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Development and Evaluation of an NTM-IGRA to Guide Pediatric Lymphadenitis Diagnosis

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Background: Diagnosis of nontuberculous mycobacteria (NTM) infections remains a challenge. In this study, we describe the evaluation of an immunological NTM-interferon (IFN)-γ release assay (IGRA) that we developed using glycopeptidolipids (GPLs) as NTM-specific antigens.

Methods: We tested the NTM-IGRA in 99 samples from pediatric patients. Seventy-five were patients with lymphadenitis: 25 were NTM confirmed, 45 were of unknown etiology but compatible with mycobacterial infection and 5 had lymphadenitis caused by an etiologic agent other than NTM. The remaining 24 samples were from control individuals without lymphadenitis (latently infected with *M. tuberculosis*, uninfected controls and active tuberculosis patients). Peripheral blood mononuclear cells were stimulated overnight with GPLs. Detection of IFN-γ producing cells was evaluated by enzyme-linked immunospot assay.

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Results: NTM culture-confirmed lymphadenitis patient samples had a significantly higher response to GPLs than the patients with lymphadenitis of unknown etiology but compatible with mycobacterial infection ($P < 0.001$) and lymphadenitis not caused by NTM ($P < 0.01$). We analyzed the response against GPLs in samples from unknown etiology lymphadenitis but compatible with mycobacterial infection cases according to the tuberculin skin test (TST) response, and although not statistically significant, those with a TST ≥5 mm had a higher response to GPLs when compared with the TST <5 mm group.

Conclusions: Stimulation with GPLs yielded promising results in detecting NTM infection in pediatric patients with lymphadenitis. Our results indicate that the test could be useful to guide the diagnosis of pediatric lymphadenitis. This new NTM-IGRA could improve the clinical handling of NTM-infected patients and avoid unnecessary misdiagnosis and treatments.

Key Words: nontuberculous mycobacteria, tuberculosis, interferon-γ release assay, glycopeptidolipids, enzyme-linked immunospot assay

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Nontuberculous mycobacteria (NTM) constitute a wide group of species,[1] which in many cases, are associated with respiratory diseases, disseminated infections, especially in immunocompromised patients, and cervicofacial lymphadenitis specifically in children.[2–4] Recently, NTM infections have increased, not only due to improved diagnosis, recording and typing but also due to rising incidence rates of diseases caused by NTM.[5]

In immunocompetent children, cervicofacial lymphadenitis is the most frequent manifestation of NTM infection; however, there is still considerable uncertainty about its management, as reflected in the American Thoracic Society/Infectious Diseases Society of America statement.[4] In the United States, about 10% of the culture-confirmed mycobacterial cervicofacial lymphadenitis in children is reported to be due to *Mycobacterium tuberculosis* (*M. tb*).[4] However, in adults, this rate is higher, reaching 90% of the culture-confirmed mycobacterial lymphadenitis.[4] A definitive etiologic diagnosis of lymphadenitis requires culture or nucleic acid amplification tests (NAATs) confirmation.[4,6]

Distinguishing lymphadenitis caused by *M.tb* from those caused by NTM is critical, as the former requires not only different drug therapy but also public health tracking.[4] However, as both cause similar clinical and radiological features and are frequently histologically alike,[7,8] their differential diagnosis can be challenging. Immunodiagnostic methods available for tuberculosis (TB) also require caution when reaching conclusions regarding NTM or *M.tb* infection. A positive tuberculin skin test (TST) is not totally specific and may be indicative of either NTM or *M.tb* infection, as the purified protein derivative (PPD) used in the TST has antigens that are present in both, NTM and *M.tb*.[9] Additionally, a small number of NTM species, such as *Mycobacterium kansasii, Mycobacterium szulgai and Mycobacterium marinum*, synthesize antigens encoded in the region of difference (RD-1), which are used in TB-interferon (IFN)-γ release assay (TB-IGRAs) (6 kD early secretory antigenic target and 10 kD culture filtrate antigen) and thus, *M. kansasii*-infected individuals could yield positive results for these tests.[10] Previous studies indicate that a negative TB-IGRA independently of the TST result could discard *M.tb* infections.[10,11] A recent revision of 4 cohorts of contact TB children under 2 years of age evidenced that none of 575 untreated children with negative IGRA test results progressed to TB disease.[12] However, a negative TB-IGRA should be interpreted cautiously, given the low sensitivity reported in some studies, especially in young children.[13,14]

Following the official American Thoracic Society/Infectious Diseases Society of America statement,[4] absence of *M.tb* in the lymph node culture provides strong presumptive evidence for the diagnosis of lymphadenitis caused by NTM. However, given the low mycobacterial load found in these types of lesions, culture sensitivity depends on the method used to collect samples.[4,7,15] This fact leaves an important number of lymphadenitis cases without a definitive confirmation of their causative agent. To date, there is no less invasive nor more reliable method to diagnose NTM infections in children with lymphadenitis.

To address this lack of NTM diagnostic methods, we evaluated the use of *Mycobacterium avium* sensitins (a mix of antigens used for in vivo assessment of *M. avium* infection) as possible molecules to stimulate the blood samples with an IGRA-based test.[16] Results were promising but further validation was required and potential cross-reactivity between mycobacterial sensitins and previous data demonstrated that discrimination between *M. avium* and *M.tb* was not possible using *M. avium* sensitins,[17,18] which made it clear that more NTM-specific antigens needed to be considered.

Glycopeptidolipids (GPLs) are a type of cell envelope glycolipids produced by a wide range of NTM such as *Mycobacterium abscessus*, *Mycobacterium smegmatis* and species from the *M. avium* complex (MAC) such as *M. avium* and *Mycobacterium intracellulare*,[19–21] but not by species from the *M.tb* complex. These NTM synthesize GPLs with a common lipopeptide core but, depending on the species, their glycosylation, methylation and acetylation patterns will be different.[19,20,22] Various functions have been attributed to GPLs such as determination of bacteria physiology: colony morphology, motility or biofilm formation and a role in virulence and host immune responses induced.[20,23,24]

In this study, we describe the use of GPLs for peripheral blood mononuclear cells (PBMCs) stimulation using an enzyme-linked immunospot assay (ELISPOT)-based NTM-IGRA. This study aims to evaluate the use of this novel developed NTM-IGRA to detect specifically NTM infections in children with lymphadenitis.

MATERIALS AND METHODS

Study Design: Settings and Patient Groups

In this cross-sectional study, children and adolescents (16 years old or younger) were prospectively recruited at 6 Spanish health care centers, 4 located in Catalonia and 2 in Madrid. The study was conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) and ethical approval was provided by the Ethics Committee of the HUGTiP (PI 10-214). Written informed consent at enrollment was obtained from parents or legal guardians, as was informed assent in patients >11 years of age. Whole blood samples were collected on site, together with a detailed questionnaire indicating demographic and clinical data.

Ninety-nine patients were included and classified as follows:

1. Patients with lymphadenitis (75/99, 75.8%). Included patients with a lymphadenitis considered for further study after following the Spanish Association of Paediatrics guidelines.[25] Recommended diagnostic procedures were performed[15] including image (echography), histology (by needle aspiration and/or excisional biopsy procedures), cultures, TST, TB-IGRAs and serology (mainly, for Epstein-Barr virus, cytomegalovirus, *Bartonella*, toxoplasma and parvovirus infections). These were further divided into 3 groups:

i. NTM culture confirmed (25/99, 25.3%). The majority with a positive culture for MAC (18/25, 72.0%), *Mycobacterium lentiflavum* (4/25, 16.0%) and *Mycobacterium malmoense* (3/25, 12.0%).
ii. Of unknown etiology without microbiological confirmation but compatible clinically, radiologically and/or histologically with mycobacterial infection[15,25] (45/99, 45.5%).
iii. Not caused by NTM (5/99, 5.1%). Lymphadenitis caused by another confirmed etiologic cause different from NTM: 2 were due to bacterial infection (1 by *Staphylococcus aureus* and the other by *Bartonella* spp.), another one was due to an Epstein-Barr infection and the last 2 corresponded with lymphoma, one of them a Hodgkin lymphoma.

2. Control individuals without lymphadenitis:

i. Pulmonary active tuberculosis (aTB) (5/99, 5.1%). Children with compatible symptoms and radiology and with a positive polymerase chain reaction and/or culture for *M.tb*.
ii. Latently infected with *M. tuberculosis* (LTBI) (13/99, 13.1%). Individuals with a positive TB-IGRA and TST, without clinical signs or symptoms of aTB.
iii. Uninfected (6/99, 6.1%). Immunocompetent children with no documented clinical history or risk factors for *M.tb* or NTM infections, with negative TB-IGRAs and TST.

Demographic and clinical data are shown in Table 1.

TST

Following Spanish guidelines for TB,[26] using the Mantoux method, 0.1 mL of PPD solution (2-TU of PPD RT23, Statens Serum Institut, Copenhagen, Denmark) was injected intradermally in the patients' forearm. The induration diameter was measured after 48–72 hours. The test was considered positive when the induration was ≥5 mm.

PBMCs Isolation

Whole blood samples were collected in 8-mL mononuclear cell preparation tubes (Becton Dickinson Diagnostics) for the subsequent PBMCs isolation for future stimulation. Briefly, collected blood was centrifuged at 1600×*g* for 30 minutes at room temperature. PBMCs were harvested and washed twice with RPMI 1640 medium with L-glutamine (Biowest, Nuaillé, France) supplemented with 10% fetal bovine serum (Biowest, France). Washed cells were resuspended in AIM V medium (Gibco, Life Technologies Ltd. Fountain Dr, Inchinnan, United Kingdom) for a final concentration of 2.5×10^6 cells/mL.

IGRAs for TB Infection Diagnosis

We used QuantiFERON-TB Gold In-Tube or QuantiFERON-TB Gold Plus (Qiagen, Düsseldorf, Germany) and T-SPOT.TB (Oxford Immunotec Limited, Abingdon, United Kingdom). The tests were performed and the results analyzed following strictly the manufacturers' recommendations. In ELISPOT, the spot forming cells (SFCs) results refer to stimulation of 250,000 cells per well.

NTM-IGRA

To test the GPLs performances, we used ELISPOT plates (Oxford Immunotec Limited, Abingdon, United Kingdom) as platform. Briefly, 250,000 PBMCs in 100 µL of AIM V were incubated overnight with purified GPLs, using media as negative control and phytohemagglutinin as positive control. Wells were washed and conjugate and substrate were added to detect SFCs. Specific stimulation was determined by subtracting SFCs from the negative control to that in the GPLs stimulation condition. For result interpretation, cutoff values were determined using receiver operating characteristic (ROC) curve analysis.

The GPLs purification procedure and protocol setup are detailed in the Material, Supplemental Digital Content 1, http://links.lww.com/INF/F348.

TABLE 1. Demographic and Clinical Data of the Patients Included

		Lymphadenitis (n = 75)					
	Overall	NTM+	Unknown Etiology	Not-NTM	LTBI	Uninfected	aTB
	(n = 99)	(n = 25)	(n = 45)	(n = 5)	(n = 13)	(n = 6)	(n = 5)
Age, average (yr) ± SD	5.6 ± 4.4	2.6 ± 1.2	4.6 ± 3.6	8.5 ± 5.0	10.2 ± 3.1	9.0 ± 5.0	10.7 ± 6,61
Sex (%)							
Female	50 (50.5)	12 (48.0)	27 (60.0)	2 (40.0)	7 (53.8)	1 (16.7)	1 (20)
Male	49 (49.5)	13 (52.0)	18 (40.0)	3 (60.0)	6 (46.2)	5 (83.3)	4 (80)
BCG vaccination (%)	14 (14.1)	0 (0.0)	2 (4.4)	1 (20.0)	9 (69.2)	2 (33.3)	0 (0.0)
TST (%)							
≥5 mm	57 (57.6)	17 (68.0)	23 (51.1)	0 (0.0)	13 (100)	0 (0.0)	4 (80)
<5 mm	41 (41.4)	7 (28.0)	22 (48.9)	5 (100)	0 (0.0)	6 (100)	1 (20)
Unknown	1 (1.0)	1 (4.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
TB-IGRA (%)*							
Positive	32 (32.3)	2 (8.0)	11 (24.4)	2 (40.0)	13 (100)	0 (0.0)	4 (80)
Negative	67 (67.7)	23 (92.0)	34 (75.6)	3 (60.0)	0 (0.0)	6 (100)	1 (20)
Site of lymphadenitis (%)							
Cervicofacial	38 (50.7)†	12 (48.0)	22‡ (48.9)	4 (80.0)	-	-	-
Submandibular/jugulodigastric	31 (33.0)†	10§ (40.0)	21¶ (46.7)	0 (0.0)	-	-	-
Other	8 (10.7)†	3‖ (12.0)	4** (8.9)	1†† (20.0)	-	-	-

*QFN-G and/or T-SPOT.TB.
†Percentage calculated based on the total number of lymphadenitis samples, n = 75.
‡One also had a submandibular lymphadenitis and another a submandibular and a jugulodigastric lymphadenitis.
§One also had a preauricular lymphadenitis.
¶Two of them also had a cervicofacial lymphadenitis and other 2 also had a preauricular lymphadenitis.
‖Inguinal (n = 1), preauricular (n = 1) and intraparotid (n = 1).
**Preauricular (n = 1), supraclavicular (n = 1), mediastinal (n = 1) and external auditive conduct and retropharyngeal (n = 1).
††Axilar (n = 1).
BCG indicates *Mycobacterium bovis* bacillus Calmette-Guérin; NTM+, NTM confirmed; LTBI, latently infected with *M. tuberculosis*; SD, standard deviation; QFN-G, QuantiFERON-TB Gold In-Tube/QuantiFERON-TB Gold Plus.

Statistical Analysis

Statistical analyses and graphical representations were performed using GraphPad Prism (v.5.0, GraphPad Software, San Diego, CA). Two-tailed Mann-Whitney *U* test was used for unpaired comparison between groups considering as statistically significant a *P* value <0.05. ROC curve analyses were performed and the area under the curve (AUC) was calculated to determine the cutoff values of the GPLs stimulation. As positive cases, we used results from patients with NTM-positive lymphadenitis and as the negative control group those results from uninfected individuals. To determine the best cutoff value for the test, we applied the Youden index.

RESULTS

NTM-IGRA for Lymphadenitis in Pediatric Population

Samples with a higher response to GPLs were those from patients with NTM culture-confirmed lymphadenitis, followed by patients with lymphadenitis of unknown etiology but compatible with mycobacterial infection ($P < 0.001$) and lymphadenitis not caused by NTM ($P < 0.01$) (Fig. 1). aTB patients, LTBI individuals and uninfected controls showed a lower response to that obtained in the NTM culture-confirmed lymphadenitis ($P < 0.001$ for aTB and $P < 0.0001$ for LTBI and uninfected controls), and lymphadenitis of unknown etiology ($P < 0.01$ for LTBI and $P < 0.05$ for aTB and uninfected controls) groups.

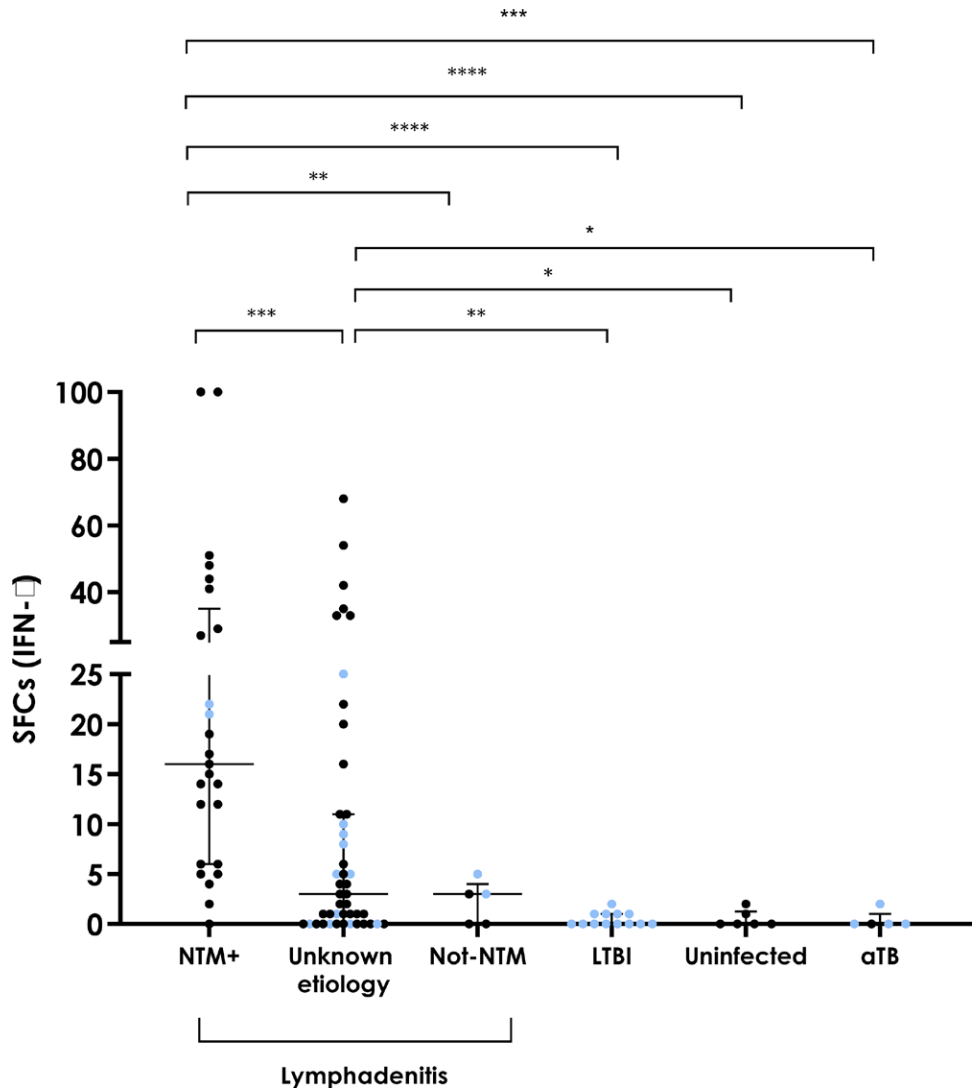

FIGURE 1. Response to stimulation with GPLs in samples from patients with lymphadenitis. Median (IQR) values in SFCs for each group were: 16 (6–35) for positive NTM lymphadenitis, 3 (0–11) for lymphadenitis of unknown etiology, 3 (0–4) for not-NTM lymphadenitis, 0 (0–1) for LTBI and 0 (0–1.25) for those uninfected. Mann-Whitney test was performed to compare between groups: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. In blue, those with a positive TB-IGRA. Results refer to the SFCs in the GPL stimulation condition after response subtraction of the negative control. The 2 samples from the NTM+ group located in the 100 SFCs had a SFCs count higher than 100 SFCs. Due to the difficulty in the counting of >100 SFCs, they have been represented here in that position. IQR: interquartile range.

Considering the wide range of response against GPLs in samples from unknown etiology lymphadenitis but compatible with mycobacterial infection, we analyzed them considering their TST result. We divided this group into 2: those with a TST result ≥5 mm and those with a TST <5 mm (Fig. 2). Although not statistically significant, those with unknown etiology lymphadenitis with a ≥5 mm TST result had a higher response against GPLs when compared with the TST <5 mm group (Fig. 2).

After ROC curve analysis, a positive value was considered when there were more than 3 SFCs (AUC = 0.97, Youden index = 0.92), obtaining 92.0% sensitivity and 100% specificity, [confidence interval (CI): 73.97%–99.02% and 54.07%–100%, respectively] (Fig. 3). Using the selected cutoff, we obtained a total of 45 positive results, the majority found in the NTM culture-confirmed lymphadenitis group (92.0%, 23/25), followed by the unknown etiology lymphadenitis group that had a TST result ≥5 mm (65.2%, 15/23) (Table 2). From the NTM-positive lymphadenitis group with a positive NTM-IGRA results (n = 23), 17 were positive for MAC, 2 for *M. malmoense* and 4 for *M. lentiflavum*. Considering MAC-positive samples alone (n = 18), 17 were positive for the test yielding a test sensitivity of 94.4%. The 2 samples from the NTM-positive lymphadenitis group with a negative NTM-IGRA were culture confirmed for MAC in one case and *M. malmoense* in the other. The positive sample from the group of not-NTM lymphadenitis was from a patient with Hodgkin lymphoma and its quantitative test result was 5 SFCs.

Taking into consideration the TB-IGRA, many samples from the lymphadenitis cohorts with a positive NTM-IGRA were negative for TB-IGRA (80.0%, 36/45). When considering the unknown etiology lymphadenitis but compatible with mycobacterial infection group (n = 45), 11 had a positive TB-IGRA. Despite not having microbiological confirmation, 5 were considered as TB following clinical criteria such as TB contact history, risk factors and epidemiology. From these, only one was positive for the NTM-IGRA. Regarding the remaining 6 samples, 5 had a positive NTM-IGRA; TB was finally ruled out by the pediatricians due to absence of TB risk factors or contact, borderline TB-IGRA results and/or TB-IGRA turning negative in follow-up visits. Regarding the aTB patients, the TB-IGRA was positive in 4 of 5 cases, while the NTM-IGRA was negative for all of them.

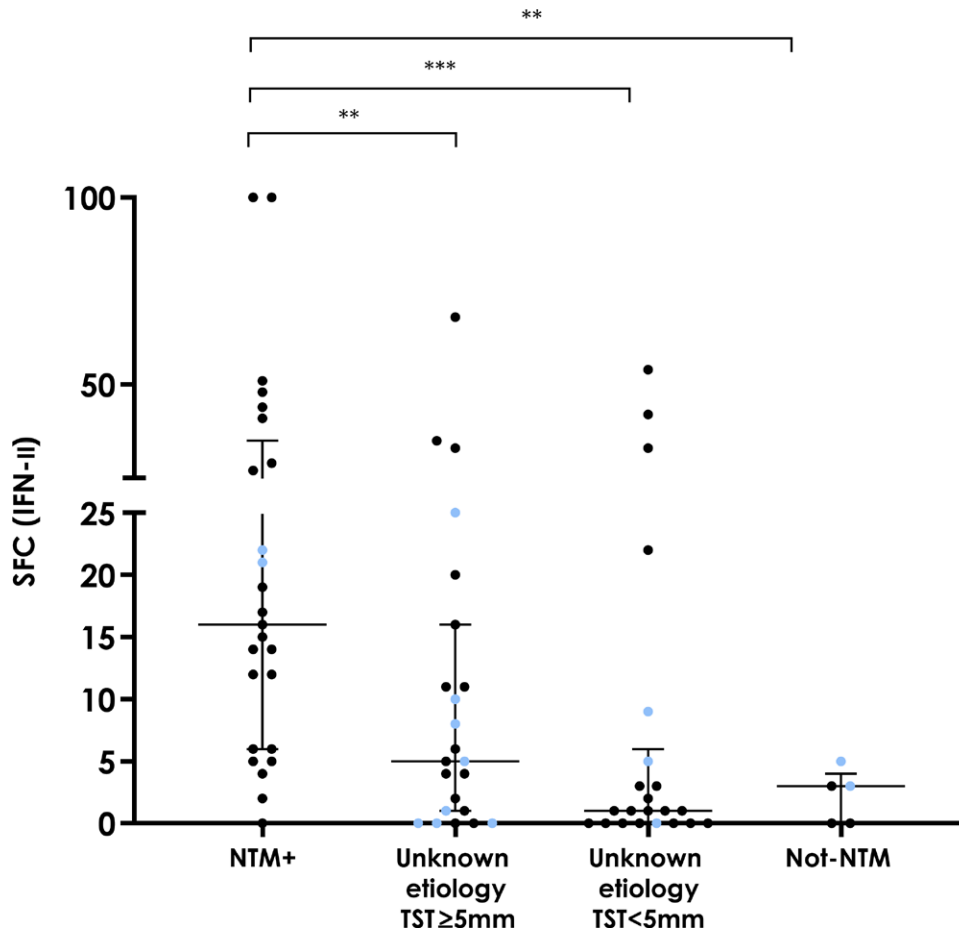

FIGURE 2. Response to stimulation with GPLs in samples from patients with lymphadenitis. Median (IQR) values in SFCs for each group were: 16 (6–35) for positive NTM lymphadenitis, 5 (1–6) for lymphadenitis of unknown etiology with a TST result ≥5 mm, 1 (0–6) for lymphadenitis of unknown etiology with a TST result <5 mm and 3 SFCs (0–4) for lymphadenitis not caused by NTM. Mann-Whitney test was performed to compare between groups: $^{**}P < 0.01$; $^{***}P < 0.001$. In blue, those with a positive TB-IGRA. Results refer to the SFCs in the GPL stimulation condition after response subtraction of the negative control. The 2 samples from the NTM+ group located in the 100 SFCs had a SFCs count higher than 100 SFCs. Due to the difficulty in the counting of >100 SFCs, they have been represented here in that position. IQR: interquartile range.

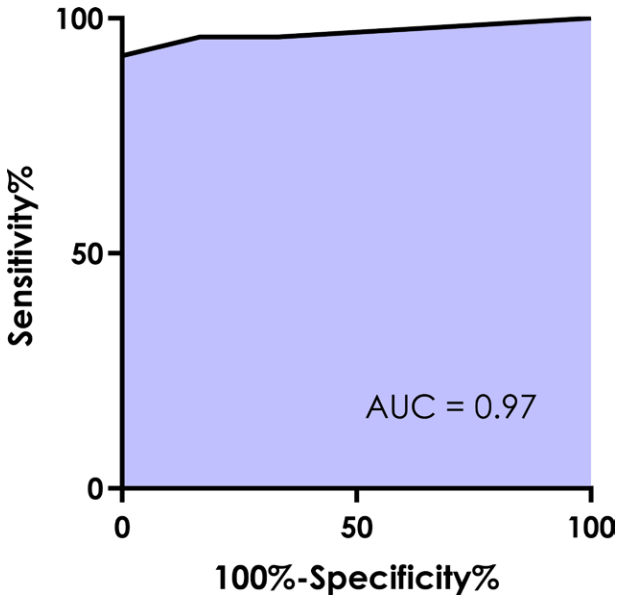

FIGURE 3. Receiver operating characteristic (ROC) curve analysis.

TABLE 2. NTM-IGRA-Positive Results Obtained Per Group

	Positive Results (%)
NTM-positive lymphadenitis (n = 25)	23 (92.0)
Lymphadenitis of unknown etiology (n = 45)	21 (46.7)
TST ≥5 mm (n = 23)	15 (65.2)
TST <5 mm (n = 22)	6 (27.3)
Not-NTM lymphadenitis (n = 5)	1 (20.0)
aTB (n = 5)	0 (0.0)
LTBI (n = 13)	0 (0.0)
Uninfected (n = 6)	0 (0.0)

LTBI indicates latently infected with *M. tuberculosis*.

DISCUSSION

NTM infection diagnosis remains a challenge and its rising incidence in the last years has arisen the importance of their correct diagnosis and management.[27] Cervicofacial lymphadenitis is the most common manifestation of NTM infections in children; however, to date, for a definitive diagnosis and characterization of the causative agent, isolation or confirmation by NAATs is needed. For this, an invasive procedure of sample collection from the lymph nodes is required and reliable results are not always obtained.[6] Here we developed an NTM-IGRA based on stimulation with GPLs, which are NTM specific, and evaluated its use in children with lymphadenitis to help guide clinical decisions.

After stimulating with GPLs, samples from NTM-confirmed lymphadenitis patients showed a higher response, followed by those from patients with unknown etiology lymphadenitis but compatible with mycobacterial infection. In the latter, those with a TST result ≥5 mm had a higher response compared with those with a TST <5 mm ($P > 0.05$). Using the selected positivity cutoff of 3 SFCs, the majority of samples from patients with NTM culture-confirmed lymphadenitis had a positive result (92.0%, 23/25) followed closely by those with unknown etiology lymphadenitis but compatible with mycobacterial infection and a TST ≥5 mm (65.2%, 15/23). These results suggest that in these 15 patients, their lymphadenitis could be due to an NTM that was not detected by culture or NAATs. This could also be the case of the 6 positive results obtained in the unknown etiology lymphadenitis group with a TST <5 mm.

Similar to our study, in 2019, Della Bella et al[28] evaluated the use of IFN-γ, Interleukin (IL)-2 and IL-17 detection by ELISPOT but using a *M. avium* lysate, also in children with lymphadenitis, to detect NTM infection. This study found that when distinguishing NTM infected from uninfected children, the IL-2 ELISPOT had sensitivity and specificity rates of 87.5% and 85.7%, respectively, while the IL-17 ELISPOT performed poorly (50.0% sensitivity and 71.4% specificity). Regarding the IFN-γ detection that relates to our present study, although using other antigens as stimuli, their test yielded sensitivity and specificity rates of 81.3% and 71.4%, respectively, using a cutoff of 63.4 SFC per million PBMCs. Our cutoff point using GPLs was lower (3 SFCs per 250,000 PBMCs, 12 SFC per million PBMCs) and the test sensitivity and specificity rates were higher (92.0% and 100%, respectively). Della Bella et al also reported an alternative analysis in which they excluded those patients who had a confirmed infection with a non-*M. avium* NTM. Doing this, their sensitivities and specificities improved in all cases. They also observed that some participants from the control group had a positive response to stimulation with the *M. avium* lysate, which could be due to an asymptomatic exposure to NTM. In our case, we did not have positive responses from the uninfected, aTB or LTBI groups, but a larger cohort will be needed to evaluate possible sensitizations of unregistered NTM infections. We also observed that 2 of the 3 cases of *M. malmoense*-positive lymphadenitis were positive for the NTM-IGRA. The presence of GPLs in this specie has not been described in the literature.[20] However, GPLs contain rhamnose, which could lead to cross-reaction with described rhamnose-rich components of the *M. malmoense* cell envelope.[29] Nevertheless, this could be also explained by an asymptomatic coexposure to other undetected NTM-containing GPLs.

Due to the cross-reaction between NTM and TB antigens present in PPD, TST can be a cause of confusion in TB infection diagnosis. In those cases with a negative TB-IGRA but positive TST despite no *Mycobacterium bovis* bacillus Calmette-Guérin vaccination, determining whether a patient is NTM infected rather than TB infected would benefit the patient's management, especially in children.[10,30–32] For this purpose, using GPLs, which are NTM specific, could aid in this situation, while sensitins and *M. avium* lysates, although shown to be useful to detect NTM sensitization, may also have higher cross-reactivity with *M.tb*. In this direction, Steindor et al[33] described an IGRA to specifically detect *M. abscessus* complex infection focusing on patients with pulmonary disease. This study used *M. abscessus*-specific proteins to stimulate T-cells using ELISPOT reaching a sensitivity and specificity of 58% and 94%, respectively.

Recently, the use of TB-IGRAs alone has been reported as suitable to distinguish lymphadenitis caused by MAC from tuberculous lymphadenitis in children.[11] When comparing 2 groups with either NTM- or TB-confirmed lymphadenitis, a specificity of 96.9% for the diagnosis of TB and positive and negative predictive values of 95.7% and 93.9% were obtained, respectively. This study presents a promising perspective for the use of TB-IGRAs in this scenario; however, this strategy should be evaluated further to test its validity in a prospective study. In our study, 2 patients with NTM culture-confirmed lymphadenitis and positive NTM-IGRA were positive for TB-IGRA. Should the TB-IGRA been performed alone in these 2 cases, valuable information for patient's care would have been missed.

Interestingly, in addition to the 2 patients with NTM-confirmed lymphadenitis, 6 samples from children with unknown etiology lymphadenitis but compatible with mycobacterial infection and 1 from the not-NTM lymphadenitis were positive for both IGRAs (NTM and TB). These results indicate that these patients, in

addition to being TB infected/sensitized, also recognized NTM antigens, suggesting that they could also be infected by NTM species. Given that the antigens used in the TB-IGRA (6 kD early secretory antigenic target and 10 kD culture filtrate antigen) are absent in the majority of NTM and the GPLs used in the NTM-IGRA are not present in *M.tb,* a cross-reaction is unlikely. Additionally, from the 11 samples with a positive TB-IGRA in the unknown etiology lymphadenitis but compatible with mycobacterial infection group, 5 were considered as TB where only one (20.0%) had a positive result of 5 SFCs for the NTM-IGRA. Most cases (83.3%, 5/6) in which TB was ruled out despite a positive TB-IGRA, the NTM-IGRA was positive.

From the clinical perspective, the selected cutoff of 3 SFCs is low as it could lead to unreliable reading and difficult interpretation of the results. Statistically, 3 SFCs is the optimal cutoff; however, following the ROC curve analysis, the next possible values would be 4.5 and 5.5 SFCs. Choosing the >4.5 cutoff value, the sensitivity and specificity would be 88.0% and 100%, respectively, with a Youden index of 0.88. By choosing the >5.5 as positivity point, the sensitivity and specificity would be 80.0% and 100%, respectively, with a Youden index of 0.8. Interestingly, when including in the ROC curve analysis the not-NTM lymphadenitis, aTB and LTBI groups as controls, the cutoff values obtained remain fairly the same: >3.5 as the optimal one followed by >4.5 and >5.5 with Youden indexes of 0.89, 0.85 and 0.80, respectively. However, from a clinical perspective, a cutoff of >5.5 spots reaches a higher specificity (100%) with a sensitivity of 80%. This identical cutoff considering different control groups for the ROC analysis reflects the robustness of the technique. Furthermore, considering a "borderline zone" between >3 and ≤6 SFCs, 12 samples would be classified as borderline: 6 from the unknown etiology lymphadenitis (5 of them with a TST >5 mm and 1 corresponding to the case considered as TB), 5 from the NTM culture-confirmed lymphadenitis (4 positive for MAC and 1 for *M. lentiflavum*) and 1 from the not-NTM lymphadenitis group. To select a definitive and more robust cutoff for the test, a bigger sample size will be necessary.

Whether IGRAs perform well in children, especially young children (<5 years), has no definitive clear answer yet; studies are scarce and reach different conclusions.[14,34–37] In 2010, Bamford et al[14] informed that only 66% of confirmed TB cases in children had a positive T-SPOT.TB. However, other studies suggest that they have a good diagnostic value.[36] In this regard, our NTM-IGRA performed well both in children under and over 5 years old, with high specificity and sensitivity rates (100% and 92.0%, respectively). Despite not having indeterminate results, the effect of decreased immune responses due to age should not be overlooked and could be, in fact, responsible for those cases with NTM-confirmed lymphadenitis that were negative for the test. In addition, evaluating the production of other cytokines and chemokines should also be considered to improve the sensitivity of the test as previously suggested for TB diagnosis.[28,38,39] Furthermore, although currently unknown, the performance of the test may vary depending on the NTM responsible for the lymphadenopathy where alternative cutoffs and cytokine patterns could be more suitable.

As limitations of this study, we found that the number of children with lymphadenitis included is small and lacks diversity in terms of NTM-isolated species. Additionally, we lack a culture-confirmed *M.tb* lymphadenitis group that could give us more information about possible cross-reaction. In this regard, all patients from the aTB group studied were NTM-IGRA negative, and considering the samples from culture-confirmed pulmonary TB patients included in Material, Supplemental Digital Content 1, http://links.lww.com/INF/F348, only 6 of 29 had a positive result for the test, which could be due to a coexposure with undetected NTM as discussed previously. To address these limitations, a bigger cohort with inclusion of a more diverse NTM-confirmed lymphadenitis group with a wider range of NTM species and considering sites with a higher prevalence of TB would be optimal to arrive to more robust conclusions. Finally, although the validation studies showed a high correlation, the use of different methodology described in Figures, Supplementary Digital Contents 2, http://links.lww.com/INF/F349 and 3, http://links.lww.com/INF/F350 (fixed GPLs vs. nonfixed) may have an effect on the results.

Overall, the results obtained in this study suggest that the NTM-IGRA evaluated could serve as a less invasive tool in the diagnosis of peripheral lymphadenitis caused by NTM in children. To our knowledge, this is the first study describing the use of NTM GPLs as stimulating antigens and we show that these could be potential candidates for an immunodiagnostic test to detect NTM infection. In this study, we have successfully evaluated their use in children with lymphadenitis, which could be used in combination with TB-IGRAs, strengthening the certainty of the results. In addition, their use could also address other populations and help guide clinical decisions in situations in which differential diagnosis is needed.

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