

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
Author(s)	Morita, Shinya; Nakamaru, Yuji; Fukuda, Atsushi; Fujiwara, Keishi; Suzuki, Masanobu; Hoshino, Kimiko; Honma, Aya; Homma, Akihiro
Citation	Otology & neurotology, 43(3), E337-E343 https://doi.org/10.1097/MAO.00000000003431
Issue Date	2022-03
Doc URL	http://hdl.handle.net/2115/88232
Rights	This is a non-final version of an article published in final form in Morita, Shinya; Nakamaru, Yuji; Fukuda, Atsushi; Fujiwara, Keishi; Suzuki, Masanobu; Hoshino, Kimiko; Honma, Aya; Homma, Akihiro 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, Otology & Neurotology: March 2022 Volume 43 Issue 3 p e337-e343
Туре	article (author version)
Additional Information	There are other files related to this item in HUSCAP. Check the above URL.
File Information	Otol Neurotol 43(3) e337-e343.pdf



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

Shinya Morita, M.D., Ph.D., Yuji Nakamaru, M.D., Ph.D., Atsushi Fuku
da, M.D., Keishi Fujiwara, M.D., Ph.D., Masanobu Suzuki, M.D., Ph.D.,
Kimiko Hoshino, M.D., Aya Honma, M.D., Ph.D., Akihiro Homma, M.
D., Ph.D.

Affiliations:

Department of Otolaryngology - Head and Neck Surgery, Faculty of Me dicine and Graduate School of Medicine, Hokkaido University, Sapporo, Japan

Corresponding author: Shinya Morita

Department of Otolaryngology - Head and Neck Surgery

Faculty of Medicine and Graduate School of Medicine, Hokkaido Univer sity.

Kita 15, Nishi 7, Kita-ku, Sapporo, Hokkaido 060-8638, Japan

Tel: +81-(0)11-706-5958; Fax: +81-(0)11-717-7566

E-mail address: <u>shinyamorita@huhp.hokudai.ac.jp</u>

Financial Support: Japan Society for the Promotion of Science, KAKENHI (Grant Number 20K09744)

Conflict of Interest: No conflicts of interest exist for any author

1 ABSTRACT

24

 $\mathbf{2}$ **Objective:** This study aimed to quantify the cell-free deoxyribonucleic acid (DNA), citrullinated-histone H3 (cit-H3)-DNA complex, and myeloperoxidase 3 (MPO)-DNA complex as extracellular trap cell death (ETosis)-derived 4 products in the middle ear fluid, and to identify diagnostic biomarkers for $\mathbf{5}$ the discrimination of antineutrophil cytoplasmic antibody 6 (ANCA)-associated vasculitis (OMAAV) from eosinophilic otitis media 7(EOM). 8 9 10 Study Design: Prospective study. 11 12Setting: Tertiary referral center. 1314Patients: OMAAV patients were eligible for inclusion in this analysis. 15Patients with EOM were examined as controls. 16 Intervention: All samples were obtained from the middle ear fluid in patients 17with OMAAV or EOM. The fluid samples were aspirated from the middle ear 1819through the anterior-inferior portion of the tympanic membrane using a 1-ml tuberculin syringe with a 24- or 26-gauge needle under a microscope. 2021Main Outcome Measures: The levels of cell-free DNA, cit-H3-DNA complex 22and MPO-DNA complex in the fluid samples were quantified using an 23

1

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	

 $\mathbf{2}$

1 INTRODUCTION

 $\mathbf{2}$ Although otitis media with effusion (OME) is a common disease, otitis media that is refractory to conventional treatment, such as otitis media with 3 antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (OMAAV) 4 and eosinophilic otitis media (EOM), is a relatively rare form and exhibits a $\mathbf{5}$ different clinical course (1-3). OMAAV presents mixed or sensorineural 6 7hearing loss rather than conductive hearing loss, which occasionally progresses to complete deafness and systemic ANCA-associated vasculitis 8 (AAV) (1). AAV is comprised of granulomatosis with polyangiitis (GPA), 9 10 microscopic polyangiitis (MPA) and eosinophilic granulomatosis with polyangiitis (EGPA), which commonly involves various organs and is a 11 12life-threatening disorder (4). Thus, early diagnosis at the otitis media stage 13is crucial to achieving good survival and hearing outcomes. However, it 14remains difficult to definitively diagnose patients with OMAAV until 15progression to the systemic organs due to the presence of ANCA-negative 16 cases and the low rate of histopathological identification based on specimens obtained from the otorhinological regions (1, 2). 17EOM is defined as intractable otitis media characterized by the presence of 1819a highly viscous yellowish effusion containing eosinophils and immunoglobulin E (3). EOM presents without systemic symptoms, such as a 2021rapidly progressive glomerular nephritis with necrotizing glomerular tufts, 22alveolar hemorrhage, interstitial pneumonia or peripheral neuropathy, 23which distinguishes it from OMAAV. However, its clinical course and 24otologic 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

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

3 A previous study has demonstrated that the detection and quantification of the ETosis-derived products in the otitis media fluid can be utilized to 4 discriminate OMAAV from OME (11). There is an absence of ETosis-derived $\mathbf{5}$ products in patients with OME caused by dysfunction of the eustachian tube. 6 Meanwhile, both OMAAV and EOM have been suggested to involve ETosis 7originating from neutrophils and eosinophils, respectively (11, 16). To date, 8 various ETosis-derived products, such as cell-free DNA, citrullinated-histone 9 10 H3 (cit-H3)-DNA complex, and MPO-DNA complex, have been used as biomarkers to evaluate the activity and severity of AAV (17-21). As NETs 11 12and EETs have almost the same basic composition, it remains unclear whether the measurement of these biomarkers can allow the differentiation 1314of neutrophil- from eosinophil-derived products. This prospective study aimed to quantify cell-free DNA, citrullinated-histone H3 (cit-H3)-DNA 1516 complex, and MPO-DNA complex in the middle ear fluid, and to identify diagnostic biomarkers for the discrimination of OMAAV from EOM. 17

18

19

 $\mathbf{5}$

1 MATERIALS AND METHODS

2 Patients and controls

3 This prospective study included patients diagnosed with OMAAV in the 4 $\mathbf{5}$ were instructed on the potential risks and benefits of the management 6 7program, and written informed consent for the use of their fluid samples and clinical data was obtained after a full explanation. This research adhered to 8 the tenets of the Declaration of Helsinki and was approved by our 9 10 Institutional Review Board (No. 020-0344).

OMAAV patients were eligible for inclusion in this analysis. OMAAV was 11 12diagnosed using the criteria proposed by the OMAAV study group of the 13Japan Otological Society as follows: 1) intractable otitis media with effusion or granulation, which was resistant to antibiotics and insertion of tympanic 1415ventilation 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 occurrence of ear symptoms; (b) positivity for serum MPO- or PR3-ANCA; (c) 17histopathologically consistent with AAV; and (d) at least one accompanying 1819 AAV-related symptoms involving organs other than the ear (eye, nose, pharynx/larynx, lung, kidney, facial palsy, hypertrophic pachymeningitis, 2021mononeuropathy and the others); and 3) exclusion of other types of 22intractable otitis media such as bacterial otitis media, cholesterol granuloma, 23cholesteatoma, malignant osteomyelitis, tuberculosis, neoplasms and EOM, 24as 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.,

 $\overline{7}$

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

9

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

The cell-free DNA content in the middle ear fluid was determined by 11 enzyme-linked immunosorbent assay (ELISA) using Cell Death Detection 12ELISA PLUS (Roche, Cat. No.: 1177442500) according to the manufacturer's 13protocol (9, 18-21). The determination was based on quantitative sandwich 1415ELISA using an anti-DNA antibody and anti-histone antibody, specifically 16 binding mono- and oligonucleosomes derived from the nuclei of eukaryotic 17cells. The optical absorbance was measured at 405 nm using an ELISA reader (Bio-Rad 680; Bio-Rad Laboratories, Tokyo, Japan). 18

19

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

The cit-H3-DNA complex level in the fluid samples was quantified using ELISA, as previously described (9, 18-21). An anti-histone H3 (citrulline R2+R8+R17) antibody (Abcam, ab5103) was coated on 96-well microtiter 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).

 $\mathbf{5}$

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

7The MPO-DNA complex level in the fluid samples was quantified using ELISA, as previously described (9, 21). A mouse anti-human MPO antibody 8 (4A4; Bio-Rad Laboratories, Tokyo, Japan) was coated on 96-well microtiter 9 10 plates. After blocking with 1% bovine serum albumin, the fluid sample was then added together with a peroxidase-labeled anti-DNA monoclonal 11 12antibody (Cell Death Detection ELISA kit; Roche, Cat. No.: 11774425001). 13After incubation, the peroxidase substrate was added according to the manufacturer's instructions. The optical absorbance was measured at 405 1415nm using an ELISA reader (Bio-Rad 680; Bio-Rad Laboratories, Tokyo, 16 Japan).

17

18 Statistical analysis

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

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

1 **RESULTS**

2 Clinical profiles of patients and controls

3 The study population consisted of 12 males and 23 females, ranging in age from 27 to 78 years (median, 66 years). Nine patients were diagnosed with 4 GPA, 5 with MPA, 11 with EGPA and 10 patients with localized forms of $\mathbf{5}$ OMAAV. Twenty-one patients were MPO-ANCA positive and 3 patients were 6 7PR3-ANCA positive, whereas 11 patients were ANCA negative at the time of sampling. 8 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 differences in background characteristics, such as age or gender distribution, 11 12between the patient and control groups. 13Cell-free DNA levels in the fluid samples 1415FIGURE. 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 density (OD) values in the patients with OMAAV ranged from 1.24 to 50.7 17OD at 405 nm (median, 8.08 OD_{405}), whereas those in the patients with EOM 18ranged from 0.01 to 42.1 OD_{405} (median, 6.97 OD_{405}). There were no 19significant differences in the quantifiable levels of cell-free DNA between the 20OMAAV and EOM patients. 2122Cit-H3-DNA complex levels in the fluid samples 23

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

cit-H3-DNA ELISA in the patients with OMAAV and EOM. The quantifiable
levels of cit-H3-DNA complex in the patients with OMAAV ranged from 0.32
to 21.8 OD₄₀₅ (median, 3.37 OD₄₀₅), whereas those in the patients with EOM
ranged from 0.03 to 20.7 OD₄₀₅ (median, 4.88 OD₄₀₅). Again, there were no
significant differences in the values of cit-H3-DNA complex between the
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_{405} (median, 0.84 OD_{405}), whereas those in the patients with EOM

13 ranged from 0.01 to 0.35 OD_{405} (median, 0.10 OD_{405}). 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

- status (FIGURE. 4). These patients showed significantly higher levels of
- 23 MPO-DNA complex compared with the patients with EOM (p<0.001 and
- 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_{405} 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	

1 DISCUSSION

 $\mathbf{2}$ Excessive formation and disordered regulation of NETs have been suggested to be involved in the pathogenesis of OMAAV (11, 22, 23). Thus, 3 novel methods for the evaluation of NETs are essential to providing a 4 definite diagnosis as well as predicting the activity and severity of OMAAV. $\mathbf{5}$ Many studies have been conducted to evaluate NETs by microscopic 6 7observation using simultaneous immunohistostained DNA and neutrophil-derived proteins (6, 21, 23). The co-localization of extracellular 8 DNA and neutrophil-derived proteins suggests the presence of NETs. The 9 10 identification of citrullinated histories as determined by immunostaining also has provided evidence of NET formation, as the induction of 11 12citrullination by peptidylarginine deiminase 4 (PAD4) has been regarded as an essential step in ETosis (23-26). Although immunostaining is easy to 13 conduct, artificial NET formation in cell cultures, and the lack of objectivity 14and quantitativity are all critical methodological drawbacks. In the case of 1516 otitis media, the middle ear fluid can be obtained as a sample more easily and less invasively than middle ear or mastoid mucosal specimens. 17Therefore, this analysis focused on the soluble extracellular trap remnants 1819 in fluid samples that could be detected using ELISA. This methodology allows the process of measurement to be completed within 24 hours, and 2021seems to be the most specific, objective, and quantitative assay for the 22monitoring ETosis available at present (27). ETosis markers, based on 23ELISA, target the components of extracellular traps, including extracellular 24DNA and citrullinated histories decorated with various enzymes.

1 It has been shown that one form of soluble NET remnant is cell-free DNA $\mathbf{2}$ (28). The serum level of cell-free DNA has been reported to increase in patients with AAV (29). In this analysis, there were no significant differences 3 in the values of cell-free DNA in the middle ear fluid between OMAAV and 4 EOM patients. Cell-free DNA has been reported to be derived from dead cells $\mathbf{5}$ other than neutrophils that undergo ETosis (30). EETs, which contain 6 7cell-free DNA derived from eosinophils, have been suggested to play a novel role in the pathogenesis of EOM (16). Thus, even after the measurement of 8 cell-free DNA it remains difficult to distinguish OMAAV from EOM. 9 10 Several studies have demonstrated that PAD4 has a critical role in NET formation (24, 25). The PAD enzymes convert arginine residues to citrulline 11 12in a variety of protein substrates (26). Reactive oxygen species generation 13 and calcium influx in activated neutrophils result in the translocation of 14PAD4 from the cytoplasm to the nucleus (31). Subsequently, histores that are coiled by DNA are citrullinated, followed by the decondensation of DNA. 1516 The PAD4-induced citrullination of histones has been regarded as an essential step in NET formation. Therefore, the presence of citrullinated 17histones could be a marker of NET formation. In this analysis, there were no 18significant differences in the cit-H3-DNA values between OMAAV and EOM 19patients. As mentioned above, the pathogenesis of EOM is thought to involve 20EETs, which are composed of extracellular DNA fibers and citrullinated 2122histones decorated with eosinophilic enzymes (6). Based on the measurement 23of the cit-H3-DNA complex, which is derived from eosinophils that undergo 24ETosis as well as neutrophils, it is difficult to distinguish OMAAV from

1 EOM.

other forms of NET remnants are complexes of DNA and $\mathbf{2}$ The neutrophil-derived proteins, such as MPO and NE (9, 23). The MPO-DNA 3 complex titer in the supernatants of neutrophils has reported to be 4 well-correlated with the rate of the neutrophil ETosis (21). Correspondingly, $\mathbf{5}$ some studies have demonstrated the elevation of the MPO-DNA complex 6 levels in sera from patients with AAV (9, 18). This analysis revealed elevated 7levels of the MPO-DNA complex in the middle ear fluid from patients with 8 OMAAV in comparison to those in middle ear fluid from patients with EOM. 9 10 Even cases that were ANCA negative at the time of sampling showed high levels of MPO-DNA complex. The values for MPO-DNA complex are thought 11 12to reflect neutrophil activation toward the formation of NETs, as well as the activity and severity of OMAAV (11, 18, 19). NETs and EETs have almost the 13 same basic composition, whereas they contain different enzymes. The 1415MPO-DNA complex contains MPO derived from neutrophils, which is absent 16 in eosinophils. Therefore, the detection and quantification of the MPO-DNA complex in the otitis media fluid may aid in distinguishing OMAAV from 17EOM. It is noteworthy that otitis media associated with EGPA accompanying 18peripheral blood and tissue eosinophilia, which have much in common 19clinically with EOM, showed high quantifiable levels of MPO-DNA complex. 20

21

22 Limitations

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

no gold standard method or markers for ETosis quantification have been 1 $\mathbf{2}$ established, researchers need to select the most appropriate method and markers according to each type of pathogenesis based on their knowledge of 3 the respective advantages and disadvantages. The validation of ELISA for 4 each ETosis-derived product, such as cell-free DNA, nucleosomes, cit-H3, $\mathbf{5}$ 6 MPO, PR-3 and neutrophil elastase, remains controversial (32). The ELISA results, in particular, may have been affected by the potential 78 cross-reactivity of the antigens and antibodies due to molecular mimicry between MPO and eosinophil peroxidase (33). Further studies based on the 9 10 evaluation of a large number of samples by various methodologies with respect to each OMAAV classification are required. 11

1 CONCLUSION

 $\mathbf{2}$ This analysis is the first to evaluate the various ETosis markers for patients with OMAAV or EOM. The levels of MPO-DNA complex in the 3 middle ear fluid from patients with OMAAV were elevated in comparison to 4 those in the middle ear fluid from patients with EOM. Meanwhile, there $\mathbf{5}$ were no significant differences in the values of cell-free DNA or the 6 cit-H3-DNA complex between OMAAV and EOM patients. It should be noted 7that it is possible for cell-free DNA and the cit-H3-DNA complex in fluid 8 samples to be derived from dead cells other than neutrophils that undergo 9 10 ETosis. Although issues concerning the standardization of ELISA remain, the detection and quantification of the MPO-DNA complex in the otitis 11 12media fluid may be clinically useful in the discrimination of OMAAV, especially in EGPA, from EOM regardless of the serum ANCA status. 1314

1 DISCLOSURE STATEMENT

2 We have no conflicts of interest to declare.

1 ACKNOWLEDGEMENT

- 2 This study received financial support from Japan Society for the
- 3 Promotion of Science, KAKENHI (Grant Number 20K09744).

REFERENCES

2	1. Harabuchi Y, Kishibe K, Tateyama K, et al. Clinical features and
3	treatment outcomes of otitis media with antineutrophil cytoplasmic antibody
4	(ANCA)-associated vasculitis (OMAAV): A retrospective analysis of 235
5	patients from a nationwide survey in Japan. Mod Rheumatol. 2017;27:87-94.
6	
7	2. Yoshida N, Iino Y. Pathogenesis and diagnosis of otitis media with
8	ANCA-associated vasculitis. Allergol Int. 2014;63:523-32.
9	
10	3. Iino Y, Tomioka-Matsutani S, Matsubara A, Nakagawa T, Nonaka M.
11	Diagnostic criteria of eosinophilic otitis media, a newly recognized middle
12	ear disease. Auris Nasus Larynx. 2011;38:456-61.
13	
14	4. Jennette JC, Falk RJ, Bacon PA, et al. 2012 revised International Chapel
15	Hill Consensus Conference Nomenclature of Vasculitides. Arthritis Rheum.
16	2013;65:1-11.
17	
18	5. Fukuda A, Morita S, Nakamaru Y, Hoshino K, Fujiwara K, Homma A.
19	Differentiation Between Eosinophilic Otitis Media and Otitis Media
20	Associated With Eosinophilic Granulomatosis With Polyangiitis.
21	<i>Otol Neurotol.</i> 2019;40:e796-e802.
22	
23	6. Brinkmann V, Reichard U, Goosmann C, et al. Neutrophil extracellular
24	traps kill bacteria. <i>Science.</i> 2004;303:1532-5.

1

2	7. Urban CF, Ermert D, Schmid M, et al. Neutrophil extracellular traps
3	contain calprotectin, a cytosolic protein complex involved in host defense
4	against Candida albicans. PLoS Pathog. 2009;5:e1000639.
5	
6	8. Garcia-Romo GS, Caielli S, Vega B, et al. Netting neutrophils are major
7	inducers of type I IFN production in pediatric systemic lupus erythematosus.
8	<i>Sci Transl Med.</i> 2011;3:73ra20.
9	
10	9. Kessenbrock K, Krumbholz M, Schönermarck U, et al. Netting neutrophils
11	in autoimmune small-vessel vasculitis. Nat Med. 2009;15:623-5.
12	
13	10. Zawrotniak M, Rapala-Kozik M. Neutrophil extracellular traps
14	(NETs)-formation and implications. Acta Biochim Pol. 2013;60:277-84.
15	
16	11. Morita S, Nakamaru Y, Nakazawa D, et al. The diagnostic and clinical
17	utility of the myeloperoxidase-DNA complex as a biomarker in otitis media
18	with antineutrophil cytoplasmic antibody-associated vasculitis. Otol
19	<i>Neurotol.</i> 2019;40:e99-e106.
20	
21	12. Goldmann O, Medina E. The expanding world of extracellular traps: not
22	only neutrophils but much more. Front Immunol. 2012;3:420.
23	
24	13. Mohanan S, Horibata S, McElwee JL, et al. Identification of macrophage

1	extracellular traplike structures in mammary gland adipose tissue: a
2	preliminary study. <i>Front Immunol.</i> 2013;4:67.
3	
4	14. Lin AM, Rubin CJ, Khandpur R, et al. Mast cells and neutrophils release
5	IL-17 through extracellular trap formation in psoriasis. J Immunol.
6	2011;187:490-500.
7	
8	15. Simon D, Hoesli S, Roth N, et al. Eosinophil extracellular DNA traps in
9	skin diseases. J Allergy Clin Immunol. 2011;127:194-9.
10	
11	16. Ueki S, Ohta N, Takeda M, Konno Y, Hirokawa M. Eosinophilic Otitis
12	Media: the Aftermath of Eosinophil Extracellular Trap Cell Death. Curr
13	Allergy Asthma Rep. 2017;17:33.
14	
15	17. Leffler J, Gullstrand B, Jönsen A, et al. Degradation of neutrophil
16	extracellular traps co-varies with disease activity in patients with systemic
17	lupus erythematosus. Arthritis Res Ther. 2013;15:R84.
18	
19	18. Söderberg D, Kurz T, Motamedi A, et al. Increased levels of neutrophil
20	extracellular trap remnants in the circulation of patients with small vessel
21	vasculitis, but an inverse correlation to anti-neutrophil cytoplasmic
22	antibodies during remission. Rheumatology (Oxford). 2015;54:2085-94.
23	
24	19. Arai Y, Yamashita K, Mizugishi K, et al. Serum neutrophil extracellular

2	transplantation. Biol Blood Marrow Transplant. 2013;19:1683-9.
3	
4	20. Wang H , Sha LL, Ma TT, Zhang LX, Chen M, Zhao MH. Circulating level
5	of neutrophil extracellular traps is not a useful biomarker for assessing
6	disease activity in antineutrophil cytoplasmic antibody-associated vasculitis.
7	PLoS One. 2016;11:e0148197.
8	
9	21. Nakazawa D, Shida H, Tomaru U, et al. Enhanced formation and
10	disordered regulation of NETs in myeloperoxidase-ANCA-associated
11	microscopic polyangiitis. JAm Soc Nephrol. 2014;25:990-7.
12	
13	22. Hakkim A, Furnrohr BG, Amann K, et al. Impairment of neutrophil
14	extracellular trap degradation is associated with lupus nephritis. Proc. Natl.
15	Acad. Sci. U. S. A. 2010;107:9813-8.
16	
17	23. Nakazawa D, Tomaru U, Suzuki A, et al. Abnormal conformation and
18	impaired degradation of propylthiouracil-induced neutrophil extracellular
19	traps: implications of disordered neutrophil extracellular traps in a rat
20	model of myeloperoxidase antineutrophil cytoplasmic antibody-associated
21	vasculitis. Arthritis Rheum. 2012;64:3779-87.
22	

trap levels predict thrombotic microangiopathy after allogeneic stem cell

1	24. Li P, Li M, Lindberg MR, et al. PAD4 is essential for antibacterial innate
2	immunity mediated by neutrophil extracellular traps. J. Exp. Med.
3	2010;207:1853-62.
4	
5	25. Leshner M, Wang S, Lewis C, et al. PAD4 mediated histone
6	hypercitrullination induces heterochromatin decondensation and chromatin
7	unfolding to form neutrophil extracellular trap-like structures. Front.
8	Immunol. 2012;3:307.
9	
10	26. Rohrbach AS, Slade DJ, Thompson PR, Mowen KA. Activation of PAD4 in
11	NET formation. Front Immunol. 2012;3:360.
12	
13	27. Masuda S, Nakazawa D, Shida H, et al. NETosis markers: Quest for
14	specific, objective, and quantitative markers. Clin Chim Acta.
15	2016;459:89-93.
16	
17	28. Zhang X. Lu X. Shu X, et al. Elevated plasma cfDNA may be associated
18	with active lupus nephritis and partially attributed to abnormal regulation
19	of neutrophil extracellular traps (NETs) in patients with systemic lupus
20	erythematosus. Intern. Med. 2014;53:2763-71.
21	
22	29. Ma TT, Ma C, Wang H, et al. High-mobility group box 1 potentiates
23	antineutrophil cytoplasmic antibody-inducing neutrophil extracellular traps
24	formation. Arthritis Res. Ther. 2016;18:2.

2	30. Bronkhorst J, Aucamp J, Pretorius PJ. Cell-free DNA: preanalytical
3	variables. <i>Clin. Chim. Acta.</i> 2015;450:243-53.
4	
5	31. Remijsen Q, Kuijpers TW, Wirawan E, et al. Dying for a cause: NETosis,
6	mechanisms behind an antimicrobial cell death modality. <i>Cell Death Differ</i> .
7	2011;18:581-8.
8	
9	32. Thålin C, Daleskog M, Göransson SP, et al. Validation of an
10	enzyme-linked immunosorbent assay for the quantification of citrullinated
11	histone H3 as a marker for neutrophil extracellular traps in human plasma.
12	Immunol Res. 2017;65:706-12.
13	
14	33. Sullivan S, Salapow MA, Breen R, et al. Eosinophil peroxidase differs
15	from neutrophil myeloperoxidase in its ability to bind antineutrophil
16	cytoplasmic antibodies reactive with myeloperoxidase. <i>Int Arch Allergy</i>
17	Immunol. 1994;105:150-4.
18	

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_{405} ; 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_{405} ; 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;

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_{405} ; 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

antineutrophil cytoplasmic antibody-associated vasculitis, OD_{405} ; optical

density at 405 nm, EOM; eosinophilic otitis media, GPA; granulomatosis

1

 $\mathbf{2}$

19 otitis media, OD_{405} ; optical density at 405 nm, AUC; area under the curve.