Supporting Information

Tuberculostearic acid (TSA)-containing phosphatidylinositols as markers of bacterial burden in Tuberculosis

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Experimental Methods

Extraction and LC-MS of amphiphilic mycobacterial lipids

Mycobacteria were grown in 7H9 media (BD Middlebrook 7H9 Broth) containing 10% OADC, 0.2% glycerol and 0.05% Tween 80 as described in the main text. Samples were washed twice with PBS and aliquots corresponding to $5.0x10^8$ bacteria in 1 mL PBS were transferred into glass tubes containing 2 mL petroleum ether (bp 60-80 °C) and 4 mL methanol (LC-MS grade) for inactivation of bacteria. After 15 min shaking at RT in a rotator, samples were centrifuged for 10 min at 400 x *g*. The upper petroleum ether phase was transferred to another vial and the methanolic phase was re-extracted one more time with 2 mL petroleum ether (Figure S13). The methanolic phase was separated from the intermediate layer and stored at -20 °C until analysis.

LC-MS analysis of the methanolic phase of the mycobacterial lipid extraction

Prior to LC-MS analysis, 100 µL of the methanolic phase were dried in vacuum and reconstituted in 100 µL of $CHCl_3$/Methanol + 0.1% ammonium acetate 86/13 (v/v) and sealed using aluminum foil. An Agilent 1100 HPLC system (Agilent, Waldbronn, Germany) was used for the separation of the phospholipids on a 150 mm BETASIL Diol-100 column with a particle size of 5 µm (Thermo Fisher, Bremen, Germany) and 0.32 mm inner diameter using 5 µL injection volumes. The solvents, gradient profile and flow rates are summarized in table S4.

The mass spectrometric analyses were performed on a Q Exactive Plus instrument (Thermo Fisher Scientific, Bremen). For phospholipid analysis, full scan MS data (*m/z* 400-1800) were acquired in the first 18 minutes of the LC-run using positive and negative ion mode switching. Instrumental parameters were as follows: source temperature at 250 °C, -3.0 kV and 4.0 kV ionization voltage for negative and positive ion mode analysis, respectively. Resolution was set to 280 k, ACG target value was set at $3.0x10^6$ and RF value was at 100%. The sheath gas was set to 5 L/min, aux and sweep gas were set to 0 L/min. Data acquisition was performed using the Xcalibur 2.4 software (Thermo Fisher Scientific, Bremen).

Data Processing

LC-MS data files were sliced in raw files containing only positive or negative mass spectra using Thermo's Slicer, Version 1.3. Raw files were converted to mzml using MSConvert[1] and files of the negative ion mode were analyzed using LipidXplorer.[2,3] Lipids were assigned by their

monoisotopic masses with a mass accuracy better than 5 ppm using LipidXplorer. Import settings and MFQL scripts will be made available at https://lifs-tools.org/.

Generation of bone-marrow derived macrophages (BMDM)

Naval Medical Research Institute (NMRI) *Wnt6* gene-deficient and *Wnt6* wild-type mice were raised and maintained under specific pathogen-free conditions.[4] In order to generate BMDMs, mice were euthanized and femora and tibia were washed with ice-cold Dulbecco's Modified Eagle Medium (DMEM). Isolated bone-marrow cells were seeded on Nunclon Delta cell culture dishes (Thermo Fisher Scientific, Waltham, USA), and cultivated in macrophage cell culture medium (10 mM HEPES, 1 mM sodium pyruvate, 2 mM glutamine, 10% of heat-inactivated fetal calf serum (FCS; Biochrom, Berlin, Germany) in DMEM) containing 50 ng/mL macrophage colony stimulating factor (M-CSF; Bio-Techne, Minneapolis, USA). After 24 h, non-adhered cells were collected, seeded on cell culture dishes (Sarstedt, Nümbrecht, Germany) and cultivated for 7 days. Cells were incubated in macrophage cell culture medium overnight before proceeding further.

CFU determination in PMN culture

After incubation of *Mtb* infected PMN for 6 h at 37 °C with 5% CO_2, one aliquot of 100 µL of the PMN culture was transferred to a new plate. Afterwards, the cell culture was centrifuged (500 x *g*, 5 min, RT) and the supernatant was discarded to remove non-phagocytized bacteria. The cells were lysed in 0.5% TritonX-100 in PBS and a 10-fold serial dilution in 0.05% Tween80 in PBS was prepared and plated on Middlebrook 7H11 agar plates. After incubation for 3-4 weeks at 37 °C colonies were counted and bacterial load per mL was calculated.

Lipid analysis of mice lung tissue homogenates and protein content determination

The lung wet weight of each tissue sample was noted before homogenization. For all extraction aliquots of each lung homogenate were inactivated in 80% methanol. For the normalization of the extraction procedure a volume corresponding 0.012 g of lung tissue was transferred in a new vial, dried in a SpeedVac and afterwards resuspended in 20 µL 50 mM ammonium acetate (data file S2). The lipid extraction was performed with a customized MTBE method as described earlier.[5] Then 270 µL methanol, containing 3% acetic acid, were added. After vortexing the mixture, 30 µL of the internal standard solution (Avanti, SPLASH® Lipidomics® Mass Spec Standard, 330707) and 1 mL MTBE were added. The solution was incubated for one hour at RT with continuous shaking at 600 rpm (Eppendorf, MixMate). For phase separation 250 µL of water was added and subsequently incubated for 10 min at RT with continuous shaking at 1300 rpm. After incubation samples were centrifuged for 10 min at 15,000 x *g* and then the upper phase was collected in a separate tube. The lower phase was re-extracted with 400 µL theoretical upper phase,[6] vortexted and incubated for 20 min at RT with continuous shaking (1300 rpm). The solution was once more centrifuged as described above. The resulting upper phase was combined and the re-extraction step with the lower phase was repeated. In the end, the combined upper phase was dried in a SpeedVac. The dried extracts were dissolved in a mixture of chloroform, methanol and water (60/30/4.5; v/v/v) and stored at -80 °C. Shotgun lipidomics was performed on Q Exactive (Thermo Fisher Scientific, Bremen, Germany) as described earlier.

The water phases and non-extractable residue left after MTBE extraction were dried under vacuum. The sample pellet is resuspended in a 150 µL sample buffer (1 g SDS and 6.25 mL Tris/HCl (0.5 M, pH 6.8) filled to 100 mL with water). Samples are mixed well and are incubated for 15 min in a shaker (650 rpm) followed by a short centrifugation (2 min at 16,000 x g) step to have all sample located at the bottom of the vial. Protein quantification was performed with the Pierce™ BCA™ Protein-Assay Kit (Thermo Fisher Scientific, Waltham, US) according to the vendor's protocol.

ROC analysis

R version 3.4.0.2 was used to perform ROC analyses using the packages cutpointr and pROC to determine and visualize test performance of the parameters. Additionally, false positive and false negative rates were calculated based on the optimal cut-off classification.

Lipid annotation

The targeted MS^2 enables to identify lipids until the level of fatty acid isomers. As characteristics ions for the PI class, we utilized *m/z* 241.01. However, as quantifier ion the acyl anion of the FA 19:0 (TSA) was utilized. For the annotation convention we followed the guidelines of Liebisch et al..[7] In this study we determined isomers on three levels:

1. bond type high resolution; PI 35:0
2. fatty acyl; PI 16:0_19:0
3. fatty acyl (*); PI 16:0_19:0 (TSA)

These levels are also depicted in view of the certainty of the structural assignment of possible isomers in Figure S4. For level 3, as shown in (Figure 1, 2), we cannot exclude *sn*-position isomerism and determine isomeric purity. However, because of the utilization of $^{13}C_{18}$-labeled OA and the methylation performed by *Mtb*, we are certain of the detection of TSA.

Figures

A

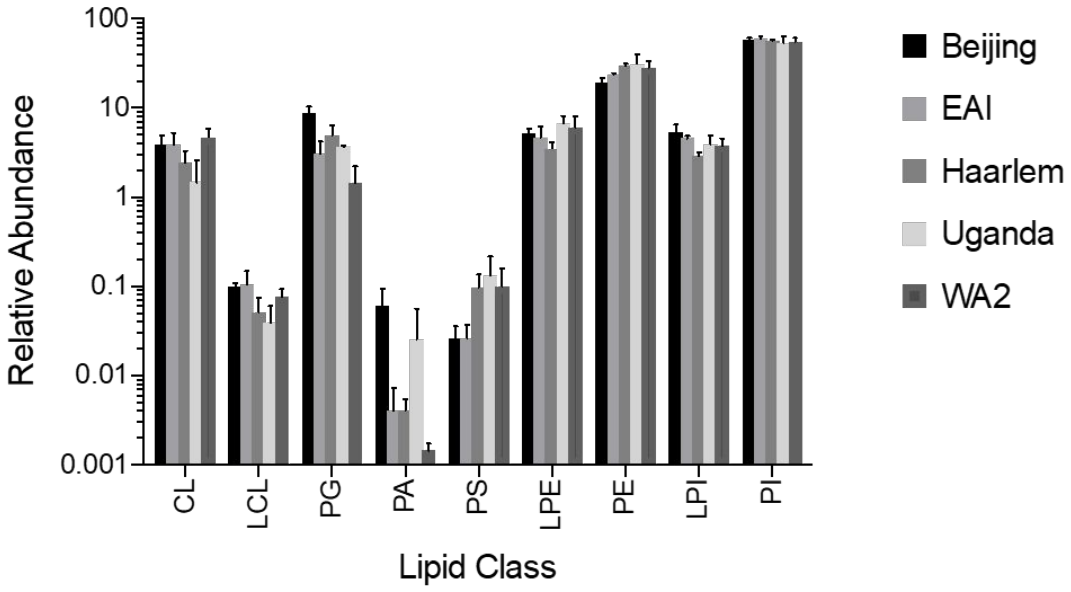

Figure S1) Overall composition of major abundant phospholipid classes of mycobacterial isolates. Lipid class distribution for all listed mycobacterial genotypes. Relative abundance represents the average of all strains of a genotype (**Table 1**). Relative abundances of lipids was determined by top down lipidomics approach using internal standards 4ME 16:0 Diether PE (1,2-di-O-phytanyl-*sn*-glycero-3-phosphoethanolamine) and PS 17:0/17:0 (both standards from Avanti Polar Lipids, Alabaster, US) for normalization of peak intensities.
CL – cardiolipin; LCL – lysocardiolipin; PG – phosphatidylglycerol; PS, phosphatidic acid; PS – phosphatidylserine; LPE – lysophosphatidylethanolamine; PE – phosphatidylethanolamine; LPI – lysophosphatidylinositol; PI – phosphatidylinositol.

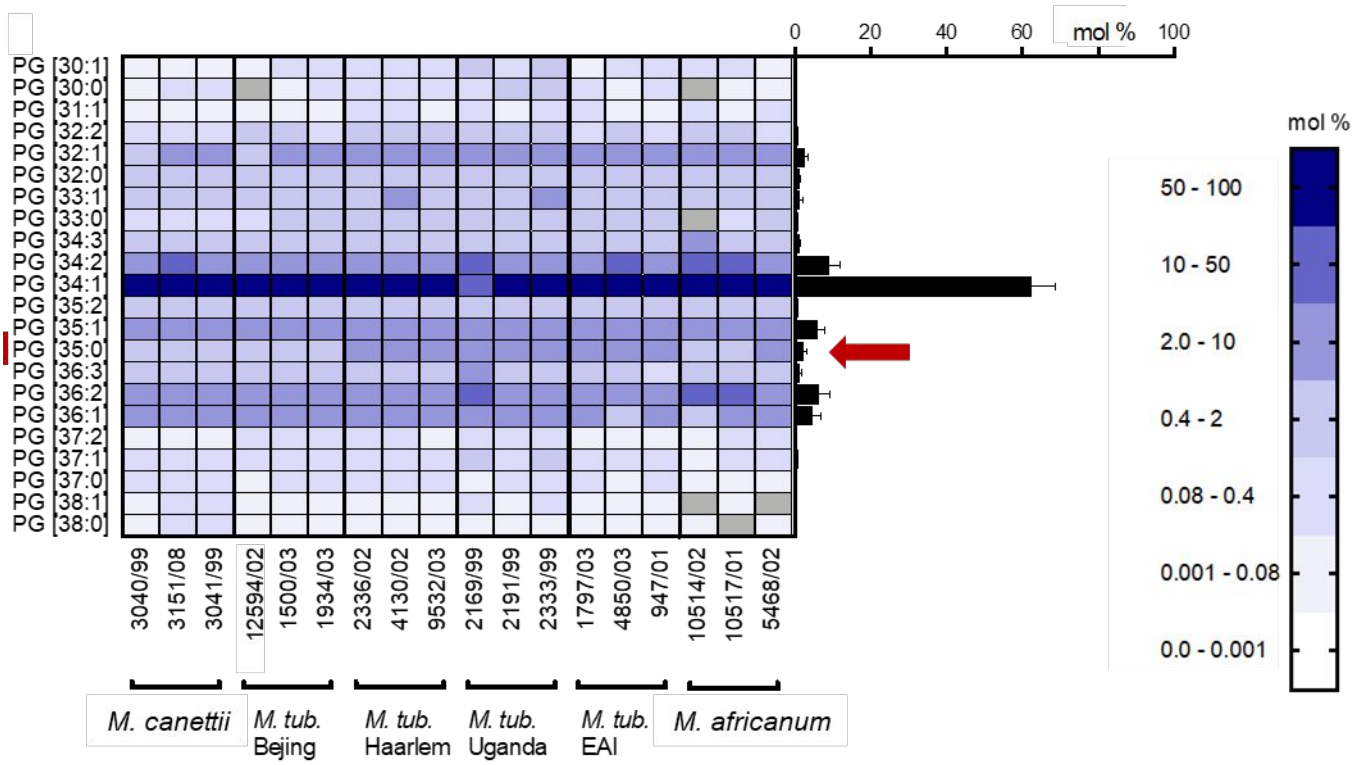

Figure S2) Lipid species distribution of major abundant mycobacterial phosphatidylglycerol (PG). Profiles of clinical isolates were determined from at least two independent isolations. Average profiles over all strains is represented in the right bar graph (Error bars represent one SD).

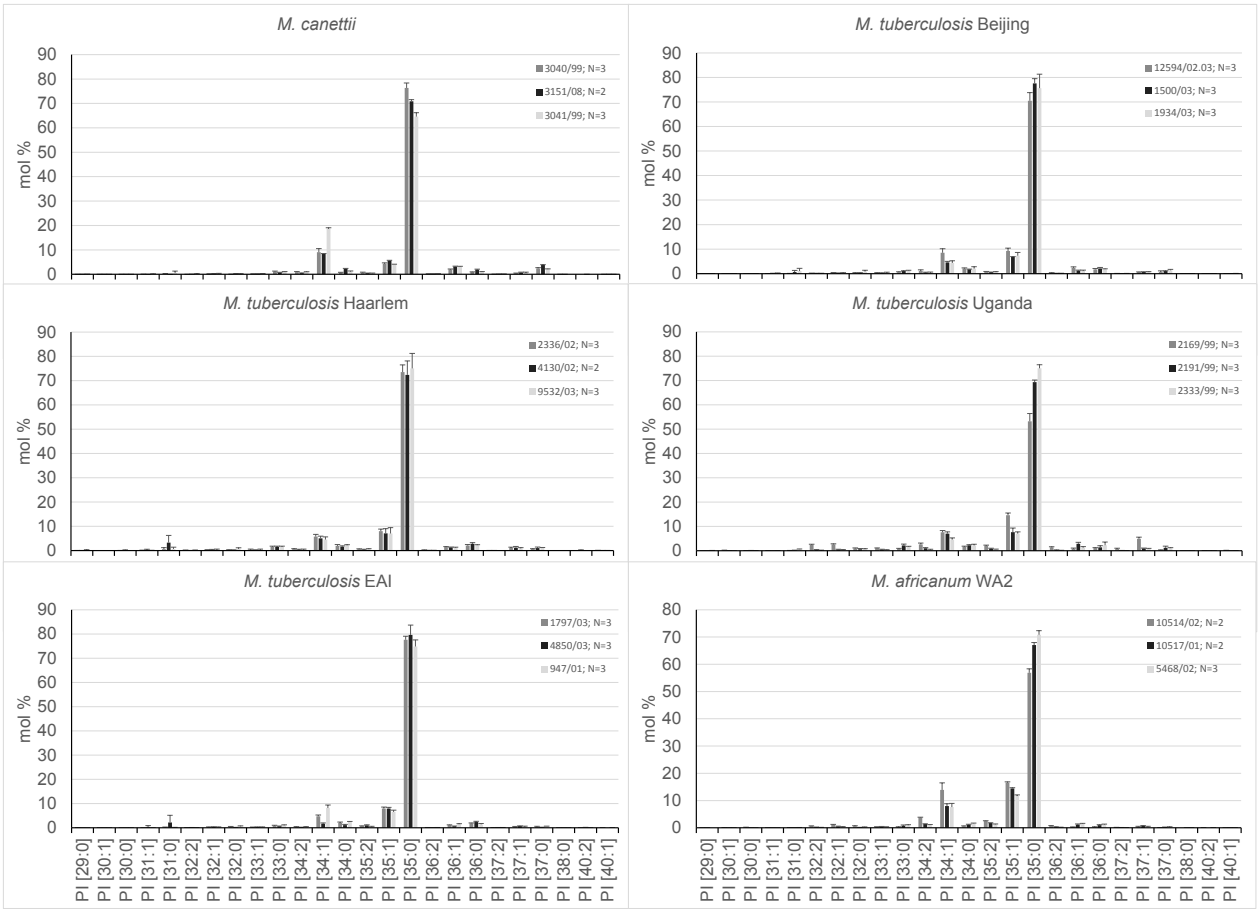

Figure S3) Distribution of PI species for 18 clinical MTBC isolates.

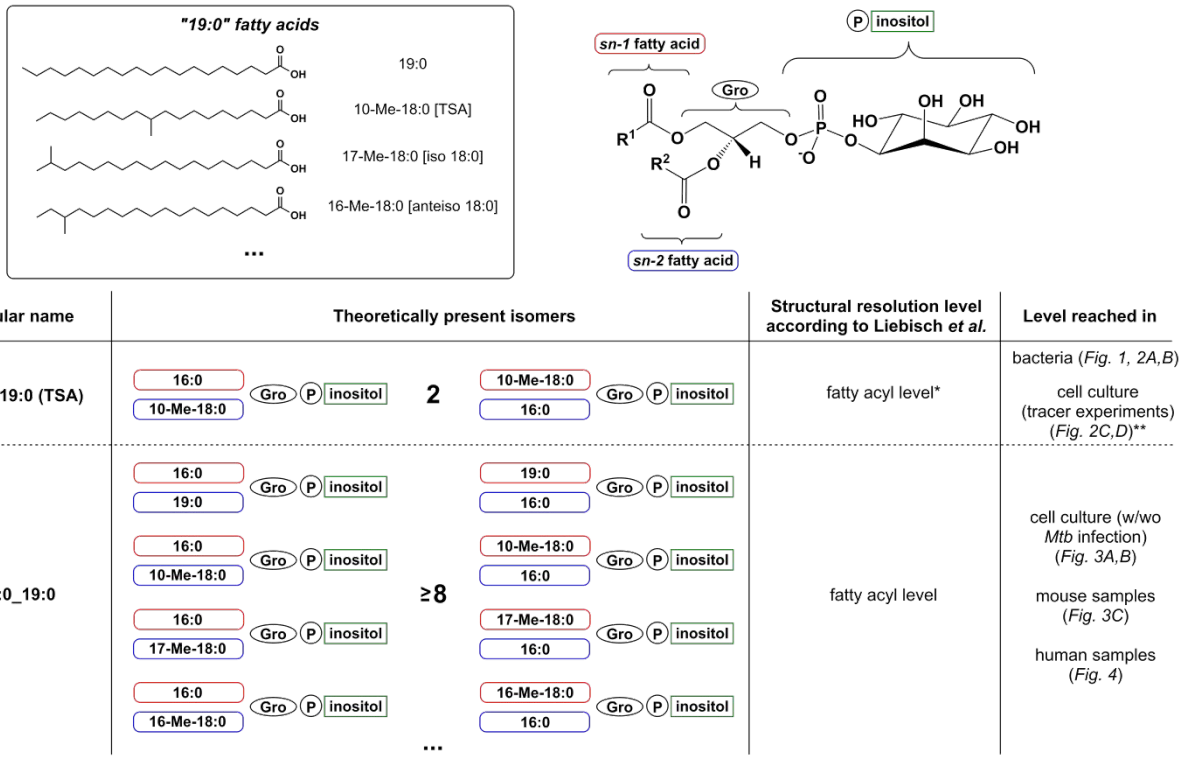

Figure S4) Structural complexity and analytical accuracy of the targeted MS/MS assay for PI 16:0_19:0 (TSA) in context of different biological matrices and model.

* PI 16:0_19:0 (TSA) one cannot exclude *sn*-position isomerism and determine isomeric purity. However, because of the utilization of $^{13}C_{18}$-labeled OA and the methylation performed by *Mtb*, we are certain of the detection of TSA.

** cell culture experiments with metabolic tracing during *Mtb* infection were demonstrated in Brandenburg et al..[8]

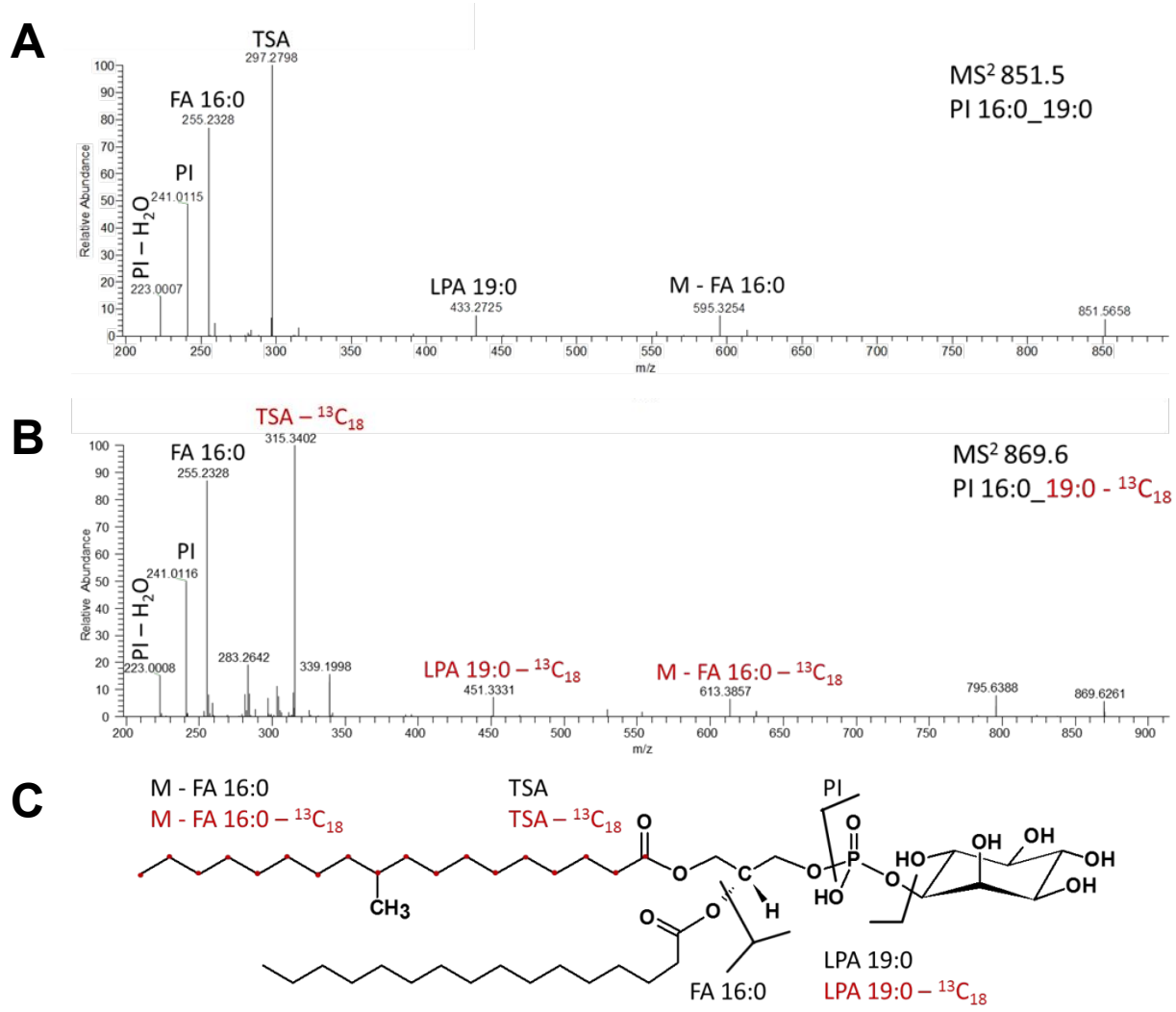

Figure S5) Chemical transformation of ^{13}C labelled oleic acid to tuberculostearic acid (TSA) by the metabolic system of *Mtb* and structural validation of PI 16:0_19:0 (TSA) by tandem mass spectrometric analysis (MS^2). A) MS^2 spectrum of PI 16:0_19:0 (TSA) without label at precursor *m/z* 851.5 and with incorporated label. **B)** MS^2 spectrum of *m/z* 869.6 for PI 16:0_19:0 ($^{13}C_{18}$-TSA). **C)** Assignment of fragment ions to chemical substructures. Fragment ions that show a shift of 18 Da due to incorporation of the $^{13}C_{18}$ aliphatic chain are coloured in red.

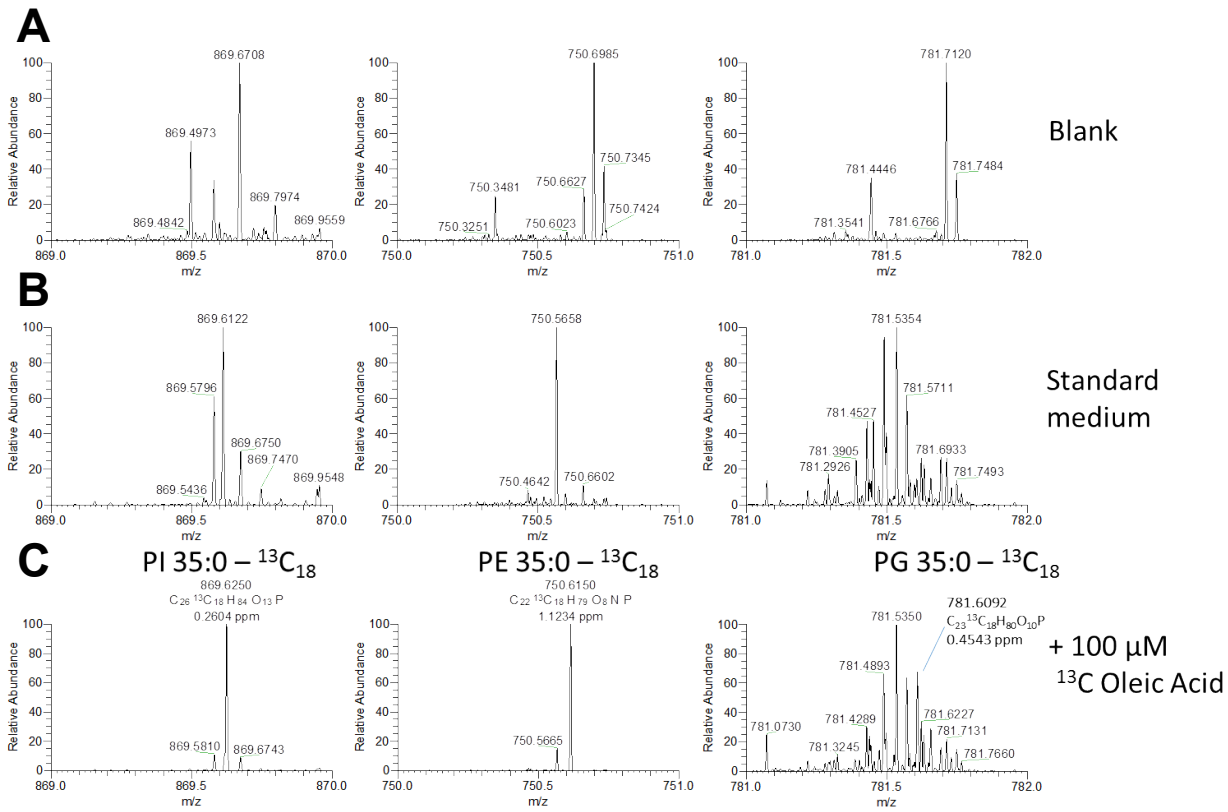

Figure S6) Mass spectrometric analysis of metabolic labelling of tuberculostearic acid (TSA).

Representative MS^1 *m/z* ranges for detection of labelled PI 35:0, PG 35:0 and PE 35:0 using single ion monitoring mode (SIM) for **A)** control extraction (Blank), **B)** *Mtb* grown under standard culture conditions and **C)** *Mtb* grown in media supplemented with 100 µM ^{13}C labelled Oleic Acid.

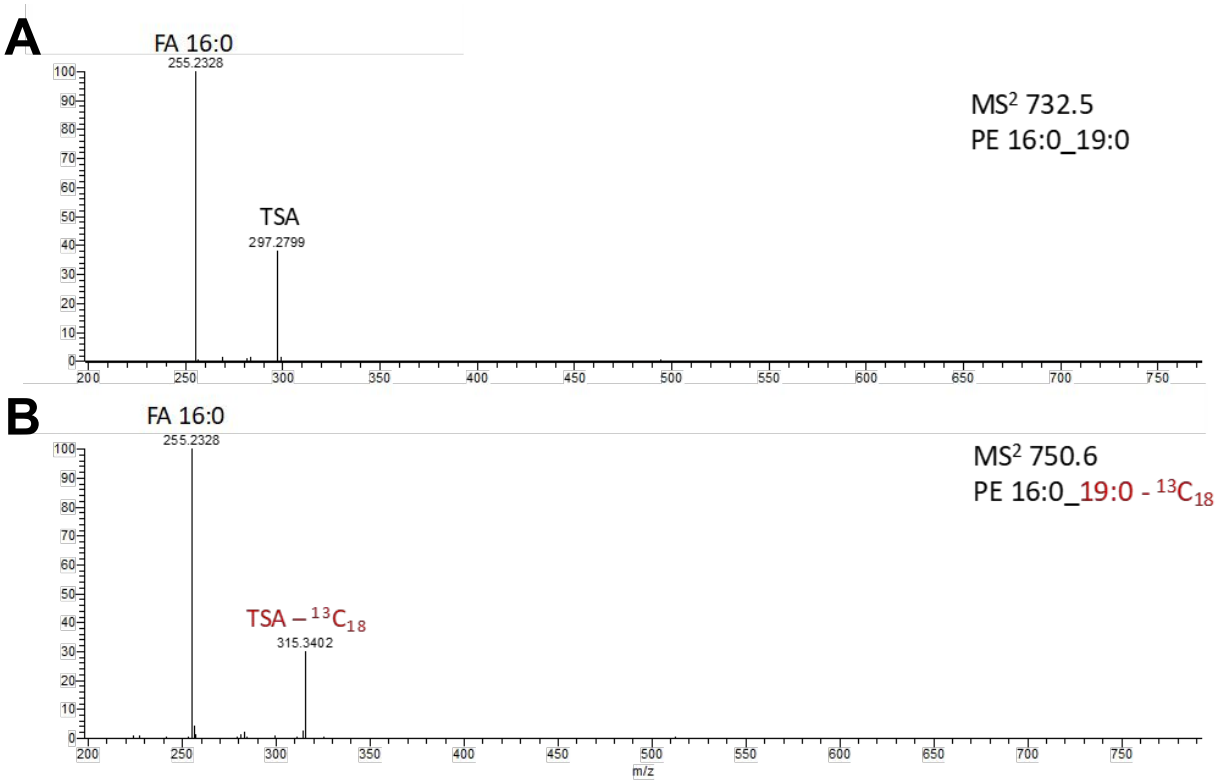

Figure S7) Incorporation of $^{13}C_{18}$ label of oleic acid into PE 16:0_19:0 (TSA). Tandem mass spectrometric analyses (MS^2) without ^{13}C labelling at the precursor *m/z* 732.5 **A)** and with labelled Oleic Acid at *m/z* 750.6 **B)** Only the TSA fragment ion showed a shift of 18 Da due to incorporation of the $^{13}C_{18}$ aliphatic chain.

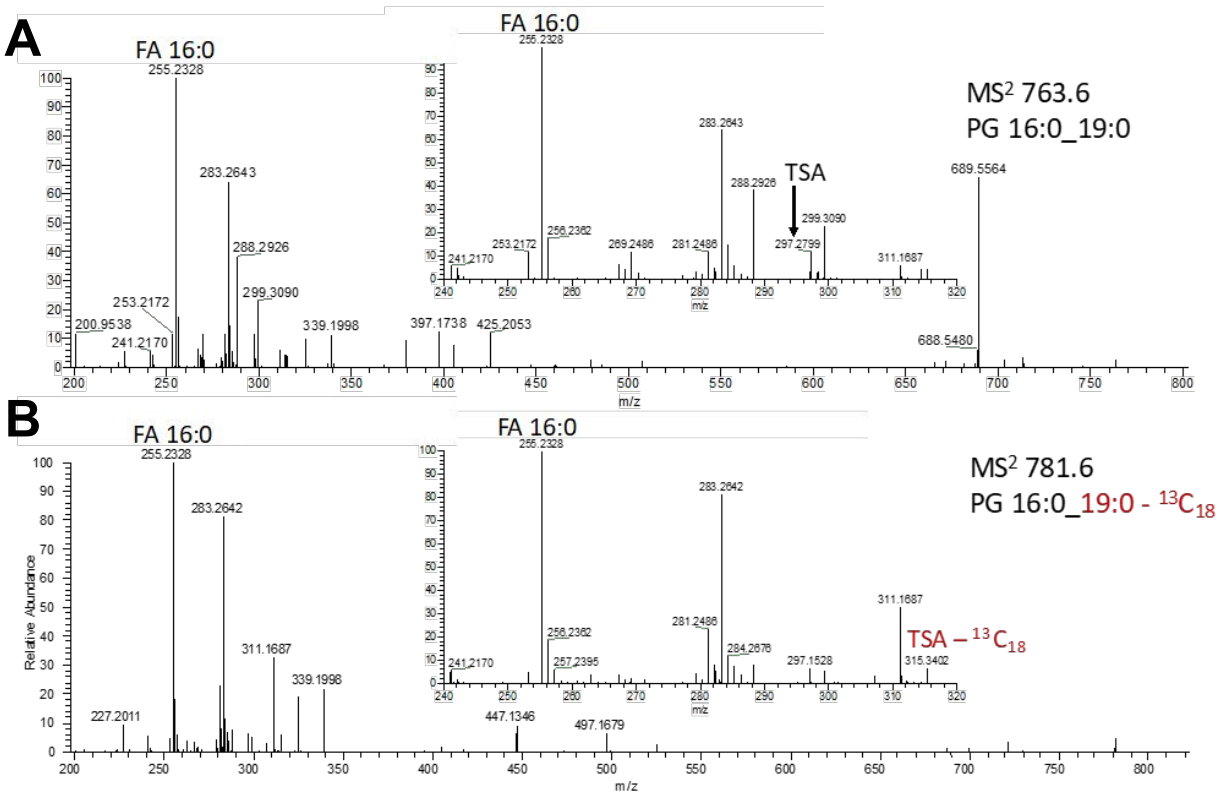

Figure S8) Incorporation of $^{13}C_{18}$ label of Oleic Acid into PG 16:0_19:0 (TSA) by *Mtb.* Tandem mass spectrometric analyses (MS^2) of PG 16:0_19:0 in lipid extracts of *Mtb* cultured in **A)** standard medium with the precursor *m/z* 763.6 and **B)** in media supplemented with $^{13}C_{18}$ labelled Oleic Acid at *m/z* 781.6. PG 16:0_19:0 is only a minor compound for all the tested MTBC isolates. Only for the TSA fragment incorporation of the label was confirmed.

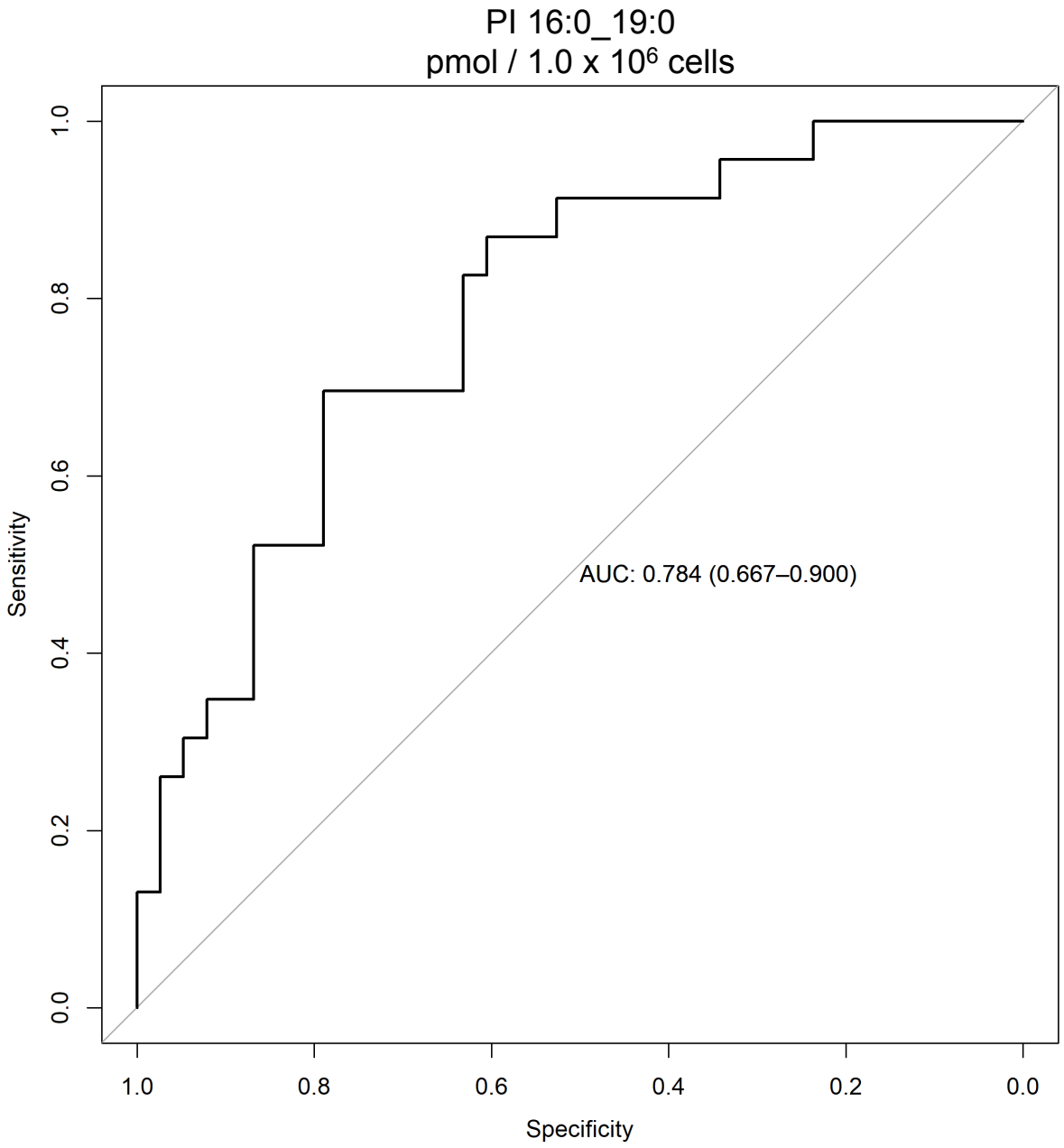

Figure S9) Receiver operating characteristic curve for PI 16:0_19:0 for the comparison of patients at study inclusion (t0 / tU) with healthy control. Details of the test are summarized in table S2.

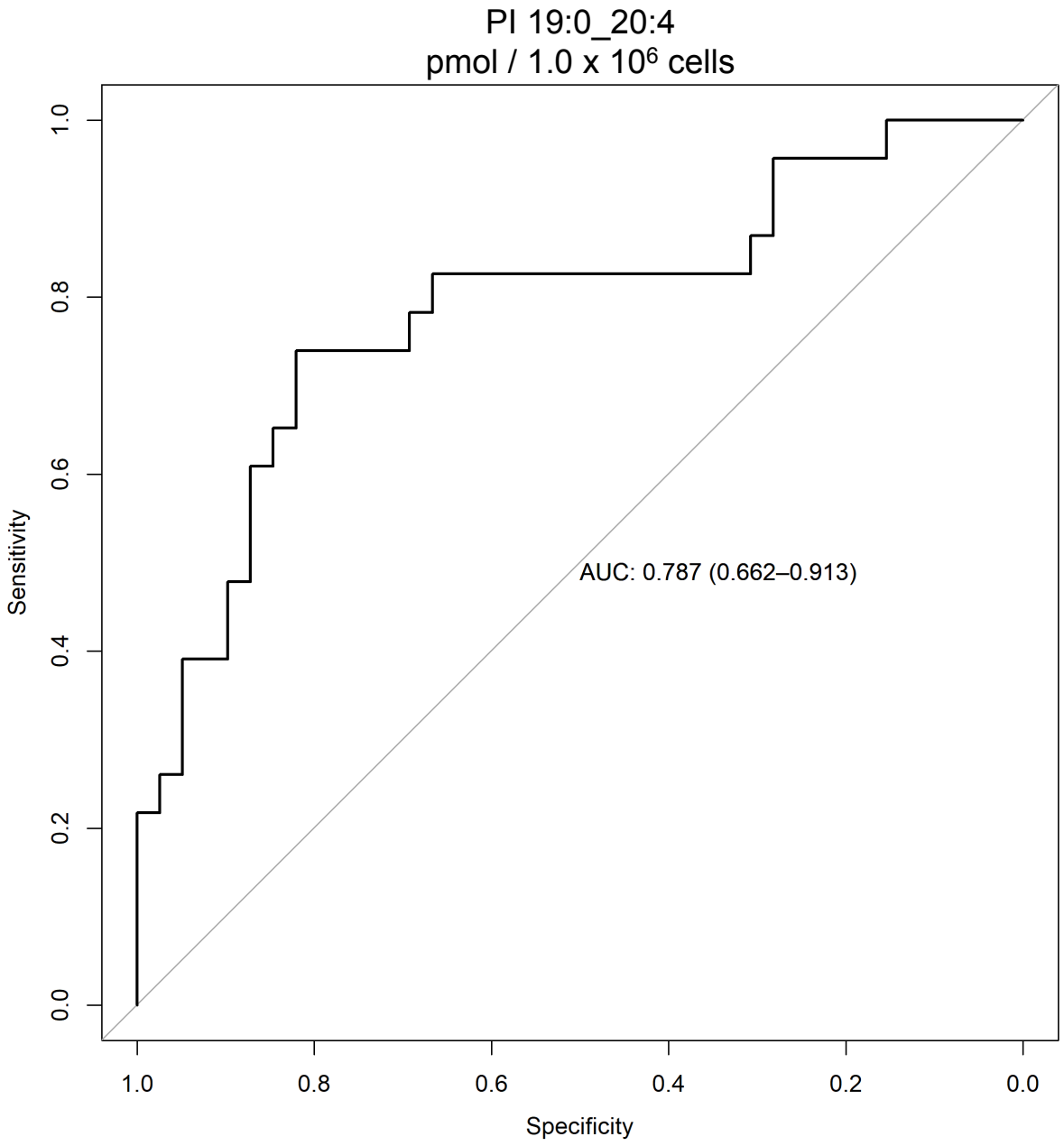

Figure S10) Receiver operating characteristic curve for PI 19:0_20:4 for the comparison of patients at study inclusion (t0 / tU) with healthy control. Details of the test are summarized in table S2.

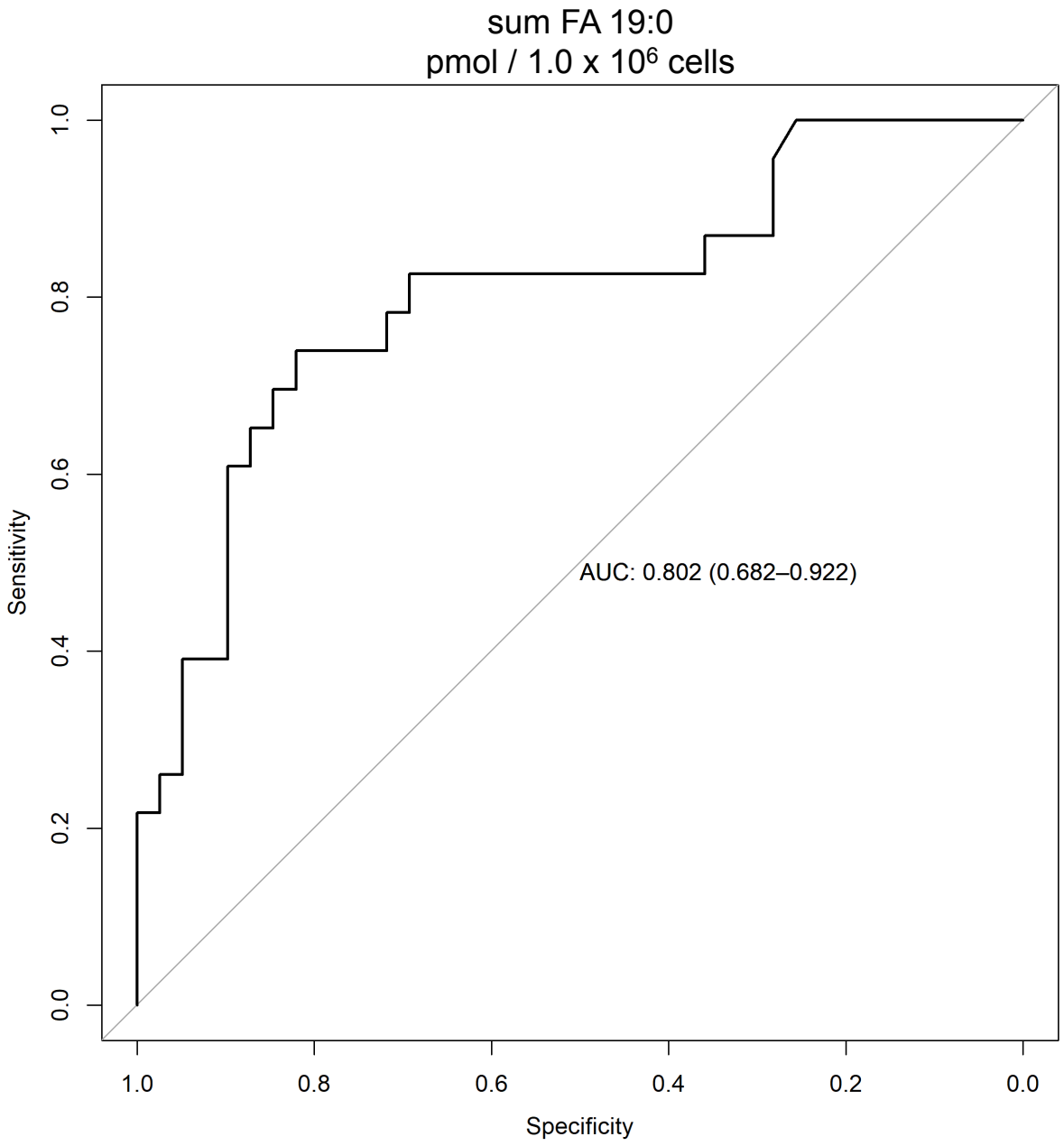

Figure S11) Receiver operating characteristic curve for the PI panel containing FA 19:0 (Table S3) for the comparison of patients at study inclusion (t0 / tU) with healthy control. Details of the test are summarized in table S2.

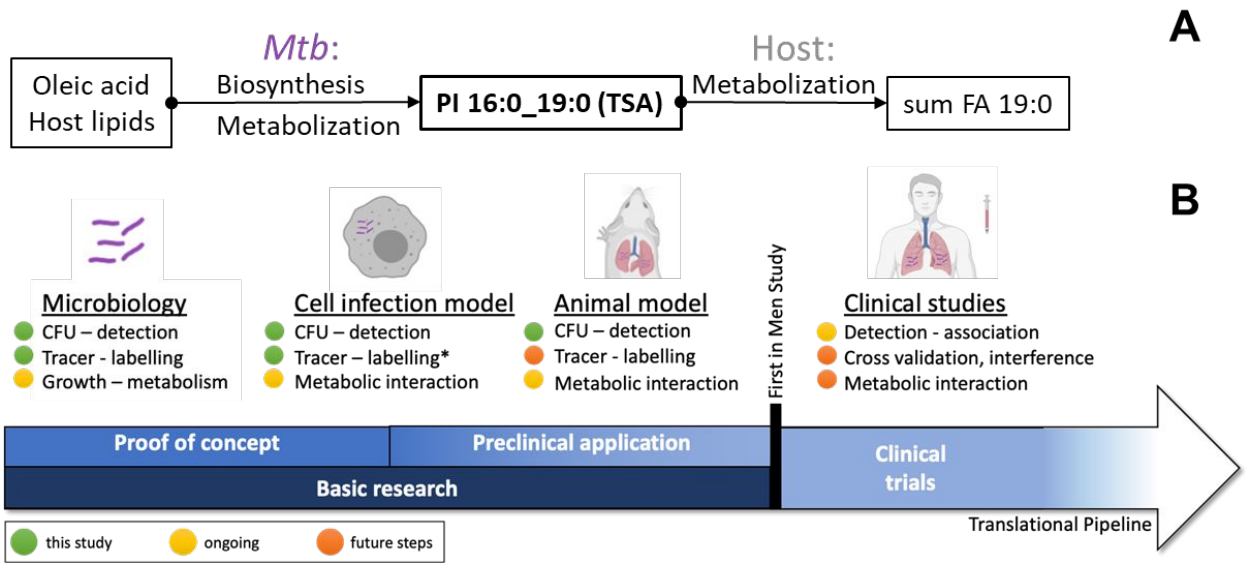

Figure S12) Implementation status for FA 19:0 (TSA)-containing mycobacterial PIs in translational TB research. A) TSA biosynthesis was observed from FA 18:1 (OA) as educt and host's complex lipids that contain OA by tracer analysis. The mycobacterial lipid PI 16:0_19:0 (TSA) itself was quantified in *in vitro* infection model system. Putative metabolization routes of TSA-containing PIs led to definition of the sum FA 19:0 marker panel. **B)** The FA 19:0 (TSA)-containing PIs of mycobacterial origin were tested in biological model systems along translational research pipeline. Studies on metabolization of PI 16:0_19:0 (TSA) in animal models are required to verify its application in the clinic. Further studies on interferences by other lung diseases and infectious diseases are required to validate TSA-containing PIs as marker for TB (* demonstrated in Brandenburg et al.[8]).

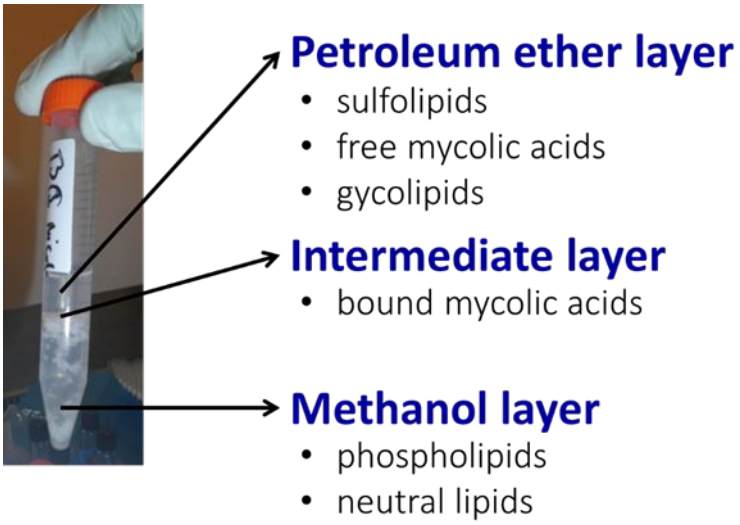

Figure S13) Phase separation and enriched lipid classes in the petroleum ether / methanol extraction system for MTBC strains.

Table S1: ROC analysis of mycobacterial derived lipid markers for TB patients at baseline and healthy control.

Lipid marker / panel	AUC	Accuracy	Optimal Cut-off	Sensitivity	Specificity	False positive rate	False negative rate
PI 19:0_16:0 (pmol / 1.0 E06 cells)	0.784 (0.667 – 0.900)	0.754	0.228	0.696	0.790	0.21	0.30
PI 19:0_20:4 pmol / 1.0 E06 cells	0.787 (0.662 – 0.913)	0.79	8.01	0.739	0.821	0.18	0.26
sum FA 19:0 pmol / 1.0 E06 cells*	0.802 (0.682 – 0.922)	0.79	8.82	0.739	0.821	0.18	0.26

* Panel of FA 19:0 (TSA) containing PIs listed in table S2.

Table S2) TSA (FA 19:0) containing PIs identified in human PBMCs utilized as marker panel.

exp. *m/z* (MS^1)	**Elemental composition**	**Error in ppm for precursor ion (MS^1)**	**Error in ppm for FA identification (MS^2)**	**Lipid species**	**Comment**
828.5693	*H71 C42 D7 O13 P1*	*0.0*	*-0.3 and -1.8*	*PI 18:1-D7 _ 15:0*	Internal standard
837.5485	H82 C43 O13 P1	-1.6	-0.8 and -1.9	PI 19:0 _ 15:0	
849.5467	H82 C44 O13 P1	-3.7	-0.9 and -1.5	PI 19:0 _ 16:1	
851.562	**H84 C44 O13 P1**	**-4.1**	**-0.8 and -1.0**	**PI 19:0 _ 16:0**	**Major abundant phospholipid in MTBC**
863.5642	H84 C45 O13 P1	-1.5	-0.8 and -0.4	PI 19:0 _ 17:1	
875.5633	H84 C46 O13 P1	-2.5	-0.8 and -0.6	PI 19:0 _ 18:2	
877.5775	H86 C46 O13 P1	-4.1	-0.8 and -0.5	PI 19:0 _ 18:1	
897.551	H82 C48 O13 P1	1.3	-0.6 and -0.5	PI 19:0 _ 20:5	
899.5662	**H84 C48 O13 P1**	**0.8**	**-0.7 and -0.4**	**PI 19:0 _ 20:4**	**Major abundant lipid of panel**
923.5577	H84 C50 O13 P1	-8.4	-0.8 and -0.2	PI 19:0 _ 22:6	
925.5739	H86 C50 O13 P1	-7.9	-0.7 and -0.1	PI 19:0 _ 22:5	

Table S3) Components of the internal standard mix for lipid analysis of human PBMCs.

Internal standard components	pmol/sample (10 µL)	pmol/sample (20 µL)
15:0-18:1(d7) PC	83.65	167.3
15:0-18:1(d7) PE	3.14	6.28
15:0-18:1(d7) PS (Na-Salt)	2.12	4.24
15:0-18:1(d7) PG(Na-Salt)	14.94	29.88
15:0-18:1(d7) PI(NH_4-Salt)	4.19	8.38
15:0-18:1(d7) PA(Na-Salt)	4.19	8.38
18:1(d7) LPC	18.89	37.78
18:1(d7) LPE	4.27	8.54
18:1(d7) Chol Ester	212.11	424.22
18:1(d7) MG	2.16	4.32
15:0-18:1(d7) DG	6.27	12.54
15:0-18:1(d7)-18:1 TG	27.64	55.28
18:1(d9) SM	16.43	32.86
Cholesterol(d7)	97.96	195.92
Ceramide C17	37.68	75.36

Internal standard mix is composed of C17 ceramide and SPLASH mixture purchase from Avanti Polar Lipids (Alabaster, US). For samples with cell counts of $1x10^6$ and $2x10^6$ 10 µL of the internal standard were added prior extraction. For samples containing the maximal cell counts of $5x10^6$ a volume of 20 µL of the standard mix were utilized.

Table S4: Gradient and flow rates used for the separation of the lipids by LC-MS.

Time (min)	% A	% B	Flow (µL / min)
0	0	100	15
1.5	0	100	15
8.2	70	30	15
11.66	70	30	15
12	80	20	15
13.3	80	20	15
13.66	0	100	15

Solvents: **A**, chloroform, methanol and ammonia solution 28-30% (86/13/1, v/v/v); **B**, chloroform, methanol and ammonia solution 28-30% (50/49/1, v/v/v)

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