Online Supplement: Host Lipidome and TB treatment Failure

Methods:

Ethics Statement

We followed US Department of Health and Human Services guidelines for human experimentation. Institutional review boards and ethics committees at BJMC (IRB 00003631), NIRT (IEC ID 2017020), Johns Hopkins University (IRB 00140325) and Columbia University (IRB AAAS0281) approved this study.

Laboratory Assessments:

Complex Lipids: Complex lipids, including ceramides, sphingomyelins, cholesterol esters, oxysterols, lyso- and phospholipids, mono-, di- and triacylglycerols, were semi-quantified using an untargeted approach by liquid chromatography with quadrupole time of flight mass spectrometry (LC-QTOF-MS). Lipids from plasma were extracted following protocols previously described[1]**.** Briefly, 1.5 mL methyl-*tert*-butyl ether (MTBE)-methanol was added to internal standard enriched plasma and vortexed for 20 s. MTBE (5 mL) was further added and incubated in a shaker at room temperature for one hour. 1.25 mL of water was added for phase separation followed by 10 min incubation in room temperature and centrifugation at 14,000 g for 2 min. The upper phase, which has the lipids without proteins or polar hydrophilic compounds, was decanted and dried in a vacuum centrifuge followed by reconstitution with 65 µl of

methanol:toluene (9:1, *v/v*) containing 1-cyclohexyl-3-uredio dodecanoic acid (CUDA; Sigma-Aldrich; St. Louis, MO) as an internal standard. For mass spectrometry analysis, residues in extracts were separated on a 2.1 mm x 100 mm, 1.7 µm Waters Acquity UPLC CSH C18 column. The extracts were detected using an Agilent 6530 QTOF-MS with resolution R=10,000 for positively charged lipids such as phosphatidyl cholines (PC), lyso-PC, phosphatidyl ethanolamines (PE) and phosphatidyl serines (PS), and Agilent 6550 QTOF-MS with resolution R=20,000 for negatively charged lipids such as free fatty acids and phosphatidylinositols. Metabolite annotations were achieved using a combination of different tools and conformed to Metabolomics Standards Initiative defined levels of compound annotation [2]. All spectra, retention times and chromatography conditions are freely available at MassBank of North America (http://massbank.us).

Oxylipins: Non-esterified oxylipins, endocannabinoids, polyunsaturated fatty acids (PUFAs) and non-steroidal anti-inflammataory drugs (NSAIDs) were isolated by liquid extraction protocol using methanol/acetonitrile mixture (1:1 v/v) from 40 µL of plasma and quantified by UPLC-MS/MS using internal standard methods. Briefly, 40 µL of plasma was mixed with 5 µL BHT/EDTA (1:1 methanol:water), 5 µL of 1250 nM deuterated oxylipins, endocannabinoids and polyunsaturated fatty acids surrogates and 5µl of an internal standard solution containing CUDA and 1-phenyl ureido 3-hexanoic acid (PUHA; kind gift from Dr. B.D. Hammock, University of California Davis) at 5µM in 1:1 methanol:acetonitrile. Next, plasma was homogenized by addition of 185 µL 1:1 methanol:acetonitrile and homogenate was centrifuge at 15,000 g for 10 min and the methanol:acetonitrile supernatant was filtered through 0.1 µm PVDF membranes. Residues in extracts were separated on a 2.1 mm x 150 mm, 1.7 µm BEH C18 column (Waters, Milford, MA) and detected by electrospray ionization with multi

reaction monitoring on a API 6500 QTRAP (Sciex; Redwood City, CA) and quantified against 7-9 point calibration curves of authentic standards using modifications of previously reported methods[3, 4]. All peak integrations were manually curated and quantification was conducted in MultiQuant v 3.0 (Sciex). The rationale to include oxylipins was due to our goal of conducting an unbiased lipidomics analysis that includes lipid families with important functions, as well as the prior literature suggesting an important role for oxylipins, such as lipoxin and leukotrienes, in impacting TB susceptibility[5].

Statistical Analyses:

Lipidomic analysis was performed on 192 individuals. 470 annotated lipids were available for analysis after removing lipids that had missing data in >70% of individuals. To be conservative, we set the threshold at 70% to minimize the influence of data imputation while maximizing the number of species covered. Missing data (0.3% of data) exceeding this threshold were imputed by singular value decomposition (SVD) imputation in Jmp v14.0 (SAS Institute, Cary NC). For the least squares regression analysis, raw data were either Johnson or log-transformed to follow an approximate normal distribution, with the optimal transformation selected by maximization of the Shapiro-Wilk W-test. Of note, quantitative and semi-quantitative data were concatenated into a single combined dataset for all statistical analysis, including FDR corrections. We used principal components analysis (PCA) for data exploration, such as visualizing separation of lipidome profile by study site using *prcomp* function from *stats* package in R 4.01.

We also performed cross validation with k=1 for classification by splitting the dataset into training (75% of data samples) and test (25% of data samples) sets. Samples were split randomly, based on information of the group classification with the function *createDataPartition* from the *caret* package in R. In log-transformed lipid data adjusted for covariates using the *sva* package [6], a machine-learning based random forest algorithm (randomForest package) was applied to the training set to identify the minimal variable set with high classification power to differentiate cases and controls, separately for the primary and subset analysis. Default parameters of random forest package were used with the following modifications to the parameters: number of trees to grow of 20000, number of permutations of 100, and number of variables randomly sampled as candidates at each split of 500. The accuracy of the lipid models was assessed by performing the receiver operating characteristic (ROC) curve and measuring the area under the curve (AUC). All results were plotted with the *ggplot* function for the barplots, and with *chordDiagram* function from *circlize* package in R 4.01.

Discussion

TGs: Interestingly, a recent study observed that ezetimibe, a drug that reduces cholesterol but was also shown to have an even stronger effect in reducing TGs, could restrict *MTB* growth in zebrafish models[7]. However, investigations into TG lowering therapy as a an adjunct therapy to improve TB cure rates [7, 8] should be made cautiously since *MTB* adaptations to long-term fenofibrate use appear to increase both macrophage lipid accumulation and the risk of TB associated mortality[9]. Regardless, our data identifies specific TGs that are associated with

increased failure and could serve as baseline indicators for increased risk of failure along with a potential utility for treatment monitoring.

Oxylipin: We also observed the oxygenated ALA metabolite 15,16-DiHODE levels were lower in failures. Data from studies of ALA show that it can actually inhibit *MTB* replication[10, 11], including through the inhibition of the *MTB* shikimate kinase enzyme[10]. Population-based studies also support a potential protective role of ALA in incidence of TB[12, 13]. Taken together, these findings suggest that ALA and its derivative 15,16-DiHODE might have differing roles in *MTB* compared to the longer chain DHA and EPA ω-3 PUFAs, and they could potentially serve as an adjunct therapy to anti-TB therapy to reduce treatment failure rates[10].

PCs: Our results on PCs could help explain why PCs have shown to have apparent conflicting roles in TB. As PCs are among the most abundant phospholipids in mammalian cells, different species of PCs are known to have different roles. In our study, we observed that baseline levels of certain PCs (e.g. 32:0) were increased among those who eventually failed treatment. Studies have shown that treatment with PC 32:0 and other PCs increased *MTB* survival in macrophages through inhibition of actin assembly, which is required for intracellular killing of *MTB[14]*. More specifically, these PCs have anti-inflammatory roles including inhibiting TNFα- gene expression and subsequent F-actin assembly[14]. Thus, higher levels of these PCs are likely linked to increased treatment failure through increased *MTB* survival and impaired immunity. In contrast, we observed PCs with longer-chains and a higher degree of unsaturation, were lower in individuals with failure. Studies have observed higher levels of some of these PCs among

individuals with TB compared to controls[15, 16]; the reasons are not clear and future studies need to address the mechanisms for how lower levels of these PCs might increase risk of failure.

Clinical Lipidomics/Metabolomics: While clinical metabolomics is an emerging technology with significant challenges, recent advances are driving development of infrastructure and technology needed to implement these efforts in a clinical setting[17-19]. Advances in high-throughput lipidomics are driving a similar focus on clinical lipidomics[20-23]. Our findings further support the potential for clinical lipidomics as an advanced diagnostic tool in the clinical setting, and efforts to provide clinicians with a routine translatable lipidomic profiling should be encouraged. In the context of TB treatment failure, our findings could lead to the development of a targeted and translatable lipid panel that can be used to monitor treatment and identity potential failures. Further, identifying these high-risk individuals for treatment failure could guide clinicians for testing potential interventions (e.g. statins or other lipid-modifying agents).

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