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Amikacin exposure and susceptibility of macrolide-resistant *Mycobacterium abscessus*

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ABSTRACT *Mycobacterium abscessus* is associated with antibiotic resistance and poor treatment outcomes. We described within-patient changes in *M. abscessus* resistance to clarithromycin and amikacin.

Patients with amikacin exposure and a >50-month interval between *M. abscessus* isolates were identified. Antimicrobial susceptibility testing was performed on the first and last isolates by broth microdilution, and genetic markers of resistance were identified.

16 patients were identified with a median amikacin exposure of 2.3 years (range 0.6–8.6 years). 15 patients also received macrolides (median 7.2 years, range 1.3–10.7 years). All initial isolates were resistant to clarithromycin (minimum inhibitory concentration (MIC) $\geqslant$8 μg·mL^{-1}). Two patients had later susceptible isolates, which were of a different subspecies (*M. abscessus* subsp. *massiliense*) than the initial isolates (*M. abscessus* subsp. *abscessus*). All initial isolates were susceptible or intermediately resistant to amikacin, and only one patient had a resistant final isolate (MIC >64 μg·mL^{-1}), accompanied by an A→G mutation at position 1408 of the 16S ribosomal RNA. Forced expiratory volume in 1 s decreased significantly over the study period, while smear quantity and the proportions of patients with elevated C-reactive protein or cavitary lesions all increased significantly.

Despite prolonged, mostly inhaled amikacin exposure, development of amikacin resistance was uncommon in this patient population; however, disease progression continued.

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Patients with long-term amikacin treatment rarely develop resistance but their disease continues to progress http://bit.ly/2V7k0kH

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Introduction

Nontuberculous mycobacteria (NTM) are environmental mycobacteria associated with chronic lung disease, with increasing prevalence in the USA, Europe and Asia [1, 2]. *Mycobacterium abscessus* is the second most common NTM species [3], with poor treatment outcomes and resistance to most antibiotics [4, 5]. *M. abscessus* is comprised of three closely related sub-taxa, which will be referred to in this paper as *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *bolletii* [6].

The 2007 American Thoracic Society NTM guidelines note that for *M. abscessus*, no drug regimen has been shown to produce long-term sputum conversion, but suggest a macrolide (clarithromycin or azithromycin) with amikacin plus cefoxitin or imipenem as noncurative therapy [7]. Cure rates may depend upon macrolide resistance, with one study finding sputum conversion in 88% of patients with primarily clarithromycin-susceptible *M. abscessus* subsp. *massiliense*, compared with 25% in patients with *M. abscessus* subsp. *abscessus*, which is commonly clarithromycin resistant [8]. Amikacin has been associated with clinical and microbiological improvement in both intravenous [8] and inhaled [9] forms.

M. abscessus exhibits both acquired and inducible forms of macrolide resistance. Acquired resistance is conferred by mutations at position 2058 (A2058G/C/T) or 2059 (A2059G/C) in the 23S ribosomal RNA (rRNA) *rrl* gene [10, 11]. Inducible resistance, expressed following macrolide exposure in *M. abscessus* subsp. *abscessus,* requires a full length and functional *erm*(41) gene [12–15]. In contrast, most *M. abscessus* subsp. *massiliense* strains lack inducible resistance due to a 274-bp deletion in the *erm*(41) gene that renders it nonfunctional [8, 14]. An intact *erm*(41) gene can also be rendered nonfunctional by a T→C mutation at position 28 [13]. Amikacin resistance is conferred by single base pair mutations in the 16S rRNA gene (*rrs*): T1406A, A1408G, C1409T and G1491T [16, 17].

Relatively little research has focused on the development of amikacin resistance within *M. abscessus* and its relevance to clinical management. One retrospective study of amikacin treatment in refractory *Mycobacterium avium* complex (MAC) and *M. abscessus* patients found a 6% prevalence of amikacin resistance initially, with no resistance development detected in *M. abscessus* patients in follow-up testing within 12 months [18]. In a trial of liposomal amikacin for inhalation (LAI), nine out of 89 (10%) MAC and *M. abscessus* patients enrolled in the study had high-level (minimum inhibitory concentration (MIC) >64 $\mu g \cdot mL^{-1}$), mutation-confirmed amikacin resistance at the start and five patients developed resistance during the study [19]. In both studies, none of the patients with amikacin resistance achieved culture conversion during the study period [18, 19]. However, most patients had MAC infections, and the development of amikacin resistance while on treatment has not been studied for *M. abscessus* alone or after long treatment durations. We conducted a pilot study to identify changes in amikacin resistance among patients with serial isolates who have undergone prolonged antibiotic treatment. Additionally, we identified genetic markers of resistance and assessed changes in clinical, radiographic and microbiological measures over the study period.

Materials and methods

Records from patients enrolled in institutional review board-approved natural history studies (www.clinicaltrials.gov identifier numbers NCT00018044 or NCT00943514) [20], for which patients provided informed consent, were queried for the period January 1, 2005 to November 28, 2016 to identify patients with *M. abscessus* isolates. For the purposes of the pilot study, we selected the 16 patients with the longest follow-up (>50 months) between sequential isolates who received ⩾3 months of treatment with amikacin sulfate as part of a multidrug regimen. Most patients received inhaled amikacin with dosing as previously described ranging from 250–500 mg once or twice daily [9]. Through selection of patients with the longest duration of amikacin exposure, we maximised the likelihood of detecting development of amikacin resistance. Data were abstracted from medical records for the visits closest in time to the initial and final cultures, with visits >365 days from sample collection excluded. C-reactive protein (CRP) was dichotomised at the upper limit of normal and severity of bronchiectasis was dichotomised as a Reiff score >3 [21]. Culture conversion was defined as three consecutive sputum cultures negative for *M. abscessus*, collected ⩾4 weeks apart [22]. For analysis of bacterial load, the highest culture quantitation at each timepoint was used [9]. Statistical significance ($p<0.05$) of differences between timepoints was assessed with a paired t-test, Wilcoxon-signed rank test or McNemar's test.

Susceptibility testing

Antimicrobial susceptibility testing was performed by broth microdilution in Mueller-Hinton medium, using Sensititre RAPMYCO plates (Trek Diagnostic Systems; Thermo Fisher Scientific, Oakwood Village, OH, USA) according to Clinical and Laboratory Standards Institute (CLSI) M24-A2 guidelines [23]. Plates were evaluated at 3–5 days; clarithromycin MICs were additionally read at 14 days to detect inducible

resistance. Consecutive isolates showing changes in amikacin resistance underwent repeat testing for confirmation.

Sequencing of 16S rRNA, 23S rRNA and erm*(41) genes*

Strains were stored at −80°C in Tween albumin broth (Remel, Lenexa, KS, USA) and subcultured onto Middlebrook 7H11 agar (Remel). DNA was extracted with the Ultra Clean microbial DNA isolation kit (MoBio Laboratories, Solana Beach, CA, USA). Partial amplification and sequencing of 16S rRNA (rrs1-F and rrs1-R [16]), 23S rRNA (23S_18F, 23S_21R, 23SrRNAF_207 and 23SrRNAR_207R [24]) and *erm*(41) (ermF and ermR1 [14]) genes was performed on all study isolates, and compared to *M. abscessus* subsp. *abscessus* ATCC 19977^{T}, *M. abscessus* subsp. *massiliense* CCUG 48898^{T} and *M. abscessus* subsp. *bolletii* BD^{T} (CIP108541) (primers from IDT, Coralville, IA, USA). For all PCR reactions, 250 ng of genomic DNA was amplified and sequenced as described previously [25]. SeqMan Pro (version 13.0.2; DNAStar, Inc., Madison, WI, USA) was used for sequence assembly. Multiple-sequence alignment was carried out using the CLUSTALW algorithm in MegAlign (version 13.0; DNAStar, Inc.). Sequencing of *secA*, *rpoB* and *hsp65* genes [26] was also conducted on all samples to help confirm that the first and last isolates for each patient were the same strain.

Results

Demographics and antibiotic exposure

99 patients were identified with *M. abscessus*. 63 patients had multiple sequential isolates available for testing, of whom 34 had >3 months of cumulative exposure to amikacin. 16 patients with the greatest duration between isolates were selected, and 32 initial and final positive sputum cultures identified. For six cultures, multiple distinct mycobacterial colony types were noted, indicating significant heterogeneity of infecting strains. Isolation of different colony morphologies for identification is CLSI-recommended practice. Thus, for these cultures, an example of each colony type was isolated and identified, for a total of 38 *M. abscessus* isolates. The average interval between samples was 7.6 years (range 4.3–10.7 years) (table 1). Of the 16 patients, two had cystic fibrosis, one had primary ciliary dyskinesia and 13 had idiopathic bronchiectasis (table 1).

During the study period, patients were treated with amikacin for a median of 2.5 years (range 0.6–8.6 years) (tables 2 and 3, and figure 1). All patients received inhaled amikacin therapy during the study period, while only three patients received intravenous amikacin. Prior to the study period, eight out of 16 patients received treatment with either inhaled amikacin, intravenous amikacin or both. Of these, five patients were treated with intravenous amikacin, including two of the three patients who received it during the study period. Intravenous amikacin use was often limited by toxicity. Macrolides were also prescribed to 15 of the 16 patients during the study period, with a median duration of 7.2 years (range 1.3–10.7 years) (tables 2 and 3). Patients were additionally treated for *M. abscessus* with combinations of other medications (table 3).

Antibiotic susceptibility test results, and sequencing of 16S rRNA, 23S rRNA and erm*(41) genes*

Most patients (15 out of 16) had both first and last isolates that tested susceptible (MIC <16 μg·mL^{-1}) or intermediately resistant (MIC 32 μg·mL^{-1}) to amikacin. Only one patient (K) had a last isolate (K2) resistant (MIC >64 μg·mL^{-1}) to amikacin; this patient's first isolate was susceptible (MIC 16 μg·mL^{-1}) (table 4). Isolate K2 had an A1408G mutation of the 16S rRNA (table S1) known to confer aminoglycoside resistance in *M. abscessus* [16].

In the time between isolates K1 and K2, this patient received 611 days of amikacin treatment, including 30 days of intravenous amikacin. However, medical records indicate that this patient had a prior resistant isolate grown from a sample taken 324 days after the initial susceptible isolate (K1) (figure 1). During these first 324 days, the patient had 241 days of inhaled amikacin treatment.

TABLE 1 Baseline patient characteristics

Patients	16
Age at initial sample years median (range)	59 (18–74)
Female sex	12 (75%)
White race/ethnicity	13 (81%)
Cystic fibrosis	2 (13%)
Primary ciliary dyskinesia	1 (6%)
Idiopathic bronchiectasis	13 (81%)
Study interval years mean±SD	7.6±1.9

TABLE 2 Exposure to selected antibiotics over the study period

Antibiotic	Patients	Time for which used years median (range)
Amikacin [#]	16 (100%)	2.3 (0.56–8.6)
Inhaled amikacin including LAI	16 (100%)	2.3 (0.56–7.7)
Intravenous amikacin	3 (19%)	0.16 (0.08–0.88)
Macrolide	15 (94%)	7.2 (1.3–10.7)

LAI: liposomal amikacin for inhalation. [#]: includes LAI in the context of a clinical trial for six patients. Of these, four patients participated in the blinded phase of this trial and their allocation is unknown. For these patients, this measure includes prescriptions for "LAI or placebo". For all four patients, "LAI or placebo" accounted for <8% of their total amikacin exposure.

All isolates tested resistant to clarithromycin (MIC $\geqslant$8 μg·mL^{-1}), except for the last isolates of two patients (E and N) (table 4). While initial isolates (E1 and N1) showed inducible clarithromycin resistance (susceptible at 3–5 days but resistant by day 14), the later isolates (E2-1, E2-2, N2-1 and N2-2), tested susceptible to clarithromycin even after 14 days (MIC $\leqslant$2 μg·mL^{-1}) and harboured truncated *erm*(41) genes. Both first isolates (E1 and N1) were identified as *M. abscessus* subsp. *abscessus* and all the last isolates (E2-1, E2-2, N2-1 and N2-2) as *M. abscessus* subsp. *massiliense*. Based on the results of genetic sequencing of four genes, *erm* (41), *rpoB*, *secA* and *hsp65*, E1 was a different strain from isolates E2-1 and E2-2; E2-1 and E2-2 were genetically indistinguishable from each other. For the N isolates, all three were genetically distinct from each other and were classified as different strains.

Of the 14 remaining patients, first and last isolates from 12 patients showed full-length *erm*(41) genes, all with a T at position 28 and thus expected to confer inducible macrolide resistance. Acquired resistance to clarithromycin was observed in the first and last isolates from patients M and P (A2058C mutation of the 23S rRNA), which had truncated *erm*(41) genes. The last isolate from patient K (K2), which had a full-length *erm*(41), also exhibited acquired resistance to clarithromycin (A2058G mutation of the 23S rRNA), along with acquired aminoglycoside resistance (table 4 and table S1).

Sequence analysis of the erm*(41) gene*

By comparison of the *erm*(41) gene sequences, all of the serial isolates for each patient grouped within their cluster except for patients E and N, whose first isolates E1 and N1 harboured a full *erm*(41) gene while their last isolates (E2-1, E2-2, N2-1 and N2-2) showed a truncated gene. 13 isolates showed a 100% match to the *erm*(41) gene of *M. abscessus* subsp. *abscessus* ATCC 19977^{T}, corresponding to sequevar 1. Nine

TABLE 3 Other medications prescribed for treatment of *Mycobacterium abscessus*

Patient	Duration of amikacin exposure years	Duration of macrolide exposure years	Other *M. abscessus*-directed medications
A	8.6	10.2	Cefoxitin, clofazimine, imipenem, linezolid, meropenem, moxifloxacin, tedizolid, tigecycline
B	4.3	8.1	Linezolid, moxifloxacin, tigecycline
C	1.6	9.2	Linezolid, meropenem, moxifloxacin
D	0.6	10.7	Linezolid, moxifloxacin, tigecycline
E	2.4	3.3	Imipenem, linezolid, meropenem, moxifloxacin
F	7.5	8.7	Linezolid, moxifloxacin
G	1.2	8.1	Linezolid, meropenem
H	2.0	7.2	Linezolid
I	2.1	7.2	Clofazimine, linezolid, meropenem, moxifloxacin, tigecycline
J	5.3	6.3	Clofazimine, imipenem, linezolid, meropenem, tigecycline
K	1.7	5.1	Bedaquiline, cefoxitin, clofazimine, imipenem, linezolid, meropenem, tedizolid, tigecycline
L	1.1	1.6	Clofazimine, linezolid, meropenem, moxifloxacin
M	5.7	0.0	Clofazimine, linezolid, meropenem, moxifloxacin, tedizolid, tigecycline
N	1.3	1.8	Meropenem
O	2.9	6.0	Clofazimine, imipenem, linezolid, moxifloxacin
P	3.0	1.2	Cefoxitin, clofazimine, linezolid, meropenem, moxifloxacin

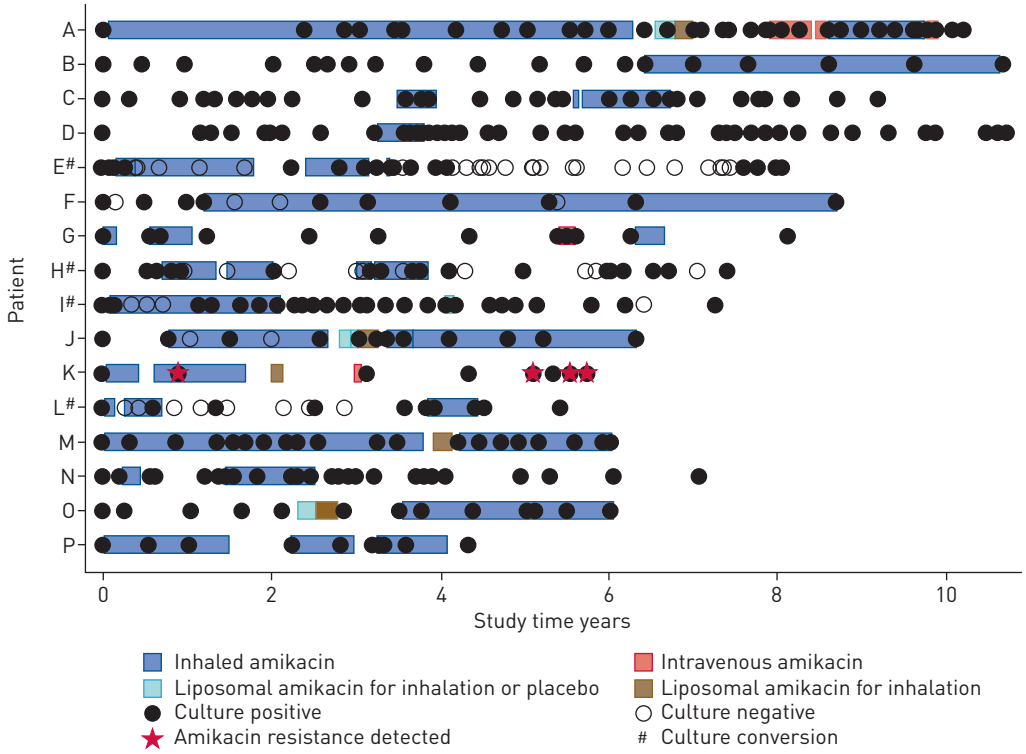

FIGURE 1 Timeline of positive and negative patient cultures over the study period relative to amikacin exposure. Amikacin resistance detected: resistance detected either through study assays or through clinical susceptibility testing. Lack of detected resistance for positive cultures is due either to lack of resistance or lack of susceptibility testing.

isolates had a 100% match to *erm*(41) gene sequevar 10 [25]. Five strains showed a 100% match to sequevar 6 and *M. abscessus* subsp. *massiliense* CI2040, while O1 showed 99.8% similarity.

The *erm*(41) genes of I1 and I2 matched 100% that of *M. abscessus* subsp. *massiliense* isolate CI8182 but were different from published *M. abscessus* sequevars (only a 98.5% match to sequevar 6). Isolates CI8182 and CI2040 were included in this manuscript as reference organisms to compare with isolates I1 and I2; CI8182, CI2040, I1 and I2 are all *M. abscessus* subsp. *massiliense* with a full *erm*(41) gene [25]. To the best of our knowledge, this is the first time multiple *M. abscessus* subsp. *massiliense* isolates with these full-length *erm*(41) sequevars have been characterised. Partial sequences of the *erm*(41) gene from strains I1, I2 and O1 were deposited in GenBank under accession numbers MG702334–MG702336, respectively. Eight isolates showed a truncated *erm*(41) gene (table S1).

Clinical, microbiological and radiographic findings

Measures of disease severity indicated disease progression in this cohort. Lung function, as measured by forced expiratory volume in 1 s, decreased significantly from a mean±SD of 89±19% predicted to 69±22% predicted, while the number of patients with elevated CRP increased from four of 16 to 11 out of 15 (table 5). Symptoms did not change significantly from the initial to the final visit. The proportion of patients with cavitary lesions increased significantly, from one out of 16 (6%) to seven out of 15 (47%), while the proportion of patients with a Reiff score indicative of severe bronchiectasis did not; most patients had severe bronchiectasis at the time of the first visit (12 out of 16, 75%) and this severity remained high at the final visit (13 out of 15, 87%). Semiquantitative acid-fast bacillus smear, a measure of bacterial load [9], increased significantly from a median of 1 (few) to 3 (many). Culture quantity also increased, from a median of 3 (scant) to 6 (heavy), although this change was not statistically significant. Four of the sixteen (25%) study patients experienced culture conversion (figure 1). Thus, although amikacin resistance did not develop in this patient population, as a whole, patients' disease did continue to progress.

Discussion

In this cohort of patients with *M. abscessus* disease and extended exposure to predominantly inhaled amikacin, only one out of 16 developed amikacin resistance, while on average, disease progressed in this patient group. Past studies have shown the potential for sporadic development of amikacin resistance in

TABLE 4 Phenotypic and genotypic antibiotic susceptibility results

Isolate	*M. abscessus* subsp.	Date of isolate	Isolate type	Clarithromycin MIC at 14 days μg·mL^{-1}	Constitutive clarithromycin resistance by 23S rRNA gene sequencing	Functional *erm*(41) by sequencing	Amikacin MIC μg·mL^{-1}	Amikacin resistance by 16S rRNA gene sequencing
A1	*abscessus*	Jan 24 2006	Rough	>16 (R)	No	Yes	16 (S)	No
A2	*abscessus*	March 22 2016	Rough	>16 (R)	No	Yes	16 (S)	No
B1	*abscessus*	Oct 12 2005	Rough	>16 (R)	No	Yes	16 (S)	No
B2	*abscessus*	June 10 2016	Rough	>16 (R)	No	Yes	32 (I)	No
C1	*abscessus*	Feb 6 2006	Rough	>16 (R)	No	Yes	16 (S)	No
C2-1	*abscessus*	April 8 2015	Rough	>16 (R)	No	Yes	32 (I)	No
C2-2	*abscessus*	April 8 2015	Rough	>16 (R)	No	Yes	16 (S)	No
D1-1	*abscessus*	Nov 8 2005	Rough	>16 (R)	No	Yes	16 (S)	No
D1-2	*abscessus*	Nov 8 2005	Smooth	>16 (R)	No	Yes	32 (I)	No
D2	*abscessus*	July 13 2016	Rough	>16 (R)	No	Yes	32 (I)	No
E1[#]	*abscessus*	Aug 26 2008	Smooth	>16 (R)	No	Yes	8 (S)	No
E2-1[#]	*massiliense*	Sept 1 2016	Rough	1 (S)	No	No	8 (S)	No
E2-2[#]	*massiliense*	Sept 1 2016	Smooth	0.12 (S)	No	No	4 (S)	No
F1	*abscessus*	Nov 29 2007	Rough	>16 (R)	No	Yes	8 (S)	No
F2	*abscessus*	Aug 4 2016	Rough	>16 (R)	No	Yes	8 (S)	No
G1	*abscessus*	July 10 2007	Rough	16 (R)	No	Yes	32 (I)	No
G2	*abscessus*	Aug 12 2015	Rough	>16 (R)	No	Yes	16 (S)	No
H1	*abscessus*	July 16 2008	Smooth	>16 (R)	No	Yes	16 (S)	No
H2-1	*abscessus*	Dec 1 2015	Rough	>16 (R)	No	Yes	16 (S)	No
H2-2	*abscessus*	Dec 1 2015	Smooth	16 (R)	No	Yes	16 (S)	No
I1	*massiliense*	Aug 5 2008	Rough	>16 (R)	No	Yes	8 (S)	No
I2	*massiliense*	Nov 4 2015	Rough	>16 (R)	No	Yes	8 (S)	No
J1	*abscessus*	May 11 2010	Smooth	>16 (R)	No	Yes	8 (S)	No
J2	*abscessus*	Sept 21 2016	Smooth	8 (R)	No	Yes	32 (I)	No
K1	*abscessus*	Sept 21 2010	Rough	>16 (R)	No	Yes	16 (S)	No
K2	*abscessus*	June 12 2016	Rough	>16 (R)	Yes	Yes	>64 (R)	Yes
L1	*abscessus*	Jan 4 2011	Smooth	>16 (R)	No	Yes	32 (I)	No
L2	*abscessus*	June 2 2016	Smooth	>16 (R)	No	Yes	16 (S)	No
M1	*massiliense*	Nov 10 2009	Rough	>16 (R)	Yes	No	16 (S)	No
M2	*massiliense*	Nov 10 2015	Rough	>16 (R)	Yes	No	8 (S)	No
N1[¶]	*abscessus*	April 2 2009	Smooth	>16 (R)	No	Yes	4 (S)	No
N2-1[¶]	*massiliense*	April 20 2016	Rough	<0.06 (S)	No	No	16 (S)	No
N2-2[¶]	*massiliense*	April 20 2016	Smooth	1 (S)	No	No	16 (S)	No
O1	*abscessus*	June 9 2010	Smooth	>16 (R)	No	Yes	16 (S)	No
O2-1	*abscessus*	June 9 2016	Smooth	>16 (R)	No	Yes	16 (S)	No
O2-2	*abscessus*	June 9 2016	Rough	>16 (R)	No	Yes	8 (S)	No
P1	*massiliense*	June 26 2007	Rough	>16 (R)	Yes	No	32 (I)	No
P2	*massiliense*	Oct 19 2011	Rough	>16 (R)	Yes	No	16 (S)	No

MIC: minimum inhibitory concentration; rRNA: ribosomal RNA; R: resistant; S: susceptible; I: intermediate. [#]: for the E isolates, based on sequencing of four genes, E1 was a different strain from isolates E2-1 and E2-2, which were genetically indistinguishable from each other; [¶]: for the N isolates, all three were genetically distinct from each other and were classified as different strains.

MAC among patients on inhaled amikacin [18, 27] or LAI [19]. However, rare development of resistance, despite long-term exposure to inhaled amikacin, suggests that this mode of exposure rarely induces resistance in *M. abscessus*, even at the extremes of treatment duration. Moreover, this study underestimates amikacin exposure, as treatment prior to the study period is not included. Further research could explore hypotheses related to the rarity of amikacin resistance in *M. abscessus*, including insufficient drug penetration or a high barrier to the development of resistance for this species.

Generalisability of these results is limited by selection for length of follow-up with positive sputum culture and thus, effectively, for treatment failure. While the culture conversion rate (for durable culture conversion without relapse) was only ~50% in a US population of *M. abscessus* patients similar to those at our centre [4], it was even lower in our study population, with only four (25%) out of 16 study patients classified as demonstrating culture conversion (with relapse or reinfection) during the study period. However, this rate is similar to other populations of patients with macrolide-resistant *M. abscessus* [8]. The worsening of disease severity during the study period is therefore not unexpected, given that we have effectively selected for patients who were culture positive at the time of their last visit, and therefore had refractory disease. Other limitations of the generalisability of these results include the almost exclusive use of inhaled amikacin sulfate and the few patients with the fibrocavitary form of the disease, which limit the applicability of the results to patients with *M. abscessus* treated with intravenous amikacin or those with fibrocavitary disease in the context of chronic obstructive pulmonary disease. While progression was not

TABLE 5 Clinical and radiographic outcomes

Symptom/severity measure	Initial visit (n=16)	Final visit (n=15[#])
Clinical		
FEV_1 % predicted mean±SD	89±19	69**±22
Elevated C-reactive protein	4 (25%)	11* (73%)
Cough	13 (81%)	15 (100%)
Haemoptysis	5 (31%)	2 (13%)
Fever	1 (6%)	3 (20%)
Night sweats	3 (19%)	5 (33%)
Fatigue	4 (25%)	3 (20%)
Sputum	13 (81%)	13 (87%)
Shortness of breath	6 (38%)	5 (33%)
Weight loss	2 (13%)	3 (20%)
Radiographic		
Cavitary	1 (6%)	7* (47%)
Reiff score ⩾3 lobes or 1 lobe with cystic BE	12 (75%)	13 (87%)
Microbiological		
Smear quantity*		
Negative	7 (44%)	4 (25%)
Few	2 (13%)	1 (6%)
Moderate	5 (31%)	1 (6%)
Many	2 (13%)	9 (56%)
Culture quantity[¶] median (IQR)	3 (3–6)	5 (3–6)

FEV_1: forced expiratory volume in 1 s; BE: bronchiectasis; IQR: interquartile range. [#]: no clinical or radiographic data were available within 365 days of the final visit for one patient, so for these variables, n=15; [¶]: according to a semiquantitative scale of 1 (liquid media only), 2 (one colony), 3 (scant), 4 (light), 5 (moderate) and 6 (heavy). *: $p<0.05$; **: $p<0.01$.

consistent across all radiographic and microbiological measures, this is likely due to the lower variability in Reiff score and smear quantity, and thus lower sensitivity to detect a change in these measures. Moreover, the effective dose of amikacin has not been established [9], and the variable doses used in this cohort may have influenced the development of resistance. Nonetheless, the lack of amikacin resistance in this subset of patients with progressing disease suggests that even in the absence of resistance, amikacin may be insufficient to decrease bacillary burden, although it may slow the rate of disease progression.

In vitro data from a hollow fibre model that mimics amikacin human pulmonary pharmacokinetics provides support for the hypothesis that lower level resistance could result in the observed poor bactericidal activity, leading to continued disease progression [28, 29]. In this model, despite a decrease in bacterial burden until day 14, bacterial burden increased above initial levels after that point. However, the amikacin-resistant subpopulation did not increase during that time, suggesting that lower level resistance could be leading to the observed poor bactericidal activity. This lack of efficacy is consistent with the described cure rates of *M. abscessus*, which range from 25% to 48% among patient populations primarily affected with the commonly macrolide-resistant subspecies *M. abscessus* subsp. *abscessus* [4, 8, 30]. Our patient population included patients with both *M. abscessus* subsp. *massiliense* and subsp. *abscessus*, but all initial isolates were resistant to macrolides. These studies highlight the need to prospectively study the efficacy of medication regimens for macrolide-resistant *M. abscessus* and its relationship to the antibiotic susceptibility of patient isolates, particularly to amikacin.

As only one patient in this study developed amikacin resistance (MIC >64 $\mu g \cdot mL^{-1}$), we cannot identify associated risk factors. This patient had cystic fibrosis and had a similar duration of exposure to amikacin compared with other subjects. Of note, the patient was one of three patients to receive intravenous amikacin during the study period; however, this amikacin was administered after the first isolate showing resistance and could not have contributed to its development. Prior to enrolment at the National Institutes of Health, this patient additionally experienced substantial amikacin exposure (including intravenous exposure).

Brown-Elliott *et al.* [13] recently described 10 *erm*(41) sequevars and the utility of *erm* sequencing to predict inducible macrolide resistance in *M. abscessus*. As noted previously, plates were evaluated at 3–5 days and 14 days to detect inducible resistance. Strains with MICs >16 $\mu g \cdot mL^{-1}$ were associated with seven sequevars (1, 4 and 6–10); the majority belonged to sequevar 1, matching the type strain (ATCC 19977^T). In our study, all 13 isolates matching sequevar 1 showed clarithromycin MICs in the resistant

range. Six isolates matching sequevar 6 and nine isolates matching sequevar 10 showed MICs ⩾16 μg·mL^{-1}, within the range reported by Brown-Elliott *et al.* [13]. Isolates I1 and I2, which harbour a full *erm*(41) gene sequence matching that of a previously reported *M. abscessus* subsp. *massiliense* strain (CI8182) with inducible clarithromycin resistance [25], also showed MICs ⩾16 μg·mL^{-1}.

While 14 of the 16 patients presumably had persistent infections with their initial strain, two patients (E and N) had first and last isolates from different subspecies. Patient E had a 3-year period of negative cultures, during which treatment was discontinued, suggesting cure and re-infection (figure 1). In contrast, patient N had consistently positive cultures while on intermittent treatment, indicating the possibility of a mixed infection. Given current culture protocols, the mixed infection may have been present since the first culture, with only the predominant strain being isolated. This finding is consistent with a past study showing 41% of patients were re-infected with NTM lung disease following successful treatment of *M. abscessus*, including five out of 14 patients who acquired a genetically distinct *M. abscessus* strain [31].

The limitations of this study include its small sample size and lack of analysis of interim isolates. The sample size was unavoidably limited by resource constraints but the examination of the remaining isolates may have identified further resistance. However, given the selection of patients for significant exposure, we maximised the chance of detecting resistance. Additionally, by analysing only the first and last isolates, we may have missed mixed infections or acquisition of new infections occurring between the first and last isolates. Given the potential for significant genetic diversity of *M. abscessus* isolates within a patient [32] and that only morphologically distinct colonies were isolated, it is also possible that additional, genetically distinct strains present in the patients were not isolated and thus excluded.

In this cohort of patients with long treatment durations, development of amikacin resistance was rare but outcomes were poor and re-infection occurred in two patients. These findings highlight the need for more effective therapies for macrolide-resistant *M. abscessus*, as well as strategies to prevent re-infection when treatment is successful.

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