To the Editor,

Tuberculosis (TB) elimination requires an effective strategy to diagnose and treat people infected with *Mycobacterium tuberculosis* (Mt) who would otherwise be at high risk of developing and transmitting active disease. The diagnostic tools for latent tuberculosis infection (LTBI) are the tuberculin skin test (TST) and the T-cell IFN-γ release assays (IGRAs). Two IGRAs are commercially available, QuantiFERON-TB Gold In Tube (QFT-GIT, Qiagen, Germantown US) and T-SPOT.TB (Oxford Immunotec, Abingdon, UK). Compared to the TST, IGRAs offer operational advantages and higher specificity in the BCG-vaccinated population[1] and they appear to be at least as sensitive for LTBI[2]. However IGRAs have limitations: a reduced sensitivity in groups at greater risk for progression to TB disease [3, 4] failure to discriminate between active TB and LTBI and poor correlation with the risk of progression to active disease[1].

QuantiFERON-TB Plus (QFT-Plus) is a new generation of QFT-IT [5] which includes an additional antigen tube (TB2). The TB1 tube contains ESAT-6 and CFP-10 derived peptides (TB-7.7, present in QFT-GIT, has been removed), designed to elicit cell mediated immune (CMI) responses from CD4+ T-helper lymphocytes. The TB2 contains newly designed shorter peptides able to stimulate INF-γ production by both CD4+ and CD8+ T-cells. Previous studies have reported higher Mtb-specific CD8+ responses in those with active TB disease compared to LTBI [6] and in those with recent Mtb exposure [6]. In addition, Mtb-specific CD8+ producing IFN-γ have been detected in active TB subjects with HIV co-infection [7] and in young children with TB disease[8].

IGRA tests primarily serve as a test for LTBI in clinical practice; however in the absence of a gold-standard test for LTBI against which to compare test efficacy, these tests are evaluated in individuals with confirmed disease i.e. active Tuberculosis. From November 2014 to September 2015, 119 consecutive individuals with active tuberculosis (TB) and 100 low risk controls were enrolled and tested with QuantiFERON-PLUS at six different sites, five located in Italy and 1 in the UK (Villa Marelli, Ospedale San Raffaele, Milan; INMI, Rome; Ospedale Sant'Orsola Malpighi Bologna; IRCCS San Matteo Pavia; Royal Free Hospital, London). The active TB group included adult patients (aged≥18) with microbiologically confirmed TB (either by Nucleic Acid Amplification (NAA) or culture), who had received less than 15 days of anti-TB drugs. HIV co-infected individuals and immunocompromised patients were not excluded. Additional information was collected regarding: results of QFT-GIT, BCG vaccination status, demographic, clinical, and microbiological data. Healthy controls were recruited among students at the time of enrolment at the University Vita-Salute in Milan (very low risk population, non BCG-vaccinated); all subjects were interviewed and screened for the absence of any risk factors for Mtb exposure. The study was approved by the Ethical Committees of all the participant
centres and informed consent was obtained from study subjects before blood sample collection.

QFT-Plus kits were donated by Qiagen and used according to the manufacturer's instructions[5]. Levels of INF-γ quantified by ELISA were converted to international units per millilitre by means of a standard curve constructed from the QFT-Plus Analysis Software provided with the kit. Test results were interpreted according to manufacturer's criteria[5]. Positivity of a single antigen tube (TB1 or TB2) was sufficient to record the QFT-Plus as positive.

Results are reported in Table 1. In the active TB group (119) three patients (2.52%) had indeterminate results. Among the 116 TB patients who had valid test results, 102 were positive leading to a sensitivity for detecting active TB of 87.93% (95% Confidence Interval CI: 80.76; 92.67%). Ninety-five of 116 were positive for TB1 and TB2, one patient was positive to TB1 tube only and six patients were positive to TB2 only.

Of the 106 subjects of the low risk control group, three had a positive QFT-Plus test, giving a specificity of 97.17% (95% CI: 92.01; 99.03) in this non BCG-vaccinated population. Two of these three positive results were positive in one antigen tube only, with IFN-γ values close to the cut-off.

Considering the quantitative data, the overall TB2 antigen IFN-γ response was higher than in TB1[median (25-75 percentile): TB1=2.09 (0.83-6.52 UI/ml; TB2 = 2.88 (1-7.89) UI/ml; p-value = 0.0002)], TB2 IFN-γ values were greater than in TB1 for 63.16% (72/114; for five patients the exact value of the tube was not available) of the TB patients and 44.34% (47/106) of the low risk control group.

As the TB1 tube elicits a CD4+ response whilst the TB2 tube elicits both a CD4+ and CD8+ response, the difference between these tubes might provide a surrogate marker of the magnitude of CD8+ responses. In active TB patients, we found that this difference was higher in smear positive compared to smear negative patients (Mann-Whitney test p-value = 0.0135)

Results of the commercially available QFT-GIT were available for 73 of the active TB group subjects. The head-to-head comparison of the QFT-GIT and the QFT-Plus in this group demonstrated agreement in results in 69 results; with 4 additional positive results using the QFT-Plus test. Four patients were scored positive only to the QFT-Plus, (3 of them scored positive at the TB2 antigens only).

This prospective multicentre study is the first independent assessment of the performance characteristics of QFT-Plus. Estimated diagnostic sensitivity (88%) was higher than the upper confidence limit reported in the most recent meta-analysis for QFT-GIT[1], suggesting that the QFT-Plus does indeed offer improved sensitivity. However the performance characteristics of this test remain insufficient for use as a rule-out test for active TB.
Immunocompromised patients were not excluded from the study population. Five HIV/TB co-infected subjects all had a positive QFT-Plus test. Of five patients with cancer undergoing chemotherapy, we found one negative result and one indeterminate. A negative result was also observed in a patient with meningeal TB receiving prednisone treatment.

Six patients resulted positive by the second antigen tube only. All these patients have smear-positive pulmonary tuberculosis (PTB), and four of them showed more than 3 cavitations. Moreover, we found that difference between TB2 and TB1 was higher in smear-positive compared to smear-negative patients. Considering difference between the two antigen tubes as surrogate marker of the magnitude of CD8+ responses, the last finding is in agreement with what observed in cyto-fluorometry studies. Mtb specific CD8+ T-cells correlate with an increased mycobacterial load and are more frequently found in smear-positive PTB individuals[9].

Finally, we observed a significant difference in IFN-γ response between the 2 tubes in QFT-Plus test, however whether this difference is due to the additional CD8+ T-cell stimulation has not be proven yet. A previous study analysed the contribution of CD4+ and CD8+ antigen-specific responses to novel peptides with HLA-I and HLA-II promiscuous binding capabilities derived from MTB genes over-expressed in an in vitro macrophage model [10]. In the study by Losi et al a small subgroup of active TB patients had a significantly higher frequency of peptide specific IFN-γ CD69+ CD4+ and INF-γ CD69+ CD8+ T-lymphocytes compared to controls[10]. Flow-cytometry studies in a larger population need to be carried out to confirm the presence of CD8+ response in the newly added tube.

In conclusion, QFT-Plus offers an improved sensitivity to detect active TB, and maintained high specificity among non-vaccinated controls. Overall there is greater IFN-γ release observed in the TB2 tube in active TB patients. The difference between TB2 and TB1 results may constitute a surrogate marker of CD8+ T-cell activation.

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