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The complexity of diagnosing latent tuberculosis infection in older adults in long-term care facilities

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SUMMARY

Objectives: In the USA, tuberculosis disease rates are highest in older adults. Diagnostic testing for latent tuberculosis infection (LTBI) has not been evaluated carefully in this group. The aim of this study was to define the relationship between tuberculin skin test (TST) results, T-SPOT.*TB* results, and T-cell responses to *Mycobacterium tuberculosis* antigens.

Methods: Long-term care facility residents with known prior TST results (positive or negative) were retested with TSTs and T-SPOT.*TB*. Prior exposure to *M. tuberculosis* was assessed by quantifying T-cell activation to mycobacterial antigens in vitro.

Results: The median age of the 37 participants was 77 years (range 57–98 years). Among 18 participants with a prior positive TST, three (16.7%) had a negative TST when retested (TST reversion); two had a negative T-SPOT.*TB*. Of the 15 who were historically and currently TST-positive, four (26.7%) had a negative T-SPOT.*TB* and one (6.7%) had a borderline result. Percentages of CD4+ T-cells responding to mycobacterial antigens were higher in participants with positive TST and T-SPOT.*TB* (18.2%) compared to those with a positive TST but negative T-SPOT.*TB* (6.4%, $p = 0.16$) and negative TST and T-SPOT.*TB* (5.9%, $p < 0.001$).

Conclusions: LTBI testing in older adults is complicated by TST reversion and TST-positive/T-SPOT.*TB*-negative discordance, *which may reflect clearance of infection or waning immunity.*

1. Introduction

An estimated 32% of the world's population, or 1.86 billion people, are infected with *Mycobacterium tuberculosis*,[1] and more than eight million new cases of tuberculosis disease (TB) occur each year.[2] Between 1993 and 2008, 21.9% of TB cases in the USA involved older adults (≥65 years of age),[3] so continued progress towards the elimination of TB in the USA will require that the substantial burden of TB in older adults is addressed. Average yearly TB rates between 1993 and 2008 were 1.5 times higher in older adults than in those aged 21–64 years and were 2.3 times higher in older adults residing in long-term care facilities (LTCF) than in those in the community.[3] Older adults comprise the fastest growing sector of the global population and are expected to account for 20% of the US population by the year 2050.[4] The population of older adults in need of long-term care is predicted to rise from eight million in 2000 to 19 million in 2050.[5] Because most TB in older adults likely results from reactivation of latent TB infection (LTBI) rather than new infection,[6,7] accurate diagnosis of LTBI in this population is critical, particularly if linked to the treatment of LTBI.

Diagnosing LTBI in older adults is complicated because of the limited specificity of the tuberculin skin test (TST) and reversion over time. TSTs are known to have reduced specificity among persons with non-tuberculous mycobacterial exposure, those with a history of residence in the southern USA, and bacillus Calmette–Guérin (BCG)-vaccinated individuals.[8–11] The use of boosted (two-step) TSTs, recommended for LTCF residents,[6,9] further reduces test specificity; a study from Hong Kong showed that boosted TST results are not predictive of TB disease.[12] The sensitivity of the TST

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in older adults may also decrease due to waning immunity,[13–17] with a 5% decline in test positivity per decade after age 65 years, up to 9% annually in those >60 years of age.[14,16] Comorbidities associated with older age, including being malnourished or underweight[12] and requiring assisted feeding,[14] can also reduce TST sensitivity. What is unknown is the degree to which the waning TST response reflects cleared infection and to what degree the waning TST response reflects a false-negative test.

Data on the use of interferon-gamma release assays (IGRAs), including the T-SPOT.*TB* and QuantiFERON-TB Gold In-Tube (QFT-GIT) assays, in older adults are limited, but the specificity for all ages is greater than the TST when BCG-vaccinated individuals are tested.[8,11,18] For low-risk populations, pooled specificity estimates range from 93% for T-SPOT.*TB* to 98% for QFT-GIT,[11] and it appears that IGRA sensitivity is less affected by age than the TST. In one study, older adults (defined as age greater than 50 years) were 3.8 times as likely to be QFT-GIT-positive/TST-negative as younger adults.[13] Older age was associated with positive IGRA (but not TST) results in persons screened prior to renal transplant,[19] Japanese healthcare workers,[20] and persons with radiographic evidence of healed TB.[21] Similarly, IGRA results were not affected by age in patients with silicosis[22] and in the older population in the UK (odds ratio 5.3, 95% confidence interval 2.9–9.8 for IGRA positivity with increasing age).[23] This suggests that with age, the TST response may wane, but the IGRA response persists.

Persistent immune responses to *M. tuberculosis* antigens can be assessed by evaluating antigen-specific memory cell response and activation. CD69 is an early activation marker on the surface of lymphocytes[24,25] and a costimulatory molecule for T-cell activation and proliferation.[25,26] The expression of CD69 on CD4+ T-cells correlates with TST induration, lymphocyte blastogenesis, and IGRA results.[25,27] CD69 has been found in greater amounts on CD4+ T-cells (after *M. tuberculosis* antigen stimulation) in persons who are TST-positive or have clinically inactive/treated TB compared with those who are TST-negative.[25,27,28] It was therefore predicted that CD69 upregulation could potentially be used to quantify T-cell activation in vitro to identify infection in older adults, thereby differentiating 'true-positive' from 'false-positive' TST and T-SPOT.*TB* results.

Given the potential for LTBI reactivation within LTCFs and spread to other residents, the diagnosis and treatment of LTBI is a public health priority. The goal of this study was to improve our understanding of LTBI diagnostics in this population and determine the relationship between TST responsiveness in older adults, T-SPOT.*TB* test results, and immunologic evidence of memory T-cell response.

2. Methods

2.1. Study location and data collection

This cross-sectional study was performed between September 2011 and August 2012 in three Boston-area LTCFs. Records were reviewed to identify all HIV-uninfected residents with a history of a positive TST performed on admission to the facility, or documented as positive prior to admission as part of the patient's routine healthcare. The specific setting and indication for prior testing was not always known and was not recorded. Controls with known prior negative TSTs (and no known positive TST) were matched to the TST-positive individuals by sex and age (within 5 years). Study personnel performed a medical records review and administered a standardized questionnaire to participants, or to the legal authorized representatives (i.e., healthcare proxies) of those persons without capacity to provide consent. Questionnaires addressed known history of TB disease, LTBI history, BCG vaccination, and comorbidities known to be associated with LTBI.

The TST was performed using the Mantoux technique with 2 TU of purified protein derivative (Sanofi Pasteur, Cambridge, MA, USA) and read by trained personnel at 48–72 h; results ≥10 mm were considered positive,[29] and any questionable result was confirmed by a second reader. TSTs were repeated ('boosted') once after 7–14 days for those persons with an initial negative TST. T-SPOT.*TB* assays were performed at the first study visit and were processed by Oxford Immunotech (Marlborough, MA, USA) in accordance with the manufacturer's guidelines. Results, interpreted by subtracting the spot count in the negative control from the spot count in panels A and B, were reported as positive (>8 spots), negative (<4 spots), borderline (5–7 spots), or invalid. Blood samples were collected at the first study visit for immunological assessment.

2.2. Immunological assays

Peripheral blood mononuclear cells (PBMCs), isolated from peripheral blood by Ficoll density gradient (Sigma-Aldrich, St. Louis, MO, USA), were cultured in 10% fetal bovine serum in RPMI 1640 supplemented with 2 mM L-glutamine and 1 mM penicillin/streptomycin (Invitrogen, Woburn, MA, USA). Cells were stimulated with *M. tuberculosis* whole cell lysate (WCL) (BEI Resources, Manassas, VA, USA) for 3 days. Cells were harvested and prepared for flow cytometry to quantify T-cell activation using anti-CD69, CD3, CD4, and CD8 antibodies (BD Pharmingen, San Diego, CA, USA). The positive control for T-cell activation was stimulation with anti-CD3/CD28 (eBioscience, San Diego, CA, USA); CD3+CD4+ T-cells and CD3+CD8+ T-cells were assessed for expression of CD69 by flow cytometry. Supernatants were analyzed for expression of interferon-gamma (IFN-γ) by ELISA (R&D Systems, Minneapolis, MN, USA).

2.3. Statistical analyses

All analyses were performed using SAS 9.1.3 (SAS Institute, Cary, NC, USA). *p*-Values were calculated using the exact Wilcoxon two-sample test for continuous variables and Fisher's exact test for categorical variables. CD69 expression on CD4+ T-cells and CD8+ T-cells was analyzed in two ways: (1) percentage expression, and (2) the ratio of percentage expression following WCL stimulation to percentage expression following stimulation with media (with a cut-off of 3 denoted as 'positive' CD69 expression). The Institutional Review Board of Boston University approved this study.

3. Results

3.1. Participant characteristics

One hundred and twenty-one persons were screened for participation and 38 persons were enrolled in the study (31.4% participation rate). Among the 83 who did not participate, the reasons for non-participation included legally authorized representative unwilling to consent (n = 32, 38.5%), inability to contact legally authorized representative despite numerous attempts (n = 19, 22.9%), subject unwilling to consent (n = 18, 21.8%), and other (n = 14, 16.9%). Laboratory data were not available for one participant. Among the 37 with data, 29 (78.4%) were male, and the median age was 77 years (range 57–98 years) (Table 1). Among those with data on race/ethnicity, 25/33 (75.8%) were black, 8/33 (24.2%) were white, and 5/34 (14.7%) were Hispanic; 12/27 (44.4%) were born outside the USA. The median duration of residence in the LTCF was 5 years (range 0–19 years).

Table 1
Demographic characteristics of the study participants ($n = 37$)

	Persons with a history of a positive TST ($n = 18$)						Persons with a history of a negative TST ($n = 19$)	
Test results at the time of study	TST-pos T-SPOT.*TB*-pos	TST-pos T-SPOT.*TB*-neg	TST-pos T-SPOT.*TB* borderline	TST boosted-pos T-SPOT.*TB*-neg	TST-neg T-SPOT.*TB*-neg	TST-neg T-SPOT.*TB* unknown	TST-neg T-SPOT.*TB*-neg or unknown	TST-pos T-SPOT.*TB*-neg
Number	10	3	1	1	2	1	18	1
Median age (range)	77 (60–88)	82 (72–83)	92	80	75.5 (67–84)	83	74 (57–98)	70
Male, *n* (%)	9 (90%)	3 (100%)	1 (100%)	0 (0%)	1 (50%)	1 (100%)	13 (72.2%)	1 (100%)
Race								
Black	8	3	0	1	2	ND	11	1
White	2	0	1	0	0		5	0
Ethnicity								
Hispanic	1	0	1	0	0	ND	3	0
Not Hispanic	9	3	0	1	2		14	1
US-born								
Yes	2	2	0	1	1	ND	10	ND
No	4	1	1	0	1		5	
Median years in LTCF (range)								
Previous LTCF	2.25 (0.5–4)	1	0	0	1.5 (1–2)	ND	3 (3–3)	ND
Current LTCF	4 (1–19)	4 (1–4)	0	7	1 (1–1)	10	6.5 (1–18)	9
Total	4 (1.5–19)	4 (2–4)	0	7	2.5 (2–3)	10	7.5 (1–18)	9

TST, tuberculin skin test; pos, positive; neg, negative; LTCF, long-term care facility; ND, unknown or not documented.

3.2. TST/T-SPOT.TB *discordance*

Of the 18 persons with a prior positive TST, 14 had a persistent positive TST (two had TSTs ≥10 and <15 mm, and 12 had TSTs ≥15 mm) and one boosted positive at the time of the study (Figure 1). Of the 14 persistent positives, three (21.4%) had a negative T-SPOT.*TB* result and one (7.1%) had a borderline positive T-SPOT.*TB* result (8 spots). Among the nine with a positive TST and T-SPOT.*TB*, the median spot count was >50 (range 16 to >50). Regarding the likelihood that their initial positive TST was a false-positive TST, none of the three people with discordant results had information on previous contact with a TB case. One was born and had lived much of life in Jamaica, but the participant's BCG vaccination status was not known. The other two persons with discordant results were unlikely to have received BCG vaccine as they were born in the USA, but they may have had exposure to non-tuberculous mycobacteria (based on location of residence). The one participant with a borderline T-SPOT.*TB* result, born in Puerto Rico and with unknown BCG vaccination history, had reportedly previously been treated for TB disease (although the timing and duration of treatment were unknown).

3.3. TST reversion over time

Of the 18 persons with a history of a positive TST, three (16.7%) had a negative TST at the time of the study, including two with a negative T-SPOT.*TB* (one was missing data) (Figure 1). None of the three had a TST test result documented at the time of admission to the LTCF and none was known to have been a contact of a TB case prior to arrival at the LTCF. Two may have had false-negative TSTs at the time of the study, as they had comorbidities that could affect TST results (rheumatoid arthritis on hydroxychloroquine and chronic renal disease).

3.4. TST boosting

One person with a history of a positive TST had a negative result followed by a boosted positive TST at the time of the study (from 0 to 10 mm). This individual, who had lived only in the USA (and hence had never been BCG-vaccinated), had a history of contact with a TB case in a family member (at unknown time). The medical history included diabetes mellitus, chronic renal disease, and dementia.

3.5. New TST-positive

Nineteen persons with a history of a negative TST (and no history of a positive TST) were enrolled; of these, one was found to be TST-positive (but T-SPOT.*TB*-negative), while the remaining were TST-negative and T-SPOT.*TB*-negative ($n = 16$) or TST-negative and not tested with T-SPOT.*TB* ($n = 2$). For the participant with a newly positive TST, the first recorded TST result was 0 mm, done 1 year prior to the study. This participant had no information available on previous TB case contacts, country of birth, previous places lived, previous BCG vaccine, or homelessness and had a history of diabetes, chronic obstructive pulmonary disease (COPD),

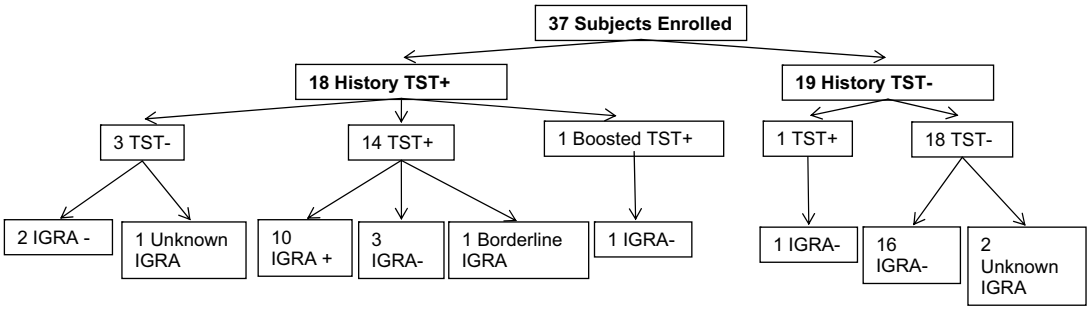

Figure 1. Flow diagram of tuberculin skin test (TST) and interferon-gamma release assay (IGRA) results for older long-term care facility residents, $n = 37$. (Note: The IGRA used was the T-SPOT.*TB* test.)

chronic kidney disease, and dementia. To the authors' knowledge, there were no new cases of TB in the LTCF during the study period, so it is unlikely that the TST conversion was the result of recent exposure.

3.6. T-cell response

The expression of CD69 on T-cells following PBMC culture with WCL was measured to determine the relative levels of TB-specific T-cell activation. Representative flow cytometry plots are shown in Figure 2A, demonstrating increased levels of CD69 following stimulation with the positive control (anti-CD3/CD28) and WCL. T-cells from all participants who were tested had increased CD69 levels following stimulation with anti-CD3/CD28 (data not shown). WCL-induced CD69 expression on CD4+ T-cells (Figure 2B) and CD8+ T-cells (Figure 2C) was higher in patients with a positive TST and T-SPOT.*TB* (CD4+ T-cells 18.2%; CD8+ T-cells 14.1%) than in those who were TST-negative and T-SPOT.*TB*-negative or T-SPOT.*TB* unknown (CD4+ T-cells 5.9%, $p = 0.0001$; CD8+ T-cells 4.1%, $p < 0.0001$). CD69 expression on CD4+ T-cells also tended to be higher in persons with a positive TST and T-SPOT.*TB* (18.2%) than in those with a positive TST but negative T-SPOT.*TB* (6.4%, $p = 0.16$) and those with a boosted-positive TST and positive T-SPOT.*TB* (4.8%, $p = 0.37$). CD69 expression on CD8+ T-cells tended to be higher in persons with a positive TST and T-SPOT.*TB* (14.1%) than in those with a positive TST but negative T-SPOT.*TB* (5.4%, $p = 0.11$) and those with a boosted-positive TST but negative T-SPOT.*TB* (4.1%, $p = 0.18$).

CD69 as a ratio measure of expression following stimulation with WCL compared to stimulation with media was also evaluated. Those who were TST-positive at the time of the study (compared to TST-negative) were more likely to be CD69-positive when defined as a three-fold increase (47% vs. 13%, $p = 0.05$). Prior positive TST status (compared to prior TST-negative) was not associated with current CD69 response (40% vs. 18%, $p = 0.24$). The one individual with a boosted TST response was not CD69-positive as defined above (their ratio of WCL/media was 2.25). Current T-SPOT.*TB*-positive status (compared to current T-SPOT.*TB*-negative) was not associated with a positive CD69 response (36% vs. 19%, $p = 0.39$).

CD69 expression on CD4+ T-cells following stimulation with WCL was found to be correlated with TST induration in millimeters ($r = 0.76621$, $p < 0.0001$) and T-SPOT.*TB* values as a continuous measure ($r = 0.68614$, $p < 0.0001$) (Figure 3A, B). CD69 expression on CD8+ T-cells following stimulation with WCL was found to be correlated with TST positivity ($r = 0.76767$, $p < 0.0001$) and with T-SPOT.*TB* values ($r = 0.61287$, $p = 0.0003$).

4. Discussion

This study of diagnostic testing for LTBI in the older LTCF population demonstrated that testing is complicated by TST reversion over time and TST-positive/T-SPOT.*TB*-negative discordance. Almost a fifth of participants with a prior positive TST had reverted to a negative TST at the time of this study. Such reversions may reflect clearance of infection (or lack of infection in the past) or false-negative reactions due to an impaired response to the test. Furthermore, a third of participants with a history of a positive TST and a persistently positive TST had a negative or borderline T-SPOT.*TB* test. Based on T-cell CD69 responses, it is possible that reversion and discordance may reflect clearance of infection.

To improve our understanding of diagnostic testing for LTBI in older adults, we must address three critical questions. First, what proportion of TST-positive responses revert over time and what

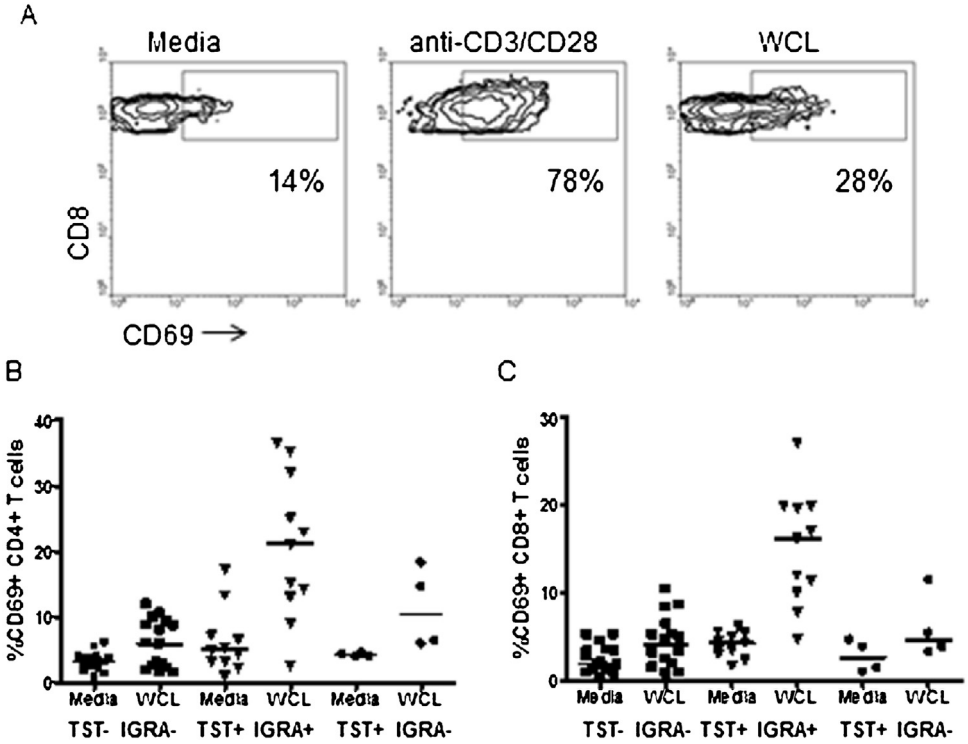

Figure 2. (a) Representative flow cytometry plots demonstrating the level of CD69 following stimulation with the positive control (anti-CD3/CD28) and whole cell lysate (WCL). (b) CD69 expression on CD4+ T-cells comparing tuberculin skin test (TST)-negative/interferon-gamma release assay (IGRA)-negative, TST-positive/IGRA-positive, and TST-positive/IGRA-negative participants. (c) CD69 expression on CD8+ T-cells comparing TST-negative/IGRA-negative, TST-positive/IGRA-positive, and TST-positive/IGRA-negative participants. (Note: The IGRA used was the T-SPOT.*TB* test.)

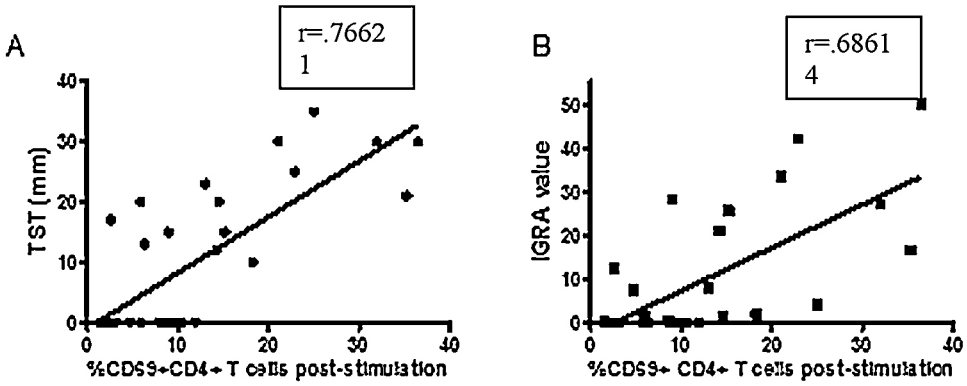

Figure 3. CD69 expression on CD4 correlations with (a). tuberculin skin test (TST) and (b). interferon-gamma release assay (IGRA) results. (Note: The IGRA used was T-SPOT.*TB* test.)

does the reversion imply? In this study, almost one-fifth of participants with a history of a prior positive TST no longer had a positive TST at the time of the study. These findings are consistent with a previous report that 9% of persons ≥60 years of age revert their TST annually.[16] Reversions are more common in persons with a smaller initial TST reaction;[30] however, the present study was limited by not having the initial TST size for the participants. Reversions suggest the possibility of cleared infection, the lack of ability to mount an immune response, or that the first TST was a false-positive. To assess the immunologic response to infection (and whether those whose TST reverted had cleared their infections), the CD69 response was evaluated. It was found that merely having a history of a positive TST was not associated with a current positive CD69 response. The fact that fewer T-cells are activated in these individuals suggests that reversion of the TST may reflect clearance of infection or waning immunity; these findings are supported by the negative T-SPOT.*TB* in these same individuals. In a 19-year follow-up study of 20 TST-positive persons, 10% reverted their TST despite a booster;[31] those who reverted had a poor blastogenic response, suggesting cleared infection. It is also possible, however, that those individuals with TST reversion in the present study were merely not able to mount an immune response; medical conditions (rheumatoid arthritis, end-stage renal disease) could have blunted the immune response in two-thirds whose TST reverted.[32–34] On the other hand, none had developed active TB despite this immunosuppression, so this makes it more likely that infection had been eliminated. A third explanation for the reverted TST, that the persons were never in fact truly infected (i.e., the previous positive test was a false-positive), is possible as none of those in the present study were known to be contacts of TB cases or to have had prior TB disease. Hence, the reversion of TSTs over time can complicate their interpretation.

The second key question when considering diagnostic testing for LTBI in older adults is how to interpret a sustained positive TST response. Does this reflect persistent infection and necessitate treatment? It was found that the magnitude of TST induration at the time of the study was correlated with the CD69 response and that those with a positive TST at the time of the study had a positive CD69 response (defined as a three-fold increase compared to media stimulation alone). These findings suggest that sustained positive TSTs may represent true infection as indicated by a sustained T-cell response. Notably, however, the one person with a new positive TST had a negative T-SPOT.*TB* and little evidence of a cellular immune response to *M. tuberculosis*. False-positive TST reactions could be explained by exposure to non-tuberculous mycobacteria, BCG vaccination (although less likely given the age of the participants), or potentially by other undetermined test characteristics in older adults.[8–10] False-positive TSTs not only result in possible treatment of individuals who are in fact not infected with *M. tuberculosis* (and the associated cost and medication side effects), but also the potential for increased testing of other facility residents and staff. Further immunologic studies are warranted to determine the true nature of a sustained (or new positive) TST response to understand which individuals require treatment.

The third key diagnostic question in this population is the significance of a boosted positive TST. In the present study, only one participant had a boosted positive TST and had little evidence of a cellular response to *M. tuberculosis* antigens. It is plausible that the boosted TST was a false-positive. This finding is in concordance with the results of a large-scale longitudinal study of LTCF residents in Hong Kong, which found that one-step but not two-step TST results predicted future TB disease.[12] At all cut-off points, two-step testing had greater sensitivity but less specificity than one-step testing. Others have reported that increasing the boosted test cut-off decreases false-positive reactions and is more predictive of developing disease.[30] Boosting is common among older persons with an initial reading between 5 and 9 mm,[15,35,36] and persons with a weak (varyingly defined as <10, <12, or <15 mm) boosted TST response are significantly more likely to revert on re-test.[31,33,37] Studies have shown that 12–13% of persons sensitized to non-tuberculous mycobacteria demonstrate boosting.[30] Hence, the use of boosted testing in the older LTCF population may result in more 'false-positives', with the associated drawbacks of unnecessary treatment.

Testing CD69 expression may be a useful adjunct to understanding TST and T-SPOT.*TB* results, particularly in conjunction with more targeted immunologic assays. It is likely that CD69 measures a different pathway for *M. tuberculosis* infection than does the T-SPOT.*TB*, because to the authors' knowledge, CD69 is not an IFN-γ-inducible gene product. A good correlation was found between the in vitro tests for CD69 expression and separately TST and T-SPOT.*TB* response—supporting earlier findings that CD69 expression correlates with *M. tuberculosis* infection status.[25,27] The present immunologic assays were limited in their capacity to evaluate T-cells specifically, due to the use of PBMCs in culture and the relatively low volume of blood collected.

This study has several limitations. First, the lack of a true gold standard for *M. tuberculosis* infection limits the interpretation of test results. Second, the study was limited by the small sample size; however, the inclusion of immunologic parameters enhances

the interpretation of the diagnostic test results. Third, it was not possible to verify the historical TST results collected prior to LTCF admission or to determine the reason for prior testing, but this lack of data mirrors what is seen in clinical practice, as one rarely has access to historical TST data when evaluating present day test results. Lastly, the interpretation of the findings is potentially limited because of antigenic differences between WCL (for CD69 expression), T-SPOT.*TB*, and TST, as antigens in WCL are not as specific for *M. tuberculosis* as those in the T-SPOT.*TB*.

In summary, this study suggests that diagnostic testing for LTBI in the older adult population is complicated by TST reversions, TST/T-SPOT.*TB* discordance, and a lack of clear immunologic correlates of infection. The CD69 analyses suggest the possibility that the reverted TST responses may reflect cleared infection, but additional immunologic analyses are needed. Accurate diagnosis of LTBI in older adults, particularly LTCF residents, is important given the potential public health implications of disease reactivation and spread within facilities. Although LTBI treatment of older adults must balance factors that increase the risk of reactivation (e.g., diabetes, use of tumor necrosis factor alpha (TNF-α) inhibitors, immunosuppression) and those that might complicate treatment (e.g., underlying liver disease),[38] as new shorter and better-tolerated regimens become available, the balance may shift in favor of treatment.

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Conflict of interest: There are no conflicts of interest for all authors involved in this study.

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