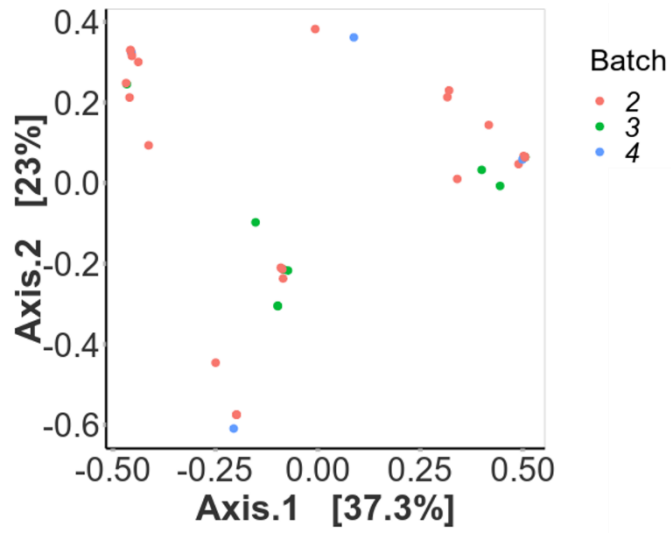


1 Supplementary data

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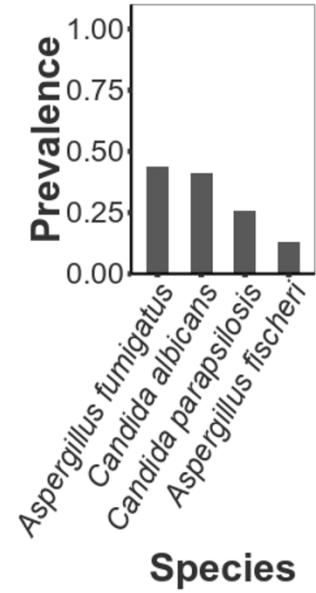
4

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

A

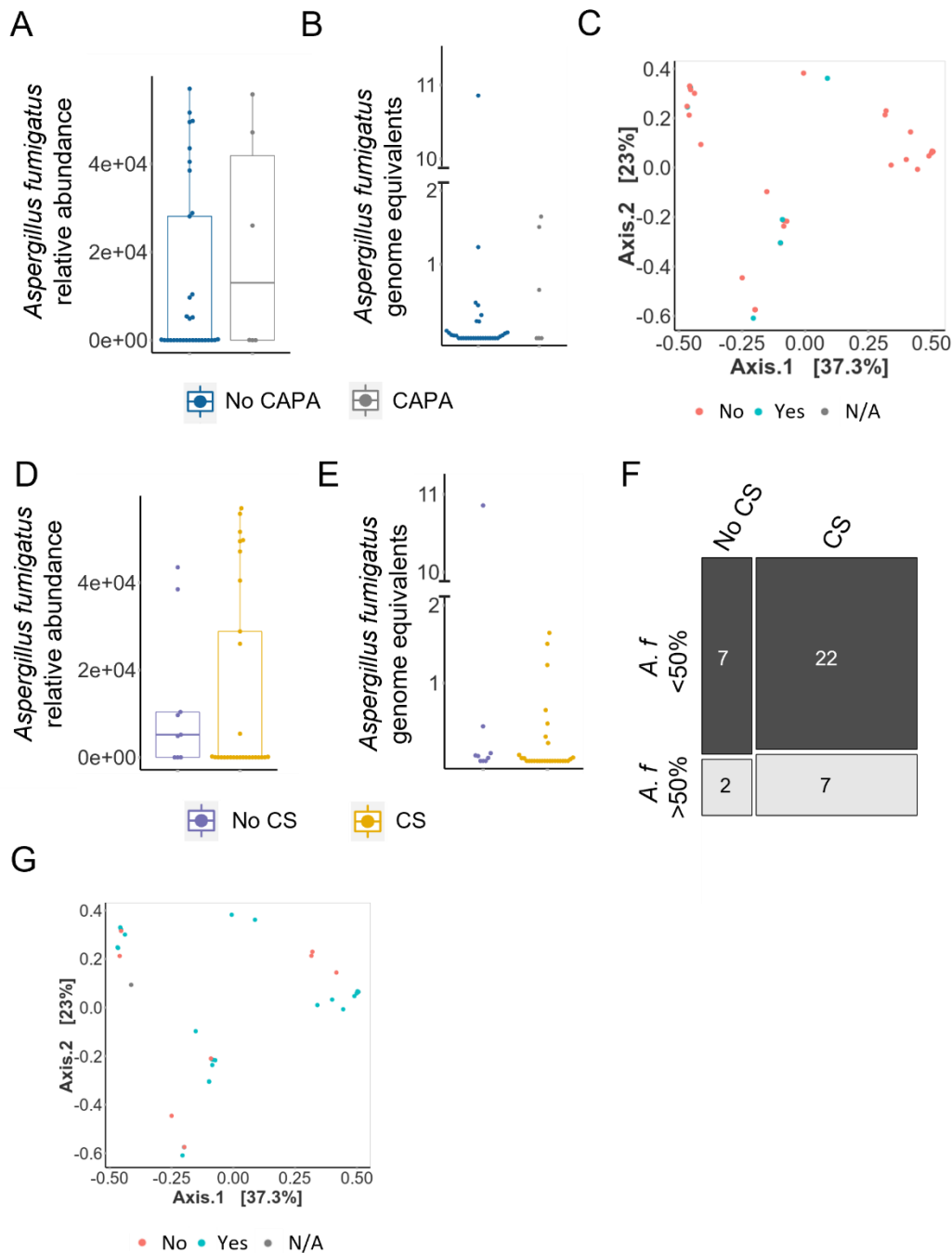


B



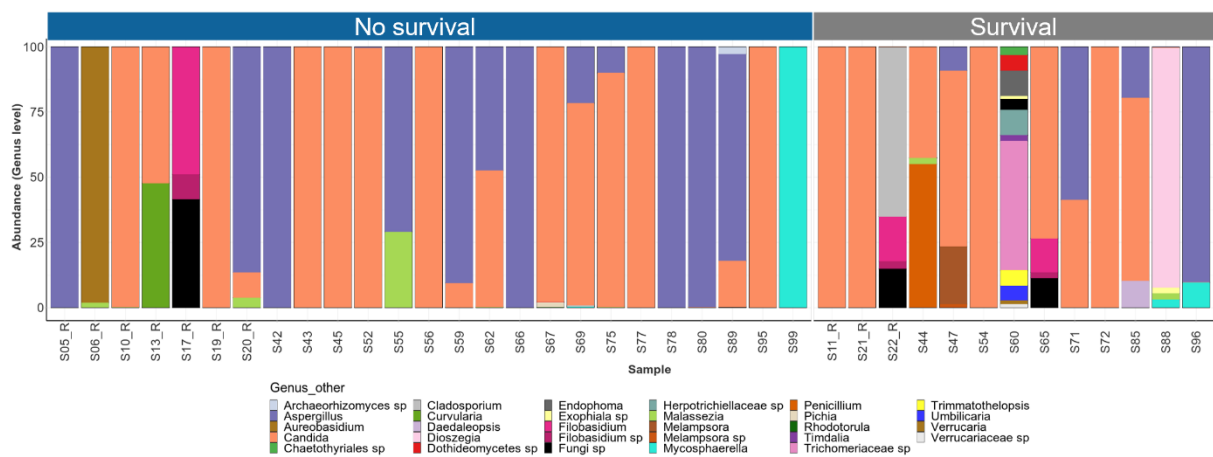
6

7 **Fig. E2. ITS1 respiratory mycobiomes at the species level.** (A) Heatmap of fungal species identified in  
 8 all samples. To remove extremely rare taxa, only those present at > 0.2% in one sample were  
 9 retained. (B) *A. fumigatus* and *C. albicans* are the most prevalent species. Taxa were counted if  
 10 present at over 5% in a sample, and only taxa found in at least 10% of samples of either group are  
 11 shown.



12

13 Fig. E3. CAPA diagnosis and corticosteroid use had no significant impact on fungal burden, *Aspergillus*  
 14 *fumigatus* levels or beta diversity. (A) Relative abundance of *A. fumigatus* in mycobiomes of those  
 15 with and without CAPA. (B) *A. fumigatus* levels in those with and without CAPA measured by qPCR.  
 16 (C) Beta diversity and CAPA status. (D) Relative abundance of *A. fumigatus* in mycobiomes of those  
 17 with and without corticosteroid use. (E) *A. fumigatus* levels in those with and without corticosteroid  
 18 use as measured by qPCR. (F) Dichotomised data for samples positive/negative for high *A. fumigatus*  
 19 burden by qPCR in patients with or without corticosteroids. (G) Beta diversity and corticosteroid use.  
 20 Boxplot data represent median and interquartile range. CS; corticosteroid use. CAPA; COVID-  
 21 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.

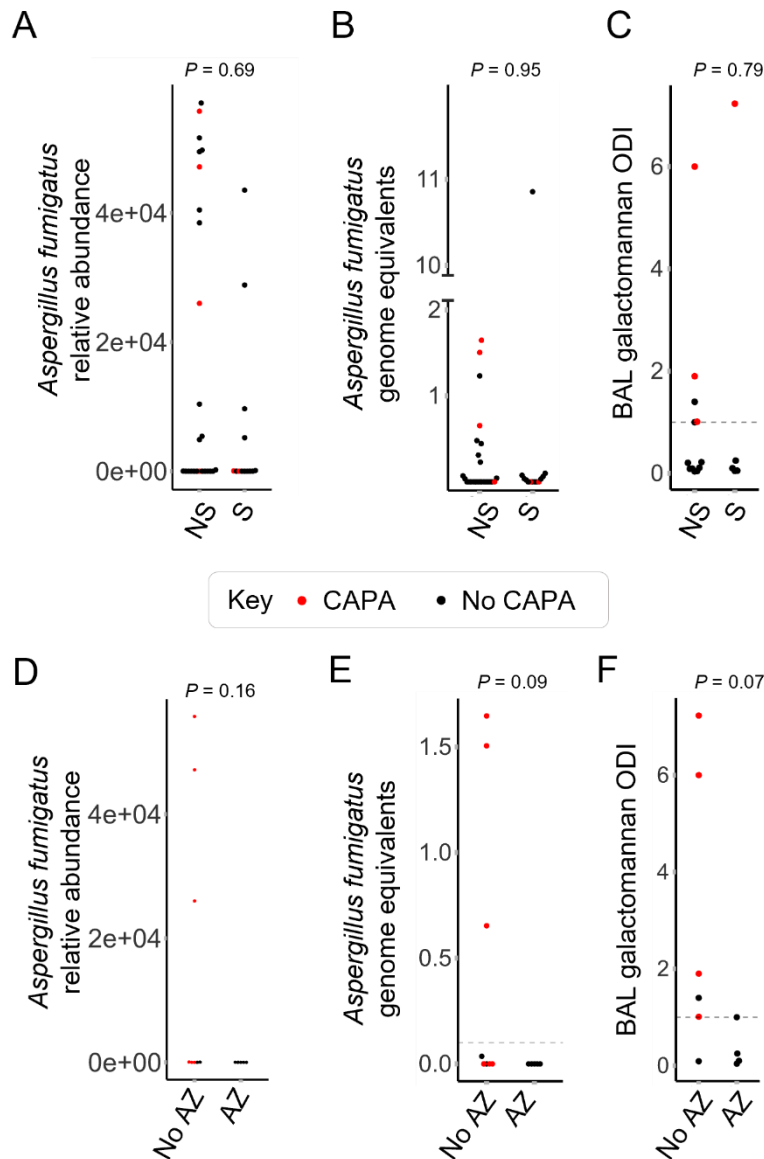
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27 **Fig. E5. Extended data for *Aspergillus fumigatus* levels in respiratory mycobionomes and BAL**  
 28 **galactomannan.** (A) *Aspergillus fumigatus* relative abundance in patients grouped by survival  
 29 outcome. (B) *A. fumigatus* burden measured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

38

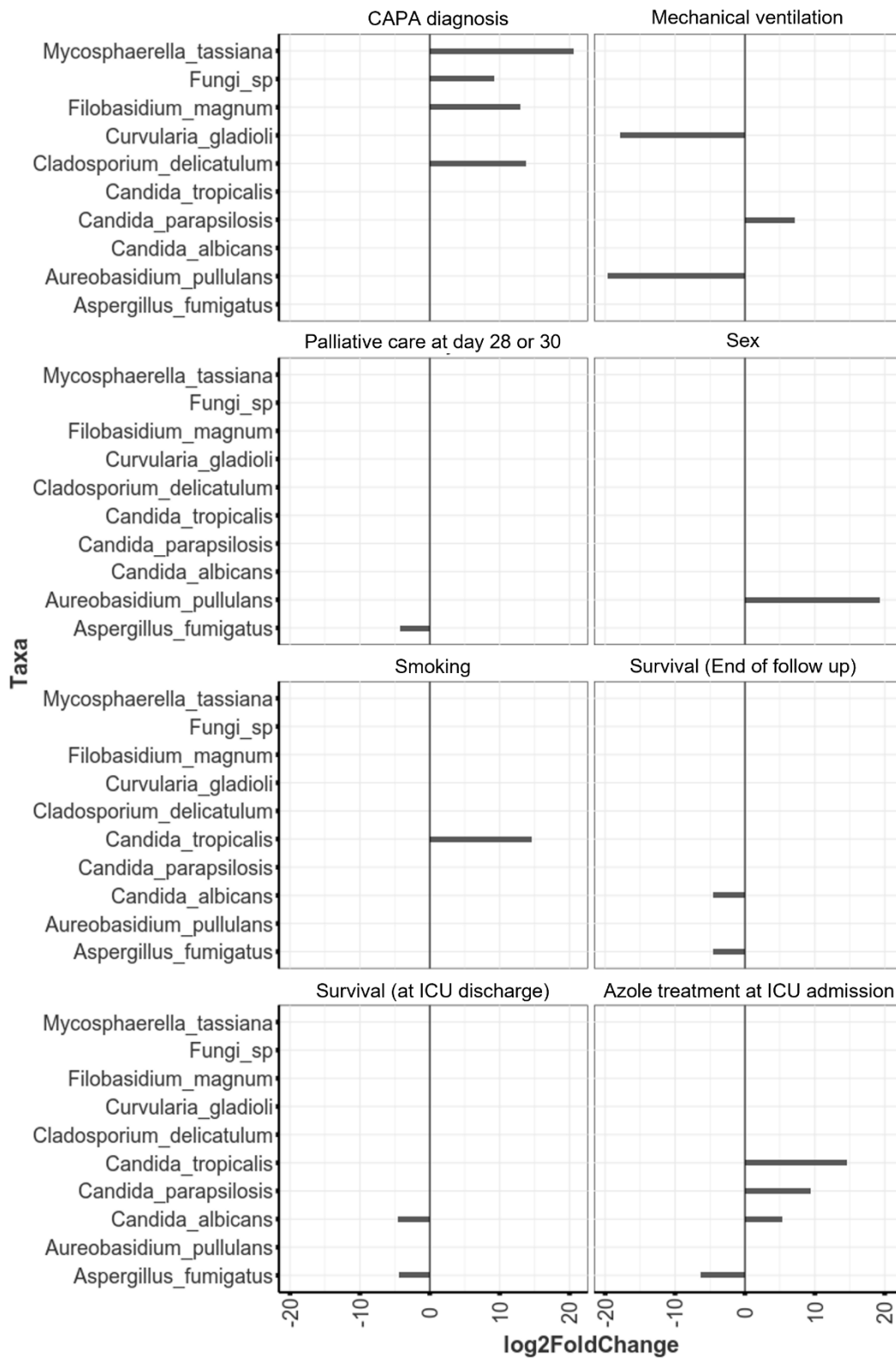
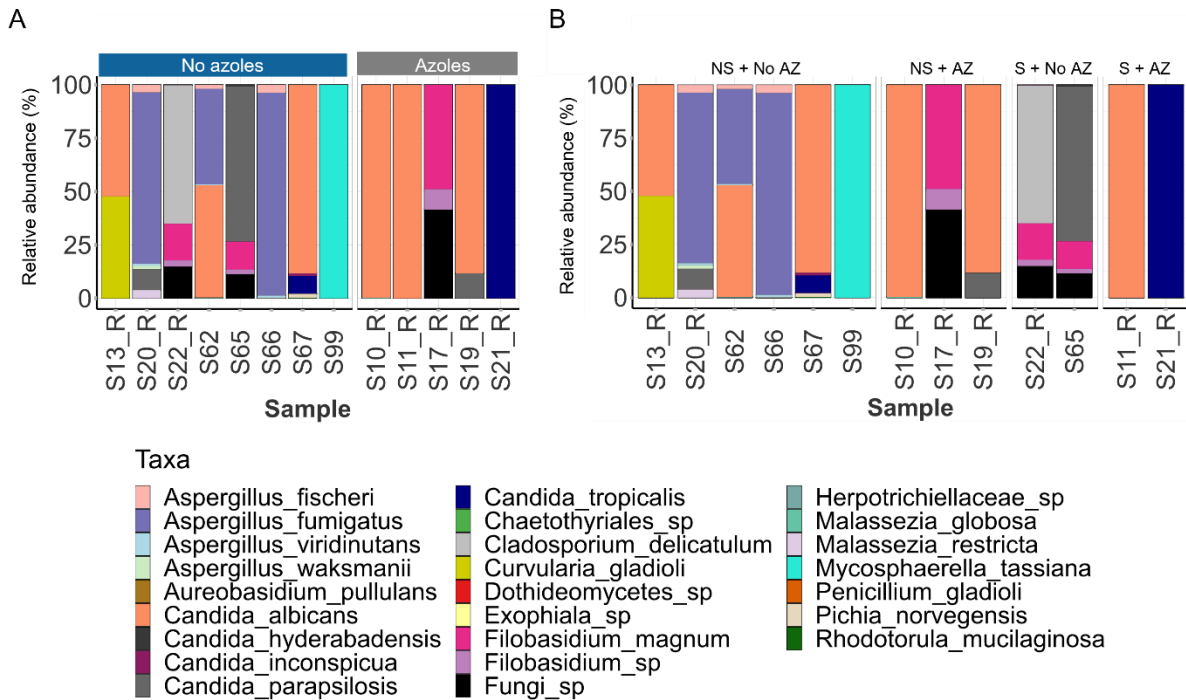


Fig. E6. Significantly differentially abundant taxa identified by DESeq2 analysis.

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43 **Fig. E7. Species data for mycobiome associations with azole treatment and mortality** (A) At the  
 44 species level, BAL ITS1 mycobiomes from COVID-19 patients which received azoles (n =5) display an  
 45 absence of *A. fumigatus*, whereas 3/8 patients not receiving azoles harboured *A. fumigatus*. (B)  
 46 When combining the use of azoles at ICU and survival outcomes, *A. fumigatus* was found in the BAL  
 47 ITS1 mycobiomes from 50% of patients which did not receive azoles or survive (3/6). No *A. fumigatus*  
 48 was present in samples from the remaining three patient groups.

49

50 **Table E1. Primer table.** Nextera XT adaptors are underlined. FunITS2-III is a Nextera XT-  
 51 compatible, modified version of ITS2<sup>25</sup> (modified bases are indicated in bold).

Primer name	Sequence (5'-3')	Reference
FunITS1-III	<u>TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG</u> TCCGTAGGT	Modified from 25
	GAACCTGCGG	
FunITS2-III	<u>GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG</u> GC	Modified from 25
	YRCGTTCTTCATCGAYGC	

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53 **Table E2. Full clinical patient demographics**

<b>Characteristics</b>	<b>N=39</b>
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)

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61 **Table E3. Full results for significantly differentially abundant taxa determined by DESeq2**  
62 **analysis when assessing CAPA diagnosis, survival, and azole treatment at ICU admission.**

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

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## 65 Supplementary methods

### 66 DNA extraction

67 BAL DNA was extracted using a cetyltrimethylammonium bromide (CTAB) method<sup>23</sup>.

68 Negative extraction controls (NECs) consisting of molecular grade Tris-EDTA (TE) buffer

69 (Promega) were included with each sample batch. First, 500 µl samples were incubated with

70 0.1% dithiothreitol (Thermo Scientific) at 37 °C for 45 minutes. To lyse fungal cells, CTAB

71 DNA Extraction Buffer (Generon Ltd) was added and a short mechanical disruption (2 x 20

72 seconds FastPrep-24) using glass beads (425-600µM, Sigma) was performed, followed by 3

73 cycles of alternated heating to 65 °C and gentle vortexing for 10 mins each. Cell lysates were  
74 treated with 4 µl RNase A (100 mg/ml, Sigma) for 30 mins prior to DNA isolation using 1:1  
75 ratio of Phenol-Chloroform (25:24:1, Merck) and 1:1 Chloroform:isoamyl alcohol (24:1,  
76 VWR). DNA was precipitated at -20 °C overnight using 2 volumes of ice-cold 100% ethanol  
77 and 0.1 volumes of 3M sodium acetate pH 4.8. Pelleted DNA was washed in 100% ethanol  
78 and twice in 70% ethanol. Final DNA was suspended in 50 µl TE buffer.

## 79 **qPCR**

80 The presence of *A. fumigatus* in respiratory samples was validated using a TaqMan probe  
81 assay targeting the ITS1 region<sup>25</sup>. Assays were performed on an Applied Biosystems 7500  
82 fast system using TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific). Standard  
83 curves were a nine sample 10-fold dilution series beginning at 100 ng *A. fumigatus* genomes  
84 per reaction.

## 85 **PCR & Sequencing**

86 The ITS1 region was amplified using Nextera XT compatible versions of ITS1<sup>24</sup> and ITS2degen  
87 (a degenerate version of ITS2 primer) (see Table E1). PCR setup used Phusion Green Hot  
88 Start II HF PCR master mix to create 25 µl reactions with 300 nM forward primer, 1.2 µM  
89 reverse primer and 2 µl DNA. Parameters included annealing temperature of 55 °C for 40  
90 cycles. PCRs included positive (*Aspergillus niger* genomic DNA), negative (molecular grade  
91 water) and negative extraction controls (NECs) from DNA extractions. Controls were  
92 processed fully and sequenced alongside the BAL samples. For sequencing library  
93 preparation, samples were processed as per Illumina fungal metagenomic demonstrated  
94 protocol (Starting at 'Clean up' section) using Nextera XT index kit v2 (Illumina). However,  
95 this protocol was modified to include sample pooling prior to the second clean up. Libraries

96 were sequenced 2x150 on an Illumina iSeq100, with a final library loading concentration of  
97 50 pM.

## 98 **Data analysis**

99 Paired end reads were subject to quality trimming at Q30 and a minimum length filter of 75  
100 nucleotides using bbduk<sup>26</sup> (BBMap v38.22). Primer sequences were removed using  
101 Cutadapt<sup>27</sup> (v1.18). Reads were mapped to UNITE database using bowtie2<sup>28</sup> (v2.3.5.1). Count  
102 data was further processed in R (v4.1.3) using the following packages: phyloseq<sup>29</sup> v1.38.0,  
103 vegan<sup>30</sup> v2.5-7, DESeq2<sup>31</sup> v1.34.0, stringr<sup>41</sup> v1.4.0, ggplot2<sup>42</sup> v3.3.5 and tidy<sup>34</sup> v1.2.0.

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

112 Manual BLAST searches confirmed the sequences assigned as *C. albicans*, *C. auris* and *A.*  
113 *fumigatus* are a 100% match to their assigned species (and *A. fumigatus* presence was also  
114 confirmed by qPCR). However, broadly, the species names described in the mycobiome data  
115 should be interpreted as candidate species designations as ITS barcoding can be unreliable  
116 for species level designations in certain cases. For example, the presence of *A. fischeri* has  
117 strong correlation with the presence of a higher abundance of *A. fumigatus* in a sample and  
118 it is likely that the *A. fischeri* sequences are a small proportion of misidentified *A. fumigatus*

119 sequences (possibly due to sequence error introduced during the PCR stages of library  
120 preparation) as this species is very closely related to *A. fumigatus* and the ITS amplicon  
121 differs by only a single nucleotide. Abundances were standardised to the median sequencing  
122 depth. Extremely low abundance taxa were removed by only retaining those occurring >  
123 0.2% in any sample. DESeq2 was used to identify significantly differentially abundant taxa  
124 (adjusted  $p$  value < 0.05 and basemean > 500). Differences in diversity (Shannon and  
125 observed OTUs) were assessed using pairwise Wilcoxon rank sum tests. PERMANOVA test was  
126 used to assess differences in Bray-Curtis ordination. A mycobiome sample was assigned as  
127 containing predominantly *A. fumigatus* if its *A. fumigatus* read counts were higher than 50%  
128 of the median sequencing depth. To determine mycobiome samples positive for high *A.*  
129 *fumigatus* burden by validating qPCR, the cutoff was 0.1 haploid genome equivalents (HGE).  
130 The resulting contingency tables for sequencing and qPCR data were visualised as mosaic  
131 plots and statistical significance tested using Fisher's exact tests.

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