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

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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.
INTRODUCTION

Lung infection with nontuberculous mycobacteria (NTM) is a diagnostic and therapeutic challenge in cystic fibrosis (CF) patients.[1] *Mycobacterium abscessus* complex (MABSC) is the dominant NTM in European CF patients and the clinical spectrum varies from colonization to invasive debilitating disease and identifying patients in need of treatment is essential.[1] New evidence of increasing incidence rates, human-to-human transmission and a detrimental effect on lung function has reinvigorated interest in this ubiquitous mycobacteria.[2–4] The pathogenesis of MABSC is not well understood, but is an urgent research priority as there might be a window of opportunity for eradication,[5] or suppression,[6] at an early stage of infection. Measuring anti-MABSC immunoglobulin G (IgG) across whole CF populations could guide clinicians in deciding how vigilant they have to be in pursuing mycobacterial cultures, a principle, which has previously been shown useful for *Pseudomonas aeruginosa* and *Aspergillus* infection in CF.[7–11] Limitations in the predictive values of such assays have also been reported,[12] and currently antibody measurements for *P. aeruginosa* are used clinically in combination with culture results, rather than as stand-alone tests.[13] Previous experiences with mycobacterial serodiagnosis are well described in *Mycobacterium tuberculosis* (TB) research with most studies focusing on assays utilizing purified single antigens such as *M. bovis* Calmette-Guérin (BCG) antigen.[14,15] Other single antigens have also been used,[16] but increasingly the recommended approach is using a combination of different antigens to improve sensitivity.[17–19] Serodiagnosis for NTM using the BCG based antigen A60 has been explored twice in CF research,[20,21] showing promising diagnostic potential.
The objective of the study was to develop and test the diagnostic accuracy of a novel multi-antigen enzyme-linked immunosorbent assay (ELISA) for measuring anti-MABSC IgG in serum from a homogenous and well-screened cohort of CF patients. We hypothesized that anti-MABSC IgG levels were correlated with disease severity and that a useful clinical application could be developed. The study proceeds in two parts: First we describe antibody serology in CF patients prospectively screened for MABSC by sputum culture at the Copenhagen CF centre in 2012 – 2014. Secondly we follow the MABSC cases, and describe antibody kinetics before and after onset of MABSC culture conversion.

METHODS

Patients and setting

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

Design and inclusion

All CF patients registered at the Copenhagen CF centre in May 2012 were eligible. Patients were consecutively enrolled during visits at the adult and paediatric outpatient clinics and admissions to the CF wards from May 2012 until February 2014. Between October 2013 and February 2014 a serum sample was collected from each patient for anti-MABSC IgG determination. Patients who died or were lung transplanted were excluded. By February 2014, the patients were divided into three groups on the basis of NTM culture results and clinical data captured from patient records: Group A were patients with MABSC pulmonary disease at the time of serum sampling...
Group B consisted of patients with previous, but not present MABSC-PD and patients with past or present infection with another NTM. Group C were patients with no known history of NTM infection. A group of healthy non-CF subjects (adults and children) was included as normal, reference persons (Group D).

**Longitudinal study of MABSC cases**

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

**NTM disease classification**

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

**Respiratory samples and isolates**

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

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

Statistical methods

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

RESULTS

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

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

<table>
<thead>
<tr>
<th></th>
<th>A (n = 19)</th>
<th>B (n = 36)</th>
<th>C (n = 252)</th>
<th>D (n = 532)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (IQR), y</td>
<td>21 (15 – 24)</td>
<td>28 (23 – 37)</td>
<td>24 (14 – 37)</td>
<td>30 (15 – 46) *</td>
</tr>
<tr>
<td>Age under 18 years, %</td>
<td>32</td>
<td>17</td>
<td>36</td>
<td>53</td>
</tr>
<tr>
<td>Female, %</td>
<td>53</td>
<td>47</td>
<td>49</td>
<td>52 **</td>
</tr>
<tr>
<td>Homozygote for Delta 508, %</td>
<td>68</td>
<td>89</td>
<td>63</td>
<td>NA</td>
</tr>
<tr>
<td>Median FEV1% of pred. (IQR), %</td>
<td>80 (63 – 84)</td>
<td>71 (62 – 80)</td>
<td>76 (53 – 94)</td>
<td>-</td>
</tr>
<tr>
<td>Chronic Gram- infection, %</td>
<td>58</td>
<td>42</td>
<td>34</td>
<td>NA</td>
</tr>
<tr>
<td>Received NTM treatment, %</td>
<td>58</td>
<td>61</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>Median positive NTM cultures (IQR), y</td>
<td>7 (4 – 16)</td>
<td>4 (1 – 10)</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>Median time positive for NTM (IQR), y</td>
<td>2 (1 – 5)</td>
<td>2 (1 – 6)</td>
<td>0</td>
<td>NA</td>
</tr>
</tbody>
</table>

Antibody levels in different groups

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

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

Reproducibility of the assay

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

Diagnostic algorithm

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

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

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

Clearing infection

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

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

Main findings

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

Need for improved diagnostic measures

The rise of MABSC in the CF population is of concern and new diagnostic measures are in demand. Our group recently showed that less than a third of Scandinavian CF patients with repeated MABSC cultures manage to clear infection and as many as a quarter are lung transplanted or die.[24] Despite an increased understanding of the pathogenic potential of
MABSC-PD, diagnostic principles have remained largely unchanged, as have their inherent obstacles: NTM culture is slow and culture failure is common typically due to insufficient material or overgrowth of Gram negative bacteria or *Aspergillus*. Additionally, radiological pathology is nonspecific in a CF setting.[22]

**Immunological tests for mycobacteria**

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

**Diagnostic delay**

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

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

Distinguishing genuine disease from mere colonization has been declared one of the most important challenges in NTM management,[1] and any objective marker of disease progression,
that would assist in making this distinction is valuable. Whether the test has other uses, such as for monitoring treatment effect in patients with MABSC disease, remains to be seen. Certainly, supplementary disease monitoring methods are in demand as there are currently no good indicators of MABSC eradication. Culture conversion is necessary, but not sufficient to stop treatment and relapse is known to occur promptly upon cessation of therapy.[32] MABSC treatment regimens are exceedingly burdensome, with hearing loss and nephrotoxicity being the most feared adverse events.[32] Any indicator that could assist in reducing unnecessary long term treatment is thus welcomed.

CONCLUSIONS

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

Funding

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Competing interests

None.
Exemption from the requirement of written patient consent was granted by the Committee on Health Research Ethics in the Capital Region of Denmark and the study was approved by the Danish Data Protection Agency.

Acknowledgements
The authors would like to thank Lena Lingren Nørregaard and Ulla Rydahl Johansen for excellent technical assistance. A special thanks to the staff of the Copenhagen Cystic fibrosis Centre for cooperation with the research project.

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

References (some automatic formatting issues that will be resolved before submission)


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Figure headings

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

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

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

Fig. 4. Diagnostic algorithm employing anti-MABSC IgG levels to stratify CF patients according to risk of MABSC infection. Raw data, multilevel predictive values and likelihood ratios are shown in the panel below.
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.
Fig 2

<table>
<thead>
<tr>
<th>Group</th>
<th>MABSC-PD</th>
<th>Other NTM and non-PD MABSC</th>
<th>No history of NTM</th>
<th>Healthy non-CF controls</th>
</tr>
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<tbody>
<tr>
<td>Number of subjects:</td>
<td>19</td>
<td>36</td>
<td>252</td>
<td>532</td>
</tr>
<tr>
<td>Median MABSC IgG (IQR)</td>
<td>434 EU (215 - 488)</td>
<td>104 EU (52 - 194)</td>
<td>64 EU (33 - 133)</td>
<td>32 EU (24 - 44)</td>
</tr>
<tr>
<td>Different (p &lt; 0.05) compared to groups:</td>
<td>B,C,D,BC</td>
<td>A,D</td>
<td>A,D,AB</td>
<td>A,B,C,AB,BC</td>
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<tr>
<td></td>
<td>95% CI</td>
<td></td>
<td></td>
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<td>--------------------------</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Sensitivity</td>
<td>95% 74 – 99</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specificity</td>
<td>73% 67 – 78</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive likelihood ratio</td>
<td>3.5 2.8 – 4.3</td>
<td></td>
<td></td>
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<tr>
<td>Negative likelihood ratio</td>
<td>0.07 0.0 – 0.5</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Positive predictive value (PPV)</td>
<td>19% 11 – 28</td>
<td></td>
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<tr>
<td>Adjusted PPV*</td>
<td>21% 13 – 32</td>
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<tr>
<td>Negative predictive value</td>
<td>100% 97 - 100</td>
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<tr>
<td>Area under ROC</td>
<td>0.90 0.84 – 0.96</td>
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<tr>
<td>Std. Error</td>
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<tr>
<td>p-value</td>
<td>&lt; 0.01 -</td>
<td></td>
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</table>
Annual CF population anti-MABSC IgG screening

- IgG result < 125 EU  
  LOW RISK  
  Continue with annual NTM culture

- IgG result 125 - 400 EU  
  INTERMEDIATE RISK  
  Repeat IgG after 6 months 
  In case of plasma level increase of > 50% repeat NTM culture

- IgG result > 400 EU  
  HIGH RISK  
  Consider NTM culture 4-12 times annually

<table>
<thead>
<tr>
<th>Serum anti-MABSC IgG (ELISA units)</th>
<th>MABSC+</th>
<th>MABSC-</th>
<th>Total</th>
<th>Predictive value (95% CI)</th>
<th>Likelihood ratio (95% CI)</th>
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<tbody>
<tr>
<td>&gt; 400</td>
<td>10</td>
<td>12</td>
<td>22</td>
<td>45% (24 - 68)</td>
<td>13 (6 - 25)</td>
</tr>
<tr>
<td>125 - 400</td>
<td>8</td>
<td>67</td>
<td>75</td>
<td>11% (5 - 20)</td>
<td>2 (1 - 3)</td>
</tr>
<tr>
<td>&lt; 125</td>
<td>1</td>
<td>209</td>
<td>210</td>
<td>0.5% (0.1 - 2.6)</td>
<td>0.1 (0.1 - 0.5)</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>288</td>
<td>307</td>
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</tr>
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</table>
Fig 5
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
SUPPLEMENTARY METHODS

Mycobacterium abscessus complex (MABSC) identification

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

Antigen preparation

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

Supplementary ELISA method

The measured (mean of double determinations) optical density (OD) values of sera were transformed to EU through the use of a standard curve plotting the titre of pooled sera from known MABSC cases in eight dilutions (1:500 to 1:64000). Outlying results were not excluded. All serum testing was performed by one laboratory technician, with expert level ELISA experience, who was blinded in regard NTM status.

Longitudinal case study setting 1987 - 2014
The patients attending the Copenhagen cystic fibrosis (CF) Centre account for approximately 70\% of the total Danish CF population. Population wide screening for nontuberculous mycobacteria (NTM) was performed once in 1988. The following 23 years, testing for NTM was only performed routinely on bronchoalveolar lavage (BAL) fluids. Sputum samples were cultured for NTM in case of clinical suspicion. In 2011, prior to the present study, systematic annual NTM screening was introduced, consisting of acid fast microscopy and mycobacterial culture performed at the International Reference Laboratory of Mycobacteriology at the State Serum Institute, Copenhagen, Denmark.

**Criteria for disease**

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

**RESULTS**

Values from CF patients with no known history of NTM were not normally distributed, but approximated normal distribution after log transformation. Geometric mean values and confidence intervals for this group and the non-CF controls were 66 EU (95 % CI: 59 – 75) and 32 EU (95 % CI: 31 – 34) respectively.
Table E1. Supplementary test characteristics of anti-\textit{Mycobacterium abscessus} complex IgG antibody ELISA* using alternative combinations of patient groups

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

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

References