Title: *In vitro* activity of imipenem/relebactam alone and in combination against cystic fibrosis isolates of *Mycobacterium abscessus*

Running Title: IMI/REL activity against CF clinical MABSC isolates

Keywords: *Mycobacterium abscessus;* Cystic fibrosis; Imipenem/relebactam; Synergy; Intracellular activity

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Abstract:

Background: *Mycobacterium abscessus* complex (MABSC) is an emerging opportunistic pathogen that causes chronic, difficult-to-treat pulmonary infections, particularly in people with cystic fibrosis (PwCF), leading to rapid lung function decline, morbidity, and mortality. Effective treatment is particularly challenging due to the pathogen's resistance mechanisms and the need for prolonged multidrug therapy, which is often hindered by poor clinical outcomes, highlighting the urgent need for novel therapeutic strategies.

Imipenem/cilastatin/relebactam (IMI/REL), a novel β-lactam-β-lactamase inhibitor combination, demonstrates *in vitro* activity against resistant MABSC strains and effective pulmonary penetration. Prior literature indicates synergistic activity of imipenem with various antibiotics against *M. abscessus*.

Methods: This study aims to evaluate the *in vitro* activity of IMI/REL, alone and in combination with other antibiotics, against MABSC clinical isolates from PwCF (n=28). Susceptibility and synergy were assessed using broth microdilution and checkerboard assays. Extracellular and intracellular time-kill assays were performed to evaluate bactericidal activity of synergistic two- and three-drug combinations.

Results: IMI/REL demonstrated potent *in vitro* activity against clinical MABSC isolates, exhibiting substantial synergy with cefuroxime, cefdinir, amoxicillin, and cefoxitin. Rifabutin, azithromycin, moxifloxacin, clofazimine, minocycline, and omadacycline also demonstrated additive effects with IMI/REL. Extracellular time-kill assays identified IMI/REL+cefoxitin+rifabutin/moxifloxacin as the most effective combinations. Rifabutin demonstrated the highest intracellular killing activity, with combination regimens involving moxifloxacin+rifabutin/omadacycline/azithromycin resulting in greater reductions in bacterial load.

Conclusion: These findings suggest that IMI/REL may offer a significant advancement in the management of MABSC infections in PwCF. The promising efficacy of multidrug regimens combining IMI/REL with agents like cefoxitin, azithromycin, moxifloxacin, rifabutin, and omadacycline highlights potential therapeutic strategies.

1. Introduction:

Cystic fibrosis (CF) is an autosomal recessive, multisystem disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which disrupts ion and water transport across epithelial cells [1-2]. This dysfunction results in increased mucus viscosity and impaired secretion, particularly in the respiratory and gastrointestinal tracts. The thickened mucus impairs mucociliary clearance and depletes airway surface liquid volume, triggering damaging cycles of airway obstruction, inflammation, and infection that progressively impair lung function, ultimately leading to respiratory failure [2]. As lung function deteriorates, people with CF (PwCF) become increasingly vulnerable to chronic bacterial infections.

Nontuberculous mycobacteria (NTM) are increasingly being isolated from the sputum of PwCF, with prevalence estimates rising from 1.3% in 1984 to 13.9% in 2019 [3-5]. The prevalence of NTM increases with age, from an average of 10% in children aged 10 years to over 30% in adults above 40 [3]. Additionally, between 2010 and 2019, the average annual incidence of NTM pulmonary infections among PwCF in the U.S. was 58.0 cases per 1,000 individuals, with a significant annual increase of 3.5% [6]. Over time, NTM can cause progressive inflammatory lung damage, a condition termed “NTM pulmonary disease” (NTM-PD). PwCF are predisposed to NTM-PD in part due to reduced CFTR-mediated reactive oxygen species generation within macrophages, leading to reduced intracellular killing [7]. Prior literature has also shown that NTM have evolved mechanisms to evade host immune responses and survive intracellularly [8-9], presenting significant challenges for antibiotics to effectively target and eradicate the infection. Prevalence surveys worldwide show that the slow-growing *Mycobacterium avium* complex and rapidly-growing *Mycobacterium abscessus* complex (MABSC) account for over 95% of NTM lung disease cases in PwCF [10]. Recent epidemiological studies have demonstrated that the presence of MABSC in the airways of PwCF is associated with more rapid lung function decline and higher mortality [3]. Furthermore, a large-scale longitudinal study identified *M. abscessus* as the most significant contributor to lung function deterioration among NTM and gram-negative bacteria in this population [11].

Treatment of MABSC pulmonary disease (MABSC-PD) remains highly challenging and necessitates prolonged therapy with multiple antibiotics. Current treatment guidelines include an initial intensive phase

49 consisting of oral azithromycin in combination with several intravenous (IV) antibiotics (e.g., amikacin,
50 tigecycline, imipenem, and cefoxitin), administered over 3-12 weeks. This is followed by a continuation phase
51 with a daily oral macrolide (preferably azithromycin), inhaled amikacin, and 2-3 additional oral antibiotics (e.g.,
52 moxifloxacin, minocycline, clofazimine, and linezolid) [12-14]. However, MABSC-PD remains a significant
53 therapeutic challenge due to the limited number of safe and effective antibiotics available for treatment, as
54 reflected in poor clinical outcomes with sputum culture conversion rates of approximately 45% [15-16]. Multi-
55 drug resistance towards existing agents creates additional challenges. *M. abscessus* is particularly difficult to treat
56 due to a combination of intrinsic, adaptive, and acquired resistance mechanisms, including permeability barriers,
57 highly efficient drug efflux systems, low-affinity antibiotic targets, and the production of drug-neutralizing
58 enzymes [17-18]. A major driver of resistance is the intrinsic expression of Bla_{mab}, a broad-spectrum β-lactamase
59 that significantly reduces the activity of β-lactam agents [19]. Recent studies have reported high overall resistance
60 rates to imipenem (55.6%) for MABSC [20]. Notably, imipenem's efficacy is significantly enhanced when
61 combined with relebactam, a potent inhibitor of Bla_{mab}, resulting in at least a twofold increase in activity [21-22].
62 The safety and tolerability of current therapies further complicate treatment efforts. A recent study reported that
63 79% of patients receiving treatment for MABSC-PD reported adverse side effects, the most severe being
64 ototoxicity, gastrointestinal distress, and myelosuppression. Such side effects – particularly those caused by
65 amikacin, tigecycline, and linezolid, respectively – required therapy modifications for 25% of patients [16]. Thus,
66 there is an urgent need for new therapeutic strategies, including novel antibiotic combinations, to effectively
67 combat MABSC-PD.

68 Imipenem/cilastatin/relebactam (IMI/REL) is a novel β-lactam-β-lactamase inhibitor combination that is
69 currently approved for use in adults with hospital-acquired and/or ventilator-associated bacterial pneumonia,
70 complicated urinary tract infections, and complicated intra-abdominal infections. It is administered as a 1.25 g
71 intravenous infusion (imipenem 500 mg, cilastatin 500 mg, relebactam 250 mg) every six hours [23]. IMI/REL
72 is generally well tolerated, with a relatively low incidence of adverse side effects [24]. Importantly, IMI/REL
73 demonstrates activity against resistant *M. abscessus* strains [21, 25-26], with pharmacokinetic data showing

74 effective pulmonary penetration in healthy volunteers [27-28]. Furthermore, imipenem has demonstrated *in vitro*
75 synergistic activity against *M. abscessus* with various antibiotics [21, 29-32], and its use in multidrug regimens
76 has been strongly associated with improved treatment outcomes for MABSC-PD, with an adjusted odds ratio of
77 2.1-2.7 [15-16].

78 The following study aims to assess the activity of IMI/REL, alongside other antibiotics, against clinical
79 MABSC isolates from PwCF. Given the prevalence of multidrug-resistant MABSC strains in clinical practice, it
80 is critical to assess the efficacy of experimental therapies against contemporary lung disease isolates.
81 Susceptibility testing was conducted on twenty-eight unique MABSC isolates to compare the effectiveness of
82 IMI/REL with ten other antibiotics. Additionally, checkerboard synergy and time-kill assays were performed to
83 identify optimal antibiotic combinations involving IMI/REL and evaluate intracellular activity. This research
84 represents the first comprehensive evaluation of IMI/REL in combination with various double and triple antibiotic
85 regimens against CF clinical MABSC isolates, offering valuable insights into potential treatment strategies for
86 MABSC in PwCF.

87

88 2. Methods:

89 2.1. Preparation of antibiotics and growth media

90 IMI/REL was provided by Merck. Amikacin, amoxicillin, cefoxitin, cefdinir, cefuroxime, moxifloxacin,
91 azithromycin, rifabutin, clofazimine, minocycline, and tigecycline were purchased from Sigma-Aldrich.
92 Tedizolid was purchased from MedChem Express. Omadacycline was obtained from Paratek Pharmaceuticals.
93 Antibiotics were prepared according to their solubility and the manufacturer's guidelines.

94 The standard liquid growth medium used for initiating bacterial cultures consisted of Middlebrook 7H9 broth,
95 supplemented with 10% (v/v) Middlebrook oleic acid-albumin-dextrose-catalase (OADC) Enrichment, 0.5%
96 (v/v) glycerol, and 0.05% (v/v) Tween 80. The standard solid growth medium was Middlebrook 7H10 agar
97 enriched with 10% (v/v) OADC. For cryogenic storage, glycerol-stock medium was formulated using
98 Middlebrook 7H9 broth supplemented with 10% (v/v) OADC, 15% (v/v) glycerol, and 0.05% (v/v) Tween 80.

99 Middlebrook 7H9 Broth Base and Middlebrook OADC Enrichment were purchased from Becton, Dickinson and
100 Company, while Middlebrook 7H10 Agar Base, glycerol, and Tween 80 were purchased from Sigma-Aldrich.

101 **2.1. Bacterial strains and culture conditions**

102 The *M. abscessus* reference strain (ATCC 19977) was obtained from the American Type Culture Collection,
103 while CF clinical isolates were acquired from National Jewish Health. Twenty-eight clinical isolates were chosen
104 from the National Jewish Health culture collection to represent the distribution of *M. abscessus* subspecies in
105 PwCF, with selection criteria informed by population genomics data from the Colorado Research and
106 Development Program [33]. For long-term storage, all strains were stored at -80°C in glycerol-stock medium.
107 Frozen stocks were streaked onto round 7H10 plates (100 × 15 mm polystyrene petri dishes) and incubated at
108 30°C for three days to promote growth, after which the plates were stored at 2-4°C. Single colonies were then
109 used to inoculate 5 mL cultures of 7H9 broth in 15 mL polypropylene culture tubes. Cultures were incubated at
110 30°C in a shaking incubator (180 rpm) for 48-96 hours to reach log-phase exponential growth.

111 **2.3. Broth microdilution assay and minimum inhibitory concentration (MIC) determination for**
112 **susceptibility testing**

113 Antibiotic susceptibility testing was performed on the *M. abscessus* ATCC 19977 reference strain and the
114 twenty-eight CF clinical isolates. The MIC of each antibiotic was determined using the broth microdilution
115 method, according to the Clinical Laboratory Standards Institute (CLSI) guidelines for NTM [34]. Exponentially
116 growing *M. abscessus* cultures were adjusted to a 0.5 McFarland standard in 1X phosphate-buffered saline (PBS;
117 Cytiva) using an Accuris SmartReader 96 spectrophotometer (Accuris Instruments) at 600 nm, then diluted 1:100
118 in Cation-Adjusted Mueller-Hinton broth (CAMHB; Sigma-Aldrich). Antibiotic solutions were prepared via two-
119 fold serial dilution in CAMHB. IMI/REL was prepared by serially diluting imipenem while maintaining a fixed
120 concentration of 4 μg/mL relebactam, consistent with prior published studies [25]. All other antibiotics were
121 diluted according to standard broth dilution methods. In a 96-well microtiter plate, 50 μL of the bacterial
122 suspension was added to each well containing 50 μL of the respective antibiotic dilutions, for a final inoculum
123 density of approximately 5×10^5 CFU/mL. Negative control wells (containing CAMHB only) were also included.

124 After incubation at 30°C for 3 days, susceptibility to the antimicrobial agents was assessed by visual inspection
125 using an inverted mirror. MICs were defined as the lowest antibiotic concentrations that completely suppressed
126 observable bacterial growth. Each antibiotic concentration was tested in triplicate. In this study, MIC_{50} was
127 defined as the lowest concentration of antibiotic that inhibited 50% of the tested CF isolates, and MIC_{90} as the
128 lowest concentration of antibiotic that inhibited 90% of the tested CF isolates.

129 The antimicrobial agents tested against ATCC 19977 included amikacin, amoxicillin, cefoxitin, cefdinir,
130 cefuroxime, IMI/REL, moxifloxacin, azithromycin, tedizolid, rifabutin, clofazimine, minocycline, tigecycline,
131 and omadacycline. Supplementary Table 1 summarizes the concentration ranges evaluated and the corresponding
132 breakpoints for the 14 antimicrobials tested in this study. Susceptible, intermediately susceptible, and resistant
133 breakpoints for amikacin, cefoxitin, imipenem, moxifloxacin, azithromycin, and minocycline followed the CLSI
134 guidelines for rapidly growing mycobacteria [34]. However, to date, there are no consensus breakpoints for
135 amoxicillin, cefdinir, cefuroxime, tedizolid, rifabutin, clofazimine, omadacycline, and tigecycline. Because of
136 this, modified susceptibility breakpoints were adopted for (i) amoxicillin, based on CLSI amoxicillin-clavulanic
137 acid breakpoints for *Nocardia* and other Actinomycetes [34]; (ii) cefdinir and cefuroxime, assumed to follow
138 CLSI breakpoints for cefoxitin as oral cephalosporins; (iii) tedizolid, assumed to be the same as linezolid [34];
139 (iv) rifabutin, based on CLSI rifabutin breakpoints for *M. marinum* [34]; (v) clofazimine, based on a molecular
140 resistance study for *M. abscessus* [35] (vi) tigecycline, based on a clinical study for *M. abscessus* [36]; and (vii)
141 omadacycline, based on a hollow fiber study for *M. abscessus* [37].

142 **2.4. Checkerboard assay and fractional inhibitory concentration (FIC) index determination for synergy**
143 **testing**

144 Checkerboard assays were conducted to evaluate the *in vitro* synergy of IMI/REL with other antibiotics
145 against *M. abscessus* ATCC 19977 and the twenty-eight CF clinical isolates, as previously described [31]. This
146 modified broth microdilution method involved preparing a series of two-fold dilution combinations of antibiotics
147 in CAMHB within a 96-well plate. The concentrations of each antibiotic ranged from 2× the respective MIC to
148 1/64× the MIC for the *M. abscessus* ATCC 19977 reference strain, with all possible combinations of these two-

149 fold dilutions evaluated. Bacterial cultures and antibiotic solutions were prepared as described above. In each
150 well, 50 µL of the bacterial suspension was combined with 50 µL of the respective antibiotic combination,
151 achieving a final inoculum density of approximately 5×10^5 CFU/mL. Negative control wells (containing
152 CAMHB only) were also included. Following a 72-hour incubation at 30°C, the plates were visually inspected
153 for bacterial growth. The FIC indices were calculated using the following formula: $FIC = (MIC_{AB}/MIC_A) +$
154 (MIC_{BA}/MIC_B). In this equation, MIC_{AB} is the MIC of drug A tested in combination and MIC_A is the MIC of drug
155 A tested alone, while MIC_{BA} is the MIC of drug B tested in combination and MIC_B is the MIC of drug B tested
156 alone [38]. An FIC index of ≤ 0.5 indicates synergy, values between > 0.5 to 1 suggest additive effects, values
157 between > 1 to 4 indicate indifference, and values ≥ 4 signify antagonism [38].

158 Initial screening of antibiotic combinations for *in vitro* synergy testing against ATCC 19977 included
159 IMI/REL with amikacin, amoxicillin, cefoxitin, cefdinir, cefuroxime, moxifloxacin, azithromycin, tedizolid,
160 rifabutin, clofazimine, minocycline, tigecycline, and omadacycline. Each antibiotic concentration was tested in
161 triplicate. Based on the screening results and a comprehensive literature review, antibiotics demonstrating strong
162 synergy with IMI/REL were prioritized for further testing in clinical isolates. These included cefoxitin, cefdinir,
163 cefuroxime, moxifloxacin, rifabutin, and minocycline, along with amoxicillin, azithromycin, clofazimine, and
164 omadacycline, which had shown synergistic potential in prior studies [21, 39-43]. Amikacin and tigecycline were
165 excluded due to significant side effects [16] and the preference for oral regimens, while tedizolid was omitted due
166 to its lack of synergy with IMI/REL. Each concentration combination was tested in singlicate, using ATCC 19977
167 as the reference quality control strain to ensure that all results for this strain were within the CLSI quality control
168 ranges [34, 44].

169 **2.5. Time-kill assays**

170 To evaluate the overall bactericidal activity of individual antibiotics and synergistic three-drug antibiotic
171 combinations with IMI/REL against *M. abscessus*, time-kill assays were conducted, as previously described [45].
172 Briefly, exponentially growing *M. abscessus* cultures were adjusted to a 0.5 McFarland standard in PBS using a
173 spectrophotometer at 600 nm, then diluted 1:100 in CAMHB. Four concentrations of each antibiotic were selected

174 based on the MICs determined for the *M. abscessus* ATCC 19977 reference strain: 16× MIC, 4× MIC, 1× MIC,
175 and 1/4× MIC. In a 96-well microtiter plate, 50 µL of the bacterial suspension was added to each well containing
176 50 µL of the respective antibiotic dilutions, for a final inoculum density of approximately 5×10^5 CFU/mL.
177 Negative control wells (containing CAMHB only) and positive growth control wells (containing bacterial
178 suspension and CAMHB only) were also included. The plates were incubated at 30°C, and 10 µL aliquots were
179 collected from each well at predetermined time points of 0, 24, 48, and 72 hours. Each aliquot was subjected to
180 five serial log dilutions, resulting in six total dilutions (10^0 to 10^{-5}) that were subsequently streaked onto square
181 7H10 agar plates. The plates were then incubated at 37°C for 72 hours, after which CFUs were counted. Each
182 antibiotic concentration was tested in duplicate. Selection of antibiotics was prioritized based on demonstrated
183 synergy with IMI/REL, intracellular penetration, activity at clinically achievable concentrations, and alignment
184 with established MABSC treatment guidelines. Thus, time-kill assays were conducted with single antibiotics and
185 combinations of IMI/REL with cefoxitin, moxifloxacin, azithromycin, rifabutin, clofazimine, and omadacycline.
186 Bactericidal activity was defined as a reduction of ≥3 $\log_{10}$ CFU/mL compared to the initial count [46]. Synergy
187 was characterized by a ≥2 $\log_{10}$ CFU/mL reduction in bacterial load for a given combination compared to the
188 most active single agent. Additivity was defined as a 1 to 2 $\log_{10}$ CFU/mL reduction in the final colony count
189 relative to the most active single agent. Indifference was indicated by a change of <1 $\log_{10}$ CFU/mL in the final
190 colony count when compared to the most active single agent [46-47].

191 **2.6. THP-1 cell culture**

192 The THP-1 cell line was purchased from ATCC and cultured at 37 °C under 5% CO_2 in RPMI 1640 medium
193 (Corning) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Genesee Scientific). Cell density
194 was maintained between 300,000 and 900,000 cells/mL. THP-1 monocytes were differentiated into macrophages
195 in the presence of 0.2 µM phorbol-12-myristate 13-acetate (PMA; Sigma-Aldrich) for 72 hours, followed by 24
196 hours of rest in PMA-free culture media prior to starting experiments. For all experiments, THP-1 cells were used
197 at passages 10-18.

198 **2.7. Intracellular time-kill assays**

199 To assess the intracellular bactericidal activity of individual antibiotics and synergistic two-drug antibiotic
200 combinations against *M. abscessus*, intracellular time-kill assays were conducted using an *in vitro* model of
201 infection in THP-1-derived macrophages, adapted from prior studies [48-52]. The individual antibiotics selected
202 for the intracellular time-kill assays included azithromycin, moxifloxacin, rifabutin, clofazimine, and
203 omadacycline. Additionally, combinations of these antibiotics were evaluated based on their MIC values against
204 the reference strain (ATCC 19977), their intracellular penetration, and their synergistic potential, as determined
205 through checkerboard and time-kill assays. Four concentrations of each antibiotic were selected based on the
206 MICs determined for the *M. abscessus* ATCC 19977 reference strain: 16× MIC, 4× MIC, 1× MIC, and 1/4× MIC.

207 THP-1 cells were seeded at a density of 10^5 cells/well into 96-well culture plates (Genesee Scientific) in RPMI
208 1640 medium (10% FBS) and differentiated as described above. Exponentially growing cultures of *M. abscessus*
209 ATCC 19977 were centrifuged (4000 rpm, 10 minutes) and resuspended in 1 mL of fresh 7H9 broth. This
210 centrifugation and resuspension process was repeated, after which the bacterial suspension was filtered through a
211 5 µm filter and adjusted to a 0.5 McFarland standard in PBS using a spectrophotometer at 600 nm. The resulting
212 bacterial suspension was centrifuged once more (4000 rpm, 10 minutes), resuspended in RPMI 1640 medium
213 (5% FBS), and adjusted to a final density of 5×10^6 CFU/mL.

214 The 96-well plates containing THP-1-derived macrophages were then centrifuged (750 rpm, 10 minutes).
215 After aspirating the media, 100 µL of the bacterial suspension was added to each well, achieving a final
216 multiplicity of infection (MOI) of 5. The plates were incubated for 4 hours (37ºC, 5% CO_2), after which they were
217 centrifuged (750 rpm, 10 minutes), the media aspirated, and the samples gently washed twice with PBS.
218 Subsequently, 100 µL of RPMI 1640 medium (5% FBS, 70 µM amikacin) were added to each well, and the plates
219 were incubated for an additional 2 hours under the same conditions.

220 Following the second incubation, the plates were again centrifuged (750 rpm, 10 minutes), the media
221 aspirated, and the samples gently washed twice with PBS. Then, 100 µL of RPMI (5% FBS) with the respective
222 antibiotic dilutions were added to the appropriate wells. Negative control wells (containing THP-1-derived

223 macrophages and RPMI only) and positive growth control wells (containing THP-1-derived macrophages, RPMI,
224 and bacterial suspension only) were also included. The plates were incubated (37ºC, 5% CO2), and cells were
225 lysed with 0.1% (v/v) Triton X-100 at predetermined time points of 0, 24, 48, and 72 hours. Lysates were
226 subjected to five serial log dilutions, resulting in six total dilutions (10^0 to 10^{-5}) that were subsequently streaked
227 onto square 7H10 agar plates. Additionally, the McFarland-adjusted bacterial suspension and the initial inoculum
228 were plated to retrospectively verify the desired bacterial density. The agar plates were then incubated at 37°C
229 for 72 hours, after which CFUs were counted. Each antibiotic concentration was tested in duplicate. Bactericidal
230 activity was defined as a reduction of ≥3 $\log_{10}$ CFU/mL compared to the initial count [46]. Synergy was
231 characterized by a ≥2 $\log_{10}$ CFU/mL reduction in bacterial load for a given combination compared to the most
232 active single agent. Additivity was defined as a 1 to 2 $\log_{10}$ CFU/mL reduction in the final colony count relative
233 to the most active single agent. Indifference was indicated by a change of <1 $\log_{10}$ CFU/mL in the final colony
234 count when compared to the most active single agent [46-47].

235 **2.8. Data and statistical analysis**

236 Statistical and graphical analyses were performed using GraphPad Prism version 10.1 (GraphPad Software,
237 Inc.). Fisher's exact tests were used to compare the susceptibility profiles of the CF clinical isolates between
238 individual antibiotics and their combinations with IMI/REL. Descriptive analysis of MICs was initially conducted
239 after $\log_2$-transformation and subsequent correction. Kruskal-Wallis tests were then applied to $\log_2$-transformed
240 MICs to compare susceptibility, stratified by subspecies, morphotype, and mutational status. Area under the curve
241 (AUC) values were calculated using the trapezoidal method. Reductions in bacterial load ($\log_{10}$ CFU/mL) and
242 AUC were evaluated using one-way ANOVA followed by Tukey's multiple comparisons test.

243

244 3. Results:

245 **3.1. *In Vitro* Susceptibility and Synergy Testing of Antibiotics Against *M. abscessus* ATCC 19977**

246 Susceptibility studies were conducted to evaluate the *in vitro* activities of IMI/REL, amikacin, amoxicillin,
247 cefoxitin, cefdinir, cefuroxime, moxifloxacin, azithromycin, tedizolid, rifabutin, clofazimine, minocycline,

248 tigecycline, and omadacycline against *M. abscessus* ATCC 19977, with results summarized in Table 1. The
249 antibiotics exhibited a range of inhibitory values. The *M. abscessus* ATCC 19977 strain displayed susceptibility
250 to imipenem, with a median MIC of 4 µg/mL across all assays. ATCC 19977 also demonstrated susceptibility to
251 clofazimine and tedizolid, intermediate susceptibility to cefoxitin and amikacin, and resistance to cefuroxime,
252 cefdinir, moxifloxacin, rifabutin, minocycline, amoxicillin, azithromycin, tigecycline, and omadacycline.

253 Checkerboard assays were conducted to evaluate the *in vitro* synergy of IMI/REL with various antibiotics,
254 and FIC indices were calculated to characterize their interactions. The MICs of the antibiotics alone and in
255 combination with IMI/REL against *M. abscessus* ATCC 19977 are also presented in Table 1, along with their
256 respective FIC indices. Synergistic interactions were observed with cefuroxime, cefdinir, cefoxitin, moxifloxacin,
257 rifabutin, and minocycline, while additive effects were noted for amoxicillin, azithromycin, tigecycline, amikacin,
258 clofazimine, and omadacycline. Indifference was observed with tedizolid, and no antagonism was detected in any
259 of the combinations tested.

260 **3.2. *In Vitro* Susceptibility Testing and Screening of Antibiotics for Synergy with IMI/REL Against *M.***
261 ***abscessus* CF Clinical Isolates**

262 A summary of the identified *M. abscessus* CF clinical isolates, detailing their subspecies classification,
263 morphology, and mutations, is provided in Supplementary Table 2. The twenty-eight isolates included 17 (60.7%)
264 *M. abscessus* subsp. *abscessus*, 7 (25.0%) *M. abscessus* subsp. *massiliense*, and 4 (14.3%) *M. abscessus* subsp.
265 *bolletii*. Among these, 3 isolates (10.7%) carried the 16S rRNA mutation at position 1408, and 6 isolates (21.4%)
266 had one or both 23S rRNA mutations at positions 2058/2059. In terms of morphotype, 15 isolates (53.6%)
267 exhibited the rough morphotype, 7 (25.0%) exhibited the smooth morphotype, and 6 (21.4%) presented
268 intermediate features of both morphotypes, leading to their classification as "unknown" along the smooth-rough
269 spectrum.

270 Susceptibility and synergy testing were performed to evaluate the *in vitro* activity of IMI/REL in combination
271 with various antibiotics against these isolates. The MIC_{50} values for the individual antibiotics, both alone and in
272 combination with IMI/REL, along with the median FIC indices, are summarized in Table 2. Supplementary Tables

273 3-12 provide these MIC values and FIC indices for each individual CF clinical isolate strain, along with the MIC_{50}
274 and MIC_{90} values. Overall, the MIC_{50} values for the CF clinical isolates of many of the antibiotics were the same
275 as the reference strain, including amoxicillin, cefuroxime, moxifloxacin, azithromycin, minocycline, and
276 omadacycline. For other antibiotics—cefoxitin, cefdinir, imipenem, rifabutin, and clofazimine—the MIC_{50}
277 differed by only a single two-fold dilution. However, several clinical isolates exhibited significantly higher MIC_{50}
278 values compared to the reference strain for various antibiotics. Outliers were identified for cefoxitin (n=3),
279 cefdinir (n=1), cefuroxime (n=1), moxifloxacin (n=1), minocycline (n=1), and omadacycline (n=2), with values
280 exceeding the MIC_{90}. Additionally, azithromycin demonstrated a broad distribution of isolates classified as
281 susceptible (21.4%), intermediate (25.0%), and resistant (53.6%) (Supplementary Table 13).

282 The combination of antibiotics with IMI/REL demonstrated varying degrees of synergistic, additive, and
283 indifferent effects across the tested CF clinical isolates (Figure 1). Cefuroxime demonstrated the highest synergy
284 (89.3%), followed by cefdinir (75.0%), cefoxitin (67.9%), and amoxicillin (57.1%), indicating strong synergistic
285 effects in most isolates. Rifabutin, azithromycin and moxifloxacin exhibited predominantly additive effects
286 (50.0%, 57.1% and 64.3%, respectively), while still achieving moderate synergy (46.4%. 42.9% and 35.7%,
287 respectively). Clofazimine and minocycline demonstrated limited synergy (14.3% and 21.4%, respectively),
288 alongside additive (71.4% and 50.0%, respectively) and indifferent (14.3% and 28.6%, respectively) effects. In
289 contrast, omadacycline lacked synergy altogether, presenting additive effects (57.1%) and a higher rate of
290 indifference (42.9%).

291 The median FIC indices revealed that many antibiotics exhibited comparable levels of synergy between the
292 reference strain and the clinical isolates, including synergistic effects with cefuroxime, cefdinir, and cefoxitin,
293 and additive effects with azithromycin, clofazimine, and omadacycline. In contrast, moxifloxacin and rifabutin,
294 which showed synergy in the reference strain, displayed more variable profiles, with balanced synergistic and
295 additive interactions across clinical isolates. Notably, amoxicillin and minocycline presented the greatest
296 variability in the degree of synergy with IMI/REL.

297 The susceptibility profiles of the 28 CF clinical isolates are provided in Supplementary Table 13 and
298 Supplementary Figure 1. Statistically significant differences were observed when comparing the susceptibility
299 profiles of isolates treated with antibiotics alone versus in combination with IMI/REL across susceptible,
300 intermediate, and resistant isolates for cefoxitin ($p < 0.0001$), cefdinir ($p < 0.0001$), cefuroxime ($p < 0.0001$),
301 azithromycin ($p = 0.00154$), rifabutin ($p = 0.0013$), and omadacycline ($p = 0.0016$). Additionally, significant
302 differences were found for cefoxitin ($p < 0.0001$), cefdinir ($p = 0.0030$), and azithromycin ($p = 0.0129$) when
303 comparing susceptible isolates to those classified as intermediate and resistant. Furthermore, comparisons
304 between the combined susceptible and intermediate isolates versus the resistant isolates revealed significant
305 differences for cefdinir ($p < 0.0001$), cefuroxime ($p < 0.0001$), azithromycin ($p = 0.0261$), and omadacycline (p
306 $= 0.0009$).

307 The distribution of MICs for individual antibiotics among CF clinical isolates were also assessed across
308 subspecies, morphotype, and RRL mutation status. Among subspecies, *M. abscessus* subsp. *abscessus* exhibited
309 significantly higher cefdinir MICs compared to *M. abscessus* subsp. *bolletii* ($p = 0.0363$), while *M. abscessus*
310 subsp. *bolletii* displayed elevated azithromycin MICs relative to *M. abscessus* subsp. *massiliense* ($p = 0.0331$).
311 No other MIC differences reached statistical significance between subspecies. When stratified by morphotype,
312 MIC distributions did not show significant variation. Although no statistically significant differences were
313 observed when stratifying by RRL mutation status, isolates with the RRL mutation had an average azithromycin
314 MIC approximately twofold higher than those without the mutation (12.7 vs. 6.2 μg/mL).

315 **3.3. Evaluation of Three-Drug Antibiotic Combinations with IMI/REL Using Time-Kill Assay**

316 **3.3.1. Initial Screening of Antibiotic Combinations Against *M. abscessus* ATCC 19977**

317 An endpoint activity assay was performed to assess the efficacy of seven individual antibiotics and nine
318 combination treatments with IMI/REL, all administered at 1× MIC. The mean bacterial loads ($\log_{10}$ CFU/mL)
319 over 72 hours for these single and combination therapies are presented in Figure 2*A*-*B*. None of the single
320 antibiotics (IMI/REL, cefoxitin, moxifloxacin, azithromycin, rifabutin, clofazimine, omadacycline) exhibited
321 bactericidal activity at 72 hours (Fig. 2*A*). However, all individual antibiotics resulted in lower bacterial counts

322 compared to the control at 72 hours ($F_{7,8} = 45.87$, $p < 0.001$). Statistically significant reductions in $\log_{10}$ CFU/mL
323 were observed for clofazimine (diff = −2.90, $p < 0.0001$), rifabutin (diff = −1.45, $p = 0.0010$), IMI/REL (diff =
324 −1.41, $p = 0.0012$), moxifloxacin (diff = −0.83, $p = 0.0200$), and cefoxitin (diff = −0.80, $p = 0.0372$). No
325 statistically significant reductions were observed with azithromycin (diff = −0.39) or omadacycline (diff = −0.23).
326 A comparison of IMI/REL in combination with other antibiotics revealed statistically significant differences
327 between treatment groups ($F_{9,10} = 28.85$, $p < 0.0001$) (Fig. 2*B*). Several combinations demonstrated bactericidal
328 activity and statistically significant reductions in $\log_{10}$ CFU/mL compared to the control at 72 hours, including
329 IMI/REL with cefoxitin + rifabutin (diff = −6.43, $p < 0.0001$), cefoxitin + moxifloxacin (diff = −4.26, $p < 0.0001$),
330 and azithromycin + clofazimine (diff = −3.89, $p = 0.0011$). In addition, the combinations of IMI/REL with
331 azithromycin + rifabutin, as well as cefoxitin + clofazimine, led to a significant reduction in bacterial load (diff =
332 −3.73, $p = 0.0016$ and diff = −2.97, $p = 0.0087$, respectively), although they did not achieve bactericidal activity.
333 Combinations of IMI/REL with cefoxitin + omadacycline and azithromycin + cefoxitin also demonstrated
334 moderate efficacy, with reductions of −1.62 and −1.50 $\log_{10}$ CFU/mL compared to the control at 72 hours,
335 respectively. The least effective combinations were IMI/REL with azithromycin + omadacycline and
336 azithromycin + moxifloxacin, yielding minimal reductions of −0.78 and −0.16 $\log_{10}$ CFU/mL, respectively.

337 **3.3.2. Kinetic Time-Kill Assay with Antibiotic Combinations Against *M. abscessus* ATCC 19977**

338 Kinetic time-kill assays were conducted to evaluate the efficacy of six individual antibiotics and four
339 combination treatments with IMI/REL against *M. abscessu*s ATCC 19977, all of which demonstrated bactericidal
340 activity in the initial screening assays, administered at 1× MIC. The mean bacterial loads ($\log_{10}$ CFU/mL)
341 measured at 24, 48, and 72 hours for both single and combination therapies are presented in Figure 3. All treatment
342 groups, including both single agents and combinations, exhibited lower CFU/mL compared to the untreated
343 control at each time point. AUC analysis revealed statistically significant reductions in bacterial load for all
344 treatment groups compared to the untreated control. Specifically, IMI/REL, cefoxitin, rifabutin, clofazimine, and
345 the combinations of IMI/REL with cefoxitin + moxifloxacin, azithromycin + clofazimine, cefoxitin + rifabutin,
346 and azithromycin + rifabutin showed highly significant effects ($p < 0.0001$). Moxifloxacin demonstrated a

347 significant reduction ($p = 0.0003$), while azithromycin exhibited a modest yet significant effect ($p = 0.0364$). At
348 the 72-hour terminal endpoint, significant reductions in bacterial load compared to the untreated control were
349 observed for IMI/REL (diff = -3.38, $p < 0.0001$), clofazimine (diff = -2.48, $p = 0.0007$), moxifloxacin (diff = -
350 2.44, $p = 0.0009$), cefoxitin (diff = -1.74, $p = 0.0027$), and rifabutin (diff = -1.71, $p = 0.0033$). In contrast,
351 azithromycin did not result in a significant reduction (diff = -0.83, $p = 0.5071$). The combination therapies
352 demonstrated further enhanced efficacy. The combinations of IMI/REL with cefoxitin + rifabutin, as well as with
353 cefoxitin + moxifloxacin, achieved both bactericidal and synergistic effects, resulting in complete bacterial
354 eradication at 72 hours (diff = -7.98, $p < 0.0001$ for both). In contrast, the combinations of IMI/REL with
355 azithromycin + clofazimine, as well as with azithromycin + rifabutin, produced significant reductions at 72 hours
356 compared to control (diff = -3.93, $p < 0.0001$ for both), but were bacteriostatic and demonstrated indifferent
357 effects compared to IMI/REL alone. Additional kinetic time-kill assays were performed to assess the efficacy of
358 these antibiotics, both individually and in combination, at concentrations of 16× MIC, 4× MIC, and 1/4× MIC,
359 revealing similar trends (Supplementary Fig. 2).

360 **3.3.3. Kinetic Time-Kill Assay with Antibiotic Combinations Against *M. abscessus* CF Clinical Isolates**
361 **CF13 and CF258**

362 Clinical isolates CF13 and CF258 were selected for additional kinetic time-kill assays with IMI/REL,
363 cefoxitin, and rifabutin based on their susceptibility to imipenem, representing MIC50 (moderately susceptible)
364 and MIC90 (least susceptible) strains, respectively (Supplementary Figs. 3 and 4). For both CF clinical isolates,
365 combination therapy (IMI/REL + cefoxitin + rifabutin) consistently resulted in the fastest eradication or the
366 greatest reduction in $\log_{10}$ CFU/mL compared to control across all concentrations. In CF13, at 16× MIC, all
367 antibiotics and the combination therapy exhibited bactericidal activity, achieving complete bacterial eradication
368 within 72 hours. Rifabutin and the combination therapy similarly demonstrated bactericidal effects at 4× MIC,
369 also leading to complete eradication within this timeframe. Although none of the treatments were bactericidal at
370 1× MIC, all displayed significant reductions in bacterial counts. Notably, rifabutin ($p = 0.0268$) and the
371 combination therapy ($p = 0.0143$) also showed statistically significant effects at 1/4× MIC. In CF258, at 16× MIC,

372 cefoxitin, rifabutin, and the combination therapy exhibited robust bactericidal activity, achieving complete
373 bacterial eradication at 16x MIC (Supplementary Figs. 3 and 4). However, no antibiotic nor the combination
374 displayed bactericidal effects at 4×, 1×, or 1/4× MIC. All treatments demonstrated statistically significant
375 reductions in $\log_{10}$ CFU/mL at 72 hours compared to the control at 4× MIC. At 1× and 1/4× MIC, IMI/REL ($p =$
376 0.0389 and 0.0485), rifabutin ($p = 0.0289$ and 0.0051), and the combination therapy ($p = 0.0011$ and 0.0005)
377 demonstrated statistically significant reductions.

378 **3.4. Evaluation of Two-Drug Antibiotic Combinations Using Intracellular Time-Kill Assay against *M.***
379 ***abscessus* ATCC 19977**

380 Kinetic intracellular time-kill assays were conducted to evaluate the efficacy of four individual antibiotics and
381 four combination treatments against *M. abscessus* ATCC 19977 administered at 1× MIC in THP-1-derived
382 macrophages. The mean intracellular bacterial loads ($\log_{10}$ CFU/mL) measured at 24, 48, and 72 hours for both
383 single and combination therapies are presented in Figure 4. All treatment groups, including both single agents
384 and combinations, exhibited lower intracellular CFU/mL compared to the untreated control at each time point.
385 AUC analysis revealed highly statistically significant reductions in bacterial load for all treatment groups
386 compared to the untreated control ($p < 0.0001$). Similarly, at the 72-hour terminal endpoint, all treatment groups
387 demonstrated significant bacterial load reductions compared to the control ($p < 0.0001$). Treatment with rifabutin
388 alone, as well as the combinations of azithromycin + omadacycline, moxifloxacin + omadacycline, moxifloxacin
389 + azithromycin, and moxifloxacin + rifabutin, led to overall decreases in bacterial counts from baseline ($\log_{10}$
390 CFU/mL reductions of -0.43, -0.46, -0.82, -0.82, -0.98, respectively). Notably, three combinations—azithromycin
391 + omadacycline, moxifloxacin + omadacycline, and moxifloxacin + azithromycin—demonstrated additive effects
392 when used together, while the combination of moxifloxacin + rifabutin exhibited indifferent effects. However, at
393 1× MIC, none of the antibiotics, either individually or in combination, exhibited bactericidal activity against
394 intracellular *M. abscessus* ATCC 19977.

395 **4. Discussion:**

396 This study explores the therapeutic potential of IMI/REL in combination with various antibiotics for treating
397 *M. abscessus*, a key pathogen responsible for severe lung infections and adverse clinical outcomes in PwCF.
398 Utilizing a diverse array of clinical isolates from CF patients alongside a reference strain, we evaluated the *in*
399 *vitro* efficacy of antibiotic combinations through susceptibility and synergy testing. Our findings were further
400 substantiated by time-kill kinetic assays, which evaluated both total and intracellular bactericidal activity. These
401 results reveal substantial synergistic interactions between IMI/REL and antibiotics from multiple drug classes,
402 indicating *in vivo* studies are warranted to evaluate clinical outcomes using these combinations for treatment of
403 these difficult-to-treat infections.

404 Susceptibility testing was performed on the *M. abscessus* ATCC 19977 reference strain using the broth
405 microdilution method, evaluating IMI/REL along with thirteen other antibiotics. Our results demonstrated a
406 strong alignment with published MIC values for ATCC 19977 obtained under similar testing conditions, with
407 exact matches for imipenem, amoxicillin, moxifloxacin, and minocycline [25, 53-55]. Additionally, MIC values
408 for amikacin, cefoxitin, cefdinir, cefuroxime, azithromycin, tedizolid, rifabutin, and omadacycline fell within one
409 to two twofold dilutions of those reported in the literature, highlighting strong consistency across studies despite
410 some minor variations [25, 53-60]. Greater variability was observed for clofazimine, with MIC values 2-3 twofold
411 dilutions higher, and for tigecycline, which showed MIC values 3-5 twofold dilutions higher than published
412 studies [53-55, 60-61]. Technical challenges contributing to MIC variability include difficulties in inoculum
413 calibration, drug instability, inherent antibiotic variability, variations in laboratory techniques, environmental
414 conditions during testing, and potential genetic drift within bacterial populations across studies. To mitigate these
415 challenges, all susceptibility testing for *M. abscessus* ATCC 19977 and CF clinical isolates was conducted
416 following CLSI guidelines, ensuring experimental consistency and facilitating more accurate comparisons with
417 literature values [34]. Furthermore, the lack of standardized interpretive criteria—particularly regarding trailing
418 effects—complicates the comparison of results across studies, as isolates with trailing endpoints can obscure MIC
419 readings and hinder interpretation. Inconsistent reporting of these techniques in some studies may also contribute

420 to the observed variations in MIC values [60]. Standard susceptibility testing was also performed on all isolates,
421 and the resulting MICs for both the *M. abscessus* ATCC 19977 reference strain and clinical isolates were
422 compared with CLSI and modified susceptibility breakpoints. Overall, the checkerboard analyses of CF clinical
423 isolates revealed that cefuroxime was the most synergistic with IMI/REL, followed by cefdinir, amoxicillin, and
424 cefoxitin, aligning with established findings on β-lactam synergy [31-32, 62]. Rifabutin, azithromycin,
425 moxifloxacin, clofazimine, minocycline, and omadacycline also demonstrated additive effects with IMI/REL.
426 Susceptibility MIC testing in the ATCC 19977 strain closely aligns with median MIC values of the CF clinical
427 isolates and indicates comparable degrees of synergy in median FIC indices. However, in line with prior published
428 studies demonstrating the genotypic and phenotypic diversity of *M. abscessus* [63-64], this study further confirms
429 substantial heterogeneity in the MICs, drug susceptibility profiles, and responses to antibiotics among clinical
430 isolates.

431 The observed high resistance rates in CF clinical isolates—particularly against β-lactams (amoxicillin,
432 cefdinir, cefuroxime), macrolides (azithromycin), fluoroquinolones (moxifloxacin), and tetracyclines
433 (minocycline)—are also expected given the established resistance mechanisms of *M. abscessus*. Cefoxitin's
434 effectiveness, despite inherent β-lactam resistance, can be attributed to its structural modifications, which increase
435 its stability against β-lactamase hydrolysis, as shown in previous studies [20]. Similarly, reduced resistance to
436 IMI/REL was observed, as expected. This can be attributed to imipenem's inherent resistance to hydrolysis, which
437 is further enhanced by its combination with the β-lactamase inhibitor relebactam [19, 22, 26]. Macrolide
438 resistance often arises from mutations in the rrl (23S rRNA) gene, as previously discussed, or the presence of the
439 *erm*(41) gene, which can induce resistance following exposure to macrolides [17-18]. *M. abscessus* subsp.
440 *abscessus* and *bolletii* harbor a functional *erm*(41) gene that confers inducible macrolide resistance and limits the
441 effectiveness of this antibiotic class, whereas subsp. *massiliense* carries a non-functional *erm(*41) gene that
442 prevents the expression of such resistance [65]. Consistent with this, we observed elevated MICs for azithromycin
443 in *M. abscessus* subsp. *abscessus* and *bolletii* compared to *massiliense*. High rates of fluoroquinolone resistance
444 in NTM can be attributed to genetic factors, specifically mutations in the quinolone resistance-determining

445 regions of the *gyr*A and *gyr*B subunits of DNA gyrase, which are the primary targets of these drugs [66]. The
446 elevated resistance to moxifloxacin observed in our study has also been reported in the literature [20].
447 Tetracycline-class resistance in *M. abscessus* is primarily mediated by ribosomal protection proteins and active
448 efflux pumps, a mechanism supported by prior studies that demonstrate the limited effectiveness of minocycline
449 and the rapid emergence of tolerance to this antibiotic [17-18, 67]. In contrast, structural modifications in
450 omadacycline enable it to evade these common resistance mechanisms, likely contributing to the observed lower
451 resistance rates against this antibiotic [53, 68]. Our analysis also revealed high rates of resistance to the rifamycin
452 antibiotic rifabutin in the CF clinical isolates. However, *M. abscessus* lacks known genetic determinants of
453 rifabutin resistance [18], suggesting that the observed resistance may be influenced more by the established
454 breakpoint than genetic factors. The breakpoint of 2 μg/mL for rifabutin susceptibility was derived from studies
455 on the slow-growing *M. avium*, which may not be applicable to rapid-growing mycobacteria like *M. abscessus*
456 [58].

457 The clinical feasibility of the antibiotics tested can be assessed by comparing their maximum achievable
458 plasma and intracellular concentrations to *M. abscessus* MICs under standard dosing regimens (Supplementary
459 Table 14) [14]. Both IMI/REL and cefoxitin achieve clinically relevant plasma levels for MABSC treatment but
460 have limited intracellular penetration [27-28, 69-70]. While azithromycin's plasma concentrations do not reach
461 clinically significant levels [71], its high intracellular penetration results in alveolar macrophage (AM)
462 concentrations exceeding those required for activity [71-72]. Moxifloxacin does not achieve significant plasma
463 concentrations alone; however, when administered with IMI/REL, its plasma levels approach those required for
464 activity, and it accumulates in AMs at levels well above the MIC for intracellular activity [73-74]. Clofazimine
465 achieves plasma concentrations that approach clinically relevant levels [75-76], with prior studies demonstrating
466 its bioaccumulation as biocrystals in alveolar macrophages [77-78]. Omadacycline reaches clinically relevant
467 plasma concentrations for most isolates and attains high AM concentrations that far exceed the MIC [79-81].
468 Rifabutin does not reach significant plasma concentrations alone but demonstrates high monocyte penetration
469 with concentrations approaching those needed for intracellular activity [82-84]. Cefuroxime [85], cefdinir [86],

470 amoxicillin [87], and minocycline [88-89] are unlikely to achieve clinically achievable plasma or intracellular
471 concentrations necessary for activity alone or in combination with IMI/REL against *M. abscessus*.

472 Intracellular time-kill assay results further highlight the clinical relevance of these antibiotics, offering a
473 deeper insight into their therapeutic potential. Compared to extracellular time-kill assays, intracellular killing was
474 markedly reduced across all antibiotics, emphasizing the challenge of achieving sufficient intracellular
475 concentrations for effective treatment. Among the antibiotics tested, rifabutin was found to be the most active in
476 terms of intracellular killing, supporting its inclusion in combination regimens. Moreover, combination antibiotic
477 regimens modestly enhanced intracellular killing, with moxifloxacin combined with rifabutin, omadacycline, or
478 azithromycin showing greater reductions in bacterial load. This suggests that rifabutin, moxifloxacin,
479 omadacycline, and azithromycin substantially enhance intracellular efficacy. Extracellular time-kill assay results
480 further reinforce these findings, with IMI/REL, cefoxitin, and rifabutin or moxifloxacin emerging as the most
481 effective combinations. While IMI/REL and cefoxitin exhibited strong synergy independently, the inclusion of
482 rifabutin or moxifloxacin with improved intracellular penetration may enhance the overall activity of the treatment
483 regimen. Together, these results underscore the critical importance of considering both extracellular and
484 intracellular pharmacodynamics when designing multi-drug regimens for *M. abscessus* infections.

485 Overall, susceptibility and synergy testing, along with time-kill assay results, underscore the *in vitro* efficacy
486 of multi-drug regimens—particularly those combining IMI/REL with cefoxitin, azithromycin, moxifloxacin,
487 rifabutin, and omadacycline—in effectively targeting *M. abscessus* infections. Cefoxitin, a guideline-
488 recommended intravenous antibiotic for the intensive phase of *M. abscessus* therapy [13], demonstrated robust
489 efficacy and consistent synergy with IMI/REL, in line with previous findings that reinforce its ongoing relevance
490 in *M. abscessus* management [90]. Azithromycin, the preferred oral macrolide for both intensive and continuation
491 phases [13], is known for its anti-inflammatory and immunomodulatory properties in PwCF [91-92], and its use
492 in multidrug regimens has been associated with improved outcomes in MABSC-PD treatment [15]. Moxifloxacin,
493 which is also recommended during the continuation phase and available orally [13], has also demonstrated
494 promising preclinical anti-inflammatory activity in CF bronchial cell models [93]. Among emerging agents,

495 rifabutin and omadacycline show considerable promise in treating *M. abscessus* infections. Rifabutin is the only
496 rifamycin shown to be effective against *M. abscessus* [58, 94-95], and its inclusion in multidrug regimens is
497 supported by its ability to reduce macrolide resistance [96]. Omadacycline, with good oral bioavailability and
498 improved tolerability compared to tigecycline [79], also demonstrates preclinical anti-inflammatory effects [97]
499 and recent Phase II clinical trial results suggest promising efficacy [98].

500 The study described herein has several limitations. First, the treatment history of tested isolates was unknown
501 and previous exposure to antimicrobials (e.g., macrolides) may have affected drug susceptibility testing results
502 [65]. Additionally, the clinical breakpoints for antibiotics evaluated in this study are not fully standardized
503 according to CLSI guidelines for rapidly growing mycobacteria, with no consensus established for amoxicillin,
504 cefdinir, cefuroxime, tedizolid, rifabutin, clofazimine, tigecycline, and omadacycline [34]. Despite efforts to
505 apply the most relevant interpretive criteria, more precise and standardized breakpoint definitions are required to
506 optimize the treatment of NTM infections. Furthermore, not all antibiotics available for *M. abscessus* treatment
507 were evaluated in this study. While IMI/REL was tested alongside 13 different antibiotics, agents such as linezolid
508 and bedaquiline were omitted due to a lack of documented synergy with other agents.

509 The stability of certain compounds, particularly IMI/REL, presents a potential concern. Certain β-lactams are
510 known to be relatively unstable, with one study reporting a degradation half-life of 16.9 hours for imipenem in
511 media [99]. To evaluate the potential impact of this instability on treatment efficacy, we tested IMI/REL
512 monotherapy with and without media replacement every 24 hours. Media replacement with IMI/REL resulted in
513 a roughly 1 $\log_{10}$ greater reduction in bacterial load (CFU/mL) compared to the non-replacement group at 16×,
514 4×, and 1× MIC concentrations, although no statistically significant differences were observed between the groups
515 in either AUC or endpoint analysis. Nevertheless, had media replacement been implemented throughout the total
516 and intracellular time-kill assays, a more pronounced effect may have been observed. This warrants further
517 investigation to better assess the impact of compound degradation on *in vitro* antimicrobial efficacy. Another
518 limitation concerns the MOI used during the intracellular time-kill assays. An MOI of 5 was chosen in our study
519 to adequately represent infection without overburdening the cells, based on previously published literature [49,

520 51]. However, other studies have suggested lower MOIs, ranging from 0.1 to 1 [48, 50, 52]. To evaluate the
521 potential impact of MOI on treatment efficacy, we tested two antibiotic combinations (omadacycline +
522 azithromycin/moxifloxacin) at an MOI of 1 but did not observe an inoculum effect or statistically significant
523 differences in efficacy. We hypothesize that the observed lack of effect may stem from a saturation effect or a
524 biological limit on the number of bacteria each macrophage can internalize at higher MOIs. Future studies are
525 warranted to explore the effects of varying MOIs on treatment efficacy and to identify the optimal conditions for
526 modeling intracellular *M. abscessus* infection, thus ensuring comparability and consistency in results. Last,
527 several previous studies have highlighted that the *in vitro* efficacy of antibiotics against *M. abscessus* may not
528 consistently translate to *in vivo* outcomes [100]. The static concentrations used in this study (16×, 4×, 1×, and
529 0.25× MIC) are typical for *in vitro* testing, but may not fully reflect dynamic pharmacokinetics in a clinical setting.
530 Future studies should utilize dynamic *in vitro* models [37, 101] and *in vivo* animal models [102-103] to better
531 simulate human conditions and confirm the clinical relevance of these findings. Despite these limitations, the
532 large-scale use of clinical isolates and antibiotic combinations in this study provides valuable data that may
533 otherwise require substantial time and resources in more complex models.
534

535 **5. Conclusion:**

536 In conclusion, IMI/REL demonstrated potent *in vitro* activity against clinical isolates of MABSC, with
537 substantial synergistic interactions observed when combined with antibiotics from multiple drug classes. These
538 findings suggest that IMI/REL may offer a significant advancement in the management of infections involving
539 MABSC in PwCF. Moreover, the promising efficacy of multidrug regimens pairing IMI/REL with agents such
540 as cefoxitin, azithromycin, moxifloxacin, rifabutin, and omadacycline highlights potential therapeutic strategies
541 for targeting *M. abscessus*. Future preclinical studies are warranted to validate these findings, optimize drug
542 combinations, and evaluate their effectiveness in treating MABSC pulmonary disease.

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8. Tables and Figures:

Table 1: Minimum inhibitory concentrations (MICs) of antibiotics and IMI/REL, both individually and in combination against the *M. abscessus* ATCC 19977 strain, along with the corresponding susceptibility interpretations, fractional inhibitory concentration (FIC) index values, and their synergism.

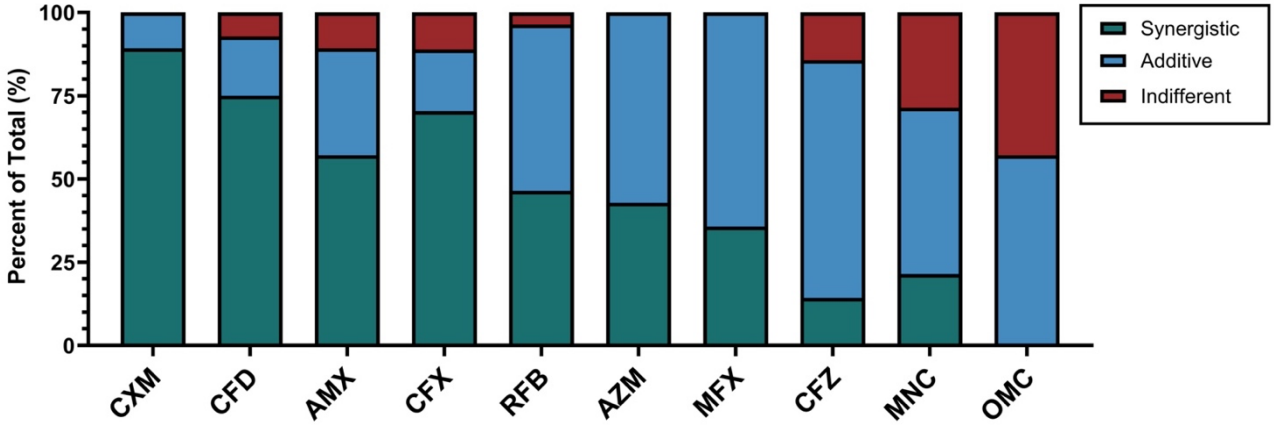
Antibacterial Agent	MIC Value (µg/mL)					FIC Index	Interaction
	Drug Alone	Susceptibility Interpretation	Drug with IMI/REL	IMI/REL Alone	IMI/REL with Drug		
Cefuroxime	512	Resistant	64	4	0.25	**0.188**	Synergistic
Cefdinir	256	Resistant	16	4	1	**0.313**	Synergistic
Cefoxitin	32	Intermediate	8	4	0.5	**0.375**	Synergistic
Moxifloxacin	16	Resistant	4	4	1	**0.500**	Synergistic
Rifabutin	16	Resistant	4	4	1	**0.500**	Synergistic
Minocycline	256	Resistant	64	4	1	**0.500**	Synergistic
Amoxicillin	2048	Resistant	16	4	2	**0.508**	Additive
Azithromycin	8	Resistant	1	4	2	**0.625**	Additive
Tigecycline	4	Resistant	2	4	0.5	**0.625**	Additive
Amikacin	32	Intermediate	16	8	4	**1.000**	Additive
Clofazimine	2	Susceptible	1	4	2	**1.000**	Additive
Omadacycline	2	Intermediate	1	4	2	**1.000**	Additive
Tedizolid	8	Susceptible	8	4	2	**1.500**	Indifferent

Table 2: Susceptibility and synergy of antibiotics against *M. abscessus* CF clinical isolates (*n*=28)

Antibacterial Agent	MIC_{50} Value (µg/mL)[a]					FIC Index	Interaction
	Drug Alone	Susceptibility Interpretation	Drug with IMI/REL	IMI/REL Alone	IMI/REL with Drug		
Cefuroxime	512 (256, 512)	Resistant	64 (32, 128)	4 (4, 8)	0.38 (0.13, 0.50)	**0.250 (0.180, 0.328)**	Synergistic
Cefdinir	192 (112, 256)	Resistant	24 (14, 40)	4 (4, 8)	1 (0.50, 1)	**0.375 (0.313, 0.502)**	Synergistic
Amoxicillin	2048 (1024, 2048)	Resistant	128 (32, 256)	8 (4, 8)	2 (1, 2.50)	**0.438 (0.352, 0.625)**	Synergistic
Cefoxitin	48 (32, 64)	Intermediate	8 (4, 8)	8 (4, 16)	2 (1, 8)	**0.500 (0.375, 0.563)**	Synergistic
Rifabutin	8 (7, 16)	Resistant	2 (1, 4)	4 (4, 8)	1 (0.88, 2)	**0.563 (0.500, 0.625)**	Additive
Azithromycin	8 (4, 16)	Resistant	2 (1, 4)	8 (8, 16)	2 (1, 4)	**0.625 (0.500, 0.750)**	Additive
Moxifloxacin	16 (8, 16)	Resistant	4 (4, 4)	6 (4, 8)	2 (2, 2)	**0.750 (0.500, 0.750)**	Additive
Clofazimine	1 (0.88, 2)	Susceptible	0.50 (0.25, 0.63)	8 (4, 8)	2 (1, 4)	**0.750 (0.625, 1.000)**	Additive
Minocycline	256 (256, 256)	Resistant	96 (64, 128)	4 (2, 5)	2 (1, 2)	**0.750 (0.609, 1.039)**	Additive
Omadacycline	2 (1, 2)	Intermediate	1 (0.50, 1)	8 (8, 10)	4 (3.5, 8)	**1.000 (1.000, 1.500)**	Additive

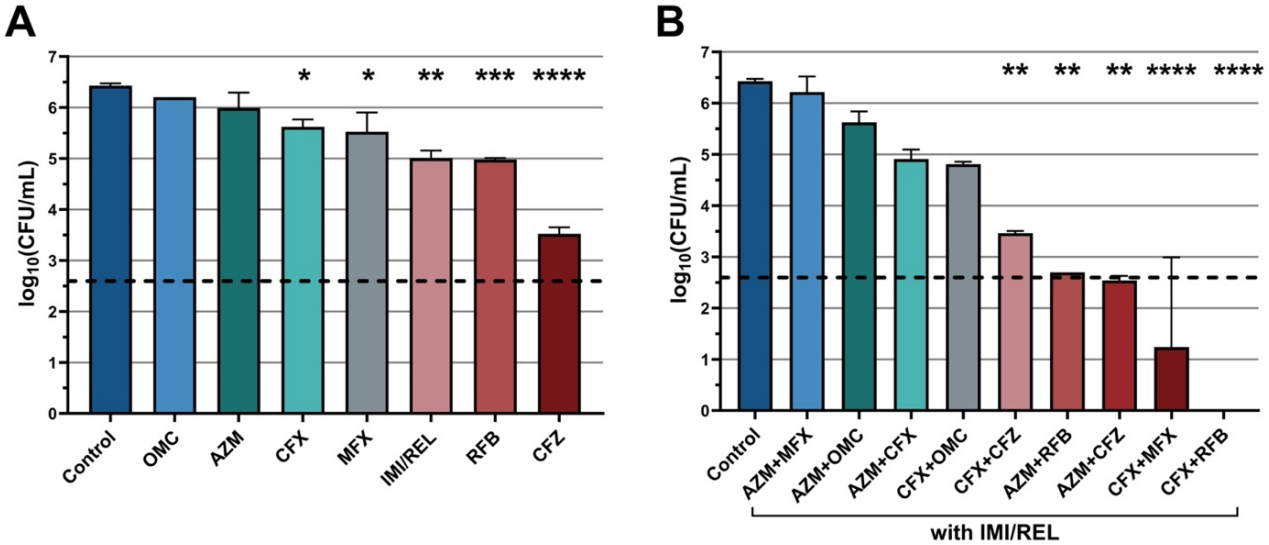
[a] Data are presented as MIC_{50} (interquartile range [IQR]).

Figure 1: Percentages of synergistic, additive, and indifferent effects of antibiotics combined with IMI/REL in *M. abscessus* CF clinical isolates



CXM = cefuroxime, CFD = cefdinir, AMX = amoxicillin, CFX = cefoxitin, RFB = rifabutin, AZM = azithromycin, MFX = moxifloxacin, CFZ = clofazimine, MNC = minocycline, OMC = omadacycline

Figure 2: Mean bacterial loads (log_{10} CFU/mL) of *M. abscessus* ATCC 1997 over 72 hours with (A) single-agent therapies and (B) three-drug combination therapies with IMI/REL

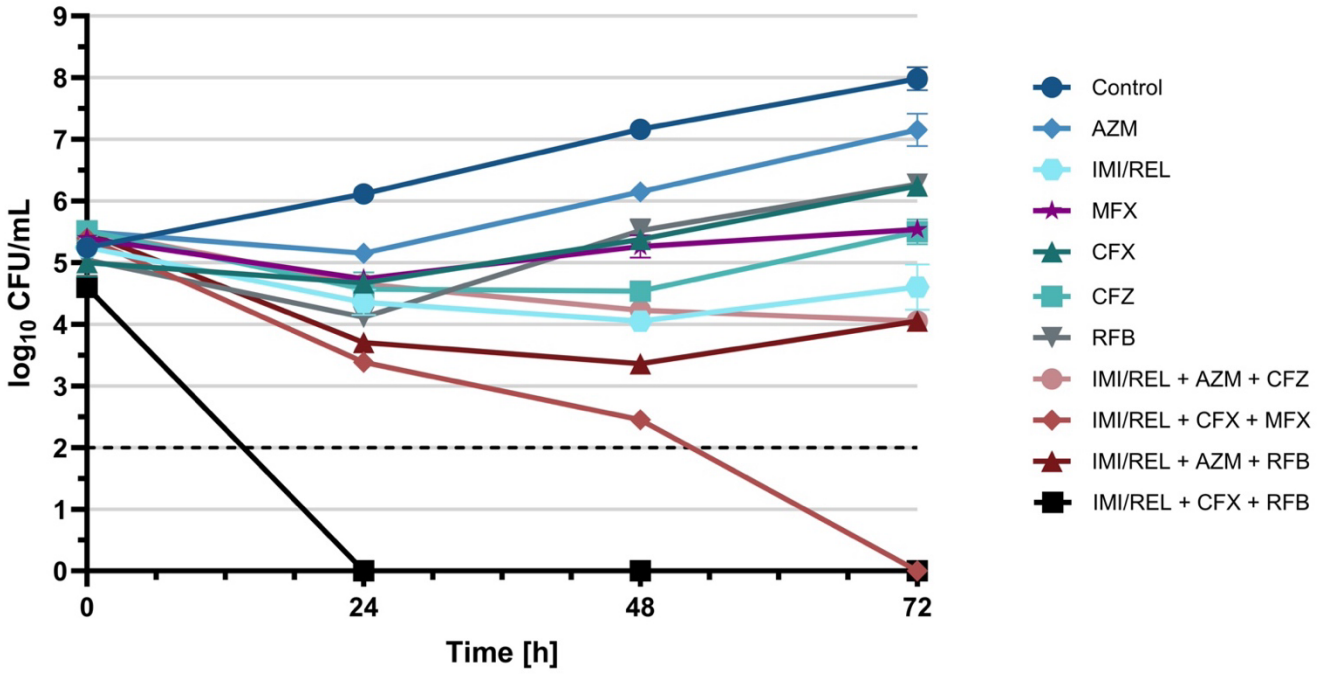


Data are presented as mean with standard errors of the mean. Asterisks denote significant differences compared to the untreated control. The horizontal dashed line marks a 3-log reduction in CFU/mL relative to the initial count of the untreated control, denoting bactericidal activity.

(*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, post-hoc Tukey's HSD test)

OMC = omadacycline, AZM = azithromycin, CFX = cefoxitin, MFX = moxifloxacin, RFB = rifabutin, CFZ = clofazimine

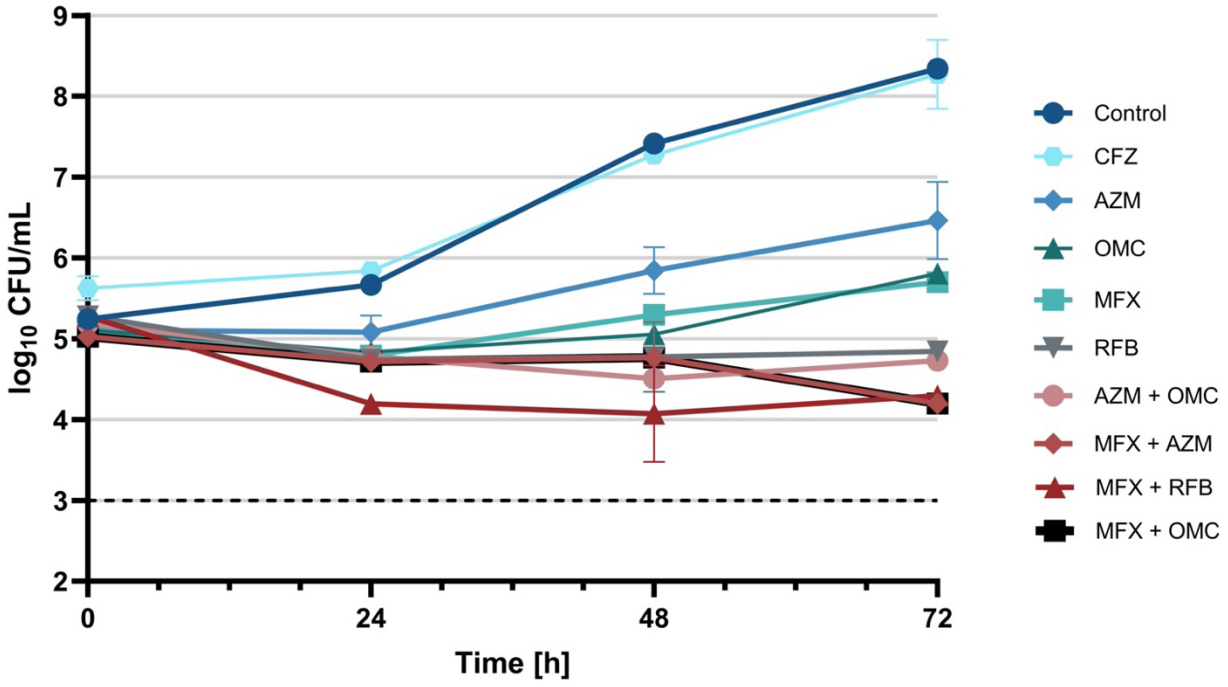
Figure 3: Bacterial load (log_{10} CFU/ml) of *M. abscessus* ATCC 19977 over 72 hours with single-agent therapies and three-drug combination therapies with IMI/REL at 1× MIC



Data are presented as mean with standard errors of the mean. The horizontal dashed line marks the lower limit of detection.

AZM = azithromycin, MFX = moxifloxacin, CFX = cefoxitin, CFZ = clofazimine, RFB = rifabutin

Figure 4: Intracellular bacterial load (log_{10} CFU/ml) of *M. abscessus* ATCC 19977 over 72 hours with single-agent therapies and two-drug combination therapies at 1× MIC



Data are presented as mean with standard errors of the mean. The horizontal dashed line marks the lower limit of detection.

CFZ = clofazimine, AZM = azithromycin, OMC = omadacycline, MFX = moxifloxacin, RFB = rifabutin

9. Supplementary:

Supplementary Table 1: Concentration ranges and breakpoints used for *M. abscessus* drug susceptibility testing by broth dilution.

Antibacterial Agent	Tested Concentration Range (µg/mL)	Breakpoints (µg/mL)		
		Susceptible	Intermediate	Resistant
Aminoglycosides				
Amikacin	0.25-32	≤ 16	32	≥ 64
β-Lactams				
Amoxicillin	4-512	≤ 8	16	≥ 32
Cefoxitin	2-256	≤ 16	32-64	≥ 128
Cefdinir	2-256	≤ 16	32-64	≥ 128
Cefuroxime	2-256	≤ 16	32-64	≥ 128
β-Lactam/β-lactamase inhibitor combinations				
IMI/REL[a]	0.125-16	≤ 4	8-16	≥ 32
Fluoroquinolones				
Moxifloxacin	0.125-16	≤ 1	2	≥ 4
Macrolides				
Azithromycin	0.25-32	≤ 2	4	≥ 8
Oxazolidinones				
Tedizolid	0.0625-8	≤ 8	16	≥ 32
Rifamycins				
Rifabutin	0.5-64	≤ 2	—	> 2
Riminophenazines				
Clofazimine	0.125-16	≤ 2	4	≥ 8
Tetracyclines				
Minocycline	4-512	≤ 1	2-4	≥ 8
Tigecycline	0.0625-8	≤ 0.5	1	≥ 2
Omadacycline	0.125-16	≤ 1	2	≥ 4

[a]The concentrations in this table refer to imipenem. IMI/REL was prepared by serially diluting imipenem while keeping the relebactam concentration fixed at 4 µg/mL.

Supplementary Table 2: *M. abscessus* CF clinical isolates identified according to their subspecies classification, morphology and mutation

CF Patient ASID	WGS Identification	CF Morphology	rrs Position 1408	rrl Position 2058	rrl Position 2059	Description
CF00006	MAB	R	A	A	A	WT
CF00013	MAB	R	A	A	A	WT
CF00016	MAB	R	A	A	A	WT
CF00017	MAB	R	G	A	A	rrs mutation
CF00023	MAB	R	A	A	A	WT
CF00038	MAB	S	A	A	A	WT
CF00040	MAB	R	A	A	A	WT
CF00041	MAB	S	A	A	A	WT
CF00043	MAB	R	A	A	A	WT
CF00136	MAB	unknown	A	C	A	rrl mutation
CF00258	MAB	unknown	A	A	C	rrl mutation
CF00855	MAB	unknown	A	G	A	rrl mutation
CF01975	MAB	R	A	A	A	WT
CF02033	MAB	R	G	G	A	rrs and rrl mutation
CF02279	MAB	R	A	A	A	WT
CF02319	MAB	R	A	A	A	WT
CF02486	MAB	S	A	A	A	WT
CF00008	MMAS	R	A	A	A	WT
CF00030	MMAS	S	A	A	A	WT
CF00035	MMAS	R	A	A	A	WT
CF00042	MMAS	S	A	A	A	WT
CF00046	MMAS	unknown	A	C	A	rrl mutation
CF00047	MMAS	S	A	A	A	WT
CF00883	MMAS	R	A	A	G	rrl mutation
CF00020	MBOL	S	A	A	A	WT
CF00113	MBOL	unknown	G	A	A	rrs mutation
CF00868	MBOL	unknown	A	A	A	WT
CF02061	MBOL	R	A	A	A	WT

MAB, *M. abscessus* subsp. *abscessus*; MBOL, *M. abscessus* subsp. *bolletii*; MMAS, *M. abscessus* subsp. *massiliense*; R, rough; S, smooth; WT, wild-type

Supplementary Table 3: Minimum inhibitory concentrations (MICs) of amoxicillin and IMI/REL, both individually and in combination against the *M. abscessus* CF clinical isolates, along with the corresponding fractional inhibitory concentration (FIC) index values and their synergism

WGS ID	CF Patient ID	MIC Value (µg/mL)				FIC Index	Interaction
		Amoxicillin Alone	Amoxicillin with IMI/REL	IMI/REL Alone	IMI/REL with Amoxicillin		
M. abscessus	CF00006	2048	256	16	8	**0.625**	Additive
	CF00013	2048	512	2	0.5	**0.500**	Synergistic
	CF00016	2048	512	8	2	**0.500**	Synergistic
	CF00017	1024	64	4	2	**0.563**	Additive
	CF00023	2048	64	4	0.5	**0.156**	Synergistic
	CF00038	2048	16	4	1	**0.258**	Synergistic
	CF00040	2048	16	8	8	**1.008**	Indifferent
	CF00041	2048	256	4	1	**0.375**	Synergistic
	CF00043	2048	256	4	1	**0.375**	Synergistic
	CF00136	2048	512	8	1	**0.375**	Synergistic
	CF00258	2048	256	8	4	**0.625**	Additive
	CF00855	2048	256	4	1	**0.375**	Synergistic
	CF01975	1024	128	16	4	**0.375**	Synergistic
	CF02033	2048	256	8	2	**0.375**	Synergistic
	CF02279	2048	64	4	2	**0.531**	Additive
	CF02319	2048	8	2	2	**1.004**	Indifferent
	CF02486	1024	256	8	4	**0.750**	Additive
M. massiliense	CF00008	2048	8	4	2	**0.504**	Additive
	CF00030	2048	64	8	0.5	**0.094**	Synergistic
	CF00035	2048	2048	8	1	**1.125**	Indifferent
	CF00042	1024	32	16	4	**0.281**	Synergistic
	CF00046	1024	256	16	8	**0.750**	Additive
	CF00047	2048	32	8	2	**0.266**	Synergistic
	CF00883	512	16	8	0.125	**0.047**	Synergistic
M. bolletii	CF00020	1024	512	8	2	**0.750**	Additive
	CF00113	1024	128	8	2	**0.375**	Synergistic
	CF00868	2048	64	8	2	**0.281**	Synergistic
	CF02061	2048	32	4	2	**0.516**	Additive

Supplementary Table 4: Minimum inhibitory concentrations (MICs) of cefoxitin and IMI/REL, both individually and in combination against the *M. abscessus* CF clinical isolates, along with the corresponding fractional inhibitory concentration (FIC) index values and their synergism

WGS ID	CF Patient ID	MIC Value (µg/mL)				FIC Index	Interaction
		Cefoxitin Alone	Cefoxitin with IMI/REL	IMI/REL Alone	IMI/REL with Cefoxitin		
M. abscessus	CF00006	64	4	16	16	**1.063**	Indifferent
	CF00013	128	32	8	2	**0.500**	Synergistic
	CF00016	64	16	8	2	**0.500**	Synergistic
	CF00017	32	8	4	2	**0.750**	Additive
	CF00023	64	16	4	1	**0.500**	Synergistic
	CF00038	32	8	16	2	**0.375**	Synergistic
	CF00040	128	8	32	32	**1.063**	Indifferent
	CF00041	32	8	4	1	**0.500**	Synergistic
	CF00043	64	8	4	1	**0.375**	Synergistic
	CF00136	32	8	4	1	**0.500**	Synergistic
	CF00258	64	8	8	1	**0.250**	Synergistic
	CF00855	128	8	64	32	**0.563**	Additive
	CF01975	32	4	64	8	**0.250**	Synergistic
	CF02033	64	8	32	4	**0.250**	Synergistic
	CF02279	64	8	4	1	**0.375**	Synergistic
	CF02319	64	2	8	2	**0.281**	Synergistic
	CF02486	32	4	8	2	**0.375**	Synergistic
M. massiliense	CF00008	64	4	16	8	**0.563**	Additive
	CF00030	32	4	16	4	**0.375**	Synergistic
	CF00035	32	4	8	8	**1.125**	Indifferent
	CF00042	16	4	16	16	**1.250**	Indifferent
	CF00046	64	4	16	8	**0.563**	Additive
	CF00047	32	4	4	1	**0.375**	Synergistic
	CF00883	16	4	8	4	**0.750**	Additive
M. bolletii	CF00020	32	4	16	4	**0.375**	Synergistic
	CF00113	64	8	4	1	**0.375**	Synergistic
	CF00868	32	8	8	2	**0.500**	Synergistic
	CF02061	32	8	4	1	**0.500**	Synergistic

Supplementary Table 5: Minimum inhibitory concentrations (MICs) of cefdinir and IMI/REL, both individually and in combination against the *M. abscessus* CF clinical isolates, along with the corresponding fractional inhibitory concentration (FIC) index values and their synergism

WGS ID	CF Patient ID	MIC Value (µg/mL)				FIC Index	Interaction
		Cefdinir Alone	Cefdinir with IMI/REL	IMI/REL Alone	IMI/REL with Cefdinir		
M. abscessus	CF00006	256	128	16	0.125	**0.508**	Additive
	CF00013	256	32	4	1	**0.375**	Synergistic
	CF00016	128	16	4	1	**0.375**	Synergistic
	CF00017	256	8	2	0.5	**0.281**	Synergistic
	CF00023	256	32	4	1	**0.375**	Synergistic
	CF00038	256	64	4	1	**0.500**	Synergistic
	CF00040	256	128	8	1	**0.625**	Additive
	CF00041	256	32	8	1	**0.250**	Synergistic
	CF00043	128	8	4	0.5	**0.188**	Synergistic
	CF00136	16	16	16	0.5	**1.031**	Indifferent
	CF00258	256	128	16	2	**0.625**	Additive
	CF00855	64	16	4	0.5	**0.375**	Synergistic
	CF01975	256	32	8	0.125	**0.141**	Synergistic
	CF02033	256	64	4	1	**0.500**	Synergistic
	CF02279	512	32	2	0.25	**0.188**	Synergistic
	CF02319	128	8	4	1	**0.313**	Synergistic
	CF02486	256	16	4	1	**0.313**	Synergistic
M. massiliense	CF00008	256	64	8	1	**0.375**	Synergistic
	CF00030	128	8	4	1	**0.313**	Synergistic
	CF00035	128	16	8	1	**0.250**	Synergistic
	CF00042	16	16	8	0.125	**1.016**	Indifferent
	CF00046	256	64	8	0.5	**0.313**	Synergistic
	CF00047	128	32	4	1	**0.500**	Synergistic
	CF00883	8	2	4	2	**0.750**	Additive
M. bolletii	CF00020	64	8	4	1	**0.375**	Synergistic
	CF00113	64	8	4	2	**0.625**	Additive
	CF00868	128	32	8	1	**0.375**	Synergistic
	CF02061	64	16	4	1	**0.500**	Synergistic

Supplementary Table 6: Minimum inhibitory concentrations (MICs) of cefuroxime and IMI/REL, both individually and in combination against the *M. abscessus* CF clinical isolates, along with the corresponding fractional inhibitory concentration (FIC) index values and their synergism

WGS ID	CF Patient ID	MIC Value (µg/mL)				FIC Index	Interaction
		Cefuroxime Alone	Cefuroxime with IMI/REL	IMI/REL Alone	IMI/REL with Cefuroxime		
M. abscessus	CF00006	512	128	8	0.5	**0.313**	Synergistic
	CF00013	512	128	4	0.125	**0.281**	Synergistic
	CF00016	256	64	16	0.5	**0.281**	Synergistic
	CF00017	512	32	4	0.5	**0.188**	Synergistic
	CF00023	512	64	4	0.5	**0.250**	Synergistic
	CF00038	64	16	4	0.125	**0.281**	Synergistic
	CF00040	512	64	8	0.5	**0.188**	Synergistic
	CF00041	256	64	4	0.25	**0.313**	Synergistic
	CF00043	256	16	4	0.125	**0.094**	Synergistic
	CF00136	128	16	16	0.125	**0.133**	Synergistic
	CF00258	256	64	8	1	**0.375**	Synergistic
	CF00855	512	128	8	0.5	**0.313**	Synergistic
	CF01975	256	128	16	1	**0.563**	Additive
	CF02033	1024	256	4	0.5	**0.375**	Synergistic
	CF02279	512	32	2	0.25	**0.188**	Synergistic
	CF02319	512	64	4	0.5	**0.250**	Synergistic
	CF02486	512	32	2	0.125	**0.125**	Synergistic
M. massiliense	CF00008	1024	128	4	0.125	**0.156**	Synergistic
	CF00030	1024	64	4	0.125	**0.094**	Synergistic
	CF00035	512	64	4	0.25	**0.188**	Synergistic
	CF00042	64	32	16	0.25	**0.516**	Additive
	CF00046	256	32	4	0.25	**0.188**	Synergistic
	CF00047	256	64	4	0.5	**0.375**	Synergistic
	CF00883	1024	512	16	2	**0.625**	Additive
M. bolletii	CF00020	512	64	8	0.25	**0.156**	Synergistic
	CF00113	2048	256	8	2	**0.375**	Synergistic
	CF00868	128	16	4	0.125	**0.156**	Synergistic
	CF02061	512	64	4	0.5	**0.250**	Synergistic

Supplementary Table 7: Minimum inhibitory concentrations (MICs) of moxifloxacin and IMI/REL, both individually and in combination against the *M. abscessus* CF clinical isolates, along with the corresponding fractional inhibitory concentration (FIC) index values and their synergism

WGS ID	CF Patient ID	MIC Value (µg/mL)				FIC Index	Interaction
		Moxifloxacin Alone	Moxifloxacin with IMI/REL	IMI/REL Alone	IMI/REL with Moxifloxacin		
M. abscessus	CF00006	8	4	4	2	**1.000**	Additive
	CF00013	16	8	8	2	**0.750**	Additive
	CF00016	32	16	16	2	**0.625**	Additive
	CF00017	16	4	4	2	**0.750**	Additive
	CF00023	16	4	8	2	**0.500**	Synergistic
	CF00038	16	4	4	2	**0.750**	Additive
	CF00040	16	4	4	1	**0.500**	Synergistic
	CF00041	16	4	4	2	**0.750**	Additive
	CF00043	8	2	4	1	**0.500**	Synergistic
	CF00136	8	2	8	2	**0.500**	Synergistic
	CF00258	16	8	4	2	**1.000**	Additive
	CF00855	8	4	8	2	**0.750**	Additive
	CF01975	8	4	4	1	**0.750**	Additive
	CF02033	16	4	8	2	**0.500**	Synergistic
	CF02279	4	2	4	0.5	**0.625**	Additive
	CF02319	16	8	4	2	**1.000**	Additive
	CF02486	16	4	8	2	**0.500**	Synergistic
M. massiliense	CF00008	16	4	8	2	**0.500**	Synergistic
	CF00030	16	4	8	2	**0.500**	Synergistic
	CF00035	8	4	8	4	**1.000**	Additive
	CF00042	16	4	4	1	**0.500**	Synergistic
	CF00046	8	4	8	2	**0.750**	Additive
	CF00047	8	4	4	1	**0.750**	Additive
	CF00883	4	2	4	2	**1.000**	Additive
M. bolletii	CF00020	8	4	8	2	**0.750**	Additive
	CF00113	8	4	4	2	**1.000**	Additive
	CF00868	16	8	8	2	**0.750**	Additive
	CF02061	16	4	8	2	**0.500**	Synergistic

Supplementary Table 8: Minimum inhibitory concentrations (MICs) of azithromycin and IMI/REL, both individually and in combination against the *M. abscessus* CF clinical isolates, along with the corresponding fractional inhibitory concentration (FIC) index values and their synergism

WGS ID	CF Patient ID	MIC Value (µg/mL)				FIC Index	Interaction
		Azithromycin Alone	Azithromycin with IMI/REL	IMI/REL Alone	IMI/REL with Azithromycin		
M. abscessus	CF00006	4	1	16	2	**0.375**	Synergistic
	CF00013	2	1	8	1	**0.625**	Additive
	CF00016	16	8	16	4	**0.750**	Additive
	CF00017	2	1	16	2	**0.625**	Additive
	CF00023	16	4	16	4	**0.500**	Synergistic
	CF00038	2	0.5	8	1	**0.375**	Synergistic
	CF00040	8	2	8	1	**0.375**	Synergistic
	CF00041	4	2	4	0.5	**0.625**	Additive
	CF00043	8	4	4	1	**0.750**	Additive
	CF00136	4	2	4	1	**0.750**	Additive
	CF00258	16	4	16	4	**0.500**	Synergistic
	CF00855	16	4	8	2	**0.500**	Synergistic
	CF01975	16	4	16	4	**0.500**	Synergistic
	CF02033	16	8	16	4	**0.750**	Additive
	CF02279	32	16	8	2	**0.750**	Additive
	CF02319	8	2	8	4	**0.750**	Additive
	CF02486	4	2	8	2	**0.750**	Additive
M. massiliense	CF00008	4	1	16	0.5	**0.281**	Synergistic
	CF00030	2	0.5	8	1	**0.375**	Synergistic
	CF00035	2	0.5	8	1	**0.375**	Synergistic
	CF00042	4	1	8	2	**0.500**	Synergistic
	CF00046	2	1	16	4	**0.750**	Additive
	CF00047	4	2	4	1	**0.750**	Additive
	CF00883	32	16	4	2	**1.000**	Additive
M. bolletii	CF00020	32	16	16	8	**1.000**	Additive
	CF00113	32	4	8	4	**0.625**	Additive
	CF00868	16	8	8	4	**1.000**	Additive
	CF02061	8	2	8	2	**0.500**	Synergistic

Supplementary Table 9: Minimum inhibitory concentrations (MICs) of rifabutin and IMI/REL, both individually and in combination against the *M. abscessus* CF clinical isolates, along with the corresponding fractional inhibitory concentration (FIC) index values and their synergism

WGS ID	CF Patient ID	MIC Value (µg/mL)				FIC Index	Interaction
		Rifabutin Alone	Rifabutin with IMI/REL	IMI/REL Alone	IMI/REL with Rifabutin		
M. abscessus	CF00006	16	8	16	2	**0.625**	Additive
	CF00013	4	2	4	0.5	**0.625**	Additive
	CF00016	16	16	16	0.125	**1.008**	Indifferent
	CF00017	4	0.5	4	2	**0.625**	Additive
	CF00023	16	8	4	1	**0.750**	Additive
	CF00038	16	4	4	1	**0.500**	Synergistic
	CF00040	16	2	4	2	**0.625**	Additive
	CF00041	8	1	4	2	**0.625**	Additive
	CF00043	16	4	4	1	**0.500**	Synergistic
	CF00136	8	2	16	0.125	**0.258**	Synergistic
	CF00258	16	4	16	2	**0.375**	Synergistic
	CF00855	4	1	4	1	**0.500**	Synergistic
	CF01975	2	1	8	0.5	**0.563**	Additive
	CF02033	8	4	4	0.25	**0.563**	Additive
	CF02279	8	4	2	1	**1.000**	Additive
	CF02319	8	2	4	1	**0.500**	Synergistic
	CF02486	16	1	4	2	**0.563**	Additive
M. massiliense	CF00008	16	4	4	2	**0.750**	Additive
	CF00030	16	4	8	2	**0.500**	Synergistic
	CF00035	4	1	8	2	**0.500**	Synergistic
	CF00042	8	2	4	1	**0.500**	Synergistic
	CF00046	16	1	4	2	**0.563**	Additive
	CF00047	8	2	8	2	**0.500**	Synergistic
	CF00883	2	0.5	4	1	**0.500**	Synergistic
M. bolletii	CF00020	2	0.5	8	1	**0.375**	Synergistic
	CF00113	16	4	4	1	**0.500**	Synergistic
	CF00868	8	4	4	0.5	**0.625**	Additive
	CF02061	8	4	4	0.5	**0.625**	Additive

Supplementary Table 10: Minimum inhibitory concentrations (MICs) of clofazimine and IMI/REL, both individually and in combination against the *M. abscessus* CF clinical isolates, along with the corresponding fractional inhibitory concentration (FIC) index values and their synergism

WGS ID	CF Patient ID	MIC Value (µg/mL)				FIC Index	Interaction
		Clofazimine Alone	Clofazimine with IMI/REL	IMI/REL Alone	IMI/REL with Clofazimine		
M. abscessus	CF00006	0.5	0.5	4	0.25	**1.063**	Indifferent
	CF00013	0.5	0.25	2	0.25	**0.625**	Additive
	CF00016	0.5	0.5	8	0.25	**1.031**	Indifferent
	CF00017	0.5	0.5	4	0.25	**1.063**	Indifferent
	CF00023	2	1	8	4	**1.000**	Additive
	CF00038	1	0.25	8	4	**0.750**	Additive
	CF00040	2	0.5	8	4	**0.750**	Additive
	CF00041	1	0.25	8	0.25	**0.281**	Synergistic
	CF00043	0.5	0.25	4	2	**1.000**	Additive
	CF00136	2	1	4	2	**1.000**	Additive
	CF00258	2	1	8	1	**0.625**	Additive
	CF00855	1	0.25	8	2	**0.500**	Synergistic
	CF01975	0.5	0.25	16	2	**0.625**	Additive
	CF02033	1	0.25	4	1	**0.500**	Synergistic
	CF02279	2	0.5	8	4	**0.750**	Additive
	CF02319	1	0.5	16	4	**0.750**	Additive
	CF02486	1	0.25	4	1	**0.500**	Synergistic
M. massiliense	CF00008	0.5	0.25	4	2	**1.000**	Additive
	CF00030	2	1	8	1	**0.625**	Additive
	CF00035	1	0.5	16	4	**0.750**	Additive
	CF00042	2	1	8	8	**1.500**	Indifferent
	CF00046	2	1	16	8	**1.000**	Additive
	CF00047	2	1	8	1	**0.625**	Additive
	CF00883	1	0.5	4	1	**0.750**	Additive
M. bolletii	CF00020	1	0.5	16	4	**0.750**	Additive
	CF00113	1	0.5	8	4	**1.000**	Additive
	CF00868	1	0.5	16	4	**0.750**	Additive
	CF02061	1	0.5	8	2	**0.750**	Additive

Supplementary Table 11: Minimum inhibitory concentrations (MICs) of minocycline and IMI/REL, both individually and in combination against the *M. abscessus* CF clinical isolates, along with the corresponding fractional inhibitory concentration (FIC) index values and their synergism

WGS ID	CF Patient ID	MIC Value (µg/mL)				FIC Index	Interaction
		Minocycline Alone	Minocycline with IMI/REL	IMI/REL Alone	IMI/REL with Minocycline		
M. abscessus	CF00006	256	128	4	2	**1.000**	Additive
	CF00013	256	128	4	1	**0.750**	Additive
	CF00016	512	256	32	8	**0.750**	Additive
	CF00017	256	128	2	2	**1.500**	Indifferent
	CF00023	512	64	4	2	**0.625**	Additive
	CF00038	256	64	4	1	**0.500**	Synergistic
	CF00040	256	128	8	4	**1.000**	Additive
	CF00041	512	128	2	2	**1.250**	Indifferent
	CF00043	256	128	2	2	**1.500**	Indifferent
	CF00136	256	64	2	1	**0.750**	Additive
	CF00258	256	128	8	2	**0.750**	Additive
	CF00855	256	128	8	2	**0.750**	Additive
	CF01975	256	64	8	2	**0.500**	Synergistic
	CF02033	256	128	4	2	**1.000**	Additive
	CF02279	256	128	2	1	**1.000**	Additive
	CF02319	256	64	2	1	**0.750**	Additive
	CF02486	1024	128	8	4	**0.625**	Additive
M. massiliense	CF00008	256	64	8	2	**0.500**	Synergistic
	CF00030	256	16	4	2	**0.563**	Additive
	CF00035	256	32	4	0.5	**0.250**	Synergistic
	CF00042	16	16	4	0.25	**1.063**	Indifferent
	CF00046	512	128	4	1	**0.500**	Synergistic
	CF00047	256	32	2	2	**1.125**	Indifferent
	CF00883	8	8	2	0.125	**1.063**	Indifferent
M. bolletii	CF00020	256	256	4	2	**1.500**	Indifferent
	CF00113	256	64	4	1	**0.500**	Synergistic
	CF00868	8	8	4	0.125	**1.031**	Indifferent
	CF02061	256	64	4	2	**0.750**	Additive

Supplementary Table 12: Minimum inhibitory concentrations (MICs) of omadacycline and IMI/REL, both individually and in combination against the *M. abscessus* CF clinical isolates, along with the corresponding fractional inhibitory concentration (FIC) index values and their synergism

WGS ID	CF Patient ID	MIC Value (µg/mL)				FIC Index	Interaction
		Omadacycline Alone	Omadacycline with IMI/REL	IMI/REL Alone	IMI/REL with Omadacycline		
M. abscessus	CF00006	2	1	8	4	**1.000**	Additive
	CF00013	2	1	8	4	**1.000**	Additive
	CF00016	2	1	8	4	**1.000**	Additive
	CF00017	2	1	4	2	**1.000**	Additive
	CF00023	1	1	4	2	**1.500**	Indifferent
	CF00038	1	1	4	2	**1.500**	Indifferent
	CF00040	1	0.5	4	2	**1.000**	Additive
	CF00041	2	1	8	8	**1.500**	Indifferent
	CF00043	2	2	16	8	**1.500**	Indifferent
	CF00136	4	2	16	8	**1.000**	Additive
	CF00258	4	4	16	16	**2.000**	Indifferent
	CF00855	2	2	16	8	**1.500**	Indifferent
	CF01975	0.5	0.5	8	4	**1.500**	Indifferent
	CF02033	1	0.5	8	8	**1.500**	Indifferent
	CF02279	2	1	8	4	**1.000**	Additive
	CF02319	2	1	4	4	**1.500**	Indifferent
	CF02486	2	1	8	4	**1.000**	Additive
M. massiliense	CF00008	1	0.5	8	2	**0.750**	Additive
	CF00030	2	0.5	8	4	**0.750**	Additive
	CF00035	0.5	0.5	8	8	**2.000**	Indifferent
	CF00042	0.5	0.5	8	4	**1.500**	Indifferent
	CF00046	2	2	8	4	**1.500**	Indifferent
	CF00047	1	0.5	8	2	**0.750**	Additive
	CF00883	1	0.5	8	4	**1.000**	Additive
M. bolletii	CF00020	2	1	16	4	**0.750**	Additive
	CF00113	2	1	8	2	**0.750**	Additive
	CF00868	2	1	16	8	**1.000**	Additive
	CF02061	2	1	16	8	**1.000**	Additive

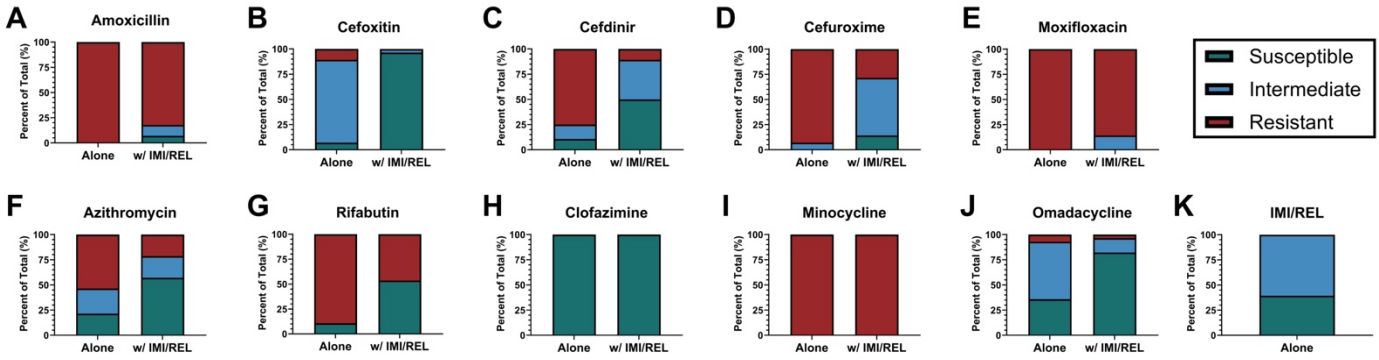
Supplementary Table 13: Numbers and percentages of susceptible, intermediate, and resistant *M. abscessus* CF clinical isolates treated with antibiotics and IMI/REL, both individually and in combination

Antibacterial Agent	Susceptible Isolates[a]		Intermediate Isolates[a]		Resistant Isolates[a]	
	Drug Alone	**Drug with IMI/REL**	Drug Alone	**Drug with IMI/REL**	Drug Alone	**Drug with IMI/REL**
Amoxicillin	0 (0.0%)	**2 (7.1%)**	0 (0.0%)	**3 (10.7%)**	28 (100.0%)	**23 (82.1%)**
Cefoxitin	2 (7.1%)	**27 (96.4%)**	23 (82.1%)	**1 (3.6%)**	3 (10.7%)	**0 (0.0%)**
Cefdinir	3 (10.7%)	**14 (50.0%)**	4 (14.3%)	**11 (39.3%)**	21 (75.0%)	**3 (10.7%)**
Cefuroxime	0 (0.0%)	**4 (14.3%)**	2 (7.1%)	**16 (57.1%)**	26 (92.9%)	**8 (28.6%)**
Moxifloxacin	0 (0.0%)	**0 (0.0%)**	0 (0.0%)	**4 (14.3%)**	28 (100.0%)	**24 (85.7%)**
Azithromycin	6 (21.4%)	**16 (57.1%)**	7 (25.0%)	**6 (21.4%)**	15 (53.6%)	**6 (21.4%)**
Rifabutin	3 (10.7%)	**15 (53.6%)**	—	**—**	25 (89.3%)	**13 (46.4%)**
Clofazimine	28 (100.0%)	**28 (100.0%)**	0 (0.0%)	**0 (0.0%)**	0 (0.0%)	**0 (0.0%)**
Minocycline	0 (0.0%)	**0 (0.0%)**	0 (0.0%)	**0 (0.0%)**	28 (100.0%)	**28 (100.0%)**
Omadacycline	10 (35.7%)	**23 (82.1%)**	16 (57.1%)	**4 (14.3%)**	2 (7.1%)	**1 (3.6%)**
IMI/REL[b]	11 (39.3%)	—	17 (60.7%)	—	0 (0.0%)	—

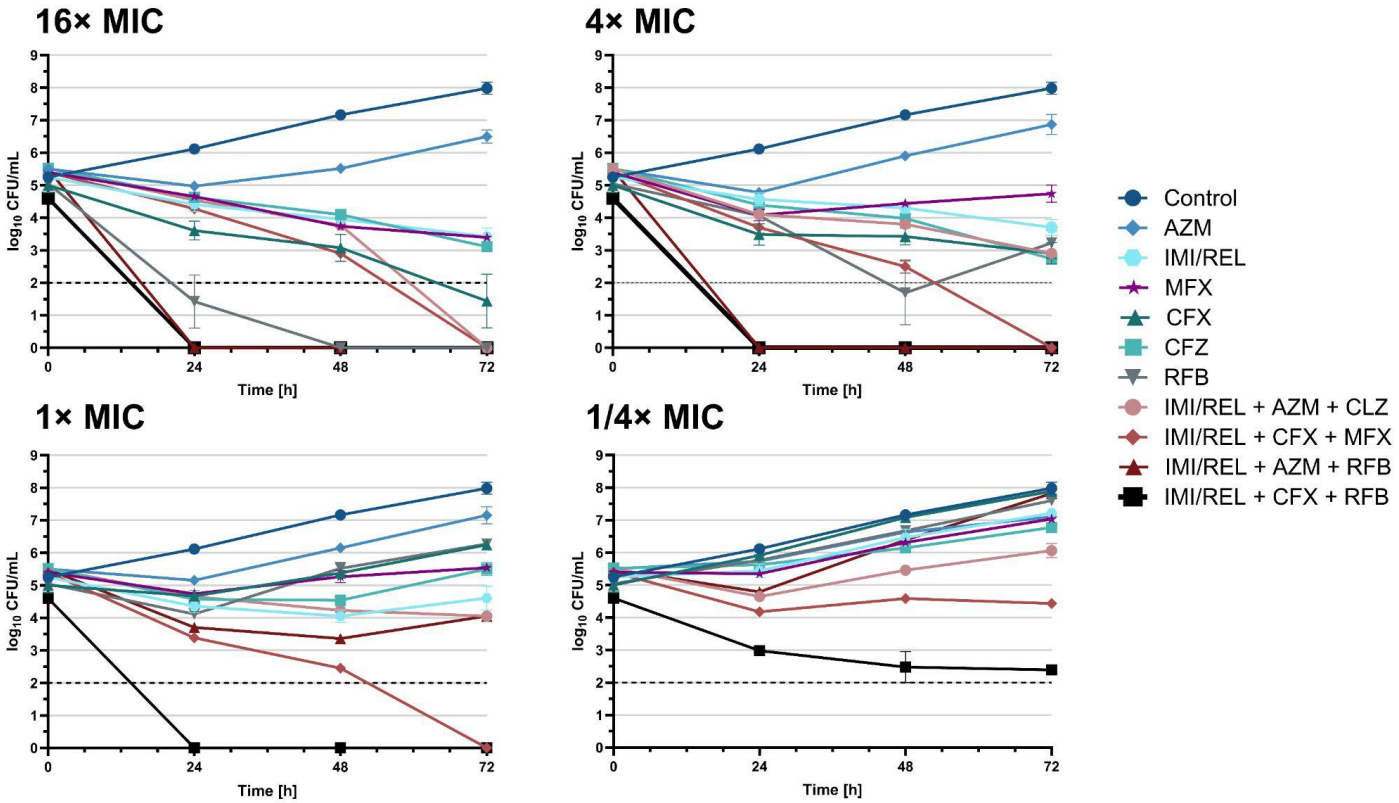
[a]Susceptibility interpretations for all isolates are reported as n (%)

[b]IMI/REL was prepared by serially diluting imipenem while keeping the relebactam concentration fixed at 4 µg/mL.

Supplementary Figure 1: Susceptibility profiles of *M. abscessus* CF clinical isolates treated with antibiotics and IMI/REL, both individually and in combination for (A) amoxicillin, (B) cefoxitin, (C) cefdinir, (D) cefuroxime, (E) moxifloxacin, (F) azithromycin, (G), rifabutin, (H) clofazimine, (I) minocycline, (J) omadacycline, and (K) IMI/REL.



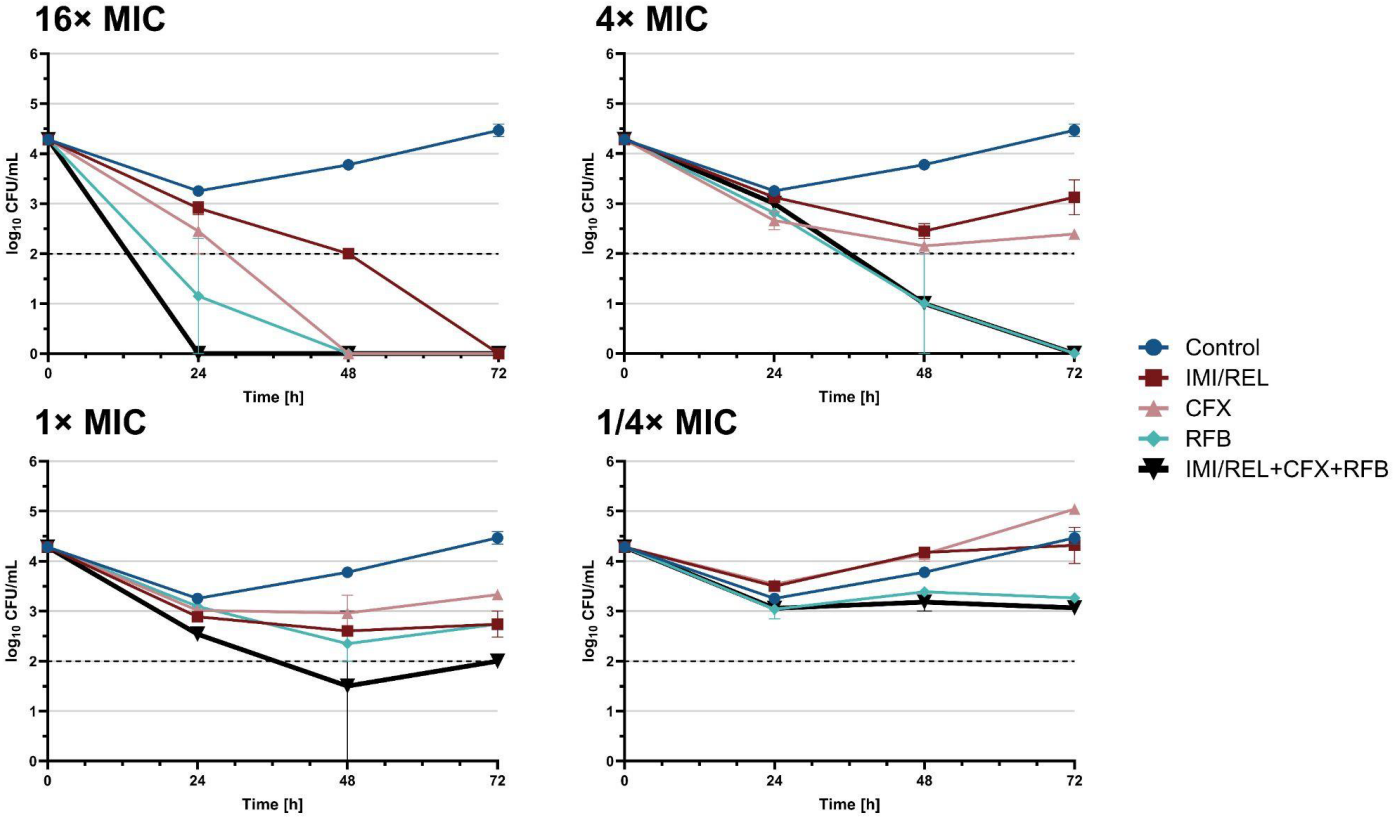
Supplementary Figure 2: Bacterial load (log_{10} CFU/ml) of *M. abscessus* ATCC 19977 over 72 hours with single-agent therapies and three-drug combination therapies with IMI/REL at 16× MIC, 4× MIC, 1× MIC, and 1/4× MIC.



Data are presented as mean with standard errors of the mean. The horizontal dashed line marks the lower limit of detection.

MIC = minimum inhibitory concentration, AZM = azithromycin, MFX = moxifloxacin, CFX = cefoxitin, CFZ = clofazimine, RFB = rifabutin

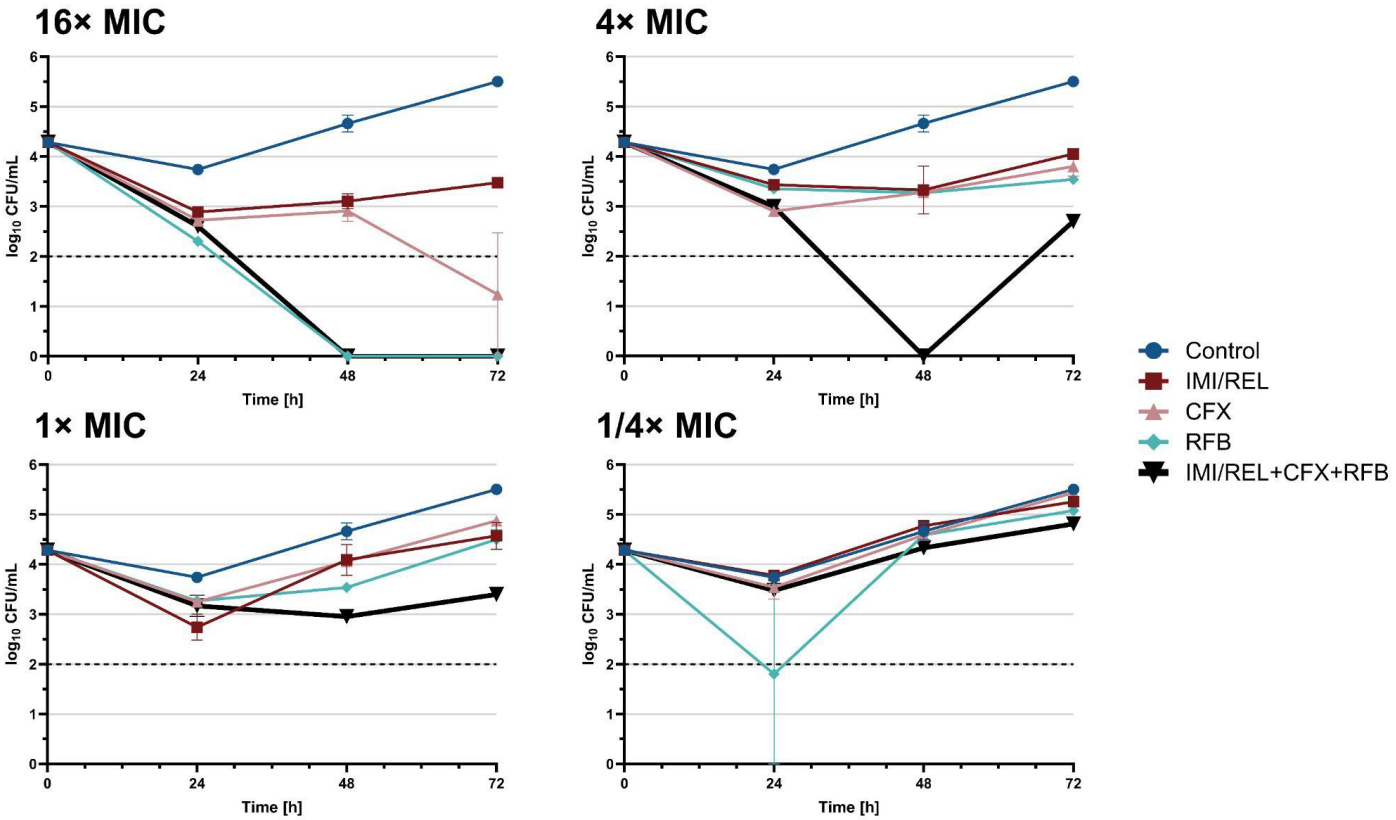
Supplementary Figure 3: Bacterial load (log_{10} CFU/ml) of *M. abscessus* CF clinical isolate 13 over 72 hours with IMI/REL, cefoxitin, rifabutin, and their combination at 16× MIC, 4× MIC, 1× MIC, and 1/4× MIC.



Data are presented as mean with standard errors of the mean. The horizontal dashed line marks the lower limit of detection.

MIC = minimum inhibitory concentration, CFX = cefoxitin, RFB = rifabutin

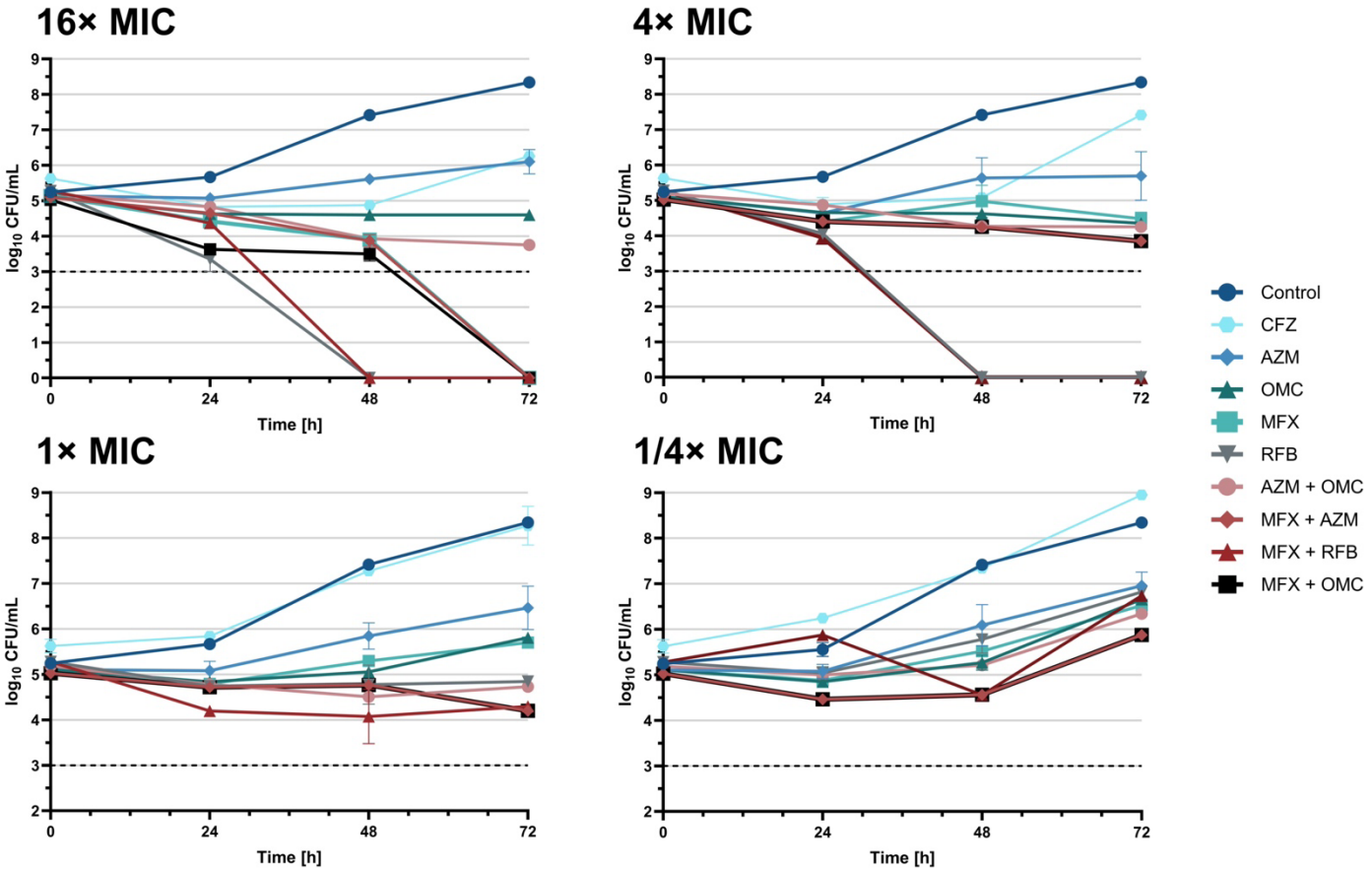
Supplementary Figure 4: Bacterial load (log_{10} CFU/ml) of *M. abscessus* CF clinical isolate 258 over 72 hours with IMI/REL, cefoxitin, rifabutin, and their combination at 16× MIC, 4× MIC, 1× MIC, and 1/4× MIC.



Data are presented as mean with standard errors of the mean. The horizontal dashed line marks the lower limit of detection.

MIC = minimum inhibitory concentration, CFX = cefoxitin, RFB = rifabutin

Supplementary Figure 5: Intracellular bacterial load (log_{10} CFU/ml) of *M. abscessus* ATCC 19977 over 72 hours with single-agent therapies and two-drug combination therapies at 16× MIC, 4× MIC, 1× MIC, and 1/4× MIC.



Data are presented as mean with standard errors of the mean. The horizontal dashed line marks the lower limit of detection.

MIC = minimum inhibitory concentration, AZM = azithromycin, OMC = omadacycline, MFX = moxifloxacin, RFB = rifabutin

Supplementary Table 14: Susceptibility of antibiotics and IMI/REL, both individually and in combination against *M. abscessus* CF clinical isolates (*n*=28), alongside maximum concentrations in plasma, epithelial lining fluid (ELF), and alveolar macrophages (AM) based on previously published data.

Antibiotic Agent	MIC_{50} (µg/mL)[a]		Plasma C_{max} (µg/mL)	ELF C_{max} (µg/mL)	AM C_{max} (µg/mL)	References
	Drug Alone	Drug with IMI/REL				
IMI/REL	**4 (4, 8)**	—	29.8	9.8	0.0	[27-28]
Cefoxitin	**48 (32, 64)**	**8 (4, 8)**	244.0	—	—	[78]
Azithromycin	**8 (4, 16)**	**2 (1, 4)**	0.4	1.2	194.0	[80]
Moxifloxacin	**16 (8, 16)**	**4 (4, 4)**	3.2	20.7	56.7	[82]
Clofazimine	**1 (0.88, 2)**	**0.50 (0.25, 0.63)**	0.4	—	—	[84-85]
Omadacycline	**2 (1, 2)**	**1 (0.50, 1)**	2.1	2.3	18.0	[90]
Rifabutin	**8 (7, 16)**	**2 (1, 4)**	0.5	~4.5	~7.8	[91-93]
Cefuroxime	**512 (256, 512)**	**64 (32, 128)**	1.1	0.0	0.0	[94]
Cefdinir	**192 (112, 256)**	**24 (14, 40)**	4.2	0.5	0.0	[95]
Amoxicillin	**2048 (1024, 2048)**	**128 (32, 256)**	6.9	0.9	0.0	[96]
Minocycline	**256 (256, 256)**	**96 (64, 128)**	3.1	—	—	[97]

[a] Data are presented as MIC_{50} (interquartile range [IQR]).