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Original article

Neutrophil-derived lipocalin-2 in adult-onset Still's disease: a novel biomarker of disease activity and liver damage

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Abstract

Objective. Liver damage is a common manifestation and can be life-threatening in adult-onset Still's disease (AOSD), an autoinflammatory disease. The hallmark of AOSD is activation of neutrophils, whose infiltration in liver is suspected to promote tissue injury. Here we aimed to identify a candidate biomarker and to validate its association with liver damage in AOSD.

Methods. Transcriptome analysis of neutrophils from treatment-naïve active AOSD patients and healthy donors was performed. Lipocalin-2 (LCN2) expression was assessed in neutrophils, plasma and liver biopsies of AOSD. The correlations of LCN2 with different variables and its ability to identify liver damage from AOSD patients were analysed.

Results. LCN2, a novel biomarker in hepatic inflammation, was found to be upregulated in AOSD neutrophils by RNA sequencing and confirmed at the mRNA and protein levels. Plasma levels of LCN2 were significantly higher in AOSD patients than healthy controls, RA and SLE patients. Plasma LCN2 levels were closely correlated with inflammatory markers, systemic score, HScore and cytokines. Moreover, LCN2 levels were increased in active AOSD with liver involvement and independently associated with liver dysfunction. Enhanced expression of LCN2 was detected in liver biopsies from three patients with ongoing liver injury. Furthermore, the area under the curve value of LCN2 for identifying AOSD with liver injury from other liver diseases was 0.9694.

Conclusion. Our results reveal that neutrophils-derived LCN2 is higher in plasma and liver tissue in AOSD patients than in healthy controls, and it could serve as a potent biomarker for identifying AOSD with systemic inflammation, especially liver damage caused by hyperinflammation.

Key words: adult-onset Still's disease, neutrophil, lipocalin-2, liver damage, biomarker

CLINICAL SCIENCE

Introduction

Adult-onset Still's disease (AOSD), a rare systemic autoinflammatory disease of uncertain aetiology, is characterized by spiking fever, evanescent rash, arthralgia or arthritis, and neutrophilia [1]. A large percentage of AOSD patients present with increased levels of hepatic enzymes [2]. Fulminant hepatic failure is a serious consequence of severe AOSD, with a very high mortality [3]. As an example, our previous study reported that hepatic failure could be one of leading causes of death in patients with AOSD [4]. The diagnosis of AOSD still relies on symptoms and nonspecific laboratory tests, and needs to rule out infectious and neoplastic diseases, including some manifesting as liver damage [5]. However, to date, specific biomarkers to separate AOSD patients with liver damage from those without are still lacking. Therefore, there is an unmet need for a

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Rheumatology key messages

- Lipocalin-2 was identified as a novel biomarker for patients with adult-onset Still's disease.
- Lipocalin-2 was increased in active adult-onset Still's disease with liver involvement, and independently associated with liver dysfunction.

sensitive biomarker to distinguish AOSD from other patients with liver dysfunction (LD) at an early stage and to predict severity of liver damage.

The pathogenesis of AOSD is still to be determined, but many lines of evidence suggest that innate immune activation could cause arthritis, skin rash and liver injury [6]. The fundamental characteristic of AOSD pathogenesis is neutrophil activation, with potent tissue-injuring and cytotoxic capacity [7]. Neutrophils are also believed to be responsible for the initiation and development of inflammation by releasing a wide variety of granule enzymes and antimicrobial proteins in AOSD [8]. During the flare of disease, >80% of patients present with a neutrophilic leucocytosis, which allows differentiation of AOSD from other fevers of unknown origin [9]. Recently, studies from our laboratory have shown that neutrophil extracellular traps, a newly defined cellular component, play a vital role in the pathogenesis of AOSD by activating NLR family pyrin domain containing 3 inflammasome and stimulating macrophages for cytokine production [10]. Importantly, increased neutrophil infiltration in the liver has been described as one of the typical histological characteristics of AOSD patients with liver involvement [11]. In this study, we explored the gene expression signature of neutrophils in AOSD patients, especially those closely related to inflammatory damage in liver. The biological pathway of neutrophil degranulation was mostly enriched and lipocalin-2 (LCN2) was significantly upregulated in active AOSD patients.

LCN2, also known as neutrophil gelatinase-associated lipocalin, is a 25 kDa protein expressed in a number of tissues and immune cells, particularly neutrophils [12]. In response to inflammatory stimuli, both expression and secretion of LCN2 are increased, which in turn aggravates inflammatory response by modulating the oxidative stress and inducing the production of pro-inflammatory cytokines [13]. It has been widely implicated as a potential non-invasive biomarker for many pathological disorders, including acute and chronic kidney injury, sepsis, cardiovascular disease, chronic obstructive pulmonary disease and various cancers [14–16]. Recently, LCN2 has been considered as a promising novel biomarker in human liver diseases [17]. Both neutrophilic inflammation and hepatic injury are shown to be associated with elevated LCN2 in liver, which can further induce accumulation of neutrophils and contribute to chronic inflammation in local tissues [18].

Based on the linkage of LCN2 to neutrophil-associated liver inflammation, we hypothesized that LCN2 could be a potential biomarker to assess disease activity and liver injury in AOSD patients. In the current study, we first investigated whether levels of LCN2 in plasma and neutrophils were elevated in AOSD patients. We also examined the relationship between plasma LCN2 levels and clinical parameters, especially liver biochemical tests. Subsequently, we analysed LCN2 expression in liver biopsies of patients with AOSD. Moreover, LCN2 expression in AOSD with LD *vs* liver injury with other reasons was measured.

Methods

AOSD patients and HC subjects

The first cohort of patients consisted of 6 treatment-naïve active AOSD patients and 6 sex- and age-matched healthy donors as controls. Neutrophils were collected and used for RNA sequencing analysis. The clinical characteristics of the first cohort are summarized in Supplementary Table S1, available at *Rheumatology* online.

The second cohort of patients and controls consisted of 109 AOSD patients (78 active and 31 inactive AOSD patients) and 120 controls [29 RA, 29 SLE patients and 62 healthy controls (HCs)]. All AOSD patients fulfilled Yamaguchi's criteria after exclusion of those with infectious, neoplastic and autoimmune disorders. All HC subjects were recruited from age- and sex-matched volunteers with no history of rheumatic or other diseases. Information on demographic and clinical data was entered into a database together with the laboratory test results. The disease activity of each AOSD patient was assessed using a modified Pouchot score [19]. AOSD-haemophagocytic lymphohistiocytosis (HLH) was defined as an AOSD patient who fulfilled the HLH-2004 criteria [20] and AOSD-macrophage activation syndrome (MAS) was defined using the 2016 EULAR/ACR/PRINTO classification criteria [21] for MAS complicating systemic JIA (JIA-MAS criteria). The HScore of each AOSD patient was calculated according to the previous report [22].

Measurement of plasma LCN2

LCN2 levels in plasma of patients were measured by commercial sandwich ELISA (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions.

Immunohistochemistry

Liver biopsies from patients with AOSD ($n = 3$), autoimmune hepatitis ($n = 2$), primary biliary cholangitis ($n = 2$), primary sclerosing cholangitis ($n = 1$), alcoholic cirrhosis ($n = 2$), drug-induced liver failure ($n = 2$), hepatic cirrhosis caused by HBV infection ($n = 2$) and HCs ($n = 2$) were analysed for protein expression by immunohistochemistry using rabbit polyclonal anti-LCN2 (absin, Shanghai, China). The liver biopsies of HCs are obtained from patients with haemangioma during surgery with normal liver histology, which could be used as the normal liver tissues according to previous studies [23]. The reaction was then visualized under light microscopy (BX51 Olympus, Tokyo, Japan).

The study was performed in accordance with the Declaration of Helsinki and the principles of Good Clinical Practice. Biological samples were obtained under a protocol approved by the Institutional Research Ethics Committee of Ruijin Hospital (ID: 2016-62), Shanghai, China. All subjects signed written informed consent.

Statistical analysis

All data were statistically analysed using the SPSS version 20.0 software (SPSS Inc., Chicago, IL, USA). Data between two groups with a Gaussian distribution were analysed by two-tailed *t* test, while nonparametric data were assessed using the Mann–Whitney *U* test. Data among three groups or more were analysed using Kruskall–Wallis test followed by *post hoc* Dunn's test. Spearman's correlation analysis was used to test the association between plasma LCN2 levels and different variables. Multivariate analyses were performed using variables with *P* values of <0.05 in the univariate analysis. The Wilcoxon signed-rank test was performed to compare plasma LCN2 levels in patients who underwent follow-up plasma sampling. Statistical significance was defined as $P < 0.05$.

Methodology describing the assessment of LD, RNA preparation, RNA sequencing, quantitative real-time PCR and immunoblotting are provided in detail in the Supplementary Material, available at *Rheumatology* online.

Results

Proinflammatory signature in neutrophils from AOSD patients

Neutrophils were isolated from the peripheral blood of six patients with treatment-naïve active AOSD and six healthy donors. The gene expression profiling was then comprehensively analysed using RNA sequencing. Principal component analysis of neutrophil transcriptomes showed clear difference between active AOSD patients and healthy donors. Using cut-offs of >2.0-fold change with $P < 0.05$, we identified 864 differentially expressed genes in neutrophils from active AOSD patients compared with healthy donors, including 442 and 422 upregulated and downregulated genes in active AOSD, respectively (Fig. 1A).

By gene ontology analysis, we identified several strongly enriched terms of biological process. The most significantly indicated gene ontology term was neutrophil degranulation (Fig. 1B). To further investigate expression of granule proteins, we identified a list of granule proteins from previously published proteomic analysis of neutrophil granule subpopulations [24]. We compared expression levels of these granule proteins depending on granule location (azurophilic, specific, gelatinase) (Fig. 1C). Genes of secreted granule proteins were significantly enriched in AOSD neutrophils, including *MMP8*, *LCN2*, *LTF*, *HP* and *PRTN3* (Fig. 1D). Of interest, among the five candidate upregulated genes, *LCN2*, which encodes LCN2 has been demonstrated to play a pathogenetic role in liver damage. We therefore concentrated on LCN2 as the target. To confirm the upregulation of LCN2, we then examined the expression of LCN2 in neutrophils. Consistently, neutrophil expression of LCN2 at the mRNA and protein levels was markedly increased in AOSD patients compared with HCs (Fig. 1E–G).

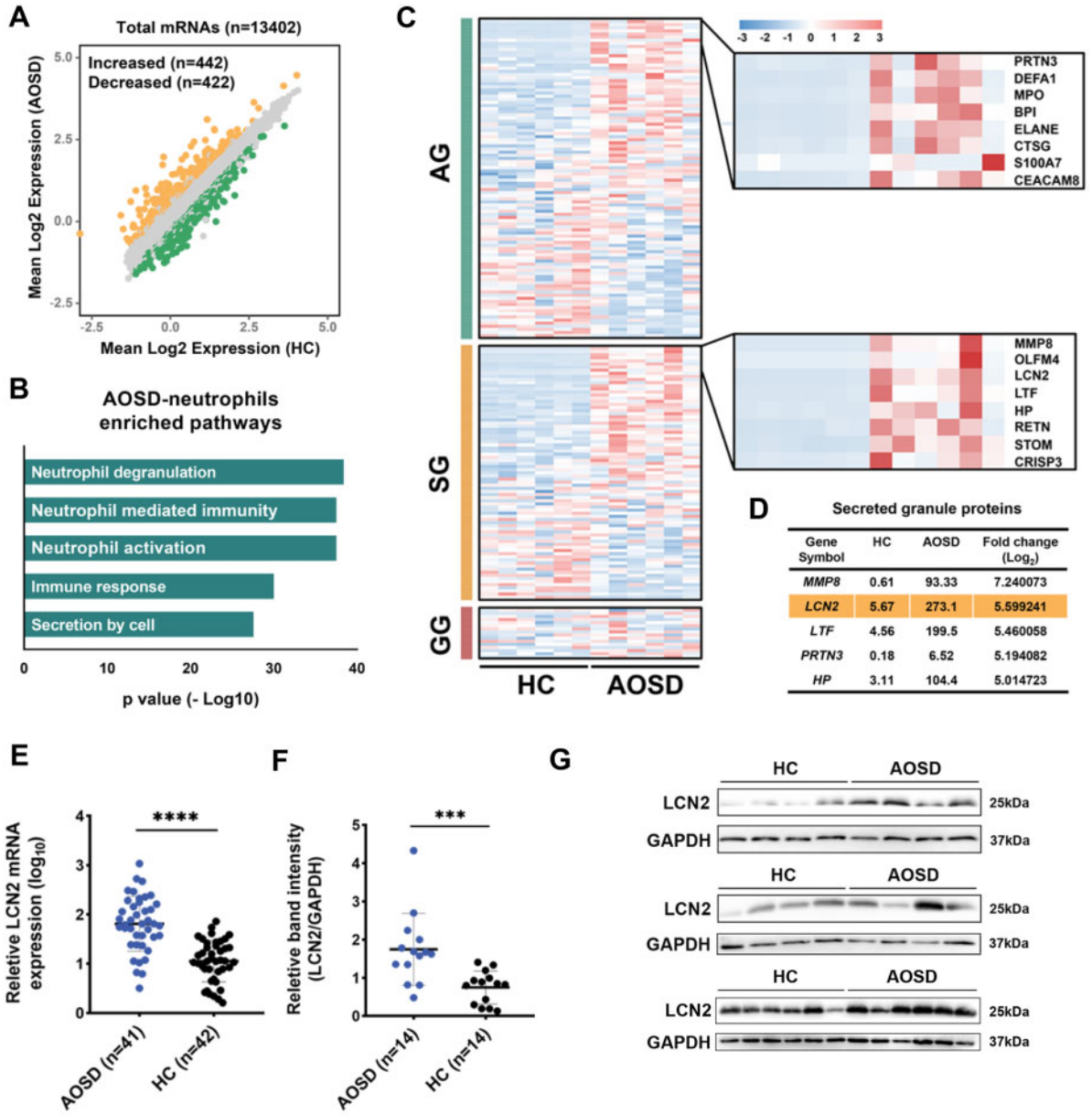
Higher levels of LCN2 in plasma of AOSD patients

To determine whether circulating LCN2 derived from neutrophils could be a potential biomarker in diagnosis of AOSD, we then measured the level of LCN2 in plasma from the second cohort. Plasma from 109 AOSD patients (78 active and 31 inactive patients) and 120 controls (29 RA, 29 SLE patients and 62 HCs) were collected. The demographic, clinical and biological characteristics of these subjects are shown in Table 1. There was no significant difference in the distribution of age and sex among subjects in each group. Compared with HCs, we detected a significantly higher level of LCN2 in plasma from AOSD patients (Fig. 2A, $P < 0.0001$). Moreover, the plasma levels of LCN2 were significantly higher in AOSD patients than RA ($P < 0.0001$) and SLE ($P = 0.0004$) patients.

Increased levels of LCN2 in active AOSD

Furthermore, we investigated plasma LCN2 levels in AOSD patients with diverse disease activity. As shown in Fig. 2B, the plasma LCN2 levels were significantly increased in active AOSD ($n = 78$) compared with those with inactive disease ($n = 31$) ($P < 0.0001$). During follow-up, LCN2 showed a significant decrease after treatment (mean ± S.D., 117.3 ± 61.42 *vs* 52.4 ± 33.39 ng/ml, $P = 0.0001$, Fig. 2C). The LCN2 levels were positively correlated with AOSD systemic disease activity ($r = 0.74$, $P < 0.0001$, Fig. 2D). We also measured the correlation of plasma LCN2 levels with routine laboratory inflammatory markers, including leucocyte counts, CRP, ESR and ferritin. The plasma LCN2 levels were strongly correlated with leucocyte counts (Fig. 2E, $r = 0.44$, $P < 0.0001$), CRP (Fig. 2F, $r = 0.55$, $P < 0.0001$), ESR (Fig. 2G,

FIG. 1 Transcriptome analysis of neutrophils from AOSD patients and identification of LCN2 upregulation in neutrophils



(**A**) Transcriptome analysis of neutrophils from patients with treatment-naïve active AOSD ($n = 6$) and healthy donors ($n = 6$) was performed. (**B**) Gene ontology pathway analysis of upregulated genes in neutrophils from AOSD. (**C**) Expression heatmap of neutrophil granule proteins across neutrophil granule subtypes in AOSD and healthy donors. (**D**) The expression level and fold-change (log2) of upregulated granule proteins. (**E**) *LCN2* mRNA level (relative expression values related to GAPDH) was measured by qRT-PCR in neutrophils. (**F**) LCN2 protein level was measured by immunoblot in AOSD neutrophils. (**G**) Representative immunoblot analysis for LCN2 in neutrophils. The results show the means ± S.D. ***$P < 0.001$, ****$P < 0.0001$. LCN2: lipocalin-2; HC: healthy controls; AOSD: adult-onset Still's disease; AG: azurophilic granules; SG: specific granules; GG: gelatinase granules; LTF: lactotransferrin; PRTN3: proteinase 3; HP: haptoglobin; qRT-PCR: quantitative real-time PCR; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

$r = 0.42$, $P < 0.0001$) and ferritin (Fig. 2H, $r = 0.57$, $P < 0.0001$). In addition, we investigated MAS, the most severe complication of AOSD, in active AOSD patients. Among 78 active AOSD patients, 6 patients (7.7%) could be diagnosed as AOSD-HLH by the HLH-2004 criteria and 24 patients (30.8%) could be diagnosed as

Table 1 Demographic and clinical characteristics of individuals with AOSD and controls

	AOSD ($n = 109$)				
	Active ($n = 78$)	Inactive ($n = 31$)	SLE ($n = 29$)	RA ($n = 29$)	HC ($n = 62$)
Age, years	40.57 ± 15.49	41.03 ± 15.75	35.69 ± 16.22	32.48 ± 10.57	34.69 ± 7.72
Sex (F/M)	55/23	26/5	21/8	21/8	41/21
Clinical manifestations					
Fever	67 (85.9)	0	8 (27.6)	3 (10.3)	
Arthralgia	58 (74.4)	0	10 (34.5)	25 (86.2)	
Skin rash	58 (74.4)	0	9 (31.0)	1 (3.4)	
Sore throat	49 (62.8)	0	1 (3.4)	0	
Lymphadenopathy	64 (82.1)	0	6 (20.7)	0	
Splenomegaly	27 (34.6)	0	2 (6.9)	2 (6.9)	
Hepatomegaly	7 (9.0)	0	1 (3.4)	0	
Myalgia	21 (26.9)	0	4 (13.8)	3 (10.3)	
Pericarditis	15 (19.3)	0	2 (6.9)	0	
Pleuritis	17 (21.8)	1 (3.2)	6 (20.7)	0	
Laboratory features					
Haemoglobin, g/l	105.0 ± 17.5	123.5 ± 10.0	110.0 ± 20.8	128.3 ± 15.0	
Leukocytes, 10^9/l	12.65 ± 6.77	9.09 ± 3.67	4.43 ± 2.30	7.01 ± 1.99	
Platelets, 10^9/l	277.7 ± 113.9	225.6 ± 69.75	154.6 ± 71.07	217.4 ± 47.30	
ESR, mm/h	58.03 ± 33.80	18.91 ± 14.35	38.56 ± 29.56	26.44 ± 22.58	
CRP, mg/l	68.33 ± 63.03	6.87 ± 6.36	10.33 ± 14.01	4.29 ± 5.57	
ALT, U/l	45.70 ± 49.27	18.52 ± 9.13	47.04 ± 111.4	16.56 ± 9.9	
AST, U/l	55.25 ± 47.94	17.38 ± 7.36	40.14 ± 75.14	42.00 ± 9.90	
Ferritin, >1500 ng/ml	41 (52.6)	0	0	0	
Creatinine	54.24 ± 13.50	61.24 ± 13.01	58.12 ± 12.94	54.73 ± 10.55	
ANA positivity	6 (7.7)	5 (16.1)	29 (100)	4 (13.8)	
RF positivity	0	0	6 (20.7)	24 (82.8)	
Treatments					
Steroid- and sDMARD-naïve	62 (79.5)	0	20 (69.0)	6 (20.7)	
Steroid monotherapy	12 (15.4)	3 (9.7)	5 (17.2)	4 (13.8)	
Steroids + sDMARD(s)	4 (5.1)	22 (71.0)	4 (13.8)	19 (65.5)	

All values are presented as numbers (with percentage) or mean ± S.D. AOSD: adult-onset Still's disease; HC: healthy control; ALT: alanine transaminase; AST: aspartate transaminase; sDMARD: synthetic DMARD; F: female; M: male.

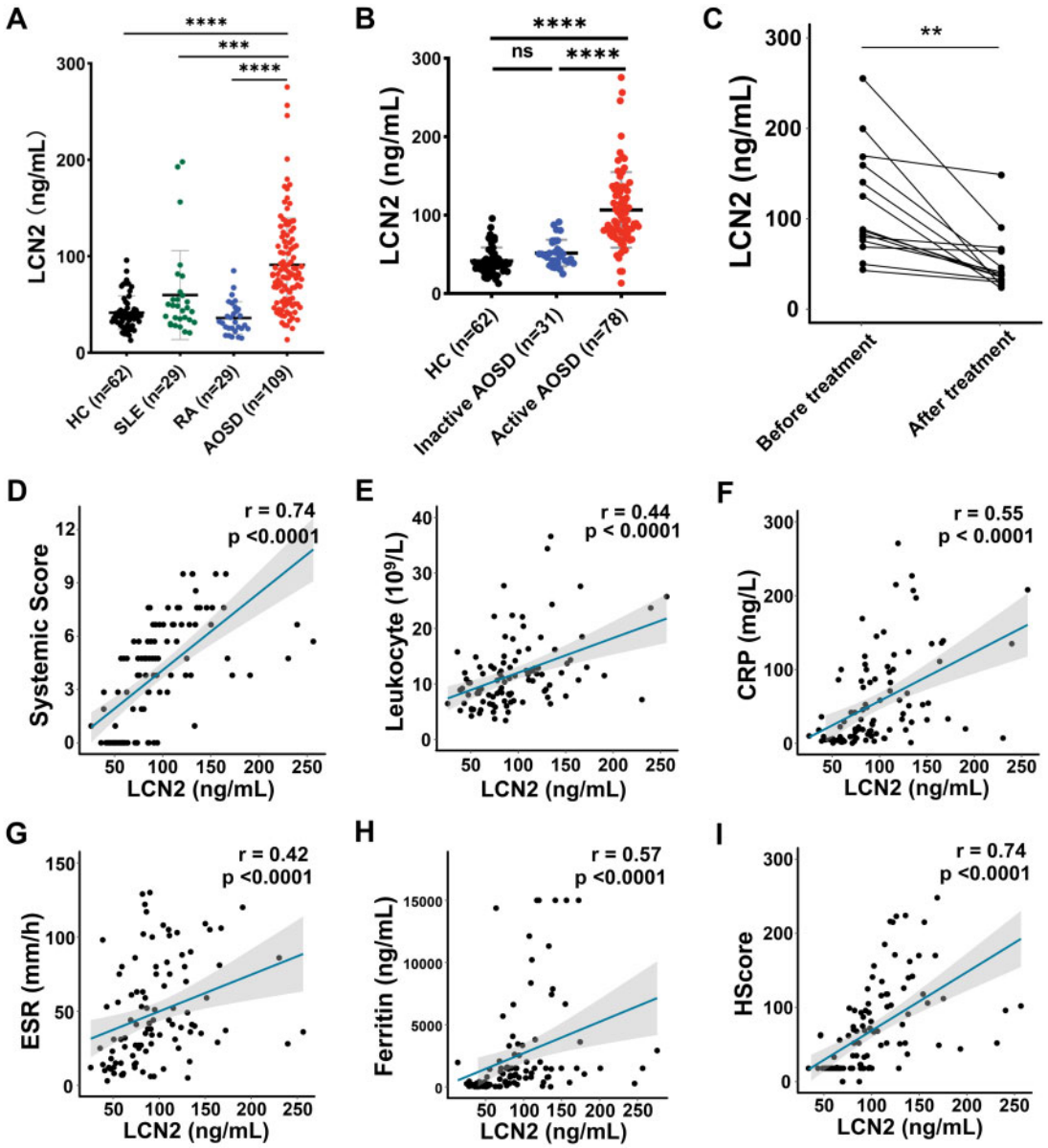
AOSD-MAS according to the systemic JIA-MAS criteria. We found AOSD-HLH and AOSD-MAS displayed significantly higher levels of LCN2 compared with those without MAS (supplementary Fig. S1, available at *Rheumatology* online). HScore, a newly developed scoring system for evaluating and diagnosing reactive hemophagocytic syndrome, was also correlated with LCN2 (Fig. 2I, $r = 0.74$, $P < 0.0001$). In view of the importance of cytokine storm in AOSD pathogenesis, we then analysed the correlation of plasma LCN2 levels with cytokine levels. We found that LCN2 levels showed a strong positive correlation with IL-1β ($r = 0.5571$, $P = 0.0017$), IL-6 ($r = 0.4980$, $P = 0.0044$), IL-10 ($r = 0.4573$, $P = 0.0097$) and IL-18 ($r = 0.5694$, $P = 0.0008$), but not TNF-α ($r = 0.3153$, $P = 0.0840$) (supplementary Table S2, available at *Rheumatology* online). These data suggest a close relationship between LCN2 and systemic inflammation.

LCN2 level was associated with liver function in AOSD

Considering the close association between LCN2 and liver inflammation, we then assessed plasma LCN2 levels in active AOSD patients with or without LD. LD in AOSD was defined by abnormal values of hepatic enzymes, including alanine transaminase (ALT), aspartate transaminase (AST), ALP and gamma-glutamyltransferase (GGT). There was no significant difference in age, sex, BMI or inflammatory index (neutrophils, CRP, ESR) between AOSD patients with and without liver damage (supplementary Table S3, available at *Rheumatology* online).

Interestingly, we found active AOSD with LD had markedly higher levels of LCN2 than active AOSD without LD ($P < 0.0001$, Fig. 3A). The receiver operator characteristic (ROC) plot of plasma LCN2, as a predictor of LD in active AOSD patients, had an area under the curve of 0.8533 ($P < 0.0001$, 95% CI 0.7688, 0.9378, S.E. 0.0431), with a specificity of 78.43% and sensitivity of 77.78% using a cut-off value of 86.86 ng/ml (Fig. 3B). In order to further evaluate the potential indicators that can be used to identify AOSD with liver damage, uni- and multivariate logistic regression analyses were performed (Table 2). The plasma LCN2 level, rather than the ferritin level (>1500 ng/ml) or routine inflammatory markers, was an independent predictive factor for LD. Serial

FIG. 2 Plasma LCN2 level is increased in AOSD and correlates with disease activity in AOSD patients

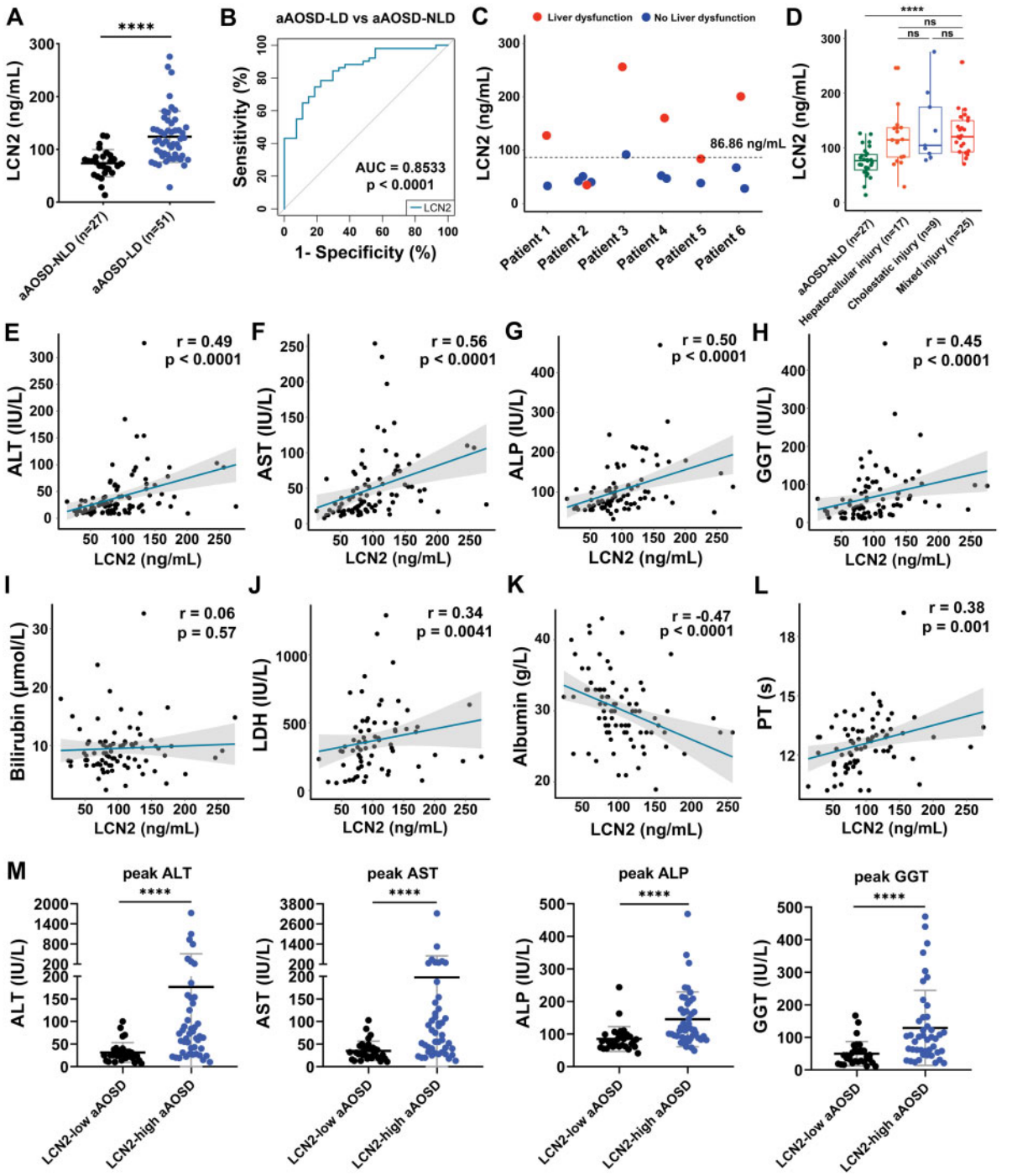


(**A**) The concentration of LCN2 in plasma of patients with AOSD ($n = 109$), RA ($n = 29$), SLE ($n = 29$) or HCs ($n = 62$) were determined by ELISA. (**B**) Comparison of LCN2 level among AOSD patients with active ($n = 78$) and inactive disease ($n = 31$) as well as in HCs ($n = 62$). (**C**) Reduced levels of LCN2 in 14 AOSD patients after treatment. (**D**) Correlation of plasma LCN2 level with systemic disease activity score in patients with AOSD. (**E**–**I**) Plasma LCN2 level was positively correlated with leucocyte counts, CRP, ESR, ferritin and HScore. ***$P < 0.001$; ****$P < 0.0001$; ns: not significant. LCN2: lipocalin-2; AOSD: adult-onset Still's disease; HCs: healthy controls.

samples from six AOSD patients were available, with liver function test data at each time point. LCN2 levels were generally higher during LD episodes compared with time points without LD (Fig. 3C).

Based on the type of liver test abnormalities, we classified liver injury into hepatocellular injury (ALT >40 U/l or AST >40 U/l), cholestatic injury (ALP >126 U/l or GGT >64 U/l) and hepatocellular-cholestatic mixed injury [(ALT >40 U/l or AST >40 U/l) and (ALP >126 U/l or GGT >64 U/l)]. Among all AOSD patients, 17 had hepatocellular injury, 9 had cholestatic injury and 25 had mixed injury. We found there was no significant difference in plasma LCN2 levels among these three groups (Fig. 3D). Considering the predictive value of LCN2 in renal involvement, we examined the correlation of LCN2 levels with creatinine levels; however, no statistical significance was established (supplementary Fig. S2, available at *Rheumatology* online).

FIG. 3 Plasma LCN2 level is increased in active AOSD with liver dysfunction



(**A**) Comparison of LCN2 level between aAOSD-LD ($n = 51$) and aAOSD-NLD ($n = 27$). (**B**) The predictive performance of LCN2 level was validated using ROC analysis and the performance was indicated by the AUC and *P*-value. (**C**) Display of LCN2 level from multiple visits in six AOSD patients with liver dysfunction. (**D**) LCN2 levels in aAOSD-LD stratified by liver function test. (**E–L**) LCN2 levels were positively correlated with ALT, AST, ALP, GGT, LDH, albumin and PT, respectively except for bilirubin. The correlations were evaluated with Spearman's test. Each plot represents an individual patient. (**M**) Comparisons of peak values of ALT, AST, ALP and GGT between active AOSD patients with different LCN2 levels. *P*-value <0.05 represents a significant difference. $^{****}P < 0.0001$; ns: not significant. LCN2: lipocalin-2; AOSD: adult-onset Still's disease; aAOSD-LD: active AOSD patients with liver dysfunction; aAOSD-NLD: active AOSD patients without liver dysfunction; ROC: receiver operator characteristic; AUC: area under the curve; ALT: alanine transaminase; AST: aspartate transaminase; GGT: gamma-glutamyltransferase; LDH: L-lactate dehydrogenase.

TABLE 2 Uni- and multivariable analysis for liver dysfunction in AOSD patients

Parameter	Univariate analysis			Multivariate analysis[a]		
	Odds ratio	95% CI	*P*-value	Odds ratio	95% CI	*P*-value
Sex (male)	2.011	0.829, 4.874	0.122			
Age, years	0.990	0.966, 1.014	0.413			
BMI, kg/m^2	1.027	0.886, 1.191	0.725			
Fever	37.784	10.330, 138.209	<0.0001	8.677	1.904, 39.533	0.005
Rash	7.722	3.198, 18.648	<0.0001			0.983
Arthralgia	3.154	1.403, 7.089	0.005			0.053
Neutrophil, 10^9/l	1.112	1.023, 1.208	0.012			0.394
ESR, mm/h	1.017	1.004, 1.030	0.009			0.201
CRP, mg/l	1.208	1.087, 1.342	0.0004			0.938
Ferritin, >1500 ng/ml	8.883	3.569, 22.107	<0.0001			0.084
Systemic score	1.840	1.479, 2.288	<0.0001			0.374
Plasma LCN2, ng/ml	1.065	1.040, 1.091	<0.0001	1.047	1.021, 1.074	0.0003

[a]Adjusted for age and sex. AOSD: adult-onset Still's disease; LCN2: lipocalin-2.

To further determine the relationship between plasma LCN2 levels and LD in AOSD, we assessed the association between plasma LCN2 levels and liver function tests. The results indicated that plasma LCN2 levels were positively correlated with ALT ($r = 0.49$, $P < 0.0001$), AST ($r = 0.56$, $P < 0.0001$), ALP ($r = 0.50$, $P < 0.0001$) and GGT ($r = 0.45$, $P < 0.0001$) (Fig. 3E–H). No significant correlation was seen between plasma LCN2 levels and bilirubin ($r = 0.06$, $P = 0.57$, Fig. 3I), possibly because few AOSD patients had elevated bilirubin. Moreover, plasma LCN2 levels had strong correlations with L-lactate dehydrogenase ($r = 0.34$, $P = 0.0041$), albumin ($r = -0.47$, $P < 0.0001$) and PT ($r = 0.38$, $P = 0.0010$) (Fig. 3J–L).

Next, we divided active AOSD patients into LCN2-low and LCN2-high groups based on the cut-off value defined by the ROC analysis. Then we compared the maximum values of liver enzymes during the hospitalization between two groups. Active AOSD with higher LCN2 displayed significant higher levels of peak ALT, AST, ALP and GGT (Fig. 3M, $P < 0.0001$ for all comparisons), indicating the potential value of LCN2 in predicting the severity of liver damage.

LCN2 overexpression in liver of AOSD patients

We next sought to explore the expression level of LCN2 in the liver of AOSD patients. As shown in Fig. 4A, AOSD patients showed interfacial hepatitis with multiple lobular necrosis, and significantly increased inflammatory cell infiltration compared with HCs. An increased number of LCN2-positive cells was found in AOSD patients, whereas LCN2 expression was barely observed in normal liver. In addition, liver biopsies from LD controls were analysed, including patients with autoimmune hepatitis ($n = 2$), primary biliary cholangitis ($n = 2$), primary sclerosing cholangitis ($n = 1$), alcoholic cirrhosis ($n = 2$), drug-induced liver failure ($n = 2$) and hepatic cirrhosis after HBV infection ($n = 2$). Despite varying degrees of inflammatory cell infiltration in LD controls, few cells were LCN2 positive.

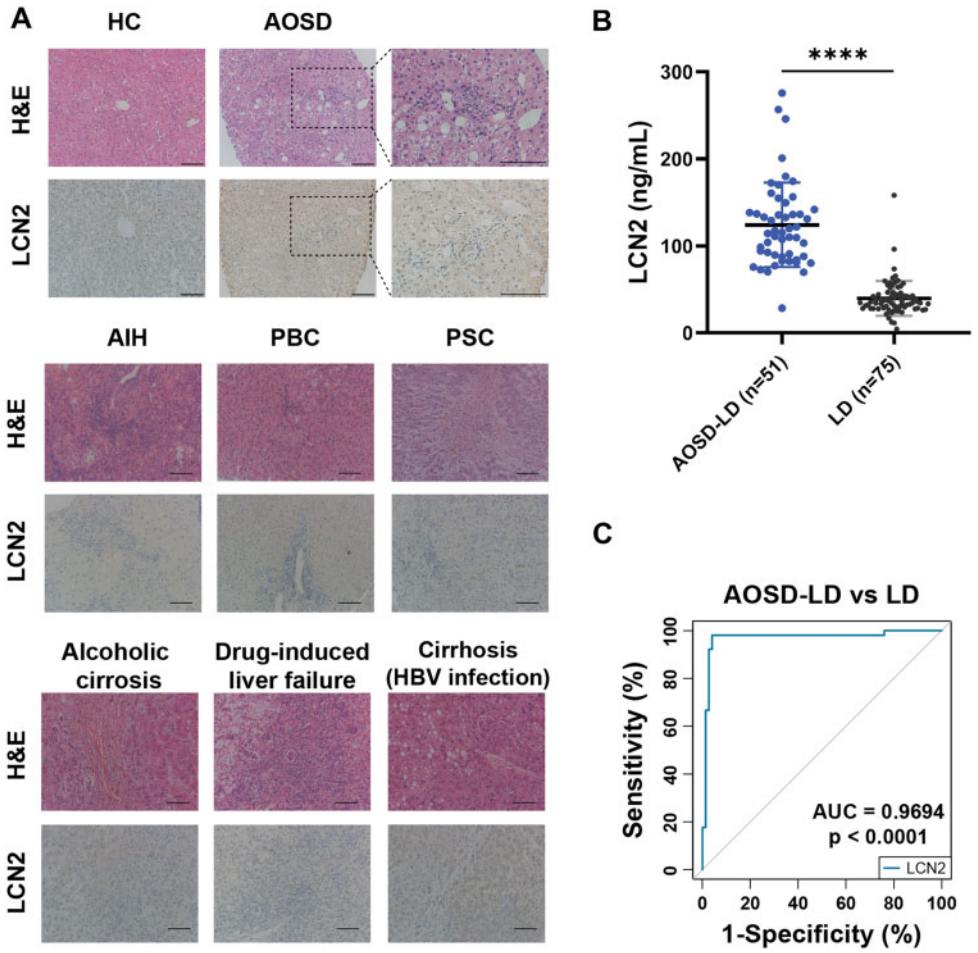
LCN2 as a potential biomarker of AOSD-LD

Ultimately, we analysed whether LCN2 concentrations were increased in AOSD patients with LD compared with LD controls ($n = 75$), including 9 patients with viral hepatitis, 16 patients with drug-induced liver injury, 11 patients with cirrhosis, 20 patients with autoimmune hepatitis and 20 patients with primary biliary cholangitis. The severity of liver damage, as assessed by routine liver function tests, was similar among these groups of patients, except bilirubin (supplementary Table S4, available at *Rheumatology* online). However, plasma LCN2 levels were significantly higher in AOSD than LD controls ($P < 0.0001$, Fig. 4B). In order to appraise the ability of plasma LCN2 to discriminate AOSD patients with liver abnormality from other LD patients, we performed ROC curve analysis. The result indicated that the area under the curve value of LCN2 was 0.9694 ($P < 0.0001$, 95% CI 0.9330, 1.0000). A cut-off point of 67.56 ng/ml was used to distinguish AOSD with LD from LD controls, yielding a sensitivity of 98.04% and a specificity of 96.00%, suggesting an important role of LCN2 in distinguishing liver damage in AOSD from other LD patients (Fig. 4C).

Discussion

The current study was performed to describe the transcriptome of neutrophils comprehensively and identify a novel biomarker for the diagnosis of AOSD with LD. Neutrophils from active AOSD patients showed a very strong enrichment of the neutrophil degranulation process, with *LCN2* as one of the top five upregulated granule genes. An increased expression level of LCN2 in neutrophils, plasma and liver in AOSD patients was confirmed, and plasma LCN2 was closely correlated to

FIG. 4 Increased LCN2 levels in liver and plasma can distinguish AOSD from liver dysfunction



(**A**) Representative H&E images and LCN2 immunohistochemistry of liver biopsies from AOSD ($n = 3$), HCs ($n = 2$) and LD controls. (**B**) Plasma LCN2 level was increased in AOSD compared with LD controls caused by other diseases. (**C**) ROC curve of LCN2 in distinguishing AOSD-LD from LD controls. ****$P < 0.0001$. LCN2: lipocalin-2; AOSD: adult-onset Still's disease; H&E: haematoxylin-eosin staining; AOSD-LD: AOSD patients with liver dysfunction; LD: liver dysfunction; HCs: healthy controls; ROC: receiver operator characteristic; AUC: area under the curve; AIH: autoimmune hepatitis; PBC: primary biliary cholangitis; PSC: primary sclerosing cholangitis.

disease activity and cytokines (IL-1β, IL-6, IL-10 and IL-18). Moreover, our study showed a markedly increased expression level of LCN2 in AOSD patients with LD compared with those in liver damage caused by other disorders. Remarkably, an indirect sign of LCN2 correlation with liver injury is also revealed by the significant decrease of plasma LCN2 levels in AOSD patients with liver involvement during follow-up. Our results suggest that LCN2 could be a potential biomarker to separate AOSD patients with liver damage from those without, and monitor ongoing hepatic damage.

AOSD is a rare systemic disease usually manifested as liver inflammation. The most frequent finding in liver of AOSD is mild hepatitis, while liver failure with massive hepatic necrosis can be seen in several cases [25, 26]. The prognosis can improved by carefully assessing the extent of hepatic damage and initiating appropriate treatment in a timely manner after diagnosis of AOSD. Several variables, such as hepatocyte-made proteins (i.e. CRP, transferrin) and endogenous damage-associated molecular patterns, are shown to reflect the severity of hepatic inflammation, while they are very common biomarkers for most acute hepatitis [27]. Evidence has suggested that serum IL-18 is elevated in AOSD patients with hepatitis, but with no statistical significance, partly because IL-18 is a nonspecific pro-inflammatory cytokine [28]. Therefore, in the clinic, routine liver function test remains the only noninvasive method to identify LD; however, elevations of liver enzymes are a nonspecific outcome of hepatic injury and a biomarker is still lacking for the diagnosis of liver damage in AOSD patients [29].

Increased neutrophil infiltration is a major pathological feature in liver of AOSD [11]. Neutrophils are the

predominant infiltrating cells in almost all acute inflammation and most chronic inflammation [30, 31]. Excessive neutrophil activation has been widely recognized to mediate tissue damage in many types of liver injury [32]. When migrating to the site of hepatic inflammation, neutrophil causes hepatocyte injury through degranulation, production of proinflammatory cytokines and reactive oxygen species, etc [33, 34]. To further understand the pathogenic role of neutrophils in acute inflammation of AOSD patients, we performed a transcriptome analysis of neutrophils from active AOSD patients. Compared with healthy donors, neutrophils from active AOSD patients displayed a proinflammatory signature including neutrophil degranulation, neutrophil activation, immune response and defence response by gene ontology pathway analysis. Interestingly, a number of differentially expressed genes have previously been demonstrated to play a pathogenetic role in AOSD, such as MMP8, S100 calcium binding protein A12 (S100A12) and calprotectin (S100A8-S100A9 proteins) [1].

In our study, *LCN2* was identified as one of the five most significantly upregulated genes encoding granule proteins in AOSD neutrophils compared with healthy donors. Indeed, several experimental studies have demonstrated that LCN2 plays a central role in hepatic inflammation and liver injury. Mechanistically, in murine models of nonalcoholic steatohepatitis, LCN2 induced infiltration of neutrophils and macrophages via the induction of CXC receptor 2, leading to hepatic damage [35]. Moreover, another study demonstrated that neutralization of LCN2 by antibody protected against neutrophilic infiltration and liver injury after acute alcohol challenge [18]. Recently, a few clinical reports have investigated altered expression of LCN2 in serum, plasma and different types of fluids and tissues in human with various liver diseases, including cirrhosis, acute-on-chronic liver failure and nonalcoholic steatohepatitis [17]. Both urine and plasma LCN2 levels were markedly increased in a cohort of $>$700 acute-on-chronic liver failure patients, and correlated with liver failure and systemic inflammation. In addition, *LCN2* gene expression was higher in patients with acute-on-chronic liver failure syndrome [36]. Furthermore, in another study, a strong relationship between neutrophil-derived LCN2 and hepatic injury was detected in patients suffering from alcoholic steatohepatitis. Serum concentrations of LCN2 were also higher in alcoholic steatohepatitis compared with non-alcoholic fatty liver disease patients [18]. Similarly, urine level of LCN2 was closely associated with liver fibrosis score in patients with chronic hepatic C infection [37]. These studies indicate that LCN2 level can be used as a potential biomarker for hepatic dysfunction. In addition, LCN2 has been well-studied as a possible biomarker for its potential clinical usefulness in kidney injury [38]. We also examined the relationship between plasma LCN2 levels and renal function in order to exclude the effect of kidney injury on LCN2.

We further demonstrated an elevated level of LCN2 in liver biopsies in AOSD patients. Importantly, LCN2 have been shown to act as chemoattractant for neutrophils and promotes cell adhesion and extravasation [39], which may partly explain the increased infiltration of neutrophils in liver of AOSD. Therefore, we suggested that LCN2 secretion from hyperactive neutrophils might play a pathogenic role in hepatic inflammation of AOSD, and it could serve as a diagnostic marker to identify AOSD in patients with unexplained LD. In this regard, we confirmed that plasma LCN2 concentrations in AOSD patients were markedly higher than patients with liver injury caused by other reasons. However, elucidation of the precise pathogenic mechanism of LCN2 in hepatic inflammation is still in the early stages, and further studies are required regarding its role in the pathogenesis of AOSD.

In conclusion, our investigation provided evidence of the effectiveness of LCN2 as a disease-specific biomarker for diagnosis of AOSD with systemic inflammation, especially liver damage.

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Supplementary data

Supplementary data are available at *Rheumatology* online.

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