J Periodontal Implant Sci. 2024 Apr;54(2):85-95
https://doi.org/10.5051/jpis.2301120056
pISSN 2093-2278·eISSN 2093-2286

JPIS
Journal of Periodontal & Implant Science

Research Article
Periodontal Science

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Decreased GCF DEL-1 and increased GCF neutrophils with increasing probing pocket depth

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OPEN ACCESS

Received: Dec 15, 2022
Revised: May 30, 2023
Accepted: Jun 4, 2023
Published online: Jul 21, 2023

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Funding
This research was funded by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, grant number 2018R1D1A1A02043024.

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

ABSTRACT

Purpose: Developmental endothelial locus-1 (DEL-1) plays a role in regulating neutrophil migration within the periodontium. The objective of this study was to evaluate the levels of DEL-1 in saliva and gingival crevicular fluid (GCF), as well as the number of neutrophils in patients with periodontitis.
Methods: Forty systemically healthy, non-smoking periodontitis patients participated in this study. Clinical periodontal parameters, including the plaque index, probing pocket depth (PPD), clinical attachment level, bleeding on probing, modified sulcular bleeding index, and marginal bone level, were measured. Levels of DEL-1, interleukin (IL)-1β, IL-6, and IL-8 in unstimulated saliva samples, as well as DEL-1 in the GCF of 3 teeth from each participant, were assessed. Neutrophil counts in oral rinse and GCF samples were recorded. Spearman correlation coefficients were used to examine the correlation between protein levels, clinical parameters, and neutrophil quantities. Participants were divided into 2 age groups (those under 50 years and those 50 years or older) in order to investigate potential age-related differences.
Results: DEL-1 levels in the GCF showed a negative relationship with PPD (sum). Neutrophils in oral rinse samples were positively correlated with PPD, IL-8, and IL-1β levels. Neutrophils in GCF exhibited a positive correlation with PPD (sum). Salivary DEL-1 levels showed correlations with IL-8 and IL-1β, but not with the clinical parameters of periodontitis.
Conclusions: The negative relationship observed between PPD and GCF DEL-1 levels is consistent with the proposed protective role of DEL-1.

Keywords: EDIL3 protein; Gingival crevicular fluid; Neutrophil; Periodontitis; Saliva

INTRODUCTION

Developmental endothelial locus-1 (DEL-1) is a multi-domain protein initially identified in embryonic endothelial cells and other cell groups involved in regulating vascular growth and remodeling [1]. The DEL-1 protein consists of 3 N-terminal epidermal growth factor (EGF)-like repeats and 2 discoidin I-like domains at the C-terminus, collectively referred to as EGF-like repeats and discoidin I-like domains 3 (EDIL3) [1]. This secreted protein has been demonstrated to be a ligand for the αVβ3 integrin receptor [1] and for the αVβ6 integrin [2].

Author Contributions

Conceptualization: Jun-Beom Park, Youngkyung Ko; Formal analysis: Eun-Mi Lee, Youngkyung Ko; Investigation: Eun-Mi Lee, Youngkyung Ko; Methodology: Seong-Ho Jin, Eun-Mi Lee, Youngkyung Ko; Project administration: Youngkyung Ko; Writing - original draft: Seong-Ho Jin, Youngkyung Ko; Writing - review & editing: Seong-Ho Jin, Jun-Beom Park, Youngkyung Ko.

DEL-1 also binds to lymphocyte function-associated antigen 1 (LFA-1; αLβ2 integrin) and Mac-1 (αMβ2 integrin), and it interacts with phosphatidylserine exposed on apoptotic cells [3-5]. DEL-1 is constitutively produced in various tissues, including the lung, brain, bone, and periodontium, and it is particularly highly expressed in endothelial cells, macrophages, and neurons [2,6]. DEL-1 is secreted by mesenchymal stromal cells, macrophages, neurons and endothelial cells, and it can regulate initiation and resolution of inflammation [7]. The ability of DEL-1 to bind to integrins and phospholipids allows it to control crucial immune functions, such as emergency myelopoiesis and the initiation and resolution of inflammation [8].

Recent findings have demonstrated the potential of DEL-1 for use in therapeutic applications. Specifically, engineered *Salmonella* capable of secreting DEL-1 may have potential in bacterial-mediated immunotherapy for cancer by reducing tumor-infiltrating neutrophils [9]. In the treatment of inflammatory arthritis, a preclinical study has shown that DEL-1 could inhibit local recruitment of neutrophils and macrophages in the joints, as well as act systemically on lymph nodes to restrain T follicular helper cell (Tfh) and germinal center B cell responses [10]. Local effects are exerted by endothelial cell-derived DEL-1, whereas stromal cell-derived DEL-1 plays an important role in systemic effects. The location of DEL-1 expression appears to be important in determining its effects. DEL-1 has also been shown to have immunomodulatory mechanisms on hypertension-associated cardiovascular damage. In a recent study using angiotensin II and deoxycorticosterone acetate-salt-induced mouse models of cardiovascular organ damage and hypertension, DEL-1 was found to prevent adverse remodeling of the aorta and heart, as well as inhibiting hypertension progression [11]. The interaction between DEL-1 and αvβ3 integrin was found to suppress the activation of pro-matrix metalloproteinase-2 and stabilize the regulatory T cell/IL-10 response. In an *in vivo* study on mice fed a high-fat diet, the administration of DEL-1 attenuated insulin resistance through sirtuin 1/sarco/endoplasmic reticulum Ca^{2+}-ATPase 2 (SIRT1/SERCA2)-mediated signaling [12].

Periodontitis is a chronic infectious inflammatory disease that affects tooth-supporting tissues and leads to gradual destruction of the alveolar bone [13]. It is not surprising that DEL-1 plays a significant role in the progression of periodontal disease. Mice deficient in DEL-1 developed spontaneous periodontitis with excessive neutrophil infiltration and IL-17 expression, and the expression of DEL-1 was inversely correlated with the susceptibility and severity of periodontal tissue breakdown in both young and old mice [6]. IL-17 has been shown to downregulate DEL-1 expression in human endothelial cells by inducing GSK-3β-dependent phosphorylation of CCAAT/enhancer binding protein β, which suppresses DEL-1 expression [14]. DEL-1 was found to be expressed in osteoclasts in the alveolar bone of mice and inhibited RANKL-induced differentiation of osteoclasts and their resorptive functions [15]. The administration of DEL-1 protein inhibited alveolar bone loss from periodontitis in mice and nonhuman primates [15]. The expression of DEL-1 was reported to be age-dependent in mice, with murine gingival tissue expression of Del-1 decreasing in old age, and this was also observed in human tissue samples [6,16].

Pathogenic bacteria residing in periodontal pockets constantly interact with host cells, leading to the activation of both innate and adaptive immunity and the subsequent release of pro-inflammatory cytokines, such as interleukins (ILs) [13]. The unrestricted production of inflammatory cytokines causes substantial damage to tissues, resulting in detrimental effects on the local environment [17]. IL-8 (chemokine [C-X-C motif] ligand 8; CXCL8) is the primary cytokine involved in the chemotaxis of neutrophils [18]. IL-8 mRNA-positive cells

https://doi.org/10.5051/jpis.2301120056

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have been topographically associated with neutrophil migration in the gingiva [19]. IL-6 is also an important cytokine in neutrophil trafficking. While IL-6 may not influence the rate of neutrophil infiltration, it may control neutrophil accumulation at local sites [20].

DEL-1 functions as an inhibitor of neutrophil recruitment by blocking LFA-1 [3] and plays a protective/homeostatic role in periodontitis. Therefore, we examined DEL-1 expression in gingival crevicular fluid (GCF) and saliva, as well as in corresponding neutrophils. We also investigated the correlations among DEL-1 levels, neutrophils, periodontal parameters, and age.

MATERIALS AND METHODS

Participants

Saliva and GCF samples were collected from 40 systemically healthy individuals diagnosed with chronic periodontitis at Seoul St. Mary's Hospital. The study protocol was approved by the Institutional Review Board of Seoul St. Mary's Hospital, the Catholic University of Korea College of Medicine (KC16TIMI0755), and was conducted in accordance with the Helsinki Declaration of 1975 (revised in 2013). The participants were enrolled between October 2017 and December 2018. All participants read and signed an informed consent form.

The inclusion criteria were the presence of interdental clinical attachment loss (CAL) with sites exhibiting more than 4mm of probing pocket depth and a whole mouth bleeding on probing (BOP) score greater than 10%, in order to exclude successfully treated stable periodontitis patients [21]. Additionally, participants were required be systemically healthy without untreated oral diseases other than periodontitis (i.e., no history of systemic diseases such as diabetes mellitus or kidney disease; no use of anti-platelet agents, calcium channel blockers, or other medications that could influence the prognosis of periodontitis; and no untreated oral diseases other than chronic periodontitis). Patients who smoked or had smoked in the past were excluded from the study. None of the participants were pregnant/breastfeeding or treated with antibiotics, antimicrobials, and/or anti-inflammatory drugs during the 3 months before the examinations and sampling. A further inclusion criterion was the presence of at least 20 teeth, excluding third molars. Saliva, GCF, and oral rinse samples were obtained from all participants, but 1 oral rinse sample was lost in the experimental process.

Periodontal examination

The following clinical parameters were assessed: the plaque index (PI) [21], gingival recession (GR), periodontal probing depth (PPD), and modified sulcular bleeding index (mSBI) [22]. The PI was measured on the buccal or lingual side, while the other parameters were measured at 6 sites. The BOP percentage (BOP%) and the sum of mSBI were calculated using mSBI measurements. The marginal bone loss percentage was determined from panoramic X-rays by measuring the ratio of the distances between the cementoenamel junction to the crestal bone and the cementoenamel junction to the root apex, as observed on the mesial or distal interproximal site of the tooth. The site with greater loss was used for analysis [23].

Saliva collection

One week following the clinical periodontal examination, 3 mL of unstimulated saliva was collected from each participant. The participants were instructed not to eat, drink, rinse their mouths, or perform any oral hygiene measures for 1 hour prior to sampling. Approximately 3 mL of non-stimulated whole saliva was obtained from each participant through expectoration

https://doi.org/10.5051/jpis.2301120056

and then centrifuged at 13,000 rpm (relative centrifugal force [rcf] 15,928 ×*g*) for 10 minutes at 4°C. The samples were stored at −80°C until further experimentation [24].

Oral rinse collection

Participants were instructed to swish 5 mL of 0.85% saline solution in their mouths for 30 seconds. The oral rinse was then centrifuged at 1,500 ×*g* for 10 minutes. The resulting precipitated pellets were resuspended in 1 mL of 4% PFA solution and stored at 4°C until use.

Gingival crevicular fluid collection

Periopaper (Oraflow Inc., Smithtown, NY, USA) was inserted in sulcus and/or pocket on four sites (mesiobuccal, mesioplatal, distobuccal, and distopalatal) of a maxillary central incisor, first premolar or first molar and left in position for 30 seconds. Supragingival plaque was removed carefully using hand instruments, and the area was isolated with cotton pellets to prevent contamination from saliva. The Periopapers were then placed in 1.5 mL microcentrifuge tubes containing 350 μL of 1% bovine serum albumin (BSA)/Dulbecco's phosphate-buffered saline (PBS) solution. The samples were centrifuged at 13,000 rpm (rcf 15,928 ×*g*) for 10 minutes at 4°C. The supernatant was stored at −80°C until analysis. The pellet was resuspended in 50 μL of 4% paraformaldehyde solution and stored at 4°C for analysis.

Neutrophil detection in oral rinse and gingival crevicular fluid samples

Immunofluorescence was performed using anti-neutrophil elastase rabbit monoclonal antibody (Abcam, Cambridge, UK) and goat anti-rabbit immunoglobulin G (IgG; H+L) secondary antibody, fluorescein isothiocyanate (FITC; Thermo Fisher, Waltham, MA, USA). The 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI; Sigma-Aldrich, St. Louis, MO, USA) was employed for nuclear staining, and fluorescent images were captured with an Axio imager M1 (Carl Zeiss Co. Ltd., Oberkochen, Germany) at ×200 magnification. Neutrophil elastase-positive signals were analyzed using ImageJ software (version 1.51, https://imagej.nih.gov/ij/). The quantity of neutrophils was determined as the percentage of pixels displaying green signals, while the quantity of cell nuclei was measured as the percentage of pixels exhibiting blue signals.

Neutrophils in oral rinse samples were measured for 39 of the 40 subjects (1 sample was lost during processing), and in GCF of 40 teeth for all 40 subjects. Immunofluorescence was utilized to measure relative neutrophil quantities in GCF or oral rinse samples. Pellet suspensions of 5 μL of 4% paraformaldehyde were placed on silane-coated slides and air-dried for 24 hours. The samples were prepared in duplicate, with oral rinse pellet suspensions diluted to one-fourth. Samples were incubated in blocking solution, consisting of 1% BSA in PBSTx (0.1% TritonX-100 in PBS), for 1 hour at room temperature. After blocking was completed, the blocking solution was removed, and samples were incubated with anti-neutrophil elastase rabbit monoclonal antibody (Abcam), diluted 1:200 in blocking solution, for 1 hour at room temperature. The samples were then washed 3 times with PBSTx and incubated with anti-rabbit IgG secondary antibody and FITC (diluted 1:50) in PBSTx, for 1 hour at room temperature. They were subsequently washed 3 times with PBSTx, and DAPI (0.5 μg/mL in PBS) was used as a nuclear counterstain for 19 minutes at room temperature, followed by a PBS rinse. All specimens were mounted with an antifade mounting medium and stored at 4°C until microscopy.

https://doi.org/10.5051/jpis.2301120056

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Detection of DEL-1, IL-8, IL-6, IL-1β in saliva and GCF samples

The levels of DEL-1, IL-8, IL-6, and IL-1β in saliva and GCF samples were determined using DuoSet enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) following the manufacturer's protocol. ELISA was performed with the DuoSet ELISA kit (R&D Systems), which included human EDIL3 (#DY6046), human IL-8/CXCL8 (#DY208), human IL-6 (#DY206), and human IL-1β/IL-1F2 (#DY201). The 3,3′,5,5′-tetramethylbenzidine dihydrochloride (TMB, #T3405; Sigma-Aldrich) was utilized as the color development substrate. DEL-1, IL-8, IL-6, and IL-1β levels were measured in the saliva of 40 participants. DEL-1 was measured in all GCF samples from all 40 subjects, totaling 120 samples. IL-8 was measured in 87 samples obtained from 29 subjects.

Statistical analysis

Data analyses were conducted using SAS version 9.4 (SAS Institute, Inc., Cary, NC, USA). Mean values and standard deviations were calculated. Clinical and laboratory data comparisons were analyzed using the independent *t*-test. Spearman correlation coefficients were used to examine the correlations between the levels of DEL-1 in saliva or GCF and the number of neutrophils in saliva and GCF with other clinical or laboratory variables. A *P* value <0.05 was considered statistically significant.

RESULTS

Characteristics of participants

The demographic and clinical characteristics of patients are presented in **Table 1**. The study population included 40 periodontitis patients with a mean age of 43.25±11.11 years, ranging from 21 to 68 years old, and a median age of 44. All participants had periodontitis, with stages ranging from I to IV according to the new classification system [25]. Four participants were classified as stage I, 16 as stage II, 19 as stage III, and 1 as stage IV.

Oral rinse neutrophils and salivary DEL-1, IL-6, IL-8, and IL-1β

We measured neutrophil levels in 39 participants, and their results for oral rinse and salivary samples, along with clinical parameters, are presented in **Table 2**. The participants were divided into 2 age groups: under 50 and 50 or older. No significant differences were observed

Table 1. Demographic and clinical characteristics of participants

Characteristics	Values
Age (yr)	43.25±11.11
Female sex	28 (70.0)
PI	2.06±0.94
PPD (mm)	2.99±0.38
GR (mm)	0.40±0.26
CAL (mm)	3.39±0.53
BOP (%)	50.59±21.03
MBL (%)	18.08±6.25
Staging	
I	4 (10.0)
II	16 (40.0)
III	19 (47.5)
IV	1 (2.5)

The data are presented as mean ± standard deviation or number (%). PI, PPD, GR, CAL, and MBP (%) per site and full-mouth BOP scores are presented.
PI: plaque index, PPD: probing pocket depth, GR: gingival recession, CAL: clinical attachment level, BOP: bleeding on probing, MBL: marginal bone level.

https://doi.org/10.5051/jpis.2301120056

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Table 2. Demographic and clinical characteristics, quantity of neutrophils, and total cell nuclei in oral rinse samples, and salivary levels of mediators grouped by age

Characteristics	Total (n=39)	Age <50 (n=26)	Age≥50 (n=13)	*P* value
Age	43.1±11.22	36.96±7.77	55.38±5.25	NA
Sex, female	27	17	10	
PI	2.06±0.95	2.08±1.06	2.02±0.69	0.832
PPD (mm)	2.97±0.37	2.91±0.41	3.09±0.23	0.175
GR (mm)	0.39±0.26	0.3±0.21	0.57±0.27	0.002[a]
CAL (mm)	3.36±0.52	3.22±0.51	3.65±0.43	0.012[a]
BOP (%)	50.07±21.04	51.6±23.51	47.02±15.35	0.529
MBL (%)	17.88±6.21	16.74±6.49	20.16±5.08	0.106
Neutrophils	3.75±9.25	4.49±10.8	2.26±4.97	0.484
All cell nuclei	3.49±4.22	3.21±3.14	4.05±5.95	0.566
Neutrophils to all cell nuclei	0.9±2.19	1.16±2.66	0.39±0.21	0.308
DEL-1 (pg/mL)	2.72±6.12	1.94±5.57	4.26±7.09	0.271
IL-6 (pg/mL)	4.1±6.78	3.52±6.38	5.27±7.65	0.456
IL-8 (pg/mL)	186.33±125.38	180.4±111.46	198.2±153.86	0.682
IL-1β (pg/mL)	140.59±58.94	134.58±55.59	152.62±65.78	0.375

The quantities of neutrophils and total cell nuclei are shown as percentages of microscopy imaging. Differences in measurements between participants <50 and ≥50 years of age were analyzed using the independent *t*-test.
NA: not applicable, PI: plaque index, PPD: probing pocket depth, GR: gingival recession, CAL: clinical attachment level, BOP: bleeding on probing, MBL: marginal bone level, DEL-1: developmental endothelial locus-1, IL: interleukin.
[a]Statistically significant difference ($P<0.05$).

between the 2 groups in terms of PI, PPD, BOP (%), and MBL (%); however, the average CAL was higher in the ≥50 group, which can be attributed to increased GR. Although there was no statistically significant difference in the quantity of neutrophils found in oral rinse samples, a trend toward a lower quantity was observed in the older group, along with a higher ratio of other oral cells. The levels of cytokines and DEL-1 did not exhibit significant differences between the groups, but all were higher in the older group.

Correlations between DEL-1 in saliva and clinical parameters, neutrophils or other cell nuclei in oral rinse, and inflammatory mediators in saliva

No clear correlation was found between salivary DEL-1 levels and the measured variables, with the exception of IL-8 and IL-1β (**Table 3**). Both IL-8 and IL-1β demonstrated positive correlations with salivary DEL-1 levels.

Table 3. Correlations between DEL-1 in saliva and demographic or clinical parameters, quantity of neutrophils or other cell nuclei in oral rinse samples, and inflammatory mediators in saliva

Parameters	Correlation with DEL-1 in saliva					
	Total (n=39)		Age <50 (n=26)		Age ≥50 (n=13)	
	r	*P* value	*r*	*P* value	*r*	*P* value
Age	0.20	0.217	0.22	0.288	−0.08	0.786
PI	−0.03	0.840	−0.03	0.900	0.06	0.841
PPD (mm)	0.05	0.776	0.11	0.577	−0.18	0.557
GR (mm)	0.03	0.843	−0.06	0.785	0.08	0.805
CAL (mm)	0.02	0.923	0.02	0.914	−0.05	0.880
BOP (%)	0.04	0.816	0.18	0.380	−0.17	0.572
MBL (%)	−0.20	0.222	−0.18	0.371	−0.32	0.290
Neutrophil (%)	0.18	0.273	0.17	0.398	0.35	0.234
All cell nuclei (%)	0.16	0.319	0.05	0.824	0.36	0.221
Neutrophils to all cell nuclei	0.00	0.994	0.11	0.599	−0.08	0.800
IL-6 (pg/mL)	0.11	0.512	−0.19	0.361	0.46	0.121
IL-8 (pg/mL)	0.34	0.034[a]	0.13	0.541	0.56	0.051
IL-1β (pg/mL)	0.32	0.047[a]	0.40	0.044[a]	0.16	0.607

Correlations were analyzed using Spearman correlation coefficients.
DEL-1: developmental endothelial locus-1, PI: plaque index, PPD: probing pocket depth, GR: gingival recession, CAL: clinical attachment level, BOP: bleeding on probing, MBL: marginal bone level, IL: interleukin.
[a]Statistically significant difference ($P<0.05$).

https://doi.org/10.5051/jpis.2301120056

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Table 4. Correlations between oral rinse neutrophil quantities and demographic characteristics, clinical parameters, and levels of DEL-1, IL-6, IL-8, IL-1β in saliva

Parameters	Correlation with neutrophils in oral rinse					
	Total (n=39)		Age <50 (n=26)		Age ≥50 (n=13)	
	r	*P* value	*r*	*P* value	*r*	*P* value
Age	−0.18	0.263	−0.12	0.559	−0.24	0.423
PI	0.15	0.378	0.23	0.256	−0.10	0.733
PPD (mm)	0.57	<0.001[a]	0.73	<0.0001[a]	0.34	0.263
GR (mm)	−0.03	0.852	−0.15	0.456	0.46	0.115
CAL (mm)	0.36	0.024[a]	0.45	0.020[a]	0.38	0.196
BOP (%)	0.21	0.195	0.41	0.038[a]	−0.30	0.317
MBL (%)	−0.26	0.107	−0.32	0.112	−0.14	0.656
DEL-1 (pg/mL)	0.18	0.273	0.17	0.398	0.35	0.234
IL-6 (pg/mL)	0.04	0.815	−0.14	0.482	0.55	0.055
IL-8 (pg/mL)	0.33	0.039[a]	0.31	0.123	0.59	0.038[a]
IL-1β (pg/mL)	0.39	0.013[a]	0.40	0.045[a]	0.52	0.074

Correlations were analyzed using Spearman correlation coefficients.
DEL-1: developmental endothelial locus-1, IL: interleukin, PI: plaque index, PPD: probing pocket depth, GR: gingival recession, CAL: clinical attachment level, BOP: bleeding on probing, MBL: marginal bone level.
[a]Statistically significant difference ($P<0.05$).

Correlations between oral rinse neutrophil quantities, demographic characteristics, clinical parameters, and DEL-1, IL-6, IL-8, and IL-1β levels in saliva

The correlations between the quantity of neutrophils in oral rinse samples and factors such as age, clinical parameters, and the levels of DEL-1, IL-6, IL-8, and IL-1β were investigated using Spearman correlation coefficients. In both the overall participant group and the group aged under 50, PPD and CAL showed statistically significant positive relationships with neutrophils in oral rinse samples which was statistically significant (**Table 4**). IL-8 and IL-1β also showed positive relationship with neutrophil oral rinse amounts.

Correlation between GCF DEL-1 and demographic and clinical parameters

In the younger group (under 50 years of age), DEL-1 levels demonstrated a significant inverse correlation with age; however, this inverse relationship was not significant in the total population or in the older group (50 and above). PPD exhibited an inverse correlation with DEL-1 levels in the total population, but this relationship was not significant when analyzed within separate age groups (**Table 5**). Each sample was analyzed individually.

Correlations between GCF neutrophils and demographic characteristics, clinical parameters, and DEL-1 levels in GCF

The quantity of neutrophils in 40 GCF samples from 40 participants demonstrated a significant positive correlation with PPD. This positive correlation was more pronounced in the younger group than in the older group. Although not statistically significant, there was a slight negative trend in the relationship between neutrophil count and age (**Table 6**).

DISCUSSION

The fifth decade of life marks a period when advancing age is associated with a significant increase in the burden of various chronic diseases [26]. Studies on the thymic generation of new T cells have demonstrated that only one-seventh of patients over 50 years of age exhibited any thymic enlargement, which is considerably less than that observed in patients in their 40s, and even less than that of younger patients [27]. A review of humoral and cellular

https://doi.org/10.5051/jpis.2301120056

Table 5. Correlations between DEL-1 in GCF and demographic or clinical parameters

Parameters	Correlation with DEL-1 in GCF								
	Total (n=120)			Age <50 (n=81)			Age ≥50 (n=39)		
	Mean±SD	*r*	*P* value	Mean±SD	*r*	*P* value	Mean±SD	*r*	*P* value
Age	43.25±11.02	−0.11	0.219	37.41±7.86	−0.32	0.004[a]	55.38±5.11	−0.23	0.162
PI	1.78±1.23	−0.11	0.225	1.89±1.3	−0.10	0.380	1.56±1.05	−0.07	0.677
PPD (sum)	17.95±3.8	−0.18	0.049[a]	17.75±4.06	−0.16	0.155	18.36±3.2	−0.24	0.136
GR (sum)	2.79±3.51	0.01	0.954	2.15±3.07	−0.12	0.277	4.13±3.99	0.22	0.172
CAL (sum)	20.74±6.2	−0.10	0.258	19.9±6.31	−0.17	0.129	22.49±5.65	0.01	0.951
mSBI (sum)	5.67±4.4	−0.28	0.002[a]	6.11±4.71	−0.26	0.017[a]	4.74±3.57	−0.28	0.086
MBL (%)	19.07±10.49	−0.14	0.140	18.2±11.43	−0.18	0.109	20.86±8.07	−0.03	0.839

Correlations were analyzed using Spearman correlation coefficients.
DEL-1: developmental endothelial locus-1, GCF: gingival crevicular fluid, SD: standard deviation, PI: plaque index, PPD: probing pocket depth, GR: gingival recession, CAL: clinical attachment level, mSBI: modified sulcular bleeding index, MBL: marginal bone level.
[a]Statistically significant difference (P<0.05).

Table 6. Correlation between GCF neutrophil quantity and demographic characteristics, clinical parameters, and DEL-1 levels in GCF

Parameters	Correlation with neutrophils in GCF								
	Total (n=40)			Age <50 (n=27)			Age ≥50 (n=13)		
	Mean±SD	*r*	*P* value	Mean±SD	*r*	*P* value	Mean±SD	*r*	*P* value
Age	43.25±11.11	−0.31	0.055	37.41±7.96	0.10	0.612	55.38±5.25	0.05	0.872
DEL-1 (pg/mL)	4.41±6.44	−0.13	0.437	5.42±7.39	−0.28	0.151	2.31±3.05	0.02	0.958
PI	2.05±1.36	0.23	0.162	2.15±1.46	0.25	0.211	1.85±1.14	0.33	0.269
PPD (sum)	20.58±3.69	0.44	0.005[a]	20.52±4.08	0.68	<0.0001[a]	20.69±2.84	0.61	0.031[a]
GR (sum)	4.9±4.19	0.01	0.932	4.04±4.23	0.58	0.002[a]	6.69±3.61	0.06	0.837
CAL (sum)	25.48±6.82	0.19	0.242	24.56±7.53	0.70	<0.0001[a]	27.38±4.75	0.43	0.144
mSBI (sum)	7.48±4.27	−0.04	0.814	8±4.75	0.13	0.516	6.38±2.9	−0.26	0.382
MBL (%)	21.66±13.94	0.19	0.235	20.45±15.8	0.68	<0.001[a]	24.18±8.97	0.44	0.138

Correlations were analyzed using Spearman correlation coefficients. PI, PPD, GR, CAL and mSBI are presented as the mean ± SD of sum of sites measured on each tooth.
GCF: gingival crevicular fluid, DEL-1: developmental endothelial locus-1, SD: standard deviation, PI: plaque index, PPD: probing pocket depth, GR: gingival recession, CAL: clinical attachment level, mSBI: modified sulcular bleeding index, MBL: marginal bone level.
[a]Statistically significant difference (P<0.05).

immunity to varicella-zoster virus revealed that reactivation, presenting as herpes zoster, is more prevalent in individuals over 50 years of age [28]. In a study based on 2 population-based samples from 2 countries, the median value for subject-based mean PD increased up to ages 45 to 49 years and then remained relatively stable across older age groups [29]. These findings were considered when choosing 50 years as the age threshold for dichotomizing the 2 subgroups in this study.

Table 2 results reveal no significant differences between the <50 and ≥50 groups in all parameters, except for GR and CAL, which indicate increased gingival recession in the older group. The difference in PPD was not statistically significant. These findings are consistent with trends observed in a population study [29]. Although salivary DEL-1 levels were higher in the older group, this difference was not statistically significant.

In this study, no correlation was found between salivary DEL-1 and all other variables studied, except inflammatory cytokines IL-8 and IL-1β (**Table 3**). A previous study involving 180 systemically healthy, non-smoking subjects with varying degrees of periodontal status discovered a statistically significant negative correlation between salivary DEL-1 and clinical parameters of PPD, CAL, PI, and BOP [30]. These differences are believed to result from the criteria used for participant selection. The previous study included a periodontally healthy (H) group, a gingivitis (G) group, a chronic periodontitis (CP) group, and a generalized aggressive periodontitis (GAP) group [30]. However, in the present study, only participants diagnosed with chronic periodontitis were included. In the previous study, salivary DEL-1

https://doi.org/10.5051/jpis.2301120056

levels were significantly higher in the H and G groups compared to both periodontitis groups and showed no significant difference between the CP and GAP groups [30]. Based on these findings, the relationship between periodontal parameters and DEL-1 may be more clearly demonstrated when a group with relatively healthy periodontal status is included among the participants. No direct relationship between IL-8 and IL-1β has been reported for DEL-1, and since these are markers for inflammation and DEL-1 is critical for immune homeostasis [7], this may be an interesting pathway to pursue.

Table 4 presents results showing a positive correlation between relative neutrophil amounts and PPD. This correlation was significant in the younger group, but not in the older group. A study comparing neutrophil counts in oral rinse samples from edentulous and dentate subjects revealed that the edentulous group had only three-fifths the number of cells [31]. Whole saliva primarily consists of fluid produced by major and minor salivary glands, with non-exocrine contributions accounting for 4.4% of the volume. These non-exocrine components include host cells, gingival fluid, potential mucosal seepage, microorganisms, food residue, and GCF, which constitutes more than half of this portion [32]. The origin of neutrophils in oral rinse samples remains unknown, which may contribute to the discrepancy between the results of this study and the expectations based on *in vitro* and *in vivo* studies.

In the GCF samples (**Table 5**), DEL-1 levels appeared to decrease with age, and this was statistically significant in the younger group. There was no relationship with PI, but the sum of PPD demonstrated an inverse relationship with DEL-1 levels overall. This correlation aligns with previous studies on the biological role of DEL-1 [6,8,14,15]. The differing correlation between DEL-1 and PPD in saliva and GCF samples seems to stem from the variation in sample sizes. A total of 120 samples were used in the correlation analysis between DEL-1 and PPD in GCF samples, which likely led to a clearer correlation than the salivary DEL-1 analysis using 39 samples. Another possible explanation is the anatomical proximity of the periodontal pocket and the GCF. Since GCF has the opportunity to be closely approximated to the periodontal tissues where periodontal disease begins, it appears to provide more accurate information than markers in saliva [33]. Additionally, molecules in saliva can also originate from salivary glands, meaning that cellular and biochemical mediators in saliva may reflect the diseases and metabolic status of glands rather than periodontal diseases [33].

The quantity of neutrophils in GCF demonstrated a positive correlation with the sum of PPD (**Table 6**). This finding was expected, as neutrophils are an essential component of the periodontal host response and constitute the vast majority (≥95%) of leukocytes recruited to the gingival crevice in response to tooth-associated biofilms [34].

There is considerable diversity among human participants in research, and chronological age may not accurately represent biological age [24]. A prospective study of a young human cohort (the Dunedin Study birth cohort) demonstrated that young individuals with the same chronological age exhibited markedly different levels of physical deterioration [24]. This could be a contributing factor to the observed lack of decline in salivary DEL-1 levels with age in the present study. Another limitation of this study is the absence of periodontally healthy patients, which can be attributed to resource constraints.

We assumed that DEL-1 found in whole unstimulated saliva (or oral fluid) primarily originates from GCF. However, DEL-1 is expressed in salivary glands, and the amount secreted from these glands may be relatively greater than the amount present in GCF, which is a component

https://doi.org/10.5051/jpis.2301120056

of whole saliva [35]. It would be intriguing to collect gland saliva and whole saliva separately at different time points during the day and examine the relative amounts of DEL-1 in these samples. To determine if mucosal seepage of DEL-1 occurs, the levels of DEL-1 in gland saliva and whole saliva could be measured in individuals without any teeth or dental implants. The limited volume of GCF samples makes it challenging to measure multiple proteins, and as a result, we were unable to study IL-17 levels. Combining GCF samples to investigate multiple cytokine levels may provide a more accurate representation of the immune-inflammatory processes occurring in the periodontium.

Salivary DEL-1 did not show correlations with clinical parameters of periodontitis; however, it exhibited a mild positive relationship with IL-8 and IL-1β. GCF DEL-1 demonstrated a negative correlation with the sum of PPD. Neutrophils in the oral rinse were positively correlated with PPD, IL-8, and IL-1β levels. Neutrophils in GCF displayed a positive correlation with the sum of PPD. The negative correlation between GCF DEL-1 and PPD levels suggests a protective role for DEL-1 in periodontal inflammation.

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