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ABSTRACT

Background: The aim of this study was to evaluate C-reactive protein(CRP), globulin and white cell count as predictors of treatment outcome in pulmonary tuberculosis.

Methods: An observational study of patients with active pulmonary tuberculosis was conducted at a tertiary centre. All patients had serum CRP, globulin and white cell count measured at baseline and two months following commencement of therapy. The outcome of interest was requirement for extension of therapy beyond 6 months.

Results: There were 226 patients included in the study. Serum globulin>45 g/L was the only baseline biomarker evaluated that independently predicted requirement for therapy extension(OR 3.59(1.79–7.57;p <0.001)). An elevated globulin level that failed to normalize at 2 months was also associated with increased requirement for treatment extension(63.9% versus 5.1%;p<0.001) and had low negative likelihood ratio(0.07) for exclusion of requirement for therapy extension. On multivariable analysis, an elevated globulin that failed to normalize at 2 months was independently associated with requirement for therapy extension (OR 6.12(2.23–16.80);p<0.001).

Conclusions: Serum globulin independently predicts requirement for treatment extension in pulmonary TB and outperforms CRP and white cell count as a predictive biomarker. Normalization of globulin at two months following treatment commencement is associated with low risk of requirement for treatment extension.

INTRODUCTION

Tuberculosis (TB) represents a major public health concern and a leading cause of morbidity and mortality worldwide.[1] Active pulmonary TB is typically treated with an intensive phase of four antimicrobial agents for two months and subsequently with dual agent continuation phase therapy for a further four months. This regimen leads to complete microbiological and clinical cure in the majority of cases.[1, 2] However, in some patients, routine therapy fails to adequately control and treat disease, leading to failure of symptomatic improvement, prolonged infectivity and requirement for extension of therapy.[3] The length of anti-tuberculous therapy can have negative implications for patient adherence and places increased pressure on health care systems.[4]

Early evaluation of the response to anti-tuberculous therapy has the potential to optimize routine clinical management of the disease and thus lead to improved outcomes. A biomarker that is predictive of likely response prior to commencement of therapy or that can be used to monitor subsequent treatment response could be invaluable to clinicians. Biomarkers measured at baseline could potentially identify patients with higher bacterial burden and/or enhanced inflammatory response that require more intensive monitoring and longer therapy regimens than those with more minimal uncomplicated disease.[5] Early treatment markers may allow identification of patients in whom ineffective therapy has led to uncontrolled bacterial replication and development of drug resistance.[5, 6] Stratification of patients with TB at diagnosis or early in therapy into those requiring different therapeutic regimens and durations could improve compliance and treatment outcome and allow health care services to focus more attention on patients with greater risk of adverse treatment outcomes.[7] An accurate predictive biomarker would also be invaluable in validation of new TB drug candidates, thereby accelerating development of novel therapies.

Currently available baseline markers of disease severity include chest radiographic findings[8-10] and sputum smear grade[9, 11] and available clinical indicators of treatment response include symptomatic improvement[12], weight gain[13], radiographic resolution[8] and sputum culture conversion[10, 14]. However, the results of microbiological tests can often be delayed and chest radiograph assessment can be difficult to standardize and

complicated by presence of chronic changes.[5, 6] Therefore, a reliable marker than can be easily measured in blood as an accurate surrogate of treatment success is particularly desirable.

A number of immune parameters in blood have been to shown to correlate with extent of disease and/or treatment response including neopterin[15, 16], c-reactive protein[17-19] and haematological parameters such as white cell count and erythrocyte sedimentation rate [20, 21]. However, these parameters have only been assessed in small studies at the onset of disease. Globulins are a collection of proteins that can be readily measured in the blood. Total globulin levels are routinely measured in serum samples and are non-specifically elevated in response to several inflammatory conditions including active tuberculosis[22]. Studies have previously shown that globulin levels in serum correlate with adverse outcomes from *Pneumocystis jiroveci* pneumonia[23] and lung cancer[24]. The value of serum globulin as a predictor of outcome in tuberculosis has not been formally evaluated previously.

The aim of this study was to assess the value of measuring serum levels of routine inflammatory biomarkers globulin, CRP and white cell count at baseline and two months following therapy commencement for prediction of outcome in patients treated for active pulmonary tuberculosis.

METHODS

Study population

We conducted an observational study of consecutive adult patients (>16 years) with active bacteriologically confirmed pulmonary TB commenced on anti-tuberculous chemotherapy at St. Mary's Hospital, London between January 2008 and January 2013. The study received local approval. Patients were included if they had sputum or bronchoalveolar lavage samples that were positive for culture of *Mycobacterium tuberculosis.*

Exclusion criteria were:

- Patients who were treated based on clinical likelihood for pulmonary tuberculosis but without evidence of positive cultures for *Mycobacterium tuberculosis*.
- Loss to follow-up or failure to complete therapy.

Measurement of inflammatory biomarkers in serum

All patients included in the study had measurement of C-reactive protein, white cell count and total globulin levels in serum samples taken at baseline (prior to initiation of anti-tuberculous therapy) with repeat measurement undertaken at 2 months following commencement of therapy. The normal ranges of the assays were: CRP 0-10 mg/L, globulin 19 – 35 g/L, white cell count 4.0 – 11.0 x 10^9 cells/L

Microbiological evaluation

Microscopy was performed in all patients who produced sputum or underwent bronchoscopy with bronchoalveolar lavage (BAL). The density of acid-fast bacilli (AFB) was graded as scanty, 1, 2 or 3+ according to standard protocols.[25] TB culture was performed by incubation of sputum or BAL samples using the Bactec™ MGIT ™ 960 system (BD, New Jersey USA) for up to 6 weeks.

Radiographic evaluation

As part of the initial diagnostic evaluation, all patients included in the study underwent standard posteroanterior chest radiograph to assess for signs of active tuberculosis including nodules, consolidation and cavitation.

Outcome

The outcome of interest was requirement for extension of antituberculous therapy beyond 6 months. The indications for extension of therapy were left to the discretion of the treating physician and included one or more of the following factors: persistent smear or culture positivity; failure of chest radiograph improvement; drug resistance; persistent symptoms; poor compliance with therapy; presence of extra-pulmonary disease and drug- induced liver injury. We also conducted a separate analysis to evaluate the outcome of persistent sputum smear and/or culture positivity (defined as > 2 months following treatment initiation).

Statistical analysis

All data were analysed using SPSS version 13.0 for windows (SPSS Inc., Chicago, Il). The chi-squared test was used to compare categorical variables. The Mann Witney U test and the Kruskal Wallis test were used to compare continuous variables between two or multiple groups respectively.

Sensitivity, specificity, positive and negative predictive values, positive and negative likelihood ratios and area under the receiver operator characteristic curve were used to assess the value of serum biomarkers for prediction of outcomes of interest.

We used multivariable logistic regression to evaluate the association of baseline and two-month levels of globulin, CRP and white cell count with outcomes of interest. The following variables were included in the regression model: age>50 years, male sex, requirement for directly observed therapy (DOT), alcohol excess, HIV, drug resistance, smear positivity, poor compliance, cavitating disease and multilobar chest radiograph changes,

A two tailed p value<0.05 was considered to be statistically significant

RESULTS

There were 226 patients included in the study. Baseline demographics of the study cohort are summarized in table 1.

Correlation of pre-therapy globulin levels with microbiological and radiological disease burden

Measurement of inflammatory biomarkers prior to commencement of anti-tuberculous therapy identified 175 patients (77.4%) with an elevated serum globulin (>35 g/dL), 155 patients (68.6%) with an elevated serum CRP (>10mg/L) and 28 patients (12.4%) with an elevated white cell count (>11.0 x 10^9/L). Figure 1 shows correlation of pre-therapy levels of these biomarkers with microbiological and radiological markers of disease burden including smear positivity (fig 1 a-c), radiographic lobar involvement (fig 1d-f) and presence of cavitatory disease (fig 1 g-i).

Predictive value of pre-therapy serum inflammatory biomarkers for requirement of therapy extension

The value of pre-therapy serum CRP, globulin and white cell count levels for prediction of the requirement for extension of anti-tuberculous therapy (>6 months) was evaluated. eTable 1 shows reasons for therapy extension (supplementary data). Table 2 shows that increasing levels of serum globulin, CRP and white cell count were all significantly associated with increased frequency of requirement for therapy extension.

The sensitivity, specificity, positive and negative predictive values, positive and negative likelihood ratios and AUCs for pre-therapy globulin>45 g/L, CRP>50 mg/dL and White cell count>11 x 10^9/L with regards to prediction of requirement for therapy extension were evaluated. All tests had poor to moderate predictive value with globulin having the highest AUC (0.70, see table 3).

Multivariable analyses

On multivariable analysis, pre-therapy globulin >45 g/L was independently associated with requirement for therapy extension OR 3.42 (1.59 – 7.32; p <0.001). Pre-therapy CRP>50 mg/L and White cell count>11 x 10^9/L were not independently associated with therapy extension (see eTable 2).

Evaluation of serial inflammatory biomarker measurements for prediction of treatment outcome in pulmonary tuberculosis

Having investigated the predictive value of pre-therapy levels of inflammatory biomarkers, we next evaluated whether measurement of repeat biomarker levels at two months following initiation of therapy could predict treatment outcome. Table 2 shows rates of requirement for therapy extension stratified according to whether or not the levels of CRP, globulin or white cell count normalized at two-month measurement. Significantly increased rates of requirement for therapy extension were observed in patients in whom globulin or CRP failed to normalize by 2 months post initiation of therapy but no significant association was observed for normalization of white cell count (see table 2).

We next formally assessed the predictive value of normalization of globulin, CRP and white cell count at two-month measurement for identification of persistent smear and/or culture positivity and requirement for therapy extension. A globulin that normalized at 2 months had a negative likelihood ratio of 0.07 for excluding requirement for therapy extension (see table 3), indicating that this represents a clinically valuable rule-out test[26]. CRP and white cell count had poor negative likelihood ratios for excluding requirement for therapy extension.

Multivariable analysis

On multivariable analysis, an elevated globulin level that failed to normalize by two months was independently associated with requirement for therapy extension OR 6.13 (2.23–16.8; p<0.001). CRP that failed to normalize was also independently associated with therapy extension OR 3.0 (1.15 – 7.82; p = 0.025)(see eTable 2). An analysis of white cell count normalization could not be carried out due to only a small number of patients having elevated levels at baseline.

Sub-group analysis of baseline and serial biomarkers for prediction of treatment extension associated with persistent smear/culture positivity or failure of radiographic improvement

In addition to evaluation of inflammatory biomarkers as predictors of treatment extension, we also carried out a sub-analysis to evaluate these tests for prediction of surrogate markers of treatment response, persistent 2-month sputum smear/culture positivity and failure of radiographic improvement. Increasing pre-therapy levels of all three biomarkers correlated significantly with increased frequency of therapy extension associated with failure of radiographic improvement but not with persistent smear and/or culture positivity (see table 2). Significantly increased rates of persistent smear and/or culture positivity were observed in patients in whom globulin, CRP or white cell count did not normalize by 2 months post initiation of therapy. Patients in whom globulin or CRP did not normalize also had increased rates of therapy extension due to failure of radiographic improvement (see table 2). Similar to the outcome of requirement for treatment extension, a globulin that normalized at 2 months also had the lowest negative likelihood ratio for excluding treatment extension associated with persistent smear or culture positivity or failure of radiographic improvement (see table 3).

DISCUSSION

In this study we evaluated the predictive value of the routinely measured serum biomarkers CRP, globulin and white cell count for prediction of treatment outcome in patients treated for active pulmonary tuberculosis. We found that baseline pre-therapy levels of all three biomarkers correlated with the extent of radiological and microbiological disease burden and increasing pre-therapy biomarker levels were associated with increased frequency of requirement for therapy extension. However, after correction for other potential confounding variables, globulin>45 g/L was the only baseline biomarker found to be independently associated with treatment outcome.

All of the tests evaluated performed poorly as pre-therapy predictors of the clinically relevant outcome of requirement for therapy extension with AUC values $\leq$ 0.7, the threshold that represents a clinically useful test. This suggests that none of these tests could be used alone to accurately predict treatment outcome at baseline. Of the three markers evaluated, pre-therapy globulin had the highest AUC value as a baseline predictor of outcome. In particular, only 28 patients (12.3%) had an elevated white cell count prior to commencement of therapy which highlights that it is extremely unlikely to be clinically useful as a predictive biomarker. This was reflected in a low AUC value of 0.58. It is perhaps unsurprising that biomarkers were poorly predictive of length of treatment. This outcome is not solely dependent on mycobacterial burden or inflammatory response, which would be expected to correlate directly with serum levels of immune markers such as globulin, but may also be determined by other unrelated factors such as poor compliance with therapy or complications such as drug-induced liver injury.

In addition to assessing the value of pre-therapy biomarker levels, we also evaluated the predictive value of repeat measurement of inflammatory biomarkers at 2 months following treatment initiation to determine whether failure of normalization of these markers correlated with requirement for therapy extension. Failure of normalization of globulin or CRP was independently associated with requirement for therapy extension. However, globulin had the lowest negative likehood ratio for excluding requirement for therapy extension. It is recognized that a threshold of likelihood ratio

<0.1 is indicative of a clinically useful rule-out test.[26] The low negative likelihood ratio of globulin normalization at 2 months suggests it is a good marker of adequate response to therapy. Our data therefore suggest that measurement of globulin in patients commenced on anti-tuberculous therapy with subsequent normalization of this blood test by 2 months is associated with very low rates of requirement for treatment extension and raise speculation that globulin may thus be a useful adjunct to clinical judgment in identifying low-risk patients. By contrast, two-month CRP and white count measurement had high negative likelihood ratios thus suggesting lack of utility in a clinical setting.

Our finding that globulin could predict requirement for therapy extension in tuberculosis raises speculation it could be a useful marker in clinical practice. Serum globulin is a simple, cheap and widely available blood test. In most centres, all patients with active TB are routinely reviewed at 2 months to assess treatment response and decide whether therapy can be altered from intensive to continuation phase therapy. Therefore, our finding that normalization of globulin levels at two months can exclude requirement for treatment extension offers a predictive test that can be rapidly and reliably measured without the need for additional hospital visits. In combination with other recognized markers of treatment response, including weight gain[13], symptomatic improvement[12] and resolution of radiographic changes [8], serum globulin provides an additional clinical marker of treatment response that can be easily assessed by clinicians and could aid decisions regarding safe and appropriate conversion to continuation phase therapy.

The length of anti-tuberculous therapy is an important endpoint as it may have negative implications for patient compliance [27]. There is historical data suggesting that patients who respond early to therapy may be safely managed with a shortened course of antibiotic therapy [28] although this remains controversial and recent studies have reported worse outcomes for four month regimens [29, 30]. A test such as globulin that could stratify patients into risk groups to guide duration of treatment could potentially improve compliance, outcomes and treatment related costs. Further studies are required to determine whether globulin, alone or in combination with other predictors, could be used in this way.

Total Globulin level reflects a combination of specific proteins including the alpha globulins (such as alpha-1-antitrypsin and haptoglobulin), transferrin, complement and immunoglobulins. Previous studies have shown that complement C4 [31] and *M.tuberculosis* specific immunoglobulins [32, 33] are elevated in serum from patients with active TB. We did not formally carry out serum protein electrophoresis in our study to distinguish which sub-components are specifically elevated in patients with active tuberculosis but data from these previous studies offers a biologically plausible explanation for our finding that total globulin is elevated in patients with active TB and correlates with treatment outcomes. Additionally, as our study was observational in nature, we could not perform all measurements in all patients. The study may also be limited by sample size, as indicated by wide confidence intervals observed with some of our analyses.

In conclusion, we report that measurement of paired serum globulin samples at baseline and 2 months into therapy can identify patients at lower risk of requirement for therapy extension. Globulin outperformed the other biomarkers evaluated in our study. When combined with other clinical measures, globulin may provide clinicians with a rapid, simple means of identifying lower risk patients. Whether measurement of globulin could be used to predict other more robust measures of treatment success such as recurrent disease and TB-related death remains unknown and further studies in independent populations are now warranted.

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Summary of Conflicts of Interests:

AS has received honoraria for speaking from GlaxoSmithKline; JDC has received honoraria for speaking from Bayer, Griffols, AstraZeneca, GlaxoSmithKline, Pfizer and Napp; AL is inventor for several patents underpinning T cell based diagnosis. The ESAT-6/CFP-10 IFN-gamma ELISpot was commercialised by an Oxford University spin-out company (T-SPOT.TB, Oxford Immunotec, Abingdon, UK), in which Oxford University and AL have minority shares of equity and entitlement to royalties; OMK has chaired an advisory board for Janssen and spoken on postgraduate educational sessions for Janssen and Otsuka Pharmaceuticals at the European Respiratory Society

All other authors report no conflicts of interest.

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TABLES

Table 1: Baseline demographics of study population	
Characteristic	**n (%) or median (IQR)**
Demographics	
Age (median (IQR)	33 (25.3-49)
Male sex	148 (65.5%)
Born in UK	53 (23.5%)
Caucasian	55 (24.3%)
Black African	49 (21.7%)
Asian	51 (22.6%)
Other	71 (31.4%)
Comorbidities	
Chronic lung disease	15 (6.6%)
Diabetes mellitus	6 (2.7%)
Alcohol excess	11 (4.9%)
HIV	7 (3.1%)
Other immunosuppression	2 (0.9%)
Chronic renal failure	2 (0.9%)
Chronic liver disease	2 (0.9%)
Smoker	21 (9.3%)
Microbiology	
Smear negative	115 (50.9%)
Smear Grade:	
Scanty AFB	32 (14.2%)
+	18 (8.0%)
++	18 (8.0%)
+++	43 (19.0%)
Persistent smear and/or culture positivity (>60 days)	20 (8.9%)
Non MDR drug resistance	19 (8.4%)
Multi drug resistance	9 (4.0%)
Radiology	
Normal chest radiograph	35 (15.5%)
Cavitating disease	76 (33.6%)
Multi-lobar changes	78 (34.5%)
Pleural effusion	31 (13.7%)
Treatment outcome	
Requirement for extension of therapy (>6 months)	86 (38.1%)
TB recurrence	2 (0.9%)
TB-related death	2 (0.9%)
Abbreviations: AFB=Acid fast bacilli; HIV=human immunodeficiency virus; TB = tuberculosis	

Table 2: Outcomes stratified according to pre-therapy and two month biomarker levels				
	n	**Requirement for therapy extension n (%)**	**Persistent smear and/or culture positivity n(%)**	**Failure of radiographic improvement n(%)**
Pre-therapy Globulin (g/L)				
≤35	51	10 (19.6%)	4 (7.8%)	3 (5.9%)
36-40	56	17 (30.4%)	4 (7.1%)	4 (7.1%)
41-45	57	19 (33.3%)	5 (8.8%)	9 (15.8%)
46-50	42	24 (57.1%)	4 (9.5%)	12 (28.6%)
>50	20	16 (80.0%)	3 (15.0%)	9 (45%)
p value		<0.001	0.88	<0.002
Globulin fails to normalize by 2 months				
Yes	97	72 (74.2%)	12 (12.4%)	27 (27.8%)
No	78	4 (5.1%)	1 (1.3%)	4 (5.1%)
p value		<0.001	0.007	<0.001
Pre-therapy CRP (mg/L)				
≤10	71	14 (19.7%)	3 (4.2%)	8 (11.3%)
11-50	70	27 (38.6%)	5 (7.1%)	9 (12.9%)
51-100	45	21 (46.7%)	4 (8.9%)	7 (15.6%)
100-150	21	14 (66.7%)	4 (19.0%)	6 (28.6%)
>150	19	10 (52.6%)	3 (15.7%)	7 (36.8%)
p value		<0.001	0.184	0.039
CRP fails to normalize by 2 months				
Yes	42	27 (61.4%)	7 (16.7%)	12 (28.6%)
No	113	44 (28.9%)	4 (3.5%)	9 (8.0%))
p value		0.006	0.0095	0.0037
Pre-therapy White cell count ($x10^9$/L)				
<4.0	10	2 (20%)	1 (10.0%)	1 (10.0%)
4-11	188	66 (35.1%)	14 (7.4%)	26 (13.8%)
11-14	18	11 (61.1%)	3 (16.7%)	6 (33.3%)
>14	10	7 (70%)	1 (10.0%)	4 (40.0%)
p value		0.015	0.596	0.029
White cell count fails to normalize				
Yes	3	3 (100%)	2 (66.7%)	3 (100%)
No	25	14 (56%)	2 (8.0%)	19 (76.0%)
p value		0.258	0.045	1.0
Abbreviations: CRP = C-reactive protein				

Table 3: Evaluation of pre-therapy and two month biomarker levels for prediction of outcome

Test	Sensitivity	Specificity	PPV	NPV	PLR	NLR	AUC
PRE-THERAPY BIOMARKER LEVELS - THERAPY EXTENSION							
Globulin > 45 g/L	46.5% (35.7-57.6%)	84.3 % (77.2-89.9%)	64.5 % (51.3%-76.4)	72.0% (64.4 –78.7%)	2.96 (1.90 -4.62)	0.63 (0.51 –0.78)	0.70 (0.63–0.77)
CRP > 50 mg/L	52.3% (41.3 – 63.2%)	71.4 % (63.2 –78.7%)	52.9% (41.8 –63.9%)	70.9% (62.9 –78.3%)	1.83 (1.32 – 2.55)	0.67 (0.52-0.85)	0.67 (0.60-0.74)
WCC > 11 X 10^9/mL	12.8% (6.3 – 22.3%)	87.8% (81.5 –92.6%)	35.7% (18.6 –55.9%)	65.7% (58.6 – 72.2%	1.05 (0.51 – 2.17)	0.99 (0.89 –1.10)	0.58 (0.50 – 0.66)
TWO MONTH BIOMARKER LEVELS							
Globulin fails to normalize							
Therapy extension	94.7% (87.1-98.7%)	74.8% (65.0-82.9%)	74.2 % (64.6-82.6%)	94.9 % (87.-98.6%)	3.75 (2.66-5.29)	0.07 (0.03-0.18)	-
Persistent smear and/or culture positivity	92.3 % (64.0–99.8)	47.0% (39.6-55.5)	12.4% (6.6-20.6)	98.7% (93.0 – 100.0)	1.76 (1.42-2.18)	0.16 (0.02 -1.07)	-
Failure of radiographic improvement	81.7% (70.2-96.4)	51.4% (42.9-59.8)	27.8 % (19.2-37.9)	94.9 % (87.4-98.6)	1.79 (1.44-2.22)	0.25 (0.10-0.64)	-
CRP fails to normalize							
Therapy extension	38.0% (26.8-50.3%)	82.1% (72.3-89.7%)	64.3 % (48.0-78.5%)	61.1 % (51.4-70.1%)	2.13 (1.23-3.68)	0.75 (0.61-0.93)	-
Persistent smear and/or culture positivity	17.7 % (3.8– 43.4%)	100 % (71.5-100%)	100 % (29.2-100%)	44 % (24.4-65.1%)	-*	0.82 (0.66-1.03)	-
Failure of radiographic involvement	57.1 % (34.0 – 78.2)	75.8 % (67.3 – 83.0)	28.6 % (15.7-44.6)	91.3% (84.6-95.9)	2.36 (1.46-3.83)	0.57 (0.34-0.94)	-
White cell count fails to normalize							
Therapy extension	17.7 % (3.8-43.4)	100 % (71.5-100)	100 % (29.2-100)	44.0 % (24.7-65.1)	-*	0.82 (0.66-1.03)	-
Delayed smear and/or culture positivity	33.3 % (0.8-90.6)	92.0 % (74.0-99.0)	33.3 % (0.8-90.6)	92.0 % (74.0-99.0)	12.0 (1.39-103.48)	0.52 (0.20-1.40)	-
Failure of radiographic involvement	13.6 % (2.9-34.9)	100 % (54.1-100.0)	100 % (29.2-100.0)	24 % (9.4-45,1)	n/a	0.86 (0.73-1.02)	-

NLR = negative likelihood ratio; PLR = positive likelihood ratio; NPV = negative predictive value; PPV = positive predictive value

* Unable to calculate

Figure Legend

Figure 1: Correlation between pre-therapy biomarker levels and microbiological and radiological markers of disease burden. Box and whisker plot displaying showing median globulin, CRP and white cell count levels stratified according to (a-c) smear grade (d-f) lobar involvement on chest radiograph and (g-i) presence of cavitating disease on chest radiograph. Comparison of groups by Kruskal Wallis test in (a) and (b) and Mann-Witney U test in (c). Abbreviations: AFB = acid-fast bacilli.