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SEROLOGICAL BIOMARKERS FOR THE DIAGNOSIS OF *MYCOBACTERIUM ABSCESSUS* INFECTIONS IN CYSTIC FIBROSIS PATIENTS

Vincent Le Moigne[1]*, Anne-Laure Roux[1,2], Hélène Mahoudo[1], Gaëtan Christien[1], Agnès Ferroni[3], Oana Dumitrescu[4,5], Gérard Lina[4,5], Jean-Philippe Bouchara[6], Patrick Plésiat[7], Jean-Louis Gaillard[1,2], Stéphane Canaan[8], Geneviève Héry-Arnaud[9], and Jean-Louis Herrmann[1,10]*

(1) Université Paris Saclay, UVSQ, Inserm, Infection et Inflammation, Montigny-le-Bretonneux, France.

(2) AP-HP, GHU Paris Saclay, Hôpital Ambroise Paré, Service de Microbiologie, Boulogne-Billancourt, France.

(3) AP-HP, GHU Paris, Hôpital Necker-Enfants Malades, Service de Microbiologie, Paris 15e, France.

(4) Hospices Civils de Lyon, Hôpital de la Croix Rousse-Centre de Biologie Nord, Institut des Agents Infectieux, Laboratoire de Bactériologie, Grande Rue de la Croix Rousse, 69004, Lyon, France.

(5) Centre International de Recherche en Infectiologie, INSERM U1111, Université de Lyon, Lyon, France.

(6) CHU, Service de Parasitologie-Mycologie, Groupe d'Etude des Interactions Hôte-Pathogène (GEIHP, EA 3142), UNIV Angers, UNIV Brest, SFR 4208 ICAT, Angers, France.

(7) Laboratoire de Bactériologie, CHRU de Besançon, UMR CNRS 6249 Chrono-Environnement, Faculté de Médecine-Pharmacie, Université de Bourgogne Franche-Comté, Besançon, France.

(8) Université Aix-Marseille, CNRS, LISM, IMM FR3479, Marseille, France.

(9) Département de bactériologie-virologie, hygiène et parasitologie-mycologie, centre hospitalier régional universitaire (CHRU) de Brest, Brest, France ; Inserm, EFS, UMR 1078 France « génétique, génomique fonctionnelle et biotechnologies », GGB, université Brest, 29200 Brest, France.

(10) AP-HP, GHU Paris Saclay, Hôpital Raymond Poincaré, Service de Microbiologie, Garches, France.

(*) Correspondance to VLM (vincent.le-moigne@uvsq.fr) and JLH (jean-louis.herrmann@aphp.fr), UMR U1173, UFR Sciences de la Santé Simone Veil and INSERM, Université Versailles-Saint-Quentin, 78180 Montigny-le-Bretonneux, France.

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ABSTRACT (244 words)

Background: Culture conditions sometimes make it difficult to detect non-tuberculous mycobacteria (NTM), particularly *Mycobacterium abscessus*, an emerging cystic fibrosis (CF) pathogen. The diagnosis of NTM positive cases not detected by classical culture methods might benefit from the development of a serological assay.

Methods: As part of a diagnostic accuracy study, a total of 173 sera CF-patients, including 33 patients with *M. abscessus* positive cultures, and 31 non-CF healthy controls (HC) were evaluated. Four *M. abscessus* antigens were used separately, comprising two surface extracts (Interphase (INP) and a TLR2 positive extract (TLR2eF)) and two recombinant proteins (rMAB_2545c and rMAB_0555 also known as the phospholipase C (rPLC)).

Results: TLR2eF and rPLC were the most efficient antigens to discriminate NTM-culture positive CF-patients from NTM-culture negative CF-patients. The best clinical values were obtained for the detection of *M. abscessus*-culture positive CF-patients; with sensitivities for the TLR2eF and rPLC of 81.2% (95% CI:65.7-92.3%) and 87.9% (95% CI:71.9-95.6%) respectively, and specificities of 88.9% (95% CI:85.3-94.8%) and 84.8% (95% CI:80.6-91.5%) respectively. When considering as positive all sera, giving a positive response in at least one of the two tests, and, as negative, all sera negative for both tests, we obtained a sensitivity of 93.9% and a specificity of 80.7% for the detection of *M. abscessus*-culture positive CF-patients.

Conclusion: High antibody titers against TLR2eF and rPLC were obtained in *M. abscessus*-culture positive CF-patients, allowing us to consider these serological markers as potential tools in the detection of CF-patients infected with *M. abscessus.*

1. INTRODUCTION

Non-tuberculous mycobacterial pulmonary infections remain frequent and, depending on the mycobacterium, severe for patients with cystic fibrosis (CF). Nineteen epidemiological studies have been published to date, representing a total number of 23,418 CF-patients, 6% of whom were found to be infected with non-tuberculous mycobacteria (NTM) (1). In fact, numbers vary from 3.3% and up to 13% from one study to another (2-9). The risk factors for acquisition of NTM include concurrent chronic infection due to another infectious agent (*Staphylococcus aureus*, *Aspergillus fumigatus*, *Stenotrophomonas maltophilia*), older CF-patients, the use of oral corticosteroids and intravenous broad spectrum antimicrobial therapy (1, 10). The main NTM isolated from CF-patients according to recently defined diagnostic criteria (11, 12) are *Mycobacterium avium* and *Mycobacterium intracellulare*, which belong to the *M. avium* complex, and *Mycobacterium abscessus sensu lato* (5, 6, 12-15).

What has become clear is that almost 50% of CF-patients with an NTM isolated from their respiratory specimens comply with the defined diagnostic criteria regarding NTM pulmonary disease (11). In addition, the pathogenicity of the isolated NTMs must now be considered (14, 16). Their clinical relevance varies according to the NTM species but also to radiological findings on chest CT (computerized tomography) and Acid-Fast Bacilli (AFB) staining, leading to the observation that only certain NTMs are or will be consistently associated with a pulmonary pathology (15), while meeting previously defined criteria (11). Finally, the impact of NTM lung infection on ventilatory function in CF-patients, and even on mortality in any population outside CF, has only recently been evaluated. Nevertheless, there is now strong evidence that, according to the causative species, NTM lung infections reduce the lung capacity of CF-patients, and that mortality of CF infected individuals at five years, is significantly higher, with or without co-morbidities, compared to the mortality calculated for the general population (17, 18).

Therefore, a reliable and accurate microbiological diagnosis is necessary. Unfortunately, a microbiological diagnosis relies on inoculation of respiratory specimens onto enriched media specific to mycobacteria, which may be rapidly contaminated by other CF pathogens, notably *Pseudomonas aeruginosa* or *Staphylococcus aureus*, and therefore unusable despite the possibility of a second step of decontamination of the respiratory specimen (12). Recent work on a new medium makes it possible to avoid the decontamination step of the respiratory sample, with a certain increase in the sensitivity of the culture (19). However, this medium is still largely a more favorable medium for the growth of rapid-growing NTM (RGM) compared to slow-growing NTM (SGM), as shown recently, with only a 50% recovery for *M. avium* and *M. intracellulare* (20).

An indirect diagnosis, based on the host's immune response during mycobacterial infection through measurement of antibody production, has not yet demonstrated its effectiveness, particularly in the context of tuberculosis (21). In the setting of NTM infections in CF-patients, data are scarce, with clinical values difficult to interpret (22, 23). A few studies focused on the characterization of *M. abscessus* lung infections by ELISA assays (24, 25). Nevertheless, when considering the common nature of the antigens evaluated in many NTMs, it seemed essential to us to develop a serological assay that would immediately detect CF-patients with the presence of antibodies against NTM, differentiating a current or past NTM infectious process. The idea was then to investigate in more detail those CF-patients considered NTM positive using the culture techniques or even molecular methods at our disposal. We took advantage of two antigenic preparations, previously described (26, 27), and for which we demonstrated, in the course of this work, their value as serological diagnostic tools for detecting NTM-CF infected patients, and more specifically CF patients with positive *M. abscessus* cultures.

2. MATERIAL and METHODS

2.1 Patients and antigens

Serum samples for antibody determination collected from 173 CF-patients (6) and 31 healthy controls (HC) were stored at −20°C for further investigation. Recombinant MAB_0555 (rPLC) was produced and purified as previously described (28). *MAB_2545c* was cloned in the plasmid pMyC and the recombinant protein, rMAB_2545c, was expressed and purified as described (28). Mycobacterial Interphase (INP) and TLR2eF preparations were obtained as described (29, 27).

2.2 ELISA assays

IgG antibodies against INP, TLR2eF, rMAB_2545c and rPLC were determined by ELISA as previously described (26). Briefly, plates were coated overnight at 4°C with 1 µg/ml of each antigen in 100 µl of carbonate-bicarbonate buffer (0.1 M, pH 9.6), except for INP that was diluted and coated in water/ethanol (50/50, v/v). Plates were then washed twice with PBS and blocked by incubation for one hour at 37°C with PBS containing 1% bovine serum albumin (PBS-BSA). Sera were added at a 1/400 dilution in PBS-Tween 20 (0.05% v/v) (PBS-T) containing 0.5% BSA (PBS-T-BSA). After incubation for 1 h 30 at 37°C followed by four washes with PBS-T, alkaline phosphatase-conjugated goat anti-human IgG or goat anti-human IgA (Southern biotechnology, Birmingham, USA) diluted in PBS-T-BSA was added. Plates were then incubated for 1 h 30 at 37°C. After four additional washes, 100 µl of 1 mg/ml of *p*-nitrophenylphosphate (Sigma, Saint Quentin Fallavier, France) in diethanolamine buffer (pH 9.8) was added, and plates were incubated in the dark at room temperature for 2 h. Plates were then read spectrophotometrically at 405 nm with a Bio-Rad PR 3100 TSC instrument (Bio-Rad France, Marnes-la-Coquette, France). The ELISA against the *Mycobacterium avium* subsp. *paratuberculosis* lipopentapeptide (L5P) was performed as previously described (30).

Results were given as optical density (OD). Each serum was tested in duplicate per plate and per series. Each serum sample was evaluated on at least two series. A strong positive and a weak positive serum were systematically added to each plate to establish inter-plate and inter-day variability. OD mean ± standard deviation was calculated for each patient group. Sensitivity and specificity of the ELISA assays were assessed by calculating the area under the receiver operating characteristic curve (AUC). The test positivity thresholds were determined using this receiver operating characteristics (ROC) curves (31). Corresponding ROC curves were presented in supplementary Figures 1A to 1D and in supplementary Figures 2A to 2D. The closest point to the upper left side corner was used to determine the cut-off value.

2.3 Data analysis.

We used Student's t test for all analyses using GraphPad Prism 6.0 software (GraphPad Software, La Jolla, CA, USA) and considered a 2-sided *P* of < 0.05 to be statistically significant (ns = non-significant, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

3. RESULTS

3.1 Distribution of serological samples according to the pathogens isolated in culture

A total of 204 sera from 173 patients with CF and 31 HC were studied (Figure 1). All sera were obtained prospectively during the 2004 survey of atypical mycobacteria infection in CF patients in France (6). Depending on the results of sputum cultures, several groups of patients were distinguished according to strain of mycobacteria, the bacteria and/or the fungi isolated (Figure 1). A first group included 33 CF-patients with a mycobacterium isolated from their sputum and belonging to the *M. abscessus* complex (Mabs, 28 *M. abscessus subspecies abscessus*, 1 *subspecies bolletii* and 4 *subspecies massiliense*). A second group included 21 CF-patients with mycobacteria belonging to the *M. avium* complex (MAC, 14 *M. avium*, 2 *Mycobacterium chimaera* and 5 *M. intracellulare* – a subgroup named Mint). A third group (NTMa) included 5 CF patients with the following mycobacteria isolated from their sputa: 2 *Mycobacterium chelonae*, 1 *Mycobacterium poriferae*, 1 *Mycobacterium lentiflavum* and 1 *Mycobacterium gordonae*. All together, we had an NTM-culture positive group of 59 CF-patients (11, 12).

Three other groups of CF-patients were defined, without mycobacteria recovered from sputum samples, including 35 CF-patients with *P. aeruginosa* detected by culture (PA), 51 CF-patients without NTM nor *P. aeruginosa*, and 28 CF-patients with fungi only (Figure 1). Thus, we had a total of 114 CF-patients with no proven NTM infection but colonized or infected by other microorganisms. These different groups were therefore compared to each other in terms of antibody response to the different antigens tested and compared to the control group of 31 HC.

Figure 1 :

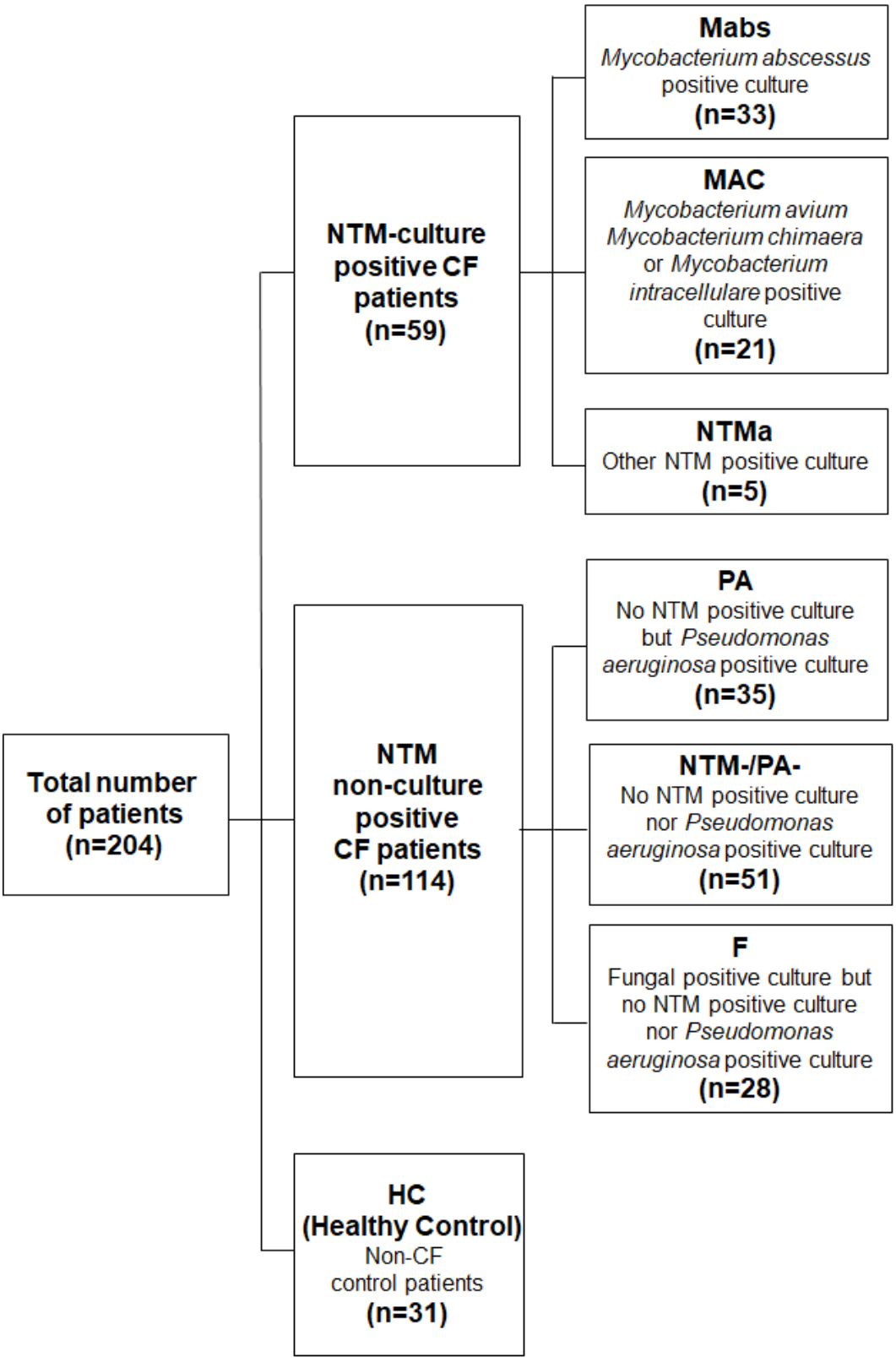


Figure 1: Flow chart. Names and compositions of the different studied groups.

3.2 Diagnostic value of the Interphase, TLR2eF, rMAB_2545c and rPLC IgG assays when comparing NTM-infected vs. NTM-non infected CF-patients

We previously characterized a complex *M. abscessus* fraction from its cell surface called Interphase (INP) that reproduced by itself the TLR2-dependent hyper-proinflammatory response observed with the R morphotype (29). In this fraction, lipoproteins have been found to be the main TLR2 agonists. To obtain larger amounts of these lipoproteins, we modified the protocol and purified another fraction from *M. abscessus* cell wall, named TLR2eF (27). Two recombinant proteins, the previously described PLC (26) and MAB_2545c, present in large quantities in the INP fraction (29), were also investigated in our study. The four antigenic extracts or recombinant proteins were then used as antigens separately in our ELISA assay.

IgG antibody titers against INP in NTM-infected group (59 CF-patients) *vs.* non-NTM infected group (114 CF-patients + 31 HC) allowed us to discriminate CF-patients with or without an NTM ($p < 0.0001$) (Figure 2A). The AUC of 0.655 allowed us to determine a cut-off value of 0.2479 (not shown). Sensitivity and specificity of INP ELISA for detecting NTM-culture positive individuals were 49.2% (95% confidence interval [CI]: 35.9-62.5%) and 82.2% (95 % CI: 75.0-88.0%), respectively.

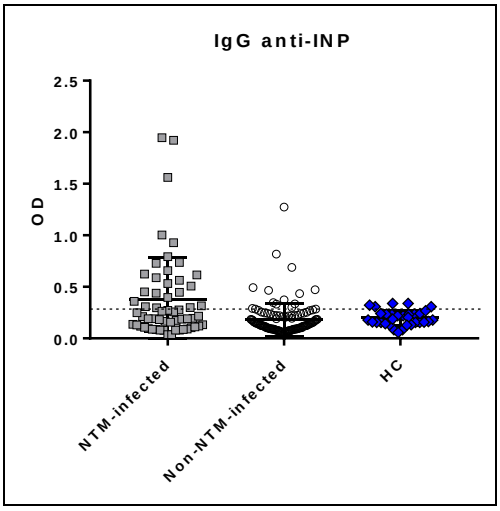
The IgG antibody titers against TLR2eF in NTM-infected group *vs.* non-NTM infected and control groups separate even more significantly both groups ($p < 0.0001$) (Figure 2B). Indeed, AUC was 0.798 with a cut-off value of 2.023 (not shown). Moreover, sensitivity and specificity of the TLR2eF ELISA for detecting NTM-culture positive individuals were 61% (95% CI: 47.73-74.59%) and 91.7% (95 % CI: 86.08-95.68%), respectively.

Results for the use recombinant proteins as specific antigens were protein dependent. IgG antibody titers against rMAB_2545c in NTM-culture positive group *vs.* non-NTM and control

groups did not lead to a differentiation between both groups (p = 0.54) (Figure 2C). In contrast, IgG antibody titers against rPLC clearly differentiated both groups (p < 0.0001) (Figure 2D). AUC was 0.779 and it allowed the determination of a cut-off value of 2.039 (not shown). Sensitivity and specificity of the rPLC ELISA for the detection of NTM-culture positive CF-patients were closed to the results obtained with TLR2eF, 64.4% (95% CI: 50.9-76.4%) and 86.9% (95 % CI: 80.3-91.9%), respectively. Positive and negative predictive values, likelihood ratios and accuracy for all antigens are given in Supplementary Tables 1A to 1D. They were calculated on the basis of a prevalence of 3.6% according to the results obtained in the previous prevalence study (6). The more interesting scores was obtained with TLR2eF as antigen.

Figure 2

A



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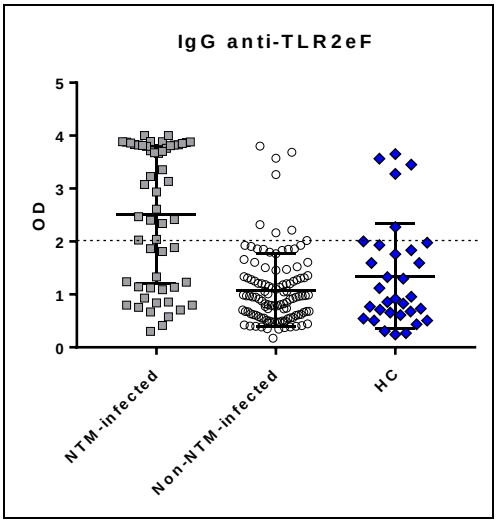
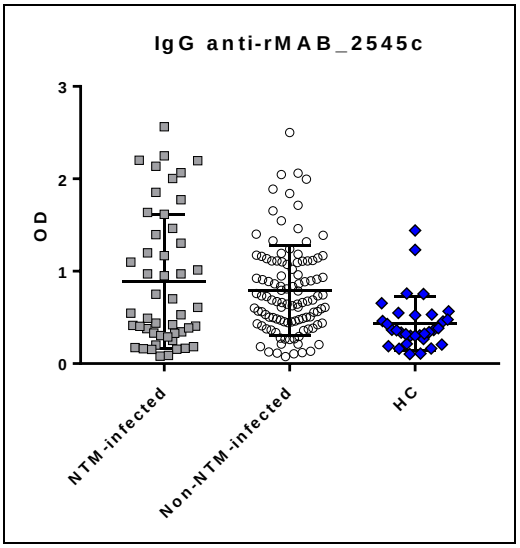


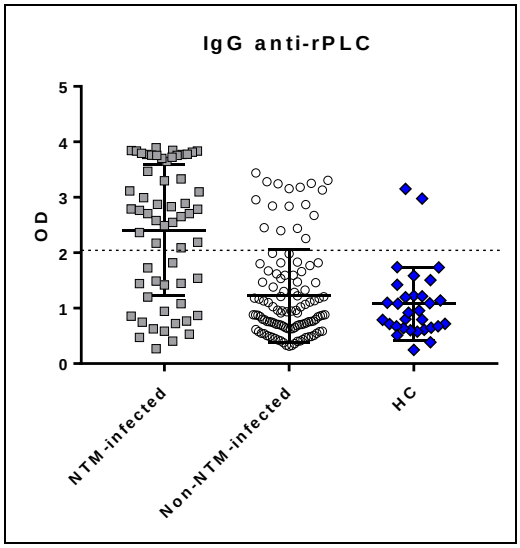
INP	NTM-positive culture	NTM-negative culture	Total
Positive serology	29	26	55
Negative serology	30	119	149
Total	59	145	204

C

TLR2eF	NTM-positive culture	NTM-negative culture	Total
Positive serology	36	12	48
Negative serology	23	133	156
Total	59	145	204

D





rMAB_2545c	NTM-positive culture	NTM-negative culture	Total
Positive serology	23	37	60
Negative serology	36	108	144
Total	59	145	204

rPLC	NTM-positive culture	NTM-negative culture	Total
Positive serology	38	19	57
Negative serology	21	126	147
Total	59	145	204

Figure 2: IgG response of NTM-positive culture, non-NTM-positive culture groups and healthy control (HC) group opposite to (A) INP or the interphase, (B) TLR2eF or the surface-extract TLR2 activating-enriched fraction, (C) rMAB_2545c and (D) rPLC. Each dot represents one patient in the scattergrams. Horizontal lines represent the mean and vertical bars SDs. Values are presented in tables 1, 2, 3 and 4 for each antigenic sample, respectively. Chosen cut-off values (test positivity threshold) are respectively 0.2479 (A), 2.023 (B) and 2.039 (D) (dotted horizontal lines). $p < 0.001$ for comparisons of NTM-culture positive groups *versus* the non-NTM group plus the HC group. Beneath each figure is represented the corresponding 2 by 2 table for NTM-culture positive patients.

3.3 Diagnostic *value of the Interphase, TLR2eF, rMAB_2545c and rPLC IgG assays when comparing mycobacterial species.*

Our ELISA assay using INP as antigen clearly differentiated CF-patients with *M. abscessus* (Mabs) positive culture from the other NTM-culture positive patient group ($p = 0.034$), as well as from CF-patients with no-NTM ($p < 0.0001$), and from HC ($p < 0.0001$) (Figure 3A). Comparison with the CF-patient group with *M. avium* complex (Mac) positive culture also revealed a significant difference ($p = 0.005$). However, when considering only CF-patients with *M. intracellulare* (Mint) positive cultures (supplementary Figure 3A), we no longer observed a significant difference in mean OD values with Mabs-culture positive CF-patients. The mean OD was lower in the Mint group than in the Mabs group, but the difference was not significant ($p = 0.29$) (supplementary Figure 3A). Sensitivity and specificity for detecting Mabs-culture positive CF-patients reached 78.8% (95% CI: 54.5-86.7%) and 81.3% (95 % CI: 75.4-87.4%), respectively. In comparison, sensitivity and specificity for detecting Mac-culture positive CF-patients were 19% and 81.3% respectively (supplementary Table 2A).

Similarly, the OD values obtained with the TLR2eF antigen also significantly separate the Mabs-culture positive CF-patients from the other patient groups ($p < 0.0001$), except for the Mint-culture positive CF-patient group ($p = 0.84$), when segregated from the Mac group (Figure 3B). In this case, sensitivity, and specificity for the detection of Mabs-culture positive CF-patients were 82% (95% CI: 65.7-92.3%) and 90% (95 % CI: 85.3-94.8%) respectively. By comparison, sensitivity, and specificity for detecting Mac-culture positive CF-patients were 23.8% and 90.6%, respectively (supplementary Table 2B). Of note, Mint-culture positive CF-patients were as responsive as Mabs-culture positive CF-patients, and more responsive than *M. avium*-culture positive CF-patients (supplementary Figure 3B).

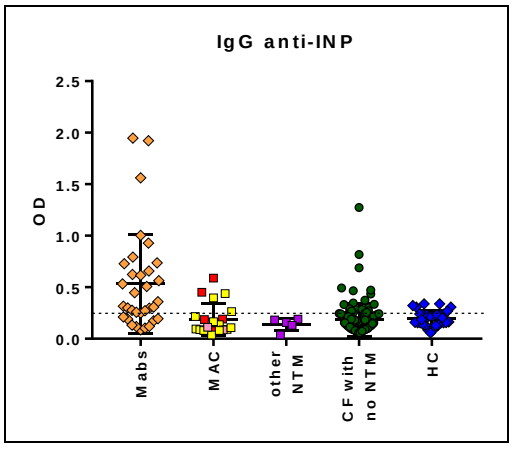
Results obtained with rMAB_2545c were disappointing since it was not possible to discriminate between groups using this antigen (Figure 3C). Sensitivity and specificity for detecting Mabs-culture positive CF-patients were 63.7% (95% CI: 43.7-78.9%) and 76.6% (95 % CI: 70.4-83.9%) respectively. By comparison, sensitivity, and specificity for detecting Mac-culture positive CF-patients were 19% and 76%, respectively (supplementary Table 2C).

Again, Mint-culture positive CF-patients showed a similar antibody response towards MAB_2545c as Mabs-culture positive CF-patients, and a higher response compared to Mac-culture positive CF-patients (supplementary Figure 3C).

Finally, the use of rPLC as antigen clearly showed significant differences between groups, with a clear separation between Mabs-culture positive CF-patients and all other patient groups ($p < 0.0001$). Sensitivity and specificity for the detection of Mabs-culture positive CF-patients were 88% (95% CI: 71.9-95.6%) and 84.5% (95 % CI: 80.6-91.5%) respectively. By comparison, sensitivity, and specificity for detecting Mac-culture positive CF-patients were 33.3% and 87.3% respectively (supplementary Table 2C). As with the two previous antigens, TLR2eF and rMAB_2545C, the OD values obtained for Mint-culture positive CF-patients were almost identical to those obtained for the Mabs-culture positive CF-patients ($p = 0.95$), and higher than those obtained for Mac-culture positive CF-patients (supplementary Figure 3D). Positive and negative predictive values, likelihood ratios and accuracy for all antigens are given in Supplementary Tables 3A to 3D, the more interesting results being also here obtained with TLR2eF antigen.

Figure 3

A



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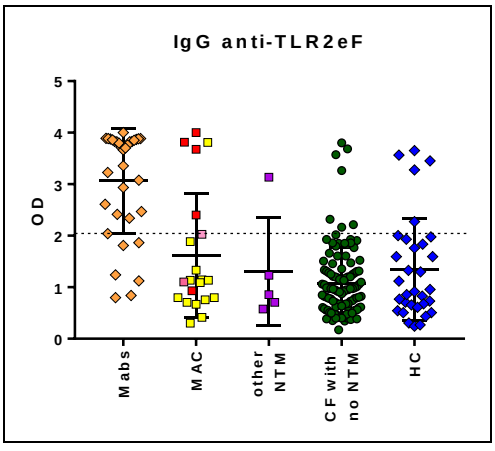


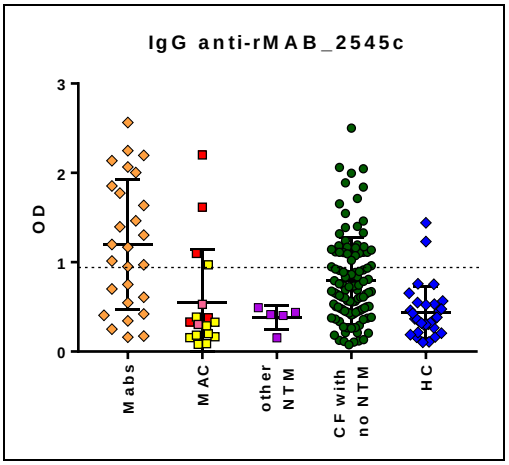
INP	Mabs-positive culture	Mabs-negative culture	Total
Positive serology	26	32	58

TLR2eF	Mabs-positive culture	Mabs-negative culture	Total
Positive serology	27	19	46

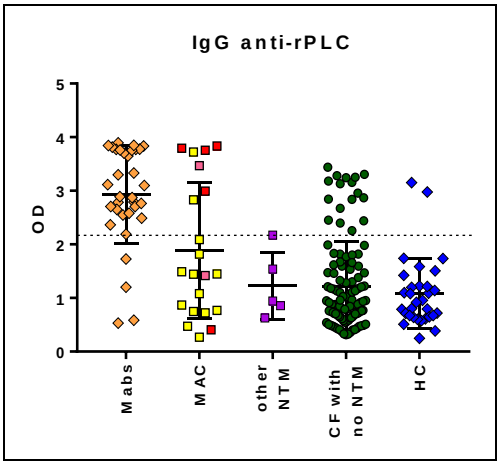
Negative serology	7	139	146
Total	33	171	204

Negative serology	6	152	158
Total	33	171	204

C



D



rMAB_2545c	Mabs-positive culture	Mabs-negative culture	Total
Positive serology	21	40	61
Negative serology	12	131	143
Total	33	171	204

rPLC	Mabs-positive culture	Mabs-negative culture	Total
Positive serology	29	26	55
Negative serology	4	145	149
Total	33	171	204

Figure 3: IgG response of the different cystic fibrosis (CF) patient groups and healthy control (HC) group opposite to: (A) INP or the interphase, (B) TLR2eF or the surface-extract TLR2 activating-enriched fraction, (C) rMAB_2545c and (D) rPLC. Each dot represents one patient in the scattergrams. In MAC group, yellow squares represent *M. avium*-, pink squares represent *M. chimaera*- and red squares *M. intracellulare*- culture positive CF-patients. Horizontal lines represent the mean and vertical bars SDs. Chosen cut-off values (test positivity threshold) are respectively 0.2479 (A), 2.034 (B), 0.950 (C) and 2.181 (D) (dotted horizontal lines). Beneath each figure is represented the corresponding 2 by 2 table, for Mabs culture positive CF-patients, and see the supplementary 2 by 2 Table 2A to 2D for MAC culture positive CF-patients.

3.4 Diagnostic value of combined results obtained with the TLR2eF extract and rPLC ELISA assays

When we review the data obtained individually, antigen by antigen, we found TLR2eF and rPLC, making it possible to separate CF-patients with NTM and/or *M. abscessus* positive culture from the other patient groups, regardless of any other bacteria or fungi isolated from their respiratory secretions. The characterization of these two antigens may be not surprising given their recently demonstrated vaccine properties (27, 28). As part of an "or" strategy, we therefore compiled results for each antigen, and categorized samples as positive when at least one of the two tests was positive, and as negative when the results were negative for both tests. Data were then compared to culture results *i.e.,* NTM-positive culture and Mabs-positive culture (Tables 1A and B). Sensitivity and specificity for the detection of CF-patients with NTM positive culture were 66.1% and 82.8% respectively (Table 1A), and were 94% and 80.7%, respectively, for the detection of CF-patients with *M. abscessus* positive culture (Table 1B). Positive and negative predictive values, likelihood ratios and accuracy for these two Tables are presented in Tables 1C and 1D, with a NPV of 99.7% when sera from Mabs-positive culture were compared to Mabs-negative culture.

Table 1: Two by two table when combining TLR2eF and rPLC serology results versus NTM culture (A) or Mabs culture (B). Corresponding values of positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (PLR), negative likelihood ratio (NLR) and accuracy and their respective confidence interval at 95% (95% CI) of results presented in C and D.

A

TLR2eF or rPLC	NTM-positive culture	NTM-negative culture	Total
Positive serology	39	25	64
Negative serology	20	120	140
Total	59	145	204

C

TLR2eF or rPLC		95% CI
PPV (%)	12.5	8.8 to 17.6
NPV (%)	98.5	97.8 to 98.9
PLR	3.8	2.6 to 5.7
NLR	0.4	0.3 to 0.6
Accuracy (%)	82.2	76.2 to 87.1

B

TLR2eF or rPLC	Mabs-positive culture	Mabs - negative culture	Total
Positive serology	31	33	64
Negative serology	2	138	140
Total	33	171	204

D

TLR2eF or rPLC		95% CI
PPV (%)	15.4	11.7 to 20.0
NPV (%)	99.7	98.9 to 99.9
PLR	4.9	3.5 to 6.7
NLR	0.08	0.02 to 0.29
Accuracy (%)	81.2	75.1 to 86.3

4. DISCUSSION

The microbiological diagnosis of NTM lung infections in patients with CF remains challenging (32). Sputum samples received in the laboratory often contain several other pathogenic bacteria (*S. aureus, P. aeruginosa, Stenotrophomonas maltophilia, Burkholderia cepacia* complex...), which grow much faster than mycobacteria. This requires a treatment step called decontamination-concentration, with a 4% sodium hydroxide solution, similar to that performed on sputum received for the diagnosis of *Mycobacterium tuberculosis* pulmonary infection (32). The purpose of this step is to eliminate these pathogenic bacteria, as well as the saprophytic associated microbial flora. However, this decontamination is not insignificant for mycobacteria, especially the RGM, leading to the loss of almost 90% of the mycobacteria present in the sample, without eliminating all other pathogenic bacteria such as *P. aeruginosa*. Therefore, a second decontamination step is often required, with oxalic acid, even more drastic for mycobacteria (33). The result, experienced daily in the mycobacteriology laboratory, is therefore either a culture contaminated by one of the associated pathogenic bacteria such as *P. aeruginosa,* or the absence of mycobacterial growth following successive sputum decontaminations, leading to a low clinical yield for the culture (33). In addition, many patients such as the youngest patients with CF cannot produce sufficient sputum samples making it challenging to identify mycobacteria, as recommended by recent recommendations (12, 34).

Alternate diagnostic methods such as serological tests have been widely criticized, particularly in the context of tuberculosis, both in terms of highly variable clinical values across tests or studies (21), and in terms of the financial costs of overtreating due to too many false positive results (35). Some meta-analyses carried out for NTM infections, for example on the diagnostic accuracy of the anti-*M. avium* IgA assay in the context of *M. avium* infections, also showed very large disparities in the clinical values (36).

Our main objective therefore was to study the presence or absence of antibodies against several antigens from *M. abscessus* in CF-patients with an NTM-positive culture. We did not consider defined clinical criteria or recommendations (11, 38), which are often poorly adapted to this patient population, or which required specific recommendations, mainly based on expert opinion (12, 34), as we wanted to see if this antibody response could discriminate CF-patients with NTM-positive cultures, from CF-patients without an NTM-positive culture, and/or with sputum culture positive for fungi or other bacteria. Serological studies in patients with CF showed promising clinical values, although somewhat surprising regarding the antigen used, which is often a mycobacterial extract containing a cocktail mixture of mycobacterial antigens, and for which a pan-mycobacterial response should be found (24, 25, 37). Nevertheless, it is important to be able to offer diagnostic alternatives to CF-patients infected by NTM, especially given the destructive potential observed in some NTM lung infections, such as those due to *M. abscessus*. Finally, we anticipate interest in such a serological test which would easily exclude CF patients with negative serology, as a rule out test, to focus solely on positive patients, particularly when considering the difficulties encountered due to the pitfalls of microbiological diagnosis by culture. Since CF patients are particularly well monitored clinically, with one NTM search per year in all sputum producing patients (12), this ELISA would also allow screening of this CF patient population to establish the risk of acquiring or developing a pulmonary NTM infection (risk stratification).

Among the antigens evaluated, only TLR2-activating enriched fraction (TLR2eF) and rPLC were performing well, with sensitivities of 61%, 64.4%, and specificities of 91.7% and 86.9% respectively for the detection of CF-patients with NTM-positive culture. These values were noticeably higher for the detection of *M. abscessus*-positive CF-patients, with sensitivities of 82%, 88%, and specificities of 89% and 84.5%, respectively.

Surprisingly, no reaction or only a low antibody response was seen for MAC-positive CF-patients, except for those CF-patients (4 out of 5) culture positive for *M. intracellulare*. This

was not due to the characteristic of the antigens extracted from *M. abscessus* since the use of a *M. avium* subspecies-specific antigen (L5P) (30) did not induce or demonstrate a strong antibody response in *M. avium*-positive CF-patients (5 out of 14) (supplementary Figure 4). The response of CF-patients with a positive culture to *M. intracellulare* and *M. chimaera* also confirms that *M. abscessus* extracts can be used to recognize other NTM-culture positive CF-patients. Perhaps this represents a serological response identifying true NTM infections. Either way, the observed responses seem to correlate well with the two pathogens, *M. abscessus* and *M. intracellulare*, the NTMs currently most monitored. Non-response, or low anti-MAC response, may simply indicate colonization rather than infection, but again this needs to be further investigated in a prospective study.

As discussed in the context of tuberculosis, serological responses may vary from one individual to another and depending on the antigen used. The use of multiple antigens has shown reasonable success in some studies (39-43) but has never been looked at in the context of NTM infections in CF patients.

The best grouping, according to our study, is to combine the results of TLR2eF and rPLC ELISA assays. Using this selection, we were able to increase the detection of CF-patients with NTM-positive culture, with sensitivity values of 66.1% and specificity values of 82.8%. Similarly, the sensitivity value was even higher when looking at CF-patients with a positive *M. abscessus* culture (94%). This allows us to consider these two antigens as an excellent diagnostic complement for the characterization of CF-patients potentially infected with NTM, and especially with *M. abscessus* and *M. intracellulare* (supplementary Figure 3B and 3D). This will allow us to study them more precisely, on the clinical, radiological, and microbiological levels to understand the details of a positive response in TLR2eF and/or rPLC ELISA assays. Indeed, we can consider that the specificity values obtained are not optimal, compared to the 90% often expected, and that the false positive rate obtained is as a consequence of what we have just described above, linking the difficulties of culturing

samples from CF-patients to the interest of an alternative approach to identify patients potentially infected by an NTM. Correlation with clinical and radiological evolution, as well as repeated microbiological examination of respiratory secretions, which is being carried out within the context of the prevalence study performed in France, will make it possible to solve the problem of specificity of these tests. Interestingly, and taking into account the "rule out test" strategy, the NPVs are very close to 99%, indicating that a negative serology is unlikely to be associated with a positive culture. This may allow us to set up a risk stratification strategy to differentiate the presence or absence of NTM infection by first performing the serological test. This strategy is currently being tested prospectively in our ongoing clinical study.

In conclusion, we present here two ELISA assays, which provide added value to the diagnostic arsenal we have at our disposal in the mycobacteriology laboratory when combining their respective results, since they improve the diagnosis of CF-patients with a NTM positive culture, and even more since they allow the serological confirmation of almost all CF-patients presenting a positive culture to *M. abscessus*. These two ELISA assays are currently being tested in a prospective study to determine the prevalence of NTM infections in CF patients (clinical trial n° ID RCB: 2017-A00025-48).

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