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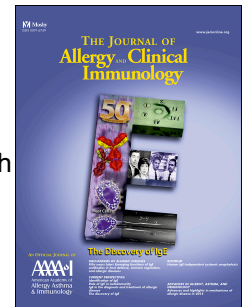
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***Aspergillus fumigatus* alkaline protease 1 (Alp1/Asp f13) in the airways
correlates with asthma severity**

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Summary

A fungal protease (Alp1/Asp f13) from *Aspergillus fumigatus* was detected in the airways of subjects with asthma but not controls, which correlated strongly with disease severity, respiratory dysfunction, and steroid use.

Keywords

Asthma; molds; *Aspergillus fumigatus*; protease; allergen; bronchial smooth muscle

Abbreviations used

Aspergillus fumigatus (Af); Alkaline protease 1 (Alp1) severe asthma with fungal sensitization (SAFS), airway hyperresponsiveness (AHR), allergic bronchopulmonary aspergillosis (ABPA), extracellular matrix (ECM), immunohistochemistry (IHC), bronchoalveolar lavage fluid (BALF), inhaled corticosteroid (ICS), Global Initiative for Asthma (GINA); short acting β -agonist (SABA); long acting β -agonist (LABA).

To the Editor:

Although fungi are ubiquitous in the environment and respiratory exposure to airborne spores occurs on a daily basis, sensitization and disease due to such exposure is relatively infrequent. *Aspergillus fumigatus* (Af) has been linked to severe, uncontrolled asthma. A potentially unique phenotype, termed “severe asthma with fungal sensitization” (SAFS) is thought to describe approximately 20-25% of individuals with uncontrolled, persistent disease.¹ Previously, we detected a known allergen serine protease from Af, Alkaline protease 1 (Alp1), in the bronchial submucosa of subjects with mild/moderate asthma but not healthy controls and in allergen-

65 challenged but not naïve mice.² We determined that Alp1 disrupted airway smooth muscle
66 (ASM)-extracellular matrix (ECM) interactions through its protease activity, which augmented
67 Ca^{2+} signaling and RhoA activation. Based on these initial findings, we hypothesized that: 1)
68 Alp1 protease contributes the pathogenesis of asthma in some patients; 2) Alp1 abundance in the
69 airways correlates with disease severity.

70 To further delineate the role of Alp1 in the lower respiratory tract in asthma, we evaluated
71 Alp1 immunoreactivity in airway sections from 36 asthmatics and 10 healthy controls and in
72 induced sputum supernatants from another 29 asthmatics and 10 healthy controls. The
73 demographics of these subjects are described in Tables E1 and E2 **in this Letter's Online**
74 **Repository**. There were an approximately equal number of male/female subjects, and the median
75 age was 34 years (range 18-68 years). The group with severe asthma was significantly older than
76 the healthy control group (median age 49, range 20-68 years; $p=0.01$). Subjects with asthma
77 were classified as mild/moderate or severe based on GINA criteria. Immunostaining of airway
78 sections with anti-Alp 1 antisera detected Alp1 immunoreactivity in bronchial smooth muscle
79 bundles of lungs of subjects with asthma but not in healthy subjects, and Alp1 quantities in the
80 bronchial smooth muscle layer increased progressively with asthma severity (**Figure 1A-B**).
81 Little to no staining of was observed when airway sections from subjects with severe asthma
82 were incubated with non-specific rabbit antisera (**Figure E1**). Surprisingly, Alp1 abundance was
83 roughly equivalent in patients with and without sensitivity to *Af* (**Figure 1C**). We also observed
84 similar patterns of Alp1 staining within the bronchial epithelium (**Figure E2**).

85 Alp1 immunoreactivity in ASM negatively correlated with pre-bronchodilator FEV_1
86 (analysis of the cohort as a whole or restricted to the asthma group only) and PC_{20} (the
87 provocative concentration of inhaled methacholine inducing a 20% decrease in FEV_1) (**Figure**

2A-B). Alp1 immunostaining in airways from patients receiving short or long acting β -agonists only (SABA/LABA, respectively) was significantly less than in the airways of patients requiring LABA plus inhaled corticosteroids (ICS), and Alp1 quantities correlated with ICS requirement (fluticasone equivalent dose) (**Figure 2C-D**).

Because we previously detected Alp1 in bronchoalveolar lavage fluid (BALF) of *Af* sensitized and challenged mice, but not naïve mice, by immunoblotting, we determined whether it could also be measured non-invasively in sputum from human subjects by immunoblotting. We detected a ~33 kDa band corresponding to the protease purified from commercial *Af* allergen extracts or from supernatants of *Af* cultures (**Figure E3A left panel**). In sputum samples from subjects with asthma, but not healthy controls, we observed an additional band at ~42-45 kDa, potentially representing an unprocessed precursor³ as detection of either band was strongly reduced or eliminated when blots were incubated with Alp1 antibody preadsorbed with antigen (**Figure E3A right panel**). This band was present only in sputum from subjects with asthma but not controls (**Figure E3B**). To determine whether Alp1 quantities in induced sputum correlated with disease severity, we generated a standard curve based on quantitative immunoblotting of defined amounts of purified Alp1 (**Figure E4A**). However, Alp1 quantities were similar in sputum from patients with mild/moderate and severe asthma (**Figure E3B**). Alp1 quantities were significantly higher in sputum from patients with *Af* sensitivity than those without, regardless of clinical severity (**Figure E4C**). Alp1 levels were also significantly correlated with sputum neutrophil, but not eosinophil counts (**Figure E4D** and data not shown). We did not detect Alp1 in sera from healthy subjects or patients with asthma. Collectively, these results suggest that Alp1 protease may contribute to severe asthma through two distinct mechanisms: 1)

allergenicity; and 2) proteolytic destruction of lung tissue, which could promote influx of neutrophils into the airway lumen.

Several observations support a pathogenic contribution of fungal colonization of the airways to severe asthma. Chronic fungal exposure is linked to asthma exacerbations in both children and adults.⁴ In experimental models of asthma in mice, chronic respiratory inoculation with fungi induces a more severe asthma phenotype than inhalation of other allergens such as house dust mite (HDM) through IL-33-dependent yet steroid-independent mechanisms.⁵ Alp1 is the major serine protease secreted by *Af*, and serine proteinase activity is crucial for *Af* to induce allergic airway inflammation and AHR.^{6, 7} Here we found equivalent Alp1 quantities in the airways regardless of *Af* sensitivity and no correlation between sputum Alp1 and eosinophils, suggesting that an IgE/type 2 response to *Af* is not strictly required for the pathogenicity of Alp1 in asthma. Fungal protease allergens may exert direct pathogenic effects on epithelial barrier function by disrupting cell-cell junctions, inducing cytoskeletal rearrangements, and promoting cytokine (e.g. IL-8) secretion.^{8, 9} Alp1 in sputum correlated well with sputum neutrophils, suggesting that it might increase epithelial secretion of neutrophil chemoattractants and thereby actively promote neutrophil migration to the airway lumen.

Alp1 may persist in the airways of patients with severe asthma due to impaired mucociliary clearance mechanisms, i.e. asthma-induced alteration of epithelium morphology and/or corticosteroid use. Alp1 also activates mucin gene expression in airway epithelial cells, which could further impede fungal clearance. Because our study was retrospective and cross-sectional, we could not address the utility of Alp1 measurements to evaluate longitudinal parameters including disease control and progression, or responses to specific treatments. However, Alp1 could be explored as a biomarker for endotype classification and/or surrogate for assessment of

therapeutic interventions as it is easily identified in the airways from subjects with asthma but is virtually undetectable in samples from healthy controls, and it strongly correlates with hard functional endpoints (FEV1, PC₂₀).

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FIGURE LEGENDS

Figure 1 Alp1 is present in human airways and correlates with asthma severity. (A) Airway sections were immunostained with anti-Alp1Ab (representative images from 10-21 subjects/group (original magnification, 40x, bar=50 µm). Arrows delineate smooth muscle areas. **(B-C)** Alp1 staining in ASM increases with asthma severity (B) but not sensitivity to Af (C). Quantified using ImageJ and represented as Alp1⁺ ASM per total ASM area. (A) **p*=0.04, *****p*<0.0001, one way ANOVA. (B)*, **, ****, ******p*=0.01; 0.004; 0.0008; <0.0001, Kruskal

Wallis.

Figure 2 Alp1 immunostaining in ASM correlates with lung functional impairment. (A-C)
 Correlation between Alp1 staining and FEV₁pre-bronchodilator (BD) in the entire cohort (A) or
 among subjects with asthma (B); Alp1 correlation between Log(PC₂₀FEV₁) (C). Alp1
 immunostaining in airways of subjects with asthma treated with ICS or SABA/LABA (**p*=0.03,
 Mann-Whitney). (D) Correlation between Alp1 staining in airways and ICS requirement
 [Log(fluticasone equivalent)].

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Figure 1 A

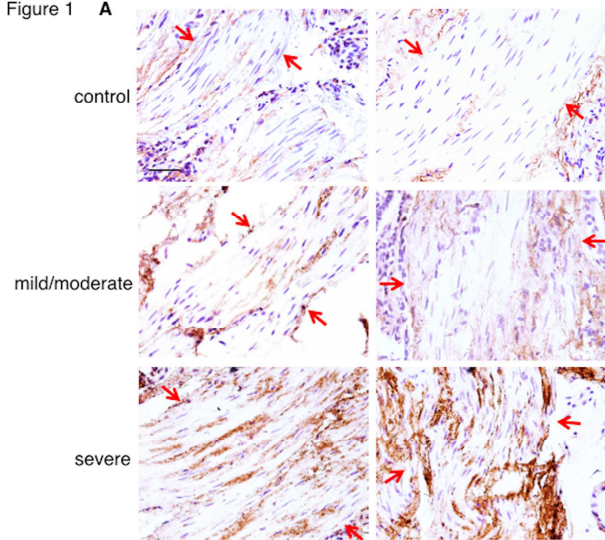


Figure 1 cont.

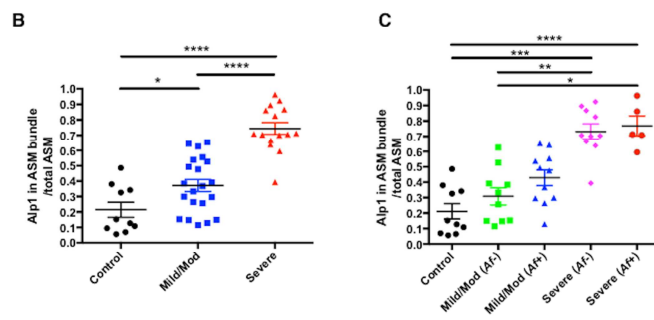
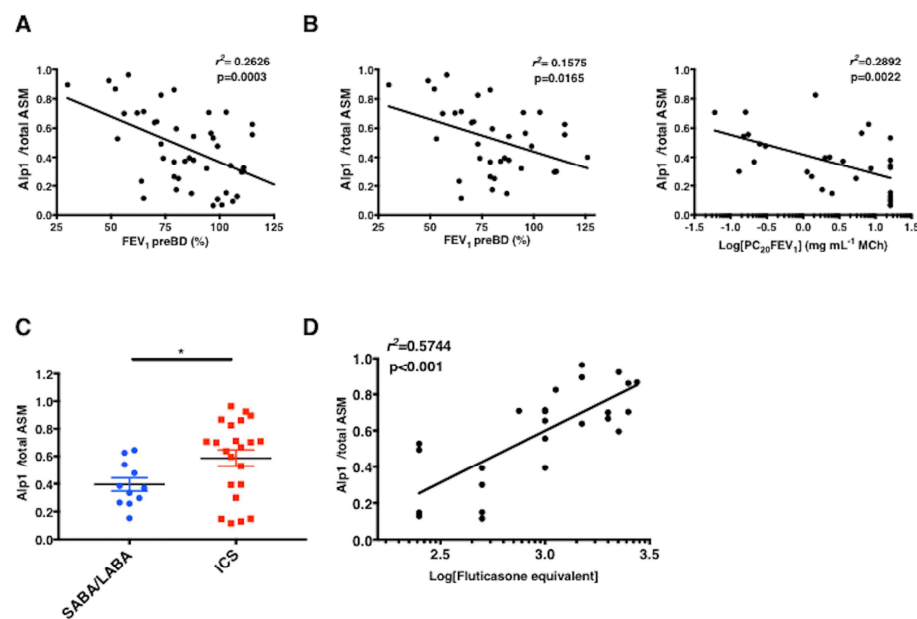


Figure 2



ONLINE DATA REPOSITORY

MATERIALS AND METHODS

Immunohistochemistry and immunoblotting

De-identified human airway specimens, induced sputum, sera, and clinical parameters were obtained by the Respiratory Health Network Tissue Bank of the Fonds de recherche du Québec – Santé, IUCPQ site (www.tissuebank.ca). The IUCPQ Ethics Committee approved the collection of tissue samples by the Tissue Bank and their use for these studies. Research Ethics Committees of the IUCPQ approved the biobank (#1200) and their use for this study (#20965). Written, informed consent was obtained from all subjects. Subjects were classified as having mild/moderate or severe asthma based on criteria established by GINA and further categorized by skin prick sensitivity to a panel of aeroallergens including *Aspergillus fumigatus*. Lung sections were deparaffinized and incubated with normal rabbit serum (ThermoFisher Scientific) or anti-Alp 1 rabbit antisera (1:1500) followed by 3,3'-Diaminobenzidine (DAB)-based detection and haematoxylin counterstaining as described previously. Images of sections were obtained with a Leica DMI4000 B microscope equipped with a Retiga 200R camera (QImaging, Canada) and acquired with Image-pro Plus software. The area of Alp1 positive staining was measured using Image J software containing the 'Color Deconvolution' plug-in to differentiate haematoxylin and DAB staining. The DAB⁺ area was normalized to the total ASM area in each field. The process was repeated to determine Alp1 staining outside of the ASM bundle (epithelial staining) and in the entire image (ASM and epithelial staining). Values for each patient were measured by taking the average of staining found in two regions of ASM within the same airway section. To detect Alp1 by immunoblotting of induced sputum supernatants, we separated proteins on NuPAGE Novex 12% Bis-Tris protein gels (ThermoFisher Scientific) in MOPS

buffer before transfer to a nitrocellulose and probing with anti-Alp1 polyclonal antisera and near infrared-conjugated anti-rabbit IgG as described previously. Images were obtained with the LiCor Oydsey Classic Imaging System and analyzed with Image Studio Software (Li-Cor).

Alp1 purification and extraction

Alp1 protease was isolated from commercial allergenic extracts (Jubilant Hollister-Stier) exactly as described previously.¹ *Af* strain B-5233 was isolated from a patient with fatal invasive aspergillosis. To obtain conidial suspensions, the *Af* strain was cultured on *Aspergillus* minimal agar for 7 days at 37°C, as described previously. The conidia were harvested in 0.01% Tween 20-phosphate-buffered saline (PBS) (T/P), collected by filtering through a sterile BD Falcon 40- μ m-pore-size cell strainer (BD Biosciences), and washed with sterile distilled water. The conidia were inoculated in liquid yeast glucose (YG) broth at a final concentration of 1×10^6 conidia/50 ml and incubated on a rotary shaker at 200 rpm for 48 h at 37°C. Secreted and excreted proteins were collected as described previously. Briefly, the culture filtrates were collected by filtering through Miracloth (Calbiochem). The supernatant was centrifuged at $12,000 \times g$ for 30 minutes at 4°C. Secreted proteins were then precipitated by the addition of 10% (w/v) trichloroacetic acid (TCA) for 12 hours at 4 °C. After centrifugation for 45 minutes at $17,000 \times g$ at 4°C, the supernatant was removed and the pellet was rinsed twice with 90% ice-cold acetone containing 0.07% (v/v) 2-mercaptoethanol at $17,000 \times g$ at 4°C for 15 minutes. The pellet was air-dried for five minutes at room temperature, resuspended in radioimmunoprecipitation (RIPA) lysis buffer supplemented with protease inhibitors and incubated for one hour at 4°C. After centrifugation at $20,000 \times g$ for 30 min, the supernatant was collected.

Statistical Analysis

All data analyses were performed by using GraphPad Prism, version 7.0 (GraphPad Software). Statistical significance between human lung samples was determined using parametric or nonparametric one-way analysis of variance depending on normality of variances and sample size and multiple comparisons correction testing using Dunn's method. Statistical significance was defined as a p value of ≤ 0.05 (two-tailed).

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ONLINE FIGURE LEGENDS

Figure E1 Specificity of anti-Alp1 antisera. Lung sections from a subject with severe asthma were immunostained with rabbit serum or anti-Alp1 antisera as indicated. Images are representative of airways from two subjects/group.

Figure E2 Alp1 immunostaining in bronchial epithelium correlates with asthma severity. Representative images of epithelial Alp1 immunostaining in bronchial epithelium (red arrows). (B) Alp1 epithelial staining correlates with asthma severity (B) but not sensitivity to *A. fumigatus* (C). (B) $*p=0.04$, $****p<0.0001$, one way ANOVA (C) $**p<0.009$, $***p=0.001$, Kruskal Wallis.

Figure E3 Alp1 detection in induced sputum. (A) Reactivity of native Alp1 antibody (left

panel) or antibody preadsorbed with purified Alp1 (right panel) prior to immunoblotting membranes containing supernatants of *A. fumigatus* cultures (“Af”) or purified Alp1. Bands corresponding to the putative full length Alp1 containing its autoinhibitory domain (~45 kDa) and the mature, cleaved protease (~33 kDa) are designated by the arrows. (B) Representative immunoblots of sputum (20 µl/lane) samples from the indicated groups probed with Alp1 antibody. Secreted supernatants from *Aspergillus* cultures (“Af”) were run as positive controls for antibody staining.

Figure E4 Induced sputum Alp1 correlates with asthma and Af sensitivity. (A) A standard curve was generated by immunoblotting known amounts of purified Alp1. Optical density values were obtained using ImageStudio software (LiCOR). (B-C) Alp1 levels in sputum samples interpolated from standard curves. * $p=0.03$, ** $p=0.002$, Mann Whitney. (D) Correlation between Alp1 levels and neutrophils (% total cells) in sputum.

Table E1 – Clinical characteristics of 48 subjects who provided bronchial biopsy specimens

	Controls	Mild/Moderate Asthma (no <i>Af</i> sensitivity)	Mild/Moderate Asthma (<i>Af</i> sensitivity)	Severe Asthma (no <i>Af</i> sensitivity)	Severe Asthma (<i>Af</i> sensitivity)
Number	10	10	11	10	5
Age[§]	23(19-35)	42.5(18-61)	28.5(20-53)	55.5(30-68)	37(20-44)
BMI*	22.84(4.85)	25.59(1.59)	25.79(1.12)	30.45(1.77)	32.51(4.49)
Male/Female	3:7	6:4	6:5	4:6	4:1
Never/current/ex-smokers	7/0/3	3/3/4	10/0/1	5/0/5	3/0/2
Atopy[⊙]	0(0)	0(0)	10(83.3)	4(40)	6(100)
+ <i>Af</i> skin prick[⊙]	0(0)	0(0)	11(100)	0(0)	5(100)
Blood eos (x10⁹/l)*	0.059(0.002)	0.14(0.05)	0.38(0.06)	0.04(0.02)	0.03(0.01)
Serum IgE (IU)*	<5 [#]	22 [#]	637(26.5)	185(106)	269(215)
FEV₁ (% predicted)[§]	102(88-111)	83.5(53-126)	88(71-115)	62(30-95)	79(58-103)
PC₂₀FEV₁ (mg/mL)[⋄]	≥16	2.2(1.1-4.4)	0.7(0.31-1.56)	0.30(-0.43-2.16)	0.16 [#]
BD response (%)*	-2(0)	11.63(4.49)	9.4(6.5)	7.9(3.22)	3(1.14)
Inhaled Corticosteroids^z	0	5(250-500)	5(250-1000)	10(1000-2750)	5(750-2500)

Af: *Aspergillus fumigatus*; BD: bronchodilator; FEV₁: forced expiratory volume in 1 second; PC₂₀FEV₁: provocative concentration of methacholine to induce a 20% decrease in FEV₁

[§]Median (range)

*Mean (± s.e.m)

[⊙]Number of subjects (%)

[⋄]Geometric mean (95% CI); [#]Value only available for one subject.

^zNumber of subjects (range equivalent of fluticasone, µg)

Table E2 – Clinical characteristics of 39 subjects who provided induced sputum supernatants

	Controls	Mild/Moderate Asthma (no <i>Af</i> sensitivity)	Mild/Moderate Asthma (<i>Af</i> sensitivity)	Severe Asthma (no <i>Af</i> sensitivity)	Severe Asthma (<i>Af</i> sensitivity)
Number	10	7	7	8	7
Age[§]	36.8(21-66)	44.1(18-61)	64.1(51-78)	47.8(25-71)	54.4(23-79)
BMI*	28.23(1.59)	30.64(2.63)	25.37(1.45)	28.40(1.24)	30.64(1.98)
Male/Female	6:4	5:2	4:3	4:4	2:5
Never/current/ex-smokers	8/0/2	2/2/3	3/2/2	3/0/5	3/0/4
Atopy[⊙]	9(90)	7(100)	5(71.43)	6(75)	7(100)
+ <i>Af</i> skin prick[⊙]	3(30)	0(0)	7(100)	0(0)	7(100)
Sputum eos (%)	3.1(2.6)	2.8(0.7)	10.96(7.2)	2.2(1.12)	13.1(6.2)
Sputum neutros (%)	30.8(6.4)	48.9(7.7)	60(9.97)	52.7(9.1)	45.7(8.6)
FEV₁ (% predicted)[§]	103.6(92-132)	75.6(47-100)	68.1(33-82)	77.7(57-104)	70.0(51-82)
PC₂₀FEV₁ (mg/mL)[♦]	≥16	2.31	0.11	5.29(3.67-7.06)	N/A
BD response (%)[*]	-2.3(1.86)	6.7(3.70)	3.4(2.38)	7.2(2.47)	6.4(2.58)
Inhaled Corticosteroids^z	0(0)	7(250-500)	7(250-500)	8(1000-3000)	7(1000-1750)

Af: *Aspergillus fumigatus*; BD: bronchodilator; FEV₁: forced expiratory volume in 1 second; PC₂₀FEV₁: provocative concentration of methacholine to induce a 20% decrease in FEV₁

§Mean (range)

*Mean (± s.e.m)

⊙Number of subjects (%)

♦Geometric mean (95% CI)

N/A (not available)

zNumber of subjects (range equivalent of fluticasone, µg)

Figure E1

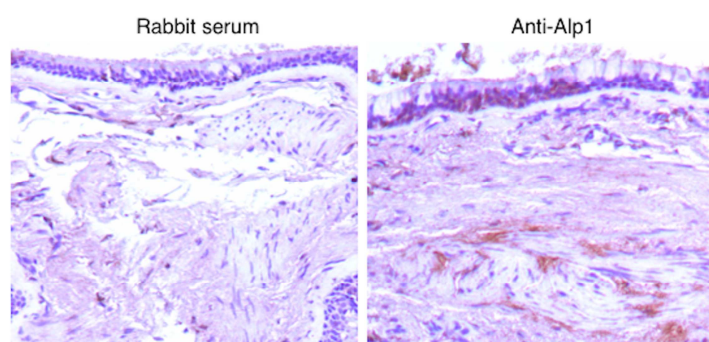


Figure E2

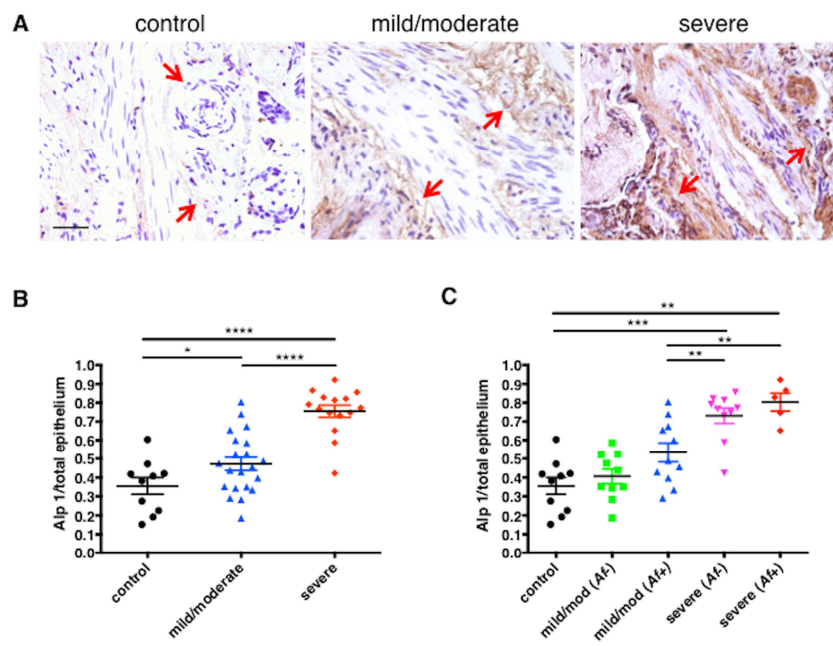


Figure E3

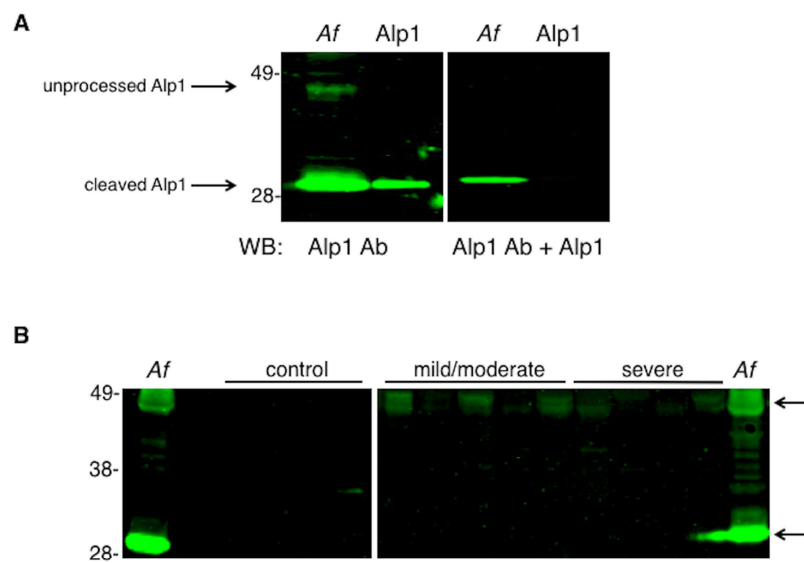


Figure E4

