



31 **Abstract**

32 **Introduction:** Diagnosis of COVID-19 associated pulmonary aspergillosis remains unclear especially  
33 in non-immunocompromised patients. The aim of this study was to evaluate seven mycological  
34 criteria and their combination in a large homogenous cohort of patients.

35 **Methods:** All successive patients (n=176) hospitalized for COVID-19 requiring mechanical  
36 ventilation and who clinically worsened despite appropriate standard of care were included over a one-  
37 year period. Direct examination, culture, *Aspergillus* qPCR (*Af*-qPCR) and galactomannan was  
38 performed on all respiratory samples (n=350). Serum galactomannan,  $\beta$ -D-glucan and plasma *Af*-  
39 qPCR were also assessed. Criteria were analyzed alone or in combination in relation to mortality rate.

40 **Results:** Mortality was significantly different in patients with 0,  $\leq 2$  and  $\geq 3$  positive criteria (logrank  
41 test,  $p=0.04$ ) with death rate of 43.1, 58.1 and 76.4% respectively. Direct examination, plasma qPCR  
42 and serum galactomannan were associated with a 100% mortality rate. Bronchoalveolar lavage (BAL)  
43 galactomannan and positive respiratory sample culture were often found as isolated markers (28.1 and  
44 34.1%) and poorly repeatable when a second sample was obtained. *Aspergillus* DNA was detected in  
45 13.1% of samples (46/350) with significantly lower Cq when associated with at least one other criteria  
46 (30.2 vs 35.8) ( $p<0.001$ ).

47 **Conclusion:** Combination of markers and/or blood biomarkers and/or direct respiratory sample  
48 examination seems more likely to identify patients with CAPA. *Af*-qPCR may help identifying false  
49 positive results of BAL galactomannan testing and culture on respiratory samples while quantifying  
50 fungal burden accurately.

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230/250 words

53

54 **Introduction**

55 Reports on Coronavirus disease 2019 (COVID-19)-associated aspergillosis (CAPA) are increasing but  
56 incidence, diagnosis and management remains unclear. Because no host factor meeting European  
57 Organization for Research and Treatment of Cancer and Mycosis Study Group Education and  
58 Research Consortium (EORTC/MSGERC) definitions is present in most patients, alternative  
59 classifications were needed (1). Despite several attempts to clarify CAPA definition by expert panels  
60 (2-4) diagnosis remains arguable (5-7). The lack of impact of both antifungal treatment and  
61 prophylaxis in many large cohort studies but the impact of prophylaxis on the incidence of CAPA  
62 cases leads to hypothesize that many of these cases may only be colonization rather than infection (8,  
63 9). Therefore, incidence and prognosis may be overestimated while impact of treatment may be  
64 underestimated in real aspergillosis cases. Minimum requirement for CAPA diagnosis according the  
65 European Confederation for Medical Mycology and International Society for Human and Animal  
66 Mycology (ECMM/ISHAM) in a COVID-19 patient presenting with acute respiratory distress  
67 syndrome includes at least one imaging characteristic (i.e. pulmonary infiltrates), one clinical  
68 characteristic (i.e. refractory fever, chest pain) and one mycological criterion (3). The first two  
69 requirements (imaging and clinical) are poorly specific and quite frequent in severe COVID-19  
70 patients, therefore CAPA diagnosis mainly relies on mycological criteria with one criterion alone  
71 being sufficient. The performances of such mycological criteria lack proper evaluation in this context  
72 mostly because the gold standard depends on histopathological data evidencing invasive hyphae in  
73 lung tissue. Lung biopsies would be difficult to justify in severe COVID-19 patients given the number  
74 of patients, infection control risks and the lack of identified infected site in most cases on imaging. In  
75 parallel, rare studies including autopsies have been published. The few autopsy data available rarely  
76 include routinely performed fungal investigations before the death of the patients and mycological  
77 tests were mostly not evaluated (10, 11). Despite, the absence of accessible gold standards, more data  
78 are needed on routinely performed individual mycological criteria, regarding their advantages and  
79 shortcomings in the context of critically ill COVID-19 patients. Mortality is known to be high in  
80 invasive aspergillosis and high mortality rate may better reflect infection over colonization in CAPA  
81 suspected patients. Therefore, the aim of our study was to evaluate the performances of seven

82 mycological criteria and their combination, analyzed in terms of clinical outcome in mechanically  
83 ventilated COVID-19 patients.

84

## 85 **Methods**

### 86 *Patients and study design*

87 A retrospective observational cohort study was conducted. All successive COVID-19 patients  
88 admitted to the four intensive care units (ICUs) of our two university hospitals between March 15<sup>th</sup>  
89 2020 and March 1<sup>st</sup> 2021 with a positive SARS-CoV-2 PCR (Cobas<sup>®</sup> SARS-CoV-2 Test, Roche,  
90 Meylan, France) and  $\geq 1$  respiratory sample (bronchoalveolar lavage [BAL], tracheal aspirate, sputum)  
91 sent to the mycology department were included. Of note, the 105 first patients included were partially  
92 analyzed to assess risk factors associated with CAPA (12). Culture and *Aspergillus* quantitative  
93 polymerase chain reaction (qPCR) were systematically performed on all types of respiratory samples  
94 (BAL, sputum, tracheal or bronchial aspirates). Direct examination of those specimens was only  
95 performed in samples received after March 26<sup>th</sup> because of previous lack of evidence regarding the  
96 risk of contamination of lab staff members. After this date, direct examinations in 0.1M KOH were  
97 performed again as SARS-CoV-2 was found to be inactivated at pH >10. Galactomannan detection  
98 (GM) was performed in the supernatant of every BAL fluid (after centrifugation) following strict  
99 recommendations of the manufacturer. If blood sample was concomitantly received, GM and  $\beta$ -D-  
100 glucan (BDG) were performed on serum and *Aspergillus* qPCR on plasma. Patients were classified as  
101 probable IPA according to the EORTC/MSGERC updated consensus criteria (1) if a host factor was  
102 present and according to the ECMM/ISHAM consensus criteria (3) otherwise. The following clinical  
103 data were collected including age, gender, length of ICU stay, prescription of antifungals and  
104 mortality.

### 105 *Mycology criteria testing*

#### 106 *Direct examination and culture of respiratory samples*

107 All mucous respiratory samples were diluted to 1:10, transferred to sterile 50 mL falcon tube,  
108 incubated at 37°C for 15 min and centrifuged 10 min at 3000 G. BAL supernatant was reserved for  
109 GM detection. A direct examination was performed by mixing 50  $\mu$ L of the pellet, 10  $\mu$ L of

110 Calcofluor White and 10  $\mu$ L of KOH and read at 475 nm with a Nikon Eclipse E600 fluorescence  
111 microscope. The other part of the pellet was seeded in parallel on BBL™ CHROMagar Candida  
112 Medium (BD, Grenoble, France), 2% MALT extract + chloramphenicol agar (VWR Chemical,  
113 Leuven, Belgium) and Sabouraud agar supplemented with chloramphenicol/gentamicin (Biorad,  
114 Marnes-la-coquette, France). *Candida* chromogenic media and one Sabouraud was incubated at 37°C.  
115 MALT plates and one Sabouraud slant was incubated at 30°C for a maximum of 3 weeks.

116

#### 117 *Aspergillus fumigatus*-qPCR (Af-qPCR)

118 Whole nucleic acids together with the internal control DiaControlDNA (Diagenode, Seraing,  
119 Belgium) was extracted from 1 mL of plasma or from half of the pellet of the respiratory sample  
120 (other half was used for culture) which was submitted to bead-beating in 1000  $\mu$ L DNA-free water  
121 using the Qiasymphony DSP virus/Pathogen Mini kit (Qiagen, Hilden, Germany) and a QIASymphony  
122 apparatus (Qiagen). All extracts were tested in duplicate using the 28S rDNA PCR assay previously  
123 reported (13, 14). PCR assay were performed on a LightCycler 480 instrument (Roche Diagnostics,  
124 Meylan, France).

125

#### 126 *GM testing*

127 GM detection was performed using Platelia Bio-Rad kit (BioRad Laboratories, CA, USA)  
128 according to the manufacturer. Each positive result was tested the day after on the same sample as  
129 recommended (15). Only samples found positive twice were considered positive. ODI >0.5 and >1  
130 were considered positive in serum and BAL, respectively [3,4].

131

#### 132 *BDG testing*

133 BDG testing was performed using Fungitell assay (Cape Cod Diagnostics, Cape Cod, USA)  
134 according to the manufacturer's instructions. BDG was considered positive when >80 pg/mL.

135

#### 136 *Statistics*

137 Data were reported in percentage, mean and standard deviation ( $\pm$ SD) if variable followed a  
138 Gaussian distribution or median and interquartiles (IQR) [Q1-Q3] otherwise. Univariate analyses were  
139 performed using Fisher's exact, Chi-2 and Wilcoxon tests as appropriate.  $P < 0.05$  (two-tailed) was  
140 considered statistically significant. Mortality analysis was performed using Kaplan-Meier survival  
141 curves and log-rank test. No statistical adjustments were made for multiple testing or confounders. All  
142 analyses were performed using Prism, version 8.0.

143

144 *Ethical statements*

145 Our institutional ethics committee approved the study (IDRCB, 2020-A00256-33; CPP, 11-  
146 20-20.02.04.68737). Written information was given to the patient or the next of kin.

147

148 **Results**

149 *Population characteristics*

150 Patients' characteristics are described in **Table 1**. Briefly, 176 patients for which 350 respiratory  
151 samples were received by the mycology department were included (**Figure 1**). Patients were mainly  
152 males (M/F sex ratio=3.6) with a median age of 64 years [IQR 58-72]. A median of 2 [IQR 1-3, range  
153 1-9] respiratory samples were received per patient. Three (1.7%) patients had EORTC/MSGERC host  
154 factor (kidney transplant, n=2; hematological malignancy, n=1). A total 42 (23.9%) and 11 (6.3%)  
155 patients could be classified as probable and possible CAPA according to ECMM/ISHAM consensus  
156 criteria (3), respectively (**Table S1**). The incidence of probable CAPA in our cohort, was estimated to  
157 7.1, 12.8 and 23.9% in patients admitted to the ICU, mechanically ventilated and who clinically  
158 worsened after initiation of mechanical ventilation, respectively. Mean delay between intubation and  
159 CAPA diagnosis was 8 (5-17) days. Thirteen patients with possible (n=2) or probable (n=11) CAPA  
160 received more than 3 days of antifungals (voriconazole, n=12; liposomal amphotericin B, n=1).  
161 Mortality in patients who received antifungals for probable CAPA was not significantly different from  
162 patients with probable CAPA who did not (76.5 vs 52.0%, p=0.11). Differences between first and  
163 second COVID-19 waves were compared (**Table S2**).

164 *Evaluation of mycological criteria in relation with patient mortality*

165 Mortality rate tended to increase in patients with probable CAPA (61.9%) compared to patients who  
166 were not classified as CAPA (46.3%, p=0.08), with no difference with possible CAPA (54.5%,  
167 p=0.60). Survival curves are shown in **Figure 2A** (logrank test, p=0.06). Mortality was significantly  
168 different in patients with 0,  $\leq 2$  and  $\geq 3$  mycological criteria (logrank test, p=0.04) with death rate of  
169 43.1, 58.1 and 76.4% respectively (**Figure 2B**).

170 All mycological criteria were studied separately and analyzed in the light of repeated samples.

171 (i) Direct examination evidenced *Aspergillus*-type hyphae in 2.5% (8/318) of samples (BAL, n=5 and  
172 tracheal aspirate, n=3) from 8/160 patients and was systematically associated with a median of 3 other  
173 mycological criteria [IQR 2-4] and a mortality rate of 100%.

174 (ii) A proportion of 11.7% (41/350) respiratory samples (BAL, n=29; tracheal aspirate, n=12) from  
175 32/176 patients grew *Aspergillus* spp. (*Aspergillus fumigatus*, n=35; *Aspergillus niger*, n=6). Overall

176 mortality in patients with a positive *Aspergillus* culture was 68.8%. Isolated positive cultures (i.e. no  
177 other positive mycological criteria) were found in 14 patients, of which 4 had a second respiratory  
178 sample. Only 50.0% (2/4) remained positive despite the absence of antifungal prescription between  
179 both samples. Mortality in this subgroup was 71.4% (10/14).

180 (iii) GM was measured in 255 BAL samples from 144 patients and 8.2% (21/255) had an index level  
181 >1.0. A total of 6 samples from 5 patients showed isolated increased GM levels ranging from 1.6 to  
182 4.3 with a median of 3.1 [IQR 2.3-3.9]. All these patients had a second BAL performed in the next 7  
183 days or less and none was positive, while only one patient received voriconazole between both  
184 samples. A proportion of 20% (1/5) of these patients died, while 62.5% (5/8) died if BAL GM was  
185 associated to at least one other positive criterion ( $p=0.26$ ).

186 (iv) *Aspergillus* DNA was detected in 13.1% (46/350 from 32/176 patients) of respiratory samples  
187 with a median Cq value of 30.7 [IQR 29.6-32.3]. Cq values were significantly lower when positive *Af*-  
188 qPCR was associated with other mycological criteria with a median of 30.2 [IQR 29.4-31.8] vs. 35.8  
189 [IQR 31.5-36.9] when found as the sole mycological criterion ( $p<0.001$ ). A ROC curve was computed  
190 to identify the best *Af*-qPCR Cq value to predict mortality (Figure S1). In our settings, including 46  
191 positive values, a cut-off value of Cq=32 was associated with the best sensitivity (88.9% [IC95 67.2-  
192 98.0]) and specificity (57.1% [IC95 32.6-78.6]) to predict mortality in case of positive *Af*-qPCR. The  
193 area under the curve was 0.73 ( $p=0.03$ ). Mortality associated with Cq cut-off values of 36 and 32 are  
194 shown in Figure 3A and 3B, respectively. While a Cq <32 was significantly associated with mortality  
195 (logrank test,  $p=0.007$ ), a Cq <36 was not (log rank test,  $p=0.19$ ). Of note, 7 patients had a Cq between  
196 32 and 36, 14.3% (1/7) only died. qPCR data acquired from BAL and non-BAL samples was  
197 compared (Figure 3C). Cq values were lower in non-BAL samples compared to BAL samples in non-  
198 survivors ( $p=0.009$ ) but not significantly different in survivors ( $p=0.30$ ). For 12 patients with positive  
199 qPCR, a second sampling was performed of which 75% (9/12) were still positive, confirming the first  
200 sample result. In the 3 patients with negative second sample, one received voriconazole between both  
201 samples. Twenty-nine positive *Af*-qPCR were performed on BAL with concomitant GM tests. As  
202 expected, a significant inverse correlation was observed between GM index and Cq values (slope=  
203  $0.60 \pm 0.20$ ;  $R^2=0.26$ ;  $p=0.005$ ) (Figure 3D). Of note, 22 respiratory samples presented positive *Af*-

204 qPCR and negative culture despite intermediate/high fungal load with a median Cq of 31.8 [30.7-  
205 35.2]. A proportion of 81.8% (18/22) of positive *Af*-qPCR/negative *Af*-culture grew *Candida* spp. in  
206 culture, in comparison to 29.1% (7/24) in positive *Af*-qPCR/positive *Af*-culture samples ( $p < 0.001$ ).

207 (v) At least one specific positive blood marker, *i.e.* serum GM tested in 73.3% (129/176) and/or *Af*-  
208 qPCR in plasma available in 50.0% (88/176) of our patients, was positive in 8 patients, systematically  
209 associated with additional mycological criteria (median=3.5 [IQR 3-5]) and a 100%-mortality rate  
210 except for one patient with GM serum level of 0.6 for whom no other samples were obtained and who  
211 survived (Table S3).

212 (vi) BDG was performed in 74.4% (131/176) of our patients. Positive BDG was associated with death  
213 in all patients (75.8 vs 47.9%;  $p = 0.006$ ).

214

## 215 Discussion

216 In this large homogeneous 1-year retrospective cohort of 176 mechanically ventilated COVID-19  
217 patients ( $n = 350$  respiratory samples), we evaluated the diagnostic and prognostic values of seven  
218 mycological criteria in CAPA. A positive examination of respiratory samples (*i.e.* *Aspergillus*-type  
219 hyphae), a serum GM index  $> 0.5$  and a positive plasma *Af*-qPCR were systematically associated with  
220 other mycological criteria and to a mortality of 100%. Conversely, BAL GM index  $> 1.0$  and positive  
221 respiratory sample culture were frequently found as isolated mycological criteria (28.5 and 34.1% of  
222 positive samples, respectively) mostly not confirmed on a second sample if performed in the absence  
223 of treatment. This raises doubts on the ability of these criteria to accurately diagnose CAPA taking  
224 into account the possibility of false positive results of culture (sample, culture contamination) and  
225 galactomannan (GM cross-reaction).

226

### 227 *Evaluation of single criteria in critically ill COVID-19 patients*

228 According to ECMM/ISHAM consensus criteria (3), BAL direct examination identifying  
229 *Aspergillus*-type hyphae, positive *Aspergillus* sp. culture of BAL, BAL GM  $> 1.0$ , BAL *Af*-qPCR  $< 36$   
230 Cq and serum GM  $> 0.5$  are each sufficient criterion to diagnose CAPA, without any superiority  
231 between them. Here, we found positive direct examination to be associated with 100% mortality rate

232 and at least two other mycological criteria highlighting the importance of this criteria most likely  
233 reflecting high fungal burden and therefore poor prognosis. Blot *et al.* algorithm using more stringent  
234 criteria to diagnose pulmonary aspergillosis in the critically ill patients included positive cytological  
235 smear showing branching hyphae as mandatory criteria (16).

236 Surprisingly, 8.4% (14/176) of patients had a single positive culture with a negative qPCR  
237 expected to be similarly or more sensitive than culture. Discrepancies can be attributed to extremely  
238 low fungal load or contamination of the sample by ubiquitous *Aspergillus* spores during sampling or  
239 handling of the sample. Therefore, cautious interpretation of isolated positive culture may be  
240 recommended even in BAL and semi-quantification or quantification of CFUs obtained from agar  
241 plate culture should be interesting to notify and evaluate.

242 GM >1.0 in BAL was found as isolated fungal criteria in 6 samples, not confirmed on a second  
243 BAL. GM is widely used to diagnose invasive aspergillosis but is hampered by variable occurrence of  
244 unreproducible positive results (15) and its concentration was not associated with a higher mortality  
245 rate in a recent multicentric study (17). Aigner *et al.* suggested heavy colonization of the respiratory  
246 tract by *Candida* spp. may trigger galactomannan positivity (18). In our study, five out of 6 isolated  
247 BAL GM were associated with a positive culture for *Candida albicans*. Overall, BAL GM seems to be  
248 a poor marker to distinguish infection and colonization and can be affected by technical issues.  
249 Positive BAL GM could be confirmed by a second positive biomarker such as fungal DNA to initiate  
250 antifungal treatment.

251 In our study, we confirmed the results of a previously published multicentric study using mortality to  
252 analyze mycological criteria characteristics (17). This study as ours showed a association between  
253 serum GM detection (>0.5) and an increased mortality rate in proven/probable CAPA cases (87.5% in  
254 serum GM positive CAPA patients versus 41.7% in serum GM negative CAPA patients).

255 Many interesting features of *Af*-qPCR are highlighted by our study. *Af*-qPCR could serve as  
256 confirmation tool to identify BAL GM and false positive culture results, while quantifying fungal  
257 burden. Its performance appears similar in BAL and non-BAL samples supporting the use of  
258 endotracheal samples to help diagnose patients with CAPA as already proposed (5). *Af*-qPCR may  
259 also help detecting *A. fumigatus* when unable to grow due to heavy *Candida* spp. load in the

260 respiratory tract preventing *Aspergillus* growth, supported by the high significant rate of *Candida*  
261 colonization in positive *Af*-qPCR/negative *Af*-culture samples in our study. However, pitfalls remains,  
262 including the potential variability in the quantification due to heterogeneity of the specimen and the  
263 variable performances of the different *Af*-qPCR assays among centers (19). PCR performances  
264 depends on many factors including DNA extraction method, primers & probe as well as DNA target  
265 amplified, reagents and thermocycler used (20, 21). A Cq value of 36, as defined by ECMM/ISHAM  
266 consensus criteria, does not appear the ideal threshold as our study reveals that this threshold does not  
267 impact the prognosis of the patient. We suggest that the threshold of 32 (*i.e.* fungal load increased by  
268 20), as significantly associated with an increased mortality rate, may be considered as a more relevant  
269 mycological criterion to diagnose CAPA. More studies are needed as any specific cut-off will have to  
270 be optimized for each specific qPCR assay.

271

272 *Does sample type really matter?*

273 Tracheal aspirates are reported to be more often positive than BAL in the critically ill COVID-19  
274 patients but to represent upper airway colonization (3). This was the trend in our study although not  
275 significant with 16.2% and 10.8% of tracheal aspirates and BAL being positive in culture, respectively  
276 ( $p=0.20$ ). Furthermore, mortality in patients with a positive culture in tracheal aspirate was similar to  
277 that in BAL (75.0% vs 72.4%;  $p=0.99$ ) raising doubts on the colonization only hypothesis. Fungal load  
278 evaluated by median *Af*-qPCR was also similar in both sample types. Bronchial aspirates were also  
279 previously suggested to be more sensitive than BAL for the diagnosis of invasive pulmonary  
280 aspergillosis in allogeneic hematopoietic stem cell transplant recipients associated with a more  
281 bronchial tropism during this type of invasive disease (22). Overall, a positive endotracheal sample  
282 should be sufficient to delineate probable aspergillosis for patient management, as suggested by  
283 others, especially if it is supported by one or more other criteria (5, 16).

284

285 *Combination of criteria for the diagnosis of CAPA*

286 According to the significant difference in the survival of patients depending on the number of  
287 positive criteria and initially proposed by White et al. to diagnose CAPA when clear recommendations

288 were lacking (23), we propose to use a combination of criteria to diagnose CAPA but our suggestion  
289 needs further discussion. In White and al.,  $\geq 2$  positive criteria were needed when much more stringent  
290 radiological evidence than ECMM/ISHAM guidelines was lacking (i.e. nodules, halo or consolidation  
291 instead of infiltrates being sufficient as currently recommended). Out of 135 screened patients, 16 had  
292 multiple *Aspergillus* criteria while 23 had a single criterion but mortality in both groups was not  
293 mentioned. Although, the number of positive criteria would reflect fungal burden, which was already  
294 suggested in immunocompromised IPA patients (24) and recently also observed in CAPA patient with  
295 no known risk factors (25), combining criteria could help overcome false positive issues and  
296 overdiagnosis of CAPA. Reflecting most probably higher fungal loads, positive direct examination,  
297 positive serum GM and *Af*-qPCR should be included in a future algorithm as criteria with higher  
298 weighting coefficient (Figure 4). Isolated BAL GM and isolated positive culture may be given less  
299 weight especially if not coupled and confirmed to *Af*-qPCR on the same sample or associated with  
300 another criteria. This could also harmonize the diagnosis regardless of the type of respiratory sample,  
301 which makes classifying patients compelling.

302

### 303 *Limits of this study*

304 Diagnosis of CAPA has been a hurdle in patients with no EORTC/MSGERC host factors preventing  
305 the identification of patients who might benefit from antifungal prophylaxis or treatment. Similarly to  
306 every study evaluating mycological criteria characteristics for the diagnosis of CAPA, our design  
307 lacks an adequate gold standard (i.e., histopathological data showing tissue-invasive hyphae).  
308 Evaluating these criteria in relationship to mortality has many biases in this population, in which death  
309 is most likely multifactorial resulting from bacterial/fungal superinfection (26), refractoriness of  
310 COVID-19, and underlying disease decompensation (27). However, only few patients received an  
311 effective and early antifungal treatment, which may be the reason why there is no difference in  
312 mortality between treated and untreated patient. Therefore, using mortality may help identify  
313 significant criteria or combination of criteria reflecting the invasiveness of the *Aspergillus* disease  
314 known to be associated with a high mortality rate.

315

316 **Conclusion**

317 Despite the lack of gold standard, evaluating available mycological criteria for CAPA diagnosis, is a  
318 priority as the pandemic is likely to continue and more data is needed. One isolated positive criterion  
319 is most likely insufficient to diagnose CAPA especially for respiratory sample criteria or should at  
320 least be reevaluated in a second sample. *Af*-qPCR to address false positive and avoid false negative in  
321 case of intense concomitant *Candida* colonization should be considered systematically. However,  
322 standardized prospective studies are needed to compare autopsy results and mycological data to  
323 confirm these findings and better target patients that could benefit from antifungals.

324

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325

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332 **Author contributions**

333 Writing original draft: SD and AA, Writing review & editing: all, Conceptualization: SD, AA.

334 Investigation: SD, ED, SF, MC; Data curation: SD, ED, SF, SV, MC. Formal analysis: SD.

335 Visualization: SD, AA. Supervision: AA.

336 **Conflict of interest**

337 The authors declare no conflict of interest related to the content of the present study

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478 **Table 1.** Patient characteristics.

Characteristics	Patients (n=176)	Samples (n=350)
Age (years), median [Q1-Q3]	64 [58-72]	
Male, n (%)	139 (78.1%)	
Sample per patient, median [Q1-Q3]	2 [1-3]	
Type of samples, n (%)		
BAL n (%)		269 (76.9%)
Tracheal aspirate n (%)		74 (21.1%)
Bronchial aspirate n (%)		7 (2.0%)
<b>Patient with sufficient diagnostic mycological criteria for</b>		
IPA <sup>‡</sup> n (%)	3 (1.7%)	
Probable CAPA* n (%)	42 (23.9%)	
Possible CAPA* n (%)	11 (6.3%)	
Patients with positive BDG only n (%)	13 (7.3%)	

479 BAL: Bronchoalveolar lavage; BDG:  $\beta$ -1,3-D-glucan; CAPA: COVID-19 associated pulmonary aspergillosis;480 IPA: invasive pulmonary aspergillosis; <sup>‡</sup> according to EORTC/MSGERC guidelines \*according to

481 ECMM/ISHAM guidelines

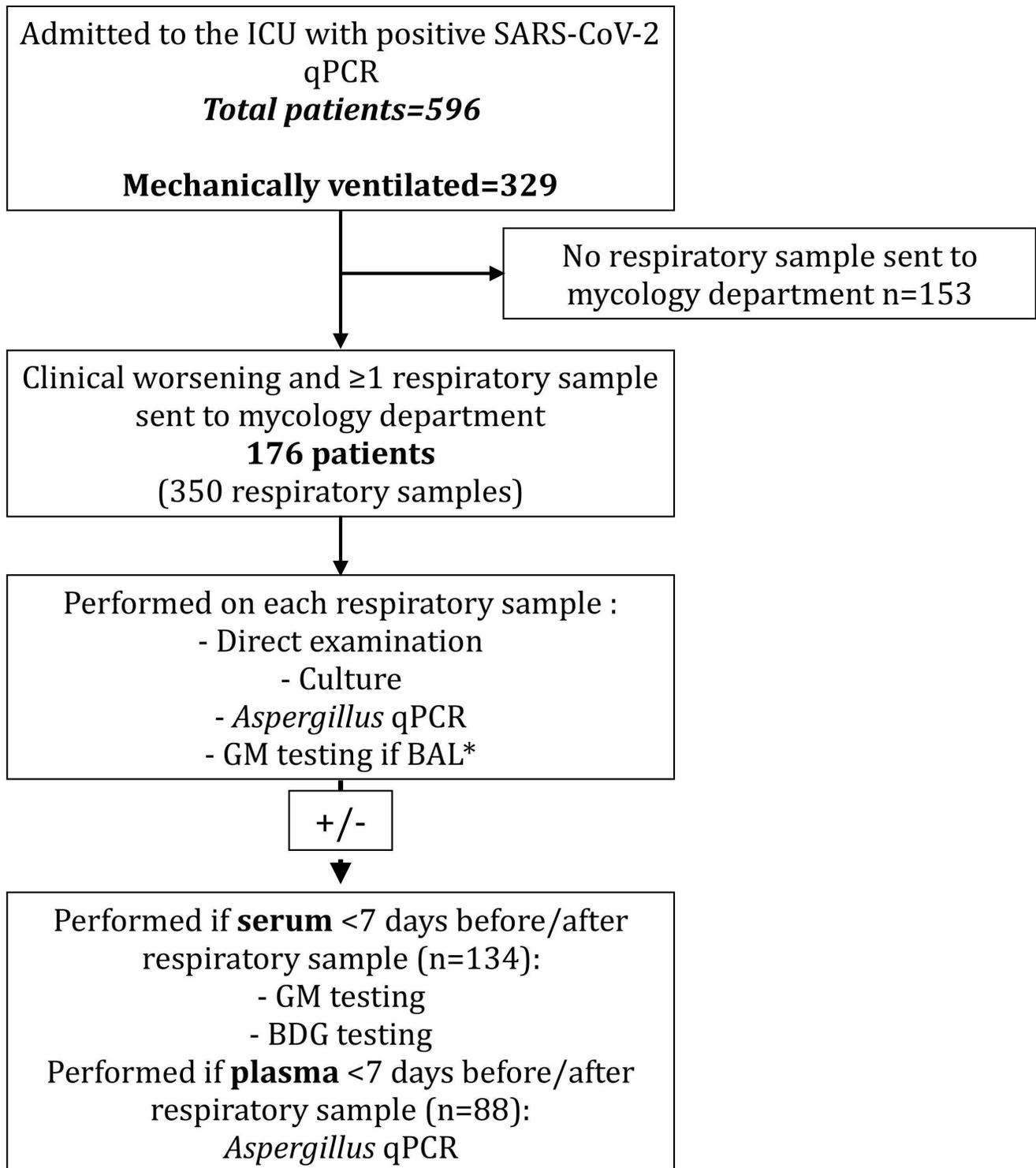
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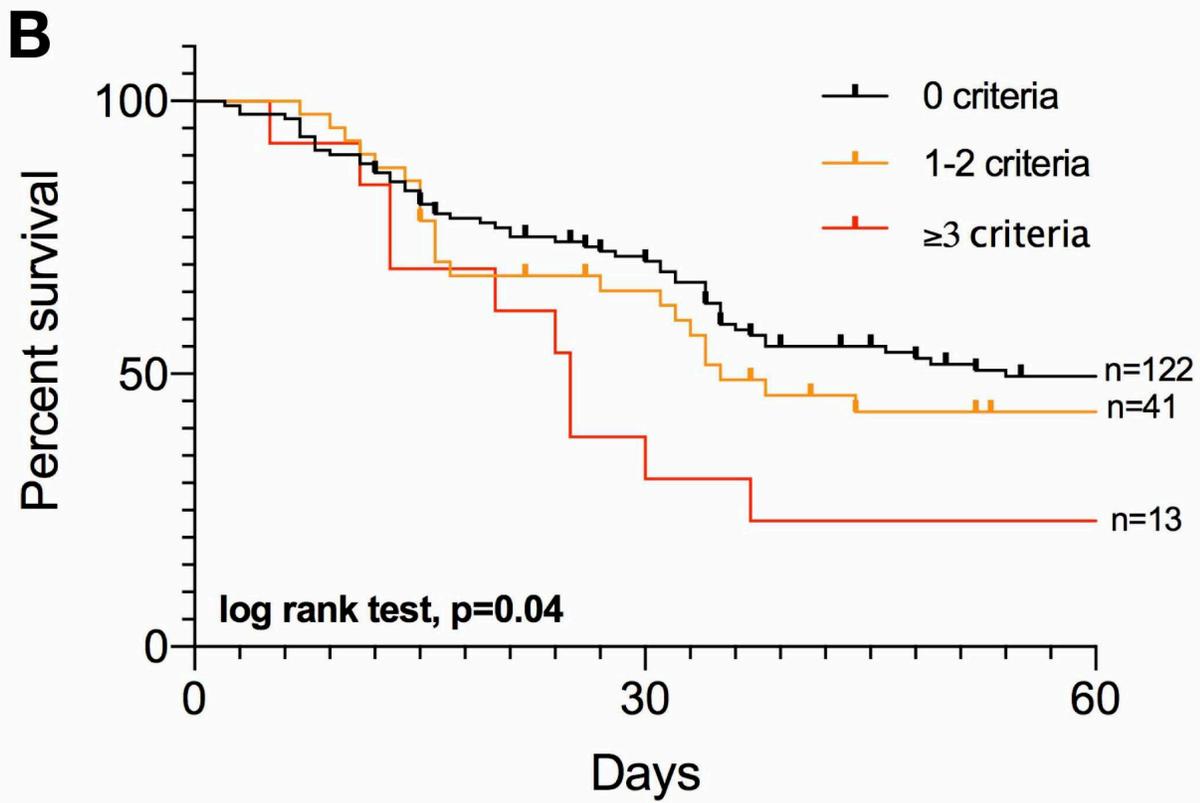
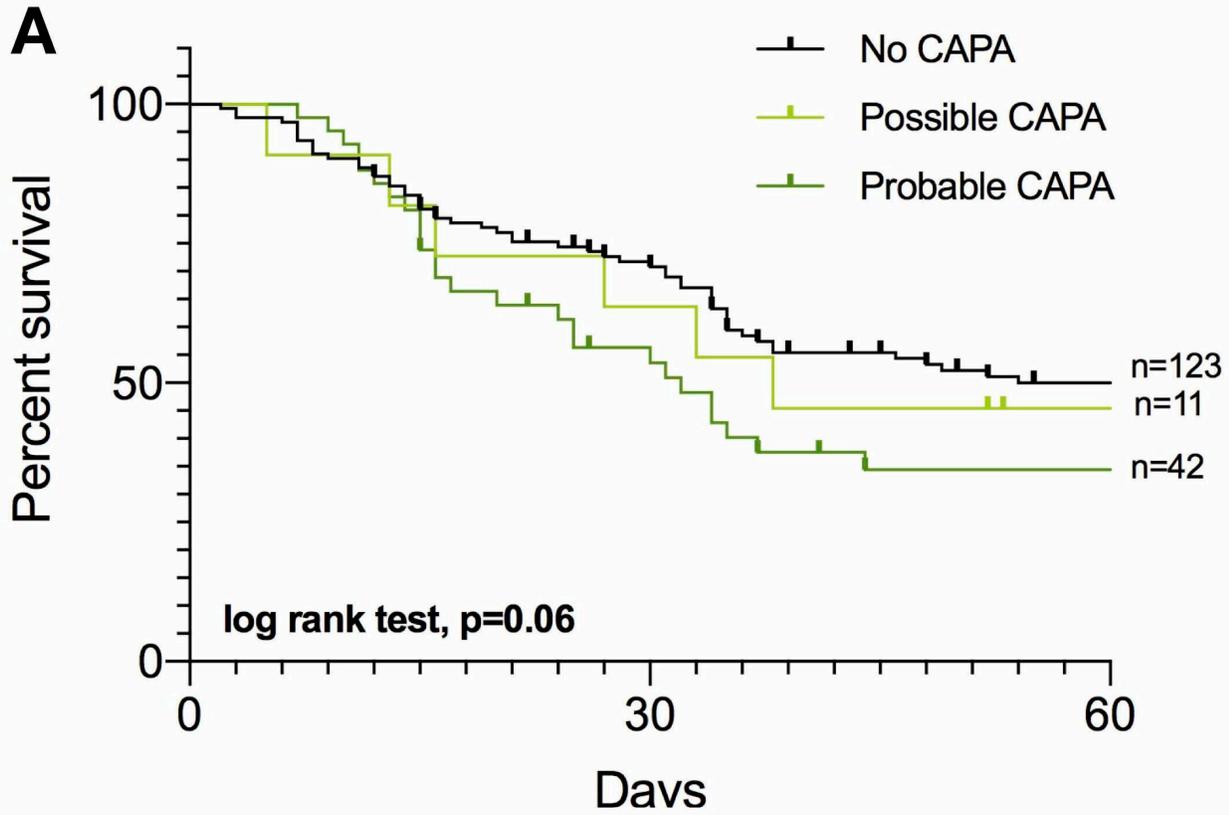
483 **Figure 1.** Study flowchart. \*as recommended by the manufacturer. BAL: bronchoalveolar lavage;  
484 BDG:  $\beta$ -1,3-D-glucans; GM: galactomannan; ICU: intensive care unit; qPCR: quantitative polymerase  
485 chain reaction.  
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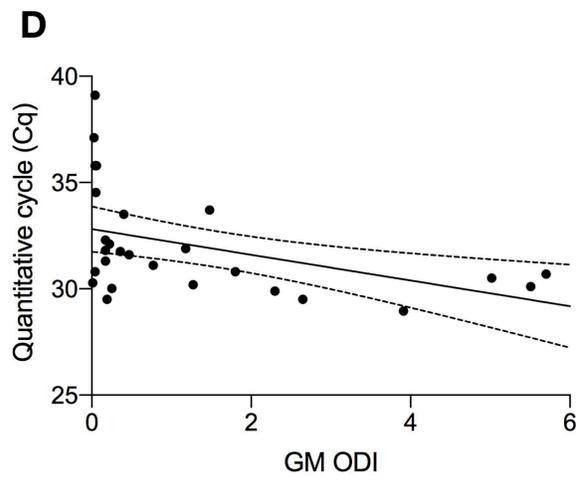
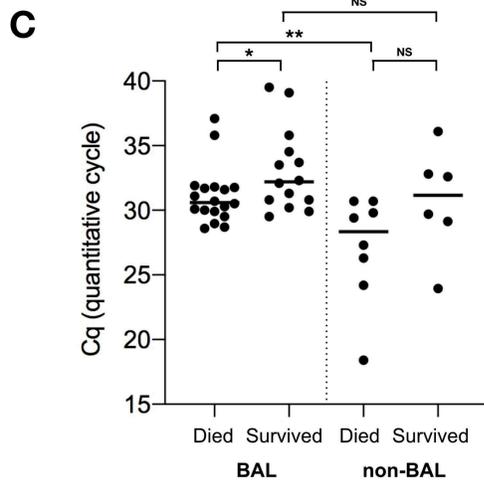
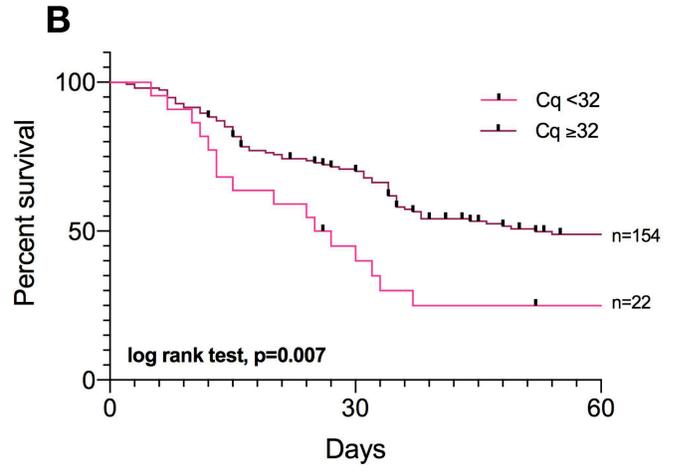
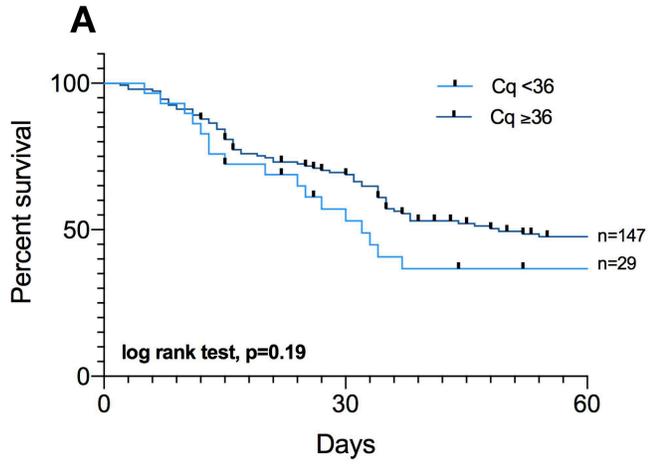
489 **Figure 2.** Kaplan-Meier survival curves for 60-day mortality of patients with (A) no COVID-19  
490 associated pulmonary aspergillosis (CAPA), possible or probable CAPA according to ECMM/ISHAM  
491 consensus criteria (logrank test,  $p=0.06$ ) and with (B) 0, 1 or 2 and  $\geq 3$  positive mycological criteria  
492 (logrank test,  $p=0.008$ ). The following were considered *Aspergillus* criteria. From respiratory  
493 specimens (i) direct respiratory sample examination with branched hyphae suggestive of *Aspergillus*-  
494 type hyphae, (ii) respiratory sample culture growing *Aspergillus* sp., (iii) galactomannan in  
495 bronchoalveolar lavage (index  $>1.0$ ) (iv) *Aspergillus* qPCR in respiratory sample with  $Cq < 36$ . From  
496 serum (i) galactomannan in blood (index  $>0.5$ ), (ii) positive *Aspergillus* qPCR in blood with  $Cq < 40$ ,  
497 and (iii) blood  $\beta$ -D-glucan (BDG)  $>80$  pg/mL.  
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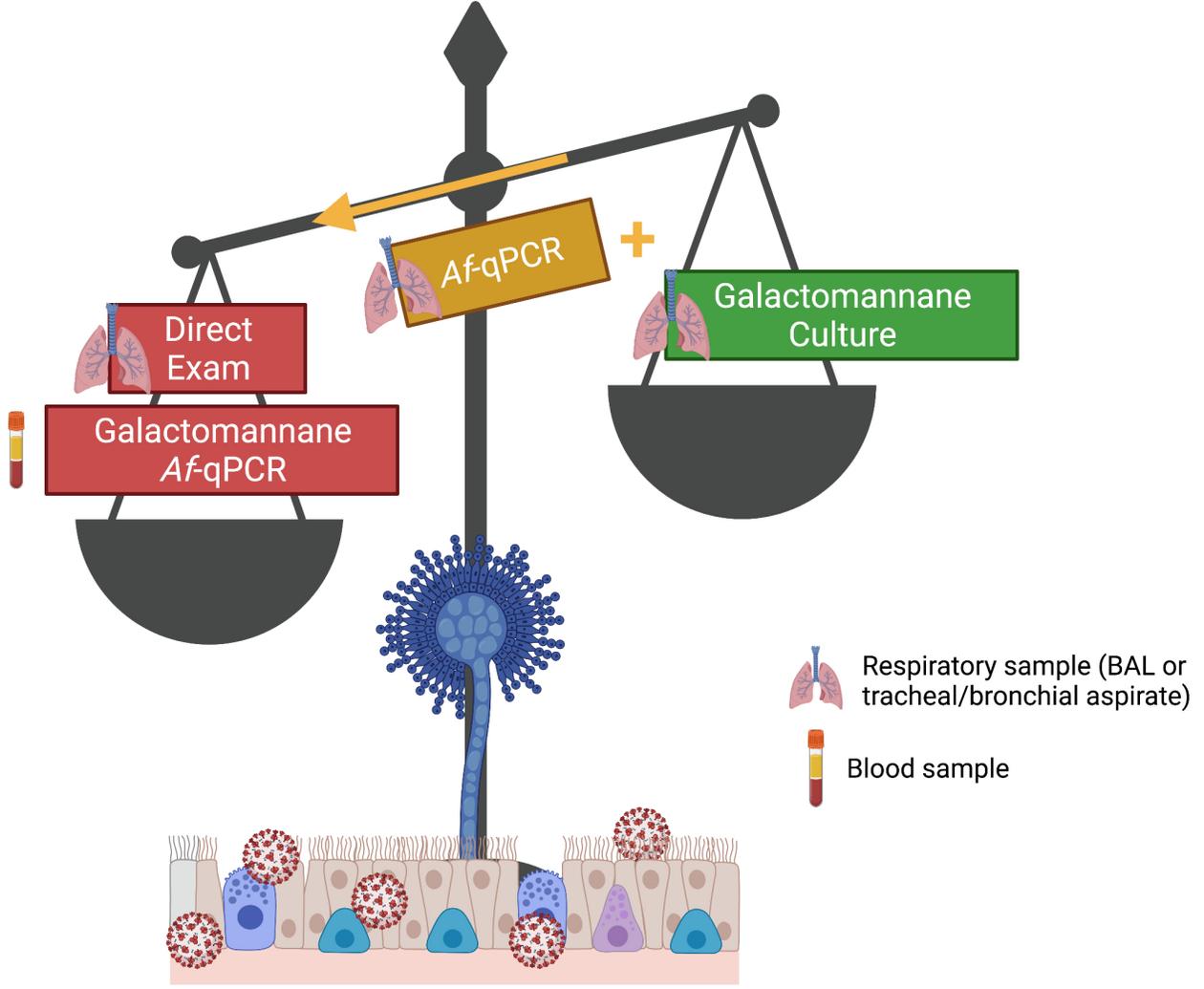
499 **Figure 3. Characteristics of *Aspergillus fumigatus*-qPCR in respiratory sample.** (A & B) Kaplan-  
500 Meier survival curves for 60-day mortality associated with a qPCR cut-off of (A) 36 quantitative cycle  
501 (Cq) and (B) 32 Cq. (C) Scatter plot of qPCR Cq values if performed in BAL and non-BAL samples  
502 depending on patient's outcome. (D) Correlation between galactomannane index (GM ODI) and  
503 quantification cycles (Cq value) in qPCR-positive BAL samples.  
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510 **Figure 4.** Combination and weight of mycological criteria for the diagnosis of COVID-19 associated  
511 pulmonary aspergillosis according to their impact on prognosis. Created with BioRender.com.  
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**March 15<sup>th</sup> - March 1<sup>st</sup> 2021**







**Table S1. Mycological criteria** characteristics of all patients who classified as possible or probable CAPA according to ECMM/ISHAM recommendations.

#	Sex	Age	Type	DE	Culture	Af-qPCR Resp Sample	Galactomannan BAL	Serum BDG	Serum GM	PCR asp	CAPA classification	EORTC_H F	ICU_Death
3	M	62	BAL	NEGATIVE	<b>A. fumigatus + C. albicans</b>	NEGATIVE	0.15	7.8	0.06	NA	Probable	No	Yes
6	M	62	Trach Asp	NEGATIVE	<b>A. Niger + C. albicans</b>	NEGATIVE	NA	<b>305</b>	<b>1.55</b>	NEGATIVE	Probable	No	Yes
14	F	74	BAL	NEGATIVE	C. albicans	<b>35.8</b>	0.04	8.3	0.05	NEGATIVE	Probable	No	No
15	M	62	BAL	NEGATIVE	C. albicans	NEGATIVE	<b>4.27</b>	36.9	0.04	NEGATIVE	Probable	No	No
20	M	71	Trach Asp	NEGATIVE	C. albicans	<b>30.7</b>	NA	<b>331</b>	0.06	NEGATIVE	Possible	Yes	Yes
26	M	63	BAL	NEGATIVE	C. albicans	NEGATIVE	<b>3.31</b>	NA	NA	NA	Probable	No	No
27	F	65	BAL	NEGATIVE	C. glabrata	<b>30.8</b>	<b>1.8</b>	12	0.03	NEGATIVE	Probable	No	No
28	F	59	BAL	NA	<b>A. fumigatus</b>	NEGATIVE	0.03	NA	0.04	NEGATIVE	Probable	No	No
34	M	57	BAL	NEGATIVE	<b>A. fumigatus + C. parapsilosis</b>	NEGATIVE	0.06	7.8	0.05	NEGATIVE	Probable	No	No
40	M	67	BAL	NEGATIVE	<b>A. fumigatus</b>	<b>31.9</b>	<b>1.18</b>	<b>177</b>	0.06	NEGATIVE	Probable	No	Yes
43	F	53	BAL	NEGATIVE	<b>A. fumigatus + C. albicans</b>	NEGATIVE	0.09	NA	NA	NA	Probable	No	Yes
44	F	63	BAL	NA	C. tropicalis + C. albicans	NEGATIVE	0.15	<b>105</b>	<b>0.51</b>	NA	Probable	No	Yes
53	M	56	BAL	NEGATIVE	C. albicans + C. parapsilosis	<b>35.8</b>	0.06	NA	NA	NEGATIVE	Probable	No	Yes
56	M	72	BAL	NEGATIVE	<b>A. fumigatus + C. albicans</b>	NEGATIVE	0.05	46	0.48	NA	Probable	No	Yes
58	M	79	BAL	NA	<b>Aspergillus fumigatus</b>	<b>34.53</b>	0.05	23	0.02	NEGATIVE	Probable	No	No
61	M	51	BAL	NEGATIVE	<b>A. fumigatus + C. albicans</b>	NEGATIVE	0.06	11	0.02	NA	Probable	No	Yes
62	M	75	BAL	NEGATIVE	C. albicans	<b>37.1</b>	0.03	<b>115</b>	0.04	NA	Possible	No	Yes
63	M	77	BAL	NA	<b>Aspergillus fumigatus</b>	<b>28.96</b>	<b>3.91</b>	<b>135</b>	0.37	NEGATIVE	Probable	No	Yes
64	F	75	Trach Asp	<b>POSITIVE</b>	<b>A. fumigatus</b>	<b>18.4</b>	NA	<b>450</b>	0.37	NEGATIVE	Possible	No	Yes
73	M	74	BAL	NEGATIVE	NEGATIVE	<b>31.7</b>	NA	NA	NA	NA	Probable	No	Yes
76	M	58	BAL	NEGATIVE	<b>A. fumigatus + C. albicans</b>	NEGATIVE	0.04	NA	NA	NA	Probable	No	Yes
82	M	76	BAL	NA	<b>A. fumigatus</b>	<b>30.28</b>	0.01	<b>184</b>	0.14	NA	Probable	No	Yes

84	M	63	Trach Asp	NEGATI VE	<b>A. fumigatus + C. albicans</b>	<b>29.8</b>	NA	<b>126</b>	0.3	NEGATIVE	Possible	No	Yes
85	M	76	BAL	NEGATI VE	<b>A. fumigatus + C. tropicalis + C. albicans + C. glabrata</b>	NEGATIVE	0.14	29	0.06	NEGATIVE	Probable	No	Yes
87	M	65	BAL	NEGATI VE	<b>A. fumigatus + C. albicans</b>	NEGATIVE	0.04	NA	NA	NA	Probable	No	Yes
91	M	58	Trach Asp	NEGATI VE	<b>A. fumigatus + C. albicans</b>	<b>27.3</b>	NA	<b>353</b>	<b>2.42</b>	<b>37.4</b>	Probable	Yes	Yes
92	M	47	Trach Asp	NEGATI VE	<b>C. albicans + A. fumigatus</b>	NEGATIVE	NA	14	0.09	NEGATIVE	Possible	Yes	Yes
96	M	58	Trach Asp	NEGATI VE	<b>C. albicans + A. fumigatus</b>	NA	NA	7.8	0.03	NA	Possible	No	No
100	M	68	Trach Asp	<b>POSITIV E</b>	<b>A. fumigatus</b>	<b>29.4</b>	NA	<b>523</b>	<b>1.81</b>	<b>30.8</b>	Probable	No	Yes
103	F	69	Trach Asp	NA	<b>Aspergillus fumigatus</b>	<b>23.94</b>	NA	7.8	0.03	NEGATIVE	Possible	No	No
104	M	56	Trach Asp	<b>POSITIV E</b>	<b>Aspergillus fumigatus</b>	<b>26.3</b>	NA	7.8	0.05	NEGATIVE	Possible	No	Yes
107	M	62	BAL	<b>POSITIV E</b>	<b>A. fumigatus + A. nidulans</b>	<b>28.7</b>	<b>6.32</b>	<b>420</b>	0.12	NEGATIVE	Probable	Yes	Yes
111	M	69	BAL	NEGATI VE	<b>C. albicans</b>	NEGATIVE	<b>1.58</b>	7.8	0.06	NEGATIVE	Probable	No	No
112	F	50	BAL	NEGATI VE	NEGATIVE	NEGATIVE	<b>2.05</b>	7.8	0.03	NEGATIVE	Probable	No	No
114	M	78	BAL	<b>POSITIV E</b>	<b>A. fumigatus</b>	<b>28.6</b>	NA	<b>185</b>	0.06	<b>36.9</b>	Probable	No	Yes
115	M	60	BAL	NEGATI VE	<b>C. albicans</b>	<b>32.1</b>	0.22	<b>266</b>	0.07	NA	Probable	No	No
119	M	67	BAL	NEGATI VE	<b>C. albicans</b>	NEGATIVE	<b>2.98</b>	7.8	0.1	NEGATIVE	Probable	No	Yes
120	M	74	BAL	<b>POSITIV E</b>	<b>A. niger + C. parapsilosis</b>	NEGATIVE	<b>4.68</b>	30.7	<b>4.63</b>	NEGATIVE	Probable	No	Yes
127	F	63	Trach Asp	NEGATI VE	<b>C. albicans</b>	<b>36.1</b>	NA	7.8	0.06	NA	Possible	No	No
131	M	32	BAL	NEGATI VE	<b>A. fumigatus + C. albicans</b>	<b>32.3</b>	0.17	NA	NA	NA	Probable	No	No
133	F	82	BAL	NEGATI VE	<b>C. albicans + C. glabrata</b>	NEGATIVE	<b>5.24</b>	<b>253</b>	0.06	NEGATIVE	Probable	No	Yes
134	M	74	BAL	NEGATI VE	<b>A. fumigatus + A. niger + C. albicans</b>	<b>29.9</b>	<b>2.3</b>	<b>383</b>	0.05	NEGATIVE	Probable	No	No
136	m	81	BAL	NEGATI VE	<b>C. albicans</b>	<b>31.8</b>	0.17	<b>381</b>	0.06	NEGATIVE	Probable	No	Yes
137	F	73	BAL	NEGATI VE	<b>A. niger + Rhizopus sp.</b>	NEGATIVE	0.08	9	0.07	NEGATIVE	Probable	No	Yes
138	M	56	BAL	NEGATI VE	<b>A. fumigatus</b>	<b>33.7</b>	<b>1.48</b>	14.4	0.18	NEGATIVE	Probable	No	No
139	F	70	BAL	NEGATI VE	<b>C. albicans + C. glabrata</b>	<b>30.8</b>	0.04	<b>486</b>	0.06	NEGATIVE	Probable	No	No

140	M	66	BAL	<b>POSITIV E</b>	<i>C. albicans</i>	<b>29.9</b>	<b>6.2</b>	55.8	<b>2.6</b>	NA	Probable	No	Yes
146	F	82	BAL	NEGATI VE	<i>C. albicans</i>	<b>31.1</b>	0.77	9	0.09	NEGATIVE	Probable	No	Yes
147	F	59	Bronchia l Asp	NEGATI VE	<i>C. tropicalis</i>	<b>32.6</b>	NA	<b>523</b>	0.08	NEGATIVE	Possible	No	No
151	M	64	Trach Asp	NEGATI VE	<b>A. fumigatus</b>	<b>29.13</b>	NA	<b>523</b>	0.1	NEGATIVE	Possible	No	No
152	M	81	BAL	NEGATI VE	<b>A. fumigatus + C. albicans + C. glabrata</b>	<b>30.02</b>	0.25	7.8	0.14	NEGATIVE	Probable	No	Yes
164	F	68	BAL	NEGATI VE	NEGATIVE	NEGATIVE	0.03	39.7	<b>0.6</b>	NEGATIVE	Probable	No	No
175	M	72	BAL	<b>POSITIV E</b>	<b>A. fumigatus + C. albicans</b>	<b>31.3</b>	0.17	46.2	0.05	NEGATIVE	Probable	No	No

**Table S2.** Patients and standard of care characteristics according to the first or second COVID-19 wave. COVID-19 standards of care and patients' characteristics evolved between the first (March 16<sup>th</sup> 2020 – June 30<sup>th</sup> 2020) and second (July 1<sup>st</sup> 2020 – February 28<sup>th</sup> 2021) pandemic waves in France. Patients admitted to the ICU were less mechanically ventilated during the second wave (67.2 vs 36.1%;  $p < 0.001$ ) and median delay for intubation after admission was longer (0 vs 2 days;  $p < 0.001$ ). Median age of patients who clinically worsened after the initiation of mechanical ventilation was higher during the second wave (63 [IQR 56-69] vs 68 [IQR 60-75];  $p = 0.002$ ). Although not significant, mortality increased in this subset of patients (44.7 vs 59.2%;  $p = 0.06$ ). Despite these differences, the incidence of CAPA and delay of antifungal introduction if  $\geq 1$  positive mycological criterion did not significantly differ.

	<b>1<sup>st</sup> wave (n=105)</b> March 15 <sup>th</sup> 2020 – June 30 <sup>th</sup> 2020	<b>2<sup>nd</sup> wave (n=71)</b> July 1 <sup>st</sup> 2020- March 1 <sup>st</sup> 2021	<i>P</i>
Age (years), median [Q1-Q3]	63 [56-69]	68 [60-75]	<b>0.002</b>
Male, n (%)	85 (80.9)	54 (76.1)	0.43
Death, n (%)	47 (44.7)	42 (59.2)	0.06
Ratio of patients intubated if admitted to the ICU	246/366 (67.2%)	83/230 (36.1%)	<b>&lt;0.001</b>
ICU to tracheal intubation time (days), median [Q1-Q3]	0 [0-2]	2 [0-4]	<b>&lt;0.001</b>
<b>Patients with sufficient diagnostic mycological criteria for</b>			
IPA {Donnelly:2019cq}	2 (1.9%)	1 (1.4%)	0.99
CAPA {Verweij:2020gy}	20 (19.0%)	15 (21.1%)	0.91
Probable CAPA {Koehler:2020ki}	23 (21.9%)	19 (26.8%)	0.46
Possible CAPA {Koehler:2020ki}	8 (7.6%)	3 (4.2%)	0.36
Introduction of antifungal after first positive criteria (BDG excluded)	1 [0-4]	0 [0-2]	0.49
Patients who received antifungal if positive criteria (BDG excluded)	8/32 (25.0%) with $\geq 1$ criterion	10/22 (45.5%) with $\geq 1$ criterion	0.15

ICU: intensive care unit

**Table S3.** Correlations between any positive *Aspergillus* criteria in plasma/serum and respiratory sample and  $\beta$ -D-glucan

Patient #	Positive serum/plasma criteria	Positive criteria in respiratory sample	$\beta$ -D-glucan
6	GM ODI=1.55	(TA) Culture*	<b>305</b>
44	GM ODI=0.51	BAL was performed but no mycological criteria were positive	<b>105</b>
91	GM ODI=2.42 / <i>Af</i> -qPCR Cq=37.4	(TA) Culture / <i>Af</i> -qPCR Cq=27.3	<b>353</b>
100	GM ODI=1.81 / <i>Af</i> -qPCR Cq=30.8	(TA) DE / Culture / <i>Af</i> -qPCR Cq=29.4	<b>&gt;523</b>
120	GM ODI=4.63	(BAL) Culture* / GM=5.37	30.7
140	GM ODI=2.6	(BAL) DE / <i>Af</i> -qPCR Cq=29.9 / GM=6.2	55.8
164	GM ODI=0.6	(BAL) None	39.7
114	<i>Af</i> -qPCR Cq=36.9	(TA) DE / Culture / <i>Af</i> -qPCR Cq=28.6	<b>185</b>

\*Culture was positive with *Aspergillus* section *nigri* unamplified by our in-house *Af*-qPCR, BAL: bronchoalveolar lavage, DE: direct examination; GM ODI: galactomannan optical density index (positive if  $\geq 0.5$ ); qPCR: quantitative polymerase chain reaction; TA: tracheal aspirate; Cq: quantification cycle

**Figure S1. Characteristics of *Aspergillus fumigatus*-qPCR in respiratory sample.** Sensitivity and specificity of Af-qPCR to predict mortality using a receiving operating curve. Of note, optimal cut-off = 32 Cq.

