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Title: Airway surfactant protein D (SP-D) deficiency in adults with severe asthma

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Abstract

Background: Surfactant protein D (SP-D) is an essential component of the innate immune defence against pathogens within the airways. In addition SP-D regulates allergic inflammation and promotes the removal of apoptotic cells. SP-D dysregulation is evident in several pulmonary diseases. Our aim was to investigate whether airway and serum levels of SP-D are altered in treatment-resistant severe asthma.

Methods: SP-D concentrations were measured in matched serum and bronchoalveolar lavage (BAL) samples collected from 10 healthy controls (HC) and 50 asthmatics (22 mild [MA] and 28 severe [SA]). These samples were also evaluated by Western blots to investigate variations in SP-D size.

Results: SP-D levels in BAL were significantly lower in SA compared to HC and MA ($P < .001$) and inversely correlated with BAL eosinophil cationic protein (ECP) concentrations in severe asthma ($P < .01$). Serum SP-D was significantly increased in SA when compared to HC and MA ($P < .001$), BAL/serum ratios were significantly lower in SA compared to HC and MA ($P < .001$). Reduced SP-D levels in in BAL with concomitant rises in serum in SA were associated with degraded fragments of SP-D in the serum and increased BAL neutrophils and lipopolysaccharide levels.

Conclusions: These findings suggest defective innate immunity within the airways in severe asthma, as reflected by low BAL SP-D concentrations and altered bacterial presence with airway neutrophilia. Furthermore, BAL SP-D leakage into the serum in severe asthmatics may provide a peripheral blood biomarker reflecting increased epithelial damage and/or epithelial permeability within the peripheral airways.

Abbreviations used

BAL:	Bronchoalveolar lavage
SP-D:	Surfactant protein D
HC :	Healthy control
MA :	Mild asthma
SA :	Severe asthma
COPD:	Chronic obstructive pulmonary disease
IPF :	Idiopathic pulmonary fibrosis
CF:	Cystic fibrosis
ELISA:	Enzyme-linked immunosorbent assay
FEV:	Forced expiratory volume
GINA:	Global initiative for asthma
FVC:	Forced vital capacity
PBS:	Phosphate buffered saline
SDS-PAGE:	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
PSG:	Pollen starch granules
W/V:	Weight to volume
V/V:	Volume to volume

BTS: British thoracic society

MPO: Myeloperoxidase

ECP: Eosinophil cationic protein

IL-(8): Interleukin-(8)

LPS: Lipopolysaccharide

INTRODUCTION

Asthma is a chronic airway disorder that is characterised pathologically by airway inflammation and structural tissue remodelling. Airway inflammation in asthma is typically eosinophilic, with elevated type 2 cytokines such as interleukin (IL)-4, IL-5 and IL-13.[1] Airway remodelling is characterised by structural airway changes associated with airway wall thickening, which is most evident in patients with severe asthma and inversely correlates with lung function measures[2]. In severe asthma there may be neutrophilic as well as eosinophilic airway inflammation.[3, 4] We have reported that neutrophilic asthma is linked to an altered bacterial profile within recovered bronchoalveolar lavage fluid (BAL).[5] As surfactant protein D is an important component of innate immunity within the distal airways, we investigated the potential that dysregulation of airway surfactant protein D is a feature of severe asthma.

Surfactant protein D has numerous actions whose deficiency could be detrimental in asthma. In the airways, SP-D binds to carbohydrates in a calcium dependent manner, orchestrating pathogen aggregation and enhancing their phagocytosis. In addition, SP-D enhances their chemotactic, phagocytic, and oxidative properties interacts with phagocytic cells, such as macrophages and neutrophils.[7] SP-D also modifies allergic responses in that it reduces the activation of basophils, mast cells and eosinophils whilst regulating the release of TGF-β, IL-10 and IL-12 and reducing IL-2 concentrations.[10, 13] In allergen challenge models, exogenous SP-D inhibits allergen-induced T lymphocyte proliferation and hypersensitivity[16] , inducing a shift from a type 2 to type 1 cytokine response, and reduces induced airway remodeling.[17, 18] Conversely SP-D deficient mice exhibit an enhanced IL-13 dependent inflammatory allergic response within the airways.[19]

The aim of this study was thus to investigate airway luminal and serum SP-D concentrations in mild and severe asthmatics, compared to healthy controls, and to examine the structural integrity of SP-D in these study groups. We hypothesized that severe asthma may be associated with a deficiency of SP-D within the airways.

MATERIALS AND METHODS

Subjects

Adult volunteers aged 18 to 65 years, recruited from the Wessex Severe Asthma Cohort and a departmental database of volunteers participated in the study, which had prior ethics approval from Southampton and West Hampshire Research Ethics Committee (A) [reference numbers 05/Q1702/165 and 08/H0502/6]. All subjects gave written informed consent. Healthy controls had no history of respiratory disease and no evidence of bronchial hyperreactivity to methacholine challenge. Asthmatic volunteers all had a physician diagnosis of asthma. In addition the mild asthmatics were all skin prick test positive to house dust mite allergen extract [*Dermatophagoides pteronyssinus*]), had abnormal airway reactivity to methacholine required to lower the FEV_1 by 20 percent (PC_{20}) of less than 8mg/mL, were life-long non-smokers and only receiving as required short acting beta agonist therapy. Severe asthma patients were on step 4 (n=12) or 5 (n=16) of GINA guidelines therapy[20] ,were poorly controlled with a six domain Asthma Control Questionnaire score of ≥1.5 and had not smoked for at least 1 year.

Bronchoscopic airway samples

Fibreoptic bronchoscopy was undertaken according to established guidelines. [21] BAL was performed by instilling 6 x 20 mL aliquots of pre-warmed normal saline into a sub-segmental bronchus of the anterior segment of the right upper lobe followed by gentle suction. BAL fluid was filtered (BD Falcon cell strainer, Marathon Laboratory Supplies, UK) then centrifuged at 1300G for 10 mins at 4°C. Cell pellets were resuspended in phosphate buffered saline (PBS) for cytospins and the supernatant was stored at -80°C for later analysis. Cells were stained with rapid Romanowsky stain (Raymond Lamb Ltd, UK) to distinguish between macrophages, neutrophils and eosinophils, 400 cells were counted blind using coded samples.

Serum sampling

Venous blood was allowed to clot for 60 minutes, and then centrifuged for 15 minutes at 1500g at 4°C. The serum layer was removed and stored at -80°C for further analysis.

SP-D enzyme-linked immunosorbent assay

Antibodies were raised in rabbits against a recombinant fragment of SP-D (neck/head) which is considered the functional domain of the protein. Briefly, SP-D was assayed in 96-Well microtiter plates (Maxisorp, Nunc) coated with rabbit anti-recombinant fragment human SP-D (α-rfhSP-D at a 1:1000 dilution as previously described and detected with biotinylated-α-rfhSP-D. Native human SP-D (0-500µg/mL) was used as a standard (full method in supplementary information) [10]

Native human SP-D was used as a standard (0-500µg/mL).[10]

SP-D western blotting

The same antibody, as described above, was used to detect 'functional' SP-D in patient samples. 20µl neat BAL or serum, (100µl serum incubated with 20µl strataclean resin™, in 500µL PBS [Agilent Technologies, Inc.] with 2mM $CaCl_2$). was incubated for 30 minutes with rotation at room temperature. Samples were then spun at 1300G and reduced according to the manufacturer's instructions (NuPAGE®). Proteins were resolved by 12% (w/v) SDS-PAGE (NuPAGE®, life technologies). Degradation of endogenous nhSP-D in BAL was visualized by immunoblotting of PVDF membranes (iBlot®, life technologies) using α-rfhSP-D antibodies.

ELISA for measures of inflammation

BAL concentrations of myeloperoxidase (MPO [Hycult Biotech, Cambridge Bioscience, Cambridge, uk.] detection range 1.6-100ng/mL), eosinophil cationic protein (ECP [Medical and microbiologies.co.ltd.] detection range 0.125-40 ng/mL), interleukin 8 (IL-8 [R&D systems, UK] detection range 0-1000pg/mL) and neutrophil elatase (NE [Cambridge Bioscience] detection range 0.4-25 ng/mL) were measured using ELISA kits as *per* the manufacturer's instructions.

Measurement of BAL Lipopolysaccharide

The *Limulus* Amebocyte Lysate (LAL [Thermo Scientific Pierce] detection range 0.1-1.0 EU/mL) Chromogenic Endotoxin Quantitation Kit was used to measure lipopolysaccharide (LPS) in BAL.

Statistical analysis

SPSS version v21 (IBM co, New York, USA) was used for statistical analysis of the data. Data that were not parametrically distributed (measures of SP-D, inflammation and LPS) were analysed using the Kruskal-Wallis test for between group comparisons, with Mann-Whitney testing between pairs of groups as appropriate. For normally distributed data, a one way ANOVA test was initially used to test for differences between groups, with an unpaired T-test used for further analyses. Linear regression analysis was undertaken to investigate biological relationships. A *P* value of equal or less than 0.05 was considered to indicate statistical significance.

RESULTS

Patient demographics

The healthy controls (n=10, 8F/2M, FEV_1 % predicted [group mean ± SD] 107.4 ± 7.1) had significantly better lung function than either the mild asthmatics (n=22, 15F/7M, FEV_1 % predicted 91.8 ± 13.3, $p<0.01$) or severe asthmatics (n=28, 20F/8M, FEV_1 % predicted 70.0 ± 24.8, $p<0.001$). Full participant characterisation is shown in e-Table 1 of the online supplement.

Measures of Inflammation

BAL cytospin differential cell counts were significantly altered in asthma from that in health, with both the mild and severe asthma groups having increased BAL eosinophils (group mean ±SD %) when compared to healthy controls (severe 3.1% ± 7.1%, mild 2.6% ± 3.2%, healthy 0.1% ± 0.3% ($p<0.01$)). In addition, the severe asthma patients had significantly increased BAL neutrophils (16.1% ± 19.1%) compared to mild asthmatics (3.7% ± 2.7%) and healthy controls, 5.0±6.3% ($p<0.05$)), with a resultant decreased BAL macrophages percentage (severe 63.6% ± 7.4%, mild 82.7% ± 9.3%, healthy 86.0% ± 12.1% ($p<0.01$ [figure 1]). BAL concentrations of MPO, IL-8 and ECP were increased in severe asthmatics compared to mild asthmatics ($p<0.02$) and healthy controls ($p<0.001$) as (shown in e-table 2, figure 2). Neutrophil elastase levels were also significantly increased in BAL from severe asthmatics compared to healthy controls ($p=0.0314$) and mild asthmatics ($p=0.0044$).

SP-D measures in matched BAL and serum samples

SP-D concentrations in BAL from healthy controls and mild asthmatics were not significantly different, (Median [IQR] 282[134-526] ng/mL compared to 268 [212 to 355] respectively) (figure 3a). BAL SP-D concentrations from severe asthma

patients were significantly decreased (Median [IQR] 42 [21 to 81] ng/mL compared to both healthy controls ($p<0.005$) and mild asthmatics ($p<0.005$). Conversely, severe asthmatics had significantly increased SP-D concentrations in serum (Median [IQR] 55 [28 to 130] ng/mL) compared to both healthy controls (16 [0 to 25] ng/mL, ($p<0.005$)) and mild asthmatics (19 [11 to 32]), $p<0.001$) (figure 3b). To integrate these measures from the 2 biological compartments, the log ratio of BAL/serum SP-D concentrations was calculated (figure 3c). There was no difference between the BAL/serum SP-D log ratio in healthy controls (Median [IQR] 1.5 [0 to 2.1]) or mild asthmatics (1.8 [1.7 to 2.1]) though in severe asthma (1.3 [0.8 to1.4]) this ratio was significantly different to the healthy controls ($p<0.001$) and mild asthmatics ($p<0.001$).

SP-D relationships in severe asthma

In severe asthma there was no significant impact of age (e-Table 3), past history of smoking, oral steroid use (e-table 1), inhaled steroid concentration or atopy on SP-D concentrations. There was, however a significant relationship to inflammation, in that serum SP-D correlated with BAL ECP (figure 4) in severe asthma (spearman's ρ, $p<0.01$, $r^2 = 0.352$).

SP-D integrity in BAL and serum

The structural integrity of SP-D was assessed by Western blotting. In BAL there was no evidence of SP-D breakdown in either healthy controls or mild asthmatics (e-Figure 1). Who demonstrated a single band at 50kDa under reducing conditions with no detectable SP-D in serum (data not shown). In contrast, in the severe asthma group in addition to the 50kDa band there were faint bands detected in the BAL at 38 and 17kDa (figure 5a) and strong bands in serum at 38, 28 and 17kDa (figure 5b).

lipopolysaccharide.

LPS was raised in severe asthmatics compared to mild ($p=0.0012$). Both BAL neutrophil elastase (spearman's ρ, $r^2=0.163$) and LPS (spearman's ρ, $r^2=0.162$) showed weak but significant inverse relationships with serum SP-D concentrations ($p<0.05$, figure 6c).

DISCUSSION

We have identified that in severe asthmatics there are reduced concentrations of surfactant protein D in BAL with an associated increase in serum concentrations. Furthermore, we have identified that the SP-D in severe asthma has 50kD forms but also fragmented bands at 17kD and 38kD in BAL and 17kD, 28kD and 30kD bands in serum.[9] These findings are all distinct from healthy and also from mild non-steroid treated asthma, suggestive of their particular relevance to the severe asthma population. All the severe asthmatics studied here had treatment resistant disease, in that despite treatment at steps 4 and 5 of the GINA guidelines they still had inadequate disease control, as reflected by a high ACQ score. All had experienced at least one disease exacerbation within the last year, though none within 8 weeks of their bronchoscopy and were in a stable phase of their asthma. This is the first study to investigate the relationship between asthma and SP-D in patients with such severe disease.

Severe treatment-resistant asthma is more complex than mild asthma, in that it is not purely a type 2 orientated disease. Consistent with this we identified that, in contrast to the mild asthmatics who had increased BAL eosinophils, the severe asthmatics had an increase in both eosinophils and neutrophils within their distal airways. Neutrophil serine proteinases can cleave SP-D within its carbohydrate recognition domain (CRD) region and render it functionally inactive.[23] Furthermore, bacterial proteases have been shown to cleave SP-D rendering the molecule incapable of binding and aggregating lung pathogens.[24] Thus in severe asthma, neutrophilic airway inflammation and possibly alteration in bacterial colonisation, may both contribute to SP-D degradation and impaired function.

In COPD reduced BAL SP-D concentrations are associated with increased concentrations of SP-D in the serum.[6] This has been interpreted as reflective of altered epithelial and/or endothelial permeability within the distal airways and alveoli, allowing leakage of SP-D from the airways into the serum. This interpretation is supported by studies of the effect of cigarette smoking. Cigarette smoke exposure reduces BAL SP-D and increases serum SP-D whilst increasing airway SP-D mRNA, indicating that the reduced airway SP-D is not due to reduced synthesis but due to enhanced leakage into the serum.[25] Furthermore, acute lung injury in animals, that induces airway inflammation, enhances spillage of SP-D molecules into the systemic circulation and increases serum SP-D. [26] The increase in serum SP-D in COPD is associated with risk of exacerbation and reduced by high dose oral steroid therapy.[27] Glucocorticoids up-regulate the expression of surfactant proteins including SP-D both *in vivo* and *in vitro*. [29] The effect of steroids in reducing serum SP-D is thus likely to be indirect, related to an anti-inflammatory effect reducing the airway leakage. Therefore, in our study it is unlikely that the altered SP-D dynamics in the severe asthmatics can be explained by their steroid therapy.

In severe asthma, the reduced distal airway SP-D concentrations may exacerbate the disease process. Although both mild asthmatics and severe asthmatics had increased eosinophil percentages within their bronchoalveolar lavage samples, compared to healthy airways, only the severe asthmatics had increased BAL ECP concentrations. SP-D interacts through its CRD with the Fc gamma II receptor on eosinophils and has been shown to inhibit eosinophil degranulation.[29] Reduced airway SP-D will thus increase the impact of airway eosinophilic inflammation and provides an explanation for the significant inverse correlation between serum SP-D and BAL ECP and the significantly higher ECP concentrations in the severe asthmatics. Altered airway concentrations of SP-D could also leave the airways

susceptible to opportunistic airway infection due to impaired bacterial clearance and enhance the IL-13 dependent allergic airway response[13]. An altered airway microbiome has been described in severe asthma, linked to neutrophilic airway inflammation and poorer lung function[5], both features evident in the severe asthma patients included in this study. Furthermore, the elevated levels of LPS present in the BAL of the severe asthmatics, as compared to the milder asthmatics, is consistent with the presence of increased gram negative bacteria within the airways. The altered innate immune profile, as reflected by defective and/or deficient SP-D, provides a potential explanation for this. The SP-D antibody used in these studies recognises the carbohydrate region of SP-D, the region responsible for interactions with pathogens and immune mediators, as such the ELISA measures reflect functional SP-D (see supplementary information).

Thus altered innate immune profile, as reflected by defective and deficient SP-D, provides a potential explanation for this.

The severe asthmatics gave a history of disease exacerbation within the last year. Exacerbations of asthma are mainly triggered by viral infections, such as rhinovirus and influenza A viruses. SP-D has been shown to directly inhibit viral activity and modulate subsequent innate and adaptive immunity and inflammation. Consistent with this, genetic mutations in SP-D that result in a reduced ability to form dodecamers or multimerise are associated with severe respiratory syncytial infections in children. [31] As SP-D enhances allergen removal and modulates allergic inflammation as well as aiding viral eradication, both allergen and viral responses may be more severe in treatment-resistant asthma. It is thus likely that the reduced SP-D levels in the airways is a significant contributor to the disease progression in these severe asthmatics characterised by decreased airflow and increased inflammatory mediators in the airway. It raises

the possibility that recombinant SP-D administration, which has been shown to restore the type1-type2 cytokine balance, may offer a therapeutic approach in patients with severe asthma unresponsive to standard therapy. Serum SP-D concentrations may also provide a peripheral blood biomarker reflecting airway inflammation in severe asthma and be used to monitor the effects of novel therapies, especially those which attempt to alter airway permeability. [32]

In summary, we demonstrate SP-D concentrations in bronchoalveolar lavage (BAL) decline and serum concentrations increase in treatment-resistant severe asthma. As reduced BAL concentrations of SP-D are reflected by elevated serum SP-D concentrations measures of serum SP-D may serve as a potential biomarker of airway events underlying treatment-resistant asthma. Furthermore, we have identified that the structural integrity of SP-D in the BAL and serum of patients with severe asthma is compromised. As SP-D is a component of the innate immune defence within the airway, contributing to pathogen clearance and resolution of inflammation, SP-D deficiency in severe asthma may have relevance to disease persistence. As such, airway replacement may offer a potential therapy in treatment-resistant asthma.

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Authorship RAM had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis, including and especially any adverse effects, RAM, CLG, LCL, CB, HWC and PHH contributed substantially to the study design, data analysis and interpretation, and the writing of the manuscript.

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Figure Legends

Figure 1 Bronchoalveolar lavage inflammatory cell counts: percentages of total cell count for (a) Macrophages, (b) neutrophils, (c) Eosinophils from healthy controls (HC [n=10]), mild asthma (MA [n=22]) and severe asthma (SA [n=28]).

Figure 2 Bronchoalveolar lavage Inflammatory mediator concentrations: concentrations in BAL of a) Myeloperoxidase (MPO) ng/mL, b) Interleukin (IL)-8 pg/mL, c) Eosinophil Cationic Protein (ECP) ng/mL from healthy controls (HC [n=10]), mild asthma (MA [n=22]) and severe asthma (SA [n=28]).

Figure 3 Surfactant protein (SP-D) concentrations, in matched BAL (a) and serum (b) samples from healthy controls (HC [n=10]), mild asthma (MA [n=22]) and severe asthma (SA [n=28]) as well as the log ratio BAL:serum SP-D concentrations (c) for the same groups.

Figure 4 Correlation between serum Surfactant protein (SP-D) and Eosinophil Cationic Protein (ECP) concentrations, in severe asthmatics. (spearman's ρ, $r^2=0.352$ $p<0.05$).

Figure 5 Western blots showing the structural integrity of Surfactant protein D (SP-D); severe asthma 1-7 matched patient (a) BAL and (b) serum samples.

Figure 6 Bronchoalveolar lavage neutrophil elastase and LPS levels and relationship to serum SP-D; (a) Neutrophil elastase levels in BAL from healthy volunteers (HC [n=10]), mild asthma (MA [n=22]) and severe asthma (SA [n=28]), (b) Lipopolysaccharide (LPS) concentrations in BAL from mild asthma (MA [n=16]) and severe asthma (SA [n=19]), (c) linear

regression plot of serum SP-D against both BAL neutrophil elastase and BAL LPS identifying significant inverse correlations (spearman's ρ, r^2 =0.163 and r^2=0.162 respectively, both p <0.05).

Supplementary information

SP-D information

e-Table 1-Patient characteristics

e-Table 2-Inflammatory markers

e-Table 3-linear regression with age as confounding factor

e-Figure- 1 Western blots showing SP-D in BAL from healthy control subjects (HC) and mild asthmatics (MA)

Figures

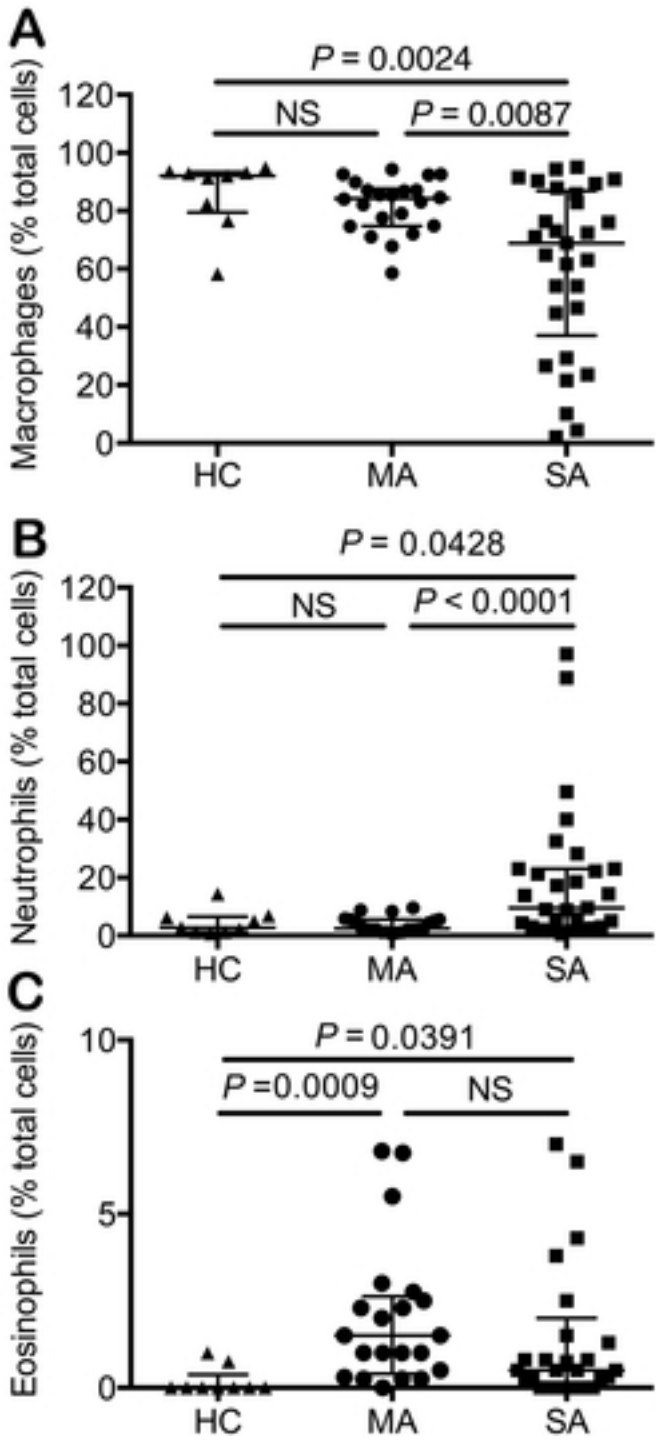

Figure 1

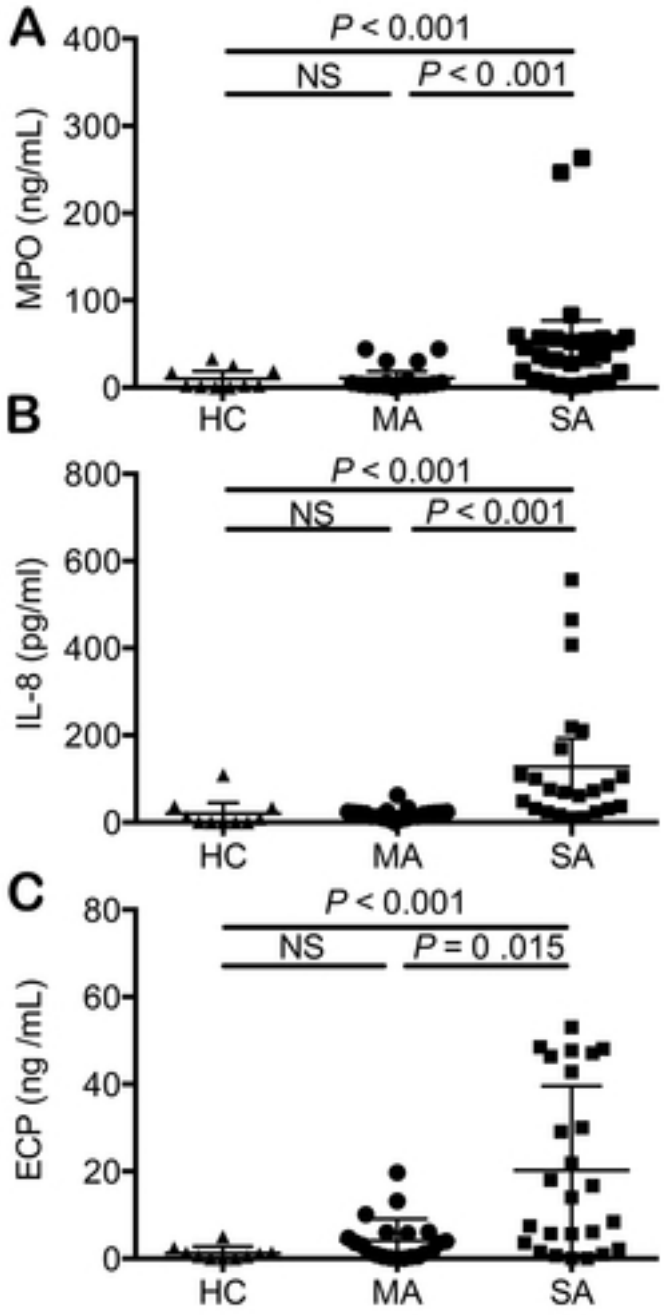
A
400
300
200
100
0
MPO (ng/mL)
P < 0.001
NS
P < 0 .001
HC
MA
SA
B
800
600
400
200
0
IL-8 (pg/ml)
P < 0.001
NS
P < 0.001
HC
MA
SA
C
80
60
40
20
0
ECP (ng /mL)
P < 0.001
NS
P = 0 .015
HC
MA
SA

Figure 2

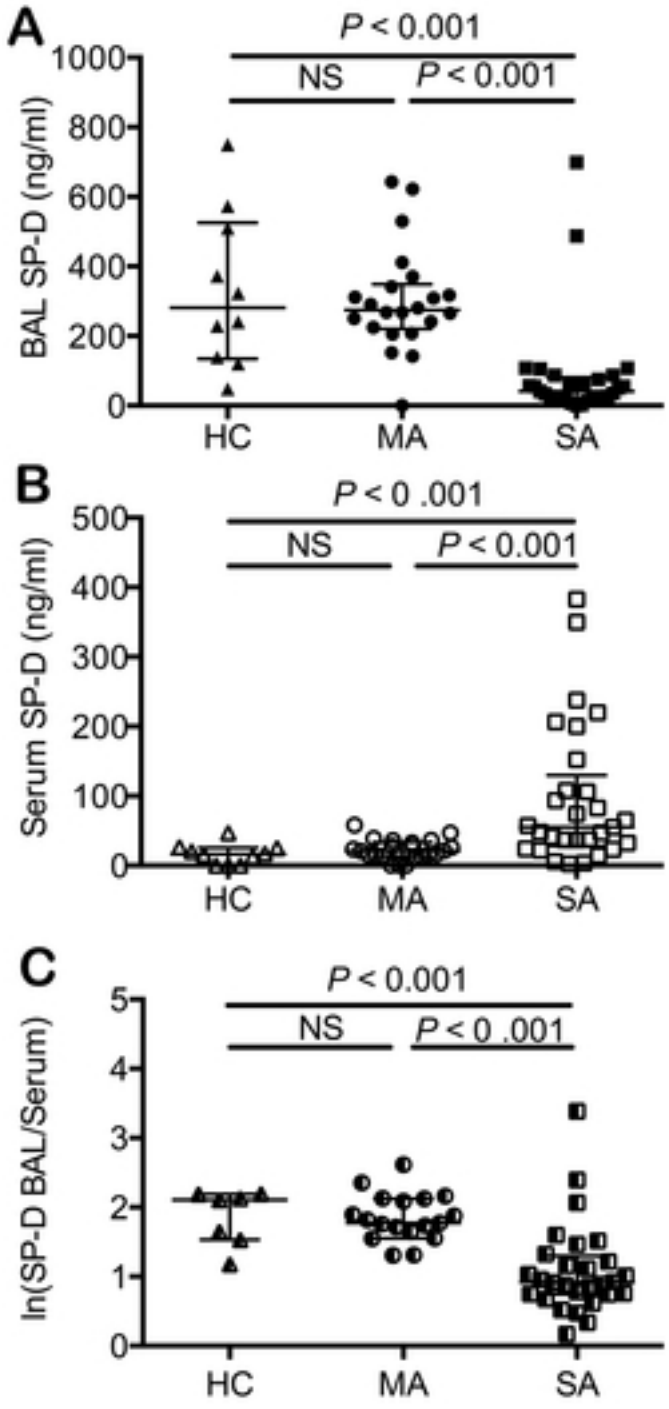
A
P < 0.001
NS
P < 0.001
BAL SP-D (ng/ml)
1000
800
600
400
200
0
HC
MA
SA
B
P < 0 .001
NS
P < 0.001
Serum SP-D (ng/ml)
500
400
300
200
100
0
HC
MA
SA
C
P < 0.001
NS
P < 0 .001
ln(SP-D BAL/Serum)
5
4
3
2
1
0
HC
MA
SA

Figure 3

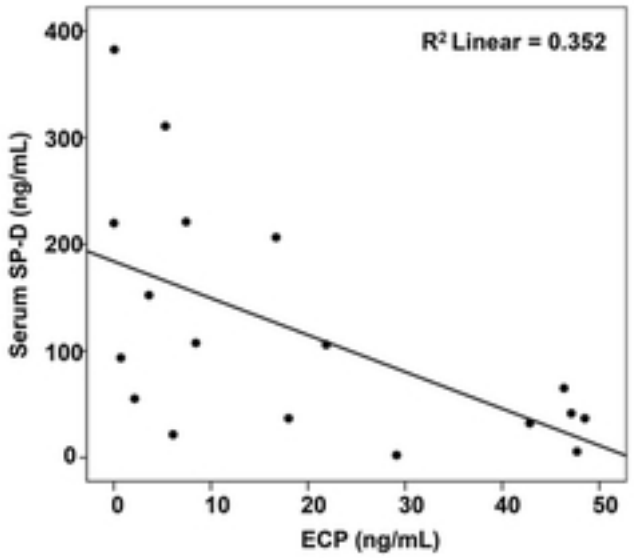

Figure 4

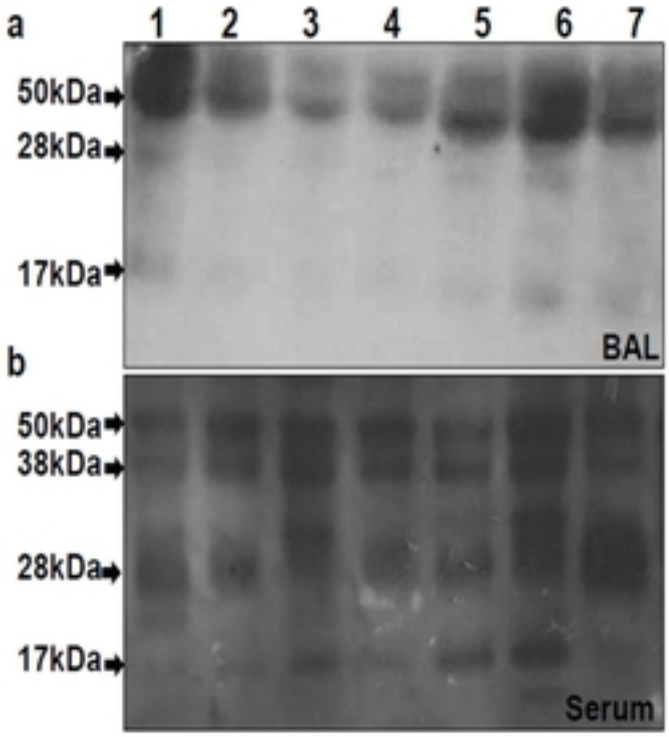

Figure 5

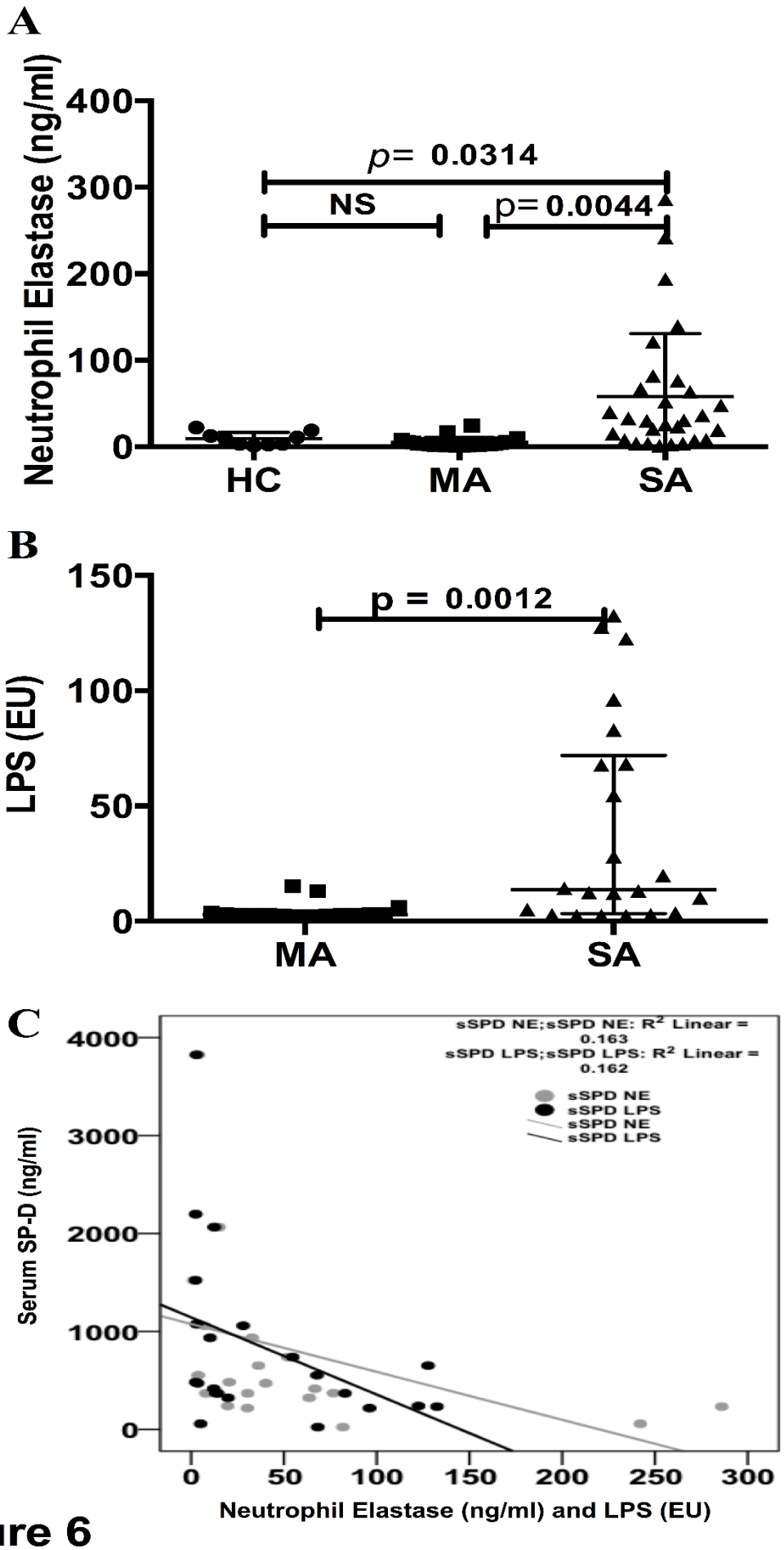
A
Neutrophil Elastase (ng/ml)
400
300
200
100
0
p= 0.0314
NS
p= 0.0044
HC
MA
SA
B
LPS (EU)
150
100
50
0
p = 0.0012
MA
SA
C
Serum SP-D (ng/ml)
4000
3000
2000
1000
0
sSPD NE;sSPD NE: R² Linear = 0.163
sSPD LPS;sSPD LPS: R² Linear = 0.162
sSPD NE
sSPD LPS
sSPD NE
sSPD LPS
0
50
100
150
200
250
300
Neutrophil Elastase (ng/ml) and LPS (EU)

Figure 6

	Healthy (n=10)	Mild (n=22)	Severe (n=28)	P value
Median MPO	2.3	4.1	37	❖ **<0.001**
				# **<0.01**
(IQR)	(0.89 to 20)	(2.4-14)	(8.4 to 57)	*** NS**
Median IL-8	4.5	20.48	72.4	❖**<0.0001**
				# **<0.01**
(IQR)	(0 to 34)	(11 to 24)	(31 to 170)	* NS
Median ECP	0.81	3.1	14	❖ **<0.0001**
				# <0.01
(IQR)	(0 to 1.7)	(0.47 to 5.9)	(2.9 to 45)	* NS
Median NE	9.7	3.0	30	❖ **<0.0001**
				# <0.01
(IQR)	(2.8 to16)	(1.9 to 5.4)	(8.1 to72)	* NS
Median LPS		2.8	14	❖ **<0.001**
	ND			
(IQR)		(2.6 to 3.6)	(3.4 to 72)	

Supplementary information.

SP-D antidodies.

The same 'in house' antibody was used as the capture antibody and for the detection of SP-D by ELISA. The antibodies were raised in rabbits against a recombinant fragment of SP-D, which consists of the head neck and a small part of the collagen-like region. This is considered to be the active part of the protein although cleavage effects functionality, the fragment is not as efficient as agglutination as the native full-length protein, it remains active. Therefore, because there is evidence of degradation only in the severe asthmatics it suggests that complete cleavage is the possible mechanism for lower levels of SP-D seen in this group.

Enzyme linked immunosorbent assay (ELISA)

Maxisorp 96-well plates (Nunc) were coated overnight at 4 ◦C with rabbit anti-SP-D antibody (d: 1/1000) in sodium carbonate coating buffer (15mM Na2CO3, 35mMNaHCO3, pH 9.6). Plates were washed 3 times with 300µl per well PBS-T and blocked for 1 hour at room temperature with 300 µl per well of blocking buffer (PBS-T with 3% BSA). Plates were washed as before and incubated for 1 hour at room temperature with 100 µl per well of biotinylated rabbit anti-SP-D antibody in blocking buffer (d: 1/1000). Plates were washed again and incubated with 100 µl per well of Horseradish peroxidase-linked anti-rabbit IgG in blocking buffer (d: 1/1000) for 1 h at room temperature. Plates were washed once more and incubated for 15 min with 100 µl per well of TMB peroxidase reagent (BioRad). The reaction was terminated with 50 µl per well of 1N H_2SO_4 and plates were read at 450 nm.

Asthma	Age Mean (range) SD	N	Male	Ex-Smokers	Atopy	Percentage predicted FEV_1 Mean (range) SD	Percentage predicted FVC Mean (range) SD	Inhaled steroid (µg) Median (IQR)	Oral steroid (n)
Healthy	30.2 (19-55) 14.1	10	2	1	2	108 (101-115) 7.1	112 (96-114) 8.8		
Mild	24.4 (18-56)7.9	22	7	0	22	90 (82-103) 15	110 (102-114) 7.6		
Severe	41.5(20-69) 13.2	28	8	14	19	70 (43-86) 26	82 (63-102) 27	1600-2400 (2000)	15

e-Table 1, Patient characteristics.

	Healthy (n=10)	Mild (n=22)	Severe (n=28)	P value
Median MPO	2.3	4.1	37	❖ **<0.001** # **<0.01**
(IQR)	(0.89 to 20)	(2.4-14)	(8.4 to 57)	* **NS**
Median IL-8	4.5	20.48	72.4	❖**<0.0001** # **<0.01**
(IQR)	(0 to 34)	(11 to 24)	(31 to 170)	* NS
Median ECP	0.81	3.1	14	❖ **<0.0001** # <0.01
(IQR)	(0 to 1.7)	(0.47 to 5.9)	(2.9 to 45)	* NS
Median NE	9.7	3.0	30	❖ **<0.0001** # <0.01
(IQR)	(2.8 to16)	(1.9 to 5.4)	(8.1 to72)	* NS
Median LPS	ND	2.8	14	❖ **<0.001**
(IQR)		(2.6 to 3.6)	(3.4 to 72)	

e-Table 2, Inflammatory mediators

	Beta	CI 95%	N	P Value
BAL SP-D	-126.0	-185.4 to -66.7	60	0.001
Serum SP-D	48.3	21.0 to 75.5	60	0.001

e-Table 3. Linear regression was use to assess age as a confounder for the SP-D results.
After adjusting for age, SP-D results were still significantly different between the groups.