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Mycobacterium abscessus: environmental bacterium turned clinical nightmare

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Abstract:

Mycobacteria are a large family of over 100 species, most of which do not cause diseases in humans. The majority of the mycobacterial species are referred to as nontuberculous mycobacteria (NTM), meaning they are not the causative agent of tuberculous (TB) or leprosy, i.e. *Mycobacterium tuberculous* complex and *Mycobacterium leprae,* respectively. The latter group is undoubtedly the

most infamous, with TB infecting an estimated 10 million people and causing over 1.2 million deaths in 2017 alone (1). TB and leprosy also differ from NTM in that they are only transmitted from person to person and have no environmental reservoir, whereas NTM infections are commonly acquired from the environment (2) (3). It took until the 1950's for NTM to be recognised as a potential lung pathogen in people with underlying pulmonary disease and another 3 decades for NTM to be widely recognised by the medical community when NTM, particularly *Mycobacterium avium* complex (MAC) was recognised as the most common group of opportunistic pathogens in AIDS patients (4). This review focusses on an emerging NTM called *Mycobacterium abscessus* (*M. abs*). *M. abs* is a rapidly growing (RGM) NTM that is responsible for opportunistic pulmonary infections in patients with structural lung disorders such as cystic fibrosis (CF) and bronchiectasis (5), as well as a wide range of skin and soft tissue infections (SSTIs) in humans (6) (7). *M. abs* is a weakly staining Gram-positive mycobacterium that is neverand is, like other NTM, most often seen in soil and aquatic environments (8). The bacillus-shaped bacterium is 1-6µm long and 0.2-0.5µm in diameter, with curved ends and the presence of cord factor, or trehalose 6-6'-dimycolate, a glycolipid found in the cell wall of virulent species of mycobacteria that results in "serpentine cord" cell morphology is sometimes observed (8) (9). On solid growth medium, *M. abs* can display either a rough (*M. abs*-R) or smooth (*M. abs*-S) morphotype, with the rough morphotype displaying a more virulent phenotype than its smooth variant (10). The rough morphotype is characterised by irregular parallel filaments that form ridges across the colony, whereas a smooth morphology is displays a wet, smooth colony with no filaments or ridges (79). This morphology is driven by cell wall glycopeptidolipid (GPL); a loss of GPL results in the reversion from rough to smooth morphotype (80) (81). Moreover, it has been shown using human tissue culture models of infection that *M. abs*-R is able to persist and multiply within the host macrophage whereas *M. abs*-S lacks this capacity, hence its role in virulence (82). Like all other mycobacteria, *M. abs* are aerobic, non-motile and acid-fast organisms with a characteristically thick, lipid-rich cell wall that is hydrophobic. Due to their unusually impermeable, thick cell wall, mycobacteria are notoriously resistant to many antibiotics, disinfectants and heavy metals (11). When the genome of *M. abs*

49 became available in 2009, elucidation of the resistance mechanisms of *M. abs* became an area of focus
50 for scientific research, as the considerable threat it poses to public health became more apparent (12)
51 (13) (14). In this review we will discuss how we came to understand the pathogen, how it is currently
52 treated, as well as a discussion of drug resistance mechanisms and novel treatments currently in
53 development.

54

55 **Introduction:**

56 *M. abs* was first isolated in 1952 by Moore and Frerichs from a 63-year-old woman's knee abscess (6)
57 and since then, our understanding of the pathogen has rapidly and somewhat turbulently expanded.
58 When it was first isolated, it was suggested by the authors that *M. abs* was an entirely new species of
59 NTM and was given its name due to its ability to produce subcutaneous abscesses. Interestingly, at
60 this point, *M. abs* was considered to be a pathogen of low virulence due to the perception that it was
61 primarily a pathogen causing cutaneous infections that appeared transient and self-limiting (6). 40
62 years after its discovery, *M. abs* was first implicated in pulmonary infections after an analysis of 154
63 patients with RGM pulmonary infections revealed that 82% of the isolates were *M. abs*; the disease
64 was considered to be slowly progressive but virulent nonetheless (15). Since its first identification, *M.*
65 *abs* nomenclature and species/subspecies identification have undergone many changes.

66 In 1952 (6), *M. abs* was believed to be identical to *Mycobacterium cholenae*, another RGM that infects
67 fish and amphibians, as it presented identical biochemical features (16). Then, in 1972, following an
68 international collaborative study by the International Working Group on Mycobacterial Taxonomy,
69 *M.abs* was designated subspecies status (16). 20 years later, in 1992, Kusunoki and Ezaki used DNA
70 hybridisation to establish that there is only 35% DNA relatedness between *M. chelonae* subsp.
71 *chelonae* and *M. chelonae* subsp. *abscessus*. In light of this, *M. abs* was finally re-elevated to species
72 status.

However, in 2004, an unusual *mycobacterium* was isolated from a patient with hemoptoic pneumonia, and researchers were unable to accurately identify the species using the techniques described above. They developed partial PCR sequencing of the *rpoB* gene and were able to demonstrate that the isolate shared 96.0% partial *rpoB* sequence similarity and a 98.0% *recA* gene sequence similarly with only the *M. abs* type strain. They had previously proposed that *rpoB* gene sequence difference of >3% and a *recA* gene sequence difference of >2% was sufficient to differentiate between different NTM species. Using this new *rpoB* gene sequencing technique aided with the more traditional biochemical assays and 16S rRNA gene sequencing, the authors were able to produce an accurate phylogenetic tree of various NTM. They concluded that this novel isolate was a new species closely related to and likely recently derived from *M. abs*. This was subsequently named *Mycobacterium massiliense* (17).

In 2006, *rpoB* gene sequencing was used on 59 clinical isolates of RGM (18), and they found that 15.3% of these isolates were novel, corresponding to 3 new species of mycobacteria. One of these species, named *Mycobacterium bolletii* by the authors, was found to share 100% 16S gene similarity and 95.6% *rpoB* gene sequence similarity with *M. abs.*

In 2011 it was proposed by Leao *et. al.,* (19) that the *M. abscessus* complex (*MABS* complex) should be amended to include *M. abscessus* subsp. *abscessus* (as before) and to combine the two subspecies to form one single subspecies, *M. abscessus* subsp. *bolletii.* Finally, in 2013, whole-genome sequencing (WGS) was used by Bryant *et. al.,* to identify transmission between patients with CF (20). The authors subjected 168 clinical isolates of *M. abs* to WGS and a phylogenetic tree produced from the isolates showed clearly, for the first time, that *M. abscessus* subsp. *abscessus, M. abscessus* subsp. *bolletii,* and *M. abscessus* subsp. *massiliense* are three distinct subspecies belonging to the *MABS* complex. The idea that *MABS* is a complex that contains 3 subspecies that are genetically very similar, but phenotypically divergent was given more traction in 2016 when Tortoli *et. al.,* (21) published an amended description of the *MABS* complex that highlighted the importance of subspecies differentiation. The authors argued that the criteria for subspecies as proposed by Wayne *et. al.,* (22)

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i.e. “genetically close organisms that diverge in phenotype” is appropriate in this case, considering the genetic similarity and the presence of an inducible and functional *erm*(41) gene conferring macrolide resistance in only *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *abscessus* isolates whereas *M. abscessus* subsp. *massiliense* has a non-functional *erm*(41) gene.

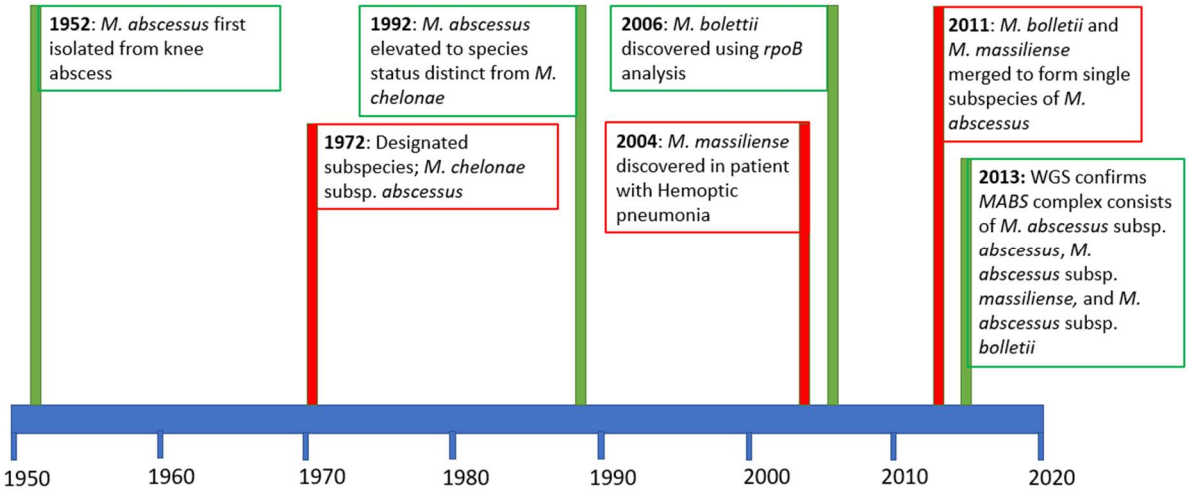

Figure 1: Timeline of *Mycobacterium abscessus* taxonomy from 1950 through to the present day. The first 50 years since its discovery, no congruent terminology was in widespread use to accurately describe and differentiate *M. abs* from other NTM. In the mid-2000s improved molecular technology resulted in the discovery of the two *M. abscessus* subspecies; *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *bolletii* in 2004 and 2006, respectively. Then in 2011 it was proposed that *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *bolletii* should be merged into one subspecies, *M. abscessus* subsp. *massiliense.* This caused some confusion within the medical community, until in 2013, when whole genome sequencing (WGS) showed genetic divisions that clearly identified the three subspecies within the *M. abs* complex.

Speciation of the *M. abscessus* complex:

Over the years, many different biochemical and molecular techniques have been employed to identify NTM species. Up until the early 2000's, the sodium chloride tolerance test was used to identify species of RGM, particularly in distinguishing between *M. abs* and *M. chelonae* species, as *M. abs* is able to grow on Löwenstein-Jensen medium with 5% sodium chloride but *M. chelonae* is not (23). However several investigators reported that this method is unreliable, likely due vague criteria and the cross-over of biochemical features between differing species of RGM (23) (24) (25). The citrate utilization assay perhaps provides more reliability, the premise being that *M. abs* is unable to use citrate as a carbon source whereas other RGM such as *M. chelonae* are (26). As is also the case with the sodium chloride test, this assay takes up to 8 weeks to complete and therefore is losing traction in the clinical setting (23). High Performance Liquid Chromatography (HPLC) has also been used to generate mycolic acid patterns and thus distinguish between RGM species, however this technique has limitations as several RGM have similar mycolic acid profiles (27). Despite its widespread use in species identification, 16S rRNA sequencing has been shown to be inadequate for species identification of mycobacteria (17). An assay with superior specificity was needed to differentiate between NTM species and subspecies.

***M. abscessus* and Cystic Fibrosis:**

NTM species are ubiquitous in the environment (unlike *M. tuberculosis* and *M. leprae* which require a living host and are transmitted patient to patient or zoonotically), suggesting that NTM exposure is extremely common, whereas NTM disease is still relatively rare. Those with pre-existing lung diseases undoubtedly have some predisposition to NTM infection, leading some to describe a "two-hit" theory of NTM disease acquisition (28). Undoubtedly, the leading population affected by *M. abs* is the CF population. However, there have also been incidences of *M. abs* infections in non-CF populations.

CF is an autosomal recessive disorder caused by mutations in the CF transmembrane conductance regulator gene (CFTR). Despite being a multi-organ disease, one of the most prominent features in CF is chronic pulmonary infection. The major pathogen associated with lung infection in CF is *Pseudomonas aeruginosa*, and unfortunately, 80 to 90% of patients with CF die from respiratory failure as a result of chronic bacterial infection (29). Even from infancy, the lungs of CF patients are already commonly colonised with a variety of organisms such as *Staphylococcus aureus* and *Haemophilus influenzae*. Before 1990, NTM infection was not often associated with CF. However, since then, reports of *M. abs* infection (along with other NTM species) have been increasingly common. Several large-scale studies have been performed over the past decade or so, revealing an NTM prevalence in CF patients in some areas as high as 20% (table 1).

Study	Location	Sample size	NTM prevalence in CF
Oliver, KN (2004) (30)	USA	750	13% (majority *M. avium* complex)
Roux, AL, et. al. (2009) (31)	France	1582	6.6% (*M. abs* most common)
Seddon, P, et. al. (2013) (32)	UK	3805 adults 3317 children	5% adults 3.3% children
Adjemian, J, et. al. (2014) (33)	USA	18,003	10-20%; depending on area
Mussaffi, H, et. al. (2005) (34)	Israel	139	8.6%

Table 1: Prevalence of non-tuberculous mycobacterial lung disease in cystic fibrosis patients in differing geographical areas between 2004 and 2014.

Age is a strong correlator of NTM infection in this group, with 40% of CF patients over the age of 40 having NTM smear positive results, as opposed to 4-20% in the under 40s population (35). Other risk factors for NTM infection in CF patients appears to be lower body mass index (BMI) values, worse forced expiratory volume (FEV_1), current infection with *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*, experience of pneumothorax requiring chest drain, the use of inhaled

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158 antibiotics and other medical interventions. (36). One study performed in Israel found a significant
159 association between *Aspergillus* species and NTM species in sputum cultures of CF patients (37).

160

161 **_M. abscessus_ infection in non-CF populations**

162 It is well documented that a risk factor for NTM pulmonary disease is patients with low body fat. The
163 mechanisms behind this are not well understood, however it is possible that leptin plays a role in NTM
164 predisposition (38).

165 Aside from pulmonary infections, *M. abs* is also able to produce skin and soft tissue infections (SSTIs)
166 in otherwise healthy hosts. There have been cases of *M. abs* outbreaks following the use of
167 contaminated needles and other surgical instruments (39) and even, as was the case in a cohort of
168 'lipotourists' (i.e., people who travel abroad for cosmetic surgery for fat removal), severe outbreaks
169 following cosmetic surgery (40). Interestingly, *M. abs* has also been linked to late-onset wound
170 infections following crush trauma sustained by Swedish survivors of the 2004 tsunami that killed over
171 200,000 people and caused serious crush injuries in another >2000 (41)

172 *M. abs* also causes serious disseminated infections following transplantation (42). A single case study
173 involving post-transplant *M. abs* SSTI resulted in disseminated pulmonary infection and eventually the
174 death of the patient, despite aggressive pre- and peri-operative anti-mycobacterial therapy (43). For
175 this reason, many have recommended that *M. abs* colonisation should be viewed as a contraindication
176 to lung transplantation. This suggestion, however, has been met with criticism. Some studies have
177 shown that it is possible to perform a lung transplant on patients with *M. abs* colonisation and that
178 subsequent clearance of infection is possible, albeit with a strong possibility of severe complications
179 (44) (45). Despite this uncertainty surrounding the outcome of lung transplantation in patients
180 colonised with *M. abs*, it is increasingly clear that effective treatments for *M. abs* lung infection must
181 be developed, as lung transplantation is a potentially life-saving therapy for end-stage lung disease
182 caused by CF and other lung disorders.

Environmental reservoirs and transmission:

NTM are ubiquitous in the environment; especially water sources and soil (4). They are prone to biofilm formation and this contributes to their ability to persist in harsh environments (46). NTM can persist in environments that are in close proximity to human populations, particularly human water sources, hospital water supplies (sinks, showerheads), and homes.

M. abs, like other NTM, is able to survive in harsh, nutrient-starved environments where other competing microorganisms would not survive, such as in chlorinated water (47). The presence of the lipid-rich cell wall results in a hydrophilic cell surface, which facilitates the formation of biofilms, their slow growth and adherence to surfaces, thus aiding their survival and providing them with a selective advantage (48) (49) (50). Furthermore, many RGM are oligotrophic, requiring low levels of two carbon sources and minimal amounts of metal ions (51), further indicating their hardiness and persistence in harsh environments. The impenetrable nature of the *M. abs* cell wall in comparison to other non-mycobacterial pathogens also contributes to its resistance to many antibiotics and disinfectants (52) (12). The ability of *M. abs* to survive in the human environment presents a huge problem for human health, with most studies up until this point suggesting that patients with CF predominately acquire NTM infection from the environment (20). This long-held belief was called into question in 2013 when Floto and his team used WGS to show possible patient to patient transmission of *M. abs* within a CF clinic in the UK (53).

In 2009, Feazel *et al* demonstrated that showerheads provide an enriched environment for NTM biofilm formation; the presence of human pathogens including NTM were >100 fold higher in showerhead biofilms compared to the background water contents (54). A study in Hawaii investigated the prevalence of NTM in household plumbing; areas such as showerheads, sinks, taps, shower drains, and refrigerator water dispensers were sampled. The authors found that 69% of households surveyed had clinically significant NTM colonisation, of which 10% was *M. abs* (55). Another 2018 study revealed

an outbreak of *M. abs* skin infections in children who were exposed to the same indoor wading pool (56). This study demonstrates the importance of identifying *M. abs* environmental reservoirs, reporting *M. abs* cases and subsequent environmental remediation in order to reduce the risk of infection.

The persistence and spread of NTM species within healthcare environments is fast becoming a serious problem and a significant threat to human health (57) . It was a long-held belief in the scientific community that NTM is transmitted to humans from the environment, and that patient to patient transmission is unlikely. Resulting in a clinical focus on reducing the risk of environmental transmission using effective sterilising techniques and other hygiene practices. Such as it is, the CF Trust published *M. abs* infection control recommendations that include general infection control measures such as hand washing and more specific recommendations such as segregation of infected patients from other patients (58).

The mode of transmission of pathogenic NTM to humans is still poorly understood, with many studies seeking evidence of human to human transmission using molecular techniques such as WGS. A study undertaken in 2001 sought to address this question; a retrospective analysis of 1062 respiratory specimens taken from 214 patients with CF revealed 5 patients with *M. abs* lung infection. These 5 patients each had isolates with a unique genotype that was not shared with any of the other patients, which led the authors to conclude that patient to patient transmission of *M. abs* was not occurring within their cohort (59).

In 2014, a small-scale study was performed on 27 *M. abs* isolates from 20 paediatric CF patients (60). The authors used a combination of epidemiology, variable number tandem repeat (VNTR) profiling and WGS to find evidence of cross-infection between paediatric CF patients. They hypothesized that patients with strains that had identical VNTR profiles would have had intense exposure to each other compared with patients with strains that had different VNTR profiles. They found little evidence of transmission between patients, except for 2 patients who were siblings and therefore had higher

intensity of exposure. They concluded that cross-infection was uncommon in their cohort, and that transmission is most likely to be from a common environmental source (60).

The biggest shift in our understanding of transmission came in 2013 when a major study was published in which WGS was used to identify transmission of *M. abs* between patients at an adult CF centre in the UK between 2007 and 2011 (20). The authors found a high level of relatedness between isolates of *M. abscessus* subsp. *abscessus*, but clusters were clearly segregated from one another, indicating that patients have independently acquired either genetically diverse strains or a dominant circulating clone. In the case of *M. abscessus* subsp. *massiliense*, however, the authors found isolates from different individuals with almost identical genomic sequences, strongly indicating transmission between patients. Analysis of the environment revealed no NTM species isolated from the water supply to the clinic, showerheads, dish washers, bronchoscopes or the local River Cam or Papworth Hospital Pond. Further investigation into possible transmission routes revealed patients with isolates from the same genetic relatedness clusters were present in the clinic at the same time as each other, further supporting their hypothesis that *M. abscessus* subsp. *massiliense* is likely transmitted from patient to patient rather than independently from the environment. This finding represents a major clinical advance which may require patients infected with *M. abs* to be segregated from *M. abs*-naïve patients to prevent onward transmission.

Following on from the localised retrospective study published in 2013 (20), a global WGS initiative was launched on 1080 isolates from 517 patients from the UK, USA, Republic of Ireland, mainland Europe and Australia (53). This study found that the majority of isolates were from densely clustered genotypes that were not diverse, suggesting a high level of human-human transmission. Phylogenetic analysis also revealed that there are 3 dominant circulating clones globally, and these clones are associated with higher virulence and poor clinical outcomes. Human-human transmission appears to have facilitated the evolution of *M. abs* from an environmental pathogen to a transmissible human pathogen.

Diagnosis and treatment:

As *M. abs* and other NTM species are ubiquitous in the environment, including drinking water supplies, the presence of culture-positive respiratory tract sample for NTM does not always indicate NTM-pulmonary disease (NTM-PD). Therefore, patients must also have characteristic symptoms, compatible radiology, and two or more positive sputum samples for the same NTM species, as well as the exclusion of other potential causes of pulmonary disease (61).

For clinical laboratory identification of NTM species, the British Thoracic Society (BTS) recommends that isolates be obtained from sputum samples, and if this is not possible (for example in children), bronchoalveolar lavage or transbronchial biopsy samples should be taken when NTM pulmonary disease is suspected (61). NTM infection can be validated in the laboratory, with the use of auramine-phenol staining and microscopy, as well as culture on solid and liquid media.

All clinical isolates of *M. abs* undergo susceptibility testing for clarithromycin, cefoxitin and amikacin. They also recommend that other antibiotics such as tigecycline, imipenem, minocycline, moxifloxacin and clofazimine are tested in this manner (61).

Treatment

When *M. abs* was first isolated in 1952, it was thought the patient was initially infected with the pathogen at the age of 14 years old. The patient's condition resolved without intervention and so for some time, treatment wasn't considered a priority in *M. abs* infections (6).

Of course, today it is well known that treatment for *M. abs* pulmonary infection is essential to give the patient the best chance of survival. Unfortunately, antimicrobial chemotherapy for *M. abs* infection is particularly difficult due to its intrinsic and acquired resistance to most of the commonly used antibiotic classes. Further complications in the treatment of *M. abs* infection is the lack of evidence that *in vitro* susceptibility of antibiotics corresponds to *in vivo* efficacy in treating pulmonary disease

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(62). Because chemotherapy-based treatment of *M. abs* infection is often unsuccessful, the American Thoracic Society advises that certain patients may have the best chance of disease regression with resectional surgery, especially if the patient exhibits a poor response to drug therapy, if macrolide-resistance develops, or if the patient is experiencing disease-related complications such as haemoptysis (62).

Current treatment guidelines from the BTS (61) recommend that treatment for *M. abs* pulmonary disease should consist of an initial phase antibiotic regimen that includes intravenous (I.V.) and oral antibiotics, followed by a continuation phase comprising of oral and inhaled antibiotics (Figure 2). Further genetic analysis of clinical isolates can provide information on the *erm*(41) (inducible macrolide resistance) and/or presence of 23S rRNA point mutation (constitutive macrolide resistance) in clinical isolates of *M. abs*, which can then be used to inform patient-specific treatment regimens.

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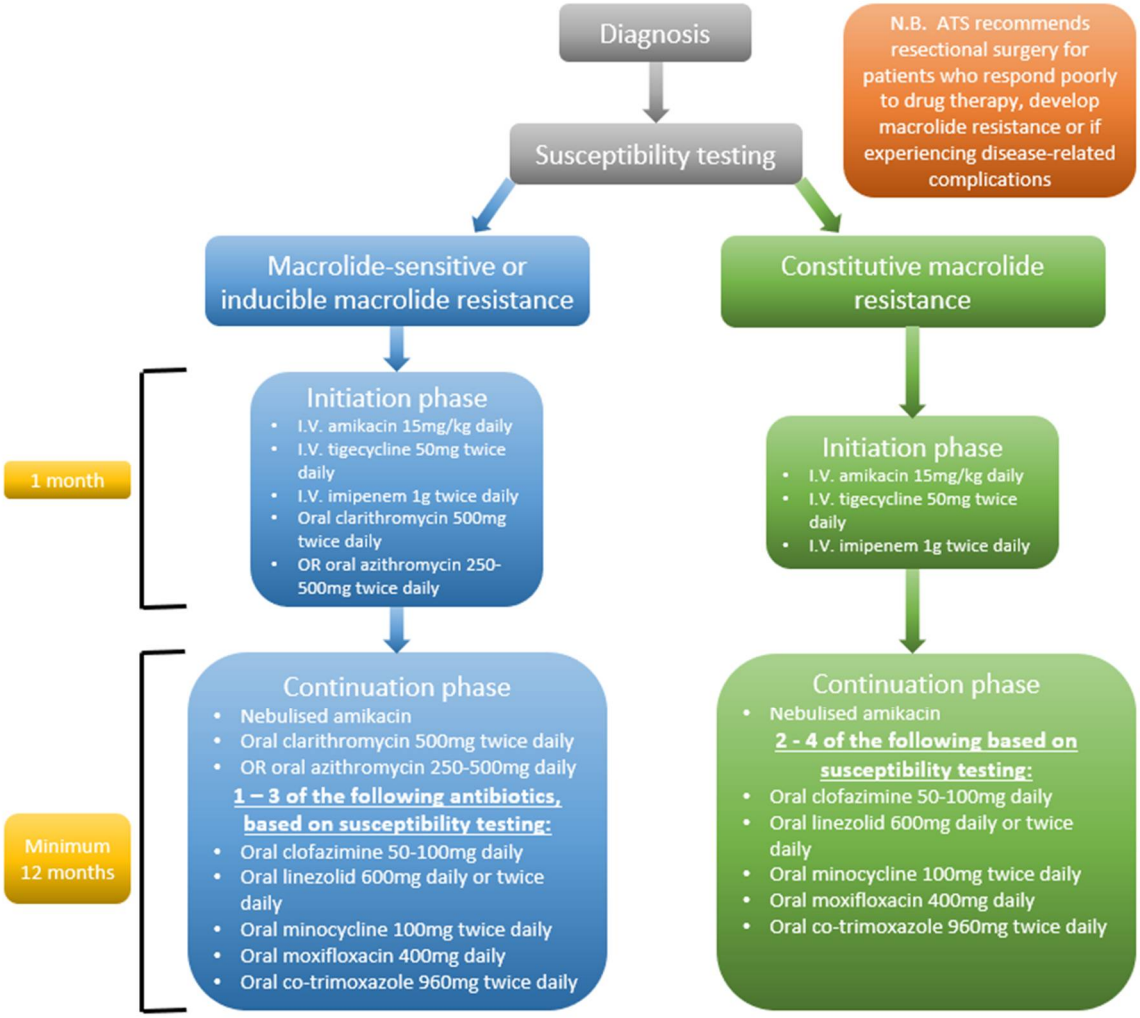

293

294 **Figure 2**: Flow chart showing treatment regimen for *M. abs*-pulmonary disease based on laboratory
295 susceptibility testing results as recommended by the British Thoracic Society. Treatment will differ
296 based on the whether the isolate displays macrolide sensitivity/inducible macrolide resistance or
297 constitutive macrolide resistance. The initial phase of treatment involves three intravenous (I.V.)
298 antibiotics, and for macrolide sensitive/inducible macrolide resistance 1 of 2 oral macrolides, and this
299 phase lasts one month. The continuation phase also depends on laboratory susceptibility testing
300 results and clinicians will typically administer 1-4 oral antibiotics over a period of at least 12 months.
301 It is also important to note that the American Thoracic Society recommends surgical resection of
302 infected area if the patient is not responding to therapy, if macrolide resistance develops, and/or if
303 the patient develops disease-related complications such as haemoptysis.

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Side effects of *M. abs* treatment are common and can be severe. A retrospective analysis of 65 patients undergoing treatment for *M. abs* lung disease in South Korea (63) revealed frequent adverse reactions to cefoxitin; 51% of patients developed leukopenia, 6% of patients developed thrombocytopenia, and 15% of patients experienced drug-induced hepatotoxicity. As a result, cefoxitin was discontinued in 60% of patients and side effects resolved. Another common side effect observed was gastrointestinal problems (nausea, anorexia, or diarrhoea), which affected 22% of patients and caused 4 patients (6%) to completely stop antibiotic treatment. A clinical recommendation was made to consider imipenem as an alternative to cefoxitin, however prolonged treatment with imipenem can cause neutropenia.

Another study that analysed treatment outcomes in 65 patients with *M. abs* in North America also found a high prevalence of side effects. IV amikacin (65% of patients) and azithromycin (71% of patients) were the most commonly used antimicrobials in this cohort. They found 74 different side effects reported in 62% of patients, most commonly nausea/vomiting (31%) and skin changes (20%). They attributed many of these side effects to amikacin or tigecycline, and as a result, of those received amikacin or tigecycline therapy, 51% and 36% of patients, respectively, had to adjust or stop medication due to severe side effects such as ototoxicity. Similar to the South Korean study, 4 patients had to totally stop treatment because of their side effects (64).

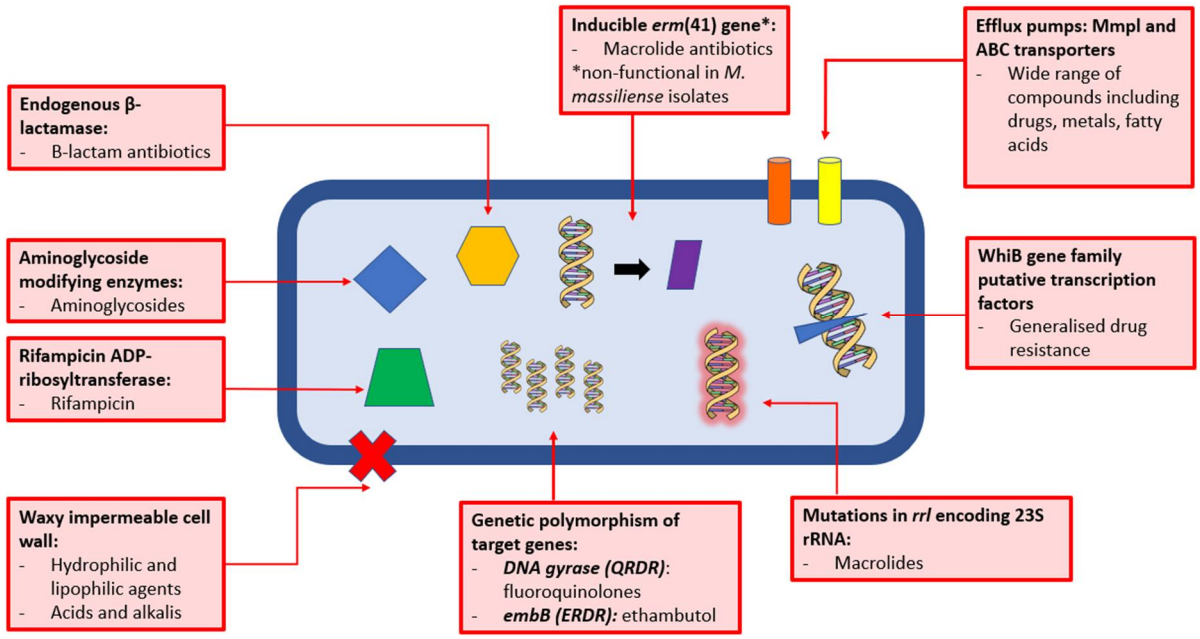
Clarithromycin is one of the most commonly used antibiotics to treat *M. abs* (35). However, clarithromycin has been associated with hearing loss, with one study citing a 7% hearing loss rate in their patients. This side effect did resolve in all but one patient, but the authors state that the patient had a pre-existing condition that hindered their ability to attribute this hearing loss solely to clarithromycin (65). A case study on an 81-year-old woman, who was being treated with clarithromycin for infective exacerbation of chronic pulmonary obstructive disease (COPD) showed another example of clarithromycin-related permanent hearing loss, despite evidence that clarithromycin is relatively well tolerated (66) (67). The major issue with using clarithromycin to treat

M. abs is the presence of a functional inducible *erm*(41) gene that confers macrolide resistance in both *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii* but not *M. abscessus* subsp. *massiliense.*

Future perspectives for *M. abscessus*

The resistance problem: why the drugs don't work:

M. abs is known for its intrinsic resistance to most chemotherapeutic agents, including all the anti-tuberculous drugs used to treat *M. tuberculosis* infection (68) (69). Furthermore, *in vitro* drug susceptibility testing on *M. abs* often proves unhelpful in guiding treatment regimens (70). There are a number of natural resistance mechanisms displayed by *M. abs* (along with other mycobacteria), including a waxy and impermeable cell wall, drug export systems, antibiotic modifying/inactivating enzymes, and genetic polymorphism of target genes (12).



345 **Figure 3:** Graphical summary of the resistance mechanisms exhibited by *Mycobacterium abscessus*
346 (*M. abs*). There are several mechanisms involving different physiological, enzymatic and genomic
347 processes that contribute to the notoriously drug-resistant profile of *M. abs*. It is likely that these
348 processes work in synergy to produce a highly resistant pathogen, such as efflux pumps and drug
349 resistance genes.

350

351 The greatest contributing factor to the lack of *M. abs* sensitivity to many major classes of antibiotic is
352 the mycobacterial cell wall, the role of which has long been studied. The high lipid content and unusual
353 thickness of the mycobacterial cell wall provides an effective barrier for hydrophilic and lipophilic
354 agents (71). In 1990 it was shown that the lack of permeability of the *M. chelonae* (then grouped
355 together with *M. abs*) cell wall plays a vital role in making the pathogen resistant to antibiotics (52).
356 The cell wall barrier is also responsible for *M.abs*' intrinsic resistance to acids and alkalis (72). The cell
357 wall of mycobacteria also contains porins, it was shown in 1990 that *M. chelonae* possesses a 59 kDa
358 cell wall protein that allows for the diffusion of small, hydrophilic solutes. This porin, however, is
359 minor, unlike that of *E. coli* where they are the most abundant cell wall protein, explaining the low
360 permeability to hydrophilic solutes (11). The cell wall cannot explain all of the intrinsic drug resistance
361 seen in *M. abs,* in fact it is known that the cell wall, particularly the porins, act synergistically with
362 internal systems that are activated by the presence of intracellular antibiotics, and that the low
363 permeability of the mycobacterial cell wall means that the bacteria has time to induce the expression
364 of drug resistance genes (73).

365 As a constituent of the mycobacterial cell wall, active efflux pumps can be described as one of the
366 main causative factors of drug resistance in mycobacteria (12) (74) (75). They primarily act to protect
367 bacteria against toxic compounds and bacterial homeostasis by transporting toxins or metabolites to
368 the extracellular environment (75). *M. abs* encodes protein members of the major facilitator family
369 ABC transporters as well as mycobacterial membrane protein large (MmpL) families (76). ABC

transporters are found in all forms of life and make use of adenosine triphosphate (ATP) to transport molecules across membranes. The MmpL transporter family is a subclass of a large family of multidrug resistance pumps known as Resistance-Nodulation-Cell-Division (RNCD) permeases. MmpLs export lipid components across the cell envelope of mycobacteria (77). The role of MmpLs in *M. abs* drug resistance is yet to be fully understood, however there is evidence that MmpL7 in *M. tuberculosis* confers resistance to isoniazid (78), suggesting that MmpLs may play a major role.

Macrolides are one of the mainstays of *M. abs* treatment (35), yet despite this, *M. abs* infections tend to respond poorly to macrolide therapy, even when they appear sensitive to clarithromycin *in vitro* (79). A study performed in 2009 revealed the presence of an inducible *erm*(41) gene in 7 out of 10 *M. abs* clinical isolates that confers resistance to macrolides with a minimum inhibitory concentration (MIC) of ≥32 µg/mL. The 3 remaining susceptible isolates had *erm*(41) gene, however it appeared to be non-functional (79). The *erm*(41) gene produces a functional 23S rRNA methylase, contributing to macrolide resistance along with point mutations in the *rrl* encoding 23S rRNA gene (80). Following on from this, it was shown that macrolides may be useful in treating approximately 20% of *M. abs* infections in the U.S., and that sequencing of the *erm*(41) gene is a potentially useful tool in predicting macrolide susceptibility (81). It is also noteworthy that *M. abscessus* subsp. *massiliense* contains a large 97 base pair deletion in *erm*(41), rendering it useless and therefore meaning *M. abscessus* subsp. m*assiliense* retains susceptibility to macrolides, except in the case of *rrl* mutants (82) (79) (83) (84). *M. abs* isolates possessing an *rrl* mutant display constitutive resistance to macrolide antibiotics. This phenomenon is known to be mediated by a mutation in *rrl* encoding the bacterial 23S rRNA gene, particularly at positions 2058 and 2059, i.e. the drug binding pocket of the gene (85).

If macrolide therapy is not advised due to evidence of constitutive resistance, there are of course other chemotherapeutic options available. However, in many of the conserved genes in *M. abs* that can potentially act as drug targets there is the presence of genetic polymorphisms, which can often confer drug resistance (12).

A 1998 study showed revealed an amino acid substitution at position 83 (Ser83Ala) in the quinolone-resistance-determining-region (QRDR) in fluoroquinolone-resistant isolates of *M. abs* (86). This substitution occurs in the region of DNA gyrase subunit *GyrA* that binds DNA, and as fluoroquinolones bind strongly to the gyrase-DNA complex, and weakly to protein or DNA alone, this mutation results in fluoroquinolone resistance (87). Genetic polymorphisms also occur within the *emb* operon that codes for several homologous arabinosyl transferases. These are enzymes involved in the polymerisation of arabinogalactan, an essential component of the mycobacterial cell wall and can be inhibited by the tuberculosis drug ethambutol. A 1997 study showed that polymorphisms at position 306 in a highly conserved *embB* gene conferred natural resistance across many species of mycobacteria, including *M. abs* (88). *M. abs* has high natural levels of resistance to ethambutol (MIC >64mg/L), and the same study transferred the *M. abs emb* region to ethambutol-susceptible *M. smegmatis* resulted in a 500-fold increase in the MIC to ethambutol (88).

M. abs also produces a number of target-modifying enzymes. Rifampicin ADP-ribosyl transferase, Arr_*Mab* inactivates rifamycins such as rifampicin. Aminoglycoside 2'-N-acetyltransferase and aminoglycoside phosphotransferases mediate the susceptibility to aminoglycoside antibiotics. *M. abs* has also been shown to produce an endogenous β-lactamase (Bla_{Mab}), that efficiently hydrolyses the β-lactam ring of β-lactam antibiotics, rendering them ineffective (89).

Aside from antibiotic-specific internal drug resistance mechanisms, a family of transcriptional regulators, the WhiB family, is excusive to actinomycetes and may be involved in conferring drug resistance in *M. abs*. Members of this family have been shown to regulate systems of drug resistance in *M. tuberculosis*, including antibiotic export and activation (90). *M. abs* has been shown to possess a homologue of the *M. tuberculosis WhiB7*. When *M. abs WhiB7* is deleted, the result is increased sensitivity to clinically relevant antibiotics that target the ribosome, such as clarithromycin, amikacin and tetracycline (91).

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Future treatments:

It perhaps goes without saying that there is an urgent, unmet need for safe and effective treatments against *M. abs* pulmonary disease. There have been instances of successful treatment of *M. abs* with already available antibiotics. One such case was reported in 2002, where a 63-year-old patient whose infection had not responded to the traditional regimen was prescribed a course of faropenem, a new member of the β-lactam antibiotic class. Treatment was successful and produced no adverse side effects (92). It is not just antimicrobials that have potential in enhancing *M. abs* treatment. In 2012, Okazaki *et. al.* reported that the use of clarithromycin, amikacin and imipenem/cilastatin to treat a case of *M. abs* pulmonary was greatly enhanced with the addition of corticosteroids. The authors recommend that the presence of organising pneumonia (a non-specific inflammatory pulmonary process) or an allergic reaction may have helped to explain the poor response to antibiotic treatment alone in some patients, and that this possibility should be considered when applicable to improve treatment outcomes (93).

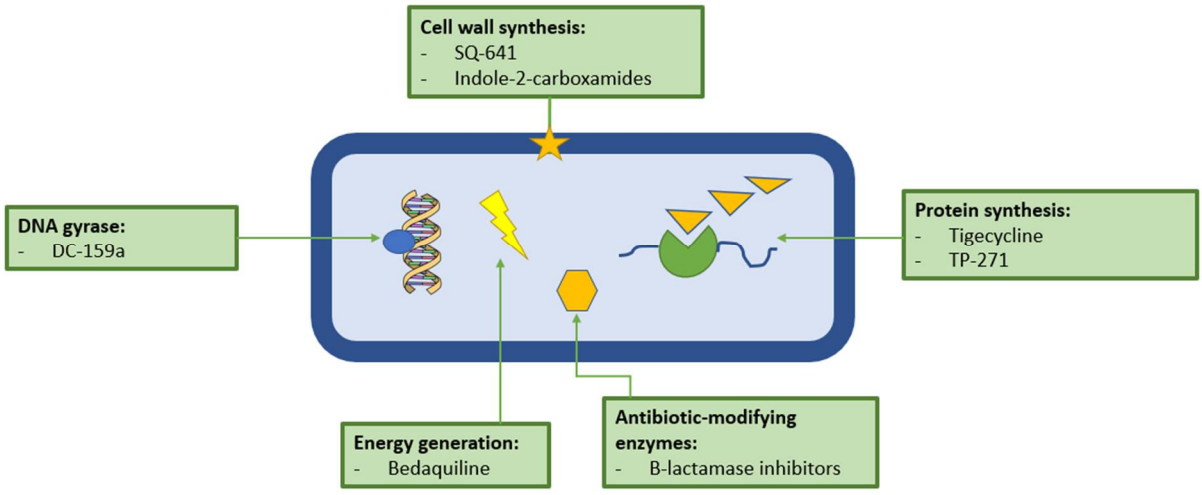
One of the enzymatic resistance mechanisms employed by *M. abs* is the production of an endogenous β-lactamase, Bla_{Mab} (Figure 3). Cefoxitin and imipenem, both β-lactam antibiotics, are commonly used to treat *M. abs*. In order to improve the efficacy of these antibiotics, a β-lactamase inhibitor may be administered in conjunction during therapeutic treatment. A 2015 study revealed that avibactam, a β-lactamase inhibitor is able to efficiently inhibit BlaMab (94), and a subsequent 2017 study showed that avibactam improves the efficacy of imipenem against *M. abs* both *in vitro* and in macrophage, and zebrafish models of infection (95).

Aside from these examples, very few case studies have reported successful treatment with repurposed antibiotics. Therefore, novel drug targets in *M. abs* must be discovered and elucidated, and novel compounds that safely and effectively inhibit these targets discovered.

There are potentially a wide variety of viable drug targets in *M. abs* (Figure 4) Many of the most promising leads against *M. abs* have come about as a result of concerted effort to find novel drugs for

445 *M. tuberculosis*, which a handful of researchers have applied to *M. abs* and other NTM species.
446 Unfortunately, only a small percentage of the novel drugs which are active against *M. tuberculosis*,
447 are also active against *M. abscessus,* further highlighting just how resistant and dangerous this
448 pathogen is proving to be.

449



450

451 **Figure 4**: Graphical summary of the exploitable drug targets in *Mycobacterium abscessus* (*M. abs*).
452 There are several potential target areas in *M. abs* including physiological, genomic, enzymatic and
453 metabolic processes. Many of the drugs with potential to be used as part of *M. abs* treatment are
454 old classes of antibiotics that have been repurposed, such as β-lactamase inhibitors, or have been
455 discovered as part of the anti-tuberculous drug discovery pipelines, such as bedaquiline.

456

457 One potential target in *M. abs* is DNA gyrase, despite the fact that *M. abs* is naturally resistant to
458 quinolones (96), a novel fluoroquinolone, DC-159a was developed in 2010 as part of the Working
459 Group on TB Drugs, and was found to be active against *M. abs* with an MIC of 16 µg/mL, which was 4
460 to 8-fold lower than the other already available quinolones tested (97). The authors stressed the
461 importance of *in vivo* testing of DC-159a, however, no publications attesting to the *in vitro* activity of
462 DC-159a against *M. abs* have been released to date.

The mycobacterial cell wall, in all its complexity, can offer an attractive range of potential antibiotic targets. The three distinct layers of the mycobacterial cell wall: core peptidoglycan, arabinogalactan and mycolic acids are each essential to the pathogen and involve a number of exploitable processes (98). A 2010 study subjected several species of NTM to a capuramycin analogue SQ641 (99). Capuramycins are a novel class of nucleoside antibiotics that work by targeting phosphor-*N*-acetylmuramyl-pentapeptide-translocase (translocase-1 or TL-1) which is essential for peptidoglycan synthesis. They found that the drug had an MIC of 0.25-1 µg/mL, as well as finding synergy between SQ641 and rifabutin and streptomycin. This drug has great potential as it is fast-acting and displays a long post-antibiotic effect (100). In 2017 a study was published in which several members of the newly synthesized MmpL3 inhibitors, indole-2-carboxamides, have shown potent activity against *M. abs*. These inhibitors have been shown to work by inhibiting the transfer of mycolic acids to their cell envelope acceptors in *M. abs* strains (101). Further work has been done on this class of inhibitors; in 2019, Pandya *et. al.* reported that oral administration of the inhibitors shows a statistically significant reduction in bacterial load in the lungs and spleens of *M. abs*-infected mice (102).

It has been demonstrated that *M. abs* displays high levels of intrinsic resistance to the tetracycline class of antibiotics via the monooxygenase, MabTetX, a *WhiB7*-independent pathway (103). This is not the end of the road for this class of antibiotics. Tigecycline, the first developed glycylcycline, a new class of tetracycline antibiotics originally developed for SSTIs, was shown in 2014 to be highly effective *in vivo* against *M. abs* pulmonary disease (104). Further work in 2018 revealed that tigecycline is a poor substrate of MabTetX and is incapable of inducing its expression, explaining its high efficacy in comparison with other tetracycline antibiotics (103). Tigecycline is now of the recommended treatment options for *M. abs* pulmonary disease, and is arguably one of the most effective, with one study citing clinical improvement in >60% patients with *M. abs* pulmonary disease when tigecycline is employed as part of the multi-drug regimen against *M. abs* (104). Tigecycline is not the only tetracycline showing activity against *M. abs*. A 2012 study tested the *in vitro* activity of a novel fluorocycline antibiotic, TP-271 (a tetracycline-related antibiotic) against 22 isolates of *M. abs*. They

found all the isolates to have an MIC of ≤1 µg/mL with an average of 0.5 µg/mL, which is decidedly superior than that of the other orally available tetracycline antibiotics such as moxifloxacin and tetracycline (105).

Bedaquilin, the latest drug indicated for the treatment of multi-drug resistant TB (MDR-TB) was approved by the FDA in 2011, and it works by targeting the ATP synthase of mycobacteria. Obregon *et. al.* (106) demonstrated an MICs of 1.0 µg/mL against *M. abs* reference strain and then in 2017, Vesenbeckh and colleagues pointed to bedaquiline as a potential antimicrobial against *M. abs* after the drug exhibited MICs of ≤1 µg/mL against 20 *M. abs* clinical isolates *in vivo.* (107)

Summary:

M. abs is increasingly being recognised as an important pathogen responsible for a wide range of infections and implicated in severe, and often untreatable pulmonary infections in people with CF and other structural lung disorders. Almost all of the currently available antibiotics are useless against the pathogen, with even official guideline treatment regimens having little to no evidence of *in vivo* efficacy. With such high treatment failure rates, clinicians are often forced to administer last-resort antibiotics in the hope of a cure. Coupled with increasing prevalence and its already extensively drug resistant profile, it is glaringly obvious that novel, effective and safe treatments are needed. Many of the novel drugs mentioned above are in various phases of clinical trial against *M. tuberculosis* and there is a significant paucity of data regarding their efficacy against *M. abs* and other NTM species. Furthermore, there is a startling lack of *in vivo* efficacy data for any of these drugs, which is particularly worrying considering the inconsistencies between *in vitro* and *in vivo* anti-*M. abs* activity. Whilst TB has many dedicated drug-discovery programmes, NTM has none. A dedicated NTM drug discovery pipeline is essential to ensure the disease burden of NTM does not become overwhelming.

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516

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526 **References:**

527 1. **Organisation, World Health.** *Global tuberculosis report.* Geneva : s.n., 2018. CC BY-NC-SA 3.0 IGO.

528 2. *Human-to-human transmission of Mycobacterium kansasii or victims of a shared source?* **Ricketts,**
529 **WM, O'Shaughnessy, TC and van Ingen, J.** 2014, European Respiratory Journal, Vol. 44, pp. 1085-
530 1087.

531 3. *Distinguishing Tuberculosis from Nontuberculosis Mycobacteria Lung Disease, Oregon, USA.*
532 **Kendall, BA, et al.** 3, 2011, Emerging Infectious Diseases, Vol. 17, pp. 506-509.

533 4. *Nontuberculous mycobacteria: opportunistic environmental pathogens for predisposed hosts.*
534 **Cook, J.** 1, 2010, British Medical Bulletin, Vol. 96, pp. 45-59.

5. *An Official ATS/IDSA Statement: Diagnosis, Treatment, and Prevention of Nontuberculous Mycobacterial Diseases.* **Griffith, DE, et al.** 4, 2007, ATS Journals, Vol. 175.

6. *An Unusual Acid-Fast Infection of the Knee with Subcutaneous, Abscess-Like Lesions of the Gluteal Region.* **Moore, M and Frerichs, JB.** 2, 1953, Journal of Investigative Dermatology, Vol. 20.

7. *Cutaneous infection with Mycobacterium abscessus.* **Fitzgerald, DA, et al.** 1995, Journal of Dermatology, Vol. 132, pp. 800-804.

8. *Laboratory Maintenance of Mycobacterium abscessus.* **Cortes, MAM, Nessar, R and Kumar Singh, A.** 2010, Current Protocols in Microbiology, Vol. 18, pp. 10D.1.-10D.1.12.

9. *Spontaneous reversion of Mycobacterium abscessus from a smooth to a rough morphotype is associated with reduced expression of glycopeptidolipid and reacquisition of an invasive phenotype.* **Howard, ST, et al.** 2006, Microbiology, Vol. 152, pp. 1581-1590.

10. *Preliminary Characterization of a Mycobacterium abscessus mutant in human and murine models of infection.* **Byrd, TF and Lyons, CR.** 1999, Infection and Immunity, Vol. 67, pp. 4700-4707.

11. *Mycobacterial cell wall: Structure and role in natural resistance to antibiotics.* **Jarlier, V and Nikaido.** 1994, FEMS Microbiology Letters, Vol. 123, pp. 11-18.

12. *Mycobacterium abscessus: a new antibiotic nightmare.* **Nessar, R, et al.** 4, 2012, Journal of Antimicrobial Chemotherapy, Vol. 67, pp. 810-818.

13. *Nontuberculous Mycobacteria in Adult Patients with Cystic Fibrosis.* **Kilby, JM, et al.** 1, 1992, Chest Journal, Vol. 102, pp. 70-75.

14. *Nontuberculous Mycobacteral Disease in Adult Cystic Fibrosis Patients.* **Aitken, ML, et al.** 4, 1993, Chest Journal, Vol. 103, pp. 1096-1099.

556 15. *Clinical Features of Pulmonary Disease Caused by Rapidly Growing Mycobacteria: An Analysis of*
557 *154 Patients.* **Griffith, DE, Girard, WM and Wallace, RJ.** 5, 1993, American Review of Respiratory
558 Disease, Vol. 147.

559 16. *A Co-operative Numberical Analysis of Rapidly Growing Mycobacteria.* **Kubica, GP, et al.** 1972,
560 Journal of General Microbiology, Vol. 73, pp. 55-70.

561 17. *Amoebal Coculture of "Mycobacterium massiliense" sp. nov. from the Sputum of a Patient with*
562 *Hemoptoic Pneumonia.* **Adekambi, T, et al.** 12, 2004, Journal of Clinical Microbiology, Vol. 42, pp.
563 5493-5501.

564 18. *rpoB gene sequence-based characterization of emerging non-tuberculous mycobacteria with*
565 *descriptions of Mycobacterium bolletii sp. nov., Mycobacterium phocaicum sp. nov. and*
566 *Mycobacterium aubagnense sp. nov.* **Adekambi, T, et al.** 2006, International Joural of Systematic and
567 Evolutionary Microbiology, Vol. 56, pp. 133-143.

568 19. *Proposal that Mycobacterium massiliense and Mycobacterium bolletii be united and reclassified*
569 *as Mycobacterium abscessus subsp. bolletii comb. nov., designation of Mycobacterium abscessus*
570 *subsp. abscessus subsp. nov. and emended description of Mycobacteri.* **Leao, SC, et al.** 2011,
571 International Journal of Systematic and Evolutionary Microbiology, Vol. 61, pp. 2311-2313.

572 20. *Whole-genome sequencing to identify transmission of Mycobacterium abscessus between*
573 *patients with cystic fibrosis: a retrospective cohort study.* **Bryant, JM, et al.** 2013, The Lancet, Vol.
574 381, pp. 1551-1560.

575 21. *Emended description of Mycobacterium abscessus, Mycobacterium abscessus subsp. abscessus*
576 *and Mycobacterium abscessus subsp. bolletii and designation of Mycobacterium abscessus subsp.*
577 *massiliense comb. nov.* **Tortoli, E, Kohl, TA and Brown-Elliot, BA.** 2016 , International Journal of
578 Systematic and Evolutionary Microbiology, Vol. 66, pp. 4471-4479.

579 22. *Report of the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics.*
580 **Wayne, LG, et al.** 4, 1987, International Journal of Systematic Bacteriology, Vol. 37, pp. 463-464.

581 23. *Variables Affecting Results of Sodium Chloride Tolerance Test for Identification of Rapidly*
582 *Growing Mycobacteria.* **Conville, P and Witebsky, FG.** 6, 1998, Journal of Clinical Microbiology, Vol.
583 36, pp. 1555-1559.

584 24. *Deoxyribonucleic Acid Relatedness Study of the Mycobacterium fortuitum-Mycobacterium*
585 *chelonae Complex.* **Levy-Frebault, V, et al.** 3, 1986, International Journal of Systematic Bacteriology,
586 Vol. 36, pp. 458-460.

587 25. *Clinical Significance, Biochemical Features and Susceptibility Patterns of Sporadic Isolates of the*
588 *Mycobacterium chelonae-Like Organism.* **Wallace, RJ, et al.** 12, 1993, Journal of Clinical
589 Microbiology, Vol. 31.

590 26. *Identification of Clinically Significant Mycobacterium fortuitum Complex Isolates.* **Silcox, VA,**
591 **Good, RC and Floyd, MM.** 6, 1981, Journal of Clinical Microbiology, Vol. 14, pp. 686-691.

592 27. *Mycobacterium immunogenum sp. nov., a novel species related to Mycobacterium abscessus and*
593 *associated with clinical disease, pseudo-outbreaks and contaminated metalworking fluids: an*
594 *international cooperative study on mycobacterial taxonomy.* **Wilson, RW, et al.** 2001, International
595 Journal of Systematic and Evolutionary Microbiology, Vol. 51, pp. 1751-1764.

596 28. *Understanding nontuberculous mycobacterial lung disease: its been a long time coming.* **Griffith,**
597 **DE and Aksamit, TR.** 2016, F1000 Research, pp. 1-8.

598 29. *Lung Infections Assocaited with Cystic Fibrosis.* **Lyczak, JB, Cannon, CL and Pier, GB.** 2, 2002,
599 Clinical Microbiology Reviews, Vol. 15, pp. 194-222.

600 30. *The natural history of nontuberculous mycobacteria in patients with cystic fibrosis.* **Olivier, KN.**
601 (Suppl A), 2004, Paediatric Respiratory Reviews, Vol. 5, pp. S213-S216.

602 31. *Multicenter study of prevalence of nontuberculous mycobacteria in patients with cystic fibrosis in*
603 *France.* **Roux, AL, et al.** 12, 2009, Journal of Clinical Microbiology, Vol. 47, pp. 4124-4128.

604 32. *Prevalence of nontuberculous mycobacteria in cystic fibrosis clinics, United Kingdom, 2009.*
605 **Seddon, P, et al.** 7, 2013, Emerging Infectious Disease, Vol. 19, pp. 1128-1130.

606 33. *Nontuberculous Mycobacteria among Patients with Cystic Fibrosis in the United States. Screening*
607 *Practices and Environmental Risk.* **Adjemian, J, Olivier, KN and Prevots, DR.** 5, 2014, American
608 Journal Critical Care Medicine, Vol. 190, pp. 581-586.

609 34. *Nontuberculous mycobacteria in cystic fibrosis associated with allergic bronchopulmonary*
610 *aspergillosis and steroid therapy.* **Mussaffi, H, et al.** 2005, European Respiratory Journal, Vol. 25, pp.
611 324-328.

612 35. *An Official ATS/IDSA Statement: Diagnosis, Treatment, and Prevention of Nontuberculosis*
613 *Mycobacterial Diseases.* **Griffith, DE, et al.** 4, 2007, American Journal of Respiratory and Critical Care
614 Medicine, Vol. 175, pp. 367-417.

615 36. *Epidemiology of nontuberculous myocbacteria (NTM) amongst individuals with cystic fibrosis*
616 *(CF).* **Viviani, L, et al.** 5, 2016, Journal of Cystic Fibrosis, Vol. 15, pp. 619-623.

617 37. *Multicenter Cross-Sectional Study of Nontuberculous Mycobacteria Infections among Cystic*
618 *Fibrosis Patients, Israel.* **Levy, I, et al.** 3, 2008, Vol. 14, pp. 378-384.

619 38. *Leptin modulates the T-cell immune response and reverses starvation-induced*
620 *immunosuppression.* **Lord, GM, et al.** 6696, 1998, Nature, Vol. 394, pp. 897-901.

621 39. *Clinical and taxonomic status of pathogenic nonpigmented or late-pigmenting rapidly growing*
622 *mycobacteria.* **Brown-Elliott, BA and Wallace, RJ.** 4, 2002, Clinical Microbiology Review, Vol. 15, pp.
623 716-746.

624 40. *Outbreak of Mycobacterium abscessus wound infections among "lipotourists" from the United*
625 *States who underwent abdominoplasty in the Dominican Republic.* **Furuya, EY, et al.** 8, 2008, Clinical
626 Infectious Diseases, Vol. 46, pp. 1181-1188.

627 41. *Late-Onset Posttraumatic Skin and Soft-Tissue Infections Caused by Rapid-Growing Mycobacteria*
628 *in Tsunami Survivors.* **Appelgren, P, et al.** 2, 2008, Clinical Infectious Diseases, Vol. 47, pp. 11-16.

629 42. *Mycobacterium abscessus infections in lung transplants: 15 year experience from a single*
630 *institution.* **Osmani, M, et al.** 2, 2018, Transplant Infectious Disease, Vol. 20, pp. 1-8.

631 43. *Mycobacterium abscessus chest wall and pulmonary infection in a cystic fibrosis lung transplant*
632 *recipient.* **Taylor, JL and Palmer, SM.** 8, 2006, Journal of Heart and Lung Transplant, Vol. 25, pp. 985-
633 988.

634 44. *Lung transplant outcomes in cystic fibrosis patients with pre-operative Mycobacterium abscessus*
635 *respiratory infections.* **Lobo, LJ, et al.** 4, 2013, Clinical Transplantation, Vol. 27, pp. 523-529.

636 45. *Lung transplantation in patients with cystic fibrosis and Mycobacterium abscessus infection.*
637 **Gillijam, M, et al.** 4, 2010, Journal of Cystic Fibrosis, Vol. 9, pp. 272-276.

638 46. *Microcolony and biofilm formation as a survival strategy for bacteria.* **Johnson, LR.** 1, s.l. : Journal
639 of Theoretical Biology, 2008, Vol. 251, pp. 24-34.

640 47. *Nontuberculosis mycobacteria and the lung: from suspicion to treatment.* **McGrath, EE, et al.** 4,
641 2010, Lung, Vol. 188, pp. 269-282.

642 48. *Health Impacts of Environmental Mycobacteria.* **Primm, T, Lucero, CA and JO, Falkinham.** 1,
643 2004, Clinical Microbiology Review, Vol. 17, pp. 98-106.

644 49. *The Envelope of Mycobacteria.* **Brennan, P and Nikaido, H.** 1995, Annual Review of Biochemistry,
645 Vol. 64, pp. 29-63.

646 50. *Physiochemical Cell Surface and Adhesive Properties of Coryneform Bacteria Related to the*
647 *Presence and Chain Length of Mycolic Acids.* **Bendinger, B, et al.** 11, 1993, Applied and
648 Environmental Microbiology, Vol. 59, pp. 3973-3977.

649 51. *Impact of human activities on the ecology of nontuberculosis mycobacteria.* **Falkinham, JO.** 6,
650 2010, Future Microbioloy, Vol. 5, pp. 951-960.

651 52. *Permeability Barrier to Hydrophilic Solutes in Mycobacterium chelonei.* **Jarlier, V and Nikaido, H.**
652 3, 1990, Journal of Bacteriology, Vol. 172, pp. 1418-1423.

653 53. *Whole-Genome Sequencing Reveals Global Spread of Mycobacterium Abscessus Clones Amongst*
654 *Patients With Cystic Fibrosis.* **Grogono, D, et al.** Washington : American Thoracic Society, 2017. C25
655 Non-Tuberculosis Mycobacteria: From Bench to Clinic. Vol. 195.

656 54. *Opportunistic Pathogens Enriched in Showerhead Biofilms.* **Feazel, LM, et al.** 38, 2009,
657 Proceedings of the National Academy of Sciences, Vol. 106, pp. 16393-16399.

658 55. *Environmental Nontuberculous Mycobacteria in the Hawaiian Islands.* **Honda, JR, et al.** 10, 2016,
659 PLOS Neglected Tropical Diseases, Vol. 10, pp. 1-17.

660 56. *First United States Outbreak of Mycobacterium abscessus Hand and Foot Disease Among*
661 *Children Associated With a Wading Pool.* **Carter, KK, et al.** 2018, Pediatric Infectious Diseases
662 Society, pp. 1-6.

663 57. *General Overview on Nontuberculous Mycobacteria, Biofilms and Human Infection.* **Faria, S, Joao,**
664 **I and Jordao, L.** 2015, Journal of Pathogens, Vol. 2015, pp. 1-10.

665 58. **Group, Cystic Fibrosis Trust Mycobacterium abscessus Infection Control Working.**
666 *Mycobacterium abscessus: Suggestions for infection prevention and control (Interim guidance -*
667 *October 2013).* s.l. : Cystic Fibrosis Trust, 2013.

668 59. *Lack of Transmission of Mycobacterium abscessus among Patients with Cystic Fibrosis Attending*
669 *a Single Clinic.* **Bange, FC, et al.** 11, 2001, Clinical Infectious Diseases, Vol. 32, pp. 1648-1650.

670 60. *Whole-Genome Sequencing and Epidemiological Analysis Do Not Provide Evidence for Cross-*
671 *transmission of Mycobacterium abscessus in a Cohort of Pediatric Cystic Fibrosis Patients.* **Harris, KA,**
672 **et al.** 7, 2015, Clinical Infectious Diseases: an official publication of the Infectious Diseases Society of
673 America, Vol. 60, pp. 1007-1016.

674 61. *British Thoracic Society Guideline for the management of non-tuberculous mycobacterial*
675 *pulmonary disease (NTM-PD).* **Haworth, CS, et al.** 2017, BMJ Open Respiratory Research, Vol. 4, pp.
676 1-12.

677 62. *An Official ATS/IDSA Statement: Diagnosis, Treatment and Prevention of Nontuberculosis*
678 *Mycobacterial Disease.* **Griffith, DE, et al.** 4, 2007, American Thoracic Society Journals, Vol. 175, pp.
679 367-417.

680 63. *Antibiotic Treatment of Mycobacterium abscessus Lung Disease.* **Jeon, K, et al.** 2009, American
681 Journal of Respiratory and Critical Care Medicine, Vol. 180, pp. 896-902.

682 64. *Treatment of Mycobacterium abscessus infection.* **Novosad, SA, et al.** 3, 2016, Emerging
683 Infectious Diseases, Vol. 22, pp. 511-514.

684 65. *Does Clarithromycin Cause Hearing Loss? A 12-Year Review of Clarithromycin Therapy for*
685 *Nontuberculous Mycobacterial Lymphadenitis in Children.* **Heffernan, CB, et al.** 2018, Annals of
686 Otology, Rhinology and Laryngology.

687 66. *Irreversible sensorineural hearing loss due to clarithromycin.* **Coulston, J and Balaratnam, N.**
688 2005, Postgraduate Medicine Journal, Vol. 81, pp. 58-59.

689 67. *Overview of the tolerability profile of clarithromycin in preclinical and clinical trials.* **Guay, DR, et**
690 **al.** 5, 1993, Drug Safety, Vol. 8, pp. 350-364.

691 68. *Clinical and Taxonomic Status of Pathogenic Nonpigmented or Late-Pigmenting Rapidly Growing*
692 *Mycobacteria.* **Brown-Elliott and Wallace, RJ.** 4, 2002, Clinical Microbiology Reviews, Vol. 15, pp.
693 716-746.

694 69. *Role of embB in Natural and Acquired Resistance to Ethambutol in Mycobacteria.* **Alcaide, F,**
695 **Pfyffer, GE and Telenti, A.** 10, 1997, Antimicrobial Agents and Chemotherapy, Vol. 41, pp. 2270-
696 2273.

697 70. *The talking Mycobacterium abscessus blues.* **Griffith, DE.** 5, 2011, Clinical Infectious Disease, Vol.
698 52, pp. 572-574.

699 71. *Mycobacterial cell wall: structure and role in natural resistance to antibiotics.* **Jarlier, V and**
700 **Nikaido, H.** 1-2, 1994, FEMS Microbiology Letters, Vol. 123, pp. 11-18.

701 72. *The envelope layers of mycobacteria with reference to their pathogenicity.* **Daffe, M and Draper,**
702 **P.** s.l. : Advanced Microbial Physiology, 1998, Vol. 39, pp. 131-203.

703 73. *Foundations of antibiotic resistance in bacterial physiology: the mycobacteria paradigm.* **Nguyen,**
704 **L and Thompson, CJ.** 7, 2006, Trends in Microbiology, Vol. 14, pp. 304-312.

705 74. *Role of mycobacterial efflux transporters in drug resistance: an unresolved question.* **De Rossi, E,**
706 **Ainsa, JA and Riccardi, G.** 2006, FEMS Microbiology Review, Vol. 30, pp. 36-52.

707 75. *A Balancing Act: Efflux/Influx in Mycobacterial Drug Resistance.* **Louw, GE, et al.** 8, 2009,
708 Antimicrobial Agents and Chemotherapy, Vol. 53, pp. 3181-3189.

709 76. *Non Mycobacterial Virulence Genes in the Genome of the Emerging Pathogen Mycobacterium*
710 *abscessus.* **Ripoll, F, et al.** 6, 2009, PLoS ONE, Vol. 4.

711 77. *Analysis of the proteome of Mycobacterium tuberculosis in silico.* **Tekaia, F, et al.** 6, 1999,
712 Tubercle and Lung Disease, Vol. 79, pp. 329-342.

713 78. *mmpL7 Gene of Mycobacterium tuberculosis Is Responsible for Isoniazid Efflux in Mycobacterium*
714 *smegmatis.* **Pasca, MR, et al.** 11, 2005, Antimicrobial Agents and Chemotherapy, Vol. 49, pp. 4775-
715 4777.

716 79. *A Novel Gene, erm(41), Confers Inducible Macrolide Resistance to Clinical Isolaes of*
717 *Mycobacterium abscessus but is Absent from Mycobacterium chelonae.* **Nash, KA, Brown-Elliot, BA**
718 **and Wallce, RJ.** 4, 2009, Antimicrobial Agents and Chemotherapy, Vol. 53, pp. 1367-1376.

719 80. *Rapid detection of mutations in erm(41) and rrl associated with clarithromycin resistance in*
720 *Mycobacterium abscessus complex by denaturing gradient gel electrophoresis.* **Liu, W, et al.** 2017,
721 Journal of Microbiological Methods, Vol. 143, pp. 87-93.

722 81. *Utility of Seqencing the erm(41) Gene in Isolates of Mycobacterium abscessus subsp. abscessus*
723 *with Low and Intermediate Clarithromycin MICs.* **Brown-Elliot, BA, et al.** 4, 2015, Journal of Clinical
724 Microbiology, Vol. 53, pp. 1211-1215.

725 82. *Rapid Molecular Detection of Inducible Macrolide Resistance in Mycobacterium chelonae and M.*
726 *abscessus Strains: a Replacement for 14-Day Susceptibility Testing?* **Hanson, KE, et al.** 5, 2014,
727 Journal of Clinical Microbiology, Vol. 52, pp. 1705-1707.

728 83. *Mycobacterium massiliense in differentiated from Mycobacterium abscesssus and*
729 *Mycobacterium bolletii by erthythromycin ribosome methyltransferase gene (erm) and*
730 *clarithromycin susceptibility patterns.* **Kim, HY, et al.** 6, 2010, Microbiology and Immunology, Vol. 54,
731 pp. 347-353.

732 84. *Assessment of clarithromycin susceptibility in strains belonging to the Mycobacterium abscessus*
733 *group by erm(41) and rrl sequencing.* **Bastian, S, et al.** 2, 2011, Antimicrobial Agents and
734 Chemotherapy, Vol. 55, pp. 775-781.

735 85. *The role of ribosomal RNAs in macrolide resistance.* **Sander, P, et al.** 3, 1997, Molecular
736 Microbiology, Vol. 26, pp. 469-480.

737 86. *Correlation between Quinolone Susceptibility Patterns and Sequences in the A and B Subunits of*
738 *DNA Gyrase in Mycobacteria.* **Guillemin, I, Jarlier, V and Cambau, E.** 8, 1998, Antimicrobial Agents
739 and Chemotherapy, Vol. 42, pp. 2084-2088.

740 87. *Crystal structure of the breakage-reunion domain of DNA gyrase.* **Morais Cabral, JH, et al.** 1997,
741 Nature, Vol. 388, pp. 903-906.

742 88. *Role of embB in Natural and Acquired Resistance to Ethambutol in Mycobacteria.* **Alcaide, F,**
743 **Pfyffer, GE and Telenti, A.** 10, 1997, Antimicrobial Agents and Chemotherapy, Vol. 41, pp. 2270-
744 2273.

745 89. *Characterization of broad-spectrum Mycobacterium abscessus class A Beta-lactamase.* **Soroka,**
746 **D, et al.** 3, 2014, Journal of Antimicrobial Chemotherapy, Vol. 69, pp. 691-696.

747 90. *WhiB7, a transcriptional activator that coordinates physiology with intrinsic drug resistance in*
748 *Mycobacterium tuberculosis.* **Burian, J, et al.** 9, 2012, Expert Review of Anti-Infective Therapy, Vol.
749 10, pp. 1037-1047.

750 91. *Mycobacterium abscessus WhiB7 Regulates a Species-Specific Repertoire of Genes to Confer*
751 *Extreme Antibiotic Resistance.* **Hurst-Hess, K, Rudra, P and Ghosh, P.** 11, 2017, Antimicrobial Agents
752 and Chemotherapy, Vol. 61, pp. e01347-17.

753 92. *Successful treatment with faropenem and clarithroymcin of pulmonary Mycobacterium abscessus*
754 *infection.* **Tanaka, E, et al.** 2002, Journal of Infection and Chemotherapy, Vol. 8, pp. 252-255.

755 93. *Successful treatment with chemotherapy and corticosteroids of pulmonary Mycobacterium*
756 *abscessus infection accompanied by pleural effusion.* **Okazaki, A, et al.** 2013, Journal of Infections
757 and Chemotherapy, Vol. 19, pp. 964-968.

758 94. *Beta-lactamase inhibition by avibactam in Mycobacterium abscessus.* **Dubee, V, et al.** 4, 2015,
759 Journal of Antimicrobial Chemotherapy, Vol. 70, pp. 1051-1058.

760 95. *Inhibition of the Beta-lactamase BlaMab by Avibactam Improves the In Vitro and In Vivo Efficacy*
761 *of Imipenem against Mycobacterium abscessus.* **Lefebvre, AL, et al.** 4, 2017, Antimicrobial Agents
762 and Chemotherapy, Vol. 61.

763 96. *Correlation between quinolone susceptibility patterns and sequences in the A and B subunits of*
764 *DNA gyrase in mycobacteria.* **Guillemin, I, Jarlier, V and Cambau, E.** 8, 1998, Antimicrobial Agents
765 and Chemotherapy, Vol. 42, pp. 2084-2088.

766 97. *In Vitro Activities of DC-159a, a Novel Fluoroquinolone, against Mycobacterium Species.*
767 **Disratthakit, A and Doi, N.** 6, 2010, Antimicrobial Agents and Chemotherapy, Vol. 54, pp. 2684-2686.

768 98. *Mycobacterial cell wall biosynthesis: a multifaceted antibiotic target.* **Abrahams, KA and Besra,**
769 **GS.** 2, 2018, Parasitology, Vol. 145, pp. 116-133.

770 99. *In vitro antimicrobial activities of capuramycin analogues against non-tuberculous mycobacteria.*
771 **Dubuisson, T, et al.** 12, 2010, Journal of Antimicrobial Chemotherapy, Vol. 65, pp. 2590-2597.

772 100. *In vitro antimycobacterial activities of capuramycin analogues.* **Reddy, VM, Einck, L and Nacy,**
773 **CA.** 2, 2008, Antimicrobial Agents and Chemotherapy, Vol. 52, pp. 719-721.

774 101. *Design, synthesis and evaluation of indole-2-carboxamides with pan anti-mycobacterial activity.*
775 **Franz, ND, et al.** 14, 2017, Bioorganic and Medicinal Chemistry, Vol. 25, pp. 3746-3755.

776 102. *Indole-2-carboxamides are Active Against an Actute Mycobacterium abscessus Infected Mouse*
777 *Model.* **Pandya, AN, et al.** 2019, Antimicrobial Agents and Chemotherapy, pp. 1-16.

778 103. *High Levels of Intrinsic Tetracycline Resistance in Mycobacterium abscessus Are Conferred by a*
779 *Tetracycline-Modifying Monooxygenase.* **Rudra, P, et al.** 6, 2018, Antimicrobial Agents and
780 Chemotherapy, Vol. 62, pp. 1-14.

781 104. *Clinical experience in 52 patients with tigecycline-containing regimens for salvage treatment of*
782 *Mycobacterium abscessus and Mycobacterium chelonae infections.* **Wallace, RJ, et al.** 7, 2014,
783 Journal of Antimicrobial Chemotherapy, Vol. 69, pp. 1945-1953.

784 105. *In Vitro Activity of TP-271 against Mycobacterium abscessus, Mycobacterium fortuitum, and*
785 *Nocardia Species.* **Cynamon, M, et al.** 7, 2012, Antimicrobial Agents and Chemotherapy, Vol. 56, pp.
786 3986-3988.

787 106. *Susceptibility of Mycobacterium abscessus to Antimycobacterial Drugs in Preclinical Models.*
788 **Obregon-Henao, A, et al.** 11, 2015, Antimicrobial Agents and Chemotherapy, Vol. 59, pp. 6904-6912.

789 107. *Bedaquiline as a potential agent in the treatment of Mycobacterium abscessus infections.*
790 **Vesenbeckh, S, et al.** 2017, European Respiratory Journal, Vol. 49.

791 108. *Proposal of Mycobacterium peregrinum sp. nov., nom. rev., and Elevation of Mycobacterium*
792 *chelonae subsp. abscessus (Kubica et al.) to Species Status: Mycobacterium abscessus comb. nov.*
793 **Kusunoki, S and Ezaki, T.** 2, 1992, International Journal of Systematic Bacteriology, Vol. 42, pp. 240-
794 245.

795 109. *Challenges of NTM Drug Development.* **Falkinham, JO.** 1613, 2018, Frontiers in Microbiology,
796 Vol. 9, pp. 1-7.

797 110. *S92 Genomic investigation unmasks evidence of transmission across mycobacterium abscessus*
798 *cystic fibrosis patients.* **Alateah, S, et al.** s.l. : Thorax, 2017. Vol. 72, p. A56.

799 111. *16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses,*
800 *Perils and Pitfalls.* **Janda, JM and Abbott, SL.** 9, 2007, Journal of Clinical Microbiology, Vol. 45, pp.
801 2761-2764.

802 112. *16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory.* **Patel,**
803 **JB.** 4, 2001, Molecular Diagnostics, Vol. 6, pp. 313-321.

804 113. *A novel gene, erm(41), confers inducible macrolide resistance to clinical isolates of*

805 *Mycobacterium abscessus but is absent from Mycobacterium chelonae.* **Nash, KA, Brown-Elliot, BA,**

806 **Wallace, RJ.** 2009, Antimicrobial Agents and Chemotherapy, Vol. 53, pp. 1367-1376.

807 114. *Challenges facing the drug discovery pipeline for non-tuberculous mycobacteria.* **Soni, I, et al.**

808 2016, Journal of Medical Microbiology, Vol. 65, pp. 1-8.

809

810