

# Platelet activation and platelet-leukocyte interaction in generalized aggressive periodontitis

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## ABSTRACT

Generalized aggressive periodontitis (GAgP) is an inflammatory disease of host response to bacterial challenge. To explore the role of platelets in host-microbial interactions in patients with periodontitis, 124 patients with GAgP and 57 healthy subjects were enrolled. Reliable indicators of subclinical platelet functional status, platelet count (PLT), platelet large cell ratio (PLCR), and mean platelet volume (MPV), were significantly lower in the GAgP group than in the control group and were negatively correlated with clinical periodontal parameters. The levels of important cytosolic protein in neutrophils, calprotectin (S100A8/A9) in plasma, and gingival crevicular fluid (GCF) were significantly higher in patients with GAgP compared with healthy subjects. Moreover, the GCF calprotectin level was negatively correlated with PLCR and MPV values. To explore the possible mechanisms of changes in platelet indices in periodontitis, flow cytometry analysis was performed, and patients with GAgP were found to have a higher status of platelet activation compared with healthy controls. *Porphyromonas gingivalis* (*P. gingivalis*) and recombinant human S100A8/A9 (rhS100A8/A9) induced platelet activation and facilitated platelet-leukocyte aggregate formation in whole blood of healthy subjects. In response to *P. gingivalis* and rhS100A8/A9, platelets from patients with GAgP increased activation and increased formation of platelet-leukocyte aggregates compared with those from healthy subjects. Platelet aggregates and platelets attached to leukocytes were found on gingival tissues from patients with GAgP, suggesting that decreased platelet size and count in the circulation might be related to consumption of large, activated platelets at inflamed gingiva. Platelets may have a previously unrecognized role in host response to periodontal infection. *J. Leukoc. Biol.* **100: 000-000; 2016.**

## Introduction

Periodontitis is an inflammatory disease involving the host response to bacterial challenge, which leads to inflammatory destruction of periodontal tissues. GAgP is a more rapid, aggressive, and generalized, severe, destructive form of periodontitis in otherwise young healthy individuals [1]. The pathologic changes of periodontitis include periodontal pocket formation and ulceration of the epithelial lining [2, 3], which contribute to dissemination of oral bacteria into the circulation, causing bacteremic episodes and systemic inflammation [4, 5].

The host response to the bacterial challenge that originates from the dental biofilm has been considered to have a major role in the initiation and tissue destruction of periodontal diseases [6]. Increasing evidence from animal models suggests that platelets may also be an important component of the host immune system [7]. In this regard, platelets may participate in a wide variety of processes involving tissue injury, immune response, and repair that underlie diverse diseases, such as atherosclerosis, autoimmune disorders, inflammatory lung and bowel disorders, host-defense responses, and sepsis [8–11]. Reliable indicators of subclinical platelet functional status, such as MPV, have been investigated in several chronic inflammatory disorders. High-grade inflammation is accompanied by a decrease of MPV, as in rheumatoid arthritis [8] and ulcerative colitis [12]. Previous work by our group also showed that the decrease of MPV was related to periodontal inflammation and that the MPV value increased after active periodontal treatment in patients with severe periodontitis [13]. However, to our knowledge, platelets in GAgP have not been investigated so far.

A previous study showed that platelets from patients with periodontitis have an increased activation status compared with platelets from healthy subjects [14]. Species of the oral microflora, such as *Streptococcus sanguinis*, can induce platelet

Abbreviations: AL = attachment loss, BI = bleeding index, BMI = body mass index, CD62P = P-selectin, CPT amount = the amount of calprotectin, CPT conc. = calprotectin concentration, FSC = forward light scatter, GAgP = generalized aggressive periodontitis, GCF = gingival crevicular fluid, MPV = mean platelet volume, NET = neutrophil extracellular trap, NEUT = number of neutrophils, NEUT% = percentage of neutrophils,

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activation in vitro and in animal studies [15]. Given the regularly occurring bacteremic episodes and systemic increase of various proinflammatory mediators in patients with periodontitis, periodontal pathogens may contribute to platelet activation. Our recent study [16] provided evidence that periodontal infection was associated with increased levels of plasma S100A8/A9. Extracellular S100A8/A9 is able to bind to receptors on target cells, including CD36 [17], RAGE [18], and TLR-4 [19]. Interestingly, both CD36 and RAGE signaling have been directly implicated in platelet activation. Therefore, the effect of S100A8/A9 on platelet activation needs to be explored.

After platelet activation, the formation of platelet-leukocyte aggregates has been proposed to be a link between platelets and inflammation [20]. The physical and functional interactions between platelets and leukocytes can have important consequences for leukocyte function. Circulating leukocytes with attached, activated platelet display a more adhesive phenotype and have an enhanced propensity for phagocytosis [21]. As described above, activated platelets drive responses in target leukocytes that modulate the host response to infection in inflammatory lung [22], bowel disorders [23], and sepsis [24]. Whether platelets become activated and participate in the host response of GAgP is unknown. Therefore, the aims of this study were to explore the role of platelets in host response to periodontal infection.

## MATERIALS AND METHODS

### Subjects

One hundred twenty-four patients with GAgP were recruited consecutively from the Department of Periodontology, Peking University School and Hospital of Stomatology. The diagnostic criteria for GAgP were according to the classification proposed at the International Workshop for a Classification of Periodontal Diseases and Conditions in 1999 [1]. The inclusion criteria of the GAgP group were as follows: 1) the onset of the periodontal disease at younger than 35 y; 2) at least 8 teeth with PD >6 mm, 3 or more of which were not first molars or incisors; and 3) radiographs showing alveolar bone loss.

In addition, 57 periodontally healthy subjects were selected from the staffs and students at Peking University School and Hospital of Stomatology; their inclusion criteria were 1) PD ≤3 mm, 2) percentage of sites with bleeding on probing <10%, 3) no AL, and 4) no bone loss on radiographs.

Exclusion criteria of all subjects were 1) current or previous smoker, 2) the presence of systemic disease or pregnancy, or 3) history of periodontal therapy or antimicrobial therapy within 6 mo or usage of any medication (including oral contraceptive drugs).

### Clinical and blood examination

All subjects completed a questionnaire and underwent a full-mouth clinical periodontal examination by a calibrated examiner, including PD and AL, using a William's periodontal probe at 6 sites of each tooth. BI was also recorded for each tooth. Sites with PD >6 mm and AL >5 mm were defined

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PD = probing depth, PDW = platelet distribution width, *P. gingivalis* = *Porphyromonas gingivalis*, PLCR = platelet large cell ratio, PLT = platelet count, PSGL-1 = P-selectin glycoprotein-1, RAGE = advanced glycation end products, rhS100A8 = recombinant human S100A8, rhS100A9 = recombinant human S100A9, S100A8/A9 = calprotectin, SSC = side scatter of light

as sites of severe periodontal destruction. Each subject received a set of full-mouth periapical radiographs.

Fasting blood samples were obtained from each subject by venipuncture using an EDTA-containing collection tube and a coagulant-containing tube. The blood samples were sent to the testing laboratories within 30 min of collection. A technician, who was blinded for case status, performed the complete blood cell analysis in a calibrated Sysmex XS-1,000 automated hematology analyzer (Sysmex, Kobe, Japan) and the biochemical analysis in a HITACHI 7180 Automatic Analyzer (HITACHI, Tokyo, Japan). Plasma was separated by centrifugation and immediately stored at -70°C.

### Collecting and processing of GCF

GCF was collected at 2 sites from 45 patients with GAgP and 29 healthy subjects by filter paper strip (Whatman, Maidstone, Kent, United Kingdom). One site was the mesial buccal aspect of the right maxillary incisor, and the other was the mesial buccal aspect of the left mandibular molar. In patients with GAgP, the PD at these sites was >4 mm with AL >1 mm. The GCF collection and volume determination were performed as previously described [25]. GCF samples were eluted from the strips by placing them in 300 μl of PBS (HyClone, Beijing, China). The S100A8/A9 levels in the GCF supernatant and plasma were measured using a commercially available ELISA kit (Phicaltest; Calpro AS, Oslo, Norway) according to the manufacturer's instructions. The amount of S100A8/A9 in each GCF sample was calculated based on the dilution as described previously [25].

### Production of recombinant human S100A8, S100A9, and S100A8/A9

The recombinant proteins were produced and purified as described previously [26].

Recombinant proteins were analyzed on SDS-PAGE gels, and it was shown that recombinant human S100A8 (rhS100A8) and S100A9 (rhS100A9) were free of contaminating bacterial proteins after purification. Equivalent amounts of rhS100A8 and rhS100A9 were mixed to form heterodimeric rhS100A8/A9 in the presence of 1.3 mM Ca<sup>2+</sup> as previously described [27].

### Bacteria culture and preparation

Preparation of *P. gingivalis* (ATCC33277) was carried out in the oral microbiology laboratory of Peking University School and Hospital of Stomatology. *Porphyromonas gingivalis* was cultured on tryptic soy agar (containing 5 μg/ml hemin, 1 μg/ml menadione, and 10% defibrinated goat blood) at 37°C under anaerobic conditions (80% N<sub>2</sub>, 10% H<sub>2</sub>, and 10% CO<sub>2</sub>). *Porphyromonas gingivalis* was diluted in Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free PBS (HyClone) to 2 × 10<sup>9</sup> CFU/ml (OD 600 nm = 1 of McFarland standard) and was further adjusted to 2 × 10<sup>8</sup> or 2 × 10<sup>7</sup> CFU/ml using PBS. *Porphyromonas gingivalis* was subjected to experimental conditions within 30 min after removal from the anaerobic environment.

### Assessment of platelet activation and platelet-leukocyte aggregate formation

Nine consecutive patients with GAgP were included, and for each patient, an age-, gender-, and race-matched healthy control was recruited, applying similar inclusion and exclusion criteria as in the cross-sectional case-control study.

Fasting venous blood samples were collected into tubes containing 3.8% citrated sodium by venipuncture of the antecubital fossa, through a 21-gauge needle without venous stasis.

To evaluate platelet activation in patients with GAgP and healthy subjects in vivo, within 10 min of blood collection 5 μl whole blood of each subject was directly measured for surface expression of P-selectin (CD62P), S100A9, and binding of PAC-1 (PAC-1 recognizes an epitope on the glycoprotein IIb/IIIa complex of activated platelets at or near the platelet fibrinogen receptor) to individual fresh platelets, and formation of platelet-leukocyte/neutrophil/monocyte aggregates by whole-blood flow cytometry.

**TABLE 1. Clinical and hematologic parameters of patients with GAgP and healthy subjects**

Variables	GAgP group (n = 124)	Control group (n = 57)	P value
Age (y)	26.50 (23.0–31.0)	24.00 (23.0–27.5)	0.061 A
Gender (F/M)	75/49	38/19	0.405 B
BMI	21.00 (19.50–24.00)	21.00 (20.00–22.00)	0.518 A
Mean PD (mm)	4.89 (4.16–5.65)	1.52 (1.29–1.88)	<0.001 A
Mean BI	3.68 (3.37–3.97)	1.15 (1.11–1.21)	<0.001 A
Mean AL (mm)	4.54 (3.65–5.85)	0.00	<0.001 A
WBC ( $\times 10^9/L$ )	6.10 (5.00–7.00)	5.50 (4.80–6.35)	0.033 A
NEUT%	63.88 $\pm$ 9.10	57.64 $\pm$ 7.05	<0.001 C
NEUT ( $\times 10^9/L$ )	3.70 (2.93–4.78)	3.00 (2.50–3.90)	<0.001 A
PLT ( $\times 10^9/L$ )	216.38 $\pm$ 51.68	234.33 $\pm$ 51.48	0.032 C
PDW (fL)	10.90 (10.30–11.90)	11.40 (10.45–12.55)	0.065 A
PLCR	0.18 (0.15–0.23)	0.21 (0.18–0.27)	0.005 A
MPV (fL)	8.90 (8.40–9.50)	9.20 (8.70–9.75)	0.008 A

Data are presented as median (lower–upper quartile), the number of subjects, or means  $\pm$  sd. Between-group comparisons were performed using the Mann-Whitney *U* test (A), the  $\chi^2$  test (B), or *t* test (C).

To measure platelet reactivity *in vitro*, 45  $\mu$ l of whole blood from healthy subjects and patients with GAgP was incubated with *P. gingivalis* ( $10^7$ ,  $10^8$ , or  $10^9$  CFU/ml at final concentration), rhS100A9 (1  $\mu$ g/ml, 2  $\mu$ g/ml, or 10  $\mu$ g/ml at final concentration) or rhS100A8/A9 (1  $\mu$ g/ml, 2  $\mu$ g/ml, or 10  $\mu$ g/ml at final concentration) for 5 min at 37°C. ADP (100  $\mu$ M/L at final concentration; Chrono-Log Corporation, Havertown, PA) was used as a positive control, and Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free PBS was used as a negative control. Expression of CD62P and S100A9, binding of PAC-1 to platelet surfaces, and formation of platelet-leukocyte/neutrophil/monocyte aggregates were subsequently assessed in whole blood by flow cytometry.

CD41a-BV510, CD62P-BV421, PAC-1 FITC, CD45-PE-CF594, and their isotype controls were obtained from Becton Dickinson Immunocytometry Systems (San Jose, CA, USA). S100A9-PE and PE-conjugated IgG<sub>1</sub> were obtained from BioLegend (San Jose, CA, USA). Blood samples were analyzed in a FACSCalibur flow cytometer (Becton Dickinson). FSC and SSC were set at logarithmic gain. Neutrophils and monocytes were identified on their characteristic light scattering on FSC/SSC dot-plot and extent of CD45-PE-CF594 expression. The threshold for positive events was set with the appropriate isotype control antibody. We calculated the percentage of positive cells for each antibody. Platelet activation was assessed by expression of CD62P and binding of PAC-1 on platelet surface. The CD41a<sup>+</sup> leukocytes/neutrophils/monocytes represent the platelet-leukocyte aggregates, the platelet-neutrophil aggregates, and the platelet-monocyte aggregates, separately.

### Measurement of the concentrations of soluble P-selectin (CD62P) in plasma samples

Concentrations of soluble (s)-CD62P in platelet-poor plasma from 9 patients with GAgP and healthy subjects were determined with an ELISA kit (Gerson, Beijing, China) according to the instructions of the manufacturer.

### Histology and immunohistochemistry of gingiva samples

To visualize platelets in gingival tissue, harvested gingival tissues from patients with GAgP and healthy subjects were fixed (10% paraformaldehyde), embedded, sectioned (5  $\mu$ m), and stained with H&E. Immunohistochemistry was performed using rabbit monoclonal antibody to human CD41 (Abcam, Milton, Cambridge, United Kingdom) according to the manufacturer's instructions. The primary antibody was replaced by species-specific

nonimmune IgG for negative controls. Images were captured on a digital microscopic system (BX51/DP72; Olympus, Tokyo, Japan).

### Statistical analysis

Shapiro-Wilk test and Levene variance homogeneity test were performed to test normality and equality of variance of the data, respectively. Continuous, normally distributed data were expressed as means  $\pm$  sd, whereas median (lower to upper quartile) was used to describe nonnormally distributed data. Student's *t* test (for percentage of neutrophils, PLT, and plasma calprotectin levels) and Mann-Whitney *U* test (for age, BMI, clinical parameters, other blood parameters, and calprotectin levels in GCF) were used to identify any differences between groups. Gender was analyzed using  $\chi^2$  test. Correlation analysis was performed using Pearson's rank or Spearman's rank correlation analysis. The percentage of CD62P<sup>+</sup>, PAC-1<sup>+</sup>, or S100A9<sup>+</sup> platelets, and platelet<sup>+</sup> leukocytes/neutrophils/monocytes among different groups were analyzed using Student's *t* test or 1-way ANOVA to check the entire difference and Holm-Sidak method to further achieve pairwise multiple comparison procedures. For some experiments with failed equal variance test, Kruskal-Wallis 1-way ANOVA on ranks, and subsequently Dunn's method were applied to make multiple comparisons vs. the control group. A 2-tailed *P* value < 0.05 was considered statistically significant. All statistical analyses of the data were performed using SPSS package (version 19.0; SPSS, Chicago, IL, USA).

### Ethics

The study protocol was approved by the Ethics Committee of Peking University Health Science Center (IRB00001052-08010), and the study was

**TABLE 2. Correlations among platelet indices and clinical periodontal parameters**

Variables	PLT		PLCR		MPV	
	<i>r</i>	<i>P</i> value	<i>r</i>	<i>P</i> value	<i>r</i>	<i>P</i> value
Mean PD	-0.163	0.028	-0.176	0.018	-0.163	0.028
Mean BI	-0.148	0.046	-0.187	0.012	-0.178	0.017
Mean AL	-0.158	0.034	-0.161	0.030	-0.144	0.053

Correlation analysis was performed using Spearman's rank correlation analyses.

**TABLE 3. Comparisons of GCF and plasma calprotectin levels between patients with GAgP and healthy subjects**

Variables	GAgP group	Control group	P value
CPT conc. in plasma (µg/ml)	2.20 ± 1.10, n = 124	1.79 ± 0.89, n = 57	0.008 A
CPT amount in GCF (ng)	4126.01 (1586.47–7127.42), n = 45	75.27 (59.42–137.26), n = 29	<0.001 B
CPT conc. in GCF (µg/ml)	2331.09 (737.72–3192.69), n = 45	625.24 (283.27–1009.51), n = 29	0.004 B

Data are presented as means ± SD or median (lower–upper quartile). Between-group comparisons were performed using the *t* test (A) or Mann-Whitney *U* test (B).

conducted in accordance with the Helsinki Declaration of 1975, as revised in 2008. Written, informed consent was obtained from each subject, and all subjects were individually informed about the risks and benefits and had the right to drop out of the study.

## RESULTS

### Clinical and hematologic characteristics

Clinical and hematologic parameters of patients with GAgP and healthy subjects are shown in **Table 1**. There were no significant differences in age, gender, or body mass index (BMI) between the 2 groups. The mean values of PD, AL, and BI were significantly higher in the GAgP group ( $P < 0.001$ ). Significantly higher WBC count, NEUT, and NEUT% were observed in the GAgP group compared with the control group, whereas PLT, PLCR, and MPV were significantly lower in the GAgP group than in the control group ( $P < 0.05$ ). In accordance with PLT, PLCR, and MPV, PDW was also relatively lower in patients with GAgP than it was in controls, although the difference did not reach significance ( $P = 0.065$ ).

### Correlations among platelet indices and clinical periodontal parameters

The negative correlations between platelet indices (including PLT, PLCR, and MPV) and clinical periodontal parameters, including mean BI, PD, and AL, are shown in **Table 2**.

### Elevated GCF and plasma S100A8/A9 levels in the GAgP group

As presented in **Table 3**, the GAgP group had significantly higher plasma S100A8/A9 concentration compared with the healthy control group ( $P < 0.05$ ). In addition, both the amount and concentration of S100A8/A9 in GCF were significantly higher in the GAgP group than they were in the healthy control group (amount in GCF,  $P < 0.001$ ; concentration in GCF,  $P < 0.05$ ).

### Correlations between S100A8/A9 levels and other parameters

The S100A8/A9 level in plasma was positively correlated with clinical periodontal parameters (mean BI, PD, AL, the percentage of severe sites), WBC number, NEUT, and NEUT% (**Table 4**). In addition, there were positive correlations among the amount and concentration of S100A8/A9 in GCF and the clinical periodontal parameters (mean BI, PD, and AL; **Table 4**). Although PLCR and MPV values were negatively correlated with GCF S100A8/A9 level (**Table 4**).

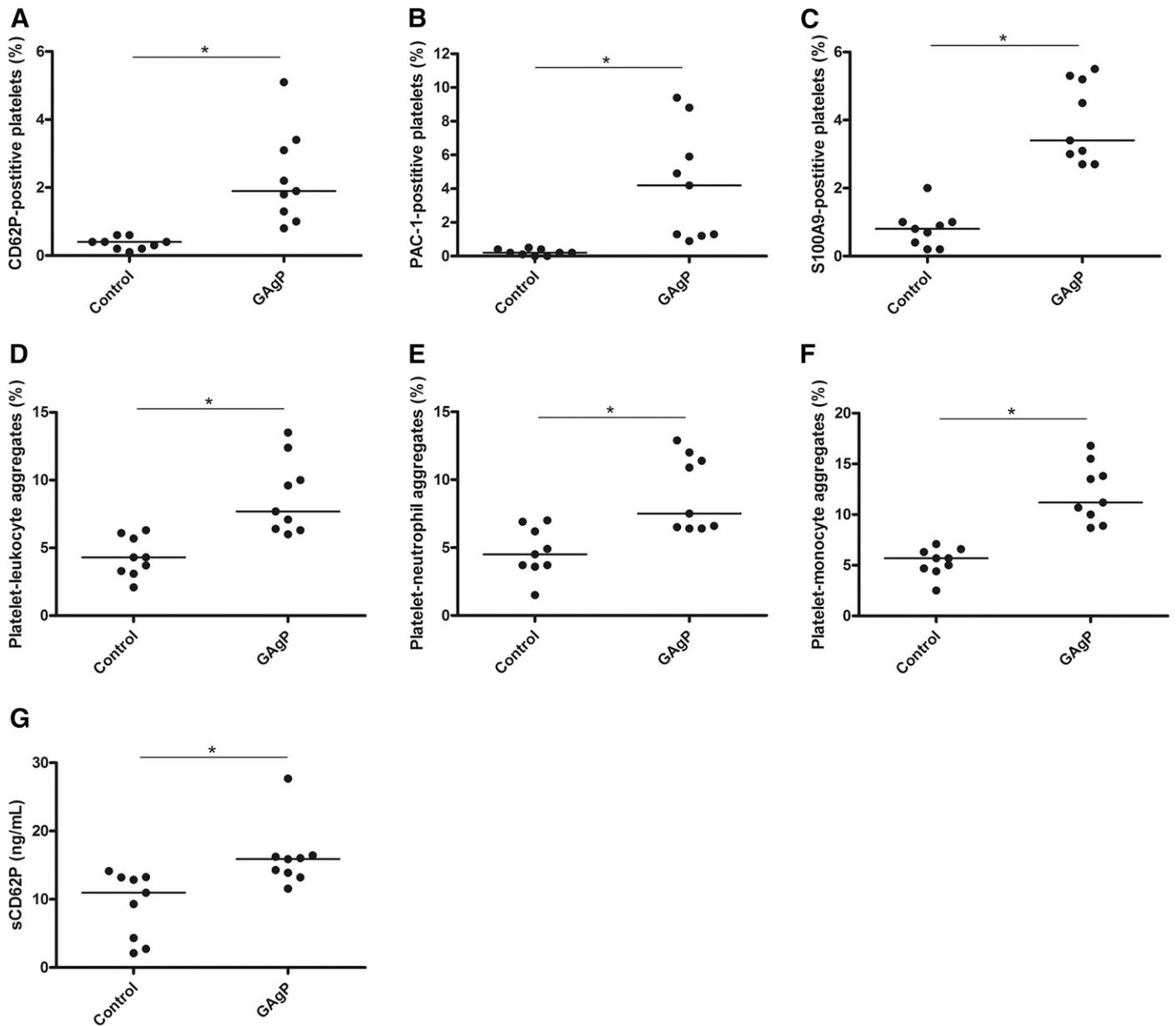
### Platelet activation and platelet–leukocyte aggregates in patients and controls

The fraction (percentage) of platelet expressing CD62P or S100A9, binding PAC-I, platelet–leukocyte/neutrophil/monocyte aggregates was significantly elevated in patients with GAgP compared with controls ( $P < 0.05$ , **Fig. 1A–F**). Plasma levels of sCD62P were also significantly elevated in patients with GAgP ( $P < 0.05$ , **Fig. 1G**).

**TABLE 4. Correlations between calprotectin levels and the variables evaluated in the study**

Variables	CPT conc. in plasma		CPT amount in GCF		CPT conc. in GCF	
	<i>r</i>	<i>P</i> value	<i>r</i>	<i>P</i> value	<i>r</i>	<i>P</i> value
Mean PD	0.314	<0.001 B	0.650	<0.001 B	0.472	<0.001 B
Mean BI	0.219	0.003 B	0.632	<0.001 B	0.473	0.001 B
Mean AL	0.277	<0.001 B	0.501	<0.001 B	0.341	0.008 B
Severe sites (%)	0.429	<0.001 B	0.209	0.251 B	0.155	0.380 B
WBC	0.225	0.002 B	0.013	0.942 B	0.083	0.531 B
NEUT%	0.223	0.003 A	0.007	0.968 B	0.188	0.155 B
NEUT	0.261	<0.001 B	0.005	0.977 B	0.093	0.484 B
PLT	−0.112	0.133 A	−0.043	0.742 B	−0.065	0.623 B
PLCR	0.015	0.845 B	−0.271	0.036 B	−0.265	0.043 B
MPV	0.019	0.796 B	−0.272	0.036 B	−0.261	0.046 B

Correlation analysis was performed using Pearson’s rank correlation analyses (A) or Spearman’s rank correlation analyses (B).



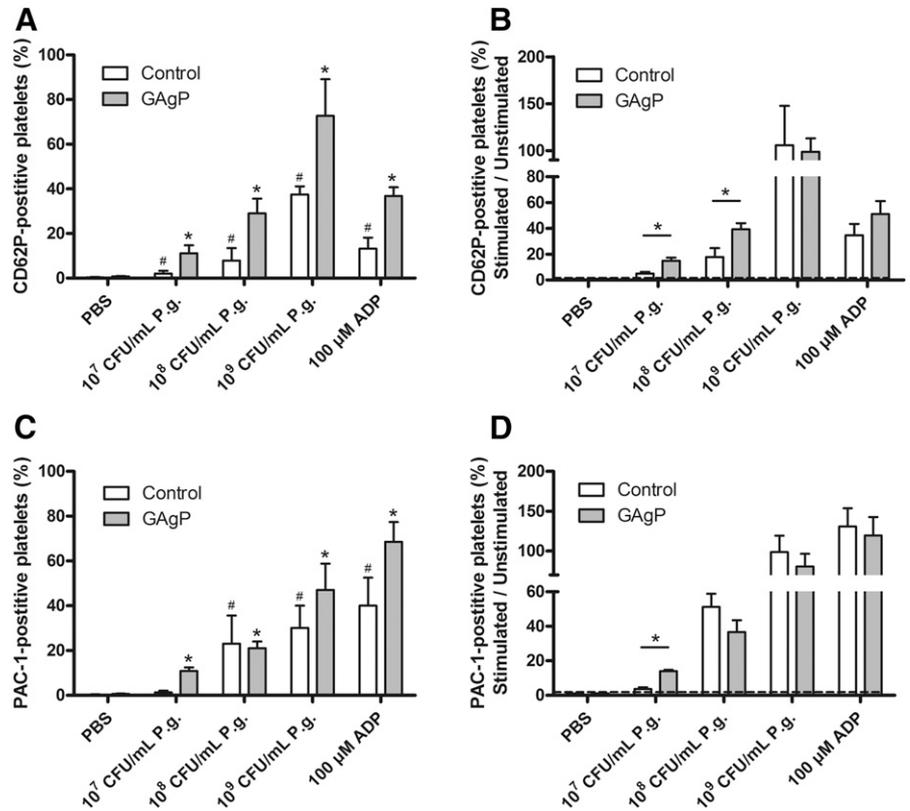
**Figure 1. Platelet activation and platelet-leukocyte aggregates in patients with GAgP ( $n = 9$ ) and controls ( $n = 9$ ).** The percentage of positive platelets expression of CD62P (A), PAC-1 (B), and S100A9 (C), and the percentage of platelet-leukocyte aggregates (D), platelet-neutrophil aggregates (E), and platelet-monocyte aggregates (F). (G) Concentrations of sCD62P in platelet-poor plasma of patients with GAgP and in healthy subjects. \* $P < 0.05$ .

### The effects of *P. gingivalis*, rhS100A9, and rhS100A8/A9 on platelet activation and platelet-leukocyte aggregates formation in vitro

In vitro, *P. gingivalis* stimulation of whole blood from healthy subjects or patients with GAgP induced platelet activation as assessed by expression of P-selectin and binding of PAC-1. After stimulation with *P. gingivalis* at  $10^7$ ,  $10^8$ , or  $10^9$  CFU/ml, the percentage of CD62P<sup>+</sup> platelets in whole blood of control subjects and patients with GAgP both increased significantly ( $P < 0.05$ ; Fig. 2A). In whole blood of patients with GAgP, stimulation with *P. gingivalis* at  $10^7$ ,  $10^8$ , or  $10^9$  CFU/ml also

increased PAC-1 binding significantly ( $P < 0.05$ , Fig. 2C). When blood from control subjects was incubated with *P. gingivalis* at  $10^7$  CFU/ml, PAC-1 binding was not increased ( $P > 0.05$ ; Fig. 2C). In response to *P. gingivalis* at  $10^7$  or  $10^8$  CFU/ml, the exposure of CD62P or PAC-1 binding on platelets increased in patients with GAgP compared with control subjects ( $P < 0.05$ ; Fig. 2B and D). When blood from healthy subjects was stimulated with 10  $\mu$ g/ml rhS100A8/A9, the percentage of CD62P<sup>+</sup> platelets increased significantly ( $P < 0.05$ ; Fig. 3A). The plasma concentration of S100A8/A9 in patients with GAgP was  $2.20 \pm 1.10$   $\mu$ g/ml (Table 3). Even stimulation with low

**Figure 2. Platelet activation in response to stimulation with *P. gingivalis* (P.g.) or ADP.** (A) *Porphyromonas gingivalis* (at  $10^7$ ,  $10^8$ , or  $10^9$  CFU/ml) or 100  $\mu$ M ADP increased the percentage of CD62P<sup>+</sup> platelets in whole blood of controls and patients (<sup>#</sup> $P < 0.05$  vs. PBS group in controls; \* $P < 0.05$  vs. PBS group in patients) ( $n = 5$ ). (B) The ratio of the percentage of CD62P<sup>+</sup> platelets in stimulated whole blood to the percentage of CD62P<sup>+</sup> platelets in unstimulated whole blood in patients and controls (\* $P < 0.05$  comparison between patients and controls). (C) *Porphyromonas gingivalis* (at  $10^7$ ,  $10^8$ , or  $10^9$  CFU/ml) or 100  $\mu$ M ADP increased the percentage of PAC-1<sup>+</sup> platelets (<sup>#</sup> $P < 0.05$  vs. PBS group in controls; \* $P < 0.05$  vs. PBS group in patients) ( $n = 5$ ). (D) The ratio of the percentage of PAC-1<sup>+</sup> platelets in stimulated whole blood to the percentage of PAC-1<sup>+</sup> platelets in unstimulated whole blood of patients and controls (\* $P < 0.05$  comparison between patients and controls).



concentrations of rhS100A9 or rhS100A8/A9 in whole blood of patients with GAgP, the percentage of CD62P<sup>+</sup> platelets also increased significantly ( $P < 0.05$ , Fig. 3A). The effects of rhS100A9 and rhS100A8/A9 stimulation on exposure of CD62P were increased in patients with GAgP compared with controls ( $P < 0.05$ ; Fig. 3B).

In vitro, stimulation with  $10^8$  or  $10^9$  CFU/ml of *P. gingivalis* induced formation of platelet–leukocyte aggregates ( $P < 0.05$ ; Fig. 4A), platelet–neutrophil aggregates ( $P < 0.05$ ; Fig. 4C), and platelet–monocyte aggregates ( $P < 0.05$ ; Fig. 4E) both in the whole blood of control subjects and for patients with GAgP. When whole blood from patients with GAgP was stimulated with low concentrations of *P. gingivalis* at  $10^7$  CFU/ml, it also significantly induced formation of platelet–leukocyte aggregates ( $P < 0.05$ ; Fig. 4A), platelet–neutrophil aggregates ( $P < 0.05$ ; Fig. 4C), and platelet–monocyte aggregates ( $P < 0.05$ ; Fig. 4E). In the presence of *P. gingivalis* at  $10^7$  CFU/ml, more platelet–leukocyte aggregates, platelet–neutrophil aggregates, and platelet–monocyte aggregates were formed in whole blood from patients with GAgP than in blood from control subjects ( $P < 0.05$ ; Fig. 4B, D, and F). In addition, 2  $\mu$ g/ml rhS100A9 stimulation also significantly increased the number of platelet–leukocyte aggregates ( $P < 0.05$ ; Fig. 5A) and platelet–neutrophil aggregates ( $P < 0.05$ ; Fig. 5C) both in whole blood of control subjects and in patients with GAgP. In response to 1, 10  $\mu$ g/ml rhS100A9, or 2, 10  $\mu$ g/ml rhS100A8/A9, the formation of platelet–leukocyte aggregates ( $P < 0.05$ ; Fig. 5A) and platelet–neutrophil aggregates

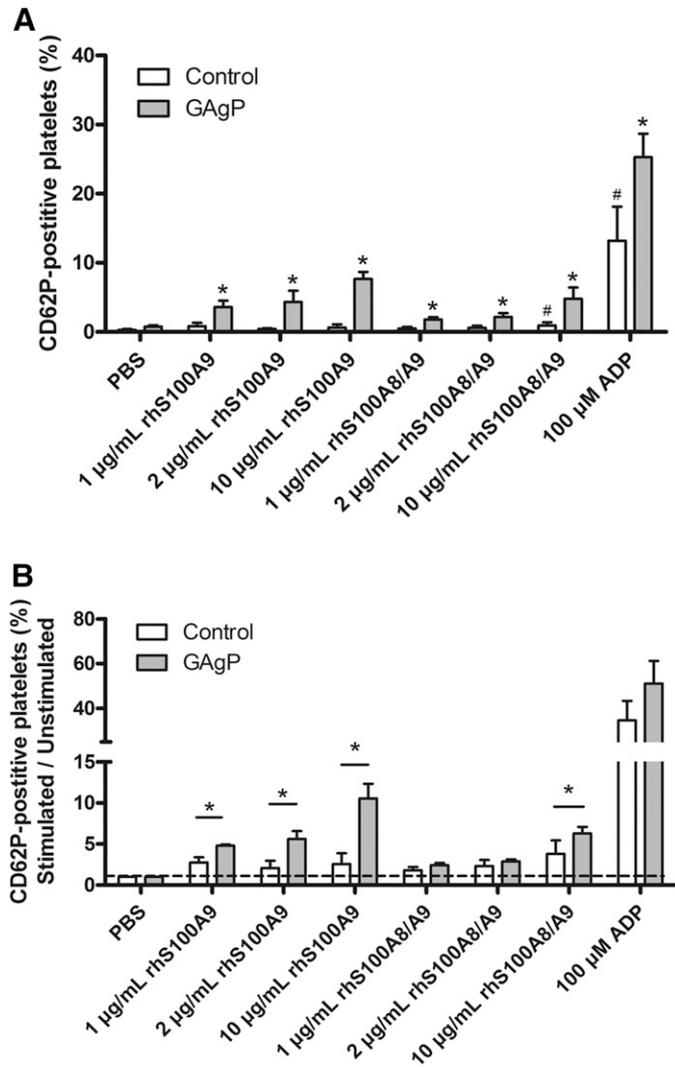
( $P < 0.05$ ; Fig. 5C) was also induced in the whole blood of patients with GAgP. In addition, stimulation with 2  $\mu$ g/ml rhS100A9 or 1 or 10  $\mu$ g/ml rhS100A8/A9, platelet–monocyte aggregates formation was induced only in the whole blood of patients with GAgP ( $P < 0.05$ ; Fig. 5E). The most marked difference was observed in response to 1  $\mu$ g/ml rhS100A9 and 1  $\mu$ g/ml rhS100A8/A9, where more platelet–monocyte aggregates formed in the whole blood from patients with GAgP than in blood from control subjects ( $P < 0.05$ ; Fig. 5F). Platelet–leukocyte aggregates and platelet–neutrophil aggregates were comparable in patients and in controls ( $P > 0.05$ , Fig. 5B and D).

### Platelet surface expression of S100A9

Platelet surface expression of S100A9 during rest and *P. gingivalis*-stimulated platelets was determined by flow cytometry. After stimulation with *P. gingivalis* at  $10^8$  or  $10^9$  CFU/ml, the percentage of S100A9<sup>+</sup> platelets was elevated significantly compared with resting platelets ( $P < 0.05$ ; Fig. 6).

### Platelet localization in inflamed gingiva from patients with GAgP

To verify that platelets were components of inflammatory cells in inflamed gingival tissues, we examined gingival tissues from patients with GAgP and from healthy subjects. There were vasodilation, vascular proliferation, and dense leukocytes infiltration in the connective tissues of inflamed gingiva from patients with GAgP. Immunohistology of 5 gingival tissues from



**Figure 3. Platelet activation in response to stimulation with rhS100A9, rhS100A8/A9, or ADP assessed by expression of CD62P on platelet surface.** (A) rhS100A9, rhS100A8/A9, or ADP increased the percentage of CD62P<sup>+</sup> platelets in whole blood of controls and patients (<sup>#</sup> $P < 0.05$  vs. PBS group in controls; \* $P < 0.05$  vs. PBS group in patients) ( $n = 5$ ). (B) The ratio of the percentage of CD62P<sup>+</sup> platelets in stimulated whole blood to the percentage of CD62P<sup>+</sup> platelets in unstimulated whole blood in patients and controls (\* $P < 0.05$  comparison between patients and controls).

patients with GAgP showed aggregates of CD41<sup>+</sup> platelets and platelet-leukocyte comigration through the vasculature. (Fig. 7E and F). The distribution of the platelet aggregates was consistent with the area of leukocytes infiltration. Some of these platelets were colocalized with, and attached to, leukocytes. Only scattered, isolated platelets were found in healthy gingival tissues (Fig. 7B and C).

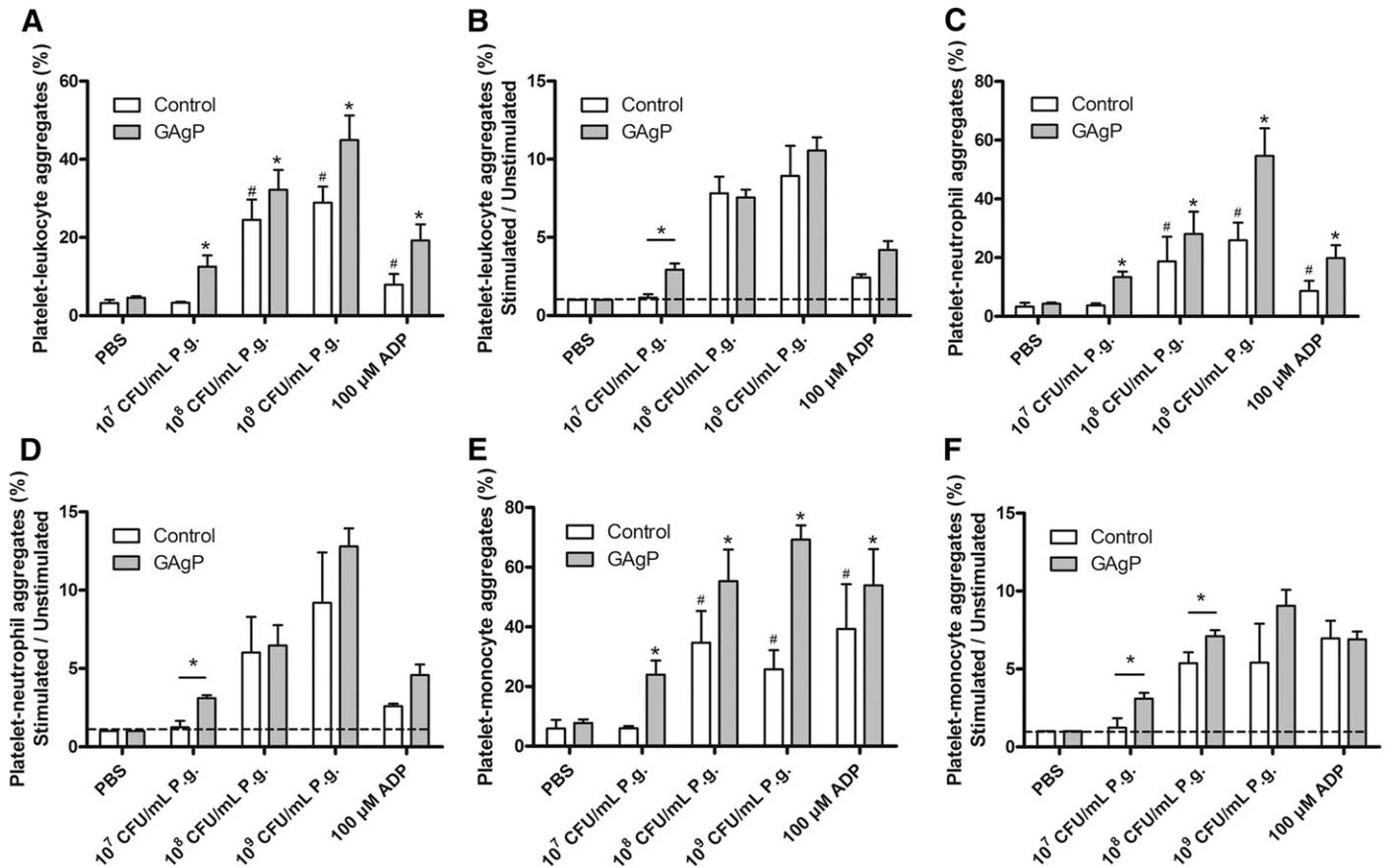
## DISCUSSION

The present study showed that the high-grade inflammatory condition GAgP is accompanied predominantly by few platelets

in peripheral circulation. Moreover, the values of PLT, PLCR, and MPV were negatively correlated with clinical periodontal parameters, such as PD and BI, indicating severe periodontal inflammation was accompanied by a decrease of platelet size in patients with GAgP. These findings suggest a link between platelet and periodontal inflammation.

Because it is a reliable indicator of platelet function and activation, platelet size has been proposed as a potential indicator of inflammation in other inflammatory disorders [28]. It seems that the size of circulating platelets is dependent on the intensity of inflammation, with contrasting features of MPV in high- and low-grade inflammatory disorders. The cause of the reduced MPV in the circulation of patients with GAgP is unknown but may be a consequence of inflammation similar to what occurs in other high-grade inflammatory diseases, such as rheumatoid arthritis [8], ulcerative colitis [12], ankylosing spondylitis [8, 29], systemic lupus erythematosus [29], and inflammatory bowel disease [30], in which low MPV may be related to an increased consumption of large, activated platelets at the sites of inflammation. The underlying mechanism of platelet consumption at inflammatory sites may be related to the interaction of platelets and leukocytes.

Immune inflammatory responses require leukocytes to roll, to adhere to, and to transmigrate through the vasculature endothelium into inflammatory tissue and thus participate in host defense. These processes can be promoted by the formation of platelet-leukocyte aggregates [21]. As platelets become activated, they express CD62P on their surfaces [31]. Membrane-expressed CD62P (on platelets) engages its receptor, PSGL-1 (on neutrophils and monocytes), which is a critical step in activation and recruitment of leukocytes to inflammatory sites [32, 33]. Selectin-mediated platelet-neutrophil interactions are a critical step in activation and recruitment of leukocytes to the lung in acute lung injury [22]. As described above, activated platelets drive responses in target leukocytes that modulate the host response to infection. This study provided evidence that patients with GAgP had a higher status of platelet activation compared with healthy controls. The infectious milieu provides a variety of signals that lead to platelet activation. It is well known that several orally derived species of bacteria interact with platelets [15]. *Porphyromonas gingivalis*, a gram-negative bacterium known to be a predominant periodontal pathogen, could induce platelet aggregation and contribute to atherothrombosis [34]. Our previous studies demonstrated that the prevalence of *P. gingivalis* was up to 94.4% and the relative abundance up to 35.88% in subgingival plaque samples from Chinese patients with GAgP [35–37], indicating that *P. gingivalis* might be a predominant pathogen and have an important role in these patients. Histopathologic observations demonstrated disruption of the epithelial integrity of the periodontal pocket and an ulcerated surface of up to 8–20 cm<sup>2</sup> [38]. With exposure of vascularized tissue to the subgingival biofilm, *P. gingivalis* may enter into blood after dental procedures, mastication, or personal oral hygiene [5]. The effect of *P. gingivalis* on platelets was, therefore, assessed in this study. In vitro, *P. gingivalis* activated platelet in the whole blood of healthy subjects and patients with GAgP, suggesting that platelet activation in patients with GAgP might indeed be partly in response to *P. gingivalis*. Interestingly,



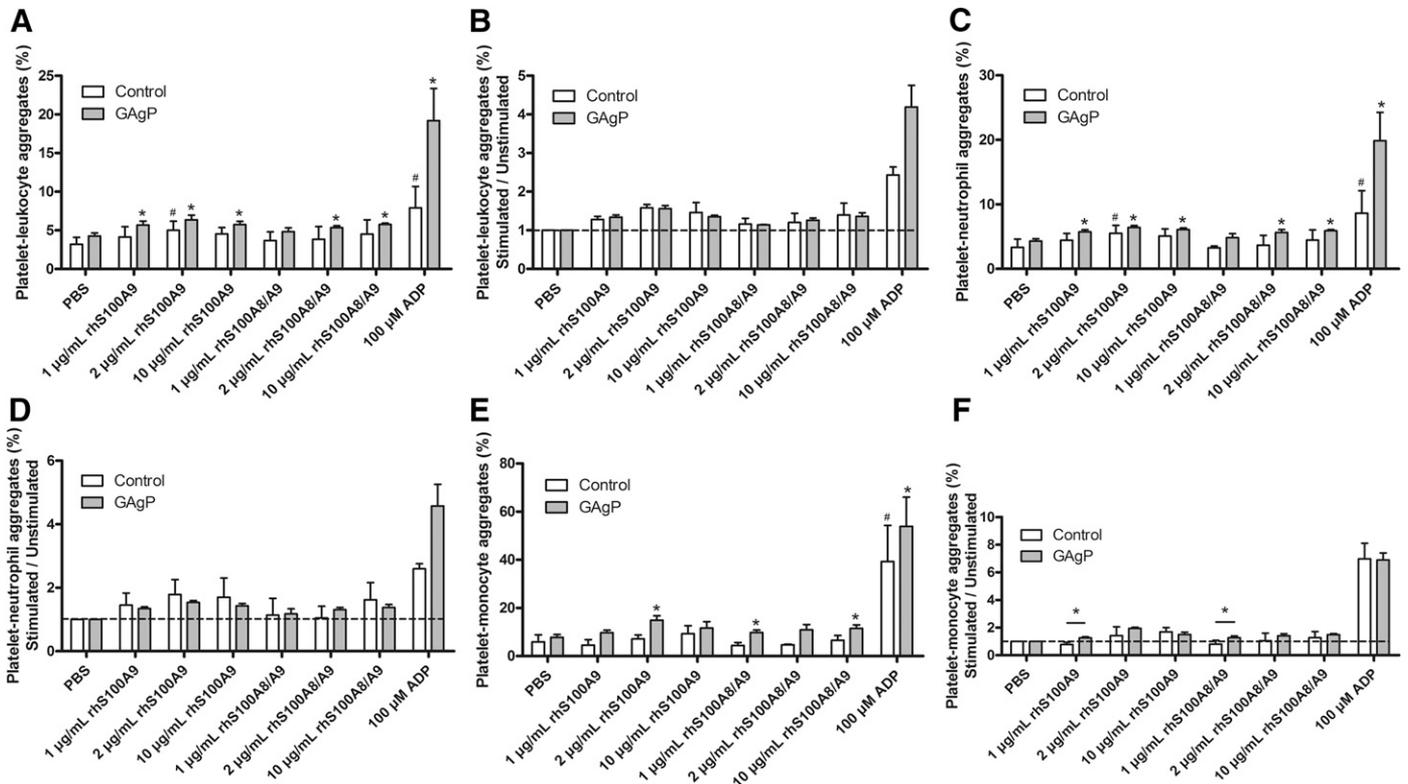
**Figure 4. Formation of platelet-leukocyte aggregates (including platelet-neutrophil aggregates and platelet-monocyte aggregates) in response to stimulation with *P. gingivalis* (P.g.) or ADP.** *Porphyromonas gingivalis* or ADP increased the percentage of platelet-leukocyte aggregates (A), platelet-neutrophil aggregates (C), and platelet-monocyte aggregates (E) in whole blood of controls and patients, respectively (<sup>#</sup>*P* < 0.05 vs. PBS group in controls; \**P* < 0.05 vs. PBS group in patients) (*n* = 5). Platelet-leukocyte aggregate (B), platelet-neutrophil aggregate (D), and platelet-monocyte aggregate (F) formation in response to *P. gingivalis* as a ratio (the percentage after stimulation to the percentage of unstimulated whole blood) from patients and controls (\**P* < 0.05 comparison between patients and controls).

platelets from patients with periodontitis have an increased sensitivity to the activation by *P. gingivalis*, which indicates the preactivation status of platelets in patients with GAgP and agrees with the findings that platelets from patients with GAgP have an increased activation status compared with platelets from healthy controls. Oral bacteria are regularly disseminated in the blood [5], and this increased sensitivity of platelets may contribute to aggravation of local and systemic inflammation in patients with GAgP.

Inflammatory mediators also can induce platelet activation [39]. Platelets express CD36 [17] and RAGE [18], which are both signals that have been directly implicated in platelet activation. Because plasma S100A8/A9 level is elevated in patients with GAgP, we also evaluated whether S100A8/A9 had an effect on platelet activation in the current study. It was verified in vitro that rhS100A9 or rhS100A8/A9 induced platelet activation in whole blood of healthy subjects and patients with GAgP. Platelets from patients with GAgP have an increased sensitivity to activation by rhS100A9 or rhS100A8/A9.

The results of the present study indicate that platelet activation in patients with GAgP might be partly due to *P. gingivalis* and

S100A8/A9 stimulation. As platelets become activated, they express CD62P on their surfaces and engage their receptor PSGL-1 on leukocytes, forming platelet-leukocyte aggregates [31–33]. In the present study, *P. gingivalis* induced platelet-leukocyte aggregate formation, including platelet-neutrophil aggregates and platelet-monocyte aggregates, in the whole blood of healthy subjects and patients with GAgP. In addition, 2 μg/ml rhS100A9, close to plasma calprotectin concentrations of patients with GAgP, induced the formation of platelet-leukocyte aggregates in whole blood of healthy subjects, indicating the effect of calprotectin in the plasma of patients with GAgP on platelets and leukocytes. Platelet-leukocyte aggregates are important for functional alterations of these cells. Formed aggregates produce proinflammatory cytokines in larger amounts than uncomplexed cells [40]. More platelet-leukocyte aggregates were formed in the blood of patients with GAgP compared with healthy subjects, and in response to *P. gingivalis*, rhS100A9 or rhS100A8/A9, formation of platelet-leukocyte (neutrophil/monocyte) aggregates significantly increased in the blood from patients with GAgP compared with healthy subjects, which can amplify the immune inflammatory response in GAgP.

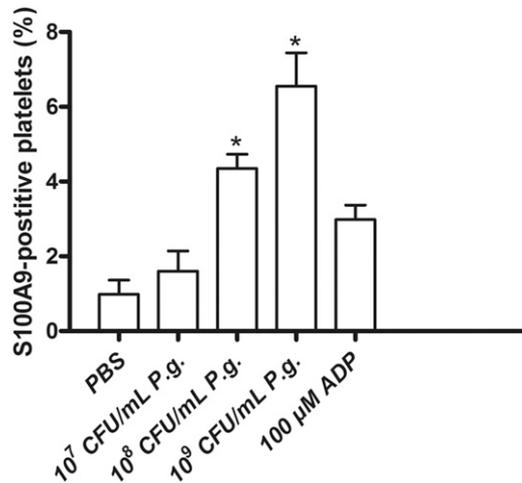


**Figure 5. Formation of platelet-leukocyte aggregates (including platelet-neutrophil aggregates and platelet-monocyte aggregates) in response to stimulation with rhS100A9, rhS100A8/A9, or ADP.** rhS100A9, rhS100A8/A9, or ADP increased the percentage of platelet-leukocyte aggregates (A), platelet-neutrophil aggregates (C), and platelet-monocyte aggregates (E) in whole blood of controls and patients, respectively ( $^{\#}P < 0.05$  vs. PBS group in controls;  $*P < 0.05$  vs. PBS group in patients) ( $n = 5$ ). Platelet-leukocyte aggregate (B), platelet-neutrophil aggregate (D), and platelet-monocyte aggregate (F) formation in response to rhS100A9 and rhS100A8/A9 as a ratio (the percentage after stimulation to the percentage of unstimulated whole blood) from patients and controls ( $*P < 0.05$  comparison between patients and controls).

Periodontitis features an intense infiltrate of leukocytes. Platelets are known to have an essential role in leukocyte recruitment in the vasculature and, hence, their trafficking into tissues. Moreover, it has recently been demonstrated in a model of experimental colitis that recruitment of leukocytes and platelets was a codependent processes in inflamed colonic venules [23]. Platelet-leukocyte aggregate formation facilitates the process of leukocyte migration to inflammatory sites [21, 41]. In this study, the histopathologic alterations that occurred within the gingival tissues of GAgP were obvious. Vasodilation and vascular proliferation were observed. There was a dense infiltration of defense cells in connective tissues, particularly neutrophils, macrophages, plasma cells, and lymphocytes. Because of the accumulation of these defense cells and the extracellular release of their destructive enzymes, there was disruption of the normal anatomy of the connective tissues, which results in collagen depletion. That platelets could be involved in periodontal inflammation was first inferred, to our knowledge, from this study, along with platelet aggregates and platelet-leukocyte comigration into extravascular tissue. Platelet movement across the vascular wall was proportional to the extent of leukocyte transmigration. The distribution of platelet aggregates was consistent with leukocyte recruitment and permeability. Many intravascular leukocytes had one or

more platelets attached to their surface, and some platelets in extravascular tissue were also attached to leukocytes. The parallel increase of platelets and leukocytes in inflamed gingival tissue suggests the possibility of interplay between platelets and leukocytes in the fight against infection. Therefore, the underlying mechanism of increased consumption of platelets in patients with GAgP might be that the periodontal infection promotes platelet activation and platelet-leukocyte aggregate formation, followed by platelet and leukocyte migration through the vasculature to the inflamed periodontal tissue, resulting in large-sized, activated platelets being consumed intensely. This study, thus, provides new insights into the interaction of platelets and leukocytes in periodontitis.

Platelet-leukocyte aggregates are of importance, not only in homing leukocytes to inflammatory sites but also in better bacterial clearance [42, 43]. Neutrophils are the first line of innate immune defense against infection. In addition to the more-traditional mechanism of phagocytosis to kill bacteria, activation of neutrophils causes the release of web-like structures of DNA (NETs). These NETs are extracellular chromatin lattices, decorated with neutrophil-derived proteins, for trapping and killing microbes in tissues. A recent study [44] demonstrated that platelets signal



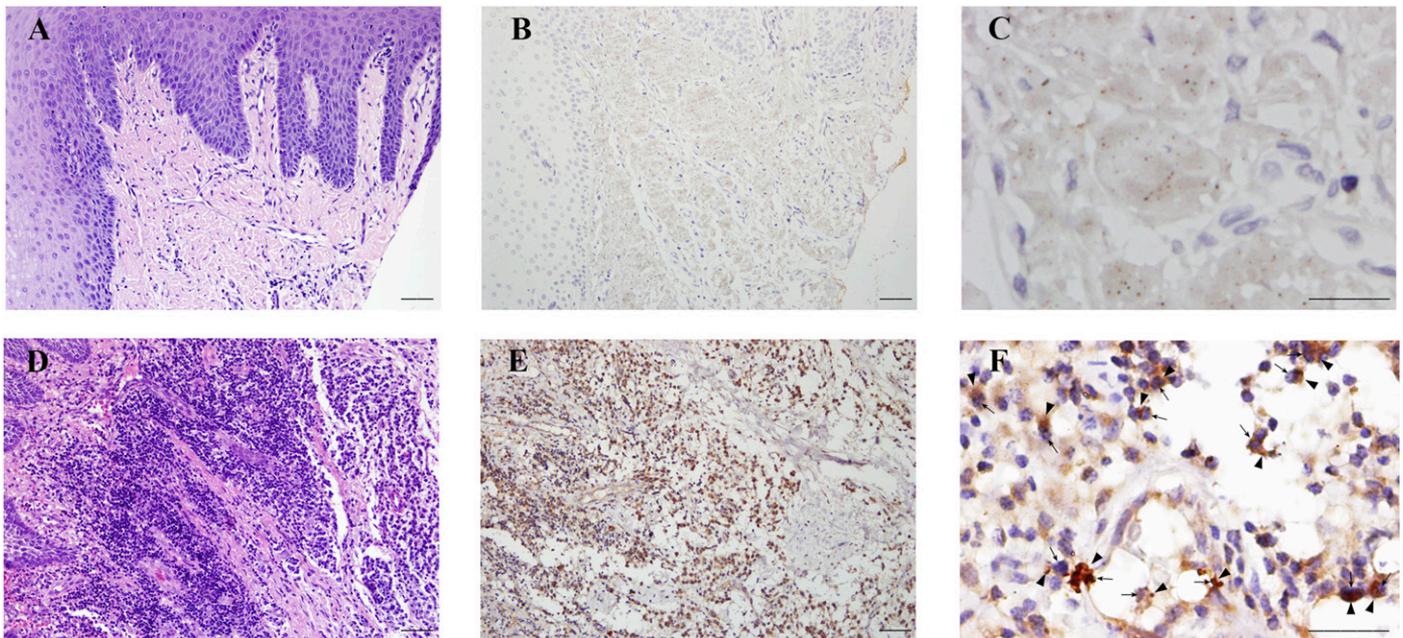
**Figure 6. Platelet surface expression of S100A9 at rest, *P. gingivalis* (P.g.), or ADP stimulated human peripheral blood.** *Porphyromonas gingivalis* (at 10<sup>8</sup>, 10<sup>9</sup> CFU/ml) increased the percentage of S100A9<sup>+</sup> platelets in whole blood of controls (\**P* < 0.05 vs. PBS group) (*n* = 5).

polymorphonuclear leukocytes to form NETs. S100A8/A9, known as the main cytoplasmic protein of neutrophils, has been identified as one of the major antimicrobial proteins in NETs [45]. In the present study, the plasma S100A8/A9 concentration increased and was correlated with the amount of WBCs and with NEUT and NEUT%. Neutrophils might be a source of S100A8/A9 in GAgP [16]. The elevated S100A8/A9 level in GCF was associated with reduced PLCR and MPV

values. Taking these observations together, we speculate that activated platelets binding to adherent neutrophils in inflammatory periodontal tissues facilitate formation of NETs and the release S100A8/A9 into GCF to clear microbes.

The data from the present study showed that about 0.98% of platelets in healthy subjects express S100A9 protein. After stimulation with *P. gingivalis* at 10<sup>8</sup> CFU/ml or 10<sup>9</sup> CFU/ml, the percentage of S100A9<sup>+</sup> platelets increased to 4.35% and 6.55%, respectively. A previous study [46] suggested that platelet activation status influenced platelet protein expression. Combined with the above-mentioned data showing that rhS100A9 facilitated platelet–leukocyte aggregate formation, these observations indicate that the elevated expression of S100A9 on platelets’ surfaces after *P. gingivalis* stimulation might induce a positive feedback loop in which S100A9 facilitated further platelet–leukocyte aggregate formation.

Although similar platelet–leukocyte interactions have been described in several diseases, codependency of platelet and leukocyte recruitment in gingiva during periodontitis has not, to our knowledge, been previously addressed. Based on our preliminary work, such a mechanism is conceivable in patients with GAgP, where platelet activation in response to *P. gingivalis* and S100A8/A9 is concurrent with the formation of platelet–leukocyte aggregates in patients with GAgP, and, thereby platelet–leukocyte aggregates contribute to better bacterial clearance through mechanisms that include recruitment of leukocytes into inflamed periodontal tissue and secretion of S100A8/A9 into the GCF.



**Figure 7. Histologic and immunohistochemical appearance of gingival tissue samples from patients with GAgP and healthy subjects.** (A) H&E staining of gingiva from a healthy subject. (B and C) Immunohistochemistry for CD41 (GpIIb) shows a few isolated platelets on healthy gingival tissue from a healthy subject. (D) H&E staining of gingiva with a dense inflammatory-cell infiltrate in the connective tissue of a patient with GAgP. (E and F) Immunohistochemistry for CD41 (GpIIb) shows many platelets (triangles) diffusely distributed on inflamed gingival tissue; most are attached to, or colocalized with, leukocytes (arrows), and some platelets and leukocytes comigrate through the vasculature. Scale bars, 50 μm (A, B, D, and E) and 20 μm (C and F).

In conclusion, the platelet activation response to *P. gingivalis* and S100A8/A9 is paralleled by increased formation of platelet-leukocyte aggregates with the recruitment of platelets and leukocytes to inflamed gingiva in patients with GAgP. The decrease in platelet size and count in patients with GAgP may be related to increased consumption of large, activated platelets at sites of inflammation of the gingiva. Our findings provide novel insights into the impact of platelets on the fight against infection as previously unsuspected conspirators of inflammation and tissue injury in GAgP. However, the roles of platelets in periodontitis still remain largely unclear and provide a fertile area for future studies.

## AUTHORSHIP

Y.Z. performed data analysis, bacterial culture, flow cytometry, ELISAs, and histology and immunohistochemistry, as well as preparing the manuscript. R.L. and H.M. analyzed the results, contributed to the conception and design of the paper, and prepared the manuscript. X.W. performed the collection of subjects and assessment of clinical parameters, and J.H. contributed to the conception and design of the paper and performed the sample collection.

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## DISCLOSURES

The authors declare no conflicts of interest.

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**KEY WORDS:**

mean platelet volume · calprotectin · *Porphyromonas gingivalis*

## Platelet activation and platelet–leukocyte interaction in generalized aggressive periodontitis

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