ABSTRACT

The increase in type 2 diabetes mellitus in countries with high tuberculosis (TB) prevalence is undermining efforts to achieve the U.N. Millennium Development Goal of reversing the rise in TB incidence by 2015. Most published studies on this topic have been limited by retrospective design, weak diagnostic criteria for diabetes and a failure to assess diabetes severity which is likely to be a critical variable. India is the country most impacted by the dual burden of TB and diabetes yet few clinical studies of this problem have been based there. The goal of the proposed Effects of Diabetes on Tuberculosis Severity (EDOTS) study is to develop a cohort for prospective investigation of the impact of diabetes on the presentation, treatment response and outcomes of pulmonary TB. We will recruit a cohort of patients newly diagnosed with pulmonaryTB, equally divided between those with and without diabetes. These patients will be followed longitudinally in a clinical study aiming to establish whether diabetes alters the clinical presentation and treatment outcome of TB disease in an Indian population. We anticipate that diabetic patients will have more severe TB disease (x-ray score, sputum smear and culture), delayed sputum conversion on TB treatment and higher rates of unfavorable outcomes (treatment failure, death, relapse) as compared euglycemic TB patients. Results of the EDOTS study will define the characteristics of diabetic patients at highest risk for unfavorable outcomes and will help refine the clinical and public health response to the dual burden of TB and diabetes. This knowledge will provide a rational basis for future interventions to improve TB outcomes in patients with diabetes. Using approaches including flow cytometry and gene expression profiling we will conduct fundamental studies in this cohort, comparing selected features of the immune response to TB in subjects with or without diabetes. We anticipate finding an exaggerated, Th1-biased immune response in diabetic patients causing the greater immune pathology that underlies the increased TB severity and unfavorable outcomes in this population. Results from these basic studies will be correlated with clinical data and are expected to provide more sensitive measures of TB disease activity than the crude clinical parameters of x-ray score and sputum smear. New knowledge produced in the fundamental study will increase our understanding of the immunological basis for TB susceptibility in people with diabetes and could inform the discovery of clinically useful biomarkers. The urgent need forthe EDOTS study is highlighted by our recently published data showing that nearly 50% of TB patients in Chennai have diabetes or pre-diabetes. Developing an effective clinical and public health response to the dual burden of TB and diabetes in Indian depends on an accurate foundation of knowledge about its scope and consequences. The EDOTS study will lay that foundation.

PROJECT NARRATIVE

a) Cohort Description

Our cohort will be recruited from patients presenting to two neighborhood TB clinics (TB Units; TBU) of the Revised National Tuberculosis Control Program (RNTCP) in Chennai, Tamil Nadu. The composition of our study population may be estimated from results of an investigation of diabetes and pre-diabetes prevalence in TB that was recently published by our Indian PI, Dr.Viswanathan [1]. In the first quarter of 2011, his group recruited 827 active TB cases from two TBUs in the Chennai District along with 3 TBUs in the adjacent Tiruvallur and Kanchipuram Districts. Based on that experience we estimate that subjects recruited to the EDOTS study willbe urban dwellers with a mean age of 41 years and a gender distribution of 70% male to 30% female. In this population, approximately 75% are literate, slightly more than 50% are employedand 75% have an annual income < 5,000 INR (< $500 USD). Current use of tobacco and alcohol in this population is < 2%.

The 4.7 million inhabitants of Chennai are served by 10 TBUs distributed throughout the different neighborhoods of the city, each with a catchment area of ~500,000 people. These clinics are typically within walking distance for patients in their catchment areas. TB screening, diagnosis and treatment are provided at no cost to the patients and clinical data are stored in a central database. The Pulianthope and Tondiarpet TBUs are in close proximity to the M.V. Diabetes Research Centre where members of the clinical research team are located. We have obtained permission from the Tamil Nadu state RNTCP for this study and enlisted the help of Dr. Lavanya K, the district TB control officer working out of the Pulianthope TBU. She will help coordinate our recruiting from TBUs.

In 2011 the Pulianthope TBU identified 286 new smear-positive TB cases, 205 new smear-negative cases and 207 new extrapulmonary TB cases. In the same year, the Tondiarpet TB units reported 241 were new smear-positives, 180 were new smear-negatives and 201 were new extrapulmonary TB. As detailed in the clinical research plan below, we will specifically recruit 300 new smear-positive TB patients; 150 with diabetes and 150 confirmed to be non- diabetic. Based on 2.5 years of data from 2010 through the second quarter of 2012, the combined total number of new smear-positive TB cases reported by both TBUs was 1,394. We expect a similarly ample pool of candidates during accrual of 300 patients for the EDOTS study. One factor relevant to our study is multi-drug resistant (MDR) TB, which is relatively uncommon in Chennai at <5% of new cases. HIV/AIDS prevalence of in this population is <5%. Counseling and HIV and testing are offered to all patients treated at the TBUs, with a 93% acceptance rate. Recruiting 827 newly diagnosed TB patients from selected TBUs in Tamil Nadu in the first quarter of 2011, Dr Viswanathan's team found that 25% had diabetes and another 25% had pre-diabetes based on WHO criteria. Mean glycosylated hemoglobin (HbA1c) for the diabetic group was 8.9%, consistent with poor glycemic control in the 3 months prior to testing.

Regarding accessibility factors, the local government provides general medical care free of charge to uninsured residents through general medical outpatient clinics, the TBUs, and inpatient care provided at several public hospitals in the city. Likewise, the government provides medically-related social services at these clinical sites. While striving to provide excellent medical care and social services, these clinical sites are constrained by limited resources and

very high demand in a population with an unemployment rate of 23% and a low median monthly income among the 54% of adults who are employed.

The patient population treated at Chennai TBUs has considerable experience participating in clinical research projects owing to the activities of the M.V. Diabetes Centre, the National Institute for Research in Tuberculosis (NIRT) and the NIH-sponsored International Center for Excellence in Research (ICER) in Chennai among other local institutions. The engagement of TBU patients with research was demonstrated by Dr. Viswanathan's experience implementing the diabetes prevalence study. Of 905 subjects who passed screening by inclusion/exclusion criteria, only 77 (8.5%) declined participation. The 91.5% response rate was notably high considering that every recruited subject agreed to oral glucose tolerance testing (OGTT) after fasting overnight. The longitudinal nature of the proposed EDOTS study might reduce the acceptance rate but we are confident in reaching the recruiting goals detailed in the Project Narrative below. The population of Chennai is not highly transient, with a majority of citizens having stable living conditions shared with multiple family members. This will facilitate our longitudinal study design and plan for 1-year follow-up after completing directly observed short- course (DOTS) treatment. Medical Social Workers (MSW) from the M.V. Diabetes Center teamexperienced with the TBU population expressed confidence that a high proportion of patients that we recruit will reside at the same address throughout the time span of the EDOTS study.

In our letter of intent, we described a plan to recruit patients from the outpatient clinic of the M.V.Diabetes Centre as well as the nearby TBUs. After a team meeting in Chennai at the end of August, the two PIs and the clinical research time members concluded that the typically older age and higher socioeconomic status of M.V. Diabetes Centre patients would lead to difficulty in matching demographic and other patient characteristics between the diabetic and non-diabetic groups. We will avoid this problem by recruiting exclusively from the TBUs. Dr. Viswanathan's diabetes prevalence study identified age another potential barrier to matching the diabetic and non-diabetic patient groups. The mean age of TB patients with diabetes was 49.3 vs. 35.6 for those without diabetes or pre-diabetes. Based on that experience we will limit recruitment to subjects $\geq$ 30 years old. Dr. Viswanathan's data also show that there will be no shortage of diabetes cases with recruiting limited to the TBUs. In the unlikely event that we have trouble meeting recruitment goals at Pulianthope and Tondiarpet we can expand recruiting to one or more of the other 8 TBUs in Chennai. With an eye to the future, we are confident that we could recruit a much larger cohort if initial success enables us to expand to fCRU status.

Unlike the clinical research plan, the fundamental study we propose requires diabetic and non-diabetic controls who do not have TB or other major illness. These subjects (n=30 per group) will be recruited from healthy family members accompanying patients to the TBU and, if needed,from the outpatient clinic at the M.V. Diabetes Centre. The recruiting effort for controls will beginafter the EDOTS cohort begins to be populated with TB patients so we can aim for frequency matching of demographics and other potentially confounding variables.

b) Scope of the Propsed Research

Clinical Research Plan

Hypotheses: Our central hypothesis is that diabetes impairs immunity, leading to increased severity of TB disease. Our objective is to test that hypothesis in an Indian population, building a well characterized cohort for longitudinal investigation. Our aims are: *1*, recruit the cohort; *2*, compare clinical features of TB disease on presentation; *3*, compare the response to TB treatment; and *4*, compare TB outcomes in a matched cohort of diabetic and non-diabetic patients with pulmonary TB.

Background: A relationship between diabetes and TB has been appreciated by clinicians for centuries [2] but has only lately become a major topic of clinical and fundamental research. Renewed interest is due to increasing rates of diabetes in Asian countries where TB was already prevalent. The dual burden of diabetes and TB clearly represents a serious global public health concern [3] but published data are incomplete and, in many cases, inconsistent. Published studies on TB susceptibility and TB severity in diabetes almost universally used retrospective data collection. A paucity of studies adjusted for potentially confounding variables, and most used weak diabetes case definitions with little or no characterization of diabetes severity. Despite these limitations, a coherent picture is emerging. Jeon and Murray [4] reported a meta- analysis of 13 observational studies on the risk for TB disease in diabetics and estimated a summary relative risk (RR) of 3.11, from a subgroup of four cohort studies. The RR across all 13 cohort and case:control studies ranged from 0.99 to 7.83. This variability may be due in part to the different populations studied, each with unique social conditions and distinct host and microbial genetic backgrounds. However, much of the variability also likely stems from deficiencies in study design and implementation, including small sample size and weak diabetes case definition (e.g. based solely on patient reported history). Jeon and Murray concluded that while the impact of diabetes on TB susceptibility is less than that caused by HIV infection, the much greater prevalence of diabetes results in a roughly equal global population-attributable TB risk for diabetes and HIV/AIDS.

In addition to increasing the risk for reactivating latent TB infection (LTBI), there is growing evidence that diabetes is also associated with greater severity of TB disease. Most reports on this topic suffer the same limitations noted for studies on diabetes and the risk of reactivating LTBI, and they similarly produced conflicting results and conclusions. The current state of research on diabetes and TB outcomes is summarized in a meta-analysis by Baker et al. [5]. Of 33 studies included in their analysis, 9 reported culture conversion at 2 o three months, 12 reported the combined outcome of treatment failure and death, 23 reported deaths, 4 reported deaths adjusted for age and some other potentially confounding factors and 5 reported relapse. Overall, the data suggest that diabetes is associated with greater radiographic severity of TB disease, delayed sputum conversion, unfavorable outcomes and increased risk of relapse. However, estimates in individual studies range from high risk to no difference between diabetic and non-diabetic TB patients. As with studies of TB reactivation risk in diabetics, the conflicting data on TB outcomes may reflect true population-specific differences in the effects of diabetes on TB severity but are also attributable at least in part to weaknesses in study design.

Another limitation in generalizing conclusions from the meta-analysis of Baker et al. to an Indian population is that only 2 of the 33 reports included in that review were from studies conducted in

India. Of those, a report by Subhash et al. [6] investigated the association of diabetes and HIV infection with drug-resistant TB in a tertiary care teaching hospital where 51% of all cases were resistant to at least one first-line drug. They found no association between drug resistant TB and diabetes. A more recent study by Banu Rekha et al. [7] investigated diabetes and HIV infection as risk factors for delayed sputum smear conversion after the intensive phase of category-I (DOTS) treatment for pulmonary TB. This retrospective study found no increased risk for delayed conversion in the diabetic group. That conclusion was shared by three other studies in the meta-analysis but five studies reported an increased risk for delayed conversion with RR ranging from 2.01 to 3.25. Banu Rekha et al. concluded that the current RNTCP policy to treat all pulmonary TB patients with or without co-morbid conditions using the standard category-I regimen is appropriate. That conclusion should be taken with caution since their study did not include data on sputum *culture* conversion and did not report on outcomes or relapse. Baker et al. reported a summary RR of 3.89 for relapse in diabetic vs. non-diabetic TB patients, based on five papers.

Despite weaknesses in prior studies linking diabetes to risk for reactivating LTBI and increased severity of TB disease, the trends identified in that literature are sobering if one multiplies the increased risk to develop active TB with the predicted effects of diabetes on TB severity. The values shown in Figure 1 reflect RR 3.0 for progression from LTBI to TB disease in diabetics, applying the estimated summary RR and odds ratio (OR) values of Baker et al. Mortality is calculated for an estimated 2-fold increased odds of death in diabetic TB patients and a 5% mortality rate for TB in the general Indian population. The latter is based on a survey conducted in Designated Microscopy Centres in Hyderabad, Krishna and Adilabad districts of Andhra Pradesh, South India, between 2005 and 2009 [8]. Considering Dr. Viswanathan's discovery that 25% of patients presenting for TB treatment in Chennai have diabetes, the potential contribution of diabetes to the clinical and public health burden of TB in South India is profound. Developing an appropriate and effective response to this challenge depends on having accurate data on its true scope and manifestations in the Indian population. While many clinical TB/diabetes studies have been reported, most were retrospective investigations with inadequate characterization of diabetic severity or consideration of other co- morbidities. Moreover, few such studies have been based in India whose diabetic population could have unique characteristics influencing the interaction between TB and diabetes.

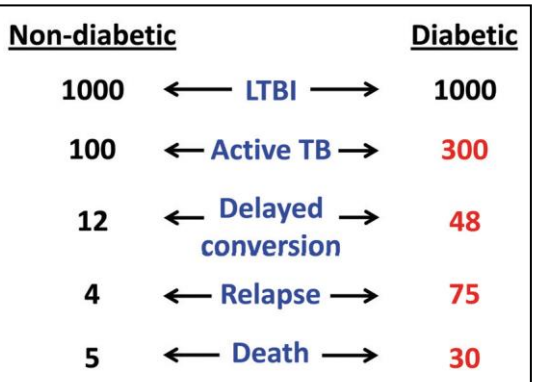

Figure 1. Predicted occurrence of active TB disease and TB outcomes in 1,000 non-diabetic and 1,000 diabetic patients with LTBI in India.

The proposed EDOTS study will produce accurate information about the impact of diabetes on the manifestations and outcomes of TB in an Indian population, applying standards for diabetes research that are unprecedented in the TB literature. Composite data from a detailed diabetes-related medical history, a diabetes-specific physical examination, and serial measurement of HbA1c will permit us to stratify based on diabetic severity and to identify any impact of co-morbid TB on glycemic control and other diabetic complications during and after TB treatment.

In light of Dr. Viswanathan's discovery that 25% of TB patients in Chennai have pre-diabetes, our plan to rigorously confirm that the non-diabetic patients in our cohort are truly euglycemic is particularly important. That high prevalence of pre-diabetes in patients newly diagnosed with TB raises the interesting question whether that condition also influences the risk of progression from LTBI to active TB and/or the severity of TB disease. Budget constraints for the dCRU proposal prevent us from including a pre-diabetic group in our cohort but we are keenly interested to investigate such patients if and when resources become available.

Diabetes is only one of several acquired TB risk factors that may impact our study population. We will exclude HIV co-infection from the EDOTS cohort but several other common comorbid factors will be assessed. Dr. Kornfeld and collaborators at NIAID and the International Tuberculosis Research Center in Korea recently analyzed the effect of diabetes on TB outcomes in 657 patients admitted to the National Masan Tuberculosis Hospital [9]. In that cohort, 25% of patients had diabetes, which was associated with a 2-fold increased hazard of death in 12 months (Table 1). Smoking ≥ 1 pack of cigarettes daily increased mortality to a comparable degree. The combination of these two independent risk factors resulted in hazard ratio of 4.25 for all deaths and 5.78 for TB-related death. Of note, none of these subjects smoked during the first four months of TB treatment when they were inpatients at the TB hospital. While cigarette smoking may be uncommon in our study population, we will collect accurate data on cigarette and bidi use in the EDOTS cohort. Vitamin D deficiency is another recognized as a TB risk factor in humans [10]. Vitamin D deficiency was also recently identified as a comorbid risk factor for diabetic foot infection [11]. The combined risks of diabetes and vitamin D deficiency could havean effect of TB severity similar to the combined effects of diabetes and smoking, thus identify a subgroup at particularly high risk for unfavorable TB outcomes. We will measure serum levels of25-hydroxy vitamin D (25(OH)D) in our cohort at the time of enrollment.

Table 1. Impact of diabetes and smoking ≥ 1 pack daily on one-year mortality with TB. *

	All Deaths		**TB-related Deaths**	
	Hazard Ratio [95% CI]	**p-value**	**Hazard Ratio [95% CI]**	**p-value**
Diabetic	2.18 [1.10, 4.34	0.026	1.98 [0.90, 4.35]	0.091
Smoker	1.95 [0.59, 6.46]	0.52	2.93 [0.70, 12.28]	0.14
Diabetic smoker	4.25 [1.06, 17.08]	0.04	5.78 [1.09, 30.56]	0.04

* Hazard vs. non-smoking subjects without diabetes adjusted for age, gender and alcohol use.

A role of dyslipidemia in TB severity is suggested by Dr. Kornfeld's studies in the mouse aerosolTB model. Hypercholesterolemia increased TB susceptibility and immune pathology [12;13]. This question has not yet been studied in a human population but it could be contributing factor in type 2 diabetes which features a characteristic pattern of dyslipidemia with high VLDL and low HDL cholesterol, as well as elevated triglycerides [14]. The EDOTS study is designed to identify any adverse (or protective) effect of tobacco and alcohol consumption, vitamin D deficiency anddyslipidemia on TB severity in diabetic and non-diabetic TB patients in India.

The perspective of virtually all TB/diabetes studies published up to now has been reasonably focused on the risk of developing active TB and the severity of TB disease. Important questions

that have not yet been addressed in clinical research is how comorbid TB diseases influences diabetes in terms of glycemic control, the progression of other diabetic complications and the potential for an episode of TB to promote diabetes in non-diabetic or pre-diabetic patients. The possible influence of diabetes on the pharmacokinetics (PK) of rifampin is recognized although data on such an effect, as with so many other diabetes/TB interactions, are conflicting [15;16]. In contrast, the influence of DOTS on the PK and optimal use of anti-diabetic medication has not been rigorously investigated despite the fact that rifampin is a potent inducer of the hepatic cytochrome p450 enzymatic system. Rifampin other rifamycins could reduce levels of oral anti-diabetes drugs including sulfonylureas, thiazolidinediones, meglintinide analogs and many biguanides. The limitations imposed by the dCRU budget make it unfeasible for us to address questions of diabetes drug management in the EDOTS study but our Specimen Repository and limited data from our clinical study may provide a basis for such studies in the future.

Study design: We will conduct a prospective cohort study, recruiting patients newly diagnosed with smear and culture-positive pulmonary TB disease from the population presenting to the Pulianthope and Tondiarpet TBUs in Chennai. We will compare selected features of TB at presentation, track the response to anti-TB treatment and compare outcomes and relapse in a cohort of 300 patients equally divided between those with and those without comorbid diabetes. A field team composed of MSWs and phlebotomists based at the M.V. Diabetes Centre will visit both TBUs daily and screen newly identified smear-positive cases. Potential recruits will be offered a screening consent to collect demographic and medical history data pertinent to the inclusion and exclusion criteria. Candidates not excluded at that stage will undergo diabetes assessment including fasting plasma glucose (FPG), OGTT and HbA1c assay. Patients with known diabetes history and currently taking any form of anti-diabetic medication will forego OGTT. All patients treated at the TBUs are offered counseling and HIV testing. Our team will test female EDOTS candidates <50 years old for pregnancy by urine hCG assay. *Inclusion criteria*: age ≥30 and current smear-positive pulmonary TB. *Exclusion criteria*: any prior episode of TB disease; treatment for current TB episode >1 week before enrollment; pre-diabetes; pregnancy or childbirth within 6 months; HIV-seropositive; and current use of immunosuppressive therapy.

Volunteers who pass the screening stage will be consented for full participation in the prospective study. They will undergo the clinical and laboratory evaluations specified in the section below, and they will be followed by the research team monthly for the 6 months of DOTS treatment, coinciding with routine TBU visits for TB treatment under the care of RNTCP staff physicians. Patients with MDR-TB or who remain smear-positive after 6 months of DOTS are routinely seen by TBU staff at additional time points and will be seen by the EDOTS team during those visits. HIV serology and initial sputum culture results will not be available at the time of enrollment; subjects found to be HIV-seropositive or culture-negative for *Mycobacterium tuberculosis* will be excluded as soon as those results are reported. Based on low rates of HIV and MDR-TB, and very high rates of positive cultures in AFB smear-positive TB patients in Chennai, we anticipate that only a small number of enrolled subjects will be excluded in the first 2 months after enrollment. Follow-up of patients after cure (negative sputum smear at 5 and 6 months) is not routine practice for the TBUs. Patients enrolled in the EDOTS study will be seen 3, 6, 9 and 12 months after the completion of TB treatment to screen for relapse, progression of initially non-diabetic patients to diabetes, and the status of glycemic control and

complications in the diabetic group. A longer post-treatment follow-up period could be considered if additional funding is secured. The timeline and sequence of evaluations for the EDOTS study are shownin Figure 2.

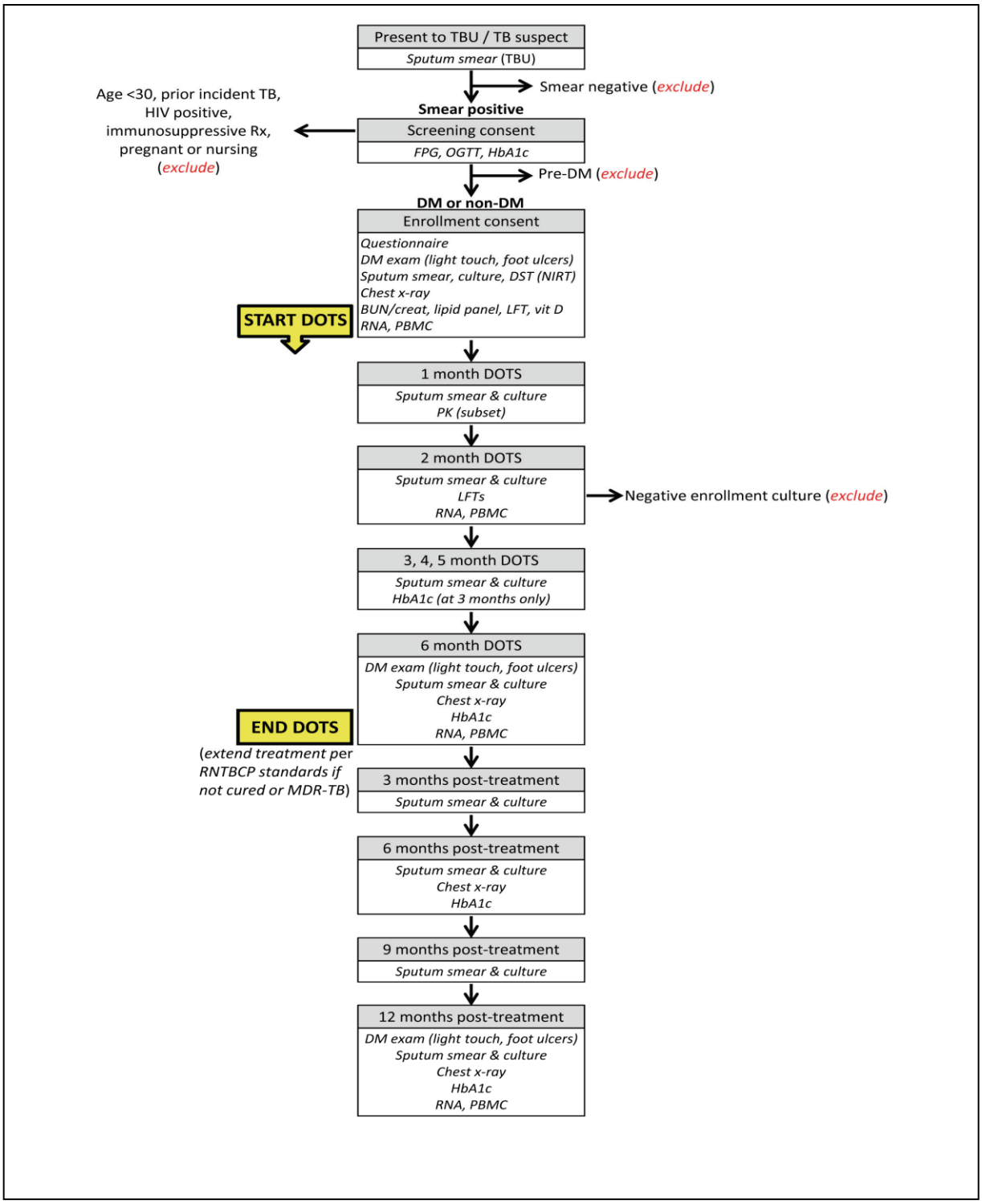

Figure 2. EDOTS study timeline. FPG, fasting plasma glucose. OGTT, oral glucose tolerance test. LFT, liverfunction tests. BUN/creat, serum urea and creatinine levels. DST, drug sensitivity test. PK, pharmacokinetics. RNA, blood sample for transcriptional studies. PBMC, blood sample for flow cytometry studies.

Clinical and laboratory evaluations: Diagnosis of diabetes and pre-diabetes will be based on FPG, OGTT and HbA1c results, using WHO definitions as described by Viswanathan et al. [1]. At the time of enrollment, subjects passing screening evaluation will complete a questionnaire for demographic information, current medications, past medical history with a detailed diabetes history (duration, treatment, complications). Information about TB risk factors (tobacco and alcohol use, incarceration or other congregate living conditions, silica exposure) will be recorded. The MSWs will perform a review of systems focused on TB symptoms and diabetic complications. They will measure height and weight, and perform a directed physical exam for signs of peripheral vascular disease, foot ulcers and neuropathy. Initial laboratory tests include blood urea, creatinine, transaminase levels, lipid profile (total and HDL cholesterol plus triglycerides), complete blood count and 25(OH)D. These tests will be performed in the clinical laboratory of the M.V. Diabetes Centre. Additional plasma and serum aliquots will be transferred to ICER and stored in the EDOTS Specimen Repository. Blood samples for T cell phenotyping and RNA isolation for the fundamental study plan will also be collected at the time of enrollment.

The TBUs do not perform sputum cultures or chest x-rays on uncomplicated, smear-positive cases. All EDOTS study participants will have 3 sputum samples collected at enrollment for smear and culture on solid media in the NIRT microbiology laboratory. Smears and cultures will be scored (scant to 4+) and undergo drug sensitivity testing. *M. tuberculosis* isolates from every EDOTS patient will be stored in the Specimen Repository. All EDOTS study participants will have a 1-view chest x-ray done at the time of enrollment using the radiology facilities of the TBUs. The resulting films will be temporarily transferred to NIRT where they will be scanned and scored by a trained and blinded panel qualified physicians.

EODTS cohort patients will be seen by research team members monthly during the 6 months of TB treatment. Two sputum samples will be collected for smear and culture at every visit. At **one month**, a subset of 50 cohort patients equally divided between those with and without diabetes will participate in a PK study. On an appointed day, those patients will report to NIRT instead of the TBU for one of their scheduled thrice weekly DOTS treatment. They will take their routine medication (HRZE) at NIRT and blood will be collected 2 hours later for measurement of all four drugs. At **two months**, in addition to sputum sampling, we will repeat transaminase assays and collect blood samples for T cell and RNA studies. At **three months** we will repeat HbA1c. At **six months**, when the majority of EDOTS subjects will complete DOTS, the chest x-ray, diabetic physical exam, HbA1c and blood sampling for T cell and RNA studies will be repeated.

After completing TB treatment, EDOTS study patients will be seen every 3 months for 1 year to screen for relapse and to reevaluate diabetic status. Sputum samples will be collected at each visit, chest x-rays repeated at the 6 and 12-month post-treatment time points, and the diabetic physical exam and HbA1c will be repeated on the final visit. Screening for relapse can be augmented by accessing the RNTCP database; this will be helpful for patients who have moved and cannot be traced by the MSWs. The EDOTS study consent will include permission to search the RNTCP database for information related to any study volunteer for the total 18 months of this study.

Outcomes will be classified using TB control definitions of the WHO, the International Union Against Tuberculosis and Lung Disease, and the Royal Netherlands Tuberculosis Association

[17]. Results of these investigations will support or refute the following predicted effects of diabetes on TB severity, which have some basis in published studies:

1. Increased radiographic severity of disease on enrollment and at the completion of treatment.
2. Higher sputum smear and culture scores on enrollment and delayed clearance on treatment.
3. Reduced levels of rifampin and possibly other first-line TB drugs.
4. Increased frequency of treatment failure.
5. Increased hazard of death within 6, 12, and/or 18 months of starting TB treatment.
6. Increased risk of retreatment for TB due to relapse or exogenous reinfection after successful complete of 6-month DOTS.
7. The risk for unfavorable outcomes and related measures of TB severity will correlate with the degree of HbA1c elevation and with a composite diabetes severity score.

Our study will also produce data addressing issues related the TB/diabetes interaction where is there is no or only a limited basis for prediction:

1. A compounded increased risk of unfavorable TB outcomes in diabetic patients with comorbid dyslipidemias.
2. A compounded increased risk of unfavorable TB outcomes in diabetic patients with comorbid vitamin D deficiency or insufficiency.
3. Increased risk for the development or progression of peripheral neuropathy in diabetic patients during TB treatment.

Statistical considerations: This longitudinal, observational study will recruit patients with incident diagnosis of pulmonary TB, targeting recruitment for an equal number of diabetics and non-diabetics (excluding pre-diabetic patients). Initial analyses will focus on the association of diabetes with disease characteristics at intake and with shorter term outcomes (18 months). If funding is secure for longer follow-up, then more long-term outcomes could be analyzed.

Baseline analyses (*associations at intake*). Initial outcomes of interest include radiographic severity, rates of MDR TB, and comorbid factors including lipid and vitamin D levels. Patient characteristics (demographics, behaviors, clinical history) will be compared between diabetics and non-diabetics for descriptive purposes and to inform multivariable models used to estimate associations. Unadjusted associations of diabetes with the outcomes will be estimated (rates of severity levels; rates of MDR-TB, mean and median lipid and 25(OH)D levels, as well as rates meeting clinical levels of interest e.g. LDL >100).

Adjusted associations will be estimated using general linear models, with the appropriate link function and error models used for each outcome. Mean lipid and 25(OH)D levels can be compared using linear regression models with examination of the distribution of residuals to determine if assumptions are appropriately met. Alternative models would examine transformation of the outcome data or the use of median regression. Rates of MDR-TB would be modeled using logistic regression. Radiographic severity will be examined as ordinal categories or dichotomized depending upon the distribution of the severity categories. A dichotomous outcome would be modeled with logistic regression; ordinal categories would examine the appropriate use of either an ordinal or multinomial logistic model. In each case, the association

of diabetes and outcome would be estimated using a multivariable model which would incorporate relevant covariates. Model building will follow the methods described by Harrell [18]. Linear associations with continuous covariates will be examined using lowess curves [19]. In addition to examining associations with diabetes, exploratory analyses would examine diabetic severity (HbA1c levels or diabetes severity score) association with baseline outcomes.

Longitudinal outcomes. Primary outcomes of interest are treatment outcome (cure, failure, relapse, death) and time to sputum conversion. Treatment outcome at 12 months (or longer if funding permits) can be examined as a multinomial outcome (cure, failure, default, death) and rates of outcomes estimated and modeled using a multinomial model. More outcome specific analyses will examine time to event (e.g., cure, death, relapse) using Kaplan-Meier estimates for unadjusted associations and Cox regression models for adjusted associations. Proportional hazard assumptions would be tested and graphically examined; if assumptions are not met, time varying hazard ratios can be estimated. With the possibility of multiple events, the use of competing risk models will be estimated and robust of associations examined. Time to sputum conversion would use similar time to event models (survival models) to estimate the association of diabetes with sputum conversion. Examination of missing data and dropouts will be made. We will strive to minimize both but, if needed, the robustness of results that account for missing data will be examined using imputation methods.

Power and Sample Size estimates. Sample size determination for longitudinal, observational studies is a balance among power, resources and feasibility. Based on power estimates across several outcomes (and resources and feasibility) we determined that recruiting 150 diabetics and 150 non-diabetics would provide sufficient power (>80%) for clinically important effect sizes. The exclusion of pre-diabetics should enhance the effect sizes.

For longitudinal outcomes we examined a combined treatment failure/death outcome where reviews indicate rates of ~8% in non-diabetics [5]. The sample of 300 provides >80% power for estimated hazard ratios (HR) ≥2.1 (approximately 8% vs. 12% rate at one year). For sputum conversion, there is >80% power for HR≥1.7 (based on an average delayed conversion of ~17% in non-diabetics across multiple studies [5]). For rates of MDR-TB there is >80% power for OR=2.0-2.5. For comparison of means the sample will provide >80% power for effect sizes of ≥0.35 or greater (changes of .35 standard deviations) which is considered a small to moderateeffect.

These preliminary calculations indicate sufficient power across a spectrum of outcomes for small to moderate effects. The cohort should provide estimates and tests of effects for examining the impact of diabetes on TB severity and outcomes, and generate estimates that will inform future studies.

Data Capture. There are several options for data collection; the final choice will be based on consultation between the PIs and member of the Scientific Working Groups. Dr. Viswanathan's recent study was conducted using paper case report forms (CRF). If that is carried over to the EDOTS study, then a data entry clerk will be hired and trained to transfer data from the CRF toa secure server at ICER. Alternatively, we may elect to use an electronic CRF. Options include REDCap which is available to Dr. Kornfeld (and his international collaborators) through UMass Medical School, along with secure server space for data storage at UMass. Alternatively, DataFax is being widely adopted by NIAID and could be employed with secure servers at ICER and NIAID. Both eCRF choices have advantages of data fidelity and security. We understand that the sponsors plan to develop a Statistical and Data Management Center to oversee and

coordinate data collection across all six funded CRUs, which could impact our plans. It would beadvantageous to develop common definitions and to use a common framework eCRF so that similar elements of the CRF used by individual CRUs would not have to be created from scratchin each case. Individual CRUs could then add project-specific elements to the framework CRF as needed.

Community engagement: Community engagement will be offered at several levels in the EDOTS study. The Indian RNTCP, through TBUs like Pulianthope and Tondiarpet is already engaged in outreach education for the community they serve. The MV Diabetes Center has an outstanding record of community engagement and the dissemination of information on diabetes prevention and treatment to the lay population it serves and to the local and national medical community. A long-term goal of Dr. Viswanathan is make diabetes awareness and screening a common element of TB control programs, with a reciprocal focus on TB case finding and management in patients presenting for diabetes care to clinics like the MV Diabetes Centre. In the course of recruiting and screening candidates for the EDOTS study, TB patients (and their families) at Pulianthope and Tondiarpet will receive information about diabetes from the MSWs. All potential study subjects who pass the inclusion and exclusion criteria will receive diabetes screening with FPB, OGTT and HbA1c at no cost. Screening of this type is not widely available through the government operated health clinics. Based on Dr. Viswanathan's data we anticipate that half of all TBU patients undergoing this screening will either have diabetes or pre-diabetes. Those patients newly identified with either condition will receive additional education on site by EDOTS team MSWs who have much experience in this practice. Steps will be taken to ensure that these patients are directed to the appropriate clinical services for subsequent diabetes management or diabetes prevention (for pre-diabetic patients). In addition to appropriate referral for newly diagnosed diabetes patients, the study team will provide transportation to the relevant clinics and ensure that patients are properly matched with effective diabetes care providers.

Academic/industry collaboration: No collaborations outside the EDOTS study team are presently in place for the clinical study. As the cohort and its associated Specimen Repository become established, we may seek academic or commercial partners to initiate new projects related to the dual burden of TB and diabetes.

Human studies approval: The EDOTS study represents collaboration between the M.V. Diabetes Centre and UMass Medical School, with participation of NIRT, ICER and NIAID. All work with human subjects will be conducted in India. Accordingly, the UMass Medical School will defer to the human studies committee of the M.V. Diabetes Centre. This has been codified by an IRB Authorization Agreement signed by institutional representatives of both parties. The other participating institutions will also sign reliance agreements for the human studies review and approval conducted by the IRB of the M.V. Diabetes. It is important to emphasize that this observational study does not involve any highly invasive procedures, nor will it interfere with thestandard care of TB patients provided by the TBUs.

Fundamental Research Plan

Hypotheses: We propose two fundamental research projects to investigate the impact of diabetes on human immunity in TB. The unifying hypothesis is that diabetes alters the immune response to TB resulting in delayed initiation but then excessive induction of Th1 and Th17

biased cell mediated immunity resulting in damaging immune pathology in the lung. Our aims are: *1*, to analyze the baseline differences in the T cell populations between active TB patients with or without diabetes; *2*, to compare and contrast the immune responses in peripheral blood of active TB patients with or without diabetes mellitus; and *3*, to compare the peripheral blood mononuclear cell (PBMC) transcriptional response in diabetic and non-diabetic TB patients on presentation, after the intensive phase of DOTS and after the completion of DOTS.

Background: The association of diabetes with an increased risk to develop active TB disease and increased severity of TB disease is now widely understood, albeit with many open questions about TB outcomes that our clinical project will address. Despite the clinical and public health significance posed by the dual burden of TB and diabetes, very little is known about the immunological and biochemical mechanisms of susceptibility.

Among the few laboratories addressing this topic the Kornfeld lab has established mouse models combining metabolic disorders including diabetes with aerosol infection by *M. tuberculosis* [12;13;20;21]. They found that like other diabetic complications, TB susceptibility develops in the setting of chronic but not acute hyperglycemia. TB defense requires the prompt induction of an adaptive immune response to *M. tuberculosis* in the lung, dominated by $CD4^+$ T cells producing interferon (IFN)-γ. Mice with chronic hyperglycemia are slow to prime the adaptive immune response after aerosol challenge owing to a delay in recruiting dendritic cells to the initial site of infection where inhaled bacilli invade resident alveolar macrophages. This delay allows *M. tuberculosis* additional days of unrestrained replication before Th1-biased immunity is expressed in the lung. The result is a >10-fold higher bacterial burden in the lung. Once adaptive immunity is expressed in the lungs of diabetic mice with TB it is both effective and exuberant. *M. tuberculosis* replication is ultimately constrained but at the expense of greatly increased immune pathology, culminating in accelerated pulmonary fibrosis. Once TB is established in the lung, diabetic mice ultimately express higher levels of protective cytokines including IFN-γ. Two different mouse models of hypercholesterolemia are also associated with increase fibrosis from TB. One of them (apolipoprotein E null mice) also exhibits a profound delay in immune priming associated with early mortality. The biochemical basis of these phenotypes is currently being explored. Studies are focusing on pathological glycation of matrix proteins influencing cell trafficking and on chronic oxidative stress impacting hematopoietic stemcells and differentiated leukocyte populations.

Data from the mouse model mirror the surprising finding that human TB patients with diabetes also over-express cytokines that are normally protective in TB. Restrepo et al. [22] measured multiple cytokines released by PPD stimulation of whole blood from TB patients with or without diabetes, finding higher expression of Th1 cytokines in the diabetic patients and a correlation with elevation of HbA1c within the diabetic group. Another research team measured cytokine production from cultured PBMC, failing to find a difference in antigen-stimulated IFN-γ production between diabetic and non-diabetic TB patients [23].

EDOTS team member Dr. Babu has preliminary data from whole blood restimulation that support and expand on the results of Restrepo et al. Dr. Babu used flow cytometry to compare T cells from diabetic and non-diabetic TB patients that were well matched for age and gender. The diabetic group had significantly elevated mean HbA1c (11.2% vs. 5.3% in controls). Peripheral blood was restimulated with different *M. tuberculosis* antigens or broadly stimulated with anti-CD3 mAb and then the frequencies of $CD4^+$ T cells expressing IFN-γ, tumor necrosis factor (TNF)-α or interleukin (IL)-2 alone or in combinations were compared (Fig. 3). Diabetic TB patients had significantly higher frequencies of T cells expressing the individual Th1 cytokines and polyfunctional T cells co-expressing TNF-α and IL-2 in response to *M. tuberculosis* antigen but not anti-CD3 stimulation. In addition to this increased frequency of antigen-specific

Th1 cells, Dr. Babu has pilot data suggesting that diabetic TB patients have an increased frequency of Th17 cells and a reduced frequency of natural regulatory (nTreg) cells. These differences in immune response between diabetic and non- diabetic TB patients may relate to differences in lung bacterial load arising from delayed initiation of a primary or secondary immune response and/or some fault of immune regulation caused by diabetes.

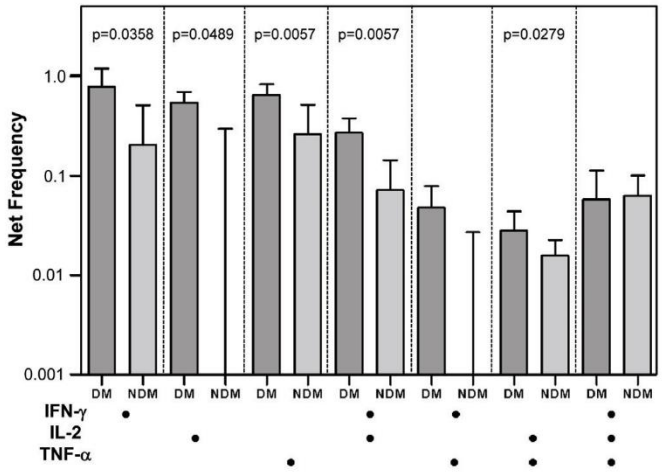

Figure 3. Increased frequency of antigen-specific mono- and polyfunctional T cells in diabetic (*DM*) vs. non-diabetic (*NDM*) TB patients. Whole blood restimulated with PPD and analyzed by flow cytometry with intracellular cytokine staining, gated on CD4. n=22 per group.

Differences in peripheral blood cytokines and chest imaging are imprecise biomarkers of the extent of TB disease. Berry et al. [24] described a peripheral blood gene expression signature that discriminated active TB disease from healthy controls, LTBI and several other infectious and non-infections inflammatory illnesses in European and African populations. A metric termed molecular distance to health (MDTH) [23] based on the composite of transcripts differing from the normal baseline was significantly higher in TB patients with radiographically advanced as compared to minimal disease. The molecular distance decreased after 2 months of TB treatment and the signature was extinguished by 12 months. The transcriptional signature of active TB measured in whole blood mainly reflected genes expressed in neutrophils and monocytes, rather than lymphocytes as was expected. Genes downstream of type 1 and type 2 IFN receptors were over-represented in the blood of TB patients vs. controls despite no difference in serum IFN protein levels. These data show that the immune response in TB produces a unique expression profile in PBMC through the systemic activation of myeloid cells in transit from bone marrow to sites of disease. Peripheral blood transcriptional profiling may be more sensitive and specific than measuring individual cytokines in the blood. It offers a window to the tissue inflammatory response in TB without resorting to invasive and costly approaches like bronchoscopy or PET scanning.

We will compare the MDTH in diabetic and non-diabetic TB patients, anticipating a higher median value in diabetics at enrollment. During treatment, diabetic TB patients are predicted to have a slower and, in some cases, incomplete normalization of MDTH. The transcriptional signature of TB and MDTH calculation are expected to provide a more accurate estimate of the burden of inflammation and the risk of incomplete sterilization of TB than is possible using clinical parameters alone. We anticipate that diabetic patients in our cohort will have a higher frequency of clinical relapse within one year of completing DOTS. We recognize, however, that relapse within 12 months might underestimate the true burden of residual disease that population. The ultimate risk of relapse could take decades to be fully realized. The pattern of peripheral blood gene expression at the completion of DOTS (6 months) might predict the risk

of relapse in a timely manner. If that is the case, this could stimulate the search for simpler biomarkers based on the expression of a restricted set of genes. We choose the approach of unbiased whole transcriptome profiling for the EDOTS study rather than focusing on the restricted set of signature genes because this offers the chance to make new discoveries of unique differences in PBMC gene expression between diabetic and non-diabetic TB patients. Finding differences beyond the already characterized TB signature could produce new insights to the mechanisms of susceptibility and immune pathology in the diabetic population.

Study design and laboratory evaluations: Definitive implementation of the fundamental studies we propose will entail costs that exceed the scope of a dCRU budget. We therefore plan to use the new resource provided by our EDOTS cohort and the available dCRU funding to conduct pilot studies. Preliminary data generated by these studies will be used to support applications for future funding opportunities.

1. **T cell phenotyping.** We will conduct a pilot study using PBMC from 50 TB patients (25 diabetic and 25 non-diabetic) at time points before, during and after DOTS treatment. PBMC samples will be isolated from the entire EDOTS cohort on presentation with TB, again after 2 and 6 months of DOTS, and once again at 12 months after the completion of DOTS. PBMC will be isolated from the whole blood by Ficoll-Hypaque density centrifugation and cryopreserved using freezing media (CTL-Cryo, Cellular Technology Ltd). Cryopreserved cells stored in the Specimen Repository and will be available for comprehensive analysis as new funding allows.

For the pilot study, 1 ml of blood will be used for ex vivo flow cytometry to quantify T cell subsets. Absolute numbers of $CD4^+$ T cells will enumerated in whole blood using BD Multiset™ 6-Color TBNK cocktail (BD Biosciences). Naïve and memory T cell phenotyping will be performed using CD45RA and CCR7 staining, gated on $CD4^+$ T cells. Naïve cells will be classified as $CD45RA^+$ $CCR7^+$; effector memory cells as $CD45RA^-$ $CCR7^-$; and central memory cells as $CD45RA^-$ $CCR7^+$. Natural regulatory T cells (nTregs) will be classified as $CD4^+$ $CD25^+$ $Foxp3^+$ $CD127^{dim}$. Ex vivo intracellular staining for Ki-67 expression on $CD4^+$ T cells will be performed to determine recent activation / proliferation. Extracellular expression of PD-1 will be measured to estimate cellular exhaustion. This will provide baseline T cell data to ascertain whether diabetes influences the T cell numbers, differentiation or expansion. If we do find baseline alterations in T cell numbers or differentiation, this would have broader implications for the diabetic complication of impaired protective immunity. In addition to TB, people with diabetes are known to be at particularly high risk for infection by certain pathogens including some, such as *Burkholderia pseudomallei*, that are highly relevant to the Indian population.

When funding is available to support further experiments, cryopreserved PBMC will be thawed and then used to determine the role of T cell subsets in immune responses to TB antigens in diabetic vs. non-diabetic patients. Our preliminary data show that the frequency of polyfunctional $CD4^+$ T cells as well as Th17 cells (expressing IL-17A and IFN-γ or IL-17A and IL-10) are increased in diabetic individuals compared to controls in response to *M. tuberculosis* antigens including PPD and ESAT-6. Our preliminary data also show a lower frequency of nTregs at baseline in diabetic individuals. We will try to elucidate the mechanism of this TB antigen- specific enhancement of $CD4^+$ T cells subsets in diabetes with the following experiments. We will examine the expression of Th1 transcription factors (T-bet and Eomes) in $CD4^+$ T cells expressing IFN-γ as well as the expression of IFN-γ in $PD\text{-}1^+$ and $PD\text{-}1^-$ cells. This would enable us to identify whether Th1 differentiation is altered in diabetes. In addition, we will examine the antigen-specific and non-specific responses of nTreg cells in response to PPD, ESAT-6, and anti-CD3. We expect increased expansion of nTregs in diabetic compared to non-diabetic TB patients. Correlations will be performed to ascertain associations between nTreg and Th1 cells. Finally, we will attempt to elucidate the role of antigen-presenting cells (APC) in the differential Th1 responses observed in diabetic-TB patients. This would

include investigation of the cytokine expression patterns in $CD14^+$ $HLA\text{-}DR^+$ or $CD14^+$ $HLA\text{-}DR^-$ monocytes as well as $CD14^+$ $CD16^-$ and $CD14^+$ $CD16^+$ monocyte subsets in response to TB antigens and LPS. We anticipate findingdifferences in the induction of IL-10, IL-12, IL-23 and heme oxygenase-1 in the monocyte populations, which would then reflect on the $CD4^+$ T cell cytokine expression. Future experiments would attempt to sort purify the APC populations as well as the nTregs from these individuals to further determine their function.

These lines of investigation will be supported by interaction with the Kornfeld lab at UMass. In preliminary studies his team discovered that purified $CD4^+$ T cells isolated from chronically diabetic mice are hyper-responsive to T cell antigen receptor signaling ex vivo and in vivo compared to non-diabetic controls. Paradoxically, their studies also revealed a reduced capacityof APC in chronically diabetic mice to activate antigen-stimulated proliferation of adoptively transferred naïve non-diabetic OT-II transgenic T cells. We anticipate that the interaction between laboratories working on T cell functions in diabetes in humans and in mice will enhanceproductivity of both groups and increase the pace of discovery.

2. **Transcriptional profiling.** This study has two main goals. The first is to use the TB signatureidentified by Berry et al. as a quantitative biomarker to compare the extent and activity of TB disease in diabetic vs. non-diabetic TB patients. We predict that the median MDTH of the TB signature genes will be higher in diabetic than non-diabetic TB patients on enrollment. We further predict that the gene expression pattern will trend towards the normal baseline more slowly in the diabetic than non-diabetic TB patients over the course of DOTS treatment. After completing DOTS, the TB signature pattern might persist in some diabetic patients. Our second goal is to use blood transcriptional profiling for an unbiased survey, anticipating that diabetic TBpatients will also show qualitative differences beyond the signature genes as compared to matched, non-diabetic TB patients. New knowledge produced by this survey may contribute to understanding the effects of diabetes on protective immunity in TB.

This transcriptional profiling study will receive critical support from Dr. Anne O'Garra who has agreed to serve as a consultant and collaborator. Based on extensive experience, she believes that a test set with 12-15 subjects per group has a very high chance to yield data that will support or refute our expectations. Sample size is an important consideration since the dCRU budget cannot cover the cost of microarrays needed for a definitive study. The dCRU budget willallow us to collect blood for RNA on all 300 subjects at four time points: before TB treatment; after the intensive phase of treatment (2 months DOTS); at the completion of 6 months DOTS; and one year after treatment. We will also collect peripheral blood for RNA from 60 control subjects without active TB or other acute illness. Of these, 50% will have diabetes and 50% will be non-diabetic (and without pre-diabetes). The control population will be recruited after we have begun to accumulate the TB cohort in order match demographic characteristics.

We plan a test set of transcriptional profiles using 14 subjects per group, including diabetic TB patients at enrollment, non-diabetic TB patients at enrollment and the two control groups. Total RNA on from these groups will be isolated using Tempus kits and validated by bioanalyzer and nanodrop (instruments available at ICER). On the recommendation of our team members at ICGEB we will have an Indian company (Bionivid) run the microarrays. At a cost of $425 USD per sample, Bionivid will transport the RNA from ICER, perform sample preparation and hybridization to Illumina HT-12 arrays, and then process data scanned from the microarray slidefor bioinformatics analysis that includes: raw data QC, normalization, list of differentially regulated genes, and pathway and gene ontology analysis. Further bioinformatic analysis will beconducted by our collaborators at ICGEB following Dr. O'Garra's approach and with her oversight. Briefly, this will include calculations of the MDTH [25], transcriptional modular analysis [26] and analysis of significance [27] and pathway analysis using Ingenuity. A detailed description of methods is available at: http://www.nature.com/nature/journal/v466/n7309/extref/nature09247- s1.pdf. We have sufficient funds in

the dCRU budget to double the size of the test set or to evaluate a second time point (e.g. 2 months DOTS) with n=14 per group. That decision will be made based on initial results. We will also apply for external funding for large scale validation studies, including the times points at 2 and 6 months of DOTS and 12 months after completion of treatment. An application for such funding should be competitive once we have established a well-defined, prospectively recruited cohort with full characterization of diabetes status.

Statistical considerations: We will use ANOVA for data resulting from flow cytometry. Dr. Babu's preliminary data provide confidence that statistically significant differences ($p<0.05$) willbe identified in a pilot study using n=25 per group. Bioinformatic methods are described and referenced above. Dr. O'Garra's recommendation for the test set sample size in gene expression studies is based on her experience working with diverse populations. Since we will have RNA samples for all patients at three time points, we can increase test set size if that is needed to confirm suggested differences between groups.

Academic/industry collaboration: The EDOTS team (MV Diabetes Centre, UMass, NIRT, ICER, ICGEB) plan collaboration with Dr. O'Garra (MRC National Institute for Medical Research). We will contract with Bionivid for microarray processing. As our CRU becomes established, a number of potential collaborators with academic interest in the dual burden of TB and diabetes, as well as commercial collaborators with interests in TB biomarkers and therapeutics, will come to light. We are eager to make the best possible use of the effort contributed by EDOTS study volunteers and the resource of our cohort and Specimen Repository to support new collaboration.

Human studies approval: This is addressed in the description of the clinical study above.

Summary: The rising global epidemic in inflammatory diseases promises to outstrip the major infectious diseases in prevalence, morbidity and economic impact. Diabetes is a leading player in this epidemic. Understanding its interaction with TB, a common infectious comorbidity in many rapidly developing regions, can provide important general insight into how inflammatory diseases influence infection and vice versa. As emphasized in this proposal, diabetes is clearly a major cofactor influencing the outcome of TB in India. The planned cohort study which brings together local as well as international experts in the fields of both diabetes and TB, affords a unique opportunity for an in-depth investigation of this important public health issue.

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