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Ability of S100 proteins and matrix metalloproteinase-9 to identify periodontitis in a ligature-induced periodontitis dog model

Mengmeng Liu[1] | Jae Won Lee[1] | Soyoung Jung[1] | Suk Ji[2] | Youngnim Choi[1]

[1]Department of Immunology and Molecular Microbiology, School of Dentistry and Dental Research Institute, Seoul National University, Seoul, Korea

[2]Department of Periodontology, Ajou University Hospital, Suwon, Korea

Correspondence
Suk Ji, Department of Periodontology, Ajou University Hospital, 164, World cup-ro, Yeongtong-gu, Suwon, Korea.
Email: sukji@ajou.ac.kr

Youngnim Choi, Department of Immunology and Molecular Microbiology, School of Dentistry and Dental Research Institute, Seoul National University, 101 Daehak-ro, Jongno-gu, Seoul 03080, Korea.
Email: youngnim@snu.ac.kr

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Abstract

Aims: The present study aimed to monitor the levels of selected salivary biomarkers during the development and treatment of periodontitis and to evaluate their ability to identify periodontitis in dogs.

Materials and methods: A total of 15 beagle dogs were divided into a control group (no ligature), group 1 (ligature on six teeth), and group 2 (ligature on 12 teeth). The experimental periods consisted of 8 weeks of periodontitis induction and 4 weeks of treatment. Clinical measurements and the sampling of saliva were performed every 4 weeks. The levels of S100A8, S100A9, S100A8/A9, and matrix metalloproteinase (MMP)-9 were measured by enzyme-linked immunosorbent assay.

Results: All experimental animals and two control animals developed periodontitis, which was successfully treated. All salivary biomarkers were significantly increased in periodontitis with high diagnostic power (c-index ≥ 0.944) and were able to identify animals with periodontitis on a single tooth. Whereas the levels of salivary S100A8/A9 recovered to levels in health, those of S100A8, S100A9, and MMP-9 in periodontitis stability remained significantly higher than in health.

Conclusion: Salivary S100A8, S100A9, S100A8/A9, and MMP-9 may be used for the screening of periodontitis in dogs, but with caution of other conditions that can affect their levels in saliva.

KEYWORDS

biomarkers, matrix metalloproteinase-9, periodontitis, saliva

1 | INTRODUCTION

Periodontitis is a prevalent inflammatory disease in humans and dogs, affecting 20%–50% of the global human population and up to 84% of dogs (Kortegaard, Eriksen, & Baelum, 2008; Nazir, 2017). Periodontitis is initiated by the dysbiosis of the subgingival plaque biofilm; however, it is the host immune responses against invading bacterial components and bacteria that predominantly mediate the destruction of periodontal tissue (Kinane, Stathopoulou, & Papapanou, 2017). As destroyed alveolar bone hardly recovers, and periodontitis often progresses without self-recognition, it is ideal to diagnose periodontitis when bone destruction is initiated at a single site. Furthermore, accumulating evidence suggests that periodontitis contributes to the overall inflammatory burden of the body and is associated with diverse systemic conditions, including type 2 diabetes, major cardiovascular events, and Alzheimer's disease (Dominy et al., 2019; Myllymäki et al., 2018; Park et al., 2019). Therefore, the importance of the early diagnosis and treatment of periodontitis is being increasingly recognized.

Currently, periodontitis is diagnosed based on radiography and clinical measurements of periodontal probing depth (PPD), clinic attachment level, and bleeding on probing (BOP). This traditional

method is time-consuming and can be performed only when patients visit dental clinics. Furthermore, all of these procedures are performed under general anaesthesia in dogs, which imposes an extra burden. With advances in sensor technology, interests in near-patient testing to diagnose periodontitis at home are increasing. Saliva is a promising biological medium for this purpose due to non-invasive, easy, and painless sample collection, even in dogs.

During the last decade, various biomarkers for periodontitis have been identified in saliva and gingival crevicular fluid (GCF), including S100A8, S100A9, S100A8/A9, and matrix metalloproteinase-9 (MMP-9) (Haigh et al., 2010; Teng, Sodek, & McCulloch, 1992). While the major source of MMP-9 in saliva is extravasated degranulating neutrophils, the major sources of S100A8 and S100A9 include both neutrophils and oral epithelial cells (Gorr, 2009; Westerlund et al., 1996). S100A8 and S100A9 are also more prevalent in the GCF of teeth with periodontitis compared with those with gingivitis in dogs (Davis et al., 2016). During inflammation, S100A8 and S100A9 are actively released, form homodimers or heterodimers (S100A8/A9), and serve a critical role in the development of inflammation (Ryckman, Vandal, Rouleau, Talbot, & Tessier, 2003). MMP-9, a zinc-dependent endopeptidase, is one of the major proteases involved in periodontal tissue destruction and regulates several functions associated with inflammation (Mäkelä, Salo, Uitto, & Larjava, 1994; McMillan et al., 2004).

A number of studies have reported that salivary S100A8, S100A9, S100A8/A9, or MMP-9 can differentiate patients with periodontitis from subjects without periodontitis with varying degrees of accuracy (Holmström et al., 2019; Karna, Shin, Kim, & Kim, 2019; Kim, Shin, Kim, Kim, & Ahn, 2016; Ramseier et al., 2009; Wu et al., 2018). Although there have been a limited number of longitudinal studies, how the levels of these biomarkers change during disease progression from health to periodontitis and following treatment of the disease remains to be elucidated. In addition, whether these biomarkers can be used for the diagnosis of periodontitis in dogs is not known. The present study aimed to monitor changes in the levels of S100A8, S100A9, S100A8/A9, and MMP-9 in saliva during the development and treatment of periodontitis and to evaluate their ability to identify periodontitis in a ligature-induced dog periodontitis model.

Clinical Relevance

Scientific rationale for the study: Association of salivary S100A8, S100A9, S100A8/A9, and MMP-9 with periodontitis has been shown in humans. However, changes in the levels of these biomarkers during the periodontal disease initiation, progression, and recovery are not elucidated.
Principal findings: All biomarkers significantly increased from health to periodontitis and decreased after treatment in a ligature-induced periodontitis dog model. Whereas the levels of salivary S100A8, S100A9, and MMP-9 in periodontitis stability remained significantly higher than in health, those of S100A8/A9 recovered to levels in health.
Practical implication: The biomarker that recovers the levels in health after treatment may have merit.

2 | METHODS AND MATERIALS

2.1 | Animals

The animal experiment was approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-130806-4-1). A total of 15 beagle dogs (six females and nine males) aged 12 months old were used in the present study. During the experiment, the dogs were individually housed at an ambient temperature of 20–25°C and a relative humidity of 30%–70% and were fed with a vitamin C-deficient diet (LabDiet). All procedures, with the exception of saliva collection and tooth brushing, were performed under general anaesthesia with a tiletamine–zolazepam mixture (15 mg/kg; Virbac Korea). Local anaesthesia was applied to the surgical site by injection of 2% lidocaine hydrochloride and 1:100,000 epinephrine (Septodont).

2.2 | Induction and treatment of periodontitis

The experiments were performed in two separate sets using female and male dogs, respectively. The overall experimental scheme is outlined in Figure 1a. In the 2-week pre-conditioning period, the periodontal status of the dogs was standardized by scaling, daily tooth brushing, and feeding with a pellet-type hard food. The dogs were then randomly divided into a control group (no ligature), group 1 (ligature on six teeth), and group 2 (ligature on 12 teeth). The dogs in group 1 received ligatures on the second, third, and fourth premolars in the left and right sides of the upper jaw (UP2, UP3, and UP4). The dogs in group 2 received ligatures on the third and fourth premolars and first molars in the left and right sides of the lower jaw (LP3, LP4, and LM1), in addition to the six maxillary premolars. A ligature was placed around the cervical region of the tooth and sutured through the inter-dental gingiva using 2–0 silk (Covidien). The ligature was checked daily and re-placed if it was no longer intact. For the 8-week periodontitis induction period, the dogs did not receive tooth brushing and were fed with a soft-moistened diet. After 8 weeks, the dogs received periodontal treatments, which included scaling of all teeth and gingivectomy for the experimental teeth on the right side (control and group1: UP2, UP3, and UP4; group2: UP2, UP3, UP4, LP3, LP4, and LM1) following removal of the ligature. For the 4-week healing period, the dogs received daily tooth brushing and were fed with a pellet-type hard diet. The effect of gingivectomy on the treatment outcome will be separately reported.

2.3 | Evaluation of clinical parameters

The clinical parameters, including plaque index (PI), gingival index (GI), BOP, PPD, and gingival recession (GR), were recorded at 0, 4,

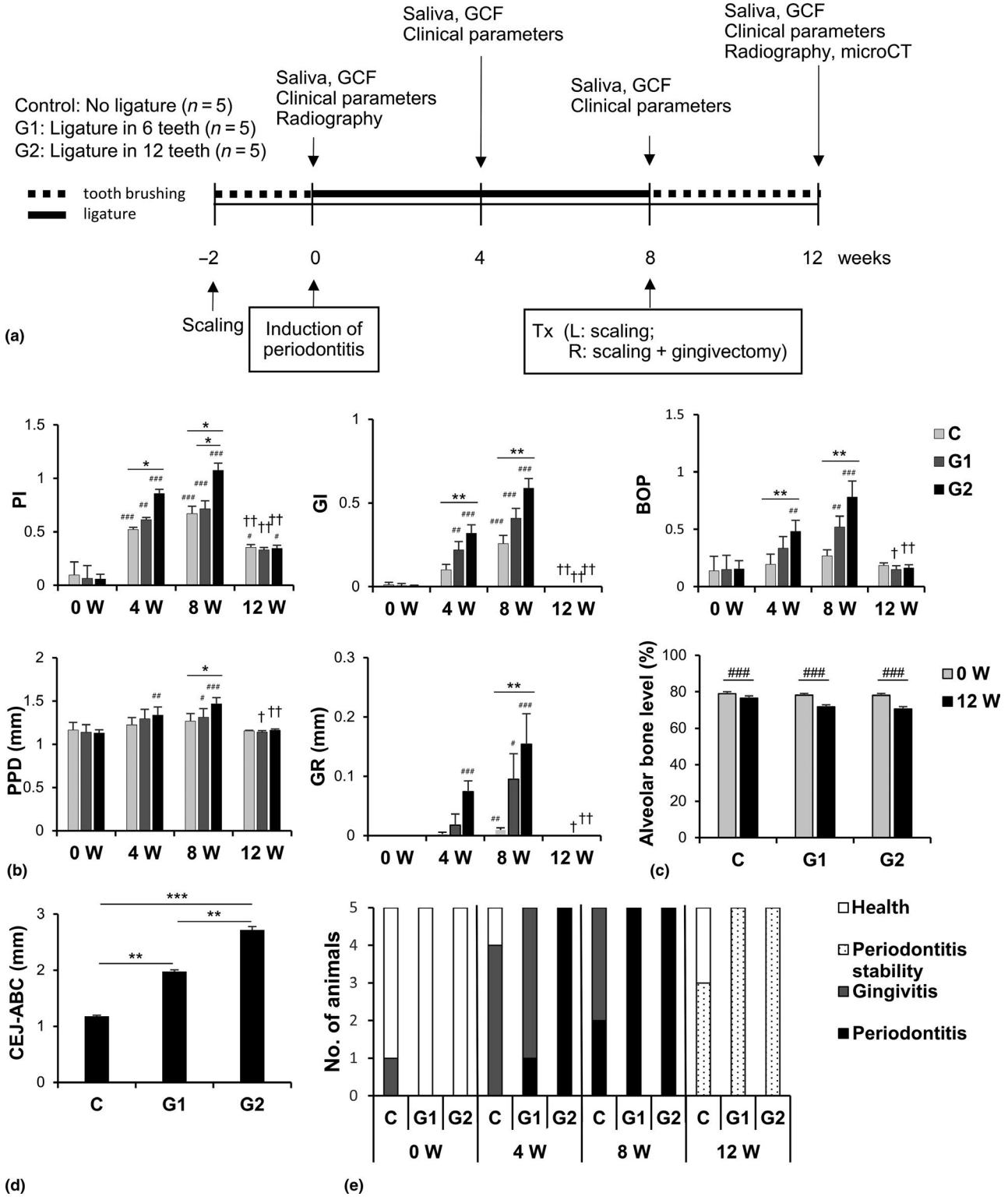


FIGURE 1 Induction and successful treatment of periodontitis in beagle dogs. (a) Outline of the overall experimental scheme. (b) Measurements of clinical parameters during the induction and treatment periods of periodontitis. (c) Alveolar bone levels of maxillary premolars were evaluated by radiography at 0 and 12 weeks. (d) Distances from the CEJ to the ABC of the 12 experimental teeth at the maxilla and mandible were measured by micro-CT at week 12. (e) Periodontal status of each animal at each evaluation time point scored according to the criteria in Table 1. In the control group, two animals were diagnosed with periodontitis at week 8 based on gingival inflammation, PPD, and GR, whereas three animals showed significant bone loss over the 12-week period on radiography, and were classified as periodontitis stability at week 12. Column graphs represent the mean ± *SE* of the mean. **p* < .05; ***p* < .01; ****p* < .0005 presenting inter-group differences. #*p* < .05; ##*p* < .01; ###*p* < .0005 compared with baseline (week 0). † *p* < .05; †† *p* < .0005 compared with week 8

TABLE 1 Scoring criteria for periodontal status

Status	Inflammation	Attachment loss	Bone loss
Health	No	No	–
Gingivitis	Bleeding on probing in at least two adjacent sites with redness and oedema		–
Periodontitis	Bleeding on probing in at least two adjacent sites with redness and oedema	>2 periodontal probing depth (>3 on canine) or >0 gingival recession in at least two adjacent sites	–
Periodontitis stability	No	No	Significant reduction compared with baseline

8, and 12 weeks. PPD, BOP, and GR were recorded at six sites per tooth, that is mesiobuccal, mid-buccal, distobuccal, mesiolingual, mid-lingual, and distolingual sites. The criteria for PI, GI, and BOP were the same as those used in humans (Löe, 1967; Newbrun, 1996). All measurements were performed by one experienced clinician using a standard periodontal probe (Frontier Dental Industrial Co.).

2.4 | Measurements of alveolar bone loss

Alveolar bone loss was evaluated by radiography and microcomputed tomography (micro-CT). Digital dental radiography was performed at 0 and 12 weeks using a bisecting technique for the maxillary experimental teeth to examine bone loss over the experimental period. The alveolar bone levels were determined as the percentage of the distance from the alveolar bone crest (ABC) to the root apex to that from the prominence of crown to the root apex, which were measured at the mesial margin of each experimental tooth using ImageJ software. As accurate identification of the cementoenamel junction (CEJ) was difficult in many cases, the prominence of the crown was used as an alternative anatomical point.

At 12 weeks, the mandibles and maxillae (from only the female set) were obtained, and the hemimaxillae and hemimandibles were de-fleshed and examined using a benchtop micro-CT system (Bruker). The sagittal plane of the hemi-jaw sample was set parallel to the X-ray beam axis. The samples were scanned at a resolution of 12 μm in all three spatial dimensions. The distances between the CEJ and the ABC at the mesiobuccal, mid-buccal, distobuccal, mesiolingual, mid-lingual, and distolingual sites of each tooth were measured, and three experimental teeth per each hemi-jaw from four hemi-jaws per dog were measured using an on-screen computer-aided measurement package. All alveolar bone loss measurements were performed by one researcher in a blinded manner using coded images.

2.5 | Collection of saliva and GCF

Saliva was collected at 0, 4, 8, and 12 weeks 1 day prior to clinical measurements between 9 and 10 a.m. Salivary secretion was stimulated by showing the animal treats, and saliva was then collected using a SalivaBio children's swab (Salimetrics) under conditions in which the dogs were considered to be completely relaxed. The salivary samples were centrifuged for 20 min at 1,000 *g*, and the supernatants were carefully collected and stored in aliquots at − 80°C.

Gingival crevicular fluid samples were collected by placing periopapers (OraFlow Inc.) into the mid-buccal and mid-lingual gingival sulcus of the left and right UP3 and UP4 for 30 s. The two periopapers from each tooth were eluted with 200 μl Dulbecco's phosphate-buffered saline and stored at −80°C.

2.6 | Enzyme-linked immunosorbent assay (ELISA)

The levels of S100A8, S100A9, S100A8/A9, and MMP-9 proteins in saliva or GCF were determined in duplicates using ELISA kits (MyBio Source). The sensitivity of each kit was 1, 1, 0.1, and 0.1 ng/ml, respectively. Matching number of samples from the three groups were analysed in one plate to minimize the effect of inter-plate variabilities.

2.7 | Statistical analyses

The data are expressed as the mean ± *SE* of the mean, unless described otherwise. Clinical parameters and biomarker concentrations were almost normally distributed within each group at one-time point but not following pooling. Therefore, two-way mixed analysis of variance with Tukey's post hoc test was used to determine intergroup differences over time, but Spearman's rho was used to determine correlations between two parameters. The diagnostic power of biomarkers was analysed by receiver operating characteristic (ROC) curve to evaluate the c-index producing the area under the curve (AUC). $p < .05$ was considered to indicate statistical significance. All statistics were performed using SPSS software version 23.0 (IBM).

3 | RESULTS

3.1 | Successful induction and treatment of periodontitis in beagle dogs

Different degrees of periodontitis were established by applying a ligature on six (group 1) or 12 teeth (group 2) in 1-year-old beagle

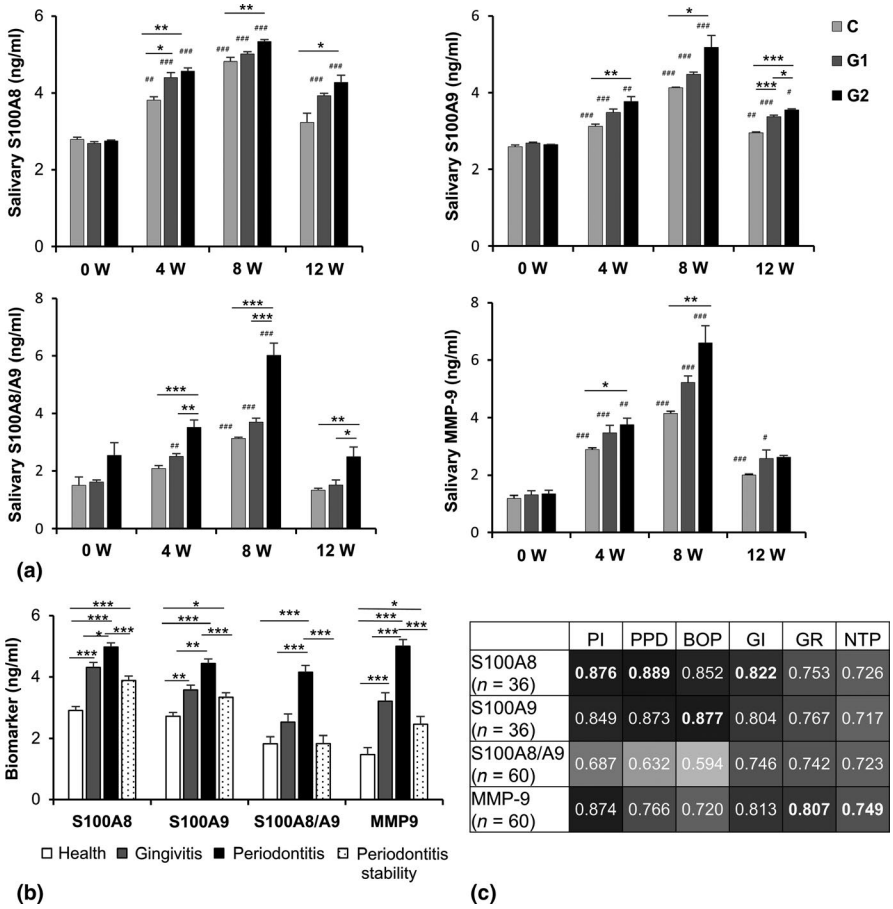
dogs. During the total 12-week period of periodontitis induction and recovery following treatment, changes in periodontal parameters for the full mouth were comprehensively monitored every 4 weeks (Figure 1a). PI significantly increased compared with the baseline level as early as 4 weeks, even in the control group. In group 1, a significant increase in the inflammation index (GI) was observed as early as week 4; however, increases in the tissue destruction index (GR) were observed at the later time point of week 8. In group 2, increases in all parameters were observed at both week 4 and 8. During the periodontitis induction period, inter-group differences in the clinical parameters were observed only between the control group and group 2. Four weeks following ligature removal and periodontal treatment, all clinical parameters returned to baseline levels, with the exception of PI, which was marginally increased, compared with the level at baseline (Figure 1b).

The comparison of radiographs taken at 0 and 12 weeks revealed that all three groups experienced alveolar bone loss over the 12 weeks (Figure 1c). The degree of bone destruction at week 12 measured in four hemi-jaws by micro-CT presented with inter-group differences: group 2 > group 1 > control (Figure 1d).

The results of 60 evaluations (15 animals at four time points) were classified into heath, gingivitis, periodontitis, and periodontal disease stability using a scoring system (Table 1) adapted from Davis et al. (2016) and Lang and Bartold (2018). Periodontitis was induced in all animals in group 2 at week 4 and in group 1 at week 8, which was effectively treated. In the control group, two animals were diagnosed with periodontitis at week 8 based on gingival inflammation, PPD, and GR, whereas three animals showed significant bone loss over the 12 week period on radiography, and were classified as periodontitis stability at week 12 (Figure 1e).

3.2 | Diagnostic power of salivary biomarkers S100A8, S100A9, S100A8/A9, and MMP-9 for periodontitis

To validate the salivary biomarkers associated with periodontitis, the levels of S100A8, S100A9, S100A8/A9, and MMP-9 in saliva were determined by ELISA. The levels of S100A8, S100A9, and MMP-9 were significantly increased in all three groups at week 4, further increased at week 8, and decreased following treatment, although not to baseline levels. By contrast, the levels of S100A8/A9 were significantly increased at week 8 and restored the baseline levels following treatment. During the periodontitis induction period, inter-group differences in the levels of salivary biomarkers were observed mainly between the control and group 2. Of note, the levels of S100A9 at week 12 showed significant inter-group differences, reflecting the degree of bone destruction (Figure 2a). The levels of each biomarker were compared according to the status of periodontal health. All biomarkers showed significant differences between periodontitis



	PI	PPD	BOP	GI	GR	NTP
S100A8 (n = 36)	**0.876**	**0.889**	0.852	**0.822**	0.753	0.726
S100A9 (n = 36)	0.849	0.873	**0.877**	0.804	0.767	0.717
S100A8/A9 (n = 60)	0.687	0.632	0.594	0.746	0.742	0.723
MMP-9 (n = 60)	0.874	0.766	0.720	0.813	**0.807**	**0.749**

(c)

FIGURE 2 Levels of salivary biomarkers S100A8, S100A9, S100A8/A9, and MMP-9. (a) Levels of biomarkers in saliva sampled at 0, 4, 8, and 12 weeks were determined by ELISA. The levels of S100A8 and S100A9 were analysed only in the second set of experiment using male dogs (n = 3 per group) because enough amounts of saliva were not collected from the first set. (b) The levels of biomarkers determined in all samples were compared according to the periodontal status scored in Figure 1e. *p < .05; **p < .01; ***p < .0005 presenting inter-group differences. #p < .05; ##p < .01; ###p < .0005 compared with baseline. (c) Correlation heat map between salivary biomarkers and clinical parameters. The numbers represent Spearman's rho. NTP, number of teeth with periodontitis; MMP, matrix metalloproteinase; ELISA, enzyme-linked immunosorbent assay

and the other three statuses (Figure 2b). In addition, the levels of all biomarkers showed significant positive correlations with clinical parameters. The inflammation indices, including BOP and GI, had the highest correlation with S100A8 and S100A9, whereas indices for tissue destruction, including GR and the number of tooth with periodontitis (NTP) had the highest correlation with MMP-9 (Figure 2c).

We also analysed the diagnostic power of each biomarker for screening periodontitis by ROC analysis. All biomarkers had an AUC ≥ 0.944. S100A8 had the greatest diagnostic power based on the AUC, whereas MMP-9 had the greatest diagnostic power based on the accuracy of diagnosis (Figure 3a and Table 2). The levels of each biomarker were plotted together with the number of teeth with gingivitis or periodontitis. The animals with gingivitis of ≥7 teeth were often false positively diagnosed. However, the duration of inflammation also appeared to influence the level of biomarkers. The samples obtained at week 4 from the animals with gingivitis of 9 or 10 teeth were correctly diagnosed by S100A9, S100A8/A9, and MMP-9. Similarly, the animals, which had developed periodontitis of six teeth at week 4, were misdiagnosed by S100A9 and S100A8/A9, whereas the animals, which had developed periodontitis at one tooth at week 8, were all correctly diagnosed (Figure 3b).

3.3 | Diagnostic power of GCF biomarkers S100A8/A9 and MMP-9 for periodontitis

In adjunct to salivary biomarkers, the kinetics of selected biomarkers in GCF samples obtained from four maxillary teeth per animal was also examined. Changes in the clinical parameters of the teeth from which the GCF were sampled were similar to those of the full mouth. However, as only experimental teeth were included for analyses, significant differences between the control group and group 1 were also observed (Figure 4a). Due to the limited amount of samples, only S100A8/A9 and MMP-9 were analysed. The levels of both S100A8/A9 and MMP-9 were significantly increased in all three groups at week 4, which further increased at week 8, then returned to baseline levels at week 12 (Figure 4b). The levels of both S100A8/A9 and MMP-9 in GCF had strong positive correlations with those in saliva (Figure 4c). When the levels of GCF biomarkers were compared according to the periodontal health status of each tooth (Figure 4d), the levels of both S100A8/A9 and MMP-9 were significantly higher in periodontitis than in the other three statuses (Figure 4e). ROC curve analysis revealed that S100A8/A9 resulted in higher AUC and accuracy of diagnosis than MMP-9 (Figure 4f and Table 2).

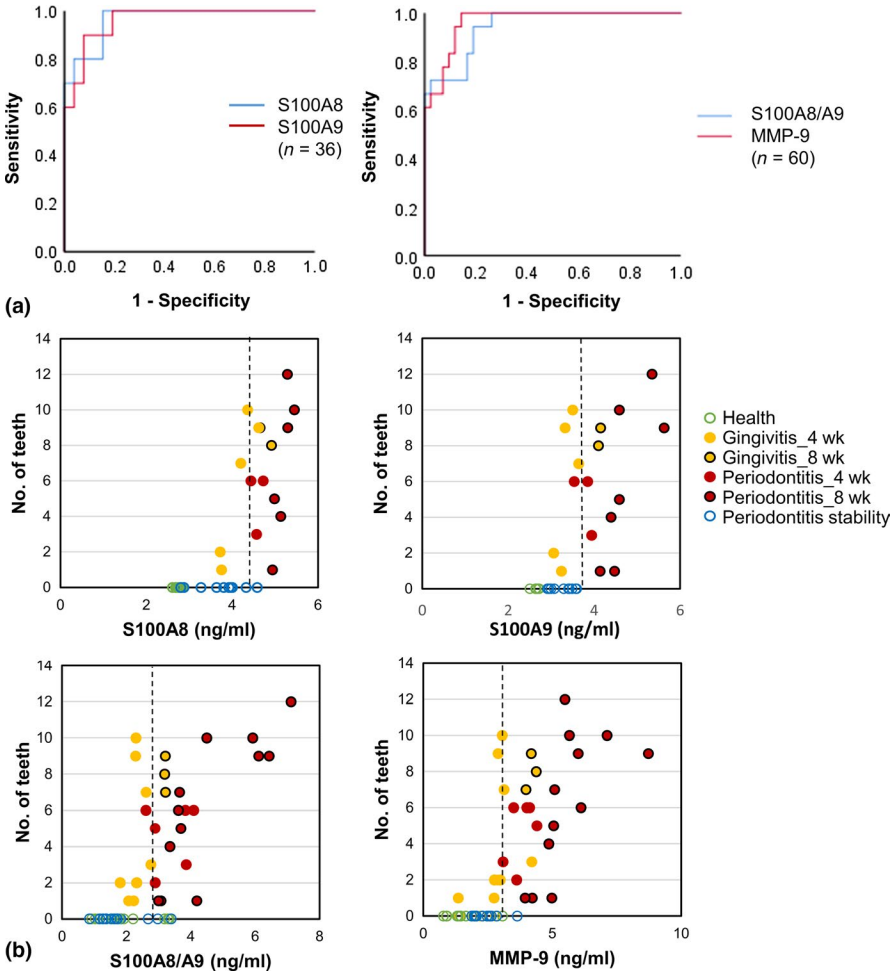


FIGURE 3 Diagnostic power and characteristics of salivary biomarkers for periodontitis identification. (a) ROC curves of salivary S100A8, S100A9, S100A8/A9, and MMP-9. (b) Scatter plots between the levels of biomarkers and the number of teeth with gingivitis or periodontitis in each sample. The vertical dotted lines indicate the threshold values for the diagnosis of periodontitis presented in Table 2. MMP, matrix metalloproteinase; ROC, receiver operating characteristic

TABLE 2 Power of salivary (s) or GCF (g) biomarkers to diagnose periodontitis

Biomarker	Area under curve	*p*	Threshold (ng/ml)	Sensitivity	Specificity	Accuracy
sS100A8	0.965 (0.913–1)	<.005	4.40	1.000	0.846	0.923
sS100A9	0.962 (0.906–1)	<.005	3.74	0.900	0.923	0.912
sS100A8/A9	0.944 (0.925–1)	<.005	2.82	0.944	0.810	0.877
sMMP9	0.964 (0.891–0.998)	<.005	3.08	1.000	0.857	0.929
gS100A8/A9	0.916 (0.876–0.955)	<.005	5.27	0.843	0.862	0.853
gMMP9	0.905 (0.867–0.944)	<.005	2.93	0.980	0.672	0.826

Abbreviations: GCF, gingival crevicular fluid; MMP, matrix metalloproteinase.

4 | DISCUSSION

The present study demonstrates the ability of salivary and GCF biomarkers to identify periodontitis in a ligature-induced periodontitis dog model.

The highest diagnostic accuracy was observed for salivary MMP-9, although all biomarkers assessed presented with an AUC ≥ 0.944, which is greater than those reported in humans (Kim et al., 2016; Ramseier et al., 2009; Wu et al., 2018). The higher diagnostic power of the same biomarkers in dogs than in humans may be attributed to the lack of common confounding variables including age, oral hygiene, smoking, drinking, and other periodontitis-associated systemic conditions. In the setting of evaluating community dogs with variable ages and systemic diseases, which can affect the levels of biomarkers in saliva, the diagnostic powers may decrease. The ELISA kit used in this study measured both pro- and active MMP-9. Although total and active MMP-9 are reported to differentiate periodontitis in humans (Kim et al., 2016; Ramseier et al., 2009; Wu et al., 2018), only active MMP-8 was effective in the diagnosis of periodontitis (Leppilahti et al., 2011; Sorsa et al., 2011). When several saliva samples were analysed for the activity of MMP-9, inter-group differences were revealed, which were not detected by total MMP-9 (Figure S1). This suggests that measurement of active MMP-9 alone would provide better diagnostic power.

Controlled experimental settings allowed examination of the pure effect of periodontal disease initiation, progression, and recovery on the levels of salivary S100A8, S100A9, S100A8/A9, and MMP-9, which is the greatest strength of the present study. As with S100A8/A9 (Nishii et al., 2013; Sorenson, Khammanivong, Guenther, Ross, & Herzberg, 2012), MMP-9 is secreted by infiltrating neutrophils and macrophages in addition to resident cells (Franco, Patricia, Timo, Claudia, & Marcela, 2017). Therefore, the rise and fall of these biomarkers during the induction and treatment periods of periodontitis may reflect the degree of immune cell infiltration. The biological functions of secreted S100A8, S100A9, and S100A8/A9 include leucocyte recruitment, induction of cytokines, antimicrobial function, anti-inflammatory function, and modulation of cell proliferation (Wang et al., 2018). In our study, GI and BOP had the highest positive correlation with S100A8 (r = .822) and S100A9 (r = .877), respectively, but only intermediate levels of correlation with S100A8/A9. This suggests that S100A8 and S100A9 predominantly serve pro-inflammatory functions in periodontal disease, whereas the functions of S100A8/A9 may be more complex, at least in the ligature-induced periodontitis dog model. In humans, S100A9 has been reported to be negatively associated with periodontitis (Karna et al., 2019), suggesting antimicrobial or anti-inflammatory function of S100A9 in human periodontitis. GR had the highest positive correlation with MMP-9 (r = .807), which coincides with the fact that MMP-9 is involved in collagen degradation, osteoclast differentiation, and bone resorption, and thus periodontal soft and hard tissue destruction (Hill et al., 1995; Lee, Aitken, Sodek, & McCulloch, 1995; Nannuru et al., 2010).

The present study also investigated the number of teeth with periodontitis required for identification with salivary biomarkers. A single tooth with periodontitis was detected by salivary biomarkers; however, time factor was involved. Three animals in the control group developed gingivitis at ≥7 teeth but no periodontitis at week 8, and these cases were misdiagnosed as periodontitis by all salivary biomarkers. One of these animals exhibited a significant alveolar bone loss over the 12 weeks. Therefore, these three animals with false positive results may have been at a status close to periodontitis. In the present study, three of the five control animals exhibited alveolar bone loss over the 12 weeks, which was higher than in a previous report, which showed 20% of beagle dogs aged between 1 and 2 years exhibited clinical attachment loss (Kortegaard et al., 2008). Feeding the dogs with a softened vitamin C-deficient diet must have contributed to the development of periodontitis.

Of note, the levels of salivary S100A8, S100A9, and MMP-9 in periodontitis stability remained significantly higher than in health, whereas those of S100A8/A9 recovered to levels as low as in health. Interestingly, the levels of MMP-9 in GCF recovered to baseline levels at week 12, and the strong correlations between the levels of biomarkers in saliva and GCF suggest that the majority of biomarkers in saliva may originate from GCF. The gingival oral epithelium might be the additional source of the salivary S100A8, S100A9, and MMP-9 that remained higher in periodontitis stability than in health. MMP-9 has been reported to be detected frequently in all the structures of gingival mucosa from patients with periodontitis (Liu, Cao, & Zhu, 2017; Şurlin et al., 2014). Whether S100A8 and S100A9 are upregulated in the gingival oral epithelium in periodontitis needs to be studied. Although the underlying mechanism currently remains unclear, one important question to be answered in the future is whether the levels of S100A8, S100A9, and MMP-9 increase further following another episode of active periodontitis and recovery.

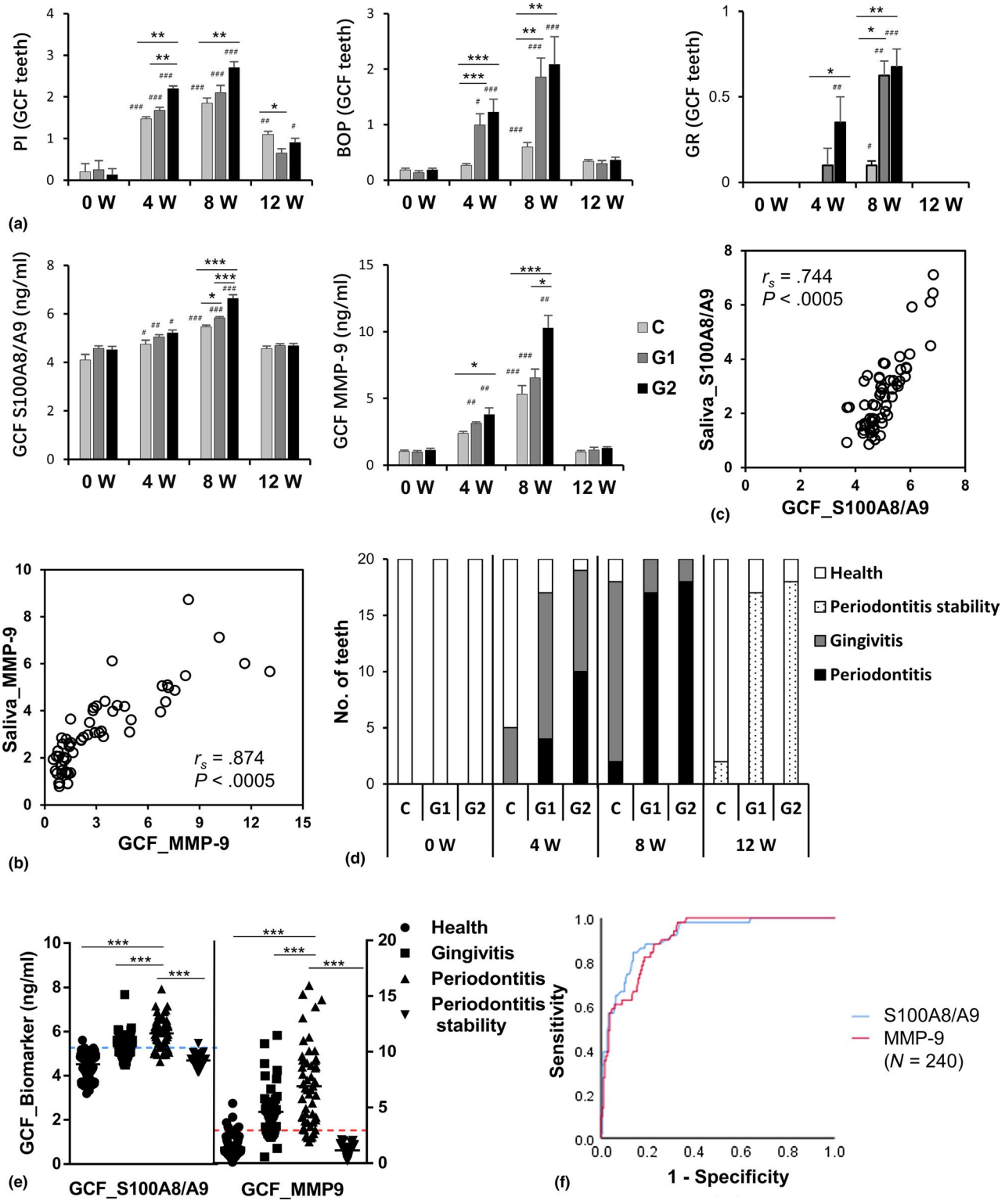


FIGURE 4 Levels and diagnostic power of GCF biomarkers S100A8/A9 and MMP-9 for periodontitis identification. (a) Measurements of clinical parameters of teeth from which the GCF were sampled during induction and treatment of periodontitis. (b) GCF samples were collected by placing periopapers into the mid-buccal and mid-lingual gingival sulcus of the left and right UP3 and UP4 for 30 s. The two periopapers from each tooth were eluted with 200 μl Dulbecco's phosphate-buffered saline. Levels of S100A8/A9 and MMP-9 in GCF sampled from four maxillary premolars per dog were determined by enzyme-linked immunosorbent assay (n = 20 per group). (c) Periodontal status of each tooth, scored according to the criteria in Table 1. (d) Levels of biomarkers in GCF depending on periodontal status. The blue and red dotted lines indicate the threshold values for the diagnosis of periodontitis presented in Table 2. (e) ROC curves of GCF S100A8/A9 and MMP-9. *p < .05; **p < .01; ***p < .0005 presenting inter-group differences. #p < .05; ##p < .01; ###p < .0005 compared with baseline. GCF, gingival crevicular fluid; ROC, receiver operating characteristic

In order to implement point-of-care (POC) testing for the diagnosis of periodontitis at a patient's home, the salivary biomarker must meet several characteristics. First, the level in saliva should be higher than the detection limit of the current technology. Second, inter-group difference in levels should be large, whereas intra-group variation should be low, particularly that for heath and periodontitis stability. Third, the levels of biomarkers should respond fast to the development or treatment of periodontitis. Fourth, the biomarker should be specific to periodontitis, that is not associated with other disease conditions. The thresholds for biomarkers assessed in the present study were all in the range of several ng/ml, which is higher than sub pg/ml – 0.1 ng/ml, the detection limits obtainable by nanotechnology-based immunosensors (Rusling, Kumar, Gutkind, & Patel, 2010; Tang et al., 2009). S100A8/A9 and MMP-9 presented with higher inter-group differences than either S100A8 or S100A9. MMP-9 identified all periodontitis cases, even at week 4, but remained at a higher level in periodontitis stability than in health. By contrast, S100A8/A9 failed to identify one periodontitis case at week 4, but recovered to the low-level present in heath following therapy. Unfortunately, neither S100A8/A9 nor MMP-9 is specific to periodontitis. S100A8/A9 is a general indicator of inflammatory activity. Inflammation in the oral cavity, including a peritonsillar abscess, can directly affect the level of salivary S100A8/A9 (Spiekermann et al., 2017). Saliva reflects plasma proteins in concentration of ~3% (Edger, 1992). Diverse systemic conditions with increased serum levels of S100A8/A9, including systemic lupus erythematous, rheumatoid arthritis, acute coronary syndrome, acute myocardial infarction, and obesity (Brun, Jonsson, & Haga, 1994; Healy et al., 2006; Mortensen et al., 2009; Tydén et al., 2013), may also have effect on the salivary level of S100A8/A9, which requires clarification. Similarly, a number of systemic conditions involving the degradation of extracellular matrix, including acute coronary syndrome, haemorrhagic transformation following ischaemic stroke, colorectal cancer, and abdominal aortic aneurysm, are associated with increased levels of circulating MMP-9 (Herszényi, Hritz, Lakatos, Varga, & Tulassay, 2012; Lin, Yokoyama, Rac, & Brooks, 2012; Takagi, Manabe, Kawai, Goto, & Umemoto, 2009; Wang et al., 2018). Salivary MMP-9 is also increased in oral squamous cell carcinoma and hypertension (Hema Shree et al., 2019; Labat et al., 2013).

5 | CONCLUSION

Periodontitis in dogs was associated with increased levels of salivary S100A8, S100A9, S100A8/A9, and MMP-9. These salivary biomarkers may be used for POC assessment of periodontitis at home, although caution is required as other oral or systemic conditions can affect these levels in saliva.

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CONFLICT OF INTEREST

All authors have no conflicts of interest to declare.

ORCID

Suk Ji https://orcid.org/0000-0001-9720-6731
Youngnim Choi https://orcid.org/0000-0002-6496-5560

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SUPPORTING INFORMATION

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