INTERFERON-GAMMA RELEASE ASSAYS DIFFERENTIATE BETWEEN *MYCOBACTERIUM AVIUM* COMPLEX AND TUBERCULOUS LYMPHADENITIS IN CHILDREN

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Conflicts of interest:

Marc TEBRUEGGE has received QuantiFERON assays at reduced pricing or free of charge for other TB diagnostics projects from the manufacturer (Cellestis/Qiagen) in the past, and has received support for conference attendance from Cepheid. The manufacturers had no influence on study design, data collection, analysis or interpretation, writing of the manuscript or decision to submit the data for publication. The remaining authors have no conflicts of interest to disclose.

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Short title: Interferon-gamma release assays differentiate MAC and TB adenitis

List of abbreviations:

BCG, bacillus Calmette-Guérin

ENSeMBLE, European NontuberculouS MycoBacterial Lymphadenitis in childrEn

IGRA, interferon-gamma release assays

IQR, interquartile range

LTBI, latent tuberculosis infection

MAC, *Mycobacterium avium complex*

MTB, *Mycobacterium tuberculosis*

NPV, negative predictive value

NTM, non-tuberculous mycobacteria

PPD, purified protein derivative

PPV, positive predictive value

pTBred, Spanish Network for the Study of Pediatric Tuberculosis

QFT-GIT, QuantiFERON Gold-in-Tube

RD1, region of difference

TB, tuberculosis

TST, tuberculin skin test

ABSTRACT

Objectives

Non-tuberculous mycobacteria (NTM) can cause subacute/chronic cervical lymphadenitis in children, and *Mycobacterium avium complex* (MAC) are the commonest causative species. We aimed to assess the performance of interferon-gamma release assays (IGRA) in the differential diagnosis between NTM and tuberculous (TB) lymphadenitis.

Study designs

Multi-center observational study comparing children with microbiologically-confirmed MAC lymphadenitis from the European NontuberculouS MycoBacterial Lymphadenitis in childrEn (ENSeMBLE) study with children with TB lymphadenitis from the Spanish Network for the Study of Pediatric TB (pTBred) database.

Results

Overall, 78 patients with MAC and 34 with TB lymphadenitis were included. Among MAC cases, 44/74 (59.5%) had positive tuberculin skin test (TST) results at the 5 mm cut-off, compared with 32/33 (97.0%) TB cases ($p<0.001$); at the 10 mm cut-off TST results were positive in 23/74 (31.1%) *vs*. 26/31 (83.9%), respectively ($p<0.001$). IGRA results were positive in only 1/32 (3.1%) MAC cases who had undergone IGRA testing, compared with 21/23 (91.3%) TB cases ($p<0.001$). Agreement between TST and IGRA results was poor in MAC (23.3%;$\kappa=0.017$), but good in TB cases (95.6%;$\kappa=0.646$). IGRA had a specificity of 96.9% (95%CI:84.3-99.8%), positive predictive value (PPV) of 95.4% (95%CI:78.2-99.8%), and negative predictive value (NPV) of 93.9% (95%CI:80.4-98.9%) for TB lymphadenitis.

Conclusions

In contrast to TST, IGRA have high specificity, NPV and PPV for TB lymphadenitis in children with subacute/chronic lymphadenopathy, and can consequently help to discriminate between TB and NTM disease. Therefore, IGRA are useful tools in the diagnostic work-up of children with lymphadenopathy, particularly when culture- and PCR-results are negative.

INTRODUCTION

Non-tuberculous mycobacteria (NTM) are ubiquitous bacteria found in the environment worldwide. In healthy young children, NTM infections are one of the most frequent causes of subacute/chronic cervical lymphadenitis, although epidemiological data remain scarce (1-3). *Mycobacterium avium* complex (MAC) has consistently been reported to be the most common causative species of NTM lymphadenitis across various geographical locations (3-5), accounting for up to 70-80% of cases (5).

In low-burden tuberculosis (TB) countries, peripheral lymphadenitis remains the commonest presentation of extrapulmonary TB (6,7). In Spain, the incidence of TB has continuously decreased over the past 20 years, to 9.34 cases/100,000 in 2019 overall (4.20/100,00 in patients aged <15 years)(8). Bacillus Calmette-Guérin (BCG) vaccination has not been part of the routine immunization program in Spain since 1980, except for the Basque Country, where neonatal vaccination continued until 2013.

Clinically it is challenging to distinguish between TB and NTM lymphadenitis, as both conditions have similar clinical features, including gradual lymph node enlargement over several weeks, and often lack of constitutional symptoms that characterize other forms of TB, such as persistent fever, malaise and weight loss (9,10). Radiological features are also very similar (9), and histologically TB and NTM lymphadenitis are often indistinguishable, although necrotizing granulomas appear more common in the former (10). Furthermore, mycobacterial cultures have suboptimal sensitivity in both diseases due to the low mycobacterial load. Previous studies have shown that mycobacterial cultures are false-negative in approximately one third of cases with NTM lymphadenitis (11-13), and similar figures have been reported for TB lymphadenitis (14,15).

In children, immune-based tests are used for the diagnosis of latent TB infection (LTBI), and also commonly as an adjunctive tool in the diagnostic work-up for TB disease. One key limitation of the tuberculin skin test (TST) is that it cannot distinguish between TB and NTM infection, as the test substance, purified protein derivative (PPD), contains antigens that are expressed by both *Mycobacterium tuberculosis* (MTB) and NTM. Based on the test design, interferon-gamma release assays (IGRA) are likely to have greater specificity for TB infection (13). IGRA are based on the detection of interferon-gamma responses to relatively MTB-specific antigens encoded in the region of difference (RD1). RD1 is absent in most NTM

species, including MAC, although there are notable exceptions, including *M. kansasii*, *M. marinum* and *M. szulgai* (16). Consequently, IGRA should have an inherent ability to distinguish between TB and NTM infections. However, to date the available data related to this issue are limited to fewer than 50 patients with microbiologically-confirmed NTM infections across different studies (3,17,18).

The primary aim of this study was to determine whether IGRA can aid to distinguish between MAC and TB lymphadenitis in children and adolescents, using two separate large cohorts of children with microbiologically-confirmed MAC and microbiologically-confirmed TB disease. Secondarily this study aimed to describe the clinical presentation, therapeutic approaches and outcomes in children with NTM and TB lymphadenitis.

PATIENTS AND METHODS

Study design

A cross-sectional study was performed within the Spanish Network for the Study of Pediatric TB (pTBred) and the European NontuberculouS MycoBacterial Lymphadenitis in childrEn study (ENSeMBLE).

pTBred is a multidisciplinary collaborative network comprising healthcare professionals managing children with TB in Spain, and currently includes 148 members across 83 institutions (19,20). Patients aged <18 years diagnosed with TB disease are eligible for inclusion in the pTBred database. Study data are collected and managed using REDCap electronic data capture tools (21), hosted at Instituto de Investigación Sanitaria Gregorio Marañón (Madrid). Approval for this study was obtained from Hospital Carlos III (Madrid) Ethics Committee (ref. P13/12). Informed consent from parents and informed assent from patients aged >12 years is obtained prior to inclusion.

The ENSeMBLE Study is a multinational retro- and prospective multi-center observational cohort study comprising centers/investigators within pTBred in Spain, the Paediatric Network European Trials Group (22-24) and the Nontuberculous Mycobacteria Network European Trials Group (NTM-NET), investigating children with peripheral NTM lymphadenitis. The inclusion criteria were: i) age <18 years, and ii) microbiologically confirmed NTM infection (by culture or PCR). Study data are collected through REDCap software. Ethics approval for this study was obtained from the Hospital Sant Joan de Déu (Barcelona) Ethics

Committee (ref. EPA-04-15). In the prospective study arm informed consent from parents or legal guardians is obtained prior to inclusion.

For the study presented here, patients with peripheral lymphadenitis and a positive culture and/or molecular assay result for MTB from any anatomical site who had been entered into the pTBred database between January 2013 and June 2018 were included, and compared to patients from the ENSeMBLE Study with culture and/or PCR-confirmed peripheral lymphadenitis caused by MAC who had been recruited by June 2018.

Immunological and microbiological tests

TSTs were performed by intradermal injection of 2 tuberculin units of PPD (RT23, Statens Serum Institut, Copenhagen, Denmark), with results read after 48–72 hours. As per national guidelines, an induration of ≥5mm diameter was considered to be positive (25). All IGRA assays (QuantiFERON-TB Gold [QFT], QFT Gold-in-Tube and QFT-Plus, Cellestis/Qiagen, Carnegie, Australia [26]; and T-SPOT.*TB*, Oxford Immunotec, Abingdon, United Kingdom [27]) were performed in fully-accredited diagnostic laboratories at each participating institution, and interpreted according to manufacturers´ instructions. Cultures and PCR-based assays for MTB and NTM were also performed at fully-accredited clinical laboratories at the participating institutions or at regional reference laboratories.

Study definitions and classifications

The clinical phenotype at presentation was classified according to affected sites, and clinical stage (I: painless, firm, adherent to overlying skin, increased vascularity; II: fluctuance; III: skin changes, violaceous discoloration, thinning of the skin, parchment-like changes, shiny appearance; and IV; fistulization [28]). Complications and/or sequelae were defined as: secondary infection, facial nerve palsy, hypertrophic scar/keloid, changes in skin color, fistulization, or adverse drug events. Paradoxical reaction was defined as clinical or radiological worsening of existing TB lymphadenitis or development of new lesions in patients receiving anti-tuberculous medication who initially improved on treatment (29).

Statistical analysis

Categorical data are presented as absolute numbers and proportions; continuous variables are expressed as median and interquartile ranges (IQRs). Groups were compared with Student's *t* tests or Mann-Whitney *U* tests for continuous variables, as appropriate, and chi-square tests for categorical variables. Total percentage agreement and Cohen kappa coefficient (κ) with standard error were used to quantify concordance between TST and IGRA results; indeterminate IGRA results were excluded from that particular analysis. Strength of agreement was defined as poor ($\kappa \leq 0.2$), fair ($0.2 < \kappa \leq 0.4$), moderate ($0.4 < \kappa \leq 0.6$), good ($0.6 < \kappa \leq 0.8$) and very good ($\kappa > 0.8$). Statistical significance was defined as a two-sided *p*-value <0.05. All analyses were performed with SPSS (Version 23.0, Chicago, IL, U.S.).

RESULTS

Between January 2013 and June 2018, 189 patients with microbiologically-confirmed NTM lymphadenitis were contributed to the ENSeMBLE study by Spanish centers, with the earliest retrospective case having been diagnosed in 1996. In 78 (43.6%; 34 female) MAC was identified as the causative agent (earliest case in 1997). By the same cut-off date, 604 TB cases had been included in pTBred, 113 (18.7%) of whom had extrapulmonary TB disease; peripheral lymphadenitis was the most common diagnosis in the latter group (53/113;46.9%). Of those 53 cases, 34 (53%; 18 female) were confirmed microbiologically (MTB, n=27; *Mycobacterium bovis*, n=7), and therefore included in this study.

A comparison of baseline characteristics and clinical presentation of MAC and TB lymphadenitis cases is shown in ***Table 1***. Patients with TB lymphadenitis were on average significantly older, more commonly born abroad, more commonly BCG-vaccinated, and more commonly had significant underlying conditions than patients with MAC lymphadenitis. In TB patients, the most common site of disease was the cervical area, while in MAC patients the submandibular/jugulodigastric location predominated; no other clinical characteristics at presentation were potentially useful to aid the distinction between the two disease entities.

Of 78 MAC cases, only three had risk factors for TB infection (known TB contact, n=2; birth in high TB burden country, n=1). The clinical stage at presentation was I, II, III and IV in 38 (48.7%), 8 (10.3%), 30 (38.5%) and 2 (2.5%) cases, respectively. Overall, constitutional symptoms were very rare in cases with MAC lymphadenitis, present in only 2 (2.6%) patients.

Among TB patients, 12 (35.3%) had constitutional symptoms at presentation, comprising fever (n=9;26.5%), asthenia (n=7;20.6%) and weight loss (n=5;14.7%). The median (IQR) lymph node diameter at presentation was 3.0 (3.0-4.0) cm. Spontaneous fistula formation had occurred in three cases (8.8%) prior to presentation. Based on radiological investigations, co-existing pulmonary disease was present in 12 (35.3%) patients (abnormal chest x-ray, n=11; abnormal chest computed-tomography scan, n=1). Six (17.6%) patients had co-existing extrapulmonary TB disease, comprising abdominal (n=2), central nervous system (n=2), and osteoarticular disease (n=1); one had a thigh abscess with inguinal lymphadenitis. All *M. bovis* cases were previously healthy children of Moroccan origin presenting with unilateral cervical lymphadenitis, without TB disease elsewhere.

Immune-based test results

The results of the immune-based tests are summarized in ***Table 2*** and ***Table 3 (online)***. IGRA were performed in 32 MAC patients (QFT,n=24; T-SPOT.*TB*,n=4; both assays,n=4); 30 patients had negative results, while one patient had a positive and one an indeterminate assay result. The overall agreement between TST and IGRA results was poor (23.3%;κ=0.017 at the 5mm cut-off; 53.3%;κ=0.067 at the 10mm cut-off). The positive QFT result occurred in a 3-year-old boy with a 3-week history of unilateral submandibular and cervical lymphadenopathy with recent close contact with a smear-positive adult with pulmonary TB; his TST result was also positive (17mm induration), but his chest x-ray was unremarkable. A lymph node biopsy grew MAC in culture; neither cultures nor molecular tests identified MTB in lymph node material or gastric aspirates.

Among TB patients, agreement between TST and IGRA results was good (95.6%;κ=0.646 at the 5mm cut-off and 85.7%;κ=0.323 at the 10mm cut-off). Both tests were negative in a previously healthy BCG-unvaccinated 12-year-old Spanish girl with submandibular TB lymphadenitis confirmed by Xpert MTB/RIF (Cepheid, Maurens-Scopont, France) performed on lymph node tissue; she was not known to be immunodeficient or receiving immunosuppressive medication. Also, a previously healthy BCG-unvaccinated 8-year-old Spanish boy with submandibular TB lymphadenitis confirmed by FluoroType-MTB (Hain Lifescience, Tübingen, Germany) performed on gastric aspirates, had a positive TST (12mm induration), but both a QFT-GIT and a T-SPOT.*TB* assay produced negative results.

A significantly larger proportion of TB patients had positive TST and/or IGRA results compared with MAC patients, and TST indurations were significantly larger in the former group (***Table 2***). Overall, IGRA assays had a sensitivity of 91.3% (95%CI:73.2-98.4%), a specificity of 96.9% (95%CI:84.3-99.8%), a positive predictive value of 95.4% (95%CI:78.2-99.8%) and a negative predictive value of 93.9% (95%CI:80.4-98.9%) for TB disease.

Microbiological test results

The sample types analyzed and the corresponding microbiological test results are summarized in ***Table 2***. In MAC cases, microbiological confirmation was obtained from samples from the site of disease (lymph node material or discharge fluid) in all cases. Cultures and molecular assays were positive in 76/78 (97.4%) and 6/20 (30.0%) cases, respectively; in four patients both were positive.

In contrast, among patients with TB lymphadenitis only 26 (76.5%) were microbiologically confirmed by testing of lymph node material or discharge fluid; in the remaining 8 (23.5%) the diagnosis of TB disease was based on detection of MTB or *M. bovis* at another site, mainly in gastric aspirates and respiratory samples.

Treatment and outcomes

The initially planned treatment in MAC lymphadenitis patients according to clinical stage at diagnosis is summarized in ***Table 4***. This mainly comprised surgical intervention (n=56;71.8%), with (n=33) or without (n=23) anti-mycobacterial treatment (***Table 5 [online]***). After initial treatment, 16 (20.5%) patients needed further treatment not planned initially (anti-mycobacterial drugs;n=4; surgical intervention;n=12). One patient was lost to follow-up. At a median follow-up of 7.5 (IQR:4.3-13.4) months, 32 (41.6%) patients had experienced complications, comprising persistent/recurrent local MAC infection (n=9), newly developed fistula (n=11), bacterial superinfection (n=3), temporary (n=8) or permanent (n=1) facial palsy; 17 (21.8%) had cosmetic sequelae (changes in skin color, keloid or hypertrophic scar). The overall incidence of facial nerve palsy (temporary or permanent) among patients who underwent surgical intervention was 16.1% (9/56). There was no statistical association between the clinical stage at presentation and the initial therapeutic approach chosen (p=0.393), the need for additional unplanned treatment (p=0.494) or the

development of complications/sequelae (p=0.351). Initial complete surgical resection was not associated with fewer complications/sequelae (p=0.375) compared to conservative management (*i.e.* observation or antibiotics alone). Additional subgroup analyses only including cases with clinical stage I or II disease at presentation produced similar results (data not shown).

In TB lymphadenitis patients, standard treatment regimens with first-line oral anti-TB drugs were used for a median duration of 28 (IQR:26-36) weeks. Patients with additional extrapulmonary TB disease at another site received significantly longer treatment courses on average (52 [IQR:49-72] weeks; p<0.0001). Fifteen patients underwent initial surgical procedures for diagnostic purposes (fine-needle aspiration or biopsy). Six underwent lymph node excision and three abscess drainage prior to the diagnosis of TB being established. During anti-TB treatment, fistula formation and paradoxical reactions occurred in seven and two patients, respectively. Four patients underwent excisional surgery due to limited response to antibiotic treatment. One patient was lost to follow-up 9 weeks after treatment initiation; the remaining 33 were cured without functional long-term sequelae, but four had developed keloid scars. Neither complications during treatment (41.6% *vs.* 27.3%;p=0.155) nor long-term sequelae (23.4% *vs.* 12.1%;p=0.176) were significantly more common in MAC than in TB cases.

DISCUSSION

This study includes two of the largest series of children and adolescents diagnosed with microbiologically-confirmed MAC and TB peripheral lymphadenitis, facilitated by ongoing research collaborations involving a very large number of healthcare institutions providing care for children with mycobacterial infections.

The clinical characteristics were similar to those described by other authors, both for TB and MAC lymphadenitis (3,30-33). Compared to MAC cases, children with TB lymphadenitis are usually older and more commonly born in TB-endemic countries, and present more commonly with constitutional symptoms and elevated inflammatory markers (9,31). Our findings confirm these clinical differences, although none of these features allow to discriminate between MAC and TB lymphadenitis.

The most common location of lymph node disease differed significantly between both groups. While submandibular/jugulodigastric lymph nodes were most commonly affected in NTM patients, the cervical

region predominated in TB patients. In contrast, we observed no significant differences in the proportions of children with bilateral involvement, or with more than one lymph node region affected, both of which have been reported to be less common in NTM lymphadenitis (5).

The diagnostic workup in our series included TST in almost all patients. Several mycobacterial peptides contained in PPD are expressed by both MTB and NTM species. Consequently, positive TST results have been reported in 30% to 60% of children with NTM disease, which aligns with the proportion of patients (59% at the 5mm cut-off; 31% at the 10mm cut-off) observed in our series (1,3,12,33). Although the TST positivity rate was lower and induration diameters were overall smaller in MAC patients, this test did not allow to distinguish between MAC and TB infection.

Despite specificity being the key advantage of IGRA over TST, data on their performance in the setting of NTM infections in the pediatric age group are scarce (3,17,18). Including data from our series (***Table 6***), only 3 (3.8%) of 78 children and adolescents with microbiologically-confirmed NTM infections were reported to have had positive IGRA results. Two of those patients had cutaneous *M. marinum* infection (rather than NTM lymphadenitis)(3), an NTM species that expresses RD-1 antigens, and those positive results are therefore to be expected. The remaining patient (current series) had MAC lymphadenitis and likely had concomitant LTBI following recent TB contact. In our study, IGRA assay specificity, positive and negative predictive values for the diagnosis of TB lymphadenitis were universally high (96.9%, 95.7% and 93.9%, respectively). These figures are in line with a meta-analysis on the performance of IGRA in childhood TB that included sensitivity estimates from eight pediatric studies (34). However, only one of those eight studies included patients with microbiologically-confirmed NTM disease as part of the control group (n=19; [17]). Our results confirm the usefulness of IGRA for discriminating between TB and MAC infections in children with subacute/chronic peripheral lymphadenitis.

Most TB and MAC cases were confirmed by culture in our series. It would not be appropriate to compare the diagnostic sensitivity of cultures or molecular methods between MAC and TB patients, as only microbiologically-confirmed cases were eligible for inclusion in both cohorts and samples other than lymph node tissue were only tested in TB cases. However, the low sensitivity (31.8%) of molecular tests in culture-confirmed MAC cases in our cohort is striking when compared to that reported in previous studies, ranging

from 72% to 91% (10,35). This could be related to the earliest MAC case in our series dating back to 1997, and validated NTM PCRs having been developed only relatively recently. In comparison, molecular tests performed far better in the TB cases, with false-negative results only occurring in approximately 1 in 5 patients. This aligns well with the pooled sensitivity estimate of 83.1% for Xpert MTB/RIF performed on TB lymph node tissue or aspirates in a recent meta-analysis (36).

The treatment of NTM-associated lymphadenitis in healthy children remains controversial (37). A meta-analysis showed that surgical excision has the highest cure rate, compared with antibiotics or watchful waiting, but that this treatment option is also associated with significant complications, such as the ones observed in our study, including facial palsy, fistulae, bacterial superinfection and cosmetic sequelae (5). However, our ambispective observational study design prevents any conclusions regarding the effectiveness of different treatment options. Approximately half of the patients both underwent surgery and received antibiotics as initial treatment, and a total of 11 different antibiotic regimens were used. We did not find differences in the rate of complications during treatment or adverse long-term outcomes in NTM patients associated with the clinical stage at presentation or the initial treatment chosen, even when only cases with early stage disease were analyzed. As expected, in TB patients surgical procedures were mainly performed for diagnostic purposes, and only rarely during antibiotic treatment due to poor response to anti-tuberculous therapy (30,32,38). Complication rates during treatment were similar in MAC and TB cases, and sequelae were less common in the latter, albeit not significantly. Similar to previous reports, facial palsy affected 9 (11.7%) patients in our MAC cohort, which was permanent in one (3). Fistula formation was the most common complication in TB lymphadenitis, frequently occurring after fine-needle aspiration performed during the diagnostic work-up.

The main limitation of this observational study lies in its ambispective design. Also, our results may not be applicable to high-burden TB settings or to lymphadenitis caused by NTM species other than MAC, particularly those that possess the RD1 region, such as *M. kansasii* (16). However, RD-1-containing NTM species are an uncommon cause of NTM lymphadenitis, accounting for <5% of the cases overall (5,39). We decided to include only microbiologically-confirmed cases, thereby inevitably reducing the number of eligible cases, to produce robust data based on the current diagnostic gold standard. A further limitation is that a variety of different PCR methods were used for the detection of MTB and NTM in clinical samples

across the different participating centers, limiting our ability to determine their precise performance characteristics.

Our study shows that certain characteristics, including demographics, presenting symptoms and location of disease, differ between children with MAC and those with TB lymphadenitis. However, none of these features allow to securely distinguish between those two disease entities. While fewer children with MAC disease had a positive TST result and TST indurations were on average smaller than in TB cases, this immune-based test did not help to distinguish between MAC and TB lymphadenitis. In contrast, our data show that IGRA can contribute substantially to making this distinction, as indicated by the finding that their specificity, positive predictive value and negative predictive value for TB lymphadenitis universally exceeded 90%. Consequently, IGRA can play an important part of the diagnostic work-up of children presenting with subacute/chronic lymphadenitis, particularly those with subsequently negative culture and PCR results. Further, prospective studies in larger pediatric study populations are needed to consolidate our findings.

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