Coagulation factors in the airways in moderate and severe asthma and the effect of inhaled steroids

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ABSTRACT

Background:
There is evidence of activation of the extrinsic coagulation cascade in the asthmatic airway and both plasma and locally-derived factors may be involved. We tested the hypothesis that the normal haemostatic balance of healthy airways sampled by sputum induction favours fibrin formation in asthmatic airways, and that inhaled corticosteroids (ICS) and plasma exudation influence this balance.

Methods:
ELISA and activity assays were used to measure alpha-2-macroglobulin (an index of plasma leakage) and coagulation factors in hypertonic saline-induced sputum of 30 stable subjects (10 controls, 10 moderate and 10 severe asthmatics). Additionally, the moderate cohort were weaned off their ICS, followed by further sputum induction 5 days after cessation of steroids.

Results:
ICS wean induced a significant rise in plasminogen (median (IQR): 13.92 (6.12-16.17) vs. 4.82 (2.14-13.32) ng/ml; 95% CI 0.003 to 8.596, p=0.0499) and tissue-plasminogen activator (tPA; 5.57 (3.57-14.35) vs. 3.88 (1.74-4.05) ng/ml; 95% CI 0.828 to 9.972, p=0.0261) levels in sputum, such that tPA in untreated moderate asthma was significantly (p=0.0029) higher than normal (2.14 (0.0-2.53) ng/ml). Severe asthmatics had significantly more alpha-2 macroglobulin (p=0.0003), tissue factor (p=0.023), plasminogen activator inhibitor (p=0.0091) thrombin activatable fibrinolysis inhibitor (p=0.0031) and fibrin degradation products (p=0.0293) in their sputum than control subjects.

Conclusion:
Untreated moderate asthma is associated with increased fibrinolysis that is corrected by ICS. Severe asthma and high dose corticosteroid therapy is associated with a pro-fibrinogenic, anti-fibrinolytic environment in the airways. Our study suggests that inhibition of fibrin deposition in severe asthma may be a therapeutic approach.
INTRODUCTION

Asthma is characterised by airway inflammation and variable tissue remodelling. It is influenced by inhaled corticosteroid (ICS) therapy, yet many asthmatics remain symptomatic, some with severe manifestations of the disease, as structural changes lead to airflow obstruction that may be irreversible. Plasma exudation from the bronchial microvasculature contributes to bronchial obstruction by fibrin deposition in mucus plugs, bronchial wall thickening, epithelial shedding, thickening of the basement membrane, hypertrophy of the smooth muscle and mucus secretion. Fibrin deposition in distal airways is also associated with increased bronchial hyperresponsiveness. We further reported that fibrin formation by bronchial epithelial cells in vitro is independent of plasma proteins, and is essential for bronchial epithelial repair.

Exposure of plasma to cell-bound tissue factor (TF), the principal activator of the extrinsic coagulation cascade initiates fibrin clot formation within minutes, although the rate of fibrin formation is determined by components of the intrinsic coagulation cascade. Thus, early, physiologically desirable fibrin formation for normal wound healing, initiated by TF-bearing cells, may propagate into exudated bulk plasma, generating excessive and physiologically undesirable fibrin that could lead to fibrosis, mucus plug formation, airway narrowing and bronchial hyperreactivity (BHR).

Levels of TF in induced sputum are higher in patients with mild asthma compared to healthy controls, and after allergen challenge. A TF-dependent increase in coagulation and plasminogen activator inhibitor (PAI-1)-dependent decrease in fibrinolysis is a feature of alveolar fibrin formation in ARDS and idiopathic pulmonary fibrosis. There has been recent interest in the role of platelets contributing to the coagulation response in patients with acute lung injury, however their role in the regulation of the extrinsic coagulation cascade in the lung, and in asthmatics is not known. Activation of the coagulation cascade has both fibrin-dependent and fibrin-independent influences on tissue fibrosis. Fibrin-dependent effects include neutralisation of surfactant, binding and accumulation of TGFβ, and provision of a matrix for fibroblast proliferation. Independent effects include activation of serine proteases, notably thrombin FVIIa and FXa, that activate protease activated receptors (PARs), present on fibroblasts and bronchial epithelial cells, to further stimulate fibrotic and inflammatory processes in the lung, and also further enhance TF expression. Thus increased levels of TF are self-amplifying when coagulation is initiated.

In the normal lung, the haemostatic balance is antithrombotic and favours fibrinolysis. We sought to test the hypothesis that the normal haemostatic balance changes in favour of coagulation in moderate and severe asthma, and that inhaled corticosteroids and plasma exudation influence this balance.

METHODS

Patients

Healthy controls and patients with moderate and severe asthma were recruited from the community and asthma clinic at Queen Alexandra Hospital, Portsmouth, UK. Asthma was confirmed by objective assessments of variable airflow obstruction and/or BHR, and together with lung function and exhaled nitric oxide measurements (FeNO), are described in the online appendix. Subjects were 18-70 years old and had stable symptoms with no need for increased asthma treatment within six weeks of study entry. Current smokers, subjects with a smoking...
history of >10 pack years, other coexisting lung disease or significant co-morbidity were excluded. ‘Moderate’ asthma was defined as mild persistent disease with low or medium daily dose ICS (200-1000 µg beclomethasone dipropionate (BDP) or equivalent) under the Global Initiative for Asthma (GINA) criteria, with additional asthma medications permitted.

‘Severe’ asthma was defined as GINA severe persistent disease with high daily dose ICS (>1000 µg of BDP or equivalent), with additional asthma drugs (including oral corticosteroids) permitted.

**Study design**

After initial screening and two week run in, patients underwent induced sputum and venous blood sampling. Furthermore, ‘moderate’ subjects underwent tapered ICS withdrawal (200 mcg beclomethasone dipropionate (BDP) equivalent per week, see appendix) one week after the first sputum induction. Continuation of long acting β₂ adrenoceptor agonists (LABA) and reliever medications was permitted. Subjects completed twice daily PEF (Wright mini peak flow meter, Clement Clark International Ltd, Harlow, UK) and diary card monitoring with weekly review until five days after complete steroid cessation when a second sputum induction was performed.

Ethical approval was granted by the Reading & Berkshire Research Ethics Committee, and all subjects provided informed consent. Sample size calculations are discussed in the appendix.

**Sputum induction and processing**

Sputum induction was performed using an ultrasonic nebuliser (Devilbiss Ultraneb 2000, Sunrise Medical, UK) and hypertonic saline, and processed with 0.05% w/v (final concentration) dithiothreitol (DTT), as described in the appendix.

**Alpha-2 macroglobulin and coagulation factors in induced sputum**

Alpha-2 macroglobulin was used as a marker of plasma exudation and assayed by an in-house ELISA described in the appendix. TF, tissue factor pathway inhibitor (TFPI), tissue plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1) were analysed by sandwich ELISA using commercially available kits (American Diagnostica, Stamford, CT, USA). Fibrinogen, factor VII (FVII), factor X (FX), factor XIII (FXIII), plasminogen and thrombin activatable fibrinolysis inhibitor (TAFI) were analysed by enzyme immunoassay using antibody pairs according to the manufacturers instructions (Affinity Biologicals, Ancaster, ON, Canada). D-dimers were assayed by sandwich ELISA (Hyphen Biomed, Andresy, France). FDPs were measured using the Thrombo-Wellcotest (Remel Europe Ltd, Dartford, UK). Enzyme activity for thrombin and plasmin were analysed using specific substrates, S2238 and S2251 respectively (Chromogenix, Milan, Italy). Quantification was with the values for normal reference plasma (Precision Biologic, Dartmouth, NS, Canada). All standards were prepared with 0.05% w/v DTT.

**Statistical analyses**

The Kruskal-Wallis test compared median levels across the three independent subgroups. Comparisons between subgroups were made using a Mann Whitney U test, and 95% confidence intervals (CI) for the difference between medians calculated. Repeated measurements on the moderate asthma cohort were analysed using a paired t test. Associations between parameters were analysed using a pooled r value from Spearman’s rank correlation coefficient after heterogeneity testing with a chi-square test. Analysis was performed using GraphPad Prism version 4.03 (GraphPad software, San Diego California, USA).
RESULTS

Baseline patient characteristics are shown in Table 1. In the moderate group, one patient failed the second sputum induction (after withdrawal of steroids), therefore, 9 participants remained in the moderate untreated group, with 9 paired sputum samples (pre and post-steroid withdrawal) available for analysis.

Table 1 Patient characteristics

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>Moderate</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>6/4</td>
<td>3/7</td>
<td>5/5</td>
</tr>
<tr>
<td>Age (years)</td>
<td>36.0 (29.5-49.0)</td>
<td>56.0 (39.0-62.0)</td>
<td>54.0 (42.5-53.5)</td>
</tr>
<tr>
<td>Smoking history: Ex</td>
<td>0</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Never*</td>
<td>10</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Pack years</td>
<td>0</td>
<td>0.75</td>
<td>2.1</td>
</tr>
<tr>
<td>Atopy (+/-)*</td>
<td>1 / 9</td>
<td>5 / 5</td>
<td>8 / 2</td>
</tr>
<tr>
<td>ICS dose (BDP equivalent, mcg)†</td>
<td>-</td>
<td>400 (200-400)</td>
<td>2000 (1800-4000)</td>
</tr>
<tr>
<td>Oral prednisolone (mg daily)</td>
<td>-</td>
<td>-</td>
<td>4.0 (0-7.5)</td>
</tr>
<tr>
<td>Additional medications:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Long acting beta agonist</td>
<td>-</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Leukotriene receptor antagonist</td>
<td>-</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Long acting antimuscarinic</td>
<td>-</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>Oral theophylline</td>
<td>-</td>
<td>-</td>
<td>6</td>
</tr>
<tr>
<td>Oral antihistamine</td>
<td>-</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>FEV₁ % predicted‡</td>
<td>103 (92-117)</td>
<td>90 (87-108)</td>
<td>60.5 (55-79)</td>
</tr>
<tr>
<td>FEV₁/FVC ratio‡</td>
<td>0.78(0.71-0.82)</td>
<td>0.76 (0.74-0.8)</td>
<td>0.62 (0.53-0.75)</td>
</tr>
<tr>
<td>Baseline FeNO (ppb)</td>
<td>15.5 (9-26)</td>
<td>18 (13-24)</td>
<td>21 (8.5-42)</td>
</tr>
</tbody>
</table>

All figures presented as median (IQR).
* p<0.05, control group vs. severe group by Fisher’s exact test.
† p<0.05 by Mann Whitney U test.
‡ p<0.05, both control and moderate group vs. severe group by Mann Whitney U test.
ICS = inhaled corticosteroid; BDP = beclomethasone dipropionate; FEV₁ = forced expiratory volume in 1 second;
FVC = forced vital capacity; FeNO = fraction of exhaled nitric oxide; ppb = parts per billion.
Blood and airway inflammation

Sputum differential cell counts are presented in Table 2 and blood results in appendix Table 1. Median values for sputum and blood eosinophilia were significantly higher in the severe group compared to the control group (sputum: 11.2 vs. 0.0, 95% CI 1.32 to 31.24, p=0.0001; blood: 0.6 vs. 0.1, 95% CI 0.10 to 0.70, p=0.0095). Patients with moderate treated asthma had significantly greater sputum neutrophilia than those with severe asthma (51.8 vs 18.2, 95% CI 10.0 to 45.6, p=0.0054). ICS wean resulted in a non-significant increase in the proportion of sputum eosinophils relative to pre-wean values (1.97 vs. 0.62, 95% CI -6.11 to 4.91, p=0.404), but a significant sputum eosinophilia compared to controls (1.97 vs. 0.00, 95% CI 0.76 to 10.4, p=0.0015). FeNO levels did not differ between the groups and were not influenced by withdrawal of ICS (18 vs. 28.5, 95% CI 3.30 to 29.5, p=0.104: appendix Table 1).

Table 2  Sputum differential inflammatory cell counts

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control n=10</th>
<th>Moderate treated n=10</th>
<th>Moderate untreated n=9</th>
<th>Severe n=10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells /g sputum (x10⁶ /g)</td>
<td>0.76 (0.51-2.18)</td>
<td>0.57 (0.28-1.88)</td>
<td>1.58 (0.51-2.76)</td>
<td>1.13 (0.40-3.2)</td>
</tr>
<tr>
<td>Squamous (%)</td>
<td>16.28 (8.35-28.69)</td>
<td>19.8 (10.3-31.7)</td>
<td>12.0 (9.83-22.8)</td>
<td>13.8 (4.59-21.7)</td>
</tr>
<tr>
<td>Macrophage (%)</td>
<td>49.65 (23.02-70.11)</td>
<td>43.1 (35.5-58.5)</td>
<td>39.9 (29.7-66.5)</td>
<td>52.6 (28.1-73.6)</td>
</tr>
<tr>
<td>Neutrophils (%)*</td>
<td>35.42 (18.38-65.43)</td>
<td>51.8 (29.2-64.9)</td>
<td>45.4 (24.7-59.7)</td>
<td>18.2 (12.0-43.1)</td>
</tr>
<tr>
<td>Eosinophils (%)†‡</td>
<td>0.0 (0.0-0.36)</td>
<td>0.62 (0.28-5.58)</td>
<td>1.97 (0.93-11.8)</td>
<td>11.2 (1.25-45.5)</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>1.31 (0.45-2.37)</td>
<td>1.26 (0.55-1.5)</td>
<td>1.11 (0.44-2.0)</td>
<td>1.3 (1.07-1.96)</td>
</tr>
<tr>
<td>Epithelial (%)</td>
<td>3.69 (1.46-5.35)</td>
<td>1.07 (0.77-2.69)</td>
<td>1.38 (0.69-2.15)</td>
<td>1.08 (0.61-1.68)</td>
</tr>
</tbody>
</table>

All data presented as median (IQR). All statistical results from Mann Whitney U test.
* p<0.01, moderate treated group vs. severe group.
† p<0.001, control group vs. severe group.
‡ p<0.01, control group vs. moderate untreated group.

Sputum alpha-2 macroglobulin levels were significantly higher in the severe cohort compared to other groups (control: 95% CI 741.3 to 4447.7, p=0.0003; moderate treated 95% CI 884.3 to 4676.0, p=0.0002: Figure 1 and appendix Table 2) and were not affected by ICS withdrawal (Figure 1 and appendix Table 3). Over all groups there was a significant positive correlation with sputum eosinophils (r=0.676, p<0.001: appendix Table 4).

Comparison of alpha-2 macroglobulin levels in induced sputum with levels in reference normal plasma (1000 μg/ml) allowed a plasma dilution factor for the samples to be calculated. Thus, plasma proteins were present in induced sputum as a percentage of normal reference.
values that was: (mean±SD) controls 0.031±0.021 %, moderate treated 0.015±0.013 %, moderate untreated 0.068±0.135 % and severe 0.38±0.45 %. Knowledge of these values allowed us to consider the provenance, either plasma exudation or local cellular synthesis, of the coagulation factors present in the airways.

**The extrinsic coagulation cascade**

The full results for all assays are provided in appendix Tables 2 and 3, with a relevant summary provided below.

**Fibrinogenesis: Tissue Factor, Factor VII, Factor X**

Tissue factor (Figure 2A) levels were significantly higher in the severe cohort compared to controls (11.85 vs. 3.95, 95% CI 1.49 to 16.05, p=0.023) and treated moderate asthma (11.85 vs. 5.15, 95% CI 1.3 to 16.21, p=0.0167), with no significant difference after weaning ICS (5.915 vs. 4.02, 95% CI -9.72 to 2.44, p=0.102). TF was significantly and positively correlated with sputum eosinophil counts (r=0.550, p=0.008).

Factor VII (Figure 2B) levels were significantly higher in controls compared to the severe cohort (2.05 vs. 0.00, 95% CI 0.091 to 3.043, p=0.0235) and untreated moderate asthma (2.05 vs. 0.07, 95% CI 0.297 to 3.061, p=0.0029). There was a significant reduction in FVII levels following withdrawal of ICS (1.14 vs. 0.07, 95% CI 0.10 to 4.697, p=0.0429: Figure 2B, appendix Table 3). Factor X (Figure 2C) levels were significantly decreased in severe asthma compared to controls (0.910 vs. 6.00, 95% CI 0.65 to 9.46, p=0.0058); with no effect following ICS withdrawal (2.62 vs. 3.43, 95% CI -6.71 to 6.03, p=0.451).

**Fibrinolysis: Plasminogen, tPA, plasmin, PAI-1, TAFI, FDPs**

Plasminogen (Figure 3A) was significantly lower in treated moderate asthma compared to controls (4.82 vs. 12.99, 95% CI 2.62 to 13.62, p=0.0115) and ICS withdrawal resulted in a significant increase in levels (4.82 vs. 13.92, 95% CI 0.003 to 8.596, p=0.0499).

Levels of tPA (Figure 3B) were significantly higher in severe and untreated asthma cohorts when compared to controls (6.26 vs. 2.14, 95% CI 1.599 to 11.975, p=0.0046 and 5.57 vs. 2.14, 95% CI 1.907 to 10.831, p=0.0015 respectively), and there was a significant increase in levels following ICS withdrawal (3.88 vs. 5.57, 95% CI 0.828 to 9.972, p=0.0261: appendix Table 3).

Significantly higher levels of PAI-1, TAFI and FDPs (Figures 3C, 3D & 3F) were observed in severe asthma compared to controls (95% CI 1.12 to 12.77, p=0.0091; 0.450 to 6.751, p=0.0031, and 0.01 to 30.1, p=0.0301, respectively). Furthermore, TAFI was significantly higher in the severe cohort as compared to treated asthmatics (95% CI 0.852 to 8.049, p=0.0052). In the severe group, FDPs levels correlated positively with sputum eosinophil count (r=0.667, p=0.003) and tPA (r=0.493, p=0.006).

**DISCUSSION**

We have compared plasma exudation and coagulation activity in the sputum of healthy controls, patients with moderate persistent asthma, before and after withdrawal of ICS, and in patients with severe persistent asthma. ICS withdrawal for five days resulted in significantly decreased FVII, increased plasminogen and tPA levels compared to pre-weaning values, and
evidence of a more fibrinolytic environment in the airways of patients with untreated moderate asthma compared to controls. Conversely, severe asthma patients had significantly higher levels of alpha-2 macroglobulin, TF, PAI-1 and TAFI compared to treated moderate asthma, indicative of a change to a pro-fibrinogenic and anti-fibrinolytic environment in severe asthma. We believe this is the first study to investigate the haemostatic balance in the airways of patients with severe asthma and following withdrawal of ICS in moderate asthma.

The significantly higher levels of alpha-2 macroglobulin in the airways of patients with stable severe asthma are indicative of on-going bronchial inflammation despite treatment with inhaled and/or oral steroids, and the strong correlation of alpha 2-macroglobulin with sputum eosinophils supports this notion.

Discussion of the study design, and cohort characteristics, including sputum differential cell counts and FeNO measurements, are in the appendix.

**Fibrinogenesis**

The higher levels of TF in the airways of patients with severe asthma, in the absence of detectable difference in TFPI levels indicates a potentially procoagulant environment. Taking into account the plasma dilution factor, most of the TF we detected in the airways is likely to derive from local cellular sources, including monocytes, macrophages, eosinophils and possibly apoptotic bronchial epithelial cells that are features of severe asthma.

Levels of FVII were significantly lower in both untreated and severe asthma. This raises the possibility that FVII may be reduced by a mechanism associated with asthma pathogenesis, rather than therapy. Our unpublished in vitro evidence indicates that FVII and FX rapidly bind to bronchial epithelial cells, supporting the notion that these factors are lower in severe asthma because coagulation is initiated on cell surfaces by TF via reactions dependent on catalytic phospholipids in the cell membranes, and our samples have examined supernatant, not cell bound FVII or FX. A strong negative correlation of these factors with observed TF levels in this study (FVII: r=-0.610, p=0.002; FX: r=-0.634, p=0.001), lends further support for this hypothesis. Therefore, our observation may reflect binding and consumption of FVII and FX in the coagulation process, rather than their absence which would completely abolish coagulation. Whilst not measured in this current study, levels of FXa activity have been shown to correlate with mucosal thickness and collagen deposition in a mouse model of asthma and blockade with FXa inhibitor reduced airway hyperresponsiveness.

Thrombin activity is normally undetectable in plasma, but was detected in all sputum samples (Figure 2D), indicating local activation of prothrombin in the airways. Considering the plasma dilution factor, thrombin in the healthy airway is likely to be largely (94%) derived from locally expressed prothrombin, activated by FXa. However, prothrombin expression by bronchial epithelial cells remains to be confirmed by RT-PCR.

Levels of fibrinogen in our controls and asthmatics were similar to those reported elsewhere, although this is the first study to report levels in stable severe asthma and a positive correlation of fibrinogen with D-dimers was observed. The levels in the control group may equate to derivation by plasma exudation, although a local source is not ruled out. However, values in the asthma groups were lower than those that would be derived from unfiltered plasma, indicating that fibrinogen is either deposited in the tissue matrix before it arrives at the epithelium or is rapidly cleaved by thrombin and deposited as insoluble fibrin on epithelial cell surfaces.
FXIII levels in the severe group (0.46 (0.0-3.1) ng/ml) were lower than predicted (~38 ng/ml) by the plasma dilution factor. This indicates that FXIII may be adsorbed by the fibrin clot that forms as FXIII cross-links fibrin monomers, and/or that FXIII is proteolytically degraded, for example by neutrophil elastase which is shown to regulate FXIII within fibrin clots.25

**Fibrinolysis**

tPA is expressed by a number of cells in the airway including mast cells, macrophages, fibroblasts7 and bronchial epithelial cells.26 We have demonstrated higher levels of tPA in the severe and untreated asthma groups, which may indicate a disease-related increase in tPA expression. D-dimers were detected in all samples, indicating fibrin formation and fibrinolysis even in normal healthy airways, as previously reported.4 27 Despite significantly higher levels of tPA in untreated moderate and severe asthma, there were no significant differences in D-dimer levels between the groups which may have been degraded by local neutrophil elastase activity.28 Fibrinolysis is negatively regulated by PAI-1 and TAFI, both of which were significantly higher in the severe group as compared to controls and calculation of a PAI-1 / tPA molar ratio demonstrated a raised ratio in the severe cohort (control group median 0.873, severe 1.455) which suggests an anti-fibrinolytic environment in the airways, related to asthma severity. The higher FDP levels in severe subjects compared to controls is likely to represent overall upregulation of the external coagulation cascade with fibrin accumulation.

Levels of plasminogen detected in sputum are lower than those predicted from plasma dilution, indicative of binding of plasminogen to fibrin and cell surfaces, which accelerates its conversion to plasin by tPA. Plasminogen expression by cells outside of the liver, including keratinocytes (reviewed in29) has been reported, but to our knowledge there are currently no reports of plasminogen expression by cells in the airway. Concentrations of tPA and PAI-1 in induced sputum are orders of magnitude higher than predicted from plasma exudation alone, and most must come from local cellular sources. TAFI was detected at levels that could be expected from plasma exudation and TAFI expression by cells other than hepatocytes and adipocytes has not been described.30

Neutrophils,31 mast cells,8 macrophages, fibroblasts and the bronchial epithelium7 are potential local sources of PAI-1. A 4G/5G polymorphism in the PAI-1 promoter region has been reported and preferential transmission of the 4G allele correlates with increased PAI-1 expression in asthma.8 32 PAI-1 and TAFI have been implicated in normal wound healing, and increased expression of both PAI-17 8 and TAFI30 33 have also been implicated in fibrin formation leading to the development of lung fibrosis. To our knowledge this is the first report of increased TAFI levels in the asthmatic airway.

**The role of corticosteroids**

Plasminogen levels were significantly lower in treated moderate asthma compared to controls and rose significantly on cessation of ICS, with no change in plasma exudation, indicating that ICS may suppress local plasminogen expression. A similar suppressive effect of ICS on local tPA expression, possibly by bronchial epithelial cells,34 was demonstrated in the same group. Lower plasminogen and tPA levels would tend to reduce fibrinolysis and support fibrin-dependent epithelial repair in the airway, revealing a possible mechanism by which ICS contribute to epithelial continuity in moderate asthma.

PAI-1 and TAFI expression is enhanced by corticosteroids30 35 and when oral and inhaled corticosteroid doses are converted to equivalent units (see appendix), a significant correlation
of PAI-1 with corticosteroid dose ($r=0.46$, $p=0.04$) was found for the moderate and severe groups. This raises the possibility that corticosteroids contribute to the increased expression of PAI-1 and TAFI in severe asthma and excessive fibrin formation that may be contributing to airway narrowing and BHR.\textsuperscript{3-8}

**CONCLUSIONS**

Defective fibrin formation and/or instability, and increased degradation of the fibrin matrix may contribute to the pathogenesis of moderate asthma, an effect that can be reversed by treatment with ICS.

Conversely, the levels of coagulation factors in severe asthma indicate a pro-coagulant, anti-fibrinolytic environment in which excessive fibrin formation and accumulation may contribute to irreversible airway narrowing and subepithelial bronchial fibrosis. Since PAI-1 and TAFI control the clearance of fibrin in the lung, and a stimulatory effect of PAI-1 on neutrophilic inflammation has been reported,\textsuperscript{31,36} our findings suggest these molecules may be possible therapeutic targets in severe asthma.

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**Competing interests:** None

**Ethics approval:** A favourable local research ethics committee opinion was obtained for the study
REFERENCES


Figure Legends

**Figure 1.** Alpha-2 macroglobulin (α2-MG) levels demonstrating plasma exudation in the airways of controls and asthmatic subjects. All data presented as data points and median with log 10 scale. Mod = moderate. *p<0.001 by Mann Whitney U test.

**Figure 2.** Profibrinogenic factors in the airways of controls and asthma subjects. All data presented as data points and median. Mod = moderate. (A) tissue factor (TF), (B) factor VII (FVII), (C) factor X (FX), (D) thrombin activity, (E) fibrinogen, (F) Factor XIII (FXIII). *p<0.05, †p<0.01 both by Mann Whitney U test; ‡p<0.05 by paired t test.

**Figure 3.** Profibrinolytic and antifibrinolytic factors in the airways of controls and asthmatic subjects. All data presented as data points and median. Mod = moderate. (A) plasminogen, (B) tissue plasminogen activator (tPA), (C) plasminogen activator inhibitor (PAI-1), (D) thrombin activatable fibrinolysis inhibitor (TAFI), (E) D-dimers (log 10 scale), (F) fibrin degradation products (FDPs). The dotted line on the FDP graph indicates the lower limit of detection (<2 μg/ml), with log 10 scale; only performed on controls and severe cohorts, see appendix. *p<0.05, †p<0.01 both by Mann Whitney U test; ‡p<0.05 by paired t test.
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APPENDIX (AS ONLINE SUPPLEMENT)
ADDITIONAL METHODOLOGY

Screening and enrolling of participants

At an initial screening visit all patients were interviewed to ensure suitability for study entry. Asthmatic subjects were assessed for frequency of symptoms and asthma control, short acting β₂ agonist use and assessment of compliance with inhaled corticosteroid (ICS) use. The moderate asthma cohort were taking low or medium daily dose ICS (200-1000 µg beclomethasone dipropionate (BDP) or equivalent), and the severe group a high daily dose ICS (>1000 µg of BDP or equivalent), with additional oral corticosteroids permitted.

Additional exclusion criteria were as follows: other pulmonary disease including an abnormal chest radiograph, treatment with any immunomodulators, pregnancy and any other significant co-morbidity. All subjects underwent a physical exam, spirometry and further investigations such as chest radiography or electrocardiogram as appropriate.

Sample size and power calculations

There is a paucity of published data on expected values of components of the clotting cascade in the airways of healthy controls and asthmatic subjects. In order to calculate sample size we made use of results reported by Gabazza et al¹ (who described levels of tissue factor (TF) in the induced sputum of fourteen stable mild asthmatics (5 taking ICS, 9 no ICS) as 1337.8 ± 297 pg/ml and healthy controls 356.1 ± 136 pg/ml), and also Xiao et al² (who reported plasminogen activator inhibitor (PAI-1) levels in thirty four mild asthmatics (7 taking regular ICS) as 4802 ±1127 pg/ml and controls 592 ± 97 pg/ml). Assuming that a logarithmic transformation would improve symmetry and homogeneity in the distribution of measurements, we determined that a sample size of 7 subjects per group would be required to detect a 50% increase in mean values when comparing asthmatics to the control group. These calculations assumed a power of 80% using a two-tailed test at the 5% significance level and a between subject standard deviation of 0.25 units on a logarithmic scale. We increased the sample sizes to 10 subjects per group to compensate for the use of non-parametric tests and multiple comparisons (although adjusted p-values have not been computed, see additional discussion below).

As this study planned to examine a number of novel parameters, we further calculated secondary variables using our own earlier observations of bronchoalveolar fluid in healthy controls, using the same power and alpha values as above and estimating a 20% difference to be of biological significance. These were tissue factor pathway inhibitor (TFPI) 0.227 ± 0.025 ng/ml, factor X (FX) 7.11 ± 1.08 ng/ml and tissue plasminogen activator (tPA) 9.39 ± 1.34 mIU/ml. This provided numbers of 6, 9 and 8 respectively, establishing that a sample size of 10 in each group would be likely to demonstrate differences in other variables of interest. Further discussion with respect to sample size and statistical analysis is considered below.

Inhaled corticosteroid withdrawal considerations

Our steroid withdrawal protocol was based on a previously described method by Gibson et al.³ Combination inhalers were split into constituent medicines to allow continuation of long acting β₂ adrenoceptor agonists (LABA) therapy where appropriate. Equivalent doses of different ICS were estimated using information adapted from the GINA guidelines⁴ and knowledge of the pharmacokinetics. A tapered withdrawal of 200 mcg beclomethasone dipropionate (BDP) equivalent per week was undertaken with weekly review, daily symptom and peak expiratory flow (PEF) monitoring and 24 hour telephone contact if necessary.
The length of time to withhold ICS (5 days) was based upon examination of the pharmacokinetics and biological action of the steroids, with mitigating increasing risk of inducing an exacerbation of asthma as a result of removing treatment.

**Lung function and exhaled nitric oxide**

Baseline lung function was recorded as forced expiratory volume in 1 second (FEV$_1$), forced vital capacity (FVC) and expiratory ratio (FEV$_1$/FVC) using a Vitalograph Compact Spirometer (Vitalograph Medical Instrumentation, Buckingham, UK) in accordance with the ATS/ERS task force standardisation of lung function testing guidelines. The fraction of exhaled nitric oxide (FeNO) was recorded using the NIOX MINO system (Aerocine AB, Solna, Sweden), using a single breath technique (50 ml/sec ±10%), in accordance with the recommendations of the ATS/ERS joint statement on exhaled nitric oxide measurement. Observations were recorded at baseline and at weekly intervals throughout the trial.

**Variable airflow obstruction, bronchial hyperresponsiveness (BHR) and the diagnosis of asthma and atopy**

In order to satisfy the diagnosis of asthma, we recorded objective assessments of variable airflow obstruction and/or BHR. This included an increase in FEV$_1$ by at least 12% after inhalation of 400 µg of salbutamol delivered by a metered dose inhaler (MDI) and spacer and the concentration of methacholine required to cause a 20 % reduction in FEV$_1$. BHR to inhaled methacholine was measured using the 5-breath procedure (described by Chai and colleagues) at visit 2. Subjects were asked not to take their short acting β$_2$-adrenoceptor agonist for at least 4 hours and LABA for 24 hours, prior to bronchial provocation. Nebulised normal saline was first administered followed by isotonic 0.03 mg/ml methacholine (Sigma Co, Poole, Dorset, UK) through a dosimeter (Spira, Electro2, Spira, Finland) in doubling dilutions up to a maximum of 16 mg/ml until the FEV$_1$ (measured at intervals of up to five minutes) dropped by at least 20% of the baseline value. BHR was expressed as the cumulative provocative concentration of methacholine that reduced the FEV$_1$ by 20% of baseline (PC20), as determined by linear interpolation on a log scale. For safety reasons, the test was not conducted on severe subjects. As all severe subjects were recruited from the difficult asthma clinic, this allowed scrutiny of the medical records, observing variations in FEV$_1$, documented wheeze by physicians and results of radiological and pulmonary function testing, including bronchodilator reversibility in all cases, to satisfy investigators (FJB & AJC) of the diagnosis of asthma.

Atopy was defined as positive skin prick tests to routine aeroallergens (Allergopharma Skin Test Solution, Diagenics Ltd, Milton Keynes, UK) and/or a raised serum total IgE >81 kU/L.

**Sputum induction**

Induced sputum collection was performed using a modification of a protocol previously described, using an ultrasonic nebuliser (Devilbiss Ultraceb 2000, Sunrise Medical, W. Midlands, UK) and hypertonic saline. Our induced sputum protocol included pre-treatment of all subjects with inhaled salbutamol (200 mcg, via MDI and spacer) and stratification of subjects into ‘low risk’ (controls and moderate subjects on ICS) and ‘at risk’ (severe and moderate subjects weaned off ICS). Low risk subjects inhaled nebulised 4.5% saline (South Devon Health Care, Devon, UK) using an ultrasonic nebuliser over four five minute periods, with assessment of any symptoms and FEV$_1$ after each five minutes. At risk subjects inhaled initially 0.9% saline (Noridem Enterprises Ltd, Nicosia, Cyprus) over short staggered time
periods (e.g. 30 sec, 1 min, 2 mins, 5 mins), with symptom and FEV₁ assessment after each stage. Assuming stability, the saline was changed to 2.7% (Fresenius Kabi Ltd, Cheshire, UK), then 4.5%. The procedure was abandoned if there was a significant fall (>20%) in FEV₁ from baseline value, or with disagreeable symptoms.

Sputum processing
Expectorated samples were processed, after removal of saliva, by adding an equal volume of 0.1% w/v dithiothreitol (DTT) prepared from 1% w/v DTT supplied as Sputolysin™ (Calbiochem, Nottinghamshire, UK). Samples were vortexed briefly followed by gentle mixing on a roller at room temperature for 20-30 minutes until homogeneous, filtered through a 70 µm cell strainer (Falcon), and cells and supernatant were separated by centrifugation for 10 minutes at 1500 rpm and 4 °C. The supernatant was stored at -80°C. The cell pellet was resuspended in phosphate buffered saline (PBS, Invitrogen Ltd, Paisley, UK) and assayed in duplicate for total cell count and percent viability, using Trypan blue stain and a haemocytometer. For differential cell counts, 70 µl of a cell suspension (1 x 10⁶/ml) was loaded into a cytopsin funnel and centrifuged at 1500 rpm for 5 minutes (Shandon Cytospin 3, Thermo Fisher Scientific Inc., MA, USA). Cytospin slides were air dried and stained with the Hema Gurr Rapid staining set (BDH, Poole, UK). Slides were fixed in methanol, stained with eosin, and air dried again before counterstaining with methylene blue, and being washed of excess stain. Slides were air dried and mounted with DPX (VWR International). Differential cell counts were based on at least 500 cells per slide.

In-house alpha-2 macroglobulin ELISA
Wells of a 96-well microtitre plate were coated overnight at 4°C with 100µl/well sheep anti-alpha-2 macroglobulin (The Binding Site, Birmingham, UK) diluted 1:500 in bicarbonate buffer, pH 9.6. The wells were washed three times with 200 µl PBS/0.05% Tween 20. Alpha-2 macroglobulin standards (Sigma, Poole, UK) were prepared in the range 2 - 1000 ng/ml in PBS/0.05% DTT/2% Tween-20 and 100 µl per well of standard and test samples containing 0.05% DTT were incubated for 90 min at 37 °C. The wells were washed three times with 200 µl PBS/0.05% Tween 20. The detection antibody, sheep anti-alpha2-macroglobulin peroxidase conjugate was custom synthesized by The Binding Site, and added at 1:1000 dilution in PBS/2% Tween, 100 µl per well, and the plate incubated for 90 min at 37°C. The wells were washed three times with 200 µl PBS/0.05% Tween 20 and 100 µl per well substrate (10 mg o-phenylene diamine (Sigma, Poole, UK) in 25 ml PBS with 40 µl 30% H₂O₂) added, and incubated for 15 min at room temp. The reaction was stopped by addition of 50 µl per well 2M and the plate read at 490 nm.

Fibrin degradation products assay
As the fibrin degradation product assay required a large sample volume (0.5 – 1 ml), the number of samples with sufficient volume was limited, and as a result analysis was restricted to the control group (n=9) and severe group (n=6). Positive agglutination of a suspension of latex particles coated in a specific FDP antibody was detected in samples containing at least 2 µg/ml FDP, therefore non-reactive samples where deemed to have a level of 1.9 µg/ml for the purposes of statistical analysis. All censored observations were in the control group and the Mann Whitney U test was used for comparison of the two groups.

Calculation of equivalent glucocorticoid doses for correlation
750 mcg of beclomethasone (BDP) is considered equivalent to 5 mg prednisolone.\textsuperscript{12} In order to be able to correlate steroid doses with measures parameters, the doses of corticosteroids were converted into the same units where 750 mcg of BDP = 1 unit, and therefore 5 mg prednisolone = 1 unit. Therefore patients taking ICS and oral steroids could be compared with those taking ICS alone. For instance if a patient was taking 2000 mcg BDP and 5 mg prednisolone, the corticosteroid units would equal \((2000/750)=2.666 + 1\) (5mg) = 3.666 units.

**ADDITIONAL RESULTS**

Seventy-five potential participants were invited to join the study; 11 declined to take part and 34 subjects met several exclusion criteria including the failure to produce adequate sputum.

Complete results from venous blood sampling and exhaled nitric oxide (FeNO) levels are presented in Table 1.

**TABLE 1. Blood parameters and exhaled nitric oxide (FeNO) values**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>Control treated</th>
<th>Moderate treated</th>
<th>Moderate untreated</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb</td>
<td>g/l</td>
<td>14.2 (13.4-15.1)</td>
<td>13.7 (12.9-15.0)</td>
<td>13.3 (12.7-15.0)</td>
<td>14.2 (13.0-15.3)</td>
</tr>
<tr>
<td>WCC*</td>
<td>x10(^9)/L</td>
<td>7.3 (5.2-8.5)</td>
<td>6.4 (5.3-6.9)</td>
<td>6.8 (5.7-7.8)</td>
<td>11.3 (7.6-13.2)</td>
</tr>
<tr>
<td>Neutrophils†</td>
<td>x10(^9)/L</td>
<td>4.0 (2.6-5.0)</td>
<td>3.5 (2.8-4.0)</td>
<td>4.0 (3.1-4.9)</td>
<td>5.9 (5.4-7.8)</td>
</tr>
<tr>
<td>Eosinophils†</td>
<td>x10(^9)/L</td>
<td>0.1 (0.0-0.1)</td>
<td>0.2 (0.1-0.4)</td>
<td>0.2 (0.0-0.45)</td>
<td>0.6 (0.2-0.85)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>g/L</td>
<td>2.6 (2.55-3.2)</td>
<td>2.95 (2.75-3.3)</td>
<td>3.45 (2.5-4.0)</td>
<td>2.7 (2.5-3.45)</td>
</tr>
<tr>
<td>D-dimer</td>
<td>µg/ml</td>
<td>0.35 (0.2-0.6)</td>
<td>0.4 (0.27-0.62)</td>
<td>0.31 (0.25-1.01)</td>
<td>0.4 (0.78-1.1)</td>
</tr>
<tr>
<td>INR</td>
<td></td>
<td>1.0 (1.0-1.1)</td>
<td>1.0 (1-1.1)</td>
<td>1.0 (0.9-1.0)</td>
<td>1.0 (0.9-1.0)</td>
</tr>
<tr>
<td>APTR</td>
<td></td>
<td>1.0 (0.9-1.0)</td>
<td>0.9 (0.8-1.1)</td>
<td>1.0 (0.9-1.1)</td>
<td>1.0 (0.9-1.1)</td>
</tr>
<tr>
<td>Na</td>
<td>mmol/L</td>
<td>138 (137-140)</td>
<td>138 (137-140)</td>
<td>139 (137-140)</td>
<td>138 (137-140)</td>
</tr>
<tr>
<td>K</td>
<td>mmol/L</td>
<td>3.8 (3.6-4.0)</td>
<td>3.9 (3.5-4.4)</td>
<td>3.8 (3.5-4.6)</td>
<td>4.1 (3.5-4.4)</td>
</tr>
<tr>
<td>Urea</td>
<td>mmol/L</td>
<td>3.6 (3.2-4.0)</td>
<td>3.5 (2.7-5.7)</td>
<td>4.2 (3.1-5.0)</td>
<td>4.85 (3.6-6.1)</td>
</tr>
<tr>
<td>Creatinine</td>
<td>µmol/L</td>
<td>74 (55-94)</td>
<td>76 (67-87)</td>
<td>81 (60-100)</td>
<td>90 (79-93)</td>
</tr>
<tr>
<td>Total IgE†</td>
<td>kU/L</td>
<td>31 (7.5-82)</td>
<td>53 (15-73)</td>
<td>-</td>
<td>98 (52-450)</td>
</tr>
<tr>
<td>FeNO</td>
<td>ppb</td>
<td>15.5 (9-26)</td>
<td>18 (13-24)</td>
<td>28.5 (11-56)</td>
<td>21 (8.5-42)</td>
</tr>
</tbody>
</table>

All data presented as median (IQR). All statistical results from Mann Whitney U test.

*\(p<0.05\), moderate treated group vs. severe group.

†\(p<0.05\), control group vs. severe group.

Hb, haemoglobin; WCC, white cell count; INR, international normalised ratio; APTR, activated prothrombin time ratio; FeNO, fraction of exhaled nitric oxide; ppb, parts per billion.
TABLE 2. Group comparison of induced sputum alpha-2-macroglobulin and coagulation factor levels

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Moderate treated</th>
<th>Severe</th>
<th>p-value (Kruskal-Wallis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha-2-macroglobulin (ng/ml)*</td>
<td>292.3 (101.7-457.5)</td>
<td>120.6 (38.47-278.2)</td>
<td>2489 (916.9-5738)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TF (ng/ml)†‡</td>
<td>3.95 (2.08-11.82)</td>
<td>5.915 (1.77-7.46)</td>
<td>11.85 (6.81-21.07)</td>
<td>0.0076</td>
</tr>
<tr>
<td>TFPI (OD)</td>
<td>0.356 (0.19-0.50)</td>
<td>0.228 (0.153-0.31)</td>
<td>0.199 (0.160-0.371)</td>
<td>0.2849</td>
</tr>
<tr>
<td>FX (ng/ml)§</td>
<td>6.00 (1.21-14.3)</td>
<td>2.62 (0.23-25.14)</td>
<td>0.910 (0.20-1.39)</td>
<td>0.0246</td>
</tr>
<tr>
<td>FVII (ng/ml)†</td>
<td>2.05 (0.56-3.41)</td>
<td>1.14 (0.03-3.52)</td>
<td>0.00 (0.00-0.86)</td>
<td>0.0341</td>
</tr>
<tr>
<td>FXIII (ng/ml)</td>
<td>2.01 (0.24-3.44)</td>
<td>1.14 (0.0-2.90)</td>
<td>0.46 (0.0-3.10)</td>
<td>0.5125</td>
</tr>
<tr>
<td>Thrombin activity (µg/ml)</td>
<td>0.386 (0.287-0.919)</td>
<td>0.372 (0.206-0.681)</td>
<td>0.672 (0.394-0.870)</td>
<td>0.1383</td>
</tr>
<tr>
<td>Fibrinogen (ng/ml)</td>
<td>634.7 (223.5-1264)</td>
<td>352.6 (248.3-599.6)</td>
<td>568.4 (202.0-1494)</td>
<td>0.0979</td>
</tr>
<tr>
<td>tPA (ng/ml)§</td>
<td>2.14 (0.0-2.53)</td>
<td>3.88 (1.74-4.05)</td>
<td>6.26 (3.12-16.58)</td>
<td>0.0048</td>
</tr>
<tr>
<td>Plasmin (µg/ml)</td>
<td>84.54 (59.45-126.1)</td>
<td>80.22 (61.30-98.44)</td>
<td>85.57 (70.3-129.1)</td>
<td>0.6507</td>
</tr>
<tr>
<td>Plasminogen (ng/ml)¶</td>
<td>12.99 (9.70-22.8)</td>
<td>4.82 (2.14-13.32)</td>
<td>10.97 (9.08-16.71)</td>
<td>0.0280</td>
</tr>
<tr>
<td>PAI-1 (ng/ml)§</td>
<td>1.81 (0.63-3.88)</td>
<td>2.43 (0.68-5.26)</td>
<td>7.23 (3.22-14.89)</td>
<td>0.0175</td>
</tr>
<tr>
<td>TAFI (ng/ml)§**</td>
<td>1.40 (0.35-3.87)</td>
<td>1.325 (0.00-2.10)</td>
<td>3.00 (1.98-9.65)</td>
<td>0.0049</td>
</tr>
<tr>
<td>D-dimer (ng/ml)</td>
<td>75.24 (28.10-191.1)</td>
<td>88.97 (16.9-210.0)</td>
<td>78.21 (29.07-266.2)</td>
<td>0.8388</td>
</tr>
<tr>
<td>FDPs (µg/ml)†</td>
<td>2.0 (1.9-3.0)</td>
<td>-</td>
<td>16 (-)</td>
<td>-</td>
</tr>
</tbody>
</table>

All data is presented as median (IQR).
* p<0.001, both control and moderate treated groups vs. severe group by Mann Whitney U test.
† p<0.05, control group vs. severe group by Mann Whitney U test.
‡ p<0.05, moderate treated group vs. severe group by Mann Whitney U test.
§ p<0.01, control group vs. severe group by Mann Whitney U test.
¶ p<0.05, control group vs. moderate treated group by Mann Whitney U test.
** p<0.01, moderate treated group vs. severe group by Mann Whitney U test.
TF, tissue factor; TFPI, tissue factor pathway inhibitor; OD, optical density; FX, factor X; FVII, factor VII; FXIII, factor XIII; tPA, tissue plasminogen activator; PAI-1, plasminogen activator inhibitor-1; TAFI, thrombin activated fibrinolysis inhibitor; FDPs, fibrin degradation products.
## TABLE 3. Induced sputum alpha-2-macroglobulin and coagulation factor levels in moderate asthma before and after ICS wean.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>p Value (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Moderate treated</td>
<td>Moderate untreated</td>
</tr>
<tr>
<td>Alpha-2-macroglobulin</td>
<td>120.6 (38.47-278.2)</td>
<td>284.3 (83.3-472.9)</td>
</tr>
<tr>
<td>TF (ng/ml)</td>
<td>5.915 (1.77-7.46)</td>
<td>4.02 (2.61-18.47)</td>
</tr>
<tr>
<td>TFPI (OD)</td>
<td>0.228 (0.153-0.31)</td>
<td>0.188 (0.158-0.258)</td>
</tr>
<tr>
<td>FX (ng/ml)</td>
<td>2.62 (0.23-25.14)</td>
<td>3.43 (0.43-19.79)</td>
</tr>
<tr>
<td>FVII (ng/ml)</td>
<td>1.14 (0.03-3.52)</td>
<td>0.07 (0.0-0.25)</td>
</tr>
<tr>
<td>FXIII (ng/ml)</td>
<td>1.14 (0.0-2.90)</td>
<td>0.18 (0.0-0.39)</td>
</tr>
<tr>
<td>Thrombin activity (µg/ml)</td>
<td>0.372 (0.206-0.681)</td>
<td>0.532 (0.355-0.589)</td>
</tr>
<tr>
<td>Fibrinogen (ng/ml)</td>
<td>352.6 (248.3-599.6)</td>
<td>616.0 (274.9-863.3)</td>
</tr>
<tr>
<td>tPA (ng/ml)</td>
<td>3.88 (1.74-4.05)</td>
<td>5.57 (3.57-14.35)</td>
</tr>
<tr>
<td>Plasmin (µg/ml)</td>
<td>80.22 (61.30-98.44)</td>
<td>67.57 (60.7-85.39)</td>
</tr>
<tr>
<td>Plasminogen (ng/ml)</td>
<td>4.82 (2.14-13.32)</td>
<td>13.92 (6.12-16.17)</td>
</tr>
<tr>
<td>PAI-1 (ng/ml)</td>
<td>2.43 (2.14-13.32)</td>
<td>1.71 (6.12-16.17)</td>
</tr>
<tr>
<td>TAFI (ng/ml)</td>
<td>1.325 (0.68-5.26)</td>
<td>1.20 (0.72-4.30)</td>
</tr>
<tr>
<td>D-dimer (ng/ml)</td>
<td>88.97 (16.9-210.0)</td>
<td>87.20 (52.16-259.7)</td>
</tr>
</tbody>
</table>

Data is presented as median (IQR) and 95% confidence interval (CI) for significant difference using the paired t test.

TF, tissue factor; TFPI, tissue factor pathway inhibitor; OD, optical density; FX, factor X; FVII, factor VII; FXIII, factor XIII; tPA, tissue plasminogen activator; PAI-1, plasminogen activator inhibitor-1; TAFI, thrombin activated fibrinolysis inhibitor.

### Tissue factor pathway inhibitor (TFPI) ELISA

In our assays, the TFPI standard failed to demonstrate immunoreactivity, despite the samples reacting appropriately. TFPI is an approximately 70 kDa protein with tandem Kunitz domains, each with 3 disulphide bonds. Thus, it is likely that the TFPI in the standard was denatured by DTT, and therefore that what was measured in the samples was bound TFPI to TF-FVII-FX- complexes, with the disulphide bonds protected from the effects DTT. It was still possible to compare relative optical densities between the groups, see Tables 2 & 3.
TABLE 4. Relationship of markers of coagulation across all three subgroups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group correlation coefficient*</th>
<th>Control</th>
<th>Moderate Treated</th>
<th>Severe</th>
<th>Pooled ‘r’</th>
<th>p=</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fibrinogenesis:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TF vs. FVII</td>
<td></td>
<td>-0.818</td>
<td>-0.661</td>
<td>-0.191</td>
<td>-0.609</td>
<td>0.002</td>
</tr>
<tr>
<td>TF vs. FX</td>
<td></td>
<td>-0.673</td>
<td>-0.740</td>
<td>-0.467</td>
<td>-0.634</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Fibrinolysis:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-dimer vs. thrombin</td>
<td></td>
<td>0.297</td>
<td>0.485</td>
<td>0.600</td>
<td>0.469</td>
<td>0.026</td>
</tr>
<tr>
<td>D-dimer vs. plasminogen</td>
<td></td>
<td>0.176</td>
<td>0.239</td>
<td>0.358</td>
<td>0.259</td>
<td>0.190</td>
</tr>
<tr>
<td>D-dimer vs. fibrinogen</td>
<td></td>
<td>0.103</td>
<td>0.411</td>
<td>0.660</td>
<td>0.417</td>
<td>0.050</td>
</tr>
<tr>
<td><strong>Inflammatory Cells:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sputum eosinophils vs. TF</td>
<td></td>
<td>0.589</td>
<td>0.433</td>
<td>0.600</td>
<td>0.550</td>
<td>0.008</td>
</tr>
<tr>
<td>Sputum eosinophils vs. A2MG</td>
<td></td>
<td>0.694</td>
<td>0.588</td>
<td>0.733</td>
<td>0.676</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Lung Function:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Predicted FEV1 vs. A2MG</td>
<td></td>
<td>0.407</td>
<td>0.115</td>
<td>0.012</td>
<td>0.184</td>
<td>0.277</td>
</tr>
</tbody>
</table>

* By Spearman’s Rank correlation coefficient.

All groups tested for heterogeneity with a chi-square test with 2 degrees of freedom. If there was significant heterogeneity present, use of a pooled r value was deemed inappropriate and therefore not tested for significance.

**DISCUSSION**

**Study design and statistical analyses**

With the small numbers in each group for this study it is arguably possible that both type I and II statistical errors may have been introduced as multiple testing increases the likelihood of type I errors (from the traditional 5%) and small samples increase the likelihood of a type II error. The use of non-parametric tests throughout (except for the paired t-test, where the differences between the moderate groups is likely to be symmetrically distributed, and are assumed so under the null hypothesis), was necessary given the likely skewness in the response variables and lack of homogeneity anticipated between the groups, especially within the asthma cohorts, with variable phenotypes. We accept that the relatively small numbers and heterogeneity within the groups make demonstration of clear mechanistic changes difficult in this instance.

Adjustment for multiple comparisons, for instance using the Bonferroni adjustment, has not been employed in this study. If adjustments of the p value for multiple comparisons were made, it would also increase the chances of making Type II errors and increase the possibility that important relationships between coagulation factors and asthma are not discovered. Additionally, due to the novel, exploratory nature of this study, with some uncertainly as to exactly how many parameters could be successfully measured in the airways, establishing an adjustment calculation a priori would have been arbitrary and likely inaccurate. Furthermore, if we had lowered the alpha level and maintained the beta level in the sample size/design of our study, this would have also greatly increased the sample size required. Never-the-less the argument for adjustment of reported p-values for multiple comparisons is acknowledged,
and appropriate care should be taken in interpretation of the values presented in this study as a result.

In an attempt to reduce the possible confounding effect of performing correlations across groups with significantly differing variables, and with disease severity potentially influencing the association, we have calculated a pooled r-value following calculation of correlation coefficients for each subgroup. As the subgroups are of the same/similar sizes, the weights are also (roughly) equal and so simple averages could be used to give answers correct to at least two decimal places. We have therefore calculated a 'pooled r' value, using Spearman's rank correlation coefficient for each independent subgroup (not including the moderate treated and untreated group together). Statistical heterogeneity between the groups was assessed by using a Chi-squared test with 2 degrees of freedom, and if present, we did not proceed to any tests of significance. Weighted averages of the homogenous coefficients were then calculated, followed by the appropriate test of significance, see Table 4.

**General considerations**

Severe asthma may have an eosinophilic or neutrophilic phenotype. In our study, patients with severe asthma had predominant airway eosinophilia and sub-group analysis shows that the 7/10 severe patients receiving OCS had significantly fewer neutrophils than those not on OCS, which is consistent with other reports.

Tapered withdrawal or reduction of ICS dose in patients with stable and difficult to control asthma, has previously been shown to be associated with induction of blood and airway eosinophilia and exacerbation of symptoms. These changes in eosinophils were slow to develop and it is thus not surprising that no significant increase in eosinophils was demonstrated in this study following steroid withdrawal for five days in moderate asthma patients. This lack of, or delayed, inflammatory cell recruitment may also account for the alpha-2 macroglobulin and FeNO levels not changing significantly following ICS withdrawal. Furthermore, it has been suggested that FeNO measurements indicate loss of disease control and this study was not designed to induce loss of asthma control, although 8/9 patients reported worsening daily symptoms following ICS cessation.

The significant differences observed between the control and severe cohort in rates of atopy (yes/no: 1/9 and 8/2 respectively) and ex-smoking history (yes/no: 0/10 and 5/5 respectively) are unlikely to confound results. Atopy and asthma are intimately related and share many of the same inflammatory mediators and cells. The prevalence of atopy in the moderate and severe cohorts is likely to be representative of a larger asthmatic population, although due to the wide phenotypic variation in asthma, caution in generalisation of the results of this study should be encouraged. Whilst half of the severe cohort had an ex-smoking history, a median 2.1 pack-years is unlikely to be of biological importance in this study.
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Coagulation factors in the airways in moderate and severe asthma and the effect of inhaled steroids

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