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## **The presence of anti-neutrophil extracellular trap antibody in patients with microscopic polyangiitis**

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## **Abstract**

**Objective** Although anti-neutrophil cytoplasmic antibody (ANCA) is the major autoantibody in patients with ANCA-associated vasculitis (AAV), previous studies have suggested the presence of anti-neutrophil extracellular trap (NET) antibody in patients with microscopic polyangiitis (MPA), one type of AAV. In this study, we aimed to determine the prevalence and pathogenic role of anti-NET antibody (ANETA) in MPA.

**Methods** We examined the presence or absence of ANETA in sera obtained from 19 MPA patients by indirect immunofluorescence. We compared the clinical parameters, including age, sex, myeloperoxidase (MPO)-ANCA, creatinine, C-reactive protein, MPO-DNA complexes, and vasculitis activity, in ANETA-positive and ANETA-negative MPA patients. We investigated the serum NET induction and degradation abilities of ANETA-positive and ANETA-negative MPA patients with reference to healthy controls (n=8). Furthermore, we assessed the relationship of ANETA with the effect of IgG depletion on the serum NET degradation ability.

**Results** ANETA was present in 10 of the 19 MPA patients. There was no significant difference in the clinical parameters in ANCA-positive and ANCA-negative MPA patients. Although the NET induction ability was higher and the NET degradation ability was lower in MPA sera than those in healthy controls, these abilities were not different between ANETA-positive and ANETA-negative MPA sera. Interestingly, the NET degradation ability in some sera with ANETA was markedly increased by IgG depletion.

**Conclusion** Some MPA patients produce ANETA and some ANETA possess an inhibitory function against the serum NET degradation ability. Although further studies are needed, ANETA is worthy of attention for understanding the pathophysiology of MPA.

**Keywords:** ANCA; ANCA-associated vasculitis; Neutrophil extracellular traps (NETs); Anti-NET antibody (ANETA)

**Key messages:**

- Anti-neutrophil extracellular trap antibody (ANETA) is present in some MPA patients.
- Some anti-neutrophil extracellular trap antibody (ANETA) inhibit the serum NET degradation ability.
- Anti-neutrophil extracellular trap antibody (ANETA) is worthy of attention for understanding the pathophysiology of MPA.

## **Introduction**

Anti-neutrophil cytoplasmic antibody (ANCA) is the pathogenic autoantibody in ANCA-associated vasculitis (AAV). ANCA binds to the antigens, such as myeloperoxidase (MPO), on the surface of neutrophils primed by proinflammatory cytokines, including tumor necrosis factor (TNF)- $\alpha$ . Simultaneously, the Fc portion of ANCA binds to the Fc $\gamma$  receptors on neutrophils, resulting in the activation of the cells. The activation of neutrophils by ANCA leads to the injury of small vessels through the release of reactive oxygen species and proteolytic enzymes [1].

Neutrophil extracellular traps (NETs) are web-like DNA decorated with antimicrobial proteins, such as MPO, which are extruded from activated neutrophils [2]. NETs can trap microorganisms by the web-like DNA and kill them using antimicrobial proteins. Although NETs are essential for innate immunity, NET formation is strictly regulated by serum factors [3] because NETs have also adverse effects on the hosts [4-7]. The impaired regulation of NETs causes the supply of NET components as autoantigens to the hosts [3, 8, 9].

ANCA can induce NETs in neutrophils primed by TNF- $\alpha$  [10, 11]. Based on the collective findings, we have hypothesized that the ANCA-NET vicious cycle is involved in the pathogenesis of AAV, including microscopic polyangiitis (MPA) [12]. We have recently noted that anti-NET antibody (ANETA) is a factor that can promote the ANCA-NET vicious cycle [12, 13]. In this study, we attempted to determine the prevalence of ANETA in MPA patients and investigated the relationship of ANETA with the serum NET induction and degradation abilities.

## **Methods**

### **Patients and healthy controls**

Nineteen patients diagnosed with MPA and treated at Hokkaido University Hospital between January 2008 and May 2014 were enrolled. The participants included 16 women and 3 men (mean age, 69.3 years). Eight healthy volunteers were employed as controls. Informed consent was obtained from the all participants. This study was conducted with the permission of the Ethical Committee of the Faculty of Health Sciences, Hokkaido University (Permission No. 15-90).

### **Blood samples**

After the acquisition of written informed consent, peripheral blood was taken without anticoagulants, and the blood was centrifuged at 1900 g for 15 min at room temperature for serum separation. At the time of blood sampling, the Birmingham Vasculitis Activity Score (BVAS), which represents the clinical activity of vasculitis [14], was assessed and the levels of MPO-ANCA, anti-nuclear antibody (ANA), creatinine (Cr), C-reactive protein (CRP), and MPO-DNA complexes (soluble form of NETs [10]) were determined. All MPA patients were positive for MPO-ANCA but negative for ANA. Enzyme-linked immunosorbent assay (ELISA) for MPO-DNA complexes was carried out as described [8, 10, 12].

### **Isolation of neutrophils**

Neutrophils were isolated from peripheral blood obtained from healthy volunteers using Polymorphprep (Axis-Shield, Dundee, Scotland). The purity of neutrophils applied to assays was more than 95%.

### **Elution of IgG from serum**

IgG was eluted from serum samples using immunoabsorbent columns (Protein G HP SpinTrap; GE Healthcare, Tokyo, Japan). The sera after the elution of IgG were used as IgG-depleted serum samples.

### **Detection of ANETA by indirect immunofluorescence**

Peripheral blood neutrophils from a healthy volunteer were seeded in the wells ( $1 \times 10^6/\text{ml}$ ) of four-well chamber slides (Thermo Fisher Scientific, Yokohama, Japan), incubated for 15 min at 37°C, and then made to react with 25 nM phorbol myristate acetate (PMA) for 2 h at 37°C. After washing with phosphate-buffered saline (PBS), the cells were fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature and then allowed to react with 250 µg/ml of the eluted IgG samples from MPA patients (n=19) and healthy individuals (n=8) for 1 h at 37°C. Alternatively, the cells were exposed to an ANA-positive serum sample [250 µg/ml IgG from a patient with systemic lupus erythematosus (SLE); ANA,  $\times 1280$ ]. After washing with PBS, the cells were exposed to Alexa Fluor 488-conjugated rabbit anti-human IgG (1:500; Santa Cruz Biotechnology, Dallas, TX) for 1 h at 37°C. Rabbit anti-citrullinated histone H3 (Cit-H3) antibody (1:250; Abcam, Tokyo, Japan) and normal rabbit IgG (1:20; Santa Cruz Biotechnology) were used as positive and negative controls, respectively. In these control cases, Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody (1:500; Invitrogen, Tokyo, Japan) was applied for the secondary antibody. The cells were then mounted with the solution containing 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO).

### **NET induction by IgG eluted from serum**

Neutrophils from a healthy volunteer were seeded in the wells of four-well

chamber slides ( $1 \times 10^6$ /ml), treated with 5 ng/ml TNF- $\alpha$  for 15 min at 37°C, and then exposed to 250  $\mu$ g/ml of the eluted IgG samples from MPA patients (n=19) and healthy individuals (n=8). After incubation for 3 h at 37°C, the supernatants were removed and then the remaining cells on the slides were fixed with 4% PFA for 15 min at room temperature. Thereafter, the samples were washed with PBS and then allowed to react with rabbit anti-Cit-H3 antibody (1:100) for 1 h at room temperature. After washing with PBS, the samples were next exposed to Alexa Fluor 594-conjugated goat anti-rabbit IgG antibody (1:500; Invitrogen) for 1 h at room temperature followed by mounting with the DAPI-containing solution. Photomicrographs (magnification,  $\times 200$ ) were taken randomly (six fields per well of chamber slides), and then the number of Cit-H3-positive and DAPI-positive NETs was counted using the ImageJ software (free software; <https://imagej.nih.gov/ij/index.html>). Data were standardized by the number of DAPI-positive neutrophils.

### **NET degradation by serum**

Neutrophils from a healthy volunteer were seeded in the wells of four-well chamber slides ( $1 \times 10^6$ /ml) and then treated with 100 nM PMA for 3 h at 37°C. After washing with PBS, the samples were incubated in 10% MPA serum (n=19), 10% serum of healthy volunteers (n=8) as positive controls, and PBS as a negative control for 6 h at 37°C. EDTA (2 mM) was added into each well to stop the serum nuclease activity, and then the samples were fixed with 4% PFA for 15 min at room temperature. After washing with PBS, the remaining cells were mounted with the DAPI-containing solution. Photomicrographs (magnification,  $\times 200$ ) were taken randomly (six fields per well of chamber slides). The DAPI-positive area was measured using the ImageJ software and determined as residual NETs. The NET degradation rate (%) was calculated as follows:  $\{(\text{residual NET area, incubated with PBS}) - (\text{residual NET area,}$

incubated with serum) / (residual NET area, incubated with PBS)}  $\times$  100.

### **Competitive binding assay**

NETs were induced and fixed as aforementioned. The cells were preincubated with anti-MPO polyclonal antibodies (1:400, rabbit IgG; Agilent, Tokyo, Japan) or normal rabbit IgG (1:40; Santa Cruz Biotechnology) overnight at 4°C. After washing with PBS, the cells were allowed to react with 250  $\mu$ g/ml of the eluted IgG samples from MPA patients. Thereafter, the cells were exposed to Alexa Fluor 488-conjugated rabbit anti-human IgG (1:500; Santa Cruz Biotechnology) for 1 h at 37°C followed by mounting with the DAPI-containing solution.

### **Statistical analyses**

Fisher's exact test was applied for the analysis of gender variance between ANETA-positive and ANETA-negative MPA patients. For comparison between groups, we first checked if the sample distribution accorded with Gaussian distribution. According to the results, Student's *t*-test and Mann-Whitney *U*-test were applied for the comparison of values between two parametric and nonparametric groups, respectively.  $p < 0.05$  was regarded as statistically significant.

## **Results**

### **Detection of ANETA in the serum**

First, we determined the presence or absence of ANETA in the sera of 19 MPA patients and 8 healthy individuals by indirect immunofluorescence. Neutrophils from a healthy volunteer stimulated by PMA exhibited DAPI-positive extracellular DNA fibers (**Figure 1a, blue**). The anti-Cit-H3 antibody, MPA sera (all MPO-ANCA positive), and the ANA-positive lupus serum (ANCA negative; ANA,  $\times 1280$ ) but not normal rabbit IgG or sera from healthy individuals bound to NETs (**Figure 1a, green**).

In this study, the Adobe Photoshop Elements 14 software was used to evaluate ANETA reactivity. Briefly, the green fluorescence intensity (GFI) that represented IgG binding to NETs was calculated using the software. Because there is no standard ANETA-positive serum, the intensity of the MPA serum, which showed the highest ELISA titer of MPO-ANCA in this study (2011 IU/ml), was set as a criterion (orange dot; GFI, 31.26), and sera showing greater intensity than the criterion were regarded as ANETA positive. As a result, we decided that ANETA was positive in 10 of the 19 MPA sera (**Figure 1b**).

### **Comparison of clinical parameters between ANETA-positive and ANETA-negative MPA patients**

The clinical parameters of MPA patients are summarized in **Table S1**. Between ANETA-positive (n=10) and ANETA-negative (n=9) MPA patients, there was no significant difference in age, gender variance, levels of MPO-ANCA, Cr, CRP, MPO-DNA complexes, and BVAS.

### **Comparison of serum NET induction and degradation abilities between**

### **ANETA-positive and ANETA-negative MPA patients**

We investigated the serum NET induction and degradation abilities of ANETA-positive and ANETA-negative MPA patients with reference to healthy controls. Although the NET induction ability was higher and the NET degradation ability was lower in MPA sera than those in healthy controls, these abilities were not different between ANETA-positive and ANETA-negative MPA sera (**Figures 1c and 1d**).

### **Effect of IgG depletion on the serum NET degradation ability**

A previous study has suggested that anti-DNA antibodies in SLE patients can inhibit the serum NET degradation ability [3]. Using this evidence as a reference, we speculated that some ANETA in MPA patients could inhibit the serum NET degradation ability similarly to anti-DNA antibodies in SLE patients. To test this, we compared the serum NET degradation ability between before and after IgG depletion concerning seven ANETA-positive and five ANETA-negative MPA sera chosen at random.

Although there was no significant difference between before and after IgG depletion in both ANETA-positive and ANETA-negative groups, three of the seven ANETA-positive MPA sera exhibited a marked increase in the NET degradation ability after IgG depletion (**Figure 2a, orange lines**). On the contrary, this tendency was not apparent in the ANETA-negative MPA sera (**Figure 2b**).

### **Distinction of the ANETA from MPO-ANCA**

To determine the distinction of the ANETA from MPO-ANCA, a competitive binding assay was carried out concerning the ANETA-positive MPA sera, in which the NET degradation ability was increased obviously after IgG depletion and reached normal levels (n=2), and ANETA-negative MPA sera (n=2) chosen at random. The binding of ANETA-positive MPA sera (both positive for ANETA and MPO-ANCA) to

NETs was not inhibited by the preincubation of NETs with excessive anti-MPO polyclonal antibodies (1:400, rabbit IgG), whereas that of ANETA-negative MPA sera (solely positive for MPO-ANCA) was inhibited by the competitor (**Figure 2c**).

## Discussion

This study demonstrated that ANETA was present in some MPA patients. It is interesting to determine if ANETA is pathogenic. However, this study could not demonstrate the direct contribution of ANETA to the pathogenesis of MPA. There is no standard ANETA-positive serum at present, and this is a critical limitation of this study. Further studies on the association of accurate ANETA titers with clinical parameters are needed to conclude the pathogenicity of ANETA.

Although a straightforward difference in NET induction was not found between ANETA-positive and ANETA-negative MPA sera, the NET degradation ability of whole serum samples was markedly increased by IgG depletion in some ANETA-positive MPA sera. The effect of IgG depletion can be reflected by ANCA depletion. Nevertheless, it is worth considering that some ANETA can inhibit the serum NET degradation ability and drive the ANCA-NET vicious cycle [12].

Currently, the antigens of ANETA are not identified. NETs consist of more than 30 kinds of nuclear, cytoplasmic, and granular components, such as histones, high-mobility group box 1, LL37, neutrophil elastase, cathepsin G, lactoferrin, MPO, and proteinase 3 [15]. We have confirmed that all sera of MPA patients are negative for ANA with routine indirect immunofluorescence. However, it is possible that some ANETA recognize nuclear antigens, which receive a certain modification during NET formation. The identification of ANETA antigens is critical and now ongoing in our laboratory. We have preliminarily identified a cell structure-related 17-kDa protein in neutrophils as a candidate of ANETA antigen.

In conclusion, we have demonstrated that ANETA is present in some MPA patients and suggested the possible inhibitory function against the serum NET degradation ability. Although further studies are needed to clarify the relationship

between ANETA and the pathogenesis of MPA, ANETA is worthy of attention for understanding the pathophysiology of MPA.

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### **Disclosures**

All the authors declared no competing interests.

### **Author Contributions**

F.H., U.T., and A.I. designed the study. F.H., D.N., K.W-K., Y.K., H.S., and S.M. performed the experiments. D.N., S.M., S.N., U.T., T.A., and A.I. analyzed the data. F.H., U.T., and A.I. wrote the manuscript.

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## Figure legends

### Figure 1. ANETA and NET induction/degradation abilities in MPA sera

(a) Detection of ANETA. NETs were reacted with the eluted IgG from MPA sera (n=19), healthy controls (n=8), and the ANA-positive lupus serum. Rabbit anti-Cit-H3 antibody and normal rabbit IgG were used as positive and negative controls, respectively. (b) Comparison of GFI between MPA sera (n=19) and healthy controls (n=8). The orange dot indicates the intensity of the MPA serum, which showed the highest ELISA titer of MPO-ANCA (2011 IU/ml). Comparison of the serum NET induction (c) and degradation (d) abilities among ANETA-positive MPA patients (n=10), ANETA-negative MPA patients (n=9), and healthy individuals (n=8). \*\* $p < 0.01$ , Mann-Whitney *U*-test, n.s.: not significant.

### Figure 2. The characteristics of ANETA

Effect of IgG depletion on the serum NET degradation ability of ANETA-positive (a, n=7) and ANETA-negative (b, n=5) MPA sera. Orange lines indicate a marked increase in the serum NET degradation ability after IgG depletion. (c) Distinction of ANETA from MPO-ANCA. NETs were preincubated with excessive anti-MPO polyclonal antibodies or normal rabbit IgG. After washing with PBS, the cells were reacted with the eluted IgG samples from the ANETA-positive MPA sera, in which the NET degradation ability was increased obviously after IgG depletion and reached normal levels (n=2), and ANETA-negative MPA sera (n=2) chosen at random. Representative photos are shown.

Figure 1

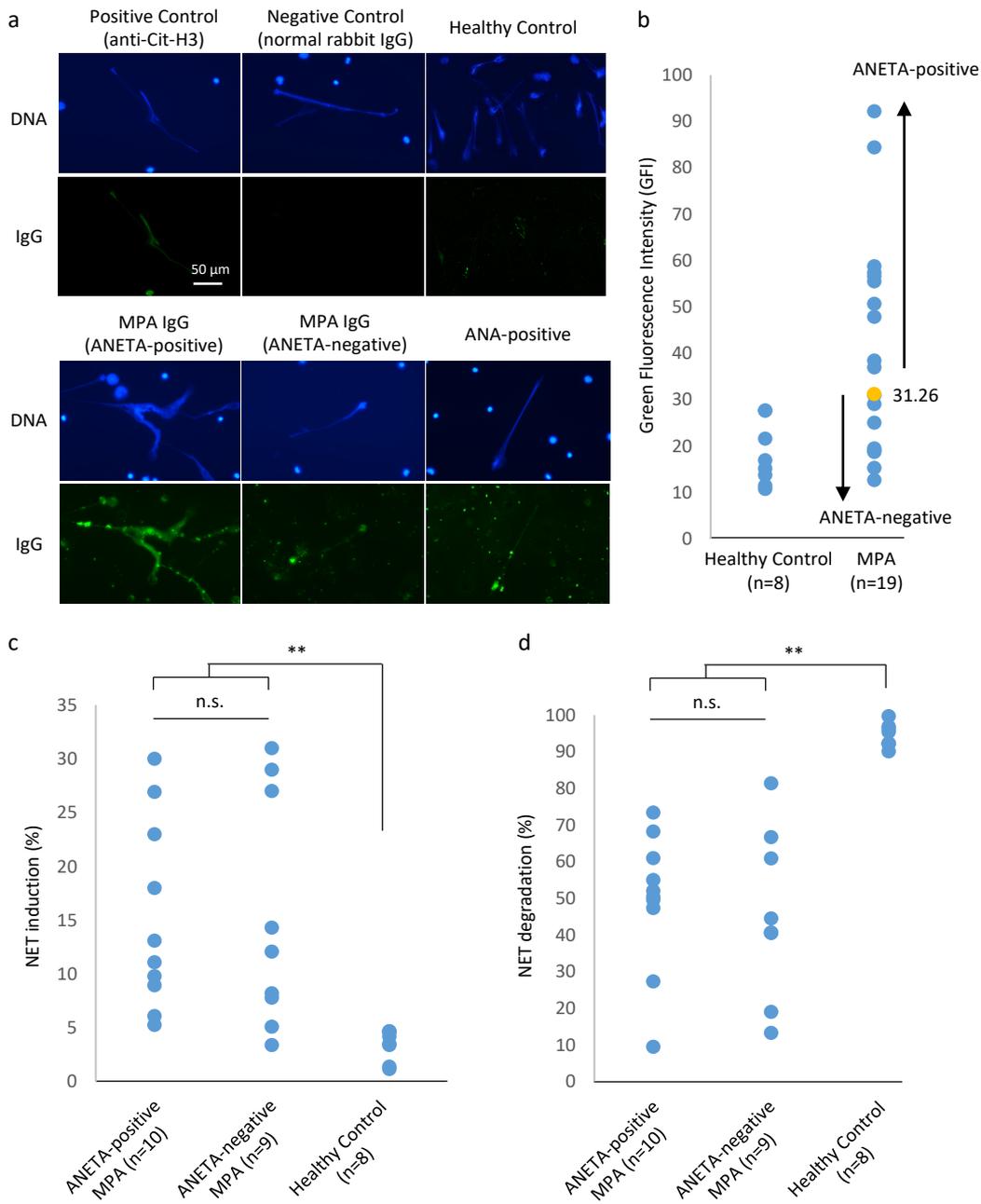
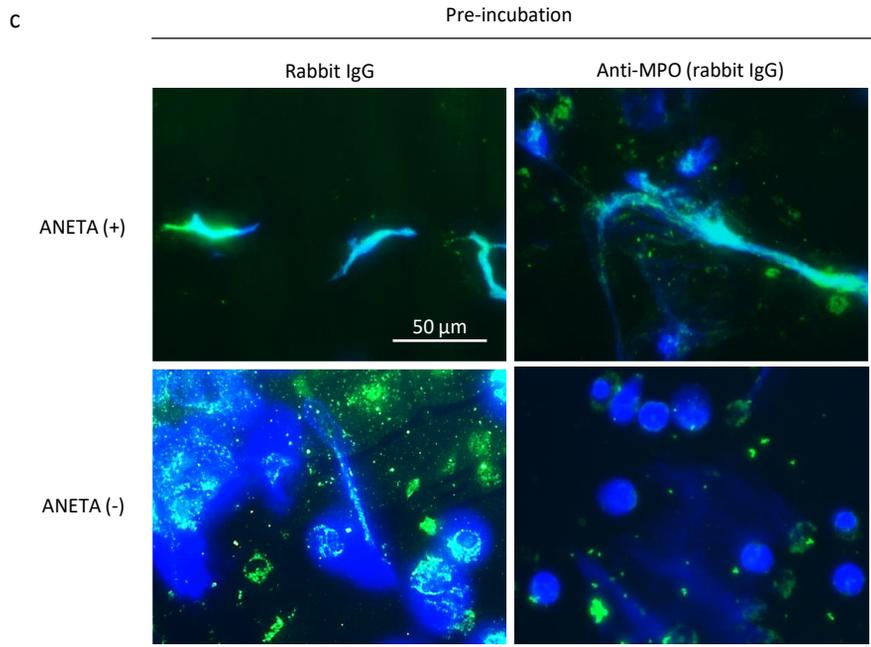
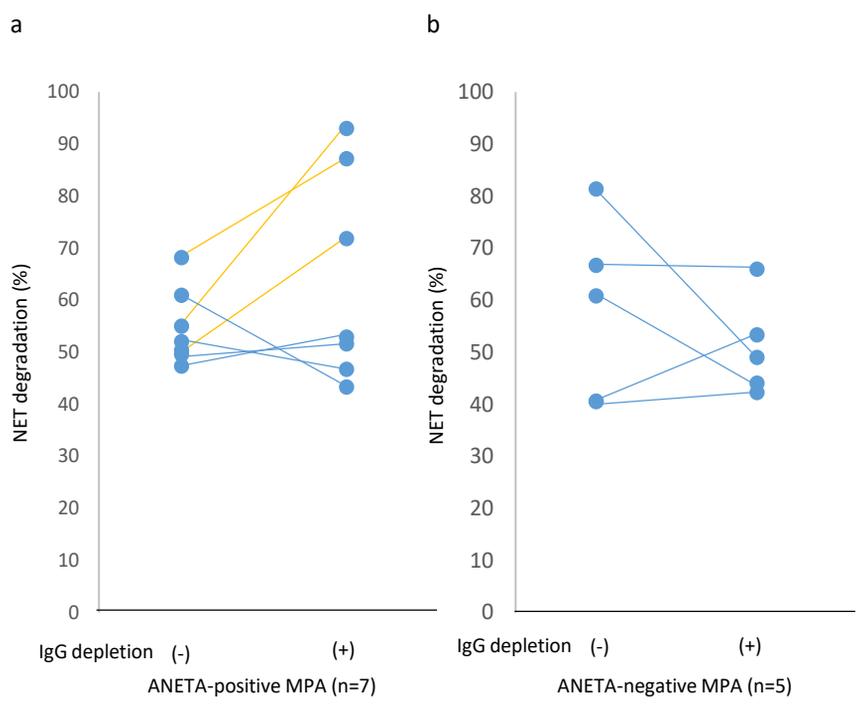


Figure 2



**Table S1. Clinical parameters of MPA patients enrolled in this study**

	ANETA positive	ANETA negative	p
Number	10	9	
Age*	70.2±5.6	68.2±13.8	0.682**
Sex (male/female)	3/7	0/9	0.087***
MPO-ANCA (IU/ml)*	280.1±273.8	313.7±686.9	0.889**
Cr (mg/dl)*	1.66±1.25	1.22±0.58	0.342**
CRP (mg/dl)*	6.22±3.37	5.55±5.37	0.747**
MPO-DNA complexes (O.D.)*	0.26±0.13	0.29±0.22	0.933**
BVAS*	14.8±6.3	11.6±6.7	0.332**

\*Mean±SD. \*\* Student's *t*-test. \*\*\* Fisher's exact test.