# A STUDY OF SERUM ACID SPHINGOMYELINASE ACTIVITY AND CLINICAL SEVERITY IN INFANTS WITH RESPIRATORY SYNCYTIAL VIRUS BRONCHIOLITIS

Shoko Yamamoto[1)2)], Atsuko Noguchi[1)], Chiaki Hatazawa[2)], Takako Hatazawa[2)], Daiki Kondo[2)], Hiroki Kubota[2)], Satoko Tsuchida[3)] and Tsutomu Takahashi[1)*]

[1)]*Department of Pediatrics, Akita University Graduate School of Medicine, Hondo 1-1-1, Akita, Akita, 010-8543, Japan*
[2)]*Division of Pediatrics, Akita Kousei Medical Center, Akita, Akita, Japan*
[3)]*Division of Pediatrics, Akita Red Cross Hospital, Akita, Akita, Japan*

(received 18 April 2022, accepted 13 June 2022)

**Abstract**
Respiratory syncytial virus (RSV) bronchiolitis is the leading cause of hospitalization in infants without any effective treatment strategies. Identification of biomarkers associated with disease severity may be significant in improving management. However, several studies have failed to identify specific biomarkers for bronchiolitis. Serum secretory acid sphingomyelinase (S-ASM) activity has been considered a biomarker of cytokine release, inflammation, and oxidative stress in various diseases. This study aimed to evaluate whether serum S-ASM activity increases and correlates with disease severity in infants with RSV bronchiolitis. Serum S-ASM activity was measured in 31 infants with RSV bronchiolitis, 9 infants with RSV-negative febrile infection, and 8 healthy infants. Laboratory data and clinical observational findings were analyzed for correlation with serum S-ASM activity. Serum S-ASM activity was significantly higher in the 31 infants with RSV bronchiolitis (9.5±5.4 nmol/mL/h) than individuals in the control groups (RSV-negative febrile infection patients : 4.3±1.9 nmol/mL/h, $p<0.005$ ; healthy infants : 4.0±1.4 nmol/mL/h, $p<0.005$). Serum S-ASM activity was negatively correlated with interferon-γ levels (rho=−0.448, $p=0.012$) but not with any other outcomes. Serum S-ASM activity was significantly higher in infants with RSV bronchiolitis than in individuals in the control groups ; however, its clinical significance requires further investigation.

**Keywords** : biomarker, bronchiolitis, infant, secretory acid sphingomyelinase, respiratory syncytial virus

## Introduction

Bronchiolitis is the most common cause of lower respiratory infections and one of the major causes of hospital admission in young children[1)]. This condition is predominantly a viral disease, and more than 70% of bronchiolitis is caused by respiratory syncytial virus (RSV), with fewer cases involving the rhinovirus[2)]. Patients in the acute period of bronchiolitis often require oxygen administration or intensive care. However, no effective treatment has been established other than supportive care[1)]. In addition, various studies have demonstrated an increasing effect of viral bronchiolitis on the development of recurrent wheezing and asthma as long-term sequelae in later childhood[3,4)]. Because bronchiolitis has these disease specificities, the prediction of its severity and long-term prognosis may be significant.

To investigate the underlying immunological mecha-

Corresponding Author : Shoko Yamamoto
Department of Pediatrics, Akita University Graduate School of Medicine, Hondo 1-1-1, Akita, Akita, 010-8543, Japan.
Tel : +81-18-884-6159
Fax : +81-18-836-2620
E-mail : tomy@doc.med.akita-u.ac.jp

nisms and identify immune biomarkers that can predict disease severity, many studies have evaluated immune biomarker concentrations in the blood of infants with bronchiolitis[5-7]. In a systematic review of the immune biomarkers, two dozen of biomarkers showed a significant association with disease severity in at least one study, such as interleukin (IL)-1, IL-4, and IL-8 as positive correlation, and interferon-γ (IFN-γ), IL-10, and IL-18 as negative correlation of the serum levels with disease severity[6]. However, comparable studies on such biomarkers have reported conflicting results[6,7], suggesting that no specific immune biomarkers can determine disease severity in infants with bronchiolitis.

Acid sphingomyelinase (ASM) is a lysosomal enzyme that plays a crucial role in sphingolipid metabolism. ASM hydrolyzes sphingomyelin, an essential cellular membrane component, into ceramide and facilitates normal membrane turnover[8]. Ceramide functions as a second intracellular messenger involved in various cellular functions ; thus, ASM activation has been reported as a proactive cellular response in many biological functions and pathological responses in humans[8]. The sphingomyelin phosphodiesterase 1 gene encodes a common precursor protein called pre-pro-ASM, which is cleaved into pro-ASM and then processed into two types of ASM—lysosomal ASM (L-ASM) and secretory ASM (S-ASM). Pro-ASM is processed into ASM through N- and C-terminal modifications and differential glycosylation in the Golgi apparatus[9]. L-ASM is transported into lysosomes, exposed to the $Zn^{2+}$ cell pool, and then saturated with $Zn^{2+}$ as a metallophosphodiesterase[9]. In contrast, S-ASM is secreted into the extracellular space where there is no $Zn^{2+}$ saturation[9]. Consequently, while L-ASM requires no additional $Zn^{2+}$ ions for activation, S-ASM requires $Zn^{2+}$ ions. Thus, S-ASM activity can be measured in various body fluids, including blood, using an assay buffer containing $Zn^{2+}$ [10]. ASM activation has been shown to be associated with the extracellular secretion of ASM, which can be detected through an increase in S-ASM activity. However, the exact mechanisms of the extracellular secretion of ASM remain to be elucidated.

In over 20 clinical studies, serum S-ASM activity has been measured in human diseases, and a significant increase in S-ASM activity was found in various diseases, such as heart disease, diabetes mellitus 2, Alzheimer's disease, alcohol dependence, and inflammatory diseases, including sepsis, systemic inflammatory response syndrome, hepatitis C, systemic vasculitis, inflammatory renal disease, lymphohistiocytosis, and coronavirus disease 2019 (COVID-19)[9,11]. These results suggest that the increase in S-ASM activity may reflect cytokine release, inflammation, oxidative stress, or vascular endothelium (which is thought to be an abundant source of S-ASM) damage[9].

In our previous study, serum S-ASM activity was measured in 50 children with acute common illness who were divided into five groups based on their clinical diagnoses : RSV bronchiolitis, adenovirus infection, streptococcal infection, asthma, and other infections of unknown origin[12]. Our results showed that serum S-ASM activity was significantly elevated by more than two-folds in a group of six patients with RSV bronchiolitis compared with that in healthy children and children in the other four groups. Our previous study preliminarily suggested an association of ASM activation in infants with RSV bronchiolitis.

The present study aimed to evaluate serum S-ASM activity in a cohort of 31 infants with RSV bronchiolitis and determine the clinical significance of serum S-ASM activity.

## Materials and Methods

### Study population

A cohort of 31 infants aged 0-11 months who were diagnosed with RSV bronchiolitis were enrolled in this study. None of patients were born prematurely (at or before 35 weeks of gestation) or had any history of diseases, including bronchopulmonary dysplasia, hemodynamically significant congenital heart diseases, neuromuscular disorders, immunocompromise, or Down syndrome. None of the patients had received palivizumab injections before admission. The demographic characteristics of patients are presented in Table 1. They were admitted to the Akita Kousei Medical Center between August 2018 and January 2020, and their diagnosis of RSV infection was made using antigen detection tests on nasopharyngeal discharges (ImunoAce RSV

Table 1. The patients' demographic characteristics in this study.

| | Acute RSV bronchiolitis ($n$=31) | RSV-negative infection ($n$=9) | Healthy control ($n$=8) |
|---|---|---|---|
| Age (months) | 3.6±3.0 (0-11) | 8.55±2.5 (3-11)** | 3.1±2.9 (0-8) |
| Sex (male) | 17 (54.8%) | 5 (55.6%) | 7 (87.5%) |
| Gestational age (months) | 38.1±1.1 | 38.4±1.1 | 38.1±1.9 |
| Birth weight (g) | 2997.1±385.9 | 3143.0±349.6 | 2953.1±401.4 |
| White blood cells ($\times 10^2/\mu L$) | 95.9±23.2 | 117.4±42.6 | 115.7±22.4 |
| C-reactive protein (mg/dL) | 0.67±1.06 | 1.98±1.50* | 0.01±0.01** |
| aspartate aminotransferase (IU/L) | 41.5±13.1 | 35.7±11.0 | 36.3±11.5 |
| alanine aminotransferase (IU/L) | 23.9±10.1 | 17.1±2.6 | 25.4±13.0 |
| lactic acid dehydrogenase (IU/L) | 323.8±50.7 | 294.1±50.8 | 342.6±89.6 |
| S-ASM (nmol/mL/h) | 9.5±5.4 | 4.3±1.9** | 4.0±1.4** |
| Interferon-γ (pg/mL) | 9.2±11.0 | 14.5±23.4*** | 1.5±0.0** |
| Interleukin-18 (pg/mL) | 431.3±276.3 | 260.9±77.5 | 240.8±137.8 |

*$p$<.05 vs. acute RSV bronchiolitis, ** $p$<.005 vs. acute RSV bronchiolitis, ***$p$<.05 vs. healthy control

Table 2. Clinical scoring system

| Score | Oxygen Saturation* (%) | Respiratory Rate (breaths/min) | Wheezing | Accessory Respiratory Muscle Utilization |
|---|---|---|---|---|
| 0 | ≥95 | <30 | None** | None |
| 1 | 91-95 | 30-45 | Terminal expiration with stethoscope only | Presence of mild intercostal in drawing (just visible), no head bobbing or tracheal tug |
| 2 | 85-90 | 46-60 | Entire expiration and inspiration with stethoscope only | Moderate amount of intercostal indrawing, no head bobbing or tracheal tug |
| 3 | <85 | >60 | Expiration and inspiration without stethoscope | Moderate or marked intercostal indrawing with presence of head bobbing or tracheal tug |

*Oxygen saturation was measured during 15 min without supplemental oxygen.
**If no wheezing is audible due to minimal air entry, score 3.

Neo, Shizuoka, Japan). The clinical diagnosis of RSV bronchiolitis was made based on the following criteria : cough, increased respiratory rate, chest retraction, prolongation of expiratory time, sibilant rhonchi, and hyperinflated lungs on chest radiography[13]. Blood was collected on admission and serum was stored at <−20°C.

Eight healthy infants and nine hospitalized infants with RSV-negative febrile infection were included as control groups in this study. The RSV-negative hospitalized febrile infants included three patients with diagnoses of acute bronchitis, two with adenovirus infections, one with hand-foot-and-mouth disease, one with febrile convulsion, one with mycoplasma infection, and one with otitis media and presented with fever and respiratory symptoms. RSV-negative infections were confirmed using an antigen detection test.

Patients with RSV bronchiolitis were scored on a scale of 0-3 points for each of the four factors—oxygen saturation of peripheral artery, respiratory rate, wheezing, and accessory respiratory muscle utilization—for a maximum of 12 points every day during hospitalization. We used a modified versions of a previously reported scoring system detailed in Table 2[14-16]. Medical records were assessed for age, sex, clinical symptoms, complications, need for oxygen administration, and laboratory data, including white blood cell count, platelet count, aspartate

aminotransferase, alanine aminotransferase, lactate dehydrogenase, hemoglobin, and C-reactive protein (CRP) levels.

Ethical approval was obtained from the Ethics Committee of Akita Kousei Medical Center and Akita University Graduate School of Medicine, Akita, Japan. Written informed consent was obtained from the parents or guardians of the patients and healthy infants, and the study was performed in accordance with the Declaration of Helsinki.

### Determination of serum S-ASM activity and IFN-γ and IL-18 levels

Serum S-ASM activity was measured as described previously and was assayed using a substrate of $^{14}$C-labeled sphingomyelin (PerkinElmer, MA, USA) and a buffer containing $Zn^{2+}$, given that S-ASM is $Zn^{2+}$-dependent[17]. A standard 200-μL assay mixture was made up of 100 μL serum, 50 μL assay buffer (1.0 M sodium acetate, pH 5.0) with 4% Triton X-100 at a final concentration of 1%, and 50 μL of substrate (20 nmol of $^{14}$C-labeled sphingomyelin ; 0.08 μCi/20 nmol) in 0.2% taurodeoxycholic acid. The assay mixture was incubated at 37°C for 6 h. The reaction was stopped with 200 μL of ice-cold 30% trichloroacetate and 400 μL of 2.5% bovine serum albumin. The mixtures were briefly vortexed and allowed to settle for 5 min before centrifugation (3,000 rpm for 5 min). The supernatant (500 μL) was carefully transferred to a glass scintillation pre-filled with 4.5 mL of Clearsol II (Nakalai Tesque, Kyoto, Japan). Radioactivity was measured using a liquid scintillation counter (LSC 950 ; Aloka, Tokyo, Japan).

Serum INF-γ level was determined using Human IFN gamma (enzyme-linked immunoassay) ELISA Kit (Thermo Fisher Scientific, Waltham, MA, USA) and the lower detection level was 1.56 pg/mL. Therefore, the INF-γ level below the detection limit was regarded as 1.5 pg/mL. Serum IL-18 level was determined using Human IL-18 ELISA Kit (MBL Inc., Tokyo, Japan) and the lower detection limit was 12.5 pg/mL.

### Statistical analysis

Data were analyzed using the IBM SPSS statistics software package (version 26.0) and the results are presented as mean±standard deviation. The Kruskal–Wallis test was used to compare the mean differences between the two groups. Spearman's rank correlation coefficient was used to examine the correlation between serum S-ASM activity and clinical study measures. Statistical significance was set at $p<0.05$.

## Results

The characteristics of patients with RSV bronchiolitis, patients with RSV-negative febrile infection, and healthy infants are summarized in Table 1. The age, sex, gestational age, and birth weight of the healthy infants were comparable with those of patients with RSV bronchitis.

### Serum S-ASM activity and IFN-γ and IL-18 levels in acute RSV bronchiolitis (Fig. 1)

The mean serum S-ASM activity was 9.5±5.4 nmol/mL/h in the 31 patients with RSV bronchiolitis, and whereas it was 4.3±1.9 and 4.0±1.4 nmol/mL/h in the 9 patients with RSV-negative febrile infection and the 8 healthy infants, respectively. Serum S-ASM activity was significantly higher in patients with RSV bronchiolitis than in patients with RSV-negative febrile infections ($p<0.005$) and healthy infants ($p<0.005$).

The mean serum INF-γ level was 9.2±11.0 pg/mL in the 31 patients with RSV bronchiolitis, whereas this level was 14.5±23.4 and 1.5±0.0 pg/mL in the 9 patients with RSV-negative febrile infection and 8 healthy infants, respectively, indicating that serum IFN-γ levels were significantly higher in patients with RSV bronchiolitis ($p<0.005$) and patients with RSV-negative febrile infection ($p<0.05$) than in healthy infants.

The mean serum IL-18 level was 431.3±276.3 pg/mL in the 31 patients with RSV bronchiolitis, whereas the 9 patients with RSV-negative febrile infection and 8 healthy infants had levels of 260.9±77.5 and 240.8±137.8 pg/mL, respectively. No significant differences were noted in IL-18 levels among the three groups of infants.

### Relationship between S-ASM activity and clinical severity score, laboratory findings, and clinical observatory findings in RSV bronchiolitis

The relationship between S-ASM activity and the clini-

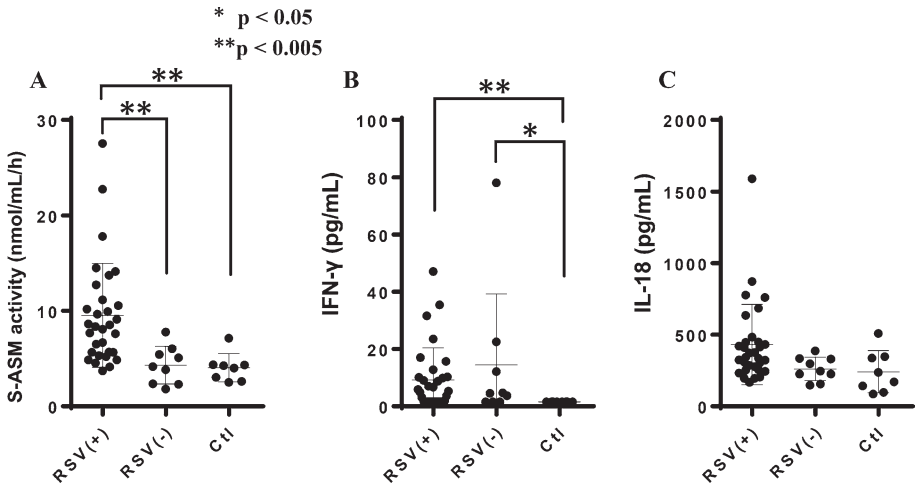


Fig. 1. S-ASM activity, and IFN-γ and IL-18 levels in acute RSV infection.
Serum S-ASM activity (A), IFN-γ levels (B), and IL-18 levels (C) are shown for the RSV(+), RSV(−), and control (Ctl) groups.

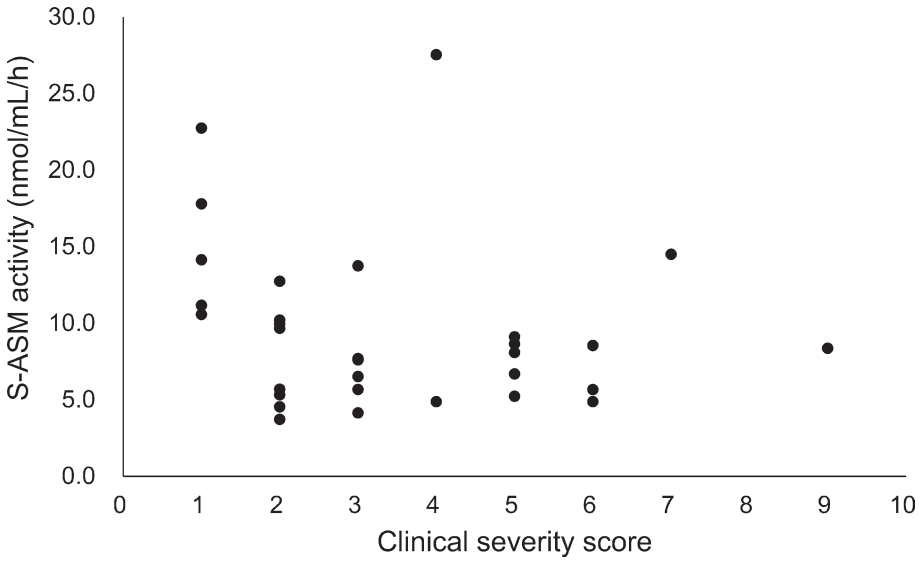


Fig. 2. Serum S-ASM activity and clinical severity score on admission in infants with RSV bronchiolitis.

cal severity score on admission was not significant (Fig. 2). Spearman's rho correlations between S-ASM activity and age, sex, clinical severity score on admission, days from the onset of fever, oxygen saturation, demand for oxygen therapy, duration of administration, administration of antibiotics, administration of steroids, white blood cells counts, CRP level, and IL-18 level were not significant (Table 3); however, Spearman's rho correlation between S-ASM activity and IFN-γ level (rho=−0.448, $p$=0.012) was significant (Table 3).

Table 3. Spearman's rho correlations of S-ASM and clinical and laboratory findings in acute RSV bronchiolitis

| | Acute RSV bronchiolitis $n=31$ | S-ASM activity | |
|---|---|---|---|
| | | ρ | ($p$) |
| Age (months) | 3.6±3.0 | −0.250 | (0.175) |
| Sex (male) | 17 | 0.080 | (0.670) |
| Clinical severity score on admission | 3.42±2.00 | −0.283 | (0.123) |
| Days from the onset of fever | 1.39±2.04 | −0.087 | (0.642) |
| Saturation of oxygen (%) | 97.1±3.0 | 0.099 | (0.595) |
| Demand on oxygen therapy | 10 | −0.054 | (0.773) |
| Duration of admission (days) | 4.9±1.5 | 0.285 | (0.120) |
| Administration of antibiotics | 5 | 0.250 | (0.175) |
| Administration of steroids | 3 | −0.073 | (0.696) |
| White blood cells ($\times 10^2/\mu L$) | 95.4±23.2 | 0.261 | (0.155) |
| CRP (mg/dL) | 0.67±1.06 | 0.179 | (0.336) |
| INF-γ* (pg/mL) | 9.2±11.0 | −0.448 | (0.012) |
| IL-18 (pg/mL) | 431.3±276.3 | −0.256 | (0.164) |

*significant correlation

## Discussion

In the present study, serum S-ASM activity in the 31 infants with RSV bronchiolitis was significantly higher than in individuals in the control groups (8 healthy infants and 9 patients with RSV-negative febrile infection). This result confirmed the findings of our previous study, wherein we found higher serum S-ASM activity in 6 hospitalized infants with RSV bronchiolitis than in 5 patients with adenovirus infection, 9 patients with streptococcal infection, 6 patients with asthma attacks, and 24 patients with other infections[12]. The present study showed elevated levels of serum S-ASM in the infants with RSV bronchiolitis about 2 times more than those in the controls, while the previous study showed elevated levels of serum S-ASM in the patients about 3 times more than those in controls. Both studies showed significant elevations of serum S-ASM in the patients, compared with the controls. The results of both these studies collectively suggest that elevated serum S-ASM activity is a characteristic pathological reaction in infants with RSV bronchiolitis. During the last two decades, serum S-ASM activity has been shown to be elevated in various human diseases[9] ; however, to the best of our knowledge, the present study is the first to confirm that elevation of serum S-ASM activity is a characteristic pathological reaction in the acute phase of a viral infection.

Only one previous study has described the relationship between viral infection and elevated serum S-ASM activity[18] ; in this study, patients with chronic hepatitis C virus infection had significantly higher serum S-ASM activity than healthy individuals. Especially in case of chronic hepatitis C infection, S-ASM activity correlated significantly with markers of hepatic injury and showed a high discriminative power. Grammatikos et al. concluded that chronic hepatitis C virus infection and non-alcoholic fatty liver disease induce the upregulation of serum ASM, which appears to be a novel biomarker in hepatopathies. None of our patients with RSV bronchiolitis had any liver dysfunction related to hepatic injury, indicating that elevation of serum S-ASM activity may originate from a different mechanism compared with that involving hepatitis C virus infection.

Increased S-ASM activity has been associated with the enhanced release of the enzyme and enhanced activation via oxidative stress, inflammation, cytokine release, or vascular endothelium damage ; therefore, it is regarded as a biomarker of these conditions[9]. In addition, S-ASM has been specifically demonstrated to be a cytokine-responsive enzyme that increases in activity upon stimula-

tion with IL-1β or tumor necrosis factor-α (TNF-α)[19]. It is well known that acute bronchiolitis caused by RSV triggers an inflammatory response through the production and release of several proinflammatory cytokines[6,7]. The two most common cytokines are TNF-α and IFN-γ[20]. In the present study, we did not measure TNF-α levels in our patients. However, numerous studies have measured TNF-α and IFN-γ levels in patients with RSV bronchiolitis, reporting various associations between these levels and bronchiolitis severity. Thus, the elevation of S-ASM may originate from a cellular reaction to the release of various cytokines in RSV bronchiolitis.

In this study, two cytokines, IFN-γ and IL-18, were determined in the subjects. The serum levels of IFN-γ were significantly increased in patients with RSV-positive bronchiolitis and RSV-negative febrile diseases, compared with the control. However, there was no significant difference between RSV-positive and -negative patients in this study. The serum levels of those cytokines have been reported to be negatively correlated with disease severity in children less than 24 months with bronchiolitis[6]. However, this study did not show any significant association of the serum levels of those cytokines and clinical severity in the disease.

In addition, the relationship between S-ASM activity and laboratory data and clinical findings, including clinical severity scores, was statistically analyzed in our study, and no positive correlation was found, with only serum IFN-γ levels being negatively correlated. This negative correlation of S-ASM with IFN-γ could be explained by some reports[21,22]. Legg et al. examined the type 1 and type 2 cytokine imbalance in RSV bronchiolitis, suggesting that deficient type 1 immunity response might contribute to the aggravation of RSV bronchiolitis. IFN-γ is well-known to play a major role in type 1 immune response, and then levels of IFN-γ might be lower in a deficient type 1 infants with RSV infection. If S-ASM is positively correlated with clinical severity in RSV bronchiolitis, S-ASM activity would be negatively correlated with IFN-γ levels. Further studies of S-ASM activity are needed to elucidate the original site and mechanism of ASM secretion in lower respiratory tract infections.

There were limitations to our study. The number of participants in our study was small and larger-scale studies are needed to confirm our findings. Furthermore, some of the infants with RSV bronchiolitis participated in our study may have been co-infected with other infections. There were many studies on cytokine fluctuations due to co-infection with RSV, and it would have been better to deny other infections as well[23].

In conclusion, the present study demonstrated that serum S-ASM activity was significantly elevated in infants with RSV bronchiolitis, suggesting an association between ASM activation and RSV bronchiolitis. In addition, serum S-ASM activity is a possible biomarker for clinical utility ; however, its clinical significance requires further study.

**Acknowledgements**

The authors are grateful to Ms. Harumi Sugawara and Ms. Maiko Ito for their technical assistance. This study was not funded by any grants.

**Author Contributions**

S.Y., A.N., and T.T. designed the study ; S.Y., A.N., C.H., T.H., D.K., H.K., and S.T. collected the data ; S.T., A.N., and T.T. analyzed the samples ; S.Y., A.N., and T.T. contributed to data analysis and interpretation ; and S.Y., A.N., and T.T. wrote the manuscript. All authors edited the manuscript and agreed with the content submitted.

**Conflict of Interest**

The authors have no conflicts of interest to declare.

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