Received: 19 July 2021 | Revised: 28 October 2021 | Accepted: 16 November 2021

DOI: 10.1002/JPER.21-0419

JOURNAL OF Periodontology

ORIGINAL ARTICLE

Pro-inflammatory profiles in cardiovascular disease patients with peri-implantitis

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Abstract

Background: To investigate the pro-inflammatory cytokine profiles in patients with or without cardiovascular disease (CVD) and with or without peri-implantitis.

Methods: Serum, peri-implant crevicular fluid (PICF), and gingival crevicular fluid (GCF) were collected from patients with (n = 82) or without CVD (n = 46) at the most severe peri-implantitis site including sites with periodontitis. A panel of proinflammatory molecules including high-sensitivity C-reactive protein (hsCRP), fibrinogen, interleukin-1 beta (IL-1β), IL-6, plasma tumor necrosis factor-alpha (TNF-α), matrix metallo-proteinase-8 (MMP-8), osteoprotegerin (OPG), vascular endothelial growth factor (VEGF), IL-17, IL-8, tissue inhibitor of metalloproteinase-2 (TIMP-2), myeloperoxidase (MPO), and prostaglandin E_2 (PGE_2) were analyzed using human custom Quantibody arrays. Krunskal-Wallis test was used to compare groups. The diagnostic ability of each biomarker was assessed using chi-square test and ROC analysis.

Results: Serum IL-1β, TNF-α and fibrinogen were significantly higher in CVD patients than those without. Serum fibrinogen displayed a trend of higher concentration in patients with radiographic bone loss (RBL) ≥2 mm (P = 0.08). PICF TNF-α exhibited a significantly higher detection level in the CVD patients that is coincided with the local peri-implant inflammation. In addition, PICF MMP-8 was significantly higher in the RBL ≥2 mm sites than the healthy implants; whereas IL-1β, IL-8, MMP-8, and TIMP-2 proved to be the significant predictors for peri-implant disease. GCF TNF-α collected from patients with periodontitis was significantly associated with CVD cases.

Conclusion: The augmented expression of local and systemic pro-inflammatory cytokines found in the current study supports the weak association between the chronic peri-implantitis with increasing severity and CVD.

KEYWORDS
cardiovascular disease, cardiovascular risk factor, dental implants, inflammation mediators, peri-implantitis, systemic inflammation

1 | INTRODUCTION

The highest global burden of non-communicable diseases (NCDs) arises from cardiovascular diseases (CVDs), which are the leading cause of mortality around the globe.[1] CVD not only displays the highest global burden of disease (>50%),[2] and is also the most relevant age-related disease (38.4% of the age-related disease burden), particularly ischemic heart disease and stroke.[3] Today, CVD or atherosclerotic cardiovascular disease (ASCVD) is used as a general term to describe a range of clinical heart and circulatory diseases. "Total cardiovascular Disease" coined by the American Heart Association (AHA) comprising coronary heart disease (CHD), heart failure, stroke, and hypertension was found around 48% of prevalence in adults.[4]

Growing evidence supports the independent association between chronic oral infection and several chronic NCDs, including CVD.[5] Severe periodontitis appears to be a modifiable non-traditional risk factor for CVD,[6] particularly among populations with multi-morbidity.[7] Current biologically plausible mechanisms of the association between periodontitis and CVD has been centered on the bacteremia and the systemic inflammatory sequalae because both inflammatory pathologies are characterized by elevated serum levels of multiple inflammatory cytokines, lipids, and thrombotic and hemostatic factors.[8] The presence of elevated serum levels of inflammatory mediators in patients with periodontitis and CVD, and reduced serum levels of these inflammatory markers after periodontal treatment support the link between periodontal inflammation and the ASCVD risk.[9–13] Another chronic oral infection, peri-implantitis, shared similar characteristics with periodontitis, including predominant plasma-cells inflammatory infiltrates, gram negative anaerobes, and increased production of local inflammatory cytokines.[14] In this study, it was hypothesized that chronic inflammation at sites with peri-implantitis may induce systemic low-grade inflammation and increase the risk of CVD. Hence, the purpose of this investigation was to assess the pro-inflammatory profiles of systemic and local peri-implant biomarkers among those CVD patients with or without peri-implantitis to understand the potential inflammatory link between CVD and peri-implantitis.

2 | MATERIALS AND METHODS

A case-control designed, cross-sectional study was conducted to investigate the association between CVD and peri-implantitis (e-pub ahead of print: JOP-21-0418.R1), details are presented in Supplementary Table S1 in the online *Journal of Periodontology*. A total of 128 subjects comprised of 82 "Cases" with CVD and 46 "Controls" without CVD were included in this study. "Cases" patients were enrolled only when the implants were placed prior to CVD diagnosis, and individuals with peri-implantitis were included only when the peri-implantitis onset was evident radiographically prior to CVD in order to test our hypothesis. Clinical assessments were recorded at six sites around the "most diseased" implant with the most severe radiographic bone loss (RBL), including peri-implant probing pocket depth (PPD), clinical attachment level (CAL) with the reference of implant crown margin, bleeding upon probing (BOP), suppuration, modified plaque index (modPI) and gingival index (modGI). All the periodontal parameters were assessed at the remaining natural teeth. The result indicated that the prevalence of peri-implantitis with a progressive RBL ≥2 mm was found significantly higher in the CVD group. In order to clearly discern the inflammatory burden caused by peri-implantitis, this more stringent cut-off threshold of peri-implantitis (BOP/suppuration and RBL ≥2 mm) was implemented in the current study and defined as moderate to severe peri-implantitis. Pro-inflammatory profiles were analyzed from an array of biomarkers collected from serum, peri-implant crevicular fluid (PICF), and gingival crevicular fluid (GCF) samples among these 128 subjects to evaluate the pro-inflammatory profile between CVD and non-CVD patients with or without peri-implantitis.

2.1 | Serum analysis

Whole blood samples were collected in the venous blood collection tubes[*] from patients with 8 hours-fasting. Blood samples were allowed undisturbed at room temperature for 30 minutes, followed by centrifuged at 2700 rpm for 15 minutes. Serum samples were immediately aliquoted into labeled polypropylene cryovials[†] and stored at −80°C freezer until the final analysis. High-sensitivity C-reactive protein (hsCRP) was measured using human CRP enzyme-linked immunosorbent assays (ELISA) assay[‡]. The concentration was determined by interpolation from a calibration curve of known concentrations with a dilution factor of 1000×. Fibrinogen was measured using human fibrinogen ELISA assay[§] via high-sensitivity antibodies[**] with a dilution factor of 2000×. Optical density was measured at 450 nm using an absorbance microplate

[*] BD Vacutainer Serum 10 mL tubes, Becton Dickinson and Company, Franklin Lakes, NJ.
[†] Eppendorf Microtubes, Eppendorf AG, Hamburg, Germany.
[‡] RayBio Human CRP ELISA assay, RayBiotech, Inc., Norcross, GA.
[§] SimpleStep ELISA kit, Human Fibrinogen ELISA assay, Abcam, Cambridge, MA.
[**] High-sensitivity RabMab antibodies, Abcam, Cambridge, MA.

reader[††]. The last part of serum samples were analyzed using commercial human custom multiplexed sandwich ELISA-based arrays[‡‡] to detect and quantify the cytokine levels including IL-1β, IL-6, TNF-α, MMP-8, and OPG according to the manufacturer's protocol. After the capture of antibodies and incubation, the target cytokine is arrayed, laser-scanned and completed the multiplex detection.

2.2 | PICF/GCF analysis

The PICF samples were collected from the implant with "greatest RBL" per patient, either from the mesio-buccal aspect of a healthy implant or the deepest implant pocket of a diseased implant. Prior to collection, supragingival plaque was removed using a sterile curet. After the implant crown was dried/isolated with sterile gauze and gentle air spray, PICF samples were collected using methylcellulose strips[§§] gently placed into the dried sulcus or pocket until mild resistance was felt for 1 minute. Methylcellulose strips[§§] contaminated with blood were discarded and the site was re-sampled after 90 seconds. GCF samples were taken from two identified sites from each patient. One was collected from the most severe periodontitis site (deepest PD) in the subjects with periodontitis and the other one was collected from a healthy site (mesio-buccal site of healthy tooth). Two healthy or gingivitis sites were randomly chosen in the subjects with healthy periodontal status. GCF samples were collected using methylcellulose strips[§§] gently placed and kept in the dried pockets until mild resistance was felt for 30 seconds after cleaning and drying. Finally, GCF and PICF samples were transferred into labeled polypropylene cryovials[†] and stored in a −80°C freezer for further analyses.

A 20 μL extraction solution containing 10 g/mL aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 0.1% human serum albumin in phosphate-buffered saline was pipetted directly onto the cellulose portion of each methylcellulose strips[§§] and secured at the top of a 12 × 75 mm polystyrene culture tube. After centrifugation at 2000 rpm at 4°C for 5 minutes, each strip was washed five times to yield a total elution volume of 100 μL. Quantitative assessments of biomarker expression in PICF and GCF samples were performed using commercial human custom multiplexed sandwich ELISA-based arrays[‡‡]. Targeted biomarkers included 12 different molecules: hsCRP, pro-inflammatory and angiogenic biomarkers including IL-1 β, IL-6, TNF-α, VEGF, T-cell modulator: IL-17, chemokine: IL-8, inflammation mediator and proteolytic enzymes: MMP-8, and biomarkers for bone metabolism: OPG and TIMP-2, and MPO. PGE_2 was separately analyzed by the human PGE_2 ELISA assay[***].

2.3 | Statistical analysis

All quantitative variables were reported in the measures of mean± standard deviation (median). One-way ANOVA or Krunskal-Wallis test was used to compare groups based on the result of Shapiro-Wilk normality test. Wilcoxon signed-rank test was performed to compared between GCF sample within the same subject. One-way ANCOVA or Quade's ranked analysis of covariance was used to compare groups adjusted for multiple relevant covariates. The diagnostic ability was further assessed using chi-square test with 2×2 contingency tables to predict the odds ratio (OR) of CVD occurrence based on the cut-off point (median) of cytokine level. Pearson or Spearman correlation coefficient was used to assess associations between variables, including BOP%. The receiver operating characteristic (ROC) curve analyses were performed to further assess the likelihood of CVD occurrence. All statistical analyses were performed using a statistics software[†††]. The differences were considered statistically significant at P-value of < 0.05.

3 | RESULTS

3.1 | Serum-derived biomarkers

The mean ± standard deviation (median) values of the pro-inflammatory cytokines and the significant differences between non-CVD and CVD group were presented in Table 1. The result showed that IL-1β, TNF-α and fibrinogen were significantly higher in the CVD group than in the non-CVD group (97 versus 58.4 pg/mL, 104 versus 56.5 pg/mL, 86.2 versus 62.3 md/dL, respectively). Although not statistically significant, IL-6 and OPG demonstrated a trend of higher concentrations in the CVD group. Conversely, the mean concentration of hsCRP was higher in the non-CVD group (6.4 vs 7.7 pg/mL). The results of chi-square analysis based on the dichotomized data showed that serum IL-1β, OPG, and TNF-α were potential predictors for the CVD occurrence (Table 2). The ROC analysis demonstrated a significantly fair accuracy of disease prediction with TNF-α (AUC 67%) and fibrinogen (AUC 65%) for CVD (Figure 1). After controlling for peri-implantitis

[††] EZ Read 400 Microplate Reader, Biochrom, Holliston, MA.
[‡‡] Human custom Quantibody Arrays, RayBiotech, Inc., Norcross, GA.
[§§] PerioPaper strips, Oraflow Inc., Smithtown, NY.
[***] SimpleStep ELISA kit, Human PGE_2 ELISA assay, Abcam, Cambridge, MA.
[†††] IBM SPSS Statistics for MAC, 24.0 version, IBM Corp., Armonk, NY.

TABLE 1 Comparison analyses of serum-derived biomarker profiles

Variables	IL-1β (pg/mL)	IL-6 (pg/mL)	MMP-8 (pg/mL)	OPG (pg/mL)	TNF-α (pg/mL)	HsCRP (mg/L)	Fibrinogen (mg/dL)
Non-CVD ($n = 46$)	58.4 ± 103.0 (17.9)	191.3 ± 362.4 (6.1)	33.2 ± 101.1 (13.2)	1044.5 ± 846.6 (758.2)	56.5 ± 152.1 (6.9)	7.7 ± 4.2 (9.2)	62.3 ± 35.7 (50.9)
CVD ($n = 82$)	97.0 ± 136.8 (33.6)	267.3 ± 570.6 (27.0)	29.1 ± 54.5 (13.2)	1287.8 ± 925.1 (968.4)	104.7 ± 161.7 (31.6)	6.4 ± 3.5 (6.5)	86.2 ± 61.5 (70.0)
P-value	**0.02**‖	0.09	0.66	0.09	**<0.01**‖	0.11	**0.03**‖
Adjusted* *P*-value	**0.05**	**0.05**	0.87	0.08	**0.005**	0.06	0.22
Adjusted[a] *P*-value	**0.02**	0.14	0.76	0.54	**0.02**	0.19	0.79
ASCVD[b] low risk ($n = 6$)	50.2 ± 88 (17.9)	104.7 ± 253.5 (4.0)	24.3 ± 20.7 (13.2)	976 ± 1123.1 (551.0)	32.3 ± 71.9 (6.9)	9.1 ± 3.8 (9.6)	67.7 ± 47.7 (56.1)
ASCVD[b] borderline risk ($n = 5$)	81.2 ± 82.4 (32.6)	150 ± 207.6 (63.7)	15.5 ± 6 (13.2)	1449.1 ± 688.3 (1390.1)	70.3 ± 91.7 (28.0)	5.1 ± 3.2 (5.5)	116.7 ± 56.7 (120.7)
ASCVD[b] borderline risk ($n = 18$)	81.2 ± 82.4 (32.6)	226 ± 582.7 (14.9)	47.9 ± 118.9 (13.2)	962.3 ± 697.9 (799.3)	91.7 ± 175.2 (6.9)	6.5 ± 3.8 (6.1)	56.0 ± 23.7 (51.3)
ASCVD[b] high risk ($n = 26$)	94.4 ± 144.8 (23.6)	292 ± 530.3 (35.1)	24.9 ± 50.1 (13.2)	1331.1 ± 997.1 (945.7)	100.8 ± 170 (27.2)	6.9 ± 3.8 (6.9)	85.8 ± 63.8 (70.3)
P-value	0.8	0.72	0.52	**0.04**‖	0.72	0.3	**0.02**‖
Healthy implant + peri-implant mucositis ($n = 74$)	102.1 ± 156.4 (19.3)	282.1 ± 553.9 (27.5)	31.2 ± 59.4 (13.2)	1238.1 ± 964.8 (1020.0)	99.0 ± 155.4 (22.5)	7.5 ± 3.9 (7.3)	75.3 ± 65.1 (52.9)
Mod-sev peri-implantitis[c] ($n = 54$)	61.6 ± 76.6 (17.9)	192.4 ± 553.9 (14.9)	29.7 ± 88.4 (13.2)	1161.4 ± 836.2 (833.8)	74.7 ± 164.4 (12.5)	6.1 ± 3.6 (6.3)	81.0 ± 41.8 (71.2)
P-value	0.44	0.34	0.30	0.87	0.53	0.08	0.08
Healthy periodontium ($n = 40$)	95.5 ± 156.6 (17.9)	251.7 ± 408.8 (27.6)	28.4 ± 63.8 (13.2)	1273.2 ± 1155.9 (886.9)	112.1 ± 207.3 (6.9)	6.5 ± 4.6 (5.8)	61.1 ± 29.9 (51.5)
Periodontitis ($n = 77$)	66.7 ± 77.7 (20.9)	222.9 ± 548.8 (19.0)	32.9 ± 84.0 (13.2)	1223.3 ± 806.8 (987.7)	62.5 ± 112.8 (16.6)	7.0 ± 3.4 (6.9)	80.3 ± 45.1 (67.7)
P-value	0.43	0.52	0.73	0.46	0.58	0.48	**0.04**‖

Data are presented with mean ± SD (median) values.

*Adjusted for peri-implantitis and periodontitis by Quade's rank analysis of covariance; bold font indicates the statistically significance ($P < 0.05$).

[a]Adjusted for age, hypertension, smoking, family history of heart attack, cLDL, peri-implantitis, and periodontitis by Quade's rank analysis of covariance; bold font indicates the statistically significance ($P < 0.05$).

[b]10-year ASCVD risk assessment: low-risk (<5%); borderline risk (5% to 7.4%); intermediate risk (7.5% to 19.9%); high risk (≥20%), the presented data was.

[c]Mod-sev peri-implantitis: defined by BOP/suppuration and progressive radiographic bone loss (RBL) ≥2 mm.

‖Bold font denotes the significant difference between groups from Kruskal-Wallis test ($P < 0.05$).

All serum biomarkers were measured in the unit of pg/mL, except hsCRP in mg/L and fibrinogen in mg/dL.

TABLE 2 Diagnostic ability of serum-derived biomarkers for predicting CVD

Biomarker	Group	Threshold	Marker level		Sensitivity	Specificity	OR	95% CI	*P*-value
			High	Low					
IL-1β	CVD	19.3 (pg/mL)	41	29	58.6%	63.2%	2.4	1.1-5.4	**0.03***
	Non-CVD		14	24					
IL-6	CVD	20.7 (pg/mL)	38	16	50.3%	57.9%	1.6	0.7-3.6	0.23
	Non-CVD		16	22					
MMP-8	CVD	13.2 (pg/mL)	15	56	21.1%	35.6%	1.2	0.4-3.2	0.74
	Non-CVD		7	31					
OPG	CVD	897 (pg/mL)	49	8	86%	45.2%	5.0	1.8-14.1	**<0.01***
	Non-CVD		17	14					
TNF-α	CVD	14.6 (pg/mL)	42	28	60.0%	65.8%	2.9	1.3-6.6	**0.01***
	Non-CVD		13	25					
hsCRP	CVD	6.8 (mg/L)	28	34	45.2%	43.3%	0.6	0.3-1.6	0.6
	Non-CVD		17	12					
Fibrinogen	CVD	64.8 (mg/dL)	38	31	55.1%	58.3%	1.7	0.8-3.9	0.19
	Non-CVD		15	21					

Abbreviation: OR, Odds Ratio.

CVD group (n = 82); non-CVD group (n = 46).

*Bold font denotes the significant difference between groups from chi-square test ($P < 0.05$).

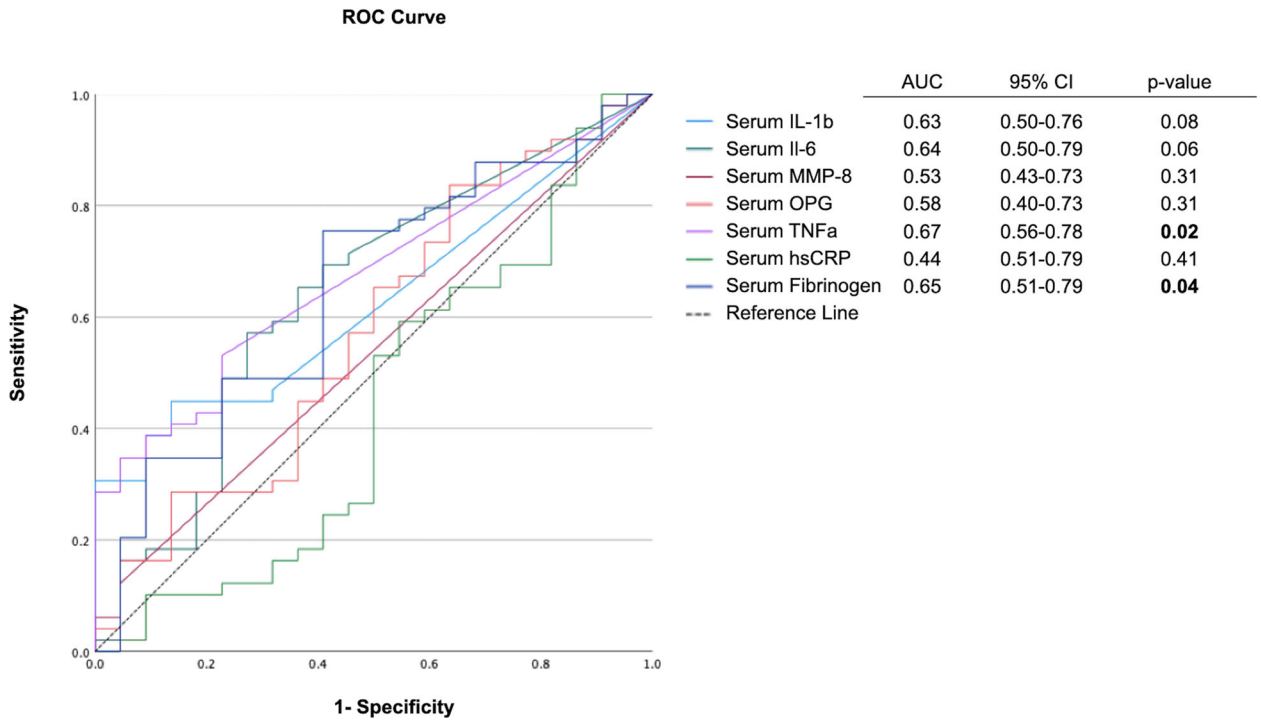

FIGURE 1 Receiver operating characteristic (ROC) curve for the diagnostic ability of serum biomarkers for predicting cardiovascular disease (CVD). AUC, Area under the curve, CI, confidence interval. Font in bold indicates a significant accuracy ($P < 0.05$)

and periodontitis by Quade's rank analysis of covariance, IL-1β, TNF-α, and IL-6 were significantly higher in the CVD group. Further adjustment was performed for the significant covariates associated with CVD outcome (Supplementary Table S1) including age, hypertension, smoking, family history of heart attack, low-density lipoprotein cholesterol (cLDL), peri-implantitis, and periodontitis. IL-1β, TNF-α maintained statistically significant higher in the CVD group.

Serum biomarker levels were also evaluated to assess the association between the serum biomarker level and the 10-year ASCVD risk (risk assessment based on

history of hypertension, diabetes, smoking, and cholesterol that predicts the likelihood of ASCVD in the next 10 years which divides into low-risk (<5%); borderline risk (5% to 7.4%); intermediate risk (7.5% to 19.9%); high risk (≥20%); definition in Supplementary Table S2 in online *Journal of Periodontology*). IL-6 and TNF-α demonstrated a concentration-dependent trend, when the inflammatory cytokine is higher, the 10-year ASCVD risk is higher.

Subgroup analyses were implemented to evaluate the impact of local (peri-implant and periodontal) inflammation on the systemic inflammatory mediators. Patients with diseased implant of RBL ≥ 2 mm demonstrated a trend towards higher serum fibrinogen level (81.0 versus 75.3 mg/dL, $P = 0.08$) compared to the healthy implants (including peri-implant mucositis [MU]). After controlling for CVD occurrence, the difference was not statistically significant ($P = 0.17$). Patients with diseased implant of RBL > 4 mm were found to have a non-significant trend of higher serum IL-1β, IL-6, MMP-8, OPG, TNF-α, and fibrinogen compared to the healthier implants ($P > 0.05$) (Supplementary Table S3 in online *Journal of Periodontology*). Finally, the serum fibrinogen level significantly increased among patients with periodontitis (80.3 versus 61.1 mg/dL, $P = 0.04$) compared to healthy periodontium. After controlling for CVD occurrence, the difference was not statistically significant ($P = 0.17$). Other comparison analyses combined are displayed in Supplementary Table S3.

3.2 | PICF analysis

The results of the 12 PICF biomarkers are shown in Table 3. TNF-α exhibited a significantly higher detection level in the CVD group ($P = 0.05$). There is a significant association between TNF-α and CVD (r = 0.17, $P = 0.05$) and the ROC curve analysis proved that PICF TNF- α has a marginally 59% AUC predictive accuracy for CVD (see Supplementary Figure S1 in online *Journal of Periodontology*). Other PICF biomarkers including MMP-8, MPO, and IL-17 seem to be higher in the CVD group, but the differences did not reach the significant level. After adjusting for peri-implantitis and periodontitis, TNF-α remained statistically significantly higher in the CVD group compared to the non-CVD group ($P = 0.03$). After adjusting for multiple CVD relevant covariates (age, hypertension, smoking, family history of heart attack, cLDL, peri-implantitis, and periodontitis), the difference of TNF-α between CVD and non-CVD group did not reach statistical significance ($P = 0.09$).

In the subgroup analysis, MMP-8 proved to be significantly higher in the sites with RBL ≥ 2 mm than the healthy implants (including peri-implant MU) (3468.7 versus 3117.3 pg/mL, $P = 0.05$) with an AUC = 60% prediction power. Although it was non-significant, the difference was amplified in the sites with RBL > 4 mm compared to healthy implants (3605.6 versus 3160.6 pg/mL, $P = 0.14$) (Supplementary Table S4 in online *Journal of Periodontology*). IL-1β, IL-6, IL-8, IL-17, MPO, OPG, TIMP-2, VEGF demonstrated the same trend. IL-1β, IL-8, MMP-8, and TIMP-2 were found higher in the local inflammation manifested as peri-implant disease (PID) (including peri-implant MU and peri-implantitis) ($P < 0.05$). In the subgroup analyses, among those patients with CVD, TNF-α and MMP-8 collected from PICF were consistently showing a non-significant tendency of higher concentration compared to non-CVD group and coinciding with the local peri-implant inflammatory status (Supplementary Table S4). The results of the Spearman correlation analysis between cytokine level and BOP% (categorized as <33%, 33-66%, >66%) showed that the MMP-8 (r = 0.22, $P = 0.01$), TIMP-2 (r = 0.17, $P = 0.05$), and PGE2 (r = 0.21, $P = 0.02$) were associated with the percentage of BOP sites at the tested implant.

3.3 | GCF analysis

Table 4 shows the mean values of GCF biomarkers collected from patients with periodontitis or with healthy periodontium. Periodontitis patients had higher hsCRP, IL-1β, IL-6, MMP-8, OPG, TIMP-2, VEGF than the healthy periodontium. Only IL-1β was statistically significant higher in periodontitis than the healthy periodontal sites (mean 160.2 versus 119.3 pg/mL, $P < 0.01$). VEGF exhibited a borderline significant difference (mean 97.5 versus 73.0 pg/mL, $P = 0.07$). IL-6 collected from patients with periodontitis was consistently higher compared to patients without periodontitis. Comparison of GCF levels between CVD and non-CVD groups at different sites is shown in Supplementary Table S5 in the online *Journal of Periodontology*. In the subjects with periodontitis, either at sites of periodontitis or healthy periodontium, TNF-α collected from GCF was higher in the CVD group. This trend can be found in patients with healthy periodontium but did not reach significance. After controlling for CVD-relevant multiple covariables, the statistical significance remained ($P = 0.02$). This pattern was not observed in other biomarkers. GCF TNF-α collected from patients with periodontitis was strongly associated with CVD cases with odds ratio of 4.4 and 4.7 (periodontitis and healthy teeth, respectively) ($P = 0.01$). The ROC curve analysis is illustrated in Supplementary Figure S2 in the online *Journal of Periodontology*. Using the GCF profile to assess the likelihood of peri-implantitis, only OPG in GCF collected from periodontitis-affected teeth was significantly higher in the RBL ≥ 2 mm

TABLE 3 Comparison analyses of PICF biomarker profiles

Variables	hsCRP	IL-17	IL-1β	IL-6	IL-8	MMP-8	MPO	OPG	TIMP-2	TNF-α	VEGF	PGE2
Non-CVD ($n = 46$)	227.9 ± 236.2 (181.5)	2.5 ± 1.6 (1.7)	129.8 ± 94.0 (118.6)	5.3 ± 14.8 (1.1)	283.8 ± 143.4 (306.1)	3144.9 ± 985.1 (3203.6)	1135.6 ± 608.8 (1070.1)	82.4 ± 127.7 (35.2)	1365.9 ± 698.2 (1352.6)	8.3 ± 3.2 (6.6)	101.0 ± 116.7 (66.0)	137.7 ± 171.1 (75.4)
CVD ($n = 82$)	169.9 ± 171.1 (130.7)	3.0 ± 5.4 (1.7)	101.8 ± 110.7 (55.0)	2.4 ± 5.1 (1.1)	275.9 ± 147.6 (293.6)	3333.2 ± 1123.4 (3476.8)	1284.1 ± 781.1 (1231.2)	78.1 ± 200.5 (15.5)	1278.8 ± 741.3 (1220.4)	11.3 ± 8.0 (6.6)	74.7 ± 101.0 (43.2)	112.9 ± 126.0 (82.6)
P-value	0.27	0.39	**0.045**[#]	0.25	0.77	0.17	0.27	0.11	0.52[**]	**0.05**[#]	0.11	0.72
Adjusted[a] *P*-value	0.21	0.95	0.21	0.1	0.91	0.2	0.67	0.48	0.85	**0.03**[#]	0.68	0.60
Adjusted[b] *P*-value	0.17	0.91	0.23	0.14	0.83	0.35	0.66	0.33	0.35	0.09	0.61	0.74
HI[c] + MU[d] ($n = 74$)	192.6 ± 172.9 (130.7)	2.6 ± 2.1 (1.7)	115.9 ± 97.3 (102.8)	2.7 ± 5.3 (1.1)	275.3 ± 138.9 (293.4)	3117.3 ± 1044.1 (3302.1)	1206.2 ± 705.2 (1111.5)	78 ± 194.8 (28.5)	1275 ± 682.5 (1243.0)	9.8 ± 5.7 (6.6)	85.4 ± 103.7 (55.0)	120.1 ± 138.3 (78.7)
Mod-sev peri-implantitis[e] ($n = 54$)	188.2 ± 229.9 (139.4)	3.1 ± 6.4 (1.7)	106.4 ± 116.5 (53.8)	4.5 ± 13.8 (1.1)	283.6 ± 155.5 (304.9)	3468.7 ± 1094.7 (3525.4)	1264.3 ± 756.6 (1239.2)	81.8 ± 151.6 (10.3)	1358.1 ± 782.5 (1362.1)	10.8 ± 8.1 (6.6)	82.5 ± 112.7 (41.5)	124.4 ± 108.0 (92.8)
P-value	*0.46*	*0.21*	*0.35*	*0.39*	*0.75*[c]	***0.05***[#]	*0.75*	*0.44*	*0.53*[**]	*0.85*	*0.23*	*0.39*
Healthy implant ($n = 12$)	121.3 ± 151.6 (74.1)	2.8 ± 1.5 (2.2)	59.1 ± 97.6 (8.1)	1.1 ± 0.1 (1.1)	171.0 ± 158.0 (114.4)	2210.7 ± 1328.6 (2176.6)	1101.9 ± 829.2 (731.7)	35.9 ± 47.4 (24.9)	688.8 ± 655.7 (435.3)	8.9 ± 3.3 (6.6)	51.1 ± 62.0 (40.1)	80.3 ± 61.4 (78.7)
PID[f] ($n = 116$)	198.0 ± 201.5 (143.8)	2.8 ± 4.6 (1.7)	117.3 ± 105.1 (85.8)	3.7 ± 10.3 (1.1)	289.9 ± 140.3 (304.0)	3374.7 ± 990.4 (3447.9)	1244.0 ± 716.0 (1186.7)	84.1 ± 185.1 (25.4)	1374.3 ± 703.0 (1337.9)	10.3 ± 7.1 (6.6)	87.6 ± 11.4 (55.6)	126.4 ± 130.5 (86.7)
P-value	0.2	**0.03**[#]	**0.01**[#]	0.44	**0.01**[#]	**<0.01**[#]	0.44	0.81	**<0.001**[**]	0.89	0.22	0.28

(Continues)

TABLE 3 (Continued)

Variables	hsCRP	IL-17	IL-1β	IL-6	IL-8	MMP-8	MPO	OPG	TIMP-2	TNF-α	VEGF	PGE2
Healthy implant ($n = 12$)	121.1 ± 151.6 (74.1)	2.8 ± 1.5 (2.2)	59 ± 97.6 (8.1)	1.1 ± 0.1 (1.1)	171 ± 158(114.4)	2210.7 ± 1328.6 (2176.6)	1101.9 ± 829.2 (731.7)	35.9 ± 47.4 (24.9)	688.8 ± 655.7 (435.3)	8.9 ± 3.3 (6.6)	51.1 ± 62(40.1)	80.3 ± 61.4 (78.7)
Peri-implant mucositis ($n = 37$)	226.1 ± 184.9 (217.0)	2.7 ± 2.6 (1.7)	129.8 ± 83.7 (146.3)	3.2 ± 6.4 (1.0)	308 ± 122.3 (309.9)	3452.3 ± 813.0 (3251.9)	1187.7 ± 761.7 (1023.5)	66.7 ± 117.1 (32.8)	1397.8 ± 526.3 (1419.7)	10.4 ± 6.4 (6.6)	91.9 ± 83.8 (60.0)	133.6 ± 121.4 (105.3)
Peri-implantitis ($n = 79$)	184.8 ± 208.6 (131.0)	2.8 ± 5.4 (1.7)	111.5 ± 113.8 (70.6)	3.9 ± 11.7 (1.1)	281.5 ± 147.9 (291.5)	3338.3 ± 1066.2 (3411.8)	1270.4 ± 697.1 (1216.8)	92.3 ± 209.7 (16.1)	1363.4 ± 774.8 (1316.6)	10.3 ± 7.4 (6.6)	85.6 ± 121.4 (44.2)	122.9 ± 135.4 (82.6)
	0.2	***0.04***[#]	***0.01***[#]	*0.38*	***0.02***[#]	***0.01***[#]	*0.57*	*0.96*	***<0.001***[**]	*0.94*	*0.1*	*0.53*

Data were presented with mean ± SD (median); all units are pg/mL, except MMP-8 is mg/L.

[a]Adjusted for peri-implantitis and periodontitis by Quade's rank analysis of covariance; bold font indicates the statistically significance ($P < 0.05$).

[b]Adjusted for age, hypertension, smoking, family history of heart attack, cLDL, peri-implantitis, and periodontitis by Quade's rank analysis of.
covariance.

[c]HI, healthy implant.

[d]MU, peri-implantitis.

[e]Mod-sev peri-implantitis, defined by BOP/suppuration and progressive radiographic bone loss (RBL) ≥2 mm.

[f]PID, peri-implant disease (including peri-implant mucositis and peri-implantitis).

[#]Bold font denotes the statistically significant difference ($P < 0.05$) from Kruskal-Wallis test.

**Indicates the results from one-way ANOVA test, and in bold indicates the statistically significant difference ($P < 0.05$).

TABLE 4 GCF biomarker profiles at healthy and periodontitis sites

Group	Sampling site[a]	hsCRP	IL-17	IL-1β	IL-6	IL-8	MMP-8	MPO	OPG	TIMP-2	TNF-α	VEGF
Periodontitis group ($n = 77$)	Perio tooth	293.5 ± 219.6	1.3 ± 0.0	160.2 ± 108.9	4.2 ± 17.7	134.8 ± 39.3	3181.6 ± 711.7	1092.9 ± 331.5	41.8 ± 72.9	1322.2 ± 532.5	13.7 ± 9.8	97.5 ± 112.3
	Healthy tooth	276 ± 165.5	1.3 ± 0.1	113.9 ± 79.6	3.4 ± 8.6	179.5 ± 376.6	3109.1 ± 664	1115 ± 324.5	30.2 ± 44.2	1311.4 ± 484.7	16.1 ± 13.4	73 ± 58.8
P-value		0.6	0.64	**0.01**[†]	0.76	0.31	0.5	0.62	0.23	0.98	0.06	0.07
Healthy group ($n = 40$) healthy sites		302.8 ± 214.6	1.4 ± 0.2	146.1 ± 112.3	3.0 ± 5.8	141 ± 41.5	3244.9 ± 616.7	1128 ± 372	47.8 ± 86.7	1423.4 ± 585.4	15.4 ± 18.4	102.4 ± 105.7

Data were presented with mean ± SD; all units are pg/mL, except MMP-8 is mg/L.

[a]sampling tooth in periodontitis group included "perio tooth": tooth with the most severe periodontitis, GCF was collected from the site with the most severe disease (deepest PD), and "healthy tooth": tooth with healthy periodontium randomly chosen from the remaining teeth, the reported data was the average concentration of two sites.

[†]Bold font denotes the statistically significant difference ($P < 0.05$) from Wilcoxon signed-rank test (median not shown).

with a 48% sensitivity and 72% specificity ($P = 0.04$) (see Supplementary Figure S3 in online *Journal of Periodontology*).

4 | DISCUSSION

The results of the present study demonstrated that IL-1β, TNF-α and fibrinogen collected from serum were significantly higher among patients with CVD. After controlling for multiple significant CVD-relevant covariables, IL-1β, TNF-α remained statistically significantly higher in the CVD group. Serum fibrinogen displayed a trend of higher concentration in those with moderate to severe peri-implantitis (RBL ≥ 2 mm) compared to healthy implants ($P = 0.08$); which remained non-significant after controlling for CVD occurrence. TNF-α collected from PICF was significantly higher in the CVD group after controlling for peri-implantitis and periodontitis; the significant difference was non-significant after controlling for multiple CVD-relevant covariables ($P = 0.09$). Higher concentration of TNF-α was found at sites with inflammation. Specifically, GCF TNF-α collected from patients with periodontitis was strongly associated with CVD cases. In addition, PICF MMP-8 was significantly higher in the RBL ≥ 2 mm sites, and strongly correlated with the peri-implant BOP prevalence. Although, PICF MMP-8 did not show significant predictive power for CVD occurrence; PICF MMP-8 were consistently showing a tendency of higher concentration among CVD group compared to non-CVD group and coinciding with the local peri-implant inflammatory status.

Cardiovascular disease (CVD) is a chronic inflammatory state of the cardiovascular system, and the majority of etiology is attributed to atherosclerosis, which is an inflammatory process involving the host's immune mechanism interacting with other risk factors to initiate, disseminate, and activate lipoprotein-driven lesions throughout the cardiovascular system.[8] It drives clinical disease sequelae through luminal narrowing or by precipitating thrombi that obstruct blood flow to the heart (CHD), brain (ischemic stroke), or lower extremities (peripheral vascular disease).[15] Yet, it cannot be fully explained by conventional risk factors.[16] Emerging evidence has demonstrated that low-grade chronic inflammation, including periodontitis, is not only associated with the increased prevalence of cardiovascular risk factors but is also an independent risk factor for the development of CVD.[17] The plausible link between periodontal infection and atherogenesis has been theorized to be associated with the dual role of systemic inflammation held in common by both diseases. Augmented local and systemic pro-inflammatory mediators via oral infection contributed to vascular inflammation

and atherosclerosis, and subsequently increased CVD) risk and severity.[18] Our previous report (e-pub ahead of print: JOP-21-0418.R1) identified the risk of CVD (especially inflammation-related atherosclerotic CVD when higher levels of inflammation were found around dental implants (moderate to severe peri-implantitis with RBL ≥ 2 mm) which was consistent with this potential inflammatory link between peri-implantitis and CVD identified in the current study.

A variety of heart diseases, including CHD, atherosclerotic heart disease and chronic heart failure (CHF), are associated with increased serum levels of proinflammatory cytokines, such as interferon-γ (INF-γ), IL-1β, IL-6, and TNF-α.[19] Therefore, it was not surprising that the concentration of serum inflammatory biomarkers IL-1β, TNF-α, and fibrinogen, were significantly higher in the CVD patients independent of the local peri-implant inflammatory status. It has been shown that interleukins mediating the signaling of leukocytes contribute to the atherosclerosis process, especially when IL-1β is associated with proatherogenic events such as upregulation of endothelial adhesion and activation of macrophages and vascular cells.[20] TNF-α is implicated as a pro-inflammatory cytokine that contributing to vascular dysfunction and upregulating the oxidative stress resulted in an adiposity-induced inflammation.[21] IL-6, together with IL-1 and TNF-α, has been shown downstream from vascular inflammatory cascade of accelerating atherosclerosis[22] that modulates immune reaction and causes stress hypoxia and tissue destruction that may lead to cardiac cachexia.[23]

Despite the lower concentration compared to plasma, the results of high sensitivity-ELISA differentiated a significant difference of serum fibrinogen level between CVD and non-CVD group. Elevated levels of serum fibrinogen has been associated with increased blood viscosity and thrombus formation[24] and linked to the development of CVD.[25] It has been reported that serum fibrinogen found in periodontitis patients was increased when compared to periodontally healthy patients with or without CVD and reduced after periodontal treatment.[26,27] In this study, serum fibrinogen was found to be higher in patients with RBL ≥ 2 mm ($P = 0.08$). Although it was not statistically significant, the difference was evident in implants with RBL > 4 mm compared to healthier implants (Supplementary Table S3). Along with other pro-inflammatory mediators (such as IL-6, MMP-8, OPG, and TNF-α) found in the RBL > 4 mm subgroup, this may imply that severe peri-implant tissue destruction might augment systemic inflammation. MMP-8 appears to promote periodontal and peri-implant lesion progression and is associated with collagen fiber destruction which may also associate with atherosclerosis and atheroma plaque instability.[28] Literature also linked the serum MMP-8 with periodontal local inflammation to the augmented systemic load,[12] which may explain why MMP-8 collected from PICF were consistently showing a tendency of higher concentration in CVD group that might coincide with the local peri-implant inflammation. In addition, serum OPG, a regulatory protein for bone metabolism and vascular calcification, has been associated with CVD pathophysiology and cardiovascular mortality and morbidity[29]; and it has been reported reflecting the increased risk of alveolar bone loss around peri-implantitis sites.[30] Lastly, serum hsCRP was not found to be associated with CVD occurrence in our results. The predictive value of hsCRP has been shown to be limited because of a lack of causative relationship as well as significant heterogeneity from genetic polymorphisms and disease-phenotype variability.[31] The average hsCRP level in the non-CVD group was higher. This is possibly associated with the higher prevalence of hypercholesterolemia (abnormal low-density lipoprotein cholesterol, LDL-C) found in the non-CVD group (Supplementary Table S1) that CRP directly bonds to atherogenic oxidized LDL-C.[32]

The current evidence of biomarkers expression in subjects with peri-implant diseases are mainly focused on PICF cytokines rather than systemic markers in serum.[33] In general, we found that IL-1β, IL-8, MMP-8, and TIMP-2 collected from PICF were strong predictors for the peri-implant disease, which is in line with the existing evidence.[34,35] However, local biomarkers in PICF did not demonstrate a capacity to differentiate between CVD and non-CVD patients. Only PICF TNF-α was significantly higher in the CVD group and coincided with the local peri-implant inflammation. Interestingly, TNF-α collected from GCF in patients with periodontitis was also strongly associated with CVD cases. TNF-α has been reported as the most common cytokine isolated from patients with severe peri-implantitis and reduced significantly after mechanical anti-infective therapy.[36] Primarily, TNF-α underlines the real-time manifestation of inflammation. When the fibroblasts in the chronic peri-implant granulation tissues are unable to switch off the pro-inflammatory pathway, both migration and retention of leukocytes may occur continuously within the sites in a self-feeding loop,[37] which may explain the local pro-inflammatory TNF-α level coincided with the systemic inflammatory burden.

Interestingly, we found these biomarkers were higher in peri-implant MU than peri-implantitis (> detectable bone loss). We may be argued that IL-1β is a robust marker of acute inflammatory changes in gingiva,[38] and it may synergistic with TNF-α to initiate and propagate inflammation.[39] IL-1β is consistently recognized as a dominant biomarker at the peri-implant inflammatory sites.[33] It regulates the degradation of extracellular matrix (ECM)

components of plasminogen system and the collagenase activity in the inflammatory response.[40] Although bursts of IL-1β can precipitate acute attack of systemic/local inflammation, it also contributes to several chronic inflammatory diseases.[41] This is in consistent with our findings that IL-1β was a dominant cytokine in PICF at >4 mm RBL sites and in GCF at the periodontitis sites. In addition, IL-8 and MMP-8 appear to be early signals of peri-implant inflammation.[42,43] IL-8 as a potent neutrophil chemotactic and activating factor was reported escalated in short period of time at the early stage of peri-implantitis. The strong correlation between MMP-8 and peri-implant tissue destruction has been reported widely in the literature,[44] which is line with the current finding that PICF MMP-8 was significantly higher at sites with moderate to severe peri-implantitis, especially it was statistically correlated with the signs of active inflammation within the peri-implant pocket. A disruption of MMP-TIMP balance may lead to a pathological process of losing ECM, such as atherosclerosis and periodontitis. Particularly, TIMP-2 may behave as an effective predictor for peri-implantitis (OR = 4.4), and further increase the predictive power when it was combined with microbial profile.[45]

Recently, Chaushu et al., has reported an increase in serum inflammatory parameters including total protein and albumin concentrations in an experimental peri-implantitis disease. It provides evidence of a stimulation of immune system and upregulation of the inflammatory pathway, which substantiates the systemic effect of the local inflammation occurred at sites with peri-implantitis.[46] Overall, it is reasonable to extrapolate the biological mechanisms of periodontitis to chronic peri-implantitis, especially when the disease severity and tissue destruction increased. In the current study, local pro-inflammatory TNF-α level at PICF was found coinciding with the systemic inflammatory burden in the CVD patients; yet the difference of PICF TNF-α between peri-implantitis (≥2 mm RBL) and healthy implants was not-significant. Only an escalating trend can be observed with increasing local and systemic inflammatory burden in the peri-implant disease and CVD. Systemic thrombotic marker, fibrinogen, was found a non-significant trend of association to the moderate to severe peri-implantitis (≥2 mm RBL).

In summary, the evidence in the current study remained weak for the potential link explaining the association between CVD and peri-implantitis. It was acknowledged that the sample size, especially in the severe peri-implantitis subgroup, might be underpowered, which could partially be the reason of weak association. Other possible reasons may be the limited number of diseased implants (25.8% [mean] were single implant) that the effect on the systemic inflammation may be weak or diluted by the background comorbidities. Another limitation of the current study is the cross-sectional observation without longitudinal monitoring of biomarker changes during the disease process. Finally, the augmented inflammatory effect from periodontitis evidenced by increased serum fibrinogen in the current result may be correlated to the CVD risk, but the potential synergistic effect between peri-implantitis and periodontitis remains unknown. It was noteworthy that pro-inflammatory mediators in PICF, including IL-1β, IL-6, IL-8, MMP-8, TIMP-2, has increased markedly from healthy to peri-implant MU, similar to the level of more severe peri-implantitis. It underlines the importance of regular implant maintenance to decrease the inflammation within peri-implant soft tissue at the early stage of disease and avoid the risk of increasing systemic inflammatory load. Future longitudinal studies on the important systemic and local pro-inflammatory mediators, especially fibrinogen, TNF-α, MMP-8, IL-1β and IL-6 with larger patient populations and multivariable controlling were warranted to understand the potential inflammatory link between CVD and peri-implantitis with higher disease severity.

5 | CONCLUSION

The augmented expression of local and systemic pro-inflammatory cytokines found in the current study supports the possible association between the chronic peri-implantitis with increasing severity and CVD. However, the concluding evidence is weak. Future longitudinal studies with larger patient populations and controlling for confounding factors and comorbidities will expand our knowledge to elucidate the role of peri-implant infection in the pathogenesis of atherosclerotic disease.

ACKNOWLEDGMENTS

This research project has been supported by the Delta Dental Research Fund from The Delta Dental Foundation at Michigan and the Graduate Student Research Fund at the University of Michigan. The authors would like to thank Dr. Shogo Maekawa, Ms. Veronica Slayton, Ms. Donna Brennan, Ms. Cynthia Miller, and Ms. Alicia Baker, Department of Periodontics and Oral Medicine, the University of Michigan, for their contributions to this investigation.

CONFLICTS OF INTEREST

The author Dr. Jeffery Johnston, Vice President, Chief Science Officer, and Director of the Research and Data Institute at Delta Dental of Michigan declared the conflicting interests that this work has been funded by Delta Dental. The remaining authors have no specific conflict of interest related to the present research.

AUTHOR CONTRIBUTIONS

I-Ching Wang, Jeffery Johnston, William V. Giannobile, Hom-Lay Wang: Contributed to the conception and design of the study, acquisition of the data, drafting of the article, critical revision of the article, and final approval of the version to be published. Jim V. Sugai, Jad Majzoub: Contributed to the acquisition of data and final approval of the version to be published. All authors gave final approval and agreed to be accountable for all aspects of the work.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Wang IC, Sugai JV, Majzoub J, Johnston J, Giannobile WV, Wang HL. Pro-inflammatory profiles in cardiovascular disease patients with peri-implantitis. *J Periodontol*. 2022;93:824–836.
https://doi.org/10.1002/JPER.21-0419