

Genomic characterization of *Achromobacter* species isolates from chronic and occasional lung infection in cystic fibrosis patients

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Abstract

Achromobacter species are increasingly being detected in cystic fibrosis (CF) patients, where they can establish chronic infections by adapting to the lower airway environment. To better understand the mechanisms contributing to a successful colonization by *Achromobacter* species, we sequenced the whole genome of 54 isolates from 26 patients with occasional and early/late chronic lung infection. We performed a phylogenetic analysis and compared virulence and resistance genes, genetic variants and mutations, and hypermutability mechanisms between chronic and occasional isolates. We identified five *Achromobacter* species as well as two non-affiliated genogroups (NGs). Among them were the frequently isolated *Achromobacter xylosoxidans* and four other species whose clinical importance is not yet clear: *Achromobacter insuavis*, *Achromobacter dolens*, *Achromobacter insolitus* and *Achromobacter aegrifaciens*. While *A. insuavis* and *A. dolens* were isolated only from chronically infected patients and *A. aegrifaciens* only from occasionally infected patients, the other species were found in both groups. Most of the occasional isolates lacked functional genes involved in invasiveness, chemotaxis, type 3 secretion system and anaerobic growth, whereas the great majority (>60%) of chronic isolates had these genomic features. Interestingly, almost all ($n=22/23$) late chronic isolates lacked functional genes involved in lipopolysaccharide production. Regarding antibiotic resistance, we observed a species-specific distribution of bla_{OXA} genes, confirming what has been reported in the literature and additionally identifying bla_{OXA-2} in some *A. insolitus* isolates and observing no bla_{OXA} genes in *A. aegrifaciens* or NGs. No significant difference in resistance genes was found between chronic and occasional isolates. The results of the mutator genes analysis showed that no occasional isolate had hypermutator characteristics, while 60% of early chronic (<1 year from first colonization) and 78% of late chronic (>1 year from first colonization) isolates were classified as hypermutators. Although all *A. dolens*, *A. insuavis* and NG isolates presented two different *mutS* genes, these seem to have a complementary rather than compensatory function. In conclusion, our results show that *Achromobacter* species can exhibit different adaptive mechanisms and some of these mechanisms might be more useful than others in establishing a chronic infection in CF patients, highlighting their importance for the clinical setting and the need for further studies on the less clinically characterized *Achromobacter* species.

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Abbreviations: ANI, average nucleotide identity; CF, cystic fibrosis; LPS, lipopolysaccharide; NG, non-affiliated genogroup; T3SS, type 3 secretion system.

Sequences have been deposited at the NCBI SRA database under project no. PRJEB40979, secondary accession: ERP124698.

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Data statement: All supporting data, code and protocols have been provided within the article or through supplementary data files. Seven supplementary tables are available with the online version of this article.

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DATA SUMMARY

The authors confirm all supporting data, code and protocols have been provided within the article or through supplementary data files.

INTRODUCTION

Achromobacter species are ubiquitous Gram-negative bacilli, widely distributed in aquatic environments and soil, and increasingly found in nosocomial settings. They act as opportunistic pathogens in certain populations, such as subjects with cystic fibrosis (CF), where these microorganisms can survive for a long time in both lower and upper airways [1]. The genus *Achromobacter* comprises 22 named species and multiple genogroups [2]; whole genome sequencing data are available for all 22 species but complete reference genomes are available only for seven of them: *Achromobacter deleyi*, *Achromobacter denitrificans*, *Achromobacter insolitus*, *Achromobacter pestifer*, *Achromobacter ruhlandii*, *Achromobacter spanius* and *Achromobacter xylosoxidans*. The type species *A. xylosoxidans* is the most often isolated *Achromobacter* species among CF patients and more than half of CF patients with airway colonization by *A. xylosoxidans* develop chronic infection, which has been associated with a decline in respiratory function and lung inflammation [1, 3–5]. Of note, an outbreak caused by another species, *A. ruhlandii*, was reported at two Danish CF centres [6, 7].

Lung infection caused by *Achromobacter* results either from acquisition from the environment or from direct/indirect transmission [7, 8] and is usually complicated by the innate and acquired multidrug resistance carried by these microorganisms. Approximately 50 drug-resistance-associated genes have been predicted in the *A. xylosoxidans* type strain [9, 10]. *A. xylosoxidans* is intrinsically resistant to aminoglycosides, many cephalosporins and aztreonam [9, 11] but only a few intrinsic resistance mechanisms have been identified to date. The resistance-nodulation-cell division (RND)-type multidrug efflux pump AxyAB-OprM is involved in resistance to cephalosporins (except cefepime), aztreonam, nalidixic acid, fluoroquinolones and chloramphenicol [12], while AxyXY-OprZ and AxyEF-OprN are responsible for resistance to aminoglycosides and levofloxacin, respectively [13, 14]. Moreover, the chromosomally encoded narrow-spectrum class D lactamase *bla*_{OXA-114} showed hydrolysis of piperacillin and ticarcillin [15]. In addition, clinical isolates of *A. xylosoxidans* may exhibit acquired resistance, especially for beta-lactams. To date, genes identified include IMP-type carbapenemase genes *bla*_{IMP-1}, *bla*_{IMP-10} and *bla*_{IMP-19} [16, 17], VIM-type carbapenemase-encoding genes *bla*_{VIM-1} [18] and *bla*_{VIM-2} [19], the extended-spectrum beta-lactamase-encoding gene *bla*_{VEB-1} [20], and the metallo-lactamase-encoding gene *bla*_{TMB-1} [21].

With regard to pathogenic mechanisms, similar to other gram-negative pathogens, *A. xylosoxidans* expresses cell membrane-bound virulence factors such as the Vi capsular polysaccharide, involved in surface adhesion and protection

Impact Statement

Achromobacter species can develop chronic lung infections in cystic fibrosis (CF) patients, which have been associated with worsening of the clinical condition. Although the frequency of infection by these opportunistic pathogens is increasing, little is still known about the mechanisms that are involved in their pathogenicity and persistence. To better elucidate these features, we analysed the genome of 54 isolates collected from both occasionally and chronically infected patients. This approach represents a novelty in the available literature, as the majority of studies have focused on chronic infection only, while the comparison with occasional isolates may allow us to identify genetic markers of persistence. Moreover, most of the available information regards the type species *Achromobacter xylosoxidans*, while data are lacking about other clinically relevant species. We identified four of them in our collection and additionally performed species-specific analyses. Our results show the occurrence of different adaptive mechanisms in terms of virulence, antibiotic resistance, within-host genomic evolution and hypermutation, suggesting that some features might be more useful than others to establish chronic infections. These findings could support the creation of diversified treatment regimens and improve the management and clinical outcome of *Achromobacter* species lung infections in CF patients.

from phagocytosis and toxins, and the O-antigen, which elicits a host immune response [22]. Lipopolysaccharide (LPS) also induces key inflammatory cytokines [23]. *A. xylosoxidans* is also equipped with various secretion systems that mediate the release of molecules to provide capability for invasion of the host cells, but little is known about its exoproducts [24, 25]. Previous *Achromobacter* comparative genomics analyses showed that virulence genes related to the type 3 secretion system (T3SS) are more common in CF isolates than in environmental strains [22, 26]. Genome analysis also showed the presence of genes encoding colicin V, a protein cytotoxic to similar bacteria, and *aepA*, involved in cellulase and protease regulation [24]. In addition, production of phospholipase C and ability to inactivate the *Pseudomonas aeruginosa* quinolone signal molecule were observed [27, 28]. Moreover, a heat-stable cytotoxic factor, associated with an increase of pro-inflammatory cytokines *in vitro*, was identified [29]. Jakobsen and colleagues also investigated the presence of secreted virulence factors known to be important for other CF pathogens such as *P. aeruginosa*, but reported the absence of extracellular proteases, chitinase and rhamnolipids in clinical strains. More recently, we detected protease secretion from *A. xylosoxidans* CF clinical isolates [30]. Regarding the biofilm mode-of-growth, *A. xylosoxidans* showed poor surface adhesion [24] but can form unattached or loosely attached aggregates, held together by polysaccharides forming

a peripheral shell around the bacterial cells [31]. This ability might help bacteria to form complex communities and survive in a hostile environment such as CF lungs.

Another mechanism contributing to the persistence of pathogens in the airways of CF patients is the ability to genetically adapt during chronic infection through accumulation of pathoadaptive mutations [25]. This phenomenon can be accelerated due to mutations in the DNA mismatch repair system, giving rise to hypermutation events and to clonal diversification within the host [32]. The mutator genes whose mutations have been associated with development of hypermutation are *mutL*, *mutS*, *pfpI*, *superoxide dismutase*, *radA*, *radC*, *rad50*, *uvrA*, *uvrB*, *uvrC* and *uvrD* [33].

To better understand the mechanisms underlying CF lung colonization by *Achromobacter* species, we sequenced the whole genome of 54 isolates from patients with both occasional and chronic infection. By using different bioinformatics tools, we performed a phylogenetic analysis and compared virulence and resistance genes, genetic variants and mutations, as well as hypermutability mechanisms between chronic and occasional *Achromobacter* isolates.

METHODS

Sample collection and identification

The samples analysed in this study have already been described in our previous work [34]. Briefly, 54 *Achromobacter* clinical isolates were collected from 53 sputum samples of 26 patients followed at the CF Centre of Verona (Italy) and were identified as *Achromobacter* species by MALDI-TOF-MS (bioMérieux). Informed consent was obtained according to projects CRCFC-CEPPO026 and CRCFC-CEPPO031 approved by the local Ethical Committee. Collection was mainly performed in two time periods: 2014–15 and 2017–18. Sputum samples were collected approximately every 3 months for microbiological analysis. Only one isolate from each sample was included in the study, except for one P09 sample from which two morphologically different isolates were identified and included (9–4 and 9–5).

The classification of occasional and chronic infection was assessed using information regarding all *Achromobacter* isolates identified between 2013 and 2018. According to the European Consensus criteria (ECC), infection was defined as chronic when at least three positive cultures with at least a 1 month interval between the samples were obtained within ≥ 6 months from the first colonization event [35]. Isolates from chronically infected patients were further classified as early (<1 year from first colonization event) and late isolates (>1 year from first colonization). A minimum of one and a maximum of six successively collected isolates (mean=2.5 isolates per patient) were recovered from chronically infected patients with a mean time delay of 197 days (range=21–1182 days). Only one isolate was recovered from each occasionally infected patient, except for P06 and P12 from which we recovered two isolates with a time delay of 112 and 155 days, respectively.

Genome sequencing

Collected isolates underwent whole genome sequencing at the Technological Platform Centre of the University of Verona as previously described [34]. Sequencing data were submitted to the NCBI SRA database with project number PRJEB40979. Genomic DNA was extracted using a QIAamp DNA Blood Mini Kit (Qiagen) and its quality was assessed using Nanodrop (Thermo Fisher Scientific) and Fragment Analyzer (Agilent Technologies). Libraries were prepared using the KAPA PCR-free kit and sequenced on a NextSeq500 Illumina (Illumina) platform generating 150 bp paired-end reads with mean reads yield of 10978104 and mean coverage of 190 \times . Read quality controls, *de novo* assembly and genome annotation were performed similarly to our previous work [30]. Details on the sequencing and *de novo* assembly are available in Table S1 (available in the online version of this article).

Phylogenetic analysis

Based on MALDI-TOF identification results, all complete *A. xylosoxidans* genomes stored in the NCBI RefSeq database [36] ($n=7$ – January 2020, accessions: GCF_900475575.1, GCF_001051055.1, GCF_001558755.2, GCF_001558915.1, GCF_001559195.1, GCF_000165835.1 and GCF_001457475.1) were considered as reference genomes for this study.

The average nucleotide identity (ANI) among all available *Achromobacter* species genomes, regardless of their assembly status ($n=142$ – NCBI RefSeq database, January 2020), and all sequenced isolates was calculated using FastANI [37] in order to ensure the correct species classification of the reference and isolate genomes. Isolates were considered to belong to the same species when ANI ≥ 95 %, whereas isolates having ANI<95% with all available *Achromobacter* species genomes were considered as unaffiliated to any species sequenced up to now and referred to as non-affiliated genogroups (NGs) in this work [38]. This was further supported by *in silico* multilocus sequence typing (MLST) scheme analysis (Table S2). After ANI calculation, some reference genomes deposited as *A. xylosoxidans* showed ANI>95% with genomes of other *Achromobacter* species (*A. insuavis*, *A. aegrifaciens*, *A. dolens* incomplete genome) suggesting their misclassification. Thus, in this study they were used as reference genomes for isolates having ANI>95%, with the exception of *A. dolens*, whose complete reference genome was not available yet. In order to avoid using incomplete genomes as reference, the *A. ruhlandii* complete reference genome was used because it had the closest phylogenetic relationship with *A. dolens*. In addition, the *A. insolitus* reference genome (accession: GCF_008245125.1) was considered for the analysis, as it showed ANI>95% with some genomes.

The phylogenetic tree was generated performing a core genome alignment using the parsnp tool of the Harvest-OSX64-v1.1.2 suite [39]. The tree file in newick format was used as input in Microreact [40] together with a metadata file for visualization.

Functional annotation of relevant genomic variants

Analysis of variants was performed according to the Bacdist pipeline [41] considering all the genome sequences coming from the same patient and belonging to the same clone type (maximum SNP distance between clonally related isolates was 217) against the closest reference genome. Details on variant analysis results are available in Table S3. The amino acid sequences of genes presenting variants or mutations (here defined as variants that cause gene loss of function) were extracted and, using eggno-mapper v1 [42], each gene product was classified in one of 25 eggno functional categories, further grouped into four eggno main categories: information storage and processing, cellular processes and signalling, metabolism, and poorly characterized.

Virulence factors and antibiotic resistance genes

In order to determine the presence of virulence factor and antibiotic resistance genes, the *de novo* assemblies were analysed using BLAST [43] against the PATRIC database (accessed January 2020) [44] and using Abricate [45] to search against the Resfinder database (accessed January 2020) [46]. Matches were filtered, thus considering matches with at least 50% gene coverage and 95% gene identity.

The presence of mutations was evaluated in two ways. To analyse genes that are present in the reference genomes, the defence mechanism category of the eggno-mapper output was taken into consideration: the presence of mutations was evaluated, and the functional impact of missense variants was predicted using the Provean protein tool. In order to analyse genes that are present in the isolates genomes but not in the reference, BLAST and Abricate outputs were considered: genes having an identity <100% were analysed by extracting the corresponding amino acid sequence and using BLASTP against the non-redundant (nr) database. The functional effect of each mutation was predicted using the Provean protein tool [47]. Heatmaps were generated for visualization purposes using the pheatmap R package v1.0.8 [48]. Fisher's exact test was performed per clone type to ascertain the significance of results of statistical testing using R v3.5.

Mutator genes

The genetic basis of hypermutation was investigated from whole genome sequencing data by analysing genes involved in this phenomenon. The following list of mutator genes taken from the literature was considered: *mutL*, *mutS*, *ppfI*, *superoxide dismutase*, *radA*, *radC*, *rad50*, *uvrA*, *uvrB*, *uvrC* and *uvrD* [33]. In order to verify the presence of hypermutator strains, a combination of factors such as the occurrence of high-impact mutations and deleterious missense in mutator genes (Table S4), the transition/transversion rate and overall and per-site mean yearly variant rate (Table S3) were taken into consideration. Additional information on the classification of the isolate is reported in Table S5.

RESULTS

Fifty-four *Achromobacter* clinical isolates were longitudinally collected over 5 years from the sputum samples of 26 patients followed at the CF Centre of Verona (Italy). Among them, 17 presented chronic lung infection while nine were occasionally colonized. Fig. 1 shows the sample collection timeline and the first colonization event for each patient (data available since 2013). In 10 patients with chronic infection (P01, P02, P05, P08, P09, P10, P13, P18, P19, P21) we collected isolates identified as early colonisers (<1 year from first colonization); the first isolate collected from four of them (P02, P09, P13, P21) coincides with the first colonization event. Moreover, we collected both early and late (>1 year from first colonization) colonizing strains from four chronically infected patients (P01, P05, P08, P10). In two of these patients (P01, P05) early and late isolates were isolated a few months apart (5 months between isolates 1–1 and 1–2; 2 months between isolates 5–3 and 5–4). From two patients with occasional infection we collected *Achromobacter* species twice during the sampling periods (~3 months apart in P06, ~5 months apart in P12).

Phylogenetic analysis

Fig. 2 shows the phylogenetic tree based on core genome alignment of 54 collected *Achromobacter* species isolates and seven reference genomes. In order to accurately classify the isolates in the genus *Achromobacter*, we calculated the ANI among all available *Achromobacter* species genomes and the genomes of our isolates. As a result of the phylogenetic analysis, five *Achromobacter* species were identified: *A. aegrifaciens*, *A. dolens*, *A. insolitus*, *A. insuavis* and *A. xylosoxidans*. The majority of isolates was identified as *A. xylosoxidans* (67%, $n=36$), followed by *A. insuavis* (13%, $n=7$), *A. dolens* (7%, $n=4$), *A. aegrifaciens* (7%, $n=4$) and *A. insolitus* (6%, $n=3$). Among a total of 26 patients, 17 (65%) were infected by *A. xylosoxidans*, three (12%) by *A. aegrifaciens*, three (12%) by *A. insolitus*, two (8%) by *A. insuavis* and one (3%) by *A. dolens*. Interestingly, four strains [3] isolated from two patients (P03, P16) showed an ANI <95% against all the analysed genomes suggesting that they likely belong to *Achromobacter* species with no genomic assembly available yet. Because solely based on phylogenetic information, in this study we refer to them as non-affiliated genogroups (NGs).

A. xylosoxidans and *A. insolitus* were collected from both patients with chronic and occasional infections. By contrast, *A. aegrifaciens* was only observed in occasionally infected patients while *A. insuavis*, *A. dolens* and NG isolates established chronic infections. For each of the patients with more than one isolate (10 with chronic and two with occasional infection) the genetic relatedness between genomes was also evaluated. As expected, all strains isolated from each chronic patient were clonally related (maximum SNP distance between clonally related isolates was 217). For the occasionally infected patients with more than one isolate, one of them (P06) harboured clonal isolates while different clones were identified in the other one (P12).

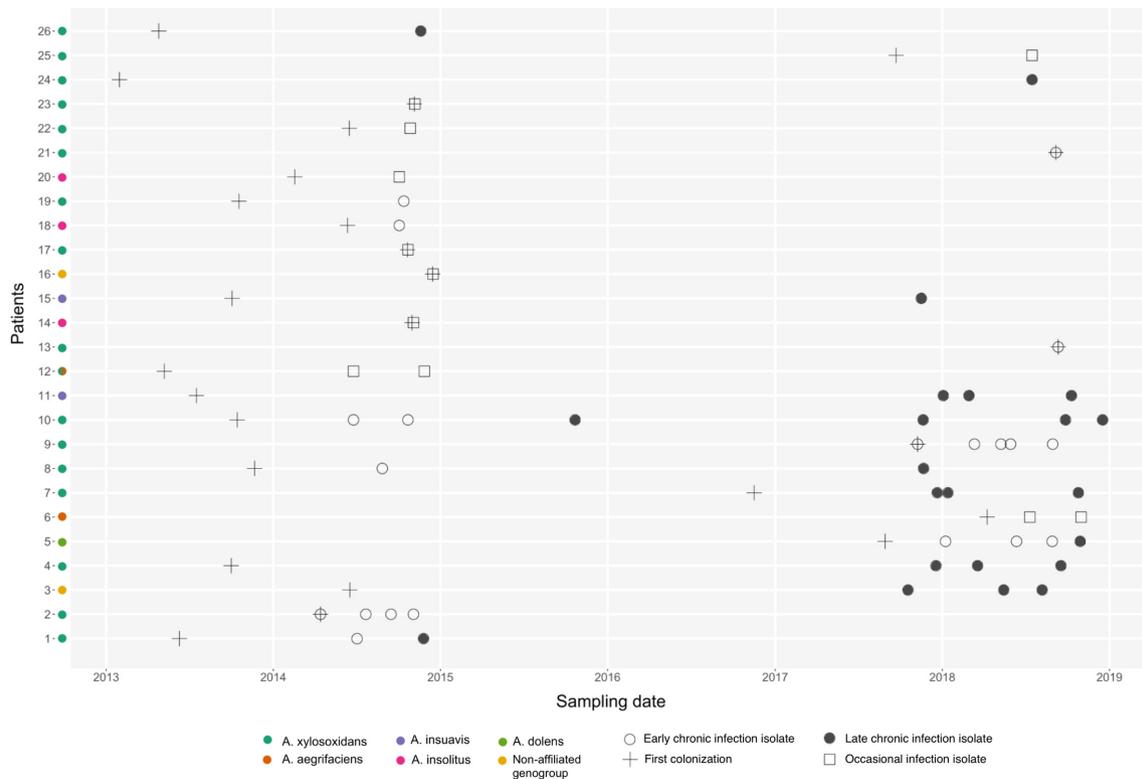


Fig. 1. Isolate collection timeline. Dates of collection of *Achromobacter* species isolates and first colonization event are shown for each patient enrolled in the study. A minimum of one isolate and a maximum of six isolates were collected from 26 patients between 2014 and 2018. +=first colonization event; □=occasional isolates; ○=early chronic isolates; ●=late chronic isolates; in some cases, isolates correspond with the first colonization event (superimposed symbols). In the left column, patient ID numbers are reported; coloured dots indicate the *Achromobacter* species of the isolates collected from each patient; a bicoloured dot indicates that isolates belonging to two species were recovered from the patient.

Virulence factors and antibiotic resistance

The results of the virulence factor analysis (Fig. 3) showed that argininosuccinate synthase, carbamoyl phosphate synthase large chain, *hfq* and large subunit ribosomal protein L36p are present in all collected isolates. These proteins are mainly involved in protein synthesis and stress response. The differences among early chronic infection isolates ($n=20$, <1 year from first colonization), late chronic infection isolates ($n=23$, >1 year from first colonization) and occasional infection isolates ($n=11$) were investigated. To evaluate differences in gene functionality, we took into account the presence of mutations in virulence factor genes (Table S6). All late chronic isolates ($n=23/23$) and 90% ($n=18/20$) of early chronic isolates carried functional genes associated with the ability to grow in an anaerobic environment (17 clonotypes, $P=0.002$), while only 45% ($n=5/11$, four clone types) of occasional isolates had this potential. Moreover, 96% ($n=22/23$) of late chronic isolates lacked genes involved in LPS production or carried mutations in these genes (10 clone types, $P=0.036$). Occasional isolates showed a significantly lower content of functional genes associated with the ability to infect cells (55%, $n=6/11$, five clone types, $P=0.028$) and, to a lesser extent, chemotactic

movement (55%, $n=6/11$, five clone types, $P=0.194$) and T3SS (73%, $n=8/11$, seven clone types, $P=0.11$).

Focusing on differences among species, the results suggested that *A. aegrifaciens* ($n=3$), *A. insolitus* ($n=3$) and all the NG isolates ($n=4$) carried a restricted number of genes related to virulence. The analysis showed the absence of respiratory nitrate reductase (anaerobic growth), GDP mannose 4,6 dehydratase (pathogenicity) and *liN* (flagellar protein) genes in *A. aegrifaciens*, *A. insolitus* and one NG isolate. T3SS genes were also absent in these isolates and in one additional *A. xylosoxidans* occasional isolate. *fliP* and *fliC* genes (flagellar proteins) were not present in *A. aegrifaciens* or *A. insolitus* genomes together with the serum resistance-linked gene *yihY*, which was also absent in all NG isolates. Endotoxin-related genes were all found to be present in some *A. xylosoxidans* genomes while a putative oxidoreductase was also detected in all *A. insuavis* isolates ($n=4$). Moreover, UDP 2 acetamido 3 amino 2,3 dideoxy D-glucuronic acid acetyltransferase gene (O antigen biosynthesis) was also found in *A. dolens* genomes ($n=4$). Finally, the putative cysteine hydrolase *ycaC* gene was found in *A. aegrifaciens* and in the majority (94%, $n=34/36$) of

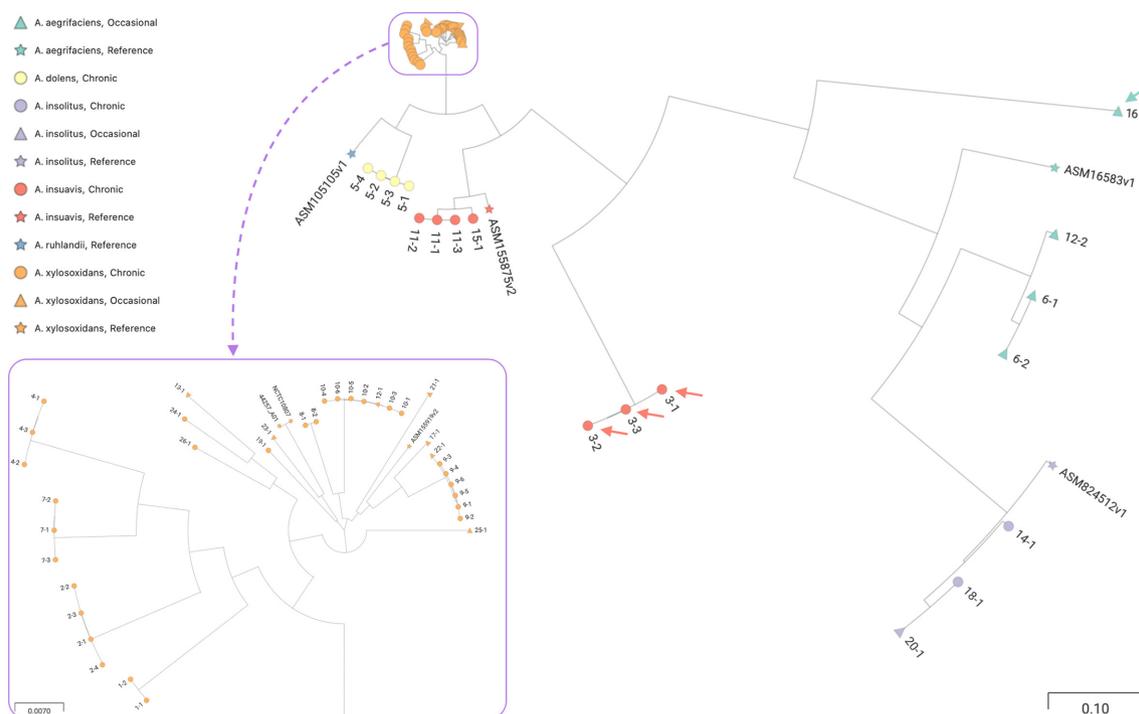


Fig. 2. Phylogenetic tree based on core genome SNPs of 54 collected *Achromobacter* species isolates and eight reference genomes. Δ indicates occasional infection isolates, \circ indicates chronic infection isolates, while \star indicates a reference genome. The symbols are coloured according to the *Achromobacter* species identified with ANI analysis. Arrows indicate genomes with ANI < 95 % against all the analysed reference genomes; in this case, symbols are coloured according to the closest *Achromobacter* species reference genome that was used for variant analysis. Genomic distance scales are reported at the bottom of the figure.

A. xylosoxidans isolates, while *motA* (flagellar motility) was only present in *A. aegrifaciens* genomes.

Differences among chronic and occasional isolates were also investigated by species. In contrast to the chronic isolates, *A. xylosoxidans* occasional isolates ($n=5/36$) all carried *fliP* and NADP-dependent malic enzyme (growth in gluconeogenic conditions) genes while occasional isolates belonging to the NGs ($n=1/4$) lacked *cheB*, *cheW*, *cheY* (chemotaxis), *fliN* (flagellar protein), *yscS* (T3SS) and respiratory nitrate reductase. Conversely, no differences were detected among *A. insolitus* chronic and occasional isolates ($n=3$).

From the results of the antibiotic resistance genes (Fig. 4), the most represented genes are the sulfonamide resistance gene *sul1* and the *aac(6')* family aminoglycoside acetyltransferase, followed by class D β -lactamase *bla*_{OXA} genes. The analysis showed a species-specific distribution of *bla*_{OXA} genes: *bla*_{OXA-114} in *A. xylosoxidans*, *bla*_{OXA-243} in *A. insuavis*, *bla*_{OXA-364} in *A. dolens*, *bla*_{OXA-2} in *A. insolitus* (except for isolate 14-1 carrying no *bla*_{OXA} genes), and no *bla*_{OXA} genes in *A. aegrifaciens* or NG isolates. Moreover, *bla*_{CARB-2} was found in isolates from patient P01. In addition to *bla*_{OXA} genes, 31% of all isolates carried other antibiotic resistance genes. Among the isolates presenting deleterious variants – compromising gene function – in antibiotic resistance genes (Table S7), 54% ($n=7/13$) carried mutations in at least one *bla* gene.

Mutator genes

The genetic basis of hypermutation was investigated by verifying the occurrence of high-impact mutations and deleterious missense within genes involved in this phenomenon: *mutL*, *mutS*, *pfpI*, superoxide dismutase, *radA*, *radC*, *rad50*, *uvrA*, *uvrB*, *uvrC* and *uvrD* [33]. Moreover, a combination of factors such as the transition/transversion rate and overall and per-site mean yearly variant rate were taken into consideration. Similarly to other CF pathogens presenting hypermutation events during chronic infection, in this study no occasional isolates of *Achromobacter* species ($n=11$) showed hypermutator characteristics, while 60% of early chronic isolates ($n=12$, 4/10 patients) and 78% of late chronic isolates ($n=18$, 8/11 patients) were classified as hypermutators. In particular, patients from whom we collected both early and late isolates ($n=4$) showed different hypermutation onset patterns: P01 and P05 presented hypermutant strains in early isolates, in P10 hypermutation developed in late isolates and P08 isolates showed no hypermutation-linked phenomena.

The results of the mutator genes analysis, reported in Fig. 5, showed that all *A. dolens* ($n=4$), *A. insuavis* ($n=4$) and NG isolates ($n=3$) presented two different *mutS* genes. Moreover, *A. insolitus* ($n=3$), *A. dolens* and NG isolates lacked *radC* while *A. dolens* and NG isolates also lacked a functional *uvr* system.

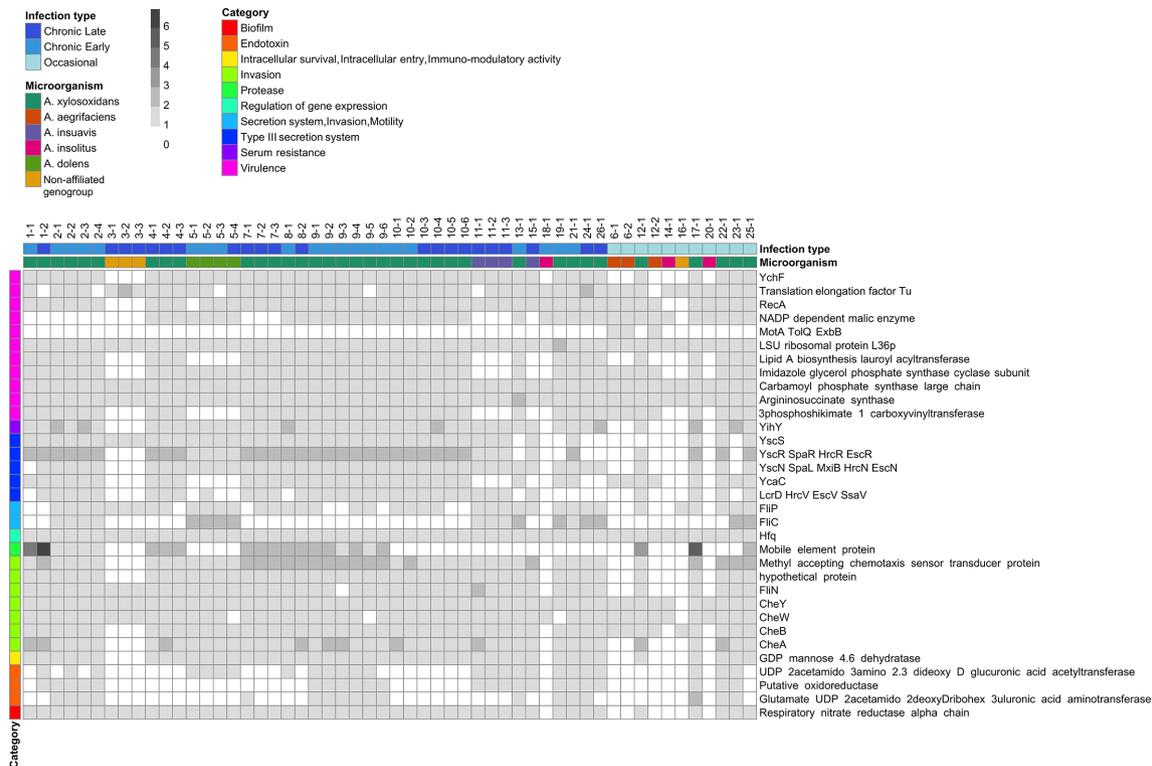


Fig. 3. Virulence factor genes identified in *Achromobacter* species isolates. The heatmap shows the presence and number of virulence factor genes on a grey scale. Additional information regarding the microorganism species, infection type and gene functional annotation (PATRIC categories) is presented in the annotation rows and column.

DISCUSSION

Achromobacter species are opportunistic pathogens that can cause lung infections in CF patients. Although chronic colonization has been associated with a decline in respiratory function, increased frequency of exacerbations and lung inflammation, little is still known about its pathogenic mechanisms. To provide new insights about *Achromobacter* virulence, antibiotic resistance and evolution, we performed bioinformatics analysis of 54 whole genome sequences and compared these features between chronic and occasional isolates, as well as among different *Achromobacter* species. Although patients carrying these isolates were defined as chronically or occasionally infected based on their clinical microbiological history since 2013, clinical isolates included in this study were mainly collected in 2014–15 and 2017–18, thus limiting the continuous observation of adaptation during these 5 years. Nonetheless, they still provide longitudinal data that can support the understanding of pathogenicity and persistence, particularly in poorly characterized microorganisms such as *Achromobacter* species. Moreover, we further compared isolates collected within the first year of chronic colonization (early chronic isolates) with those collected later (late chronic isolates) in order to observe early changes occurring during the very first phase of chronic colonization, when eradication is still likely to be feasible, and identify markers of

persistence that might be associated with an enhanced risk of eradication therapy failure.

As expected, the majority of isolates belong to *A. xylosoxidans* (65%) while none was identified as *A. rhulandii*, the second most frequently recovered species in the USA and Argentina and previously reported to be responsible for outbreaks in Danish CF centres [49–51]. We also identified four other species whose clinical importance is less clear: *A. aegrifaciens*, *A. dolens*, *A. insolitus* and *A. insuavis*. The prevalence of *A. dolens* (3%) and *A. insolitus* (12%) in our patients was comparable with those reported in previous studies (2–17%) while dissimilarities were observed for *A. aegrifaciens* (12%) with respect to the literature (5%) [49, 50, 52–54]. Also, the prevalence of *A. insuavis* (8%) is similar to that described in Argentina (5%) but differs from Denmark (24%) [49, 50]. Interestingly, four isolates showed a low ANI (<95%) against all the available *Achromobacter* genomes, and thus we were unable to classify them within known species. This suggests that they might belong to *Achromobacter* species not yet sequenced or even to new species. In this study we considered them as non-affiliated genogroups. Predictably, all the longitudinal isolates from chronic patients belonged to the same *Achromobacter* species and were clonally related. Interestingly, the same situation also occurred in one of the occasionally infected patients (P06).

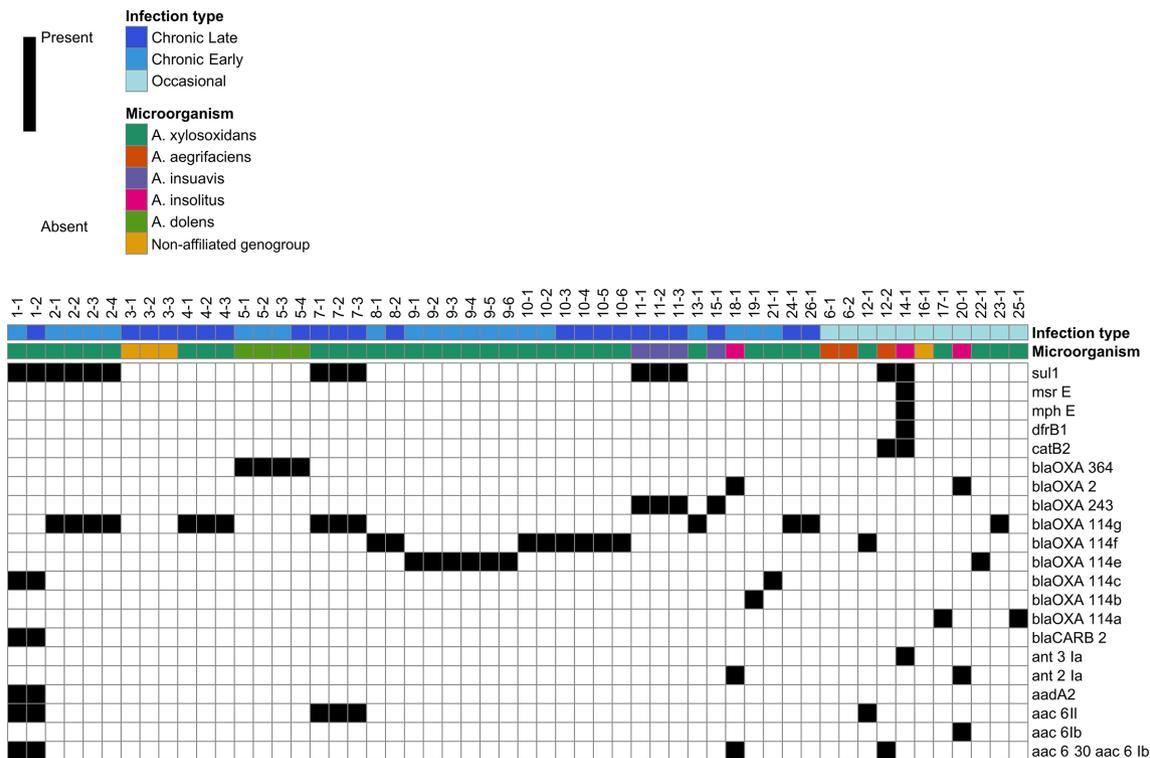


Fig. 4. Antibiotic resistance genes identified in *Achromobacter* species isolates. The heatmap shows the presence of antibiotic resistance genes on a black and white scale. Additional information regarding the microorganism species and the infection type is presented in the annotation rows.

Considering the species affecting chronic and occasional patients, *A. insuavis* and *A. dolens* were isolated only from chronically infected patients, *A. aegrifaciens* only from occasionally infected patients, while all the other species (*A. xylosoxidans*, *A. insolitus* and NGs) were found in both groups. For some species we only have a few isolates, and this may suggest that some *Achromobacter* species might be more likely to establish a chronic infection in CF patients. In particular, *A. aegrifaciens* – found in two occasional patients – lacks various genes related to virulence: this could limit its ability to colonize CF lungs. Indeed, *A. aegrifaciens* as well as the majority of occasional isolates showed a lack of functional genes related to the ability to infect cells, chemotactic movement, T3SS and anaerobic growth, whereas the great majority of chronic isolates have these potentials. In particular, anaerobic growth ability confers to the cells the ability to locate deeper within the mucous layer or within biofilm structures or in more hypoxic regions of the lung, where antibiotics can be dramatically less effective due to anaerobic conditions [55]. This can favour resistance of bacterial cells to antibiotic therapies and attack from neutrophils. Additionally, almost all ($n=22/23$) late chronic isolates lacked functional genes involved in LPS production, probably leading to a reduced recognition by the host defence system. For example, a reduction in the number of LPS lipid A acyl chains by other bacteria was shown to modulate the recognition of LPS by

toll-like receptors [56]. These might represent evolutionary mechanisms that favour bacterial persistence.

Regarding antibiotic resistance, we observed a species-specific distribution of *bla*_{OXA} genes. While the specificity of *bla*_{OXA-114}, *bla*_{OXA-243} and *bla*_{OXA-364} respectively for *A. xylosoxidans*, *A. insuavis* and *A. dolens* has already been reported in the literature [57], we observed no *bla*_{OXA} genes in *A. aegrifaciens*, NGs or one *A. insolitus* isolate (out of three). Additionally, we identified *bla*_{OXA-2} in two *A. insolitus* isolates. However, we found that *bla*_{OXA-2} does not follow a species-specific distribution when analysing all the available genome assemblies belonging to this species ($n=16$). Among the isolates presenting deleterious variants in antibiotic resistance genes ($n=13$), 54% carried mutations in at least one *bla* gene. These mutations have been bioinformatically predicted to compromise protein function, probably reverting an antibiotic-resistant phenotype to a sensitive one, potentially influencing the results of antibiotic profiling with implications for therapies.

No significant difference in resistance genes was found between chronic and occasional isolates. Besides *bla*_{OXA}, the most well-represented antibiotic resistance genes among all isolates are the sulfonamide resistance gene *sul1* and the *aac*(6') family aminoglycoside acetyltransferase, both frequently found within mobile genetic elements such as integrons, plasmids

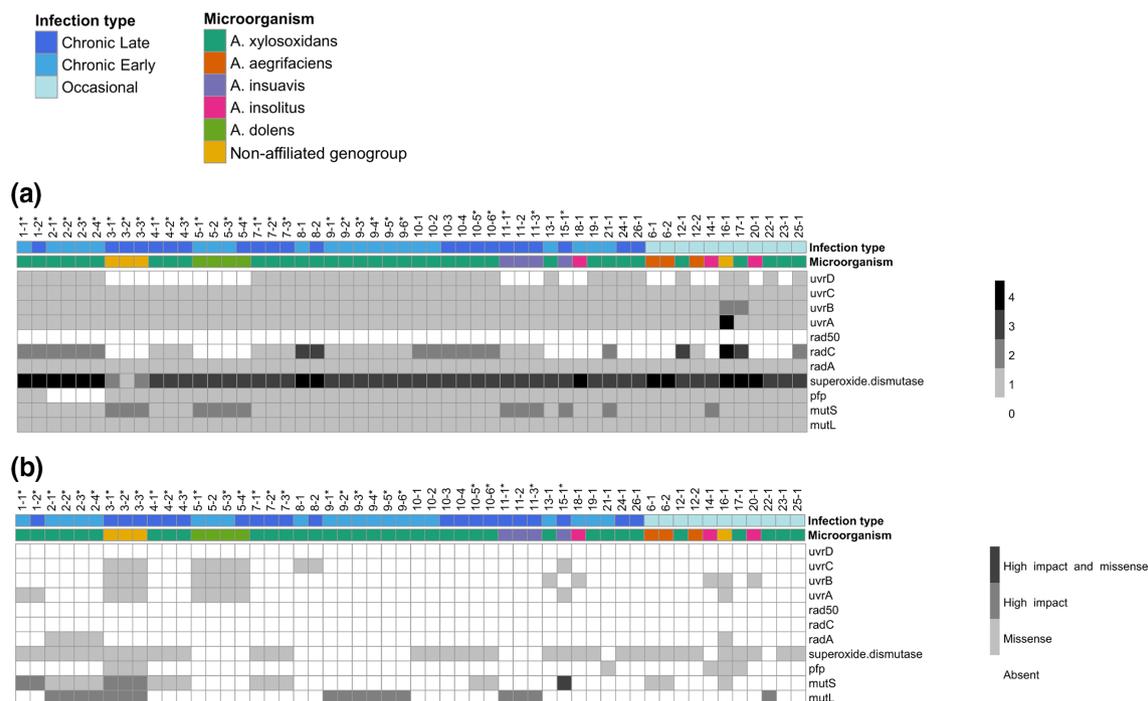


Fig. 5. Mutator genes identified in *Achromobacter* species isolates. Heatmap (a) shows the presence and number of mutator genes on a grey scale. Heatmap (b) shows the presence and type of mutations in these genes on a grey scale. Additional information regarding the microorganism species and the infection type is presented in the annotation rows. *Hypermutable isolates.

and transposons carried by other Gram-negative opportunistic pathogens [58]. Moreover, all the isolates belonging to one chronically infected patient (P01) carried the *bla*_{CARB-2} gene, usually found in *P. aeruginosa*, another microorganism frequently involved in CF lung infections. Interestingly, in this patient both *bla*_{CARB-2} and *sul1* were found to be carried by an integron. Moreover, we previously observed that these isolates carry various *P. aeruginosa* insertion sequences [34], supporting the possibility of inter-species genetic transfer. Further analysis of *Achromobacter* resistance-related mobile genetic elements could provide additional insights into antibiotic resistance acquired through its interaction with other microorganisms.

Overall, we detected a small number of genes linked to antibiotic resistance while a previous study using a multiple-database-based annotation approach predicted up to 50 drug-resistance associated genes in the *A. xylosoxidans* type strain [9]. However, that study analysed only one genome, using an approach that is hardly applicable to a large number of genome sequences due to the intensive manual effort required. To overcome this limitation, we first evaluated and compared the content of multiple databases to select the one able to ensure the most comprehensive outcome achievable with a more standard single-database-based approach. Nonetheless, the available databases report a restricted number of robustly annotated genes, not specific for *Achromobacter* species, probably leading to an underestimation of their presence. This lack currently limits the possibility of performing a more accurate

analysis of the antibiotic resistance genes on a large number of strains belonging to this specific genus and highlights the need of a more specific database.

Regarding bacterial evolution, we focused on the study of hypermutation – in which defects in DNA repair processes promote an increased mutation rate, one of the main mechanisms favouring bacterial persistence in CF airways. The occurrence of hypermutation during chronic colonization of the CF lung has been demonstrated for various microorganisms such as *P. aeruginosa* [32, 59–61], *Burkholderia cepacia* complex [62] and *A. xylosoxidans* [30, 63]. Similarly, no occasional isolates showed hypermutator characteristics while 60% of early chronic and 78% of late chronic isolates were classified as hypermutators. Interestingly, the results of analysis of the mutator genes showed that all *A. dolens*, *A. insuavis* and NG isolates presented two different *mutS* genes (mismatch repair). To our knowledge, this peculiarity has not previously been reported. To validate this finding, we analysed *mutS* genes present in the available reference genomes of *A. dolens* ($n=6$) and *A. insuavis* ($n=3$): while the former presented two copies of the gene, in *A. insuavis* its copy number (one or two) was variable among the assemblies. One could hypothesize that the presence of an additional *mutS* could decrease the hypermutability potential. However, we identified hypermutator isolates carrying mutations in a single *mutS* gene, thus suggesting that both genes are needed for effective mismatch repair and indicating that they have a complementary rather

than compensatory function. Further studies are needed to elucidate the role of *mutS* genes in these species, their involvement in hypermutability and their importance for pathogenicity.

The presence of hypermutators in the bacterial populations of the CF airways is often associated with the parallel occurrence of different subpopulations with different phenotypic traits [32]. The study of subpopulations requires the isolation of more colonies – preferably, morphologically different – from the same sample. In this study, we were able to identify morphologically different clones only in one sample (isolates 9–4 and 9–5), while a single isolate was collected from each of all the other samples. However, we cannot exclude that morphologically similar clones were present in the collected samples. Since the design of the isolate collection limited our ability to investigate *Achromobacter* subpopulations in CF airways, the variations that we observed over time within longitudinal isolates may reflect longitudinal evolution as well as intra-patient diversity. The most accurate approach to study intra-patient bacterial adaptation would be to analyse successively recovered clonally related isolates coming from the same patient against the first isolate recovered from that patient. However, due to a technological limit in the sequencing strategy (short-read technology) a complete genome of the first isolates could not be obtained. Thus, analysis of the isolates here was performed using a same-patient same-clone type approach; that is, we analysed all the isolates coming from the same patient and having the same clone type using the closest reference genome. In particular, for the majority of identified species the reference was a complete reference genome of the same species, while for *A. dolens* and NG isolates the reference was the closest complete reference genome available, since no complete reference genomes are available yet (e.g. *A. dolens* isolates were analysed against the *A. ruhlandii* reference genome).

In conclusion, the variety of virulence and antibiotic resistance genetic profiles observed in the different species underlines the importance of accurate species identification to properly manage the infection and apply diverse therapeutic regimens. Lung colonization not only by *A. xylosoxidans* but also by other species has already revealed clinically relevant consequences such as a high risk of pulmonary exacerbation [51] or high transmissibility among patients [6, 7]. Our results support that *Achromobacter* species can exhibit different adaptive mechanisms and suggest that some of them might be more useful to establish a chronic infection in CF patients, highlighting their importance for the clinical setting and the need for further studies of the less clinically characterized *Achromobacter* species.

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Author contributions

Conceptualization: A. S.; Methodology L. V.; Validation L. V., G. M.; Formal analysis L. V., A. S.; Investigation L. V., A. S., C. P.; Resources P. M., G. M., M. M. L.; Data curation L. V.; Writing – Original Draft Preparation L. V., A. S.; Writing – Review and Editing C. P., P. M., G. M., M. M. L.; Visualization L. V., A. S.; Supervision G. M., M. M. L.; Project Administration G. M., M. M. L.; Funding Acquisition M. M. L.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

Informed consent of all patients was obtained according to projects CRCFC-CEPPO026 and CRCFC-CEPPO031 approved by the local Ethical Committee.

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