

Increased vascular permeability precedes cellular inflammation as asthma control deteriorates

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Summary

Background Airway microcirculation is abnormal in asthma but the role of vascular changes in asthma deteriorations remains poorly defined. We prospectively assessed the vascular changes accompanying worsening of asthma control by using an inhaled corticosteroid (ICS) dose-reduction model.

Objectives To evaluate airway vascularity, vascular permeability and expression of vascular endothelial growth factor (VEGF) in early asthma deterioration induced by ICS back-titration. **Methods** Twenty mild-to-moderate persistent symptomatic asthmatics on low-to-moderate ICS were recruited and treated with 4 weeks of high-dose fluticasone propionate (1000 µg/day) to achieve symptom control. This was followed by dose reduction to half of the pre-study doses for 4–8 weeks until the symptoms began to return. Endobronchial biopsy and bronchoalveolar lavage (BAL) samples were obtained after both treatment periods.

Results Vascularity as measured by the number and size of blood vessels, as well as VEGF expression did not change following ICS reduction. Even on high-dose ICS, perivascular albumin staining and BAL microalbumin levels in asthmatic subjects, as markers of permeability, were elevated when compared with normal subjects and both further increased significantly after ICS reduction. There was a significant association between changes in vascular leakiness and clinical deterioration. Increases in airway albumin correlated with previously reported increases in airway wall infiltration with T lymphocytes.

Conclusions Our results suggest that airway vascular leakage is a major pathophysiologic feature of early asthma deterioration, occurring before recrudescence of cellular inflammation.

Keywords asthma, vascular endothelial growth factor, vascular remodelling

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Introduction

Asthma is a chronic inflammatory airway disorder characterized by intermittent airflow obstruction with symptoms of wheeze, chest tightness and cough. Airway inflammation has been regarded as central to asthma, the key feature being cellular infiltration.

In more recent years, airway wall remodelling has been recognized as another core abnormality, including a marked increase in blood vessel number in the lamina propria [1, 2]. In the traditional asthma paradigm, inflammatory cells play an important role in the pathogenesis of asthma deterioration and this is certainly true after challenges with an inhaled allergen [3, 4]. However, recent studies of asthma deterioration following withdrawal or

reduction of inhaled corticosteroids (ICS) demonstrate conflicting results with respect to inflammation [5–9]. There are also inconclusive data on the association of exhaled nitric oxide and increased sputum inflammatory cell counts with spontaneous asthma exacerbations [7–10]. Furthermore, there have been very few studies of the pathological changes in the airway as asthmatic subjects enter the early-phase of asthma deterioration and those studies that have been performed suggest that increased cellular inflammation is not a prominent feature [6]. The key underlying pathological events during the very early stages of asthma exacerbation, therefore, remain unclear.

There is increasing evidence that changes in airway microcirculation are a major contributor to asthma pathogenesis. Important vascular changes include increased

permeability, hypervascularity and ongoing active angiogenesis. These vascular changes are likely to increase airway wall thickness and contribute to airflow limitation and airway hyper-reactivity (AHR) [11–13]. Vascularity of the airway increases with the severity of asthma [1, 14, 15]. The role of the airway microcirculation during clinical deteriorations or frank exacerbations in asthma is unknown, but the system has the potential for rapid change, especially if leakiness causes oedema and a major volume change in the lamina propria, which would have a profound influence on the luminal obliterative action of contraction of the surrounding muscle layers.

Assessment of airway inflammatory events during asthma exacerbations is difficult and most investigators have attempted to artificially model exacerbations by using allergen challenge tests. However, these models assess cellular events over a very short time period, which is somewhat different from the majority of naturally occurring exacerbations, which occur over several days and are rarely related to allergen exposure. It is also difficult to study naturally occurring exacerbations prospectively, particularly in the very earliest phases of deterioration, as subjects normally present once they are in the established stage of exacerbation.

In this study, we have therefore chosen to model early exacerbation events by dose-reduction of ICS in mild-to-moderate persistent symptomatic asthmatic subjects first rendered very well controlled with a high-dose ICS therapy. We have previously reported that cellular inflammation is not a feature of early asthma deterioration. This had led us to the hypothesis that the earliest pathological changes in the airways associated with clinical deterioration in asthma may be related to abnormal blood vessels, and especially to increasing leakiness with oedema formation. In the current study, we have focused on airway vasculature changes, including airway vascularity, vascular permeability and vascular endothelial growth factor (VEGF) expression, in relation to asthma deterioration.

Methods

Subjects

This study was approved by the Institutional Ethics Committee and all subjects gave written informed consent. Twenty atopic, mild-to-moderate persistent asthmatics receiving low-to-moderate doses of ICS [100–500 µg/day of fluticasone propionate (FP) equivalent] were recruited to undergo baseline assessment (see Table 1 for full details of recruitment). All subjects had a history of symptomatic asthma for the last 12 months. None were current smokers and seven were ex-smokers, having given up for a median of 9 years (interquartile range 3–18 years). None had received oral or nasal corticosteroids for the previous 12 months before the study. Twenty-two

Table 1. Baseline characteristics of the study subjects (*n* = 20)

Age±SD (years)	39±12
Gender (male/female)	12/8
Duration of asthma±SD (years)	29.0±13.0
ICS dose at baseline (µg of FP, or equivalent)	343.75±121.9
Number of skin prick positives±SD	2.9±1.2
Smoking history	
Current smoker, <i>N</i> (%)	0 (0)
Ex-smoker, <i>N</i> (%)	7 (35)
Non-smoker, <i>N</i> (%)	13 (65)
Lung functions	
FEV ₁ ±SD (L)	2.84±0.76
FEV ₁ ±SD (% predicted)	82±14
FVC±SD (L/min)	4.12±1.11
FVC±SD (% predicted)	101±16
FEV ₁ /FVC ratio±SD	0.70±0.13
PD ₂₀ methacholine (mg/mL)	0.18±0.46
Daily monitoring	
Morning PEFs±SD (L/min)	457±123
Evening PEFs±SD (L/min)	477±123
Symptom scores±SD (per week)	8.7±5.6
Bronchodilator usage±SD (per week)	13.5±11.0

SD, standard deviation; PEF, peak expiratory flow; ICS, inhaled corticosteroid; FP, fluticasone propionate; FEV₁, forced expiratory volume in 1 s.

healthy, non-smoking, non-atopic volunteers with normal spirometry were recruited to provide bronchoscopic biopsy and BAL samples for an external control.

Study design

The study outline is summarized in Fig. 1. This was the same study as described previously [6], where asthmatic subjects were assessed both clinically and bronchoscopically using an ICS dose-reduction protocol, with the intention of assessing subjects responding well on a high-dose ICS and then again when symptomatic control began to deteriorate. Bronchoscopy with bronchial biopsies and bronchoalveolar lavage (BAL) was performed as described previously at a maximum of 2 days after physiological assessment at each time-point [16].

Clinical measures

Spirometry was performed according to the American Thoracic Society criteria [17]. Daily diary cards were recorded for peak expiratory flow (PEF), relief bronchodilator use and asthma symptoms according to a five-point scale for each day and night. The total symptom score for each week was then summated for each individual.

Enzyme-linked immunosorbent assays

Standard sandwich ELISA was performed for albumin (Abbott Diagnostics, USA) in unconcentrated cell-free

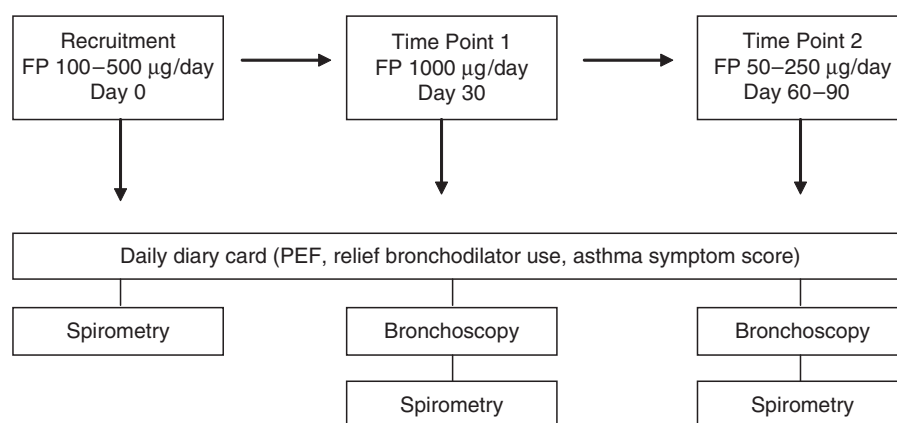


Fig. 1. Schematic summary of study design. FP, fluticasone propionate; PEF, peak expiratory flow.

BAL fluids. The absorbance was measured at a wavelength of 450 nm.

Immunohistochemistry and quantification

Biopsy tissue processing and immunohistochemistry staining were performed as described previously [6]. Anti-VEGF monoclonal antibodies were used for the assessment of epithelial, smooth muscle, inflammatory cells and blood vessel staining. Anti-von Willebrand Factor (vWF) (Dako, Australia) and anti-CD34 Class II (Dako) antibodies were used for quantification of total and immature blood vessels, respectively. For the assessment of vascular leakage, anti-albumin polyclonal antibody (ABCAM, Australia) was used with counter staining with anti-vWF antibody (Dako).

Airway biopsy sections were analysed to a depth of 150 µm below the epithelial basement membrane using a computerized image analyser (Image Pro V4.1, Media Cybernetics, USA). The quantification of blood vessels was performed as described previously [18, 19]. The blood vessels were evaluated for number, vascular area, 'sprouting' [20], growth factor expression and vascular leakage. To evaluate vascular leakage, the perivascular albumin staining area was measured within a radius of 10 µm around blood vessels (to avoid overlapping of areas). The measurement was expressed as perivascular albumin staining (percentage) = (area of positive albumin staining/total perivascular area) × 100. Positive VEGF staining intensities for epithelial and smooth muscle cells were scored as areas of weak or strong staining, and were expressed as a percentage of the total area.

Statistical methods

Statistical analysis was performed using Graphpad Prism (v5, Graphpad Software, USA). Parametric distributions were analysed with *t*-tests for comparisons of two groups

and with analysis of variance, followed by the Tukey *post hoc* test for multiple groups. Correlations were assessed using Pearson's test. For non-parametric data, Mann-Whitney or Kruskal-Wallis tests, followed by Dunn's post-test were used for two or multiple comparisons, respectively. Non-parametric correlations were assessed using Spearman's test. Results are expressed as median and interquartile range (unless specified otherwise). Significance was accepted as $P < 0.05$.

Results

The full details of the clinical, physiological and airway inflammatory profiles at time-point 1 and time-point 2 for all 20 subjects have been presented previously, and are represented in the appendices (Table 1) [6]. Asthma control improved with a treatment of high-dose FP. Overall, subjects experienced a small but significant increase in symptoms and deterioration in spirometry and peak flows after ICS reduction. Fifteen subjects (termed 'deteriorators') demonstrated consistent deterioration in spirometry (both FEV₁ and PEF), symptom scores and bronchodilator use after a reduction in ICS treatment while five subjects remained largely stable (non-deteriorators). As already reported previously, there was no significant change in airway inflammatory cells in either BAL fluid or biopsies following ICS reduction, apart from an increase in CD4⁺ T lymphocytes and the ratio of CD4⁺/CD8⁺ T lymphocytes in airway biopsies and a reduction in the number of stained mast cells, but these changes were not significantly related in regression analyses to the changes in the clinical measures of asthma control [6].

Airway vascularity

The evaluation of both immature and mature blood vessels using CD34 showed no significant changes in

overall airway vascularity ($P=0.93$), number ($P=0.73$) and size ($P=0.76$) of blood vessels after reduction of FP treatment. Similarly, no significant changes were found in these indices for evaluation of mature blood vessels using vWF. The number of angiogenic sprouts per square millimetre of lamina propria also did not change using either CD34 or vWF staining after dose reduction of ICS (CD34: $P=0.93$, vWF: $P=1.00$) [19].

Immunoreactivity of vascular endothelial growth factor

The number of VEGF-positive blood vessels was not different between time-point 1 and time-point 2 ($P=0.64$), and there was no change in VEGF staining either in the epithelium or of lamina propria inflammatory cells following reduction of FP dose.

Airway vascular permeability

Despite having clinically well-controlled asthma on high-dose FP, the asthmatic subjects had a significantly higher percentage of perivascular albumin staining and microalbumin concentration in BAL fluid as compared with normal subjects (biopsy: $P<0.02$, BAL: $P<0.02$; Figs 2 and 4). Overall, following ICS reduction, there was a further significant increase in perivascular albumin staining in bronchial biopsy tissue from 27.1% (10.5–31.7%) to 38.5% (26.9–46.8%) ($P<0.0005$, Figs 2 and 3). There was also an accompanying significant increase in the microalbumin concentration in BAL fluid following a reduction of the ICS dose, from 35.0 mg/L (28.0–47.0 mg/L) to 40.0 mg/L (30.0–53.0 mg/L) ($P=0.03$, Fig. 4).

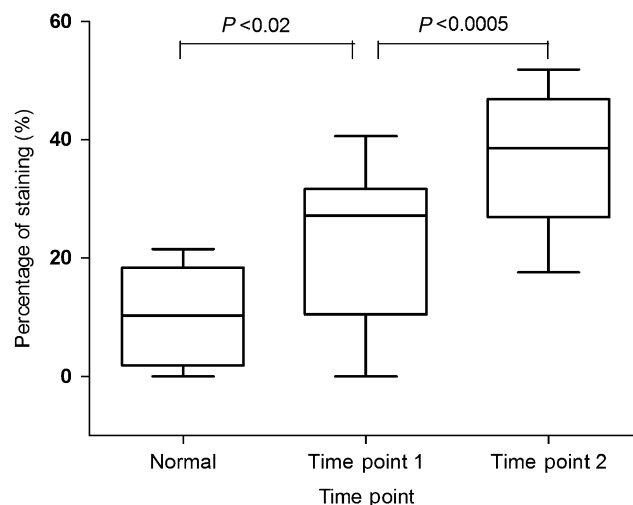


Fig. 2. Percentage of perivascular albumin staining in biopsy for normal subjects, and asthmatic subjects after high dose FP treatment (time point-1) and after reduction of FP dose (time point-2).

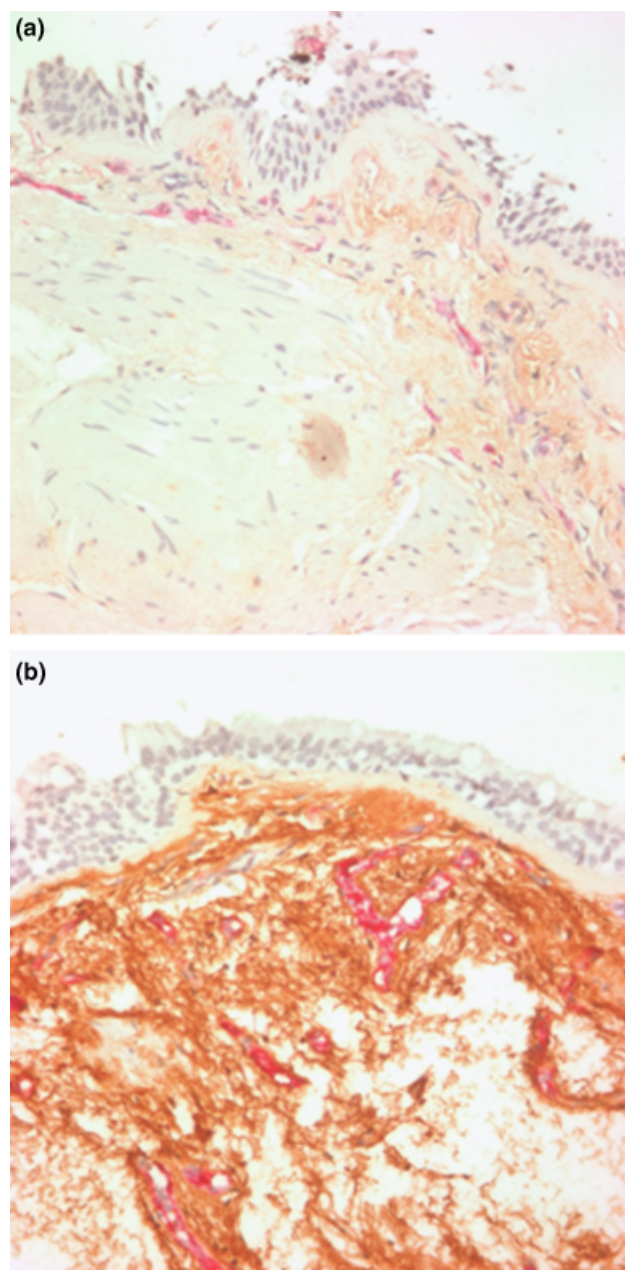


Fig. 3. Increased perivascular albumin staining in bronchial biopsy tissue after ICS reduction (b) compared after ICS (a). Brown, albumin staining; red, vWF staining.

Deteriorator vs. non-deteriorator subgroup analysis

Deteriorators showed significantly larger increase in the percentage of positive perivascular albumin staining in biopsy after FP dose reduction than the non-deteriorators ($P=0.01$, Fig. 5), but there was no significant difference in the change in microalbumin levels in BAL fluid between the two groups ($P=0.40$). Other vasculature indices were not significantly different between deteriorators and non-deteriorators (i.e. vascularity, vessel number or size and VEGF expression).

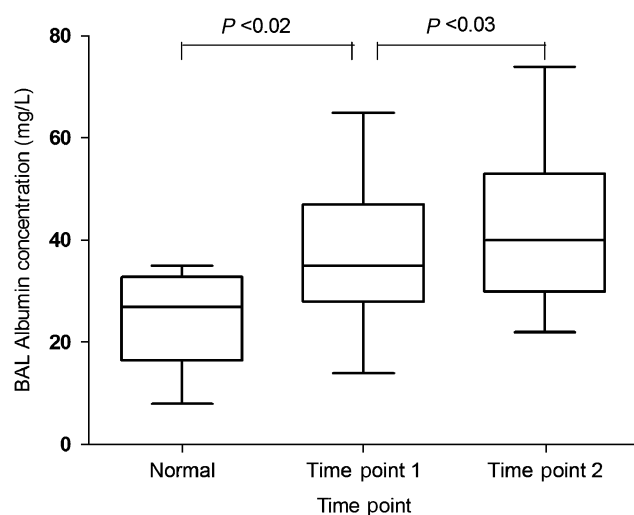


Fig. 4. BAL fluid microalbumin concentration in mg/L for normal subjects, and asthmatic subjects after high dose FP treatment (time point-1) and after reduction of FP dose (time point-2).

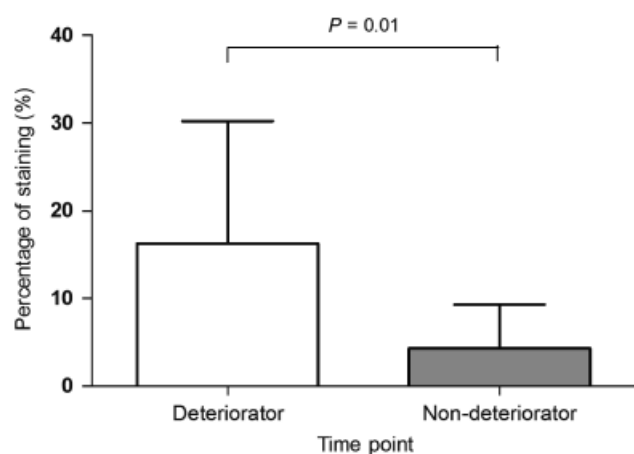


Fig. 5. Change in percentage of perivascular albumin staining in biopsy after reduction of FP between deteriorators and non-deteriorators.

Correlations

No relationship was found between changes in VEGF expression and airway vasculature after the dose-reduction treatment. Similarly, no relationships were observed between changes in VEGF expression and indices of airway vascular leakage. No significant relationship was found between changes in perivascular albumin staining in biopsies and microalbumin concentrations in BAL fluid between the two treatment periods.

However, changes in microalbumin concentrations in BAL fluid correlated significantly with the previously reported changes in $CD4^+$ T lymphocytes ($r=0.60$, $P=0.02$) and $CD4^+/CD8^+$ T lymphocyte ratio ($r=0.67$, $P=0.007$). A significant correlation was also present between changes in perivascular albumin staining in biopsies and the increase in symptom scores ($r=0.55$, $P=0.02$, Fig. 6)

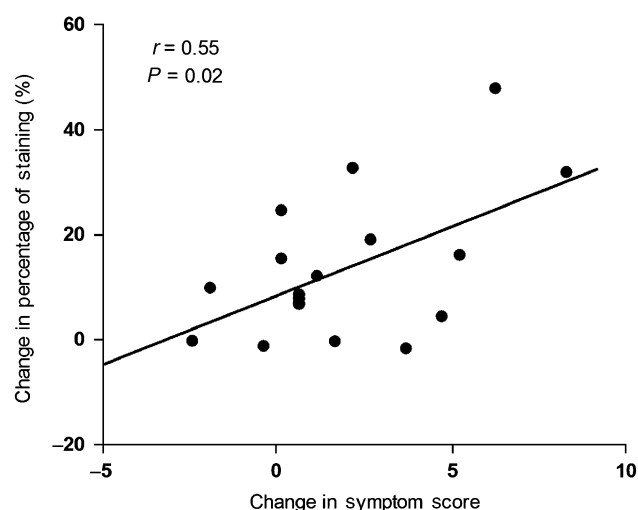


Fig. 6. Correlation between change in percentage of perivascular albumin staining and change in asthma symptoms score.

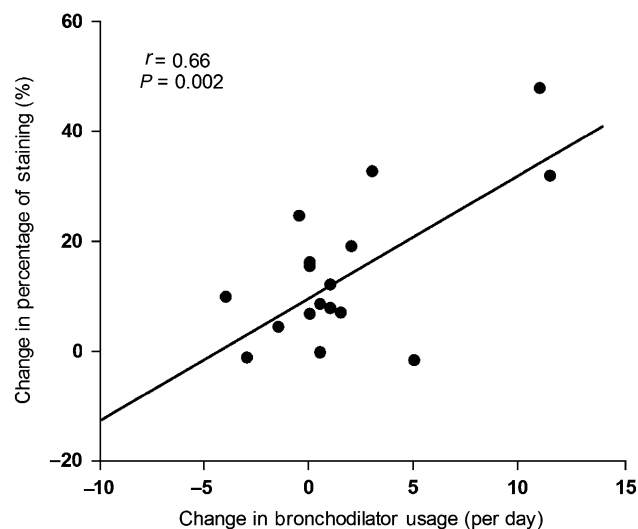


Fig. 7. Correlation between change in percentage of perivascular albumin staining and change in daily bronchodilator usage.

and the increase in bronchodilator usage ($r=0.66$, $P=0.002$, Fig. 7).

Discussion

In this study, we have demonstrated that very early deterioration in asthma control after a reduction of ICS dosage was associated with increased airway microvascular leakage of albumin, in the absence of evidence for worsening cellular inflammation. There was no associated change in vessel number or size, suggesting that this enhanced leakiness was not related to angiogenesis. Furthermore, vascular leakage was marked even with high-dose FP therapy and in the setting of good asthma control with a decrease in AHR. Our data suggest that

increased airway vascular permeability is central to asthma pathophysiology as well as the very earliest stages of asthma deterioration associated with ICS reduction.

Uncontrolled or untreated asthma is characterized by airway obstruction, intense airway inflammation and hyperaemia of the airway mucosa. Vascular changes in asthma include increased vessel numbers per unit area, vasodilatation and active new vessel formation (angiogenesis). However, little data exist on vascular leakage. Most studies on vascular leakage in asthma are based on animal models or acute stimulation models [20–24]. The accumulation of all these vascular changes has been interpreted as potentially important contributors to increased airway wall thickness, so that when the lamina propria is swollen, the effect of contraction of the smooth muscle layer outside it on luminal obliteration will be disproportionately much greater [1, 25–27]. The current study takes these insights further and indicates that increased vascular permeability does play an important role during the early stages of asthma deterioration before the enhancement of inflammatory cellular changes, but without evidence of active angiogenesis and probably without oedema being a main pathophysiological driver.

Previous attempts to assess vascular permeability and plasma exudation have measured solutes in sputum and BAL fluid of patients with asthma exacerbations [28] or after an allergen challenge [20, 21]. However, none of these studies have directly visualized vascular leakage in the airway wall. We assessed vascular permeability by staining airway tissue sections for albumin as a marker and measuring the circumferential area of positive staining as a percentage of perivascular area. In addition, we also assessed protein leakage into the airway lumen by evaluating BAL microalbumin levels. Both indices were elevated in well-controlled asthmatic subjects following high-dose ICS therapy, although they did not correlate with one another. BAL is likely to sample small airways while our biopsies were from relatively large airways, and these two airway compartments are not necessarily acting synchronously.

This ICS dose-reduction protocol used worked well, and allowed us to study the earliest changes in the activation of the asthmatic disease process associated with clinical deterioration. We did not use a placebo limb as we wanted to mimic current clinical practice guidelines and it was difficult to know what we were trying to control for externally. Given that the individual subject responses to reduced ICS therapy were likely to be variable, as they were, we felt that this variability would provide adequate internal control, and the outcomes vindicated our approach. Indeed, at present there are no validated criteria for following back-titration of ICS objectively, and our data would suggest that routine spirometry and monitoring of conventional symptoms are good at reflecting the onset of the earliest underlying pathologic changes. Our

protocol for dose reduction was simple, for pragmatic reasons, but our research nurse contacted subjects frequently to check on symptoms, and a further investigation occurred when there was an increase in reported symptoms, but there was a cap of 8 weeks when subjects were brought back for formal assessment anyway.

A limitation of this study was the lack of bronchoscopic data before entering the high-dose FP phase of the study. This would have been beneficial in establishing the potential improvement in vascular permeability along with improvement in asthma symptoms with high-dose ICS treatment. However, this was not performed to limit the number of bronchoscopies to only two, so as not to over-burden the good will of subjects and to prevent dropout.

The exuded plasma represented by the excess airway albumin we observed will be rich in pro-inflammatory mediators capable of initiating several events, including the activation of sensory neural pathways, inflammatory cell recruitment, inhibition of surfactant function and compromise of epithelial integrity and ciliary function [12, 29–31]. Plasma protein leakage has been shown to be associated with asthma exacerbations [32]. Plasma exudation into the interstitium may cause mucosal oedema, and thus reduced airway calibre and worsening airflow obstruction. In our study, increased microvascular leakage of albumin was not obviously associated with histological appearances suggestive of marked mucosal oedema, which is consistent with the lack of a significant change in AHR that was reported in our first report on this study [6], and that seemed paradoxical at the time. This suggests that the likely major mechanism of vascular leakage contributing to asthma deterioration in this specific situation was the transfer of pro-inflammatory mediators, rather than physical swelling of the mucosa. Clearance mechanisms either through lymphatics or out through the epithelium into the airway lumen seemed able to cope with the extra water that would have emerged into the tissues with the extra solutes. A previous study revealed lymphangiogenesis in chronic airway inflammation, which may provide conduits for extravasated fluid from leaky blood vessels [33].

The mechanisms contributing to the increased vascular permeability in asthma that we observed could include vasodilatation and microvascular congestion, endothelial and epithelial cell injury and impaired lymphatic clearance of extravasated proteins [12, 29, 31, 34]. It could be that the coincidental increase in CD4⁺ T lymphocytes [6] produces mediators that induce increased vascular permeability. One potentially important mediator is VEGF, which is a potent vascular permeability factor highly expressed in asthmatic airways [18, 35, 36]. VEGF levels in induced sputum have been found to be correlated with airway vascular permeability in asthma [36, 37]. In our study, VEGF expression remained unchanged after

reduction of the ICS dose. This suggests that increased vascular permeability in our subjects was not directly VEGF-dependent, although we have previously shown that the VEGF receptor system seems to be quite plastic [19], and we have not completely excluded the involvement of this complex system in asthma deterioration, as yet. Other mediators contributing to vascular leakage include angiopoietin-1, histamine, bradykinin, substance P, leukotrienes, platelet-activating factor and 5-hydroxytryptamine may contribute [11, 12, 29, 31, 38] – further research should assess these. Another mechanism of increased vascular permeability could be endothelial and epithelial cell injury via interaction with activated leucocytes, in particular neutrophils. However, in our study, the absence of BAL and biopsy neutrophilia suggests that this was not a significant cause, and previous studies have demonstrated that plasma exudation can occur across an intact epithelial lining [31, 39, 40].

Corticosteroids have profound anti-inflammatory actions and they remain the most effective anti-asthma drug available. Corticosteroids have been shown to reduce airway vascular changes in asthmatics [41, 42]. They are likely to exert their effects through both genomic and non-genomic mechanisms. By modulating gene expression, ICS down-regulate the expression of airway inflammatory cytokines, including VEGF [19]. ICS therapy has been shown to significantly reduce the sputum angiopoietin-1 level in asthmatics and the level was inversely correlated with a vascular permeability index [43]. A study based on the rat trachea showed that dexamethasone treatment reduced plasma extravasation produced by mediators of neurogenic inflammation, such as tachykinins and substance P [44]. In addition, evidence suggests that corticosteroids decrease airway blood flow by modulating the sympathetic control of vascular tone, through potentiation of noradrenergic neurotransmission in the airway vasculature to cause vasoconstriction [45–47]. Although such suppression of airway hyper-perfusion by ICS could theoretically reduce microvascular permeability, vessel size did not change after ICS reduction and thus, vasoconstriction is unlikely to be a central component in the control mechanisms of airway vascular permeability.

β_2 -agonists are potent bronchodilators. Short-acting β_2 -agonists are commonly used as required for immediate relief of bronchoconstriction, while long-acting β_2 -agonists (LABA) may be added to ICS for optimal management of persistent asthma. Apart from relaxation of airway smooth muscles, β_2 -agonists have been shown to have anti-inflammatory effects via actions on resident airway cells and circulating inflammatory cells [48–50]. In addition, β_2 -agonists, particularly LABA, have been demonstrated to inhibit extravasation of plasma proteins [51–53]. Although the importance of the anti-permeability effects of β_2 -agonists is unclear, it has been suggested

that the target for the anti-permeability action of β_2 -agonists is likely to be the endothelial cells of the post-capillary venules and collective venules [53]. In this study, there was an increased vascular leakage despite an increased use of short-acting bronchodilator, but further studies should be performed focusing on LABA in this context.

In conclusion, our study provides evidence that after the reduction of the ICS dose, airway vascular permeability is increased in parallel with early clinical deterioration of asthma control preceding airway inflammatory cellular changes and angiogenesis. Changes in vascular permeability were not related to VEGF expression, which suggests that alternative mechanisms underlie vascular leakage in the natural history of asthma deterioration after back-titration of ICS dose. The clinical effects seen were not likely to be related primarily to tissue oedema, and our study has provided novel insights into the potential central role of vascular leakiness of asthmagenic mediators in the pathogenesis of early asthma deterioration. This should stimulate further research into the importance of vascular leakiness and plasma exudation in asthma. The novel statistical relationship between increases in CD4⁺ T lymphocytes and changes in vessel permeability deserves further investigation of potential mechanistic links.

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Competing interests: Y. H. K., A. K. Y. T., S. M. L., D. C. Q. M., S. W., D. W. R. and E. H. W. declare that they have no conflict of interest.

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