Mycobiome analyses of critically ill COVID-19 patients

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- Analysis and interpretation of data: DW, SG, MH, MJB and PB
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Abstract

Rationale: COVID-19-associated pulmonary aspergillosis (CAPA) is a life-threatening complication in patients with severe COVID-19. Previously, acute respiratory distress syndrome in patients with COVID-19 has been associated with lung fungal dysbiosis, evidenced by reduced microbial diversity and *Candida* colonisation. Increased fungal burden in the lungs of critically ill COVID-19 patients is linked to prolonged mechanical ventilation and increased mortality. However, specific mycobiome signatures associated with severe COVID-19 in the context of survival and antifungal drug prophylaxis have not yet been determined and such knowledge could have an important impact on treatment.

Objectives: To understand the composition of the respiratory mycobiome in critically ill COVID-19 patients with and without CAPA and the impact of antifungal use in patient outcome.

Methods: We performed a multi-national study of 39 COVID-19 patients in intensive care units (ICU) with and without CAPA. Respiratory mycobiome was profiled using ITS1 sequencing and *Aspergillus fumigatus* burden was further validated using qPCR. Fungal communities were investigated using alpha diversity, beta diversity, taxa predominance and taxa abundances.

Results: Respiratory mycobiomes of COVID-19 patients were dominated by *Candida* and *Aspergillus.* There was no significant association with corticosteroid use or CAPA diagnosis and respiratory fungal communities. Increased *A. fumigatus* burden was associated with mortality and, the use of azoles at ICU admission was linked with an absence of *A. fumigatus.*

Conclusions: Our findings suggest that mould-active antifungal treatment at ICU admission may be linked with reduced *A. fumigatus*-associated mortality in severe COVID-19. However, further studies are warranted on this topic.

Introduction

COVID-19 is a pulmonary disease caused by severe acute respiratory syndrome coronavirus 2. There have been over 700 million confirmed cases of COVID-19 since December 2019 with mortality ~7 million[1]. Around 5% of patients with COVID-19 require admission into the intensive care unit (ICU)[2,3] and, 50% of those patients need mechanical ventilation[4], thus increasing the risk of hospital-acquired pneumonia[5]. The pulmonary microbiome and its associations with disease outcomes in COVID-19 patients has been explored since the beginning of the pandemic[6–8]. However, our knowledge on the role of fungi in the pathophysiology of COVID-19 is limited. Specifically, the association between respiratory mycobiome composition and patient outcome, and the interplay of antifungal use, is yet to be investigated.

Mycobiome sequencing of the upper respiratory tract (nasopharyngeal swabs) suggests COVID-19 infection significantly reduces fungal diversity, with a higher abundance of *Alternaria* and *Cladosporium* spp., and a lower abundance of other taxa including *Candida* and *Aspergillus*[9]. In the lower respiratory tract (tracheal aspirates), bacterial and fungal microbiome analyses of patients with severe COVID-19 have shown changes over time that might be linked to antimicrobial pressure[10]. A variety of respiratory mycobiome clusters

were identified, including those dominated by *Candida* and *Cladosporium.* Using 18S qPCR in bronchoalveolar lavage (BAL) samples, it has been reported that critically ill COVID-19 patients with high fungal burdens are less likely to be liberated from mechanical ventilation[11]. However, the taxa responsible for this outcome remains unclear. Lastly, mycobiome sequencing of BAL found COVID-19 patients with acute respiratory distress syndrome (ARDS) to be associated with reduced fungal diversity and an increase in *Candida* colonisation[12]. In patients without *Candida* colonisation, an increased abundance of an unclassified *Ascomycota* species was identified.

COVID-19-associated pulmonary aspergillosis (CAPA) is an important complication of COVID-19, mainly described in critically ill patients. Multicentre cohort studies of CAPA conducted in the ICU setting report incidence rates varying between 10-15%[14–16]. Nevertheless, mortality rates in patients with CAPA were double that observed in critically ill COVID-19 patients without CAPA[17,18]. Airway epithelial cell damage due to viral replication and COVID-19 associated downregulation of interferon γ signalling pathway, aberrant immune responses due to ARDS, corticosteroids, azithromycin, or the use of immunomodulators have been linked with susceptibility to CAPA[19–22]. With a view to investigating the impact of the respiratory mycobiome in the outcome of COVID-19, we performed a multi-national mycobiome analysis of 39 respiratory samples from critically ill COVID-19 patients with and without CAPA.

Methods

Study design, participants, and sample collection

This study was based on a multinational retrospective study on the prevalence of invasive pulmonary aspergillosis in critically ill COVID-19 patients in ICUs during 2020[15]. Inclusion

104 criteria consisted of: PCR confirmed COVID-19 infection, bronchoscopy or tracheal aspiration
105 performed during routine clinical investigations, and chest imaging available seven days
106 before or after respiratory samples were collected. Patients less than 18 years of age were
107 excluded. Respiratory samples not passing quality control (described in Supplemental
108 methods) were also excluded. Respiratory specimens obtained at ICU admission or during
109 ICU stay were collected, aliquoted (at least 1ml) and stored at -80 °C. Criteria for defining
110 aspergillosis were according to previous guidelines[23] with the following modifications:
111 COVID-19 requiring ICU admission was included as an additional host factor, tracheal
112 aspirates were equated to BAL fluid for microbiological tests, and serum and BAL GM was
113 added as entry criterion. For a summary of full patient demographics, see Table E1.

114 IRB approval was obtained at each participating center: Medical University of Graz EC #32-
115 296 ex 19/20. University of Genoa Liguria Region Ethics Committee registry number
116 163/2020. Rennes Teaching Hospital N ° 16-117.

117 **Sample processing**

118 BAL DNA was extracted using a cetyltrimethylammonium bromide (CTAB) method[24]. Full
119 details on sample processing are provided in the online supplement. Briefly, for mycobiome
120 analysis, the ITS1 region was amplified using Nextera XT compatible versions of ITS1[25] and
121 ITS2degen (a degenerate version of ITS2 primer) (see Table 1). The presence of *A. fumigatus*
122 in respiratory samples was validated using a TaqMan probe assay targeting the ITS1 region[26].

123 **Data analysis**

124 Paired end reads were subject to quality trimming at Q30 and a minimum length filter of 75
125 nucleotides using bbduk[27] (BBMap v38.22). Primer sequences were removed using
126 Cutadapt[28] (v1.18). Reads were mapped to UNITE database using bowtie2[29] (v2.3.5.1). Count

127 data was further processed in R (v4.1.3) using the following packages: phyloseq[30] v1.38.0,
128 vegan[31] v2.5-7, DESeq2[32] v1.34.0, stringr[33] v1.4.0, ggplot2[34] v3.3.5 and tidyr[35] v1.2.0.

129 Abundances were standardised to the median sequencing depth. Extremely low abundance
130 taxa were removed by only retaining those occurring > 0.2% in any sample. DESeq2 was used
131 to identify significantly differentially abundant taxa (adjusted *p* value < 0.05 and basemean >
132 500). Differences in diversity (Shannon, Chao1 and observed OTUs) were assessed using
133 pairwise Wilcoxon rank sum tests. PERMANOVA test was used to assess differences in Bray-
134 Curtis ordination.

135

136 Results

137 **Patient cohort**

138 The respiratory mycobiome of 91 critically ill COVID-19 patients in the intensive care unit
139 (ICU) was analysed using internal transcribed spacer 1 (ITS1) amplicon sequencing of BAL.
140 Samples from 39 patients harboured significant fungal communities which passed quality
141 control (See Data analysis section in supplementary methods). Table 1 describes
142 demographic and clinical characteristics of the 39 patients maintained in the mycobiome
143 analysis, stratified by CAPA diagnosis. Six patients had CAPA (15%). Patients were from
144 Genoa, Graz and Rennes (64%, 26% and 10%, respectively). Mean age of those with and
145 without CAPA was 61 and 64, respectively. Most patients received systemic corticosteroids
146 (83% of those with CAPA and 73% of those without CAPA). No patients diagnosed with CAPA
147 received azole treatment at the time of ICU admission. Mortality at end of follow-up in
148 patients with CAPA was 33% (2/6) while mortality in patients without CAPA was also 33%
149 (11/33).

150 **Table 1.** Clinical and demographic characteristics of patients with and without probable

151 CAPA diagnosis.

Variable	Patients with CAPA (N=6)	Patients without CAPA (N=33)
Age		
Mean (SD)	61 (3.4)	64 (7.9)
valid (missing)	6 (0)	32 (1)
Sex		
male	100% (6)	79% (26)
female	0% (0)	18% (6)
missing	0% (0)	3% (1)
Ethnicity		
Caucasian	100% (6)	91% (30)
other		6.1% (2)
missing	0% (0)	3% (1)
BMI > 30		
yes	50% (3)	18% (6)
no	50% (3)	79% (26)
missing	0% (0)	3% (1)
Smoking		
yes	33% (2)	12% (4)
no	67% (4)	85% (28)
missing	0% (0)	3% (1)
Institution		
Graz	33% (2)	24% (8)
Genoa	17% (1)	73% (24)
Rennes	50% (3)	3% (1)
Hematology oncology		
yes	17% (1)	9.1% (3)
no	83% (5)	88% (29)
missing	0% (0)	3% (1)
Solid organ transplant		
yes	17% (1)	3% (1)
no	83% (5)	94% (31)
missing	0% (0)	3% (1)
Cardiovascular disease		
yes	67% (4)	45% (15)
no	33% (2)	52% (17)
missing	0% (0)	3% (1)
Pulmonary disease		
yes	33% (2)	24% (8)
no	67% (4)	73% (24)
missing	0% (0)	3% (1)
Diabetes Mellitus		

yes	17% (1)	9.1% (3)
no	83% (5)	88% (29)
missing	0% (0)	3% (1)
Corticosteroids		
yes	83% (5)	73% (24)
no	17% (1)	24% (8)
missing	0% (0)	3% (1)
Tocilizumab		
yes	17% (1)	6.1% (2)
no	83% (5)	91% (30)
missing	0% (0)	3% (1)
Azithromycin		
yes	33% (2)	33% (11)
no	67% (4)	61% (20)
missing	0% (0)	6.1% (2)
Azole treatment at ICU admission		
yes	0% (0)	15% (5)
no	100% (6)	6.1% (2)
missing	0% (0)	79% (26)
Life Support		
mechanical	100% (6)	70% (23)
ECMO		3% (1)
noninvasive		6.1% (2)
mechanical & noninvasive		12% (4)
none		6.1% (2)
missing	0% (0)	3% (1)
Duration ICU (days)		
Mean (SD)	27 (11)	32 (28)
valid (missing)	6 (0)	32 (1)
Palliative (Day 28 or 32)		
yes	67% (4)	48% (16)
no	33% (2)	48% (16)
missing	0% (0)	3% (1)
Survival (at end of follow up)		
yes	33% (2)	33% (11)
no	67% (4)	64% (21)
missing	0% (0)	3% (1)
Days from ICU admission to CAPA		
Mean (SD)	6.2 (3.5)	-
BAL GM (ODI > 1)		
positive	83% (5)	9.1% (3)
negative	17% (1)	82% (27)
missing	0% (0)	9.1% (3)

BAL PCR		
positive	50% (3)	0% (0)
negative	0% (0)	58% (19)
missing	50% (3)	42% (14)
BAL culture		
positive	50% (3)	0% (0)
negative	50% (3)	97% (32)
missing	0% (0)	3% (1)
BAL LFD		
positive	17% (1)	6.1% (2)
negative	0% (0)	9.1% (3)
missing	83% (5)	85% (28)
Tracheal Aspirate GM		
positive	0% (0)	3% (1)
negative	0% (0)	3% (1)
missing	100% (6)	94% (31)
Tracheal Aspirate PCR		
positive	33% (2)	0% (0)
negative	17% (1)	21% (7)
missing	50% (3)	79% (26)
Tracheal Aspirate Culture		
positive	17% (1)	0% (0)
negative	33% (2)	3% (1)
missing	50% (3)	97% (32)
Serum GM (> 0. 5)		
positive	33% (2)	0% (0)
negative	50% (3)	42% (14)
missing	17% (1)	58% (19)
Bronchial Aspirate culture		
positive	17% (1)	0% (0)
negative	33% (2)	21% (7)
missing	50% (3)	79% (26)

CAPA; COVID-19 associated pulmonary aspergillosis. BMI; body mass index. ICU; intensive care unit. ECMO; extracorporeal membrane oxygenation. BAL; bronchoalveolar lavage. GM; galactomannan. LFD; lateral flow device.

Candida and *Aspergillus spp.* dominate respiratory mycobiomes in critically ill COVID-19 patients

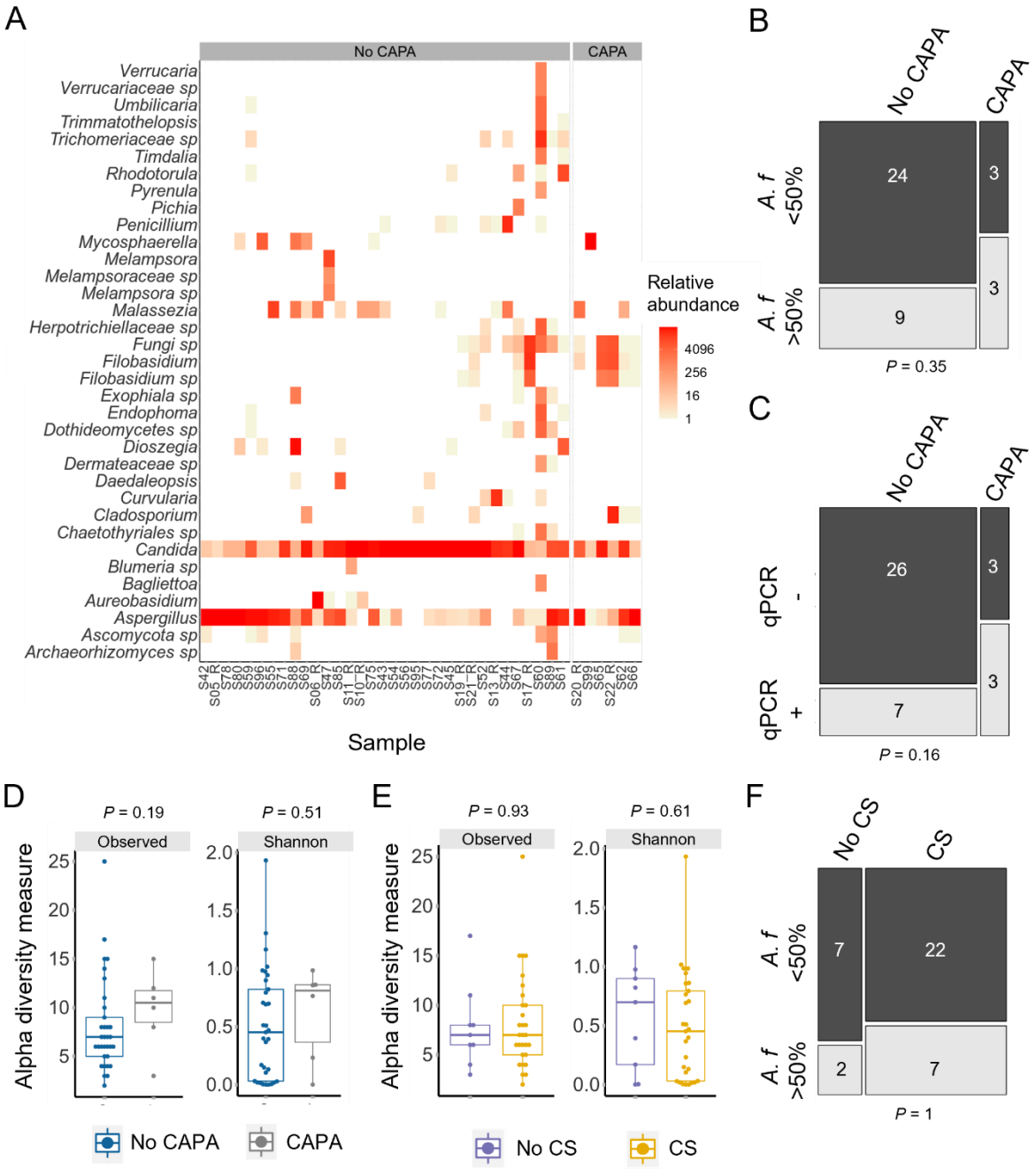
Median read counts per sample for the 39 samples that passed quality control was 57,913 (range 6,970 – 342,640). There were 36 Genera in total and a median of 5 Genera per

159 sample (range 1 – 22). There was no significant clustering between sample batches (Fig. E1),

160 suggesting that sample processing had no impact on mycobiome communities. Mycobiomes

161 predominantly consisted of *Candida* and *Aspergillus* (Fig. 1A). *Candida albicans*, *Aspergillus*

162 *fumigatus* and *Candida parapsilosis* were the most abundant species (Fig. E2A-B).



163

164 **Fig. 1. *Aspergillus* and *Candida spp.* dominate the respiratory mycobiome in critically ill COVID-19**
165 **patients.** (A) *Aspergillus* and *Candida* were the main genera observed in the lungs from COVID-19

166 patients included in the study (n = 39). Samples are grouped based on CAPA status. (B) *A.*
167 *fumigatus w*as the predominant species in the mycobiomes of 50% of patients with COVID-19
168 associated pulmonary aspergillosis (CAPA), compared to 27% of those without CAPA. (C) Fifty percent
169 of CAPA patients were *A. fumigatus*-positive by species specific qPCR, compared to 21% of those
170 without CAPA. (D) Alpha diversity measures (Observed OTUs and Shannon) trended towards higher
171 diversity in CAPA patients (E) Corticosteroid treatment caused no apparent effect on alpha diversity as
172 measured by Observed OTUs or Shannon diversity. (F) *A. fumigatus w*as the predominant species in
173 the mycobiomes of 24% of patients receiving corticosteroids, compared to 22% of those without
174 corticosteroids. Hypothesis testing was applied using Wilcoxon Rank Sum tests (D,E) or Fisher's exact
175 tests (B,C,F). CS: Corticosteroids. Boxplot data represent median and interquartile range.

176

177 **No significant correlation is found between mycobiome communities and CAPA status or**
178 **corticosteroid use**

179 In our study, higher median *A. fumigatus* levels were observed in CAPA when assessed by
180 read count (~16,700 vs. 35) and *A. fumigatus* specific qPCR (0.3 vs. 0 genome equivalents)
181 (Fig. E3A-B), but there was overlap between the patient groups and statistical significance
182 was not reached. *A. fumigatus* burden in patients without CAPA was varied, some patients
183 had little to no *A. fumigatus* and others contained particularly high burden (Fig. 1A, Fig. E3A-
184 B). As *A. fumigatus* levels appeared to be bimodal, we dichotomised these data into two
185 groups to assess either the predominance of *A. fumigatus* (present at over 50% of a
186 mycobiome sample) or high *A. fumigatus* burden (qPCR positive at over 0.1 haploid genome
187 equivalents (HGE)). Analysing the data in this manner also found no significant difference
188 between *A. fumigatus* predominance (Fig. 1B) or high burden (Fig. 1C) in patients with and
189 without CAPA. Furthermore, species differential abundance analysis did not find significant
190 differences between *A. fumigatus* levels based on CAPA status. Instead, *Cladosporium*
191 *delicatulum, Mycosphaerella tassiana* and *Filobasidium magnum* were found to be at higher
192 abundance in CAPA patients (Fig. E6). In addition, probable CAPA patients had higher median

193 α diversity, however, this difference was not significant, and no significant effect on β
194 diversity was observed (Fig. 1D, Fig. E3C).

195 Corticosteroid use resulted in lower median α diversity (Shannon only), however, this
196 difference was not significant (Fig. 1E). Median levels of *A. fumigatus* were lower in the
197 corticosteroid treated group as assessed by sequencing (8 vs. ~5,800 reads) and, qPCR (0 vs.
198 0.1 genome equivalents) (Fig.E3D-E). However, patients on corticosteroids were highly
199 heterogenous in terms of *A. fumigatus* abundance. There was no significant difference
200 between *A. fumigatus* predominance (Fig. 1F) or high burden (Fig. E3F) in patients with and
201 without corticosteroid treatment. Species differential abundance analysis found no
202 significant differences between corticosteroid usage. In addition, corticosteroid use had no
203 significant impact on β diversity (Fig. E3G).

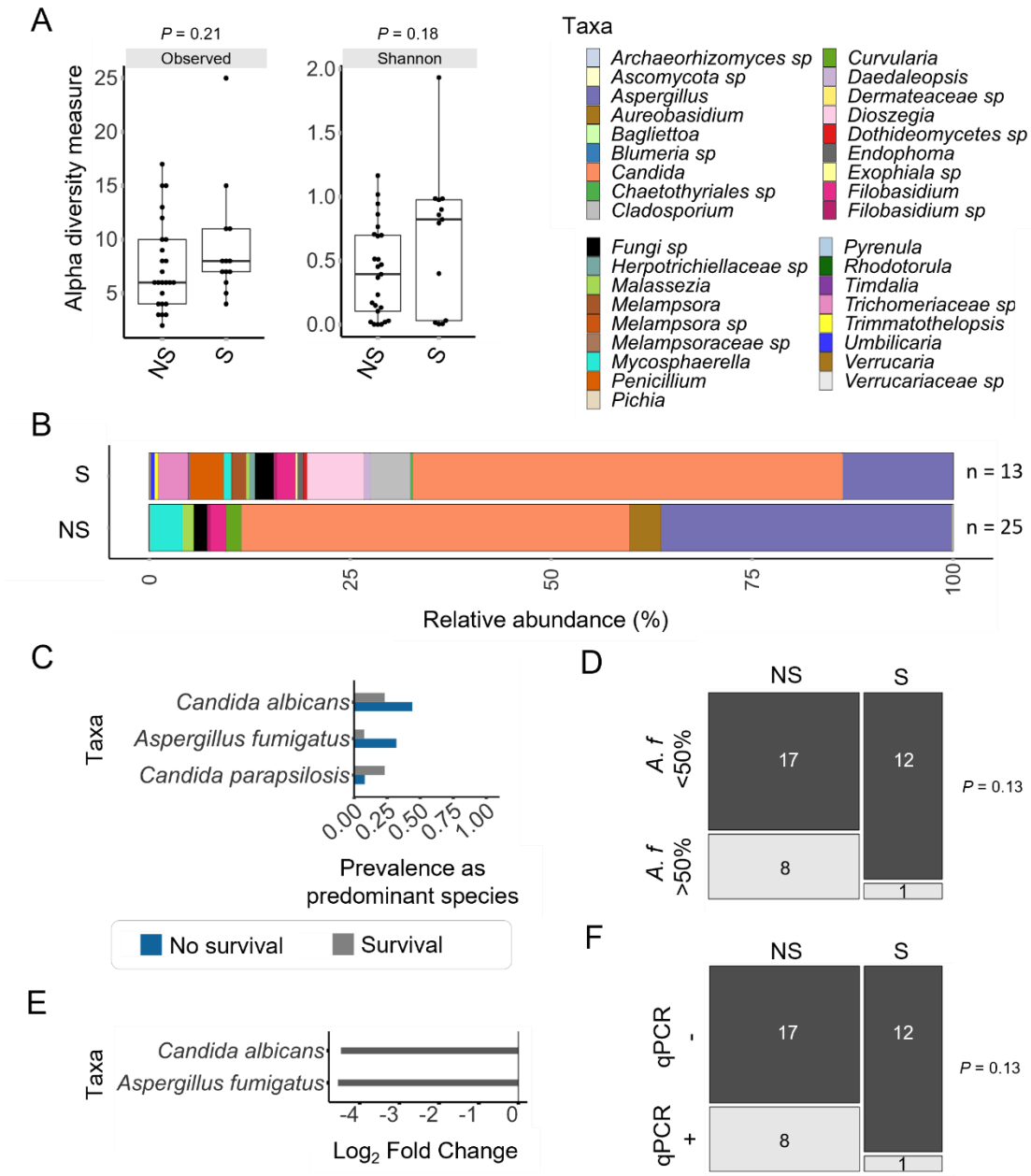
204 **Increased *A. fumigatus* burden is associated with mortality**

205 The mycobiome of surviving individuals showed a trend towards higher median α diversity
206 (Fig. 2A). Grouped mean abundances indicated a lower level of *Aspergillus* was present upon
207 survival (Fig.2B). At the individual sample level, many mycobiomes of non-surviving patients
208 were predominated by *Aspergillus* (Fig. E4). Species predominance analysis suggested that
209 this difference was due to *A. fumigatus,* with 32% (8/25) of non-surviving patients'
210 mycobiomes being dominated by this species compared to only 8% (1/13) of patients which
211 survived (Fig. 2C-D). Furthermore, mycobiome differential abundance analysis found *A.*
212 *fumigatus* and *C. albicans* to be significantly less abundant upon survival, with log fold
213 change values of -4.3 and -4.6, respectively (*padj* < 0.05) (Fig.2E). Quantitative PCR data also
214 showed 32% of patients which did not survive displayed a high burden of *A. fumigatus*
215 compared to only 8% of surviving patients (Fig. 2F). All datapoints for *A. fumigatus* relative

216 abundance and qPCR burden are shown in Fig. E5A-B. BAL galactomannan index values were

217 not significantly different between the patient groups, with an outlier in the survival group

218 having a very high index (Fig.E5C).



219

220 **Fig. 2. A higher *A. fumigatus* burden is associated with mortality in critically ill COVID-19 patients.**
221 (A) Alpha diversity measures (Observed OTUs and Shannon) trended towards higher diversity in
222 critically ill COVID-19 patients which survived. Data represent median and interquartile range. (B) At
223 the genus level, pooled relative abundance mycobiome data from patients which survived (n =13)
224 indicated a lower proportion of *Aspergillus*, and an apparent increase in the number of observed taxa
225 overall. (C) *C. albicans* and *A. fumigatus* were prevalent as the predominant species in a mycobiome

226 in more patients which did not survive (blue, prevalence 0.44 and 0.32, respectively) than those that
227 did survive (grey, prevalence 0.32 and 0.08, respectively). Taxa were counted if present at over 50% of
228 total counts, and only taxa found in at least 10% of samples of either group are shown. (D) *A.*
229 *fumigatus* was the predominant species in 8/25 (32%) patients which did not survive, compared to
230 1/13 (8%) of patients which did survive. (E) Analysis using DESeq2 identified *A. fumigatus* and *C.*
231 *albicans* to be at significantly lower abundance in patients which survived. (F) Thirty-two percent of
232 patients which did not survive were *A. fumigatus*-positive by species specific qPCR, compared to 7%
233 of those which survived. Hypothesis testing in was applied using Wilcoxon Rank Sum tests (A),
234 Fisher's exact tests (D,F), or DESeq2 (E). S: Survival; NS: No survival.

235

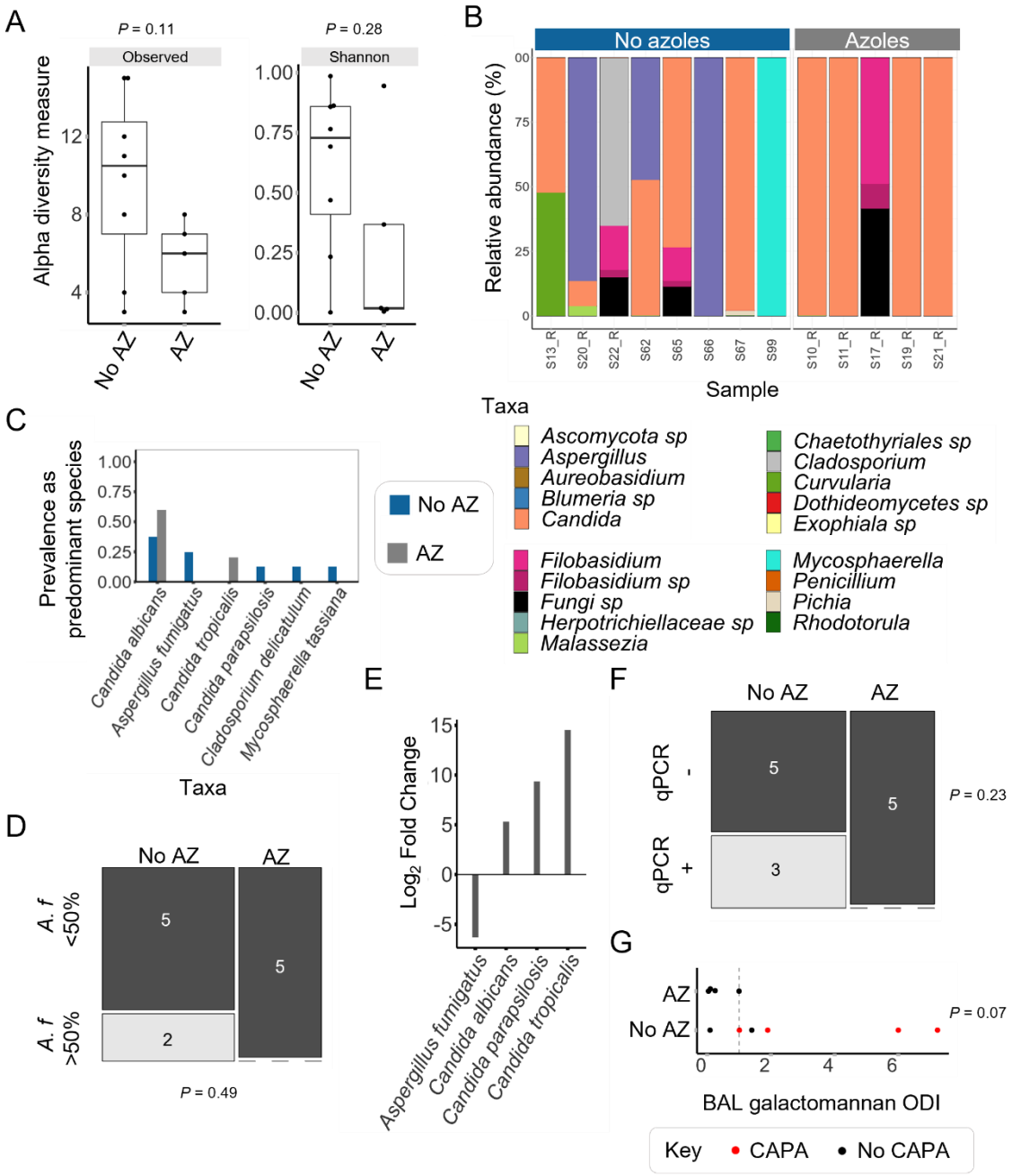
236 **Azole treatment at intensive care unit admission is associated with reduced *A. fumigatus***

237 **burden in critically ill COVID-19 patients and COVID-19 survival**

238 Use of azole treatment at ICU admission in critically ill COVID-19 patients resulted in
239 significantly reduced α diversity when analysing raw mycobiome data. Upon removal of very
240 rare taxa, there was a trend towards reduced median α diversity upon azole treatment (Fig.
241 3A). There was a lack of *Aspergillus* in the mycobiomes of patients with azole treatment, and
242 *Aspergillus* was present at a considerable relative abundance in ~38% (3/8) patients
243 receiving azoles (Fig. 3B). At the species level, *A. fumigatus* was the predominant species in
244 25% patients without azole treatment, whereas this species was not detectable in patients
245 receiving treatment (Fig. 3C-D). Furthermore, differential abundance analysis of mycobiome
246 data found a significant reduction of *A. fumigatus* in patients which received azole treatment
247 (LFC -6.3, *padj* 0.04) (Fig. 3E). This analysis also found *Candida albicans, Candida parapsilosis*
248 and *Candida tropicalis* had significantly higher abundance in COVID-19 patients receiving
249 azole treatment (LFC 5.3, 9.4 and 14.5, respectively). Quantitative PCR (qPCR) data found
250 38% of patients which did not receive azoles displayed a high burden of *A. fumigatus*
251 compared to no patients on azole treatment (Fig. 2F). Datapoints for *A. fumigatus* relative
252 abundance and qPCR burden with and without azole treatment at ICU admission are shown
253 in Fig. E5D-E. BAL galactomannan index was not statistically different between patients with

254 or without azole treatment, however, all individuals receiving treatment were GM negative

255 whereas only half of the patients without treatment were GM negative (Fig. 3G).



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257 **Fig. 3. *A. fumigatus* is associated with the absence of azole treatment at intensive care unit**

258 **admission in critically ill COVID-19 patients.** (A) Alpha diversity measures (Observed OTUs and

259 Shannon) trended higher diversity in COVID-19 patients without azoles at ICU admission (AZ). (B) At

260 the genus level, BAL ITS1 mycobiomes from COVID-19 patients which received azoles (n =5) display an

261 absence of *Aspergillus,* whereas 3/8 patients not receiving azoles harboured *Aspergillus.* (C) *C.*

262 *albicans* was prevalent as the predominant species of a mycobiome in more patients which received

263 azoles (grey, prevalence 0.6) than those which did not receive azoles (blue, prevalence 0.38). *A.*
264 *fumigatus* was prevalent as the predominant species in 25% of patients receiving azoles. *A. fumigatus*
265 was not prevalent in any patients which received azoles. Taxa were counted if present at over 50% of
266 total counts, and only taxa found in at least 10% of samples of either group are shown. (D) *A.*
267 *fumigatus* was the predominant species in 2/8 (25%) patients which did not survive, compared to
268 none of the patients which did survive. (E) Analysis using DESeq2 identified *A. fumigatus* to be at
269 significantly lower abundance in patients which received azoles. *C. albicans, C. parapsilosis* and C.
270 *tropicalis* were all at significantly higher abundance in patients receiving azoles. (F) Thirty eight
271 percent (3/8) of patients which did not receive azoles were *A. fumigatus*-positive by species specific
272 qPCR, compared to no patients which did receive azoles. (G) All patients receiving azoles were BAL
273 galactomannan negative (ODI 1 or lower). Two thirds (4/6) of patients not receiving antifungals were
274 galactomannan positive. Hypothesis testing in was applied using Wilcoxon Rank Sum tests (A, G),
275 Fisher's exact tests (D,F), or DESeq2 (E). AZ: Azoles; No AZ: No azoles; ODI: Optical density index.

276 Our findings suggest an association between *A. fumigatus* abundance and mortality in
277 critically ill COVID-19 patients, and that azole treatment at ICU significantly reduces *A.*
278 *fumigatus* levels. Therefore, we combined these factors to assess the association between *A.*
279 *fumigatus* and survival outcomes depending on the presence or absence of azole treatment.
280 It was apparent that *Aspergillus* was associated with mortality only in COVID-19 patients
281 who had not received azole treatment (Fig. 4A). Presence of *A. fumigatus* in only those
282 patients that did not survive or receive azole treatment was confirmed by qPCR (Fig. 4B).
283 Therefore, these findings suggest azole treatment at ICU admission may have been
284 protective against *A. fumigatus*-associated mortality in this severe COVID-19 patient cohort.

285 **Fig. 4. *A. fumigatus* is associated with mortality in patients with COVID-19 who have not received**
286 **azole treatment at intensive care unit admission.** (A) When combining the use of azoles at ICU and
287 survival outcomes, *Aspergillus* was found in the BAL ITS1 mycobiomes from 50% of patients which did
288 not receive azoles or survive (3/6). No *Aspergillus* was present in samples from any other groups. (B)
289 *A. fumigatus* levels (measured by qPCR) did not differ significantly between groups (Wilcoxon Rank
290 Sum test); however, *A. fumigatus* burden was observed only in the patient group which did not
291 survive or receive azole treatment at ICU admission. AZ: Azoles. S: Survival. NS: No survival.

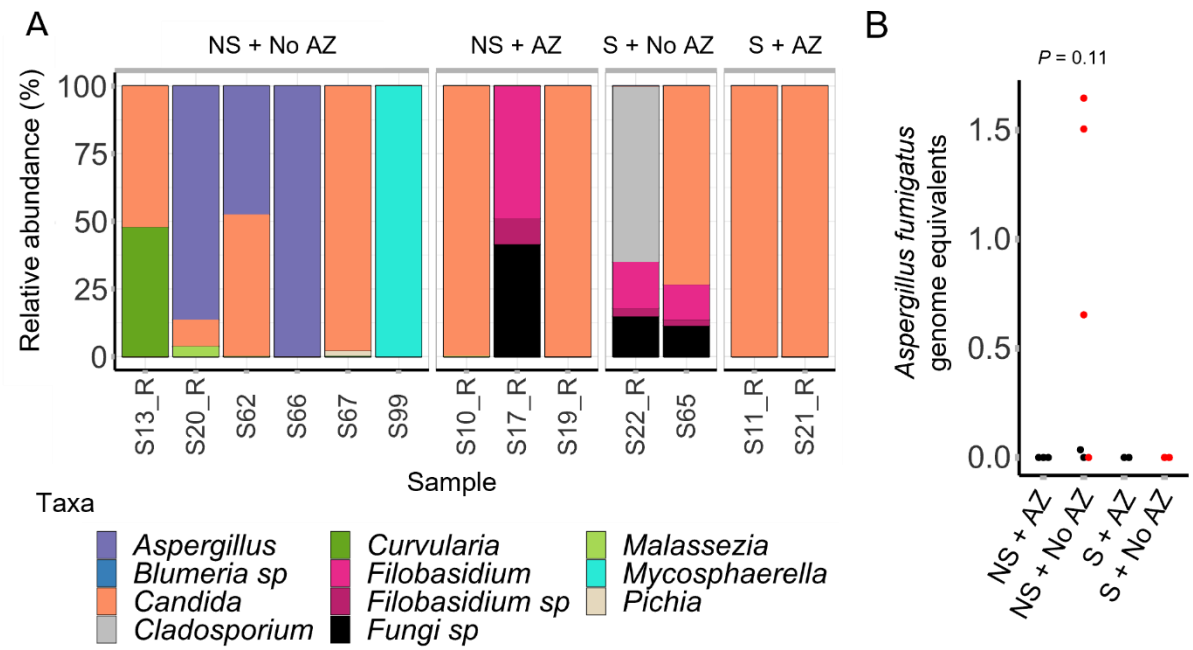

Fig. 4. *A. fumigatus* is associated with mortality in patients with COVID-19 who have not received azole treatment at intensive care unit admission. (A) When combining the use of azoles at ICU and survival outcomes, *Aspergillus* was found in the BAL ITS1 mycobiomes from 50% of patients which did not receive azoles or survive (3/6). No *Aspergillus* was present in samples from any other groups. (B) *A. fumigatus* levels (measured by qPCR) did not differ significantly between groups (Wilcoxon Rank Sum test); however, *A. fumigatus* burden was observed only in the patient group which did not survive or receive azole treatment at ICU admission. AZ: Azoles. S: Survival. NS: No survival.

Discussion

This multinational study found higher *Aspergillus fumigatus* levels in critically ill COVID-19 patients were associated with increased mortality. In addition, the association of *A. fumigatus* with mortality was found only in patients who did not receive azole treatment at ICU admission, suggesting that the use of prophylactic mould-active antifungals in severe COVID-19 patients is potentially valuable for the reduction of *A. fumigatus*-associated mortality in this cohort.

309 The respiratory mycobiomes of critically ill COVID-19 patients described here were
310 dominated by *Candida* and *Aspergillus.* Previous studies using similar patient groups have
311 also reported lung fungal communities to be dominated by *Candida*[10,12]*.* Furthermore, one
312 study identified a significant increase in unidentified Ascomycota spp. in patients without
313 *Candida* colonisation[12]. Due to the reported incidence of CAPA in severe COVID-19, the
314 authors hypothesised that *Aspergillus* could be present in these patients and, although
315 mycobiome samples were mostly negative for *Aspergillus*, the presence of *Aspergillus* was
316 confirmed by PCR in follow up BAL samples in over 20% of patients.

317 A recent report suggests higher fungal burden in the lung microbiota of patients with
318 proven/probable CAPA[11]. However, considerable overlap in fungal burdens between those
319 with and without CAPA was noted. Our study found CAPA patients had higher median levels
320 of *A. fumigatus* and trended towards higher fungal diversity. However, these findings did not
321 meet statistical significance, which may have been driven by the low sample size (n = 6 in
322 CAPA group). Some patients within the non-CAPA group harboured considerable levels of *A.*
323 *fumigatus.* It is known that high *Aspergillus* burdens can be found in the lungs of healthy
324 individuals[36]. These observations suggest that if a sufficient level of *Aspergillus* is present in
325 the lung, other factors such as disease susceptibility or strain virulence in the context of CAPA
326 may be more important than burden in the outcome of infection. This study was limited to
327 one sample time point, and it would be interesting to assess how *Aspergillus* burden changes
328 during CAPA or COVID-19 infection.

329 It has been suggested that corticosteroid treatment increases lung fungal burden
330 (particularly *A. fumigatus*)[13] and lowers Shannon diversity[37] in asthma. In contrast, no
331 significant differences were found between mycobiome diversity or taxa abundance in

332 respiratory fungal communities of COPD patients with or without inhaled corticosteroid
333 treatment[38]. Another recent study found that alterations in the airway mycobiome in COPD
334 were not significantly affected by corticosteroid use[39]. Large cohort studies suggest systemic
335 corticosteroids are a risk factor for CAPA[16,19], however, there are no specific reports of the
336 influence corticosteroid use has on lung fungal communities in COVID-19. In our study,
337 patients receiving corticosteroids displayed lower median levels of *A. fumigatus* and lower
338 fungal diversity compared to those not receiving corticosteroids. However, these differences
339 did not meet statistical significance. As most (~74%) patients received corticosteroids in this
340 cohort, this may have contributed to low power in these statistical comparisons, warranting
341 further data on this topic.

342 A high fungal burden has previously been associated with a lower likelihood of release from
343 mechanical ventilation and increased mortality risk in severe COVID-19 patients[11]. However,
344 as this study utilised pan-fungal qPCR to identify burden, there was no indication of the
345 specific fungal taxa responsible for this association. Our findings suggest that higher levels of
346 *A. fumigatus* are associated with increased mortality in severe COVID-19. There are no
347 previous reports on the impact of antifungal use on the respiratory mycobiome in COVID-19
348 patients. In this study, the use of azoles at ICU admission was associated with an absence of
349 *A. fumigatus* and appeared protective against *A. fumigatus*-associated mortality.

350 Our study investigated the composition of respiratory fungal communities in critically ill
351 COVID-19 patients with and without CAPA. *Candida* and *Aspergillus* were predominant in the
352 respiratory communities. CAPA diagnosis was associated with higher median *A. fumigatus*
353 level and fungal diversity, and a higher prevalence of *A. fumigatus* was associated with
354 mortality and a lack of azole treatment at ICU admission. Our data suggests that the

355 potential use of prophylactic antifungals (with anti-*Aspergillus* activity) in seriously ill COVID-
356 19 patients is worthy of further consideration for the possible prevention of *A. fumigatus*-
357 associated mortality. However, a limitation of this study is the small number of patients
358 included, particularly the low number of CAPA cases. In addition, incomplete clinical data
359 with respect to azole use reduced the sample sizes for this comparison, which may have
360 resulted in low power for these statistical tests. Therefore, study of a larger cohort would be
361 valuable to improve our understanding of the association between prophylactic azole use,
362 the presence of *A. fumigatus* in the respiratory mycobiome and patient outcome in COVID-
363 19 critical care.

364

365 Conflicts of Interest

366

367 MH received research funding from Gilead, Astellas, MSD, Euroimmune, IMMY, Scynexis,
368 Pulmocide, F2G and Pfizer, outside the submitted work.

369 MJB is a former employee and has previously received research funding from F2G outside
370 the submitted work.

371 In the past 5 years SG has received speaker fees from Gilead Sciences and research grant
372 support from Pfizer outside of the submitted work.

373 Outside the submitted work, DRG reports investigator-initiated grants from Pfizer, Shionogi,
374 and Gilead Italia and speaker and/or advisor fees from Pfizer and Tillotts Pharma.

375 Outside the submitted work, MB reports research grants and/or personal fees for
376 advisor/consultant and/or speaker/chairman from Bayer, BioMérieux, Cidara, Cipla, Gilead,
377 Menarini, MSD, Pfizer, and Shionogi. In the past 5 years JPG has received speaker fees from

Gilead Sciences, MundiPharma, Pfizer, and Shionogi outside of the submitted work. In the past 5 years HG has received speaker fees from Gilead Sciences. JP has received speakers' fees from Gilead Sciences, Pfizer, Swedish Orphan Biovitrum, Associated of Cape Cod outside of the submitted work, served at advisor boards Gilead Sciences and Pfizer, and holds stocks of NovoNordisk and AbbVie Inc. All remaining authors declare no competing interests.

Acknowledgements

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484

485

486 Supplementary data

487 Raw sequence data has been deposited at the NCBI sequence read archive (SRA) under accession
488 number PRJNA905224. Code used for analysis is available at
489 https://github.com/Danweaver1/COVID_respiratory_mycobiome.

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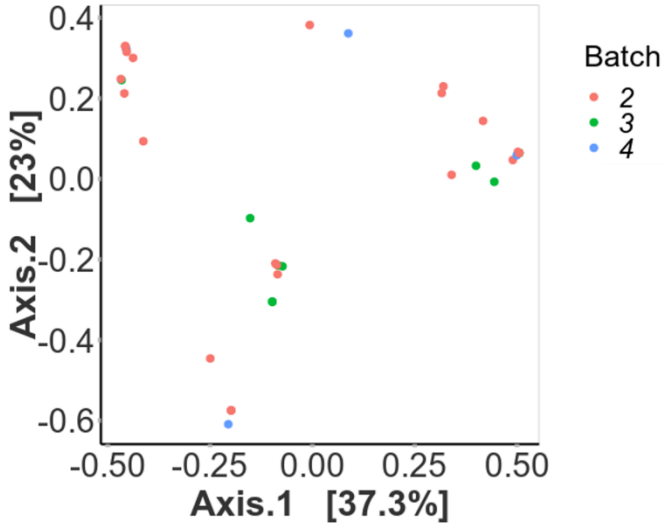


Fig. E1. Mycobiome Bray-Curtis ordination by sample batch

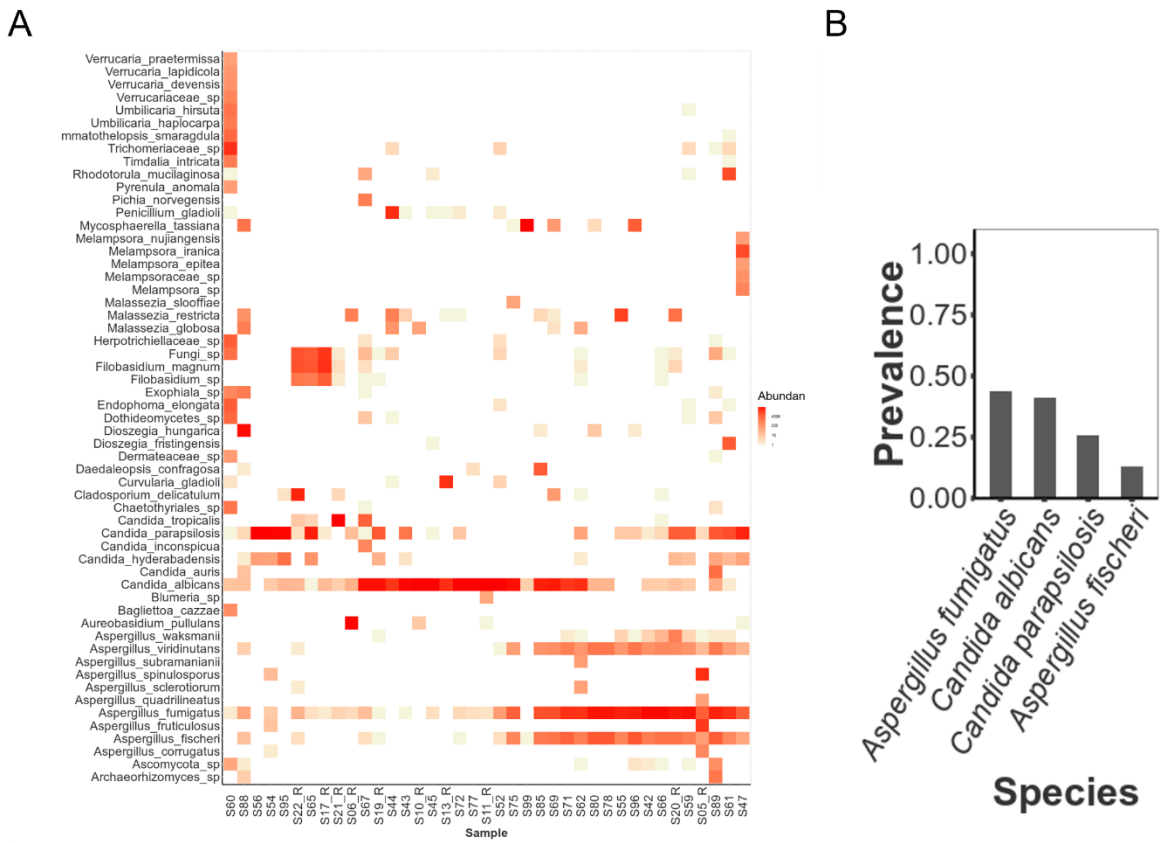
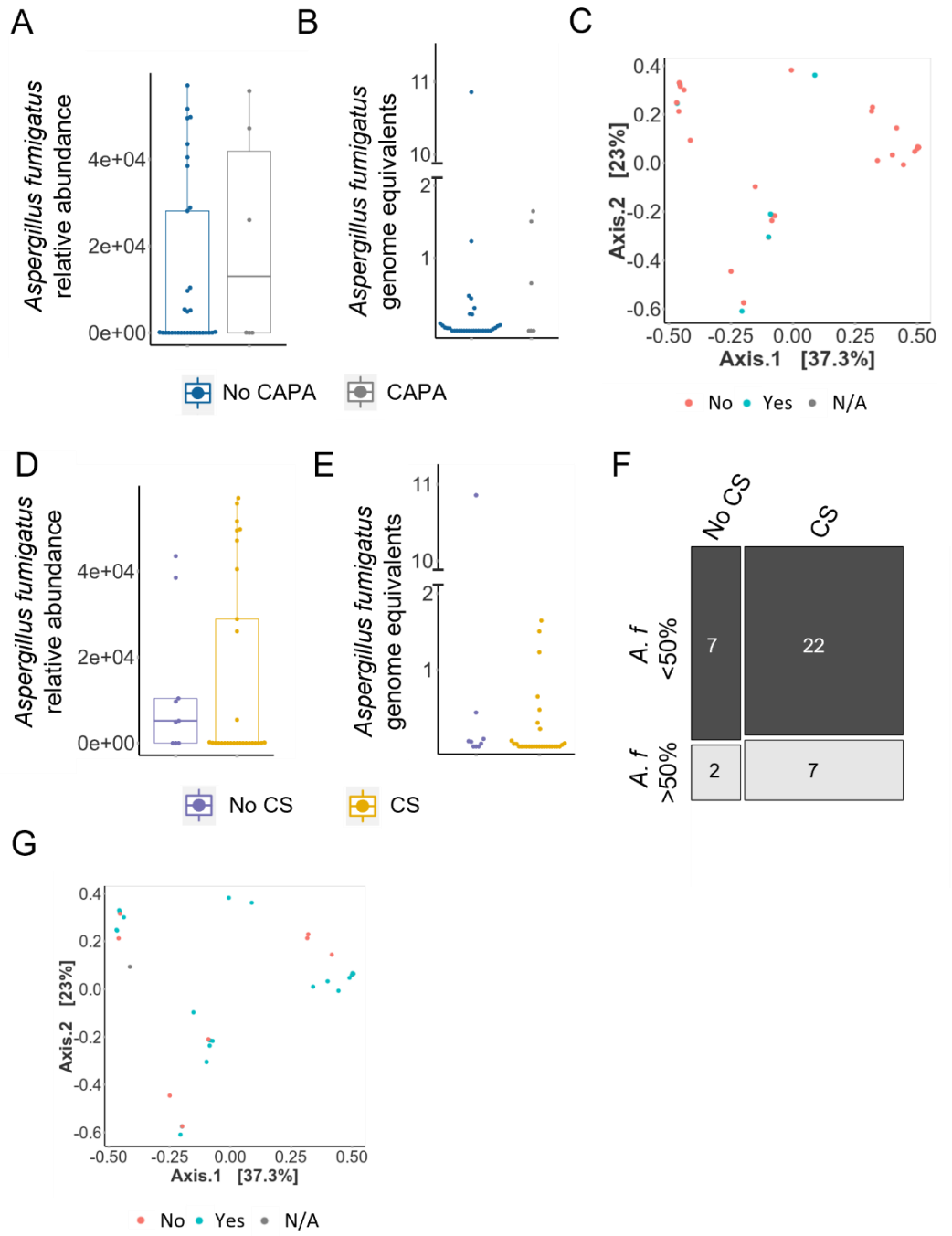


Fig. E2. ITS1 respiratory mycobiomes at the species level. (A) Heatmap of fungal species identified in all samples. To remove extremely rare taxa, only those present at > 0.2% in one sample were retained. (B) *A. fumigatus* and *C. albicans* are the most prevalent species. Taxa were counted if

496 present at over 5% in a sample, and only taxa found in at least 10% of samples of either group are
497 shown.



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499 Fig. E3. CAPA diagnosis and corticosteroid use had no significant impact on fungal burden, *Aspergillus*
500 *fumigatus* levels or beta diversity. (A) Relative abundance of *A. fumigatus* in mycobiomes of those
501 with and without CAPA. (B) *A. fumigatus* levels in those with and without CAPA measured by qPCR.
502 (C) Beta diversity and CAPA status. (D) Relative abundance of *A. fumigatus* in mycobiomes of those
503 with and without corticosteroid use. (E) *A. fumigatus* levels in those with and without corticosteroid
504 use as measured by qPCR. (F) Dichotomised data for samples positive/negative for high *A. fumigatus*
505 burden by qPCR in patients with or without corticosteroids. (G) Beta diversity and corticosteroid use.

506 Boxplot data represent median and interquartile range. CS; corticosteroid use. CAPA; COVID-
507 associated pulmonary aspergillosis.

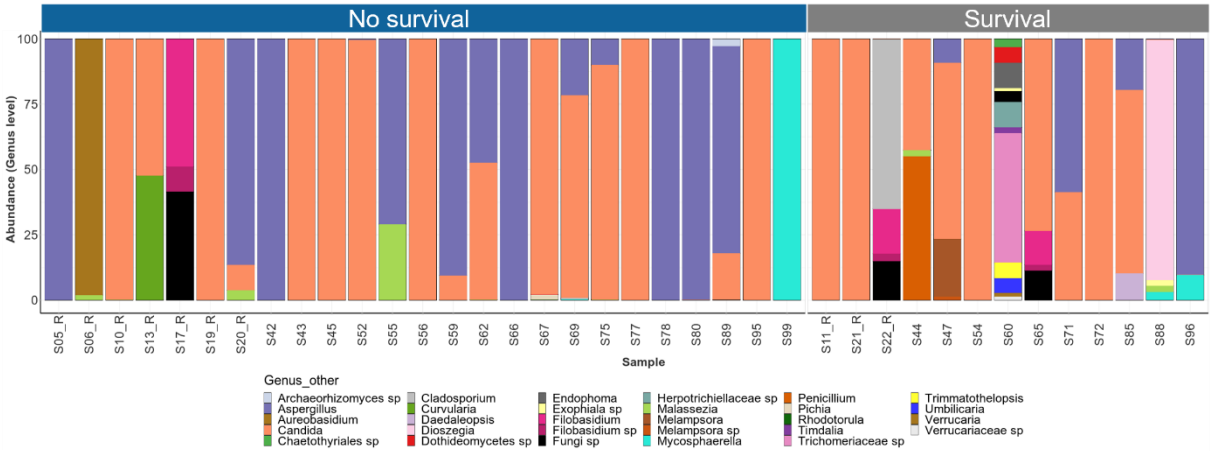

Fig. E4. Sample data for ITS1 mycobiomes grouped by survival outcome. Relative abundance of fungal Genera identified in each sample grouped by survival outcome. Taxa present below 1% were removed prior to visualisation.

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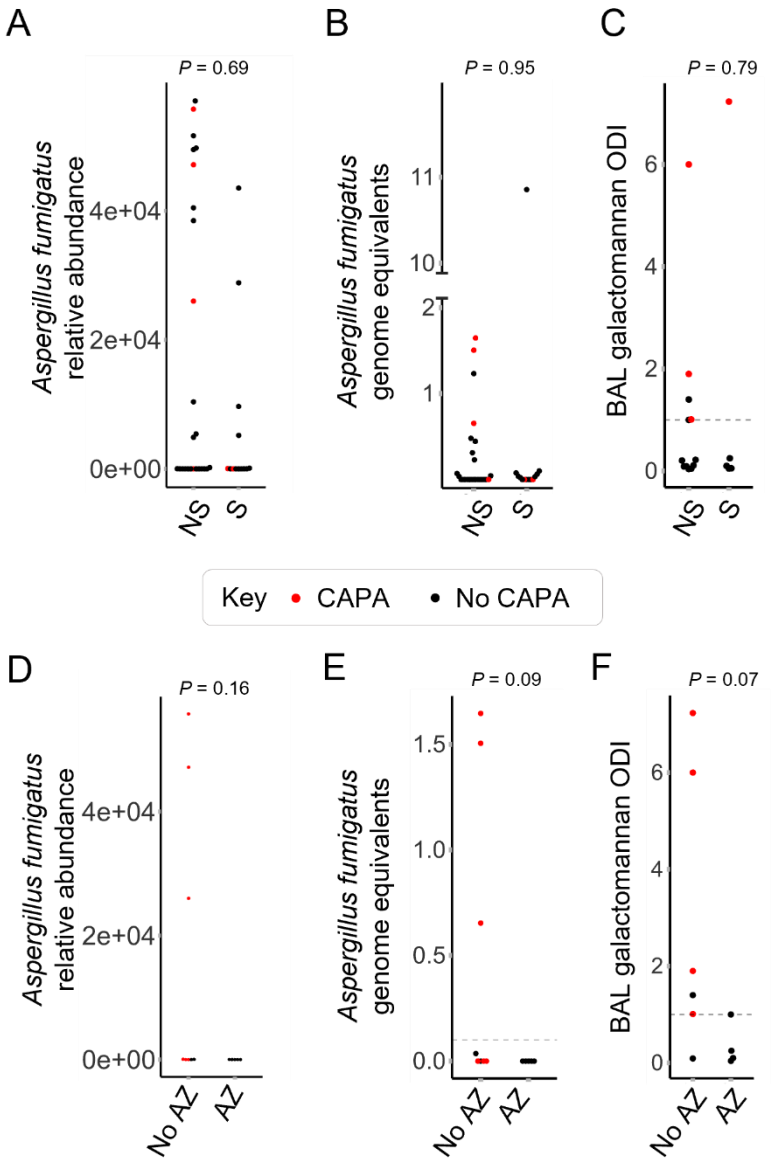

Fig. E5. Extended data for *Aspergillus fumigatus* levels in respiratory mycobiomes and BAL galactomannan. (A) *Aspergillus fumigatus* relative abundance in patients grouped by survival outcome. (B) *A. fumigatus* burden measured by qPCR in patients which did not survive appeared biomodal, with some patients exhibiting a high burden (>0.1 genome equivalents). Excluding one outlier with an extremely high burden, all patients which survived displayed a low burden of 0.1 or below. (C) BAL galactomannan levels of patients grouped by survival outcome. (D) *Aspergillus fumigatus* relative abundance in patients grouped by azole treatment at ICU admission. (E) *A. fumigatus* burden measured by qPCR in patients which did or did not receive azole treatment at ICU admission. (F) All patients receiving azole treatment were BAL galactomannan negative (1 or lower). Half of patients not receiving azole treatment were galactomannan positive. CAPA: COVID-associated pulmonary aspergillosis. AZ: Azole treatment; No AZ: No azole treatment; S: Survival; NS: No survival.

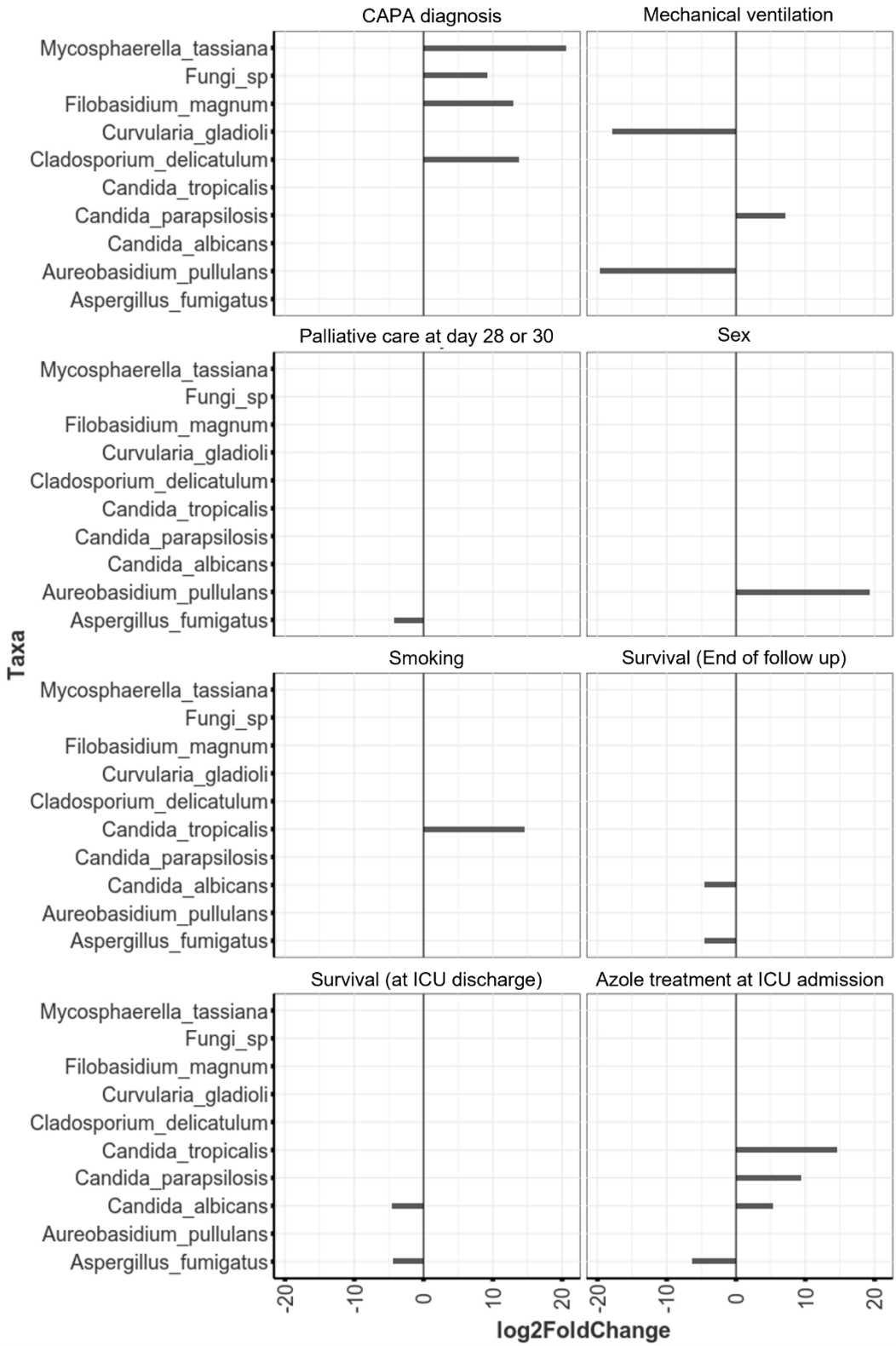

Fig. E6. Significantly differentially abundant taxa identified by DESeq2 analysis.

Table E1. Full clinical patient demographics

Characteristics	N=39
Age	
Mean (SD)	64 (7.4)
valid (missing)	38 (1)
Sex	
male	82% (32)
female	15% (6)
missing	2.6% (1)
Ethnicity	
Caucasian	92% (36)
other	5.1% (2)
missing	2.6% (1)
BMI > 30	
yes	23% (9)
no	74% (29)
missing	2.6% (1)
Smoking	
yes	15% (6)
no	82% (32)
missing	2.6% (1)
Institution	
Graz	26% (10)
Genoa	62% (24)
Rennes	13% (5)
Hematology oncology	
yes	10% (4)
no	87% (34)

missing	2.6% (1)
Solid organ transplant	
yes	5.1% (2)
no	92% (36)
missing	2.6% (1)
Cardiovascular disease	
yes	49% (19)
no	49% (19)
missing	2.6% (1)
Pulmonary disease	
yes	26% (10)
no	72% (28)
missing	2.6% (1)
Diabetes Mellitus	
yes	10% (4)
no	87% (34)
missing	2.6% (1)
Corticosteroids	
yes	74% (29)
no	23% (9)
missing	2.6% (1)
Tocilizumab	
yes	7.7% (3)
no	90% (35)
missing	2.6% (1)
Azithromycin	
yes	33% (13)
no	62% (24)
missing	5.1% (2)

Azole treatment at ICU admission	
yes	13% (5)
no	21% (8)
missing	67% (26)
Life Support	
mechanical	74% (29)
ECMO	2.6% (1)
noninvasive	5.1% (2)
mechanical & noninvasive	10% (4)
none	5.1% (2)
missing	2.6% (1)
Duration ICU (days)	
Mean (SD)	31 (26)
valid (missing)	38 (1)
Palliative (Day 28 or 32)	
yes	51% (20)
no	46% (18)
missing	2.6% (1)
Survival (at end of follow up)	
yes	33% (13)
no	64% (25)
missing	2.6% (1)
Antifungal Treatment Outcome	
yes	5.1% (2)
no	5.1% (2)
partial	0% (0)
missing	90% (35)
Primary Treatment	
yes	5.1% (2)

no	0% (0)
missing	95% (37)
Antifungals initiated for CAPA	
yes	18% (7)
no	0% (0)
missing	82% (32)
Antifungal initiated	
Posa Isa	5.2% (2)
Isav	0% (0)
IsavCasp	2.6% (1)
LAmb	0% (0)
Voriconazole	10% (4)
missing	82% (32)
BAL GM (ODI > 1)	
positive	21% (8)
negative	72% (28)
missing	7.7% (3)
BAL LFD	
positive	7.7% (3)
negative	7.7% (3)
missing	85% (33)
BAL PCR	
positive	7.7% (3)
negative	49% (19)
missing	44% (17)
BAL culture	
positive	7.7% (3)
negative	90% (35)
missing	2.6% (1)

Bronchial Aspirate culture	
positive	2.6% (1)
negative	23% (9)
missing	74% (29)
Tracheal Aspirate culture	
Positive	2.6% (1)
negative	7.7% (3)
missing	90% (35)
Tracheal Aspirate GM	
positive	2.6% (1)
negative	2.6% (1)
missing	95% (37)
Tracheal Aspirate PCR	
positive	5.1% (2)
negative	21% (8)
missing	74% (29)
Serum GM (> 0.5)	
positive	5.1% (2)
negative	44% (17)
missing	51% (20)

Table E2. Full results for significantly differentially abundant taxa determined by DESeq2 analysis when assessing CAPA diagnosis, survival, and azole treatment at ICU admission.

Variable	Taxa	base-Mean	log2FoldChange	lfcSE	stat	p	Adjusted p
CAPA diagnosis	Cladosporium delicatulum	4389.2	13.71	4.00	3.42	0.000619	0.003719
	Filobasidium magnum	1165.8	12.93	3.13	4.13	3.59E-05	0.000368
	Fungi sp	1017.7	9.18	2.62	3.51	0.000456	0.003719
	Mycosphaerella tassiana	312608.4	20.62	4.01	5.15	2.65E-07	3.62E-06
Survival (at end of follow up)	Aspergillus fumigatus	8266.3	-4.55	1.30	-3.50	0.000462	0.017097
	Candida albicans	83190.1	-4.48	1.42	-3.15	0.001611	0.029809
Azole treat-ment at ICU admission	Aspergillus fumigatus	943.1	-6.33	2.43	-2.6036	0.009225	0.042435
	Candida albicans	66157.1	5.32	2.13	2.499698	0.01243	0.047648
	Candida parapsilosis	8112.9	9.35	2.68	3.486701	0.000489	0.003749
	Candida tropicalis	51931.1	14.52	2.99	4.85916	1.18E-06	1.36E-05

Supplementary methods

DNA extraction

BAL DNA was extracted using a cetyltrimethylammonium bromide (CTAB) method[24]. Negative extraction controls (NECs) consisting of molecular grade Tris-EDTA (TE) buffer (Promega) were included with each sample batch. First, 500 µl samples were incubated with 0.1% dithiothreitol (Thermo Scientific) at 37 °C for 45 minutes. To lyse fungal cells, CTAB DNA Extraction Buffer (Generon Ltd) was added and a short mechanical disruption (2 x 20 seconds FastPrep-24) using glass beads (425-600µM, Sigma) was performed, followed by 3 cycles of alternated heating to 65 °C and gentle vortexing for 10 mins each. Cell lysates were

549 treated with 4 µl RNase A (100 mg/ml, Sigma) for 30 mins prior to DNA isolation using 1:1
550 ratio of Phenol-Chloroform (25:24:1, Merck) and 1:1 Chloroform:isoamyl alcohol (24:1,
551 VWR). DNA was precipitated at -20 °C overnight using 2 volumes of ice-cold 100% ethanol
552 and 0.1 volumes of 3M sodium acetate pH 4.8. Pelleted DNA was washed in 100% ethanol
553 and twice in 70% ethanol. Final DNA was suspended in 50 µl TE buffer.

554 **qPCR**

555 The presence of *A. fumigatus* in respiratory samples was validated using a TaqMan probe
556 assay targeting the ITS1 region[26]. Assays were performed on an Applied Biosystems 7500
557 fast system using TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific). Standard
558 curves were a nine sample 10-fold dilution series beginning at 100 ng *A. fumigatus* genomes
559 per reaction.

560 **PCR & Sequencing**

561 The ITS1 region was amplified using Nextera XT compatible versions of ITS1[25] and ITS2degen
562 (a degenerate version of ITS2 primer) (see Table 1). PCR setup used Phusion Green Hot Start
563 II HF PCR master mix to create 25 µl reactions with 300 nM forward primer, 1.2 µM reverse
564 primer and 2 µl DNA. Parameters included annealing temperature of 55 °C for 40 cycles.
565 PCRs included positive (*Aspergillus niger* genomic DNA), negative (molecular grade water)
566 and negative extraction controls (NECs) from DNA extractions. Controls were processed fully
567 and sequenced alongside the BAL samples. For sequencing library preparation, samples were
568 processed as per Illumina fungal metagenomic demonstrated protocol (Starting at 'Clean up'
569 section) using Nextera XT index kit v2 (Illumina). However, this protocol was modified to
570 include sample pooling prior to the second clean up. Libraries were sequenced 2x150 on an
571 Illumina iSeq100, with a final library loading concentration of 50 pM.

572 **Data analysis**

573 Paired end reads were subject to quality trimming at Q30 and a minimum length filter of 75
574 nucleotides using bbduk[27] (BBMap v38.22). Primer sequences were removed using
575 Cutadapt[28] (v1.18). Reads were mapped to UNITE database using bowtie2[29] (v2.3.5.1). Count
576 data was further processed in R (v4.1.3) using the following packages: phyloseq[30] v1.38.0,
577 vegan[31] v2.5-7, DESeq2[32] v1.34.0, stringr[40] v1.4.0, ggplot2[41] v3.3.5 and tidyr[35] v1.2.0.

578 To minimise contamination in the mycobiome data, samples were subject to a multi-staged
579 quality control process using NEC samples, along with qPCR and sequencing data. Firstly,
580 samples from a DNA extraction batch with *A. fumigatus* detected (Ct <40) in the
581 corresponding NEC by qPCR were excluded. Secondly, samples were excluded if they
582 produced less than 500 fungal reads (read count filter) and/or a lower number of fungal
583 reads than the corresponding NEC sample (NEC count filter). Of 99 initial samples, 22 were
584 removed due to contamination of an extraction batch, 35 samples did not pass the read
585 count filter and 3 did not pass the NEC count filter.

586 Abundances were standardised to the median sequencing depth. Extremely low abundance
587 taxa were removed by only retaining those occurring > 0.2% in any sample. DESeq2 was used
588 to identify significantly differentially abundant taxa (adjusted p value < 0.05 and basemean >
589 500). Differences in diversity (Shannon and observed OTUs) were assessed using pairwise
590 Wilcoxon rank sum tests. PERMANOVA test was used to assess differences in Bray-Curtis
591 ordination. A mycobiome sample was assigned as containing predominantly *A. fumigatus* if
592 its *A. fumigatus* read counts were higher than 50% of the median sequencing depth. To
593 determine mycobiome samples positive for high *A. fumigatus* burden by validatory qPCR,
594 the cutoff was 0.1 haploid genome equivalents (HGE). The resulting contingency tables for

595 sequencing and qPCR data were visualised as mosaic plots and statistical significance tested

596 using Fisher's exact tests.

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