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Mycobacterium tuberculosis-stimulated whole blood culture to detect host biosignatures for tuberculosis treatment response

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Abstract

Host markers to monitor the response to tuberculosis (TB) therapy hold some promise. We evaluated the changes in concentration of Mycobacterium tuberculosis (M.tb)-induced soluble biomarkers during early treatment for predicting short- and long-term treatment outcomes. Whole

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blood samples from 30 cured and 12 relapsed TB patients from diagnosis, week 1, 2, and 4 of treatment were cultured in the presence of live M.tb for seven days and patients followed up for 24 weeks after the end of treatment. 57 markers were measured in unstimulated and antigen-stimulated culture supernatants using Luminex assays. Top performing multi-variable models at diagnosis using unstimulated values predicted outcome at 24 months after treatment completion with a sensitivity of 75.0% (95% CI, 42.8 – 94.5%) and specificity of 72.4% (95% CI, 52.8 – 87.3%) in leave-one-out cross validation. Month two treatment responder classification was correctly predicted with a sensitivity of 79.2% (95% CI, 57.8 – 92.9) and specificity of 93.3% (95% CI, 64– 99.8%). This study provides evidence of the early M.tb-specific treatment response in TB patients but shows that the observed unstimulated marker models are not outperformed by stimulated marker models. Performance of unstimulated predictive host marker signatures is promising and requires validation in larger studies.

Keywords

Antigen-specific; biomarkers; relapse; slow responders; treatment response; tuberculosis

INTRODUCTION

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*M.tb*), is one of the top 10 causes of death worldwide [1]. A quarter of the world is estimated to be infected with *M.tb*, and 5–10% of individuals will progress to active disease [1,2]. The standard six-month treatment is effective in treating TB; however, cases of rifampicin-resistant, multidrug resistant and extensively-drug resistant TB are increasing [1].

Host markers have been investigated to diagnose latent, incipient and active infection, to predict who will progress to active TB disease, as well as to monitor the response to therapy [3–5]. However, limited information is available on biomarkers to differentiate between cured and relapsed patients at diagnosis, or between fast and slow responders to TB therapy, as defined by sputum smear or culture conversion at week 8 of treatment [6,7]. However, with the use of host biomarkers, treatment response could be predicted earlier, possibly at diagnosis.

Slow responders (positive sputum smear or culture at week 8 of treatment) with a high risk for relapse could receive targeted treatment from diagnosis, reducing tissue damage and risk of drug resistance. Treatment shortening of appropriate patients may be possible through clinical characteristics or by laboratory results [8]. Shortening treatment could improve drug adherence and alleviate the burden on the health care system. Additionally, the stratification of patients into similar risk groups would reduce the cohort size, cost, and duration of chemotherapy clinical trials, as well as accelerate approval of new anti-tuberculosis drugs [4,9,10].

The benefit of stimulation with *M.tb* or its proteins prior to host marker detection is not clear. Although transcriptomic data is available on the present cohort [11], few studies have measured serum or culture supernatant levels of soluble protein host markers in the first four weeks of treatment in cured and relapsed patients (determined by outcome at 24 months) or

in fast and slow responders according to week 8 sputum culture conversion. Thus, this study aimed to investigate the changes in the concentration of antigen-stimulated markers during early treatment and to evaluate the predictive ability of such markers for short- and long-term treatment outcome.

METHODOLOGY

Study setting and participants

Ethical clearance was obtained from the Health Research Ethics Committee of Stellenbosch University (Reference number 99–039). Samples were obtained from the Surrogate Marker Study, details of which have been published by Hesseling et al. [12]. The cohort in the present study is a subgroup of the TB treatment outcome serum biomarker publication by Ronacher et al. [5]. Briefly, participants were longitudinally recruited between 15 May 1999 and 15 July 2002 from five primary health care TB clinics (Ravensmead, Uitsig, Adriaanse, Elsiesriver and Leonsdale) near Tygerberg Academic Hospital, Western Cape, South Africa. Participants were eligible if they were between the ages of 20 and 65, had two positive sputum smears, were willing to participate in the study, undergo HIV-testing and gave informed consent. Patients were excluded if they were pregnant, had previous history of TB infection, HIV-positive, resistant to both rifampicin and isoniazid, used systemic steroids, or had diabetes, malignancies, lung cancer, chronic bronchitis, or sarcoidosis.

Samples from 42 patients were selected from the larger study-specific sample bank; twelve patients who relapsed within 24 months of initial cure (relapse group) were matched to thirty patients who remained cured within 24 months after initial cure (cured group). Matching of cured group patients was done according to sex, age and extent of disease on CXR to the relapse cases. All patients received the standard six-month clinic-based directly observed treatment (DOTS) as recommended by the South African National Tuberculosis Programme. The drug regimen consisted of a two-month intensive phase of rifampicin, isoniazid, pyrazinamide and ethambutol, followed by rifampicin and isoniazid for four months. The intensive phase was prolonged to three months if sputum smear conversion had not yet occurred at month two. Treatment adherence was monitored by research nurses who reviewed the pill doses taken and who recalled participants, on the same day, if a dose was missed.

Sputum culture was done using the BACTEC 460 radiometric mycobacterial broth culture system (Becton Dickinson, NJ, USA) and the time to positivity (TTP) was recorded. Mycobacterial drug susceptibility testing for resistance to both first- and second-line drugs was carried out on samples taken prior to treatment initiation as well as at the end of treatment if cultures remained positive. *M.tb* strains were identified by standardized restriction fragment length polymorphism (RFLP) banding patterns after Southern Blot hybridization with an IS6110 probe in all patients with recurrent TB. All relapse patients had identical strain patterns between the first and second episodes of TB. The presence of *M.tb* was confirmed in positive cultures with ZN staining and IS6110 DNA fingerprinting, and contamination was ruled out with culture on blood agar.

Laboratory Procedures

Blood samples were collected from the 42 patients at diagnosis (pre-treatment), week 1, 2, and 4. Blood was collected into sodium heparin tubes (Becton Dickenson), and 1 ml was transferred into 50 ml tissue culture flasks and diluted with 9 ml of Roswell Park Memorial Institute medium (RPMI)-1640 (Gibco). This was cultured in the presence of live *M.tb* (1×10^5 cfu/ml) at 37 °C and 5% CO_2 for seven days. Identical cultures were incubated for seven days in the absence of *M.tb* (unstimulated/nil). Supernatants were harvested, aliquoted and frozen at −80 °C. Immune markers were measured in unstimulated and *M.tb*-stimulated culture supernatants using Luminex technology, according to the manufacturer's instructions.

A total of 57 markers were evaluated in the supernatants using Milliplex multiplex immunoassays (Millipore, St. Charles, Missouri, USA). Four kits were used that contained the following markers: **27-plex kit:** Eotaxin, Epidermal Growth Factor (EGF), Fibroblast Growth Factor-2 (FGF-2), FMS-Like Tyrosine kinase-3 (FLT-3-ligand), Fractalkine, Granulocyte Colony Stimulating Factor (G-CSF), Granulocyte Macrophage Colony Stimulating Factor (GM-CSF), Growth Regulated Oncogene (GRO), Interferon inducible protein 10 (IP-10), Interferon-α2 (IFN-α2), Interleukin (IL)-1α, IL-3, IL-9, IL-12p40, IL-15, IL-17, Interleukin 1 Receptor Antagonist (IL-1ra), Macrophage Derived Chemokine (MDC), Macrophage Inflammatory Protein-1α (MIP-1α), Macrophage Inflammatory Protein-1β (MIP-1β), Monocyte Chemotactic protein 1 (MCP-1), Monocyte Chemotactic protein 3 (MCP-3), soluble CD40 Ligand (sCD40L), Transforming Growth Factor-α (TGF-α), Tumour Necrosis Factor-α (TNF-α), TNF-β and Vascular Endothelial Growth Factor (VEGF); **13-plex high sensitivity kit:** GM-CSF, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IFN-γ and TNF-α; **14-plex soluble receptor kit:** soluble CD30 (sCD30), soluble Epidermal Growth Factor Receptor (sEGFR), soluble glycoprotein 130 (sgp130), soluble Interleukin-1 Receptor-1 (sIL-1R1), sIL-1R2, soluble Interleukin-2 Receptor-α (sIL-2Rα), soluble Interleukin-4 Receptor (sIL-4R), sIL-6R, soluble Receptor for Advanced Glycation End products (sRAGE), soluble Tumour Necrosis Factor Receptor 1 (sTNFR1), sTNFR2, soluble Vascular Endothelial Growth Factor Receptor 1 (sVEGFR1), sVEGFR2 and sVEGFR3; **3-plex acute phase proteins kit:** Serum Amyloid protein A (SAP A), Serum Amyloid Protein P (SAP P) and C-reactive Protein (CRP).

The 27-plex and 13-plex high sensitivity kit had a standard curve range of 3.2–10000 pg/ml and 0.13–2000 pg/ml, respectively. The analytes in the 14-plex soluble receptor kit can be grouped into three groups; sCD30, sgp130, sIL-1R1, and sIL-2Rα had a standard curve of 24.4–100 000 pg/ml; sIL-4R, sIL-6R, sRAGE, sTNFR1 and sTNFR2 had a standard curve of 12.2–50 000 pg/ml; and sEGFR, sIL-1R2, sVEGFR1, sVEGFR2 and sVEGFR3 had a standard curve of 122.1–500 000 pg/ml. For the acute phase proteins, the standard curve ranged between 0.08–250 ng/ml for SAP A and SAP P, and between 0.016–50.0 ng/ml for CRP.

Statistical Analysis

GM-CSF and TNF-α were measured in two kits; however, only the values of the high sensitivity kit are reported. In all cases the unstimulated (Nil) values were subtracted from

the antigen-stimulated (Ag-Nil) values. Antigen-stimulated values lower than the unstimulated values were changed to 0. Biomarker values that fell outside the highest or lowest point on the standard curve were assigned the lowest or highest value that could be extrapolated by the Bio-Plex Manager Software (Version 4.11). Up to 80% of extrapolated values or antigen-stimulated values lower than the unstimulated values were allowed before markers were excluded. Eighteen host markers had very low or undetectable stimulated and unstimulated levels in supernatants (G-CSF, IL-3, IL-9, IL-15, IL-17, MIP-1α, IL-2, IL-13, sCD30, sIL-1R1, sRAGE, sVEGFR3, IL-5, GM-CSF, FGF-2, TGF-α, IL-12p40 and IL-1α), and were excluded from further analysis.

A mixed model ANOVA with LSD *posthoc* test was used to determine if marker levels were significantly different between time points. A two-way mixed model ANOVA, with patient as random effect and time as fixed effects, was used to determine if individual marker levels were significantly different at different time points between cured and relapse patients, and between fast and slow responders. General discriminant analysis was used to determine if multi-variable models could predict treatment outcome and responder classification at diagnosis. Prediction accuracy was estimated using leave-one-out cross validations. This method was used due to the small sample size and lack of a validation cohort. Values greater than three standard deviations from the mean were Winsorized (statistics based on 10% trimmed values). Spearman's rank correlation test was used to determine associations among biomarker levels. Statistica version 9 (Statsoft; Ohio, USA) was used to analyse data, and significance was determined at $p \leq 0.05$. As this was a screening experiment, no FDR correlations were implied, as any positive result would subsequently be validated in a larger study.

Heatmaps were produced using grouped means for each time point of the various markers. These included BoxCox corrected means for non-normally distributed markers. We performed unsupervised hierarchical clustering using average linkage as clustering method and Pearson's correlation coefficients as the distance measuring method on heatmapper.com. Resultant clusters were used in subsequent pathway analysis to determine their possible combined biological functions. Pathway analysis was done using g:Profiler, a web based tool for functional annotation of omics data (University of Tartu; Institute of Computer Science; Bioinformatics, Algorithmics and Data Mining Group BIIT; https://biit.cs.ut.ee/gprofiler/gost) [13]. Clusters were queried from sources including Gene Ontologies, KEGG, Reactome, and Wikipathways. Over-representation analysis (ORA) was performed, and the significance of pathways was determined using the g:SCS algorithm at a p-value threshold of 0.05. Clusters were queried against annotated genes, excluding those inferred from electronic annotation. Pathways that were significant for the complete set of measured markers (background) were removed, producing a list of cluster specific significant pathways.

Linear discriminant analysis (LDA) was used for investigating the ability of combinations of markers to predict treatment outcome. Best subsets LDA was used for marker selection. Leave-one-out cross validations were used to select the optimal number of predictors (p^*) to include (capped at a maximum of five markers due to the small sample size). Within all the p^* models, the one with smallest Wilk's lambda was reported, and from the top 20 p^*

model, counts of how many times a marker appeared in the top models were reported. This provided an indication of which markers consistently appeared in the 20 best models.

RESULTS

There was no difference in age, sex or chest X-ray severity between groups as the groups were matched on these parameters. The cured group had a mean age of 37.3 ± 10.7 (18 – 64), and the relapsed group had a mean age of 39.0 ± 13.7 (22 – 63). Twenty-three patients (76.7%) were male in the cured group, and nine (75.0%) in the relapsed group. Culture and smear results of the participants are indicated in Table 1. In the remaining samples, 16 patients in the cured group and eight patients in the relapsed group had positive sputum cultures at Week 8 (slow responders). The mean total TTP increased with treatment, although no significant differences were observed.

1. Treatment response patterns of markers

1.1. Treatment Outcome—The blood samples were cultured with (stimulated/Ag-Nil) or without (unstimulated/nil) *Mycobacterium tuberculosis* for seven days and the supernatants were tested using multiplex Luminex kits. In cured patients, 28 unstimulated and three stimulated (unstimulated subtracted from stimulated) marker concentrations changed significantly during treatment. Three clusters could be classified: Cluster A was highest at diagnosis, Cluster B was highest at Week 4, and Cluster C was highest at Week 1. Cluster A consisted of GRO_{Nil}, EGF_{Nil}, FLT-3-$ligand_{Nil}$, IL-$1ra_{Nil}$, $Eotaxin_{Nil}$, SAP A_{Nil}, CRP_{Nil}, IL-7_{Nil}, TNF-α_{Nil}, IL-8_{Nil}, IL-6_{Nil}, $sTNFR2_{Nil}$, IL-$6_{Ag\text{-}Nil}$, sIL-$2R\alpha_{Nil}$, IL-$1\beta_{Ag\text{-}Nil}$, TNF-β_{Nil}, MCP-3_{Nil}, $Fractalkine_{Nil}$, $sCD40L_{Nil}$ and SAP P_{Nil}. Cluster B included $sVEGFR1_{Nil}$, MDC_{Nil}, $sTNFR1_{Nil}$, IFN-$\alpha2_{Nil}$, $sIL\text{-}1R2_{Nil}$, IL-4_{Nil} and $sEGFR_{Nil}$. Cluster C consisted of $sgp130_{Nil}$, IL-$8_{Ag\text{-}Nil}$, $sIL\text{-}6R_{Nil}$ and $sIL\text{-}4R_{Nil}$ (Figure 1A). Pathway analysis indicated that Cluster A was primarily involved in TNF superfamily functions, NFkB regulating mechanisms, and IL-1 signalling regulation, as well as glycosylation of MUC1, Vitamin D function, lipid storage, and adipogenesis. Significant pathways of Cluster B included IL-13 regulation and IL-1 receptor function. Only two of the three analytes were available for Cluster C, therefore the pathways are not discussed (Supplementary Figure 1).

Marker levels in relapsed patients had a similar pattern to those in cured patients (Figure 1B). Relapsed patients had significantly lower levels of some markers at baseline (IL-$6_{Ag\text{-}Nil}$ and SAP A_{Nil}) or Week 2 (IFN-$\alpha2_{Nil}$ and $sEGFR_{Nil}$), and significantly higher levels at baseline (IL-6_{Nil}), Week 1 (IL-7_{Nil}), Week 2 ($sTNFR2_{Nil}$ and $Fractalkine_{Nil}$) and Week 4 (IL-$1\beta_{Ag\text{-}Nil}$ and MCP-3_{Nil}). GRO_{Nil} and TNF-α_{Nil} were significantly higher at baseline and Week 4 in relapsed patients.

1.2. Responder Classification—Patients were classified into fast and slow responders based on week 8 sputum culture conversion. In fast responders, 29 markers showed significant changes with treatment. These markers had a similar treatment response pattern in fast and slow responders (Figure 2), although six markers had significant differences at different time points. MCP-$3_{Ag\text{-}Nil}$, IL-$1\beta_{Ag\text{-}Nil}$ and SAP P_{Nil} were significantly lower at diagnosis while SAP $P_{Ag\text{-}Nil}$ was significantly lower at Week 2, in slow responders. Slow

responders had higher levels than fast responders of sIL-2Rα$_{Nil}$ and sVEGFR2$_{Nil}$ at Week 2 and Week 4, respectively.

2. Individual markers for prediction of treatment response:

2.1. Treatment outcome—When comparing marker concentrations at diagnosis using two-way mixed model ANOVA with an LSD *posthoc* test, six markers were significantly different between subsequently cured and relapsed patients as assessed at month 24 after treatment completion. The levels of MIP-1β$_{Ag-Nil}$, IP-10$_{Ag-Nil}$, IL-10$_{Nil}$ and Eotaxin$_{Ag-Nil}$ were significantly increased, while the levels of sgp130$_{Nil}$ and IL-6$_{Ag-Nil}$ were significantly decreased in relapsed patients. When investigating the diagnostic accuracy of individual markers using receiver operator characteristics (ROC) curve analysis, only four of these markers (MIP-1β$_{Ag-Nil}$, IP-10$_{Ag-Nil}$, sgp130$_{Nil}$ and IL-10$_{Nil}$) showed promise with an AUC of ≥ 0.67 (Table 2).

2.2. Responder classification—For responder classification, five markers were significantly different between slow and fast responders at diagnosis (two-way mixed model ANOVA with an LSD post-hoc test). The level of sgp130$_{Nil}$ was significantly increased, while the values of Fractalkine$_{Nil}$, SAP P$_{Nil}$, IL-1β$_{Ag-Nil}$ and CRP$_{Nil}$ were significantly decreased in slow responders. Four of these markers (sgp130$_{Nil}$, Fractalkine$_{Nil}$, SAP P$_{Nil}$ and IL-1β$_{Ag-Nil}$) had an AUC of ≥ 0.67 (Table 3)

3. Multi-marker models for predictions of treatment outcome:

Using general discriminant analysis, the top-performing multi-variable models (maximum of five variables) were identified to predict treatment outcome (Table 4) and responder classification (Table 5) at diagnosis using unstimulated and antigen-stimulated markers. The ROC curves and most frequently incorporated markers to predict treatment outcome and responder classification are presented in Figure 3 and 4, respectively.

DISCUSSION

This study investigated the early TB treatment response of patients who subsequently remained cured or who relapsed, as well as slow and fast treatment responders to evaluate the potential of seven day whole blood *M.tb*-stimulated soluble host marker levels as predictors of outcome. Additionally, we investigated the use of individual and multi-marker models to predict treatment outcome and responder classification.

Treatment response patterns

Few studies have assessed the treatment response of antigen-stimulated markers. In the present study, only three antigen-stimulated markers indicated a significant change with treatment in cured patients in univariate analysis; IL-6$_{Ag-Nil}$ and IL-1β$_{Ag-Nil}$ were high at diagnosis and decreased by Week 1, and IL-8$_{Ag-Nil}$ was highest at Week 1. IL-6 induces production of CRP and serum amyloid A [14], IL-1β is a pro-inflammatory cytokine, and IL-8 assists with granuloma formation [15]. Sahiratmadja et al. [16] investigated the change in antigen-stimulated levels of biomarkers at diagnosis, and after 8 and 24 weeks of treatment. Marker levels were measured in whole blood and peripheral blood mononuclear

cells, stimulated with *M.tb*, lipopolysaccharide (LPS) or phytohemagglutinin. When stimulated with *M.tb* and lipopolysaccharide, TNF-α and IL-12p40 levels did not change significantly with treatment, even though the levels were significantly higher when stimulated with LPS. IL-10 was undetectable when stimulated with *M.tb*, and when stimulated with LPS there was no significant change with therapy. In contrast, *M.tb*-stimulated IFN-γ levels increased significantly with treatment. *M.tb* antigen stimulation has demonstrated utility for diagnosis of pulmonary TB [17], differentiation of active and latent TB [18], and to predict relapse in transcriptomic studies [11]; however, antigen-stimulation provided limited advantage over unstimulated biomarker levels in the present treatment response study.

Of the 28 unstimulated markers that indicated a significant change with treatment, 13 overlapped with the results of Ronacher et al. [5], in a study that investigated serum marker levels of the same participants as for the present report. However, CRP, SAP A and SAP P were the only markers that followed a similar treatment response pattern (decrease with treatment) in both studies. The differences in results between these two studies can be explained by the different assays used and different markers assessed (72 markers compared to 57 markers in the present study). The difference between processing methodologies (*ex vivo* versus 7-day culture) in the two studies could also explain differences in marker levels due to continued secretion or degradation of some markers in the longer-term culture assay. Additionally, different sample types were analysed (serum compared to whole blood cultured for 7 days in the present study), and more patients were included in the study published by Ronacher et al. [5] (78 cured patients compared to 30 cured patients in the present study).

The biomarkers analysed could be grouped into three clusters based on their pattern of response following treatment. Cluster A consisted of 20 biomarkers produced at high concentrations at diagnosis, decreased at week 1, and remaining at low levels up to Week 4. These biomarkers included chemokines (GRO_{Nil}, $Eotaxin_{Nil}$, $IL\text{-}8_{Nil}$, $MCP\text{-}3_{Nil}$ and $Fractalkine_{Nil}$), growth factors (EGF_{Nil}), cytokines ($FLT\text{-}3\text{-}ligand_{Nil}$, $IL\text{-}1ra_{Nil}$, $IL\text{-}7_{Nil}$, $TNF\text{-}\alpha_{Nil}$, $TNF\text{-}\beta_{Nil}$ and $sCD40L_{Nil}$), soluble receptors ($sTNFR2_{Nil}$ and $sIL\text{-}2R\alpha_{Nil}$) and acute phase proteins ($SAP\ A_{Nil}$, $SAP\ P_{Nil}$, CRP_{Nil} and $IL\text{-}6_{Nil}$). Few studies have investigated the treatment response within the first four weeks of treatment, and few significant changes have been reported. Lawn et al. [19] observed a significant decrease in serum CRP and sIL-2Rα levels by Week 4 of treatment, and Ronacher et al. [5] observed a significant reduction in serum sIL-2Rα at Week 4. Other studies have also observed a significant decrease in CRP [5,15,16,20–25] and sIL-2Rα [21,26,27] levels with treatment at later time points. In contrast to the present study's findings, Djoba Siawaya et al. [28] reported a significant increase in eotaxin and EGF at Week 1, although limited studies of these markers have been conducted. A reduction in these biomarker levels is likely due to a reduction in bacterial load with treatment. Acute phase proteins, such as CRP, are present at high levels during systemic inflammation, and decrease with resolving infection [29]. A reduced bacterial load results in a decrease of antigen-presentation, which reduces cell-to-cell signalling. This leads to a decreased level of pro-inflammatory cytokines, regulatory cytokines and chemokines [30].

Clusters B and C consisted of markers increasing at Week 4 and Week 1, respectively; soluble receptors (sIL-$6R_{Nil}$, sIL-$4R_{Nil}$ and $sgp130_{Nil}$) increased at Week 1, while anti-inflammatory cytokines (IL-4_{Nil} and IFN-$\alpha2_{Nil}$), selected soluble receptors ($sVEGFR1_{Nil}$, $sEGFR_{Nil}$, sIL-$1R2_{Nil}$ and $sTNFR1_{Nil}$) and MDC_{Nil} increased at Week 4 of treatment. Few studies have assessed the levels of these biomarkers within the first four weeks of treatment, and no significant changes have been reported for IL-4 levels [28,31]. However, a significant increase in IL-4 levels was observed in epithelial lining fluid obtained through broncho-alveolar lavage after 6 months of treatment [32]. While pro-inflammatory cytokines are essential in eliminating bacteria, anti-inflammatory cytokines downregulate the immune response to limit tissue injury. The TH1 immune response produces pro-inflammatory cytokines such as TNF, IL-1 and IFN-γ. When bacterial numbers are reduced during treatment, anti-inflammatory cytokines, such as IL-4, inhibits the TH1 immune response [33], thereby limiting immunopathology. Additionally, soluble receptors regulate immune responses through inhibition of cytokine signalling [34]. Type I IFNs are anti-inflammatory cytokines that may hinder the host's ability to reduce bacterial replication [35]. Although the mechanisms are still unclear, *M.tb* induces Type I IFN expression [36]. Treating patients with pegylated IFN-α was also associated with reactivation of tuberculosis [37].

Biomarker differences between treatment outcome groups

While the treatment response of cured and relapsed patients was broadly similar, 12 biomarkers were significantly different at particular time points. It is noteworthy that five of the markers (IL-$6_{Ag\text{-}Nil}$, SAP A_{Nil}, IL-6_{Nil}, GRO_{Nil} and TNF-α_{Nil}) were already significantly different at baseline. Limited studies have distinguished between the treatment response of cured and relapsed patients; Ronacher et al. [5] observed higher levels of sIL-2ra (diagnosis, week 2 and week 4) and CRP (diagnosis and week 2) in relapse patients compared to cured patients. These serum samples were from the same cohort as the present study.

Biomarker differences between responder groups

Few studies have focused on the differences between fast and slow responders. The biomarker closest to qualifying as a surrogate endpoint for clinical trials is 2-month sputum conversion as a predictor of relapse risk [38]. In the present study, slow responders had higher levels of sIL-$2R\alpha_{Nil}$ and $sVEGFR2_{Nil}$, and lower levels of MCP-$3_{Ag\text{-}Nil}$, IL-$1\beta_{Ag\text{-}Nil}$, SAP P_{Nil} and SAP $P_{Ag\text{-}Nil}$ compared to fast responders. Three of these markers (MCP-$3_{Ag\text{-}Nil}$, IL-$1\beta_{Ag\text{-}Nil}$ and SAP P_{Nil}) were already significantly altered at baseline. While studies have not reported differences in these biomarkers, reports have been published on differences in IL-8, IL-1ra and CRP between fast and slow responders. Lee and Chang [15] investigated 13 fast and five slow responders (depending on Week 8 smear results), and observed that IL-8 and IL-1ra levels were significantly higher at baseline, Week 8 and Week 24 in slow responders. There was no difference in CRP levels [15]. Almeida et al. [20] classified patients as fast, intermediate or slow responders (smear negative at Week 3, 5, and 8, respectively), and observed significantly higher CRP in slow responders after 3 weeks of treatment compared to fast responders.

Prediction of treatment outcome

In the present study, few individual markers showed promise with AUC > 0.70, and few published reports have found acceptable individual markers [39,40]. MIP-1$\beta_{Ag\text{-}Nil}$ was the most successful individual marker to predict treatment outcome with an AUC of 0.75 (95% CI, 0.57 – 0.92). MIP-1β, or CCL4, is a chemokine that attracts natural killer cells and monocytes to the infection site and stimulates the release of IL-6 and TNF-α [41]. For responder classification, $sgp130_{Nil}$ was most useful (AUC = 0.70). Sgp130 is a transmembrane protein that forms part of the IL-6 receptor and is necessary for signal transduction [42]. IL-6 inhibits IFN-γ function, which can result in ineffective autophagy of *M.tb* [43].

Several studies have investigated cytokine, chemokine, or soluble factor levels as possible markers to detect early treatment response and responder classification. The composition of white blood cells can also be used to predict treatment outcome; Chedid et al. [44] observed that white blood cell counts and lymphocyte proportions could predict treatment outcome at baseline with an AUC of 0.84 (95% CI, 0.72 – 0.96). It has been noted that a combination of clinical parameters and marker levels would provide a better prediction model for treatment outcome and responder classification. Ronacher et al. [5] observed that TTP and BMI, as well as the serum levels of TNF-β, sIL-6R, IL-12p40 and IP-10 at baseline could classify treatment outcome with a sensitivity of 75% (95% CI: 0.38–1.0), specificity of 85% (95% CI: 0.75–0.93) and an AUC of 0.82 (95% CI: 0.68–0.93; p-value = 0.0037) in the training set. Moreover, Jayakumar et al. [21] observed that age, baseline sTNF-R1 and Week 8 CRP levels could classify 39 patients into fast and slow responders with an accuracy of 85% (cross-validated C-statistic of 0.76, Hosmere-Lemeshow p-value > 0.2). The current study did not combine clinical parameters, since limited information was available and as time to detection in liquid culture, male sex, body mass index, sputum smear grading and number of cavities on chest X-ray were already reported on the complete cohort for this study [12]. The unstimulated multi-marker models could, however, predict treatment outcome with AUC of 0.81 (95% CI, 0.64 – 0.99), sensitivity of 75.0% (95% CI, 42.8 – 94.5%) and specificity of 72.4% (95% CI, 52.8 – 87.3%), and responder classification with AUC of 0.97 (95% CI, 0.93 – 1.00), sensitivity of 79.2% (95% CI, 57.8 – 92.9%) and specificity of 92.3% (95% CI, 64.0 – 99.8%) after leave-one-out cross-validation. These findings indicate that using antigen-stimulated multi-marker models provided only slightly higher AUC values, indicating that live *M.tb*-stimulated markers are not substantially superior to unstimulated markers, although they require significantly more infrastructure, including biosafety level 3 laboratories and a 7-day delay in obtaining samples for cytokine measurement.

There were several limitations in this study. A healthy control group was not available for comparison, and a validation cohort was not available to test the prediction models. Additionally, a small sample size was available, as only 12 patients were included in the relapse group, a reflection of the very limited sample repositories available across the globe to conduct such investigations. Finding biomarkers in a small discovery cohort needs to be validated in larger cohorts, when such become available, in future research. While this study aimed to investigate the early treatment response, measurements of antigen-specific

biomarkers at the end of treatment might yield better results, which could be investigated in future studies.

CONCLUSION

In conclusion, this study provided evidence of the early *M.tb*-specific treatment response of cured and relapsed patients, as few studies have measured biomarker levels in the first four weeks of treatment. Early identification of risk for poor treatment outcome would offer opportunities for individualized interventions and risk-specific treatment regimens or duration. Moreover, individual and multi-marker models were identified, which could predict treatment outcome and responder classification at diagnosis with promising performance. Additionally, this study provides evidence that *M.tb*-stimulated markers do not offer advantages over unstimulated markers when assessing treatment response. Live *M.tb*-stimulated cultures, which involve greater logistical complexity therefore appear unnecessary for the investigated marker selection. Limiting testing to unstimulated levels would reduce the need for work in a BSL III facility, where live *M.tb* is used as stimulant, and consequently reduce the cost, time, and exposure risk to laboratory staff. Future, larger studies should evaluate markers through to the end of treatment. A test indicating that sufficient antibiotic therapy has been administered would greatly impact TB treatment and guide decisions on the duration of therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights:

- *Mycobacterium tuberculosis*-specific response in early TB treatment
- Stimulated marker models do not outperform unstimulated marker models
- Performance of predictive host marker signatures is promising

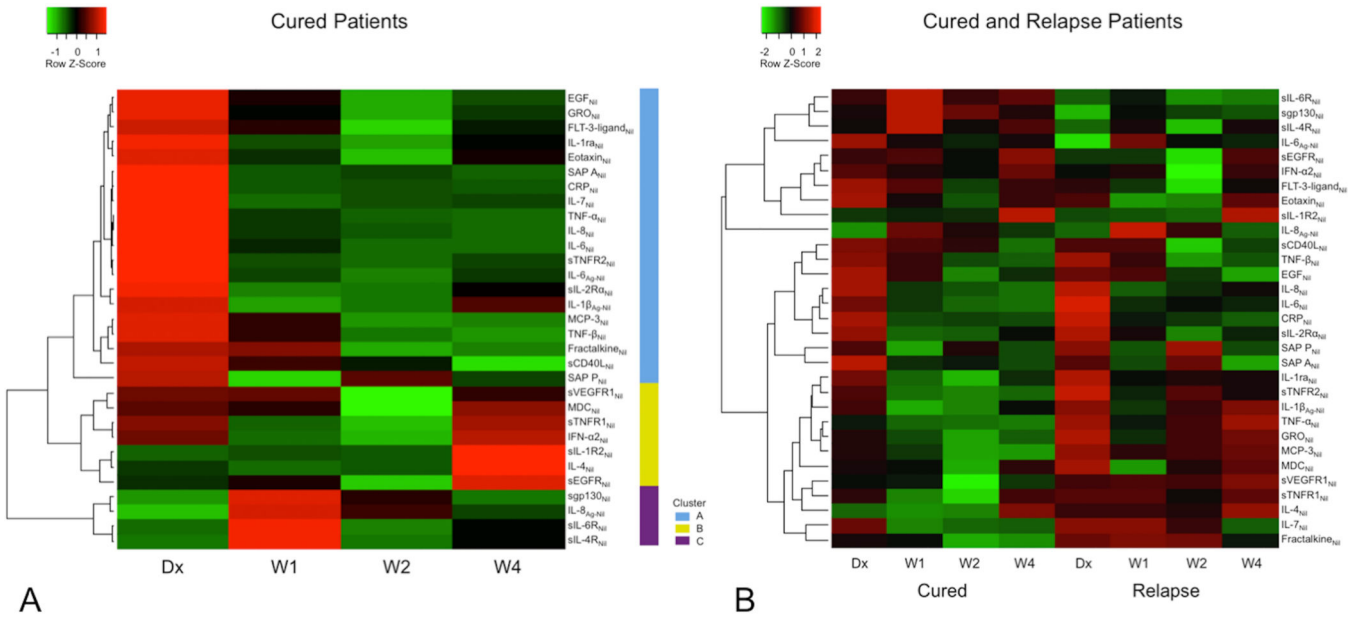

Figure 1. Heatmaps of Least Squares (LS) mean values of host marker concentrations in unstimulated (Nil) and live *M. tb* stimulated (Ag minus unstimulated; Ag-Nil) 7-day whole blood culture assays, indicating A) three clusters observed in cured patients, and B) treatment response of cured and relapsed patients.

LS means were first $\log_{10}$ transformed, and unsupervised hierarchical clustering was performed using average linkage as the clustering method and Pearson's as the distance measuring method. Green indicates downregulation from the mean (black), and red indicates upregulation.

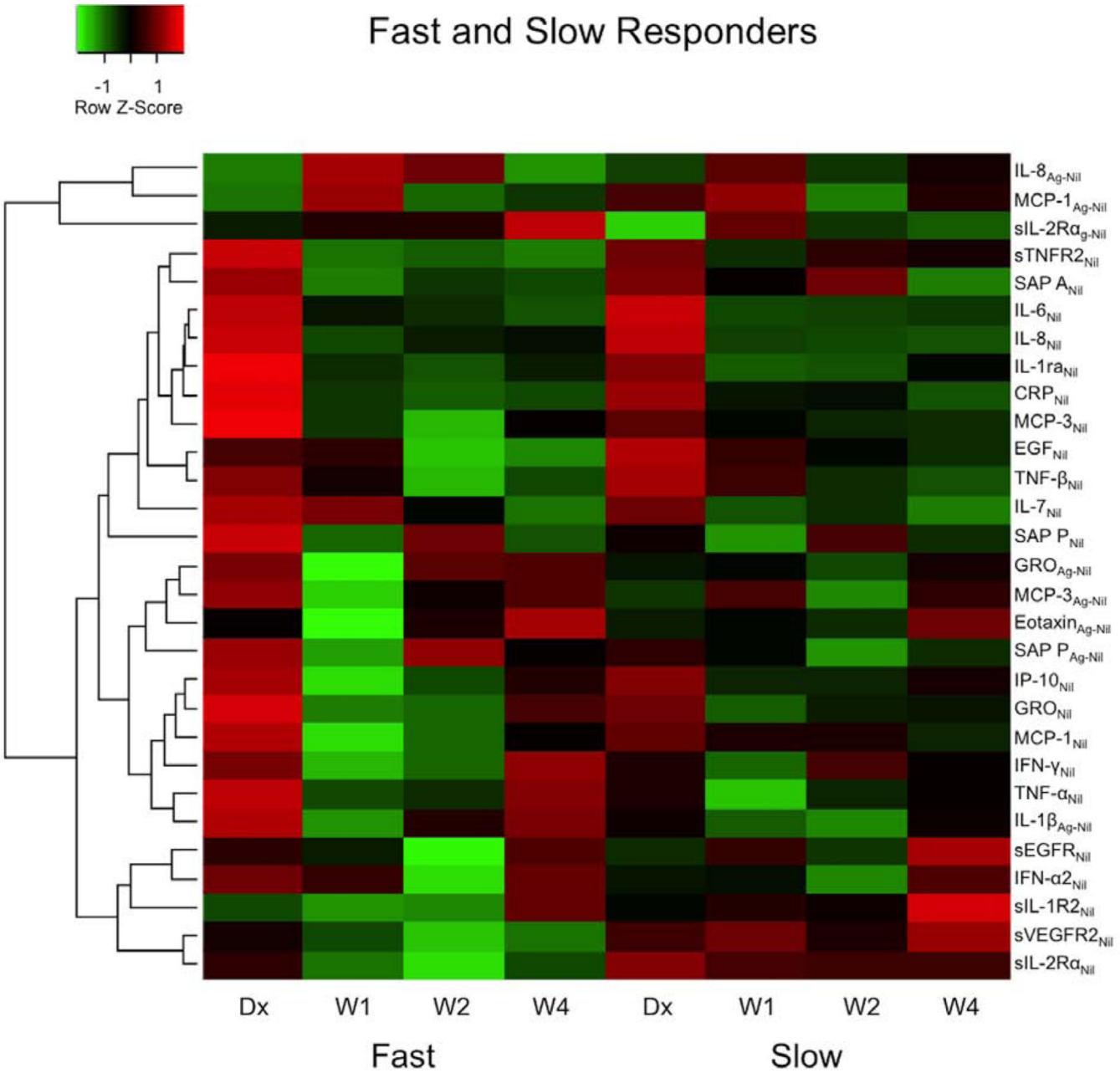

Figure 2. Heatmap of LS mean values of host marker levels in unstimulated (Nil) and live *M. tb* stimulated (Ag minus unstimulated; Ag-Nil) 7-day whole blood culture assays, indicating treatment response patterns of fast and slow responders.

LS means were first $\log_{10}$ transformed, and unsupervised hierarchical clustering was performed using average linkage as clustering method and Pearson's as the distance measuring method. Green indicates downregulation from the mean (black), and red indicates upregulation.

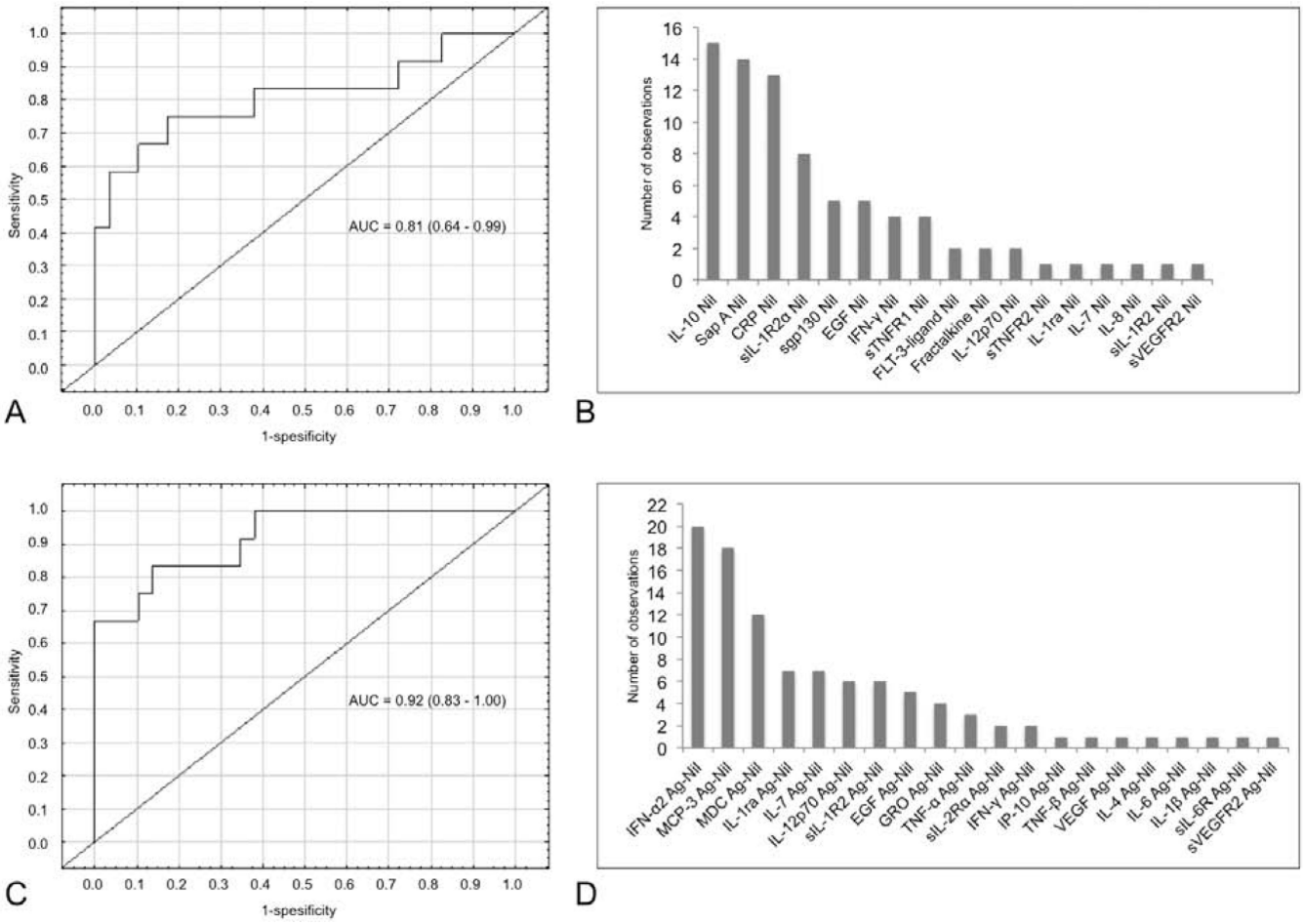

Figure 3. Multi-marker model prediction of treatment outcome.

A) Receiver operator characteristics curve for optimal four-marker model using unstimulated values ($IL\text{-}10_{Nil}$, $sIL\text{-}2R\alpha_{Nil}$, $sTNFR1_{Nil}$, and EGF_{Nil}), B) Frequency of markers in the top general discriminant analysis models using unstimulated values, C) Receiver operator characteristics curve for optimal five-marker model using stimulated values ($GRO_{Ag\text{-}Nil}$, $IFN\text{-}\alpha2_{Ag\text{-}Nil}$, $IL\text{-}1ra_{Ag\text{-}Nil}$, $MCP\text{-}3_{Ag\text{-}Nil}$ and $MDC_{Ag\text{-}Nil}$), and D) Frequency of markers in the top general discriminant analysis models using stimulated values.

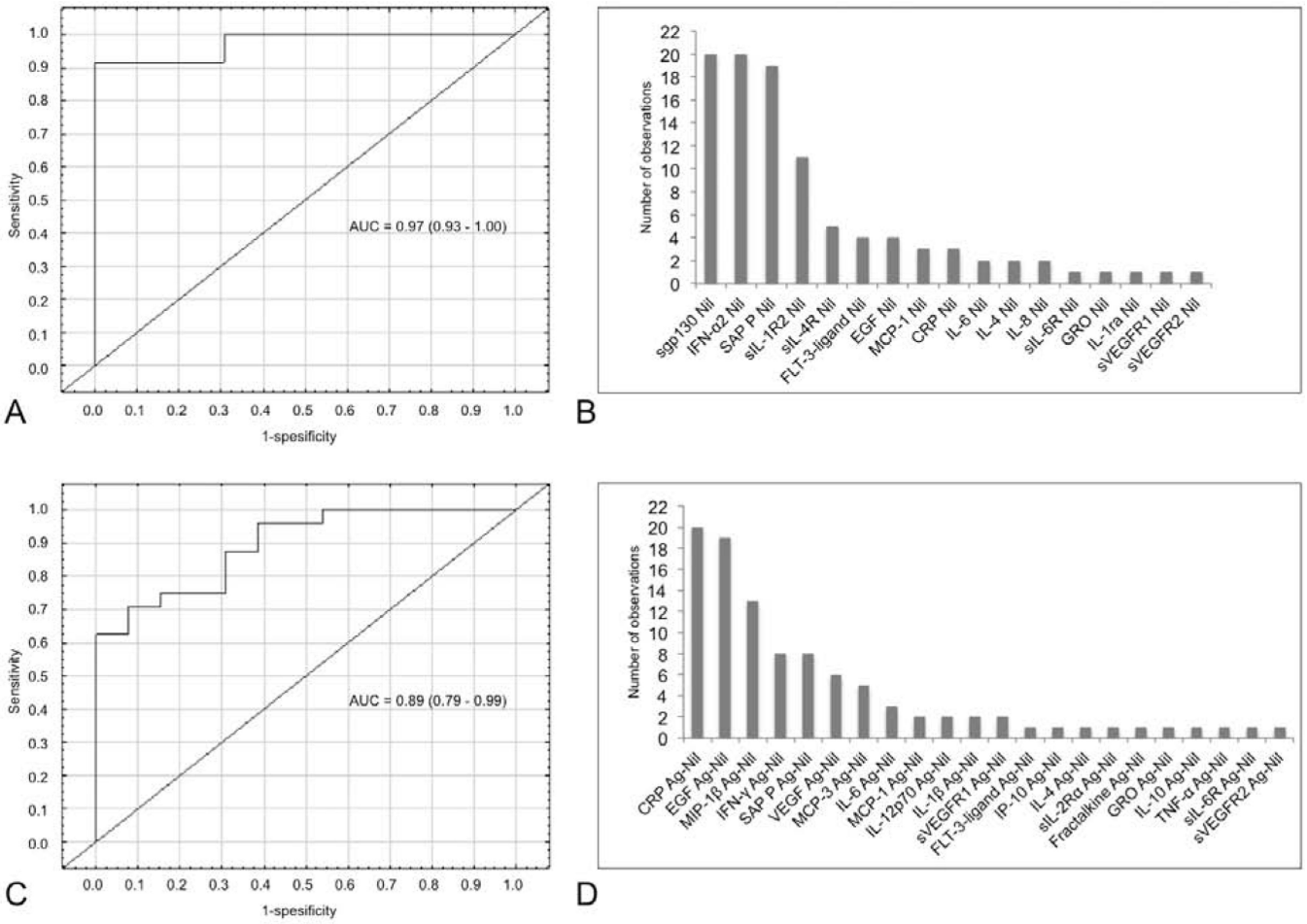

Figure 4. Multi-marker model prediction of responder classification.

A) Receiver operator characteristics curve for optimal five-marker model using unstimulated values ($sgp130_{Nil}$, SAP P_{Nil}, IFN-$\alpha 2_{Nil}$, sIL-$1R2_{Nil}$ and EGF_{Nil}), B) Frequency of markers in the top general discriminant analysis models using unstimulated values, C) Receiver operator characteristics curve for optimal four-marker model using stimulated values ($EGF_{Ag\text{-}Nil}$, MCP-$3_{Ag\text{-}Nil}$, MIP-$1\beta_{\mathbf{Ag}\text{-}Nil}$, IFN-$\gamma_{Ag\text{-}Nil}$ and $CRP_{Ag\text{-}Nil}$), and D) Frequency of markers in the top general discriminant analysis models using stimulated values.

Table 1.

Smear and culture results of cured and relapsed patients at diagnosis, Week 1, Week 2, Week 4 and Week 8.

		Diagnosis	Week 1	Week 2	Week 4	Week 8
Cured	Smear positive[a]	29 (96.7%)	26 (86.7%)	22 (73.3%)[c]	14 (46.7)[c]	7 (23.3%)
	Culture positive[a]	30 (100%)	30 (100%)	27 (90.0%)[c, d]	26 (86.7%)[c, d]	16 (53.3%)[d]
	TTP[b]	3.5 ± 1.8 (1–7)	8.6 ± 3.5 (2–19)	10.7 ± 4.9 (3–27)	13.4 ± 5.1 (3–29)	18.0 ± 4.2 (13–29)
Relapsed	Smear positive[a]	12 (100%)	12 (100%)	12 (100%)	11 (91.7%)[c]	6 (50.0%)
	Culture positive[a]	12 (100%)	12 (100%)	11 (91.7%)[d]	11 (91.7%)[c]	8 (66.7%)
	TTP[b]	2.6 ± 3.2 (1–12)	7.0 ± 2.6 (2–12)	8.7 ± 2.7 (4–15)	11.3 ± 3.1 (5–15)	18.3 ± 6.5 (9–27)

[a]Values expressed as n (%)

[b]Values expressed as Mean ± Standard Deviation (Min - Max)

[c]One missing value, Cured group Week 4 had 2 missing smear and culture values

[d]One contaminated sample, Cured group Week 8 had four contaminated samples (TTP) Total time to positivity

Table 2.

Levels of markers at diagnosis for treatment outcome prediction.

Marker	% >MDC*	Cured	Relapsed	AUC p-value	AUC (95% CI)	Optimal cut-off	Sensitivity	Specificity	PPV	NPV
MIP-1β_{Ag-Nil}**	87.3%	72.24	168.79	0.01	0.75 (0.57 – 0.92)	> 129.78	66.7%	86.2%	0.67	0.86
IP−10$_{Ag-Nil}$	96.8%	4440.53	7227.55	0.02	0.71 (0.54 – 0.89)	> 8069.18	66.7%	72.4%	0.50	0.84
sgp130$_{Nil}$	100%	8356.29	6773.55	0.05	0.67 (0.49 – 0.84)	< 7034.34	58.3%	72.4%	0.81	0.47
IL-10$_{Nil}$**	92.4%	2.59	5.47	0.05	0.67 (0.46 – 0.88)	> 2.97	66.7%	65.5%	0.44	0.83
Eotaxin$_{Ag-Nil}$	59.5%	1.03	2.37	0.05	0.66 (0.47 – 0.85)	> 0.68	75.0%	62.1%	0.45	0.86
IL-$_{Ag-Nil}$	79.1%	90.64	26.57	0.05	0.66 (0.49 – 0.82)	< 4.94	66.7%	58.6%	0.81	0.40

*% above the minimum detectable concentration

**Winsorized

Table 3.

Levels of markers at diagnosis for responder classification prediction.

Marker	% >MDC*	Fast	Slow	AUC p-value	AUC (95% CI)	Optimal cut-off	Sensitivity	Specificity	PPV	NPV
$sgp130_{Nill}$	100%	6439.51	8358.11	0.02	0.70 (0.51 – 0.89)	> 7504.60	75.0%	76.9%	0.86	0.63
$Fractalkine_{Nil}$**	82.3%	54.99	29.19	0.04	0.68 (0.50 – 0.86)	< 50.89	79.2%	53.8%	0.58	0.76
SAP P_{Nil}	100%	7337.55	5769.64	0.04	0.67 (0.48 – 0.87)	< 6550.98	70.8%	69.2%	0.56	0.81
IL-$1\beta_{Ag\text{-}Nil}$**	94.9%	10.41	5.48	0.04	0.67 (0.49 – 0.86)	< 9.12	66.7%	61.5%	0.50	0.76
CRP_{Nil}	74.7%	81144.20	59439.12	0.05	0.66 (0.47 – 0.86)	< 85154.78	79.2%	53.8%	0.58	0.76

*% above the minimum detectable concentration

**Winsorized

Table 4.

Multi-marker models for treatment outcome prediction at diagnosis.

Treatment Outcome		**Resubstitution classification matrix**			
Markers (all unstimulated)	AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	PPV	NPV
$IL\text{-}10_{Nil}$ * $sIL\text{-}2R\alpha_{Nil}$ * $sTNFR1_{Nil}$ * EGF_{Nil}	0.81 (0.64 – 0.99)	75.0% (42.8 – 94.5%)	75.9% (56.5 – 89.7%)	0.56	0.88
		Leave-one-out crossvalidation			
		Sensitivity (95% CI)	Specificity (95% CI)	PPV	NPV
		75.0% (42.8 – 94.5%)	72.4% (52.8 – 87.3%)	0.53	0.88
		Resubstitution classification matrix			
Markers (all stimulated)	AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	PPV	NPV
$GRO_{Ag\text{-}Nil}$ * $IFN\text{-}\alpha2_{Ag\text{-}Nil}$ * $IL\text{-}1ra_{Ag\text{-}Nil}$ * $MCP\text{-}3_{Ag\text{-}Nil}$ * $MDC_{Ag\text{-}Nil}$ *	0.92 (0.83 – 1.00)	75.0% (42.8 – 94.5%)	89.7% (72.6 – 97.8%)	0.75	0.90
		Leave-one-out crossvalidation			
		Sensitivity (95% CI)	Specificity (95% CI)	PPV	NPV
		66.7% (34.9 – 90.1%)	86.2% (68.3 – 96.1%)	0.67	0.86

*Winsorized

Table 5.

Multi-marker models for responder classification prediction at diagnosis.

Responder Classification		**Resubstitution classification matrix**			
Markers (all unstimulated)	AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	PPV	NPV
$sgp130_{Nil}$ SAP P_{Nil} IFN-$\alpha 2_{Nil}$ * sIL-$1R2_{Nil}$ * EGF_{Nil}	0.97 (0.93 – 1.00)	87.5% (67.6 – 97.3%)	100.0% (79.4 – 100.0%)	1.00	0.81
		Leave-one-out crossvalidation			
		Sensitivity (95% CI)	Specificity (95% CI)	PPV	NPV
		79.2% (57.8 – 92.9%)	92.3% (64.0 – 99.8%)	0.95	0.71
		Resubstitution classification matrix			
Markers (all stimulated)	AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	PPV	NPV
$EGF_{Ag\text{-}Nil}$ MCP-$3_{Ag\text{-}Nil}$ * MIP-$1\beta_{Ag\text{-}Nil}$ * IFN-$\gamma_{Ag\text{-}Nil}$ * $CRP_{Ag\text{-}Nil}$ *	0.89 (0.79 – 0.99)	70.8% (48.9 – 87.4%)	84.6% (54.6 – 98.1%)	0.89	0.61
		Leave-one-out crossvalidation			
		Sensitivity (95% CI)	Specificity (95% CI)	PPV	NPV
		66.7% (44.7 – 84.4%)	76.9% (46.2 – 95.0%)	0.84	0.56

* Winsorized