

Cystic fibrosis patients with pulmonary disease caused by *Mycobacterium abscessus* complex have elevated specific antibody levels

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TQ: Study design, data collection, data analysis, writing the article.

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What is the key question?

Can antibodies be used to identify cystic fibrosis patients with *Mycobacterium abscessus* complex?

What is the bottom line?

Patients with pulmonary disease caused by *M. abscessus* complex had significantly elevated antibody levels specific for this pathogen, compared to patients with previous or no infection.

Why read on?

The new antibody assay was easy to perform and the test quickly identified high risk patients in need of increased diagnostic vigilance and more frequent culture for *M. abscessus* complex.

Ideas for reviewers

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ABSTRACT

Background: Early signs of pulmonary disease with *Mycobacterium abscessus* complex (MABSC) can be missed in patients with cystic fibrosis (CF), due to subtle presentation, ample differential diagnoses and mycobacterial culture being a suboptimal gold standard. A serological method could potentially help stratify patients according to risk, a principle which has previously been shown clinically useful.

Objectives: The aim of this article was to test the diagnostic accuracy of a novel multi-antigen method for investigating immunoglobulin G (IgG) activity against MABSC.

Methods: All patients attending the Copenhagen CF Centre were cultured for MABSC during a 22 month period and then screened in a cross-sectional study of anti-MABSC IgG serum levels determined by enzyme-linked immunosorbent assay (ELISA). Culture positive patients had stored serum samples examined for antibody kinetics before and after culture conversion.

Results: 307 CF patients had 3,480 respiratory samples cultured for nontuberculous mycobacteria and were then tested with the anti-MABSC IgG ELISA. Patients with pulmonary MABSC disease had median anti-MABSC IgG levels six-fold higher than patients with no history of NTM infection (434 vs. 64 ELISA units; $p < 0.001$). Test sensitivity was 95 % (95 % CI: 74 – 99) and specificity 73 % (95 % CI: 67 – 78). A diagnostic algorithm was constructed to stratify patients according to risk.

Conclusion:

The test accurately identified patients with pulmonary disease caused by MABSC and was suited to be used clinically as a complement to mycobacterial culture.

2 INTRODUCTION

3 Lung infection with nontuberculous mycobacteria (NTM) is a diagnostic and therapeutic
4 challenge in cystic fibrosis (CF) patients .[1] *Mycobacterium abscessus* complex (MABSC) is
5 the dominant NTM in European CF patients and the clinical spectrum varies from colonization to
6 invasive debilitating disease and identifying patients in need of treatment is essential.[1] New
7 evidence of increasing incidence rates, human-to-human transmission and a detrimental effect on
8 lung function has reinvigorated interest in this ubiquitous mycobacteria.[2–4] The pathogenesis
9 of MABSC is not well understood, but is an urgent research priority as there might be a window
10 of opportunity for eradication,[5] or suppression,[6] at an early stage of infection. Measuring
11 anti-MABSC immunoglobulin G (IgG) across whole CF populations could guide clinicians in
12 deciding how vigilant they have to be in pursuing mycobacterial cultures, a principle, which has
13 previously been shown useful for *Pseudomonas aeruginosa* and *Aspergillus* infection in CF.[7–
14 11] Limitations in the predictive values of such assays have also been reported,[12] and currently
15 antibody measurements for *P. aeruginosa* are used clinically in combination with culture results,
16 rather than as stand-alone tests.[13] Previous experiences with mycobacterial serodiagnosis are
17 well described in *Mycobacterium tuberculosis* (TB) research with most studies focusing on
18 assays utilizing purified single antigens such as *M. bovis* Calmette-Guérin (BCG) antigen.[14,15]
19 Other single antigens have also been used,[16] but increasingly the recommended approach is
20 using a combination of different antigens to improve sensitivity.[17–19] Serodiagnosis for NTM
21 using the BCG based antigen A60 has been explored twice in CF research,[20,21] showing
22 promising diagnostic potential.

23 The objective of the study was to develop and test the diagnostic accuracy of a novel multi-
24 antigen enzyme-linked immunosorbent assay (ELISA) for measuring anti-MABSC IgG in serum
25 from a homogenous and well-screened cohort of CF patients. We hypothesized that anti-MABSC
26 IgG levels were correlated with disease severity and that a useful clinical application could be
27 developed. The study proceeds in two parts: First we describe antibody serology in CF patients
28 prospectively screened for MABSC by sputum culture at the Copenhagen CF centre in 2012 –
29 2014. Secondly we follow the MABSC cases, and describe antibody kinetics before and after
30 onset of MABSC culture conversion.

31 32 **METHODS**

33 **Patients and setting**

34 The Copenhagen CF Centre cares for 100 children and 216 adults with CF. Since the
35 establishment of the centre in 1968, CF patients have been seen for microbiological and clinical
36 examinations in the outpatient clinic every four weeks. During visits clinical parameters are
37 registered in a clinical database and serum samples for antibody determination are collected at
38 least once a year and stored at -80° C for further investigation.

39 **Design and inclusion**

40 All CF patients registered at the Copenhagen CF centre in May 2012 were eligible. Patients were
41 consecutively enrolled during visits at the adult and paediatric outpatient clinics and admissions
42 to the CF wards from May 2012 until February 2014. Between October 2013 and February 2014
43 a serum sample was collected from each patient for anti-MABSC IgG determination. Patients
44 who died or were lung transplanted were excluded. By February 2014, the patients were divided
45 into three groups on the basis of NTM culture results and clinical data captured from patient
46 records: Group A were patients with MABSC pulmonary disease at the time of serum sampling

47 (MABSC-PD). Group B consisted of patients with previous, but not present MABSC-PD and
48 patients with past or present infection with another NTM. Group C were patients with no known
49 history of NTM infection. A group of healthy non-CF subjects (adults and children) was
50 included as normal, reference persons (Group D).

51 **Longitudinal study of MABSC cases**

52 We applied the ELISA method developed above on previously stored serum samples from all
53 patients with previous or ongoing MABSC from 1987 onwards. Lung transplanted patients were
54 not excluded in this analysis of antibody kinetics, before, during and after MABSC infection.

55 **NTM disease classification**

56 The CF Centre uses the American Thoracic Society and Infectious Disease Society of America
57 (ATS/IDSA)'s criteria to classify NTM patients.[22] In this paper we use the term MABSC-PD
58 to describe patients, who fulfilled both the ATS/IDSA's clinical, radiological and
59 microbiological criteria for pulmonary disease. We defined clearing MABSC-PD as 12 months
60 of culture negativity after culture conversion to negative (based on at least four separate MABSC
61 negative cultures).

62 **Respiratory samples and isolates**

63 Sputum samples/laryngeal suction samples were collected and *Burkholderia cepacia* selective
64 agar (BCSA) were used as growth medium according to a previously described method.[23]
65 Identification of MABSC is described in the data repository. The longitudinal study also relied
66 on previously collected NTM culture data from 1987 to 2012 according to methods previously
67 described in detail.[24]

68 **ELISA method**

69 Mycobacterial antigen preparation is described in detail in the online data repository. Anti-
70 MABSC IgG levels were determined by ELISA, modified from a previously described method
71 from our laboratory,[7,26] and expressed in ELISA units (EU). The test positivity threshold was
72 determined using a receiver operating curve (ROC). For validation purposes a commercial
73 ELISA kit (A60, Anda Biologicals, Strasbourg, France) was also tested.[15] Intraplate, plate-to-
74 plate, and day-to-day variations were performed on samples from 10 patients, covering the
75 spectrum of high to low antibody titres and were expressed using coefficient of variation (CV)
76 and standard deviation (SD). Clinical data were extracted from patient files and the Danish CF
77 registry.

78 **Statistical methods**

79 The manuscript was prepared in accordance the guidelines for reporting studies on diagnostic
80 accuracy (STARD). Baseline data were reported as medians and interquartile ranges (IQR) for
81 non-normally distributed continuous variables, and percentages for categorical variables. Group
82 comparisons were made using Kruskal Wallis non-parametric tests and Dunn's multiple
83 comparisons test. Pearson's correlation coefficient (r) was used to compare A60 and anti-
84 MABSC assays. A p value ≤ 0.05 was considered statistically significant. SPSS version 19.0
85 (SPSS Inc, Chicago, IL, USA) and GraphPad Prism 6.0 (GraphPad Software, La Jolla, Ca, USA)
86 was used for data analysis. The study was approved by the Committee on Health Research Ethics
87 in the Capital Region of Denmark (H-3-2012-098) and the Danish Data Protection Agency
88 (2007-58-0015).

89

90 **RESULTS**

91 A flow diagram of the study design is shown in Fig. 1.

92 [Fig. 1 here]

93 The Copenhagen CF cohort consisted of 316 patients of which 307 were included. From these
94 patients 3,480 NTM cultures were performed between May 2012 and February 2014. The
95 average number of cultures per patient was 11 (range: 1 – 23). All 307 patients had one serum
96 sample collected between Oct. 2013 and Feb. 2014. Characteristics of patients and healthy non-
97 CF controls are presented in Table 1.

98 **Table 1. Characteristics of 307 patients at the Copenhagen cystic fibrosis centre in Feb.**
99 **2014 stratified by nontuberculous mycobacteria status and 532 healthy non-CF controls**

100

	A	B	C	D
	MABSC-PD	Other NTM	No history	Healthy non-
		or non-PD	of NTM	CF controls§
	(n = 19)	MABSC	(n = 252)	(n = 532)
		(n = 36)		
Median age (IQR), y	21 (15 – 24)	28 (23 – 37)	24 (14 – 37)	30 (15 – 46) *
Age under 18 years, %	32	17	36	53
Female, %	53	47	49	52 **
Homozygote for Delta 508, %	68	89	63	NA
Median FEV1 of pred. (IQR), %	80 (63 – 84)	71 (62 – 80)	76 (53 – 94)	-
Chronic Gram- infection, %	58	42	34	NA
Received NTM treatment, %	58	61	0	NA
Median positive NTM cultures (IQR)	7 (4 – 16)	4 (1 – 10)	0	NA
Median time positive for NTM (IQR), y	2 (1 – 5)	2 (1 – 6)	0	NA

101 CF = cystic fibrosis, MABSC = *M. abscessus* complex, PD = pulmonary disease (as defined by ATS/IDSA criteria),
102 MAC = *M. avium* complex, NTM = nontuberculous mycobacteria, IQR = interquartile range, FEV1% = forced
103 expiratory volume in 1 second as percent of predicted for age, height and sex, ATS/IDSA = American Thoracic
104 Society / Infectious Disease Society of America. * = age missing for 64/250 paediatric and 64/250 adult controls. **
105 = sex data missing for 184/282 paediatric controls, § from frozen sera left from a previous study

106

107 **Antibody levels in different groups**

108 Median anti-MABSC IgG was four times higher in patients with MABSC-PD than in patients
109 with another NTM species or non-PD MABSC (p = 0.03) and six times higher than CF patients
110 without any NTM history (p < 0.001) (Fig. 2). Healthy, non-CF controls had the lowest IgG
111 levels with 95 % of subjects demonstrating values below 77 EU.

112 [Fig. 2 here]

113 **Test performance**

114 For the purpose of evaluating test performance patients with MABSC-PD were defined as cases
115 and all other CF patients as controls (Groups B and C combined, n = 288). A receiver operating
116 curve (ROC) was created based on different thresholds of test positivity (Fig. 3). A cut-off of 125
117 ELISA units was chosen based on its proximity to the upper left corner of the ROC (perfect
118 classification point), and its high sensitivity, given the intended clinical use of the test, designed
119 to be used in conjunction with culture (high specificity). Supplementary test performance is
120 summarized in Table E1 in the online data repository. Comparison with the antigen A60 method
121 was performed on 39 patient serum samples and revealed good correlation between the two
122 methods: Pearson's correlation coefficient (r) was 0.88 (95 % CI: 0.78 – 0.93) ($R^2 = 0.77$, p-
123 value < 0.0001).

124 [Fig. 3 here]

125 **Reproducibility of the assay**

126 Intraplate, day-to-day and inter-plate variation measurements were 5 %, 16 % and 11 %
127 respectively (IQR: 3 – 9 %, 10 – 27 % and 5 – 14 %, respectively).

128 **Diagnostic algorithm**

129 A diagnostic algorithm was constructed (Fig. 4), based on the principle of risk stratification. The
130 premise of the algorithm was one routine serum sample for anti-MABSC IgG measurement per
131 patient, per year, and compatibility with current ATS/IDSA screening recommendations.[22]

132 [Fig. 4 here]

133 **Longitudinal study of MABSC cases**

134 Fig. 5 shows anti-MABSC IgG levels before and after first positive culture in 26 MABSC cases
135 that fulfilled criteria for MABSC-PD and six patients who only had one positive MABSC
136 culture.

137 [Fig. 5 here]

138 Eleven out of the 26 patients became IgG positive prior to first positive culture. The majority of
139 these patients (9/11) were found MABSC culture positive pre 2012, when screening was less
140 frequent. Noticeably, for four of these cases, their first positive MABSC culture was also their
141 first ever NTM culture. Seven patients (27 %) had increases in antibody titres prior to culture
142 conversion, despite previous negative NTM cultures, suggesting various degrees of diagnostic
143 delay. Overall 85 % of patients that went on to develop MABSC-PD had sharp increases in
144 antibodies either before or after culture positivity validating the diagnostic potential of the test.
145 Three had slowly rising antibody levels and did not reach the cut-off of 125 EU within three
146 years of being classified as having MABSC-PD. The majority of patients with only one positive
147 MABSC culture did not become IgG positive (Fig. 5 B).

148 **Clearing infection**

149 Seven patients received extensive antimycobacterial treatment prior to clearance and two of the
150 seven were also lung transplanted. Five patients (56 %) had a convincing overlie (+/- 12 months)
151 of falling IgG levels and time of clearance, defined as 12 months of culture negativity in at least
152 four cultures. Two remained antibody positive, despite consistently being culture negative and
153 having clinical and radiological signs of arrested disease progression (Fig 5 C).

154

155 **DISCUSSION**

156

157 The study is the largest examination of antibodies against NTM in CF to date. The primary
158 strength is the prospective design in a homogeneous and well-screened CF cohort.

159

160 **Main findings**

161 In a large centre based cohort of CF patients in Denmark comprehensively screened for NTM
162 infection, anti-MABSC IgG was found to be significantly elevated in patients with MABSC-PD
163 and the test demonstrated good diagnostic accuracy. Longitudinal measurements of known
164 MABSC cases revealed patterns of early antibody kinetics indicative of significant diagnostic
165 delay, suggesting a potential for earlier intervention. This is the largest study of antibodies
166 against NTM in CF and key strengths include the prospective design in a homogeneous CF
167 population screened on a monthly basis. The potential for verification bias [27] was reduced with
168 an average of 11 cultures per patient and concomitant clinical evaluations. Through the use of
169 stored serum samples dating back 27 years, the MABSC case study could visualize antibody
170 kinetics longitudinally, providing a unique opportunity to retrospectively assess MABSC-PD
171 pathogenesis. Among study weaknesses are the changes in mycobacterial culture techniques and
172 screening procedures in the period 1987 – 2011. During this period, changing methodology
173 could confound the overall incidence rates of NTM. Validation wise, intra-plate variation was
174 good, while inter-plate variation was only acceptable.[31]

175 **Need for improved diagnostic measures**

176 The rise of MABSC in the CF population is of concern and new diagnostic measures are in
177 demand. Our group recently showed that less than a third of Scandinavian CF patients with
178 repeated MABSC cultures manage to clear infection and as many as a quarter are lung
179 transplanted or die.[24] Despite an increased understanding of the pathogenic potential of

180 MABSC-PD, diagnostic principles have remained largely unchanged, as have their inherent
181 obstacles: NTM culture is slow and culture failure is common typically due to insufficient
182 material or overgrowth of Gram negative bacteria or *Aspergillus*. Additionally, radiological
183 pathology is nonspecific in a CF setting.[22]

184 **Immunological tests for mycobacteria**

185 Traditionally, immunological testing for mycobacteria has been based on measurements of
186 cellular immune responses. Thus, the tuberculin skin test (TST) and later interferon- γ release
187 assays have been widely used.[28,29] However TST has low sensitivity and is affected by BCG
188 vaccination and interferon- γ release assay are non-reactive to almost all NTM species and have
189 no clinical applicability in CF. Serodiagnosis has re-emerged as a field of interest,[17] and two
190 NTM studies have proposed clinical utility in CF.[20,21] Our results suggest that both our own
191 ELISA IgG test based on a cocktail of MABSC antigens and a commercially available kit, based
192 on a purified BCG antigen, are feasible approaches to serodiagnosis for NTM.

193 **Diagnostic delay**

194 The longitudinal study of MABSC cases revealed patterns of early antibody kinetics indicative
195 of significant diagnostic delay. *Martiniano et al.*[6] have previously shown that a low FEV1
196 prior to first positive NTM culture was associated with whether the subsequent infection was
197 clinically significant. The authors avoid speculating if the causality involved was either poor
198 lung function leading to increased susceptibility to NTM, or reversely, that non-detected NTM
199 itself caused the lung function decline in the year leading up to its first isolation (diagnostic
200 delay). Our study of anti-MABSC IgG kinetics supports the second hypothesis, that NTM itself
201 is more likely to be the cause of poor lung function. The rise of antibody levels prior to culture
202 positivity suggests significant, albeit clinically undetected exposure.

203 **Usefulness of risk algorithm**

204 Serological screening can expedite diagnosis by selectively increasing diagnostic vigilance in
205 high risk patients. *P. aeruginosa* serology has proved a useful clinical tool in supporting clinical
206 decision making.[7,9–11] The principle has limitations,[12] and treatment decisions should not
207 be based on *P. aeruginosa* antibody levels alone.[13] In this paper we suggest a risk algorithm
208 that can selectively increase the mycobacterial culture frequency of patients at highest risk of
209 MABSC infection. Conventional culture remains the gold standard and is required for the final
210 diagnosis of MABSC infection. However, an annual serum screening would allow patients to be
211 stratified according to risk, permitting clinicians to readily differentiate between patients in need
212 of intensified observation and those who can await routine annual culture. This sequence of first
213 serum screening, then culture, allows for the optimal combination of high sensitivity (serology)
214 and subsequent high specificity (culture). This explains our choice of a relatively low positivity
215 threshold (125 EU) for the IgG screening. While a higher PPV could be achieved at the 400 EU
216 cut-off, the 125 cut-off had the advantage of minimizing the false negative rate (5 %). The trade
217 off on PPV is acceptable as antibody screening will always be followed by culture. Applying the
218 algorithm to our MABSC negative population, 73 % were IgG negative resulting in no clinical
219 consequence, 23 % had intermediate values between 125 – 400 EU warranting a new antibody
220 measurement after three-six months to monitor for an increase and just four percent were high
221 risk patients, who would be subjected to more frequent NTM culturing. With a positive
222 likelihood ratio of 13 for patients with anti-MABSC IgG over 400 ELISA units, the test fulfils
223 the prerequisites of a useful diagnostic test for this purpose.[30]

224 Distinguishing genuine disease from mere colonization has been declared one of the most
225 important challenges in NTM management,[1] and any objective marker of disease progression,

226 that would assist in making this distinction is valuable. Whether the test has other uses, such as
227 for monitoring treatment effect in patients with MABSC disease, remains to be seen. Certainly,
228 supplementary disease monitoring methods are in demand as there are currently no good
229 indicators of MABSC eradication. Culture conversion is necessary, but not sufficient to stop
230 treatment and relapse is known to occur promptly upon cessation of therapy.[32] MABSC
231 treatment regimens are exceedingly burdensome, with hearing loss and nephrotoxicity being the
232 most feared adverse events.[32] Any indicator that could assist in reducing unnecessary long
233 term treatment is thus welcomed.

234

235 **CONCLUSIONS**

236 Antibody levels against *M. abscessus* complex were significantly elevated in CF patients with
237 MABSC pulmonary disease. Anti-MABSC IgG screening proved to be an accurate diagnostic
238 tool and can help clinicians identify CF patients in need of more frequent mycobacterial culture,
239 thus reducing the problem of diagnostic delay.

240

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245

246 **Competing interests**

247 None.

248

249 **Patient consent**

250 Exemption from the requirement of written patient consent was granted by the Committee on
251 Health Research Ethics in the Capital Region of Denmark and the study was approved by the
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253

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258

259 **List of abbreviations**

260 MABSC = *Mycobacterium abscessus* complex, PD = pulmonary disease, CF = cystic fibrosis,
261 NTM = nontuberculous mycobacteria, MAC = *Mycobacterium avium* complex, ATS = American
262 Thoracic Society, IDSA = Infectious Disease Society of America, IgG = immunoglobulin G,
263 ELISA = enzyme-linked immunosorbent assay, TB = *Mycobacterium tuberculosis*, BCG =
264 *Mycobacterium bovis* Calmette-Guérin, HRCT = High resolution computer tomography, PCR =
265 polymerase chain reaction, PBS = phosphate-buffered saline, OD = optical density, ROC =
266 receiver operating curve, CV = coefficient of variation, SD = standard deviation, IQR =
267 interquartile range, CI = confidence interval, TST = tuberculin skin test

268

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369 **Figure headings**

370

371 **Fig. 1. Study design.** MABSC = *M. abscessus* complex, MABSC-PD = MABSC pulmonary
372 disease (as defined by American Thoracic Society / Infectious Disease Society of America
373 criteria), NTM = nontuberculous mycobacteria

374

375 **Fig. 2. Median (IQR) anti-MABSC IgG levels among 3 groups of CF patients (A, B, C) and**
376 **healthy non-CF controls (D).** Wide horizontal lines = median, short horizontal lines = IQR,
377 IQR = interquartile range, IgG = immunoglobulin, NTM = nontuberculous mycobacteria,
378 MABSC = *Mycobacterium abscessus* complex, PD = pulmonary disease (as defined by the
379 American Thoracic Society / Infectious Disease Society of America's criteria), EU = ELISA
380 Units, AB = combination of group A and B, BC = combination of group B and C. Only non-
381 significant comparison was group B vs. C ($p = 0.66$).

382

383 **Fig. 3. Receiver operating curve (ROC) for anti-*Mycobacterium abscessus* complex**
384 **(MABSC) IgG ELISA.** The star represents the chosen cut-off (125 ELISA units). Panel below
385 shows test characteristics for patients with MABSC pulmonary disease ($n = 19$) compared to all
386 other cystic fibrosis patients ($n = 288$). * = Adjusted analysis where patients with previous
387 MABSC or another nontuberculous mycobacteria are excluded ($n = 252$).

388

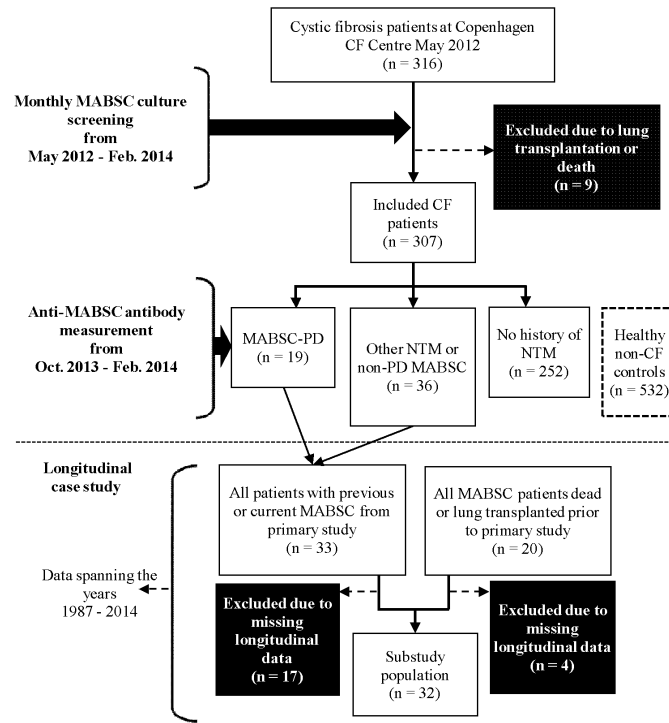
389 **Fig. 4. Diagnostic algorithm employing anti-MABSC IgG levels to stratify CF patients**
390 **according to risk of MABSC infection.** Raw data, multilevel predictive values and likelihood
391 ratios are shown in the panel below.

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Fig. 5 Anti-*Mycobacterium abscessus* complex (MABSC) antibody kinetics in serum from 26 cystic fibrosis (CF) patients who develop pulmonary MABSC disease (A) and 6 CF patients with only 1 positive MABSC culture (B). C shows anti-MABSC IgG kinetics in 9 CF patients who cleared active pulmonary MABSC infection. In A, B, C the dotted horizontal line is 125 ELISA units (test positivity threshold). In A and B vertical lines represent the date of first positive culture, and in C the date of last positive culture before clearance of MABSC pulmonary disease. Squares represent intravenous courses of amikacin combined with a carbapenem. Heavy dotted lines represent post-lung transplantation data.

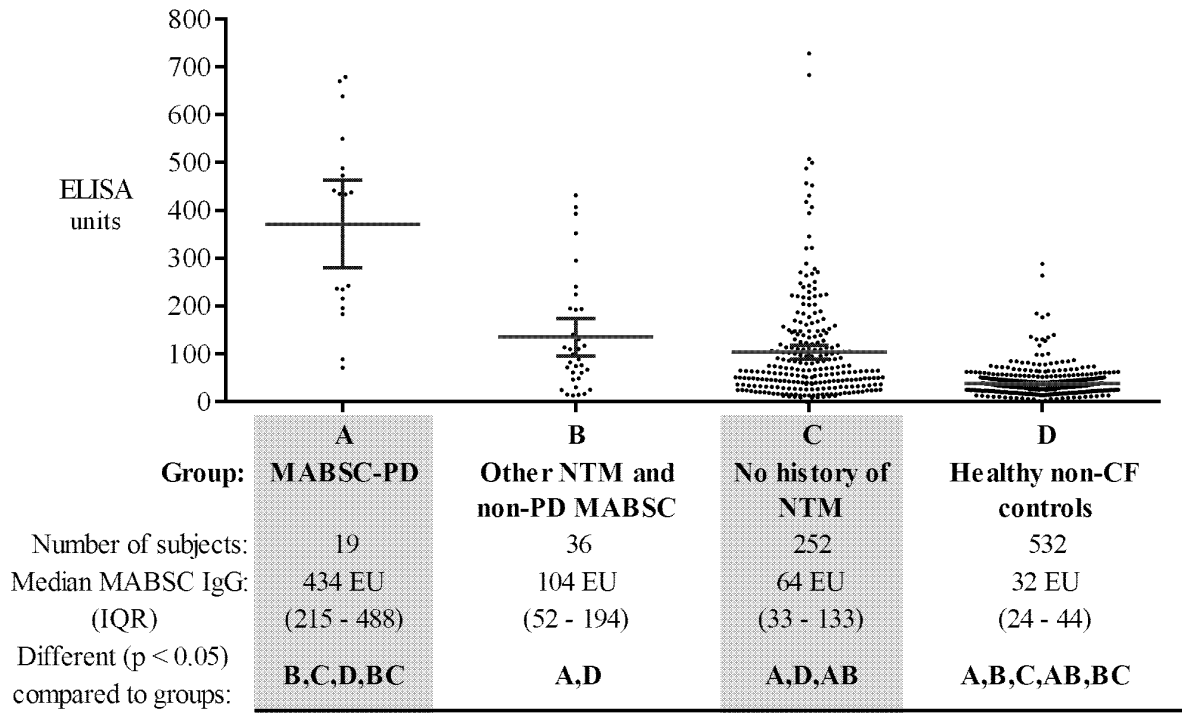
402

403 Fig 1

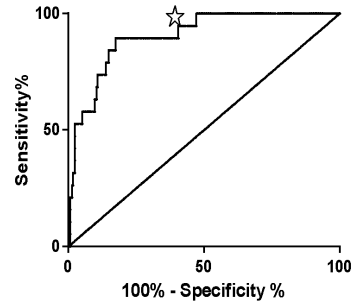


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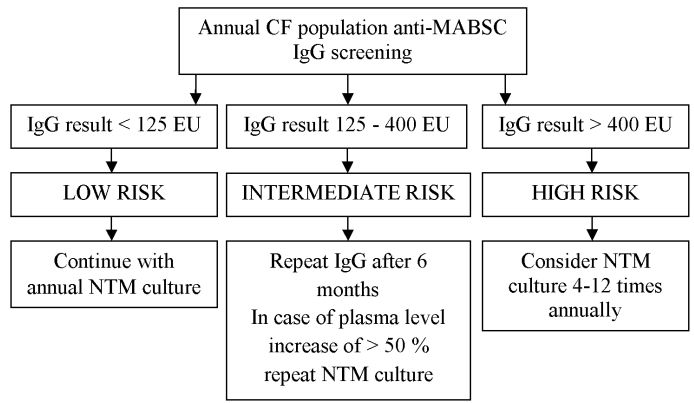
405 Fig 2



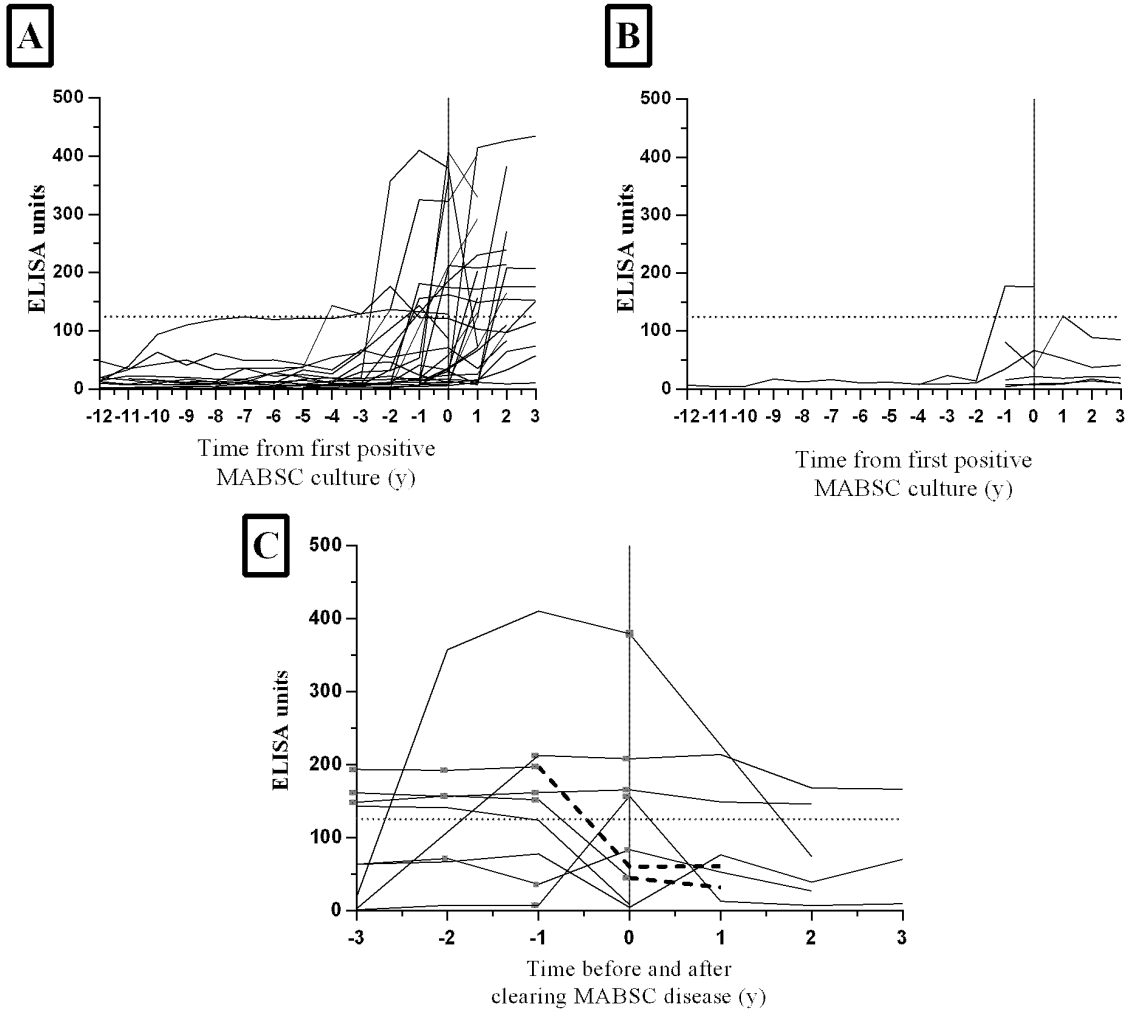
406



		95 % CI
Sensitivity	95 %	74 – 99
Specificity	73 %	67 – 78
Positive likelihood ratio	3.5	2.8 - 4.3
Negative likelihood ratio	0.07	0.0 - 0.5
Positive predictive value (PPV)	19 %	11 – 28
Adjusted PPV *	21 %	13 – 32
Negative predictive value	100 %	97 - 100
Area under ROC	0.90	0.84 - 0.96
Std. Error	0.03	-
p-value	< 0.01	-



		MABSC+	MABSC-	Total	Predictive value (95 % CI)	Likelihood ratio (95 % CI)
Serum anti-MABSC IgG (ELISA units)	> 400	10	12	22	45 % (24 - 68)	13 (6 - 25)
	125 - 400	8	67	75	11 % (5 - 20)	2 (1 - 3)
	< 125	1	209	210	0.5 % (0.1 - 2.6)	0.1 (0.1 - 0.5)
		19	288	307		



409

410 Fig 5

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Online data repository

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419

**Cystic fibrosis patients with pulmonary disease caused by *Mycobacterium abscessus*
complex have elevated specific antibody levels**

Tavs Qvist, Tania Pressler, David Taylor-Robinson, Terese L Katzenstein, Niels Høiby

420

421 **SUPPLEMENTARY METHODS**

422

423 ***Mycobacterium abscessus* complex (MABSC) identification**

424 Identification of MABSC was based on acid-fast staining followed by identification by both
425 mass spectrometry (MALDITOFF) and r16RNA gene sequencing. First isolates from each
426 patient were confirmed by the Reference Laboratory of Mycobacteriology at the State Serum
427 Institute, Copenhagen, Denmark.

428 **Antigen preparation**

429 Mycobacterial antigen preparation was performed using a *M. abscessus sensu stricto* serovar
430 obtained from a patient known to be infected with MABSC. Cells were grown in Souton's
431 medium for 2 weeks and harvested by centrifugation, washed in phosphate-buffered saline
432 (PBS), X-press disrupted at maximal force (200 MPa at -20°C) and then sonicated with a
433 Sonopuls Ultrasonic Homogenizer (Bandelin, Berlin, Germany) as described by Closs et al. [1]
434 The protein concentration was set at 2.67 mg/ml as determined by refractometry (Atago, Tokyo,
435 Japan). The antigen preparation was stored at -80° C.

436 **Supplementary ELISA method**

437 The measured (mean of double determinations) optical density (OD) values of sera were
438 transformed to EU through the use of a standard curve plotting the titre of pooled sera from
439 known MABSC cases in eight dilutions (1:500 to 1:64000). Outlying results were not excluded.

440 All serum testing was performed by one laboratory technician, with expert level ELISA
441 experience, who was blinded in regard NTM status.

442 **Longitudinal case study setting 1987 - 2014**

443 The patients attending the Copenhagen cystic fibrosis (CF) Centre account for approximately 70
444 % of the total Danish CF population. Population wide screening for nontuberculous
445 mycobacteria (NTM) was performed once in 1988. The following 23 years, testing for NTM was
446 only performed routinely on bronchoalveolar lavage (BAL) fluids. Sputum samples were
447 cultured for NTM in case of clinical suspicion. In 2011, prior to the present study, systematic
448 annual NTM screening was introduced, consisting of acid fast microscopy and mycobacterial
449 culture performed at the International Reference Laboratory of Mycobacteriology at the State
450 Serum Institute, Copenhagen, Denmark.

451 **Criteria for disease**

452 The American Thoracic Society and Infectious Disease Society of America (ATS/IDSA)'s
453 criteria to classify NTM patients were: Pulmonary symptoms, nodular or cavitary opacities on
454 chest radiograph, or a HRCT scan that shows multifocal bronchiectasis with multiple small
455 nodules after appropriate exclusion of other diagnoses. In addition, positive culture results from
456 at least two separate sputum samples or positive culture results from at least one bronchoalveolar
457 lavage. Information from patient files was used to confirm historical NTM cases and classify
458 when patients fulfilled ATS/IDSA criteria for pulmonary disease (PD).

459

460 **RESULTS**

461 Values from CF patients with no known history of NTM were not normally distributed, but
462 approximated normal distribution after log transformation. Geometric mean values and
463 confidence intervals for this group and the non-CF controls were 66 EU (95 % CI: 59 – 75) and
464 32 EU (95 % CI: 31 – 34) respectively.

465

466 **Supplementary Table**

467

468 **Table E1. Supplementary test characteristics of anti-*Mycobacterium abscessus* complex IgG**
 469 **antibody ELISA* using alternative combinations of patient groups**

	Group AB vs. C	95 % CI	Group AB vs. D	95 % CI	Group A vs. D	95 % CI	Group A vs. BC	95 % CI
Sens, %	56	(42-70)	56	(42-70)	89	(67-98)	89	(67-98)
Spec, %	74	(68-79)	98	(96-99)	98	(96-99)	73	(67-78)
PLR	2.2	(1.6-2.9)	25.0	(13.6-45.8)	40.0	(22.2-70.9)	3.3	(2.6-4.2)
NLR	0.6	(0.4-0.8)	0.5	(0.3-0.6)	0.1	(0-0.4)	0.2	(0-0.5)
PPV, %	32	(23-42)	72	(56-85)	59	(39-76)	18	(11-27)
NPV, %	89	(83-93)	96	(94-97)	100	(99-100)	99	(97-100)

470 * = applying the 125 ELISA unit cut-off. Group A = cystic fibrosis (CF) patients with *M. abscessus*
 471 complex (MABSC) pulmonary disease (PD), Group B = CF patients with other nontuberculous
 472 mycobacteria (NTM) and patients with previous MABSC-PD, Group C = CF patients with no history of
 473 NTM disease, Group D = healthy non-CF controls, IgG = immunoglobulin, ELISA = enzyme-linked
 474 immunosorbent assay, CI = confidence interval, sens = sensitivity, spec = specificity, PLR = positive
 475 likelihood ratio, NLR = negative likelihood ratio, PPV = positive predictive value, NPV = negative
 476 predictive value.

477

478 **References**

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