Coagulation and fibrinolysis in tuberculosis, melioidosis and beyond
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Pulmonary tuberculosis induces a systemic hypercoagulable state

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Submitted
Chapter 2

ABSTRACT

Background
Human tuberculosis (TB) remains an important cause of death globally and Bangladesh is one of the most affected countries. Small studies have linked TB to increased risk of deep venous thrombosis. We here aimed to investigate the impact of pulmonary TB on pro- and anticoagulant mechanisms in the circulation.

Methods
This prospective study was conducted in Chittagong, Bangladesh. We performed an in-depth analysis of coagulation activation and inhibition in plasma obtained from 64 patients with primary lung TB and 11 patients with recurrent lung TB and compared these with 37 healthy controls. Additionally, in nine patients coagulation activation was studied in bronchoalveolar lavage fluid (BALF) harvested from the site of infection and compared with BALF from a contralateral unaffected lung subsegment.

Results
Relative to uninfected controls, primary and recurrent TB was associated with a systemic net procoagulant state, as indicated by enhanced activation of coagulation (elevated plasma levels of thrombin-antithrombin complexes, D-dimer and fibrinogen) together with impaired anticoagulant mechanisms (reduced plasma levels of antithrombin, protein C activity, free protein S, and protein C inhibitor, prolonged clotting times). Moreover, TB patients demonstrated clear evidence of endothelial cell activation. Activation of coagulation did not correlate with plasma concentrations of established TB biomarkers. Coagulation activation could not be detected at the primary site of infection in a subset of TB patients.

Conclusions
Pulmonary TB is associated with a systemic hypercoagulable state.
INTRODUCTION

Tuberculosis (TB), caused by the acid-fast bacterium *Mycobacterium (M.) tuberculosis*, is one of the most devastating infectious diseases worldwide, with one-third of the world population being infected\(^1,2\). In 2011, globally 8.7 million people became infected and 1.4 million people died from this disease\(^2\). Bangladesh is one of the most affected countries with an annual incidence in 2011 of 225 new cases per 100000 inhabitants and an overall mortality rate of 45 per 100000\(^2\). Treatment of TB involves prolonged antibiotic regimens. *M. tuberculosis* bacilli that are not fully eradicated from the lungs remain a potential danger to the infected individual and his/her surrounding people\(^1,3\). This emphasizes the importance of understanding host response mechanisms during TB.

There is ample evidence that during severe acute pulmonary infections, in addition to activation of inflammatory pathways, haemostatic changes occur\(^4,5\). These changes include increased procoagulant activity, decreased expression of anticoagulant factors, and suppression of the fibrinolytic system, which in most severe cases can result in disseminated intravascular coagulation and microvascular thrombosis\(^4,5\). In patients with acute lower respiratory tract infections, procoagulant changes are also detected at the primary site of infection, in the bronchoalveolar space\(^6-9\). Previous investigations have indicated that pulmonary TB may be associated with activation of coagulation in the circulation, as reflected by elevated plasma levels of fibrinogen\(^10,11\). Several case reports and small series have suggested a link between TB and deep venous thrombosis, further pointing to a procoagulant state in these patients\(^12-16\).

Thus far, detailed analyses of haemostatic disturbances in patients presenting with TB have not been reported. Therefore, in this prospective study we aimed to get more insight into activation of pro- and anticoagulant mechanisms in the circulation of patients with pulmonary TB. In addition, we measured activation of the coagulation system in bronchoalveolar lavage fluid (BALF) obtained from a subset of patients who underwent a diagnostic bronchoscopy because of clinically suspected TB.

MATERIALS AND METHODS

Study design and population

In this observational prospective study we aimed to investigate systemic (Part A) and local (Part B) host responses with respect to coagulation, anticoagulation, and fibrinolysis during active pulmonary TB. Patients were screened at the Tuberculosis Clinic of Chittagong General Hospital, Chittagong, Bangladesh and in the Chittagong Medical College & Hospital, Chittagong, Bangladesh. Written informed consent was obtained from all study subjects or next-of-kin by a native Bengali speaker. Inclusion criteria for study enrolment were: (a) 18-80 years of age; (b) confirmed *M. tuberculosis* infection; (c) ability to give written informed consent prior to study-specific procedures. For study enrolment, pulmonary TB was considered confirmed when at least two out of three
sputum samples collected on two consecutive days, including an early morning sample, tested posi-
itive on Ziehl-Neelsen (ZN)-staining. Subsequent diagnostic confirmation was done batch-wise
on stored specimens by a PCR-method for \textit{M. tuberculosis} (GeneXpert, Cepheid, Solna, Sweden)
performed at the Department of Medical Microbiology in the Academic Medical Center in
Amsterdam, the Netherlands. Patients were divided into two study groups: patients with a
primary pulmonary TB who had never been treated for TB before and patients with recurrent
pulmonary TB who had been treated for TB in the past. Exclusion criteria and further study details are
described in the ‘Supplementary Material’. Blood samples (citrated, EDTA and heparinized blood)
were taken at first presentation of the patient. In Part B of the study this was followed by a bilateral
bronchoalveolar lavage (BAL) to obtain BALF in a subset of patients with negative Ziehl-Neelsen
(ZN) results of sputum, but with a strong clinical suspicion of pulmonary TB and with chest
X-ray abnormalities consistent with TB. For Part A, local healthy blood donors served as controls.
The study was approved by the National Research Ethics Committee (NREC), Bangladesh Medical
Research Council, Bangladesh and the Oxford Tropical Research Ethics Committee, University of

\textbf{Assays}
BALF was centrifuged at 300g for ten minutes. Blood was centrifuged at 1500g for ten minutes.
Supernatants were snap-frozen in liquid nitrogen and stored at -80ºC until analysis. All assays are
described in detail in the ‘Supplementary Material’.

\textbf{Statistical analysis}
Comparisons between groups were performed using the Mann-Whitney \textit{U} test. Comparisons
between paired BALF samples were performed using a Wilcoxon signed rank test. Analyses were
done using GraphPad Prism version 5.01 (San Diego, CA). Correlations were calculated using the
Spearman rho test in SPSS statistical package version 16.0 (Armonk, NY). \textit{P}-values < 0.05 were
considered statistically significant.

\textbf{RESULTS}

\textbf{Patient characteristics}
For Part A of the study, 37 healthy blood donors, 64 patients with primary TB and 11 patients
with recurrent TB were recruited. In all patients TB was confirmed by PCR of sputum. For Part
B, 23 consecutive sputum ZN-negative patients with a clinical suspicion of TB underwent bron-
choscopy with bilateral BAL. Of these, nine patients (39\%) tested positive for TB in BALF by
\textit{M. tuberculosis} PCR. Patient characteristics and clinical features are summarized in Table 1.
### Table 1. Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>PART A</th>
<th>PART B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healthy controls</td>
<td>Primary TB</td>
</tr>
<tr>
<td><strong>n = 37</strong></td>
<td><strong>n = 64</strong></td>
<td><strong>n = 11</strong></td>
</tr>
<tr>
<td><strong>Demographics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>28 (24-34)</td>
<td>28 (22-42)</td>
</tr>
<tr>
<td>Male sex (n, %)</td>
<td>29 (78%)</td>
<td>45 (70%)</td>
</tr>
<tr>
<td>BCG-vaccinated (n, %)</td>
<td>24 (65%)</td>
<td>23 (36%)</td>
</tr>
<tr>
<td>HIV-positive (n, %)</td>
<td>0 (0%)</td>
<td>2 (3%)</td>
</tr>
<tr>
<td><strong>Symptoms</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fever (n, %)</td>
<td>0 (0%)</td>
<td>64 (100%)</td>
</tr>
<tr>
<td>Night sweats (n, %)</td>
<td>0 (0%)</td>
<td>24 (38%)</td>
</tr>
<tr>
<td>Weight loss (n, %)</td>
<td>0 (0%)</td>
<td>47 (73%)</td>
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<tr>
<td>Fatigue (n, %)</td>
<td>1 (3%)</td>
<td>33 (52%)</td>
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<tr>
<td>Shortness of breath (n, %)</td>
<td>1 (3%)</td>
<td>7 (11%)</td>
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<tr>
<td>Productive cough (n, %)</td>
<td>4 (11%)</td>
<td>58 (91%)</td>
</tr>
<tr>
<td><strong>Signs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>36.9 (36.5-37.1)</td>
<td>37.2 (36.8-38.1)**</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>83 (79-93)</td>
<td>80 (70-87)*</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>80 (78-86)</td>
<td>90 (80-100)**</td>
</tr>
<tr>
<td>Respiratory rate (brpm)</td>
<td>20 (20-23)</td>
<td>24 (22-28)**</td>
</tr>
<tr>
<td>BMI (w/l²)</td>
<td>23.9 (22.2-25.9)</td>
<td>17.6 (15.3-19.6)**</td>
</tr>
</tbody>
</table>

Abbreviations: BMI body mass index, expressed as weight (w) divided by length (l)²; bpm beats per minute; brpm breaths per minute; MAP mean arterial blood pressure; n total number; TB tuberculosis. Percentages given are within study group. Data are medians with interquartile ranges. *P < 0.05, ***P < 0.001 for the difference between primary TB or recurrent TB-patients versus controls.
Systemic coagulation is activated in patients with pulmonary TB
Primary TB was associated with activation of coagulation as reflected by elevated plasma concentrations of thrombin-antithrombin complexes (TATc; \( P < 0.01 \) for primary TB patients versus controls) and D-dimer (\( P < 0.001 \) for primary TB patients versus controls; Figure 1A-B). D-dimer levels were also significantly elevated in recurrent TB patients compared to controls (\( P < 0.001 \), Figure 1B). Plasma fibrinogen levels were strongly elevated in patients with primary and recurrent TB (both \( P < 0.001 \) versus controls; Figure 1C). In addition, both prothrombin time (PT) and activated partial thromboplastin time (aPTT) were prolonged in primary and recurrent TB patients compared to controls (\( P < 0.001 \) and \( < 0.05 \) for the differences between primary and recurrent TB patients respectively compared to controls; Figure 1D-E). To further determine the relative contribution of the different clotting factors in the systemic procoagulant response during pulmonary TB, we measured concentrations of the coagulation proteins affecting PT and aPTT (Figure 2). Of the proteins affecting PT, factor VII (Figure 2A) and factor X (Figure 2B) were decreased in both patients with TB and recurrent TB. Factor II was unaffected (Figure 2C), while factor V was increased in primary TB patients (Figure 2D). Strikingly, of the factors affecting aPTT (factors XI, IX, VIII, X, V, II), only factors XI and X were decreased in recurrent and both primary and recurrent TB patients, respectively (Figure 2E-F). In contrast, factor VIII and factor V were increased in primary TB-patients (Figure 2D) and in both primary and recurrent TB patients (Figure 2G), respectively.

Systemic anticoagulant pathways are downregulated during pulmonary TB
Activation of coagulation in pulmonary TB was associated with a depression of anticoagulant pathways: antithrombin and protein C levels were lower in both primary and recurrent TB patients compared to controls (Figure 3A-B). The calculated TATc/protein C ratio was increased in primary TB patients (\( P < 0.001 \), data not shown), which illustrates a misbalance in pro- and anticoagulant factors, representing a net procoagulant state. Levels of protein C inhibitor, a sensitive marker for activated protein C (APC) generation\(^\text{17}\), were significantly decreased in both primary and recurrent TB patients (Figure 3C). Total protein S concentrations were slightly enhanced in primary TB patients compared to controls (Figure 3D; \( P < 0.05 \)), whereas free protein S was strongly decreased in patients with primary TB (Figure 3E; \( P < 0.001 \)). Levels of C4b-binding protein, which binds protein S\(^\text{18}\), were increased in both primary and recurrent TB patients when compared to controls (Figure 3F; \( P < 0.001 \) and 0.01 respectively). In addition, levels of soluble endothelial protein C receptor (EPCR) were significantly decreased in both primary and recurrent TB patients compared to controls (\( P < 0.01 \) for both comparisons; Figure 3G), whereas levels of soluble thrombomodulin did not show significant differences between the study groups (Figure 3H).

Influence of pulmonary TB on endogenous thrombin potential
To investigate the functionality of the detected changes in coagulation factors, we determined the endogenous thrombin potential, a measure of the coagulation potential of plasma induced by tissue factor\(^\text{19}\). As shown in Figure 4, the lag-time and time-to-peak were prolonged both in primary and
Figure 1. Plasma coagulation is activated during pulmonary TB. Significant increases in (A) plasma levels of thrombin-antithrombin complexes (TATc) and (B) D-dimer levels were seen in primary (black dots; n = 64) and recurrent (black squares; n = 11) TB patients compared to healthy controls (open dots; n = 37). In addition, primary and recurrent TB-patients showed strongly elevated levels of fibrinogen when compared to healthy controls (C). In addition, increases in (D) prothrombin time (PT) and (E) activated partial thromboplastin time (aPTT) were detected in both primary and recurrent TB patients as compared to controls. Data are expressed as dot plots with medians. *P < 0.05, **P < 0.01, ***P < 0.001 for the difference between primary TB and recurrent TB patients versus controls.

Figure 2. Clotting factors in plasma during pulmonary TB. Clotting factors (F) (A) VII, (B) X, (C) II, (D) V, (E) XI, (F) IX and (G) VIII were measured and expressed in percentages of change compared to normal pooled plasma. Primary (black dots; n = 64) and recurrent (black squares; n = 11) TB patients were compared with healthy controls (open dots; n = 37). Data are expressed as dot plots with medians. *P < 0.05, **P < 0.01, ***P < 0.001 for the difference between primary TB and recurrent TB patients versus controls. #P < 0.05 for the difference between primary and recurrent TB patients.
Figure 3. Systemic anticoagulant pathways are downregulated during pulmonary TB. Both primary (black dots; n = 64) and recurrent (black squares; n = 11) pulmonary TB was associated with a decrease in (A) antithrombin and (B) protein C (PC) when compared to healthy controls (open dots; n = 37). Levels of protein C inhibitor (PCI; C) were strongly decreased both in primary and recurrent TB patients. Levels of (D) total protein S (PS tot) and (E) free protein S (PS free) were elevated in primary, but not in recurrent TB patients. In addition, levels of (H) C4b-binding protein (C4bBP) were strongly enhanced in both primary and recurrent pulmonary TB patients when compared to healthy controls. Finally, levels of (G) soluble endothelial protein C receptor (sEPCR) were significantly decreased in both primary and recurrent TB patients when compared to controls, while levels of (H) soluble thrombomodulin (sTM) did not display any differences between groups. Data are expressed as dot plots with medians. *P < 0.05, **P < 0.01, ***P < 0.001 for the difference between primary TB and recurrent TB patients versus controls. Levels of antithrombin, protein C, free and total protein S, PCI and C4bBP are expressed in percentages of change compared to normal pooled plasma.
Coagulation in tuberculosis

recurrent TB patients (Figure 4A-B), in line with the observed prolonged PTs in these patients. In contrast, the peak (Figure 4C) and area-under-the-curve (AUC; Figure 4D) were significantly higher in primary TB patients, suggesting that although initiation of coagulation may be delayed, the amount of thrombin that can be formed is higher in these patients. These higher amounts of thrombin are most likely the result of the affected anticoagulation pathways, since antithrombin is an important determinant of the endogenous thrombin potential19.

Activation and inhibition of systemic fibrinolysis in pulmonary TB

Earlier studies in TB patients demonstrated evidence of activation and inhibition of fibrinolysis, as reflected by elevated plasma concentrations of tissue-type plasminogen activator (tPA) and plasminogen activator inhibitor type I (PAI-1)10,11. Our data confirm these results by showing elevated tPA levels in both primary and recurrent TB patients in comparison to controls (P < 0.01 and < 0.05 respectively; Figure 5A) and elevated levels of α2-antiplasmin in primary TB patients (P < 0.001; Figure 5B). No differences in PAI-1 could be detected between the study groups (Figure 5C).

Activation of the vascular endothelium during pulmonary TB

During proinflammatory conditions, the endothelium becomes activated resulting in attraction of leukocytes and thrombus formation20. The large multimer von Willebrand factor, secreted by endothelial cells, is an acute phase protein and capable of binding platelets and clotting factors20. Under normal circumstances ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) regulates von Willebrand factor levels by proteolytical degradation of the multimers20. Both primary and recurrent pulmonary TB were associated with decreased ADAMTS13 concentrations (P < 0.001 for both groups compared to controls; Figure 6A) and, consequently, with enhanced levels of von Willebrand factor antigen (P < 0.001 for both groups, Figure 6B), suggesting that pulmonary TB is associated with systemic activation of the vascular endothelium.

Systemic coagulation activation in pulmonary TB patients correlates weakly with circulating levels of TB biomarkers

We next sought to examine whether the extent of coagulation activation in TB patients was correlated with the plasma levels of established TB biomarkers. For this we used plasma TATc, D-dimer and the TATc/protein C ratio as readout for systemic activation of coagulation. As biomarkers, we assessed plasma IL-6 and IL-8 (elevated in patients with active TB21), as well as soluble IL-2 receptor-α, soluble intercellular adhesion molecule 1, soluble tumour necrosis factor receptor type 1 and type 2 and C-reactive protein (all established markers for severity of disease)21; this analysis was restricted to patients with primary TB. In accordance with previous research3,21, patients with TB showed elevated plasma levels of all biomarkers measured (Table 2). Only C-reactive protein and both soluble tumour necrosis factor receptor subtypes showed significant yet weak correlations with plasma TATc, D-dimer or TATc/PC ratio (Spearman’s rho < 0.35; Table 3).
Figure 4. Influence of pulmonary TB on endogenous thrombin potential (ETP). Both primary (black dots; n = 64) and recurrent (black squares; n = 11) pulmonary TB was associated with an increase in (A) lag-time, (B) time-to-peak, (C) peak and (D) area-under-the-curve (ETP) when compared to controls (open dots; n = 37). Data are expressed as dot plots with medians. *P < 0.05, ***P < 0.001 for the difference between primary and recurrent TB patients versus controls. AUC area-under-the-curve.

Figure 5. Activation and inhibition of systemic fibrinolysis in pulmonary TB. Activation and inhibition of systemic fibrinolysis in primary and recurrent TB patients was reflected by elevated plasma concentrations of (A) tissue-type plasminogen activator (tPA) and (B) α2-antiplasmin (α2AP) in primary (black dots; n = 64) and recurrent (black squares; n = 11) TB patients compared to healthy controls (open dots; n = 37). No differences were seen for (C) levels of plasminogen activator inhibitor type I (PAI-1). Data are expressed as dot plots with medians. *P < 0.05, **P < 0.01, ***P < 0.001 for the difference between primary TB and recurrent TB patients versus controls. Levels of α2AP are expressed in percentages of change compared to normal pooled plasma.
Figure 6. Activation of the endothelium during pulmonary TB. Both primary (black dots; \( n = 64 \)) and recurrent (black squares; \( n = 11 \)) pulmonary TB was associated with a strong decrease in (A) plasma ADAMTS13 and a profound increase in plasma von Willebrand Factor (vWF Ag) levels (B) when compared to healthy controls (open dots; \( n = 37 \)). Data are expressed as dot plots with medians. ***\( P < 0.001 \) for the difference between primary or recurrent TB patients versus controls. Levels of ADAMTS13 and vWF Ag are expressed in percentages of change compared to normal pooled plasma.

Figure 7. Lung TB does not result in enhanced coagulation at the primary site of infection. In patients (\( n = 9 \)) BALF of \textit{M. tuberculosis} positive lung subsegments (black dots) was compared with BALF of contralateral control lung subsegments (white dots). With respect to proteins involved in coagulation, only TATc (A) and PAI-1 (B) were detectable, and no differences were found between the infected lung subsegment and the contralateral control subsegment. Paired data per patient are shown.
We next studied the extent of coagulation activation at the primary site of infection. For this we performed bilateral BAL in nine patients with negative ZN smears of sputum in whom pulmonary TB was confirmed by *M. tuberculosis* PCR of BALF. BALF was harvested from the area showing an infiltrate on the chest X-ray and from the unaffected contralateral site. Of the proteins involved in the regulation of coagulation and fibrinolysis measured in plasma, only TATc and PAI-1 were detectable in BALF, but no significant differences were found between the infected and non-infected lung (Figure 7A-B).

### DISCUSSION

There is ample evidence that acute infection can result in systemic activation of the coagulation system. However, knowledge of alterations in the haemostatic mechanism during chronic infection in general and during pulmonary TB in particular is limited. We here demonstrate that pulmonary TB is associated with a net procoagulant state in the circulation, as reflected by enhanced activation of coagulation and the vascular endothelium with concurrent impairment of anticoagulant pathways. Despite this obvious prothrombotic state at the systemic level, TB patients did not exhibit increased risk of thrombosis.

### Table 3. Correlations of plasma TATc, D-dimer and the TATc/protein C ratio with TB biomarkers in patients with primary lung TB

<table>
<thead>
<tr>
<th></th>
<th>TATc</th>
<th>D-dimer</th>
<th>TATc/PC ratio</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>( r_s )</td>
<td>( P )</td>
<td>( r_s )</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.206</td>
<td>0.112</td>
<td>0.094</td>
</tr>
<tr>
<td>IL-8</td>
<td>0.206</td>
<td>0.112</td>
<td>0.004</td>
</tr>
<tr>
<td>sIL-2RA</td>
<td>0.204</td>
<td>0.122</td>
<td>-0.105</td>
</tr>
<tr>
<td>sICAM-1</td>
<td>0.183</td>
<td>0.158</td>
<td>0.155</td>
</tr>
<tr>
<td>sTNFR1</td>
<td>0.327</td>
<td>0.010</td>
<td>0.285</td>
</tr>
<tr>
<td>sTNFR2</td>
<td>0.229</td>
<td>0.073</td>
<td>0.300</td>
</tr>
<tr>
<td>CRP</td>
<td>0.270</td>
<td>0.032</td>
<td>0.299</td>
</tr>
</tbody>
</table>

Abbreviations: CRP C-reactive protein; IL interleukin; PC protein C; \( r_s \) Spearman rank coefficient of correlation adjusted for multiple comparisons using Bonferroni’s procedure; sICAM-1 soluble intercellular adhesion molecule-1; sIL-2RA soluble interleukin-2 receptor antigen; sTNFR1,2 soluble tumour necrosis factor receptor-1 and -2; TATc thrombinantithrombin complexes. Values are median (IQR).
demonstrate clear evidence for coagulation activation at the primary site of infection.

Our study is the first to present a detailed analysis of pro- and anticoagulant mechanisms in pulmonary TB patients. Using a series of assays we documented a hypercoagulable state in patients with TB, as indicated by elevated plasma levels of TATc, D-dimer, and fibrinogen. Moreover, prolonged clotting times (PT and aPTT) in this setting of infection are also consistent with coagulation activation and a hypercoagulable state. While the increase in PT may be explained by the observed decrease in factors VII and X, an explanation for the prolonged aPTT (influenced by factors XI, IX, VIII, X, V, II) is more difficult, as only factors XI and X were (moderately) decreased while factors VIII and V were increased in TB patients. Possibly, aPTT prolongation was the result of the inhibitory effects of high fibrinogen concentrations in TB patients considering that hyperfibrinogemia may suppress thrombin generation. The functionality of these changes was evaluated by the endogenous thrombin potential, wherein the prolonged lag-time and time-to-peak in TB patients are in accordance with the prolonged PT and aPTT. The higher endogenous thrombin potential ‘peak’ levels in TB patients suggest that once coagulation is initiated this response is stronger than in healthy controls, which is in line with the higher TATc levels in these patients. Our detailed analyses build on previous studies that reported elevated fibrinogen levels in TB patients.

Under normal circumstances the coagulation system is balanced by adequate anti-coagulation. However, during proinflammatory conditions, such as acute pneumonia and sepsis, derangement of anticoagulant mechanisms has been observed both in the lungs and systemically. Apart from antithrombin, APC is an important anticoagulant with also coagulation-independent anti-inflammatory properties. Activation of protein C into APC is initiated by binding of thrombin to thrombomodulin and this activation is strongly augmented by EPCR. Protein S serves as a cofactor for protein C. Our data show that during pulmonary TB anticoagulant pathways are disrupted, as we measured significant decreases in antithrombin, protein C, and free protein S. The reduced free protein S concentrations can be explained at least in part by the increased levels of C4b-binding protein in TB patients since ~70% of protein S circulates in complex with C4b-binding protein. Moreover, we show strongly decreased protein C inhibitor levels in TB-patients. Since this protein can be used as a marker for APC-activation, these data suggest that the availability of APC is altered during pulmonary TB. Altogether we provide clear evidence that lung TB is associated with an impairment of anticoagulant mechanisms at the systemic level.

Plasma soluble EPCR concentrations were decreased in pulmonary TB patients. Soluble EPCR is generated upon shedding from membrane-bound EPCR, which is accelerated under pro-inflammatory conditions. Recently, we found decreased endothelial cell-associated EPCR expression in lung granulomas of TB patients. While membrane-bound EPCR clearly is important for anticoagulation, the function of soluble EPCR remains to be established. Recent evidence from our laboratory showed that EPCR and the protein C system do not play a role in immune defence against M. tuberculosis infection in mice, suggesting that the procoagulant changes described here are not
part of a protective host response mechanism during TB.

Patients with TB demonstrated elevated circulating levels of tPA (the main activator of plasminogen), α2-antiplasmin (the main inhibitor of plasmin) and D-dimer (a fibrin degradation product), pointing to both activation and inhibition of fibrinolysis. Our data confirm an earlier study showing increased tPA levels in pulmonary TB patients\textsuperscript{10}. However, we could not confirm previous reports on the association of elevated plasma levels of PAI-1 (the main inhibitor of plasminogen activators) and pulmonary TB\textsuperscript{10,11}.

Activation of the vascular endothelium controls the recruitment of leukocytes to the site of infection, but might induce also a procoagulant environment favouring thrombus formation\textsuperscript{20}. Endothelial cells secrete large von Willebrand factor multimers into the circulation upon activation, which are cleaved by ADAMTS13\textsuperscript{20}. Acute severe infections, such as sepsis, has been associated with decreased ADAMTS13 activity, resulting in increased von Willebrand multimer levels accompanied by coagulation activation and formation of microthrombi\textsuperscript{29}. We here demonstrate for the first time that during pulmonary TB the endothelium becomes activated, as reflected by increased von Willebrand factor and decreased ADAMTS13 levels, which are expected to further augment the hypercoagulable state in these patients.

Acute infection of the lower airways is associated with local activation of coagulation\textsuperscript{7-9,23,24}. We could not demonstrate any significant differences in parameters for coagulation between affected and non-affected subsegments of \textit{M. tuberculosis} infected lungs. We cannot exclude that local coagulation activation does occur in patients with lung TB since we studied only a limited number of patients with ZN-negative sputa who had limited radiologic abnormalities in their lungs. Based on the observed differences in nine patients, it can be calculated that a sample size of 44 and 81 patients would be required to show a similar but significant difference between affected and non-affected subsegments in TATc and PAI-1 levels respectively. In accordance, a previous study also reported a trend towards enhanced procoagulant activity in BALF of patients with lung TB\textsuperscript{30}. Taken together it is unlikely that local haemostatic alterations are the driving force behind the hypercoagulable state at the systemic level in patients with lung TB.

In conclusion, in this study we demonstrated that pulmonary TB is associated with systemic activation of coagulation with concurrent impairment of anticoagulant mechanisms, resulting in a net procoagulant state. The main parameters indicative for the hypercoagulable state in TB patients showed only a weak albeit significant correlation with a number of established TB biomarkers\textsuperscript{21} suggesting that coagulation parameters do not fully reflect the extent of disease. Several case reports and small series have suggested an association between TB and deep venous thrombosis\textsuperscript{12-16}. The observed altered haemostatic balance in our study will indeed favour thrombus formation.
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Chapter 2

REFERENCES

SUPPLEMENTARY MATERIAL

MATERIALS AND METHODS

Study design and population
In this observational study we aimed to investigate the systemic (Part A) and local (Part B) host responses with respect to coagulation, anticoagulation and fibrinolysis during active pulmonary TB. For Part A, patients were recruited prospectively in the Tuberculosis Clinic of Chittagong General Hospital, Chittagong, Bangladesh and in the Chittagong Medical College & Hospital, Chittagong, Bangladesh. Inclusion criteria were: (a) 18-80 years of age; (b) confirmed \textit{M. tuberculosis} infection; (c) ability to give signed and dated informed consent prior to study-specific procedures. Pulmonary TB was considered confirmed when at least two out of three sputum samples collected on two consecutive days, including an early morning sample, tested positive on Ziehl-Neelsen (ZN)-staining. TB-positivity was confirmed by PCR on \textit{M. tuberculosis} (GeneXpert, Cepheid, Solna, Sweden) in the Laboratory of Microbiology in the Academic Medical Center in Amsterdam, the Netherlands. Exclusion criteria were: (a) concomitant disease or clinical condition which could interfere with the conduct of the study; (b) unwillingness or inability to comply with the study protocol for any other reason. Patients were divided into two study groups: patients with a primary pulmonary TB who had never been treated for TB before and patients with a recurrent pulmonary TB who had been treated for TB in the past. Healthy blood donors were recruited from the Chittagong Medical College & Hospital and served as controls. Of all patients and controls blood samples (citrated, EDTA and heparinized blood) were taken directly at presentation.

For Part B patients were recruited prospectively in the Tuberculosis Clinic of Chittagong General Hospital, Chittagong, Bangladesh. Inclusion criteria were: (a) 18-65 years of age; (b) clinical suspicion of pulmonary TB, according to the WHO-based National Guidelines for Bangladesh\textsuperscript{1}, as presented by persistent cough for three weeks or more, with or without production of sputum despite the administration of non-specific antibiotics or if three of the following clinical signs and symptoms were present: shortness of breath, chest pain, coughing up of blood, unintended weight loss (at least 10% of body weight), loss of appetite, fever (body temperature > 38.5°C), night sweats; (c) three consecutive sputum samples tested negative for TB on ZN-staining; (d) no TB treatment started; (e) unilateral abnormalities on chest X-ray suspect for TB; (f) able to give signed and dated informed consent prior to any study-specific procedures. Exclusion criteria were: (a) concomitant disease or clinical condition which could interfere with the conduct of the study, or due to which a bronchoscopy cannot be performed; (b) unwillingness or inability to comply with the study protocol for any other reason. All patients were tested for human immunodeficiency virus (HIV) infection by a Determine\textsuperscript{®} HIV 1/2 test (Alere, Tilburg, The Netherlands). Of all patients and controls blood samples (citrated, EDTA and heparinized blood) were taken directly at presentation, followed by a bilateral bronchoalveolar lavage (BAL) to obtain BALF.
We considered it unethical to perform bronchoscopies in patients with ZN-positive sputum samples as this would not contribute to the diagnosis; therefore we decided to include ZN-negative patients, with a high clinical suspicion for TB. The final diagnosis ‘TB’ was made by ZN-staining of BALF or by PCR on *M. tuberculosis* (GeneXpert) of BALF. The aim of Part B of this study was to compare BALF from a diseased lung subsegment with BALF from a control lung subsegment in the same patient. Before the bronchoscopy was performed, the exact location of the diseased lung subsegment was identified by a chest X-ray. Then, a bilateral BAL was performed by well-qualified pulmonologists in a standardized fashion according to the guidelines of the American Thoracic Society, using a flexible direct bronchoscope (Olympus type C30C, P20D and P40, Shinjuku, Tokyo, Japan). Eight successive 20-mL aliquots sterile saline 0.9% were instilled at the uninfected side in a subsegment of the middle lobe or lingula and aspirated immediately with low suction. This was immediately followed by instillation and aspiration of the same amount of aliquots in the contralateral, diseased lung subsegment. The study was approved by the National Research Ethics Committee (NREC), Bangladesh Medical Research Council, Bangladesh and the Oxford Tropical Research Ethics Committee, University of Oxford, Oxford, UK (OXTREC 35-09). Written informed consent was obtained from all study subjects or next-of-kin by a native Bengali speaker.

**Assays**

BALF was centrifuged at 300 g for ten minutes. Blood was centrifuged at 1500 g for ten minutes. Supernatants were snap-frozen in liquid nitrogen and stored at -80°C until analysis. All coagulation assays were performed in citrated plasma, cytokine measurements were performed in EDTA-plasma. Prothrombin time (PT), activated partial thromboplastin time (aPTT), α2-antiplasmin, antithrombin, and coagulation factor II, V, VII, VIII, IX, X and XI activity, and D-dimer levels were measured using an automated blood coagulation analyzer (BCS® XP, Siemens Healthcare Diagnostics, Marburg, Germany) using reagents and protocols of the manufacturer. Fibrinogen levels were derived from the change in optical signal in the PT. ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) activity was determined as described. Thrombin-antithrombin complexes (TATc; Affinity Biologicals, Ancaster, Ontario, Canada), soluble endothelial protein C receptor (R&D systems, Minneapolis, MN) and soluble thrombomodulin (Cell Sciences, Canton, MA) were measured using commercial available enzyme-linked immunosorbent assays (ELISA). Protein C activity was measured by a kinetic assay (Coamatic, Chromogenix, Mölndal, Sweden). Total Protein S and von Willebrand factor-antigen levels were determined with in-house ELISAs using antibodies from Dako (Glostrup, Denmark). Free Protein S was measured by precipitating the C4b-binding protein-bound fraction with polyethylene glycol 8000 and measuring the concentration of free Protein S in the supernatant. C4b binding protein levels were determined by ELISA using a combination of monoclonal antibodies against C4b binding protein (8C11 and horse-radish peroxidase labeled 9H10). Protein C inhibitor (PCI) was determined by ELISA using a monoclonal antibody against PCI (API-93) as capturing antibody and rabbit polyclonal anti-PCI serum as secondary antibody. Levels of PAI-1 and tPA were measured by ELISA.
as described earlier. Endogenous thrombin potential and associated measurements (lag time, time-to-peak, peak, area-under-the-curve) were assayed on the Calibrated Automated Thrombogram (Fluoroskan Ascent, ThermoLab systems, Helsinki, Finland) and Thrombinoscope software (Thrombinoscope BV, Maastricht, The Netherlands) as previously described. Coagulation was triggered by recalcification in the presence of 5 pM recombinant human tissue factor (Innovin®, Siemens, Marburg, Germany), 4 μM phospholipids and 417 μM fluorogenic substrate Z-Gly-Gly-Arg-AMC (Bachem, Bubendorf, Switzerland). In BALF, only antithrombin, α2-antiplasmin, D-dimer, protein C activity, total and free protein S, protein C inhibitor, PAI-1, TATc and von Willebrand factor Ag were measured. Levels of the following cytokines, chemokines and other inflammatory markers were measured in plasma by a multiplex assay (Luminex, Austin, TX) using reagents from Bio-Rad (Bio-Rad Laboratories Veenendaal, The Netherlands): IL-6, chemokine (C-X-C motif) ligand (CXCL) 8, soluble IL-2 receptor subunit-α (sIL-2RA), soluble intercellular adhesion molecule 1 (sICAM-1), soluble TNF receptor-1 and -2 (sTNFR1, sTNFR2). C-reactive protein (CRP) was measured in heparinized plasma samples with the C-Reactive Protein Gen.3 test kit (Roche Diagnostics, Mannheim, Germany), an immunoturbidimetric method, on the Hitachi Modular P-800 module (Hitachi, Hitachinaka, Japan).

REFERENCES