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Sputum ADAM8 expression is increased in severe asthma and COPD

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Clinical
&
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Summary

Background Severe asthma and chronic obstructive pulmonary disease (COPD) are chronic inflammatory airway diseases in which the mechanisms are not fully understood. A disintegrin and metalloproteinase domain 8 (ADAM8) is an enzyme expressed on most leucocytes and may be important for facilitating leucocyte migration in respiratory disease.

Objective To investigate ADAM8 mRNA and protein expression in asthma and COPD and its relationship between asthma severity and inflammatory phenotypes.

Methods Induced sputum was collected from 113 subjects with asthma (severe $n = 31$, uncontrolled $n = 39$ and controlled $n = 35$), 20 subjects with COPD and 21 healthy controls. Sputum ADAM8 mRNA expression was measured by qPCR, and soluble ADAM8 (sADAM8) protein was measured in the sputum supernatant by validated ELISA.

Results ADAM8 mRNA correlated with ADAM8 protein levels ($r = 0.27$, $P < 0.01$). ADAM8 mRNA ($P = 0.004$) and sADAM8 protein ($P = 0.014$) levels were significantly higher in both asthma and COPD compared with healthy controls. ADAM8 mRNA ($P = 0.035$) and sADAM8 protein ($P = 0.002$) levels were significantly higher in severe asthma compared with controlled asthma. Total inflammatory cell count ($P < 0.01$) and neutrophils ($P < 0.01$) were also elevated in severe asthmatic sputum. Although ADAM8 mRNA was significantly higher in eosinophilic and neutrophilic asthma ($P < 0.001$), sADAM8 did not differ between asthma inflammatory phenotypes. ADAM8 expression positively correlated with sputum total cell count and sputum neutrophils.

Conclusions and Clinical Relevance ADAM8 expression is increased in both severe asthma and COPD and associated with sputum total cell count and neutrophils. ADAM8 may facilitate neutrophil migration to the airways in severe asthma and COPD.

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Introduction

Asthma and chronic obstructive pulmonary disease (COPD) are both globally prevalent chronic inflammatory conditions of the airways [1–4]. Severe asthma accounts for up to 10% of the asthma population [5–7], but disproportionately accounts for up to half the healthcare costs and social burden of asthma [6, 8, 9]. Severe asthma, by definition, is poorly controlled with current therapies such as long-acting β_2 -agonists (LABA) and corticosteroids (CS) [10]. The airway inflammatory response in asthma is heterogeneous and may be dominated by eosinophils and/or neutrophils. While eosinophilic asthma is generally responsive to CS, airway neutrophilia is not CS responsive [11–14]. There is an overlap between clinical and pathological features between COPD and asthma, suggesting that the

diseases may have some linked pathophysiology [15]. Both severe asthma and COPD are typically characterized by increased airway inflammation including neutrophil influx. The mechanisms of this linked pathophysiology remain to be defined.

A disintegrin and metalloproteinase domain 8 (ADAM8), also known as CD156, is a highly conserved and complex type I transmembrane protein [16, 17]. In the airway, ADAM8 is expressed on eosinophils, neutrophils, macrophages, monocytes, dendritic cells, B cells and the epithelium [18–23]. The primary function of ADAM8 is the cleavage of substrate proteins via the matrix metalloproteinase (MMP) ‘shedase’ domain [24]. Inflammatory cell migration into the lung occurs via the leucocyte adhesion cascade, which involves inflammatory cells binding to the endothelium and transmigration into target tissues. The leucocyte adhesion

cascade is defined by unique sequential stages, each involving different adhesion proteins [25–28]. Several leucocyte adhesion proteins have been identified as ADAM8 substrates [29]. It has been proposed that ADAM8 cleaves the early adhesion cascade protein L-selectin, allowing subsequent adhesion steps to occur, thus facilitating neutrophil migration into tissues [21]. This is likely the case for all leucocyte extravasation as disrupting ADAM8 function in mice impairs leucocyte migration [19, 30].

A study of ADAM8 knockout mice has demonstrated the importance of ADAM8 in an allergic airway disease model of asthma. ADAM8-deficient mice had no airway hyperresponsiveness (AHR), a key feature of asthma, and decreased cellular infiltration into lung tissues [30]. ADAM8 mRNA expression is up-regulated in murine models of asthma [19, 20] and in mild to moderate human asthma [31] compared with controls. ADAM8 has seldom been explored in the human airway. ADAM8 expression is higher in bronchial biopsies from moderate and severe asthmatics compared with mild asthma and controls [32]. The protein expression of ADAM8 in airway samples, such as induced sputum, and its relation to airway cell influx are unknown. In COPD, a single study reported that ADAM8 was not expressed in bronchial biopsies [32]. The requirement of ADAM8 for both eosinophil and neutrophil infiltration [21, 27] suggests that ADAM8 may have a significant role in the pathogenesis of asthma and COPD [31, 32].

This study investigated the level of ADAM8 gene expression and protein concentration measured in induced sputum from subjects with asthma and COPD and healthy controls. We further examined whether ADAM8 levels are associated with asthma severity and inflammatory phenotype. We hypothesized that ADAM8 levels will be higher in COPD, severe asthma and asthma characterized by neutrophilic inflammation compared with control groups.

Materials and methods

Participants

Participants with asthma ($n = 113$) and COPD ($n = 20$) were recruited from the John Hunter Hospital Ambulatory Care Clinic, and the Hunter Medical Research Institute volunteer register, NSW, Australia. Healthy controls ($n = 21$) were recruited from the community by advertisement. Asthma was diagnosed according to American Thoracic Society guidelines based upon current (past 12 months) episodic respiratory symptoms, doctor's diagnosis and AHR to hypertonic saline. COPD participants were physician-diagnosed with persistent airflow limitation assessed by post-bronchodilator spirometry of $FEV_1/FVC\% < 70$ and $FEV_1\%$ predicted < 80 .

Participants were excluded if they had recent (past month): respiratory tract infection, exacerbation or changes in maintenance therapy. Other exclusion criteria were current smoking, inadequate sputum production, sputum squamous cell proportion $> 50\%$ or sputum cell viability $< 40\%$. Healthy control participants had no previous asthma or COPD diagnosis with normal lung function ($FEV_1\%$ predicted > 80 and/or $FEV_1/FVC\% > 70$). Atopy was assessed by skin prick testing and a positive (> 3 mm weal) reaction to aeroallergens: *Aspergillus fumigatus*, *Alternaria tenuis*, Dust Mite (*Dermatophagoides pteronyssinus*), Cockroach and Grass Mix (Stallergens, Antony, Hauts-de-Seine, France). Fractional exhaled nitric oxide (FeNO) was measured at a controlled flow rate of 50 mL/s according to ATS guidelines (NIOX, Aerocrine, Solan, Sweden) [33]. Airway hyperresponsiveness was measured by dose–response slope (DRS) using the calculation: $DRS = [(FEV_1 \text{ at baseline} - FEV_1 \text{ post-challenge})/FEV_1 \text{ at baseline}] \times 100/(\text{volume of hypertonic saline, mL})$. This study was approved by the Hunter New England Area Health Service (07/10/24/5.03, 30/11/2007) and The University of Newcastle Research ethics committees (H-2008-0083, 01/05/2008). All subjects gave written informed consent.

Spirometry, sputum induction and sputum processing

Spirometry was performed in accordance with American Thoracic Society guidelines [34] (KoKo PD Instrumentation). $FEV_1\%$ predicted and $FEV_1/FVC\%$ were calculated from a forced expiratory flow volume curve using Knudson values [35]. Sputum induction and processing were performed as previously described [36]. Briefly, sputum mucocellular portions (plugs) were separated from saliva. For mRNA analysis, 100 μ L sputum plugs was stored in 700 μ L of RLT buffer with β -mercaptoethanol (Qiagen, Hilden, Germany) at -80°C until extraction. Sputum plugs were also homogenized with diluted Sputolysin (Dithiothreitol, Merck Millipore, Billerica, Massachusetts, USA) for 30–60 mins. Sputum cell suspensions were filtered (60 μ m) (Merck Millipore), and viability and total cell counts (TCC) were performed. Cell-free supernatants were collected and stored at -80°C until analysis. Sputum cytospin slides were prepared and stained with May–Grunwald Giemsa (MGG), and inflammatory cell counts were generated from 400 non-squamous cells.

Asthma severity classification

Severe asthma (SA) subjects had uncontrolled asthma (measured by Juniper Asthma Control Questionnaire (ACQ), score ≥ 1) and/or poor lung function ($FEV_1\%$ predicted ≤ 80 and $FEV_1/FVC\% \leq 70$) despite prescription of high-dose inhaled corticosteroids (ICS)

(> 1000 µg beclomethasone equivalents per day) in combination with LABA. If participants had poor lung function or poor symptom control, but did not meet the treatment requirements for severe asthma, they were classified as uncontrolled asthma (UA). Controlled asthma (CA) was defined as normal lung function (FEV₁% predicted > 80 and/or FEV₁/FVC% > 70) and controlled symptoms (ACQ score < 1). Eight participants could not have their asthma severity determined by these criteria and were not included in the statistical analysis by severity.

Asthma inflammatory phenotype classification

Eosinophilic asthma (EA) was defined by normal levels of sputum neutrophils < 61% and increased sputum eosinophils ≥ 3%. Neutrophilic asthma (NA) was defined as increased sputum neutrophils ≥ 61% and normal sputum eosinophils < 3%. Paucigranulocytic asthma (PGA) was defined by normal levels of sputum neutrophils < 61% and eosinophils < 3%, and mixed granulocytic asthma (MGA) had increased neutrophils ≥ 61% and eosinophils ≥ 3% [14].

ADAM8 mRNA analysis

Sputum RNA was extracted using the RNeasy Mini kit or the AllPrep DNA/RNA/Protein kit (Qiagen). A total of 200 ng of RNA was reverse-transcribed to cDNA with the High Capacity cDNA Reverse Transcription kit [Life Technologies (Applied Biosystems), Carlsbad, California, USA]. Gene expression was measured with Taqman Gene Expression Master Mix and Probes: ADAM8 (Hs 00174246_m1) and the reference house-keeping gene β-Actin (NM_001101.2) (Life Technologies). Real-time PCR results were determined using the $2^{-\Delta\Delta C_t}$ calculation method. The relative gene expression results were calculated as the cycle threshold (Ct) of the target relative to the Ct of the house-keeping gene β-actin (ΔCt) and the mean ΔCt of the healthy control group (ΔΔCt).

sADAM8 protein analysis

Protein levels of human sADAM8 in sputum supernatant were measured by a commercial DuoSet ELISA as per manufacturer's instructions (R&D Systems, Minneapolis, Minnesota, USA). The standard curve of this assay ranged from 62.5 to 4000 pg/mL. This sADAM8 assay was firstly validated for the measurement of sputum supernatant, because sputolysin used to process sputum samples contains the active chemical dithiothreitol (DTT) that can affect assay antibody binding [37, 38]. The assay was validated in terms of the effect of DTT on the standard curve, sputum

supernatant sample matrix effect and spiked sADAM8 protein recovery.

Statistical analyses

Statistical analyses were performed in STATA IC 11 (STATA Corporation, College Station, Texas, USA) and GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Parametric data had Gaussian distribution, and group variance was tested with one-way ANOVA (analysis of variance) followed by Bonferroni post hoc test. Nonparametric data had skewed distribution, and group differences were measured by the Kruskal–Wallis test followed by Dunn's comparison post hoc test. Categorical data were tested with the Pearson's chi-square (χ^2) test. All data were correlated with Spearman's rank correlation. For significance, *P* values were < 0.05.

Results

Sample population

This study investigated mRNA and protein expression of ADAM8 in 113 participants with asthma, 20 participants with COPD and 21 healthy controls. All 113 (100%) asthma participants had a sputum inflammatory cell count and ADAM8 mRNA analysis, whereas 106 (94%) had sADAM8 protein measured. All COPD participants (*n* = 20, 100%) had a sputum inflammatory cell count and sADAM8 protein measured, and 16 (80%) had ADAM8 mRNA analysis. Of the 21 healthy control participants, 19 (90%) had inflammatory cell counts, 20 (95%) had ADAM8 mRNA analysis, and 12 (57%) had sADAM8 protein measured.

Sputum supernatant validation for the measurement of sADAM8 protein

Inhibitory effects of DTT (5 mg/mL) were negated when diluted at 1/10 (Fig. 1a). Matrix effects were observed when samples (*n* = 4) were run at neat and lower dilutions; however, these effects were reduced at sample dilutions of 1/10 and above (Fig. 1b). The average recovery of spiked sADAM8 protein (*n* = 6) was 81%.

Clinical characteristics and inflammatory cell counts in asthma and COPD

Clinical descriptors and sputum cell counts for participants with asthma, participants with COPD and healthy controls are listed in Table 1. Both the asthma and COPD groups were significantly older than the healthy control population. Participants with COPD

were characterized by a greater smoking history, increased neutrophil proportions and worsened lung function than both asthmatics and healthy controls. There were 50% ($n = 10$) in GOLD grade II, 40% ($n = 8$) in GOLD grade III and 10% ($n = 2$) in GOLD grade IV. Participants with asthma had significantly lower lung function than healthy controls and a median (IQR) Juniper asthma control score of 0.9 (0.4, 1.4).

ADAM8 expression in asthma and COPD

ADAM8 mRNA (Fig. 2a) and sADAM8 protein (Fig. 2b) were significantly higher in the sputum from participants with asthma or COPD compared with healthy controls. There was no significant difference in ADAM8 mRNA or protein expression between asthma and COPD.

Clinical characteristics and inflammatory cell counts in severe asthma

There were 31 participants with severe asthma (SA), 39 with UA and 35 with CA. Their clinical characteristics and inflammatory cell counts are shown in Table 2. Fractional exhaled nitric oxide was significantly higher in uncontrolled asthma compared with both controlled asthma and healthy controls. The severe asthma group was significantly more atopic than other groups. Both severe asthma and uncontrolled asthma groups had significantly greater AHR, as measured by DRS, compared with controlled asthma (Table 2). The severe asthma group had higher TCC and proportion and number of neutrophils compared with controlled asthma (Table 2). Eosinophil proportion and number were increased in uncontrolled asthma compared with controlled asthma.

ADAM8 expression in severe asthma

ADAM8 mRNA was significantly elevated in severe asthma compared with controlled asthma group (Fig. 2c; $P = 0.035$). The sADAM8 protein concentration was significantly higher in severe asthma group compared with both uncontrolled asthma and controlled asthma groups (Fig. 2d; $P = 0.002$).

Clinical characteristics and inflammatory cell counts in asthma inflammatory phenotypes

There were 37 participants with EA, 30 with NA, 37 with PGA and 9 with MGA. Their clinical characteristics and inflammatory cell counts are shown in Table 3. Participants with EA had higher ACQ scores, elevated FeNO and worsened lung function. The EA participants had significantly greater AHR, measured by DRS, compared with PGA. Participants with NA also had higher

ACQ scores (Table 3). Sputum total cell count was increased in NA compared with both EA and PGA.

ADAM8 expression in asthma inflammatory phenotypes

ADAM8 gene expression was significantly increased in eosinophilic and neutrophilic asthma compared with paucigranulocytic asthma (Fig. 2e; $P < 0.001$). The sADAM8 protein concentration was similar across inflammatory phenotypes (Fig. 2f; $P = 0.172$).

ADAM8 correlations

ADAM8 mRNA correlated with ADAM8 protein levels ($r = 0.27$, $P < 0.01$). Induced sputum total inflammatory cells correlated with both ADAM8 mRNA ($r = 0.26$, $P = 0.006$) and protein ($r = 0.42$, $P < 0.01$, Fig. 3a). Similarly, induced sputum neutrophils correlated with ADAM8 mRNA ($r = 0.37$, $P < 0.001$) and sADAM8 protein levels ($r = 0.54$, $P < 0.001$, Fig. 3b). Neither ADAM8 mRNA ($r = 0.02$) nor sADAM8 protein ($r = -0.04$) correlated with induced sputum eosinophils. No correlation was found between participant's age and either sADAM8 ($r = 0.10$, $P = 0.275$) or sputum neutrophils ($r = 0.03$, $P = 0.743$). Additionally, there were no significant correlations between ICS dose and ADAM8 mRNA ($r = -0.14$, $P = 0.162$) or sADAM8 ($r = 0.12$, $P = 0.244$). DRS did not correlate with asthmatic ADAM8 mRNA ($r = 0.06$, $P = 0.581$) or sADAM8 ($r = 0.17$, $P = 0.094$).

ADAM8 expression with ICS treatment

To determine whether there were differences in ADAM8 expression with ICS use, the airway disease participants were divided into those currently using ICS as treatment and those not using ICS. ADAM8 mRNA expression was significantly elevated in participants using ICS ($P = 0.007$), and there was no significant difference in sADAM8 protein expression with ICS use ($P = 0.135$).

Discussion

This study measured the levels of ADAM8 mRNA and soluble protein in induced sputum samples collected from the chronic airway diseases asthma and COPD, and further investigated whether ADAM8 was associated with severity and inflammatory phenotype of asthma. ADAM8 mRNA and sADAM8 protein levels were significantly increased in asthma, severe asthma and in COPD compared with controls. The levels were associated with the intensity of inflammatory cell influx and neutrophils in the airways, suggesting that ADAM8 may be functionally important in promoting cellular influx in these airway diseases.

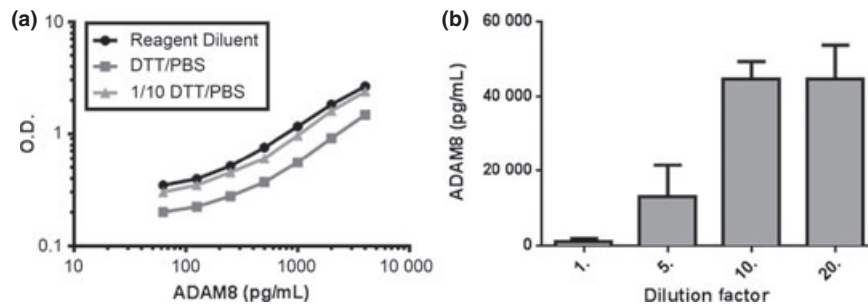


Fig. 1. Validation of sADAM8 ELISA measurement in induced sputum samples. Dithiothreitol (5 mg/mL) used to process the sputum samples lowered the standard curve of the assay; however, this effect was negated at a further 1/10 dilution (a). The sputum supernatant matrix effect on sADAM8 protein detection in sputum was removed after the samples were diluted at 1/10 or greater, $n = 4$ (b). Data are shown with the bar at median and the upper error of quartile 3.

Table 1. Clinical characteristics and sputum cell counts by respiratory disease

	Asthma	COPD	Healthy	P value
<i>n</i>	113	20	21	
Gender, Male <i>n</i> (%)	56 (50)	15 (43)	7 (33)	0.381
Age, Mean (SD)	58 (14)*	63 (3)*	41 (20)	< 0.001
Smoker, Ex <i>n</i> (%)	54 (48)	15 (75)*	5 (24)	0.005
Pack years, Median (Q1, Q3)	7.2 (1.7, 25.0)	33.0 (18.3, 58.0)*	9.0 (2.9, 30.0)	0.010
FeNO, Median (Q1, Q3)	22.0 (14.3, 41.0)	16.6 (9.8, 23.8)	16.0 (12.0, 22.7)	0.017
ICS use, Yes <i>n</i> (%)	87 (77)	17 (85)	n/a	0.424
ICS dose, Median (Q1, Q3)	1000 (500, 2000)	2000 (800, 2000)	n/a	0.223
FEV ₁ % predicted, Mean (SD)	76 (20)*	53 (17) [†] *	102 (9)	< 0.001
FEV ₁ /FVC%, Mean (SD)	67 (10)*	55 (11) [†] *	79 (7)	< 0.001
Atopy, Yes <i>n</i> (%)	74 (65)	11 (55)	8 (38)	0.054
Sputum cell viability, Median (Q1, Q3)	78.9 (65.1, 90.9)	78.2 (67.2, 88.3)	72.7 (67.2, 86.1)	0.788
TCC $\times 10^6$ cells/mL, Median (Q1, Q3)	3.6 (2.3, 6.6)	4.4 (2.2, 5.9)	3.8 (1.8, 5.1)	0.624
Neutrophil%, Median (Q1, Q3)	44.5 (22.8, 70.1)	64.8 (43.9, 80.9)*	29.5 (13.8, 56.0)	0.009
Neutrophils $\times 10^4$ cells/mL, Median (Q1, Q3)	144.3 (54.8, 324.0)	232.7 (81.8, 411.4)	93.7 (33.1, 194.4)	0.114
Eosinophil%, Median (Q1, Q3)	1.0 (0.3, 6.8)	1.3 (0.4, 4.1)	0.3 (0.3, 1.0)	0.113
Eosinophils $\times 10^4$ cells/mL, Median (Q1, Q3)	5.2 (0.3, 37.5)	6.4 (1.3, 20.0)	1.4 (0.3, 2.7)	0.069

Post hoc significant: * vs. healthy and [†] vs. asthma.

COPD, chronic obstructive pulmonary disease; FeNO, fractional exhaled nitro oxide; ICS, inhaled corticosteroid; FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity; TCC, total cell count.

While ADAM8 has been previously implicated in asthma pathogenesis in murine models [19, 20, 39], ADAM8 has seldom been explored in human asthma. Matsuno *et al.* reported that the levels of sADAM8 in serum and bronchoalveolar lavage vary for different types of human eosinophilic pneumonia [40, 41]. Paulissen *et al.* [31] found ADAM8 mRNA was increased in mild to moderate asthmatic sputum compared with healthy controls. To our knowledge, only one group has published ADAM8 results in the context of human severe asthma [32]. Foley *et al.* [32] reported increases in ADAM8 mRNA expression and an increase in ADAM8-positive cells in severe asthma bronchial biopsy samples, supporting our findings. We extend these findings to show ADAM8 mRNA expression and levels of sADAM8 protein from non-invasive induced sputum samples in asthma. Poor asthma control is a feature of both severe

and non-severe uncontrolled asthma. In this study, we show that elevated ADAM8 is more pathogenically relevant for severe asthma compared with uncontrolled asthma group, because sADAM8 protein levels were significantly increased in severe asthma. These findings also link ADAM8 with neutrophil influx, which is a feature of severe asthma, and associated with the known biological function of ADAM8.

We detected similar levels of sADAM8 protein in the supernatant from asthma and COPD participants, both of which had significantly greater sADAM8 compared with healthy controls. A previous study has shown that bronchial biopsy samples from patients with COPD had no significant ADAM8 mRNA or protein expression. The authors concluded ADAM8 was specific for asthma pathogenesis [32]. Our results conflict with this assumption, and it is difficult to ascertain the reason as the

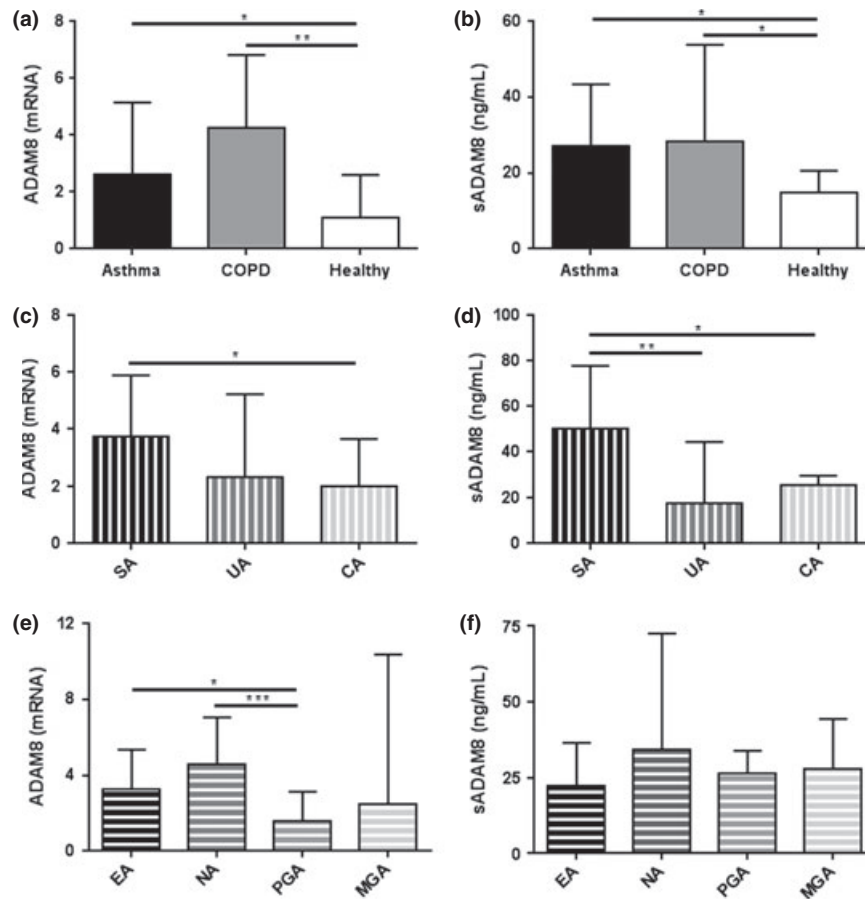


Fig. 2. ADAM8 mRNA (a) and sADAM8 protein (b) is significantly greater in asthma and chronic obstructive pulmonary disease compared with healthy control expression. ADAM8 mRNA (c) and sADAM8 protein (d) expression is increased in severe asthma. ADAM8 mRNA (e) but not sADAM8 protein (f) was increased in eosinophilic and neutrophil asthma compared with paucigranulocytic asthma. Data are shown with the bar at median and the upper error of quartile 3. Significant post hoc analysis results are shown, where *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$.

COPD bronchial biopsy results were not included in the journal article [32]. Considering that ADAM8 is implicated in the migration of neutrophils and was strongly correlated with sputum neutrophil cell count, it is likely that ADAM8 is a marker of airway neutrophil inflammation in airway disease. To our knowledge, these are the only reported instances where ADAM8 expression had been explored in the context of COPD.

Corticosteroid treatment has been reported to have no effect on ADAM8 expression in epithelial cells *in vitro*, as corticosteroid did not induce ADAM8 mRNA [32]. We found no significant correlation between ICS dose and sputum ADAM8 mRNA or soluble protein expression, therefore supporting this finding. We have also explored the ADAM8 mRNA and sADAM8 expression between airways disease participants currently medicating with ICS and those not using ICS as treatment. We found sputum ADAM8 mRNA was significantly elevated in participants taking ICS, but no difference was found for sADAM8 protein. This finding may suggest an increase in transcription of ADAM8 mRNA; however, further studies are

required to assess the effects of corticosteroid therapy on airway ADAM8 expression by investigating isolated airway cell types and patients before and after ICS treatment.

Protein expression is reported for the soluble form of ADAM8. ADAM8 is initially translated as a transmembrane protein. The activation of ADAM8 is autocatalytic, occurring with ADAM8 protein–protein interactions. ADAM8 activation occurs in the endoplasmic reticulum, and the active form is then expressed on the cytoplasm after stimulation or endothelial adhesion [21, 24, 29]. The sADAM8 protein is generated by a 'shedase mechanism' where the sADAM8 domain may be self-cleaved from the plasma membrane, this auto-cleavage may also occur in tandem, with shedding of substrate protein [21, 40]. Soluble ADAM8 contains the MMP domain and is thought to retain its catalytic shedase activity for substrate proteins *in vitro* [24, 42]. There is much still unknown about the leucocyte adhesion cascade, as the role of membrane and soluble ADAM8 during leucocyte extravasation is not clearly

Table 2. Clinical characteristics and sputum cell counts by asthma severity

	Asthma			P Value
	Severe	Uncontrolled	Controlled	
<i>n</i>	31	39	35	
Gender, Male <i>n</i> (%)	15 (48)	23 (59)	15 (43)	0.369
Age, Mean (SD)	60 (12)	57 (17)	60 (11)	0.467
Smoker, Ex <i>n</i> (%)	17 (55)	22 (56)	11 (31)	0.063
Pack years, Median (Q1, Q3)	15.0 (1.2, 25.0)	5.9 (1.7, 24.0)	4.8 (1.5, 20.0)	0.841
ACQ, Median (Q1, Q3)	1.4 (1.0, 2.3)*	1.3 (0.9, 1.9)*	0.3 (0.3, 0.4)	< 0.001
FeNO, Median (Q1, Q3)	19.3 (16.0, 35.8)	33.2 (16.3, 57.7)*	17.7 (13.4, 27.6)	0.019
ICS use, Yes <i>n</i> (%)	31 (100) [†] *	25 (64)	24 (69)	0.001
ICS dose, Median (Q1, Q3)	2000 (1000, 2000) [†] *	500 (400, 900)	1000 (500, 2000)	< 0.001
FEV ₁ % predicted, Mean (SD)	63 (18)*	68 (14)*	94 (16)	< 0.001
FEV ₁ /FVC%, Mean (SD)	62 (9)*	64 (10)*	74 (6)	< 0.001
Atopy, Yes <i>n</i> (%)	28 (90) [†] *	22 (56)	20 (57)	0.004
DRS, Median (Q1, Q3)	2.6 (1.4, 4.7)*	2.2 (0.7, 11.2)*	0.5 (0.2, 1.3)	< 0.001
Sputum cell viability, Median (Q1, Q3)	87.1 (73.9, 92.0)	78.4 (63.9, 90.9)	74.2 (60.0, 89.7)	0.119
TCC × 10 ⁶ cells/mL, Median (Q1, Q3)	5.5 (2.9, 11.7)*	3.4 (2.4, 5.0)	3.6 (1.6, 4.7)	0.004
Neutrophil%, Median (Q1, Q3)	57.8 (34.5, 75.5)*	41.3 (21.8, 67.3)	39.3 (17.0, 57.0)	0.041
Neutrophil × 10 ⁴ cells/mL, Median (Q1, Q3)	257.4 (119.7, 642.7)*	121.7 (55.4, 307.8)	70.5 (25.8, 191.6)	0.002
Eosinophil%, Median (Q1, Q3)	1.5 (0.0, 6.8)	4.5 (0.5, 19.0)*	0.3 (0.0, 2.0)	< 0.001
Eosinophil × 10 ⁴ cells/mL, Median (Q1, Q3)	6.5 (0.0, 51.6)	14.9 (2.0, 52.9)*	0.6 (0.0, 4.7)	0.001

Post hoc significant: * vs. controlled asthma and [†] vs. uncontrolled asthma.

ACQ, Asthma Control Questionnaire; FeNO, fractional exhaled nitro oxide; ICS, inhaled corticosteroid; FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity; DRS, dose-response slope; TCC, total cell count.

defined. In rheumatoid arthritis, sADAM8 is present in inflammatory joint exudate and levels correlate with disease activity markers [21]. Also, sADAM8 correlates with cleaved soluble substrate proteins VCAM-1 and CD23 in the bronchoalveolar lavage fluid of eosinophilic pneumonia [40]. Taken together, these findings suggest that the measurement of sADAM8 in sputum supernatant is likely to be a measure of ADAM8 activity in the airway. We therefore assessed correlations between ADAM8 and disease characteristics and have shown a negative correlation between ADAM8 expression with lung function FEV₁% predicted ($r = 0.49$, $P < 0.05$, data not shown) in COPD. These findings may infer increased ADAM8 expression, and therefore, activity in the airway relates to lung function impairment.

Our data show that ADAM8 mRNA and protein expression were significantly correlated with the number and proportion of neutrophils and the total sputum inflammatory cell count. Others have identified the relationship between ADAM8 mRNA and both sputum neutrophils and eosinophils in mild to moderate asthmatics [31] and to neutrophil migration, supporting our findings. Human peripheral blood eosinophils and monocytes express minimal ADAM8 on the cell surface at baseline conditions, and stimulation with leukotriene B₄ (LTB₄) increased ADAM8 two- to threefold [43]. LTB₄ is a proinflammatory mediator produced from leucocytes that is able to induce the adhesion and activation of leucocytes on the endothelium, facilitating

leucocyte migration [44]. LTB₄ is most often reported with the migration of neutrophils [45–48]. Neutrophil migration relies on ADAM8 to cleave endothelial adhesion proteins, facilitating neutrophil migration [21, 26–28].

The severe asthma and neutrophilic asthma (ADAM8 mRNA only) groups in this study had increased ADAM8 expression and inflammatory cells in their sputum. Also, the severe asthma and COPD groups had higher neutrophils, relating ADAM8 to neutrophilic burden in the airways. While this study has shown strong correlations between ADAM8 and total cellular infiltration and inflammation in the airway, this finding is likely driven by the association of ADAM8 with neutrophils. ADAM8 is required for the migration of both neutrophils and eosinophils in humans [21, 27] and in a murine model ADAM8 deficiency led to reduced numbers of eosinophils, macrophages and dendritic cells [30]. While ADAM8 is likely to be involved in the migration of other leucocytes, we did not find significant associations of sputum ADAM8 with any other leucocyte besides the neutrophil. Implying that the sputum ADAM8 expression we detected is related primarily to neutrophil migration. To our knowledge, sADAM8 protein in sputum has not previously been measured. This article is also the first to relate ADAM8 together with neutrophils in severe asthma and to report ADAM8 expression for inflammatory phenotypes of asthma.

Table 3. Clinical characteristics and sputum cell counts by asthma inflammatory phenotype

Asthma phenotype	Eosinophilic	Neutrophilic	Paucigranulocytic	Mixed granulocytic	P Value
<i>n</i>	37	30	37	9	
Gender, Male <i>n</i> (%)	15 (41)	17 (57)	20 (37)	4 (44)	0.531
Age, Mean (SD)	57 (18)	59 (11)	59 (12)	62 (10)	0.706
Smoker, Ex <i>n</i> (%)	16 (43)	16 (53)	15 (41)	7 (78)	0.195
Pack years, Median (Q1, Q3)	0.7 (0.4, 12.5)	8.3 (4.3, 31.3)	9.5 (2.0, 25.0)	20.0 (6.0, 30.0)	0.041
ACQ, Median (Q1, Q3)	1.1 (0.9, 1.7)*	1.1 (0.7, 2.0)*	0.4 (0.3, 0.7)	0.9 (0.7, 1.4)	< 0.001
FeNO, Median (Q1, Q3)	52.4 (32.1, 71.0) [†] *	20.7 (13.0, 26.2)	16.5 (12.3, 22.0)	18.4 (16.0, 24.5)	< 0.001
ICS use, Yes <i>n</i> (%)	28 (76)	25 (83)	27 (73)	7 (78)	0.787
ICS dose, Median (Q1, Q3)	1000 (500, 1250)	1000 (400, 2000)	1000 (800, 2000)	1000 (400, 2000)	0.302
FEV ₁ % predicted, Mean (SD)	70 (16)*	71 (23)	86 (20)	76 (18)	0.003
FEV ₁ /FVC%, Mean (SD)	64 (10)	65 (11)	71 (7)	67 (8)	0.231
Atopy, Yes <i>n</i> (%)	29 (78)	16 (53)	23 (62)	6 (67)	0.182
DRS, Median (Q1, Q3)	2.6 (1.4, 11.2)*	1.4 (0.6, 2.7)	0.6 (0.3, 2.0)	0.8 (0.6, 3.1)	0.007
Sputum cell viability, Median (Q1, Q3)	74.1 (58.6, 84.8)	91.0 (84.6, 94.7) [†] *	72.6 (60.8, 86.5)	89.5 (73.9, 92.6)	< 0.001
TCC × 10 ⁶ cells/mL, Median (Q1, Q3)	3.4 (2.6, 5.0)	7.0 (3.5, 13.4) [†] *	2.6 (1.6, 3.9)	4.1 (2.1, 4.7)	< 0.001
Neutrophil%, Median (Q1, Q3)	26.0 (17.8, 41.3)	77.0 (73.5, 85.5) [†] *	31.3 (20.3, 46.8)	67.3 (66.5, 70.8) [†] *	< 0.001
Neutrophil × 10 ⁴ cells/mL, Median (Q1, Q3)	95.6 (47.8, 177.4)	590.0 (307.8, 964.8) [†] *	70.4 (28.7, 156.3)	296.6 (139.2, 324.0)*	< 0.001
Eosinophil%, Median (Q1, Q3)	17.3 (6.8, 32.8) [†] *	0.6 (0.0, 1.0)	0.3 (0.0, 0.5)	3.5 (3.0, 5.0) [†] *	< 0.001
Eosinophil × 10 ⁴ cells/mL, Median (Q1, Q3)	57.7 (29.8, 105.7) [†] *	3.0 (0.0, 8.0)	0.2 (0.0, 1.0)	14.9 (13.5, 23.4)*	< 0.001

Post hoc significant: * vs. paucigranulocytic asthma, [†] vs. neutrophilic asthma, [‡] vs. eosinophilic asthma.

ACQ, Asthma Control Questionnaire; FeNO, fractional exhaled nitro oxide; ICS, inhaled corticosteroid; FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity; DRS, dose-response slope; TCC, total cell count.

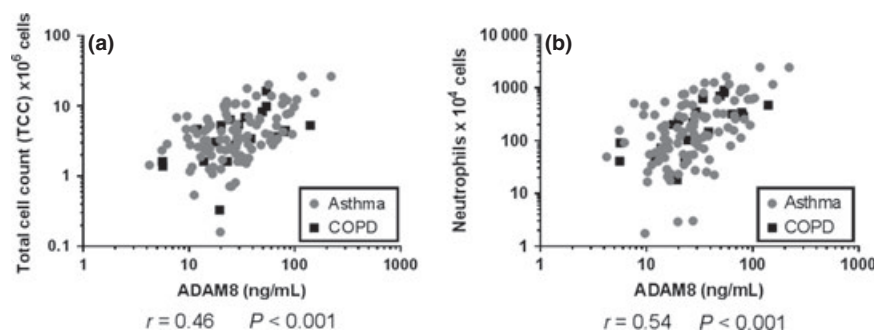


Fig. 3. sADAM8 protein levels correlate with induced sputum total inflammatory cell count (TCC) (a) and neutrophils (b). Correlations are given for asthma and chronic obstructive pulmonary disease only, healthy controls are not included in the analysis.

A recent publication by Knolle *et al.* [43] concluded that ADAM8 has anti-inflammatory properties, reducing AHR and allergic airway inflammation in mice. This protective effect of ADAM8 was shown to occur by ADAM8 initiating intrinsic apoptosis in leucocytes, promoting clearance of inflammatory cells from the lung. Knolle *et al.* report different results to others who have found ADAM8 function is important for murine model allergic inflammation and AHR [19, 20, 30]. Further reduced apoptosis of neutrophil and eosinophils has been reported in the airways of human asthma [49, 50]. Additionally, the mouse model used by Knolle *et al.* [43] is unlikely to mimic the human airway as differences in ADAM8 expression on macrophages were reported between human cells and murine cells from their models. Therefore, further work is required to validate their findings in a human population.

In this study, we did not find a significant correlation between ADAM8 mRNA or sADAM8 protein expression and the AHR outcome, DRS. This was despite the severe asthma population having increased AHR. The severe asthma group in our study had a higher proportion of neutrophils in the airway; however, we have previously shown that AHR is increased in the eosinophilic phenotype of asthma. Others have shown neutrophilic phenotypes of asthma have lower AHR compared with eosinophilic phenotypes [51]. Hence, the heterogeneity of inflammation in our asthma population may explain why there was no significant correlation between ADAM8 and AHR.

A limitation of this study was the age difference between the healthy control group and both the asthma and COPD cohorts. To remove any potential confounding effects, the healthy control data were removed from all correlations. While we have shown ADAM8 correlated with neutrophils and neutrophils have been shown to increase with age [52], we did not find any significant correlations between age and neutrophils in this cohort. The lack of relationship between age and neutrophils in this study is likely due to the narrow age range of the airway disease populations; therefore, this

study population is limited for demonstrating the effect of airway neutrophil accumulation with age. As sADAM8 did not correlate with age, it is unlikely that the age difference between the asthma and COPD groups compared with the healthy controls affected ADAM8 expression. Induced sputum is a heterogeneous sample type, consisting of many varied cellular populations. This study has utilized inflammatory cell counts to account for differences between major cell populations in sputum samples, but to fully understand the functional mechanisms of ADAM8 for specific cell types, individual populations need to be isolated and studied.

Future work should focus on further understanding the role of both membrane-bound and soluble ADAM8, the functional role for ADAM8 during the entire migration process and whether the pathway can be targeted therapeutically in humans. ADAM8 has been detected on almost every type of human inflammatory cell [18–23] and is important for the migration of inflammatory cells, especially neutrophils, to the airway. ADAM8-deficient mice were protected against airway inflammation, and ADAM8 knockout mice remain unaffected physiologically with the gene deletion [19, 30]. Taken together, these findings indicate that future therapeutic targets directed at ADAM8 may significantly attenuate asthma or COPD symptoms by reducing inflammatory cell, primarily neutrophil, migration and have few adverse physiological consequences. Future ADAM8 therapies may also provide alternative treatment for neutrophilia in asthma and COPD where corticosteroids are not responsive.

Asthma and COPD are both chronic inflammatory respiratory diseases in which treatments are not optimal and mechanisms remain unclear. This study has provided evidence that ADAM8 is involved in the pathology of severe asthma and COPD and may relate to neutrophil migration to the airway. Future work will determine whether targeting ADAM8 therapeutically may reduce neutrophilic inflammation in severe asthma and COPD, leading to improved symptoms and quality of life.

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Conflict of interest

The authors declare no conflict of interest

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