT* gene that encodes for erythromycin-clindamycin-inducible resistance [7]. These are the main biomarkers that provide preliminary evidence for the lineage and could (especially the ERY-CLI^{ind} resistance) be useful for rapid epidemiological surveillance. In conformity with these characteristics, the ERY- CLI^{ind} resistance phenotype was observed in 87.5 % of our CC398 MSSA isolates, and 75 % of them were IEC-C and 50 % carried the *ermT* gene. But only two of the isolates matched all three markers. This lineage has been increasing its importance as a public health problem by acquiring virulence and AMR genes, which indicates that the evolution of these lineages has been produced by the acquisition of prophages and other mobile elements [46]. Furthermore, t571 was the *spa*-type most frequent among our MSSA isolates. In a systematic review of several studies on nasal MSSA carriers, the *spa*-type t571 (animal-independent clade) was the most frequently identified [49] and it is associated with bloodstream infection in Spain [7].

The gene encoding the Pantone-Valentine leukocidin was not detected among the CC398 MSSA strains of our study. As a matter of fact, PVL is very unusual among *S. aureus*-CC398 isolates [46,49].

The prevalence of MRSA nasal carriage among medical students at ULPGC was 12.2 %, higher than in other Spanish studies (0.6 %) [24], (1.25 %) [10], (2.15 %) [9]. Rates of nasal colonization with MRSA have been reported to range from 0 % [8] to 29 % [10].

It was found that 12 of the 13 MRSA had the same AMR phenotype and were of the *spa*-type t2226/CC5. A common origin of this strain could be suspected, as the socio-demographic data and predisposing factors were analyzed: none had been hospitalized, 6 had done clinical practice, 5 lived with family members working in health care facilities, 6 had pets (dogs) and 6 had taken antibiotics. In Spain, MRSA of the *spa*-type t2226 has been linked to clinical settings [50,51], however, two of the students had not done clinical practice, did not live with family members working in healthcare facilities, and had not been hospitalized. The only factor they had in common was that they were students in the same faculty. These results suggest that some of the students may have contracted the MRSA-CC5-t2226 through contact with the hospital during their clinical practice or by living with a family member who had MRSA and then passed it on to their fellow students.

The differences in the results found between the studies of *S. aureus* and MRSA nasal carriage in medical students might be due

to several factors: the sampling and culture techniques and the population studied (different ethnics); geographical location, differences in infection control and prevention policies, risky environmental exposures, control of antimicrobial use, students' knowledge about MRSA epidemiology among others.

This study has some limitations, viz: Not all the students participated in the study, 102 out of the 127 enrolled, moreover, some of them did not fill the survey and others did not identify the samples in order to relate them to the surveys, so we only had 98 participants. The students collected their own samples as a part of their laboratory practices and sometimes they were cultured more than recommended time.

5. Conclusion

High rates of *S. aureus* and MRSA nasal carriage were obtained from this study. The predominance of the CC398 lineage (an emergent invasive clade) among the MSSA represents a relevant finding of public health concern. Based on these, it could be inferred that the medical students are a potential source of MRSA and MSSA-CC398 isolates that should be evaluated.

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Ethical approval

The study was conducted in accordance with the Declaration of Helsinki and the research was reviewed and approved by the FUNCANIS' ethics committee (Code CEI/CEIm: 2019-203-1).

Informed consent statement

Informed consent was obtained from all subjects involved in the study.

Data availability

Data will be made available on reasonable request to the corresponding author.

Declaration of competing interest

The authors declare that they have no competing interests.

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