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Sputum ANA Serves as a Biomarker for Severe Asthma

Running title: Autoantibody in Sputum of Severe Asthma

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4 Health and Welfare.

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6 **Conflict of interest**

7 The authors confirm that there is no conflict of interest.

To the Editor,

Autoimmune diseases are characterized by persistent self-reactivity and tissue damage mediated by autoantibodies against self-antigens in which antinuclear antibody (ANA) is a major autoantibody.^{1,2} A recent study demonstrated the evidence of autoimmune response with localizing autoantibodies in the airway secretion of asthmatics and that increased IgG to eosinophil peroxidase and/or ANA was associated with increased type 2 airway inflammation and eosinophilic degranulation, especially in patients with severe asthma (SA).^{1,2} In addition, increased levels of C3c and deposition of C1q-bound/IL-5-bound IgG were noted in mepolizumab-resistant asthmatics, suggesting that the presence of airway autoimmunity may be associated with asthma exacerbation and treatment response.³ In the present study, we evaluated ANA levels in the sputum of 46 adult asthmatics associated with clinical (including lung function, blood eosinophils, total IgE, and sputum eosinophil/neutrophil counts) and inflammatory parameters in sputum, and compared between the SA and nonsevere asthma (non-SA) groups. Levels of myeloperoxidase (MPO), eosinophil-derived neurotoxin (EDN), matrix metalloproteinase (MMP)-9, and tissue inhibitor of metalloproteinase-1 (TIMP-1) as well as ANA in sputum were measured using ELISA. Detailed information on the study subjects and methods is described in the online Appendix.

The SA group had significantly higher ANA levels as well as MMP-9/TIMP-1 levels in sputum than the non-SA group, while lung FEV₁ (%), FVC (%), FEV₁/FVC, and MMEF (%) were significantly lower in the SA group than in the non-SA group ($P < .050$ for all) (Table 1, Figure 1A). When compared between eosinophilic (higher than 300 peripheral eosinophils) and non-eosinophilic type (less than 300 eosinophils) in severe asthmatics, the SA group with eosinophilic asthma had higher sputum eosinophils/EDN levels than that with noneosinophilic asthma ($P = .032$, $P = .039$, respectively), while no significant differences were noted in sputum ANA levels ($P = .922$; Table 1). The cutoff value of sputum ANA level predicting the SA group is 111.8 U/mL with a statistical significance ($P = .002$, 70.6% sensitivity, 72.4% specificity; Figure 1B). No differences were noted in serum ANA levels (data not shown) or sputum EDN/MPO levels between the 2 groups. When asthmatics were classified into the ANA_{high} and ANA_{low} groups according to their cutoff value, the ANA_{high} group had a significantly higher prevalence of SA, and higher sputum MMP-9/MPO levels than the ANA_{low} group ($P < .050$ for all, Table S1). Significantly positive correlations were noted between sputum MPO and MMP-9/TIMP-1 levels ($r = 0.815$, $P < .001$; $r = 0.770$, $P < .001$); significantly negative correlations were found between

39 FEV₁ (%) and sputum MMP-9 /TIMP-1 levels ($r = -0.341$, $P < .025$; $r = -0.489$, $P < .001$; Figure
40 S1), indicating that activated neutrophils may involve ANA-associated inflammation in the
41 asthmatic airway, where MMP-9/TIMP-1 are associated with airway remodeling. Persistently
42 higher blood neutrophil counts were noted in severe asthmatics even with anti-asthmatic
43 medications.⁴ Severe asthmatics had a higher count of neutrophil DNA extracellular trap (NET)-
44 forming neutrophils than nonsevere asthmatics. NETs (derived from severe asthmatics) enhanced
45 the expression of epithelial autoantigens and induced epithelial activation/dysfunction which was
46 mediated by reactive oxygen species.⁵ MPO is a component of NETs. Among asthmatics, the
47 levels of MMP-9 and TIMP-1 (airway remodeling-related cytokines) were higher in uncontrolled
48 status than in controlled status.⁶ The present study demonstrated a significant association between
49 sputum MPO (not EDN) and ANA levels. The levels of MMP-9 and TIMP-1 were higher in the
50 sputum of the SA group than in the non-SA group with a negative correlation with FEV₁ (%), but
51 a positive correlation with sputum MPO levels. Taken together, activated neutrophils (especially
52 NET-induced inflammation) may increase MMP-9/TIMP-1 production in the asthmatic airway,
53 contributing to airway remodeling and progression to SA.

54 This study has a limitation. This is a single-center observational study; however, we demonstrate
55 a significant association of sputum ANA levels with lung function and inflammatory parameters
56 (including MPO) according to the severity of asthma.

57 In conclusion, neutrophil/NET-induced inflammation is associated with ANA-associated
58 autoimmune response in the asthmatic airway, contributing to asthma severity and lung function
59 declines in which MMP-9/TIMP-1 are involved. Further studies are needed to evaluate whether
60 epithelial biologics (anti TSLP or IL-33 antibodies) could overcome this autoimmune cascade.

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Table 1. Comparison of clinical and inflammatory parameters between the severe and nonsevere asthma groups.

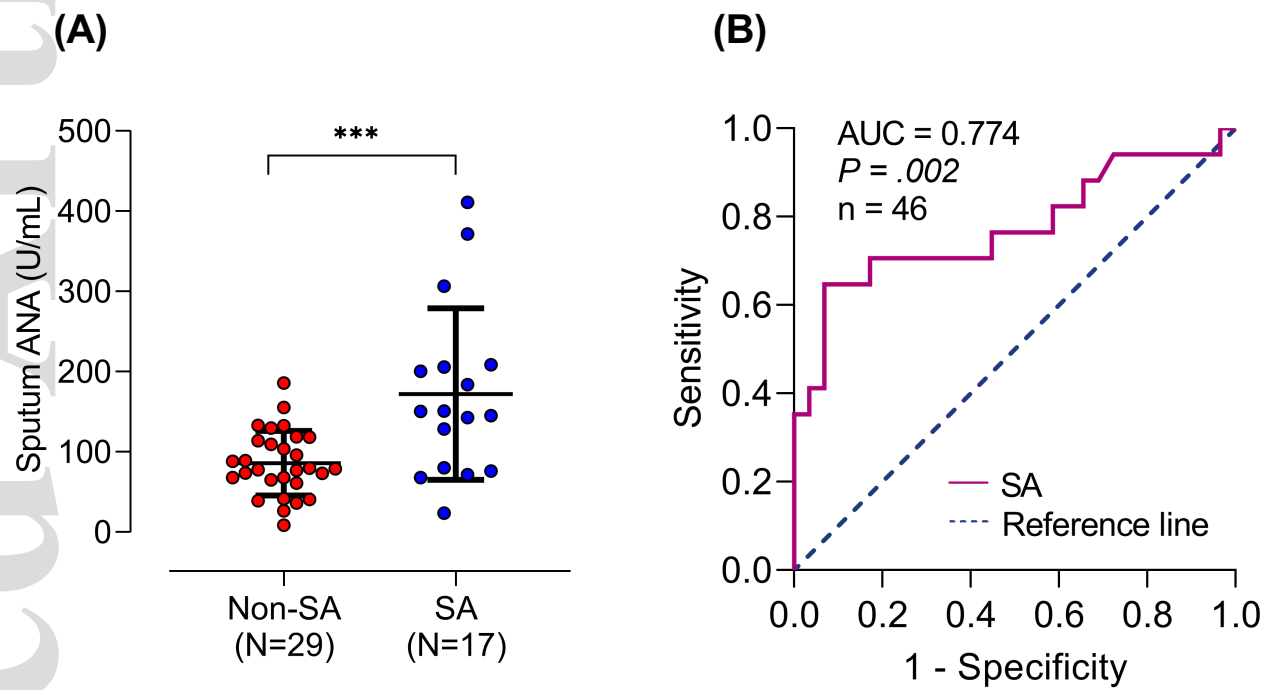
Variables	Nonsevere asthma (n=29)	Severe asthma (n=17)	<i>P</i> -value	Severe asthma		
				Noneosinophilic asthma	Eosinophilic asthma	<i>P</i> -value
				(n=7)	(n=10)	
Age (years)	54.7 ± 13.6	55.7 ± 11.3	.991	59.1 ± 16.7	53.3 ± 5.1	.063
Female sex (%)	62.1	47.1	.369	28.5	60.0	.335
Atopy (%)	31.0	66.7	.030	57.1	75.0	.608
Baseline FEV ₁ (%)	95.3 ± 16.2	74.5 ± 22.8	.001	78.6 ± 19.1	71.3 ± 25.9	.560
Baseline FVC (%)	93.4 ± 15.0	79.2 ± 19.9	.019	81.6 ± 17.3	77.3 ± 22.5	.791
FEV ₁ /FVC ratio	84.4 ± 38.3	76.1 ± 9.1	.002	76.6 ± 10.1	75.7 ± 8.9	.874
MMEF (%)	75.9 ± 24.0	51.8 ± 24.6	.004	59.11 ± 25.4	46.0 ± 23.7	.315
Serum total IgE (kU/L)	242.0 ± 259.2	410.0 ± 444.3	.218	152.3 ± 113.7	555.0 ± 478.8	.005

Total eosinophil count (cells/ μ L)	317.2 $\pm$ 187.2	482.4 $\pm$ 340.0	.118	145.5 $\pm$ 86.3	1349.9 $\pm$ 1580.0	.001
Sputum eosinophil count (%)	19.6 $\pm$ 27.7	26.8 $\pm$ 37.2	.844	0.5 $\pm$ 0.8	46.5 $\pm$ 39.2	.032
Sputum neutrophil count (%)	67.9 $\pm$ 30.0	65.3 $\pm$ 37.6	.955	91.5 $\pm$ 4.1	45.6 $\pm$ 40.0	.039
Sputum ANA (U/mL)	86.0 $\pm$ 40.3	172.2 $\pm$ 107.1	.002	134.9 $\pm$ 121.8	164.5 $\pm$ 121.8	.922
Sputum EDN (ng/mL)	1031.0 $\pm$ 780.0	1467.2 $\pm$ 1152.0	.234	693.1 $\pm$ 779.8	2094.5 $\pm$ 1180.6	.039
Sputum MPO (pg/mL)	937.9 $\pm$ 1053.0	1262.0 $\pm$ 1076.0	.162	1304.4 $\pm$ 1052.5	1288.0 $\pm$ 1221.0	.906
Sputum MMP-9 (ng/mL)	437.0 $\pm$ 675.3	1181.0 $\pm$ 1365.0	.031	753.8 $\pm$ 693.0	1439.4 $\pm$ 1848.2	.922
Sputum TIMP-1 (ng/mL)	327.9 $\pm$ 414.7	953.3 $\pm$ 1054.0	.021	533.7 $\pm$ 390.4	713.2 $\pm$ 806.5	.770

Values are given as n (%) for categorical variables and as mean $\pm$ SD for continuous variables. P values were applied by Pearson chi-square test for categorical variables and Mann–Whitney U-test for continuous variables. ANA, antinuclear antibodies; EDN, eosinophil-derived neurotoxin; IgE, immunoglobulin E; EA, eosinophilic asthma; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; MMEF, maximal mid-expiratory flow; MMP-9, metalloproteinases-9; MPO, myeloperoxidase; non-EA, noneosinophilic asthma; SA, severe asthma; TIMP-1, tissue inhibitor of metalloproteinase-1.

84 **FIGURE LEGENDS**

85 **Figure 1. Sputum ANA levels for discriminating severe asthma (SA) group from the non-SA**
86 **groups.** Comparisons of sputum ANA levels between the two groups **(A)**. Receiver operating
87 characteristic curve of sputum ANA levels for discriminating the SA group from the non-SA
88 group **(B)**. The data are represented as mean values. *P*-value was obtained by Mann–Whitey U-test.
89 ANA, antinuclear antibody; AUC, area under the curve.

**Figure 1_Quang et al.**

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18APPENDIX INFORMATION

19Study subjects and methods

20Forty-six patients with asthma were enrolled at the Department of Allergy and Clinical
21Immunology, Ajou University Hospital (Suwon, South Korea). Various clinical parameters
22from asthmatics were collected, including blood total eosinophil counts, sputum eosinophil
23/neutrophil counts, skin prick test, serum total IgE measurements as well as spirometry results
24for FEV₁ (%), FVC (%), FEV₁/FVC ratio, and MMEF %. A diagnosis of asthma was based
25on the Global Initiative for Asthma (GINA) guidelines.¹ Severe asthma was defined
26according to the International Guidelines of the European Respiratory Society/American
27Thoracic Society.² Atopic status was defined by skin prick test results with 55 common
28inhalant allergens (Bencard Co., Brentford, UK) and serum total IgE was measured using the
29ImmunoCAP system (ThermoFisher Scientific, Waltham, CA, USA).³ Lung function, FEV₁
30(%), FVC (%), and MMEF % were measured as previously described.³ The phenotype of
31eosinophilic asthma was defined if patients had ≥ 300 peripheral eosinophil counts at the
32enrollment. All the asthmatic patients were in stable conditions, and those having any
33possible autoimmune disease or immunodeficiencies were excluded. This study was
34approved by the Institutional Review Board of Ajou University Hospital (AJIRB-BMR-SUR-
3515-498) and all the subjects provided written informed consent.

36

37Induced sputum analysis

38After induction of sputum by using 3% saline, sputum was prepared as follows. Sputum was
39placed in a pre-weighed Eppendorf tube. Eight volumes of Dulbecco's phosphate-buffered
40saline (PBS) or 4 volumes of 0.1% dithiothreitol (Sigma-Aldrich, St. Louis, MO, USA) plus
41PBS were added to one volume sample weight. The total count of cells stained with Trypan
42blue was calculated by using a hemacytometer. The sputum cells were collected by

43cytocentrifugation and 500 cells were examined after staining with Diff-Quick (American
44Scientific Products, Chicago, USA). Differential cell counts (including eosinophils and
45neutrophils) were determined after removing epithelial cells.⁴ The remainder of the
46homogenized sputum sample was centrifuged at 12000 rpm for 5 min, and the supernatant
47was collected and transferred to a cryotube containing protease inhibitor cocktail solution
48(100x) (Cell Signaling Technology, Danvers, MA, USA) and ethylenediaminetetraacetic acid
49(Sigma-Aldrich). Samples were frozen and stored at -80°C for ELISA.

50

51**ELISA**

52The concentrations of myeloperoxidase (MPO), matrix metalloproteinase (MMP)-9 and
53tissue inhibitor of metalloproteinase-1 (TIMP-1) in induced sputum were measured by
54ELISA kit (R&D, Inc., Minneapolis, MN, USA). Antinuclear antibody (ANA) ELISA kit was
55purchased from IMTEC (Max-Planck-Ring, Wiesbaden, Germany), eosinophil-derived
56neurotoxin (EDN) was purchased from SKIMS-BIO Co. (Seoul, Korea) and measured
57according to the manufacturer's instructions.

58

59**Statistical analysis**

60 The Mann-Whitney *U* test was used to compare continuous variables, while Pearson's chi-
61square or Fisher's exact tests were used to comparing categorical variables. The
62sample correlation was analyzed by using Spearman's rank coefficient. All statistical analyses
63were performed with SPSS software, version 22.0 (SPSS Inc., Chicago, IL, USA). GraphPad
64Prism 5.0 software (GraphPad Inc., San Diego, CA, USA) was used for graphs, with values
65presented as the mean $\pm$ standard deviation (SD).

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Table S1. Comparison of demographic characteristics between ANA_{low} and ANA_{high} (the cutoff point at 111.8 U/mL, 70.6%

sensitivity, 72.4% specificity)

Variables	ANA _{low} (n=26)	ANA _{high} (n=20)	P value
Age (y)	53.0 ± 14.5	57.8 ± 9.5	.262
Female sex (%)	53.8	60.0	.769
Atopy (%)	29.2	60.0	.066
Severe asthma (%)	19.2	60.0	.006
Baseline FEV ₁ (%)	88.23 ± 22.0	87.4 ± 20.4	.963
Baseline FVC (%)	87.8 ± 18.7	89.1 ± 17.6	.722
FEV ₁ /FVC ratio	82.3 ± 9.1	80.2 ± 7.0	.223
MMEF (%)	70.8 ± 28.6	62.6 ± 23.7	.260
Total IgE (kU/L)	222.5 ± 263.6	398.3 ± 405.8	.058
TEC (cells/μL)	326.9 ± 230.8	445.0 ± 292.9	.160
Sputum eosinophil count (%)	24.3 ± 35.2	20.9 ± 28.7	.710
Sputum neutrophil count (%)	66.1 ± 35.1	67.5 ± 31.5	.955
Sputum EDN (ng/mL)	1176.9 ± 1048.6	1198.6 ± 796.1	.566
Sputum MPO (pg/mL)	838.2 ± 937.6	1397.8 ± 1176.1	.045
Sputum MMP-9 (ng/mL)	496.9 ± 1004.9	997.4 ± 1062.9	.010
Sputum TIMP-1 (ng/mL)	398.5 ± 534.4	771.3 ± 978.0	.118

Values are given as n (%) for categorical variables and as mean ± SD for continuous variables. *P* values were applied by Pearson chi-square test for categorical variables and Mann–Whitney U-test for continuous variables. ANA, antinuclear antibodies; EDN, eosinophil-derived

83neurotoxin; FEV₁, forced expiratory volume in 1s; FVC, forced vital capacity; IgE, immunoglobulin E; MMEF, maximal mid-expiratory

84flow; MMP-9, metalloproteinases-9; MPO, myeloperoxidase; TEC, total eosinophil count; TIMP-1, tissue inhibitor of metalloproteinase-1

85 SUPPLEMENTARY FIGURES

86 **Figure S1. Correlations between FEV₁ (%) and inflammatory parameters in sputa of**
87 **asthmatics.** Correlations between sputum MPO and MMP-9/TIMP-1 levels **(A-B).**
88 Correlations between FEV₁ (%) and sputum MMP-9/TIMP-1 levels **(C-D).** The data are
89 presented as Spearman correlation coefficient r (P -value). FEV₁, forced expiratory volume in
90 l; MMP-9, metalloproteinases 9; MPO, myeloperoxidase; TIMP-1, tissue inhibitor of
91 metalloproteinase-1

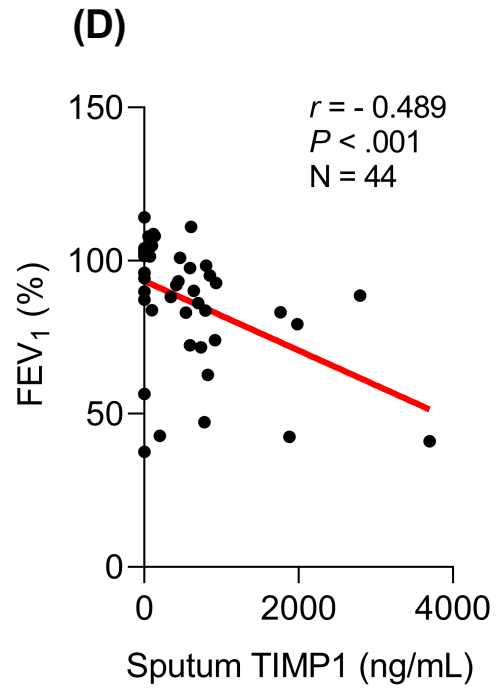
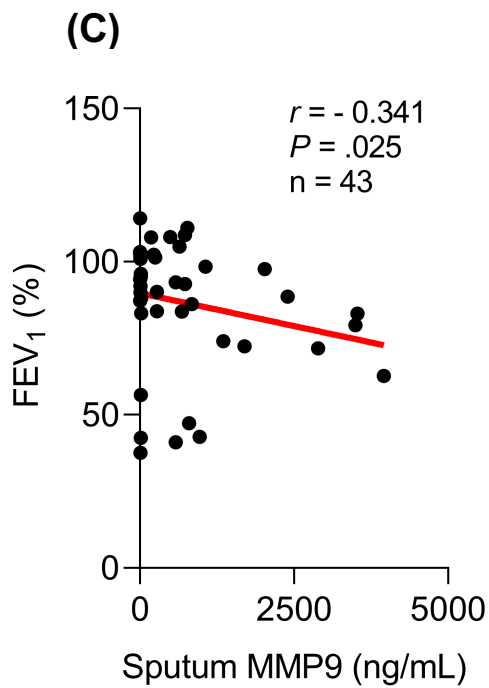
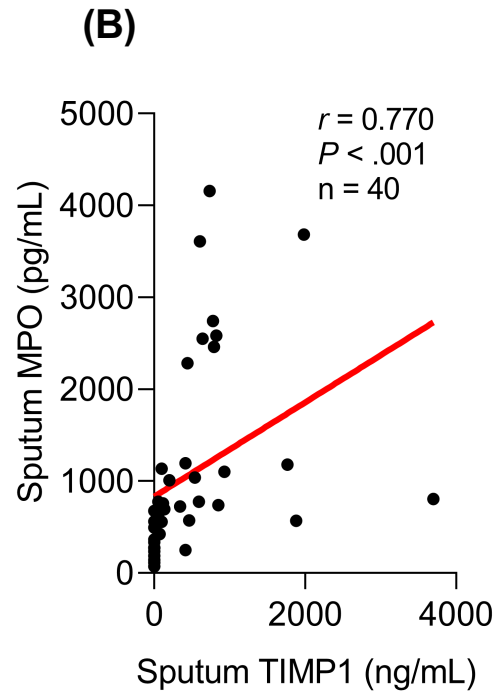
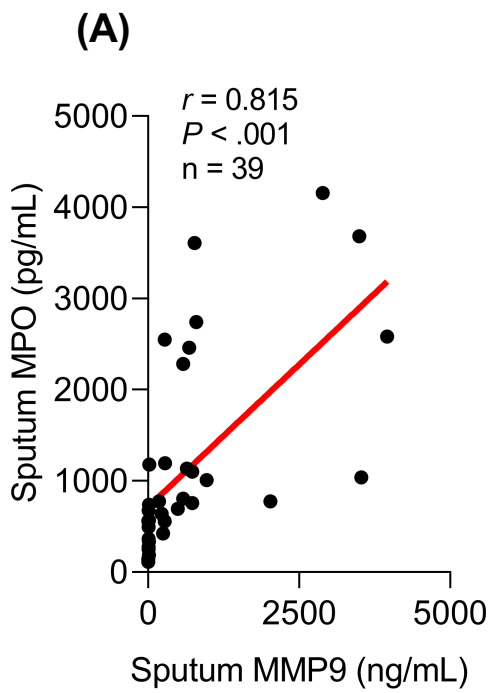


Figure S1_Quang et al.