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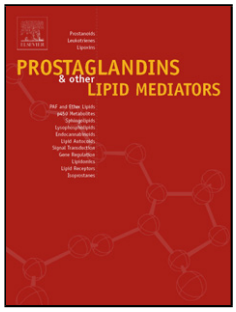

Lipid Mediators of Inflammation and Resolution in Individuals with Tuberculosis and Tuberculosis-Diabetes

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Running Head: Lipid mediators in TB and TB-DM

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Highlights

- Targeted analysis of lipid mediators of inflammation and resolution in individuals with Tuberculosis (TB) and TB-Diabetes (TB-DM)
- Most abundant pro-inflammatory and pro-resolving lipid mediators in circulation identified
- Individuals with TB-DM had a distinct mediator correlation and network profile compared to individuals with TB alone

ABSTRACT

Individuals with concurrent tuberculosis (TB) and Type 2 diabetes (DM) have a higher risk of adverse outcomes. To better understand potential immunological differences, we utilized a comprehensive panel to characterize pro-inflammatory and pro-resolving (i.e., mediators involved in the resolution of inflammation) lipid mediators in individuals with TB and TB-DM.

A nested cross-sectional study of 40 individuals (20 newly diagnosed DM and 20 without DM) was conducted within a cohort of individuals with active drug-susceptible treatment-naïve pulmonary TB. Lipid mediators were quantified in serum samples through lipid mediator profiling. We conducted correlation-based analysis of these mediators. Overall, the arachidonic acid-derived leukotriene and prostaglandin families were the most abundant pro-inflammatory lipid mediators, while lipoxins and maresins families were the most abundant pro-resolving lipid mediators in individuals with TB and TB-DM. Individuals with TB-DM had increased correlations and connectivity with both pro-inflammatory and pro-resolving lipid mediators compared to those with TB alone. We identified the most abundant lipid mediator metabolomes in circulation among individuals with TB and TB-DM; in addition, our data shows a substantial number of significant correlations between both pro-inflammatory and pro-resolving lipid mediators in individuals with TB-DM, delineating a molecular balance that potentially defines this comorbidity.

Keywords: Tuberculosis, Diabetes, lipids, inflammation, specialized pro-resolving mediators, prostaglandins, leukotrienes, lipoxins, resolvins

Introduction

Prevalence of type 2 diabetes (DM) among those with active tuberculosis (TB) disease is substantial, including in various settings with a high burden of TB[1-5]. Existing data suggests that DM increases the risk for adverse TB treatment outcomes[1,6,7]. Recent studies have investigated the biological basis behind these results, with a focus on potential differences in immunity between individuals with TB and TB-DM[8].

Heightened pro-inflammatory cytokines in individuals with TB-DM compared to TB alone have been observed in various studies[9-11]. Furthermore, individuals with TB-DM have increased Th1 immune responses, a higher bacillary load, defective phagocytosis, and increased oxidative stress[8,12,13]. Studies to date, however, have not yet characterized potential differences in lipid mediators of inflammation by TB and TB-DM status. These include lipid mediators that are important during the inflammatory process (e.g., pro-inflammatory mediators such as leukotrienes, prostaglandins and thromboxanes), along with the lipid mediators that are important for the resolution of inflammation: specialized pro-resolving mediators (SPMs), including resolvins, lipoxins, protectins and maresins[14,15] (Figure 1).

While arachidonic acid (AA)-derived pro-inflammatory lipids, such as leukotrienes and prostaglandins, are well-known mediators of inflammation, SPMs – derived mostly from Eicosapentaenoic acid (EPA), Docosahexaenoic acid (DHA) and n-3 Docosapentaenoic acid (DPA) – were discovered more recently with their roles in human pathology remaining of interest. Limited studies have characterized SPMs as actively involved in the resolution of inflammation (including reduction of pro-inflammatory cytokines and lipids), with the different SPM families having unique functions regulating various aspects of immunity such as phagocytosis, neutrophil infiltration, and microbial clearance[16].

Prior studies of specific lipid mediators in individuals with TB alone have shown that mediators including leukotriene B_4 (LTB_4), lipoxin A_4 (LXA_4), prostaglandin E_2 (PGE_2), and prostaglandin $F_2\alpha$ ($PGF_{2\alpha}$) play important roles in TB susceptibility and pathogenesis[17-20]. The role of SPMs other than LXA_4 (and aspirin triggered LXA_4 (15-epi-LXA_4)) in TB are not well understood; one study showed that individuals with TB disease had higher levels of resolvins compared to healthy controls[21] and another study detected SPMs in cerebrospinal fluid of

individuals with TB meningitis[22]. In studies of individuals with diabetes alone, levels of LTB_4 and PGE_2 were important immune factors and could affect susceptibility to other infections[23,24]; however, an analysis of additional lipid mediators in circulation, beyond LTB_4, LXA_4, resolvin, PGE_2 and $PGF_{2\alpha}$ has not been performed in those with TB alone or with TB-DM. Even the most abundant lipid mediators in circulation among those with TB have not been characterized and it is not known whether there are any differences by TB and TB-DM status. Thus, we conducted a study to characterize circulating levels of lipid mediators of inflammation, with a specific focus on relationships between lipid mediators, in serum of TB patients with and DM, by using a comprehensive panel that quantified both pro-inflammatory and pro-resolving lipid mediators[22].

Materials and Methods

Study Population

HIV-negative adults ≥18 years of age with newly diagnosed drug-susceptible pulmonary TB were enrolled in a cohort study conducted at BJ Government Medical College-Sassoon General Hospital (BJGMC-SGH) and Dr. DY Patil Medical College (DYPMC) in Pune, India. Pulmonary TB was diagnosed based on results from acid-fast bacilli (AFB) smear microscopy, Xpert MTB/RIF assay (Cepheid, CA) or Mycobacteria Growth Indicator Tube (MGIT, BD, MD) liquid culture. Individuals with resistance to rifampicin, previous history of TB, or on anti-TB treatment for greater than 7 days were excluded from enrollment into the cohort. The cohort has enrolled 796 individuals since December 2013.

We nested a cross-sectional study at baseline (pre-treatment) within this active TB cohort to assess and compare pre-treatment serum levels of lipid mediators of inflammation between

individuals with newly diagnosed diabetes (TB-DM, n=20) and those without diabetes (TB, n=20). For TB-DM patients, we focused on a random sample of individuals who had glycated hemoglobin (HbA1c) ≥ 8.0% but who did not have previous DM and were not on any DM medications. We only focused on those with newly diagnosed DM for the TB-DM group in order to exclude any effects of DM treatment and complications on these lipid mediators. HbA1c was measured by high-performance liquid chromatography (BioRad Laboratories, CA). TB only patients were randomly selected from the cohort and were matched on age and gender with TB-DM individuals. Study participants provided written informed consent and this study was approved by Institutional Review Boards (IRBs) at the participating institutions of BJGMC-SGH, DYPMC, Johns Hopkins University, and Columbia University. All methods were performed in accordance with the relevant guidelines and regulations.

Laboratory assessment

Serum Collection:

Serum was collected through BD plain vacutainer blood collection tubes. Serum was stored in -80°C until they were shipped to William Harvey Research Institute for lipid mediator assessment.

Targeted lipid mediator profiling: All samples were extracted using solid-phase extraction columns as previously described[25]. Prior to sample extraction, deuterated internal standards, representing each region in the chromatographic analysis (500 pg each) were added to facilitate quantification in 4V of cold methanol. Samples were kept at -20°C for a minimum of 45 min to allow protein precipitation. Supernatants were subjected to solid phase extraction, methyl formate and methanol fractions were collected, brought to dryness and suspended in phase (methanol/water, 1:1, vol/vol)

for injection on a Shimadzu LC-20AD HPLC and a Shimadzu SIL-20AC autoinjector, paired with a QTrap 6500 plus (Sciex). For identification and quantitation of products eluted in the methyl formate an Agilent Poroshell 120 EC-C18 column (100 mm x 4.6 mm x 2.7 μm) was kept at 50°C and mediators eluted using a mobile phase consisting of methanol-water-acetic acid of 20:80:0.01 (vol/vol/vol) that was ramped to 50:50:0.01 (vol/vol/vol) over 0.5 min and then to 80:20:0.01 (vol/vol/vol) from 2 min to 11 min, maintained till 14.5 min and then rapidly ramped to 98:2:0.01 (vol/vol/vol) for the next 0.1 min. This was subsequently maintained at 98:2:0.01 (vol/vol/vol) for 5.4 min, and the flow rate was maintained at 0.5 ml/min. QTrap 6500 plus were operated using a multiple reaction monitoring method as previously described[25].

In the analysis of peptide-lipid conjugated mediators eluted in the methanol fraction Agilent Poroshell 120 EC-C18 column (100 mm x 4.6 mm x 2.7 μm) was kept at 50°C and mediators eluted using a mobile phase consisting of methanol-water-acetic acid at 55:45:0.1 (vol:vol:vol) over 5 min, that was ramped to 80:20:0.1 (vol:vol:vol) for 2 min, maintained at 80:20:0.1 (vol:vol:vol) for the next 3 min and ramped to 98:2:0.1 (vol:vol:vol) over 3 min. This was kept at 98:2:0.1 (vol:vol:vol) for 3 min. A flow rate of 0.60 ml/min was used throughout the experiment. QTrap 6500 plus was operated in positive ionization mode using multiple reaction monitoring (MRM) coupled with information-dependent acquisition and enhanced product ion scan[25].

Each LM was identified using established criteria (Supplementary Figure 3) including matching retention time to synthetic and authentic materials and at least 6 diagnostic ions[25]. Calibration curves were obtained for each using synthetic compound mixtures at 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100, and 200 pg that gave linear calibration curves with an r^2 values of 0.98–0.99.

Statistical analysis

To assess the overall balance between pro-inflammatory mediators and pro-resolving mediators in the CSF we combined the concentrations of pro-resolving mediators, combining the concentrations of the DHA-derived RvD (RvD1, RvD2, RvD3, RvD4, RvD5, RvD6, 17R-RvD1 and 17R-RvD3) PD (PD1, 10S,17S-diHDHA, 17R-PD1 and 22-OH-PD1) PCTRs (PCTR1, PCTR2 and PCTR3) and MaR (MaR1, 7S, 14S-diHDHA, MaR2, 4S, 14S-diHDHA, 14-oxo-MaR1 and 22-OH-MaR1) MCTRs (MCTR1, MCTR2 and MCTR3), the n-3 DPA-derived RvT (RvT1, RvT2, RvT3 and RvT4), $RvD_{n\text{-}3\ DPA}$ ($RvD1_{n\text{-}3\ DPA}$, $RvD2_{n\text{-}3\ DPA}$ and $RvD5_{n\text{-}3\ DPA}$), $PD_{n\text{-}3\ DPA}$ ($PD1_{n\text{-}3\ DPA}$ and 10S, 17S-diHDPA) and $MaR_{n\text{-}3\ DPA}$ ($MaR1_{n\text{-}3\ DPA}$ and 7S, 14S-diHDPA), the EPA-derived RvE (RvE1, RvE2 and RvE3) and the AA-derived LX (LXA_4, LXB_4, 5S, 15S-diHETE, 15R-LXA_4, 15R-LXB_4 13,14-dehydro-15-oxo-LXA_4 and 15-oxo-LXA_4), AA-derived LT (LTB_4, 5S, 12S-diHETE, D6-trans-LTB_4, D6-trans, 12-epi-LTB_4 and 20-OH-LTB_4), cysLT (LTC_4, LTD_4 and LTE_4), PG (PGD_2, PGE_2 and $PGF_{2\alpha}$) and Tx (TxB_2).

As we had multiple markers but a limited sample size, this specific analysis focused on characterizing the most abundant lipid mediators and the relationships between markers, rather than a focus on any specific mediator. We removed thromboxane B_2 (TxB_2) from our analyses, because TxB_2 levels increase during platelet activation, which happens during sample collection of serum; thus the observed TxB_2 levels are due to the sample collection method rather than a reflection of the circulating levels[26]. In initial exploratory analysis, individual lipid mediators were used in orthogonal partial least squares discriminant analysis (OPLS-DA) and outliers were identified. Then, log-transformed data of lipid mediators were used to construct heat maps based on abundances of a) lipid mediator and b) metabolome groups. Separation between TB and TB-

DM patients based on their overall abundance profiles of lipid mediators and metabolomes were tested using hierarchical cluster analysis. Parametric t tests for univariable analysis and logistic regression for multivariable analysis were used to study the association of individual lipid metabolomes with TB-DM.

To better understand the correlation profiles of those with TB and TB-DM, statistically significant spearman correlations between metabolome groups were used to construct heatmaps and conduct network analysis, as previously described[27]. Network density values were compared between study groups using permutation test (100 permutations were performed)[27]. We also conducted node analysis where the number of connections of each metabolome marker in the network was quantified for each study group[28].

The statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA), JMP 13.0 (SAS, Cary, NC, USA) and R 3.1.0 (R Foundation, Vienna, Austria) programs.

Results

Study population characteristics

Our cross-sectional study included 40 individuals with treatment-naïve pulmonary TB (20 with TB and 20 with TB-DM). Their median age was 43.0 years (interquartile range (IQR): 36-49), 90% of the study participants were male and the median body mass index (BMI) was 18.0 (IQR: 17.0-21.5) (Table 1). As we matched individuals with TB and TB-DM based on age and sex, they had similar age and sex distributions. BMI, however, was different (p=0.0001) between those with TB (median: 17.0, IQR: 15.5-18.0) and TB-DM (median: 21.0, IQR: 19.0-23.5) (Table

1). Microbiological profile, as assessed by baseline sputum mycobacterial growth indicator tube (MGIT) culture time-to-detection were similar (p=0.91) between those with TB (median days: 9, IQR: 6-15) and TB-DM (median: 10, IQR: 7-13). Finally, based on the design of the study, there were expected differences in baseline HbA1c between those with TB (median: 5.5, IQR: 5.3-5.7) and TB-DM (median: 11.3, IQR: 9.7-12.7) (Table 1).

Lipid mediator profile in TB and TB-DM

To gain insights into ongoing inflammation and resolution processes within these patients, we assessed abundance levels of lipid mediators from the four major bioactive metabolomes (arachidonic acid, eicosapentaenoic acid, n-3 docosapentaenoic acid, and docosahexaenoic acid) in serum samples from 40 individuals: 20 with TB and 20 with TB-DM. We identified mediators from all four major fatty acid metabolomes, including mediators from the lipoxygenase and cyclooxygenase biosynthetic pathways (Supplementary Table 1). The abundance heatmap for all 40 individuals (Figure 2A) shows that while the overall profile varied between individuals, a few lipid mediators were the most abundant in serum and present in the majority of individuals. The most abundant lipid mediators were LTB_4 and 20-OH-LTB_4 (Figure 2C). In addition, PGE_2, LTE_4, LXB_4, PGF_{2a} and PGD_2 were other markers that were also abundant in most individuals (Figure 2C). We then performed hierarchical cluster analysis (HCA) to determine whether TB and TB-DM individuals could group separately based on their lipid mediator profile. Our results (Figure 2A and Supplementary Table 1) did not show a distinct abundance profile by TB and TB-DM status.

We next sought to determine whether there were differences in the regulation of individual lipid mediator families. For this, we assessed the abundance of each of the lipid mediator families

from the four bioactive metabolomes (*see methods* for details of the mediator families). As seen in Figures 2B and 2C, the abundance profiles were dominated by AA-derived LT, PG and LX. Since PG, LT and LX-based mediators were the most abundant individual mediators, it is not surprising that they would be the most abundant metabolome groups in the majority of individuals. Similar to the results with individual mediators, we did not observe a distinct profile by TB and TB-DM status in the HCA of metabolome abundances (Figure 2B).

Given the potent biological actions played by SPM in the regulation of both bacterial infections and metabolic inflammation, we next focused our attention on the SPM biosynthetic pathways (Figure 3). The abundance profile shows that LXB_4 is the most abundant SPM (Figure 3A). In addition, we can also see that 5S, 15S-diHETE and MCTR3 were also abundant and present in most individuals, although the levels varied (Figure 3A). In line with the individual mediators, the most abundant SPM metabolome groups were AA LX (LXB_4 and 5S, 15S-diHETE – the LX pathway marker) and DHA MaR (MCTR3) (Figure 3B). Once again, the HCA profiles were similar between those with TB and TB-DM (Figure 3A and 3B).

In exploratory analysis, comparison of levels of lipid mediator metabolomes showed that individuals with TB-DM had higher levels of both pro-inflammatory and pro-resolving lipid mediators relative to those with TB alone (Figure 2D). However, we were underpowered for this specific analysis and these differences were not statistically significant (Supplementary Table 1). In multivariable models adjusting for age, gender and body mass index (BMI), higher levels of DPA RvT (adjusted odds ratio (aOR): 118.8; 95% CI: 1.3-1124.4) and lower levels of AA PG (aOR: 0.02; 95% CI: 0.01-0.78) metabolomes were associated with TB-DM status relative to TB alone. As previous studies have suggested that the balance between lipid mediators may be

important in TB pathogenesis[17-20], we tested the ratio of PG:LX or PG:LT metabolomes but did not find any difference by TB and TB-DM status (Supplementary Figure 1).

Distinct lipid mediator correlation profiles between TB and TB-DM

In recent studies, we have shown that profiles based on correlations between inflammatory markers can help distinguish between disease states[29-33]. Extending these investigations to our analysis of lipid mediators of inflammation, we studied the correlation between the various mediator families among individuals with TB and those with TB-DM. The heatmaps presented in Figure 4 (displaying only statistically significant correlations) show that the correlation profiles differed between those with TB and TB-DM. Notably, only positive correlations between markers were found to be statistically significant. We next assessed whether there was a correlation between mediator families within each of the bioactive metabolomes. When comparing correlations within DHA or n-3 DPA metabolomes, the profiles between those with TB and TB-DM were the same. In contrast, when looking at the correlation profiles within the AA metabolomes (bottom right quadrant), those with TB-DM have a greater number of correlations compared to those with TB alone.

Since different mediator families from distinct metabolomes utilize the same biosynthesis enzymes (e.g., $RvD_{n\text{-}3\ DPA}$ and the DHA-derived RvD both utilize ALOX5 and ALOX15), we next investigated whether there were any correlations in the concentrations of mediators from different metabolomes. Correlations between n-3 DPA and DHA metabolomes (middle left quadrant) showed that those with TB-DM have a greater number of correlations between the n-3 DPA and DHA metabolomes relative to those with TB alone. We also assessed correlations between AA metabolomes with n-3 DPA and DHA metabolomes. The top right quadrants clearly show that for

individuals with TB-DM, the AA metabolomes (e.g., LX, LT and PG) have significant correlations with a greater number of metabolomes from the DHA and n-3 DPA-derived SPMs compared to individuals with TB alone. In summary, relative to those with TB alone, those with TB-DM had correlation profiles with a greater number of correlations within the various AA metabolomes, as well as between the AA metabolomes with those of DHA and n-3 DPA metabolomes.

We also assessed the correlations between lipid mediator metabolomes and baseline TB characteristics. TB bacterial load, chest x-ray (CXR) cavity and smear grade are indicators of TB disease severity, where increased bacterial load, presence of cavity and higher smear grade is reflective of a more severe disease. Among those with TB-DM, PG metabolome was positively correlated with smear grade and in those with TB alone, PG and DHA RvD were positively correlated with CXR cavity (Supplementary Figure 2).

Network profile of lipid mediators

We also conducted network analysis based on correlations between the lipid mediator metabolomes. Network analysis is another approach to visualize relationships based on correlations; an added strength of this approach is that it is easier to visualize the differences in number of correlations and identify markers with similar correlation profiles[34]. The relationship in the networks are different between those with TB and TB-DM, and for individuals with TB alone, two distinct major networks are apparent (Figure 5A). One subgroup (red circles) includes a network dominated by SPMs while the other subgroup (green circles) includes a network of both pro-inflammatory AA-derived PG and a SPM (DHA MaR). In contrast, the network profiles for those with TB-DM looked different (Figure 5A), with greater number of connections for the various subgroups relative to TB alone. Similar to individuals with TB alone, there was one

subgroup of SPMs alone (blue circle) while there was another subgroup (green circle) with a combination of pro-inflammatory AA-derived PG and SPMs. However, the specific metabolome groups within each subgroup was different and more importantly, there were a greater number of connections for each subgroup (Figure 5A). Unlike individuals with TB alone, there was also an additional subgroup (purple circle) in individuals with TB-DM, which included correlations between AA LT and n-3 DPA PD, with LT having multiples connections (Figure 5A).

These observations were further confirmed through the higher network density values in TB-DM relative to those with TB alone ($p<0.0001$; Figure 5B). Increased network density reflects more significant correlations in those with TB-DM; this suggests a more coordinated response, with TB-DM individuals exhibiting similarly high levels of more than one marker. While further studies are needed to better characterize the inter-relationships between the lipid mediator metabolomes, based on our previous studies of network density[27,35], we hypothesize that this coordinated response might reflect an underlying biological phenomenon. Finally, we also quantified the number of significant correlations for each mediator (node analysis) among those with TB and TB-DM (Figure 5C). Node analysis has been utilized to identify markers that are more connected and involved in the condition of interest (e.g., disease pathogenesis)[28]. While some differences are observed for various lipid mediator metabolomes (e.g., AA LX and AA PG), the clearest difference in the number of connections between those with TB and TB-DM was observed for AA LT, with a high number of connections in those with TB-DM relative to the low number of connections in those with TB alone (Figure 5C).

In summary, relative to those with TB alone, those with TB and newly diagnosed DM have increased connectivity between the lipid mediator metabolomes, suggesting their participation in TB-DM pathogenesis.

Discussion

In this study of TB patients with and without newly diagnosed DM, we characterized circulating levels of pro-inflammatory and pro-resolving lipid mediators. AA-derived LT and PG were the most abundant metabolomes, while among the SPMs, the AA-derived LX and DHA-derived MaR metabolome families were the most abundant. Interestingly, analysis on the relationship between the lipid mediators suggested that individuals with TB and newly diagnosed DM had increased connectivity between mediators of inflammation, with increased correlations and network connectivity between both pro-inflammatory and pro-resolving lipid mediators, compared to those with TB alone.

While limited studies have previously assessed specific lipid mediators in the context of TB[17-20], this is the first study to comprehensively characterize both pro-inflammatory and pro-resolving lipid mediators. We also extended these investigations for the first time into those with TB-DM. We observed a serum profile dominated by many AA-derived pro-inflammatory lipids including LTB_4, 20-OH-LTB_4, PGE_2, LTE_4, PGF_{2a} and PGD_2 among individuals with TB and TB-DM. The most abundant SPMs were AA-derived LXB_4 and 5S,15S-diHETE along with MCTR3.

Prior studies in TB have mainly focused on the roles of three lipid mediators: LTB_4, LXA_4 and PGE_2. Existing data indicates that PGE_2 promotes bacterial control while LXA_4 can increase bacterial growth[36]. Further, PGE_2 provided a balance between type I interferon (IFN) and IL1 levels in TB, which are two counter-regulatory cytokines that can impact the control of mycobacterium tuberculosis (Mtb) infection outcome[19,37]. However, the concept of balance between pro-inflammatory and pro-resolving lipid mediators in TB is also important, as an imbalance in either the pro-resolving LXA_4 or pro-inflammatory LTB_4 can both result in

development of active TB[18,20,38]. Our results show that PGE_2 and LTB_4 are abundant in circulation while LXA_4 is not one of the more abundant lipid mediators.

Among the other abundant pro-inflammatory mediators (20-OH-LTB_4, LTE_4, PGF_{2a} and PGD_2), only PGF_{2a} has been previously studied in the context of TB with data suggesting a similar role to PGE_2 as protective in TB susceptibility and pathogenesis[19]. Interestingly, we also observed that PG metabolomes were correlated with disease severity in both TB and TB-DM. This is in line with recent findings demonstrating that cerebrospinal fluid PG concentrations are linked with increased disease severity in TBM[39] and given that PG display immunosuppressive actions in infections[40]. Thus, we hypothesize that this increase in PG mediators may be at least in part linked with the higher bacterial load, an aspect that will need to be further elaborated in future studies. The specific role that the other mediators might have in the context of TB or TB-DM is not known. The abundance of 20-OH- LTB_4 and LTB_4 is in line with the activation of peripheral blood neutrophils during the coagulation process that leads to the activation of PLA_2 enzymes that release esterified arachidonic acid[41] and ALOX5, which is central to LTB_4 biosynthesis. Similarly, the most abundant SPMs (LXB_4, 5S,15S-diHETE and MCTR3) have not been previously studied in the context of TB. Of note, MCTR3 activates the host immune response to uptake and kill bacteria[42]; this may reflect an immune response from the host to try to control active TB. Recent studies have implied a role for LXB_4 in the protective actions of pioglitazone (a diabetes drug)[43], but our data show similar levels in individuals with TB regardless of DM status. As abundance of individual mediators does not always predict function, future studies will need to extend these investigations to understand the role and function that both abundant and less abundant mediators play in TB susceptibility and pathogenesis.

Our exploratory analysis on the association of each lipid mediator with TB-DM status showed that both pro-inflammatory and pro-resolving metabolomes were increased in those with TB-DM relative to TB alone. However, our study was not designed or powered to look at individual markers or metabolomes; future well-powered studies will need to assess whether levels of lipid mediators are significantly different, in parallel with observed increases in protein cytokines that suggest higher levels of connectivity between inflammation markers in those with TB-DM relative to TB alone. In addition, although the analysis was exploratory in nature with very wide confidence intervals, it was interesting to note that lower levels of prostaglandin metabolomes were associated with TB-DM status in multivariable models. Given that PGE_2 is important for Mtb control[36], further investigation is warranted as this may partly help explain the increased bacillary load and pro-inflammatory cytokine milieu in TB-DM relative to TB alone[8,12,13]. We also observed that n-3 DPA-derived RvT levels were higher in those with TB-DM in the exploratory multivariable analysis. Of note, RvT display both leukocyte and endothelial-directed actions. These mediators counterregulate the production of inflammatory mediators by leukocytes and upregulate the production of endothelial protective mediators such as prostacyclin[44]. Whether RvT is increased as a response to the increased pro-inflammatory milieu in TB-DM will need to be tested in future studies. As our TB-DM population were all newly diagnosed, future studies will also need to study these lipids in those with existing diabetes as well as any effect TB or diabetes treatment has on these lipid mediators. Furthermore, it would also be of interest to assess whether these lipid mediators are different between those who may have hyperglycemia that is (i.e. transient hyperglycemia) or is not reversible with treatment.

The correlation profiles were different between those with TB and TB-DM. In individuals with TB-DM, there were more correlations of the AA metabolomes with each other, and also with

DHA and DPA metabolomes. These data and results from network analysis suggest that among TB-DM individuals, there was an increase in both pro-inflammatory (driven by LT and PG) and pro-resolving (driven by LX and MaR) connectivity. One potential pathway for an increase in both pro-inflammatory and pro-resolving connectivity may involve an initial increase in inflammation for those with TB-DM, driven by LT and PG, and mirroring increases in pro-inflammatory cytokines, followed by an increase of pro-resolving mediators as a response to this heighted inflammation; future studies are needed to better understand this. While most prior studies have only focused on pro-inflammatory markers, the dysregulation of inflammation (i.e., increases in both pro and anti-inflammatory markers) in TB-DM has also been observed in limited studies of cytokines comparing those with TB and TB-DM[9,45].

A limitation of our study is that we did not have data from individuals that did not have TB disease, with and without diabetes. Due to the nested design of this study, we were limited by the parent study design, which only focused on individuals with TB. While there are lipid mediator data from other published studies on individuals without TB (e.g. healthy or DM), directly comparable data are lacking. For example, a study of diabetics in Australia showed that diabetics have higher concentrations of E-series resolvins (RvE1, RvE2), D-series resolvins (RvD1, RvD2) and Maresin 1 compared to our TB-DM population[46]. However, the sample matrix used were different as our study assessed lipid mediators in serum samples while the Australian study used plasma samples[46]; we have previously shown that lipid mediators profiles in serum and plasma are quite different and not comparable[41]. In another study that assessed lipid mediators in the same matrix (i.e. serum samples), healthy individuals had higher concentrations of most lipid mediators (e.g. the D-series and E-series resolvins), and lower levels of some other mediators (e.g. LXB_4, PGE_2 and $PGF_{2\alpha}$) compared to our study (both TB and TB-DM populations)[41]. It is important to

note, however, that while these differences could be partly explained by biology (i.e. due to TB or TB-DM status), other factors that were different between the two studies, such as sample processing (e.g. blood collection procedures), storage condition (e.g. duration) and study population characteristics[41,47], could also explain these differences. Thus, it is not possible to attribute these differences between studies to biology alone. Despite these limitations, our study provides useful information through a direct comparison among those with TB and TB-DM and suggests that future larger studies that include non-TB controls are needed.

Conclusion:

In summary, our results suggest that LT, PG, LX and MaR were among the most common circulating lipid mediators in serum of individuals with TB and TB-DM, and individuals with TB and newly diagnosed DM have increased connectivity between lipid mediators of inflammation reflected by increased correlations between both pro-inflammatory and pro-resolving lipid mediators compared to individuals with TB alone.

Footnote page

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Conflicts of interest: None declared.

Author contributions: RS designed the study, and oversaw the scientific and logistical aspects of the study. He also wrote the primary version of the manuscript. JD, RAC, FM conducted the laboratory assessments of lipid mediators, conducted initial data analysis, provided interpretation of the findings, and contributed to manuscript writing and review. SG, SC, MB, RL, DK, SD, RB,

AK, NP, SD, SA, TS, AK, VK, SR, NS, AG, AG and NG contributed to cohort study design and implementation, data collection and data management. BBA delineated and supervised the data curation and analyses and provided interpretation of the findings. KFF performed all the systems analyses, created the statistical scripts used to plots the analyses and graphs. MBA contributed to data analysis. JEG and VM are the parent study PIs. They obtained funding and contributed to study design and manuscript review. All authors read and approved the final manuscript.

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Figure 1 Title: Families of lipid mediators of inflammation and their precursor lipids

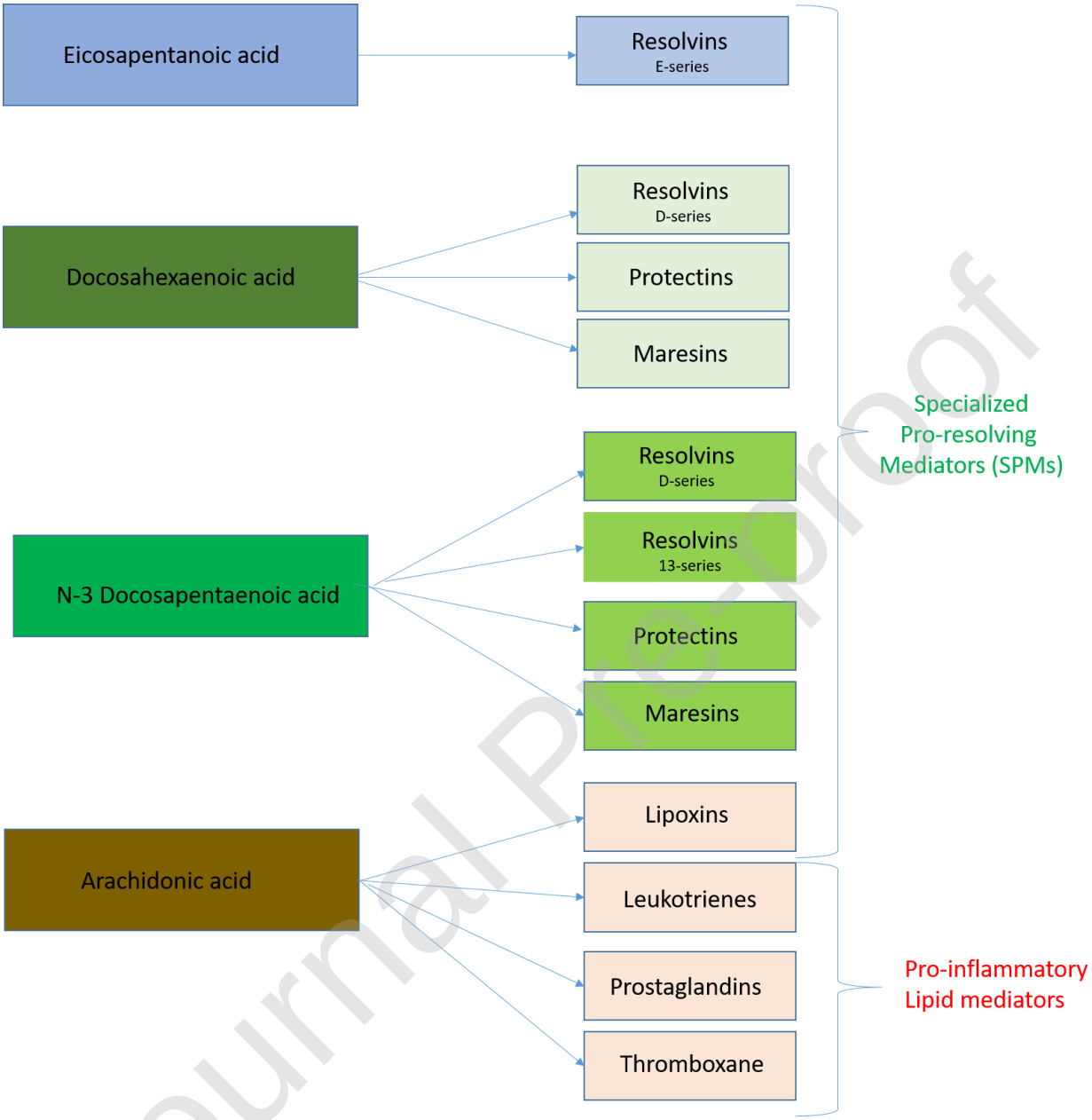

Figure 1 Legend: Eicosapentanoic acid (EPA), Docosahexaenoic acid (DHA), Docosapentaenoic acid (DPA) and arachidonic acid (AA) are the precursor molecules to various families of pro-inflammatory and Specialized Pro-resolving lipid mediators (SPMs). SPMs include resolvins, protectins, maresins and lipoxins while pro-inflammatory lipid mediator families include leukotrienes, prostaglandins and thromboxane.

Figure 2 Title: Lipid mediator abundance profile of individuals with TB and TB-DM

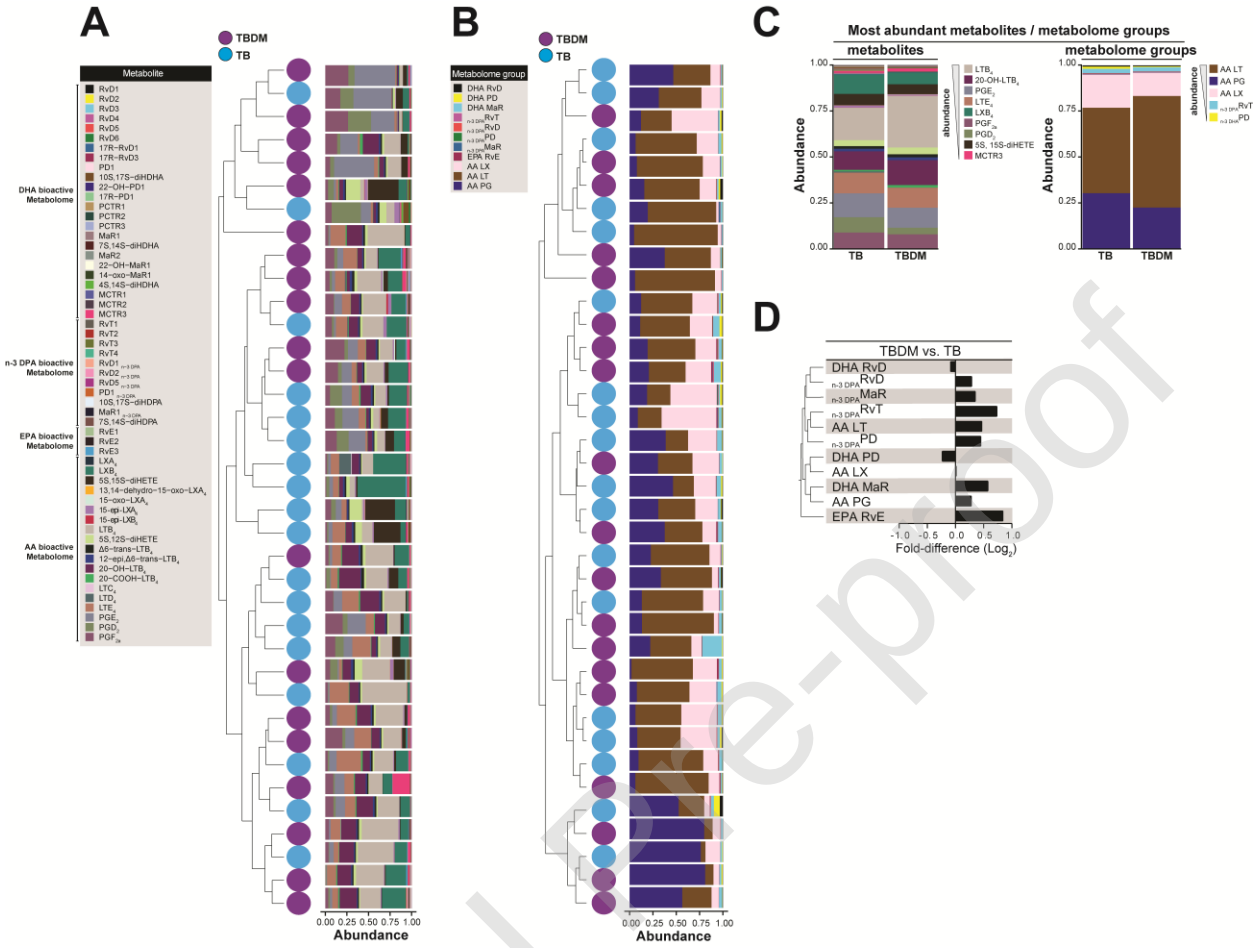

Figure 2 Legend: Heat map showing average abundances of log-transformed data for A) lipid mediators or B) lipid mediator metabolomes for each patient. Hierarchical clustering was performed to test whether they was grouping by DM status based on overall abundance profiles of the lipid mediator (A) or lipid mediator metabolome (B). The color of each mediator or metabolome group is shown in the figure legends. Individuals with TB-DM are represented by purple circles while individuals with TB alone are represented by blue circles. In C), the most abundant lipid mediators and metabolomes are shown, and in D) the relative abundance (log_2 fold-change) of each lipid mediator metabolome group in those with TB-DM relative to TB alone is shown.

Figure 3 Title: SPM abundance profile of individuals with TB and TB-DM

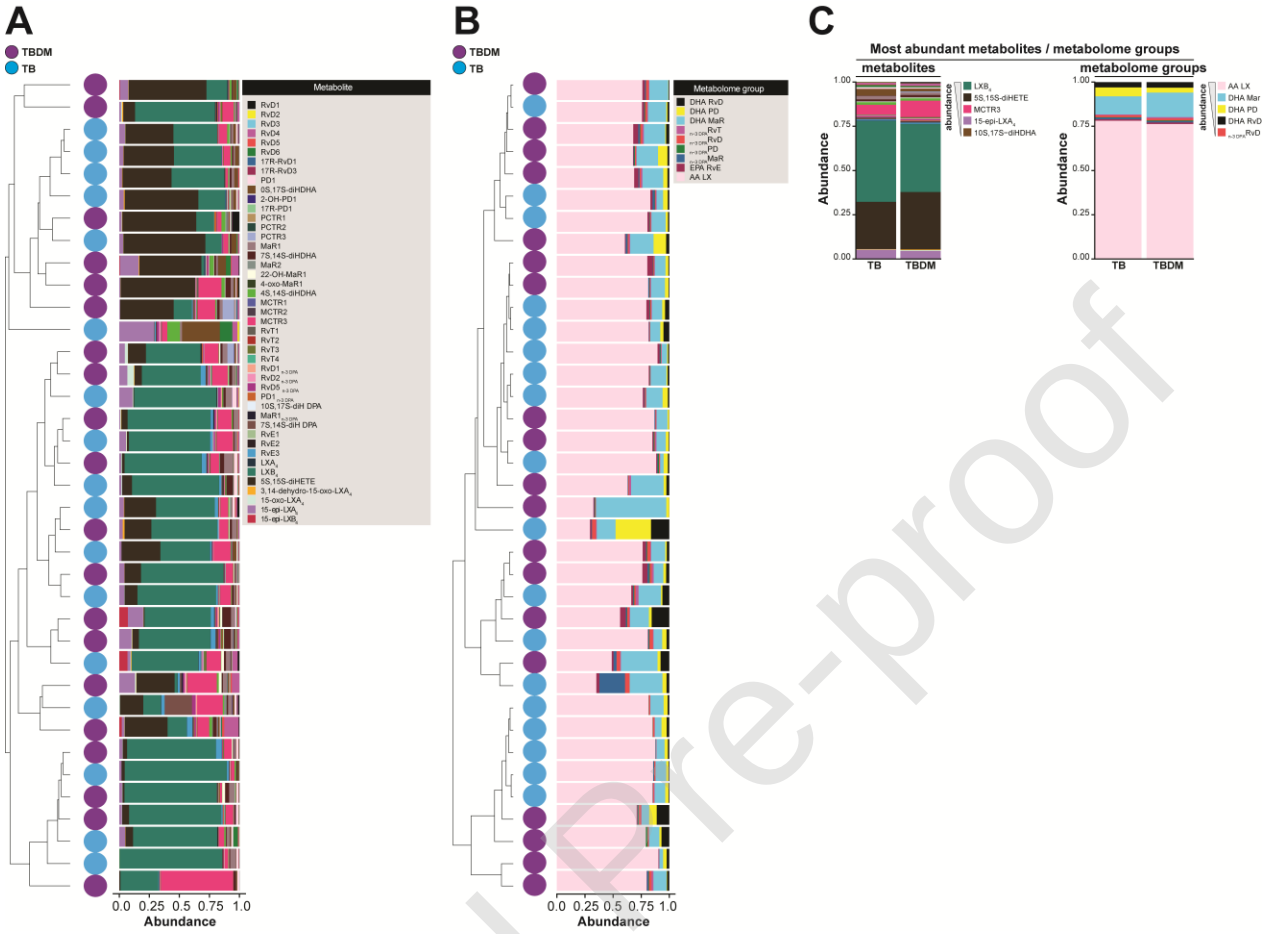

Figure 3 Legend: Heat map showing average abundances of log-transformed data for A) SPMs or B) SPM metabolomes for each patient. Hierarchical clustering was performed to test whether they was grouping by DM status based on overall abundance profiles of the lipid mediator (A) or lipid mediator metabolome (B). The color of each mediator or metabolome group is shown in the figure legends. Individuals with TB-DM are represented by purple circles while individuals with TB alone are represented by blue circles. In C), the most abundant SPMs mediators and metabolomes are shown.

Figure 4 Title: Individuals with TB and TB-DM have distinct correlation profile of lipid mediator metabolomes

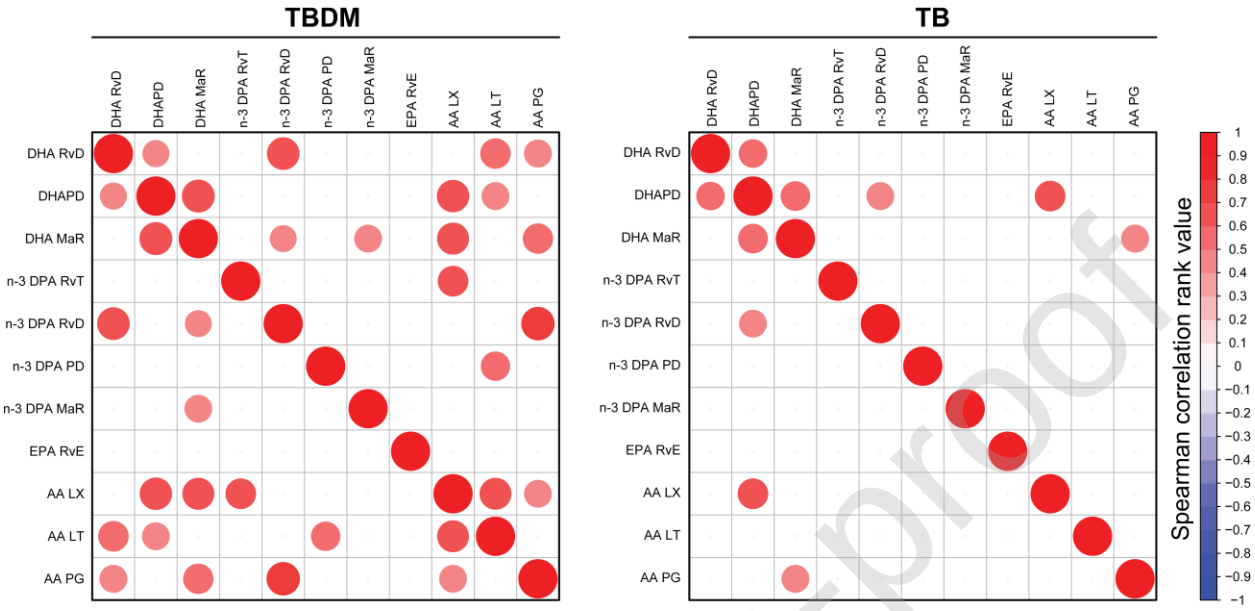

Figure 4 Legend: Spearman correlation analyses were used to identify statistically significant associations between the cumulative levels of lipid mediator metabolomes in individuals with TB and TB-DM. The heatmaps only show statistically significant correlations ($p<0.05$). Colors infer the strength and direction of the correlation. Of note, only positive correlations between the markers were found to be statistically significant.

Figure 5 Title: Network analysis of the lipid mediator metabolomes in individuals with TB and TB-DM

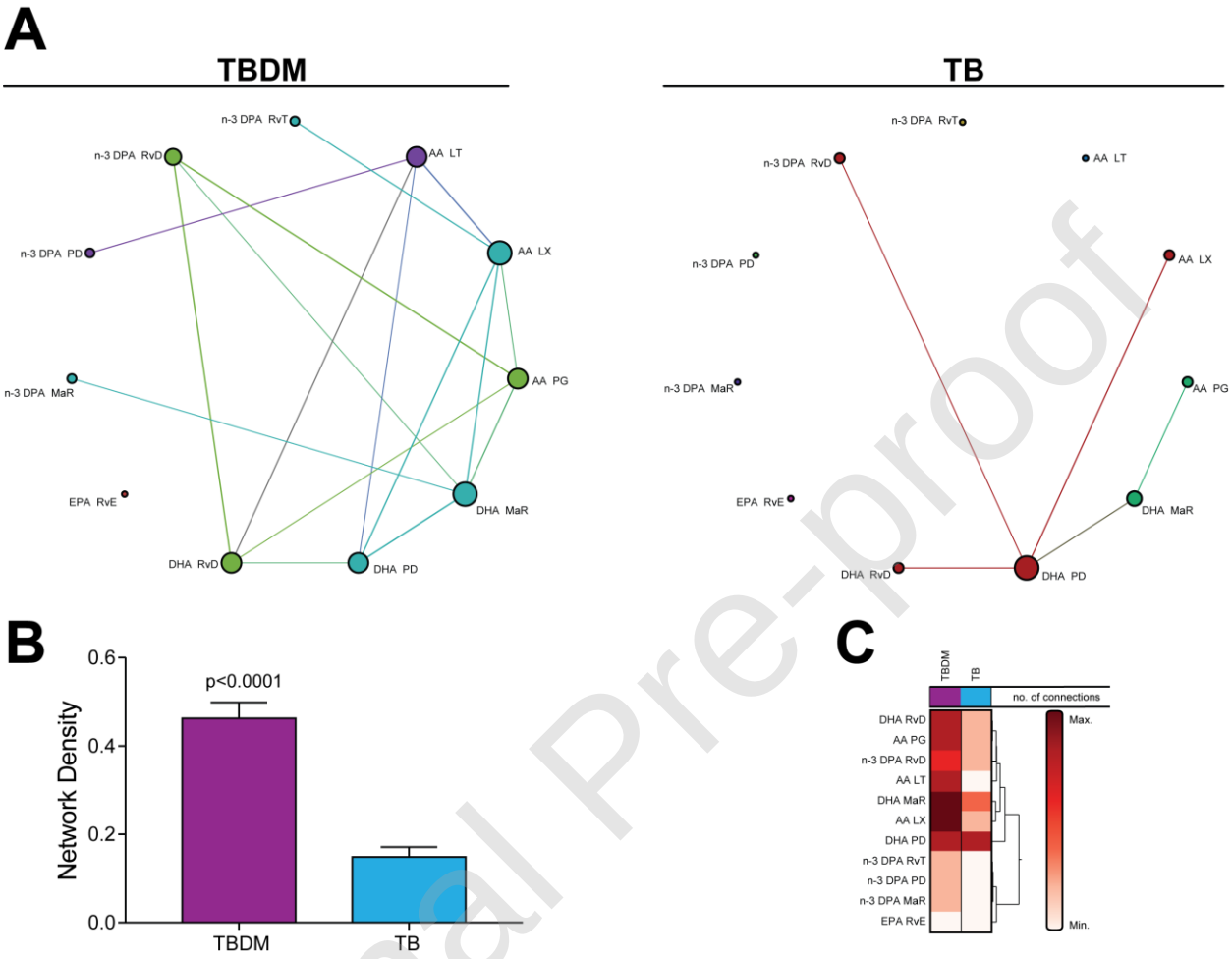

Figure 5 Legend: A) Network analysis based on spearman correlation matrices of circulating concentrations of lipid mediator metabolomes is shown. Only statistically significant correlations ($p<0.05$) are displayed. Circle sizes are proportional to the number of connections to each node. Circle colors represent distinct subgroups of parameters that exhibited a similar correlation profile. The parameters from each subgroup are linked through lines of the same color. Correlations between markers from different subgroups are highlighted in different colors. B) Network density values were compared between the study groups using permutation test (100 permutations were performed). Data represent mean and SD values of network densities per permutation. C) Node

analysis: We quantified the number of connections of each marker in the networks for each study groups. Heatmaps show hierarchical cluster analysis (Ward's method) of the number of connections of each marker in each study group, red highlights the lipid mediator groups which exhibited the highest number of connections whereas blue identifies those which displayed the lowest number of connections.

Figures and Tables:

Table 1: Characteristics by TB and TB-DM status

Characteristic	**All** **n=40**	**TB** **n=20(50%)**	**TB-DM[#]** **n=20(50%)**	**P-value***
Age (Years)	43(36-49)	43(37-48)	44(35-49)	0.82
Body mass index (kg/m^2)	18.0(17.0-21.5)	17.0(15.5-18.0)	21.0(19.0-23.5)	0.0001
MGIT culture time-to-detection (days)	10(7-13)	9(6-15)	10(7-13)	0.91
HbA1c (%)	7.1(5.6-11.3)	5.5(5.3-5.7)	11.3(9.7-12.7)	<0.0001
Gender				
Male	36(90)	18(50)	18(50)	0.99
Female	4(10)	2(50)	2(50)	

Table 1 Legend: Data are presented at no. (%) of subjects or median value (interquartile range)

Abbreviations: MGIT, Mycobacteria Growth Indicator Tube liquid culture; HbA1c, glycated hemoglobin

[#]All TB-DM patients in this study had HbA1c ≥ 8.0%

*P-values were calculated used Fisher's exact test for categorical variables and Wilcoxon rank-sum test for continuous variables to determine the difference between cases and controls