Molecular assessment of mycobacterial burden in the treatment of nontuberculous mycobacterial disease

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Shareable abstract (@ERSpublications)
Molecular assays have been developed that are capable of quantifying NTM DNA in sputum. The assays were applied to 410 samples obtained from a cohort including patients on NTM treatment. The assays provide a fast and accurate measure of molecular burden. https://bit.ly/3TwmNmG

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Abstract

Introduction Nontuberculous pulmonary disease causes significant morbidity and mortality. Efforts to tackle infections are hampered by the lack of reliable biomarkers for diagnosis, assessment and prognostication. The aim of this study was to develop molecular assays capable of identifying and quantifying multiple nontuberculous mycobacterial (NTM) species and to examine their utility in following individual patients' clinical courses.

Methods DNA was extracted from 410 sputum samples obtained longitudinally from a cohort of 38 patients who were commencing treatment for either *Mycobacterium abscessus* or *Mycobacterium avium* complex or who were patients with bronchiectasis who had never had positive cultures for mycobacteria. NTM quantification was performed with quantitative PCR assays developed in-house.

Results The molecular assays had high *in vitro* sensitivity and specificity for the detection and accurate quantification of NTM species. The assays successfully identified NTM DNA from human sputum samples (*in vivo* sensitivity: 0.86–0.87%; specificity: 0.62–0.95%; area under the curve: 0.74–0.92). A notable association between NTM copy number and treatment (Friedman ANOVA (df)=22.8 (3), $p \leqslant 0.01$ for *M. abscessus* treatment group) was also demonstrated.

Conclusion The quantitative PCR assays developed in this study provide affordable, real-time and rapid measurement of NTM burden, with significant implications for prompt management decisions.

Introduction

Nontuberculous mycobacterial (NTM) pulmonary infection is increasing in prevalence throughout the world [1–3] and is associated with significant morbidity and mortality [4–6]. Mycobacteria are ubiquitous in the environment and their impact on individual patients is heterogeneous. Determining when and if to treat patients can be difficult. This is compounded by the fact that treatment involves multiple antibiotics over many months with disappointing success rates. Efforts to tackle infections in NTM are hampered by a lack of reliable biomarkers for diagnosis, assessment of disease activity and prognostication [7]. Consequently, decisions are currently based on mycobacterial culture results. Mycobacteria, however, are slow growing and significantly can take up to 8 weeks to culture. Data-backed treatment decisions are therefore not timely and are based on out-of-date information. In addition, culture-based results have significant issues with sensitivity owing to high false negative rates [8].

Culture-independent tests based on direct DNA detection have the potential to significantly improve the current situation. Previous studies using DNA-based approaches to directly detect and identify NTM species without the need for culture from respiratory studies have, however, provided disappointing results. The reported sensitivities are low, with NTM detected in only 29–76% of culture-positive samples [9–11]. In addition, these results continue to provide binary information with regards to the presence of

mycobacteria. No techniques have been developed to accurately quantify NTM burden. To provide real-time data for the latter would enable more accurate assessment of the impact of treatment on the clinical state and subsequent treatment management decisions.

This study describes the development of a quantifiable molecular test for the six most commonly pathogenic NTM species in the UK and the longitudinal application of the assays targeting *Mycobacterium avium* complex (MAC) and *M. abscessus* in a patient cohort over a period of 18 months.

Methods

Ethical approval

Ethical approval was obtained through the North West – Liverpool Central Research Ethics Committee (reference 16/NW/0849).

Recruitment

A total of 38 patients with either cystic fibrosis (CF) or non-CF bronchiectasis were recruited to the study. Patients were assigned to one of three separate groups based on the following inclusion and exclusion criteria:

1. Patients due to start treatment for *M. abscessus* complex or MAC (termed "*M. abscessus* treatment" and "*M. avium* treatment", respectively).
2. Patients with positive cultures for *M. abscessus* or MAC on more than one occasion but who were treatment naïve (termed "*M. abscessus* control" and "*M. avium* control", respectively).
3. Patients with bronchiectasis (CF and non-CF) who had never had positive cultures for mycobacteria (termed "control").

Patients were reviewed on a monthly basis to monitor treatment compliance.

Sample collection

Patients requiring treatment provided a sputum sample at screening, then on a weekly basis for the first month, then monthly samples up to and including 12 months with final samples at 15 months and 18 months. *M. abscessus* control, *M. avium* control and control patients provided a sputum sample at screening, then on a weekly basis for the first month followed by monthly samples up to and including at 3 months.

Initial screening sputum samples (for participants in the treatment groups, these samples were obtained before treatment commenced) were divided with a sterile scalpel blade into four. One aliquot was sent immediately for mycobacterial culture and one sample frozen immediately at −80°C. The two other samples were kept at room temperature for 24 h before being sent for culture or being frozen at −80°C. A 24-h freezing delay was performed to account for changes in microbiological communities that may occur between sputum expectoration and receipt of future samples (subsequent sputum samples were sent to the laboratory *via* first class postal delivery). All sputum samples were obtained spontaneously.

Upon receipt of subsequent samples, these were again divided into two aliquots with one aliquot sent for mycobacterial culture and the other frozen at −80°C.

Stored sputum samples were thawed prior to DNA extraction. Details of DNA extraction can be found in the supplementary methods.

Assay development

Owing to its relatively large interspecies variability and its preservation between isolates of the same species, the *hsp65* gene was selected as an assay target. Custom primers and probes (table 1) for the detection and quantification of the six most commonly pathogenic mycobacteria were designed in-house. The probes were tested in singleplex and multiplex. A series of pilot experiments was performed which demonstrated that the probes had a greater limit of detection when used in singleplex. NTM quantification was subsequently performed in singleplex. For details of primer and probe design, including sensitivity, specificity and level of quantitation feasible, please see the supplementary methods.

Quantitative PCR testing

DNA-extracted samples were diluted in a 1:50 ratio with PCR grade water. For each quantitative PCR (qPCR) reaction, 5 µL of diluted sample was combined with the assay. For quantification standards, 5 µL aliquots of serially diluted plasmids were used. Each standard and extracted sample was tested in triplicate to ensure reliability of results.

TABLE 1 Custom designed primers and probes used for identification and quantification of six nontuberculous mycobacterium species

Sequence (5′ to 3′)	Function in assay
CGAGACCAAGGASCAGATC	Forward primer
GCAGGCCGAAGGTGTTGG	Reverse primer
^{FAM}TGCCACCGCGGCCATC^{BQ1}	*M. avium* probe
^{HEX}GATTTCGGCGGGCGACC^{BQ2}	*M. intracellulare* probe
^{CY5}GGCCACGGCCGGTATCTCC^{BQ2}	*M. abscessus* complex probe
^{FAM}CGCGACCGCCGCGATCTCG^{BQ1}	*M. malmoense* probe
^{HEX}CATCTCCGCGGGTGACCAGG^{BQ2}	*M. xenopi* probe
^{CY5}GGCGACCGCGGCCATCTCCGCC^{BQ2}	*M. kansasii* probe

Superscript text represents the names of each fluorophore (5′ end) and the name of each quencher (3′ end) used.

Each sample was tested in singleplex against probes targeting *M. abscessus* complex, *M. avium* and *M. intracellulare* (the latter two forming the MAC). Positive qPCR results required positivity in triplicate. Assay constituents as well as PCR cycling conditions are detailed in the supplementary methods.

Results

Clinical characteristics of the cohort

A total of 410 sputum samples were obtained from the 38 patients (table 2). Of these, 15 patients had recurrent isolates of MAC and eight of these patients (53.3%) were started on treatment. A further 18 patients had recurrent isolates of *M. abscessus* and 14 of them (77.8%) were started on treatment. MAC treatment regimens were based on guideline-based therapy with rifampicin, ethambutol and either clarithromycin or azithromycin. The treatment of *M. abscessus* was also based on guidelines with intravenous meropenem, amikacin and tigecycline and oral clarithromycin or azithromycin for the initiation phase [12–14]. The continuation phase was more varied owing to patient antibiotic intolerance as indicated in table 2.

Eight of the 14 patients who commenced treatment for *M. abscessus* were culture negative 12 months after initial culture conversion (figure 1). These patients were therefore deemed to be cured by British Thoracic Society (BTS) and American Thoracic Society (ATS) criteria [12–14]. A total of 10 patients in the treatment group had positive cultures for more than one mycobacterial species, illustrating the frequency of mixed isolates and the inherent difficulty faced when selecting treatment regimes. Four of the eight patients who commenced treatment for MAC were culture negative 12 months after initial culture conversion (figure 1) and hence deemed to be cured by BTS and ATS criteria [12–14].

In the *M. abscessus* control and *M. avium* controls, all but one patient (patient 44) had a positive culture for the expected NTM species at a time point in the study period (figure 1). Individual NTM culture results, however, were inconsistent, with 28 of the 70 samples having negative cultures for NTM. All the patients recruited to the NTM culture-negative group (control) remained negative for the duration of the study. No patients in the control groups were maintained on prophylactic macrolides.

Impact of room temperature storage of samples

For each patient, screening sputum samples were taken prior to the commencement of treatment with subsequent samples being sent by patients to the laboratory *via* Royal Mail. To investigate the potential impact of the delay between sputum production and laboratory receipt and storage at −80°C, the screening sputum sample was aliquoted into two samples (see Methods, aliquots designated time point 1 and 2) and the NTM copy number (defined as the number of *hsp65* gene copies identified by qPCR) was compared between the two time points.

For the *M. abscessus* culture-positive patients, increases in *M. abscessus* copy number between time points 1 and 2 were observed in 12 of the 18 patients but they were not statistically significant (t-test p=0.53) (figure 2a).

For MAC culture-positive patients, eight of the 15 patients had an increase in MAC copy number between the two time points but again this was not statistically significant (t-test p=0.32) (figure 2b).

TABLE 2 Clinical characteristics of the study cohort

	M. avium		*M. abscessus*		Control	p-value[#]
	Treatment	Control	Treatment	Control		
Gender						0.9
Male	7	3	7	3	2	
Female	1	4	7	1	3	
Mean age at recruitment (years)	56.81	49.86	30.43	47.75	44.4	0.7
Smoking history						0.2
Current	1	1	0	0	0	
Previous	3	0	0	0	0	
Never	4	6	14	4	5	
Disease						0.01
Bronchiectasis	7	4	2	1	2	
Cystic fibrosis	1	3	12	3	3	
Medications[¶]						
Inhaled corticosteroids	3	4	11	3	4	0.8
Oral corticosteroids	0	0	1	3	0	0.4
Immunomodulation	1	0	0	0	0	0.09
Pulmonary function[+]						
FEV_1 % predicted (%)	65.49	72.16	75.36	50.58	47.9	0.4
FVC % predicted (%)	88.89	91.73	89.98	79.4	66.58	0.3
Body mass index ($kg \cdot m^{-2}$)	21.46	21.13	20.68	22.26	22.11	0.4
***M. avium* treatment**						
Rif, Eth, AZT	6					
Rif, Eth, Clari	2					
***M. abscessus* initiation phase**						
Clari, Amik (*i.v.*), Mero (*i.v.*), Tige			12			
AZT, Amik (*i.v.*), Mero (*i.v.*), Tige			2			
***M. abscessus* continuation phase**						
Clari, Amik (neb), Mino, Cipro			5			
Clari, Amik (neb), Mino, Clof			2			
Clari, Amik (neb), Mino, Septrin			1			
Clari, Amik (neb), Doxy, Cipro			1			
Clari, Amik (neb), Mino, Clof			1			
Clari, Mero (neb), Doxy, Clof			1			
Clari, Amik (neb), Doxy, Moxi			1			
AZT, Amik (neb), Mino, Cipro			1			
AZT, Amik (neb), Doxy, Cipro			1			
AZT, Colo (neb), Mino, Cipro, Septrin			1			

Data are presented as n or mean per group, unless otherwise indicated. FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; Rif: rifampicin; Eth: ethambutol; AZT: azithromycin; Clari: clarithromycin; Amik: amikacin; *i.v.*: intravenous; Mero: meropenem; Tige: tigecycline; neb: nebulised; Mino: minocycline; Cipro: ciprofloxacin; Clof: clofazamine; Septrin: co-trimoxazole; Doxy: doxycycline; Colo: colomycin. [#]: Kruskal–Wallis rank sum test; [¶]: use of medication that potentially causes increased susceptibility to infection (immunomodulation was the use of monoclonal antibodies); [+]: prior to treatment initiation.

M. abscessus *treatment group: quantitative assessment of mycobacterial burden*

A total of 192 samples was obtained for the *M. abscessus* treatment cohort (n=14 patients). Of these, 46 of the samples were culture positive for *M. abscessus*. In addition, 11 samples were culture positive for *M. avium*, four samples were positive for *M. intracellulare*, two samples were positive for *M. xenopi* and one sample was positive for *M. kansasii* (figure 1).

For the *M. abscessus* treatment cohort, overall burden decreased significantly during antibiotic treatment (figures 3a and 4) (Friedman ANOVA (df): F_r=22.8 (3), p⩽ 0.01) with significant differences between time points 1 and 3, time points 2 and 3 (p=0.02) and time points 2 and 4 (p=0.04) (Wilcoxon signed rank test). In contrast, for the *M. abscessus* control cohort the molecular load was more stable (Friedman ANOVA (df): F_r=2.2 (3), p=0.5) (supplementary figure S2).

There was an association between qPCR results, negative culture and treatment adherence (figure 3a–c, respectively). Four patients experienced intolerable treatment side-effects (patients 2, 18, 23 and 35) and

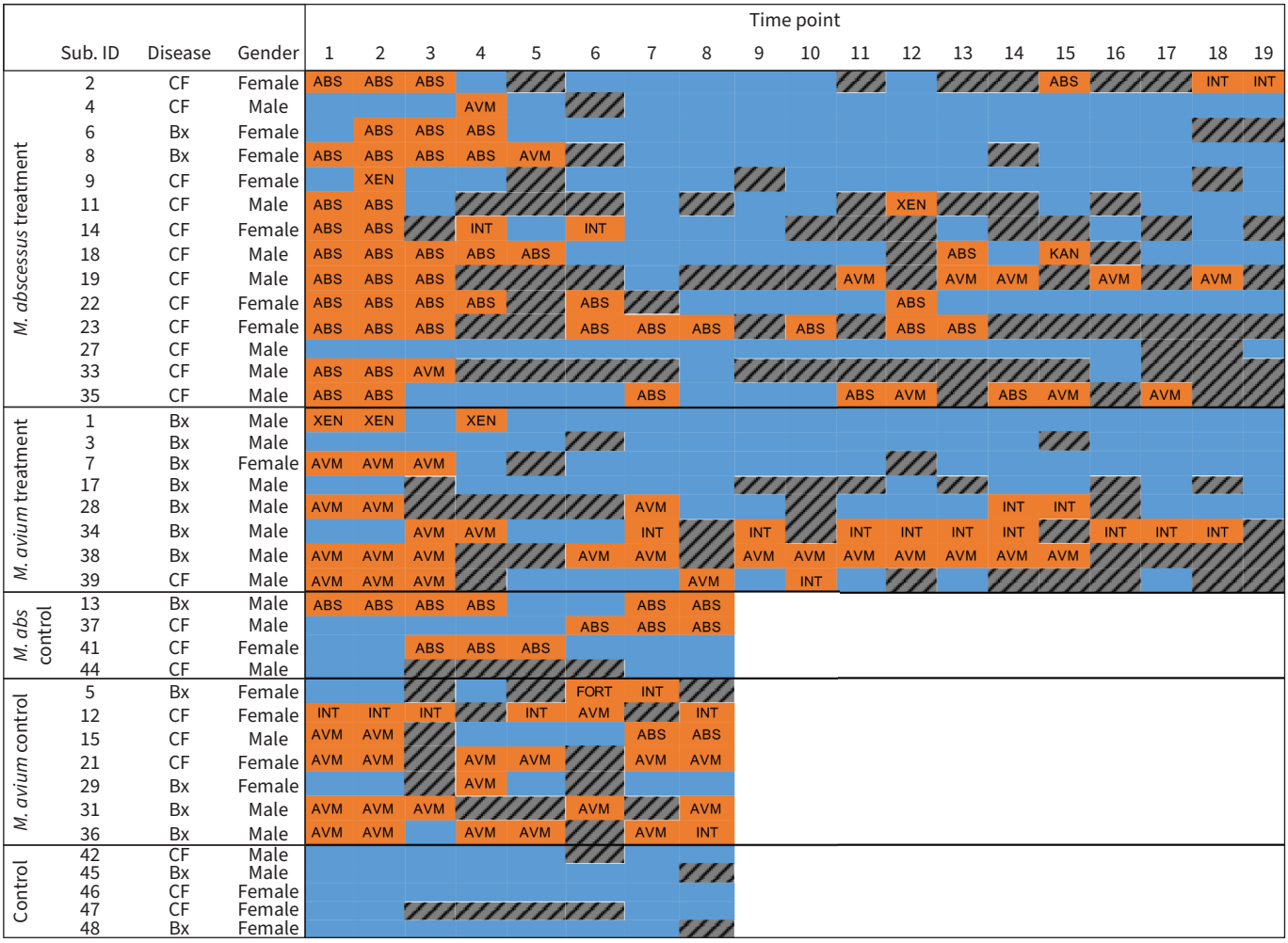

FIGURE 1 Mycobacterial culture results by patient and time point. Blue squares indicate a negative culture result while orange indicate a positive result. The name inside the orange box is the nontuberculous mycobacterial species. Hashed boxes indicate that no sample was available for the patient. Time points 1 and 2 were the pre-initiation of treatment; 3–6 were weeks 1–4; 7–17 were months 2–12; 18 was month 15; and 19 was month 18. CF: cystic fibrosis; Bx: bronchiectasis; ABS: *Mycobacterium abscessus*; AVM: *M. avium*; INT: *M. intracellulare*; XEN: *M. xenopi*; FORT: *M. fortuitum*.

therefore had varying compliance. These four were the only patients who were culture and qPCR positive beyond time point 6 (1 month of treatment). For three of the patients, *M. abscessus* burden appeared to be resolved by implementation of an alternative oral antibiotic regime. For the fourth patient (patient 23), *M. abscessus* copy number increased beyond and remained greater than pretreatment levels at time point 6 and was only addressed by the reinstatement of an initiation therapy at time point 12.

MAC treatment group: quantitative assessment of mycobacterial load

For the MAC treatment cohort (*M. avium* treatment, n=8), a total of 117 samples were obtained. Of these, 24 were culture positive for *M. avium*, 12 for *M. intracellulare* and three for *M. xenopi* (figure 1).

For the majority of patients, treatment resulted in a reduction of MAC burden (figure 5). The effect demarcation was not as clear as that seen for the *M. abscessus* treatment cohort (figure 4), most likely due to the absence of an intravenous induction phase in the MAC cohort. The decrease in MAC copy number during treatment did not reach significance (Friedman ANOVA (df): F_r=3.75 (3), p=0.3) (figure 5).

No decrease in MAC copy number was observed among the MAC control (untreated) group (Friedman ANOVA (df): F_r=3.01 (3), p=0.4) (supplementary figure S3).

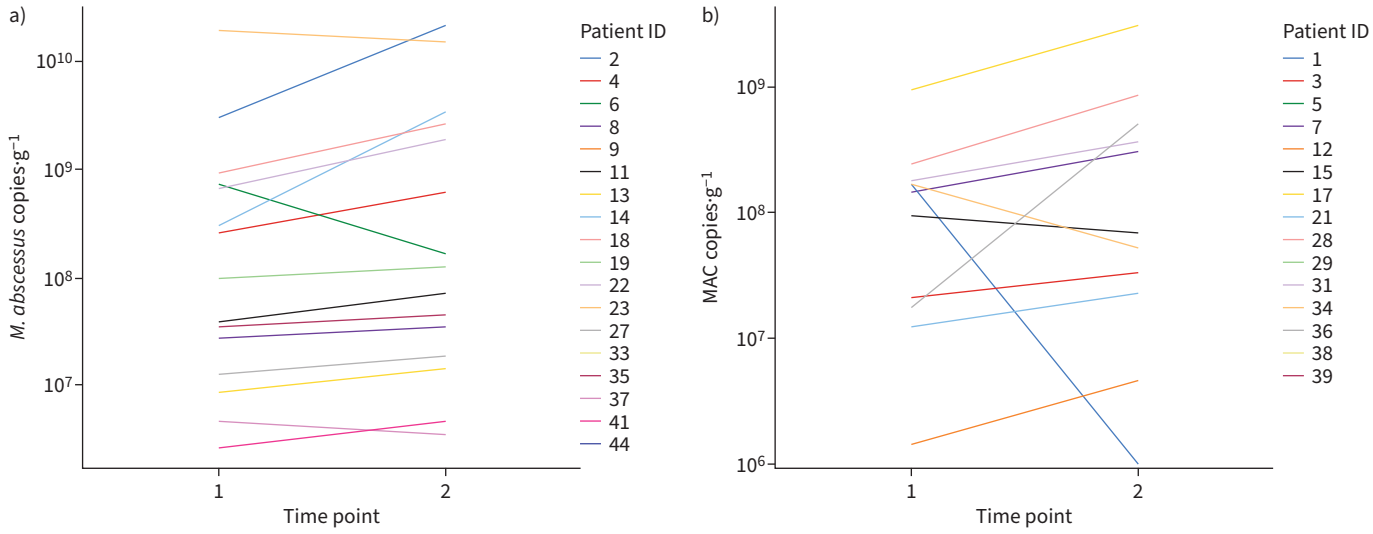

FIGURE 2 Samples were derived from the same sputum sample but sample time point 2 incurred a 24 h time delay before processing. a) *Mycobacterium abscessus* copy number between time points 1 and 2. Mean±SD copy number at time point 1 was $1.39\times10^{9}\pm4.44\times10^{9}$ copies·g^{-1} sputum and at time point 2 was $2.49\times10^{9}\pm5.79\times10^{9}$ copies·g^{-1} sputum (p=0.53). b) *M. avium* complex (MAC) copy number between time points 1 and 2. Mean±SD copy number at time point 1 was $8.2\times10^{7}\pm2.39\times10^{8}$ copies·g^{-1} sputum and at time point 2 was $2.32\times10^{8}\pm7.8\times10^{8}$ copies·g^{-1} sputum (p=0.32).

For three of the patients (patients 1, 3 and 17) there were clear disparities between culture and molecular results, with the latter showing positive results despite the culture-based analysis being negative throughout the clinical course (figure 6b).

Some association with clinical course and patient compliance was also observed. Three patients (patients 34, 38 and 39) experienced problems at various stages of treatment that resulted in the temporary cessation of treatment for a period of ⩾2 months (figure 4). These points of treatment cessation were associated with peaks in MAC DNA copy number that in one patient (patient 39) were not replicated in culture analysis.

The control group: quantitative assessment of mycobacterial load

A total of 33 samples were available from the control cohort (n=5 patients). No samples were culture positive for NTM species (figure 1). No positive results were obtained when the samples were screened using the qPCR assay targeting *M. abscessus*.

For the MAC qPCR assay, positive results were obtained for samples from three patients (patients 42, 45 and 46). These results were not reciprocated by culture analysis. No significant difference was found in MAC copy number between time points (Friedman ANOVA (df): F_r=5.69 (3), p=0.13) (supplementary figure S4).

Concordance between culture and molecular results

Because diagnosis of NTM disease currently relies on culture of the pathological mycobacterial species [12, 13], we next examined the concordance between the mycobacterial culture (the reference standard) results and qPCR assay results (table 3 and supplementary results).

The custom qPCR assay for *M. abscessus* displayed excellent test characteristics, with a sensitivity of 0.87, specificity of 0.95, positive predictive value of 0.76 and negative predictive value of 0.98, resulting in an area under the curve (AUC) of 0.923 (table 3 and supplementary figure S1a).

Owing to a significant lack of concordance between culture-based results and the qPCR assays targeting *M. avium* and *M. intracellulare*, the assays were combined to form a MAC qPCR assay, whereby MAC copy number was equal to the sum of the *M. avium* and *M. intracellulare* qPCR assays. This was performed to mitigate for misidentification of culture-based samples. It is important to note that culture samples were identified using a commercial test that is subject to misidentification of samples rather than whole genome sequencing. When combined, the assays showed 78% concordance with positive culture results with a test sensitivity of 0.86, specificity of 0.62, positive predictive value of 0.35 and a negative predictive value of 0.95, resulting in an AUC of 0.741 (table 3 and supplementary figure S1d).

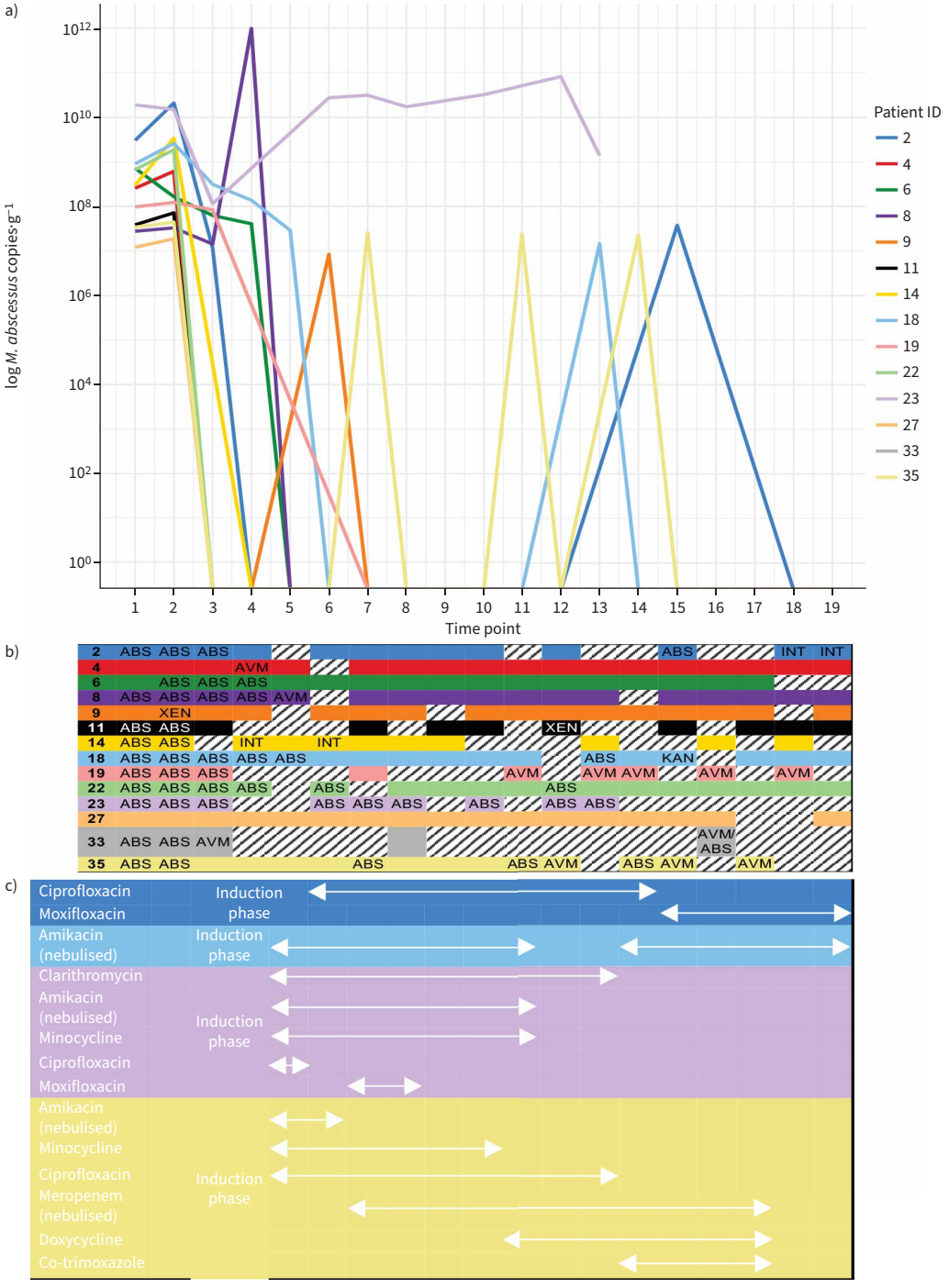

FIGURE 3 a) Custom assay with *Mycobacterium abscessus* probe used with samples from the *M. abscessus* treatment group. b) Patient culture results by time point are displayed below quantitative PCR data. Hashed boxes indicate unavailable samples. c) Interrupted treatment regimens relating to each sample time point and patient. Interruptions in treatment correlated with increased nontuberculous mycobacterial copy number. Only regimes that were interrupted are illustrated. ABS: *Mycobacterium abscessus*; INT: *M. intracellulare*; AVM: *M. avium*; XEN: *M. xenopi*; KAN: *M. kansaii*.

Discussion

A diagnosis of NTM pulmonary disease and the subsequent monitoring of treatment rely upon culturing of the pathological bacteria. The shortfalls in this technique are demonstrated by the failure to culture the

same NTM species in samples originating from the same sputum (patients 6 and 9, figure 1). This study describes the design of a custom qPCR assay and its application to a total of 410 samples from patients with treated and observed NTM pulmonary lung disease. The results demonstrate the potential clinical utility of this assay for monitoring and treatment assessments in these conditions.

The molecular assays described are of potential clinical value because they enable the provision of rapid and real-time biomarker data during the course of treatment, enabling clinicians to make treatment decisions with real-time data. Target NTM DNA demonstrated high copy numbers prior to initiation of treatment, with significant progressive reductions preceding culture conversion in several patients over the first time points. This was well demonstrated particularly in the *M. abscessus*-treated cohort, in which a reduction in molecular load provided evidence of initial treatment success. There was also some indication that DNA levels could be used to assess the need for or to establish the effectiveness of treatment changes. Several patients demonstrated increases in copy number accompanying interruption of treatments (figure 3c). This is illustrated in patient 18 who, after 8 months of antibiotic therapy, ceased nebulised amikacin therapy owing to shortness of breath. After the drug was held for 2 weeks the patient became

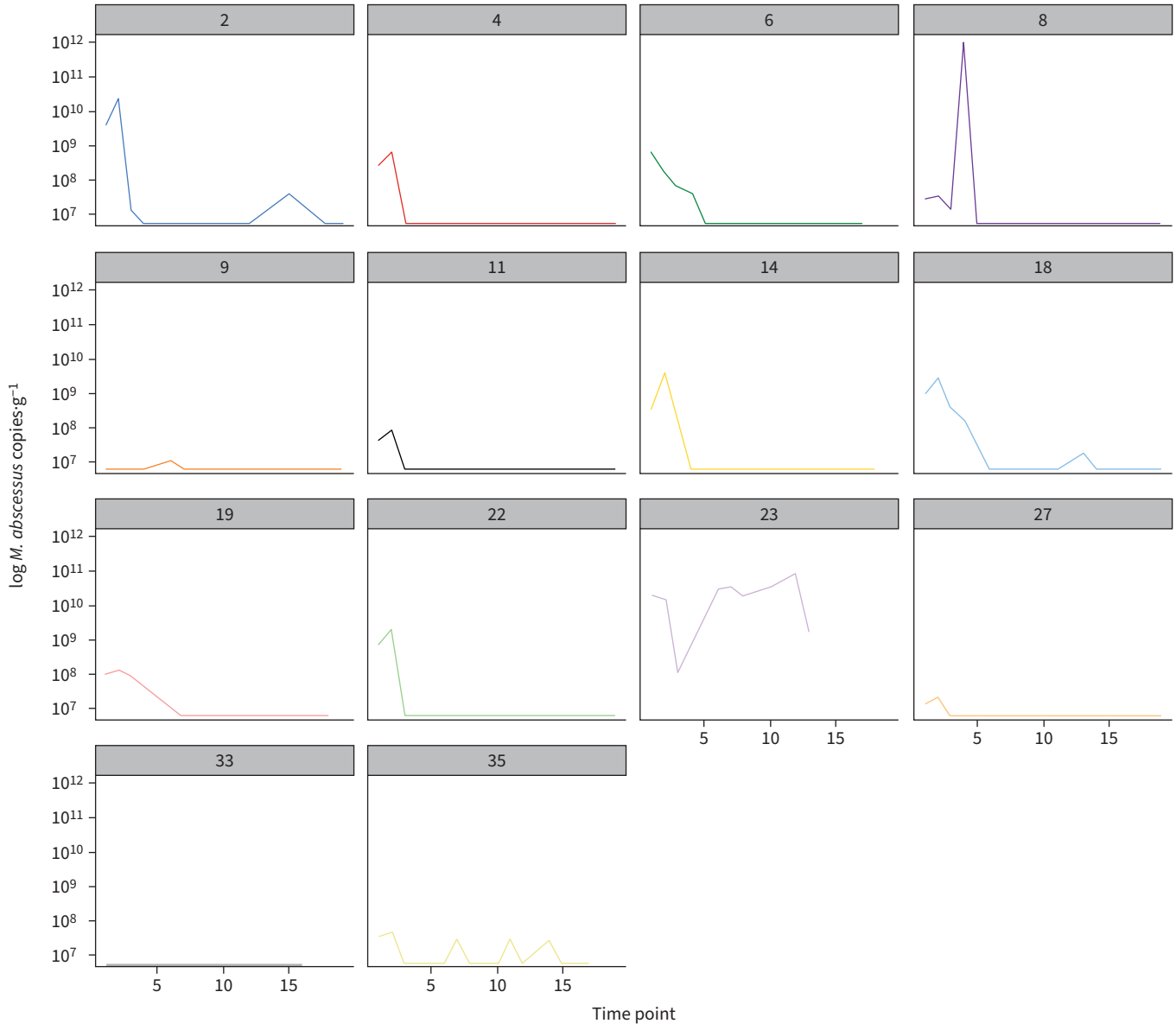

FIGURE 4 Individual patient results using the custom assay with a *Mycobacterium abscessus* probe used with samples from the *M. abscessus* treatment group.

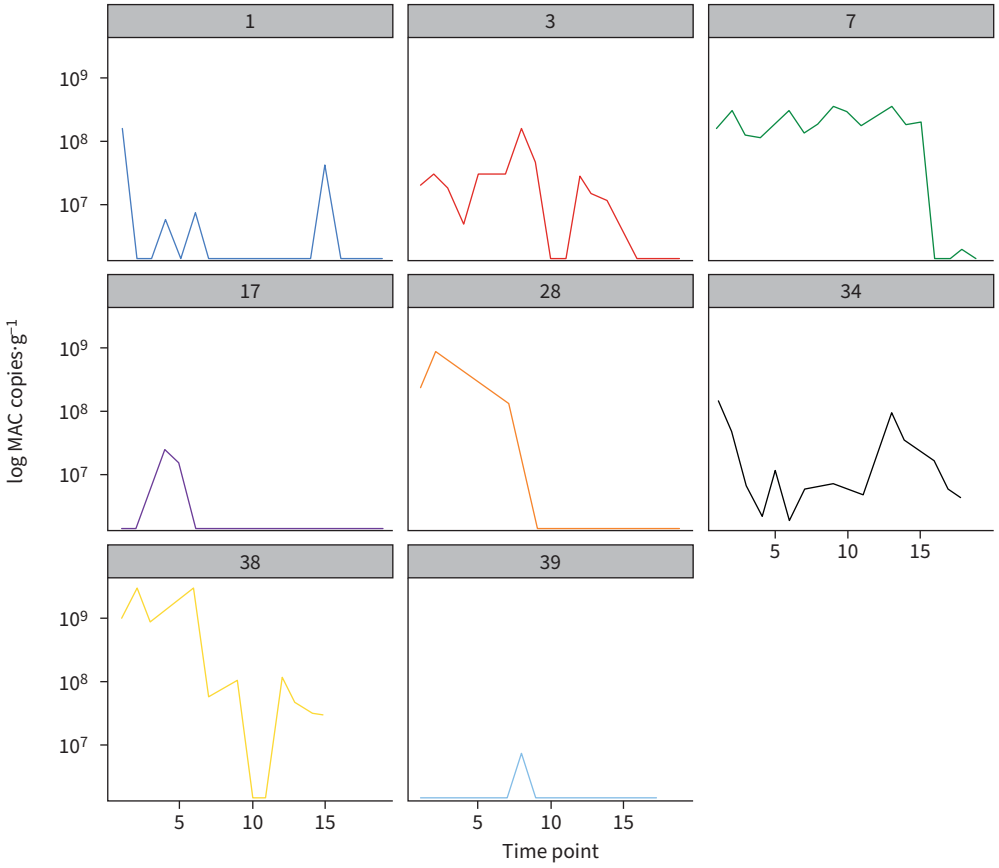

FIGURE 5 Individual patient results using the custom assay with a *Mycobacterium avium* complex (MAC) probe used with samples from the MAC treatment group.

culture and qPCR positive for *M. abscessus*. Similarly, patients 23 and 35 both showed recurrent isolates of *M. abscessus* during treatment following poor compliance. It may also be possible to use such data to determine the effectiveness of additional antibiotics to help patients with refractory disease. Presently treatment decisions are reliant on culture data that provide binary results and take many weeks to obtain. A longitudinal improvement in NTM copy number would serve to reassure clinicians of treatment efficacy when culture is persistently positive (see patient 6). Similarly the delay incurred by culture techniques may hinder patient improvement and may allow disease progression.

The treatment regimes of MAC differ significantly from *M. abscessus* not just in terms of differing antibiotics but also in that there is no initiation phase of treatment. Consequently, there is no initial rapid decline seen in MAC copy number. Instead MAC DNA copy numbers remain high often for several months before reducing. The slower replication rates associated with MAC in addition to the lack of an initiation phase could explain the difference in mycobacterial elimination.

In addition to providing rapid and quantitative data, there is evidence to suggest that the qPCR assays are also more sensitive than standard culture alone. Although there was generally good concordance between molecular and culture data, there were occasions in which negative culture results were accompanied by positive qPCR results. For samples taken from individuals undergoing treatment that exhibited this phenomenon, it could be postulated that the assay was detecting DNA from dead bacteria. However, the phenomenon was noted in samples not exposed to treatment. Samples from patient 6 at time points 1 and 2 (samples generated from the same divided sputum sample) demonstrated an incongruity between culture results (sample 1 was culture negative whereas sample 2 cultured *M. abscessus*). Molecular analyses of these samples were both qPCR positive for *M. abscessus*, demonstrating the potential for false negatives in culture-based techniques and suggesting that molecular techniques (once refined) will have better sensitivity than culture alone. Improved sensitivity is important with regards to decisions pertaining to the

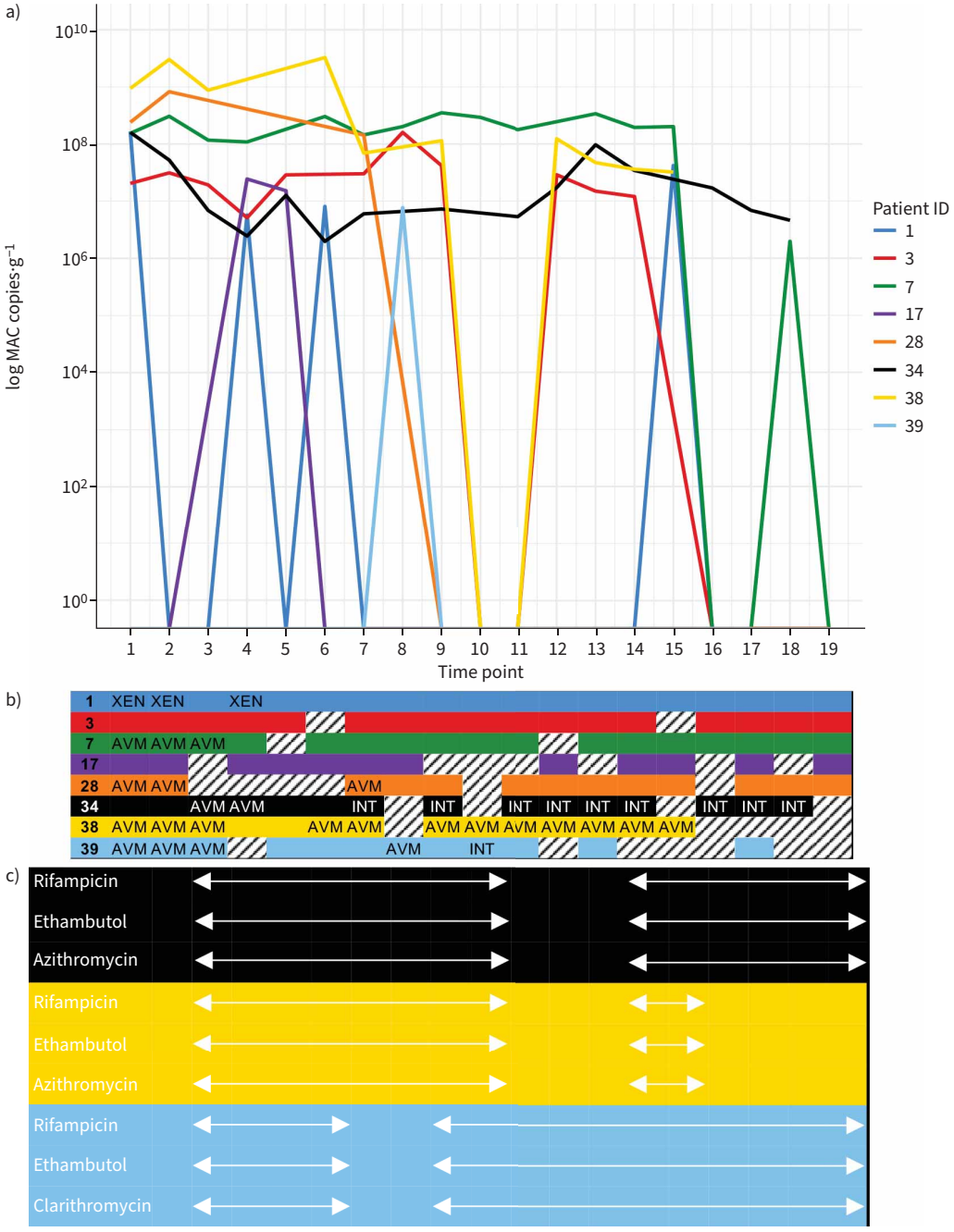

FIGURE 6 a) Custom assay with a *Mycobacterium abscessus* probe used with samples from the *M. abscessus* treatment group. b) Patient culture results by time point are displayed below quantitative PCR data. Hashed boxes indicate unavailable samples. c) Interrupted treatment regimens relating to each sample time point and patient. Interruptions in treatment correlated with increased nontuberculous mycobacterial copy number. Only regimes that were interrupted are illustrated. XEN: *M. xenopi*; AVM: *M. avium*; INT: *M. intracellulare*.

completion of treatment and culture conversion definitions. For example, patient 7 became culture negative after 1 week of anti-MAC treatment but was qPCR positive up until 10 months of treatment (time point 15) (figure 6). In these circumstances it is entirely possible that the patient's medication will have been stopped prematurely, a factor that is believed to be linked to relapse of disease and accounts for the successful eradication of MAC lung disease in only 60–80% of patients, with 20–40% failing to respond to treatment, and a significant proportion of successfully treated patients experiencing disease recurrence [15–18].

TABLE 3 Individual probe characteristics based on sputum sample culture-based results

	Probe			
	M. abscessus	*M. avium*	*M. intracellulare*	MAC
True positive	52	30	15	67
False positive	16	78	82	127
True negative	334	277	305	205
False negative	8	25	8	11
Sensitivity	0.87	0.58	0.65	0.86
Specificity	0.95	0.78	0.79	0.62
PPV	0.76	0.28	0.15	0.35
NPV	0.98	0.92	0.97	0.95
AUC	0.923	0.673	0.301	0.741

Each probe result was compared to the corresponding sample culture result, which was used as the reference standard test. MAC: *Mycobacterium avium* complex; PPV: positive predictive value; NPV: negative predictive value; AUC: area under the curve.

A further area of disparity between the culture and molecular data relates to multiple species detection by PCR. This is in line with previous data [19, 20], demonstrating that mycobacteria appear in communities rather than as individual isolates. Culture methods carry an inherent bias in favour of the reporting of fast growers whereas molecular methods, without the need for culture, would have less bias in this respect.

This study also looked at the impact of delays on NTM burden within sputum samples before their arrival at the laboratory for processing. This is currently extremely important with the huge increase in remote consultation and management, with many patients sending sputum samples *via* post. To control for samples incurring a delay while they are sent to the laboratory, screening samples were divided, with one sample undergoing a 24-h delay in freezing. Molecular analysis of NTM copy number between these samples showed no significant changes, indicating robustness of the assay with regard to sputum samples that cannot immediately be frozen. The impact of delayed freezing of sputum samples on culture-independent microbiological analyses has previously been investigated, with a significant decrease in the abundance of anaerobes seen at 12 h [21]. NTM are, however, aerobic bacteria with notoriously slow growth rates, which may account for the differences between the current study and the previous study.

Mycobacterial culture results for the control group revealed that all patients remained culture negative for the duration of the study. Three of the five patients, however, showed qPCR-positive results for MAC. This is likely attributable to the high failure rate associated with traditional culture techniques, which results in it having its own high false negative rate [22]. This study has demonstrated that culture techniques provide false negative results (see patient 6, samples 1 and 2) while other studies have shown histological evidence of NTM infection in asymptomatic patients with predisposing conditions [23].

There are some limitations of this study. For example, there were a few instances of false negatives in the presence of a positive sputum culture, suggesting some optimisation of the molecular tests may be needed going forward. In addition, because recruitment was only *via* one referral centre, the study size is small. Nevertheless, this study has significant strengths, including the development of an assay to enable rapid screening of sputum samples, and the high-frequency sputum sample testing conducted within the study that has provided insights into and a much better temporal understanding of the mycobacterial response to antibiotics.

Provenance: Submitted article, peer reviewed.

Conflict of interest: M.R. Loebinger reports the following relationships outside the submitted work: consulting fees received from Insmed, Savara, Parion, Armata, Chiesi, Zambon and Astra Zeneca; lecture fees received from Grifols and Insmed; Infection Group Chair for the European Respiratory Society. The remaining authors have nothing to disclose.

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