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1   **Title:** The impact of anti-mold prophylaxis on *Aspergillus* PCR blood testing for the  
2   diagnosis of invasive aspergillosis.

3

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30 **Key words:** *Aspergillus* PCR, blood, antifungal prophylaxis

31

32 **Running title:** Impact of antifungal prophylaxis on *Aspergillus* PCR performance

## 33 **SYNOPSIS**

### 34 **Background**

35 The performance of the galactomannan enzyme immunoassay (GM-EIA) is impaired in  
36 patients receiving mold-active antifungal therapy. The impact of mold-active antifungal  
37 therapy on *Aspergillus* PCR testing needs to be determined.

### 38 **Objectives**

39 To determine the influence of anti-mold prophylaxis (AMP) on the performance of PCR  
40 blood testing to aid the diagnosis of proven/probable invasive aspergillosis (IA).

### 41 **Methods**

42 As part of the systematic review and meta-analysis of 22 cohort studies investigating  
43 *Aspergillus* PCR blood testing in 2912 patients at risk of IA, subgroup analysis was  
44 performed to determine the impact of AMP on the accuracy of *Aspergillus* PCR. The  
45 incidence of IA was calculated in patients receiving and not receiving AMP. The impact of  
46 two different positivity thresholds, requiring either a single PCR positive test result or  $\geq 2$   
47 consecutive PCR positive test results, on accuracy was evaluated. Meta-analytical pooling  
48 of sensitivity and specificity was performed by logistic mixed-model regression.

### 49 **Results**

50 In total, 1661 (57%) patients received prophylaxis. The incidence of IA was 14.2%,  
51 significantly lower in the prophylaxis group (11-12%) compared to non- prophylaxis group  
52 (18-19%) ( $P < 0.001$ ). The use of AMP did not affect sensitivity, but significantly decreased  
53 specificity (Single PCR positive threshold: 26% reduction ( $P: 0.005$ ;  $\geq 2$  consecutive PCR  
54 positive threshold: 12% reduction ( $P: 0.019$ )).

### 55 **Conclusions**

56 Contrary to its influence on GM-EIA, AMP significantly decreases *Aspergillus* PCR  
57 specificity, without affecting sensitivity, possibly a consequence of AMP limiting the clinical

58 progression of IA and/or leading to false negative GM-EIA results, preventing the  
59 classification of probable IA using the EORTC/MSGERC definitions.

60

## 61 INTRODUCTION

62 There is convincing evidence showing that both the sensitivity and specificity of the  
63 galactomannan enzyme immunoassay (GM-EIA) are impaired in patients receiving mold-  
64 active antifungal therapy (AFT) [1, 2]. Previous exposure to AFT also needs to be  
65 considered when interpreting *Aspergillus* PCR results, as animal studies and clinical trials  
66 both indicate that AFT may adversely affect test performance. [3, 4]. Recently, a  
67 systematic review and meta-analysis investigating *Aspergillus* PCR blood testing to aid the  
68 diagnosis of invasive aspergillosis (IA) in immunocompromised patients was performed [4].  
69 Most patients had a haematological malignancy, had undergone hematopoietic stem cell  
70 transplantation (HSCT) or were solid organ transplant (SOT) recipients. The mean  
71 prevalence of proven or probable IA was 16.3 % (769/4718 patients) [4]. Pooled data  
72 showed that PCR has moderate diagnostic accuracy when used as a screening test for IA  
73 in high-risk patient groups. [4] The sensitivity and specificity of PCR for the diagnosis of IA  
74 varied according to the interpretative criteria used to define a test as positive. Considering  
75 a single positive test result as significant the sensitivity and specificity were 79.2% and  
76 79.57%, respectively, changing to 59.6% and 95.1% when requiring two consecutive  
77 positive results. Diagnostic odd ratios (DORs), negative (NPV) and positive predictive  
78 values (PPV) were 14.8/28.8, 95%/92% and 42/70%, respectively for a single positive test,  
79 and two consecutive positive tests. [4]

80 As part of the systematic review and meta-analysis of cohort studies investigating  
81 *Aspergillus* PCR blood testing in patients at risk of IA, subgroup analysis was conducted  
82 and included an evaluation of the impact of anti-mold prophylaxis (AMP) on the diagnostic  
83 accuracy of *Aspergillus* PCR. This manuscript describes those findings.

## 84 PATIENTS AND METHODS

### 85 Meta-Analytical Review

86 The index tests included PCR testing of blood specimens (whole blood or serum/plasma)  
87 and subsequently methodological heterogeneity was evident (different DNA extraction  
88 methods and PCR methods (e.g. nested, PCR-ELISA, qPCR)). Depending on the original  
89 date of publication, proven/probable IA was defined using either the original (2002) or the  
90 revised (2008) EORTC/MSG consensus definitions of invasive fungal disease (IFD) [5, 6].  
91 At the time of analysis there had been no studies using the recently published second  
92 revision of the EORTC/MSG consensus definitions, subsequently *Aspergillus* PCR was not  
93 a mycological criterion for defining IA. [7]. Systemic AMP was defined when patients  
94 received itraconazole, voriconazole, posaconazole, amphotericin B or caspofungin.

95 The cumulative incidence of IA was calculated in both patients receiving and not receiving  
96 AMP. The impact of two different positivity thresholds, requiring either a single PCR  
97 positive test result or  $\geq 2$  consecutive PCR positive test results, on diagnostic accuracy was  
98 evaluated, as the latter threshold is associated with increased specificity. A meta-analytical  
99 pooling of sensitivity and specificity was performed by logistic mixed-model regression,  
100 where the dependent variable was the positivity of the PCR test, and the covariates were  
101 “IA”, AMP (yes/no), and itraconazole versus other AMP [8]. The final comparison included  
102 as the efficacy of itraconazole prophylaxis could be inferior to other AMP. As post-  
103 estimation results, DOR, positive likelihood ratio (LR +tive), and negative likelihood ratio  
104 (LR -tive) were obtained. PPV and NPV were calculated using the Bayes’ rule as indicated  
105 by WHO, using sensitivity, specificity and prevalence data [9]. For this purpose, the  
106 considered incidence was the value calculated for each of the four individual groups,  
107 according to positivity threshold and prophylaxis. Logistic mixed-model regression analysis  
108 was used over conventional meta-analytical pooling (Supplementary Table 2) as it

109 preserves the randomization of each individual study, thereby limiting any confounding  
110 bias introduced through simple data pooling [8]. Calculations were performed with Stata v.  
111 16.0 and MS Excel

112

## 113 RESULTS

114 Of the 29 primary studies included in the primary meta-analysis [4], 12 used AMP across  
115 the entire population or in subsets of patients, 17 studies did not use AMP, although four of  
116 these studies used fluconazole for prophylaxis against certain *Candida* species. The  
117 sensitivity/specificity data for *Aspergillus* PCR associated with EORTC/MSGERC defined  
118 IA and the administration of antifungal prophylaxis was available from 22 primary studies  
119 (Supplementary table 1). Ten studies administered AMP to all patients (n=1438), 10  
120 studies did not administer prophylaxis to any patients (n=1027) and two studies  
121 differentiated patients receiving (n=223), or not receiving prophylaxis (n=224). In total,  
122 1661 patients received prophylaxis and 1251 did not receive prophylaxis.

123 The overall incidence of IA was 14.2% (95% CI: 13.0-15.5). The incidence of IA was  
124 significantly lower in the prophylaxis group compared to non- prophylaxis group: 11.9%  
125 (164/1373, 95% CI:10.3-13.8) vs 18.7% (216/1156, 95% CI: 16.5-21.0) in studies using a  
126 single PCR positivity threshold, and 11.4% (155/1356, 95% CI: 9.8-13.2) vs 18.0%  
127 (72/401, 95% CI: 14.5-22.0) in those requiring  $\geq 2$  consecutive positive test results; the  
128 differences were statistically significant ( $P < 0.001$ ), irrespective.

129 The use of AMP had no relevant effect on sensitivity, LR -tive, and NPV, but decreased  
130 specificity, LR +tive, and PPV (Table 1). When examining data under the criterion “single  
131 positive test result” (21 studies, 2529 patients, 1373 receiving prophylaxis and 1156  
132 without prophylaxis) the use of AMP decreased specificity (from 0.86 to 0.60;  $P$ : 0.005),  
133 PPV (from 0.57 to 0.22) and DOR (from 25.7 to 7.60;  $P$ : 0.01) (Table 1). Requiring  $\geq 2$   
134 consecutive positive results (12 studies, 1757 patients, 1356 receiving prophylaxis and



135 401 without prophylaxis), AMP use decreased specificity (from 0.98 to 0.86; *P*: 0.019),  
136 PPV (from 0.87 to 0.37) and DOR (from 98.1 to 11.8; *P*: 0.02), but again had no significant  
137 impact on sensitivity. Excluding studies with itraconazole prophylaxis did not significantly  
138 affect the effect performance (data not shown).

139

## 140 **DISCUSSIONS**

141 Sensitivity and specificity data were determined in subgroups of patients receiving or not  
142 receiving AMP from 22 cohort studies reporting the diagnostic accuracy of PCR testing of  
143 blood for the diagnosis of IA in immunocompromised patients. As expected, the cumulative  
144 incidence of IA was significantly lower in patients receiving AMP. Prophylaxis significantly  
145 decreased the specificity of PCR, irrespective of the interpretative criteria used to define  
146 positivity. Conversely, AMP had no significant impact on sensitivity. Likewise, AMP  
147 decreased PPVs considerably, but had no relevant effect on NPVs. A decrease in DOR  
148 with both interpretative criteria was observed. One limitation of the study is the effect of  
149 AMP on the PCR performance of assays compliant with FPCRI methodological  
150 recommendations was not performed. Given most studies predated the availability of  
151 these recommendations, the number of compliant methods will be limited and subsequent  
152 additional analysis will be needed to determine if optimal methods minimized the effect of  
153 AMP.

154 Data from clinical trials and systematic review show that mold-active antifungals affect the  
155 accuracy of GM-EIA [1, 2, 10]. However, in these studies the effect of anti-mold drugs was  
156 heterogeneous, depending on the incidence of breakthrough infections, time of drug  
157 administration, and positivity threshold used for the GM-EIA test. In one study, AMP had  
158 only a minor effect on sensitivity and decreased specificity, but the pretest probability of IA  
159 was very low (1.9%) [2]. By contrast, other observations suggest that receipt of mold-  
160 active antifungal drugs decrease sensitivity, without any relevant effect on specificity [1,

10]. This can be explained by antifungal drugs limiting the detectable burden of GM antigen, through inhibition of growth and reducing the *Aspergillus* hyphal load able to shed the antigen, which is only released into the circulation during infection, when the fungus invades the endothelial compartment [11]. Indeed, GM is no longer recommended for routine blood screening in patients receiving mold-active AFT or prophylaxis [12, 13]. The effect of antifungal therapy on the sensitivity of PCR assays for IA has long been debated. There is some evidence from animal models and clinical trials that a mold-active antifungals limit PCR detection, but this effect is not consistent across studies [3,14-17]. Variation in the antifungal administered, the incidence of IA, and the study population will influence the pretest probability of IA and potential assay performance. Our findings, based on a considerable number of trials and patients, did not show a significant reduction in the sensitivity of *Aspergillus* PCR testing of blood from patients receiving AMP. Contrary, AMP reduced the proportion of EORTC/MSGERC defined proven/probable cases of IA, and lowered specificity (i.e. increased PCR false positivity) [5, 6]. It is possible that active AMP reduces the clinical progression of IA, limiting the manifestations typically associated with IA that are essential when classifying probable IA using the EORTC/MSGERC definitions. Furthermore, given AMP has been associated with reduced GM-EIA sensitivity, the use of AMP could result in false negative GM-EIA results preventing cases of possible IA becoming probable IA and compromising PCR specificity. Conversely, the shedding of genomic material into the circulation still occurs during early infection, with the release of DNA (DNAemia) potentially being enhanced by antifungal therapy disrupting the fungal cell membrane or wall and detection of this target could define probable IA cases that would be otherwise missed using GM-EIA. Recently, *Aspergillus* PCR has been included in the updated EORTC/MSG definitions for IFD, as it provides a robust diagnostic test for screening and confirming the diagnosis of *Aspergillus* infection. [7] While the use of AMP may limit the diagnostic specificity of a single PCR

187 positive test, the specificity for multiple PCR positive tests (as required in the  
188 EORTC/MSGERC definitions) remains excellent, suitable for confirming a diagnosis of IA.  
189 Given the reasons above and that clinical/radiologic manifestations typical of overt IFD are  
190 required to achieve a classification of probable IA, the presence of *Aspergillus* PCR  
191 positivity in this setting will likely continue to provide sufficient mycological specificity. From  
192 a clinical perspective *Aspergillus* PCR of blood is best used to exclude IA, based on an  
193 adequate sensitivity, which from this study appears to be unaffected by the use of AMP.

194

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197 Conference Nice, 11-14 October 2019 Cruciani M, et al. Potential impact of anti-mold  
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200

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## 203 **Conflicts of Interest**

204 **PLW:** Performed diagnostic evaluations and received meeting sponsorship from Bruker,  
205 Dynamiker, and Launch Diagnostics; Speakers fees, expert advice fees and meeting  
206 sponsorship from Gilead; and speaker and expert advice fees from F2G and speaker fees  
207 MSD and Pfizer. Is a founding member of the European *Aspergillus* PCR Initiative.

208 **JL:** Is a founding member of the European *Aspergillus* PCR Initiative and is head of the  
209 FPCRI

210 **DB:** received research grants from Gilead Sciences and Pfizer, served on the speakers'  
211 bureau of Gilead Sciences, Merck Sharp & Dohme/Merck and Pfizer and received travel  
212 grants from Merck Sharp & Dohme/Merck and Pfizer.

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214 University of Exeter, and has received research support from Gilead and served at the speaker's  
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216 **TRR:** Served on the advisory board and at the speaker's bureau of Pfizer Healthcare Ireland,  
217 Gilead Sciences, and Menarini Pharma.

218 **JPD:** Has provided consultancy for F2G and Gilead and served at the speaker's bureau of Gilead  
219 and Pfizer. Is a founding member of the European *Aspergillus* PCR Initiative.

220 **RAB:** Is a founding member, treasurer and steering committee member of the Fungal PCR  
221 Initiative.

222 **MC, CM, COM, LK, JM, WJH, BW, DL, BJ and CC:** No conflicts declared

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**Table 1.** Effect of anti-mold prophylaxis on Aspergillosis incidence and diagnostic test estimates as determined by logistic mixed-model regression. PPV and NPV values were calculated using the Bayes' rule.

Parameter	Positivity Threshold: 1 positive PCR test		Positivity threshold: $\geq 2$ positive PCR tests	
	Prophylaxis		Prophylaxis	
	Yes	No	Yes	No
<b>Incidence (n/N, %)</b>	164/1373, 11.94	216/1156, 18.68 <sup>a</sup>	155/1356, 11.43	72/401, 17.96 <sup>a</sup>
<b>Sensitivity (95% CI)</b>	0.83 (0.72, 0.91)	0.81 (0.70, 0.88)	0.67 (0.51, 0.79)	0.70 (0.50, 0.84)
<b>Specificity (95% CI)</b>	0.60 (0.43, 0.75)	0.86 (0.75, 0.92) <sup>b</sup>	0.86 (0.71, 0.93)	0.98 (0.91, 0.99) <sup>c</sup>
<b>DOR (95% CI)</b>	7.60 (3.77, 15.31)	25.69 (13.32, 49.54) <sup>d</sup>	11.80 (4.39, 31.69)	98.06 (20.79, 462.60) <sup>e</sup>
<b>LR +tive (95% CI)</b>	2.10 (1.30, 2.90)	5.72 (2.64, 8.79)	4.62 (1.21, 8.02)	30.29 (NE, 72.30)
<b>LR -tive (95% CI)</b>	0.28 (0.14, 0.41)	0.22 (0.12, 0.32)	0.39 (0.23, 0.56)	0.31 (0.13, 0.49)
<b>PPV (95% CI)</b>	0.22 (0.15, 0.28)	0.57 (0.38, 0.67)	0.37 (0.13, 0.51)	0.87 (NE, 0.94)
<b>NPV (95% CI)</b>	0.96 (0.95, 0.98)	0.95 (0.93, 0.97)	0.95 (0.93, 0.97)	0.94 (0.90, 0.97)

**Footnote:** PPV, positive predictive value; NPV, negative predictive value; DOR, Diagnostic odds ratio; LR +tive, Likelihood ratio positive; LR -tive, Likelihood ratio negative; NE, no estimate available. 95% CI, 95% Confidence interval.

<sup>a</sup> Difference in incidence of IA with and without prophylaxis was significant ( $P < 0.001$ ). <sup>b</sup> The specificity was significantly lower under prophylaxis ( $P = 0.005$ ). <sup>c</sup> The specificity was significantly lower under prophylaxis ( $P = 0.019$ ). <sup>d</sup> The DOR was significantly lower under prophylaxis ( $P = 0.013$ ) <sup>e</sup> The DOR was significantly lower under prophylaxis ( $P = 0.022$ ).