

**Title: Initial periodontal treatment affects nucleotide-binding domain leucine-rich repeat-containing protein 3 inflammasome priming in peripheral blood mononuclear cells**

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**Running title:** Periodontitis and inflammasome priming

**Highlights:**

- Expression of *IL-1 $\beta$*  and *ASC* in PBMCs decreased after initial periodontal treatment
- PBMCs stimulated with silica crystals secreted IL-1 $\beta$
- IL-1 $\beta$  secretion by PBMCs decreased after treatment in low BOP percentages group
- IL-1 $\beta$  secretion by PBMCs increased after treatment in high BOP percentages group

**Abstract**

**Objective:** Accumulating evidence suggests an association between periodontitis and several systemic diseases, such as atherosclerosis. In the lesions of these diseases, nucleotide-binding domain leucine-rich repeat-containing protein 3 (NLRP3), apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC) and caspase-1 form inflammasome complex, which leads to the functional maturation of interleukin (IL)-1 $\beta$  via cleavage of caspase-1 in macrophages. IL-1 $\beta$  plays a critical role in the etiology of these diseases; however, inflammasome priming—specifically, *IL-1 $\beta$*  and *NLRP3* upregulation—is necessary for effective IL-1 $\beta$  production.

We investigated the effect of initial periodontal treatment on the inflammasome priming of peripheral blood mononuclear cells (PBMCs).

**Methods:** Twenty-two patients with chronic periodontitis were enrolled in this study and given initial periodontal treatment. Peripheral blood samples were collected at baseline and re-evaluation

(41.1 ± 29.1 d after the treatment), and the relative expression of *IL-1β*, and three inflammasome components, *ASC*, *NLRP3* and *Caspase-1*, mRNA was determined using quantitative reverse transcription PCR. PBMCs were stimulated with silica crystals, and the IL-1β secretion was measured via enzyme-linked immunosorbent assay.

**Results:** Probing pocket depth and bleeding on probing (BOP) were significantly improved after the treatment. Expression of *IL-1β* and *ASC* in the PBMCs decreased after the treatment. PBMCs stimulated with silica crystals secreted IL-1β. The treatment attenuated IL-1β secretion by PBMCs in low BOP percentages group whereas IL-1β secretion was increased in high BOP percentages group.

**Conclusion:** Periodontal treatment altered the inflammasome priming status of the PBMCs, however, the effects on systemic diseases need to be further investigated.

**Keywords:** chronic periodontitis; inflammation; inflammasome; interleukin-1β; mononuclear leukocyte

**Abbreviation:**

danger-associated molecular patterns (DAMPs), probing pocket depth (PPD), bleeding on probing (BOP), nucleotide-binding domain leucine-rich repeat-containing protein 3 (NLRP3), apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC), interleukin (IL), peripheral blood mononuclear cells (PBMCs), quantitative reverse transcription–

polymerase chain reaction (qRT-PCR), enzyme-linked immunosorbent assay (ELISA),

glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)

## 1. Introduction

Periodontitis is a chronic inflammatory disease that causes destruction of periodontal tissue. Bacteria in the dental plaque trigger host inflammatory responses and induce the recruitment of leukocytes into the periodontal tissue. The infiltrated leukocytes release inflammatory cytokines that promote inflammation in the periodontal tissue. Growing evidence suggests that periodontitis is associated with several systemic inflammatory diseases, such as atherosclerosis, type 2 diabetes mellitus, rheumatoid arthritis, preterm birth, and Alzheimer's disease (Falcao & Bullón, 2019; Gaur & Agnihotri, 2015). A meta-analysis found that periodontal treatment improves endothelial function and leads to a reduction in the biomarkers of atherosclerotic disease (Teeuw et al., 2014). An experimental mouse model showed that periodontitis exacerbated aspects of Alzheimer's disease (Ishida et al., 2017). These findings suggest the causal role of periodontitis in these systemic diseases. A common characteristic of periodontitis-associated systemic diseases is a noninfectious inflammation in the disease lesions. This sterile inflammation is triggered by cholesterol crystals, islet amyloid polypeptide, ATP, hypoxia, reactive oxygen species, uric acid, saturated fatty acids, glucose, or amyloid  $\beta$  (Patel et al., 2017). These molecules are called danger-associated molecular patterns (DAMPs). DAMPs activate the nucleotide-binding domain leucine-rich repeat-containing protein 3 (NLRP3) in the cytosol, and they promote the recruitment of apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC) and caspase-1 into a multiprotein complex known as the inflammasome (He, Hara, & Núñez, 2016). Assembly of the

NLRP3 inflammasome promotes self-cleavage of caspase-1, which in turn cleaves pro-interleukin (IL)-1 $\beta$  to facilitate its secretion and biological activity. Inflammasome-derived mature IL-1 $\beta$  is a potent proinflammatory mediator and has been shown to promote sterile inflammatory disease pathogenesis at multiple levels (Lukens, Gross, & Kanneganti, 2012). For example, IL-1 $\beta$  can directly cause tissue destruction, altered fibroblast proliferation, and collagen deposition.

Engagement of the IL-1 $\beta$  and IL-1 receptors promotes the production of secondary proinflammatory molecules, such as tumor necrosis factor- $\alpha$  and IL-6, which can trigger additional immune cell recruitment and activation. Stroke patients treated with an IL-1 receptor antagonist exhibited reduced levels of proinflammatory cytokines and improved cognitive functions (Emsley et al., 2005). Low-density lipoprotein receptor-deficient mice (genetically prone to atherosclerosis) that are reconstituted using bone marrow cells from mice lacking *NLRP3*, *ASC*, or *IL-1 $\beta$*  are remarkably resistant to atherosclerotic plaque formation (Duewell et al., 2010). Combined, this evidence indicates that NLRP3 inflammasome formation and IL-1 $\beta$  production play critical roles in the pathogenesis of these systemic diseases.

Before a functional NLRP3 inflammasome can be formed, transcriptional and posttranslational mechanisms must achieve a reasonable level of *NLRP3* expression within the cell, and a suitable level of *IL-1 $\beta$*  must also be achieved (Patel et al., 2017). This process is called inflammasome priming. Expression of *IL-1 $\beta$*  and *NLRP3* can be induced by priming with microbial components, such as Toll-like receptor ligands, or endogenous molecules, such as tumor necrosis

factor- $\alpha$  or IL-1 $\beta$ , through the activation of NF- $\kappa$ B (He et al., 2016). The oral cavity, and in particular periodontal pockets, could act as a natural reservoir of microorganisms (Falcao & Bullón, 2019).

These oral bacteria and their products, such as endotoxins, may enter the circulation and prime the inflammasome. Alternatively, the inflammatory cytokines or inflammatory mediators induced in diseased periodontium may have the potential to prime the inflammasome. Therefore, we hypothesized that inflammation in the periodontal tissue primes the inflammasome in peripheral blood mononuclear cells (PBMCs) and contributes to the exacerbation of systemic diseases. The aim of this study was to investigate the effect of initial periodontal treatment on inflammasome priming in PBMCs.

## 2. Materials and methods

### 2.1. Sample collection

Twenty-five patients with periodontitis who visited Nagasaki University Hospital between 2016 and 2017 were enrolled in this study, although three dropped out during the initial periodontal treatment, leaving 22 (4 males and 18 females, mean age:  $60.5 \pm 15.2$  y) whose samples were analyzed. The inclusion criteria were: 1) possession of  $\geq 15$  teeth; 2) an age of  $\geq 20$  y; and 3)  $\geq 2$  teeth with attachment loss and alveolar bone resorption. The exclusion criteria were: 1) pregnancy or lactation; 2) renal or liver disease; 3) treatment with antibiotics within the previous month; 4) periodontal treatment within the previous three months; and 5) participation in another clinical study within the previous four months. Three periodontally and systemically healthy volunteers (2 males and 1 female, mean age:  $29.6 \pm 1.5$  y) were recruited to obtain the control saliva and blood samples. Written informed consent was obtained from all participants. The design of the study and procedures for obtaining informed consent were approved by the Institutional Review Board of Nagasaki University. The study conforms to STROBE guidelines.

As part of a routine periodontal examination, probing pocket depth (PPD) and bleeding on probing (BOP) were measured at six aspects of each tooth before any periodontal treatment was performed and after the initial periodontal treatment. One mL of saliva was collected from each participant via passive secretion into a sterile tube. The saliva samples were stored at  $-80$  °C until use.



Human PBMCs were isolated from the venous blood before and after periodontal treatment.

The PBMCs were isolated from a density gradient prepared using Ficoll-Paque Plus (GE Healthcare Bio-Science AB, Pittsburg, PA), and resuspended in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin.

## **2.2. Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analysis**

To analyze *IL-1β*, *NLRP3*, *ASC*, and *Caspase-1* mRNA levels, total RNA was extracted from the PBMCs using the RNeasy Mini Kit (Qiagen, Hilden, Germany) with on-column DNase treatment according to the manufacturer's instructions. For each sample, 24 ng of total RNA was converted to first-strand cDNA using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) at 25 °C for 10 min, followed by 50 min at 42 °C, and then 15 min at 70 °C, using a Takara PCR thermal cycler (Takara Bio, Otsu, Japan). The cDNA was treated with RNase H and purified using the QIAprep Spin Miniprep Kit (Qiagen). The primer sequences used were as follows: *IL-1β* forward, 5'-GCTGGAGGACTTTAAGGGTTAC-3', and reverse, 5'-GATGTCTGGGTCTTGGTTCTC-3'; *NLRP3* forward, 5'-AGCACCAGCCAGAGTCTAAC-3', and reverse, 5'-CCCCAACCACAATCTCCGAAT-3'; *ASC* forward, 5'-AACCCAAGCAAGATGCGGAAG-3', and reverse, 5'-TTAGGGCCTGGAGGAGCAAG-3'; *Caspase-1* forward, 5'-CAAGGGTGCTGAACAAGG-3', and reverse, 5'-GGGCATAGCTGGGTTGTC-3'; glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) forward, 5'-

CATGTTCGTCATGGGTGTGA-3, and reverse, 5'-CAGGGGTGCTAAGCAGTTGG-3' (Feria, Taborda, Hernandez, & Rugeles, 2018; Tzach-Nahman et al., 2017). Comparative quantification by PCR was performed with SYBR Premix Ex Taq using the Mx3000P qPCR System (Agilent technologies, Santa Clara, CA). The amplification conditions were as follows: 95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 20 s, followed by a final cycle of 95 °C for 1 min, 55 °C for 30 s, and 95 °C for 30 s. A melting curve analysis was used to confirm that the proper PCR products were amplified in all samples. The relative expression ratios of *IL-1 $\beta$* , *NLRP3*, *ASC*, and *Caspase-1* mRNA were calculated based on PCR efficiency and the threshold cycle difference for the test sample (PBMCs from periodontitis patients) versus a calibrator (PBMCs from one healthy volunteer). Target gene expression was normalized using *GAPDH* gene expression (Pfaffl, 2001). The mRNA level of the calibrator was set to 1.

### **2.3. IL-1 $\beta$ production assay**

PBMCs were plated at a density of  $2.0 \times 10^5$  cells per well in a 96-well dish in triplicate and stimulated with 500  $\mu\text{g}/\text{mL}$  silica crystals or PBS. Following incubation for 4 h, the cell-free supernatant was harvested and analyzed for IL-1 $\beta$  secretion using an enzyme-linked immunosorbent assay (ELISA) kit (R&D, Minneapolis, MN) according to the manufacture's protocol.

### **2.4. Quantification of bacteria**

The levels of a species of periodontopathic bacterium, *Porphyromonas gingivalis*, and total bacteria in each saliva sample were determined as described by Yoshioka et al. (Yoshioka, Yoshimura, Kaneko, Golenbock, & Hara, 2008), with slight modifications. Briefly, bacterial DNA was extracted from 20 µL of the saliva samples, using the DNeasy tissue kit (Qiagen), and quantified using the Mx3000P qPCR System. The sequences of the specific primers and probes used for each bacterium and for total bacteria have been described elsewhere (Maeda et al., 2003; Pan et al., 2017). Standard curves for each organism were plotted for each primer set using threshold cycle values obtained by amplifying 10-fold serial dilutions from 1 to 10<sup>7</sup> CFU of previously quantified bacterial DNA. Bacterial DNA standards and sample DNA were run in triplicate, and their means were used to calculate the bacterial concentrations.

## **2.5. Statistical analysis**

Comparisons of the values at baseline and re-evaluation were performed using paired-sample *t*-tests.

All calculations were conducted using StatMate IV software (ATMS, Tokyo, Japan).

### 3. Results

#### 3.1. Clinical parameters and bacterial levels of the saliva samples

The mean percentage of sites with PPD = 4–5 mm was 15.9% before treatment and 8.5% after treatment (Fig. 1A). The mean percentage of sites with PPD  $\geq$ 6 mm decreased from 4.3% to 1.4% (Fig. 1B), and that of BOP decreased from 20.1% to 11.8% (Fig. 1C). The mean levels of *P. gingivalis* in the saliva decreased from 220 to 78 after treatment (Fig. 2A), whereas it was not detected in the saliva of three healthy subjects (data not shown). The level of total bacteria did not change (Fig. 2B). These results indicate the effectiveness of the initial periodontal treatment.

#### 3.2. Expression levels of IL-1 $\beta$ , NLRP3, ASC, and Caspase-1 mRNA in PBMCs

PBMCs were collected from the patients before and after the treatment, and their expression levels of *IL-1 $\beta$* , *NLRP3*, *ASC*, and *Caspase-1* mRNA were analyzed using qRT-PCR. The mRNA levels of *IL-1 $\beta$*  and *ASC* were significantly lower after the initial periodontal treatment (Fig. 3A and B). However, the mRNA levels of *NLRP3* and *Caspase-1* did not change (Fig. 3C and D). To further analyze the effect of periodontal treatment on inflammasome priming, the patients were classified according to their BOP percentage. Patients with BOP  $\geq$ 16% are known to have a higher periodontal disease activity (Lang, Joss, Orsanic, Gusberti, & Siegrist, 1986). The levels of *IL-1 $\beta$*  mRNA in the PBMCs decreased except two samples and the levels of *ASC* and *NLRP3* mRNA significantly decreased after treatment in the BOP <16% (n = 10) group (Fig. 4A–C). The levels of *Caspase-1* mRNA in the

PBMCs did not change in the same group (Fig. 4D). The levels of *IL-1 $\beta$* , *ASC* and *Caspase-1* mRNA in the PBMCs also decreased after treatment in the BOP  $\geq 16\%$  (n = 12) group (Fig. 4 E, F and H). However, the level of *NLRP3* mRNA in the PBMCs slightly increased after treatment in the BOP  $\geq 16\%$  group (Fig. 4G).

### **3.3 IL-1 $\beta$ secretion by PBMCs stimulated with silica crystals**

The PBMCs were then stimulated with PBS or silica crystals. Silica crystals were used as stimulant because they engage the NLRP3 inflammasome without priming the cells (Dostert et al., 2008).

PBMCs stimulated with PBS did not secrete any IL-1 $\beta$ , whereas those stimulated with silica crystals did (Fig. 5A and B). However, the levels of IL-1 $\beta$  thus induced did not change significantly during the treatment in the whole samples. Similarly to *NLRP3* mRNA levels, the induction of IL-1 $\beta$  by silica crystals slightly decreased after treatment in the BOP  $< 16\%$  group but increased in the BOP  $\geq 16\%$  group (Fig. 5C and D).

## Discussion

In this study, we analyzed the effects of initial periodontal treatment on inflammasome priming in PBMCs. Periodontal treatment reduced the percentage of sites with deep PPD and/or BOP in every patient. The levels of *P. gingivalis* in the saliva samples decreased after the treatment, confirming the effectiveness of the treatment (Saygun et al., 2011). Concurrently, mRNA expression levels of *IL-1 $\beta$*  and *ASC* in the PBMCs decreased after the treatment, suggesting that it contributed to the downregulation of *IL-1 $\beta$*  and *ASC* mRNA in the PBMCs. There are many factors that can activate the inflammasome, such as ATP from dead cells, metal powder, and ultraviolet B (Eisenbarth, Colegio, O'Connor, Sutterwala, & Flavell, 2008; Feldmeyer et al., 2007; Mariathasan et al., 2006). The expression levels of *IL-1 $\beta$* , *NLRP3*, *ASC* and *Caspase-1* mRNA varied widely, indicating varying levels of inflammasome priming. Nevertheless, *IL-1 $\beta$*  and *ASC* mRNA levels decreased after the periodontal treatment, suggesting the substantial contribution of periodontal diseases to the inflammasome priming of PBMCs.

However, *NLRP3* and *Caspase-1* mRNA did not decrease after treatment. Unassociated regulation of *NLRP3* and *ASC* expression was found in the previous studies by other investigators. *NLRP3* expression was enhanced but *ASC* expression was downregulated in Mono-Mac-6 cells stimulated with *P. gingivalis* (Bostanci et al., 2009). *NLRP3* expression level was elevated in periodontally diseased groups but the expression level of *ASC* was similar between groups (Aral et al., 2019). To explore the reason for the unchanged *NLRP3* mRNA levels, the patients were classified

into two groups based on their BOP percentage. BOP reflects the histological, clinical, and bacteriological alterations associated with periodontal diseases (Greenstein, 1984). *ASC* and *NLRP3* mRNA expression of patients with BOP <16% significantly decreased, whereas *IL-1 $\beta$*  and *caspase-1* mRNA expression did not. *IL-1 $\beta$*  and *caspase-1* mRNA levels (0.90 and 0.48, respectively) at baseline were lower than *ASC* and *NLRP3* mRNA levels (1.32 and 1.70, respectively). Less up-regulation of *IL-1 $\beta$*  and *caspase-1* mRNA at baseline might result in non-significant changes after the treatment. *NLRP3* mRNA expression levels of patients with BOP  $\geq$ 16% slightly increased, whereas those of patients with BOP <16% slightly decreased after treatment. Because the BOP percentage of the former group remained high, the treatment was not able to completely eliminate the inflammation in their periodontal tissue, and the residual inflammation might have contributed to the slightly increased *NLRP3* mRNA expression in their PBMCs. Alternatively, it has been shown that subgingival biofilms containing *P. gingivalis* down-regulates NLRP3, but not ASC and caspase-1 expression (Belibasakis, Guggenheim, & Bostanci, 2013). As shown in Figure 2, the level of *P. gingivalis* decreased after the treatment. The reduction of the level of *P. gingivalis* might attenuate the inhibitory effect and contribute to the up-regulation of NLRP3 of patients with BOP  $\geq$ 16%. In addition, chronic inflammation in the periodontal tissue might cause innate immune cells to be reprogrammed to resist to the reduction of *NLRP3* mRNA and IL-1 $\beta$  protein levels. It has been shown that long-term epigenetic reprogramming of innate immune cells, also termed “trained immunity,” causes prolonged altered cellular functionality for more than 4 weeks (Christ et al.,

2018). The PBMCs in this study were collected  $41.1 \pm 29.1$  d after treatment, which may not have been long enough to resolve the inflammasome priming. The long-term effects of periodontal treatment on inflammasome priming should therefore be investigated in future studies with a longer observational period.

PBMCs stimulated with silica crystals secreted IL-1 $\beta$ , whereas those stimulated with PBS did not. Because silica crystals do not prime the cells (Dostert et al., 2008), these results suggest that the PBMCs had already been primed prior to blood sampling. The secretion of IL-1 $\beta$  by silica crystals slightly decreased after treatment in the BOP <16% group but increased in the BOP  $\geq$ 16% group. The decrease in the levels of IL-1 $\beta$  secretion in the BOP <16% group might have been due to the reduction of *ASC* and *NLRP3* mRNA expression. The increase in the levels of IL-1 $\beta$  secretion in the BOP  $\geq$ 16% group might have been due to the increase of *NLRP3* mRNA expression. Periodontal pathogens can trigger IL-1 $\beta$  production and upregulate the IL-1 $\beta$  concentrations of the gingival crevicular fluids and serum of patients with periodontitis (Hegde & Awan, 2018; Zhu, Lin, Zheng, & Chen, 2015). In addition, bacteria harbored in the periodontal tissue can gain access to the circulatory system, resulting in a state of transient bacteremia. These bacteria or bacterial products can stimulate the infiltrated macrophages to induce IL-1 $\beta$  production in the lesions of systemic diseases (Falcao & Bullón, 2019). However, circulating IL-1 $\beta$  or bacteremia can affect not only the disease lesions but also any tissue in the body. In contrast to the circulating IL-1 $\beta$  or bacteria, primed PBMCs would not induce inflammation other than the target organs. DAMPs such as monosodium



urate, cholesterol, and  $\beta$ -amyloid crystals activate the NLRP3 inflammasome in the lesions of gout, atherosclerosis, and Alzheimer's disease. Without this stimulation by DAMPs, primed PBMCs would not secrete IL-1 $\beta$ , so their effects would be limited to the target organs. We have demonstrated that the inflammasome priming status of PBMCs were altered by periodontal treatment. The results of the present study are the first evidence suggesting that inflammation in the periodontal tissue can prime PBMCs, which may affect the symptoms of systemic diseases.

In conclusion, the initial periodontal treatment improved periodontal conditions and altered the inflammasome priming status of the PBMCs, in which *IL-1 $\beta$* , *ASC*, *NLRP3* and *Caspase-1* mRNA expression was differentially regulated in patients with BOP <16% and BOP  $\geq$ 16%. The distinct inflammasome priming status by BOP percentages could be due to the residual inflammation in periodontal tissue or the effects of *P. gingivalis* in dental plaque. It is necessary to use a long observational period to confirm the effects of complete periodontal treatment, including periodontal surgery, on inflammasome priming.

### **Conflicts of interest**

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

### **Acknowledgements**

This work was supported by a Grant-in-Aid for Scientific Research (C) 16K11836 from the Japan Society for the Promotion of Science, Tokyo, Japan.

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## Figure legends

### Figure 1.

#### Periodontal condition of the patients at baseline and re-evaluation

Percentage of sites with PPD = 4–5 mm (A), PPD  $\geq$ 6 mm (B), or BOP (C), for each patient measured at baseline and re-evaluation.

### Figure 2.

#### Bacterial levels in saliva samples collected at baseline and re-evaluation

Saliva samples were collected from each patient before and after treatment. The levels of *P. gingivalis* (A) and total bacteria (B) in 1  $\mu$ L of each saliva sample were determined using real-time PCR.

### Figure 3.

#### Expression of *IL-1 $\beta$* , *ASC*, *NLRP3* and *Caspase-1* mRNA in the PBMCs before and after periodontal treatment

PBMCs were collected from the patients before and after the initial periodontal treatment. Total RNA was extracted from a portion of the PBMCs, and the relative expression of *IL-1 $\beta$*  (A), *ASC* (B), *NLRP3* (C), and *Caspase-1* (D) mRNA was determined using qRT-PCR.

#### **Figure 4**

##### **Expression of *IL-1 $\beta$* , *ASC*, *NLRP3*, and *Caspase-1* mRNA in the PBMCs by BOP percentages**

The patients were classified into two groups according to their BOP percentages. The relative expression of *IL-1 $\beta$*  (A, E), *ASC* (B, F), *NLRP3* (C, G), and *Caspase-1* (D, H) mRNA levels in the BOP <16% group (A–D) and BOP  $\geq$ 16% group (E–H) were shown.

#### **Figure 5.**

##### **IL-1 $\beta$ secretion levels before and after treatment**

PBMCs were collected from the patients before and after the treatment and stimulated with PBS (A) or silica crystals (B–D) for 4 h, and IL-1 $\beta$  concentrations of the culture supernatants were measured via ELISA. The patients were classified into two groups according to their BOP percentages. The IL-1 $\beta$  secretion levels in the BOP <16% group (C) and BOP  $\geq$ 16% group (D) were shown.



Figure 1

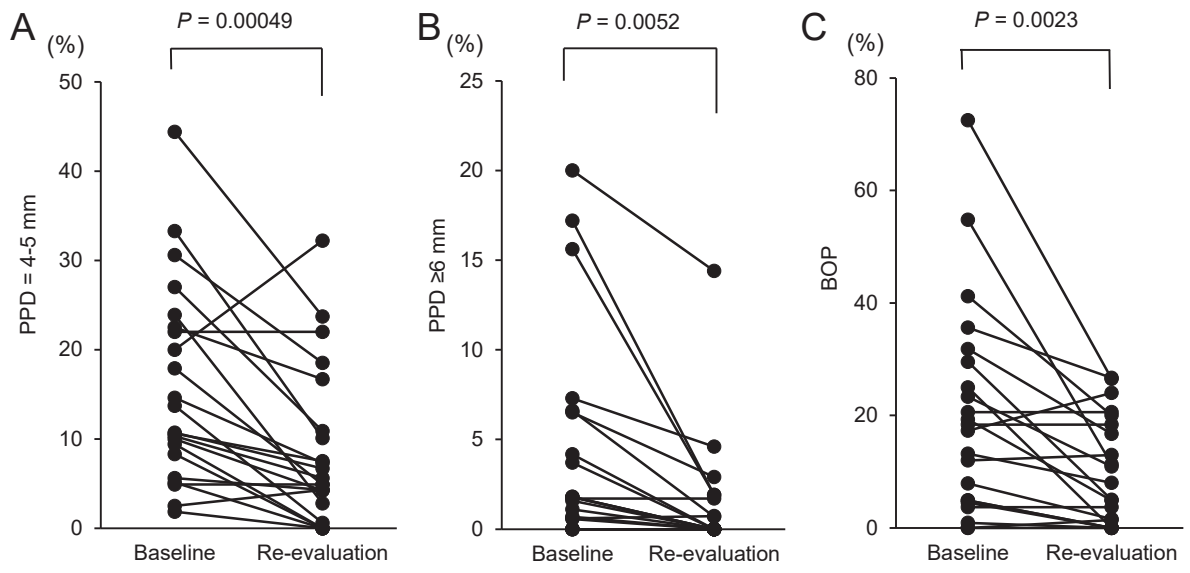




Figure 3

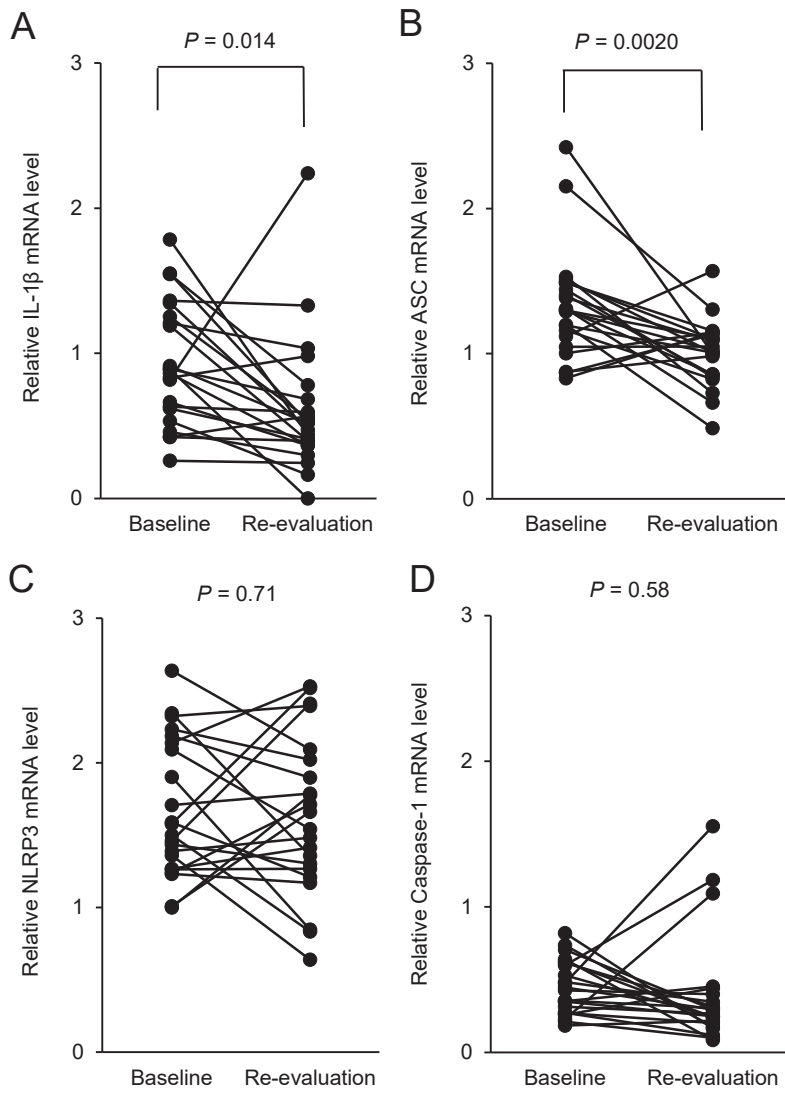
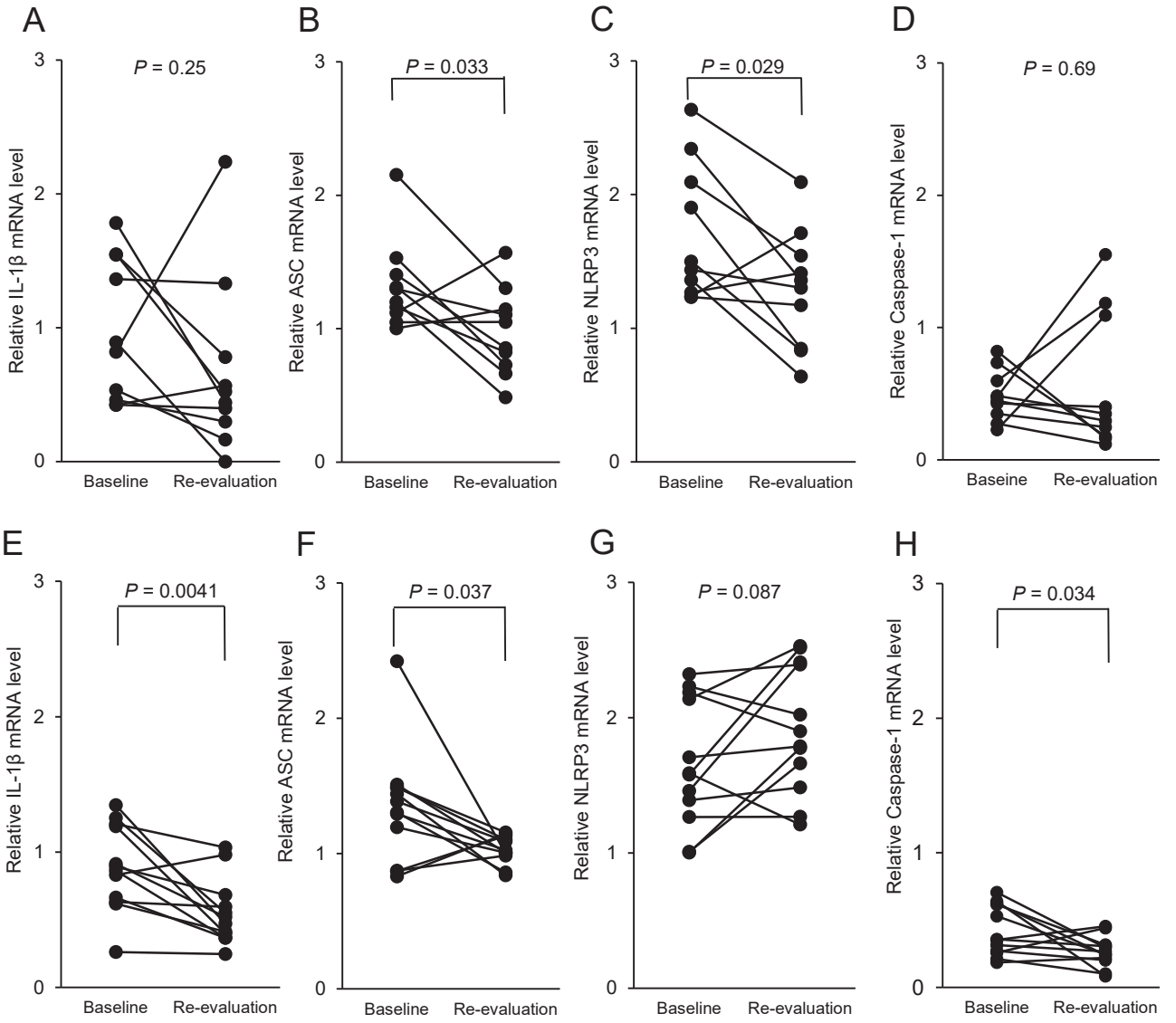


Figure 4



# Figure 5

