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Activin-A is over-expressed in severe asthma and is implicated in angiogenic processes

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**Take Home Message**

The anti-angiogenic effects of activin-A, over-expressed in severe asthma, may be compromised by reduced receptor signalling

**Keywords:** activin-A, severe asthma, VEGF, airway angiogenesis

**Abbreviations:**

Act-RIIA : Activin receptor type IIA  
ALK : Activin-like kinase  
AHR : Airway hyperresponsiveness  
BAL : Bronchoalveolar lavage fluid  
DAB : 3, 3’- Diaminobenzidine  
DAPI : 4’,6-diamidino-2-phenylindole
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<th>Abbreviation</th>
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<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Forced expiratory volume in 1 second</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HC</td>
<td>Healthy controls</td>
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<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
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<tr>
<td>HPMEC</td>
<td>Human pulmonary microvascular endothelial cell</td>
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<td>HUVEC</td>
<td>Human umbilical vain endothelial cell</td>
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<tr>
<td>ICS</td>
<td>Inhaled corticosteroids</td>
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<tr>
<td>MMA</td>
<td>Mild-moderate asthmatics</td>
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<tr>
<td>OCS</td>
<td>Oral corticosteroids</td>
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<tr>
<td>PAS</td>
<td>Periodic acid–Schiff</td>
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<tr>
<td>RBM</td>
<td>Reticular basement membrane</td>
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<td>SA</td>
<td>Severe asthmatics</td>
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<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
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<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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ABSTRACT

Introduction: Activin-A is a pleiotropic cytokine that regulates allergic inflammation. Its role in the regulation of angiogenesis, a key feature of airways remodelling in asthma, remains unexplored.

Objectives: Investigate the expression of activin-A in asthma and its effects on angiogenesis in vitro.

Methods: Expression of soluble/immunoreactive activin-A and its receptors was measured in serum, bronchoalveolar lavage fluid (BALF) and endobronchial biopsies from 16 healthy controls, 19 mild/moderate and 22 severe asthmatics. In vitro activin-A effects on baseline and VEGF-induced human endothelial cell angiogenesis, signalling and cytokine release were compared with BALF concentrations of these cytokines in vivo.

Measurements and Main Results: Activin-A expression was significantly elevated in serum, BALF and bronchial tissue of the asthmatics, while expression of its protein receptors was reduced. In vitro, activin-A suppressed VEGF-induced endothelial cell proliferation and angiogenesis, inducing autocrine production of anti-angiogenic soluble (s)VEGFR1 and IL-18, while reducing production of pro-angiogenic VEGFR2 and IL-17. In parallel, BALF concentrations of sVEGFR1 and IL-17 were significantly reduced in severe asthmatics in vivo and inversely correlated with angiogenesis.

Conclusions: Activin-A is over-expressed and has anti-angiogenic effects in vitro that are not propagated in vivo, where reduced basal expression of its receptors is observed especially in severe asthma.
INTRODUCTION
Severe asthma is increasingly recognised as a distinct disease entity associated with high morbidity and healthcare costs and represents a major unmet therapeutic need [1]. In addition to airways inflammation, remodelling changes are implicated in the pathogenesis of severe asthma and can lead to irreversible airflow obstruction [2]. Local airways angiogenesis is increasingly acknowledged to play a role in the pathophysiology of airway remodelling changes associated with asthma [3]. Disordered angiogenesis may occur early in life and lead to accelerated lung function decline, prolonged symptoms, impaired quality of life and, even, death [3;4]. Thus, amelioration of this process represents a likely valid target for therapeutic intervention and is indeed already the subject of elaborate research [5;6].

Angiogenesis is a multifaceted process regulated by the balance between the actions of numerous pro and anti-angiogenic factors [7-12], such that, no single mediator can be considered solely responsible [13]. VEGF promotes endothelial cell (EC) proliferation and differentiation and represents a key-inducer of bronchial angiogenesis in asthma through the activation of its membrane receptors, VEGFR1 and VEGFR2 [14;15]. Other pro-angiogenic factors, such as IL-17 and IL-13, have also been identified [10;16;17]. Among angiogenesis inhibitors, soluble VEGF receptor-1 (sVEGFR1), IL-18 and IL-32, exhibit potent potential effects, although, their possible roles in the context of severe asthma have not been explored [7-9;18;19]. Understanding the balance of interactions between pro and anti-angiogenic activities in the inflammatory milieu of the severe asthmatic airways remains a considerable challenge.

Activin-A is a pleiotropic cytokine, belonging to the TGF-β superfamily [20]. While TGF-β1 signalling occurs via the TβRII, activin-A signals primarily through
binding to the activin-A receptor type IIA (Act-RIIA), which results in phosphorylation of the type I receptor (termed activin-like kinase [ALK]-4) [20]. Downstream signalling occurs via phosphorylation of receptor-regulated Smads (R-Smads) that translocate to the nucleus to initiate gene transcription [20]. Both TGF-β and activin-A signalling pathways have been shown to be activated upon allergen provocation in asthma [21;22]. Emerging evidence underscores the potential impact of activin-A on the regulation of airway inflammation and remodelling [22-27].

Previous studies by our group and others have demonstrated an anti-inflammatory role for activin-A in various settings [28-31], while other studies have shown opposing effects [23;24]. Interestingly, although the effects of activin-A on airway vascularization have never been addressed, investigation of its angiogenic effects on other tissues has also produced conflicting results [32-34]. These discrepancies highlight the possibility that, although activin-A may be over-expressed in inflammation, its end-effects may be diverse or even ablated by modification of its signalling in particular inflammatory environments in vivo.

In the present study, we set out to investigate the expression and possible role of activin-A in regulating airways vascularity in severe asthma. We hypothesized that activin-A is produced to excess in the asthmatic bronchial mucosa by a variety of infiltrating and structural cells, including vascular endothelial cells. We further hypothesized that activin-A has the potential to regulate endothelial cell proliferation and angiogenesis but that its end effects are influenced by the integrity of its signalling pathway and the presence of other locally expressed mediators. To achieve this, we investigated the expression and origins of activin-A and its signalling molecules in the airways of patients with asthma of varying severity as well as healthy
controls. We also examined both the production of activin-A and its interactions with other cytokines regulating angiogenesis in cultured human endothelial cells \textit{in vitro}. 
METHODS

Full methodological details are provided in the Methods section in this article’s Online Supplement.

Study population

A total of 57 non-smoking subjects, including 16 non-atopic healthy controls (HC), 19 patients with persistent mild-moderate (MMA) and 22 patients with persistent severe asthma (SA), participated in the study (Table 1). All asthmatic patients had a physician confirmed diagnosis and asthma severity was defined according to recent GINA and ERS/ATS guidelines [35;36]. Patients had been taking anti-asthmatic therapy at a stable dosage for at least for 3 months prior to study enrolment. Subjects underwent fiberoptic bronchoscopy with endobronchial biopsy and BAL collection at steady state. Serum samples were collected from patients at steady state and if and when an exacerbation occurred during a two-year follow up period (n=17), prior to the administration of oral corticosteroids (OCS) (see flow-chart in supplement, Figure E1). Asthma exacerbations were defined as episodes of severe symptom deterioration and elevated rescue medication usage that required the administration of systemic corticosteroids or at an elevated dosage for at least three days [37]. Prior to participation in the study, all subjects provided informed written consent and the protocol was approved by the hospital’s Research Ethics Committee and the Greek National Organization for Medicines. All studies were conducted according to the principles of the Declaration of Helsinki. More information regarding the study population is available in the supplement.

Fiberoptic bronchoscopy and sample collection
Bronchoscopy was performed on an outpatient basis, as previously described [38]. After inspection of the bronchial tree, lavage was performed and BAL samples with a fluid recovery of ≥ 60% were retained for further analysis (HC n=13, MMA n=12, SA n=19), according to the ERS Task Force guidelines regarding measurements of acellular components in BAL [39].

**Cytokine measurements**

Commercially available ELISA kits were used to determine the concentration of human activin-A, IL-17A, IL-18, IL-32, VEGF and sVEGFR1 in the serum, BAL and endothelial cell culture supernatants according to the manufacturers’ instructions (see supplement).

**Tissue staining and measurements**

Paraffin sections 3-4 μm thick were utilised for H&E and immunohistochemical staining and 6 μm thick frozen sections for immunofluorescence. Immunostaining for activin-A, ALK-4, Act-RIIA and subsequent quantification was performed as previously described [22;30;38]. Immunofluorescent staining for cellular markers was also performed as previously described [38]. Blood vessels were identified with the vascular endothelial cell marker CD31 (goat polyclonal anti-CD31, sc-1506, 1:200, Santa Cruz, USA), and the total numbers of vessels divided by the biopsy area to determine the numbers of vessels per unit area (mm²) of the sections. Extensive methodological information on tissue processing and analysis is presented in the online supplement.

**Endothelial cell cultures, proliferation and in vitro angiogenesis assays**
Human umbilical vein (HUVEC) and human pulmonary microvascular (HPMEC) endothelial cell lines were purchased from Merck Millipore (Darmstadt, Germany) and PromoCell (Heidelberg, Germany), respectively. Their proliferation was measured 48h after plating (>90% confluence) using a commercially available kit (WST-8, Cayman Chemicals), as previously described [40]. For the in vitro angiogenesis assay, the V2A kit (ZHA 4000, TCS Cellworks, Buckingham, UK) was employed as previously described [40]. Recombinant human (rh)-IL-13 (10 ng/mL, R&D Systems), rh-activin-A (200 ng/mL, R&D Systems) and rh-VEGF (2 ng/mL, BioLegend) were applied for 48h at concentrations similar to previously described studies [34;41] after performing dose response and kinetic studies (see supplement, Figure E2). A human polyclonal anti-activin-A antibody was used for blocking activin-A (15 μg/mL, R&D). All experiments were repeated three times in tetraplicate wells. The results were expressed as percentages of medium control values.

**RNA extraction and Real-Time PCR**

Total RNA was isolated from HUVEC in TRI Reagent (MRC, OH, US) following the manufacturer’s instructions (see supplement, Table E1).

**Statistical analysis**

Data are expressed as median and interquartile range, unless specified otherwise. Comparisons between subject groups were performed using the Kruskal-Wallis one-way analysis of variance accompanied by Dunn’s post hoc correction. The Wilcoxon matched-pair test was applied to compare activin-A expression in the stable state and during exacerbation. Correlation coefficients were calculated using the Spearman’s rank method. Regarding the in vitro data, comparisons between three or more groups
were analyzed by one way ANOVA followed by the Mann-Whitney U test to compare specific pairs for significant differences. A statistical software package was used for all data analysis and graph preparation (Prism v5; GraphPad, San Diego, CA, USA). A $p$ value of $\leq 0.05$ was considered significant.
RESULTS

For median values and interquartile ranges please refer to Table 1.

**Activin-A is increased in the airway of asthmatics and peaks during asthma exacerbations**

Activin-A concentrations were measured in the serum and BAL fluid of asthmatics with distinct disease severities. Patients with MMA and SA had significantly elevated serum activin-A compared to HC (Figure 1A). In addition, activin-A was significantly increased in the BAL fluid of MMA and SA compared to HC (Figure 1B). Interestingly, activin-A was markedly further increased in the serum of asthmatics during exacerbations compared with the steady-state (Figure 1C). No associations were found between activin-A levels in the serum, BALF or bronchial tissue and the risk of exhibiting an exacerbation among asthma patients (data not shown). Still, there was a trend for a negative association between the body mass index (BMI) and activin-A levels in the serum and BALF which however did not reach statistical significance (see supplement, Figure E3).

Expression of activin-A immunoreactivity was markedly elevated in epithelial and subepithelial infiltrating inflammatory cells in asthmatics (Figure 1D). Quantitative analysis revealed that the median number of activin-A immunoreactive cells was significantly increased in the bronchial epithelium in MMA and SA, compared to HC (Figure 1E). Furthermore, activin-A expression by resident and infiltrating cells in the lamina propria was significantly elevated in SA compared to both MMA and HC (Figure 1F). No significant differences were observed in the serum, BALF or airway expression of activin-A between atopics and non-atopic asthmatics (see supplement, Figure E4) or in the severe asthmatics treated or not
treated with oral corticosteroids (see supplement, **Figure E5**). Double immunofluorescence coupled with confocal microscopy in severe asthmatics revealed that mast cells, neutrophils, macrophages, endothelial and smooth muscle cells express activin-A, which in some cases seems to have intranuclear localization (Figures 1G to 1J).

**Activin-A signalling pathways are dysregulated in severe asthma**

In contrast to activin-A, bronchial mucosal expression of immunoreactivity for its receptor proteins was reduced, especially in severe asthmatics. The median number of cells expressing Act-RIIA immunoreactivity was significantly reduced in the bronchial epithelium and lamina propria in MMA and SA compared to HC (**Figure 2A**). ALK-4 expression was significantly reduced in epithelial and subepithelial cells only in SA compared to HC (**Figure 2B**), with intermediate expression in MMA. No significant differences were observed in the airway expression of activin-A and its receptors ALK-4 and Act-RIIA between atopic and non-atopic asthmatics (see supplement, **Figure E4**) nor in the severe asthmatics treated or not treated with oral corticosteroids (see supplement, **Figure E5**).

**Activin-A in the asthmatic bronchial mucosa correlates with angiogenesis**

Evaluation of the vascularity of the bronchial mucosa demonstrated that MMA and SA patients had significantly elevated median numbers of blood vessels per unit area in the lamina propria compared to HC (**Figure 3A**). Interestingly, our data revealed a strong correlation between the degree of angiogenesis and activin-A expression in epithelial (**Figure 3B**) and subepithelial cells (**Figure 3C**). In contrast, bronchial mucosal expression of both activin-A receptors, ALK-4 and Act-RIIA inversely
correlated with angiogenesis (Figures 3D-E and 3F-G, respectively), suggesting that activin-A signalling is decreased in patients with prominent angiogenesis. Airways angiogenesis inversely correlated with FEV₁, and although no correlation was observed with activin-A, increased airways expression of ALK-4 and Act-RIIA was associated with improved lung function (see supplement, Figure E6).

**Activin-A suppresses spontaneous and VEGF-induced proliferation of human pulmonary endothelial cells**

Immunofluorescence analysis of biopsies from severe asthmatics revealed that the majority of mucosal peribronchial endothelial cells expressed immunoreactivity for activin-A (Figure 4A). ALK-4 and Act-RIIA were also expressed, albeit, by fewer of the pulmonary EC (Figure 4B and data not shown).

Corresponding in vitro experiments using HUVEC and, more pertinently, HPMEC [42], confirmed that, as in vivo, immunoreactivity for activin-A and its receptors was expressed in the resting state by sub-populations of HPMEC (Figure 4C and 4D) and HUVEC (not shown). To mimic the pro-allergic in vivo setting, we exposed HPMEC to IL-13, a critical inflammatory mediator in asthma with an active signalling pathway in endothelial cells [43;44]. IL-13 exposure significantly increased the mean percentages of HPMEC expressing activin-A, but decreased the mean percentages of ALK-4⁺ and Act-RIIA⁺ cells (Figure 4D). Moreover, activin-A was detectable in culture supernatants of HPMEC at rest and its secretion significantly enhanced by IL-13 (Figure 4E). Exposure of endothelial cells to exogenous activin-A significantly reduced both spontaneous and VEGF-enhanced HPMEC proliferation, even in the presence of the inflammatory cytokine, IL-13 (Figure 4F). Similar findings were obtained using HUVEC (data not shown). In an
opposite approach, we evaluated the effects of activin-A neutralization on HPMEC proliferative responses. Blockade of activin-A resulted in enhanced HPMEC responses to VEGF and IL-13 (Figure 4F).

**Activin-A inhibits VEGF-induced angiogenesis**

To further explore the effects of activin-A on the regulation of human endothelial cell responses, we utilized an *in vitro* model of angiogenesis. In this assay, exogenous VEGF strongly promoted spontaneous angiogenesis, reflected by a significant elevation of all output parameters investigated, such as the number of junctions and tubules, as well as the total tubule length (Figure 5A and 5B). Activin-A did not alter spontaneous angiogenesis but significantly inhibited VEGF-induced angiogenesis (Figure 5B). Interestingly, activin-A significantly reduced VEGF-enhanced angiogenesis also in the presence of IL-13 (Figure 5B), while neutralization of activin-A resulted in enhanced angiogenic responses to VEGF and IL-13 (Figure 5C).

**Activin-A regulates the balance of VEGF receptors and induces an anti-angiogenic cytokine milieu**

Stimulation of HUVEC with activin-A resulted in a significant (approximately 3-fold) increase in expression of mRNA encoding sVEGFR1 and mVEGFR1, and to lesser extent VEGFR2, compared with control (Figure 6A-B). VEGF significantly increased expression of mRNA encoding VEGFR2, while it had no discernible effects on mRNA encoding sVEGFR1 (Figure 6A and 6C). When employed together, the effects of activin-A prevailed over those of VEGF (Figure 6A-C), so that, whereas VEGF induced a high VEGFR2/sVEGFR1 mRNA ratio, this was reversed in the
presence of activin-A (Figure 6D). Similar findings were observed with the VEGFR2/mVGEFR1 ratio (Figure 6E). Consistent with the mRNA data, stimulation of endothelial cells with activin-A significantly increased the release of sVEGFR1 protein into the culture supernatants in the presence or absence of VEGF (Figure 6F). No significant differences were observed in VEGFR2 protein levels between the groups studied (data not shown).

Incubation of endothelial cells with activin-A in vitro resulted in significantly elevated mean release of IL-18 into culture supernatants, compared with medium control, whereas VEGF significantly reduced mean IL-18 release (Figure 6G). In the additional presence of activin-A this effect of VEGF was reversed (Figure 6G). In contrast, activin-A significantly reduced mean release of the pro-angiogenic cytokine IL-17 in medium control and VEGF-treated cells (Figure 6H). IL-32 release by human ECs was not altered in the presence of activin-A or VEGF (see supplement, Figure E7).

**IL-18 and sVEGFR1 are decreased in the BAL and inversely correlate with bronchial tissue angiogenesis in severe asthma**

In an effort to translate our findings into the clinical setting, we investigated the concentrations of the afore-mentioned mediators in the BALF of patients with asthma of varying severity. The median concentration of IL-18 and sVEGFR1 were significantly lower in the BAL fluid of SA compared to HC (Figure 7A and 7B). Conversely, VEGF was significantly increased in SA (Figure 7C). No significant differences were observed in BALF median concentrations of IL-17 and IL-32 between the groups examined (see supplement, Figure E8). In contrast to VEGF, BALF concentrations of IL-18 and sVEGFR1 inversely correlated with the degree of
airway angiogenesis (Figure 7D to 7F). Moreover, IL-18 and sVEGFR1 levels in the BAL tended to inversely correlate with those of activin-A (see supplement, Figure E9). No significant correlations were found between VEGF, IL-18 and sVEGFR1 and activin-A levels in the serum (data not shown).
DISCUSSION

Our study clearly demonstrates that activin-A is increased in the serum, BAL fluid and bronchial tissue in patients with asthma and further increases during exacerbations. Our preliminary data further suggest that its mass production is not susceptible to systemic glucocorticoid inhibition. The expression of activin-A is more prominent in the subepithelium of the bronchial mucosa of severe asthmatics, where it is produced by a variety of structural and inflammatory cells, including bronchial endothelial cells in a “T\(_{H2}\) high” environment. The hypothesis that endothelial cells are a major source of activin-A production in asthma in vivo is supported by our observed correlation between airways vascularity (the degree of which was in turn associated with more severe disease) and activin-A expression. Bronchial epithelial cells also appear to be a potent source. Our hypothesis that activin-A may exert regulatory effects on angiogenesis in asthma in vivo is supported by the fact that endothelial cells express activin-A receptors, albeit in a potentially attenuated form in the more severe patients.

No significant differences regarding activin-A levels were observed in the serum or BALF of MMA and SA patients. It is not uncommon that the expression of certain mediators involved in severe asthma and associated airway remodelling processes does not differ between severe and mild/moderate asthmatics, as in the case of matrix metalloproteinases, eotaxin, IL-5 and IL-13 [45-47], or even in the case of established biomarkers, such as sputum eosinophils and FeNO [46;48]. Nevertheless, as in the case of IL-13 [47], our findings did reveal a significant increase in the numbers of resident and infiltrating cells expressing activin-A in the lamina propria of severe asthmatics. The observation that activin-A exerts effects on endothelial cells in the airways, even though, it is predominantly expressed by bronchial epithelial cells,
corroborates the pleiotropic nature of activin-A in the context of asthma. Predominance of expression of a certain cytokine by the bronchial epithelium does not preclude a pathogenic role for the corresponding factor in other cell types and processes. In fact, several mediators involved in asthma pathophysiology, such as, TGF-β [49;50], osteopontin [38;51], pentraxin-3 (PTX3) [52], resistin-like molecule-β [53], are prominently expressed in the bronchial epithelium and exert effects on subepithelial cells. Interestingly, and in agreement with other studies, our findings demonstrated that activin-A is also localized in the nucleus of subepithelial cells in the bronchial mucosa, possibly reflecting other, yet unidentified, roles for this cytokine in gene regulation [54;55].

A prominent finding of our study is that activin-A has the ability to suppress human pulmonary endothelial cell proliferation and angiogenesis in vitro, even in the presence of a robust endothelial cellular activator, such as VEGF. VEGF exerts its angiogenic effects in the asthmatic airway through its two main receptors VEGFR1 and VEGFR2 [14], which have similar structures but divergent functions in vivo [18]. We demonstrate here that the anti-angiogenic effects of activin-A may be propagated partly through its ability to invert the ratio of VEGFR2/VEGFR1 mRNA expression by endothelial cells, thereby modulating VEGF signalling. Furthermore, we show that activin-A stimulation has the potential to alter endothelial cell production of cytokines that affect angiogenesis, in particular by increasing the secretion of the anti-angiogenic IL-18 [7;9] while reducing that of the pro-angiogenic IL-17 [10], both at baseline and in the presence of VEGF. Finally, we also demonstrate an additional, novel biological effect of activin-A on the production of the inhibitory, circulating form of the VEGFR1 by endothelial cells, through which it may indirectly further mitigate VEGF signalling [19].
The impact of activin-A on bronchial angiogenesis has not previously been explored, although its effects on vascularisation have been investigated in other tissues with conflicting results [32-34]. Our data throw considerable light on the possible reasons for these conflicting data. The fact that angiogenesis appears to proceed in severe asthmatics in the face of elevated expression of activin-A raises the possibility that its effects may not be propagated at least partly because of modification of its signalling pathway. This hypothesis is supported by our finding that, in contrast to activin-A, the expression of its receptors, ALK-4 and Act-RIIA, was reduced, at least at the level of reduced immunoreactivity, in the bronchial tissue of severe asthmatics.

It is possible that these changes are brought about by the highly inflammatory environment of the bronchial mucosa in asthma. In support of this, we show that stimulation with IL-13 markedly reduces the expression of Act-RIIA and, to a lesser extent, ALK-4 in human pulmonary endothelial cells. IL-13 is a critical inflammatory mediator in asthma with an active signalling pathway in endothelial cells [43;44] and the ability to induce VEGF production [16;17] and mediate its effects in vivo [56]. Inhibition of activin-A signalling by other inflammatory mediators locally in the airways, such as LPS and TNFα, has similarly been previously reported [57]. A variety of cytokines with increased expression in the bronchial mucosa of severe asthmatics, including VEGF as observed herein and by others [11], may counteract the upregulation of angiogenic inhibitors, such as sVEGFR1 and IL-18, through which activin-A partly mediates its effects. Our observation that both sVEGFR1 and IL-18 concentrations were decreased in the BAL fluid of severe asthmatics, whereas that of VEGF was increased even in the presence of high concentrations of activin-A, are in agreement with this hypothesis (see Figure 8). Decreased ALK-4 expression
has been also reported in the airway epithelium of chronically challenged mice [58], suggesting that allergen exposure per se could modify the expression pattern of activin-A’s signalling pathway. Although we observed no differences regarding the expression of activin-A, ALK4 and Act-RIIA in the lung biopsies from atopic and non-atopic asthmatics, the possibility that allergen exposure influences their expression cannot be excluded.

The current study presents certain limitations. In an observational cross-sectional study, there is no longitudinal follow-up and it is difficult to pursue specific postulated mechanisms of disease, for which longer term studies and/or “models” may sometimes be more appropriate. Secondly, the activin-A concentrations used in our study for the stimulation of endothelial cell lines, although optimized by preliminary in vitro experiments and similar to those used by others, are higher than those observed in the BALF and serum of our asthma patients ex vivo. Finally, we did not utilize fresh primary bronchial endothelial cells for our in vitro experiments and, in the absence of an established cell line based on human peribronchial endothelial cells, we have utilized a human pulmonary microvascular endothelial cell line, as the most appropriate readily available cell line for studying human lung diseases [42;59;60].

In summary, our studies expand our knowledge of the scope of activities of activin-A in asthma, adding potential anti-angiogenic effects to its already versatile functions as a cytokine. We also demonstrate the principle, likely applicable to any complex, chronic inflammatory process, that over-expression of a cytokine such as activin-A in asthma does not inevitably result in the same end effects as observed in vitro because of complex, local environmental interactions in vivo. Our findings provide the groundwork for future research aiming to better understand these complex
interactions in order to evaluate activin-A as a possible target for affecting the course of angiogenesis in asthma.
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Authors’ Contributions
M.G. and G.X. defined the research theme, designed the experiments and contributed to the assessments and interpretation of results. K.S., E.Z., E.E. and M.G. performed patient recruitment, bronchoscopy and sampling. K.S., N.P., M.S., I.M., S.T., performed all experiments. K.S., N.P. and E.Z. performed the data analysis. K.S. and G.X. wrote the manuscript. D.S.R., H.H.K., C.J.C., S.Y. and D.B. participated in the study design, data interpretation and edited the manuscript. All authors have read and approved the final version for submission. K.S., N.P., M.G. and G.X. had unrestricted access to the data and have final responsibility for the manuscript.
References


Figure legends

Figure 1. Activin-A is increased in asthma. a) Activin-A concentrations in the serum and b) BAL fluid at steady state, and c) serum during exacerbation. d) Representative microphotographs (x200) demonstrating activin-A expression, as indicated (blue arrow: epithelial staining, red arrows: subepithelial staining x400). e-f) Quantification of activin-A+ cells in the bronchial mucosa. g-j) Confocal microscopic images from bronchial tissue of severe asthmatics demonstrating co-localization (yellow) of activin-A (red) expression by subepithelial cell markers (green, x400) and specifically g) mast cells, h) neutrophils, i) macrophages and j) smooth muscle cells. Data are presented as median (interquartile range). HC: healthy controls, MMA: mild/moderate asthmatics, SA: severe asthmatics, DAPI: 4’,6-diamidino-2-phenylindole, α-SMA: α-smooth muscle actin, * p < 0.05, ** p < 0.01, *** p < 0.001

Figure 2. Expression of activin-A receptors Act-RIIA and ALK-4 is markedly reduced in severe asthmatics. Representative microphotographs (x200) and quantitative analysis of a) Act-RIIA and b) ALK-4 in bronchial biopsy sections, as indicated. Data are presented as median (interquartile range). HC: healthy controls, MMA: mild/moderate asthmatics, SA: severe asthmatics, * p < 0.05, ** p < 0.01, *** p < 0.001

Figure 3. Airway angiogenesis correlates with activin-A expression and is associated with reduced tissue expression of ALK-4 and Act-RIIA. a) Quantification of airway angiogenesis in all groups. b-c) Correlation of angiogenesis with epithelial and subepithelial activin-A expression in all subjects. d-g) Inverse
correlations of angiogenesis with epithelial and subepithelial expression of activin-A’s receptors ALK-4 and Act-RIIA in all subjects, respectively. Data are presented as median (interquartile range). HC: healthy controls, MMA: mild/moderate asthmatics, SA: severe asthmatics, ** $p < 0.01$, *** $p < 0.001$

**Figure 4. Activin-A inhibits the proliferation of HPMEC in vitro.** Representative photomicrographs (x400) demonstrating a) activin-A (red) and b) ALK-4 (red) in CD34+ ECs (green) in severe asthmatics. Co-localization is shown in yellow (arrows). c) Representative photomicrographs (x200) and d) quantification of immunoreactivity for von Willebrand factor (vWF), activin-A, ALK-4, Act-RIIA and pSmad2/3 on HPMEC in the presence or absence of rh-IL-13, as indicated. e) Activin-A concentrations in HPMEC supernatants in the presence (+) or absence (−) of IL-13. f) Proliferation of HPMEC, as indicated. Data are presented as mean ± SEM from three independent experiments in tetraplicate wells. DAPI: 4’,6-diamidino-2-phenylindole, HPMEC: human pulmonary microvascular endothelial cells, rAct-A: recombinant activin-A, aAct-A: anti-activin-A, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

**Figure 5. Activin-A inhibits VEGF-induced angiogenesis in vitro.** a) Representative photomicrographs (x4) showing the formation of endothelial tubular structures (black) at day 14, as indicated. Computer-assisted quantification of the number of junctions, tubules, and tubule length demonstrating the effects of stimulation with b) anti-activin-A neutralizing antibody or Ig control and c) recombinant activin-A on VEGF and IL-13 induced angiogenic processes. Data are presented as mean ± SEM from six different photomicrographs per parameter and are
representative of three independent experiments in tetraplicate wells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

**Figure 6. The anti-angiogenic effects of activin-A are associated with enhanced sVEGFR1 and decreased VEGFR2.** Quantitative real-time PCR analysis of a) sVEGFR1, b) mVEGFR1 and c) VEGFR2 mRNA expression by HUVEC relative to GAPDH upon stimulation for 96h, as indicated. d) Log ratios of VEGFR2/sVEGFR1 and e) VEGFR2/mVEGFR1 mRNA expression, as indicated. f) Soluble VEGFR1 concentrations in culture supernatants. g) IL-18 and h) IL-17 concentrations in culture supernatants, as indicated. Data are presented as mean ± SEM from three independent experiments in duplicate wells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

**Figure 7. BAL fluid IL-18 and sVEGFR1 concentrations inversely correlate with airways vascularity in SA.** a) Soluble IL-18, b) sVEGFR1 and c) VEGF concentrations in the BAL fluid. Correlations of d) IL-18, e) sVEGFR1 and f) VEGF with angiogenesis in the airways of all subjects. Data are presented as median (interquartile range). HC: healthy controls, MMA: mild/moderate asthmatics, SA: severe asthmatics, * $p < 0.05$, ** $p < 0.01$

**Figure 8. Proposed model for activin-A-mediated inhibition of angiogenesis in the airways of asthmatic individuals.** In healthy individuals, upon exposure to environmental antigens, bronchial epithelial cells release activin-A which signals through its receptors on airway endothelial cells and inhibits VEGF-induced angiogenesis. This is associated with an enhanced release of the anti-angiogenic mediators, IL-18 and VEGFR1. However, in asthmatics, in the presence of
inflammatory cytokines in the airways (such as, IL-13, IL-5, etc) the expression of activin-A receptors is decreased and, thus, its signalling pathways are disrupted. As an effect, activin-A, although overexpressed in the airways of asthmatics, cannot inhibit VEGF-induced angiogenesis or increase IL-18 and VEGFR1 levels.
Table 1. Subject characteristics and results of quantification of activin-A, Act-RIIA, ALK-4 and airway angiogenesis in all groups examined. Data are depicted as median and interquartile range, unless otherwise specified.

<table>
<thead>
<tr>
<th>Demographics</th>
<th>Healthy Controls</th>
<th>Mild/Moderate Asthmatics</th>
<th>Severe Asthmatics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects n</td>
<td>16</td>
<td>19</td>
<td>22</td>
</tr>
<tr>
<td>Age yrs</td>
<td>46.5 (38.7 – 56.7)</td>
<td>43 (38 – 61)</td>
<td>50.5 (39.4 – 59)</td>
</tr>
<tr>
<td>Males/females n</td>
<td>10/6</td>
<td>6/13</td>
<td>3/19</td>
</tr>
<tr>
<td>Asthma duration yrs</td>
<td>NA</td>
<td>30 (2 – 63)</td>
<td>34 (12 – 63)</td>
</tr>
<tr>
<td>BMI</td>
<td>26.7 (24.4 – 28.8)</td>
<td>27.3 (23.4 – 29.9)</td>
<td>29.3 (25 – 31.6)</td>
</tr>
<tr>
<td>FEV₁ pred (%)</td>
<td>92 (85.3 – 103.1)</td>
<td>82.2 (68 – 89.8)‡</td>
<td>67.2 (52.8 – 79.4)‡</td>
</tr>
<tr>
<td>Atopy n</td>
<td>0</td>
<td>11</td>
<td>17</td>
</tr>
<tr>
<td>IgE</td>
<td>33 (19.5 – 43.7)</td>
<td>73 (31 – 168)†</td>
<td>120 (63 – 366)†</td>
</tr>
<tr>
<td>LABA n</td>
<td>16</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>LTRA n</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICS (mcg/day) §</td>
<td>NA</td>
<td>600 ± 55</td>
<td>1800 ± 89</td>
</tr>
<tr>
<td>OCS (n, mg/day) #</td>
<td>NA</td>
<td>NA</td>
<td>12 (9 ± 1)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Activin-A levels (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
</tr>
<tr>
<td>103 (63-169)</td>
</tr>
<tr>
<td>BAL</td>
</tr>
<tr>
<td>334 (102-391)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Airway epithelial cells (% of total epithelial cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activin-A+</td>
</tr>
<tr>
<td>4 (2-19.2)</td>
</tr>
<tr>
<td>Act-RIIA+</td>
</tr>
<tr>
<td>64 (48-75)</td>
</tr>
<tr>
<td>ALK-4+</td>
</tr>
<tr>
<td>58.5 (43.2-75.5)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Airway subepithelial cells (per mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activin-A+</td>
</tr>
<tr>
<td>10 (4-27.1)</td>
</tr>
<tr>
<td>Act-RIIA+</td>
</tr>
<tr>
<td>240 (193-305)</td>
</tr>
<tr>
<td>ALK-4+</td>
</tr>
<tr>
<td>203 (139-333)</td>
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<tr>
<th>Angiogenesis (ves/mm²)</th>
</tr>
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<tbody>
<tr>
<td>80.5 (59.7-99.7)</td>
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</table>


*: p < 0.5 compared to HC, †: p < 0.001 compared to HC, ‡: p < 0.01 compared to mild/moderate asthmatics

§: Beclometasone dipropionate CFC equivalent dosage, mean ± SEM
#: Prednisolone equivalent dosage, mean ± SEM
FIGURE 1

d) ISOTYPE CONTROL | HEALTHY CONTROL

SEVERE ASTHMATIC | SEVERE ASTHMATIC (4-MU)

e) 

f) 

D) DAPI| Activin-A
h) DAPI| Activin-A

i) DAPI| Activin-A
j) DAPI| Activin-A

Mast cell tryptase Merge | Neutrophil Elastase Merge

Macrophage (CD68) Merge | α-SMA Merge
FIGURE 4

(a) DAPI, Activin-A, CD34, Merge

(b) DAPI, ALK-4, CD34, Merge

(c) HPMEC with positive controls (vWF, Act-A, ALK-4, Act-RIIA)

(d) Bar graph showing the % of positive cells with IL-13

(e) Bar graph showing Activin-A levels with IL-13

(f) Bar graph showing HPMEC proliferation with IL-13 and other treatments
FIGURE 6