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Research Paper

Host Protein Biomarkers Identify Active Tuberculosis in HIV Uninfected and Co-infected Individuals

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ABSTRACT

Biomarkers for active tuberculosis (TB) are urgently needed to improve rapid TB diagnosis. The objective of this study was to identify serum protein expression changes associated with TB but not latent *Mycobacterium tuberculosis* infection (LTBI), uninfected states, or respiratory diseases other than TB (ORD). Serum samples from 209 HIV uninfected (HIV^-) and co-infected (HIV^+) individuals were studied. In the discovery phase samples were analyzed via liquid chromatography and mass spectrometry, and in the verification phase biologically independent samples were analyzed via a multiplex multiple reaction monitoring mass spectrometry (MRM-MS) assay. Compared to LTBI and ORD, host proteins were significantly differentially expressed in TB, and involved in the immune response, tissue repair, and lipid metabolism. Biomarker panels whose composition differed according to HIV status, and consisted of 8 host proteins in HIV^- individuals (CD14, SEPP1, SELL, TNXB, LUM, PEPD, QSOX1, COMP, APOC1), or 10 host proteins in HIV^+ individuals (CD14, SEPP1, PGLYRP2, PFN1, VASN, CPN2, TAGLN2, IGFBP6), respectively, distinguished TB from ORD with excellent accuracy (AUC = 0.96 for HIV^- TB, 0.95 for HIV^+ TB). These results warrant validation in larger studies but provide promise that host protein biomarkers could be the basis for a rapid, blood-based test for TB.

1. Introduction

Accurate biomarkers for active tuberculosis (TB) are urgently needed for the development of additional rapid diagnostic tests (Rylance et al., 2010). Approximately a third of the world's population is infected with *Mycobacterium tuberculosis* (Mtb) (World Health Organization, 2014). While about 90% of infected individuals remain asymptomatic, in a state of latent Mtb infection (LTBI), approximately 10% develop TB over their lifetime. This risk is about 20-40 times higher in HIV-infected (HIV^+) and otherwise immunocompromised individuals (World Health Organization, 2011). In 2013, almost 9 million new TB cases were diagnosed worldwide with almost one million TB-associated deaths among HIV uninfected (HIV^-) and ~0.43 million among HIV^+ individuals (World Health Organization, 2014). Cornerstones of TB control are prevention and reduction of transmission, both of which require timely TB diagnosis and treatment initiation (World Health Organization, 2011). This need is especially high for relatively early disease stages as these are the most challenging to diagnose. The current tests depend on the detection of Mtb, and require specimens from the site of disease, typically sputum, which is not always readily obtainable and not helpful for the diagnosis of extrapulmonary TB. Sputum microscopy and culture are further hampered, respectively, by a lack of sensitivity or incubation times over weeks (Pai et al., 2009). More recently, novel nucleic-acid based amplification methods have provided an alternative rapid method for the detection of Mtb (Boehme et al., 2010, 2011), albeit at the need of a sample containing a certain quantity of Mtb.

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Exploring the human host response to TB provides opportunity for the discovery of biomarkers, and the development of additional diagnostics that do not require samples from the site of disease and that are not based on the detection of Mtb. Mass spectrometry (MS) allows rapid quantitative assessment of various analytes in clinical laboratories, and portable MS technology is becoming more available (Wiley et al., 2013; Hendricks et al., 2014; Creamer et al., 2014). Furthermore, the technological base exists to develop and deploy multi-analyte tests using multiple reaction monitoring MS (MRM-MS) (Hunter and Paramithiotis, 2010), and immunoassays for the detection and quantification of specific proteins can be easily developed for settings where fewer resources are available. Tests based on host protein panels have already demonstrated diagnostic capability and prediction of disease severity in conditions such as dengue fever and ovarian, endometrial and lung cancers (Farias-Eisner et al., 2010; Li et al., 2013; Nosov et al., 2009; Oikonomopoulou et al., 2008; Villar-Centeno et al., 2008). The overall objective of this study was the identification of protein expression patterns that are associated with TB but not with Mtb uninfected states, LTBI, or respiratory diseases other than TB (ORD). Such biomarkers might ultimately also be able to serve as surrogates for antituberculous treatment response. In this study we focussed on subjects living in the US as these are often diagnosed at earlier disease stages than individuals living in more resource-limited settings (Achkar et al., 2010), such as many regions in Sub-Saharan Africa and Asia, where mycobacterial cultures are not always available and TB is typically not diagnosed until it is sputum smear-positive and highly transmissible (Reid and Shah, 2009; Steingart et al., 2007). Due to the known impact of HIV co-infection on the pathogenesis and clinical presentation of TB (reviewed in (Achkar and Jenny-Avital, 2011)), we hypothesized that the host response in TB will differ according to HIV status. In this work we show that TB leads to changes in blood protein expression, and demonstrate that modestly sized panels of protein biomarkers that differed according to HIV status were able to accurately distinguish TB from LTBI, and ORD.

2. Methods

2.1. Study Design and Subjects

Subjects were categorized by HIV status and compared in a case control design with biologically independent sample sets in the discovery and the verification phase (Fig. 1). Sample size was determined by availability of serum samples. Subjects were enrolled at 4 public hospitals in New York City from 2007–2011. They included symptomatic patients suspected of having TB as well as asymptomatic controls (Table 1). Symptomatic subjects were consecutively enrolled and represented the spectrum of respiratory diseases that would undergo TB diagnostic testing in the clinical setting. *Inclusion criteria*: 1) symptomatic subjects: age ≥21 yrs old, and clinical suspicion with diagnostic work-up for TB; 2) asymptomatic controls: age ≥21 yrs old, with tuberculin skin-test (TST) and/or interferon gamma release assay (IGRA) for routine occupational screening with normal chest X-ray in TST+ and/or IGRA+ subjects. *Exclusion criteria*: unknown HIV status at the time of data analysis, or technical sample issues (Fig. 1); antituberculous treatment for more than 7 days, or history of TB or TB treatment within 1 year prior to enrollment. Of note, none of the TB patients in the discovery phase had received any prior treatment, and TB patients in the verification phase had received a median of 2 days (range 0–7 days) of

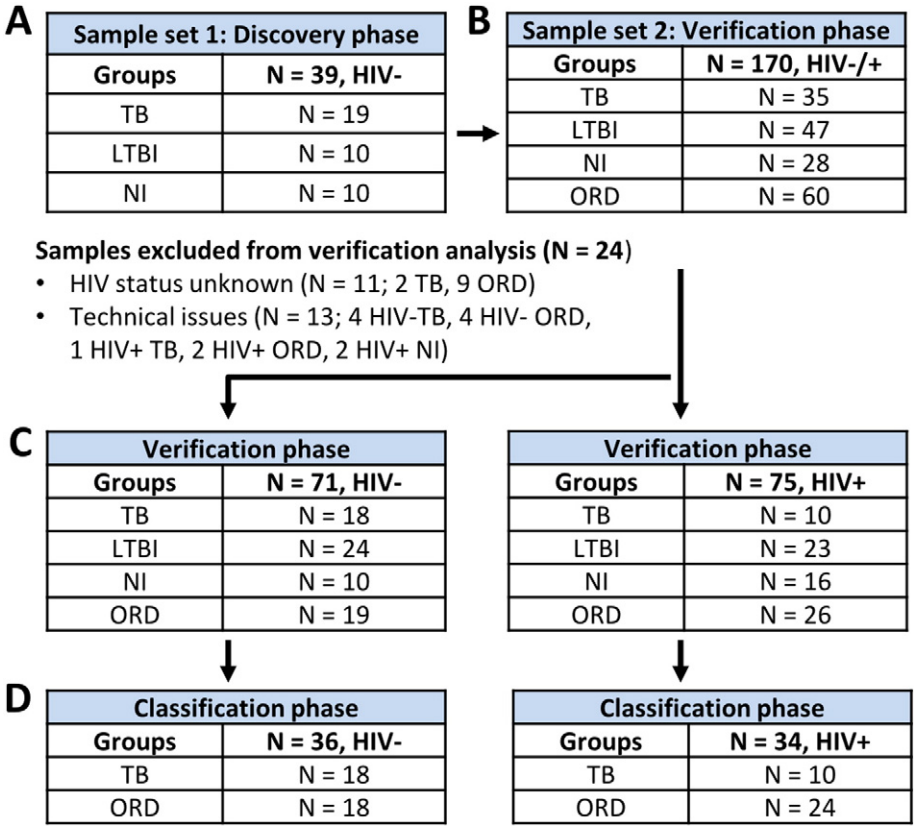

Fig. 1. Study design outline. An initial set of HIV-samples was used to identify proteins differentially expressed in TB compared to controls (*M. tuberculosis* uninfected and latent *M. tuberculosis* infection). (A). The expression of a selected subset of 87 proteins was confirmed using an independent set of HIV^- and HIV^+ samples (B). Samples were excluded from the verification analysis if their HIV status remained unidentified (N = 11) or if there were technical issues (N = 13). The remaining samples were used to verify the protein expression in sample groups stratified by HIV status (C) as well as the ability of the biomarker candidates and their combinations to discriminate between TB and other respiratory diseases (D). The classification of TB versus other respiratory diseases was established via nested cross-validation using the classification phase sample data split at random into 5 test and training sets. TB = active tuberculosis, LTBI = latent *M. tuberculosis* infection, NI = non-infected, ORD = other respiratory diseases.

Table 1
Characteristics of subjects included in discovery and verification phase analysis.

	Discovery phase		Verification phase			
	TB	Controls	TB	ORD[a]	Controls	p
	($n = 19$)	($n = 20$)	($n = 28$)	($n = 45$)	($n = 73$)	(TB vs ORD)
Age, mean (±SD)	42 (±15)	42 (±10)	40 (±13)	46 (±13)	44 (±12)	.001[f]
Male (%)	13 (68)	7 (35)	21 (75)	35 (78)	38 (52)	.78[g]
Race (%)						
Asian	7 (41)	5 (25)	4 (14)	4 (10)	11 (15)	.28[g]
Black	3 (18)	0	11 (39)	13 (31)	25 (35)	
Caucasian	1 (6)	12 (60)	1 (4)	8 (19)	13 (18)	
Hispanic	6 (35)	3 (15)	12 (43)	17 (40)	23 (32)	
Foreign-born[b] (%)	14 (74)	11 (55)	20 (71)	16 (36)	41 (56)	.004[g]
BCG vaccinated (%)	14 (74)	9 (45)	20 (71)	15 (33)	39 (53)	.004[g]
TST+[c] (%)	NA	10 (50)	NA	NA	47 (64)	
HIV$^+$ (%)	0	0	10 (36)	26 (58)	39 (53)	.07[g]
HAART (%)	NA	NA	2 (20)	15 (58)	33 (85)	.15[g]
CD4 if HIV$^+$, median (range)	NA	NA	145 (20–483)	129 (4–1070)	511 (11–1260)	.84[h]
Symptom duration[d], median weeks (range)	4 (0–52)	NA	4 (0–52)	2 (0–24)	NA	<.001[h]
AFB+[e] (%)	8 (42)	NA	10 (37)	5 (12)	NA	
M.tb culture+ (%)[i]	14 (74)	NA	23 (85)	0	NA	
TB location (%)						
Pulmonary	16 (84)	NA	17 (61)	NA	NA	
Pulmonary & extrapulmonary	19 (16)	NA	22 (18)	NA	NA	
Extrapulmonary	0	NA	6 (21)	NA	NA	

[a] ORD: other respiratory diseases than TB including community acquired pneumonia (n = 24), bronchitis (n = 8), non-tuberculous lung diseases (n = 5; 4 *M. avium* complex, 1 *M. kansasii*), and other diseases such as *Pneumocystis jiroveci* pneumonia, lung abscess and lung cancer (n = 8).
[b] Countries of origin for foreign-born individuals included African, Asian, South and Middle American countries, as well as the Caribbean Islands without Puerto Rico. Of note proportions for foreign-born and race overlap.
[c] TST: tuberculin skin-test.
[d] Self-reported TB-associated symptoms such as cough, fever, night sweats and weight loss.
[e] AFB: sputum smears for acid-fast bacilli, considered positive if at least one of initial three smears was positive.
[f] *t* test.
[g] Chi-square test.
[h] Mann–Whitney *U* test.
[i] Culture-negative TB was defined as the initial 3 sputum smears negative for acid fast bacilli and *M. tuberculosis* in cultures. Diagnosis was confirmed by a positive culture or nucleic acid amplification assay for *M. tuberculosis* in another specimen than the initial 3 sputa (e.g. bronchoalveolar lavage or lymph node biopsy in 3/19 (16%) in the discovery and 3/28 (11%) in the verification phase, by histology in 1/19 (5%) in the discovery phase, and by clinical and radiologic response in 1/19 (5%) subjects in the discovery and 2/28 (7%) in the verification phase).

antituberculous treatment. *Reference standards*: 1) TB cases were confirmed by either a positive culture for Mtb on one of the initial 3 sputum samples, or in culture-negative cases (defined as the initial 3 sputum samples negative for AFB on microscopy and negative for Mtb by mycobacterial culture) by positive culture and/or nucleic acid amplification assay and/or histology in another sample other than the initial 3 sputa, and/or by clinical response on antituberculous therapy (footnote Table 1). Cases diagnosed as culture-negative TB typically had TB risk factors, extensive diagnostic work-up to exclude other bacterial, fungal or parasitic diseases, and were unresponsive to empiric antibacterial treatment. The diagnosis of culture-negative TB was based on the criteria of the American Thoracic Society and the Centers for Disease Control and Prevention (American Thoracic Society, 2000; Blumberg et al., 2003). This included chest-CT findings such as milliary or nodular lung abnormalities consistent with TB. All of our culture-negative TB cases were reported to the New York City Department of Health (DOH), and the distribution of our TB cases was representative of that reported by the DOH (New York City Department of Health and Mental Hygiene, 2013). 2) Symptomatic controls consisted of patients with signs and symptoms of TB who were ultimately diagnosed with an ORD based on diagnostic work-up and as per physicians discharge note (footnote Table 1). 3) Asymptomatic controls without abnormalities on chest X-ray were categorized by TST results (Table 1). Due to the CDC recommendation for LTBI screening with either TST or IGRA (Mazurek et al., 2010), and the high costs of IGRAs, all of our controls had TST results but only some had IGRA results available. Thus, TST+ subjects were classified as possible LTBI. Of note, 9/10 TST+ subjects in our discovery phase also had a positive IGRA results available while most subjects in our verification phase had no IGRA test. *Ethics statement*: Approval for human subjects' research was obtained from the Internal Review Boards at the New York University School of Medicine, NY, NY, and the Albert Einstein College of Medicine, Bronx, NY. All subjects provided written informed consent prior to enrolment.

2.2. Sample Processing

Subjects were bled at the time of enrolment, and serum aliquots were stored at −80 °C until testing. Sample processing, liquid chromatography MS (LC–MS) and multiplex MRM-MS were performed by individuals blinded to the classification and diagnosis of subjects. Samples were grouped into blocks containing each of the disease groups. The order of the samples within each block was randomized. Samples were depleted of abundant proteins using an HSA/IgG column (Agilent Technologie, Mississauga, ON) in tandem with an IgY14 and Supermix (Sigma, Oakville, Ont) column and the flow through digested with trypsin (Promega, Madison, WI) at a trypsin to protein ratio of 1:10. The digested samples were freeze-dried, resolubilized, and treated with TCEP (tris(2-carboxyethyl)phosphine) to reduce disulfide bonds. Samples were desalted by solid phase extraction using a 3 M Empore C18 desalting plate and distributed into 96-well plates and vacuum evaporated. Peptides were stored at −20 °C until use.

2.3. Tandem Mass Spectrometry Analysis

Samples were resuspended in 92.5/7.5 water/ACN + 0.2% formic acid and analyzed by LC–MS (nanoAcquity pump HPLC and Q-TOF mass spectrometer, Waters, Mississauga, Ont). Peptide separation was achieved using a Waters nanoAcquity Symmetry UPLC Trap column (180 μm × 20 mm, 5 μm particle size) and a Waters nanoAcquity UPLC BEH300 analytical column (150 μm × 100 mm, 1.7 μm particle

size). The mobile phases were (A) 0.2% formic acid in water and (B) 0.2% formic acid in acetonitrile. For each sample approximately 2.5 μg was loaded onto the trap column for 4 min at a flow rate of 6 μL/min. Peptides were separated using a linear gradient (92.5% A to 75% A) at 0.6 μL/min for 57 min. MS spectra were acquired from 400–1600 Da.

Components were detected and matched across all samples using the Elucidator software version 3.3.0.1.SP4.25 (Rosetta Biosoftware, Seattle, WA) and compared for relative peak intensity. All intensity values were log (base e) transformed, and after transformation values <0 were replaced by 0. Intensity data was normalized to account for small differences in protein concentration between samples. A subset of the samples was used to create a reference sample against which all samples were then normalized. The normalization factors were chosen so that the median of log ratios between each sample and the reference sample over all the peptides was adjusted to zero. For batch-effect correction, a one-way ANOVA model $I_{ij} = M + D_i + \varepsilon_{ij}$ (I: intensity, M: overall interception, and D: batch–factor) was solved and parameters D_i ($i = 1,2$) under the constraint of $\Sigma_{i=1}^{2} (N_i{}^{*}D_i) = 0$ were obtained; the D_i's were then subtracted from the normalized intensities to form the 'batch-effect corrected' intensities. Intensities below a threshold (IT; IT = 30) were transformed to avoid spurious large fold changes: intensities in the range of (0, IT) were linearly mapped to the range of (IT/2, IT). ANOVA analysis was then applied to identify differentially expressed peptides. ANOVA model: $I_{ij} = M + C_i + \varepsilon_{ij}$ where I is peptide intensity, M is overall average intensity, C is 'clinical group' factor, and ε random error (Keeping, 1995; Montgomery, 2001). False detection rate and q-value were calculated based on the p-values obtained from the ANOVA using Storey's method to make multiple testing adjustments (implemented in MATLAB) (Storey, 2002). 'Post-hoc' contrast analyses were conducted using Tukey's hsd method to calculate p-values for each pair wise comparison (Hochberg and Tamhane, 2008). Protein identification was done by analysis of tandem mass spectrometry (LC–MS/MS). Differentially expressed peptides were targeted for sequencing, and the resulting fragmentation patterns were matched to the corresponding sequences found in a Human protein database downloaded on 20120531 from Uniprot, for which only reviewed entries were kept, using Mascot software version 2.2.06 (Matrix Science, Boston, MA). The following parameters were used for the searches: enzyme = trypsin, allowed missed cleavages = 2, peptide tolerance = 20 ppm, MS/MS tolerance = 0.4 Da, variable modifications = Deamination (N), Oxidation (M). A decoy reverse database was used to evaluate false positive error rate. Peptide/Protein Teller helped derive the simplest list of proteins to explain observed peptides, at a FDR calculated to be 6.3%. A protein level analysis was then applied by introducing a 'peptide factor' in the ANOVA used above: $I_{ijk} = M + C_i + P_j + \varepsilon_{ijk}$ where I is protein intensity, M overall constant, C 'clinical group', and P peptide factor. The number of the levels for P is protein-dependent, equal to the number of peptides for the protein. Proteins were considered to be differentially expressed if they met the following thresholds: p- and q-values <0.05 and differential intensity (DI) >1.1-fold change.

2.4. Multiple Reaction Monitoring Mass Spectrometry

Two-hundred-forty-four (244) peptides representing 87 host proteins and 2 Mtb proteins were selected for the MRM-MS assay and were synthesized (JPT Peptide Technologies, Berlin, Germany). Synthesized peptides were resolubilized in 25/75 water/DMSO (v/v), pooled and diluted with water + 0.2% formic acid to a concentration of 200 ng/mL. This peptide mix was used to develop the MRM-MS assay on a NanoAcquity pump (Waters) coupled to a QTRAP 5500 mass spectrometer (AB Sciex). Peptide separation was achieved using a BioBasic C18 column (Thermo) (320 μm × 150 mm, 5 μm particle size). Gradient time was 30 min and the flow rate was 10 μL/min. The optimal 2 transitions (combination of peptide precursor and fragment ion mass-to-charge ratio that are monitored by the mass spectrometer) per peptide were determined using selected reaction monitoring (SRM)-triggered MS/MS on a QTRAP 5500 instrument (AB Sciex). The MRM-MS/MS method was developed by calculating, for each peptide, the precursor mass of the doubly and triply charged peptide ions and the first and second y fragment ion with an m/z greater than [m/z (precursor) + 20 Da]. If these calculated transitions were observed during the MRM-MS scan, the instrument switched automatically to MS/MS mode and acquired a full MS/MS spectrum of the precursor peptide ion. The two most intense fragment ions (b or y fragment ions only) in the MS/MS spectrum and its elution time were determined for each acquired peptide. After, the collision energy (CE) was optimized for each of the chosen transitions. The developed MRM-MS assay was then applied for the analysis of the study samples. A detailed description of the final MRM-MS assay can also be found at http://www.peptideatlas.org/passel/.

Expression analysis of MRM-MS data acquired from the verification samples was performed using R version 2.14.0, platform x86_64-pc-mingw32/x64 (64-bit). The calculation of q-values was done using function "qvalue" from Storey's package "qvalue" version 1.24.0 (Storey, 2002). An intensity threshold (IT) below which the measure is deemed less reliable was determined empirically and set to 10,000 pre-normalization. A detection rate (DR) was defined as the proportion of samples within a group with a raw intensity value greater or equal to the IT. Transitions with DR below 50% for one of the two groups being compared were excluded from expression analysis. Differential intensity (DI) ratios were calculated in pair wise comparisons for each transition as the ratio of the median normalized intensities of each group. Prior to calculating the DI ratios, all intensity values that were below IT in the raw data prior to normalization were replaced by the half-IT value. Student's *t*-test was applied for the expression analysis. Protein-level statistics were also computed by linearly combining the transitions of a given protein into a single variable and then applying a *t*-test.

2.5. Network Analysis

Data were analyzed using Ingenuity Pathway Analysis (IPA; Ingenuity® Systems, Redwood City, CA). Differential expression results ($DI < 1.1$, $p < 0.05$, $q < 0.05$) were analyzed independently for HIV^{-} and HIV^{+} backgrounds. Proteins from the dataset that were associated with biological functions and/or diseases in the Ingenuity Knowledge Base were considered for the analysis. Right-tailed Fisher's exact test was used to calculate a p-value determining the probability that each biological function and/or disease assigned to that data set is due to chance alone.

2.6. Panel Selection and Receiver Operating Characteristics

The area under the curve (AUC) was the result of a nested cross-validation procedure that used stratified sampling to split the data at random into five test sets, each with a proportion of TB to ORD samples as close as possible to that of the full data set. For each test set, the remaining four fifths of the data were defined to be that test set's corresponding training set. To facilitate the selection of panels of interest, the following procedure was carried out for all possible panels with up to four proteins out of all the candidate biomarkers. Each training set was again split at random by stratified sampling into two halves. One half was used to fit a logistic regression model, which was then used to calculate out-of-sample predictive scores for the other half. This random half-and-half split procedure was repeated a number of times equal to three times the sample size of the training set; out-of-sample predictive scores and the corresponding true outcomes were aggregated over all random splits and AUCs were estimated from these. Since there are five training sets, five such AUC estimates were generated for each panel, which were then averaged. Panel selection was carried out by examining various summaries of protein performance, and also direct examination of the panels with the best AUC estimates. To compute the final AUC estimates of the selected panels, each test set was

scored by a logistic regression model fit to the corresponding training set; the resulting out-of-sample predictive scores and true outcomes aggregated over all five test sets, forming the final set from which AUCs were estimated.

2.7. Data Submission

Data submissions conformed to published guidelines (Martinez-Bartolome et al., 2013). LC–MS data are accessible at http://proteomecentral.proteomexchange.org, identifier PXD000517. MRM-MS data are available at http://www.peptideatlas.org/passel/, identifier PASS00336 for the MRM-MS assay qualification data on the discovery samples, and PASS00338, and PASS00339 part 1 and part 2 for the verification sample data. Expression data are accessible at http://enews.patricbrc.org/niaid-clinical-proteomics/, identifier ZFY for the discovery data, ZBK for the MRM-MS qualification data, and ZFX and ZGO for the verification phase data.

3. Results

3.1. Serum Protein Changes Associated With TB

In the discovery phase we compared HIV$^-$ TB cases (n = 19) to Mtb uninfected and LTBI controls (n = 20; Fig. 1 and Table 1). As anticipated, these two groups differed in most of their demographic and clinical variables. They were compared in order to capture the full spectrum of TB associated changes, and to generate a complete picture of the host biology affected by the disease. One hundred sixty five proteins were significantly differentially expressed in HIV$^-$ TB compared to Mtb uninfected and LTBI controls (Fig. 2). Since only two proteins, FTL and SHBG, showed a different pattern of expression between Mtb uninfected and LTBI controls, these groups were combined for greater statistical power. The differentially expressed proteins identified segregated into a small number of biological processes, such as the immune response, lipid transport and regulation, and tissue development and repair (Fig. 2). Approximately a third of the differentially expressed proteins represented different aspects of the immune response such as the movement of phagocytes, neutrophils, and granulocytes, inflammation, stress response, and the innate immune response. Most of the proteins associated with the immune response were up regulated in the TB subjects, consistent with a host response to an active infection. In contrast, most proteins associated with lipid processing were down regulated. Proteins involved in tissue development and repair were up or down regulated, and associated with wound healing, mechanical damage to lung tissue, and extracellular matrix protein biology.

After excluding proteins that were components of the acute phase response or had been repeatedly observed to be differentially expressed in other non-TB diseases (Ray et al., 2014), 87 host proteins were selected for inclusion into a multiplex MRM-MS assay for the verification phase. These included 18 proteins derived from the discovery phase of studies assessing protein biomarkers in more advanced Brazilian HIV$^-$ and South African HIV$^+$ TB patients. The additional proteins complemented the small US sample number of advanced HIV$^-$ and the lack of HIV$^+$ TB subjects in our discovery phase. The studies were not combined because they had different designs, and the Brazilian and South African studies were not completed at the time of data analysis. Of note, the majority of proteins selected for the verification phase overlapped between the various sites. Two Mtb culture filtrate proteins (Malate synthase and MPT51) were included because they were known to induce a humoral immune response in our HIV$^-$ and HIV$^+$ TB groups (Achkar et al., 2010; Yu et al., 2012) but no Mtb proteins were detected in any of the serum samples in the discovery phase.

3.2. Candidate Serum Protein Biomarkers for TB

An independent set of serum samples were used in the verification phase to validate the discovery data, and determine the clinically relevant biomarkers that could distinguish TB from ORD. After exclusion of samples due to remaining unknown HIV status or technical issues, these samples were comprised of HIV$^-$ and HIV$^+$ subjects with either TB (n = 28), no Mtb infection or LTBI (n = 73), or subjects suspected of having TB but ultimately diagnosed with ORD (n = 45; Fig. 1 and Table 1). Due to our hypothesis that the host protein expression in TB will differ according to HIV status, the HIV$^-$ and HIV$^+$ groups were analyzed separately. The differential expression of the host proteins measured by LC–MS in the first sample set was comparable to their corresponding expression when quantified by MRM-MS in the second sample set though the magnitude of the changes observed were greater in the MRM-MS tested sample set, consistent with the increased sensitivity of the MRM-MS assay (Supp. Table 1).

As anticipated, the comparison of TB to ORD resulted in different expression patterns than the comparison to LTBI (Fig. 3). The presence of an HIV co-infection reduced the overall magnitude of the host response, and induced other more subtle changes. The MRM-MS data was then split into training and test sets to derive biomarker panels able to distinguish TB from ORD (Fig. 1). In the HIV$^-$ and HIV$^+$ groups the best single markers had a classification performance (AUC) of up to 0.80 and 0.77, respectively. When used in combinations, a subset of 10 candidates for the HIV$^-$ and 8 for the HIV$^+$ groups, distinguished TB from ORD with excellent accuracy (AUC 0.96 and 0.95, respectively;

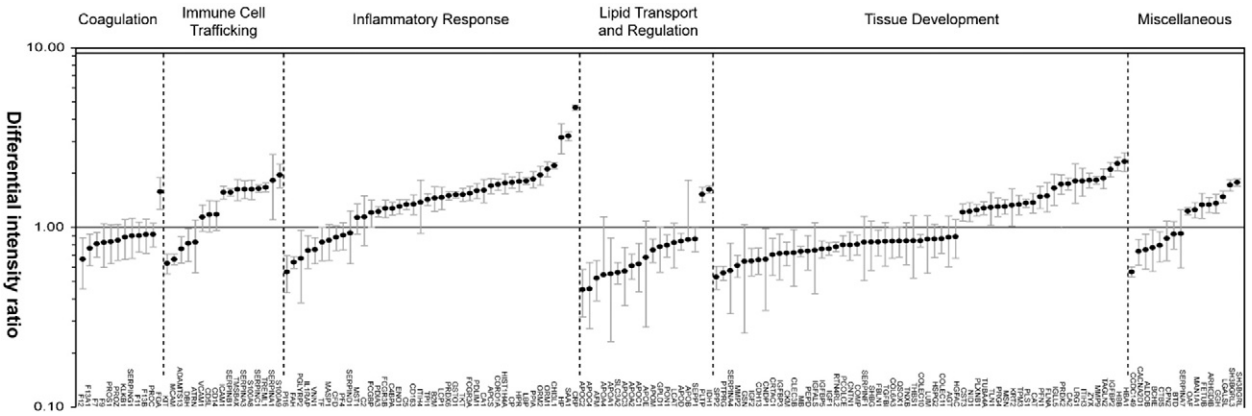

Fig. 2. Differentially expressed proteins in sera of HIV$^-$ subjects with active tuberculosis (TB) relative to controls (*M. tuberculosis* uninfected and latent *M. tuberculosis* infection). The differentially expressed proteins are indicated by dots and categorized by functional groups. Intensity values per protein were normalized and the ratios derived are displayed in logarithmic scale (y-axis). Mean and standard deviation of the expression ratio of TB versus controls are shown.

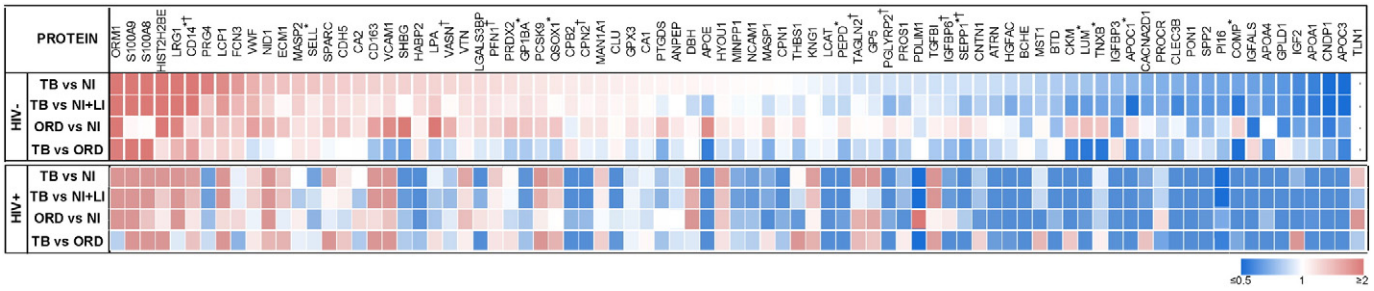

Fig. 3. Differential expression of candidate TB biomarkers in sera. Protein expression ratios expressed as fold change in subjects with active tuberculosis (TB) relative to uninfected controls (NI) and latent *M. tuberculosis* infection (LI), or relative to subjects with other respiratory diseases (ORD), in HIV$^+$ or HIV$^-$ infected groups. The numerators and denominators per comparison and the color scale are indicated. The ratio range was from 0.2 to 6.6. The HIV$^-$ and HIV$^+$ panel members are indicated by * and † respectively.

Table 2 and Fig. 4). The AUC reported are average out of sample values and larger studies are needed to establish accurate confidence intervals. The composition of the panels differed according to HIV status but two of the candidates (CD14 and SEPP1) were present in both groups. Of note, we did neither observe significant differences in the biomarker protein expression between smear-positive and smear-negative TB, culture-positive and culture-negative TB, nor pulmonary and extrapulmonary TB, although these subgroup comparisons were limited by very small sample size. Furthermore, our spectrum of ORD included 5 nontuberculous mycobacterial (NTM) diseases in HIV$^+$ patients (Table 1). Comparison of those 5 HIV$^+$ NTM to the 10 HIV$^+$ TB patients showed that 5/8 candidate proteins (SEPP1, PGLYRP2, VASN, CPN2, TAGLN2) were significantly differentially expressed between these two groups. Thus, there appears to be both a partial overlap as well as a TB specific signature of our protein biomarker candidates. Larger studies are needed to determine the accuracy of certain panel proteins to distinguish between TB and NTM disease.

4. Discussion

Our data demonstrate that the physiological changes associated with TB, including the effects of HIV co-infection, are reflected by the human host protein patterns in blood. Small panels of proteins could accurately distinguish TB from LTBI and ORD in HIV$^-$ and HIV$^+$ individuals. Particularly relevant for clinical practice was that our biomarker signature was detectable in both smear-positive and smear-negative TB, including culture-negative TB, indicating a high sensitivity for early as well as more advanced TB. Furthermore, the high specificity was estimated based on TB suspects ultimately diagnosed with an ORD, the clinically relevant comparator group. In addition, following the expression levels of host proteins during antituberculous therapy might allow rapid assessment of the treatment response, and offer advantages over the currently used culture method. Although these results require validation in larger studies, our data hold promise that the detection of host protein changes could provide the basis for adjunctive rapid TB diagnostics, either by immunoassay or by using portable MS devices that are becoming more available (Wiley et al., 2013; Hendricks et al., 2014; Creamer et al., 2014).

Table 2
Composition of the host protein biomarker panels distinguishing active tuberculosis from other respiratory diseases.

Functional category	HIV$^-$ TB panel	HIV$^+$ TB panel
Immune response	CD14, SEPP1, SELL	CD14, SEPP1, PGLYRP2
Tissue development & repair	TNXB, LUM, PEPD, QSOX1, COMP	PFN1, VASN[a]
Lipid metabolism	APOC1	–
Other	GP1BA	CPN2, TAGLN2, IGFBP6[a]

[a] Proteins introduced from discovery phase of Brazilian and South African samples (unpublished data).

The panel proteins were derived from the most prominently observed TB related host processes — the immune response, tissue repair, and lipid metabolism. As combinations of antituberculous and host-modulating therapies hold promise to improve bacterial clearance (Gonzalez-Juarrero, 2012; Maiga et al., 2012; Martins, 2011), the host protein changes described in this work could also offer insights into which host/pathogen combination therapies may be promising. Soluble CD14 (sCD14) and SEPP1 were present in the TB panels for HIV$^+$ and HIV$^-$ subjects. CD14 is a marker of pulmonary inflammation (Anas et al., 2010). Consistent with our findings, membrane and soluble CD14 become up regulated in TB (Druszczynska et al., 2013; Lawn et al., 2000; Hoheisel et al., 1995). Other infections or respiratory diseases also induce elevated sCD14, albeit usually at lower levels than TB (Hoheisel et al., 1995; Lawn et al., 2000), also consistent with our observations. SEPP1, which was down regulated in TB, is involved in selenium transport and homeostasis. SEPP1 deficient mice experience greater morbidity and mortality in Trypanosoma infection (Burk and Hill, 2009), apparently due to increased tissue injury associated with enhanced production of reactive oxygen species (Bosschaerts et al., 2008).

Among the other candidate proteins in the HIV$^-$ group, GP1BA is involved in pulmonary inflammation (McNicol and Israels, 2008), and both SELL and LUM are involved in leukocyte homing and infiltration (Wedepohl et al., 2012; Lee et al., 2009). TNXB is commonly expressed by myofibroblasts located in and underneath newly formed epithelia at the foci of recent lung lesions (Kaarteenaho-Wiik et al., 2000; Paakko et al., 2000) where it promotes the disassembly of focal adhesions and stimulates cell migration (Murphy-Ullrich et al., 1991; Petit and Thiery, 2000). Fibrogenesis and matrix turnover are enhanced in TB compared to other granuloma-generating diseases such as sarcoidosis (Kaarteenaho-Wiik et al., 2007), which underscores the physiological relevance of TNXB. The other three biomarker candidates involved in

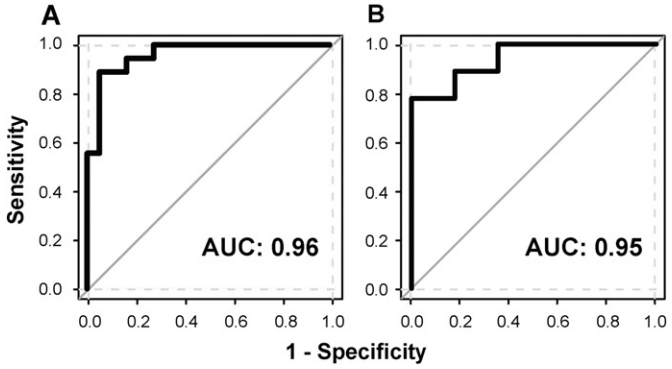

Fig. 4. Discrimination of active tuberculosis from other respiratory diseases. Area under the receiver operating curve (AUC) is shown for the biomarker panels in HIV$^-$ (A) and HIV$^+$ (B) subjects.

tissue repair, COMP, PEPD, and QSOX1, all have specific roles in morphogenesis and remodeling of the extracellular matrix (Haleem-Smith et al., 2012; Ilani et al., 2013; Kitchener and Grunden, 2012).

APOC1 has known roles in lipid transport and regulation (Jong et al., 1999). It has also been shown to enhance lipopolysaccharide (LPS)-induced inflammatory responses which depend on CD14/Toll-like receptor 4 signaling (Berbee et al., 2010). Mtb infection induces upregulation of host genes involved in lipid metabolism (Kim et al., 2010; Russell et al., 2010; Singh et al., 2012). Host lipid metabolism is also affected systemically by the acute phase response. For example, high density lipoprotein (HDL) levels become reduced and their composition is also altered (Khovidhunkit et al., 2004; Garcia-Gomez et al., 2014). Thus, our observed changes associated with lipid metabolism could reflect the sum of these metabolic adjustments. Also consistent with our observations, changes in lipid metabolism have been observed in sera and sputa of TB subjects by metabolomics (du Preez and Loots, 2013; Weiner et al., 2012).

In the HIV^+ group, the panel proteins reflected predominantly immune response and tissue repair functions. The down regulation of VASN is associated with arterial injury and contribution to neointimal formation (Ikeda et al., 2004). PFN1, which was also down regulated, contributes to wound healing by increasing cellular motility (Bae et al., 2010). CPN2 is a negative regulator of multiple inflammatory response proteins (Skidgel and Erdos, 2007). PGLYRP2, a protein associated with innate immune responses (Lee et al., 2012), was also reduced. The remaining two proteins in the HIV^+ panel, IGFBP6 and TAGLN2 have unknown roles in infectious diseases. Of note, ligands of IGFBP6 are reduced in sera and pleural fluids of subjects with tuberculous and non-tuberculous pleuritis (Le Bouc et al., 1997).

The different compositions of the protein biomarker panels for HIV^- and HIV^+ subjects is consistent with the effect of HIV co-infection on TB pathogenesis and disease presentation (reviewed in (Achkar and Jenny-Avital, 2011)). HIV^+ individuals commonly have a diminished inflammatory response to TB (Achkar and Jenny-Avital, 2011). Consistent with our data indicating less tissue damage and repair in comparison to the HIV^- TB group, TB in HIV^+ individuals is often associated with granuloma impairment resulting in a lack of pulmonary cavity formation (Achkar and Jenny-Avital, 2011). Although most of the panel proteins had known functions relevant to the host response to TB, their up or down regulation could be induced by conditions other than TB. The panel specificity thus resided in using combinations of biomarkers that represented multiple TB-associated host responses. This suggests that a holistic consideration of the host response would be required to design a TB-specific test if based on host proteins, which is consistent with the findings in other host biomarker studies of TB as well as other complex and chronic diseases (Lesho et al., 2011; Maertzdorf et al., 2011; Lu et al., 2011; Berry et al., 2010; Anderson et al., 2014; Kaforou et al., 2013; De Groote et al., 2013; du Preez and Loots, 2013; Nahid et al., 2014; Weiner et al., 2012; Li et al., 2013).

Due to the urgent need for additional tools to rapidly diagnose TB, drug resistance, and treatment response, TB biomarker research remains an active field that utilizes multiple approaches (reviewed in (Walzl et al., 2015). To identify potentially clinically relevant TB biomarkers and limit bias of diagnostic accuracy, we analyzed samples from consecutively enrolled TB suspects and compared TB cases to patients with respiratory diseases other than TB in our verification phase. Using a broadly comparable study design, Xu et al. compared TB cases to patients with either pneumonia or lung cancer, albeit with the limitations that i) these patients appeared to be selected rather than consecutively enrolled TB suspect, and ii) no information on HIV testing results was provided (Xu et al., 2015). Samples were analyzed by iTRAQ, a labeling technique that attaches mass tags to the sample peptides prior to MS analysis. The differences between iTRAQ and the label free quantitative MS we used have been described (Bantscheff et al., 2012; Patel et al., 2009), and center around the greater resolution and dynamic range of the label free method, as also indicated by the greater number of differentially expressed proteins identified in our work. In addition, Xu et al. pooled samples for discovery and did biomarker verification by ELISA. We used label-free MS for both discovery and verification, did not pool samples, and also employed statistical methods specifically designed to reduce overfitting. Despite these differences, 24 of 34 differentially expressed proteins reported by Xu et al. were also detected in the HIV-groups of our study, and 17 of these were also differentially expressed in the same direction although not always with the same magnitude. The differences in the final panel biomarkers we report are probably a result of the different analytical and statistical methods used. An earlier work from the same group compared sera from TB patients to healthy controls, and identified 4 candidate biomarkers that were not differentially expressed in their latter work, although 2 of them (APOCII, CD5L) were differentially expressed in our study (Xu et al., 2014). Another proteomics study of comparable design used label free MS to compare TB cases to a mixture of patients with non-respiratory medical problems (Song et al., 2014). Of the 21 proteins reported to be differentially expressed in that study, 18 (86%) were also detected in our work. Most of those proteins were associated with inflammatory responses consistent with a host response to an active infection. However, since TB patients were compared to patients with non-respiratory medical problems, the specificity of these proteins for TB in comparison to ORD could not be readily assessed.

Of the earlier studies, Zhang et al. compared TB to healthy controls and ORD, and identified ORM1 as a candidate biomarker (Zhang et al., 2012). Agranoff et al. compared TB subjects to ORD and inflammatory diseases, and concluded that serum amyloid (SAA1), C reactive protein (CRP), and transthyretin (TTR) were the most useful candidate protein biomarkers along with neopterin, a metabolite (Agranoff et al., 2006). Liu et al. (BMC Infect. Dis 2013; 13:506) compared TB cases to healthy controls and ORD, and identified a degradation product of fibrinogen A (FGA) as a candidate biomarker (Liu et al., 2013). ORM1, SAA1, CRP, and FGA were also found upregulated by TB in our study. However, since they were components of the non-specific acute phase response or coagulation pathway, we did not pursue them further as candidate biomarkers. TTR was removed by the abundant protein depletion column we used. The results of proteomic studies that did not provide candidate peptide or protein biomarker identifications could not be readily compared (Deng et al., 2011; Liu et al., 2010, 2015).

Targeted proteomics studies that used aptamer-based enrichment have been done to identify biomarkers associated with response to treatment (De Groote et al., 2013; Nahid et al., 2014). Although these studies a priori targeted a different set of proteins than our study, and had a different objective, many of the candidate biomarkers identified were associated with the main biologies described in our study — immune response and tissue repair, which supports that these are important elements of the host response to TB. Transcriptional profiling studies of blood cell RNA were also able to identify signatures specific to TB through analysis of changes related to immune cell activation, although comparatively large panels of 44–86 biomarker genes were necessary to distinguish TB from other diseases (Anderson et al., 2014; Kaforou et al., 2013; Berry et al., 2010).

Our study was limited to enrollment at sites in New York City. Nevertheless, our subjects revealed a broad spectrum in demographics, particularly race and country of origin. This is not surprising, as TB in the US, and particularly in New York City, is a disease predominantly diagnosed in foreign-born individuals who typically are infected during childhood with the Mtb strains prevalent in their country of origin (New York City Department of Health and Mental Hygiene, 2013; CDC, 2013). Our subjects originated from various countries of North, Central and South America, Africa and Asia. We therefore believe that our results are neither restricted to one part of the world nor to one particular Mtb strain. Another relevant limitation was the small sample size. Yet, we were able to demonstrate statistically significant differences between TB cases and controls, as well as highly reproducible data when comparing the discovery to the verification protein

expression patterns, both supporting the robustness of our data. Further studies using larger sample sizes from both HIV^- and HIV^+ subjects from various regions are warranted to validate the robustness of our panel compositions as well as identify any potential regional variations.

In summary, our data are to a large part consistent with the field's data, while, through the use of label-free MS, we expanded the number of host proteins changing as a result of TB in comparison to ORD that mimic the clinical presentation of TB. Furthermore, in contrast to prior studies, we have analyzed samples in both immune competent and immune compromised hosts, and have identified moderately sized panels of host protein biomarkers with diagnostic potential in both HIV negative as well as HIV-associated TB. We conclude that our data hold promise that the detection of host protein changes could provide the basis for adjunctive rapid TB diagnostics. Larger studies from various geographic regions are warranted to validate the robustness and potential clinical value of our identified TB biomarker panels in relevant populations.

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Conflicts of Interest

EP, LC, PC, CY, MM, IR, MS, YZ, RA, and JH were employees of Caprion Proteomics at the time the work was performed. A patent application with JMA, EP, and PC as inventors was also submitted.

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