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-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, the impact of antifungal use and its role 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 prevalence and taxa abundances.

Results: Respiratory mycobiomes 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. The use of antifungals at ICU admission was associated with an absence of *A. fumigatus.*

Conclusions: Our findings suggest that systemic antifungal treatment at ICU admission may be protective against *A. fumigatus*-associated mortality in CAPA.

58

59 Introduction

60 COVID-19 is a pulmonary disease caused by severe acute respiratory syndrome coronavirus 2.
61 There have been over 500 million confirmed cases of COVID-19 since December 2019 with
62 mortality > 6 million[1]. Around 5% of patients with COVID-19 require admission into the
63 intensive care unit (ICU)[2,3] and, 50% of those patients need mechanical ventilation[4], thus
64 increasing the risk of hospital-acquired pneumonia[5]. The pulmonary microbiome and its
65 associations with disease outcomes in COVID-19 patients has been explored since the
66 beginning of the pandemic[6–8]. However, our knowledge on the role of fungi in the
67 pathophysiology of COVID-19 is limited. Specifically, the association between respiratory
68 mycobiome composition and patient outcome, and the interplay of antifungal use, is yet to be
69 investigated.

70 Mycobiome sequencing of the upper respiratory tract (nasopharyngeal swabs) suggests
71 COVID-19 infection significantly reduces fungal diversity, with a higher abundance of
72 *Alternaria* and *Cladosporium* spp.*,* and a lower abundance of other taxa including *Candida* and
73 *Aspergillus*[9]*.* In the lower respiratory tract (tracheal aspirates), bacterial and fungal
74 microbiome analyses of patients with severe COVID-19 have shown changes over time that
75 might be linked to antimicrobial pressure[10]. A variety of respiratory mycobiome clusters were
76 identified, including those dominated by *Candida* and *Cladosporium.* Using 18S qPCR in
77 bronchoalveolar lavage (BAL) samples it has been reported that critically ill COVID-19 patients
78 with high fungal burdens are less likely to be liberated from mechanical ventilation[11].
79 However, the taxa responsible for this outcome remains unclear. Lastly, mycobiome
80 sequencing of BAL found COVID-19 patients with acute respiratory distress syndrome (ARDS)

81 to be associated with reduced fungal diversity and an increase in *Candida* colonisation[12]. In
82 patients without *Candida* colonisation, an increased abundance of an unclassified *Ascomycota*
83 species was identified.

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

93 Methods

94 **Study design, participants, and sample collection**

95 This study was based on a multinational retrospective study on the prevalence of invasive
96 pulmonary aspergillosis in critically ill COVID-19 patients in ICUs during 2020[15]. Inclusion
97 criteria consisted of: PCR confirmed COVID-19 infection, bronchoscopy or tracheal aspiration
98 performed during routine clinical investigations, and chest imaging available seven days
99 before or after respiratory samples were collected. Patients less than 18 years of age were
100 excluded. Respiratory samples not passing quality control (described in Supplemental
101 methods) were also excluded. Respiratory specimens obtained at ICU admission or during ICU
102 stay were collected, aliquoted (at least 1ml) and stored at -80 °C. Criteria for defining
103 aspergillosis were according to previous guidelines[23] with the following modifications: COVID-

104 19 requiring ICU admission was included as an additional host factor, tracheal aspirates were
105 equated to BAL fluid for microbiological tests, and serum and BAL GM was added as entry
106 criterion. For a summary of full patient demographics, see Table E1.

107 IRB approval was obtained at each participating center: Medical University of Graz EC #32-296
108 ex 19/20. University of Genoa Liguria Region Ethics Committee registry number 163/2020.

109 **Sample processing**

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

115 **Data analysis**

116 Paired end reads were subject to quality trimming at Q30 and a minimum length filter of 75
117 nucleotides using bbduk[27] (BBMap v38.22). Primer sequences were removed using Cutadapt[28]
118 (v1.18). Reads were mapped to UNITE database using bowtie2[29] (v2.3.5.1). Count data was
119 further processed in R (v4.1.3) using the following packages: phyloseq[30] v1.38.0, vegan[31] v2.5-
120 7, DESeq2[32] v1.34.0, stringr[33] v1.4.0, ggplot2[34] v3.3.5 and tidyr[35] v1.2.0.

121 Abundances were standardised to the median sequencing depth. Extremely low abundance
122 taxa were removed by only retaining those occurring > 0.2% in any sample. DESeq2 was used
123 to identify significantly differentially abundant taxa (adjusted p value < 0.05 and basemean >
124 500). Differences in diversity (Shannon, Chao1 and observed OTUs) were assessed using

125 pairwise Wilcoxon rank sum tests. PERMANOVA test was used to assess differences in Bray-
126 Curtis ordination.

127

128 # Results

129 **Patient cohort**

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

141 **Table 1.** Clinical and demographic characteristics of patients with and without probable
142 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)
Systemic antifungals 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 sample (range 1 – 22). There was no significant clustering between sample batches (Fig. E1), suggesting that sample processing had no impact on mycobiome communities. Mycobiomes predominantly consisted of *Candida* and *Aspergillus* (Fig. 1A). *Candida albicans*, *Aspergillus fumigatus* and *Candida parapsilosis* were the most abundant species (Fig. E2A-B).

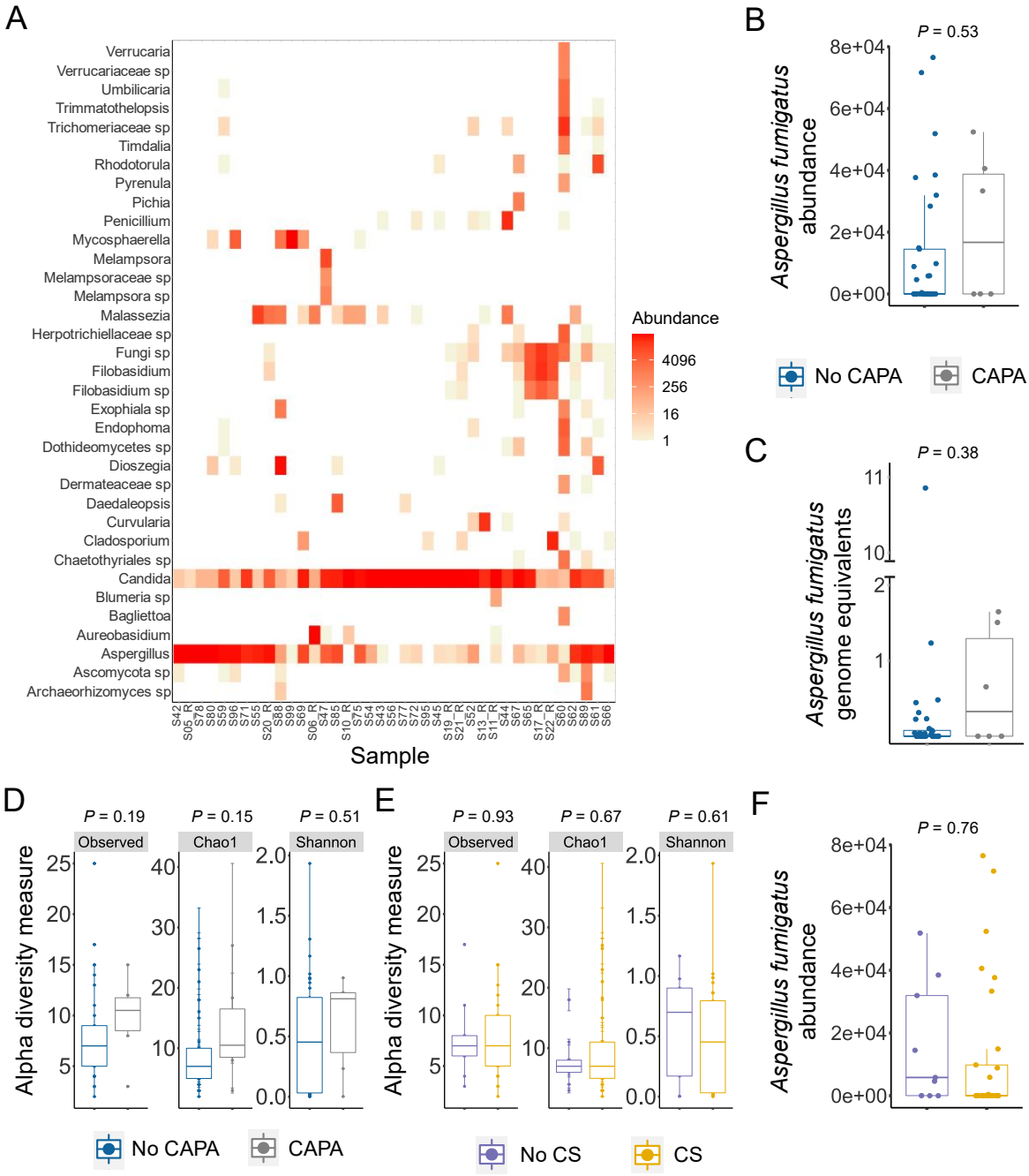

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Fig. 1. *Aspergillus* and *Candida spp.* dominate the respiratory mycobiome in critically ill COVID-19 patients. (A) *Aspergillus* and *Candida* were the main genus observed in the lungs from COVID-19 patients included in the study (n = 39). Median *A. fumigatus* abundance was higher in patients with coronavirus associated pulmonary aspergillosis (CAPA) compared to those without CAPA, measured as (B) Raw sequence reads generated by ITS1 sequencing (Median ~16,700 vs. 35) and (C) *A. fumigatus* specific qPCR (Median 0.33 vs. 0 genome equivalents) As one outlier (Grubbs test, $P < 0.0001$) had a genome equivalents value > 10, a Y axis break is included for ease of visualising majority of data points. (D) Alpha diversity measures (Observed OTUs, Chao1 and Shannon) trended towards higher diversity in CAPA patients (E) Corticosteroid treatment caused no apparent effect on alpha diversity as measured by Observed OTUs and Chao1. Shannon diversity trended towards lower diversity in patients receiving corticosteroids. (F) Median *A. fumigatus* abundance (sequence reads) was lower in patients receiving corticosteroids (Median 8 reads compared to 5,800 in those without corticosteroids). Hypothesis testing was applied using Wilcoxon Rank Sum tests. CS: Corticosteroids. Boxplot data represent median and interquartile range.

155 **CAPA patients have higher median *A. fumigatus* abundance and trend towards lower**
156 **mycobiome diversity whereas corticosteroid use has a less apparent effect on the**
157 **mycobiome**

158 In our study, higher median *A. fumigatus* levels were observed in CAPA when assessed by read
159 count (~16,700 vs. 35) and *A. fumigatus* specific qPCR (0.3 vs. 0 genome equivalents) (Fig. 1B-
160 C), but there was overlap between the patient groups and statistical significance was not
161 reached. *A. fumigatus* burden in patients without CAPA was varied, some patients had little
162 to no *A. fumigatus* and others contained particularly high burden. Furthermore, species
163 differential abundance analysis did not find significant differences between *A. fumigatus*
164 levels based on CAPA status. Instead, *Cladosporium delicatulum, Mycosphaerella tassiana* and
165 *Filobasidium magnum* were found to be at higher abundance in CAPA patients (Fig. E4). In
166 addition, probable CAPA patients had higher median α diversity, however, this difference was
167 not significant, and no significant effect on β diversity was observed (Fig. 1D, Fig. E3).

168 Corticosteroid use resulted in lower median α diversity (Shannon only), however, this
169 difference was not significant (Fig. 1E). Median levels of *A. fumigatus* were lower in the
170 corticosteroid treated group as assessed by sequencing (8 vs. ~5,800 reads). and qPCR (0 vs.
171 0.1 genome equivalents) (Fig.1F, Fig.E3). However, patients on corticosteroids were highly
172 heterogenous in terms of *A. fumigatus* abundance. Species differential abundance analysis
173 found no significant differences between corticosteroid usage. In addition, corticosteroid use
174 had no significant impact on β diversity (Fig. E3).

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

176 The mycobiome of individuals surviving CAPA showed a trend towards higher median α
177 diversity (Fig. 2A). Grouped mean abundances indicated a lower level of *Aspergillus* was

178 present upon survival (Fig.2B). Differences in species prevalence suggested this difference was
179 due to a reduction in *A. fumigatus* (Fig. 2C). Furthermore, mycobiome differential abundance
180 analysis found *A. fumigatus* and *C. albicans* to be significantly less abundant upon survival,
181 with log fold change values of -4.3 and -4.6, respectively (*padj* < 0.05) (Fig.2D). Although *A.*
182 *fumigatus* counts and haploid genome equivalents (HGE, determined by qPCR) trended
183 towards lower levels upon survival, these changes were not significant (Fig. 2E-F). Notably, *A.*
184 *fumigatus* qPCR burden in those that did not survive appeared to be bimodal; 8 samples had
185 a burden above a threshold of 0.1 HGE, 3 of which were patients with probable CAPA. Most
186 patients which survived had HGE =< 0.1, excluding one outlier which had extremely high
187 burden (>10). In addition, BAL galactomannan index values were not significantly different
188 between the patient groups, with an outlier in the survival group having a very high index
189 (Fig.2G).

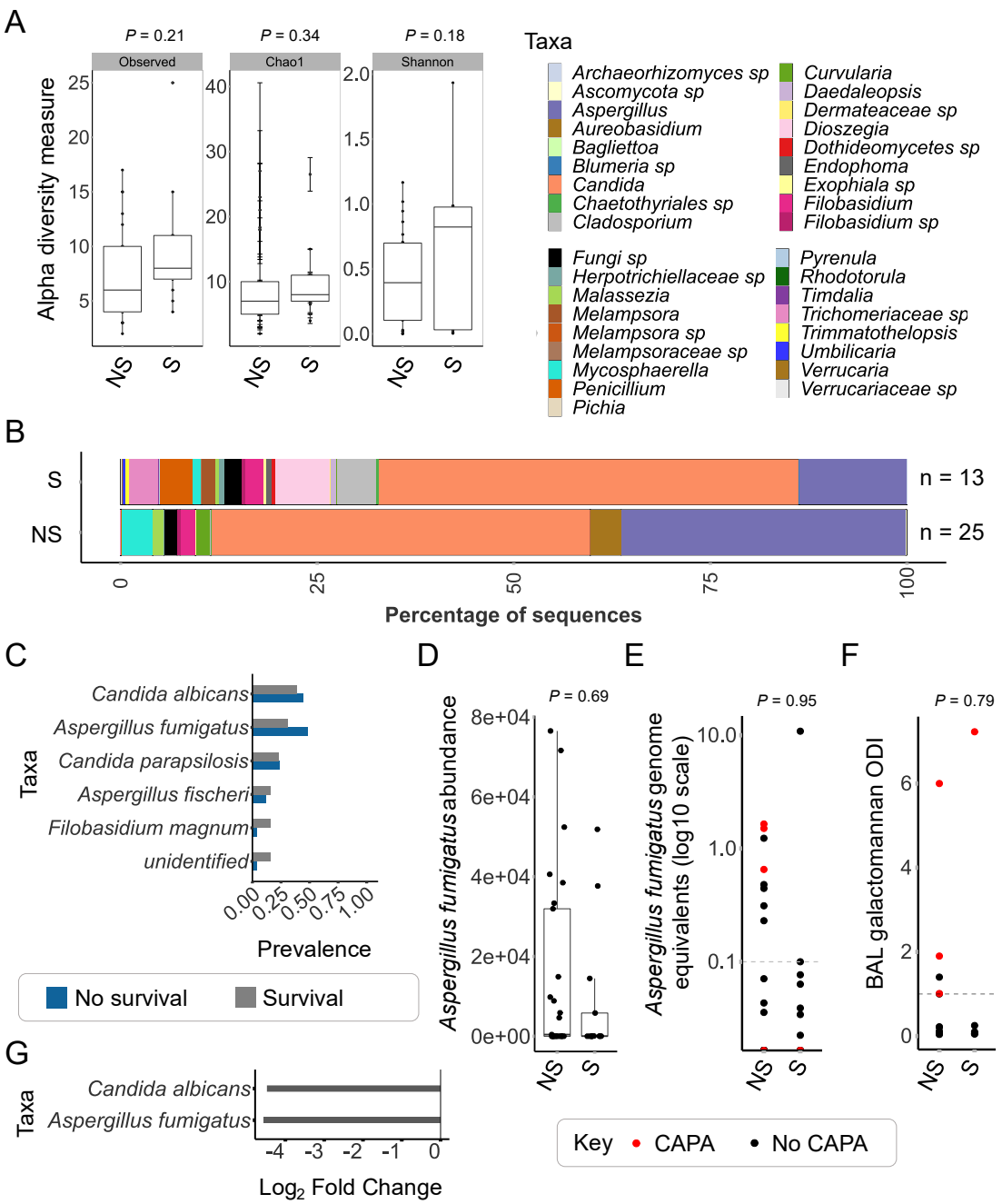
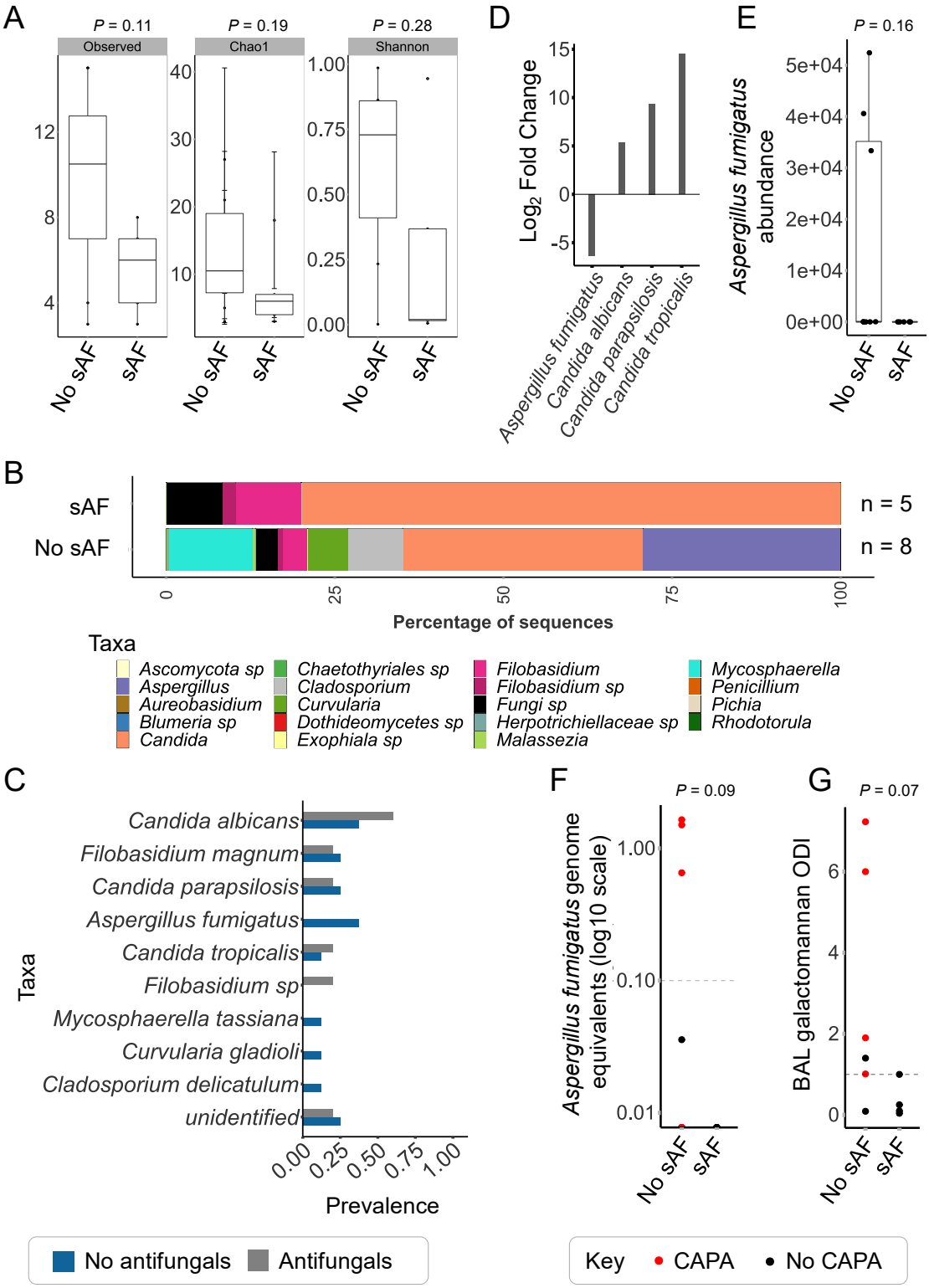


Fig. 2. A higher *A. fumigatus* burden is associated with mortality in critically ill COVID-19 patients. (A) Alpha diversity measures (Observed OTUs, Chao1 and Shannon) trended towards higher diversity in critically ill COVID-19 patients which survived. (B) At the genus level, BAL ITS1 mycobiomes from patients which survived (n =13) indicated a lower proportion of *Aspergillus*, and an apparent increase in the number of observed taxa overall. (C) Increased *A. fumigatus* burdens in lungs of COVID-19 patients which did not survive (blue, 0.5 prevalence) compared to those that did survive (grey, 0.3 prevalence). Taxa were counted if present at over 5% in a sample, and only taxa found in at least 10% of samples of either group are shown. (D) Analysis using DESeq2 identified *A. fumigatus* and *C. albicans* to be at significantly lower abundance in patients which survived. S: Survival; NS: No survival. Data represent median and interquartile range. (E) Median *A. fumigatus* abundance (raw sequence reads) was not significantly different between the patients. (F) *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). (G) BAL galactomannan levels between patient groups were not significantly different. Hypothesis testing in A and D-F was applied using Wilcoxon Rank Sum tests.

191 **Systemic antifungal use at intensive care unit admission is associated with reduced *A.***
192 ***fumigatus* burden in critically ill COVID-19 patients and COVID-19 survival**

193 Use of systemic antifungals at ICU admission in critically ill COVID-19 patients resulted in
194 significantly reduced α diversity when analysing raw mycobiome data. Upon removal of very
195 rare taxa, there was a trend towards reduced median α diversity upon systemic antifungal use
196 (Fig. 3A). There appeared to be a strong reduction in *Aspergillus* counts that correlated with
197 treatment (Fig. 3B). At the species level, the most prevalent taxa in patients without systemic
198 antifungal treatment was *A. fumigatus*, whereas this species was not detectable in patients
199 receiving treatment (Fig. 3C). Furthermore, differential abundance analysis of mycobiome
200 data found a significant reduction of *A. fumigatus* in patients which received systemic
201 antifungal treatment (LFC -6.3, *padj* 0.04) (Fig. 3D). This analysis also found *Candida albicans,*
202 *Candida parapsilosis* and *Candida tropicalis* had significantly higher abundance in COVID-19
203 patients receiving antifungal treatment (LFC 5.3, 9.4 and 14.5, respectively). Although no
204 significant difference was found between *A. fumigatus* counts or qPCR burden in groups with
205 or without systemic antifungals, three patients not receiving antifungals did have high *A.*
206 *fumigatus* levels and were CAPA positive (Fig. 3E-F). BAL galactomannan index was not
207 statistically different between patients with or without systemic antifungal use, however, all
208 individuals receiving treatment were GM negative whereas only half of the patients without
209 treatment were GM negative (Fig.3G).



210

211 **Fig. 3. *A. fumigatus* is associated with the absence of systemic antifungals** at **intensive care unit**

212 **admission in critically ill COVID-19 patients.** (A) Alpha diversity measures (Observed OTUs, Chao1 and

213 Shannon) trended higher diversity in COVID-19 patients without systemic antifungals at ICU admission

214 (sAF). (B) At the genus level, BAL ITS1 mycobiomes from COVID-19 patients which received sAF (n =8)

215 display an absence of *Aspergillus.* (C) Species prevalence in each group indicated a complete lack of *A.*
216 *fumigatus* in patients received sAF (grey) compared to those that did not (blue). Taxa were counted if
217 present at over 5% in a sample, and only taxa found in at least 10% of samples of either group are
218 shown. (D) Analysis using DESeq2 identified *A. fumigatus* to be at significantly lower abundance in the
219 mycobiome of patients who received antifungals, compared to patients who did not. In contrast, three
220 *Candida* species were found to be significantly more abundant in the patients receiving antifungals. (E)
221 Median *A. fumigatus* abundance (raw sequence reads) was not significantly different between the
222 patients. (F) *A. fumigatus* burden measured by qPCR in COVID-19 patients which did not receive sAF
223 appeared bimodal, with three patients exhibiting a high burden (~1 genome equivalents). Notably
224 these patients were all diagnosed with COVID19-associated pulmonary aspergillosis (CAPA)(red). (G)
225 All COVID-19 patients receiving antifungals where BAL galactomannan negative (1 or lower). Half of
226 patients not receiving antifungals were galactomannan positive. Hypothesis testing in A and E-G was
227 applied using Wilcoxon Rank Sum tests. sAF: Systemic antifungals.

228 Our findings suggest an association between *A. fumigatus* abundance and mortality in
229 critically ill COVID-19 patients, and that systemic antifungals at ICU significantly reduces *A.*
230 *fumigatus* levels. Therefore, we combined these factors to assess the association between *A.*
231 *fumigatus* and survival outcomes depending on the presence or absence of systemic
232 antifungal treatment. It was apparent that a higher level of *Aspergillus* was associated with
233 mortality only in COVID-19 patients who had not received systemic antifungal treatment (Fig.
234 4A). Presence of *A. fumigatus* in only those patients that did not survive or receive systemic
235 antifungal treatment was confirmed by qPCR (Fig. 4B). Therefore, these findings suggest
236 systemic antifungal treatment at ICU admission may have been protective against *A.*
237 *fumigatus*-associated mortality in patients with severe COVID-19.

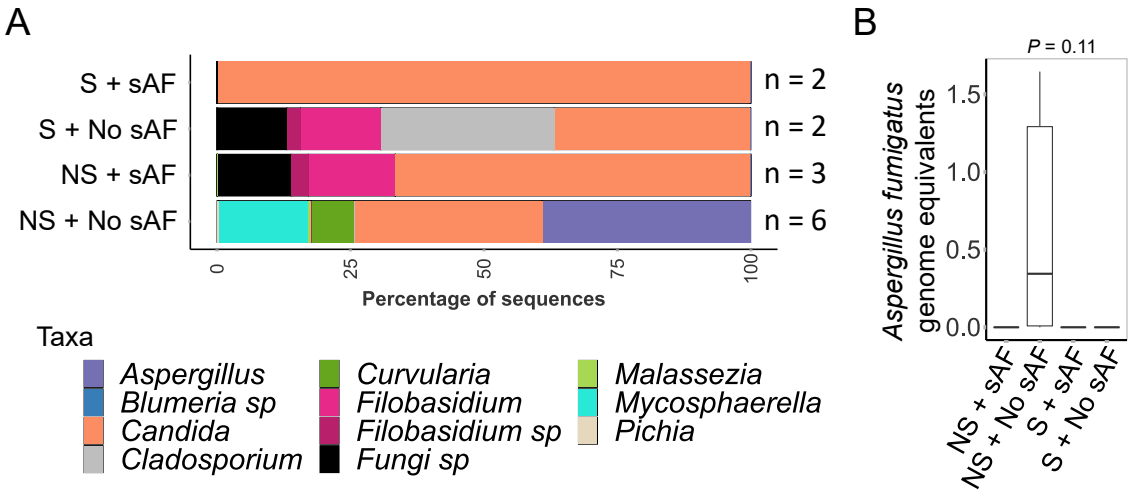


Fig. 4. *A. fumigatus* is associated with mortality in patients with COVID-19 who have not received systemic antifungals at intensive care unit admission. (A) When combining the use of systemic antifungals at ICU and survival outcomes, *Aspergillus* was only found in the BAL ITS1 mycobiomes from patients which did not receive sAF or survive (n=6). (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 systemic antifungals at ICU admission. sAF: Systemic antifungals. S: Survival. NS: No survival.

Discussion

This multinational study found higher *Aspergillus fumigatus* burdens 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 antifungal treatment at ICU admission, suggesting prophylactic antifungal use in severe COVID-19 patients may reduce *A. fumigatus*-associated mortality in this cohort.

The respiratory mycobiomes of critically ill COVID-19 patients described here were dominated by *Candida* and *Aspergillus.* Previous studies using similar patient groups have also reported lung fungal communities to be dominated by *Candida*[10,12]. Furthermore, one study identified a significant increase in unidentified Ascomycota spp. in patients without *Candida* colonisation[12]. Due to the reported incidence of CAPA in severe COVID-19, the authors hypothesised that *Aspergillus* could be present in these patients and, although

253 mycobiome samples were mostly negative for *Aspergillus*, the presence of *Aspergillus* was
254 confirmed by PCR in follow up BAL samples in over 20% of patients.

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

267 It has been suggested that corticosteroid treatment increases lung fungal burden
268 (particularly *A. fumigatus*)[13] and lowers Shannon diversity[37] in asthma. In contrast, no
269 significant differences were found between mycobiome diversity or taxa abundance in
270 respiratory fungal communities of COPD patients with or without inhaled corticosteroid
271 treatment[38]. Another recent study found that alterations in the airway mycobiome in COPD
272 were not significantly affected by corticosteroid use[39]. Large cohort studies suggest systemic
273 corticosteroids are a risk factor for CAPA[16,19], however, there are no specific reports of the
274 influence corticosteroid use has on lung fungal communities in COVID-19. In our study,
275 patients receiving corticosteroids displayed lower median levels of *A. fumigatus* and lower

276 fungal diversity compared to those not receiving corticosteroids. However, these differences
277 did not meet statistical significance. As most (~74%) patients received corticosteroids in this
278 cohort, this may have contributed to low power in these statistical comparisons, warranting
279 further data on this topic.

280 A high fungal burden has previously been associated with a lower likelihood of release from
281 mechanical ventilation and increased mortality risk in severe COVID-19 patients[11]. However,
282 as this study utilised pan-fungal qPCR to identify burden, there was no indication of the
283 specific fungal taxa responsible for this association. Our findings suggest that higher levels of
284 *A. fumigatus* are associated with increased mortality in severe COVID-19. There are no
285 previous reports on the impact of antifungal use on the respiratory mycobiome in COVID-19
286 patients. In this study, the use of antifungals at ICU admission appeared protective against *A.*
287 *fumigatus*-associated mortality.

288 Our study investigated the composition of respiratory fungal communities in critically ill
289 COVID-19 patients with and without CAPA. *Candida* and *Aspergillus* were predominant in the
290 respiratory communities. CAPA diagnosis was associated with higher median *A. fumigatus*
291 level and fungal diversity, and a higher prevalence of *A. fumigatus* was associated with
292 mortality and a lack of systemic antifungals at ICU admission. Our data provides evidence
293 suggesting the use of prophylactic antifungals in seriously ill COVID-19 patients may prevent
294 *A. fumigatus*-associated mortality. A limitation of this study is the small number of patients
295 included, particularly the low number of CAPA cases. In addition, incomplete clinical data
296 with respect to antifungal use reduced the sample sizes for this comparison, which may have
297 resulted in low power for these statistical tests. Therefore, a further study of a larger cohort
298 is warranted to further understand the association between prophylactic antifungal use, the

presence of *A. fumigatus* in the respiratory mycobiome and patient outcome in COVID-19 critical care.

Conflicts of Interest

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

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

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

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

Outside the submitted work, MB reports research grants and/or personal fees for advisor/consultant and/or speaker/chairman from Bayer, BioMérieux, Cidara, Cipla, Gilead, 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.

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References

1. World Health Organisation. WHO Coronavirus Dashboard. https://covid19.who.int/.

2. Wiersinga, W. J., Rhodes, A., Cheng, A. C., Peacock, S. J. & Prescott, H. C. Pathophysiology, Transmission, Diagnosis, and Treatment of Coronavirus Disease 2019 (COVID-19): A Review. *JAMA* **324**, 782–793 (2020).

3. Osuchowski, M. F. *et al.* The COVID-19 puzzle: deciphering pathophysiology and phenotypes of a new disease entity. *Lancet. Respir. Med.* **9**, 622–642 (2021).

4. Dongelmans, D. A. *et al.* Characteristics and outcome of COVID-19 patients admitted to the ICU: a nationwide cohort study on the comparison between the first and the consecutive upsurges of the second wave of the COVID-19 pandemic in the Netherlands. *Ann. Intensive Care* **12**, (2022).

5. Grasselli, G. *et al.* Hospital-Acquired Infections in Critically Ill Patients With COVID-19. *Chest* **160**, 454–465 (2021).

6. Merenstein, C. *et al.* Signatures of COVID-19 Severity and Immune Response in the Respiratory Tract Microbiome. *MBio* **12**, (2021).

7. Lloréns-Rico, V. *et al.* Clinical practices underlie COVID-19 patient respiratory microbiome composition and its interactions with the host. *Nat. Commun. 2021 121* **12**, 1–12 (2021).

345 8. Ren, L. *et al.* Dynamics of the Upper Respiratory Tract Microbiota and Its Association with
346 Mortality in COVID-19. *Am. J. Respir. Crit. Care Med.* **204**, 1379–1390 (2021).

347 9. Gupta, A. *et al.* Mycobiome profiling of nasopharyngeal region of SARS-CoV-2 infected
348 individuals. *Microbes Infect.* 105059 (2022) doi:10.1016/J.MICINF.2022.105059.

349 10. Ruiz-Rodriguez, A. *et al.* Bacterial and fungal communities in tracheal aspirates of intubated
350 COVID-19 patients: a pilot study. *Sci. Reports 2022 121* **12**, 1–10 (2022).

351 11. Kullberg, R. F. J. *et al.* Lung Microbiota of Critically Ill COVID-19 Patients are Associated with
352 Non-Resolving Acute Respiratory Distress Syndrome. *Am. J. Respir. Crit. Care Med.* 1–74
353 (2022) doi:10.1164/rccm.202202-0274OC.

354 12. Viciani, E. *et al.* Critically ill patients with COVID-19 show lung fungal dysbiosis with reduced
355 microbial diversity in patients colonized with Candida spp. *Int. J. Infect. Dis.* **117**, 233–240
356 (2022).

357 13. Fraczek, M. G. *et al.* Corticosteroid treatment is associated with increased filamentous fungal
358 burden in allergic fungal disease. *J. Allergy Clin. Immunol.* 1–8 (2017)
359 doi:10.1016/j.jaci.2017.09.039.

360 14. Janssen, N. A. F. *et al.* Multinational Observational Cohort Study of COVID-19–Associated
361 Pulmonary Aspergillosis. *Emerg. Infect. Dis.* **27**, 2892 (2021).

362 15. Prattes, J. *et al.* Risk factors and outcome of pulmonary aspergillosis in critically ill coronavirus
363 disease 2019 patients—a multinational observational study by the European Confederation of
364 Medical Mycology. *Clin. Microbiol. Infect.* **28**, 580–587 (2022).

365 16. Gangneux, J. P. *et al.* Fungal infections in mechanically ventilated patients with COVID-19
366 during the first wave: the French multicentre MYCOVID study. *Lancet Respir. Med.* **10**, 180–
367 190 (2022).

368 17. Bartoletti, M. *et al.* Epidemiology of Invasive Pulmonary Aspergillosis Among Intubated
369 Patients With COVID-19: A Prospective Study. *Clin. Infect. Dis.* **73**, e3606–e3614 (2021).

370 18. Salmanton-García, J. *et al.* COVID-19–Associated Pulmonary Aspergillosis, March–August 2020
371 - Volume 27, Number 4—April 2021 - Emerging Infectious Diseases journal - CDC. *Emerg.*
372 *Infect. Dis.* **27**, 1077–1086 (2021).

373 19. Leistner, R. *et al.* Corticosteroids as risk factor for COVID-19-associated pulmonary
374 aspergillosis in intensive care patients. *Crit. Care* **26**, 1–11 (2022).

375 20. Koehler, P. *et al.* Defining and managing COVID-19-associated pulmonary aspergillosis: the
376 2020 ECMM/ISHAM consensus criteria for research and clinical guidance. *Lancet Infect. Dis.*
377 **21**, e149–e162 (2021).

378 21. Feys, S. *et al.* Lung epithelial and myeloid innate immunity in influenza-associated or COVID-
379 19-associated pulmonary aspergillosis: an observational study. *Lancet Respir. Med.* **10**, 1147–
380 1159 (2022).

381 22. Hoenigl, M. *et al.* COVID-19-associated fungal infections. *Nat. Microbiol. 2022 78* **7**, 1127–
382 1140 (2022).

383 23. Blot, S. I. *et al.* A clinical algorithm to diagnose invasive pulmonary aspergillosis in critically ill
384 patients. *Am. J. Respir. Crit. Care Med.* **186**, 56–64 (2012).

385 24. Fraczek, M. G. *et al.* The cdr1B efflux transporter is associated with non-cyp51a-mediated
386 itraconazole resistance in Aspergillus fumigatus. *J. Antimicrob. Chemother.* **68**, 1486–1496
387 (2013).

388 25. White, T. J., Bruns, T., Lee, S. & Taylor, J. Amplification and direct sequencing of fungal
389 ribosomal RNA genes for phylogenetics. *PCR Protoc.* 315–322 (1990) doi:10.1016/B978-0-12-
390 372180-8.50042-1.

391 26. Walsh, T. J. *et al.* Molecular Detection and Species-Specific Identification of Medically
392 Important Aspergillus Species by Real-Time PCR in Experimental Invasive Pulmonary
393 Aspergillosis. *J. Clin. Microbiol.* **49**, 4150 (2011).

394 27. Bushnell, B. BBMap. sourceforge.net/projects/bbmap/ (2018).

395 28. Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads.
396 *EMBnet.journal* **17**, 10 (2011).

397 29. Langmead, B. & Salzberg, S. L. Fast gapped-read alignment with Bowtie 2. *Nat. Methods* **9**,
398 357–359 (2012).

399 30. McMurdie, P. J. & Holmes, S. phyloseq: An R Package for Reproducible Interactive Analysis
400 and Graphics of Microbiome Census Data. *PLoS One* **8**, e61217 (2013).

401 31. Dixon, P. VEGAN, a package of R functions for community ecology. *J. Veg. Sci.* **14**, 927–930
402 (2003).

403 32. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for
404 RNA-seq data with DESeq2. *Genome Biol.* **15**, 1–21 (2014).

405 33. Wickham, H. stringr: Simple, Consistent Wrappers for Common String Operations. R package
406 version 1.4.0. https://CRAN.R-project.or at (2019).

407 34. Wickham, H. ggplot2: Elegant Graphics for Data Analysis. at (2019).

408 35. Wickham, H. & Henry, L. tidyr: Tidy Messy Data. R package version 1.1.0. https://CRAN.R-
409 project.or at (2020).

410 36. Nguyen, L. D. N., Viscogliosi, E. & Delhaes, L. The lung mycobiome: an emerging field of the
411 human respiratory microbiome. *Front. Microbiol.* **6**, 89 (2015).

412 37. Huang, C. *et al.* Fungal and bacterial microbiome dysbiosis and imbalance of trans-kingdom
413 network in asthma. *Clin. Transl. Allergy* **10**, 1–13 (2020).

38. Martinsen, E. M. H. *et al.* The pulmonary mycobiome-A study of subjects with and without chronic obstructive pulmonary disease. *PLoS One* **16**, 1–16 (2021).

39. Tiew, P. Y. *et al.* A high-risk airway mycobiome is associated with frequent exacerbation and mortality in COPD. *Eur. Respir. J.* **57**, (2021).

40. Wickham, H. stringr: Simple, Consistent Wrappers for Common String Operations. at https://cran.r-project.org/package=stringr (2019).

41. Wickham, H. ggplot2: Elegant Graphics for Data Analysis. *Springer-Verlag New York* (2016).

Supplementary data

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

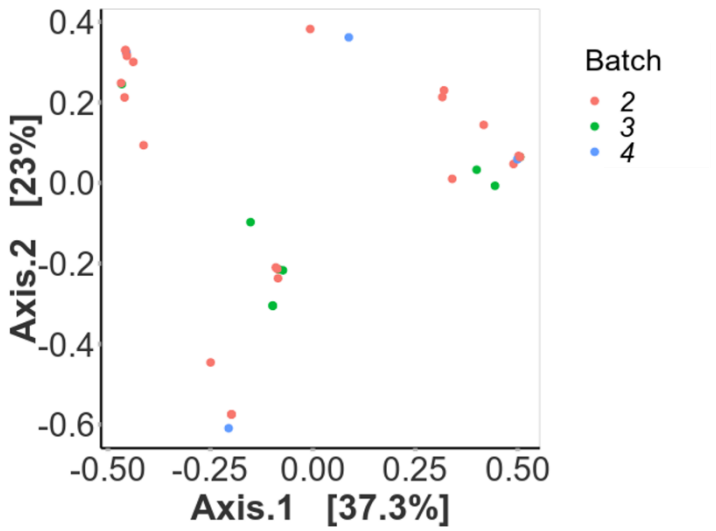
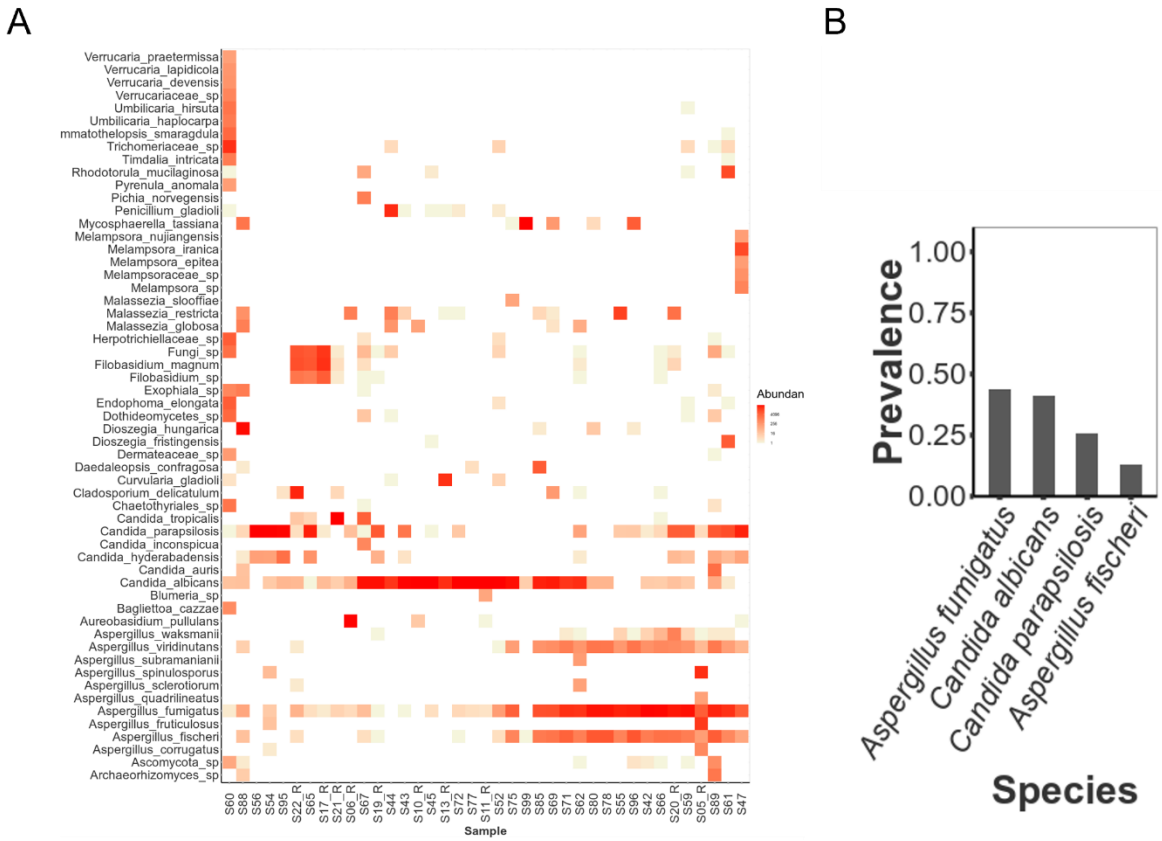


Fig. E1. Mycobiome Bray-Curtis ordination by sample batch

429



430

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

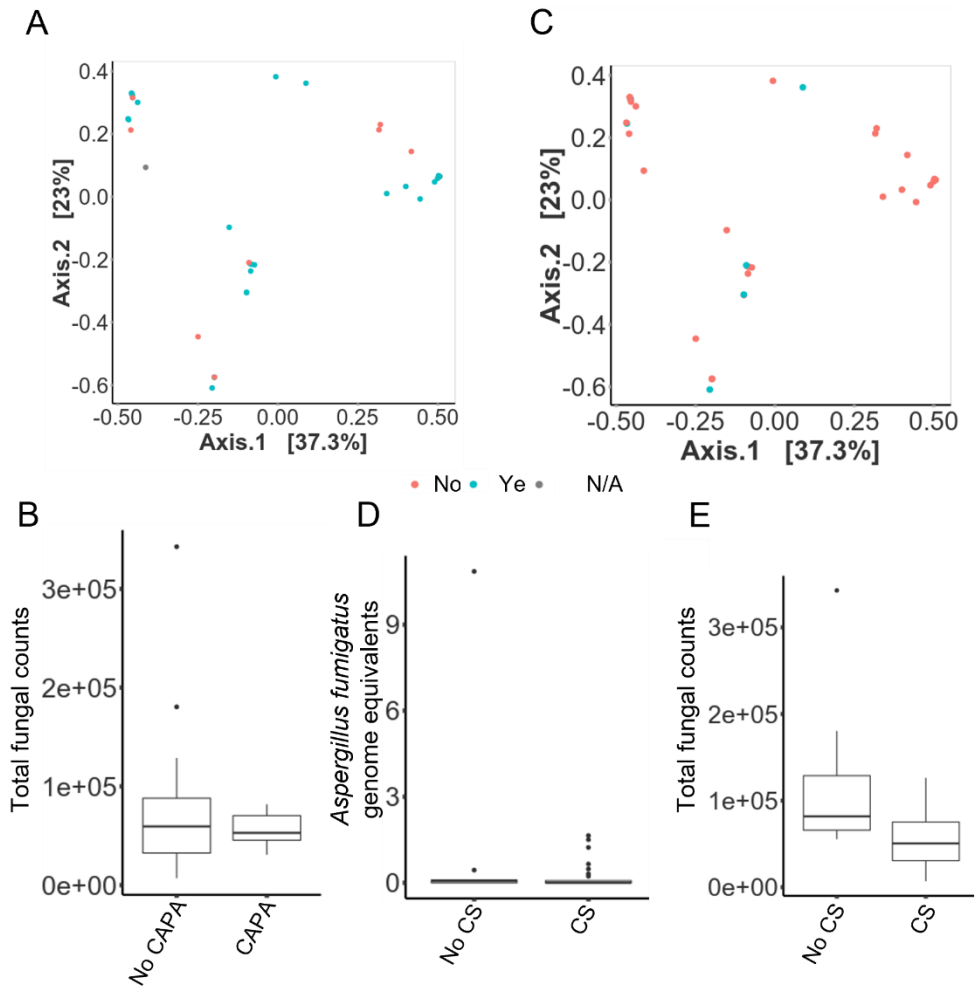
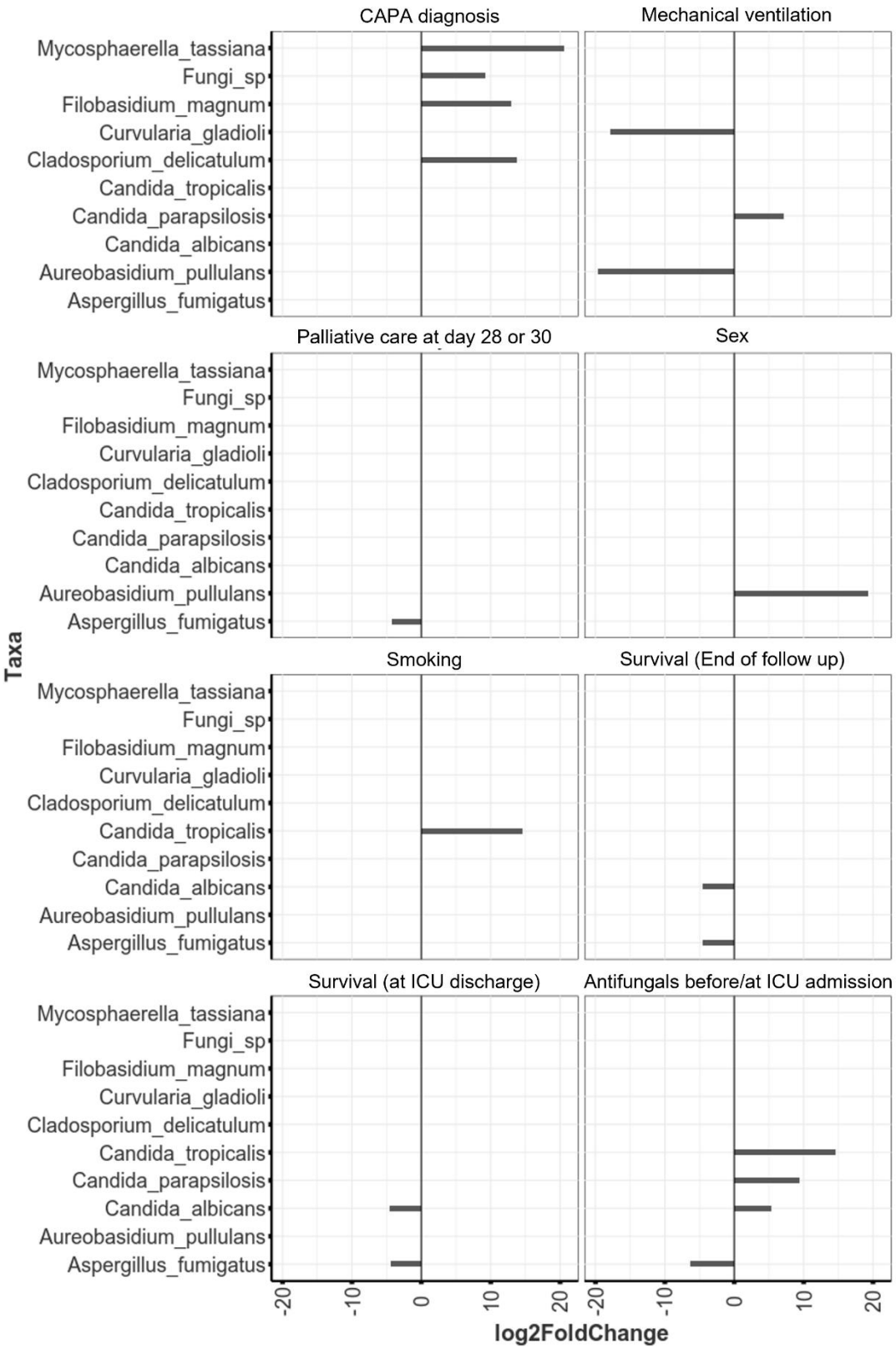


Fig. E3. CAPA diagnosis and corticosteroid use had no significant impact on fungal burden, *Aspergillus fumigatus* levels or beta diversity.



435

436 **Fig. E4. Significantly differentially abundant taxa identified by DESeq2 analysis.**

437

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)

Systemic antifungals 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 systemic antifungal use at ICU admission.

Variable	Taxa	base-Mean	log2Fold-Change	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
Systemic anti-fungals 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 treated with 4 µl RNase A (100 mg/ml, Sigma) for 30 mins prior to DNA isolation using 1:1 ratio of Phenol-Chloroform (25:24:1, Merck) and 1:1 Chloroform:isoamyl alcohol (24:1, VWR). DNA

459 was precipitated at -20 °C overnight using 2 volumes of ice-cold 100% ethanol and 0.1 volumes
460 of 3M sodium acetate pH 4.8. Pelleted DNA was washed in 100% ethanol and twice in 70%
461 ethanol. Final DNA was suspended in 50 µl TE buffer.

462 **qPCR**

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

468 **PCR & Sequencing**

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

480 **Data analysis**

481 Paired end reads were subject to quality trimming at Q30 and a minimum length filter of 75
482 nucleotides using bbduk[27] (BBMap v38.22). Primer sequences were removed using Cutadapt[28]
483 (v1.18). Reads were mapped to UNITE database using bowtie2[29] (v2.3.5.1). Count data was
484 further processed in R (v4.1.3) using the following packages: phyloseq[30] v1.38.0, vegan[31] v2.5-
485 7, DESeq2[32] v1.34.0, stringr[40] v1.4.0, ggplot2[41] v3.3.5 and tidyr[35] v1.2.0.

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

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

500

501