Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Clinical Relevance for Circulating Cold Inducible RNA-Binding Protein (CIRP) in Patients with ASD

Yuya Fujita
Fukushima Kenritsu Ika Daigaku
Toru Yago
Fukushima Kenritsu Ika Daigaku
Tomoyuki Asano
Fukushima Kenritsu Ika Daigaku
Haruki Matsumoto
Fukushima Kenritsu Ika Daigaku
Naoki Matsuoka
Fukushima Kenritsu Ika Daigaku
Jumpei Temmoku
Fukushima Kenritsu Ika Daigaku
Shuzo Sato
Fukushima Kenritsu Ika Daigaku
Makiko Furuya
Fukushima Kenritsu Ika Daigaku
Eiji Suzuki
Ohta Nishinouchi Hospital: Ota Nishinouchi Byoin
Hiroshi Watanabe
Fukushima Kenritsu Ika Daigaku
Atsushi Kawakami
Nagasaki University: Nagasaki Daigaku
Kiyoshi Migita (migita@fmu.ac.jp)
Fukushima Medical University School of Medicine

Research article

Keywords: Adult Still's disease (ASD), danger-associated molecular patterns (DAMPs), Cold inducible RNA-binding protein (CIRP)

Posted Date: November 18th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-105955/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Abstract

Background: Adult Still's disease (ASD) is a systemic autoinflammatory disease, in which danger-associated molecular patterns (DAMPs)-mediated inflammasome activation seems to be involved in the disease pathogenesis. Cold inducible RNA-binding protein (CIRP) belongs to a family of cold-shock proteins that respond to cellular stress and has been identified as a DAMP that triggers the inflammatory response. The aim of this study is to investigate the clinical significance of serum CIRP levels in ASD.

Methods: Serum samples were obtained from 42 patients with active ASD or 50 patients with rheumatoid arthritis (RA) and 15 healthy control patients (HCs). Serum levels of CIRP and IL-18 were determined using enzyme-linked immunosorbent assay and compared with 42 patients with ASD or 50 patients with RA and 15 HCs.

Results were also analyzed according to the clinical features of ASD.

Results: Serum CIRP levels were significantly higher in patients with ASD compared with patients with RA (median: 9.6 ng/mL, IQR [5.7–14.4] versus 3.2 ng/mL, IQR [1.9–3.8]; $p < 0.001$) and with HCs (2.8 ng/mL, [IQR; 1.4–4.9], $p < 0.001$). There was a significant positive correlation between serum CIRP levels and ASD disease activity score (Pouchet's score $r=0.45$, $p=0.003$) as well as between ASD-specific biomarkers ferritin and IL-18. However, there was no significant difference in the serum CIRP levels among ASD patients with three different disease phenotypes. Intracellular CIRP expression on $CD14^+$ monocytes was elevated in patients with ASD compared with those in patients in RA.

Conclusion: These results suggest that CIRP may play a significant role in the pathophysiology of ASD and could be a potential biomarker for monitoring the disease activity of ASD.

Introduction

Cold-induced RNA-binding protein (CIRP) is a highly conserved 172-amino acid nuclear protein that belongs to the family of cold shock proteins [1]. CIRP functions as an RNA chaperon facilitating RNA translation and is ubiquitously expressed in various tissues [2]. CIRP is induced in response to stress, such as hypothermia, irradiation, and hypoxia, and is released into circulation resulting in cytokine induction by stimulating immune cells [3]. Whereas serum CIRP was undetectable or minimum in the HCs, its levels were elevated in the sera of patients with sepsis or organ-targeted ischemia [4]. In addition, increased tissue and serum levels of CIRP have been reported in several inflammatory diseases [5].

CIRP is expressed in various types of cells, including innate immune cells such as macrophages neutrophils, epithelial, and endothelial cells [6]. CIRP is released as extracellular CIRP (eCIRP) from these cells by lysosomal exocytosis pathway and passively by cellular necrosis [7]. eCIRP is a pro-inflammatory molecule that triggers inflammatory responses and organ damage, suggesting a role for CIRP in immune responses and inflammatory pathways [8]. CIRP is important as a damage-associated molecular protein

(DAMPs) and is implicated in the inflammatory diseases [9]. For example, CIRP is involved in nuclear factor-κB (NF-κB) activation and the regulation of interleukin-1β expression [10].

Adult Still's disease (ASD) is a systemic inflammatory disorder of unknown etiology [11]. Although pathogenesis of the disease is not fully clarified, inflammasome activation and subsequent IL-1β induction are involved in the disease pathogenesis in ASD [12]. Inflammasomes are multimeric protein complexes that typically comprise a sensor for PAMPs and DAMPs [13]. Recent studies demonstrated that eCIRP is important as a DAMPs [9]. The involvement of CIRP in ASD, however, is not well known. In this study, we hypothesized that the release of CIRP contributes to the inflammatory processes in ASD through the macrophage activation and subsequent cytokine production. Here we found that the serum levels of CIRP were elevated in patients with ASD and correlated with disease activity.

Materials And Methods

ASD patients and controls subjects

A total of 42 ASD patients and 15 healthy controls (HCs) were included in the training and validation sets. All patients diagnosed with ASD at Department of Rheumatology, Fukushima Medical University Hospital from 2005 to 2020 were enrolled. Patients included had to be 17 years old or older to be diagnosed as ASD according the diagnostic criteria of Yamaguchi after exclusion of those with infectious, neoplastic and autoimmune disorders [14]. As controls, 15 healthy subjects (5 males, 10 females, median age 38 years, interquartile range [IQR]; 32–45 years) were included. An additional independent set consisting of 50 patients with rheumatoid arthritis (RA) was used to determine the specificity of the values of CIRP in ASD patients. Among 50 patients with RA, 39 (78.0%) were female and their median age was 67 years, [IQR]; 62–74 years. The majority of the RA patients were taking disease-modifying anti-rheumatic drugs (DMARDs), mostly methotrexate (26/50, 52%), and biologics (17/50, 34%). Median DA28-ESR was 3.43 (IQR; 2.96–4.03).

Clinical Investigation And Data Collection

In the patient group, clinical, demographic, laboratory features and medical histories were collected by reviewing electronic medical records. The following demographic and clinical ASD-related characteristics were collected using a standardized form; gender, date of birth, age at diagnosis, duration of disease, past or family history of rheumatic diseases, presence of Still's disease-related rash, arthralgia, arthritis, myalgia, fever characteristics, lymphoadenopathy, and visceral involvement (serositis, liver damage). The following laboratory data were recorded: leukocyte and thrombocyte counts, hemoglobin, C-reactive protein (CRP), transaminase, lactate dehydrogenase (LDH), ferritin, and markers for hemophagocytosis (hypertriglyceridemis, hypofrbrinogenemia, hemophagocytosis in the bone narrow). The AOSD disease activity of each patient was assessed using a modified Pouchot score [15]. Data were collected were collected from using a stand data extraction form and were double-checked by two rheumatologists.

Patients were classified as having two disease pattern, the systemic or the articular manifestations as described previously [16].

Elisa Methods

Serum concentrations of CIRP and IL-18 were measured using enzyme-linked immnunosorbent assay (ELISA) kits (MBL, Nagoya, Japan) according to the manufacturer's instruction.

Isolation Of Cd14 Positive Monocytes

Peripheral blood was obtained from 2 patients with active ASD or RA. The peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood using Lymorphoprep TM (Axis-Shield, Oslo, Norway) cushion and isolated using density sedimentation according to the manufacturer’s instructions. CD14 positive monocytes were further sorted using anti-human CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) by positive sorting according to the manufacturer’s protocol.

Immunoblot Analysis

CD14 positive monocytes were harvested and lysed by RIPA Lysis Buffer (Sigma-Aldrich, St. Louis, MO, USA) supplemented with proteinases inhibitor cocktail on ice. The cellular lysates were centrifuged at 10,000 g for 10 minutes at 4 °C and the supernatant was collected. The cellular lysates were fractionated on 4–12% Bis-Tris gels (Thermo Fisher Scientific, Tokyo, Japan) and transferred to nitrocellulose membranes, which were blocked for1 h at room temperature with 5% bovine serum albumin. The membrane was incubated with primary antibodies against human CIRP or β-actin antibodies (Sigma-Aldrich, St.Louis, MO, USA) and then incubated with secondary antibodies at room temperature, followed by visualization using ECL reagent (Amersham, Little Chalfont, UK). Immunoblot detection was achieved by LAS-3000 Imaging System (Fuji Film, Tokyo Japan).

Statistical analysis

Results were non-normally distributed and are presented throughout the manuscript with median and 25–75th centiles [median, IQR] and were compared by the Mann-Whitney U test. Correlations between continuous variables were analyzed by the Spearman’s rank correlation test. Paired data were analyzed by Wilcoxon signed rank test. All data entry and statistical analyses were performed using SPSS Statistics version 22.0 (IBM, Armonk, NY). In all the analyses, a 2-tailed $p < 0.05$ was considered statistically significant.

Results

Clinical characteristics of patients with ASD

Serum samples were obtained from patients with ASD. Table 1 summarizes the baseline characteristics and laboratory data from the patients. The principal clinical symptoms of ASD included a high spiking fever (40/42 95.2%), skin rash (30/42 71.4%), arthralgia (27/42 64.2%), sore throat (13/42 30.9.9%), and splenomegaly (16/42 37.8%), respectively. After initial investigation, all patients were treated with corticosteroids, and 6 (14.3%) of them also received at least 1 biologics (Table 1).

Serum Levels Of Cirp In Patients With Asd

Serum levels of CIRP were determined by ELISA in patients with ASD, patients with RA, and HCs. As shown in Fig. 1, serum levels of CIRP were significantly higher in patients with ASD (median: 9.6 ng/mL, IQR [5.7–14.4]) compared to those in patients with RA (3.2 ng/mL, IQR [1.9–3.8]; $p < 0.001$) and in HCs (2.8 ng/mL, [IQR; 1.4–4.9], $p < 0.001$). Three patients with ASD were complicated with hemophagocytosis syndrome. There were no significant difference in serum levels of CIRP among patients with ASD with and without hemophagocytosis syndrome (median: 7.3 ng/mL, IQR [6.2–22.0] versus median: 9.6 ng/mL, IQR [5.9–13.9] $p = 0.92$). We also compared serum levels of CIRP according to the disease activity of ASD. In the subgroup patients with active ASD, serum levels of CIRP were significantly higher compared to those in inactive ASD patients (Fig. 2).

Relationship between serum levels of CIRP and laboratory parameters in patients with ASD

The correlation between serum levels of CIRP and laboratory parameters were evaluated in patients with ASD. As illustrated in Fig. 3, serum levels of CIRP showed a significant correlation with serum ferritin levels ($r = 0.47$, $p = 0.002$), but not with CRP levels (Fig. 4). As shown in Fig. 5, serum levels of CIRP were positively correlated with the ASD disease activity score (Pouchet's score $r = 0.46$, $p = 0.003$). Positive correlation was also demonstrated between serum levels of CIRP and IL-18 ($r = 0.33$, $p = 0.03$) in patients with ASD (Fig. 6). To determine whether serum CIRP could be used to differentiate ASD phenotypes, we compared serum levels of CIRP among 3 phenotypes of ASD. However, there was no significant difference in serum levels of CIRP among ASD patients with 3 different phenotypes (Fig. 7).

Longitudinal Observation Of Serum Levels Of Cirp

To explore the longitudinal changes of CIRP, we included 8 ASD patients with 2 longitudinal samples (at least 1 month apart). In the longitudinal study, 8 patients with active ASD were followed until they became inactive and then resampled. Serum levels of CIRP significantly declined in patients with ASD after immunosuppressive therapies (Fig. 8A), paralleling to the ASD disease activity score (Fig. 8B) as well as to serum levels of ferritin (Fig. 8C).

Intracellular Expressions Of Cirp In Cd14 Monocytes

Finally, to address the underlying mechanism for the elevated levels of circulating CIRP in ASD, we analyzed the intracellular expressions of CIRP in innate immune cells. We isolated $CD14^+$ monocytes from 2 patients with active ASD or RA. In the immunoblotting examination, intracellular CIRP expression on $CD14^+$ monocytes was elevated in patients with ASD compared with those in patients in RA.

Discussion

This is the first known study investigating the serum levels of CIRP in an autoinflammatory disease, ASD. Based on the current understanding of the molecular pathogenesis of ASD, a genetic background would confer the activation of innate immunity in response to environmental factors [12]. Damage-associated molecular patterns can activate innate immunity [17], and it was hypothesized that changes in several DAMPs are associated with ASD and mediate autoinflammatory cascade by activation of inflammasome [13]. CIRP is induced by cellular stresses and functions as a DAMP molecule that promote inflammatory responses [4]. We found that extracellular serum levels of CIRP, which is an endogenous DAMP, was elevated in patients with ASD. Increased serum and synovial fluid levels of CIRP were also reported in patients with RA and osteoarthritis, and the increased synovial levels of CIRP correlated with disease activity of RA [18, 19]. However, there was no significant difference in serum levels of CIRP between patients with RA and healthy controls in our study. In contrast, patients with ASD had significantly higher serum CIRP levels compared with those in patients with RA. The serum levels of CIRP were positively correlated with Pouchet's score, a disease activity score for ASD. Furthermore, the elevated levels of CIRP correlated with serum levels of IL-18, a signature cytokine for ASD [20]. Our data expanded the role of CIRP in an autoinflammatory disorder, ASD.

Intracellular CIRP (iCIRP) and eCIRP may have different functions. iCIRP has its role in the regulation of cellular stress responses, including mRNA stability, cell proliferation cell survival, and tumor formation [21]. In contrast to iCIRP, eCIRP is presumed to act as a DAMP-promoting inflammation and tissue injury [22]. Our preliminary data indicated the increased intracellular expressions of CIRP in monocytes isolated from patients with ASD. These findings suggest that the activated status of innate immune cells in ASD may contribute to the intracellular translocation and extracellular release of CIRP, leading to the elevated levels of eCIRP in patients with ASD. Inflammatory stimuli or cellular stress may trigger the translocation of CIRP from the nucleus to the cytosol and its release to the extracellular space in patients with ASD.

eCIRP was able to induce proinflammatory cytokines through activating innate immune cells [9]. For example, macrophage treated with recombinant CIRP releases TNF-α and HMGB-1 [23]. Moreover, previous studies demonstrated that CIRP induces the activation of NLRP3 inflammasome [24], resulting in the release of IL-1β, a critical cytokine in ASD.. Dysregulated NLRP3 inflammasome activation is associated with hereditary autoinflammatory diseases as well as with an acquired autoinflammatory diseases, ASD [25]. It is possible, therefore, that elevated serum CIRP may activate NLRP3 inflammasome and subsequent activated IL-1β induction implicated in the pathogenesis of ASD.

NLRP3, a pattern recognition receptor, is activated by DAMPs released from cells during cellular stress [26]. In ASD, PAMPs or DAMPs, in response to infections or environmental factors, transmit to innate immune cells through pattern recognition receptors which activate the NLRP3 inflammasome under the predisposing genetic background [12]. The underlying mechanism by which CIRP-induced inflammation has been proposed [27]. These findings suggest that extracellular CIRP could be an endogenous proinflammatory DAMP that triggers autoinflammation. Additional studies are warranted to confirm the mechanism through which CIRP could mediate autoinflammation seen in ASD.

This study has limitations. First, due to limited CIRP immunoblot data acquired from a small number of patients, it is difficult to determine the association between extracellular (serum) and intracellular CIRP expressions. Second, we conducted a single center retrospective cohort study with small sample size of patients; more studies based on larger cohort in additional sites are necessary to verify our findings.

In conclusion, serum concentrations of CIRP were elevated in patients with ASD. Furthermore, serum concentrations of CIRP correlated with serum levels of ferritin and disease activity score in patients with ASD. Our results suggest that CIRP may be implicated in the pathophysiology of ASD and be a potential marker for autoinflammation seen in ASD. These data provided us new insights into the role of CIRP in ASD and highlighted the therapeutic potential targeting of CIRP in the regulation of autoinflammation.

Abbreviations

ASD
adult Still’s disease
CIRP
cold inducible RNA-binding protein
DAMPs
danger-associated molecular patterns
IL-18
interleukin-18
NLRP3
NLR family pyrin domain containing 3
RA
rheumatoid arthritis,

Declarations

Ethical Approval

Ethical approval for this study (No. 2889) was provided by the Ethics Committee of Fukushima Medical University.

Consent for publication

Not applicable

Availability of supporting data

Not applicable

Competing interests

KM has received research grants from Chugai, Pfizer, and AbbVie. Rest of the authors declares that they have no competing interests

Funding

The study was supported by the Japan Grant-in-Aid for Scientific Research (20K08777).

Authors' contributions

YF, TA, NM, JT, SS, HM, MF, ES, HK, HW were involved in acquisition of clinical data. YF, TY and KM drafted manuscript.

YF, TY, KM carried out the biochemical studies, participated in the sequence alignment and drafted the manuscript. TY, AK, KM participated in the sequence alignment and drafted the manuscript. TY, AK, KM participated in the design of the study, FY performed the statistical analysis. All authors read and approved the final manuscript.

Acknowledgements

We are grateful to Ms Kanno Sayaka for her technical assistance in this study.

References

1. Nishiyama H, Higashitsuji H, Yokoi H, Itoh K, Danno S, Matsuda T, Fujita J. Cloning and characterization of human CIRP (cold-inducible RNA-binding protein) cDNA and chromosomal assignment of the gene. Gene. 1997;204(1–2):115–20.
2. Zhu X, Buhrer C, Wellmann S. Cold-inducible proteins CIRP and RBM3, a unique couple with activities far beyond the cold. Cell Mol Life Sci. 2016;73(20):3839–59.
3. Zhang F, Brenner M, Yang WL, Wang P. A cold-inducible RNA-binding protein (CIRP)-derived peptide attenuates inflammation and organ injury in septic mice. Sci Rep. 2018;8(1):3052.
4. Qiang X, Yang WL, Wu R, Zhou M, Jacob A, Dong W, Kuncewitch M, Ji Y, Yang H, Wang H, et al. Cold-inducible RNA-binding protein (CIRP) triggers inflammatory responses in hemorrhagic shock and sepsis. Nat Med. 2013;19(11):1489–95.
5. Zhong P, Huang H.Zhong P, et al. Recent progress in the research of cold-inducible RNA-binding protein. Future Sci OA. 2017;3(4):FSO246.

6. Bolognese AC, Sharma A, Yang WL, Nicastro J, Coppa GF, Wang P. Cold-inducible RNA-binding protein activates splenic T cells during sepsis in a TLR4-dependent manner. Cell Mol Immunol. 2018;15(1):38–47.
7. De Leeuw F, Zhang T, Wauquier C, Huez G, Kruys V, Gueydan C. The cold-inducible RNA-binding protein migrates from the nucleus to cytoplasmic stress granules by a methylation-dependent mechanism and acts as a translational repressor. Exp Cell Res. 2007;313(20):4130–44.
8. Liao Y, Tong L, Tang L, Wu S. The role of cold-inducible RNA binding protein in cell stress response. Int J Cancer. 2017;141(11):2164–73.
9. Aziz M, Brenner M, Wang P. Extracellular. CIRP (eCIRP) and inflammation. J Leukoc Biol. 2019;106(1):133–46.
10. Brochu C, Cabrita MA, Melanson BD, Hamill JD, Lau R, Pratt MA, McKay BC. NF-kappaB-dependent role for cold-inducible RNA binding protein in regulating interleukin 1beta. PLoS One. 2013;8(2):e57426.
11. Li S, Zheng S, Tang S, Pan Y, Zhang S, Fang H, Qiao J. Autoinflammatory Pathogenesis and Targeted Therapy for Adult-Onset Still's Disease. Clin Rev Allergy Immunol. 2020;58(1):71–81.
12. Feist E, Mitrovic S, Fautrel B. Mechanisms, biomarkers and targets for adult-onset Still's disease. Nat Rev Rheumatol. 2018;14(10):603–18.
13. Roh JS, Sohn DH. Damage-Associated Molecular Patterns in Inflammatory Diseases. Immune Netw. 2018;18(4):e27.
14. Yamaguchi M, Ohta A, Tsunematsu T, et al. Preliminary criteria for classification of adult Still's disease. J Rheumatol. 1992;19:424–30.
15. Pouchot J, Sampalis JS, Beaudet F, et al. Adult Still's disease: manifestations, disease course, and outcome in 62 patients. Medicine. 1991;70:118–36.
16. Gerfaud-Valentin M, Jamilloux Y, Iwaz J, Sève P. Adult-onset Still's disease. Autoimmun Rev. 2014;13:708–22.
17. Joshi H, Morley SC. Cells under stress: The mechanical environment shapes inflammasome responses to danger signals. J Leukoc Biol. 2019;106(1):119–25.
18. Yu L, Li QH, Deng F, Yu ZW, Luo XZ, Sun JL. Synovial fluid concentrations of cold-inducible RNA-binding protein are associated with severity in knee osteoarthritis. Clin Chim Acta. 2017;464:44–9.
19. Yoo IS, Lee SY, Park CK, Lee JC, Kim Y, Yoo SJ, Shim SC, Choi YS, Lee Y, Kang SW. Serum and synovial fluid concentrations of cold-inducible RNA-binding protein in patients with rheumatoid arthritis. Int J Rheum Dis. 2018;21(1):148–54.
20. Girard C, Rech J, Brown M, Allali D, Roux-Lombard P, Spertini F, Schiffrin EJ, Schett G, Manger B, Bas S, et al. Elevated serum levels of free interleukin-18 in adult-onset Still's disease. Rheumatology. 2016;55(12):2237–47.
21. Lleonart ME. A new generation of proto-oncogenes: cold-inducible RNA binding proteins. Biochim Biophys Acta. 2010;1805(1):43–52.

22. Tan C, Gurien SD, Royster W, Aziz M, Wang P. Extracellular CIRP Induces Inflammation in Alveolar Type II Cells via TREM-1. Front Cell Dev Biol. 2020;8:579157.
23. Denning N-L, Aziz M, Gurien SD. Ping Wang. DAMPs and NETs in Sepsis. Front Immunol. 2019;10:2536.
24. Yang WL, Sharma A, Wang Z, Li Z, Fan J, Wang P. Cold-inducible RNA-binding protein causes endothelial dysfunction via activation of Nlrp3 inflammasome. Sci Rep. 2016;6:26571.
25. Tartey S, Kanneganti TD. Inflammasomes in the pathophysiology of autoinflammatory syndromes. J Leukoc Biol. 2020;107(3):379–91.
26. Bortolotti P, Faure E, Kipnis E. Inflammasomes in Tissue Damages and Immune Disorders After Trauma. Front Immunol. 2018; 9:1900.
27. Zhong P, Peng J, Yuan M, Kong B, Huang H. Cold-inducible RNA-binding protein (CIRP) in inflammatory diseases: Molecular insights of its associated signalling pathways. Scand J Immunol. 2020:e12949.

Tables

Table 1 Characteristics of ASD patients

Characteristics	Value
Number, n	42
age(years),median(IQR)	40 (27.3–56.5)
Age at onset(years), median(IQR)	39 (25.8–56.3)
Male, n(%)	13 (31)
WBC(/μL), median(IQR)	10600 (8375–16400)
Ferritin(ng/mL), median(IQR)	872 (284.5-3441.5)
CRP(mg/dL), median	5.5 (2.8–10.3)
ALT(IU/L), median(IQR)	32 (18-63.5)
Pouchot's score, median(IQR)	3 (2-4.3)
PSL(mg/day), median(IQR)	40 (40–55)
Corticosteroid pulse therary, n(%)	21 (50)
Immunosuppressant, n(%)	30 (71.4)
Biologics, n(%)	6 (14.3)
Polycyclic systemic type, n(%)	21 (50)
Monocyclic systemic type, n(%)	15 (35.7)
Chronic arthritis type, n(%)	6 (14.3)
ASD = adult Still's disease, WBC = white blood cell, CRP = C reactive protein, ALT = alanine aminotransferase, PSL = prednisolone, IQR = interquartile range	

Figures

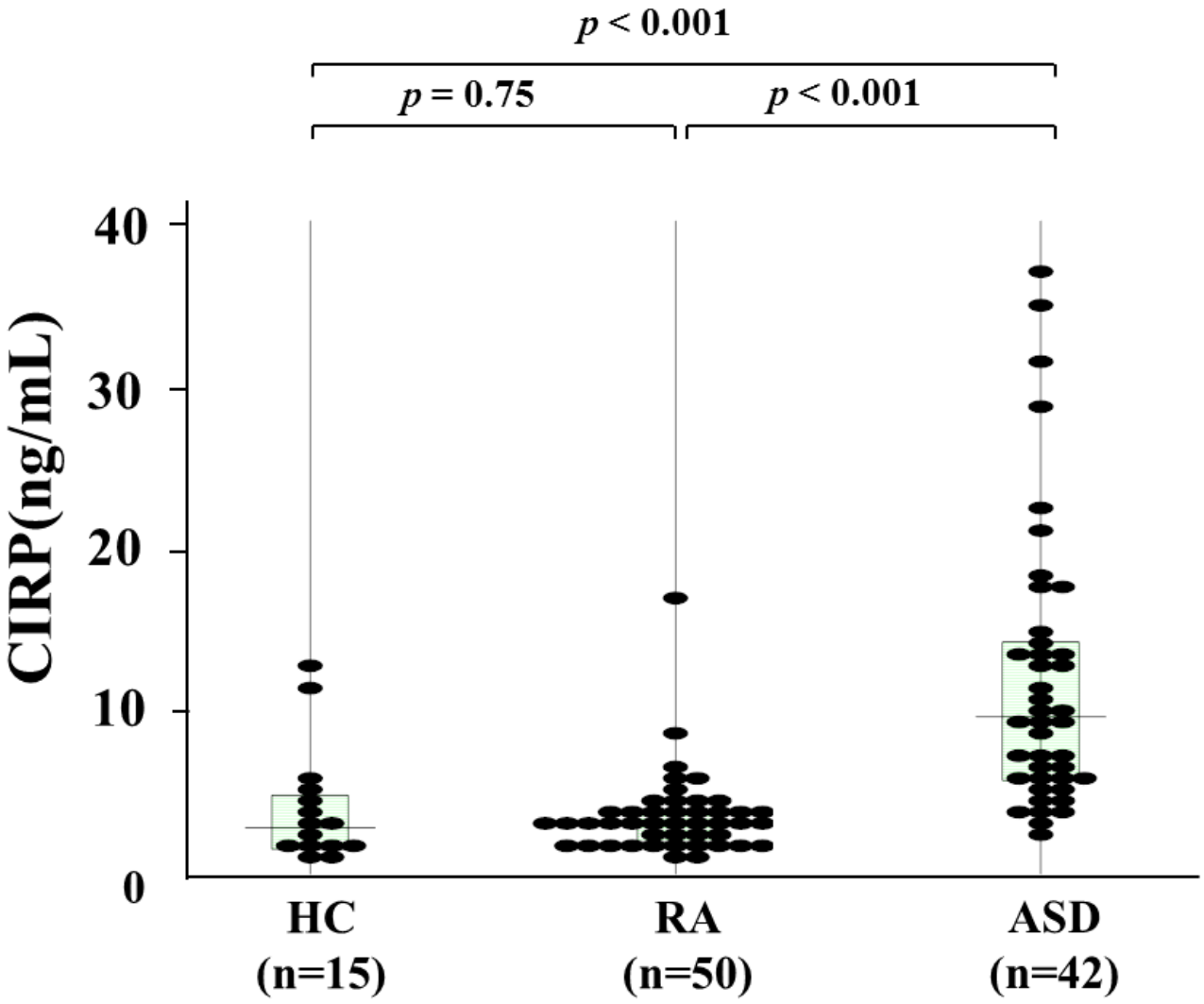

Figure 1

Serum levels of CIRP in patients with ASD Serum levels of CIRP in ASD patients (n=42) were significantly higher compared to those in RA patients (n=50) or healthy controls (n=15). Results were presented with median and were compared by the Mann-Whitney U test.

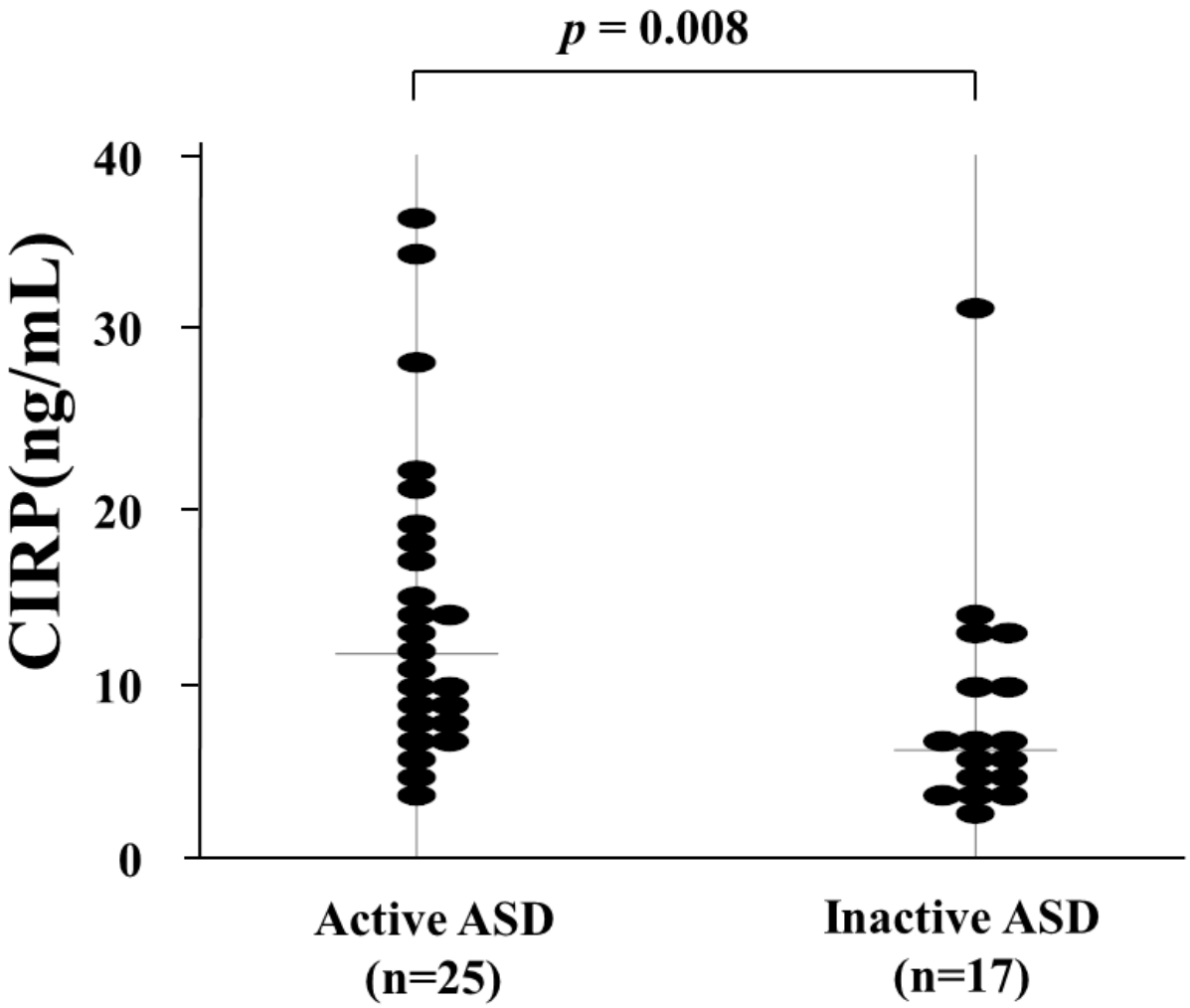

Figure 2

Comparison of serum levels of CIRP levels between active and inactive ASD patients. Serum levels of CIRP were significantly higher in active ASD patients than those in inactive ASD patients. Inactive ASD is defined when Pouchot's score is below 3 points. Results were presented with median and were compared by the Mann-Whitney U test.

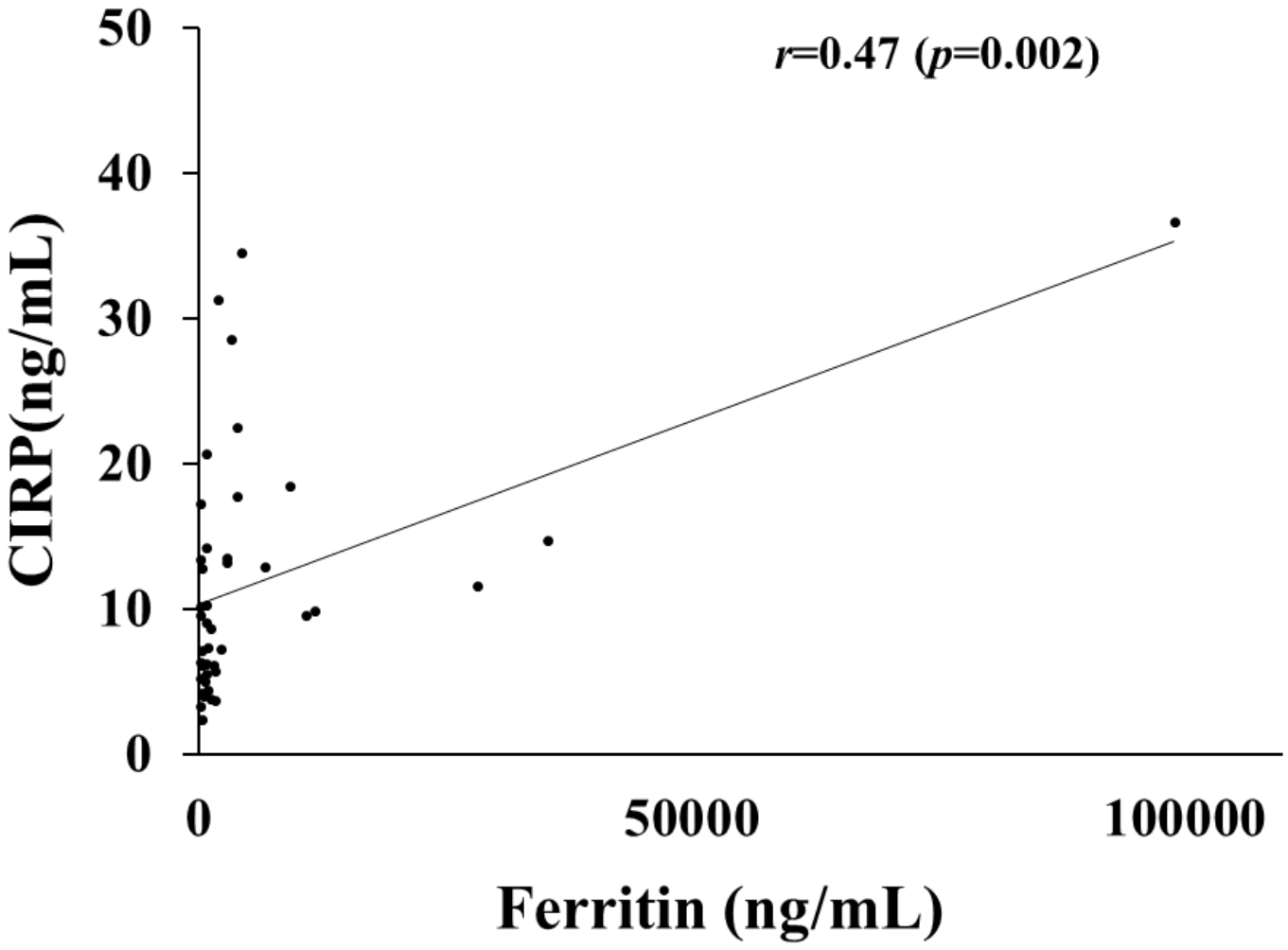

Figure 3

Relationship between serum levels of CIRP and ferritin in patients with ASD Correlation analysis of serum levels of CIRP and ferritin showed a significant positive correlation in ASD patients.

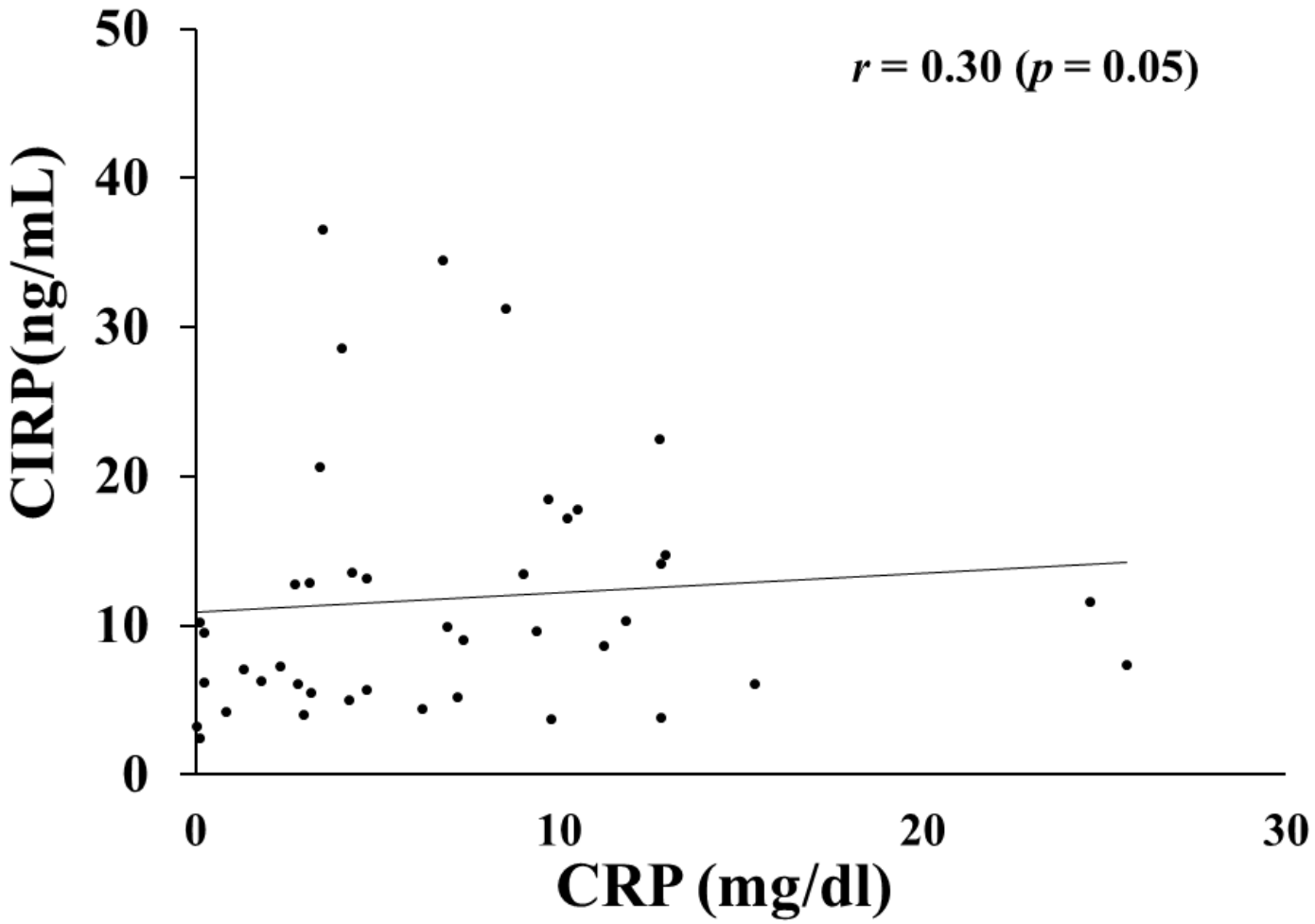

Figure 4

Relationship between serum levels of CIRP and CRP in patients with ASD Correlation analysis of serum levels of CIRP and CRP did not show a significant correlation in ASD patients.

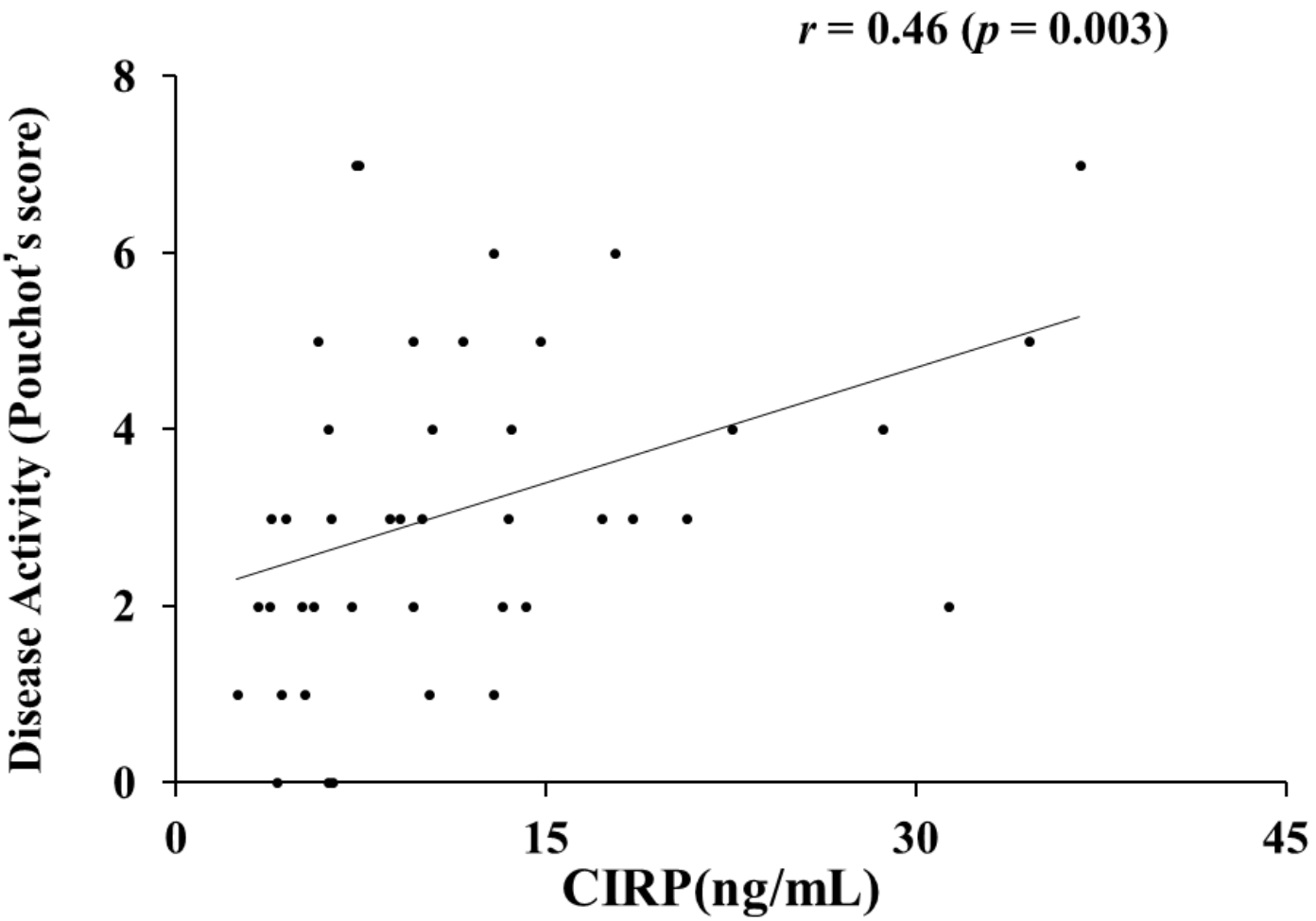

Figure 5

Correlation between serum levels of CIRP and disease activity score (Pouchot's score) in patients with ASD Correlation analysis of serum levels of CIRP and disease activity scores (Pouchot's score) showed a significant positive correlation in ASD patients.

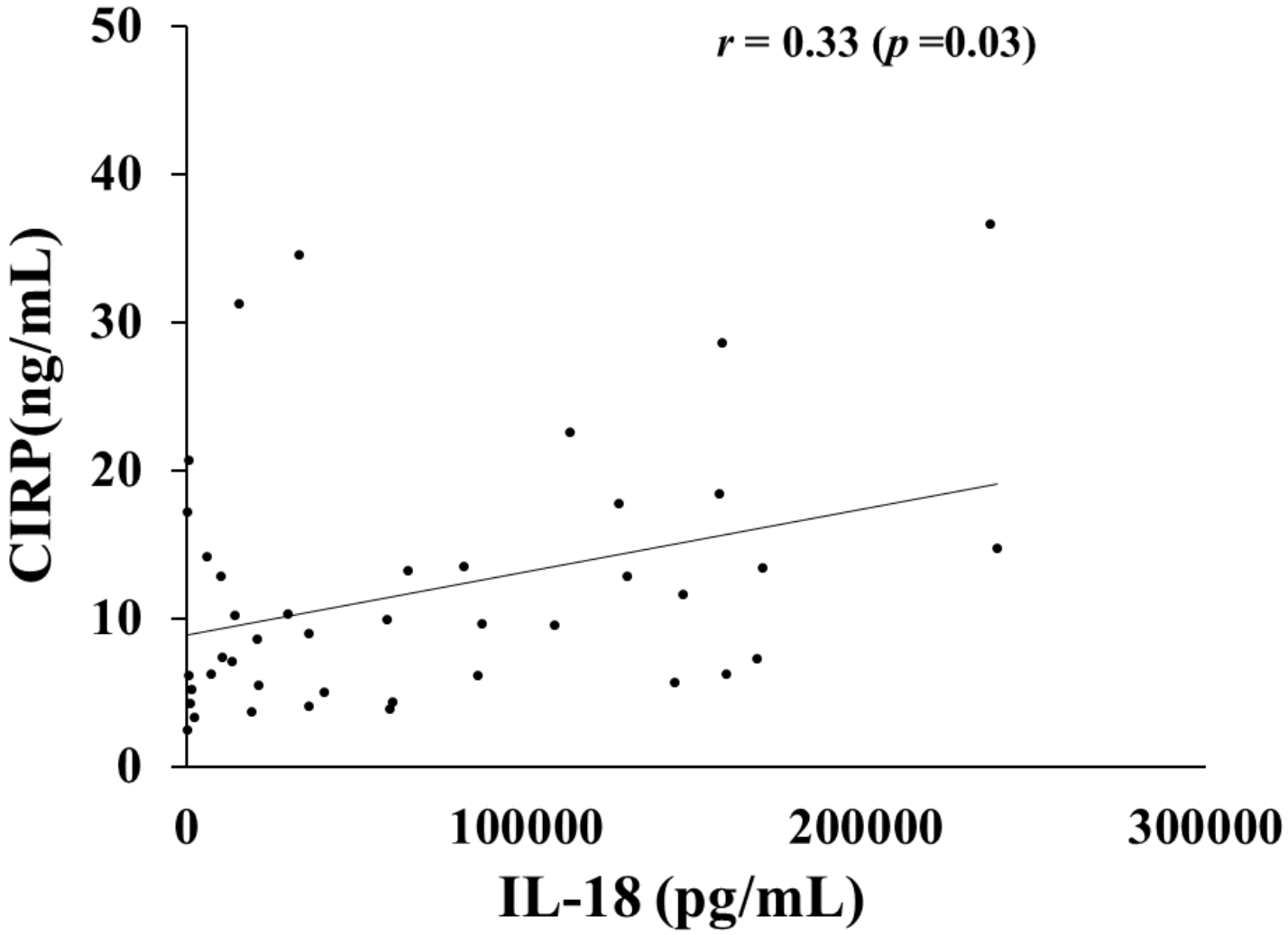

Figure 6

Relationship between serum levels of CIRP and IL-18 in patients with ASD Correlation analysis of serum levels of CIRP and IL-18 showed a significant positive correlation in ASD patients.

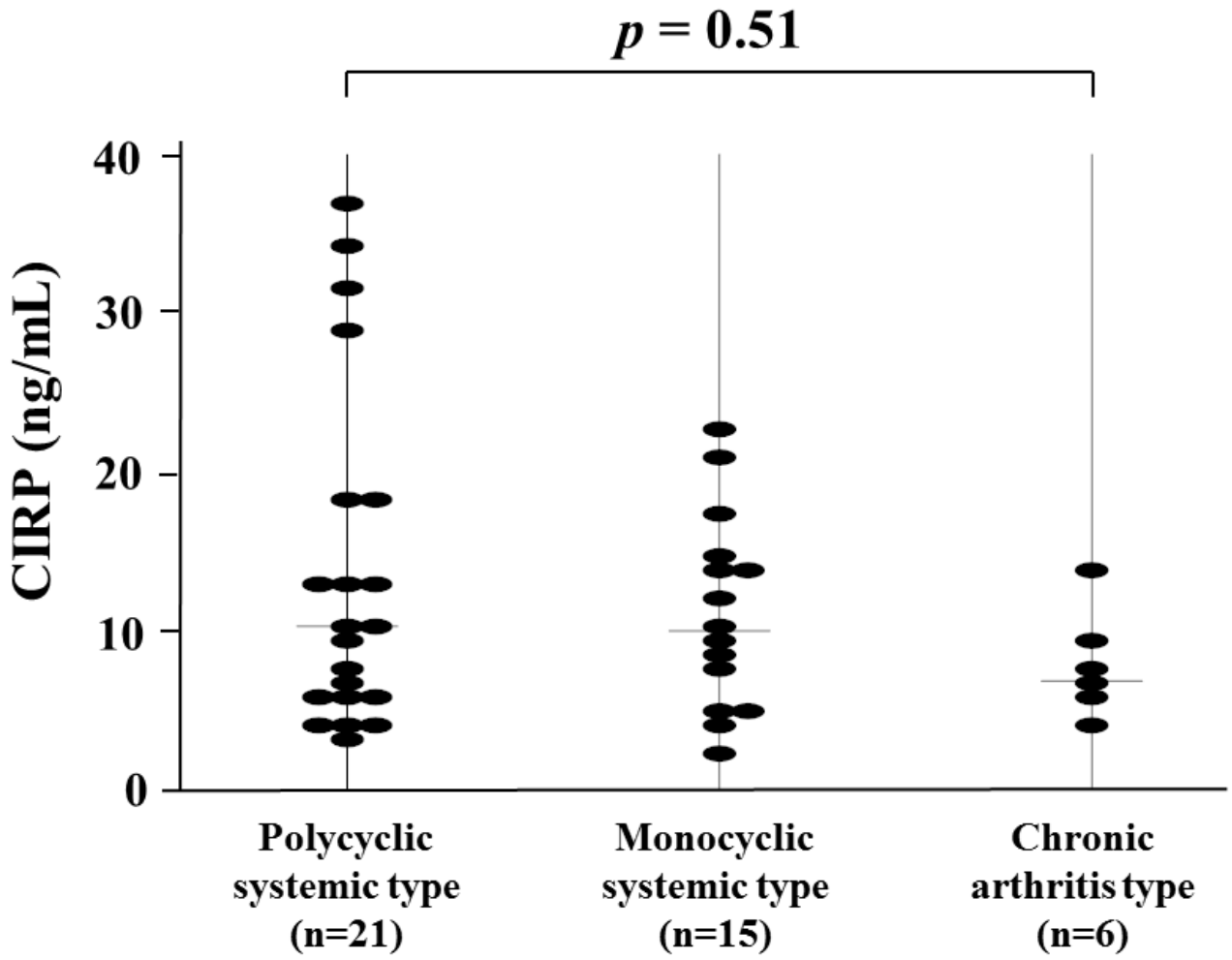

Figure 7

Serum levels of CIPR in ASD patients with three different phenotypes We compared serum levels of CIRP among ASD patients with three different disease phenotypes. There was no significant difference in serum levels of CIRP among ASD patients with three different disease phenotypes. Kruskal–Wallis test was used for continuous variables for comparisons between three groups.

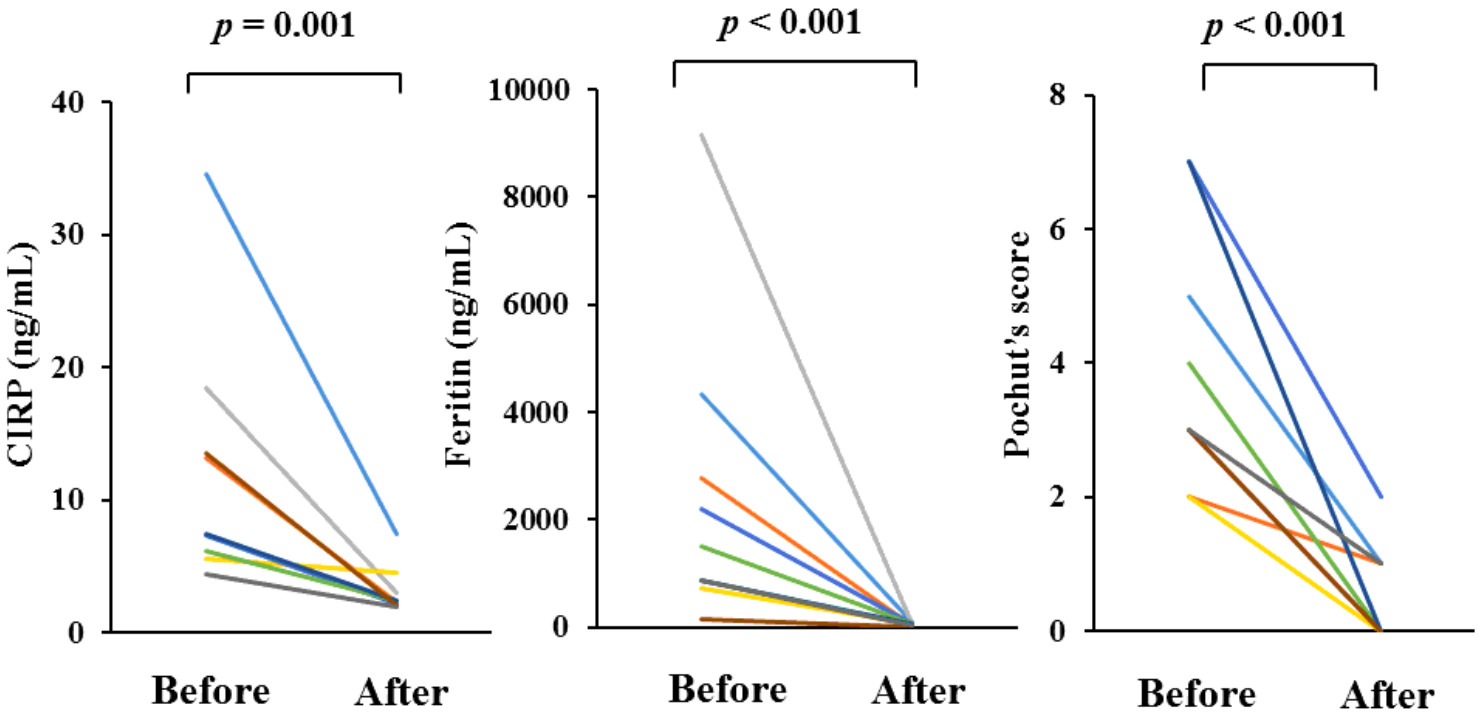

Figure 8

Longitudinal changes of serum levels of CIRP (A), ferritin (B) and disease activity score (C Pouchot's score) in 8 patients with active ASD before and after immunosuppressive treatments. Paired samples from the same subjects (n=8) were compared by Wilcoxon signed rank test.

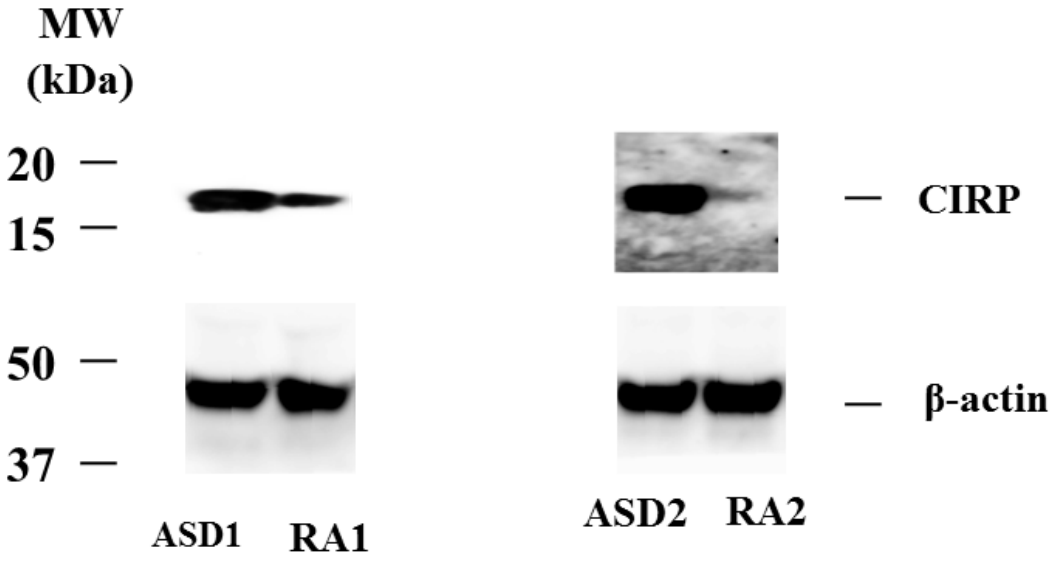

Figure 9

CIRP immunoblot analysis using the cellular lysates of CD14 positive monocytes isolated from active ASD or RA patients CD14 positive monocytes were isolated form peripheral blood in active patients with ASD or RA. Cellular lysates were analyzed by immunoblotting with antibodies that recognize CIRP. -actin was the loading control. Three experiments were performed using two different paired patients (ASD and RA).