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Association between periodontitis and anti-cardiolipin antibodies in Buerger disease

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Abstract

Aim: Anti-cardiolipin (CL) antibodies can be induced in Buerger disease (BD), an inflammatory occlusive disorder affecting peripheral blood vessels, in response to bacteria bearing homology to the TLRVYK peptide of a phospholipid-binding plasma protein β-2-glycoprotein I. TLRVYK homologies are present in *Porphyromonas gingivalis* (TLRIYT) and *Treponema denticola* (TLALYK). This study investigated the association between periodontal infection and anti-CL antibodies in BD patients.

Material and Methods: Periodontal conditions were examined in 19 BD patients and 25 systemically healthy control subjects. All subjects were heavy smokers. Serum anti-CL, anti-TLRVYK, anti-TLRIYT, and anti-TLALYK antibodies were assessed using the enzyme-linked immunosorbent assay.

Results: BD patients had a significantly higher prevalence of periodontitis, more severe periodontal destruction and increased titres of serum anti-CL, anti-TLRVYK, anti-TLRIYT, and anti-TLALYK antibodies compared with healthy subjects. The levels of anti-CL antibodies positively correlated with those of the three anti-peptide antibodies. Anti-CL antibody titres were significantly associated with the percentage of sites with clinical attachment level $\geqslant$4 mm in BD patients.

Conclusion: Elevated anti-CL antibody levels were associated with periodontal destruction in BD patients. Periodontopathic bacteria may serve as exogenous antigens that stimulate the anti-CL antibody production through molecular mimicry between the bacterial peptides and a host plasma protein.

Key words: anti-cardiolipin antibody; β-2-glycoprotein I; Buerger disease; molecular mimicry; periodontitis

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Buerger disease (BD) is an inflammatory occlusive disorder affecting small- and medium-sized arteries and veins, and occurs predominantly in males younger than 40–45 years (Buerger 1952, Mills & Porter 1993, Olin 2000, Olin & Shih 2006). Pathological findings showed well-organized intra-luminal vascular thrombosis in the surgically resected arterial specimens from patients with BD (Shionoya 1990, Tanaka 1998). Although the cause of BD remains unclear, there is a strong association between heavy tobacco use and BD (Allen & Brown 1928, Olin et al. 1990, Shionoya 1998). A high prevalence of anti-cardiolipin (CL) antibodies and increased anti-CL antibody titres were reported in patients with BD (Olin et al. 1996, Maslowski et al. 2002).

Anti-CL antibodies and lupus anticoagulant are different classes of anti-phospholipid antibodies associated with the anti-phospholipid antibody syndrome (Ginsburg et al. 1992, Nahass 1997, Greaves 1999). Anti-phospholipid antibodies interact with a phospholipid-binding plasma protein, β-2-glycoprotein I (β2GPI) (Galli et al. 1990, McNeil et al. 1990, Roubey et al. 1992), and may lead to a thrombotic predisposition. Some phospholipid-binding viral and bacterial proteins function like β2GPI in inducing the production of anti-phospholipid antibodies and anti-β2GPI anti-

Conflict of interest and source of funding statement

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bodies (Gharavi et al. 1999, 2002, Blank & Shoenfeld 2004), suggesting a mechanism of molecular mimicry. Blank et al. (2002) demonstrated that pathogenic anti-β2GPI antibodies could be induced by immunizing mice with *Hemophilus influenzae* or *Neisseria gonorrheae*, which possesses peptide sequences that are homologous to the TLRVYK peptide of β2GPI.

Schenkein et al. (2003, 2007) reported that the prevalence of patients with chronic periodontitis and a generalized aggressive periodontitis positive for anti-CL antibodies was greater than that in healthy controls. In addition, systemic markers of vascular inflammation in patients with aggressive periodontitis are associated with elevated levels of β2GPI-dependent anti-CL antibodies. We reported previously that patients with BD exhibited severe periodontitis and higher serum IgG titres against periodontopathic bacteria, suggesting that periodontal infection may also be associated with BD (Iwai et al. 2005, Chen et al. 2007).

According to the Swiss Prot database, sequences homologous to the TLRVYK peptide of β2GPI are present in the arggingipain protease of *Porphyromonas gingivalis* (TLRIYT) and the phosphoglycerate kinase of *Treponema denticola* (TLALYK). We hypothesized that homologous peptides in periodontopathic bacteria could induce the production of pathogenic anti-CL antibodies in patients with BD. The aim of the present study was to examine whether periodontal infection was associated with increased anti-CL antibody titres in patients with BD.

Material and Methods

Study population

Twenty-six BD and 38 healthy control subjects were recruited during September 2005–December 2006. Nineteen male patients with a diagnosis of BD, based on Shionoya's criteria and angiographic findings from the Clinic of Vascular and Applied Surgery in the Tokyo Medical and Dental University, were included in this study (participation rate = 73.1%). One female BD patient, four with hypertension, and two with diabetes mellitus were excluded. All patients had the typical characteristics of BD, including a history of smoking, disease onset before the age of 50 years, occlusive lesions in the infrapopliteal artery, either upper limb involvement or phlebitis migrans, and an absence of risk factors for atherosclerosis (with the exception of smoking). Occluded arterial segments were removed during surgery, and histopathological examinations were performed to confirm a diagnosis of BD.

Twenty-five healthy control subjects, defined as those without BD or any other systemic disease, were matched against the patient population by age, gender, smoking status, and systemic status (participation rate = 65.8%). Seven females and six non-smokers were excluded. Information about their current health status, medical history, drug use, and smoking behaviour was obtained via a questionnaire. Subjects were excluded if they had received antibiotics in the last 3 months or treatment for periodontal disease in the last 6 months. The socio-economic status of the subjects was homogenous without a considerable difference between BD patients and control subjects. All subjects provided informed consent, and all protocols were approved by the Ethical Committee of Tokyo Medical and Dental University.

Clinical periodontal examinations

Periodontal status was evaluated using various clinical parameters by a well-trained calibrated periodontist (M. U.). Periodontal probing depth (PD) and clinical attachment level (CAL) were recorded at six points for each tooth. CAL was calculated from the sum of the PD and the gingival margin. Participants who presented with at least one site with PD $\geqslant$ 4 mm and CAL $\geqslant$ 4 mm in each quadrant were defined as patients with periodontitis. The number of residual teeth was also recorded. Periodontal examinations were performed before patients received surgical treatment.

Determination of serum anti-CL IgG antibodies

Whole-blood samples were taken from all study subjects at the time of the dental visit. Blood samples were centrifuged at 1500 g for 10 min at 4°C. The serum was filtered and immediately stored at -80°C until analysis. Serum anti-CL antibodies were measured using the AngioMax anti-CL IgG ELISA kit (Assaypro, St. Charles, MO, USA) according to the manufacturer's instructions, and the results are presented as GPL units, which is the international standard for the measurement of anti-CL IgG antibodies (Harris 1990).

Determination of serum IgG antibody titres against TLRVYK, TLRIYT, and TLALYK peptides

Serum anti-TLRVYK, anti-TLRIYT, and anti-TLALYK antibodies were determined using an enzyme-linked immunosorbent assay method, as described previously (Wang et al. 2008). Briefly, 96-well micro-titre plates were coated with TLRVYK, TLRIYT, or TLALYK peptides (10 μg/mL) and blocked with 3% bovine serum albumin. In order to prepare a positive control, rabbits were immunized with TLRVYK peptide, and the anti-TLRVYK rabbit IgG was purified by affinity chromatography with a sepharose column conjugated with TLRVYK peptide. After washing with phosphate-buffered saline (PBS), a twofold serial dilution (50 to 0.098 ng/mL) of rabbit anti-TLRVYK IgG was added to the top two rows of the plate as a standard. Diluted serum samples (1:128) from subjects were added in duplicate to the remaining rows of the plate, followed by overnight incubation. After washing with PBS, peroxidase-conjugated protein A was added to bind both rabbit and human IgG antibody, and the binding was detected using the tetramethylbenzidine liquid substrate system (Sigma, St. Louis, MO, USA). The reaction was stopped with 1 M H_2SO_4 (Sigma) after 30 min. The optical density at 450 nm for each well was determined using a Microplate Reader (SOFT Max™, Sunnyvale, CA, USA).

Statistical analysis

Descriptive statistics and statistical analyses were performed using a computerized statistical package (SPSS). The Kolmogorov–Smirnov normality test and the Levene variance homogeneity test were applied to study the distribution normality of the data. The statistical difference of the periodontitis prevalence between the two groups was examined using Fisher's exact test. The Mann–Whitney *U*-test was applied to detect differences in the percentages of sites with PD and CAL, number of residual teeth, and serum IgG antibody titres. Spearman's rank-correlation test was applied to test the correlations between anti-CL antibodies and the per-

centage of sites with PD ⩾ 4 mm or CAL ⩾ 4 mm, and three anti-peptide IgG antibodies. Statistical significance was set at $p < 0.05$. Multivariate logistic regression analysis was performed to examine the association between BD and several risk factors.

Results

Characteristics and the periodontal status of participants

The demographics of BD patients and healthy control subjects are summarized in Table 1. There were no significant differences in age, gender, and smoking status on comparing the two groups. Because smoking was regarded as an important risk factor for both BD (Allen & Brown 1928, Shionoya 1998, Olin & Shih 2006) and periodontitis (Haber et al. 1993, Bergström et al. 2000, Tomar & Asma 2000, Hyman 2006), all subjects in our study were heavy smokers. The prevalence of periodontitis and the percentage of sites with PD ⩾ 4 mm and CAL ⩾ 4 mm were significantly higher in patients with BD than in the control subjects ($p < 0.001$, $p < 0.001$, and $p = 0.037$, respectively). Furthermore, patients with BD had fewer residual teeth when compared with healthy controls, but the difference between the two study groups was not statistically significant ($p = 0.216$).

Table 1. Characteristics of the study subjects

	Buerger disease patients	Control subjects
Number of subjects (*n*)	19	25
Age (years; mean ± SD)	56.6 ± 11	59.6 ± 14
Gender (male/female)	19/0	25/0
Heavy smoker† (*n*)	19	25
Prevalence of periodontitis**	89.5% (17/19)	28% (7/25)
Percentage of sites with PD ⩾ 4 mm**	14.3%	2.4%
Percentage of sites with CAL ⩾ 4 mm*	22.6%	13.2%
Number of residual teeth	20.2	24.2

†Heavy smoker: subject who has or had a history of smoking, as defined by ⩾ 20 cigarettes per day for > 20 years.
PD, probing depth; CAL, clinical attachment level.
Significantly different between the study groups at
**$p < 0.001$, *$p = 0.037$.

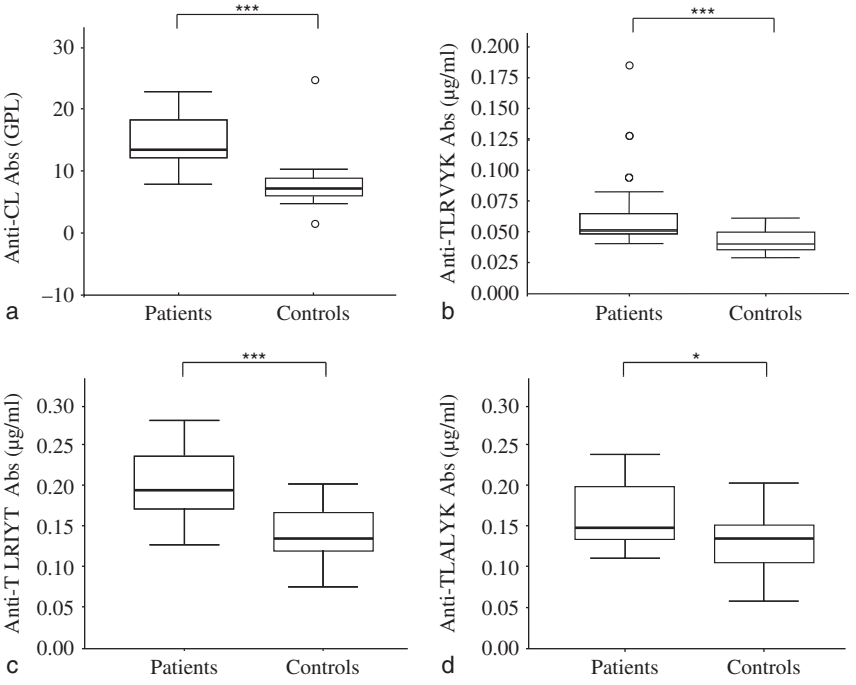

Fig. 1. Serum IgG levels of anti-cardiolipin (CL) (a), anti-TLRVYK (b), anti-TLRIYT (c), and anti-TLALYK (d) antibodies in 19 patients with Buerger disease and 25 healthy control subjects. Box plots show medians with 25th and 75th percentiles, and outliers are marked as open circles. ***$p < 0.001$, *$p < 0.05$ (Mann–Whitney *U*-test).

Serum anti-CL, anti-TLRVYK, anti-TLRIYT, and anti-TLALYK antibodies

Patients with BD had significantly higher anti-CL antibody titres when compared with control subjects ($p < 0.001$, Fig. 1a). Further, anti-TLRVYK (Fig. 1b), anti-TLRIYT (Fig. 1c), and anti-TLALYK (Fig. 1d) antibody titres were significantly higher in patients with BD than in control subjects ($p < 0.001$, $p < 0.001$, and $p = 0.017$, respectively). Figure 2 illustrates the correlations between anti-CL IgG antibody titres and three anti-peptide antibody titres. Anti-CL antibody titres positively correlated with anti-TLRVYK, anti-TLRIYT, and anti-TLALYK antibody titres ($p < 0.001$, $p = 0.002$, and $p = 0.028$, respectively).

Association between anti-CL antibody titres and periodontal status

Overall, anti-CL antibody titres significantly correlated with the percentage of sites with CAL ⩾ 4 mm and the percentage of sites with PD ⩾ 4 mm (Fig. 3a, all subjects, $r = 0.428$, $p = 0.004$; and Fig. 3b, all subjects, $r = 0.426$, $p = 0.004$; respectively). In BD patients, anti-CL antibody titres positively correlated with the percentage of sites with CAL ⩾ 4 mm (Fig. 3a, $r = 0.471$, $p = 0.042$). Although the trend for an association between anti-CL antibody titres and the percentage of sites with PD ⩾ 4 mm was observed, it did not reach a level of statistical significance (Fig. 3b, $r = 0.175$, $p = 0.474$).

Association between BD and risk factors

Logistic regression analysis was performed to examine the association between BD and several risk factors. BD was associated with anti-CL antibodies [odds ratio (OR) = 1.43, $p = 0.008$)] and periodontitis (OR = 7.71, $p = 0.046$) (Table 2).

Discussion

The association between anti-CL antibodies and BD has been reported pre-

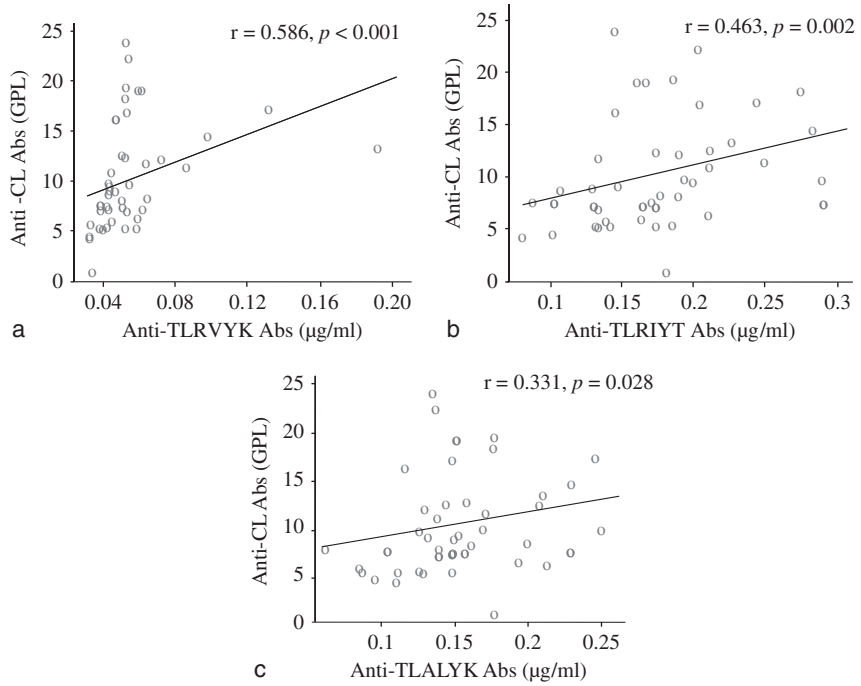

Fig. 2. The plot shows associations between serum anti-cardiolipin (CL) antibody levels and anti-TLRVYK (a), anti-TLRIYT (b), and anti-TLALYK (c) antibody levels in all subjects. Each subject is shown as an open circle, and the regression line represents the trend. ''*r*'' represents the correlation coefficient; $p<0.05$ represents statistical significance (linear regression analysis; Spearman's rank-correlation test).

viously (Olin et al. 1996, Olin 2002, Maslowski et al. 2002). Our present study also found that patients with BD displayed significantly higher anti-CL antibody titres when compared with control subjects. Logistic regression analysis confirmed that increased levels of anti-CL antibodies were related to BD. In addition, our results showed that periodontitis was strongly associated with BD. All subjects in the present study were heavy smokers. Therefore, we could not evaluate the effect of periodontitis on BD in non-smokers. It is possible that the correlation between periodontitis and BD may be limited only to the smokers.

In this study, BD patients exhibited severe periodontitis, and the anti-CL antibody titres significantly correlated with the percentage of sites with CAL $\geqslant$ 4 mm, implying that the elevated anti-CL antibody levels may be related to periodontal destruction in patients with BD. These findings are consistent with observations from previous studies suggesting that periodontitis may increase serum β2GPI-dependent anti-CL antibody titres (Schenkein et al. 2003, 2007). However, the mechanistic link between periodontal infection and anti-CL antibodies was not investigated in those studies. Because most BD patients in our study had periodontitis, further studies comparing BD patients with periodontitis with BD patients without periodontitis are warranted to confirm the contribution of periodontitis on BD pathogenesis through elevated levels of anti-CL antibodies.

We previously reported that *P. gingivalis* and *T. denticola* DNA were frequently detected in arterial specimens taken from patients with BD (Iwai et al. 2005), and that IgG antibody titres against *P. gingivalis* and *T. denticola* were significantly increased in patients with BD. These observations implicate a possible aetiologic linkage between BD and periodontitis (Chen et al. 2007). Indeed, bacteria that possess peptide sequences homologous to the TLRVYK peptide of β2GPI were demonstrated to induce pathogenic anti-β2GPI antibodies in mice (Blank et al. 2002). TLRVYK homologies are present in *P. gingivalis* (TLRIYT) and *T. denticola* (TLALYK). In this study, patients with BD had increased antibody titres against TLRVYK, TLRIYT, and TLALYK peptides, suggesting that infection with *P. gingivalis* and *T. denticola* may induce anti-TLRIYT, anti-TLALYK, and anti-TLRVYK antibodies. TLRVYK is a peptide on the β2GPI molecule, and anti-TLRVYK antibody is believed to be a part of the anti-β2GPI or anti-CL antibodies. Our results showed that the levels of serum anti-TLRVYK, anti-TLRIYT, and anti-TLALYK antibodies positively correlated with the levels of serum anti-CL antibodies. Recently, we have demonstrated that *Aggregatibacter actinomycetemcomitans* infection can elicit and modify the anti-TLRVYK antibody response via molecular mimicry between the SIRVYK peptide in *A. actinomycetemcomitans* leucotoxin c and the TLRVYK peptide of β2GPI (Wang et al. 2008). Therefore, the findings in the present study supported that periodontal infection by *P. gingivalis* and *T. denticola* may augment antibody responses against CL and/or β2GPI via molecular mimicry in patients with BD.

Molecular mimicry between periodontopathic bacteria and host-antigens has been reported in other disease (Tabeta et al. 2000, 2001, Yamazaki et al. 2004). Yamazaki et al. (2004) showed that antibody levels to human heat shock protein 60 (HSP60) and *P. gingivalis* HSP60 (GroEL) were the highest in patients with atherosclerosis, followed by periodontitis patients and healthy subjects. It was also demonstrated that patients with atherosclerosis had HSP60-reactive as well as GroEL-reactive T cells and that atherosclerotic lesions were infiltrated with HSP60 reactive T cells. These studies suggested the mechanism of molecular mimicry between self-antigens and periodontopathic bacteria as a possible link between periodontitis and vascular diseases. Although the scope of our present study was limited to BD, it is reasonable to assume that periodontitis may influence other systemic diseases via a similar mechanism of molecular mimicry. Further studies are warranted to investigate the association between periodontitis and other anti-CL antibody-related diseases.

In conclusion, our study demonstrated that periodontal destruction was associated with increased anti-CL antibody titres in patients with BD. The periodontopathic bacterial peptides homologous to the TLRVYK peptide of β2GPI, which are presented in *P. gingivalis* (TLRIYT) and *T. denticola* (TLALYK), may induce the production of anti-CL antibodies in BD patients. Since the improvement of anti-phospholipid antibody syndrome after *Helico-*

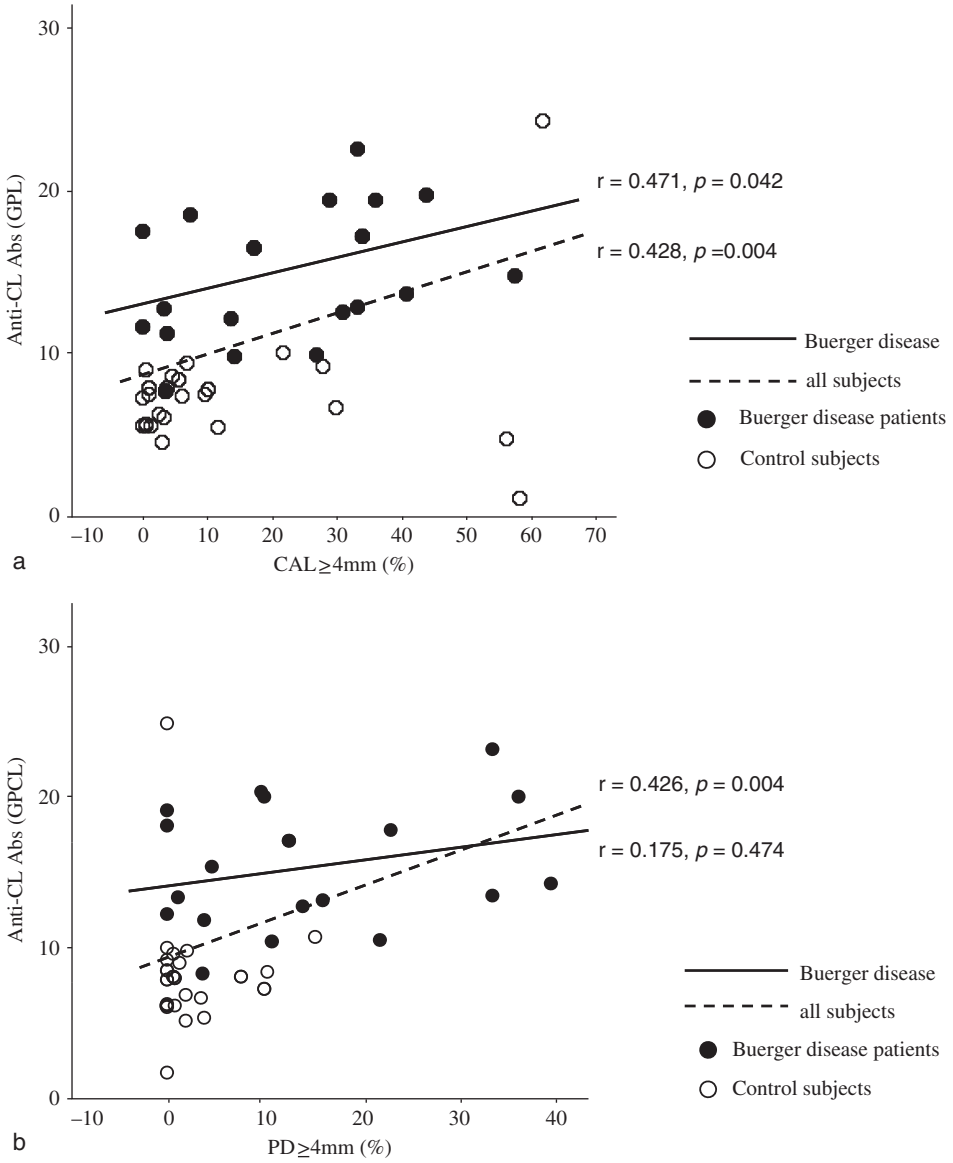

Fig. 3. The plot shows associations between serum anti-cardiolipin (CL) antibody levels and the percentage of sites with clinical attachment level (CAL)$\geqslant$4 mm (a) or probing depth (PD)$\geqslant$4 mm (b). Patients with Buerger disease are shown as black dots, and the straight line represents the trend. Control subjects are shown as open circles, and the dashed line represents the trend. ''*r*'' represents the correlation coefficient; $p<0.05$ represents statistical significance (linear regression analysis; Spearman's rank-correlation test).

Table 2. Association of risk factors with Buerger disease in a logistic regression model. Independent variables include anti-CL antibodies and periodontitis

Independent variables	Dependent variable: Buerger disease odds ratio (95% CI)	*p*-value
Anti-CL antibodies	1.43 (1.10–1.85)	0.008**
Periodontitis	7.71 (1.04–57.24)	0.046*

95% CI: 95% confidence interval. *$p<0.05$, **$p<0.01$.

bacter pylori eradication was reported (Cicconi et al. 2001), periodontal treatment to eliminate periodontopathic bacteria may reduce anti-CL antibody titres leading to an improvement of the BD condition.

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Clinical Relevance

Scientific rationale for the study: Evidences suggest that periodontitis can influence systemic diseases, including BD. However, the mechanism of this interaction remains unclear.

Principal findings: Periodontal destruction is associated with an increased production of anti-CL antibodies in patients with BD. Periodontopathic bacteria may act as exogenous antigens to stimulate the production of anti-CL antibodies through molecular mimicry between the bacterial peptides and a plasma protein β2GPI.

Practical implications: This study implies an association between periodontitis and BD. Periodontal treatment to eradicate periodontopathic bacteria may reduce anti-CL antibody titres, leading to an improvement of the BD condition.