**Subgingival Microbiota in Caucasian Patients With Desquamative Gingivitis: A Cross-Sectional Study**

**Subgingival microbiota in Caucasian patients with desquamative gingivitis: a cross-sectional study.**

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One sentence summary: Differences in the subgingival microbiota between desquamative and plaque-related gingivitis may suggest a possible association between periodontal pathogens and this rare autoimmune gingival condition.

**Abstract**

Background: The presence of epithelial desquamation, erythema, and erosions on the gingival tissue is usually described in literature as desquamative gingivitis (DG). A wide range of autoimmune/dermatological disorders can manifest as DG, although the two more common are oral lichen planus and mucous membrane pemphigoid. The aim of this study was to investigate the prevalence of 11 periodonto-pathogenic microorganisms in DG cases and to compare it with microbiologic status of individuals affected by plaque-induced gingivitis (GI).

Methods: Cross-sectional clinical and microbiologic data were obtained from a total of 66 patients (33 in each group). Subgingival plaque samples were analysed with a semi-quantitative PCR analysis.

Results: A statistically significant difference, but with little clinical significance, was observed in gingival conditions between the two groups, probably due to the worse home control hygiene of DG patients. The prevalence and levels of *Aggregatibacter actinomycetemcomitans, Eikenella corrodens* and *Fusobacterium nucleatum/periodonticum* were statistically higher in samples from DG than GI patients. In multivariate regression models, the subgingival colonization of *Aggregatibacter actinomycetemcomitans* and *Fusobacterium nucleatum/periodonticum* was not statistically associated with DG, whereas, high levels of *Eikenella corrodens* were associated with a 13-fold increased odds for DG.

Conclusions: Microbiologic differences were found in subgingival plaque between DG and GI patients. This may suggest a possible association between periodontal pathogens and DG.

**Key words:** cross-sectional study; desquamative gingivitis; PCR; *Aggregatibacter actinomycetemcomitans; Fusobacterium nucleatum; Eikenella corrodens.*

Gingival injuries in autoimmune/dermatological diseases are usually characterized by diffuse erythematous zones, blisters, erosions, and ulcerations, mainly located on the attached gingiva and/or palatal mucosa; the occurrence of epithelial desquamation, erythema, and erosive lesions on the gingival tissue is described in literature as “desquamative gingivitis” (DG).[1-3]

A wide range of disorders can manifest as DG, although the two more common are oral lichen planus (OLP) and mucous membrane pemphigoid (MMP).[2,3]

It has been reported that DG could play a role in increasing the long-term risk for periodontal tissue breakdown at specific sites, however there is scarce evidence to support this.[4-6] Quite recently, we showed that patients diagnosed with MMP have higher levels of gingival inflammation and periodontal parameters than healthy control patients,[1] and similar data were also observed for cases of gingival OLP.[7] Authors detailed that the worse periodontal status in DG patients, when compared with healthy controls, was attributable to substantial differences in home oral hygiene.[1,7]

The causal association of anaerobic Gram-negative bacteria and periodontal infections is documented, and different periodontal pathogens have been detected in patients from different age and ethnic groups, both in subgingival and supragingival plaque samples, to find a putative association with severity and progression of periodontal diseases.[8,9] Although present in smaller number in healthy periodontal sites, pathogenic species tend to increase as a healthy periodontal condition shifts to a diseased periodontal status.[10] This tendency was demonstrated in previous papers in which the authors compared the microbiota of healthy, gingivitis and initial periodontitis sites.[11,12]

To date, the role of periodonto-pathogenic microorganisms in cases of DG has not been fully clarified, with only 2 studies, with low sample sizes and no suitable control group, addressing this issue.[9,13]

Therefore, the aim of this study was to investigate the prevalence of detection of 11 periodonto-pathogenic microorganisms in DG cases and to compare it with microbiologic status of control patients affected by plaque-induced gingivitis (GI).

## MATERIALS AND METHODS

### *Description of the Study Population*

This cross-sectional study was conducted at the University of Turin, C.I.R. Dental School, Department of Surgical Sciences, University of Turin from January to June 2016. Participants were recruited consecutively among Caucasian patients resident in Piedmont region, Northwest Italy, and attending the Oral Medicine Section and the Periodontology Section of the C.I.R. Dental School. Each participant accepted and signed an informed consent form (approved by the local ethics committee of the "AOU Città della Salute e della Scienza", Turin, Italy: No.1015/2016) and read the Helsinki Declaration before entering the study.

Participants in the DG group had:

-Histological diagnosis of OLP on the basis of WHO criteria:[14] hyperkeratosis of the superficial epithelial layers, vacuolar degeneration of the germinative layer of the epithelium and band-like sub-epithelial lymphocytic inflammatory infiltrate.

-Histopathological diagnosis of MMP, which revealed the sub epithelial blistering process, also confirmed by direct immunofluorescence analysis.

-Presence of signs of gingival lesions related to the previous 2 autoimmune disorders, detailed as follow: white lesion; and/or mild erythema (< 3 mm from gingival margins); and/or bulla, or marked erythema (> 3 mm from gingival margins); and/or erosion or ulcer.[15]

The exclusion criteria were:

-Presence of histological signs of dysplasia;

-Therapy for OLP and MMP in the 3 months prior to the study.

The second group of patients presented with GI, without any history of DG or other oral signs related to OLP or MMP, and good general health. The patients were selected according to the clinical and radiographic criteria proposed by the 1999 International Workshop for the Classification of Periodontal Diseases and Conditions.[16] The inclusion criteria were no clinical evidence of periodontitis [with probing depth (PD) ≤ 3 mm and no attachment loss caused by periodontal destruction], and bleeding on probing (BoP) at sites ≥20%.

Individuals were excluded from either group for use of antibiotics in the past 3 months, etiological periodontal therapy in the past 6 months before the sampling and recording, smoking habits, pregnancy and lactation.

***Clinical Evaluation***

All the study participants received an oral examination performed by two calibrated and experienced clinicians (P.G.A, F.Ro), together with a comprehensive periodontal examination for the whole dentition as previously reported.[1]

To perform the examiners calibration, periodontal examination of 8 non-study patients was repeated after 2 days showing intra and inter-examiners

reproducibility scores higher than 0.85 for PD (intra-class correlation coefficient) and higher than 0.90 for clinical attachment level (CAL).

The following parameters were recorded: full-mouth plaque scores (FMPS); full-mouth bleeding score (FMBS); PD, defined as the distance between the free gingival margin and the bottom of the gingival sulcus; gingival recession (REC), recorded as the distance from the free gingival margin to the cemento-enamel junction (CEJ); CAL, calculated as the sum of PD and REC; tooth mobility; molar furcation involvement; and number of missing teeth, determined by subtracting the number of teeth present from 32.

Full-mouth clinical periodontal measurements were recorded on six sites per tooth (mesiobuccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual, and disto-lingual), using a 1-mm marked periodontal probe[§] and rounded to the nearest mm. A standardized digital sheet was used for recording the periodontal parameters as well as the presence and the exact location (site by site) of DG lesions.

***Sampling of Subgingival Plaque***

Subgingival plaque samples were taken from 4 periodontally affected sites, one in each quadrant, with signs of gingival inflammation (BoP positive) and a PD of ≤ 3 mm, in both groups. The deepest site in each quadrant was selected.[17] In patients with DG, sampled sites must also present clinical evidence of DG lesions.

Before sampling, supra-gingival deposits were carefully removed with sterile curettes[‖] and the sites were isolated with sterile cotton rolls and gently air-dried. Subgingival microbial samples were obtained by inserting two

[§]PCPUNC15, Hu-Friedy®, Chigago, IL, USA
[‖]Hu-Friedy®, Chigago, IL, USA

standardized No. 30 sterile paper points into the bottom of the selected sites for 20 s. For each patient, all paper points were pooled into a sterile Eppendorf tube and stored for at maximum 3 days until DNA extraction.[18]

***Microbiologic Evaluation***

For microbiologic analysis of pooled samples a commercially available PCR DNA probe test kit[¶] aiming at the combined identification of eleven periodonto-pathogenic bacterial species was used, which is based on the DNA strip technique.[19] The following species were analysed: *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*, *Treponema denticola*, *Parvimonas micra*, *Fusobacterium nucleatum/periodonticum*, *Campylobacter rectus*, *Eubacterium nodatum*, *Eikenella corrodens*, and *Capnocytophaga spp.* (*C. gingivalis, C. ochracea, C. sputigena*). The microbiologic analysis was performed at the Division of Microbiology and Virology, A.O.U. CDSS-Molinette Hospital, Turin, Italy.

*DNA extraction*

The samples were lysed by adding 200 µl Lysis Buffer solution and then incubated for 30 min at 56°C. After centrifugation for 5 min at full speed, the lysate was collected for DNA extraction. The bacterial DNA was extracted and purified by an automated system[#], based on separation with magnetic beads. The final elution volume was 90 µl.

*DNA amplification*

For each sample, two separate multiplex amplification reactions were performed with biotinylated primers. In the amplification mix 2.5 µL of DNA

[¶]Micro-IDent®plus11, Hain Lifescience GmbH, Nehren, Germany
[#]QIAsymphony System, Quiagen, Hilden, Germany

were added. PCR cycling was carried out in a thermocycler[**]. The cycling conditions comprised an initial denaturation step at 95 °C for 5 min, 10 cycles at 95 °C for 30 s and at 58 °C for 2 min, 20 cycles at 95 °C for 25 s, at 53 °C for 40 s and at 70 °C for 40 s, and a final extension step at 70 °C for 8 min. Positive and contamination controls were included in each batch of samples.

*Hybridization*

The amplicons from each sample were hybridized to nitrocellulose strips containing specific probes for periodontal pathogens. Briefly, the biotinylated amplicons were denatured and incubated at 45 °C with hybridization buffer. After PCR products had bound to their respective complementary probe, a highly specific washing step removed any not specifically bound DNA. Streptavidin-conjugated alkaline phosphatase was added, the samples were washed, and hybridization products were visualized by adding a substrate for alkaline phosphatase. Results could be obtained after 5 h.

*Evaluation*

Two variables were analysed for each periodontal pathogen: prevalence (i.e., detection of the pathogen or no detection) and semi-quantitative classification (values 0 to 4) as follows:

- (value 0) = $x < 10^3$ cells

+ (value 1) = $10^3 < x < 10^4$ cells

++ (value 2) = $10^4 < x < 10^6$ cells

+++ (value 3) = $10^6 < x < 10^7$ cells

++++ (value 4) = $x > 10^7$ cells

If detected as value 0, species was considered to be absent.

[**]SeeAMP, Seegene Inc., Seoul, Korea

***Statistical Analyses***

Data were described as absolute frequency and percentage or mean and standard deviation for qualitative and quantitative variables, respectively.

Bacteria values were treated both as continuous and categorical variables (dividing into three categories: “absent”, value = 0; “low presence”, value = 1 or 2; “high presence”, value = 3 or 4).

Differences between DG and GI groups and among different types of non-plaque-induced gingivitis were tested using the Wilcoxon sum rank test, for quantitative variables, or Chi-square or Fisher exact test, for qualitative variables, as appropriate.

Odds Ratios (OR) and 95% Confidence Interval (95% CI) were obtained from univariate and multivariate logistic regression analyses [adjusted for age, BMI (body max index), FMPS, FMBS, number of missing teeth and PD] to model the relationship between DG and bacterial exposure. No adjustment for CAL was performed due to its collinearity with PD. A statistical software program was used for the data analysis[††] and a 5% level of significance was considered for two-tails tests.

**RESULTS**

A total of 88 individuals were initially screened for eligibility but 14 patients were excluded because of a diagnosis not fully conform to the criteria of the study (6 DG and 8 GI patients), and 8 patients were excluded because they refused to sign the informed consent. Finally, a total of 66 patients were enrolled in the present study (33 in each group).

[††]SAS version 9.2, SAS Institute, Inc, Cary, NC

Demographic characteristics and full-mouth periodontal parameters are shown in Table 1. In the DG group, the mean age was 68.85 years (± 13.55), showing a high percentage of affected patients in the $6^{th}$ (10 patients), $7^{th}$ (8 patients) and $8^{th}$ decades (8 patients). As expected, GI patients were slightly younger than DG cases because of the nature of the plaque-induced gingivitis, which usually affect young to middle-aged individuals, and of the inclusion criteria. DG patients presented with significantly higher mean FMPS ($P < 0.0001$), FMBS ($P < 0.0001$), and PD ($P < 0.0001$) values compared with the control group. However, these differences, probably due to the worse compliance of DG patients in daily oral hygiene, had little clinical significance.

The detection frequency and levels of all tested bacteria in both groups are summarized in Table 2.

The PCR results showed that at least one species of periodonto-pathogens was found in each patient. Considering the total sample, *Parvimonas micra* and *Campylobacter rectus* were the only 2 species less frequently detected in DG than in control patients. *Aggregatibacter actinomycetemcomitans* and *Eikenella corrodens* were found in statistically higher prevalence and levels in subgingival samples from DG than GI patients. For *Fusobacterium nucleatum/periodonticum,* the highly positive samples (semiquantitative classification of bacterial counts: values 3 and 4) were higher in the DG group. Table 3 shows crude and adjusted $OR_s$ for 5 selected bacteria (those with positive values of < 0.07; see Table 2). After adjustment for age, BMI, FMPS, FMBS and number of missing teeth, the subgingival colonization of *Aggregatibacter actinomycetemcomitans* and *Fusobacterium nucleatum/periodonticum* was not yet significantly associated with DG,

whereas the OR of having *Eikenella corrodens* at levels ≥ $10^6$ in subgingival plaque and DG was 15.70. When PD was also entered in the multivariate regression model, the significantly positive association between DG and *Eikenella corrodens* was still present (adjusted OR=12.78).

Table 4 describes the differences in bacteria frequency detection and levels in the DG group in relation to the underlying autoimmune pathology. It comprised 19 patients with OLP and 14 patients with MMP. No statistical difference in the microbiologic profile was detected between them.

***DISCUSSION***

The current body of literature lacks controlled studies that have investigated the association between the main periodontal pathogens in subgingival biofilm samples of patients with desquamative and plaque-induced gingivitis. To the best of the authors' knowledge, this is the first study addressing this issue.

Previously, Lo Russo and co-workers[7] analysed a series of patients affected by DG (8 OLP and 4 MMP patients) in order to evaluate the relation patterns among clinical parameters relevant to plaque induced periodontitis and periodontal microbiologic data. They reported a significant correlation for the presence of gingival OLP lesions and *Aggregatibacter actinomycetemcomitans*, highlighting the need for further investigations of periodontal clinical and microbiologic aspects of disorders causing DG, to better clarify their potential interference with plaque-related periodontitis. Additionally, Ertugrul and co-workers[13] reported higher levels of detection of *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia* and *Treponema denticola* in OLP than in non-OLP patients, arguing that this greater tendency to be infected by

periodontopathogenic microorganisms may help identify the importance of such bacterial species in the progress of periodontal diseases in OLP. In spite of the lack of a control group, these two studies were useful for identifying the subgingival microbial profile in patients with oral autoimmune diseases.

In the present study, a PCR technique was used to detect 11 periodontal pathogens in subgingival pooled samples of patients with desquamative or plaque-induced gingivitis. The pooling of plaque sample increases the probability of detecting existing pathogens.[18] It was demonstrated that sampling of the deepest pocket in each quadrant increases the bacterial counts compared to separate samples when RNA probe or PCR is used.[20] In addition, pooled analysis of subgingival plaque samples from four sites is as good as from six sites to describe subgingival periodontal pathogens on the patient level.[21]

In this study *Fusobacterium nucleatum* was found in statistically higher levels in subgingival samples from DG than GI patients, followed by *Eikenella corrodens* and *Aggregatibacter actinomycetemcomitans* which displayed also higher detection frequency in the DG cases (66.7% and 24.2%, respectively). In spite of the high detection rate and counts for the other tested bacteria in DG lesions, except for *Parvimonas micras*, no statistically significant differences could be observed when compared with healthy GI patients. Data from previous studies are conflicting. While a study detected *Aggregatibacter actinomycetemcomitans* in all the subgingival samples taken from DG-positive sites in OLP patients[7], another study failed to detect it[10]. Only a study reported a prevalence of 48.1% for *Eikenella corrodens* at oral sites with asymptomatic non-ulcerative mucosal lesions.[22]

Compared to the numbers of studies describing the microbiota of periodontitis, there are relatively few comprehensive studies describing the microbiota of gingivitis in healthy adults. The high prevalence detected for *Fusobacterium nucleatum* (78.8%) and *Campylobacter rectus* (57.6%) was in line with the values observed in other studies.[23-26] Published data have shown that *Fusobacterium nucleatum* is one of the most abundant Gram negative anaerobes in mature supragingival and subgingival plaques of both healthy and periodontitis patients.[27] These results confirm its ubiquity.

Among the examined bacteria, the coinfection of red complex species, *Tannerella forsythia, Treponema denticola* and *Porphyromonas gingivalis*, was detected in 18.2% of patients and was in the range defined by other authors (6% - 26%).[24]

The less prevalent microorganism was *Aggregatibacter actinomycetemcomitans* affecting only one GI patient. This result was similar to that obtained in other studies performed in Europe[12,26], but markedly lower than studies in American countries.[23,28,29] It is possible that the differences in the detection percentages found in our study for this important periodontal pathogen may be due, in part, to racial and geographical differences.

It is worth noting that significant differences were observed between DG and GI group in relation to the percentage of sites with plaque and bleeding on probing and PD. The periodontal parameters in DG patients are similar to those reported in previous studies from our group, confirming that their periodontal status is consistently worse than controls, regardless of their healthy or inflamed periodontal tissues.[1,30,31] Patients with DG, resulting from OLP or MMP, may find it difficult to perform effective oral hygiene practices

due to the clinical symptoms associated with the lesions; henceforth they present more severe gingival inflammation and periodontal breakdown.

In order to distinguish DG from GI, based on microbiologic parameters, multivariate logistic regression analysis was performed after simultaneously adjusting for confounding factors, including demographic and periodontal parameters. Due to the collinearity between CAL and PD, the model was adjusted only for PD values as they markedly influence the subgingival environment; mean values of subgingival species differ at sites of different PD.[9] Collinearity can seriously distort the interpretation of a model. Generally, if some of the collinear variables are redundant, in terms of providing no extra useful information, or are simply duplicate measurements of the same variable, a solution is to remove one of these variables from the model.[32]

The multivariate model showed a statistically significant association between high subgingival levels of *Eikenella corrodens* and DG manifestations. Individuals with ≥ $10^6$ *Eikenella corrodens* cells in subgingival plaque seemed to have a 13-fold increased odds for DG. There was a crude positive association also between DG and both *Aggregatibacter actinomycetemcomitans* and *Fusobacterium nucleatum*; however, the statistical significance disappeared in the multivariate model.

It is known that periodontal pathogens exist as complex in the subgingival environment.[33] *Eikenella corrodens* makes one complex with *Campylobacter concisus* and *Aggregatibacter actinomycetemcomitans.* Another study showed that existence of *Eikenella corrodens* was associated with *Capnocytophaga spp* or *Aggregatibacter actinomycetemcomitans*.[34] The environment as a complex may be favourable to harbouring *Eikenella*

*corrodens* in the subgingival biofilm.

Moreover, previous findings showed that *Eikenella corrodens* may participate in the degradation of extracellular matrix components by activating the fibroblasts to produce enzymes such as gelatinases.[35] Ertugrul and co-workers also found higher levels of gelatinases in crevicular fluid and gingival tissues of OLP patients, suggesting that this increased enzyme levels in combination with poor oral hygiene increase periodontal tissue breakdown.[36] The present microbiologic findings further provide scientific grounds for the promotion of professional oral hygiene sessions at more strict intervals in DG than in GI patients.

Limitations of the present study are the limited sample and the wide interval confidence of some of the associations detected. However, it is important to point out that OLP and MMP are rare inflammatory and mucocutaneous disease, affecting less than 1% of the population, and DG manifestations occur only in half of those patients.

**CONCLUSIONS**

There is a considerable deficiency in present knowledge of the pathogenesis and risk factors of DG. In this cross-sectional study, microbiologic differences were found in subgingival plaque between autoimmune and plaque-induced gingivitis. This may suggest a possible association between periodontal pathogens and DG, as well as provide new knowledge to the field of oral manifestations of autoimmune systemic diseases. For this reason, affected patients should be advised regarding the possible risk of periodontal complications and should be informed to have routinely dental check-ups to avoid a deterioration of the condition.

Because of the cross-sectional data, no conclusions regarding causal relationship between periodontal bacteria and DG manifestations can be drawn on the ground of the present study. Future larger prospective studies could possibly give more valuable information.

## Author Disclosure Statement

No competing financial interests exist.

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