



Title	The Quantification of Extracellular Trap Cell Death-Derived Products as Diagnostic Biomarkers for Otitis Media With Antineutrophil Cytoplasmic Antibody-Associated Vasculitis and Eosinophilic Otitis Media
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**The quantification of extracellular trap cell death-derived products as diagnostic biomarkers for otitis media with antineutrophil cytoplasmic antibody-associated vasculitis and eosinophilic otitis media**

Short running head: ETosis in OMAAV and EOM

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1 **ABSTRACT**

2 **Objective:** This study aimed to quantify the cell-free deoxyribonucleic acid  
3 (DNA), citrullinated-histone H3 (cit-H3)-DNA complex, and myeloperoxidase  
4 (MPO)-DNA complex as extracellular trap cell death (ETosis)-derived  
5 products in the middle ear fluid, and to identify diagnostic biomarkers for  
6 the discrimination of antineutrophil cytoplasmic antibody  
7 (ANCA)-associated vasculitis (OMAAV) from eosinophilic otitis media  
8 (EOM).

9

10 **Study Design:** Prospective study.

11

12 **Setting:** Tertiary referral center.

13

14 **Patients:** OMAAV patients were eligible for inclusion in this analysis.  
15 Patients with EOM were examined as controls.

16

17 **Intervention:** All samples were obtained from the middle ear fluid in patients  
18 with OMAAV or EOM. The fluid samples were aspirated from the middle ear  
19 through the anterior-inferior portion of the tympanic membrane using a 1-ml  
20 tuberculin syringe with a 24- or 26-gauge needle under a microscope.

21

22 **Main Outcome Measures:** The levels of cell-free DNA, cit-H3-DNA complex  
23 and MPO-DNA complex in the fluid samples were quantified using an  
24 enzyme-linked immunosorbent assay.

1

2 **Results:** Patients with OMAAV showed significantly higher levels of  
3 MPO-DNA complex compared to patients with EOM, regardless of the serum  
4 ANCA status at the time of sampling ( $p<0.001$  and  $p<0.001$ , respectively).  
5 Meanwhile, there were no significant differences in the values of cell-free  
6 DNA or cit-H3-DNA complex between the OMAAV and EOM patients.

7

8 **Conclusion:** The findings of this study suggest that the detection and  
9 quantification of MPO-DNA complex in the otitis media fluid can be utilized  
10 to discriminate OMAAV, especially in cases of eosinophilic granulomatosis  
11 with polyangiitis, from EOM regardless of the serum ANCA status. It should  
12 be noted that it is possible for cell-free DNA and cit-H3-DNA complex in fluid  
13 samples to be derived from dead cells other than neutrophils that undergo  
14 ETosis.

15

16 **Key Words:** extracellular traps – myeloperoxidase-deoxyribonucleic acid  
17 complex – cell-free deoxyribonucleic acid – citrullinated-histone  
18 H3-deoxyribonucleic acid complex – otitis media with antineutrophil  
19 cytoplasmic antibody-associated vasculitis – eosinophilic otitis media.

20

21

## 1 INTRODUCTION

2 Although otitis media with effusion (OME) is a common disease, otitis  
3 media that is refractory to conventional treatment, such as otitis media with  
4 antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (OMAAV)  
5 and eosinophilic otitis media (EOM), is a relatively rare form and exhibits a  
6 different clinical course (1-3). OMAAV presents mixed or sensorineural  
7 hearing loss rather than conductive hearing loss, which occasionally  
8 progresses to complete deafness and systemic ANCA-associated vasculitis  
9 (AAV) (1). AAV is comprised of granulomatosis with polyangiitis (GPA),  
10 microscopic polyangiitis (MPA) and eosinophilic granulomatosis with  
11 polyangiitis (EGPA), which commonly involves various organs and is a  
12 life-threatening disorder (4). Thus, early diagnosis at the otitis media stage  
13 is crucial to achieving good survival and hearing outcomes. However, it  
14 remains difficult to definitively diagnose patients with OMAAV until  
15 progression to the systemic organs due to the presence of ANCA-negative  
16 cases and the low rate of histopathological identification based on specimens  
17 obtained from the otorhinological regions (1, 2).

18 EOM is defined as intractable otitis media characterized by the presence of  
19 a highly viscous yellowish effusion containing eosinophils and  
20 immunoglobulin E (3). EOM presents without systemic symptoms, such as a  
21 rapidly progressive glomerular nephritis with necrotizing glomerular tufts,  
22 alveolar hemorrhage, interstitial pneumonia or peripheral neuropathy,  
23 which distinguishes it from OMAAV. However, its clinical course and  
24 otologic symptoms have some similarities with OMAAV, particularly in

1 EGPA patients (5). Both EOM and EGPA patients present with  
2 accompanying asthma, chronic sinusitis, and peripheral blood and tissue  
3 eosinophilia. The features of their hearing loss include deterioration of the  
4 bone conduction thresholds and progression to deafness within a short period  
5 of time. As mentioned above, the differentiation between EOM and otitis  
6 media associated with EGPA is challenging in the early stages, as  
7 ANCA-positivity has been observed in only 30-50% of cases of EGPA (5).  
8 Thus, the identification of biological markers for the discrimination of  
9 OMAAV from EOM at initial diagnosis is required.

10 In 2004, Brinkmann et al. demonstrated that neutrophil extracellular  
11 traps (NETs) were released as a result of extracellular trap cell death  
12 (ETosis), which is a unique form of programmed neutrophil cell death  
13 distinct from apoptosis and necrosis (6). NETs are large web-like structures  
14 composed of extracellular deoxyribonucleic acid (DNA) fibers and histones  
15 H1, H2A, H2B, H3, and H4 decorated with various enzymes including  
16 myeloperoxidase (MPO), proteinase 3 (PR3), and neutrophil elastase (6-8).  
17 The formation of NETs induces vessel wall inflammation and promotes  
18 pathogenic ANCA, all of which can activate neutrophils and create a vicious  
19 circle resulting in the progression of AAV (9, 10). Thus, NETs have been  
20 suggested to have a novel role in the pathogenesis of OMAAV (11).  
21 Meanwhile, recent research has shown that extracellular traps can also be  
22 generated by cells other than neutrophils, such as macrophages, mast cells  
23 and eosinophils (12-15); and these traps are termed macrophage  
24 extracellular traps, mast cell extracellular traps and eosinophil extracellular

1 traps (EETs), respectively. EETs have been suggested to be involved in the  
2 pathogenesis of EOM (16).

3 A previous study has demonstrated that the detection and quantification  
4 of the ETosis-derived products in the otitis media fluid can be utilized to  
5 discriminate OMAAV from OME (11). There is an absence of ETosis-derived  
6 products in patients with OME caused by dysfunction of the eustachian tube.  
7 Meanwhile, both OMAAV and EOM have been suggested to involve ETosis  
8 originating from neutrophils and eosinophils, respectively (11, 16). To date,  
9 various ETosis-derived products, such as cell-free DNA, citrullinated-histone  
10 H3 (cit-H3)-DNA complex, and MPO-DNA complex, have been used as  
11 biomarkers to evaluate the activity and severity of AAV (17-21). As NETs  
12 and EETs have almost the same basic composition, it remains unclear  
13 whether the measurement of these biomarkers can allow the differentiation  
14 of neutrophil- from eosinophil-derived products. This prospective study  
15 aimed to quantify cell-free DNA, citrullinated-histone H3 (cit-H3)-DNA  
16 complex, and MPO-DNA complex in the middle ear fluid, and to identify  
17 diagnostic biomarkers for the discrimination of OMAAV from EOM.

18

19



## 1 MATERIALS AND METHODS

### 2 *Patients and controls*

3 This prospective study included patients diagnosed with OMAAV in the  
4 Department of Otolaryngology, Head and Neck Surgery, #####  
5 ##### between April 2018 and March 2021. All patients  
6 were instructed on the potential risks and benefits of the management  
7 program, and written informed consent for the use of their fluid samples and  
8 clinical data was obtained after a full explanation. This research adhered to  
9 the tenets of the Declaration of Helsinki and was approved by our  
10 Institutional Review Board (No. 020-0344).

11 OMAAV patients were eligible for inclusion in this analysis. OMAAV was  
12 diagnosed using the criteria proposed by the OMAAV study group of the  
13 Japan Otological Society as follows: 1) intractable otitis media with effusion  
14 or granulation, which was resistant to antibiotics and insertion of tympanic  
15 ventilation tubes, accompanied by progressive hearing loss; 2) at least one of  
16 the following four findings: (a) diagnosis of GPA, MPA and EGPA before the  
17 occurrence of ear symptoms; (b) positivity for serum MPO- or PR3-ANCA; (c)  
18 histopathologically consistent with AAV; and (d) at least one accompanying  
19 AAV-related symptoms involving organs other than the ear (eye, nose,  
20 pharynx/larynx, lung, kidney, facial palsy, hypertrophic pachymeningitis,  
21 mononeuropathy and the others); and 3) exclusion of other types of  
22 intractable otitis media such as bacterial otitis media, cholesterol granuloma,  
23 cholesteatoma, malignant osteomyelitis, tuberculosis, neoplasms and EOM,  
24 as well as exclusion of other autoimmune diseases and vasculitis other than

1 AAV, such as Cogan's syndrome and polyarteritis nodosa among others (1).

2 Patients with EOM were examined as controls. EOM was diagnosed  
3 according to the criteria proposed by Iino et al. in 2011 (3). The major  
4 criterion; i.e., the presence of OME or chronic otitis media with  
5 eosinophil-dominant effusion, and at least two of the following minor criteria  
6 should be fulfilled for confirmation of a diagnosis of EOM: 1) highly viscous  
7 middle ear effusion, 2) resistance to conventional treatment for otitis media,  
8 3) association with bronchial asthma, and 4) association with nasal  
9 polyposis.

10 The exclusion criteria for subjects and controls were as follows: 1) fluid  
11 samples of less than 0.1 ml which cannot provide a quantifiable level of  
12 NETosis-derived products; 2) a history of definitive ear disease such as  
13 familial hearing loss, chronic noise exposure, ototoxic drug intake, head  
14 trauma, radiation therapy, acoustic neuroma or inner ear malformation; 3) a  
15 history of cancer, diabetes, deep vein thrombosis, acute coronary syndrome,  
16 ischemic stroke or other systemic autoimmune diseases such as Cogan's  
17 syndrome, systemic lupus erythematosus, rheumatoid arthritis,  
18 IgG4-related disease, sarcoidosis or aortitis syndrome, in which NETs may  
19 be involved (8, 17); and 4) current pregnancy or aged under 20 years.

20

### 21 *Sample collection*

22 All samples were obtained from the middle ear fluid in patients with  
23 OMAAV or EOM. Tympanic membrane anesthesia using iontophoresis was  
24 applied to the external auditory canal with 4% lidocaine (AstraZeneca Co.,

1 Ltd., London, UK). The fluid samples were aspirated from the middle ear  
2 through the anterior-inferior portion of the tympanic membrane using a 1-ml  
3 tuberculin syringe with a 24- or 26-gauge needle under a microscope. The  
4 supernatants were centrifuged at 1500 rpm for 5 minutes and stored at  
5 -80 °C until analysis. The levels of extracellular traps were quantified by  
6 detecting the major components, such as cell-free DNA, cit-H3-DNA complex,  
7 and MPO-DNA complex in the fluid samples, which is consistent with the  
8 method used in most previous studies (9, 18-21).

9

#### 10 ***Evaluation of the cell-free DNA level in the fluid samples***

11 The cell-free DNA content in the middle ear fluid was determined by  
12 enzyme-linked immunosorbent assay (ELISA) using Cell Death Detection  
13 ELISA PLUS (Roche, Cat. No.: 1177442500) according to the manufacturer's  
14 protocol (9, 18-21). The determination was based on quantitative sandwich  
15 ELISA using an anti-DNA antibody and anti-histone antibody, specifically  
16 binding mono- and oligonucleosomes derived from the nuclei of eukaryotic  
17 cells. The optical absorbance was measured at 405 nm using an ELISA  
18 reader (Bio-Rad 680; Bio-Rad Laboratories, Tokyo, Japan).

19

#### 20 ***Evaluation of the cit-H3-DNA complex level in the fluid samples***

21 The cit-H3-DNA complex level in the fluid samples was quantified using  
22 ELISA, as previously described (9, 18-21). An anti-histone H3 (citrulline  
23 R2+R8+R17) antibody (Abcam, ab5103) was coated on 96-well microtiter  
24 plates, with 1% bovine serum albumin used for blocking. The fluid sample,

1 together with a peroxidase-labeled anti-DNA monoclonal antibody (Cell  
2 Death Detection ELISA kit; Roche, Cat. No.: 11774425001), was then added.  
3 The optical absorbance was measured at 405 nm using an ELISA reader  
4 (Bio-Rad 680; Bio-Rad Laboratories, Tokyo, Japan).

5

### 6 *Evaluation of the MPO-DNA complex level in the fluid samples*

7 The MPO-DNA complex level in the fluid samples was quantified using  
8 ELISA, as previously described (9, 21). A mouse anti-human MPO antibody  
9 (4A4; Bio-Rad Laboratories, Tokyo, Japan) was coated on 96-well microtiter  
10 plates. After blocking with 1% bovine serum albumin, the fluid sample was  
11 then added together with a peroxidase-labeled anti-DNA monoclonal  
12 antibody (Cell Death Detection ELISA kit; Roche, Cat. No.: 11774425001).  
13 After incubation, the peroxidase substrate was added according to the  
14 manufacturer's instructions. The optical absorbance was measured at 405  
15 nm using an ELISA reader (Bio-Rad 680; Bio-Rad Laboratories, Tokyo,  
16 Japan).

17

### 18 *Statistical analysis*

19 Statistical analyses were performed using GraphPad Prism software  
20 (version 6.0; GraphPad Software Inc.; La Jolla, CA, U.S.A.). Statistical  
21 differences were analyzed using the Mann-Whitney U-test for two  
22 independent groups and Kruskal-Wallis test for three or more independent  
23 groups, with a  $p$  value of less than 0.05 considered statistically significant.  
24 The receiver operating characteristic (ROC) curve was constructed from the

1 level of ETosis-derived products for differentiating OMAAV patients from  
2 EOM patients to determine the area under the curve (AUC) as a measure of  
3 predictive accuracy, and Youden's index was used to verify the optimal cutoff  
4 value for the ETosis-derived products. The sensitivity, specificity, positive  
5 predictive value and negative predictive value were calculated based on the  
6 cutoff values determined from the ROC curves.

7

8

## 1 RESULTS

### 2 *Clinical profiles of patients and controls*

3 The study population consisted of 12 males and 23 females, ranging in age  
4 from 27 to 78 years (median, 66 years). Nine patients were diagnosed with  
5 GPA, 5 with MPA, 11 with EGPA and 10 patients with localized forms of  
6 OMAAV. Twenty-one patients were MPO-ANCA positive and 3 patients were  
7 PR3-ANCA positive, whereas 11 patients were ANCA negative at the time of  
8 sampling.

9 The EOM group comprised 13 subjects, consisting of 5 males and 8 females,  
10 ranging in age from 21 to 82 years (median, 65 years). There were no  
11 differences in background characteristics, such as age or gender distribution,  
12 between the patient and control groups.

13

### 14 *Cell-free DNA levels in the fluid samples*

15 FIGURE. 1A and B shows the levels of extracellular traps based on the  
16 cell-free DNA ELISA in the patients with OMAAV and EOM. The optical  
17 density (OD) values in the patients with OMAAV ranged from 1.24 to 50.7  
18 OD at 405 nm (median, 8.08 OD<sub>405</sub>), whereas those in the patients with EOM  
19 ranged from 0.01 to 42.1 OD<sub>405</sub> (median, 6.97 OD<sub>405</sub>). There were no  
20 significant differences in the quantifiable levels of cell-free DNA between the  
21 OMAAV and EOM patients.

22

### 23 *Cit-H3-DNA complex levels in the fluid samples*

24 FIGURE. 2A and B shows the levels of extracellular traps based on the

1 cit-H3-DNA ELISA in the patients with OMAAV and EOM. The quantifiable  
2 levels of cit-H3-DNA complex in the patients with OMAAV ranged from 0.32  
3 to 21.8 OD<sub>405</sub> (median, 3.37 OD<sub>405</sub>), whereas those in the patients with EOM  
4 ranged from 0.03 to 20.7 OD<sub>405</sub> (median, 4.88 OD<sub>405</sub>). Again, there were no  
5 significant differences in the values of cit-H3-DNA complex between the  
6 OMAAV and EOM patients.

7

### 8 *MPO-DNA complex levels in the fluid samples*

9 FIGURE. 3A shows the levels of extracellular traps based on the  
10 MPO-DNA ELISA in the patients with OMAAV and EOM. The quantifiable  
11 levels of MPO-DNA complex in the patients with OMAAV ranged from 0.08  
12 to 3.41 OD<sub>405</sub> (median, 0.84 OD<sub>405</sub>), whereas those in the patients with EOM  
13 ranged from 0.01 to 0.35 OD<sub>405</sub> (median, 0.10 OD<sub>405</sub>). The values of  
14 MPO-DNA complex in the patients with OMAAV were significantly higher  
15 than those in the patients with EOM ( $p<0.001$ ).

16 FIGURE. 3B shows the levels of extracellular traps from the MPO-DNA  
17 ELISA based on AAV classifications. Patients with GPA, MPA, EGPA as  
18 well as localized OMAAV showed higher levels of MPO-DNA complex  
19 compared with the patients with EOM ( $p=0.001$ ,  $p=0.004$ ,  $p<0.001$  and  
20  $p=0.001$ , respectively).

21 Patients with OMAAV were divided into subgroups based on serum ANCA  
22 status (FIGURE. 4). These patients showed significantly higher levels of  
23 MPO-DNA complex compared with the patients with EOM ( $p<0.001$  and  
24  $p<0.001$ , respectively), regardless of their serum ANCA status at the time of

1 sampling.

2       FIGURE 5 shows the ROC curves obtained for evaluating the sensitivity  
3 and specificity of MPO-DNA level for differentiating between OMAAV and  
4 EOM. ROC analysis demonstrated an AUC of 0.94 (95% confidence interval:  
5 0.87-1.00). A cutoff value of 0.14 OD<sub>405</sub> according to the ROC curve showed a  
6 sensitivity of 97.1%, specificity of 76.9%, positive predictive value of 91.9%  
7 and negative predictive value of 90.9% for the diagnosis of OMAAV.

8

9

10



## 1 DISCUSSION

2 Excessive formation and disordered regulation of NETs have been  
3 suggested to be involved in the pathogenesis of OMAAV (11, 22, 23). Thus,  
4 novel methods for the evaluation of NETs are essential to providing a  
5 definite diagnosis as well as predicting the activity and severity of OMAAV.  
6 Many studies have been conducted to evaluate NETs by microscopic  
7 observation using simultaneous immunohistostained DNA and  
8 neutrophil-derived proteins (6, 21, 23). The co-localization of extracellular  
9 DNA and neutrophil-derived proteins suggests the presence of NETs. The  
10 identification of citrullinated histones as determined by immunostaining  
11 also has provided evidence of NET formation, as the induction of  
12 citrullination by peptidylarginine deiminase 4 (PAD4) has been regarded as  
13 an essential step in ETosis (23-26). Although immunostaining is easy to  
14 conduct, artificial NET formation in cell cultures, and the lack of objectivity  
15 and quantitativity are all critical methodological drawbacks. In the case of  
16 otitis media, the middle ear fluid can be obtained as a sample more easily  
17 and less invasively than middle ear or mastoid mucosal specimens.  
18 Therefore, this analysis focused on the soluble extracellular trap remnants  
19 in fluid samples that could be detected using ELISA. This methodology  
20 allows the process of measurement to be completed within 24 hours, and  
21 seems to be the most specific, objective, and quantitative assay for the  
22 monitoring ETosis available at present (27). ETosis markers, based on  
23 ELISA, target the components of extracellular traps, including extracellular  
24 DNA and citrullinated histones decorated with various enzymes.

1 It has been shown that one form of soluble NET remnant is cell-free DNA  
2 (28). The serum level of cell-free DNA has been reported to increase in  
3 patients with AAV (29). In this analysis, there were no significant differences  
4 in the values of cell-free DNA in the middle ear fluid between OMAAV and  
5 EOM patients. Cell-free DNA has been reported to be derived from dead cells  
6 other than neutrophils that undergo ETosis (30). EETs, which contain  
7 cell-free DNA derived from eosinophils, have been suggested to play a novel  
8 role in the pathogenesis of EOM (16). Thus, even after the measurement of  
9 cell-free DNA it remains difficult to distinguish OMAAV from EOM.

10 Several studies have demonstrated that PAD4 has a critical role in NET  
11 formation (24, 25). The PAD enzymes convert arginine residues to citrulline  
12 in a variety of protein substrates (26). Reactive oxygen species generation  
13 and calcium influx in activated neutrophils result in the translocation of  
14 PAD4 from the cytoplasm to the nucleus (31). Subsequently, histones that  
15 are coiled by DNA are citrullinated, followed by the decondensation of DNA.  
16 The PAD4-induced citrullination of histones has been regarded as an  
17 essential step in NET formation. Therefore, the presence of citrullinated  
18 histones could be a marker of NET formation. In this analysis, there were no  
19 significant differences in the cit-H3-DNA values between OMAAV and EOM  
20 patients. As mentioned above, the pathogenesis of EOM is thought to involve  
21 EETs, which are composed of extracellular DNA fibers and citrullinated  
22 histones decorated with eosinophilic enzymes (6). Based on the measurement  
23 of the cit-H3-DNA complex, which is derived from eosinophils that undergo  
24 ETosis as well as neutrophils, it is difficult to distinguish OMAAV from

1 EOM.

2 The other forms of NET remnants are complexes of DNA and  
3 neutrophil-derived proteins, such as MPO and NE (9, 23). The MPO-DNA  
4 complex titer in the supernatants of neutrophils has reported to be  
5 well-correlated with the rate of the neutrophil ETosis (21). Correspondingly,  
6 some studies have demonstrated the elevation of the MPO-DNA complex  
7 levels in sera from patients with AAV (9, 18). This analysis revealed elevated  
8 levels of the MPO-DNA complex in the middle ear fluid from patients with  
9 OMAAV in comparison to those in middle ear fluid from patients with EOM.  
10 Even cases that were ANCA negative at the time of sampling showed high  
11 levels of MPO-DNA complex. The values for MPO-DNA complex are thought  
12 to reflect neutrophil activation toward the formation of NETs, as well as the  
13 activity and severity of OMAAV (11, 18, 19). NETs and EETs have almost the  
14 same basic composition, whereas they contain different enzymes. The  
15 MPO-DNA complex contains MPO derived from neutrophils, which is absent  
16 in eosinophils. Therefore, the detection and quantification of the MPO-DNA  
17 complex in the otitis media fluid may aid in distinguishing OMAAV from  
18 EOM. It is noteworthy that otitis media associated with EGPA accompanying  
19 peripheral blood and tissue eosinophilia, which have much in common  
20 clinically with EOM, showed high quantifiable levels of MPO-DNA complex.

21

## 22 *Limitations*

23 The results of this analysis might have been affected by the small number  
24 of samples, as well as by the NET detection and quantification methods. As

1 no gold standard method or markers for ETosis quantification have been  
2 established, researchers need to select the most appropriate method and  
3 markers according to each type of pathogenesis based on their knowledge of  
4 the respective advantages and disadvantages. The validation of ELISA for  
5 each ETosis-derived product, such as cell-free DNA, nucleosomes, cit-H3,  
6 MPO, PR-3 and neutrophil elastase, remains controversial (32). The ELISA  
7 results, in particular, may have been affected by the potential  
8 cross-reactivity of the antigens and antibodies due to molecular mimicry  
9 between MPO and eosinophil peroxidase (33). Further studies based on the  
10 evaluation of a large number of samples by various methodologies with  
11 respect to each OMAAV classification are required.

12

1 **CONCLUSION**

2 This analysis is the first to evaluate the various ETosis markers for  
3 patients with OMAAV or EOM. The levels of MPO-DNA complex in the  
4 middle ear fluid from patients with OMAAV were elevated in comparison to  
5 those in the middle ear fluid from patients with EOM. Meanwhile, there  
6 were no significant differences in the values of cell-free DNA or the  
7 cit-H3-DNA complex between OMAAV and EOM patients. It should be noted  
8 that it is possible for cell-free DNA and the cit-H3-DNA complex in fluid  
9 samples to be derived from dead cells other than neutrophils that undergo  
10 ETosis. Although issues concerning the standardization of ELISA remain,  
11 the detection and quantification of the MPO-DNA complex in the otitis  
12 media fluid may be clinically useful in the discrimination of OMAAV,  
13 especially in EGPA, from EOM regardless of the serum ANCA status.

14

1 **DISCLOSURE STATEMENT**

2 We have no conflicts of interest to declare.

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1 **FIGURE LEDGENDS**

2 FIG. 1. The levels of cell-free DNA in the patients and controls (A). The levels  
3 of cell-free DNA in OMAAV patients by AAV classification (B).

4 DNA; deoxyribonucleic acid, OMAAV; otitis media with antineutrophil  
5 cytoplasmic antibody-associated vasculitis, AAV; antineutrophil cytoplasmic  
6 antibody-associated vasculitis, OD<sub>405</sub>; optical density at 405 nm, EOM;  
7 eosinophilic otitis media, GPA; granulomatosis with polyangiitis, MPA;  
8 microscopic polyangiitis, EGPA; eosinophilic granulomatosis with  
9 polyangiitis.

10

11 FIG. 2. The levels of cit-H3-DNA complex in the patients and controls (A).  
12 The levels of cit-H3-DNA complex in OMAAV patients by AAV classification  
13 (B).

14 cit-H3-DNA; citrullinated-histone H3-deoxyribonucleic acid, OMAAV; otitis  
15 media with antineutrophil cytoplasmic antibody-associated vasculitis, AAV;  
16 antineutrophil cytoplasmic antibody-associated vasculitis, OD<sub>405</sub>; optical  
17 density at 405 nm, EOM; eosinophilic otitis media, GPA; granulomatosis  
18 with polyangiitis, MPA; microscopic polyangiitis, EGPA; eosinophilic  
19 granulomatosis with polyangiitis.

20

21 FIG. 3. The levels of MPO-DNA complex in the patients and controls (A). The  
22 levels of MPO-DNA complex in OMAAV patients by AAV classification (B).

23 MPO-DNA; myeloperoxidase-deoxyribonucleic acid, OMAAV; otitis media  
24 with antineutrophil cytoplasmic antibody-associated vasculitis, AAV;

1 antineutrophil cytoplasmic antibody-associated vasculitis, OD<sub>405</sub>; optical  
2 density at 405 nm, EOM; eosinophilic otitis media, GPA; granulomatosis  
3 with polyangiitis, MPA; microscopic polyangiitis, EGPA; eosinophilic  
4 granulomatosis with polyangiitis.

5

6 FIG. 4. The levels of MPO-DNA complex in OMAAV patients by ANCA  
7 status.

8 MPO-DNA; myeloperoxidase-deoxyribonucleic acid, OMAAV; otitis media  
9 with antineutrophil cytoplasmic antibody-associated vasculitis, ANCA;  
10 antineutrophil cytoplasmic antibody, OD<sub>405</sub>; optical density at 405 nm, EOM;  
11 eosinophilic otitis media.

12

13 FIG. 5. The ROC curves obtained for evaluating the sensitivity and  
14 specificity of the MPO-DNA complex for the differentiation of OMAAV and  
15 EOM.

16 ROC; receiver operating characteristic, MPO-DNA;  
17 myeloperoxidase-deoxyribonucleic acid, OMAAV; otitis media with  
18 antineutrophil cytoplasmic antibody-associated vasculitis, EOM; eosinophilic  
19 otitis media, OD<sub>405</sub>; optical density at 405 nm, AUC; area under the curve.