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Vascular endothelial growth factor and cysteinyl leukotrienes in sputum supernatant of patients with asthma

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KEYWORDS
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Angiogenesis;
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Summary
Background: Vascular endothelial growth factor (VEGF) is considered to be the most important angiogenic factor in asthma. Cysteinyl leukotrienes (Cyst-LTs) have been implicated in vascular permeability in asthma. Cyst-LTs receptor antagonists modulate vascular permeability by reducing VEGF expression.
Objective: We aimed to determine the levels of VEGF and Cyst-LTs in sputum supernatants of patients with asthma and to investigate possible associations within them and with airway vascular permeability (AVP) index. Possible confounding factors were also assessed.
Methods: One hundred twenty one patients with asthma (38 with severe refractory asthma, 41 smokers) and 30 healthy subjects (15 smokers) were studied. All subjects underwent lung function tests, and sputum induction for cell count identification and VEGF, Cyst-LTs, measurement in supernatants. AVP index was also assessed.
Results: Both VEGF & Cyst-LTs (pg/ml) levels were significantly elevated in patients with asthma compared to normal subjects (median, interquartile ranges 845 [487–1034] vs. 432 (327–654) and 209 [171–296] vs. 92 [75–114] respectively, $p < 0.001$ for both). Multivariate regression analysis in the whole group showed a significant association of Cyst-LTs levels in sputum supernatants with VEGF levels in sputum supernatants and AVP index. A similar positive

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association was observed between VEGF levels in sputum supernatants and AVP index. The presence of Severe asthma was a significant covariate for both associations.
Conclusion: Our results indicate that Cyst-LTs may modulate vascular permeability by up-regulating VEGF expression. The above effect seems to be affected by asthma severity.

Introduction

Angiogenesis is a complex multiphase process, potentially involving a great number of growth factors, cytokines, chemokines and numerous other mediators but the specific role of each molecule has not been clearly defined. During chronic inflammation, the vascular remodeling process is the consequence of a pro-angiogenetic action, in which many growth factors and inflammatory mediators are involved [1]. Airway remodeling in asthma also involves an increase in angiogenesis, a process most likely to be mediated by several angiogenic mediators including vascular endothelial growth factor (VEGF) [2].

VEGF is considered to be the most important angiogenetic factor, that induces vascular endothelial cell proliferation, tubule formation and increases microvascular permeability [3]. The latter is a common feature of vascular remodeling in asthma and is modulated by the release of different inflammatory mediators, cytokines, proteases and growth factors.

Cysteinyl leukotrienes (Cyst-LTs) are important molecules that promote both airway inflammation and remodeling [4]. Evidence suggests that Cyst-LTs play an important role in the airway remodeling observed in persistent asthma that includes increases of airway goblet cells, mucus, blood vessels, smooth muscle, myofibroblasts, and airway fibrosis. Cyst-LTs can transcriptionally activate VEGF production via cysLT1 receptors, indicating that Cyst-LTs may be important in the angiogenic process of airway remodeling [5]. Furthermore in vitro and in vivo studies support that the administration of a Cyst-LTs antagonist leads to alterations of VEGF levels [6,7].

In the present study, we aimed to determine the levels of both Cyst-LTs and VEGF in sputum supernatants of patients with asthma and to investigate possible associations with airway vascular permeability as assessed by airway vascular permeability (AVP) index. Furthermore, we wanted to determine whether significant confounding factors such as underlying severity, atopy and smoking significantly affect the above mediators and processes.

Materials & methods

Subjects

Patients were recruited from an open cohort of asthmatic patients who were followed up in the asthma clinics of the 1st and 2nd Respiratory Medicine University Departments in Athens for at least 2 years. The recruitment period was between June 2008 and September 2012. The diagnosis of asthma was established according to GINA guidelines [8]. The diagnosis of Severe Refractory asthma (SRA) was established according to ATS criteria [9]. Patients with symptoms of acute rhinitis, nasal congestion, nasal polyps, or a history of aspirin hypersensitivity were excluded since these conditions are related to eicosanoid inflammation [10–12]. Patients receiving leukotriene modifiers were also excluded. Subjects with any other respiratory disease or any concomitant malignant, heart, renal, liver or collagen disease were excluded. Patients with a respiratory tract infection or asthma exacerbation in the past 8 weeks prior to admission were also excluded. The study was approved by the ethics committees of both hospitals and all subjects provided an informed consent.

Induced sputum

Sputum was induced as previously described using all the modifications for safe measurements according to the underlying asthma severity [13,14]. Briefly, patients inhaled 3% saline at room temperature nebulized by an ultrasonic nebulizer (DeVilbiss Co., Heston, UK) at the maximal saline output (4 ml/min). The total period of sputum induction was 15 min. Subjects were encouraged to cough deeply at 3-min intervals until the 15-min induction time had been completed. Sputum was processed using selected plugs as previously described [15]. Dithiothreitol (DTT) was added in a volume equal to four times the weight of the sputum specimen and it was further diluted with phosphate buffered saline (PBS) in a volume equal to the sputum plus DTT. Total cell counts were performed on a hemacytometer using Trypan blue stain. Slides were prepared by using cytospin (Shandon, Runcorn, UK) and were stained with May-Grunwald and Giemsa for differential cell counts. Cell counting was performed by an observer blind to the clinical characteristics of the subjects. At least 500 inflammatory cells were counted in each sample. A sample was considered adequate when the patient was able to expectorate at least 2 ml of sputum and the slides contained $<$10% squamous cells on differential cell counting. Total cell count expressed as the number of cells $\times$ 10^6 and the percentage (%) of sputum inflammatory cells were used for our analysis. Sputum supernatants were kept at -70 °C for further measurement of Cyst-LTs, VEGF and albumin.

Lung function

Forced expiratory volume in 1 s (FEV_1) and forced vital capacity (FVC), were measured using Master Screen Body (Viasys Healthcare, Jaeger, Hoechberg, Germany) according to the American Thoracic Society guidelines [16].

Atopic status

A positive skin prick test to any of twenty common aeroallergens (including mites, grasses, trees, fungus, domestic animals) was used to confirm atopy.

Mediator assays

VEGF was measured using an enzyme-linked immunosorbent assay kit (ELISA, R&D systems, Minneapolis, Minnesota, USA with detection limits of 9 pg/ml). Cyst-LTs were measured using an ELISA kit (detection limit 13 pg/ml, Cayman Chemical, Ann Arbor MI, USA). The intra and inter assay variability were assessed according to the manufacturer's instructions for all the mediators measured and were within acceptable Coefficient of variation (CV) % (4% and 5.5% respectively for VEGF and Cyst-LTs). The recovery and linearity of the assays after appropriate spiking experiments produced samples with values within the dynamic range of the assay. Blood was drawn for determination of serum albumin. The airway vascular permeability (AVP) index was calculated as the ratio of albumin concentrations in induced sputum and serum [17].

Study design

On day 1, all subjects underwent medical history and physical examination by an experienced respiratory physician, lung function measurements, BMI measurement, and skin prick tests. The day after, blood samples were taken and sputum induction was performed.

Statistical analysis

Normally distributed data was presented as mean ± standard deviation (SD), whereas skewed data was presented as median (interquartile ranges). Normality of distribution was checked with Kolmogorov–Smirnov test. Statistical comparisons between groups were performed with one way analysis of variance (ANOVA) for normally distributed data and with Kruskal–Wallis tests for skewed data, accompanied by appropriate post-hoc tests for multiple comparisons (Bonferroni and Dunn's, respectively). Differences in numerical variables within two groups were evaluated with unpaired *t*-tests or Mann–Whitney *U*-tests for normally and skewed data respectively, whereas comparisons of proportions were performed using chi-square tests. In order to examine the association between VEGF & Cyst-LTs, inflammatory cells, AVP index and lung function tests, multivariate linear regression analysis was performed using VEGF & Cyst-LTs, as the dependent variables. Linear regression analysis was performed in one model using presence of SRA, smoking, age, gender, BMI, atopy, history of rhinitis, duration of the disease and treatment regimens as covariates. Data were interpreted as standardized coefficients with 95% confidence intervals. A p value <0.05 (2-sided) was considered significant. Statistical analysis was performed using SPSS

Table 1 Subjects characteristics and inflammatory variables.

	Asthma (all)	Normals	*P* value
	N = 121	*N* = 30	
Age	53 [42–63]	46 [39–56]	0.165
Gender F/M	70/51	18/12	0.450
Atopy	81/121	NA	ND
Smokers	41/121	15/30	0.315
Pack years	50 [30–55]	42 [31–49]	0.126
Duration of asthma (years)	29 [21–42]	NA	ND
BMI kg/m^2	27 [24–29]	27.5 [23.5–28]	0.587
FeNO ppb	19 [14–28]	11 [8–16]	**<0.001**
FEV_1% pred	82 ± 17	97 ± 11	**<0.001**
FEV_1/FVC	68 [64–78]	94 [86–101]	**<0.001**
Cells × 10^6/ml	1.7 [1–3]	0.9 [0.6–1.2]	**0.030**
Eosinophils%	4 [2–9]	0.5 [0–1]	**<0.001**
Neutrophils%	35 [22–44]	27 [21–34]	**0.020**
Macrophages%	57 ± 17	67 [54–75]	**0.010**
Lymphocytes %	1 [0–2.2]	0.5 [0–1]	0.689
VEGF (pg/ml)	845 [487–1034]	432 (327–654)	**<0.001**
Cyst-LTs (pg/ml)	209 [171–296]	92 [75–114]	**<0.001**
AVP index	0.036 [0.02–0.053]	0.012 [0.007–0.0122]	**<0.001**
Treatment regimens			
ICS	121	N/A	
LABA	97		
Oral Cs	19		
Omalizumab	9		

Normally distributed data are presented as mean ± standard deviation (SD), whereas skewed data are presented as median (interquartile ranges). Abbreviations: M = Male, F = Female, BMI = Body mass index, FeNO = Fraction of exhaled nitric oxide, FEV_1 = Forced expiratory volume in 1 s, FVC = Forced vital capacity, AVP: Airway vascular permeability, VEGF = Vascular endothelial growth factor, Cyst-LTs = Cysteinyl leukotrienes. Bold numbers indicate significant differences across the two groups. ICS = inhaled corticosteroids, LABA = Long acting β_2 agonists, Cs-Corticosteroids, NA = Not applicable, ND = Not done. Bold numbers indicate significant differences across the two groups.

16.0 (Chicago, Illinois, USA) and Graph Pad Prism 5 (Graph Pad Software, California, USA).

Results

One hundred twenty one patients with asthma were recruited. Thirty eight of them (31%) had SRA while the remaining eighty three (69%) had mild to moderate asthma. Among asthmatic patients, 41 (33%) were smokers. Thirty healthy, non-atopic, subjects (15 smokers) comprised the control group. Subjects' demographic characteristics and inflammatory variables are summarized in Table 1. Similar data for the three asthma sub-groups are provided in Table 2. SRA patients were all receiving >1200 μg budesonide/day or equivalent plus long acting beta-2 agonists (LABA), whereas patients with mild to moderate asthma were receiving <800 μg budesonide/day or equivalent. Among SRA patients, 11 were also receiving 5 mg prednisolone/day while 6 were receiving 7.5 mg prednisolone/day with the remaining 2 receiving 10 mg prednisolone/day. Sputum induction was well tolerated by all patients.

VEGF and Cyst-LTs levels [pg/ml] were significantly higher in patients with asthma compared to normal subjects (Table 1, Figs. 1 and 2). Patients with SRA had significantly higher levels for both VEGF and Cyst-LTs compared to milder forms of the disease, both in smoking and in non-smoking subjects (Table 2, Figs. 1 and 2). Smoking significantly increases the levels of Cyst-LTs in patients with mild to moderate asthma (Table 2, Figs. 1 and 2). The AVP index was significantly higher in patients with asthma compared to normal subjects and was significantly affected by both underlying severity and smoking habit (Table s 1 and 2, Fig. 3).

Eosinophils (%) in induced sputum were significantly higher in patients with SRA compared to smoking and non-smoking mild to moderate ones (Table 2). Both patients with SRA and smoking asthmatics had significantly higher percentages of neutrophils in sputum compared to non-smoking ones with milder form of the disease (Table 2).

Associations

Major findings from regression analysis are summarized in Table 3. Multivariate regression analysis in the whole group showed a significant association of Cyst-LTs levels in sputum supernatants with VEGF levels in sputum supernatants and AVP index. A similar positive association was observed between VEGF levels in sputum supernatants and the AVP index. The presence of SRA was a significant covariate for both associations (Table 3). No other significant associations were observed. In normal subjects the aforementioned associations were not observed.

Table 2 Subjects characteristics and inflammatory variables in asthma subgroups.

	SRA	Mild to moderate non-smoking asthma	Mild to moderate smoking asthma	*P* value
	N = 38	*N* = 42	*N* = 41	
Age	52 [43–59]	55 [42–60]	47 [34–60]	0.205
Gender F/M	24/14	25/17	21/20	0.101
Atopy	27/38	28/42	25/41	0.632
Smokers/Pack years	0	0	50 [30–55]	ND
Duration of asthma (years)	28 [20–38]	30 [23–39]	28.5 [22–39]	0.705
BMI kg/m^2	27 [25–30]	27 [24–30]	26.5 [23–28]	0.619
FEV$_1$% pred	64 ± 14[a,b]	87 ± 13	91 ± 12	**<0.001**
FEV$_1$/FVC	64 ± 7[a,b]	73 ± 10	74 ± 9	**<0.001**
Cells × 10^6/ml	2.75 [1.4–3.7][a,b]	1.2 [0.7–2.3]	1.7 [1.1–2.9]	**0.005**
Eosinophils%	10 [4–16][a,b]	4 [2–8]	2 [2–5]	**<0.001**
Neutrophils%	40 [30–46][a]	23 [20–40][b]	38 [23–44]	**0.002**
Macrophages%	48 ± 15[a,b]	61 ± 17	59 ± 15	**0.010**
Lymphocytes %	1 [0–2]	1.75 [1–3]	1 [0–2.5]	0.554
VEGF (pg/ml)	1055 [909–1563][a,b]	567 (483–860)	673 [444–875]	**<0.001**
Cyst-LTs (pg/ml)	270 [213–387][a,b]	169 [148–198][b]	209 [181–328]	**<0.001**
AVP index	0.045 [0.031–0.057][a,b]	0.024 [0.017–0.042][b]	0.036 [0.028–0.066]	**<0.001**
Treatment regimens				
ICS	38	42	41	ND
LABA	38	28	31	
Oral Cs	19	0	0	
Omalizumab	9	0	0	

Normally distributed data are presented as mean ± standard deviation (SD), whereas skewed data are presented as median (interquartile ranges). Abbreviations: M = Male, F = Female, BMI = Body mass index, FeNO = Fraction of exhaled nitric oxide, FEV$_1$ = Forced expiratory volume in 1 s, FVC = Forced vital capacity, AVP: Airway vascular permeability, VEGF = Vascular endothelial growth factor, Cyst-LTs = Cysteinyl leukotrienes. ICS = Inhaled corticosteroids, LABA = Long acting β_2 agonists, Cs = Corticosteroids, ND = Not done. Bold numbers indicate significant differences across the three groups.

[a] Statistically significant difference compared to mild to moderate non-smoking asthma.

[b] Statistically significant difference compared to mild to moderate smoking asthma.

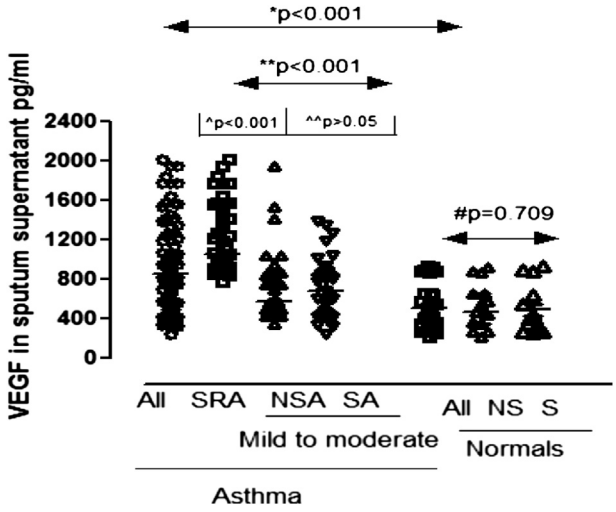

Figure 1 VEGF values [pg/ml] in patients with asthma [All, Severe refractory (SRA), mild to moderate (non-smoking (NS), smoking (S)] and healthy subjects [all, non-smoking (NS), smoking (S)], $*p < 0.001$ in favor of asthma, $**p < 0.001$-values indicate differences across the 3 groups, $\hat{}p < 0.001$ in favor of SRA, $\hat{}p < 0.001$ in favor of smoking asthma, $\#p = 0.709$ indicate no significant difference. Values are presented as median interquartile ranges.

Discussion

The current study showed that both VEGF and Cyst-LTs were up-regulated in sputum supernatant of patients with asthma, being more increased in patients with SRA compared to those with mild to moderate asthma. Furthermore, they were both closely associated with the AVP index, but these associations were mainly affected by the presence of SRA.

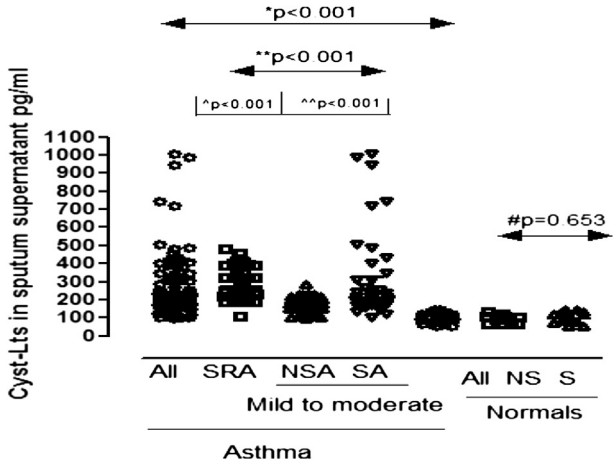

Figure 2 Cyst-LTs values [pg/ml] in patients with asthma [All, Severe refractory (SRA), mild to moderate (non-smoking (NS), smoking (S)] and healthy subjects [all, non-smoking (NS), smoking (S)], $*p < 0.001$ in favor of asthma, $**p < 0.001$-values indicate differences across the 3 groups, $\hat{}p < 0.001$ in favor of SRA, $\hat{}p > 0.05$, $\#p = 0.653$ indicate no significant difference. Values are presented as median interquartile ranges.

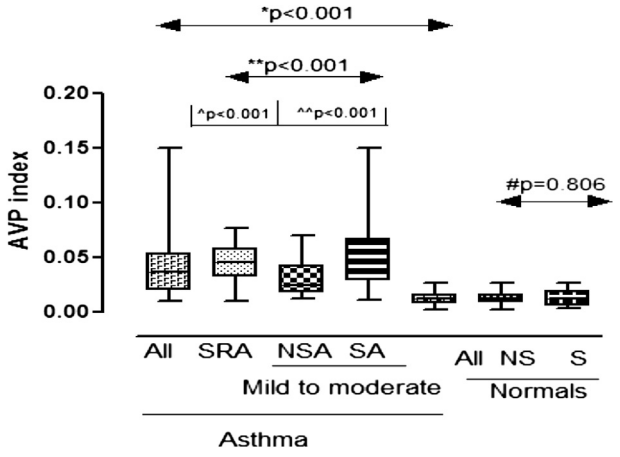

Figure 3 AVP index [All, Severe refractory (SRA), mild to moderate (non-smoking (NS), smoking (S)] and healthy subjects [all, non-smoking (NS), smoking (S)], $*p < 0.001$ in favor of asthma, $**p < 0.001$-values indicate differences across the 3 groups, $\hat{}p < 0.001$ in favor of SRA, $\hat{}p < 0.001$ in favor of smoking asthma, $\#p = 0.806$ indicate no significant difference. Values are presented as median interquartile ranges.

Angiogenesis is a complicated multi-step process which is regulated by several angiogenic factors. It is well known that VEGF is the most potent angiogenic mediator [18]. It is increased in the airways of patients with asthma [19—21], it is implicated in the microvascular permeability process and finally correlates with mucosal vascularity [19]. By increasing vascular permeability, VEGF permits the leakage of many mediators in the extravascular space which indirectly leads to mucosal edema and airway obstruction [21].

Cyst-LTs are major eicosanoid products of activated eosinophils, mast cells, basophils and monocytes, and have been implicated in the pathogenesis of asthma. The contribution of Cyst-LTs in the remodeling process seen in asthma involves new vessel formation which is considered a characteristic of angiogenesis seen in vascular remodeling [4]. An in vitro study supports that Leukotriene C4 is, at least, one of the important factors involved in angiogenesis during inflammatory processes by stimulating tube formation and endothelial cell migration [22]. In another study, leukotrienes C4 and D4 promoted angiogenesis in the chick chorioallantoic membrane in vivo via a receptor-mediated interaction [23]. In a recently published study where human monocytes and bronchial smooth muscle cells were purified from peripheral blood obtained from normal donors, Cyst-LTs and specifically LTD4 transcriptionally activated VEGF production via Cyst-LT1 receptors, suggesting the importance of cysLTs in the angiogenic process of airway remodeling[5].

Mast cells are considered key cells for Cyst-LTs production. Mast cells also enhance the migration of endothelial cells in growing capillaries supporting a significant roles of mast cells in angiogenesis [24]. Moreover, mast cells produce and secrete VEGF, which has been shown to stimulate mast cell migration at sites of angiogenesis [25]. According to the above published data, Cyst-LTs and VEGF may have a common cellular source, the mast cell, which may partially explain their association and contribution to angiogenesis.

Table 3 Multivariate Regression analysis (in one model-major findings) between VEGF & Cyst-LTs, inflammatory cells, AVP index and co-variates in the whole group of patients with asthma. The values of Table 3 need to be aligned.

Variables	Cyst-LTs (pg/ml)			VEGF (pg/ml)		
	β standardized coefficient [95% CI]	Adjusted R^2	*p* value	β standardized coefficient [95% CI]	Adjusted R^2	*p* value
Total cells	−0.036 [-17,11]	0.0008	0.774	−0.047 [−16, 8]	0.001	0.550
Eosinophils %	−0.007 [−11, 11]	0.0009	0.836	−0.125 [−6, 2]	0.003	0.117
Neutrophils%	−0.006 [−5.5, 3]	0.0008	0.503	0.026 [−3, 5]	0.005	0.723
Cyst-LTs [pg/ml]	N/A	N/A	N/A	0.289 [0.36, 1]	0.299	<0.001[a]
AVP index	0.284 [30.161]	0.168	0.004[a]	0.237 [1629, 7269]	0.197	0.020[a]
VEGF [pg/ml]	0.289 [0.36, 1]	0.299	<0.001[a]	N/A	N/A	N/A
Presence of SRA	0.564 [214, 462]	0.415	<0.001[a]	0.704 [449, 762]	0.635	<0.001[a]
Smoking	−0.007 [−1353, 1246]	0.005	0.736	0.160 [−8, 5]	0.098	0.095
Duration of the disease	0.075 [−325, 781]	0.007	0.427	0.13 [−1, 4]	0.002	0.167
BMI	0.006 [−281, 704]	0.006	0.324	0.17 [−17, 8]	0.011	0.218
Presence of Rhinitis	0.011 [−311, 801]	0.004	0.526	−0.18 [−67, 4]	0.810	0.090
Atopy	0.034 [−342, 679]	0.007	0.308	−0.04 [−43, 27]	0.004	0.657
Cs per os	0.026 [−27, 73]	0.002	0.170	−0.035 [−23, 103]	0.005	0.604

Regression analysis was performed in one model using age, gender, BMI, atopy, duration of the disease, presence of SRA, presence of rhinitis, smoking and treatment regimens as covariates.

[a] Indicate significant association. Abbreviations: AVP = Airway Vascular Permeability, VEGF = Vascular endothelial growth factor, Cyst = LTs-Cysteinyl leukotrienes, SRA = Severe refractory asthma, BMI = Body mass index, CI = Confidence intervals, NA = Not applicable.

On the other hand, Cyst-LTs might influence the angiogenesis process through the enhancement of the production of an angiogenetic factor such as VEGF. This direct linkage has been supported in a previous experimental study at a cellular level, where. LTD4 induced the expression of VEGF, via the Cyst-LT1 receptor, at both the mRNA and protein levels in two cell types relevant to asthma and airway remodeling processes, namely human monocytes and bronchial smooth muscle cells, indicating that Cyst-LTs led to increased angiogenesis via the up-regulation of VEGF [5]. In our study, using a direct assessment of the airways inflammatory process such as sputum induction, we observed a close relationship between VEGF and Cyst-LTs. The above close relationship was further associated with an index of vascular permeability. Our results support the speculation that Cyst-LTs may induce VEGF expression which then drives the vascular permeability process.

In our study, we observed that SRA was a significant covariate for the significant associations using a multivariate regression analysis. This finding is partially supported by previous reports where Cyst-LTs are up-regulated in severe asthma [20,26], as well as by the increased VEGF levels in severe asthma and the specific role of VEGF in inducing vascular permeability in a group of SRA patients [27].

Despite the fact that asthmatic smokers had increased sputum levels of Cyst-LTs, smoking was not a significant covariate in the regression analysis. This might be attributed mainly to the underlying asthma severity and the response to treatment. However published data supports that smoking significantly influences the concentration of Cyst-Lts in different biological fluids [28,29]. In an animal study, bronchial epithelial cells isolated from asthmatic rats reacted differently to chronic smoking exposure compared to cells from normal rats, leading to an increase of VEGF levels [30]. However, lower sputum levels of VEGF in asthma have been related to underlying smoking history compared to non-smoking asthmatics [31]. The increased AVP index in smoking asthmatics and its significant association with Cyst-LTs might be explained by an airway vascular process which works independently of VEGF.

Cyst-LTs receptor antagonists lead to diminished airway levels of VEGF in both murine asthma models and asthmatic patients. Pranlukast, a selective leukotriene receptor antagonist decreased airway microvascular permeability through, at least in part, a decrease in airway VEGF levels in steroid-untreated asthmatic patients [7]. In an experimental study using a mouse model, similar results were observed [6]. Our patients were optimally treated with ICS and most of them were also receiving LABA. Despite their optimal treatment, some of them still presented an increased inflammatory response mainly toward the direction of the vascular process, presenting elevated VEGF sputum levels. Thus, Cyst-LTs antagonists could be considered an additive treatment regime for the more chronic persistent asthma, characterized by airway remodelling and mainly SRA due to their effects in vascular remodeling.

In conclusion, the measurement of sputum Cyst-LTs and VEGF not only provides insights into the underlying inflammatory mechanisms and particularly those related to the vascular remodeling process, but with further validation, it may also provide information that will facilitate the clinical assessment and the optimization of therapeutic strategies in asthma.

Conflicts of interest statement

All the authors declare that they have no conflict of interest related to the content of this manuscript.

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