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Salivary and gingival CXCL8 correlation with periodontal status, periodontal pathogens, and smoking

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Abstract

Objectives: Neutrophil granulocytes have been proposed to play a major role in the mediation of periodontitis-associated tissue destruction. Their recruitment and activation are regulated by the chemokine CXCL8. This study aimed to delineate the dependency of CXCL8 expression in gingival crevicular fluid (GCF) and saliva on periodontal status, bacterial infection, and smoking, in patients with periodontitis.

Methods: The study cohort comprised 279 subjects with untreated periodontitis. Probing pocket depth (PPD), gingival recession, bleeding on probing (BOP), plaque index, and bone loss were evaluated. CXCL8 was determined in saliva and GCF using flow cytometry.

Results: Considering the entire study sample, CXCL8 levels were correlated with the mean PPD ($\rho = 0.131$; $p = 0.029$), severity of periodontitis ($\rho = 0.121$; $p = 0.043$), BOP ($\rho = 0.204$; $p = 0.001$), and smoking ($\rho = -0.219$; $p < 0.0001$) in GCF; and, in whole saliva, with mean PPD ($\rho = 0.154$; $p = 0.010$) severity of periodontitis ($\rho = 0.140$; $p = 0.020$), gender ($\rho = 0.178$; $p = 0.003$), and smoking ($\rho = -0.156$; $p = 0.010$). Subgroup analysis among non-smokers revealed significantly higher amounts of CXCL8 in GCF ($p = 0.012$) and saliva ($p = 0.026$) comparing subjects with mean PPD ≤3mm and >3mm.

Conclusion: The current study revealed a strong dependency of CXCL8 expression in GCF on the severity and activity of periodontal disease. Smoking causes a significant reduction in CXCL8 expression in saliva and GCF.

KEYWORDS
attachment loss, chemokine, neutrophils, periodontitis

1 | INTRODUCTION

Recent etiologic models have perceived a central role for neutrophil granulocytes in the manifestation and perpetuation of chronic inflammatory diseases including not only periodontitis, but also rheumatoid arthritis and inflammatory bowel disease (Apel et al., 2018; Colgan, 2015; Jorch & Kubes, 2017). Most importantly, neutrophils have now been recognized to be responsible for the mediation of inflammatory tissue destruction (Rijkschroeff et al., 2018). The severity of periodontitis, as represented by the destruction of tooth supporting connective and osseous tissue, has been associated with the intensity of neutrophil recruitment and

their exaggerated activation (Cortes-Vieyra et al., 2016; Herrmann & Meyle, 2015). Differently from their classical role as first and mostly non-specific line of defense against invading bacteria, more recent data have shown considerable versatility of neutrophils additionally mediating immunoregulatory functions through the expression of an array of proinflammatory and anti-inflammatory cytokines and chemokines on both, innate and adaptive immune response (Mantovani et al., 2011; Odobasic et al., 2016). Among others, neutrophils have the capacity to recruit and support the survival of B- and T-lymphocytes (Kalyan & Kabelitz, 2014; Perobelli et al., 2016; Scapini et al., 2005). Conversely, Th17 cells indirectly enhance the recruitment and chemotaxis of neutrophils under the mediation of neutrophil-activating chemokines (NACs) (Linden, 2001; Rajarathnam et al., 2019; Sadik et al., 2011; Sanz & Kubes, 2012). In fact, the phenotype and function of neutrophils have been proposed to be dynamic and to result from interactions with its environment. In this regard, particularly the qualitative and quantitative signal, as represented by the specific pattern of different neutrophil-activating chemokines within a defined tissue compartment, seems to play a central role.

In humans, seven neutrophil-activating chemokines, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, and CXCL8, have been identified which are recognized by two different receptors, that is, CXCR1 and CXCR2 (Ahuja et al., 1996; Tintinger et al., 2013). Chemokines are expressed by a variety of cells of the innate and adaptive immunity and in addition by many epithelial cells, that is, intestinal and airway epithelial and oral keratinocytes (Linden, 2001). These mediators are monomeric and dimeric proteins of small molecular weight (8–10 kDa). The subgroup of neutrophil-activating chemokines shares the ELR motif preceding the first conserved cysteine residue.

Considering the recruitment of neutrophils in periodontitis, CXCL8 plays a central role (Garlet et al., 2003). This chemokine signals via the CXCR2 receptor the adhesion to the endothelium and acts as chemotactic stimulus (Kolaczkowska & Kubes, 2013). Albeit its crucial role in addressing infectious challenges, it has been previously proposed that an excessive expression of CXCL8 and an inappropriate recruitment of neutrophils at diseased sites might cause a significant destruction of periodontal tissue (Gamonal et al., 2001; Konopka et al., 2012). Yet, there exists considerable controversy on the expression of CXCL8 in the gingival crevicular fluid and the affected periodontal tissue (Chung et al., 1997; Goutoudi et al., 2012). A recent meta-analysis reported higher CXCL8 expression within the gingival tissue at periodontitis-affected sites but not in gingival crevicular fluid (Finoti et al., 2017). As an explanation, it has been suggested that the individual immune response and the associated expression of chemokines stay under the influence of different modifying factors, as smoking (Persson et al., 2001). In fact, smoking is commonly accepted as risk factor increasing the individual susceptibility and clinical severity of periodontitis (Haber et al., 1993; Walter et al., 2012). Inappropriate CXCL8 expression, resulting in impaired neutrophil recruitment induced by smoking, might be responsible for an exaggerated destruction of periodontal tissue.

This study aimed to delineate the impact of clinical severity, bacterial infection, and smoking on the expression of CXCL8 in gingival crevicular fluid and saliva in patients with periodontitis.

2 | METHODS

2.1 | Study cohort

The patients were consecutively recruited among the outpatients attending the Department of Restorative Dentistry and Periodontology of our University Hospital from 2011 until 2016. Prior to enrollment into the study, all study subjects received detailed information about the objectives and methods of this study and gave their written informed consent. The study conformed to the ethical guidelines of the Helsinki Declaration and was approved by the Ethics Committees of the Medical Faculty of our University (No.025–11). Individuals with a history of severe medical disorders, that is, diabetes mellitus, immunological disorders, increased risk for bacterial endocarditis, and pregnant patients were not included in the study. The inclusion criteria were age 18 years or older, identification of the patients as a periodontal case and ability and willingness to give written informed consent. The definition of a periodontal case was based on the PSI scores, introduced by the American Dental Association for all dental patients as an integral part of routine oral diagnostic examinations and endorsed by the American Academy of Periodontology (Covington et al., 2003; Rams & Loesche, 2017) and the presence of interdental clinical attachment loss. A periodontal case was defined as presenting a PSI ≥3 or interdental clinical attachment loss ≥1mm.

2.2 | Clinical and radiographic examination

All study subjects received a standard periodontal examination including the determination of (1) probing pocket depth at six sites per tooth (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual, disto-lingual), (2) furcation involvement using a Nabers probe, and (3) bone loss as assessed by panoramic tomograms. Probing pocket depth was measured as the distance from the free gingival margin to the base of the periodontal pocket, placing the probe into the pocket until soft tissue resistance appeared. Probing depths were recorded in full millimeter steps. Bleeding on probing was dichotomously registered as being absent or present for each tooth and expressed as a percentage of all present teeth in an individual. Plaque values were determined analogously to the Plaque Control Record Index (PCR) according to O'Leary et al. and expressed as a percentage of all teeth surfaces in an individual. (O'Leary et al., 1972). Furcation defects were evaluated by horizontal probing from the furcation entrance to the base of the defect. The furcation involvement was classified according to the protocol of Hamp et al (Hamp et al., 1975). Radiographic examination of patients was done only if a panoramic tomogram was

indicated due to therapeutic reasons. It was, of course, performed in all patients diagnosed with periodontitis, but not in all the excluded ones. The individual severity of periodontitis has been retrospectively characterized referring to the new Classification of Periodontal Diseases (Papapanou et al., 2018). Different from the original procedure, patients have been classified into three stages herein, considering the deepest probing pocket depth only. Since the original classification assigns maximum periodontal pocket depth ≥6 mm to stage III or IV, these stages have been grouped together as *severe periodontitis*. For analysis, patients classified with stage I or II have been grouped together as *mild/moderate periodontitis*. The dental examinations were performed by calibrated study investigators. Patients did not receive subgingival debridement or antimicrobial treatment within 2 years prior to the examination. In addition, patients had to answer a standardized questionnaire concerning their health status and smoking habits. Current smokers and former smokers which quit smoking for <1 year were assigned to the group of smokers; never smokers and former smokers for more than 1 year have been classified as non-smokers herein.

2.3 | GCF samples for CXCL8 and bacterial testing

Gingival crevicular fluid samples were obtained from the deepest periodontal pocket of each quadrant using absorbing paper strips (Dentognostics GmbH, Jena, Germany). Immediately prior to the placement of the strip, the site to be sampled was gently air dried. The strips were inserted in the periodontal pocket until some resistance was encountered and kept in place for 30 s. The strips were collected in a cryotube. Samples were aliquoted and stored at −80°C. The bacterial samples were collected from the same periodontal pockets, using sterile paper points (VDW GmbH, Munich, Germany) kept in place for 10 s. The samples were collected in a cryotube and stored at −20°C.

2.4 | Saliva samples

Before sample collection, the patients were advised to rinse with drinking water, for 30 s. For the collection of the saliva specimens, patients were then instructed to rinse thoroughly with 10 ml of distilled water for 30 s and the mouth-rinse was transferred in a sterile 50 ml Falcon. The samples were aliquoted and stored at −80°C.

2.5 | Determination of CXCL8 in GCF and saliva samples

The saliva samples were taken from the aliquots described above. The GCF samples were thawed and suspended in 400 µl of phosphate-buffered saline, incubated for 5 min at room temperature, and vortexed. The paper strips were removed and the content aliquoted for further analyses. The cytokine concentration was measured on the BD FACSCanto™ flow cytometer and further analyzed by FCAP Array™ Software (BD Biosciences). The buffers and reagents were provided in the two kits BD™ CBA (Cytometric Bead Array) Human IL-8 Flex Set (BD™ Biosciences, Franklin Lakes, New Jersey, USA) and BD™ CBA Master Buffer Kit, and they were used following the manufacturer's instructions.

2.6 | Determination of periodontal pathogens in GCF samples

The isolation of bacterial DNA from the samples was performed using the MagNA Pure DNA Isolation Kit III (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions. Briefly, for the preisolation, the paper points were suspended in 169 µl bacteria lysis buffer and 20 µl proteinase K (Roche Diagnostics GmbH). After an incubation of 10 min in a shaking water bath at 65°C, and 10 min at 95°C, 100 µl were transferred for the automated isolation to the MagNA Pure LC Instrument (Roche Diagnostics GmbH). The amplification of the DNA, through the Parident-kit (AMPLEX Diagnostics GmbH, Gars am Inn, Germany) was carried on as in Table S1. For each bacteria group, 5 µl of the DNA sample was mixed with 45 µl of the respective master mix. For *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, and *Fusobacterium nucleatum*: the PAF-Primer from the Parident Kit was used. For *Tannerella forsythia*, *Prevotella intermedia*, and *Treponema denticola*, the PMT Primer, also provided in the kit, was used. There followed an ELISA-based identification and detection of the bacteria using the Parident ELISA Kits (AMPLEX GmbH, Deutschland), according to the manufacturer's instructions. The analyses were performed in duplicates. Varioskan 3.00.7 (Thermo Fisher Scientific, Waltham, MA, USA) was used for spectrophotometric analyses.

2.7 | Statistical analysis

Post-hoc power analysis was performed using the G-Power Calculator (version 3.1) using the salivary CXCL8 amount in individuals with mild/moderate (stage 1 & 2) or severe (stage 3 & 4) periodontitis as reference. The power was 0.63 accordingly. For all continuous variables, the mean and standard deviation have been calculated. Categorical data are presented as relative frequencies. Kolmogorov-Smirnov procedure has been used to test the normal distribution of data within groups. Since the data were not normally distributed, univariate analysis of differences between groups has been performed with Kruskal-Wallis and Mann-Whitney test where appropriate. For categorical data, Fisher's exact test has been applied. Statistical analyses have been done either on a patient or a site level, as singularly specified in the tables. Additional separate subgroup analyses have been done

following to stratification of the study group according to gender, smoking status, presence of periodontal pathogens, and severity of periodontitis. Rank-based correlation analysis has been done using the Spearman-Rho coefficient to determine interrelations between gender, age, smoking status, severity of periodontitis, probing pocket depth (mean), bleeding on probing, plaque, periodontal pathogens, and the expression of CXCL8 in saliva and GCF. To determine the impact of periodontitis on the CXCL8 expression (GCF and saliva), binary logistic regression analysis has been performed using the severity of periodontitis, age, gender, and smoking as independent variables. Odds ratios (OR) and 95% confidence intervals and the effect size f according to Cohen et al. (Cohen, 1988) have been calculated. For logistic regression analysis the CXCL8 expression in saliva and GCF has been transformed into dichotomous categories according to the data quartiles (lowest 25% vs. highest 25% of CXCL8 expression). *p*-values <0.05 have been considered significant. All test procedures were two-tailed and have been performed using SPSS software version 26.0 (SPSS Inc., Chicago, IL, USA).

3 | RESULTS

3.1 | Study sample

The study group comprised a total of 279 patients (130 females, 149 males) with periodontitis, among which the severity was classified as mild/moderate (stage I&II) in 21.9% of the patients and as severe (stage III&IV) in 78.1% of the patients. The mean age among patients with mild/moderate periodontitis was 58.2 (±14.0) years and 55.0 (±12.1) years for patients with severe periodontitis ($p = 0.066$). The male to female ratio ($p = 0.061$) and the frequency distribution of smokers and non-smokers ($p = 0.094$) were not significantly different between groups (Table 1).

3.2 | CXCL8 expression depending on clinical and radiographic findings

Categorizing study subjects according to the probing pocket depth (mean of deepest site in each quadrant), the amount of CXCL8 in both, saliva and GCF, increased with increasing mean probing pocket depth (Table 2). In cases with PD of 2 and 3 mm, the patients were diagnosed as having periodontitis due to clinical attachment loss of more than 1mm. In fact, correlation analysis showed a significant but weak correlation in saliva (ρ: 0.236; $p < 0.0001$) and GCF (ρ: 0.216; $p < 0.0001$) (Table 3). The comparison between subjects with mean probing pocket depth ≤3 mm and >3mm again showed considerably higher CLCX8 expression in saliva and GCF, but the difference did not reach significance (Table 4). Considering the CXCL8 expression depending on the relative frequency of bleeding on probing, there was found a significantly lower amount of CXCL8 in saliva ($p = 0.020$) and GCF ($p = 0.024$) comparing patients with bleeding on probing at <10%

TABLE 1 Characteristics of the study groups; *p*-values as obtained with Mann-Whitney and Fisher's exact test

	Total	Mild/moderate (stage I&II)	Severe (stage III&IV)	*p*-value (mild/moderate vs. severe)
Mean age (years ±SD)	55.7 (±12.6)	58.2 (±14.0)	55.0 (±12.1)	0.066
Gender				
(Males/females) (in %)	53.4/46.6	42.6/57.4	56.4/43.6	0.061
Smoking				
(Smokers/non-smokers) (in %)	33.7/66.3	24.6/75.4	36.2/63.8	0.094
Clinical parameters	**Males**	**Females**		***p*-value (male vs. female)**
Mean ppd (mm ±SD) (all sites)	3.06 (±0.71)	2.83 (±0.71)		0.010
Mean ppd (mm ±SD) (maximum per quadrant)	6.08 (±1.56)	5.75 (±1.59)		0.098
Full mouth BOP (% ± SD)	38.3 (±20.2)	37.1 (±20.8)		0.580
Clinical parameters	**Smokers**	**Non-smokers**		***p*-value (smokers vs. non-smokers)**
Mean ppd (mm ±SD) (all sites)	3.22 (±0.77)	2.82 (±0.65)		<0.0001
Mean ppd (mm ±SD) (maximum per quadrant)	6.21 (±1.45)	5.78 (±1.63)		0.012
Full mouth BOP (% ± SD)	36.2 (±20.5)	38.5 (±20.4)		0.377

Note: Mean ppd (all): mean probing pocket depth of all pockets (patient level). Mean ppd (maximum per quadrant): mean probing pocket depth of the deepest pocket per quadrant (site level). Full mouth BOP: mean bleeding on probing of all areas.

TABLE 2 Mean probing pocket depth (ppd) at a tooth level: deepest site per quadrant; and mean CXCL8 (±SD) in pg/ml in samples of saliva and gingival crevicular fluid (GCF) of these sites, as obtained in the entire study cohort

		Saliva	GCF
Mean ppd (mm)	Cases (*n*)	CXCL8 (mean ± SD)	CXCL8 (mean ± SD)
2	1	56.12 (±0.0)	69.50 (±0.0)
3	14	125.89 (±163.94)	131.00 (±117.99)
4	43	160.98 (±160.82)	122.51 (±94.60)
5	66	160.99 (±189.54)	257.55 (±462.72)
6	77	285.21 (±711.54)	206.49 (±345.28)
7	41	282.56 (±426.59)	198.21 (±195.78)
8	19	322.76 (±225.79)	234.65 (±257.72)
9	9	300.22 (±286.18)	294.57 (±196.14)
10	7	636.66 (±679.66)	370.43 (±250.91)
11	1	138.61 (±0.0)	868.24 (±0.0)
12	1	728.19 (±0.0)	5917.59 (±0.0)

of sites with subjects showing bleeding on probing at ≥10% of sites. The amount of CXCL8 in saliva and in GCF was significantly different between patients with mild/moderate periodontitis (stage 1&2) and patients with severe periodontitis (stage 3&4) ($p = 0.021$ for saliva and $p = 0.044$ for GCF). In female patients, a significantly lower CXCL8 expression was observed in saliva ($p = 0.003$) than in male subjects, but it was not different in GCF. All the data are reported in Table 4.

3.3 | CXCL8 expression depending on periodontal pathogens

The amount of CXCL8 in saliva ($p = 0.300$) and GCF ($p = 0.239$) was not different when comparing patients with <30% or >30% sites showing clinical detectable plaque. Analyzing the specific periodontal pathogens within the periodontal pocket, a significant dependency of CXCL8 expression in GCF was not found for any of the tested bacteria (Table 5).

TABLE 3 Correlation analysis for the association between the amount of CXCL8 in saliva and gingival crevicular fluid (GCF) and gender, age, smoking status, severity of the disease, mean probing pocket depth (ppd) (all sites-patient level), mean probing pocket depth (ppd) (mean of deepest site per quadrant-tooth level), full mouth bleeding on probing

	CXCL8 (saliva)		CXCL8 (GCF)	
	ρ	*p*-value	*ρ*	*p*-value
Gender	0.178	**0.003**	−0.015	0.806
Age	0.088	0.145	0.060	0.318
Smoking	−0.156	**0.010**	−0.219	**<0.0001**
Severity of disease	0.140	**0.020**	0.121	**0.043**
ppd (mean; all sites)	0.154	**0.010**	0.131	**0.029**
ppd (mean; deepest per quadrant)	0.236	**<0.0001**	0.216	**<0.0001**
Full mouth BOP (mean)	0.087	0.149	0.204	**0.001**

Note: *p*-values and correlation coefficient *ρ* according to Spearman-Rho analysis. Bold values indicates significant differences.

TABLE 4 Amount of CXCL8 in pg/ml (mean ± SD) in samples of saliva and gingival crevicular fluid (GCF) as obtained following stratification of the study cohort according to gender, mean probing pocket depth (ppd) (all sites-patient level), severity of the disease and full mouth bleeding on probing

	Saliva		GCF	
Gender	**CXCL8**	***p*-value**	**CXCL8**	***p*-value**
Females	172.56 (±220.92)	0.003	224.46 (±345.20)	0.806
Males	293.32 (±572.08)		238.29 (±553.18)	
Probing pocket depth				
≤3 mm	198.28 (±238.42)	0.202	203.09 (±326.87)	0.084
>3 mm	294.14 (±630.01)		270.51 (±606.30)	
Severity of disease				
Mild/moderate (stage I&II)	141.01 (±139.01)	0.021	136.60 (±149.98)	0.044
Severe (stage III&IV)	266.24 (±499.51)		258.49 (±519.73)	
Bleeding on probing				
≤10% of sites	126.42 (±147.56)	0.020	114.23 (±118.27)	0.024
>10% of sites	249.59 (±465.82)		243.08 (±485.99)	

Note: *p*-values as obtained with Mann-Whitney test.

TABLE 5 Amount of CXCL8 in pg/ml (mean ± SD) in samples of saliva and gingival crevicular fluid (GCF), depending on the presence/absence of infection with pathogenic bacteria

	Saliva		GCF	
	CXCL8 (mean ± SD)	*p*-value	CXCL8 (mean ± SD)	*p*-value
Plaque				
≤30% of sites	216.99 (±189.29)	0.300	252.39 (±380.83)	0.239
>30% of sites	243.85 (±486.82)		226.42 (±484.99)	
Porphyromonas gingivalis				
Absent	208.64 (±266.66)	0.480	241.12 (±382.35)	0.653
Present	255.04 (±522.20)		226.74 (±508.77)	
Aggregatibacter actinomycetemcomitans				
Absent	234.37 (±464.54)	0.696	212.50 (±339.36)	0.335
Present	258.88 (±363.41)		327.31 (±854.10)	
Tannerella forsythia				
Absent	195.04 (±245.62)	0.163	267.72 (±614.15)	0.621
Present	284.03 (±587.87)		194.66 (±228.81)	
Treponema denticola				
Absent	174.15 (±185.78)	0.135	256.13 (±668.58)	0.243
Present	276.92 (±545.05)		217.41 (±289.03)	

Note: Plaque: at a patient level. *p*-values as obtained with Mann-Whitney-test.

3.4 | CXCL8 expression depending on smoking

Considering the entire study sample, smoking had a highly significant impact on the CXCL8 levels in saliva ($p = 0.010$) and GCF ($p < 0.0001$), leading to a strong attenuation as compared to non-smoking individuals (Table 6). Subgroup analysis among non-smoking individuals showed considerably higher CXCL8 levels in saliva ($p = 0.026$) and GCF ($p = 0.012$) for subjects with a mean probing pocket depth >3 mm as compared to patients with mean probing depth of ≤3mm. This difference was not found for smokers. There was also found significant difference when considering the CXCL8 levels depending on the severity of the disease (stage I & II vs. stage III & IV) among non-smokers but not among smokers. The results are reported in Table 6.

3.5 | Comparing 25% lowest with 25% highest CXCL8 expression in saliva and GCF

A considerable higher proportion of subjects with stage 1 or 2 periodontitis was found among the patients with the 25% lowest expression of CXCL8 in saliva ($p = 0.011$) and GCF ($p = 0.021$) in comparison to the patients with the 25% highest CXCL8 expression (Table 7). According to binary logistic regression analysis using CXCL8 expression in saliva or GCF (25% lowest vs. 25% highest) as a dependent variable, the presence of stage III and IV periodontitis significantly increased the chance for patients to be among the subjects expressing the 25% highest CXCL8 levels in saliva (OR: 1.89; 95% CI: 1.14–3.14; $p = 0.013$) or GCF (OR: 2.32; 95% CI: 1.33–4.04; $p = 0.003$) (Table 8). Moreover, assignment of patients to the group with 25% lowest or 25% highest CXCL8 expression in saliva is significantly influenced by gender ($p = 0.001$), age ($p = 0.039$ and smoking ($p = 0.020$), whereas in GCF only smoking reached significance ($p < 0.0001$) in addition to the disease severity.

4 | DISCUSSION

Recruitment and activation of neutrophils proportionally to the bacterial challenge has been identified as a critical step to sufficiently encounter gingival and/or periodontal infection and to maintain the integrity of periodontal tissues (Hajishengallis, 2020). Insufficiently controlled, and thus excessive infiltration of neutrophils into periodontitis-affected tissue, has been associated with host-tissue damage (Nussbaum & Shapira, 2011). Among the neutrophil-activating chemokines, specifically CXCL8 is involved in the regulation of the transit of neutrophils from the gingival capillaries to the gingival crevice (Kolaczkowska & Kubes, 2013).

Among other marker molecules, the determination of CXCL8 level in gingival crevicular fluid and/or saliva was suggested for diagnosis and monitoring of periodontitis. Yet, there exists only inconclusive information on the changes in CXCL8 expression probably leading to dysregulated activation of neutrophils in periodontitis.

In this regard, it is mostly anticipated that the gingival expression of CXCL8 is correlated with the severity and activity of the disease. In fact, herein, higher CXCL8 activity was found for severe periodontitis than for moderate periodontitis and the concentration of CXCL8 in GCF and saliva was correlated with the mean of the maximal pocket depth per quadrant in periodontitis patients. Moreover, comparing patients with a mean probing pocket depth ≤3 mm and

TABLE 6 Amount of CXCL8 in pg/ml (mean ± SD) in samples of saliva and gingival crevicular fluid (GCF) among smokers and non-smokers in the entire study cohort and depending on the mean probing pocket depth (ppd) and the severity of the disease

	Saliva		GCF	
Total	**CXCL8**	***p*-value**	**CXCL8**	***p*-value**
Non-smokers	277.91 (±531.98)	0.010	240.04 (±340.23)	<0.0001
Smokers	162.39 (±186.97)		215.71 (±650.37)	
Non-smokers				
Mean ppd ≤3 mm	207.77 (±243.30)	0.026	212.32 (±309.17)	0.012
Mean ppd >3 mm	417.04 (±840.07)		292.47 (±389.55)	
Smokers				
Mean ppd ≤3 mm	168.84 (±223.04)	0.727	174.46 (±379.58)	0.250
Mean ppd >3 mm	157.82 (±158.58)		244.95 (±790.56)	
Non-smokers				
Stage I&II	155.88 (±150.17)	0.036	154.57 (±166.41)	0.024
Stage III&IV	319.18 (±604.25)		268.33 (±376.93)	
Smokers				
Stage I&II	95.42 (±85.86)	0.118	81.50 (±55.54)	0.248
Stage III&IV	175.11 (±198.32)		241.19 (±706.86)	

Note: p-values as obtained with Mann-Whitney test.

TABLE 7 Frequency distribution of severity of periodontitis among subjects with 25% lowest and 25% highest expression of CXCL8 in saliva and GCF

CXCL8 saliva	**CXCL8 25% lowest (%)**	**CXCL8 25% highest (%)**	***p*-value**
Stage I&II	30.4	11.6	0.011
Stage III&IV	69.6	88.4	
CXCL8 GCF	**CXCL8 25% lowest (%)**	**CXCL8 25% highest (%)**	***p*-value**
Stage I&II	23.2	8.6	0.021
Stage III&IV	76.8	91.4	

Note: p-values as obtained with Fisher's exact test.

>3 mm at all sites, again higher amounts of CXCL8 have been found in the latter group. Inline, a correlation between clinical periodontal parameters, particularly the probing pocket depth was found in a recent cross-sectional study (Lutfioglu et al., 2016). In addition, it has been previously shown that the level of CXCL8 in gingival crevicular fluid and at the transcriptional level also in gingival tissue samples is linked to the disease activity and is reduced at periodontitis-affected sites following to subgingival debridement (Tsai et al., 1995; Venza et al., 2010). Apart from the severity as determined by the periodontal attachment loss, the actual periodontal disease activity is reflected by gingival bleeding upon probing (Lang et al., 1996). According to the recent classification of periodontal diseases, the critical threshold between health and disease has been defined as 10% bleeding sites upon probing (Chapple et al., 2018). Using this limit herein, the amount of CXCL8 in saliva and gingival crevicular fluid was significantly higher in patients showing bleeding on probing at >10% sites. Again, this is consistent with recent observations in patients with localized and generalized aggressive periodontitis, showing linkage between crevicular CXCL8 levels and bleeding on probing (Martins et al., 2019). Contradictory, a recent meta-analysis reported lower amounts of CXCL8 in GCF in periodontitis patients compared with healthy controls (Finoti et al., 2017). As an explanation, several authors have proposed that the CXCL8 might be cleaved or its expression attenuated by some bacteria of the subgingival microflora, particularly *P. gingivalis* (Jin et al., 2000; Zhang et al., 1999). In the current study, CXCL8 tends to higher levels in saliva and gingival crevicular fluid in patients having an infection with *P. gingivalis* although the difference did not reach significance.

This study further analyzed the effect of smoking on CXCL8 levels in saliva and gingival crevicular fluid. Clinically, in smokers there were found significantly stronger tissue defects, as reflected by the mean probing pocket depth regarding the deepest site per quadrant

TABLE 8 Results of binary logistic regression analysis using the expression of CXCL8 in saliva or GCF (25% lowest vs. 25% highest) as dependent variable

	OR (95% CI)	Regression coefficient B	*p*-value	Effect size f
CXCL8 saliva (lowest 25% vs. highest 25%)				
Severity of periodontitis (stage I&II vs. stage III&IV)	1.89 (1.14–3.14)	0.639	0.013	0.32
Smoking status	2.74 (1.18–6.38)	1.008	0.020	
Gender	3.71 (1.68–8.23)	1.312	0.001	
Age	1.03 (1.00–1.07)	0.033	0.039	
CXCL8 GCF (lowest 25% vs. highest 25%)				
Severity of periodontitis (stage I&II vs. stage III&IV)	2.32 (1.33–4.04)	0.841	0.003	0.30
Smoking status	5.02 (2.25–11.22)	1.614	<0.0001	
Gender	0.92 (0.43–1.94)	−0.088	0.818	
Age	1.01 (0.98–1.05)	0.014	0.381	

Note: Significance of regression coefficient B has been tested with Wald test, results are presented as *p*-values. The effect size f has been calculated with Nagelkerke's R-squared.

Abbreviations: CI, confidence interval; OR, odds ratio.

and at all sites. On the contrary, non-smoking individuals showed a trend for a higher rate of bleeding on probing, which is commonly explained by an impaired perfusion of the periodontal pocket due to the exposure to smoke (Farina et al., 2013). Considering the entire study cohort, the CXCL8 levels were significantly lower in smokers than in non-smokers. This observation is in accordance with several previous reports. A significantly lower amount of CXCL8 was found for smokers in comparison to non-smokers at both, healthy and diseased sites in patients with severe chronic periodontitis (Tymkiw et al., 2011). Moreover, also in a study on early onset periodontitis (aggressive periodontitis) sulcular CXCL8 expression tended to be lower in patients and healthy controls among smokers compared with non-smokers (Kamma et al., 2004). Considering the transcriptional level, a significant attenuation of CXCL8 expression was found in smokers in gingival tissue samples from patients with a diagnosis of chronic periodontitis (Souto et al., 2014). An opposite trend was found in a recent study showing elevated CXCL8 in gingival crevicular fluid, irrespective from the individual periodontal status (Lutfioglu et al., 2016).

Intriguingly, subgroup analysis of the present data revealed considerably stronger differences in CXCL8 expression between subjects with mean probing pocket depth ≤3 mm and >3 mm among non-smokers as compared to smokers. Conceivably, smoking in fact attenuates the CXCL8 activity within the periodontal tissue. The stronger inflammatory stimulus at sites with higher probing pocket depth might be partially attenuated by smoking ultimately leveling the difference in CXCL8 expression between subjects with mean probing pocket depth ≤3 mm and >3 mm in smokers. A limitation of our study, in this regard, is the non-inclusion of a control group of non-periodontitis patients. Another limitation concerns possible differences among the smoker group due to a possibly diverse exposure to smoking, in quantitative terms. Also, some sites underwent PD, GCF and microbiological assessments. These might also have an interference on the measured CXCL8 levels.

In addition to analytical methods, various methods are commonly used for the determination of the intensity of periodontal inflammation, that is, the protein level of marker molecules in gingival crevicular fluid, saliva or serum and the transcriptional activity within the affected tissue itself (Darveau, 2010). Measuring the CXCL8 chemokine could be used as an adjunct in diagnosis and decision-making regarding the treatment. Differently from saliva, the gingival crevicular fluid reflects the actual inflammatory activity in the periodontal tissue more precisely, since it is not biased by other intraoral pathological conditions (Dede et al., 2013; Mauramo et al., 2018). Herein, the amount of protein expression of CXCL8 has been evaluated in samples of the gingival crevicular fluid and saliva. Interpreting the current data, the determination of the CXCL8 level in the gingival crevicular fluid in fact reveals more precise results for comparisons between various disease stages and/or smoking and non-smoking individuals than saliva. Yet, when comparing the relative frequencies of patients with mild/moderate (stage I&II) or severe periodontitis (stage III&IV) in the group of subjects showing the lowest 25% or highest 25% of CXCL8 expression in saliva or GCF considerably more patients with severe periodontitis were found in the group showing the 25% highest CXCL8 expression. Obviously, severe periodontitis increases the odds to be assigned to the group with 25% highest CXCL8 expression roughly 2-fold considering saliva and GCF. These results might corroborate the use of CXCL8 as salivary or gingival marker molecule for the diagnosis and monitoring of periodontitis.

5 | CONCLUSIONS

Taken together, the current study confirmed a positive correlation of the CXCL8 expression in gingival crevicular fluid and saliva with the severity and activity of periodontal disease, independently from the

individual smoking status. Obviously, smoking and disease activity have antagonistic effects on the CXCL8 expression, probably leading to an inappropriate activation of CXCL8 and neutrophils.

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CONFLICT OF INTEREST

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AUTHOR CONTRIBUTION

Iris Frasheri: Data curation; Formal analysis; Validation; Visualization; Writing-original draft; Writing-review & editing. **Richard Heym:** Conceptualization; Investigation; Software; Supervision; Writing-review & editing. **Christina Ern:** Conceptualization; Investigation; Methodology; Writing-review & editing. **Burkhard Summer:** Investigation; Writing-review & editing. **Till Hennessen:** Data curation; Investigation; Software; Writing-review & editing. **Christof Hoegg:** Investigation; Writing-review & editing. **Franz-Xaver Reichl:** Conceptualization; Methodology; Writing-review & editing. **Matthias Folwaczny:** Conceptualization; Data curation; Formal analysis; Methodology; Validation; Visualization; Writing-original draft; Writing-review & editing.

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