

1 **Cross-transmission is not the source of new *Mycobacterium***
2 ***abscessus* infections in a multi-centre cohort of cystic fibrosis**
3 **patients**

4
5 **Running title: *M. abscessus* transmission in CF patients**

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28 **Keywords:** Nontuberculous mycobacteria, whole-genome sequencing, transmission, cystic
29 fibrosis, phylogenomics.

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31
32 **Key points:**

- 33 • Whole genome sequencing should replace current molecular typing used routinely in
34 clinical microbiology laboratories.
35 • Patient-to-patient spread of *M. abscessus* is not common.
36 • Environmental screening may provide a better understanding acquisition of *M.*
37 *abscessus* infections.

38 **Abstract**

39 **Background:**

40 *Mycobacterium abscessus* is an extensively drug resistant pathogen that causes pulmonary
41 disease particularly in cystic fibrosis (CF) patients. Identifying direct patient-to-patient
42 transmission of *M. abscessus* is critically important in directing infection control policy for the
43 management of risk in CF patients. A variety of clinical labs have used molecular epidemiology
44 to investigate transmission. However there is still conflicting evidence as to how *M. abscessus*
45 is acquired and whether cross-transmission occurs. Recently labs have applied whole-genome
46 sequencing (WGS) to investigate this further and in this study we investigate whether WGS
47 can reliably identify cross-transmission in *M. abscessus*.

48 **Methods:**

49 We retrospectively sequenced the whole genomes of 145 *M. abscessus* isolates from 62 patients
50 seen at four hospitals in two countries over 16 years.

51 **Results:**

52 We have shown that a comparison of a fixed number of core single nucleotide variants (SNVs)
53 alone cannot be used to infer cross-transmission in *M. abscessus* but does provide enough
54 information to replace multiple existing molecular assays. We detected one episode of possible
55 direct patient-to-patient transmission in a sibling pair. We found that patients acquired unique
56 *M. abscessus* strains even after spending considerable time on the same wards with other *M.*
57 *abscessus* positive patients.

58 **Conclusions:**

59 This novel analysis has demonstrated that the majority of patients in this study have not
60 acquired *M. abscessus* through direct patient-patient transmission or a common reservoir.
61 Tracking transmission using WGS will only realise its full potential with proper environmental
62 screening as well as patient sampling.

63 **Background**

64 *Mycobacterium abscessus* (recently renamed as *Mycobacteroides abscessus*) [1], is a group of
65 three closely related subspecies *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp.
66 *massiliense* and *M. abscessus* subsp. *bolletii* [1,2]. These rapidly-growing, non-tuberculous
67 mycobacteria cause chronic pulmonary disease, particularly in patients with cystic fibrosis
68 (CF) and other chronic lung diseases. *M. abscessus* is an important pathogen that has emerged
69 in the CF patient population that has been associated with poor clinical outcomes, especially
70 following lung transplantation [3–5]. This is due, at least in part, to the extensive antibiotic
71 resistance that makes infections with this organism difficult to treat [2,6]. CF patients infected
72 with *M. abscessus* are frequently not listed for transplant, therefore the acquisition of this
73 pathogen is considered to be a serious complication in this group.

74

75 The epidemiology of *M. abscessus* strains has been studied using Variable Nucleotide Tandem
76 Repeats (VNTR) and Multi Locus Sequence Typing (MLST) [7]. The clustering of globally
77 spread sequence types was confirmed with whole genome sequencing (WGS) and has provided
78 greater resolution in how the various lineages are related as well as predicting possible
79 transmission routes [8,9]. A dominant method of transmission of *M. abscessus* remains
80 contested [10,11], with evidence for and against patient-to-patient transmission being the
81 common route [8,12–14]. *M. abscessus* is ubiquitous in the environment with its niche
82 hypothesised to be free-living amoeba [15,16], but due to the difficulties in isolating the
83 organism, little has been done to track environment-to-patient acquisition. Confirmation of
84 direct patient-to-patient transmission is important as it influences management of high-risk
85 patients it could increase the effectiveness of infection control interventions by directing the
86 use of limited resources.

87

88 In this retrospective study we assessed utility of using WGS to characterise subspecies,
89 antimicrobial resistance (AMR) profiles and typing of *M. abscessus* isolates. We also wanted
90 to utilise the data to investigate the scale of patient-to-patient transmission and whether
91 identification of single nucleotide variants (SNVs) by WGS can confirm transmission. To do
92 this we have sequenced the genomes 145 *M. abscessus* clinical isolates from a well
93 characterised cohort of 62 patients from four hospitals in two countries over 16 years.

94 **Methods**

95 **Patients and Samples collection**

96 We collected 33 *M. abscessus* isolates from 30 patients at Hospital de la Santa Creu I Sant Pau
97 (bcn_hsp), Hospital Clínic (bcn_hcl) and Hospital Vall d'Hebron (bcn_hvh), Barcelona, Spain
98 and 112 isolates from 32 patients from Great Ormond Street Hospital (ldn_gos), London, UK
99 (Supplementary table 1). Demographic and patient location data were obtained from the patient
100 administration system and microbiological data from the laboratory information management
101 system using SQL and Excel spreadsheets. Additional sources of information included CF and
102 transplant databases. American Thoracic Society consensus guidelines were used to verify
103 evidence of non-tuberculous mycobacterial infection [17]. All investigations were performed
104 in accordance with the Hospitals Research governance policies and procedures.

105

106 **DNA extraction and Whole-Genome Sequencing**

107 One hundred and forty-five *M. abscessus* isolates from 62 patients were analysed using whole-
108 genome sequencing. Briefly, DNA was extracted from all isolates as previously described [18]
109 with some modifications: DNA was extracted from all isolates using Qiagen EZ1 Blood
110 extraction kit with a previous step of bead-beating (Qiagen, Crawley, United Kingdom). Then
111 total DNA concentration was determined using a Qubit fluorometer (ThermoFisher). Fifty
112 nanograms of DNA was prepared using Nextera Library Preparation kit (Illumina) and post-
113 PCR clean-up was carried out using Ampure XP beads (Beckman). Library size was validated
114 using the Agilent 2200 TapeStation with Agilent D5000 ScreenTape System (Willoughby,
115 Australia) and 150bp paired-end reads were sequenced on a NextSeq 550 system (Illumina).
116 Raw sequencing reads have been deposited on ENA (study accession PRJEB31559).

117

118 **Multi Locus Sequence Typing (MLST) analysis**

119 We used a custom bash script to extract the alleles of the multi-locus sequence typing (MLST)
120 profile from the mapped reads to the reference genome. The MLST profile was obtained using
121 the Institut Pasteur MLST database
122 (<http://bigsd.bpasteur.fr/mycoabscessus/mycoabscessus.html>).

123

124 **Read mapping and variant calling**

125 Sequenced reads for all samples were first mapped to *M. abscessus* subsp. *abscessus* ATCC
126 19977 using BMap v37.90 (Joint Genome Institute). Single nucleotide variants (SNVs) were
127 called against the reference genome using freebayes v1.2.0 [19] and variants were filtered to
128 only include those at sites with a mapping quality >30, a base quality >30, at least five
129 supporting reads, where the variant was present on at least two forward and reverse strand reads
130 and present at the 5' and 3' end of at least two reads.

131

132 **Phylogenetic analysis**

133 Potential regions of recombination were identified from the consensus genome sequences using
134 Gubbins v2.3.1 [20]. Regions within the genome with low coverage (< 5x) were masked on a
135 per sample basis and regions with low coverage across 75% of samples were masked across
136 the entire dataset. A maximum likelihood tree was inferred from all samples using RAxML
137 v8.2.4 [21] using a GTRCAT model with 99 bootstraps. Sub-species were identified for each
138 sample based on their position upon this tree.

139

140 Separate sub-trees were also inferred for *M. abscessus* subsp. *massilense* sequences, as well as
141 for *M. abscessus* subsp. *abscessus* ST-1 and ST-26 sequences. All samples in each sub-tree
142 were mapped against a suitable reference. *M. abscessus* subsp. *massilense* str. GO 06 was used
143 as the reference sequencing for study *massilense* sequences and the *de novo* assembly of the
144 earliest ST-26 study sequence (ldn_gos_2_520) was used as a reference for other ST-26

145 samples. *M. abscessus* subsp. *abscessus* ATCC 19977 was again used as the reference for ST-
146 1 sequences as it is the same sequence type. All sub-trees were generated using the same
147 method outlined above, apart from ST-26 subtree, which did not use Gubbins but instead
148 variants were filtered if 3 SNVs were found within a 100bp window.

149

150 **Sequence clusters**

151 Sequence clusters to infer possible transmission were generated using three different methods
152 on each subtree. First we used a SNV threshold that was based on the upper bounds of all within
153 patient diversity applied to complete linkage hierarchical clustering based on pairwise SNV
154 matrix. Secondly we assigned clusters using the R package rPinecone as it incorporates SNV
155 thresholds and root-to-tip distances and so has been useful when applied to clonal populations
156 [22]. Lastly we also used hierBAPS [23] to assign clusters, however due to the fact that all
157 samples are included in the sequence clusters we found it was not appropriate for this study
158 question. We made the assumption that any strains taken from different patients that were
159 within sequence cluster constituted a possible transmission event.

160

161 ***De novo* assembly**

162 All samples underwent *de novo* assembly of bacterial genomes using SPAdes and pilon
163 wrapped in the Unicycler v0.4.4 package [24]. Assembled contigs were annotated using prokka
164 v1.13 [25] and comparison of the accessory genome was generated using roary v3.12.0 [26].
165 To generate a list of genes that could be used to differentiate isolates we filtered the annotated
166 genes to remove coding sequences (CDS) greater than 8000 bp and less than 250 bp, as well
167 as those only present in a single sample and those present in every sample.

168 **Results**

169 ***M. abscessus* population distribution**

170 We obtained whole genome sequences for 145 *M. abscessus* isolates from 62 patients. Thirty-
171 three *M. abscessus* from Barcelona subdivided into 24 *M. abscessus* subsp. *abscessus*, two *M.*
172 *abscessus* subsp. *bolletii* and seven *M. abscessus* subsp. *massiliense*. A hundred and twelve *M.*
173 *abscessus* from UK subdivided into 78 *M. abscessus* subsp. *abscessus*, one *M. abscessus* subsp.
174 *bolletii* and 33 *M. abscessus* subsp. *massiliense*. Sample MLST definitions, VNTR and AMR
175 associated mutations are shown in supplementary table 2.

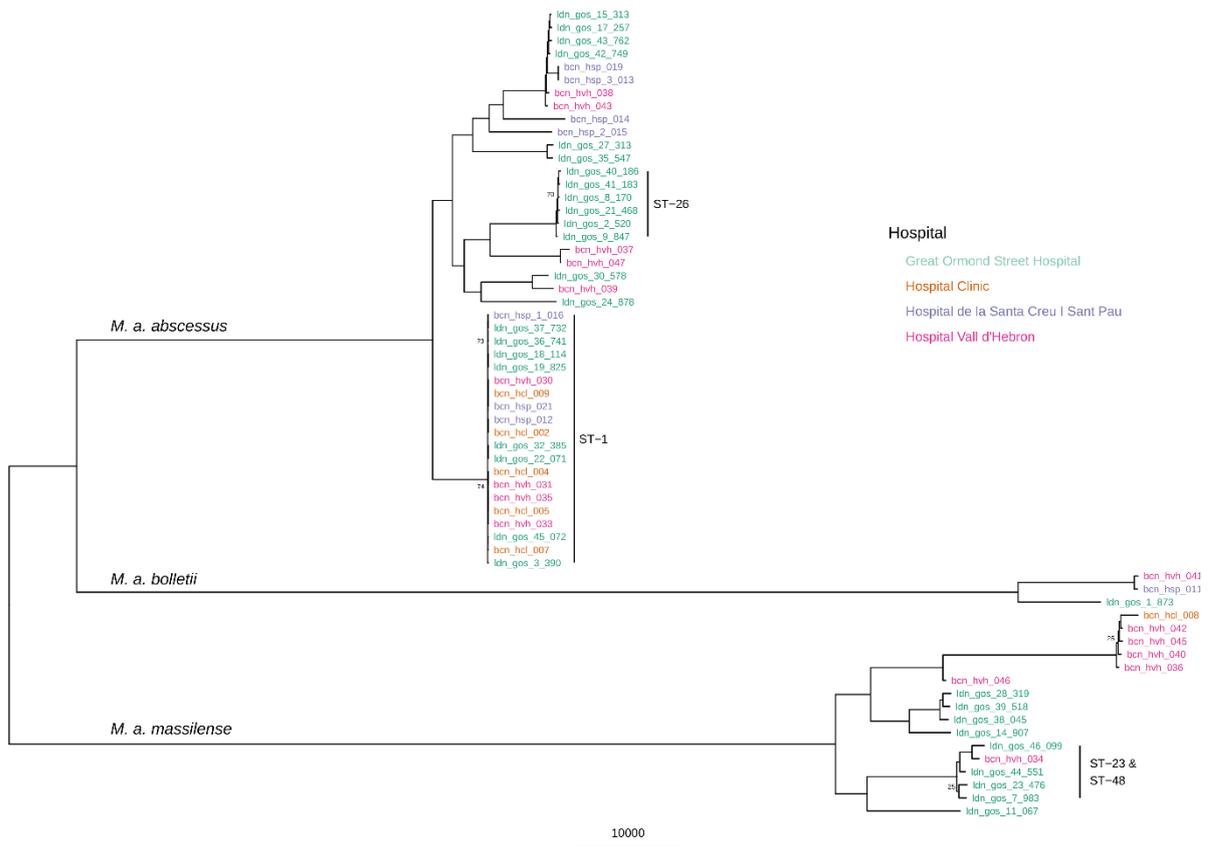
176

177 **Possible transmission within *M. abscessus* clusters**

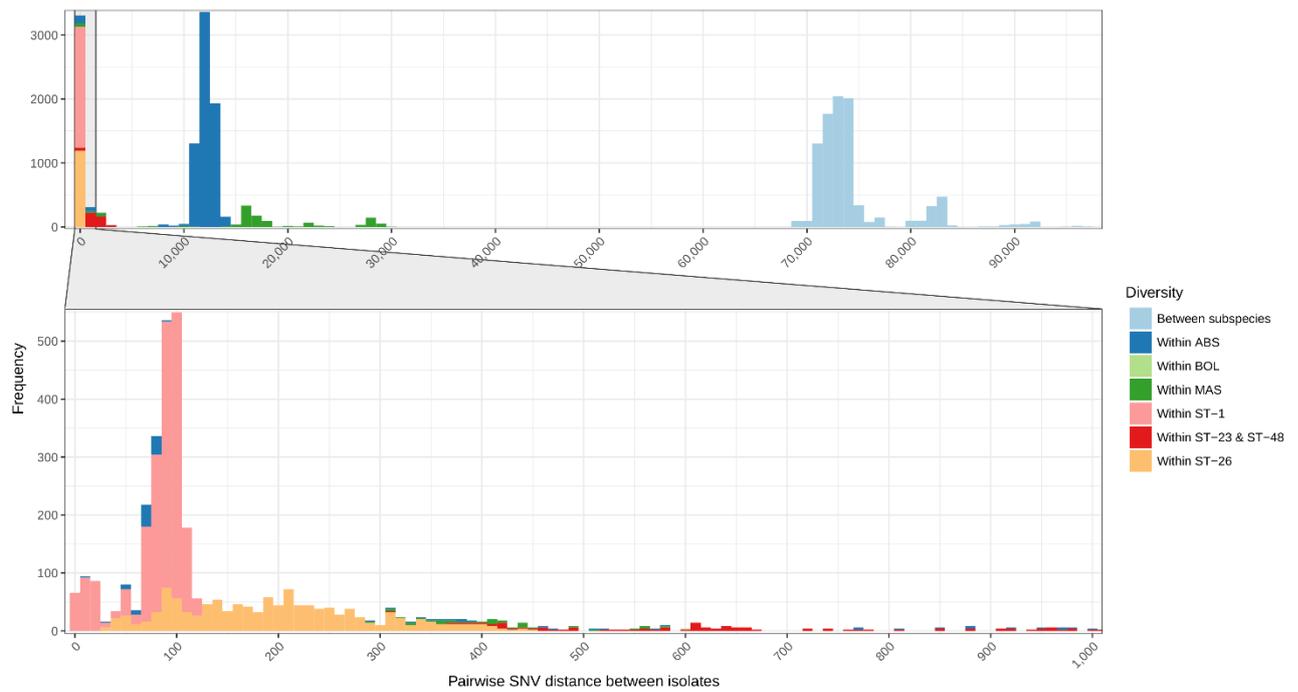
178 To confirm possible transmission between patients we required their isolate genomes to be
179 clustered together by two independent methods and epidemiological evidence that both patients
180 were at the same hospital during the same time period. Using WGS data we inferred a
181 phylogenetic tree from reference genome SNV matrix for all patients (Figure 1). We observed
182 two low variant clusters of isolates that corresponded to ST-1 and ST-26 Pasteur MLST profiles
183 (VNTR II and I respectively), as well as other closely related *M. abscessus* *susp. massilense*
184 isolates between patients. We used a SNV matrix from mapping against a reference (*M.*
185 *abscessus* subsp. *abscessus* ATCC19977), as well as hierBAPS and rPinecone to predict
186 sequence clusters. The sequence clusters generated from the single reference SNV matrix
187 provided no further information than the MLST profiles, and in many cases provided spurious
188 findings with large groups of isolates clustered with no epidemiological link (Supplementary
189 Figure 1). This included large sequence clusters relating to a single MLST type which included
190 isolates from different hospitals and countries.

191 Mapping to a single reference genome led to the inability of a single SNV cut-off, or model, to
192 exclude unrelated isolates from sequence clusters because the number of pairwise SNV
193 distances varied greatly between both subspecies and specific lineages which (Figure 2). For

194 example, the pairwise median (interquartile range) SNV distance between just ST-1 isolates
195 was 73 (62 – 81) compared to 29589 (27701 – 63703) for all *M. abscessus subsp. abscessus*
196 isolates. The same differences were seen in *M. abscessus subsp. massilense* as well with a
197 pairwise median (IQR) SNV distance between ST-23 and ST-48 isolates of 2084 (960 – 7274)
198 compared to 70545 (59947 – 71891) across all isolates from the subspecies.



199
 200 **Figure 1. Maximum likelihood single nucleotide variant (SNV) tree using only the earliest**
 201 **isolated sample from all 62 patients. SNVs were identified from mapping reads to**
 202 *ATCC19977 M. abscessus subsp. abscessus* reference genome. Sample names are highlighted
 203 in colour based on what hospital they were isolated from: Great Ormond Street Hospital,
 204 London, UK, Hospital Clínic, Barcelona, Spain, Hospital de la Santa Creu i Sant Pau,
 205 Barcelona, Spain, and Hospital Vall d'Hebron, Barcelona, Spain. The scale bar represents the
 206 number of single nucleotide variants and node bootstrap scores below are shown if below 75.
 207



208

209 **Figure 2. Frequency of pairwise single nucleotide variant (SNV) distances between all**
210 **isolates.** SNVs were identified from mapping sequence reads to *M. abscessus* subsp. *abscessus*
211 ATCC19977. The full plot includes all samples while the bottom subsidiary plot only includes
212 isolates that have a pairwise difference between zero and 1000 SNVs.

213

214 **Sub-tree sequence clusters**

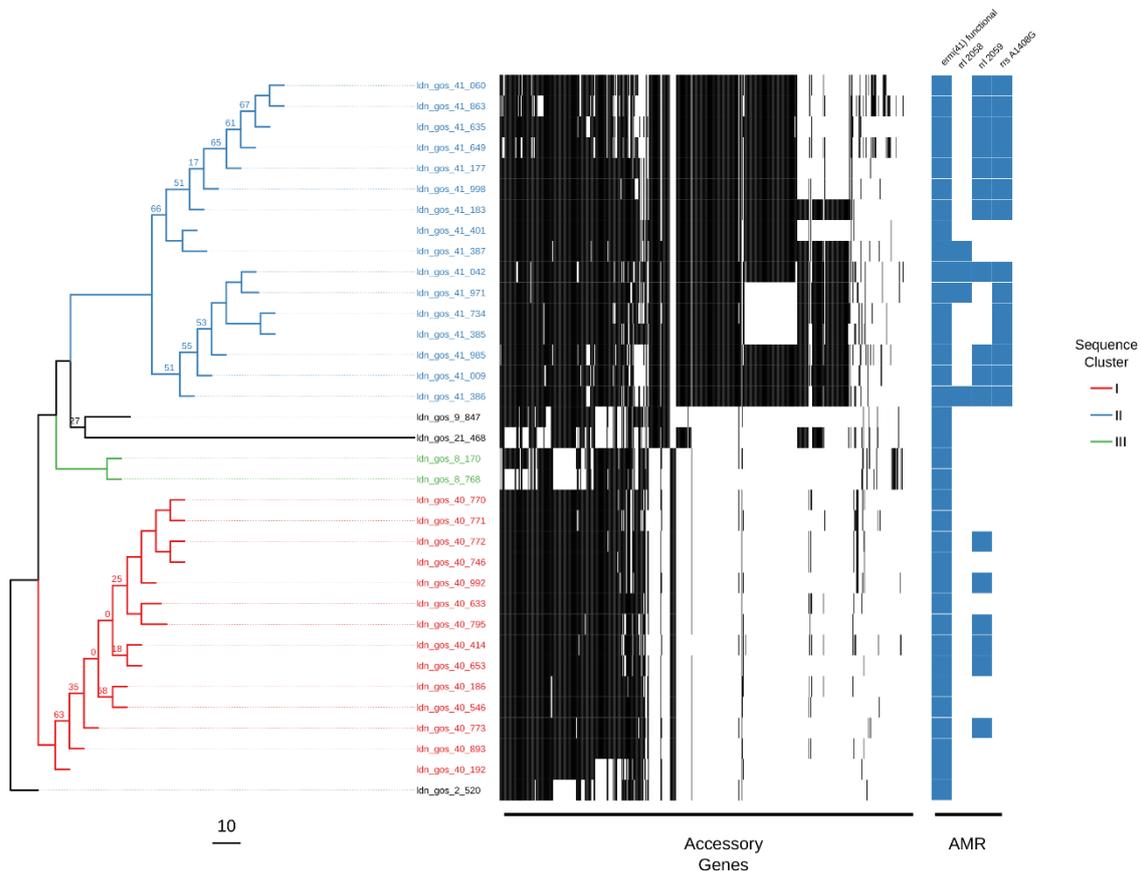
215 The variation in the scale of diversity within subspecies and sequence type hampered efforts to
216 capture possible transmission events. In order to improve accuracy of sequence clustering,
217 multiple sub-trees were made for closely related isolates using a more suitable reference
218 sequence. We separated *M. abscessus subsp. abscessus* and *M. abscessus subsp. massilense*
219 isolates, as well as further sub-trees for ST-1 (VNTR II), ST-26 (VNTR I) and ST-23/ST-48
220 (VNTR III) isolates. We also integrated the presence of accessory genes when interrogating
221 possible sequence clusters for transmission (Figures 3, 4 & 5). Sequence clusters were assigned
222 for each sub-tree using both a single SNV threshold (Supplementary Figure 2) and rPinecone.
223 Overall we found that predicting transmission from the sub-trees reduced the number of
224 different patients clustered together from 46 to 19 and the number of possible sequence clusters
225 suggesting patient-to-patient transmission from 11 to seven.

226 A total of 18 sequence clusters (I – XVIII) were identified (listed in supplementary table 2), 15
227 of these were within the sub-trees (I – XV), and seven clusters contained samples from more
228 than one patient (IV, V, VI, VIII, XIV, XVI & XVII). All sequence clusters contained isolates
229 from a single country with no evidence of international transmission. We found no evidence of
230 transmission between patients within ST-26. (Figure 3). Within ST-1, four clusters (IV, V, VI
231 and VIII) containing samples from more than one patient were found. Three of these clusters
232 (IV, V and VI) contained isolates from nine patients from multiple hospitals within Barcelona.
233 Only two of these patients were in hospital during the same time period (cluster VI:
234 bcn_hcl_009 and bcn_hvh_30), but both were treated in different hospitals. Cluster VIII
235 suggested transmission between two patients (ldn_gos_18 and ldn_gos_19) who were siblings
236 with previously assumed either direct transmission or common reservoir [13] (Figure 4). A
237 single cluster (XIV) containing samples from two patients (ldn_gos_46 and ldn_gos_7) was
238 found among ST-23 isolates. However the two strains were isolated from samples taken nine
239 years apart (Figure 5). Patient ldn_gos_7 was already positive for *M. abscessus* on first

240 admission to GOSH, and the two patients were present at the lung function lab within a month
241 of each other on two occasions, but never in the same location at the same day, and never
242 admitted to the same ward.

243 All samples found within their respective clusters also contained similar accessory gene
244 profiles with the median (IQR) shared percentage of accessory genes within a sequence cluster
245 being 89% (79% – 94%) compared to 18% (12% - 37%) for isolates not in the same sequence
246 cluster.

247 For the 32 GOSH CF patients included in the study, 16 became infected with *M. abscessus*
248 after their first visit to clinic (Supplementary Table 1), however transmission confirmed by both
249 WGS and epidemiological data could only be identified in one case (gos_19) thus suggesting
250 a different route of acquisition for the rest of these patients.



251

252 **Figure 3. Maximum likelihood single nucleotide variant (SNV) tree for all ST-26 isolates.**
253 SNVs were identified from mapping reads to a de-novo assembled study isolate genome
254 (*ldn_gos_2_520*). Samples are highlighted based on inclusion in sequence clusters. The tree is
255 annotated with the presence (black) and absence (white) of accessory genes as well as the
256 presence of AMR associated genes and mutations. This included presence of a functional
257 *erm(41)* gene conferring inducible resistance to macrolides, presence of two *rrl*
258 conferring high level macrolide resistance and the presence of mutation in *rrs* conferring high
259 level amikacin resistance. The scale bar represents the number of single nucleotide variants and
260 node bootstrap scores below are shown if below 75.

281 Discussion

282 This study has shown that whole genome sequencing of *M. abscessus* isolates can determine
283 sub-species, identify previously reported AMR associated mutations and provide common
284 typing definitions in a single workflow. This single method can replace the multiple existing
285 molecular assays used in clinical microbiology laboratories to provide the same information
286 and could be used to predict novel resistance variants [27]. We used the WGS data to investigate
287 the likelihood of cross-transmission and found 43 (69%) patients had unique isolates that did
288 not cluster with other patients. We identified seven sequence clusters from the remaining 19
289 patients but only one pair of patients (ldn_gos_18 and ldn_gos_19) had a plausible
290 epidemiological link to support possible patient-to-patient transmission, as they were siblings.
291 All other patients with genetically similar strains were either isolated in different countries,
292 different hospitals or isolated from samples that were taken years apart, making direct
293 transmission of these strains extremely unlikely.

294 Every *M. abscessus* isolated from a GOSH patient was sequenced and so the dataset generated
295 represents a complete picture of *M. abscessus* infection in this hospital, which is vital for
296 inferring transmission. Most of these patients were only attending clinics at GOSH, therefore
297 this study has captured all of their *M. abscessus* isolates and they are unlikely to have been in
298 contact with *M. abscessus* positive patients at other hospitals (Supplementary table 1).
299 Therefore, if direct patient-patient transmission was occurring frequently we would expect to
300 see evidence of it here. In contrast to this we found that the majority of patients in this study
301 had unique strains and the majority of sequence clusters were multiple isolates from the same
302 patients. This study confirms previous findings that despite many *M. abscessus* negative
303 patients spending considerable time on the same wards as patients with ongoing *M. abscessus*
304 infections they did not subsequently acquire genetically similar isolates.

305 We have therefore found that a fixed number of SNVs cannot be reliably used to infer cross-
306 transmission across all *M. abscessus* isolates as there seems to be irreconcilable differences in

307 the substitution rate between both sub-species and dominant clones. These difficulties are
308 similar to those seen in *Legionella pneumophila* outbreaks where the majority of cases can
309 belong to only a few sequence types [26]. *L. pneumophila* can also display different scales of
310 genetic diversity within different sequence or genotypes and so it is also recognised that a single
311 SNV threshold cut-off will not provide sufficient discriminatory power [27]. When using WGS
312 to infer relatedness in *M. abscessus* there has previously been an attempt to find an absolute
313 threshold which can rule in or rule out strains into a transmission event. This has previously
314 been placed as below 25-30 SNVs [8,14,28,29]. From our findings we would advocate using a
315 suitable genetically similar reference sequence when carrying out core genome SNV calling,
316 especially for the dominant clones such as ST-1 and ST-26. There is a large amount of variation
317 within the genomes of *M. abscessus* [30] and so the use of a single reference such as *M.*
318 *abscessus subsp. abscessus* ATCC 19977 will mask many differences between strains and
319 generate spurious clusters of genetically similar sequences. Where a suitable reference is not
320 available we recommend using a high quality draft de-novo assembly of the first isolated
321 sample to compare other isolates against as in the example of the ST-26 samples in this study
322 (Figure 3).

323 In addition to core genome SNV analysis we have also found the integration of accessory
324 genome information is a useful indicator of relatedness within *M. abscessus* isolates that can
325 be used to further interrogate assigned sequence clusters. Generally there was good
326 concordance between the proportion of putative genes shared and the SNV distance between
327 two samples. This is helped by using a closely related reference sequences to map sequence
328 reads against. We have seen in this study, and previously [31], diversity in the accessory
329 genome profiles as well as in the number of SNPs and AMR associated mutations taken from
330 multiple samples from the same patient on the same day. However we have always found inter-
331 patient diversity to be greater than that seen within the same patient. This would suggest that
332 any direct transmission between patients of even minority populations would still be identified

333 by WGS and, taken together, the data suggests that person-to-person transmission of *M.*
334 *abscessus* in paediatric patients in our institution is very uncommon. In this study we have an
335 example of two patients with transmission predicted by genomic epidemiology (ldn_gos_7 and
336 ldn_gos_46) that had attended a lung function laboratory on three occasions within a month of
337 each other. In this case, the only way transmission could have occurred is if ldn_gos_7 who
338 was already infected contaminated the environment and this then transmitted to ldn_gos_46.
339 The predominant view [8] that human-to-human transmission occurs via contamination of
340 fomites by respiratory secretions could explain this, although no other instances of this
341 appeared to have occurred, despite numerous other CF patients attending the unit over many
342 years. What is harder to explain is that for this to be the case, the interval between exposure
343 and culture positivity was nine years. It could be that *M. abscessus* remains present but
344 undetectable by conventional methods for this time period, or intriguingly could cause latent
345 infection, like what occurs with *Mycobacterium tuberculosis*. To the best of our knowledge,
346 this has never been a demonstrated part of the pathogenesis of *M. abscessus* infection, and
347 maybe worthy of further investigation.

348 In agreement with previous studies we have found an international distribution of *M. abscessus*
349 dominant clones [8]. We have found WGS to be useful to confirm whether different patient's
350 strains are unrelated, even within the dominant clones, but it has been far more difficult to reach
351 definite conclusions about cross-transmission. Without environmental samples we cannot rule
352 out the possibility of intermediate sources of infection and so WGS as a tool for tracking cross-
353 transmission in *M. abscessus* will only realise its full potential with proper screening of
354 environmental sources alongside longitudinal patient sampling.

355

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365

366

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Supplementary material

Supplementary table 1. Study patient information.

Supplementary table 2. Information on all individual *M. abscessus* isolates included in this study.

Supplementary Figure 1. Maximum likelihood single nucleotide variant (SNV) tree for all isolates in this study. The tree is annotated with sequence clusters that are defined either by (from left-to-right) MLST, SNV threshold, hierBAPS and rPinecone as well as the presence of AMR associated gene and mutations. This included presence of a functional *erm(41)* gene conferring inducible resistance to macrolides, presence of two *rrl* mutations conferring high level macrolide resistance and the presence of mutation in *rrs* conferring high level amikacin resistance. The scale bar represents the number of single nucleotide variants and node bootstrap scores below are shown if below 75.

Supplementary Figure 2. Frequency of pairwise single nucleotide variant (SNV) distances between samples after sub-tree analysis. Figure 3A shows pairwise differences from the ST-1 subtree. Figure 3B shows pairwise differences from the ST-26 subtree. Figure 3C shows pairwise differences from the ST-23 and ST-48 subtree.