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An Exploratory Study of Neutrophil Extracellular Traps in Children with Kawasaki Disease

Kiyoshi Yamakawa[1)], Md. Monirul Islam[1)2)3)], Hiromitsu Mori[1)], Yoshiteru Azuma[1)], Hideyuki Iwayama[1)], Hirokazu Kurahashi[1)], Eizo Watanabe[2)], Naoshi Takeyama[2)] and Akihisa Okumura[1)]

[1)] *Department of Pediatrics, Aichi Medical University, Aichi, Japan*
[2)] *Department of Emergency and Critical Care Medicine, Aichi Medical University, Aichi, Japan*
[3)] *Department of Biochemistry and Biotechnology, University of Science and Technology Chittagong, Bangladesh*

Purpose: This study aimed to verify whether or not neutrophil extracellular traps (NETs) are related to the severity of Kawasaki disease (KD).

Materials and Methods: We examined 30 children with KD and 13 controls. The children with KD were divided into two groups using prediction scores for unresponsiveness to intravenous immunoglobulin (IVIG): IVIG and IVIG＋prednisolone (PSL) groups. We measured cell-free DNA (cf-DNA), myeloperoxidase-DNA complex (MPO-DNA), and neutrophil elastase-DNA complex (NE-DNA) as NETs markers. Blood was sampled before and after IVIG administration and during the convalescent period. We compared NETs markers levels among controls, the IVIG group, and the IVIG＋PSL group.

Results: This study classified 17 children into the IVIG group and 13 into the IVIG＋PSL group. Serum sodium level was lower, and serum ALT and procalcitonin levels were higher in the IVIG＋PSL group than in the IVIG group. All NETs markers were insignificantly elevated in the IVIG＋PSL group at any time of blood sampling, whereas MPO-DNA and NE-DNA were significantly elevated in the IVIG group before IVIG administration.

Conclusion: This study found no evidence to support the relationship between severity of KD and NETs.

Key words: kawasaki disease, neutrophil extracellular traps

INTRODUCTION

Kawasaki disease (KD) is a systemic vasculitis of unknown cause that primarily affects infants and young children. The most important complication is coronary artery lesions (CALs), which can cause coronary artery stenosis. A Japanese study reported that 19% of KD patients with CAL subsequently developed coronary artery stenosis, 7.5% developed myocardial infarction and 3% died[1)]. Therefore, CALs prevention is vital in KD treatment. The standard treatment is intravenous immunoglobulin therapy (IVIG), although there are some cases of inadequate efficacy. In cases of IVIG unresponsiveness, additional treatment is required to prevent CALs. In Japan, several prediction scores of unresponsiveness to IVIG have been proposed[2)~4)] to prevent CALs, and concomitant use of steroids is recommended in cases of IVIG unresponsiveness is predicted. This suggests that cases with predicted IVIG unresponsiveness have a high degree of vascu-

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lar inflammation.

Neutrophil extracellular traps (NETs) have been suggested to be related to the pathogenesis of KD. NETs are a novel innate immune response of neutrophils reported by Brinkmann et al[5]. DNA, histones, and antimicrobial proteins, such as neutrophil elastase, are released in a mesh-like pattern to physically capture pathogens and kill them with antimicrobial proteins. NETs contribute to biological defense through an extracellular bactericidal mechanism. However, excessive NETs formation has been reported to aggravate various diseases[6)~11)] and induce vascular endothelial damage[12)13)]. Some studies of NETs in KD have suggested that NETs are related to KD pathogenesis[14)15)].

As neutrophil activation is observed in KD, we hypothesized that NETs are involved in the pathogenesis of KD and verified whether or not NETs are related to the severity of KD.

MATERIALS AND METHODS

1. Patient characteristics

The subjects of this study were 30 children with KD who were admitted to the Department of Pediatrics of Aichi Medical University Hospital between November 2021 and March 2023. KD was diagnosed based on the Diagnostic Guidelines for KD (2020 revision)[16)] after excluding viral and bacterial infections, toxic shock syndrome, and cervical lymphadenitis. Children with KD were initially treated with 2 g/kg IVIG and acetyl salicylic acid 50 mg/kg/day in our hospital. Before initial treatment, unresponsiveness to IVIG was estimated using the prediction scores proposed by Kobayashi et al [2], Egami et al [3] and Sano et al [4]. When a patient met at least one of these prediction scores, the patient was presumed to be at high risk of unresponsiveness to IVIG and prednisolone (PSL) was added to IVIG as initial treatment. Thirteen children with short stature were recruited as controls, because it is very difficult to obtain blood samples from the age-matched healthy children due to ethical issues. These children had undergone growth hormone (GH) stimulation tests in our hospital and had no pituitary dysfunction. Control subjects had no disorders other than short stature, and were not expected to have excessive NETs formation. The ethics committee of the Aichi Medical University Hospital approved this study (approval no. 2021-098). Written informed consent was obtained from the parents of children with KD and controls.

2. Blood sample collection

We used the residual blood samples which were collected for clinical evaluation. In our hospital, blood samples of children with KD were routinely collected at three time points: before IVIG, after IVIG therapy (mostly 24 h after IVIG), and during the convalescent period (approximately 3 days after IVIG). Blood samples from control subjects were collected during the GH stimulation test. Samples were centrifuged at 5,000×g for 10 min, and serum was collected and stored at −80℃.

3. Measurement of NETs markers

We measured cell-free DNA (cf-DNA), myeloperoxidase-DNA complex (MPO-DNA), and neutrophil elastase-DNA complex (NE-DNA) as NETs markers.

Serum cf-DNA levels were quantified using the Quant-iT PicoGreen dsDNA assay (Life Technologies, Carlsbad, CA, USA) at 485-nm excitation and 538-nm emission as previously described[17)]. Calf thymus DNA standards (0–2 μg/mL) were diluted with Quan-iT picoGreen reagent and incubated for 2 min at room temperature before fluo-rescence measurement to generate a standard curve. Fluorescence intensity representing the concentration of available

DNA in the serum samples was then measured using a QUBIT® 2.0 Fluorometer (Life Technologies), and the data obtained were expressed in ng/ml.

Serum levels of MPO-DNA and NE-DNA were measured according to the method reported by Islam et al[18]. In brief, quantitative detection of MPO-DNA and NE-DNA was performed using a "sandwich" enzyme-linked immunosorbent assay with specific monoclonal capture antibodies; anti-MPO (Merck Millipore Corp, catalog #07-496-I) or anti-NE (Merck Millipore Corp, catalog #MABS461), and a monoclonal detection anti-body; peroxidase-conjugated anti-DNA antibody (Roche Diagnostics, Indianapolis, Ind; Cell Death Detection enzyme-linked immunosorbent assay (ELISA) #1154467500: bottle 2). The microtiter plate wells were coated with specific monoclonal capture antibodies to capture DNA-associated MPO and DNA-associated NE. A chromogenic ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) peroxidase substrate was added, which reacted with the bound peroxidase to yield a soluble green end product detected spectrophotometrically at 405 nm. Absorbance readings were proportional to the amount of bound horseradish peroxidase-labeled anti-DNA monoclonal antibody, and the results were expressed in arbitrary units.

4. Data Collection

Demographic information was collected from patients' medical records, including sex, age, and duration of hospitalization. We gathered the following laboratory data before IVIG administration: white blood cell, neutrophil, platelet counts, serum levels of total bilirubin, sodium, aspartate aminotransferase (AST), alanine aminotransferase (ALT), D-dimer, procalcitonin, C-reactive protein (CRP), N-terminal fragment brain natriuretic peptides (NT-proBNP), and interleukin-6. These laboratory values are included in the prediction scores of IVIG unresponsiveness[2)~4)] and/or have been reported as potential biomarkers of IVIG unresponsiveness in previous studies[19)~26)]. CALs were defined when the Z-score of the right coronary artery, left main trunk, left anterior descending artery, and circumflex coronary artery was ≥ 2 at any time point. In our hospital, echocardiograms were routinely performed before and after IVIG administration and during the convalescent period in all patients with KD.

5. Statistical analysis

The children with KD were divided into two groups: those treated with IVIG alone (IVIG group) and those co-treated with IVIG and PSL (IVIG+PSL group). We hypothesized that the inflammatory response related to KD was more severe in the IVIG+PSL group than in the IVIG group because the IVIG+PSL group required more intense treatment to prevent CALs. To clarify the relationship between KD severity and NETs markers, we compared the cf-DNA, MPO-DNA, and NE-DNA values among controls, the IVIG group, and the IVIG+PSL group. Although blood sampling was performed only once in controls, the values served as a reference in all timings of blood sampling of children with KD. The Kruskal-Wallis test was applied to compare the numerical variables among these three groups. For multiple comparisons among the three groups, the Steel-Dwass test was used when the Kruskal-Wallis test showed a p value <0.05. We also compared demographic and laboratory data between the IVIG and IVIG+PSL groups. The Fisher's exact test and Mann-Whitney U test were used to compare categorical and numerical variables, respectively. In addition, we examined the correlation between age and cf-DNA, MPO-DNA, and NE-DNA values among

controls using Pearson's correlation coefficient. All statistical analyses were performed using EZR ver. 1.61[27] (available at http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmed.html).

RESULTS

KD included twenty-one boys and nine girls with a median age of 26.7 months (3.4–104.5 months) and controls included nine boys and four girls with a median age of 72.6 months (25.1–128.4 months). There was no significant

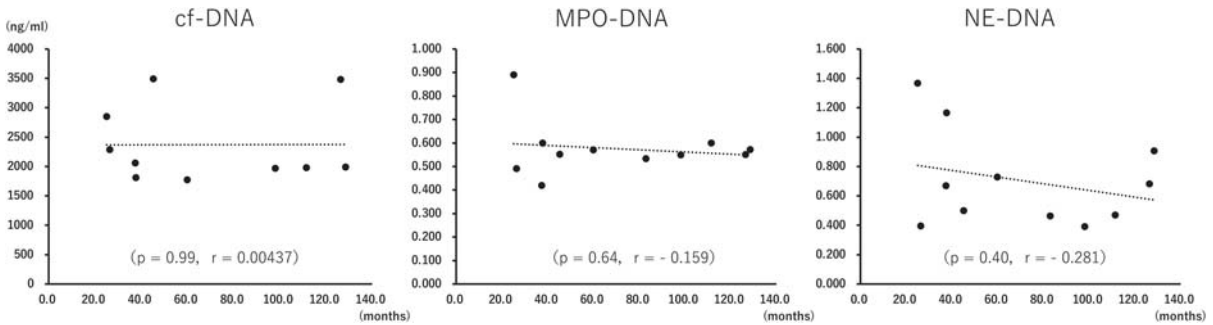

Figure 1. Correlation between age and NETs markers in the control group
cf-DNA, cell-free DNA; MPO-DNA, myeloperoxidase DNA complex; NE-DNA, neutrophil elastase DNA complex.

Table 1. Demographic and laboratory data of patients with Kawasaki disease

	IVIG (n=17)	IVIG+PSL (n=13)	p-value
Sex (M:F)	10:7	11:2	0.229
Age (months)	33.9 (3.4–104.5)	26.4 (5.7–76.4)	0.967
Duration of hospitalization (day)	8 (5–10)	8 (6–11)	0.306
Days of illness of blood sampling			
Before IVIG	4 (3–9)	4 (3–16)	0.965
After IVIG	7 (5–12)	7 (5–19)	0.599
Convalescent period	10 (7–20)	9 (7–26)	0.915
Coronary artery lesions	2 (11.8%)	1 (7.7%)	>0.99
WBC (/μL)	14,750 (5,300–22,400)	12,800 (6,000–24,400)	0.483
Neutrophil (/μL)	9,390 (3,965–16,643)	8,265 (4,500–21,472)	0.934
Platelet ($\times 10^3$/μL)	373 (207–499)	292 (122–728)	0.232
Total bilirubin (mg/dL)	0.52 (0.23–1.11)	0.69 (0.36–5.90)	0.217
Sodium (mmol/L)	134 (131–138)	132 (127–135)	<0.001
AST (U/L)	37 (21–82)	51 (26–931)	0.094
ALT (U/L)	20 (9–71)	39 (9–389)	0.049
D-dimer (μg/mL)	1.44 (1.04–5.68)	1.82 (1.25–8.14)	0.054
Procalcitonin (ng/mL)	0.57 (0.21–3.53)	2.66 (0.13–25.29)	0.048
CRP (mg/dL)	7.13 (2.36–2.61)	5.27 (2.84–14.06)	0.563
NT-pro BNP (pg/mL)	284 (43–4,441)	497 (26–2,434)	0.391
IL-6 (pg/mL)	110.3 (110.0–287.2)	81.8 (32.7–94.8)	0.100

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein; IL-6, interleukin-6; IVIG, intravenous immunoglobulin; NT-pro BNP, N-terminal fragment brain natriuretic peptides; PSL, prednisone; WBC, white blood cell counts.

difference in the sex ratio between controls and KD. The age of the controls was significantly higher than the KD (p＝0.000841), but there was no significant correlation between age and cf-DNA, MPO-DNA and NE-DNA values (Figure 1).

Among the 30 children with KD, 17 were classified into the IVIG group and 13 into the IVIG＋PSL group. Table 1 shows the demographic and laboratory data of the IVIG and IVIG＋PSL groups. The median age was 33.9 months (3.4–104.5 months) in the IVIG group and 26.4 months (5.7–76.4 months) in the IVIG＋PSL group. The demographic data between the IVIG and IVIG＋PSL groups were insignificantly different. The days of illness were indifferent between the IVIG and IVIG-PSL groups. CAL was observed in two children in the IVIG group and one in the IVIG＋PSL group. Among the laboratory data, serum sodium level was lower, and serum ALT and procalcitonin levels were higher in the IVIG＋PSL group than in the IVIG group.

Figure 2 shows the cf-DNA, MPO-DNA, and NE-DNA levels before and after IVIG administration and during the convalescent period. Before IVIG administration, cf-DNA levels were higher in the IVIG group (median 3,167 ng/ml, range 2,227–5,007 ng/ml) than in controls (median 2,025 ng/ml, range 1,777–3,493 ng/ml), whereas no significant difference was found between the IVIG＋PSL group (median 2,793 ng/ml, range 1,587–7,727 ng/ml) and controls. MPO-DNA was lower in the IVIG＋PSL group (median 0.497, range 0.437–0.577) than in the IVIG group (median 0.805, range 0.376–1.868) or controls (median 0.552, range 0.420–0.891). NE-DNA was higher in the IVIG group (median 1.165, range 0.517–1.753) than in the IVIG＋PSL group (median 0.427, range 0.309–0.852) or

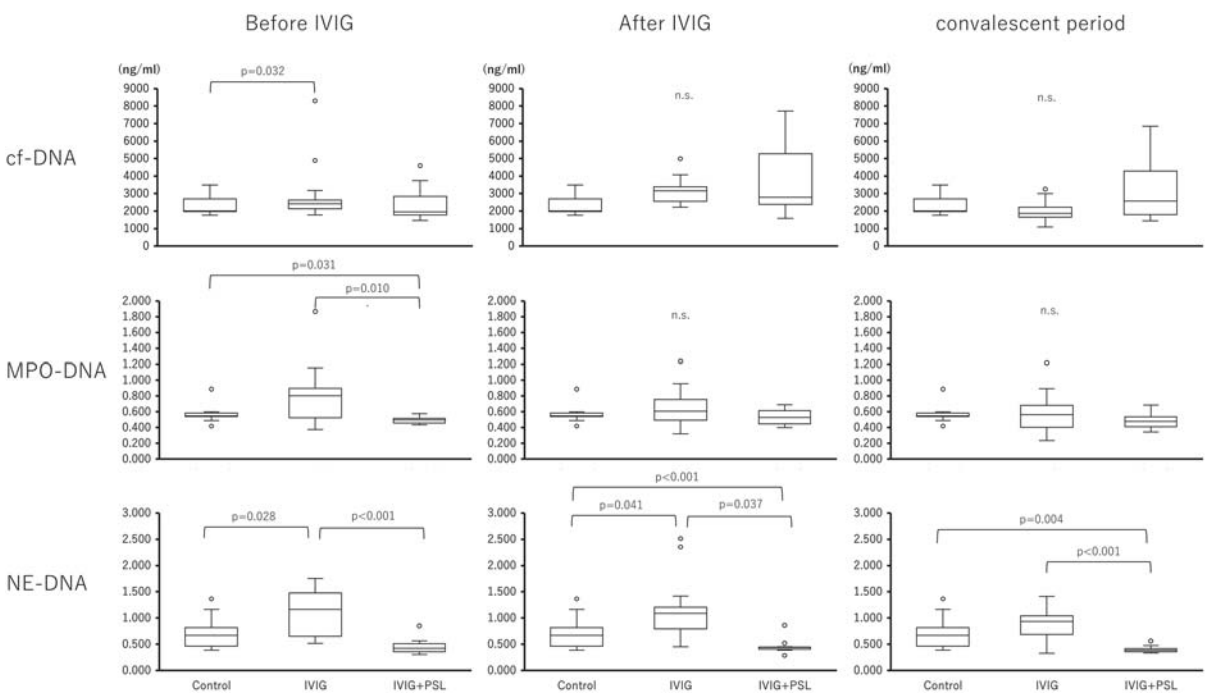

Figure 2. cf-DNA, MPO-DNA, and NE-DNA levels

cf-DNA, cell-free DNA; IVIG, intravenous immunoglobulin; MPO-DNA, myeloperoxidase DNA complex; NE-DNA, neutrophil elastase DNA complex; NETs, neutrophil extracellular traps; n.s., not signifcant; PSL, prednisone.

controls (median 0.669, range 0.392–1.368).

After IVIG administration, no significant differences were found in cf-DNA or MPO-DNA among the groups. NE-DNA was higher in the IVIG group (median, range 0.453–2.516) than in the IVIG+PSL group (median 0.441, range 0.291–0.864) or controls (median 0.669, range 0.392–1.368).

During the convalescent period, no significant differences were found in cf-DNA or MPO-DNA among the groups. NE-DNA was lower in the IVIG+PSL group (median 0.388, range 0.337–0.564) than in the IVIG group (median 0.937, range 0.333–1.416) or controls (median 0.669, range 0.392–1.368).

Table 2 shows correlations between clinical laboratory data and NETs markers in KD before IVIG administration. There were no significant correlations between any laboratory values and NETs markers. In addition, there were no positive correlations between any of the predictive scores of IVIG unresponsiveness and NETs markers (Table 3).

DISCUSSION

In this study, we measured NETs markers in children with KD and examined their relationship with KD severity determined based on prediction scores of IVIG unresponsiveness. Some NETs markers were elevated in the IVIG group,

Table 2. Correlation of laboratory data with NETs markers in patients with Kawasaki disease before intravenous immunoglobulin

	cf-DNA		MPO-DNA		NE-DNA	
	p	r	p	r	p	r
WBC	0.46	−0.143	0.57	0.11	0.213	0.238
Neutrophil	0.71	−0.0707	0.547	−0.115	0.876	0.0297
Platelet	0.926	−0.018	0.236	0.227	0.086	0.324
Total bilirubin	0.69	0.076	0.301	−0.195	0.127	−0.285
Sodium	0.76	0.0581	0.0987	0.307	0.103	0.304
AST	0.0913	0.314	0.411	−0.156	0.389	−0.163
ALT	0.416	0.154	0.214	−0.234	0.348	−0.178
D-dimer	0.506	−0.129	0.505	−0.129	0.403	−0.161
Procalcitonin	0.411	−0.156	0.215	−0.233	0.142	−0.274
CRP	0.671	−0.0808	0.545	−0.115	0.552	0.113
NT-pro BNP	0.263	−0.27	0.122	0.367	0.438	0.189
IL-6	0.961	−0.0261	0.252	0.556	0.165	0.647

ALT, alanine aminotransferase; AST, aspartate aminotransferase; cf-DNA, cell-free DNA; CRP, C-reactive protein; IL-6, interleukin-6; MPO-DNA, myeloperoxidase DNA complex; NE-DNA, neutrophil elastase DNA complex; NT-pro BNP, N-terminal fragment brain natriuretic peptides; WBC, white blood cell counts.

Table 3. Correlation between the predictive scores of IVIG unresponsiveness and NETs markers

	cf-DNA		MPO-DNA		NE-DNA	
	p	r	p	r	p	r
Kobayashi score	0.119	0.291	0.0104	−0.461	0.000716	−0.583
Egami score	0.366	0.171	0.0129	−0.449	0.029	−0.399
Sano score	0.914	0.0206	0.44	−0.146	0.923	0.0185

cf-DNA, cell-free DNA; MPO-DNA, myeloperoxidase DNA complex; NE-DNA, neutrophil elastase DNA complex.

but there was no significant increase in NETs markers in the IVIG＋PSL group at any blood collection time point. There was no significant correlation between NETs markers and any laboratory values which are presumed to reflect the severity of inflammation in patients with KD. These results suggest that NETs markers may not closely correlate with KD severity.

In this study, a correlation between NETs markers and KD severity was not demonstrated. In the IVIG group, elevated cf-DNA levels were observed before IVIG administration and elevated NE-DNA levels were observed before and after IVIG administration. On the other hand, in the IVIG＋PSL group, no increase in NETs markers was observed at any of the blood sampling times. These results suggest that NETs may have occurred in some children with KD, but were not consistent. The results of this study were inconsistent with previous studies showing NETs formation in children with KD. Yoshida et al. measured the number of NETs formed and the titers of cf-DNA and NE-DNA in vitro using neutrophils isolated from the peripheral blood of 37 children with KD and 6 healthy controls[14]. Spontaneous NETs formation was observed in neutrophils isolated from children with KD, and the number of NETs formed was significantly higher in the acute period of KD than in the convalescent period of KD and healthy controls. Hu et al. also cultured neutrophils isolated from the peripheral blood of seven KD children and seven healthy controls and analyzed the number of NETs formed using flow cytometry[15]. The number of NETs formed during the acute period of KD was significantly increased compared to healthy controls and decreased after IVIG treatment compared to the acute period. These studies suggested the involvement of NETs in KD. The discrepancies between our study and previous studies may be explained by the following reasons. First, the assessment procedures for NETs are different. We measured NETs markers directly in clinical samples, whereas previous studies have evaluated NETs in vitro. It is difficult to perfectly replicate the in vivo environment in vitro. NETs that do not form in vivo may have formed in vitro. Second, the timing of blood sampling was different between the previous studies and our study. The blood samples in the acute period were collected at 4 to 6 days of illness in the study by Yoshida et al., at 1 to 10 days of illness in the study by Hu et al., and at 3 to 16 days of illness in our study. It is speculated that the formation of NETs changes dynamically over time from the onset of KD. Thus, the timing of blood sampling may affect the assessment of NETs.

Despite the lack of correlation between NETs markers and the severity of KD in this study, it is remarkable that NETs markers were elevated in some patients with KD, especially those in IVIG group before IVIG. This suggests that NETs may be involved to some extent in the development of KD. The previous studies did not examine the correlation between NETs markers and the severity of KD[14)15)]. In the reports by Yoshida et al[14)], NETs formation was surely enhanced in patients with KD, but not in all patients. Enhanced NETs formation was not seen in several patients. It is presumed that the effects of NETs will not be uniform among patients independent of the severity of KD, although NETs may contribute to the development of KD.

We divided patients with KD based on the prediction scores of IVIG unresponsiveness as we hypothesized that these scores will reflect the severity of inflammation. It has been estab-

lished that patients with KD who are unresponsive against IVIG have an elevated risk of CAL. Laboratory values adopted in these prediction scores include day of illness at initial treatment, age, percentage of neutrophils, platelet count, total bilirubin, AST, ALT, sodium and C-reactive protein. In fact, in this study, serum sodium level was lower, and serum ALT and procalcitonin levels were higher in the IVIG+PSL group, i.e. patients fulfilling at least one of IVIG unresponsiveness prediction scores. Therefore, it seems justified that we classified severity of KD according to these predictive scores. No laboratory values showed significant correlation with NETs markers in this study. These results suggest that NETs markers will not be suitable for determining severity of KD.

There are several limitations to our study. First, the number of children with KD in the study population is small. Our study included children with KD from a single hospital. Therefore, the results of this study should be validated in a larger number of children with KD. Second, because our study was a retrospective study, we could not uniformize the timing of blood sampling among children with KD. Therefore, the evaluation of NETs formation may be inadequate. Prospective studies with scheduled blood sampling are necessary for the evaluation of NETs formation in children with KD. Third, the controls in our study may have been inappropriate. All control samples were collected during growth hormone-stimulating tests, which may have affected NETs formation. In addition, controls were older than children with KD. However, there was no significant correlation between age and NETs markers. Therefore, the impact of age differences on our study results is considered relatively small. It is difficult to collect blood samples from age-matched healthy children, but studies with more appropriate controls are desirable.

CONCLUSIONS

In our study, NETs markers were not correlated with KD severity throughout the clinical course, and no findings to support the involvement of NETs in KD were obtained. As our study has various limitations, it is premature to conclude that NETs formation is not involved in KD. Prospective studies analyzing the detailed time course of NETs markers with a large number of cases and appropriate controls are needed to clarify the involvement of NETs in KD.

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