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Comparison of serum biomarkers for the diagnosis of macrophage activation syndrome complicating systemic juvenile idiopathic arthritis

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ABSTRACT

Our study aimed to compare the accuracy of serum biomarkers for the diagnosis of macrophage activation syndrome (MAS) complicating systemic juvenile idiopathic arthritis (s-JIA). Serum cytokine levels (neopterin, IL-18, and CXCL9 and soluble tumor necrosis factor receptor type I (sTNFR-I) and II) were determined by enzyme-linked immunosorbent assay in 78 patients with s-JIA, including 21 with MAS. Receiver operating characteristic curve analysis revealed area under the curve values and cut off values of neopterin, IL-18, CXCL9, sTNFR-II/I ratio and ferritin were 0.9465/19.5 nmol/l, 0.8895/69250 ng/ml, 0.9333/3130 pg/ml, 0.9395/3.796 and 0.8671/2560 ng/ml, respectively. Serum neopterin levels were significantly elevated in patients with MAS and those were correlated positively with disease activity. In conclusion, serum neopterin levels may be used as a promising indicator of disease activity in s-JIA and MAS and for evaluating it. It may also be a useful marker to diagnose the transition to MAS from active-phase s-JIA.

1. Introduction

Macrophage activation syndrome (MAS) is a severe life-threatening condition that complicates systemic juvenile idiopathic arthritis (s-JIA). Clinical features of MAS are characterized by fever, splenomegaly, haemorrhages; and involvement of liver, central nervous system, and kidney; and eventually, multiple organ failure [1]. Laboratory findings include decreases in haemoglobin and white blood cell and platelet counts, hypertransaminasaemia, hyperferritinaemia, and evidence of intravascular coagulation [1]. A proper diagnosis of MAS is essential to start appropriate therapeutic interventions and change unfavourable outcomes. However, it is often difficult to distinguish MAS from s-JIA flares, sepsis, or haemophagocytic lymphohistiocytosis (HLH), especially in the early stage of MAS. Differentiating MAS from these conditions is essential for selecting appropriate therapeutic interventions in a timely manner. However, there is no definite clinical or laboratory parameter that can effectively diagnose MAS.

The hallmark of MAS includes uncontrolled and dysfunctional immune responses involving continual activation and expansion of T lymphocytes and macrophages, which in turn lead to marked hypercytokinaemia [2]. Interleukin (IL)-1, IL-6, and IL-18 play important roles in s-JIA pathogenesis [3]. However, the role of interferon (IFN)-γ and tumor necrosis factor (TNF)-α becomes dominant over that of IL-6 and IL-1β as MAS develops [4,5]. IL-18 and IL-6 overproduction might be associated with MAS development through NK cell dysfunction [6,7]. Moreover, IFN-γ and TNF-α is a key cytokine in the pathogenesis of MAS as well as primary and other secondary HLH [8–13]. Recent reports have shown that serum levels of IFN-γ and IFN-γ-induced chemokines, soluble TNF receptor type II /I ratio (sTNFR-II/I) are markedly elevated in patients with MAS compared with those in patients with active-phase s-JIA without MAS [4,5,14].

Neopterin, a 2-amino-4-hydroxy-(1′,2,′3′-trihydroxypropyl)-pteridine, is produced by activated monocytes/macrophages from guanosine triphosphate (GTP) via GTP cyclohydrolase I [15]. The activity of this enzyme is greatly enhanced by IFN-γ and, to a lesser extent, by IFN-α, other cytokines, and endotoxins [15]. IFN-γ, which is released by activated helper T lymphocytes type 1 and natural killer cells, is the most potent inducer of neopterin production. However, measuring

Abbreviations: MAS, macrophage activation syndrome; s-JIA, systemic juvenile idiopathic arthritis; IL, interleukin; sTNFR, soluble tumor necrosis factor receptor; TNF, tumor necrosis factor; EBV-HLH, Epstein–Barr virus-induced haemophagocytic lymphohistiocytosis; KD, Kawasaki disease; HCs, healthy controls; IFN, interferon; PSL, prednisolone; PBMCs, peripheral blood mononuclear cells

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neopterin levels is advantageous over measuring IFN-γ levels; IFN-γ quickly binds to target structures or gets neutralized by soluble receptors, whereas neopterin is biochemically inert and stable in serum [16]. Therefore, serum neopterin levels are closely linked to the activity of cellular immune system, demonstrating T cell–macrophage interplay.

In this study, to compare the accuracy of serum biomarkers for the diagnosis of MAS complicating s-JIA, we measured serum levels of neopterin, IL-18, CXCL9, sTNFR-II/I and ferritin in 78 patients with s-JIA including 21 with MAS and compared the results. Furthermore, to confirm clinical significance of serum neopterin levels as a promising indicator of disease activity in s-JIA and MAS, these levels were measured in 125 patients with s-JIA, including 30 with MAS, and determined their correlation with disease activity and severity.

2. Materials and methods

2.1. Patients and samples

To compare the accuracy of serum biomarkers for the diagnosis of MAS complicating s-JIA, serum cytokine levels (neopterin, IL-18, CXCL9, sTNFR-II/I and ferritin) were determined in 78 patients with s-JIA. Of the 78 patients with s-JIA, 21 presented with MAS, 8 of whom presented with MAS at the time of study referral and 13 developed complicating MAS during the acute disease phase and after starting steroid therapy. Serum samples from the 21 patients with s-JIA were obtained at the time of MAS diagnosis. Serum samples were obtained from 13 of these 21 patients during acute-phase s-JIA before developing MAS complication. In addition, serum samples were obtained from 57 patients with acute-phase s-JIA without MAS. Clinical characteristics of these 70 patients with acute-phase s-JIA were shown in Table 1. Of 70 patients in total, 49 received no treatment, whereas 21 were treated with prednisolone (PSL). In addition to PSL, four patients received cyclosporine, one received methotrexate and tacrolimus, and five received tocilizumab. Clinical characteristics of patients with MAS are shown in Supplementary Table 1. Of 21 patients with MAS, 7 received no treatment, 14 were treated with PSL, one was treated with methylprednisolone pulse therapy, four were treated with cyclosporine, and six were treated with tocilizumab.

Next, to confirm clinical significance of serum neopterin levels as a promising indicator of disease activity in s-JIA and MAS, serum neopterin levels were measured in 125 patients with s-JIA, including 15 with Epstein–Barr virus-induced HLH (EBV-HLH), 15 with Kawasaki disease (KD), and 28 age- and sex-matched healthy controls (HCs). Of the 125 patients with s-JIA, 30 presented with MAS, 13 of whom presented with MAS at the time of study referral and 17 developed complicating MAS during the acute disease phase and after starting steroid therapy. Serum samples from the 30 patients with s-JIA were obtained at the time of MAS diagnosis. Serum samples were obtained from 17 of these 30 patients during acute-phase s-JIA before developing MAS complication. In addition, serum samples were obtained from 95 patients with acute-phase s-JIA without MAS. Clinical characteristics of 112 patients with acute-phase s-JIA are shown in Table 2. Of 112 patients in total, 80 received no treatment, whereas 32 were treated with PSL. In addition to PSL, eight patients received cyclosporine, one received methotrexate and tacrolimus, and four received tocilizumab. Clinical characteristics of patients with MAS are shown in Supplementary Table 2. Of 30 patients with MAS, 11 received no treatment, 18 were treated with PSL, one was treated with methylprednisolone pulse therapy, four were treated with cyclosporine, and six were treated with tocilizumab.

s-JIA was diagnosed based on the International League of Associations for Rheumatology criteria [17], and MAS was diagnosed based on the 2016 European League Against Rheumatism/American College of Rheumatology/ Pediatric Rheumatology International Trials Organization classification criteria [18,19]. Criteria for acute-phase s-JIA were defined based on following characteristics: active arthritis, fever, rash, hepatosplenomegaly, generalized lymphadenopathy, active uveitis, serositis, increased erythrocyte sedimentation rate, and increased C-reactive protein levels. Patients with sepsis or severe bacterial infection were excluded. Some patients had minimal joint disease at s-JIA onset, and the presence of arthritis was confirmed later. EBV-HLH was diagnosed based on the diagnostic criteria for EBV-HLH [20]: positivity for EBV genome in the blood, bone marrow, and other tissues (determined by PCR, Southern blot and/or in situ hybridization for EBV-encoded RNA) and presence of anti-viral capsid antigen-specific-IgG. KD diagnosis was made based on the classic clinical criteria [21]. Serum samples were separated from the blood, divided into aliquots,

Table 1
Clinical characteristics of 70 patients with acute-phase s-JIA to compare the accuracy of serum biomarkers for the diagnosis of MAS complicating s-JIA.

Patients	70
Age	7.1 ± 4.5
Sex (male; female)	36; 34
Disease duration (months)	5.3 ± 10.6
Clinical symptoms	
Fever	65
Rash	47
Hepatomegaly	5
Splenomegaly	4
Lymphadenopathy	16
Pleuritis	2
Pericarditis	6
Affected joint counts	1.5 ± 2.2
Laboratory findings	
CRP (mg/dl) (n = 112)	10.5 ± 8.2
AST (IU/l) (n = 104)	46.1 ± 53.1
LDH (IU/l) (n = 95)	389.6 ± 191.9
Ferritin (ng/ml) (n = 93)	2765.7 ± 7635.1
Treatments	
Prednisolone	21 (0.9 ± 0.6 mg/kg/day)
Cyclosporine	4
Metrexate and Tacrolimus	1
Tocilizumab	5

s-JIA, systemic juvenile idiopathic arthritis; CRP, C-reactive protein; AST, aspartate aminotransferase; LDH, lactate dehydrogenase.

Table 2
Clinical characteristics of 112 patients with acute-phase s-JIA to confirm clinical significance of serum neopterin levels as a promising indicator of disease activity in s-JIA and MAS.

Patients	112
Age	6.9 ± 5.1
Sex (male; female)	59; 53
Disease duration (months)	5.4 ± 10.0
Clinical symptoms	
Fever	112
Rash	45
Hepatomegaly	10
Splenomegaly	7
Lymphadenopathy	26
Pleuritis	2
Pericarditis	9
Affected joint counts	1.8 ± 3.5
Laboratory findings	
CRP (mg/dl) (n = 112)	10.9 ± 7.5
AST (IU/l) (n = 104)	43.4 ± 45.8
LDH (IU/l) (n = 95)	378.0 ± 193.8
Ferritin (ng/ml) (n = 93)	2362.2 ± 6202.7
Treatments	
Prednisolone	32 (1.1 ± 1.1 mg/kg/day)
Cyclosporine	8
Metrexate and Tacrolimus	1
Tocilizumab	4

s-JIA, systemic juvenile idiopathic arthritis; CRP, C-reactive protein; AST, aspartate aminotransferase; LDH, lactate dehydrogenase.

frozen, and stored at −80 °C until analysis. This study was approved by the Institutional Review Board of the Kanazawa University, and informed consents were obtained from all participants.

2.2. Measurement of serum cytokine levels

Serum neopterin, IL-18, CXCL, sTNFR-I, sTNFR-II and IL-6 levels were measured using commercial enzyme-linked immunosorbent assay kits, according to the manufacturer's instructions (neopterin: IBL, Hamburg, Germany; IL-18: MBL, Nagoya, Japan; IL-6, CXCL9, sTNFR-I and sTNFR-II: R&D Systems, Inc., Minneapolis, MN, USA).

2.3. Statistical analysis

Multiple comparisons between groups were performed using Kruskal–Wallis test and Dunn's test. Within-group comparisons were performed using Mann–Whitney *U* test. The comparison of serum neopterin levels between acute-phase s-JIA and MAS was performed using paired *t*-test. Correlations were expressed using Spearman rank correlation coefficient. *P* values of < 0.05 were considered significant.

3. Results

3.1. Comparison of serum biomarkers for the diagnosis of MAS complicating s-JIA

To compare the accuracy of serum biomarkers for the diagnosis of MAS complicating s-JIA, we measured serum levels of neopterin, IL-18, CXCL9, sTNFR-II/I and ferritin in 78 patients with s-JIA including 21 with MAS and compared the results. As shown in Table 3, receiver operating characteristic (ROC) curve analysis revealed cut off values of neopterin, IL-18, CXCL9, sTNFR-II/I ratio and ferritin were 19.5 nmol/l, 69,250 ng/ml, 3130 pg/ml, 3.796 and 2560 ng/ml, respectively. Area under the ROC curve values of neopterin, IL-18, CXCL9, sTNFR-II/I ratio and ferritin were 0.9465, 0.8895, 0.9333, 0.9395 and 0.8671, respectively.

3.2. Elevation in serum neopterin levels in MAS complicating s-JIA

ROC curve analysis revealed serum neopterin levels had the highest area under the ROC curve value and neopterin is most accurate biomarker for the diagnosis of MAS complicating s-JIA. Therefore, we measured serum neopterin levels in patients with s-JIA during acute and MAS phases and compared them with those observed in patients with EBV-HLH or KD.

As shown in Fig. 1A, serum neopterin levels were significantly elevated during the acute phase in patients with active s-JIA [median, 12.2 (range, 1.9–78.0) nmol/L, $P < .0001$], MAS [median, 50.3 (range, 18.0–240.0) nmol/L, $P < .0001$], EBV-HLH [median, 72.0 (range, 44.0–280.0) nmol/L, $P < .0001$], and KD [median, 13.5 (range, 7.0–50.0) nmol/L, $P < .0001$] compared with those in HCs [median, 4.35 (range, 1.80–9.50) nmol/L]., Moreover, serum neopterin levels were significantly elevated in patients with MAS compared with those in patients active s-JIA ($P < .0001$) and KD ($P < .0001$); they were also significantly elevated in patients with EBV-HLH compared with those in patients with active s-JIA ($P < .0001$), MAS ($P < .05$), and KD ($P < .0001$).

Table 3
Receiver operating characteristic curve analysis of serum biomarkers for the diagnosis of MAS complicating s-JIA.

Biomarkers	Cut off values	Area under the ROC curve values
Neopterin	19.5	0.9465
IL-18	69,250	0.8895
CXCL9	3130	0.9333
sTNFR-II/I	3.796	0.9395
Ferritin	2560	0.8671

ROC: Receiver operating characteristic.

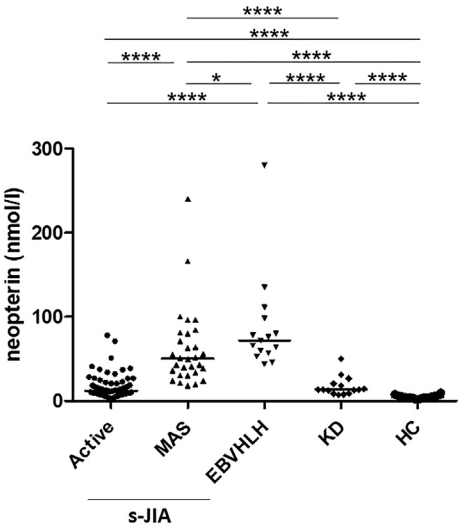

Fig. 1. Serum neopterin levels in different patient groups.
Bars represent median values. Statistically significant differences among the patient groups are shown as $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$. Active, patients with acute-phase systemic juvenile idiopathic arthritis; MAS, macrophage activation syndrome; EBV-HLH, Epstein–Barr virus-associated haemophagocytic syndrome; KD, Kawasaki disease; HC, healthy control.

ROC curve to differentiate between active s-JIA and MAS was > 29.25; at this level, the sensitivity and specificity were 83.3% and 92.0%, respectively. The area under the ROC curve was 0.9548, odds ratio was 57.2 (95% confidence interval, 17.63–185.8), and the likelihood ratio was 10.37.

3.3. The time course of changes in serum neopterin levels in MAS complicating s-JIA

We compared serum neopterin levels during acute-phase s-JIA before developing MAS and at the time of MAS diagnosis in 17 patients with MAS complicating s-JIA. As shown in Supplementary Fig. 1, serum neopterin levels were significantly elevated in the MAS phase compared with those in the acute phase. To investigate the relevance of serum neopterin levels in the pathogenesis of MAS complicating s-JIA, serum neopterin levels were serially monitored in three patients with MAS complicating s-JIA (Fig. 2A–C), which revealed a marked and rapid increase as MAS developed.

3.4. Correlation between serum neopterin levels and measures of disease activity in patients with MAS complicating s-JIA

Serum ferritin, aspartate aminotransferase (AST), and lactate dehydrogenase (LDH), IL-18, and IL-6 levels were clinically used as indicators of disease activity in s-JIA. We assessed the correlation between serum neopterin levels and these indicators. Results showed that neopterin levels positively correlated with ferritin, AST, LDH, and IL-18 levels (Fig. 3A–D) but did not correlate with IL-6 levels (Fig. 3E).

4. Discussion

In clinical settings, a timely and prompt diagnosis of MAS and differentiation of MAS from s-JIA flares or other secondary HLH are essential for selecting appropriate therapeutic interventions. In this study, we compared the accuracy of serum biomarkers including IFN-γ related molecules, neopterin, CXCL9 and IL-18, TNF-α related molecules,

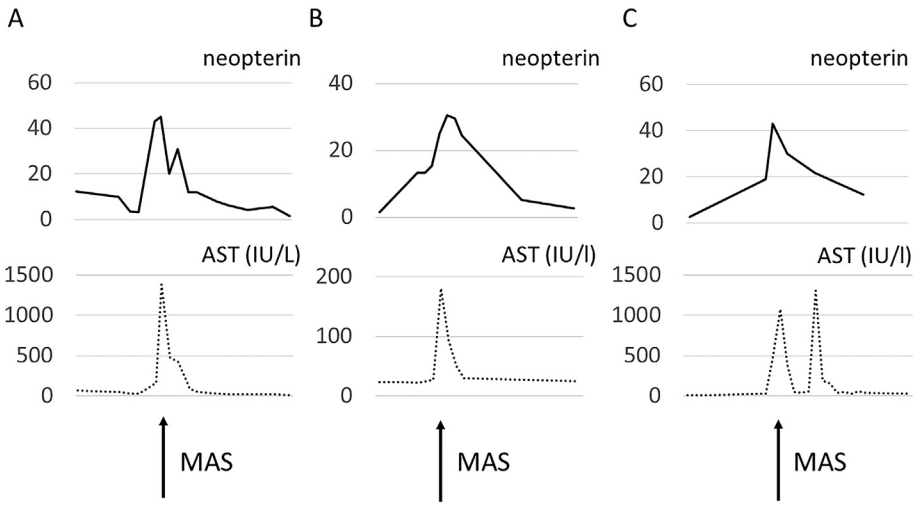

Fig. 2. Longitudinal follow-up of serum neopterin levels in three patients with MAS.
Changes in serum neopterin levels (solid lines) are shown in upper panels and AST levels are shown in lower panels.
MAS, macrophage activation syndrome; AST, aspartate aminotransferase.

sTNFR-I, sTNFR-II and ferritin for the diagnosis of MAS complicating s-JIA. ROC curve analysis revealed serum neopterin levels had the highest area under the ROC curve value, indicating that neopterin is most accurate biomarker for the diagnosis of MAS complicating s-JIA.

MAS closely resembles HLH and is classified as secondary HLH [22]. Primary HLH is caused by mutations in genes encoding molecules, including PRF1, UNC13D, STXBP2, STX11, and RAB27A, that are involved in granule exocytosis, leading to defective cytotoxic T cells and NK cells [23]. Pathogenesis and genetic basis of MAS are not completely understood; however, recent studies have shown that profoundly depressed NK cell function associated with abnormal perforin expression is observed in patients with MAS and that these abnormalities are related to causative genes of primary HLH [24–28]. These findings indicate that MAS and primary HLH have a shared mechanism.

IFN-γ plays a pivotal role in primary HLH pathogenesis. High circulating IFN-γ levels are found in patients with primary HLH and

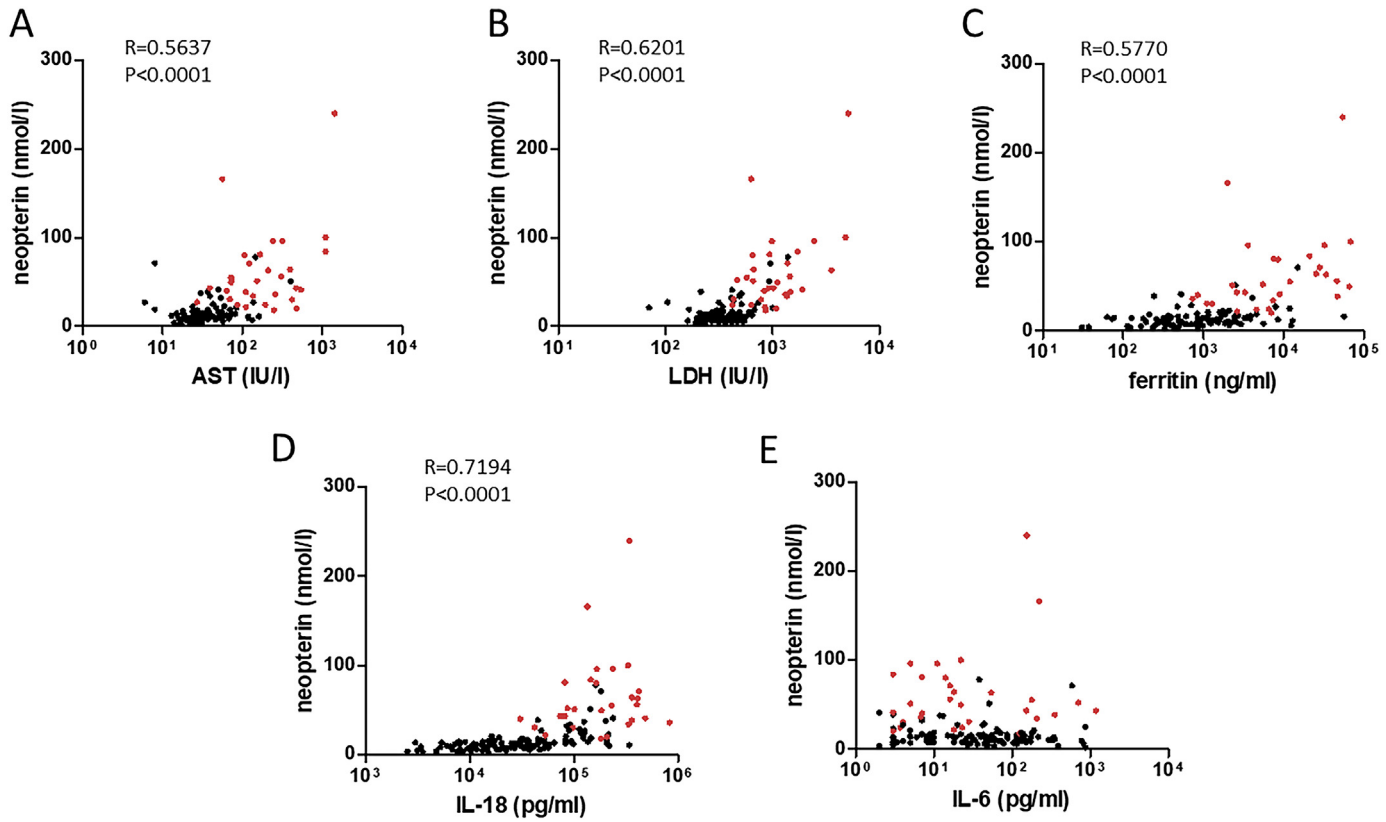

Fig. 3. Correlations between serum neopterin levels and other measures of disease activity.
(A) AST, (B) LDH, (C) ferritin, (D) IL-18, and (E) IL-6. Patients complicated with MAS are shown as red circles.
AST, aspartate aminotransferase; LDH, lactate dehydrogenase; IL, interleukin; MAS, macrophage activation syndrome. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

infection-related secondary HLH [9–11]. High circulating IFN-γ levels are also observed in animal models of primary HLH [12,13]. Furthermore, clinical symptoms of HLH have been shown to be inhibited by anti-IFN-γ antibody treatments in these models [12]. Although the role of IFN-γ in MAS remains mostly unclear, recent reports have shown that serum levels of IFN-γ and IFN-γ-induced chemokines are markedly elevated in MAS and that these levels are significantly correlated with disease activity [4,14]. Furthermore, in the animal model of MAS, serum ferritin levels have been shown to be significantly correlated with CXCL9 mRNA levels in the liver and spleen [4]. Taken together, these findings suggest that IFN-γ plays a pivotal role in the pathogenesis of MAS as well as other HLH.

On the other hand, the role of IFN-γ in s-JIA seems controversial. The results of previous reports measuring serum IFN-γ levels in patients with s-JIA are not always in agreement. Gattorno et al. have reported that serum IFN-γ levels are significantly elevated in patients with active s-JIA compared with those in HCs [29], whereas Put et al. have reported that plasma IFN-γ levels are moderately elevated in patients with s-JIA, although these results are not statistically significant [14]. Furthermore, de Jager et al. have reported that plasma IFN-γ levels do not increase in patients with s-JIA [30]. Microarray analysis has demonstrated no evidence of IFN-γ-induced gene expression in peripheral blood mononuclear cells (PBMCs) obtained from patients with active s-JIA [14,31,32]. Upon stimulating these PBMCs with IFN-γ in vitro, a normal response has been noted [14,33]. These findings suggest that IFN-γ plays a minimal role in s-JIA and that it is not required for s-JIA development.

We have previously reported that serum IL-18 levels are highly elevated in patients with s-JIA, and abnormal IL-18 production appears to be specific to s-JIA [34]. IL-18 is a signature IFN-γ-inducing cytokine; therefore, a slight increase in serum IFN-γ levels is in sharp contrast with highly elevated IL-18 levels in s-JIA. de Jager et al. have reported a defective IFN-γ production by NK cells upon stimulation with IL-18 in patients with s-JIA [6]. IL-18 may impair adequate functioning of NK cells. In the pathogenesis of MAS complicating s-JIA, IFN-γ becomes dominant over IL-6 and IL-1β [4]. Although the source of serum IFN-γ is still unclear, Put et al. have reported that endothelial cells and fibroblasts express IFN-γ-induced proteins in situ in the lymph node staining results of a patient with MAS [14]. These findings suggest that in addition to PBMCs, these cells can contribute to an IFN-γ dominant profile in MAS. In the same study, PBMCs from HLH patients have shown hyporesponsiveness to IFN-γ, which might have been due to functional exhaustions [14]. Taken together, these findings suggest that IFN-γ hyperproduction from various cells, including endothelial cells and fibroblasts, might be closely related to MAS pathogenesis.

We have previously reported that serum IL-18 levels are significantly higher in patients with active-phase s-JIA who later develop MAS than in those who do not develop MAS [35]. However, no significant difference in serum IL-18 levels has been observed patients with MAS complicating s-JIA measured either before or during MAS phase. These findings indicated that serum IL-18 levels are a useful predictor of MAS development during acute-phase s-JIA, but they are not valuable in terms of diagnosing the transition from acute-phase s-JIA to MAS. Highly elevated serum IFN-γ and IFN-γ-induced protein levels have been observed in MAS and HLH compared with those in active s-JIA [4,14]. These findings suggest that a combined monitoring of the levels of IL-18 and IFN-γ or IFN-γ related molecules are useful for diagnosing MAS.

As shown in Fig. 1, serum neopterin levels were significantly elevated in patients with MAS and EBV-HLH compared with those in patients with active s-JIA, KD, and HCs. Furthermore, serum neopterin levels in patients with EBVHLH characterized by IFN-γ producing $CD8^+$ T cell clonal expansion were significantly elevated compared to those in patients with MAS complicating s-JIA. These results indicated that serum neopterin levels reflect the degree of lymphohistiocytosis in MAS. Furthermore, as shown in Supplementary Fig. 1, serum neopterin levels significantly increased during the MAS phase compared with those during active-phase s-JIA. As shown in Fig. 2, these levels profoundly and rapidly increased as MAS developed. These findings suggested that serum neopterin levels may represent a promising indicator of disease activity in MAS and may also be a useful marker to predict the transition to MAS from acute-phase s-JIA. Specifically, patients with s-JIA with serum neopterin levels > 29.25 nmol/L, were at high risk of developing MAS. As shown in Fig. 3, serum neopterin levels were positively correlated with disease activity measures, such as ferritin, AST, LDH, and IL-18 levels. They significantly increased in patients with severe MAS with highly elevated AST, LDH, and ferritin levels. These results indicated that IFN-γ overproduction may be closely related to a wide range of tissue damage associated with MAS pathogenesis.

In conclusion, serum neopterin levels may represent a promising indicator of disease activity in MAS and may also be a useful marker to predict the transition to MAS from acute-phase s-JIA. A combined monitoring of IL-18 and neopterin levels might be useful for a timely and prompt diagnosis of MAS and for differentiating MAS from s-JIA flares or other secondary HLH.

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Declaration of Competing Interest

The authors have no conflicts of interest to disclose. The authors have no financial relationship to this article to disclose.

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