

Research Article  
Periodontal Science



# Association between periodontal bacteria and degenerative aortic stenosis: a pilot study

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OPEN ACCESS

Received: Sep 6, 2020

Revised: Dec 15, 2020

Accepted: Mar 5, 2021

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## ABSTRACT

**Purpose:** Although several reports have described the relationship between periodontal disease and cardiovascular disease, information about the association between periodontal disease and the progression of degenerative aortic stenosis (AS) is lacking. Therefore, we performed a retrospective, single-center, pilot study to provide insight into this potential association.

**Methods:** Data from 45 consecutive patients (19 men; median age, 83 years) with mild or moderate degenerative aortic stenosis were analyzed for a mean observation period of 3.3±1.9 years. The total amount of *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* and titers of serum immunoglobulin G (IgG) against periodontal bacteria and high-sensitivity C-reactive protein (hs-CRP) were evaluated. Aortic valve area (AVA), maximal velocity (Vmax), mean pressure gradient (mean PG), and the Doppler velocity index (DVI) were evaluated. The change in each parameter per year ( $[\text{Parameter}_{\text{LATEST}} - \text{Parameter}_{\text{BASELINE}}] / \text{Follow-up Years}$ ) was calculated from the retrospective follow-up echocardiographic data (baseline vs. the most recently collected data [latest]).

**Results:** No correlation was found between the concentration of periodontopathic bacteria in the saliva and AS status/progression. The anti-*P. gingivalis* antibody titer in the serum showed a significant positive correlation with AVA and DVI. Additionally, there was a negative correlation between the anti-*P. gingivalis* IgG antibody titer and mean PG. The hs-CRP concentration showed positive correlations with Vmax and mean PG. Meanwhile, a negative correlation was observed between the anti-*P. gingivalis* IgG antibody titer and  $\Delta\text{AVA}/\text{year}$  and  $\Delta\text{mean PG}/\text{year}$ . The hs-CRP concentration showed positive correlations with Vmax and mean PG, and it was significantly higher in patients with rapid aortic stenosis progression ( $\Delta\text{AVA}/\text{year} < -0.1$ ) than in their counterparts.

**Conclusions:** Our results suggest that periodontopathic bacteria such as *A. actinomycetemcomitans* and *P. gingivalis* are not directly related to the status/progression of degenerative AS. However, inflammation and a lower immune response may be associated with disease progression.

**Keywords:** Aortic valve stenosis; C-reactive protein; Periodontal diseases; *Porphyromonas gingivalis*

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**Funding**

The author(s) disclose receipt of the following financial support for the research, authorship, and/or publication of this article: the work was supported by the Japan Society for the Promotion of Science (16K21382 to A.K. and 26463128 to S.K.) and grants from the Cardiovascular Research Foundation, Tokyo, Japan, and the Epidemiological Research grant from St. Luke's International University, Tokyo, Japan, to A.K.

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**Conflict of Interest**

No potential conflict of interest relevant to this article was reported.

**INTRODUCTION**

Periodontal disease is an inflammatory disorder caused by pathogenic Gram-negative oral microorganisms that can cause both local inflammation, which destroys the alveolar bone and soft tissues around the teeth, and systemic inflammation [1-3]. Periodontal bacteria present in the dental plaque possess various virulence factors, including lipopolysaccharide, fimbriae, and enzymes, that can trigger inflammation in the periodontal tissues [4]. A previous study reported elevated levels of systemic inflammatory mediators in patients with severe periodontal disease [5]. In addition, bacteria originated from the periodontal pocket can cause bacteremia [6]. Furthermore, considering that the bacterial flora of the oral cavity differs from that of the gut [7], swallowed bacteria may affect the composition of the gut microbiome. Therefore, periodontal infection has long been associated with an increased risk of various diseases, including cardiovascular diseases [2,3,8], type 2 diabetes [9], and non-alcoholic fatty liver disease [10].

Degenerative aortic stenosis (AS), which is a common and progressive disease that makes a large contribution to mortality in the current aging population, is most often caused by an active disease process characterized by inflammation, lipid accumulation, and calcification [11]. Leaflet calcification and fibrosis eventually occur once AS is initiated, resulting in reduced leaflet motion and leading to a progressive reduction of the aortic valve area (AVA) [12].

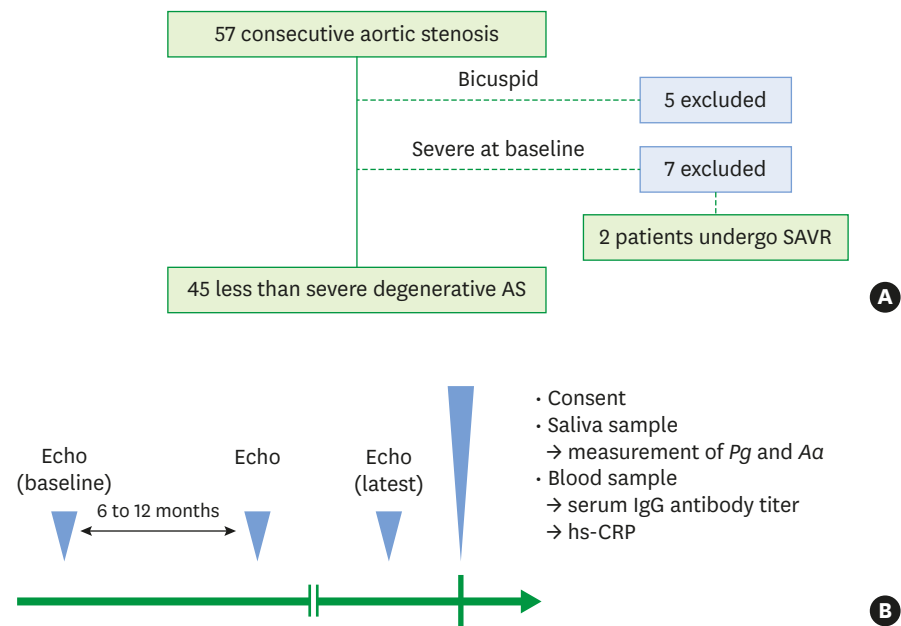
Given that degenerative AS is a systemic inflammatory disorder, we hypothesized that it could be associated with periodontal disease, similarly to other cardiovascular diseases [2,3,8,13,14]. However, in contrast to other cardiovascular diseases, information regarding the relationship between degenerative AS and periodontal disease as a chronic inflammatory disorder is lacking.

Therefore, the aim of the present study was to conduct a preliminary analysis using retrospective patient data to evaluate the potential association between periodontal disease and AS by focusing on *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* as representative periodontal bacteria.

**MATERIALS AND METHODS**

**Study design and population of the An evaluation of the association between PERiodontal baCteria and prOgression of degenerative aorTic stenosis (APRICOT) study**

In this retrospective pilot study, we screened 57 consecutive patients who were diagnosed with degenerative AS at Teikyo University Hospital between November 2017 and April 2018 and were part of the larger APRICOT study, which is a collaboration network on AS involving the Department of Medicine, Division of Cardiology, Teikyo University, and the Department of Periodontology, Tokyo Medical and Dental University. Among the 57 eligible patients, 12 were excluded due to bicuspid AS (n=5) and severe AS at baseline (n=7). Therefore, a total of 45 patients (median age, 83 years; interquartile range, 77–86 years; 42% men) were included for the main analysis (Figure 1A). In addition, 2 patients with severe AS who were excluded from the main analysis and underwent surgical aortic valve replacement were included for analyses of the aortic valve specimen to evaluate the presence of periodontal bacteria in the valves.



**Figure 1.** Diagram showing patient details and data collection protocols.

(A) Subjects were selected according to the criteria shown in the flowchart and (B) data were collected according to the protocols.

SAVR: surgical aortic valve replacement, AS: aortic stenosis, Echo: echocardiography, *Pg*: *Porphyromonas gingivalis*, *Aa*: *Aggregatibacter actinomycetemcomitans*, IgG: immunoglobulin G, hs-CRP: high-sensitivity C-reactive protein.

The study protocol was developed in accordance with the 1975 Declaration of Helsinki (revised in 2013) and was approved by the Institutional Review Board committees of Teikyo University and the Tokyo Medical and Dental University, Department of Dentistry (approval numbers TEIRIN15-236, TEIRIN16-102, and D2016-066). All patients provided informed written consent for participating in the study. This trial was registered with the University Hospital Medical Information Network with the number UMIN000024251.

### Data collection

The data collection protocol is outlined in Figure 1B. All patients were diagnosed with AS using standard 2-dimensional B-mode and Doppler transthoracic echocardiography at baseline and then followed up every 6 to 12 months according to the guidelines of the American College of Cardiology/American Heart Association [15]. Conventional and AS diagnostic parameters such as the AVA, maximal velocity (Vmax), mean pressure gradient (mean PG), and Doppler velocity index (DVI) were measured at each visit according to the guidelines of the American Society of Echocardiography [16]. The change in each parameter per year ( $[\text{Parameter}_{\text{LATEST}} - \text{Parameter}_{\text{BASELINE}}] / \text{Follow-up Years}$ ) was calculated from the retrospective follow-up echocardiographic data using baseline and the most recently collected data during follow-up (latest). Saliva and blood samples were collected at the time of enrollment after signed consent forms were provided. Saliva and plasma components obtained from centrifuged blood samples were frozen at  $-80^{\circ}\text{C}$  and subsequently were sent to the laboratory at Tokyo Medical and Dental University for analysis.

### Cultivation of *P. gingivalis* and *A. actinomycetemcomitans*

The ATCC 33277 strain of *P. gingivalis* was cultured as described previously [17]. In brief, *P. gingivalis* cells were maintained on trypticase soy agar (Difco Laboratories, Detroit, MI, USA)

supplemented with 10% defibrinated horse blood, hemin (5 µg/mL), and menadione (0.5 µg/mL) at 37°C under anaerobic conditions (10% CO<sub>2</sub>, 10% H<sub>2</sub>, and 80% N<sub>2</sub>). After 2 days of incubation, the cells were inoculated into trypticase soy broth supplemented with 0.5% yeast extract, menadione, and hemin under anaerobic conditions.

*A. actinomycetemcomitans* (the ATCC 43718 strain) was inoculated in ATCC medium 44 (brain heart infusion broth) and cultured anaerobically (AnaeroPack<sup>®</sup>-Anaero for Susceptibility, Mitsubishi Gas Chemical Company Inc., Tokyo, Japan) at 37°C for 24 hours [18].

### **Measurement of serum anti-*P. gingivalis*/*A. actinomycetemcomitans* immunoglobulin G (IgG) antibody titers**

Specific serum IgG titers were measured using enzyme-linked immunosorbent assay (ELISA) as described previously [18,19]. In brief, 96-well microplates (EIA plates; Costar, Corning, NY, USA) were coated with 10 µg/mL sonicated *P. gingivalis* or *A. actinomycetemcomitans* extracts in carbonate buffer and incubated for 2 hours at 37°C. After blocking with 2% bovine serum albumin in carbonate buffer, the plates were washed with phosphate-buffered saline (PBS)-Tween<sup>®</sup> (1× PBS, 0.05% Tween 20<sup>®</sup>, pH 7.2). Serially diluted, reference-pooled, positive-control serum samples obtained from healthy subjects (2<sup>5</sup>-2<sup>15</sup>, 200 µL per well) and single diluted patient serum samples (2<sup>10</sup>, 200 µL per well) were added to each well, and the plates were further incubated for 1 hour at 37°C and washed again. Subsequently, 200 µL of alkaline phosphatase-conjugated goat anti-human IgG (Sigma, St. Louis, MO, USA) was added to each well. After incubation, the plates were washed and developed with phosphatase substrate (Sigma), and the optical density at 450 nm was read using a microplate reader (SoftMAX; Molecular Devices, Sunnyvale, CA, USA). Antibody titers were calculated according to a previously described method [20].

### **DNA isolation and detection of *P. gingivalis* and *A. actinomycetemcomitans* in saliva samples**

A NucleoSpin DNA tissue kit (Takara Bio Inc., Kusatsu, Japan) was used to extract DNA from the saliva according to the manufacturer's instructions. To detect *P. gingivalis* and *A. actinomycetemcomitans* DNA, the extracted DNA was subjected to quantitative polymerase chain reaction (qPCR) using a TaqMan probe (5'-FAM-TGCGTAACGCGTATGCAACTTGCC-TAMRA-3' for *P. gingivalis* and 5'-FAM-ACACGTGCTACAATGGCGTATACAGAGGGT-TAMRA-3' for *A. actinomycetemcomitans*) and primers (forward: 5'-TAGCTTGCTAAGGTCGATGG-3', reverse: 5'-CAAGTGATGCGGTTTTAGT-3', for *P. gingivalis*; and forward: 5'-GTCATCATGCGCCCTTACGAGTAG-3', reverse: 5'-CCCCATCGCTGTTGGT-3' for *A. actinomycetemcomitans*) using the thermal cycler.Dice Real-time System II.

### **Evaluation of high-sensitivity C-reactive protein (hs-CRP) concentrations in serum samples**

The concentration of hs-CRP in the serum was measured using a commercially available kit (C-reactive protein human ELISA kit; Helica Biosystems, Inc., Santa Ana, CA, USA) according to the manufacturer's protocols.

### **Detection of periodontal bacteria in the aortic valve**

Detection of *P. gingivalis* and *A. actinomycetemcomitans* in the exenterate aortic valve was performed in specimens from the patients who underwent surgical aortic replacement due to symptomatic severe AS. DNA was extracted from the aortic valve using the NucleoSpin DNA tissue kit. qPCR was performed according to the same protocol described above.

### Statistical analysis

Data distribution was assessed using the Shapiro-Wilk test. Correlations between the amount of periodontal bacteria, anti-periodontal bacteria IgG titers, and AVA, Vmax, mean PG, and DVI were evaluated using Spearman rank correlation coefficients. The Wilcoxon signed-rank test was used to compare the status between baseline and follow-up, while the Mann-Whitney U test was used for comparisons between the rapid and non-rapid AS progression groups. The statistical analysis was performed using SPSS version 22.0 (IBM Corp., Armonk, NY, USA). A *P* value <0.05 was considered to indicate statistical significance.

## RESULTS

### Subject characteristics

The mean observation period in this study was 3.3±1.9 years. Table 1 and Table 2 show the basic characteristics of the subjects along with their echocardiographic data at baseline and at the most recent follow-up. Both the AVA and DVI were significantly lower at follow-up than at baseline (*P*<0.001), whereas the Vmax and mean PG values were significantly higher at follow-up than at baseline (*P*<0.001).

**Table 1.** Clinical characteristics of patients

Characteristics	All subjects (n=45)
Age (yr)	83 (77–86)
Male	19 (42.2)
Body surface area (m <sup>2</sup> )	1.52 (1.39–1.66)
Hypertension	40 (88.9)
Diabetes mellitus	15 (33.3)
Smoking	
None	30 (66.7)
Former smoker	11 (24.4)
Current	4 (8.9)
Dyslipidemia	23 (51.1)
Chronic kidney disease	8 (17.8)
COPD	4 (8.9)
Coronary artery disease	12 (26.7)
Previous stroke	2 (4.4)
Atrial fibrillation	9 (20.0)

Values are median (interquartile range) or number (%).  
COPD: chronic obstructive pulmonary disease.

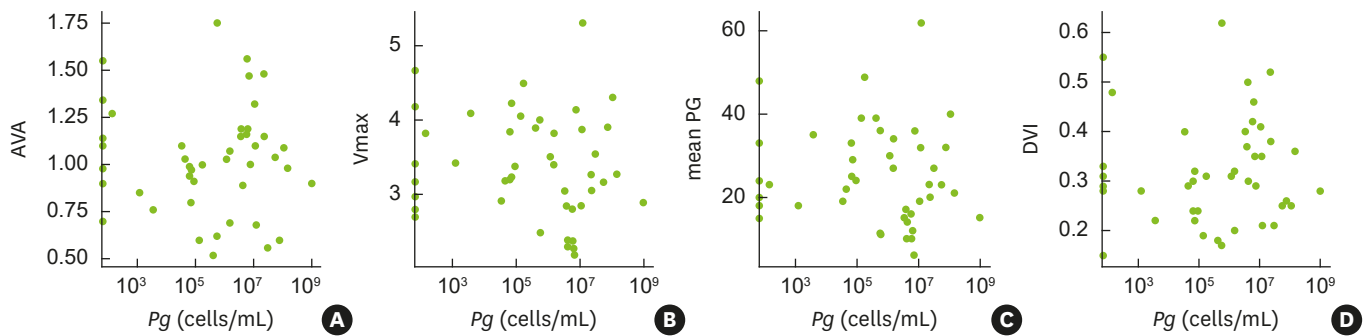
**Table 2.** Echocardiographic data of patients at baseline and follow-up

Echocardiographic data	Baseline	Latest follow-up	<i>P</i> value
AVA (cm <sup>2</sup> )	1.34±0.36	1.03±0.28 <sup>a)</sup>	<0.001
Peak velocity (m/s)	2.8±0.6	3.4±0.7 <sup>a)</sup>	<0.001
Mean PG (mmHg)	18.7±10.4	25.5±11.4 <sup>a)</sup>	<0.001
DVI	0.43±0.13	0.32±0.10 <sup>a)</sup>	<0.001
LVEF (%)	60.4±8.8	60.4±7.5	0.856
Moderate or severe AR	4 (8.9)	3 (6.7)	0.695
Moderate or severe MR	4 (8.9)	1 (2.2)	0.169
Moderate or severe TR	3 (6.7)	3 (6.7)	1.000

Values are number (%) or mean±standard deviation.

AVA: aortic valve area, PG: pressure gradient, DVI: Doppler velocity index, LVEF: left ventricular ejection fraction, AR: aortic regurgitation, MR: mitral regurgitation, TR: tricuspid regurgitation.

<sup>a)</sup>*P*<0.05 compared with baseline.



**Figure 2.** Correlations between the amount of *Pg* in the saliva and AS parameters. Correlations between *Pg* in the saliva and (A) AVA, (B) Vmax, (C) mean PG, and (D) DVI. *Pg*: *Porphyromonas gingivalis*, AS: aortic stenosis, AVA: aortic valve area, Vmax: maximal velocity, PG: pressure gradient, DVI: Doppler velocity index.

### Correlation between periodontal biochemical parameters and AS parameters

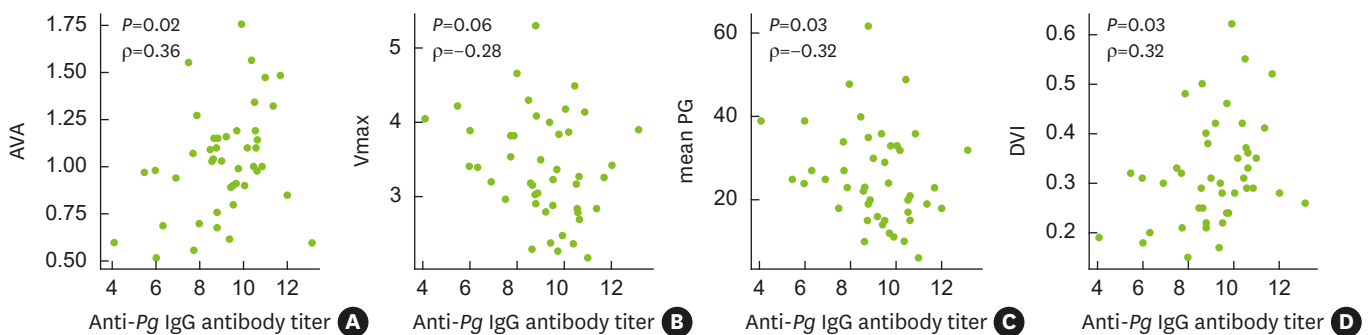
First, we evaluated the correlation between the amount of *P. gingivalis* or *A. actinomycetemcomitans* in the saliva and AS parameters at the latest follow-up (when saliva and serum samples were collected) and we found no significant correlation (Figure 2A-D, and Supplementary Figure 1A-D). Interestingly, the anti-*P. gingivalis* antibody titer in the serum showed a significant positive correlation with AVA (Figure 3A) and DVI (Figure 3D). In addition, there was a negative correlation between the anti-*P. gingivalis* IgG antibody titer and mean PG (Figure 3C). No significant correlation was observed between the anti-*P. gingivalis* IgG antibody titer and Vmax (Figure 3B). On the other hand, the anti-*A. actinomycetemcomitans* IgG antibody titer did not show any significant correlation with AS parameters (Supplementary Figure 2A-D).

### Correlation between hs-CRP concentration and AS parameters

The hs-CRP concentration showed positive correlations with Vmax (Figure 4B) and mean PG (Figure 4C). Conversely, there were no significant correlations between the hs-CRP concentration and AVA (Figure 4A) or DVI (Figure 4D).

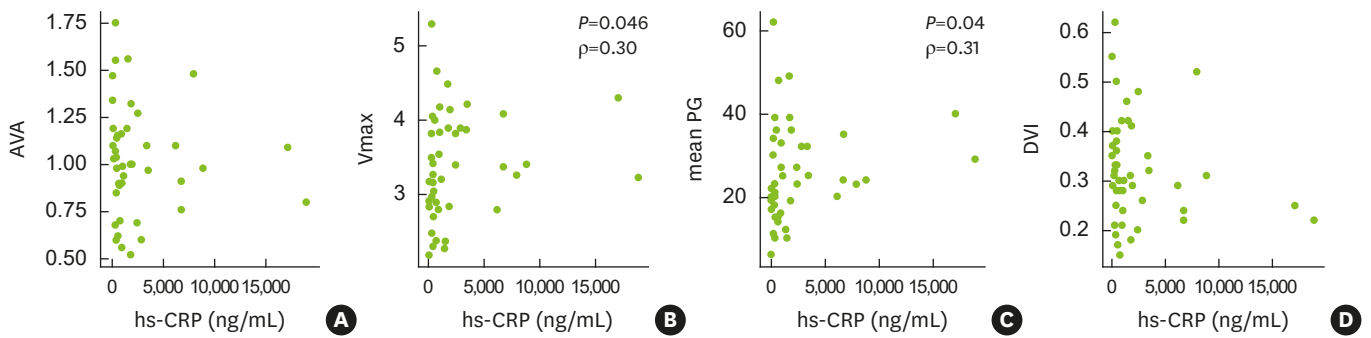
### Correlation between the anti-*P. gingivalis* IgG antibody titer and AS progression

Since the anti-*P. gingivalis* IgG antibody titer showed a significant correlation with AS parameters, we evaluated the relationship between anti-*P. gingivalis* IgG antibody titer and AS progression. Although the anti-*P. gingivalis* IgG antibody titer showed no significant



**Figure 3.** Correlations between anti-*Pg* IgG antibody titer in the serum and AS parameters. Correlations between the anti-*Pg* IgG antibody titer and (A) AVA, (B) Vmax, (C) mean PG, and (D) DVI. *Pg*: *Porphyromonas gingivalis*, IgG: immunoglobulin G, AS: aortic stenosis, AVA: aortic valve area, Vmax: maximal velocity, PG: pressure gradient, DVI: Doppler velocity index.





**Figure 4.** Correlations between the hs-CRP concentration and AS parameters.

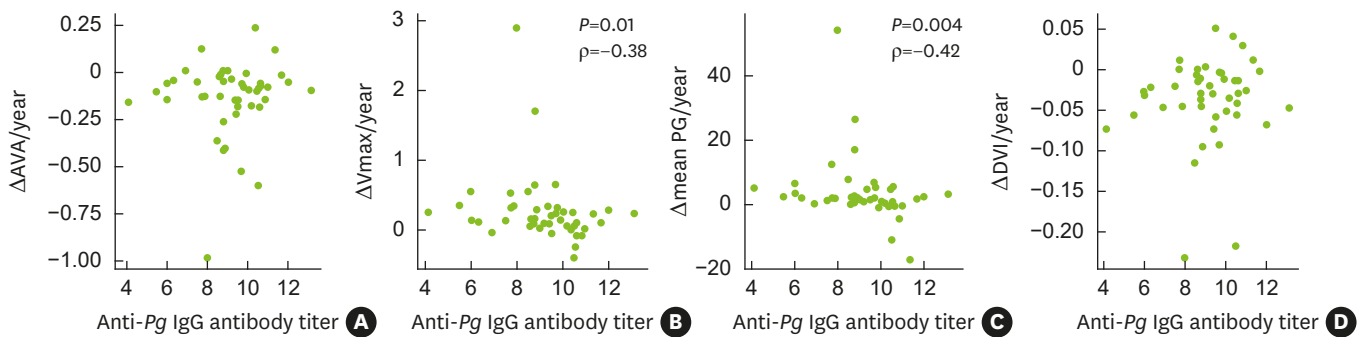
Correlations between the hs-CRP concentration and (A) AVA, (B) Vmax, (C) mean PG, and (D) DVI.

hs-CRP: high-sensitivity C-reactive protein, AS: aortic stenosis, AVA: aortic valve area, Vmax: maximal velocity, PG: pressure gradient, DVI: Doppler velocity index.

correlation with  $\Delta$ AVA/year (Figure 5A) or  $\Delta$ DVI/year (Figure 5D), a negative correlation was observed with  $\Delta$ AVA/year (Figure 5B) and  $\Delta$ mean PG/year (Figure 5C).

### Correlation between the hs-CRP concentration and AS progression

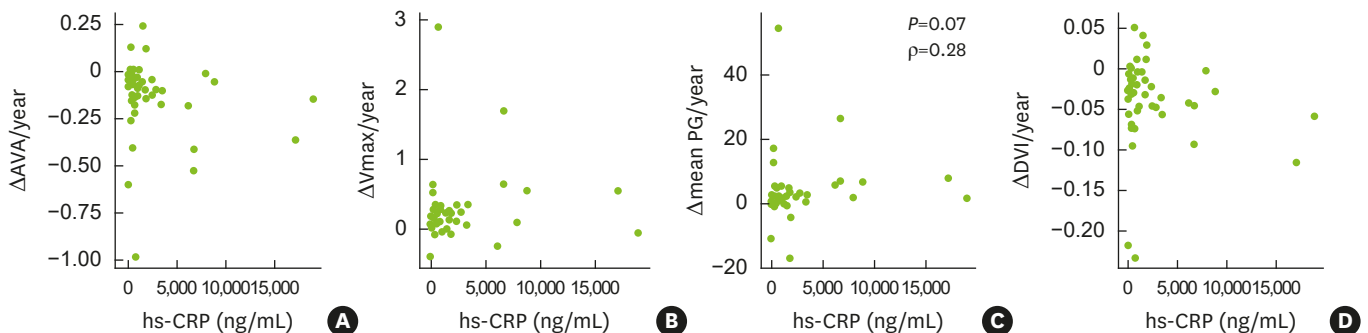
The hs-CRP concentration did not show a significant correlation with  $\Delta$ AVA/year (Figure 6A),  $\Delta$ Vmax/year (Figure 6B), or  $\Delta$ DVI/year (Figure 6D). However,  $\Delta$ mean PG showed a non-significant positive correlation ( $P=0.07$ ) with the hs-CRP concentration (Figure 6C).



**Figure 5.** Correlations between the anti-Pg IgG antibody titer in the serum and AS progression.

Correlations between the anti-Pg IgG antibody titer and (A)  $\Delta$ AVA, (B)  $\Delta$ Vmax, (C)  $\Delta$ mean PG, and (D)  $\Delta$ DVI.

Pg: *Porphyromonas gingivalis*, IgG: immunoglobulin G, AS: aortic stenosis, AVA: aortic valve area, Vmax: maximal velocity, PG: pressure gradient, DVI: Doppler velocity index.



**Figure 6.** hs-CRP levels according to the progression of degenerative AS.

Correlations between the hs-CRP concentration and (A)  $\Delta$ AVA, (B)  $\Delta$ Vmax, (C)  $\Delta$ mean PG, and (D)  $\Delta$ DVI.

hs-CRP: high-sensitivity C-reactive protein, AS: aortic stenosis, AVA: aortic valve area, Vmax: maximal velocity, PG: pressure gradient, DVI: Doppler velocity index.

Twenty-one subjects with  $\Delta$ AVA/year below  $-0.1 \text{ cm}^2/\text{year}$  were categorized into the rapid AS progression group, and 24 subjects with  $\Delta$ AVA/year greater than or equal to  $-0.1 \text{ cm}^2/\text{year}$  were categorized into the non-rapid AS progression group (Table 3). Among all characteristics evaluated, only the hs-CRP level was significantly higher in the rapid AS progression group than in the non-rapid AS progression group ( $P=0.022$ ) (Table 3 and Figure 7).

### Evaluation of periodontal bacteria in the aortic valve

One of the severe degenerative AS cases was a 71-year old woman, and the other was a 74-year old woman. The pathology of both valve specimens showed collagenous fiber growth with vitrification and formation of nodular calcification nests that were consistent with degenerative AS. The exenterate aortic valve specimens from both subjects were negative for *P. gingivalis* and *A. actinomycetemcomitans* DNA.

## DISCUSSION

*P. gingivalis* and *A. actinomycetemcomitans* are representative periodontal bacteria. *P. gingivalis* belongs to the “red complex,” a group of bacteria associated with periodontal disease [21],

**Table 3.** Differences between rapid and non-rapid AS progression groups

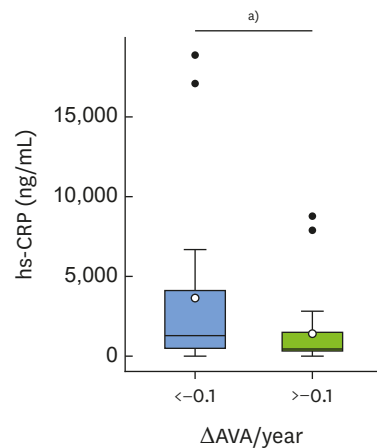
Variables	Non-rapid AS progression (n=24)	Rapid AS progression (n=21)	P value
Clinical background characteristics			
Age (yr)	82 (76–86)	84 (78–86)	0.828
Male	10 (41.7)	9 (42.9)	0.936
Body surface area (m <sup>2</sup> )	1.50 (1.43–1.63)	1.57 (1.35–1.68)	0.963
Hypertension	21 (87.5)	19 (90.5)	0.754
Diabetes mellitus	9 (37.5)	6 (28.6)	0.530
Smoking			
None	17 (70.8)	13 (61.9)	0.278
Former smoker	5 (20.8)	6 (28.6)	0.551
Current	2 (8.3)	2 (9.5)	0.889
Dyslipidemia	12 (50.0)	11 (52.4)	0.874
Chronic kidney disease	4 (16.7)	4 (19.0)	0.836
COPD	3 (12.5)	1 (4.8)	0.368
Coronary artery disease	6 (25.0)	6 (28.6)	0.789
Previous stroke	0 (0)	2 (9.5)	0.126
Atrial fibrillation	4 (16.7)	5 (23.8)	0.554
Echocardiographic data at baseline			
AVA (cm <sup>2</sup> )	1.26±0.30	1.43±0.40	0.091
Peak velocity (m/s)	2.7±0.5	2.9±0.7	0.362
Mean PG (mmHg)	18.2±11.8	19.1±8.9	0.425
DVI	0.43±0.11	0.42±0.15	0.532
LVEF (%)	60.3±7.8	60.4±10.1	0.606
Moderate or severe AR	3 (12.5)	1 (4.8)	0.368
Moderate or severe MR	1 (4.2)	3 (14.3)	0.239
Moderate or severe TR	1 (4.2)	2 (9.5)	0.477
Aa (cells/mL)	0 (0–0)	0 (0–0)	0.598
Pg (cells/mL)	1.4×10 <sup>6</sup> (4.8×10 <sup>4</sup> –6.1×10 <sup>6</sup> )	5.5×10 <sup>5</sup> (5.3×10 <sup>4</sup> –1.4×10 <sup>7</sup> )	0.741
Anti-Aa IgG antibody titer	9.4±1.5	9.7±2.0	0.592
Anti-Pg IgG antibody titer	9.4±1.7	8.7±1.8	0.245
hs-CRP, ng/mL	464 (266–1,452)	1,760 (506–6,293) <sup>a</sup>	0.022

Values are median (interquartile range), mean±standard deviation, or number (%).

AS: aortic stenosis, COPD: chronic obstructive pulmonary disease, AVA: aortic valve area, PG: pressure gradient, DVI: Doppler velocity index, LVEF: left ventricular ejection fraction, AR: aortic regurgitation, MR: mitral regurgitation, TR: tricuspid regurgitation, Aa: *Aggregatibacter actinomycetemcomitans*, Pg: *Porphyromonas gingivalis*, IgG: immunoglobulin G, hs-CRP: high-sensitivity C-reactive protein.

<sup>a</sup> $P<0.05$  compared with non-rapid AS progression subjects.





**Figure 7.** hs-CRP concentrations between rapid AS progression and non-rapid AS progression groups. hs-CRP: high-sensitivity C-reactive protein, AS: aortic stenosis, AVA: aortic valve area.  
<sup>a)</sup>P=0.022

and is the most common bacterium in periodontal infections. A previous study showed that periodontitis due to *P. gingivalis* increased the risk of developing peripheral arterial disease by 5-fold [13]. In contrast, *A. actinomycetemcomitans*, which harbors both endotoxins and exotoxins [22], was frequently detected in patients with severe periodontitis and was associated with aggressive periodontitis [23,24]. *A. actinomycetemcomitans* infection was suggested to play an important role in the development of acute coronary syndrome in the Japanese population [14]. In this study, saliva samples were collected and evaluated for the presence of *P. gingivalis* and *A. actinomycetemcomitans* by qPCR. Microbial composition depends on the collection site and sample type [25]. Therefore, subgingival plaque might be more suitable than saliva for evaluating periodontal status. However, the presence and relative abundance of *P. gingivalis* in the saliva have been found to be associated with periodontitis [26]. In addition, unstimulated saliva was found to be representative of pooled subgingival plaque samples, making it a useful tool for the detection of *A. actinomycetemcomitans* in the mouth [27].

Many reports have shown the relationship between periodontal disease and cardiovascular disease [3,5,10,11]. According to the American Heart Association, periodontal disease is associated with cardiovascular disease independently of other confounding factors, although the causative relationship is unclear [28]. Interestingly, the anti-*P. gingivalis* IgG antibody titer showed a negative correlation with the progression or clinical status of AS, unlike previous reports about the relationship between periodontal disease and cardiovascular disease. Patients with coronary heart disease showed higher anti-*P. gingivalis* serum IgG antibody titers than those without coronary heart disease [29]. In addition, the prevalence of heart failure was higher in subjects with high anti-*P. gingivalis* serum IgG antibody titers [30]. The discrepancy among these results may reflect differences in host immunity. In this study, there was no significant correlation between the anti-*P. gingivalis* IgG antibody titer and the amount of *P. gingivalis* in the saliva (data not shown). However, generally, *P. gingivalis* infection increases the anti-*P. gingivalis* IgG antibody titer, which decreases after periodontal treatment [31]. IgG antibody production is based on host immunity. Subjects with AS are generally quite old and their immune system may be disrupted [32]. Therefore, the titers of IgG antibodies against periodontopathic bacteria might not have reflected periodontopathic bacterial infections in this study. This might explain why our results are different from those of previous studies on the relationship between periodontitis and cardiovascular diseases.

hs-CRP is a well-known biomarker of low-grade inflammation, and several studies have reported hs-CRP to be a prognostic marker of cardiovascular events, including for patients with AS [11,33-35]. Furthermore, a high hs-CRP level was associated with an increased risk of aortic valve replacement in patients with AS [36]. However, in the present study, only  $\Delta V_{max}/year$  and  $\Delta mean PG/year$  showed significant associations with hs-CRP, whereas  $\Delta AVA/year$  and  $\Delta DVI/year$  did not. AVA and DVI are calculated using a formula with echocardiographic parameters such as the time velocity index at the aortic valve and left ventricular outflow tract, and the left ventricular outflow tract diameter. Therefore, AVA and DVI are more vulnerable to measurement errors than  $V_{max}$  and mean PG, and are not recommended as a first choice for the diagnosis of severe AS by the American College of Cardiology/American Heart Association guideline [15]. This may also be a reason for the inconsistent correlations with hs-CRP in this study. Our results also showed that the hs-CRP level in the rapid AS progression group was significantly higher than that in the non-rapid AS progression group, indicating that some inflammatory mechanisms might be associated with disease progression. Although we could not identify the cause of the inflammation, including the indirect involvement of periodontal bacteria, a previous report showed that degenerative AS had 2 distinct phases: early initiation and propagation [37,38]. Pathophysiologically, the early initiation phase is dominated by valvular lipid deposition, injury, and inflammation, with similarities to atherosclerosis, while the propagation phase is characterized by the emergence of pro-calcific and pro-osteogenic factors, which initiate the self-perpetuating processes of calcification and ultimately drive AS progression. Taken together, a prospective study in the future using calcium-detecting imaging modalities such as cardiac computed tomography (CT) and  $^{18}F$  positron emission CT will be required to clarify the mechanism of AS progression.

Several significant findings emerged from this pilot study. First, echocardiographic parameters during follow-up showed that AS had progressed significantly. Second, the anti-*P. gingivalis* IgG antibody titer showed a negative correlation with the progression of degenerative AS in this population. Third, despite the absence of any significant correlation between the hs-CRP level and  $\Delta AVA/year$ , the hs-CRP level increased significantly in the rapid AS progression group compared with the non-rapid AS progression group. Finally, the DNA of periodontal bacteria was not detected in the 2 aortic valve specimens, which is in line with a previous pilot study reporting lack of periodontal pathogens in the aortic valve specimens and blood samples of patients with AS [39]. Therefore, irrespective of the progression of degenerative AS, these bacteria did not emerge as significant risk factors in our cohort.

This study has several limitations. First, this was a retrospective, single-center, pilot study. The sample size was thus small and the observation period was not long enough to comprehensively test the hypothesis. Second, we investigated the concentrations of periodontopathic bacteria and the serum IgG titers at a single time point. Oral bacteria in the saliva are considered to be stable without causing acute disease or immune deficiencies, which were not present in any of the patients included in this study cohort. Third, we did not perform standard dental examinations, as the study was performed at our institution's cardiology department. However, a previous report showed that the anti-*P. gingivalis* IgG antibody levels of periodontitis patients were significantly higher than those of healthy controls, and the anti-*P. gingivalis* IgG titer was associated with the severity of periodontitis [40]. A multi-center study with a larger population, longer follow-up period, and dental examinations should address these limitations.

To the best of our knowledge, this is the first study to examine the relationship between periodontal bacteria and the progression of degenerative AS using periodontological and immunological approaches. Our results suggest that periodontopathic bacteria such as *P. gingivalis* and *A. actinomycetemcomitans* are not directly related to the progression of degenerative AS, in contrast to their well-known association with other atherosclerotic cardiovascular diseases. However, some inflammatory mechanisms may be associated with AS progression and warrant further investigation.

## ACKNOWLEDGEMENTS

We thank Ms. Aki Takahashi, who is a technician at Teikyo University, for providing technical support.

## SUPPLEMENTARY MATERIALS

### Supplementary Figure 1

Correlations between amount of *Aa* in the saliva and AS parameters.

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### Supplementary Figure 2

Correlations between anti-*Aa* IgG antibody titer in the serum and AS parameters.

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