Abstract. Background/Aim: We evaluated the relationship between serum alarmin levels and disease-specific indices in patients with anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV). Patients and Methods: Sera and data from 79 patients were utilized. For AAV-specific indices, Birmingham vasculitis activity score (BVAS), five-factor score (FFS), and vasculitis damage index (VDI) were collected and serum levels of four alarmins (hepatoma-derived growth factor, high mobility group box protein 1, S100A9, and S100A12) were measured using enzyme-linked immunosorbent assay. Associations between alarmin levels, AAV-specific indices, and inflammatory laboratory markers were assessed. Results: S100A9 levels were significantly correlated with C-reactive protein levels ($r=0.316$, $p=0.005$) and S100A12 levels correlated with VDI ($r=0.232$, $p=0.040$), which was consistent in a subgroup of patients with myeloperoxidase (perinuclear)-ANCA positivity. No other associations were found between alarmin levels and BVAS, FFS, and VDI. Conclusion: The serum S100A12 level was associated with organ damage in AAV, especially in myeloperoxidase (perinuclear)-ANCA-positive patients.

Alarmins, often used interchangeably with the term 'damage-associated molecular patterns' are defined as an endogenous group of molecules that are released due to cellular injury or damage (1). First described as damage-associated molecular patterns in 2004 by Seong et al. (2), alarmins are expressed constitutively in various cells and are thought to play a critical role in cellular regeneration and remodeling. Alarmins may also act as potent mediators of inflammation by promoting chemotaxis and inducing the expression of proteins implicated in immune activation when they are secreted extracellularly (3). Mechanistically, secreted alarmins bind to pattern recognition receptors, i.e. Toll-like receptors and receptors for advanced glycation end-products, to elicit their physiological effects. Dysregulation of this signaling can trigger aberrant immune responses (4, 5).

Previous studies have indicated that among the alarmins, hepatoma-derived growth factor (HDGF), high mobility group box protein 1 (HMGB1), and S100 proteins can play important roles in autoimmunity (6-8).

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is a rare multisystem autoimmune condition that belongs to a group of primary systemic vasculitides. A typical pathological feature of AAV is the presence of necrotizing inflammation in small-sized vessels, such as intraparenchymal arteries, arterioles, capillaries, and venules, and various organ involvement may be present in AAV (9). AAV comprises three distinct diseases: Microscopic polyangiitis, granulomatosis with polyangiitis, and eosinophilic granulomatosis with polyangiitis. These diseases can be differentiated according to their clinico-pathological and laboratory findings of ANCA serology. As mentioned above, based on the crucial role of alarmins in the immune system, several previous studies have demonstrated that the serum level of alarmin is associated with disease-specific.
measures in inflammatory disorders. The level of HMGB1 has been found to be relevant to disease activity indices of ankylosing spondylitis, psoriasis, and systemic lupus erythematosus (10-12). In addition, S100 protein concentration in serum has also been proposed as a biomarker for indicating inflammation in systemic autoimmune diseases, and it is unaffected by age and sex (13). Similar to other inflammatory disorders, alteration of immunity is considered to be responsible for triggering inflammation in AAV, these studies emphasize the fact that the expression of alarmin in serum may have clinical implications in AAV. However, only limited studies investigating the association of alarmin levels with disease-specific indices in patients with AAV have been conducted. Therefore, the present study aimed to assess the serum level of alarmin in patients with AAV and its correlation with disease-specific indices.

**Patients and Methods**

**Patient inclusion.** We utilized serum samples and data from 79 patients who were included in the Severance Hospital ANCA-associated VasculitidEs (SHAVE) cohort, which is an observational registry of patients with AAV established at Severance Hospital, Republic of Korea (14). Patients were classified into AAV subtypes according to the 2007 European Medicines Agency algorithm (15) and the 2012 revised International Chapel Hill Consensus Conference Nomenclature of Vasculitides (16). The blood samples of patients enrolled in the SHAVE cohort were collected every 3 to 6 months after obtaining written informed consent. The samples were then immediately isolated and stored at −80°C until further use. On the date of blood collection, the patients underwent routine laboratory tests, and AAV-specific indices [Birmingham vasculitis activity score (BVAS; version 3) (17), five-factor score (FFS) (2009) (18), and vasculitis damage index (VDI) (19)] were assessed by the attending physician. Moreover, patients with serious infections, malignancies, or secondary vasculitis other than AAV, as described in the entry criteria of the 2007 European Medicines Agency algorithm, were excluded. Informed consent was provided by all patients during the initial blood collection, and the study procedures were carried out according to the procedures specified by the Institutional Review Board of Severance Hospital (4-2016-0901).

**Data collection and measurement of serum alarmins.** The collected data consisted of clinical and laboratory variables, such as demographic data, AAV-specific indices, ANCA serology, disease subtypes, organ involvement, and inflammatory laboratory markers, which were obtained during serum collection. For clinical variables, demographic data comprising age, disease duration, sex, and treatment with immunosuppressive drugs (glucocorticoid, cyclophosphamide, rituximab, methotrexate, azathioprine, tacrolimus, and mycophenolate mofetil) were assessed as well as AAV-specific indices BVAS version 3, FFS, and VDI. Patterns of organ involvement were searched and distributed according to the subcategories of BVAS. Disease duration after the initial diagnosis was categorized into three different groups: i) ≤1 Month, ii) 1 month to 2 years, and iii) <2 years.

Laboratory data investigated included ANCA serology, which was evaluated according to the detection of myeloperoxidase or perinuclear (P)-ANCA and proteinase 3 or cytoplasmic (C)-ANCA

| Table I. Baseline data of the study patients (n=79). |
|------------------------------------------------------------------|
| **Demographic data**                                             | **Value** |
| Age, years                                                       | Mean±SD   | 60.4±14.6 |
| Disease duration, months                                        | Mean±SD   | 18.7±37.7 |
| Gender, n (%)                                                   | Female    | 46 (58.2) |
| Immunosuppressive drug treatment, n (%)                         | Yes       | 68 (86.1) |
| AAV-specific indices, mean±SD                                   | BVAS      | 9.7±2.5   |
|                                                               | FFS       | 1.4±1.0   |
|                                                               | VDI       | 3.1±1.8   |
| Disease subtypes, n (%)                                         | MPA       | 40 (50.6) |
|                                                               | GPA       | 25 (31.6) |
|                                                               | EGPA      | 14 (17.7) |
| Organ involvement pattern, n (%)                                 | General   | 28 (35.4) |
|                                                               | Cutaneous | 8 (10.1)  |
|                                                               | Mucous/eye| 3 (3.8)   |
|                                                               | ENT       | 33 (41.8) |
|                                                               | Pulmonary | 46 (58.2) |
|                                                               | Cardiovascular | 3 (3.8) |
|                                                               | Abdominal | 1 (1.3)   |
|                                                               | Renal     | 43 (54.4) |
|                                                               | Nervous   | 21 (26.6) |
| ANCA serology, n (%)                                            | P-ANCA-positive | 50 (63.3) |
|                                                               | C-ANCA-positive | 9 (11.4)  |
|                                                               | ANCA-negative | 22 (27.8) |
| Inflammatory markers, mean±SD                                   | WBC count, n/mm³ | 8,603.5±4,255.2 |
|                                                               | ESR, mm/h  | 42.8±36.1 |
|                                                               | CRP, mg/l  | 20.8±41.6 |
| Serum alarmins                                                  | HDGF, ng/ml | Mean±SD 2.5±0.6 |
|                                                               | HMGB1, ng/ml | Mean±SD 0.4±0.5 |
|                                                               | HMGB1, n (%) | Positive 42 (53.2) |
|                                                               | S100A9, pg/ml | Mean±SD 2,543.0±3,638.6 |
|                                                               | S100A12, ng/ml | Mean±SD 129.4±121.9 |
| AAV: Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis; BVAS: Birmingham vasculitis activity score; C: cytoplasmic (proteinase 3); ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; EGPA: eosinophilic granulomatosis with polyangiitis; ENT: ear, nose, and throat; FFS: five-factor score; GPA: granulomatosis with polyangiitis; HDGF: hepatoma-derived growth factor; HMGB1: high mobility group box protein 1; MPA: microscopic polyangiitis; P: perinuclear (myeloperoxidase); VDI: vasculitis damage index; WBC: white blood cell.

(20). Inflammatory markers assessed include white blood cell count, erythrocyte sedimentation rate, and C-reactive protein level. HDGF and HMGB1 levels were measured using enzyme-linked immunosorbent assay kits (Cloud-Clone Corp, Katy, TX, USA and Arigo Biolaboratories Corp., Hsinchu City, Taiwan, respectively), while S100A9 and S100A12 levels were evaluated using Human Magnetic Luminex® assay (R&D Systems, Minneapolis, MN, USA).

**Statistical analysis.** All statistical analyses were performed using SPSS software (version 25 for Windows; IBM Corp., Armonk, NY, USA). Continuous variables are expressed as the mean±standard deviation and categorical variables as numbers with percentages.
Correlations between the continuous variables were evaluated using Pearson correlation analysis, whereas the correlation coefficient between HMGB1 and continuous variables was calculated using Spearman’s rho, as HMGB1 was not detectable in 37 patients. The differences between continuous variables were estimated using Student’s t-test and analysis of variance, as appropriate. Two-tailed p-values of less than 0.05 were considered statistically significant.

### Results

**Baseline patient data.** The characteristics of the 79 patients included in the study are described in Table I. The mean age and disease duration of the patients were 60.4 years, and 18.7 months, respectively. A total of 46 patients (58.2%) were female, and 68 (86.1%) were on immunosuppressive drug treatment. The mean BVAS, FFS, and VDI were 9.7, 1.4, and 3.1, respectively. In addition, microscopic polyangiitis (50.6%) was the most frequent diagnosis, most frequently with pulmonary manifestation (58.2) observed. P-ANCA positivity was observed in 50 (63.3%) patients. The mean serum levels of HDGF, HMGB1, S100A9, and S100A12 were found to be 2.5, 0.4, 2.5, and 129.4 ng/ml, respectively.

**Correlations among alarmin levels, AAV-specific indices, and levels of inflammatory markers.** On assessing the relationship of alarmin levels with AAV-specific indices and inflammatory marker levels, S100A9 level was found to be significantly positively correlated with that of C-reactive protein (CRP; r=0.316, p=0.005), and S100A12 level was significantly positively associated with VDI (r=0.232, p=0.040). This association was consistent and the correlation coefficient was numerically high in a subgroup of patients with P-ANCA positivity (S100A9 and CRP r=0.477, p<0.001; S100A12 and VDI, r=0.308, p=0.030). No other associations were found regarding alarmins and the remaining variables evaluated (Table II).

**Comparison of serum S100A12 levels according to disease subtype, ANCA serology, and organ involvement.** Apart from the association between serum S100A12 level and VDI, there was no association between alarmin levels and disease-specific indices. Hence, we compared whether there were differences in serum S100A12 levels among the AAV subtypes and according to ANCA serology. However, no difference was observed in the level of serum S100A12 according to disease subtypes and the presence of ANCAs (Figure 1). Moreover, serum S100A12 levels were found to be comparable regardless of the pattern of organ involvement (Figure 2). Similarly, no difference was noted in the levels of HDGF, HMGB1, and S100A9 based on disease subtypes, ANCA serology, or organ involvement.

**Difference in alarmin levels based on disease duration and treatment status.** Next, we analyzed the levels of the four different alarmins according to disease duration and treatment status. When the patients were divided according to disease duration of ≤1 month, 1 month to 2 years, and >2 years, 40, 21, and 18 patients were included in the corresponding groups, respectively. There was no statistical difference in the levels of alarmins according to disease duration and treatment status (Figures 3 and 4).

**Discussion**

Recently, studies have identified that alarmins can act as potential danger signals in the human body and may induce autoimmunity or perpetuate chronic inflammation in systemic autoimmune diseases. Therefore, this study was conducted to evaluate whether the level of serum alarmins might be utilized as disease-specific biomarkers in patients with AAV. Our data revealed that the serum S100A9 level was correlated with CRP level, and S100A12 level was correlated with VDI in patients with AAV and that the
correlation coefficient was markedly higher in AAV patients with P-ANCA positivity, but no other associations were found. Furthermore, serum alarmin levels were not affected by other factors such as disease subtypes, ANCA serology, organ involvement, disease duration, or treatment status.

Alarmins primarily participate in immune response by modulating the activation and recruitment of immune cell subsets involved in the innate immune system, such as neutrophils and macrophages (3). Although the dysregulation of both the innate and adaptive immune systems is broadly observed and is thought to play a crucial role in AAV pathogenesis, neutrophils are traditionally regarded as major effector cells at the initial stage of AAV development (21, 22). Upon inflammatory stimuli, neutrophils are primed and
activated by ANCAs, which are responsible for the induction and maintenance of vascular inflammation (23). Interestingly, ANCAs also induce the release of neutrophil extracellular traps (NETs), which are now increasingly recognized to play an essential pathogenic role in AAV (21). NETs are composed of DNA and various proteins, including alarmins (3, 24), which may, in turn, lead to ANCA production and facilitate endothelial injury. In addition, NETs can be formed even in the absence of ANCAs (25, 26). Based on these findings, we hypothesized that the levels of serum alarmins may reflect disease-specific indices of AAV.

Unexpectedly, no significant association was found between serum HMGB1 level and disease-specific indices of AAV in this study. Previously, several studies, which evaluated the role of circulating HMGB1 levels in the blood of patients with AAV and other forms of vasculitis, reported discordant results. A study by Wang et al. reported that the plasma HMGB1 level might reflect BVAS and renal involvement in patients with AAV (27). In line with this finding, another study reported that serum HMGB1 levels were higher in patients with systemic vasculitis than in healthy controls, and they were associated with BVAS and renal involvement (28), which differs from the results of the present study. Our data are consistent with the observations made by Souza et al., who reported that the serum HMGB1 level is not a useful biomarker in AAV; in addition, a similar result was found in patients with Behçet's disease (29, 30).

Regarding serum S100A8/A9 levels in AAV, it was reported that changes in S100A8/A9 levels were associated with disease relapse in patients with C-ANCA positivity, and the changes were relevant to disease relapse and a gradual decrease in kidney function in patients with AAV (31, 32). Meanwhile, Pepper et al. reported that S100A8/A9 expression was higher in renal biopsy tissues, which increased after the cessation of treatment. S100A8/A9 expression was also higher in patients who would experience a future relapse, suggesting that S100A8/A9 level may be a potential biomarker in AAV (33). Even though a prognostic potential has been suggested through previous studies, it was not clearly indicated whether serum S100A8/9 is correlated with disease-specific indices of AAV. Our data show that serum S100A9 levels were associated with those of CRP, particularly in P-ANCA-positive patients, suggesting at least a partial relationship between serum S100A9 level and high disease burden in AAV. Since it is inconclusive whether the S100A9 level is associated with AAV-specific indices, the role of S100A8/A9 in AAV should be investigated in-depth by future studies.

Komatsuda et al. demonstrated that the serum S100A12 level was associated with BVAS and clinical and laboratory

Figure 3. Alarmin levels according to disease duration. Patients were divided into three groups according to the duration of vasculitis (≤1 month, 1 month to 2 years, and >2 years) and the levels of alarmins were compared. No difference was noted in the levels of the four different alarmins evaluated. Lines and bars indicate the mean±standard deviation. HGF: Hepatoma-derived growth factor; HMGB1: high mobility group box protein 1.
parameters in 46 patients with P-ANCA-associated glomerulonephritis (34). In contrast, Brown et al. suggested the serum S100A12 level to be elevated in patients with childhood systemic vasculitis, particularly in those positive for C-ANCA (35). In the present study, we only found an association between serum S100A12 level and VDI. However, similar to the study conducted by Komatsuda et al., which showed a specific association with subgroups of AAV, we found the correlation efficient of serum S100A12 levels and VDI was higher in a subset of patients with P-ANCA positivity (r=0.308). The precise cause of the discrepant results compared to the previous study is unclear. However, it is possible that the clinical implications of S100A12 in AAV vary according to the disease evaluated, ethnicity, or even geographic region, which should be explored further. Alternatively, consistent with the fact that alarmin levels are increased following injury or damage, it is also possible that the S100A12 level is increased because of increased organ damage (VDI) in AAV and this might be a biomarker indicating organ damage accrual in AAV. Nevertheless, the correlation coefficient (r=0.232) was low and various factors of aging, disease course, and the sequelae of treatment may have also influenced the VDI of the patients (36). Therefore, it is unclear whether the serum S100A12 level might serve as a serological marker reflecting organ injury in AAV, and further studies to validate our data are warranted.

Our study has several limitations. Firstly, the number of patients included in this study was relatively small. Secondly, a direct relationship between S100A12 level and VDI was not found through the results of this study. Thirdly, the associations between alarmins and disease-specific indices were evaluated cross-sectionally, and serial tests of alarmins to examine their dynamics were not performed. Therefore, we believe that additional research is necessary to improve our understanding of the role of alarmins in AAV.

In conclusion, among the four alarmins investigated, we found that the serum S100A12 level was associated with VDI in patients with AAV, and this association was pronounced in a subset of patients with P-ANCA positivity. Further investigations are necessary to provide comprehensive insights into the role of alarmins in AAV.

Figure 4. Alarmin levels according to immunosuppressive drug treatment status. There was no significant difference in serum level of alarmins according to treatment status. Lines and bars indicate the mean±standard deviation. HDGF: Hepatoma-derived growth factor; HMGB1: high mobility group box protein 1.
Conflicts of Interest

The Authors report no conflicts of interest in regard to this study.

Authors’ Contributions

Conceptualization: S.S.A. and S.W.L. Data curation: S.S.A. and T.Y. Formal analysis: S.S.A. and T.Y. Methodology: S.S.A. J.J.S. and S.W.L. Project administration: S.S.A., T.Y. and S.W.L. Supervision: J.J.S., Y.B.P. and S.W.L. Writing-original draft: S.S.A., T.Y. and S.W.L. Writing-review and editing: S.S.A., T.Y., J.J.S., Y.B.P. and S.W.L.

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