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Subgingival biodiversity in subjects with uncontrolled type-2 diabetes and chronic periodontitis

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Background and Objective: There is a bidirectional relationship between periodontal disease and type-2 diabetes mellitus (DM). Inflammatory mediators may negatively affect glycemic control, and increased glucose levels and resultant glycation end-products may alter the host response against bacterial infection. However, no agreement has been reached regarding the effect of DM on periodontal subgingival microbiota. Therefore, the purpose of the present study was to compare the subgingival biodiversity in deep periodontal pockets of subjects with chronic periodontitis and either uncontrolled type-2 diabetes or no diabetes using 16S rRNA gene cloning and sequencing.

Material and methods: Twelve subjects with uncontrolled type-2 diabetes (glycated hemoglobin > 8%) and eleven nondiabetic subjects presenting severe and generalized chronic periodontitis were selected. Subgingival biofilm from periodontal pockets > 5 mm were assessed using the 16S rRNA gene cloning and sequencing technique.

Results: Significant differences were observed in subgingival microbiota between diabetic and nondiabetic subjects. Diabetic subjects presented higher percentages of total clones of *TM7, Aggregatibacter, Neisseria, Gemella, Eikenella, Selenomonas, Actinomyces, Capnocytophaga, Fusobacterium, Veillonella* and *Streptococcus* genera, and lower percentages of *Porphyromonas, Filifactor, Eubacterium, Synergistetes, Tannerella* and *Treponema* genera than nondiabetic individuals ($p < 0.05$). Moreover, some phylotypes, such as *Fusobacterium nucleatum, Veillonella parvula, V. dispar* and *Eikenella corrodens* were detected significantly more often in diabetic subjects than in nondiabetic subjects ($p < 0.05$).

Conclusion: Subjects with uncontrolled type-2 diabetes and chronic periodontitis presented significant dissimilarities in subgingival biodiversity compared with nondiabetic subjects.

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Chronic periodontitis is caused by complex microbial communities present in the subgingival environment. Moreover, subjects presenting with diabetes mellitus (DM), particularly with a poorly controlled glycemic status, have a more severe and generalized periodontitis (1). Hyperglycemia and the resultant formation of advanced

glycation end-products, one of the pathways that is thought to lead to the vascular complications of DM, seem also to be involved in the pathophysiology of periodontitis in DM subjects (1), being associated with the imbalance of pro-inflammatory and anti-inflammatory mediators (2,3) and osteoclastogenesis-related factors (4).

Previous studies have focused on the role of periodontal infection and the composition of dental biofilm in patients with DM (5–7). However, the results are still inconclusive. Although some studies have shown that the microbiota associated with DM do not differ substantially from the microbiota of Non-DM patients (5–7), there is evidence that DM subjects may harbor higher levels of some bacterial species than do Non-DM subjects (8).

A plausible explanation for differences in the biodiversity between DM subjects and Non-DM subjects may be that hyperglycemia could alter the oral environment (e.g. saliva and gingival crevicular fluid) and favor the colonization of some specific species. It has been established in DM subjects that the glucose concentration in gingival crevicular fluid is correlated with the glucose concentration in serum (9). Therefore, glycemia might affect the composition of periodontal microbiota, as different levels of plasma glucose can result in different availabilities of this carbohydrate in the subgingival area, which can alter environmental and microbiota profiles. Therefore, elevated levels of glucose in saliva and in gingival crevicular fluid might increase the number of bacteria associated with caries and periodontal disease in the saliva and the supragingival and subgingival biofilm of patients with DM (8).

A previous study showed that *Capnocytophaga* species are more prevalent in the microbiota of DM patients (10), whereas Campus *et al.* (11) found a positive association between DM and the presence of *Porphyromonas gingivalis* and *Tannerella forsythia*. Recently, our research group found a higher prevalence of *T. forsythia* in the periodontal pockets of DM patients than in the periodontal pockets of Non-DM patients, but no differences were found in the prevalence of *Aggregatibacter actinomycetemcomitans* and *P. gingivalis* (12). In addition, we showed that subjects with type-2 diabetes and chronic periodontitis had a high prevalence of *Candida* spp. in periodontal pockets. Also, *Candida dubliniensis* was more commonly present than *Candida albicans* in DM subjects when compared with Non-DM subjects (13).

Despite some evidence regarding the subgingival microbiota of DM subjects, to date, it is still unclear if, and to what extent, an altered microbiota could contribute to an increased incidence or severity of periodontal disease in subjects with type-2 DM. Virtually no data are available regarding subgingival colonization by unculturable microorganisms in DM patients with chronic periodontitis.

Also, to the best of our knowledge, none of the recent molecular fingerprinting techniques have been used to compare the microbiota of DM patients with that of Non-DM patients. Instead, 16S rRNA gene cloning and sequencing, a technique that examines several host-associated ecosystems, has been employed for this purpose. The presence of large tracts of variable sequences within the 16S rRNA gene provides unique bacterial 'signatures' that allow for accurate identification of bacteria, whereas sequencing a finite number of clones from each sample provides quantitative information on the relative abundance of each organism within a community. Therefore, the aim of the present study was to determine the subgingival biodiversity of subjects with chronic periodontitis, either with or without uncontrolled type-2 DM, using the 16S rRNA gene cloning and sequencing technique.

Material and methods

Patient selection and groups

Type-2 DM and Non-DM subjects presenting generalized chronic periodontitis were consecutively selected from the population referred to the Periodontal Clinic of Guarulhos University and Piracicaba Dental School from July 2007 to February 2010. All eligible subjects were provided with detailed information on the nature, potential risks and benefits of their participation in the study and signed an informed consent form. The study protocol was previously approved by Guarulhos University's Ethics Committee on Clinical Research. All patients received a complete periodontal examination, including full-mouth periodontal probing, a radiographic examination and complete anamnesis.

The study inclusion criteria were: a diagnosis of chronic periodontitis, according to the criteria of the 1999 International Classification (14); the presence of at least 15 teeth; ≥ 30% of the sites and at least nine teeth presenting a probing pocket depth of ≥ 5 mm with bleeding on probing (with two or more of these teeth having a probing pocket depth of ≥ 7 mm); uncontrolled type-2 DM, determined by a glycated hemoglobin (HbA1c) concentration of > 8% (15) (in the group of subjects with DM); and age > 35 years. Patients were excluded if they were pregnant or lactating, required antimicrobial premedication in order to complete the periodontal examination, were suffering from any other systemic diseases (e.g. cardiovascular, pulmonary, liver or cerebral diseases), had received antimicrobial treatment in the previous 3 mo, were taking long-term anti-inflammatory drugs and/or had received a course of periodontal treatment within the last 6 mo.

The patients were placed into one of two groups: Non-DMs (patients diagnosed with generalized chronic periodontitis who were in good general health); and DM (patients diagnosed with generalized, chronic periodontitis who had a diagnosis of uncontrolled type-2 DM for at least the past 5 years, which was confirmed by a physician's examination).

Clinical examination

The following clinical parameters were assessed by calibrated examiners (TMB and VRS; interexaminer intraclass correlation = 92% in clinical attachment level): full-mouth plaque index, according to Ainamo & Bay (16) and

full-mouth bleeding score, according to Muhlemann & Son (17). These indices were calculated after dichotomously assessing the presence of dental biofilm at the site or bleeding on probing from the bottom of the pocket when probing with a manual probe (PCP-15; Hu-Friedy, Chicago, IL, USA). In addition, a full-mouth clinical examination was performed to evaluate the following parameters: probing pocket depth (the distance from the bottom of the pocket to the margin) and clinical attachment level (the distance from the bottom of the pocket to the cemento–enamel junction).

Glycemic status

Peripheral blood samples were collected from DM subjects for assessment of the levels of HbA1c (using high-performance liquid chromatography) and fasting plasma glucose (using the glucose oxidase method).

Subgingival biofilm analysis

Subgingival biofilm collection— After a full-mouth examination, five noncontiguous pockets that presented a probing pocket depth of > 5 mm, a clinical attachment level of > 6 mm and bleeding on probing were randomly chosen. For all potential sites with these characteristics, a paper code was generated and placed in an opaque envelope. Afterward, through a paper draw, five sites were selected to undergo subgingival biofilm sampling and analysis. In order to avoid any bias, all teeth that presented furcation lesions, endodontic pathology and/or extensive crown destruction were excluded from the draw. Following careful removal of the supragingival biofilm, the areas were washed with a water spray, isolated with cotton rolls and gently dried.

A sterile paper point (#35; Tanari, Manaus, AM, Brazil) was inserted into the bottom of the periodontal pocket for 30 s. The paper points were placed in sterile tubes containing 300 μL of reduced transport fluid. They were then placed separately in plastic tubes containing 0.01 M Tris–EDTA solution, pH 8. DNA collection and extraction were performed as previously described by Casarin *et al.* (18). For each patient, samples from the selected sites were pooled together to permit cloning and sequencing of the 16S rRNA gene.

Cloning and sequencing— First of all, the 16S rRNA gene was amplified using a universal primer set (27f and 1492r), as previously described (19). Cloning procedures were performed using a TOPO-TA cloning kit (Invitrogen, San Diego, CA, USA). Initially, the amplicons generated from universal amplification were cloned into *Escherichia coli*. Then, *E. coli* containing the amplicons were seeded onto LuriaBertani plates and the bacterial colonies obtained after culture were transferred to Luria–Bertani broth media (LB-Top Agar; Sigma-Aldrich, Buchs, Switzerland). Vectors were extracted, purified (QIAprep Spin Miniprep Kit®; Qiagen, Quebec, QC, Canada) and sequenced (CHUQ, Centre Hospitalier Universitaire de Québec, Université Laval, Québec, QC, Canada).

After sequencing, a partial sequence of 600 bp was generated. The sequences were initially aligned, and a similarity matrix was constructed from the alignments using the method described by Jukes & Cantor (20). Phylogenetic trees were constructed via the neighbor–joining method. Sequences were compared using the HOMD database (21), with a level of 98.5% sequence identity as the cut-off. Sequences presenting similarity, within a genus, of ≥ 98% were considered to be the same species.

Data management and statistical analysis

Clinical parameters were analyzed using the Student's *t*-test (for baseline intergroup comparisons). The sample size was based on previous studies (12,13,22). For microbiological data, a variance-stabilizing transformation described in Shchipkova *et al.* (22) was used, promoting a normal distribution of the data that was previously tested using the Shapiro–Wilk test.

The proportion (p) of each species in the periodontal biofilm community of each subject was expressed as:

$$X = \sin -1(\sqrt{p})$$

A two-tailed Student's *t*-test was used to compare the means of this transformed variable (X) between DM and Non-DM subjects. A chi-square test was used to test for the presence or absence of species and genera (release 9.02; SAS Institute Inc., Cary, NC, USA). A 5% level of significance was used in clinical and microbiological data analyses.

Results

Table 1 shows the characteristics of the DM (n = 12) and Non-DM (n = 11) subjects included in this study. No differences in gender, age or full-mouth periodontal clinical parameters (clinical attachment level and probing pocket depth) were noted ($p > 0.05$). DM subjects had greater biofilm accumulation and a lower bleeding index than Non-DM subjects ($p < 0.05$). Moreover, the levels of HbA1c (13.8 ± 2.2%) and fasting plasma glucose (268.2 ± 69.4 dg/μL) highlighted the poor glycemic control of DM subjects evaluated in this study.

In total, 2012 clones were identified (representing a mean number of clones per subject of 87.4, range: 80–101 clones), representing 79 species/phylotypes in Non-DM subjects and 113 in subjects with uncontrolled type-2 diabetes. As shown in Table 2, there was a difference in the distribution of phyla between DM and Non-DM subjects. DM subjects showed higher percentages of total clones of *Actinobacteria, Deferribacteres, Fusobacteria, Proteobacteria* and *TM7*. Conversely, they presented a lower percentage of *Bacteroidetes, Spirochaetes* and *Synergistetes* ($p < 0.05$). With regard to the culture status of clones, no statistically significant difference was detected ($p > 0.05$).

Figure 1 shows the distribution of genera in Non-DM and DM subjects. The genera that were statistically highly detected in Non-DM subjects, such as *Porphyromonas, Filifactor, Eubacterium, Synergistetes, Tannerella* and *Treponema* ($p < 0.05$) are shown on the left of the graph. The most prevalent genera among subjects with DM were *TM7, Aggregatibacter, Neisseria, Gem-*

Table 1. Characteristics of subjects. [mean ± standard deviation (range)]

Characteristic	Nondiabetic (n = 11)	Diabetic (n = 12)
Age (years)	47.8 ± 4.9 (41–62)	51.8 ± 11.3 (40–70)
Gender (% male)	55	42
Full-mouth plaque index (%)*	82.6 ± 3.1	90.5 ± 16.9
Full-mouth plaque score (%)*	78.4 ± 4.1	48.9 ± 31.3
Probing depth (mm)	3.5 ± 0.8	3.7 ± 1.0
Clinical attachment level (mm)	4.1 ± 0.9	4.5 ± 1.0
Sites with periodontal probing depth ≥ 5 mm (%)	28.9 ± 8.5	24.6 ± 7.5
HbA1c (%)	–	13.8 ± 2.2 (10–17)
Fasting plasma glucose (dg/μL)	–	268.2 ± 69.4 (200–35)

HbA1c, glycated hemoglobin.
*Statistically significant difference (Student's t-test, $p < 0.05$).

Table 2. Distribution (percentage of total clones) regarding phylum and culture status (%) in diabetic and nondiabetic subjects

	Nondiabetic	Diabetic
Phylum		
*Actinobacteria**	2.1	8.1
*Bacteroidetes**	27.9	11.9
*Deferribacteres**	0	1.4
Firmicutes	46.5	52.9
*Fusobacteria***	3.8	8.7
*Proteobacteria**	3.5	14.3
*Spirochaetes**	7.2	1.7
*Synergistetes**	9.1	0.2
*TM7***	0	0.8
Culture status		
Cultured	73	82

Statistically significant difference between groups (chi-square test: **$p < 0.001$, *$p < 0.0001$).

ella, Eikenella, Selenomonas, Actinomyces, Capnocytophaga, Fusobacterium, Veillonella and *Streptococcus* ($p < 0.05$) and are shown on the right of the graph.

The TOP-15 phylotypes (i.e. the phylotypes with the highest values in DM and Non-DM subjects, considering the transformed X-value) are described in Table 3. The prevalence of several species differed between DM and Non-DM subjects. Subjects with uncontrolled type-2 DM had higher percentages of total clones of *Streptococcus mitis, Eikenella corrodens, Veillonella parvula* and *Veillonella dispar* ($p < 0.05$) than did systemically healthy individuals. Higher percentages of *Fusobacterium nucleatum* were also observed in subjects with DM than in Non-DM subjects, whereas higher percentages of *P. gingivalis, T. forsythia, Filifactor alocis* and *Synergistetes* clone BH017 were found in the periodontal pockets of Non-DM subjects ($p < 0.05$).

Discussion

The bidirectional link between DM and periodontal disease has been extensively described since the early 1990s, although no conclusion has been reached regarding how it occurs. The present study hypothesized that specificities in the microbial profile could also play an important role in the alterations promoted by DM on periodontitis development. Therefore, the microbial profiles of DM and Non-DM subjects presenting with chronic periodontitis were screened. In general, the results showed evident dissimilarities between DM and Non-DM subjects that could directly influence the severity of periodontitis in subjects with uncontrolled DM.

In agreement with the findings of the present study, differences in subgingival microbial profiles between DM and Non-DM subjects have been previously reported (10,23). Sandholm *et al.* (23) found more gram-negative anaerobes in DM subjects than in Non-DM subjects. Moreover, some studies focused on the role of glycemic control in the subgingival frequency of certain organisms. Recently, Casarin *et al.* (24) showed that glycemic control influenced the prevalence of herpes viruses, especially Epstein–Barr virus-1, in the periodontal pockets of DM individuals. This finding was consistent with that of Makiura *et al.* (25), who found a relationship between *P. gingivalis* colonization and different levels of

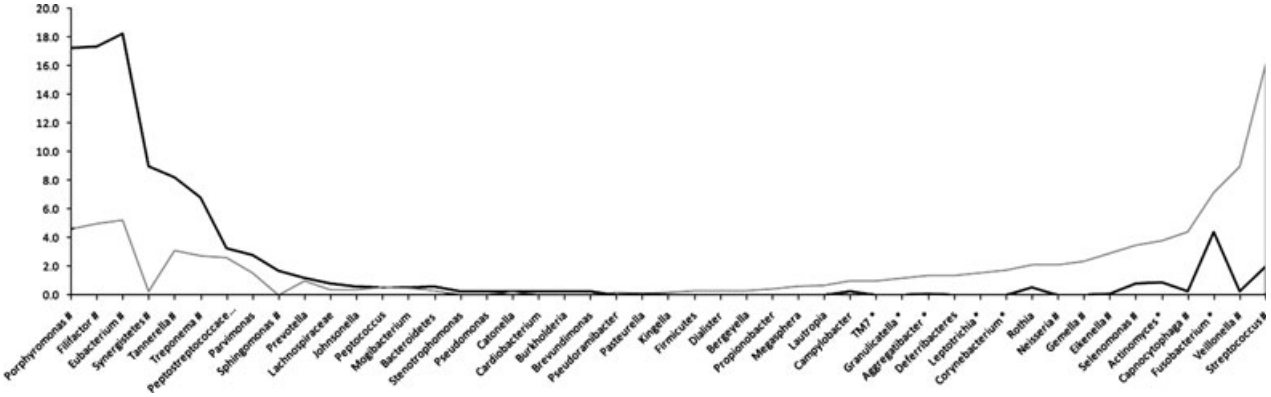

Fig. 1. Distribution (percentage of total clones) of types of bacterial genus in diabetic and nondiabetic subjects. Black line, nondiabetic subjects; gray line, diabetic subjects (Student's t test, *$p < 0.05$, $^{\#}p < 0.01$).

Table 3. Top-15 phylotypes in diabetic and nondiabetic subjects

	Phylotype	Nondiabetic	Diabetic	*p**
1	*Filifactor alocis*	0.13 ± 0.04	0.05 ± 0.06	0.00
2	*Porphyromonas gingivalis*	0.09 ± 0.08	0.02 ± 0.04	0.01
3	*Eubacterium [XI][G-5] saphenum*	0.10 ± 0.07	0.00 ± 0.01	0.00
4	*Streptococcus mitis*	0.00 ± 0.00	0.07 ± 0.05	0.00
5	*Synergistetes [G-3] sp.|Oral Taxon 360|Clone BH017*	0.04 ± 0.04	0.00 ± 0.02	0.00
6	*Peptostreptococcaceae [XI][G-4] sp.|Oral Taxon 369|Clone MCE10_174*	0.03 ± 0.04	0.02 ± 0.02	0.03
7	*Porphyromonas endodontalis|Oral Taxon 273|Clone BB13*	0.02 ± 0.04	0.02 ± 0.03	0.55
8	*Fusobacterium nucleatum*	0.04 ± 0.04	0.08 ± 0.05	0.05
9	*Veillonella parvula*	0.00 ± 0.00	0.06 ± 0.05	0.00
10	*Veillonella dispar*	0.00 ± 0.00	0.04 ± 0.05	0.01
11	*Eikenella corrodens*	0.00 ± 0.00	0.04 ± 0.16	0.01
12	*Rothia dentocariosa*	0.01 ± 0.02	0.07 ± 0.04	0.27
13	*Tannerella forsythia*	0.09 ± 0.05	0.04 ± 0.04	0.02
14	*Capnocytophaga gingivalis*	0.01 ± 0.04	0.03 ± 0.04	0.20
15	*Parvimonas micra*	0.02 ± 0.03	0.03 ± 0.04	0.11

Data represent mean ± standard deviation.
*Student's *t*-test ($p < 0.05$).

glycemic control. Aly *et al*. (26) and Nowakowska *et al*. (27) reported higher levels of *Candida* in subjects with poorly controlled diabetes than in subjects with well-controlled DM or in Non-DM subjects. In addition, high levels of HbA1c were positively correlated with the presence of *Streptococcus mutans* and *Lactobacilli*, as well as with the occurrence of caries, in DM patients.

However, it is important to note that the aforementioned studies used microbiological techniques to identify only a few species. Recently, nontargeted techniques have been used to describe the subgingival microbiota of subjects with periodontitis (22,28,29). To the best of our knowledge, this is the first study to employ an open-ended technique (16S rRNA cloning) to identify the biodiversity of oral microorganisms in subjects with uncontrolled type-2 DM subjects. Previous studies used this methodology to provide representational information on previously unknown and unsuspected bacteria associated with periodontal health and disease (22,28,29). The data of the present study clearly showed that subjects with uncontrolled type-2 diabetes and chronic periodontitis harbored a significantly different microbiota in periodontal pockets compared with Non-DM subjects.

In the present study, *Capnocytophaga* spp. were more prevalent in DM subjects than in Non-DM subjects. In a sample of patients with type-1 DM, Mashimo *et al*. (10) found a high proportion of *Capnocytophaga* spp. They also reported that 30% of the patients harbored *A. actinomycetemcomitans* concomitantly. In the present study, the higher counts of *Capnocytophaga* spp. found in DM subjects confirmed the findings reported by Ciantar *et al*. (30) using PCR identification of cultured samples. *Capnocytophaga* spp. are saccharolytic organisms, some of which manifest an increased biomass and proteolytic potential when grown in elevated glucose conditions (31). This might explain the higher frequency of this species in DM subjects.

Moreover, DM individuals also had higher percentages of total clones of *Aggregatibacter, Fusobacterium* and *TM7* genera, as well as *E. corrodens*, disease-associated genus/species (32). *E. corrodens* has been associated with chronic (32,33) and aggressive (34) periodontitis. It has also been found to be closely associated with other bacteria that are often found in periodontitis (35,36).

In addition, high numbers of *Fusobacterium* spp., particularly *F. nucleatum*, were detected in DM subjects. This species has been frequently associated with periodontitis (32,33) and evidence shows that it can play a role in disease by providing the anaerobic environment that is necessary for the growth of pathogens (37).

Interestingly, in the present study, *TM7* clones, a genus composed entirely of nonculturable clones, was detected only in DM subjects (specifically, in five of 12 diabetic subjects, $p < 0.05$). Considering that this genus was previously associated with sites presenting periodontitis signs (32,33,38), its importance should be considered and studied in future analyses. Moreover, as this is the first study to harvest subgingival samples from a selected population of DM subjects, further studies should be conducted to better understand the specific role of *TM7* clones in periodontal breakdown, especially in the periodontitis related to DM.

Another interesting result of the present microbiological assessment was that DM subjects exhibited significantly higher percentages of clones of *Veillonella*, *Streptococcus* and *Neisseria* than did Non-DM subjects. These genera are reportedly abundant in biofilm associated with healthy periodontal conditions, and their levels decrease in the presence of disease (28,29). However, it is important to consider that the increased glucose content in serum, and subsequently in gingival crevicular fluid, of DM subjects could perhaps stimulate the selective growth of bacteria, facilitating the survival of facultative gram-positive fermenting bacteria, such as streptococci, lactobacilli and *Actinomyces* (9). This hypothesis could partially explain the higher percentage of these health-compatible genera in the subgingival environment of the DM subjects enrolled in the present study.

The consequences of these findings should be investigated further as gram-positive species could also promote immunological responses in periodontal tissues through their antigenic patterns. Several studies showed that teichoic acids, for example, are present in the cell wall of gram-positive species, being able to induce experimental periodontal disease in rats (39) and stimulate the release of inflammatory mediators in cell culture (40). Thus, the subgingival presence

of these genera could play a role in the progression and the severity of periodontal disease, particularly in a group at risk of infection, such as subjects with uncontrolled diabetes.

Surprisingly, in the present study some well-established periodontal pathogens, such as *T. forsythia* and *P. gingivalis*, were more prevalent in Non-DM subjects than in subjects with DM. A previous study, using PCR amplification, did not show a significant difference between DM and Non-DM subjects in the prevalence of *P. gingivalis* (41). Hintao *et al*. (8), using a DNA–DNA hybridization technique, identified some differences in supragingival microbiota between DM and Non-DM subjects. However, in the subgingival environment, no differences were found. Therefore, it may be suggested that other nonsuspicious and not-well-known phylotypes could represent a higher proportion of the subgingival microbiota of subjects with type-2 diabetes and play a more important role in periodontal breakdown than the well-recognized periodontal pathogens. Thus, the study of the microbiome in DM subjects with chronic periodontitis has an increased importance to precisely assess the subgingival bacterial profiles in these subjects. However, some limits of the present study should be considered.

The main limitation of our study was the number of subjects in each group. Only 12 subjects with uncontrolled type-2 DM and 11 Non-DM subjects were included. It is important to note that our inclusion criteria were very restrictive. Individuals from both groups had to present generalized, severe periodontal disease in at least 15 teeth. These criteria, especially the minimum number of teeth in subjects with type-2 DM, complicated the selection process. Moreover, specifically with respect to subjects with type-2 DM, we looked for subjects who presented uncontrolled disease with an HbA1c level of > 8%. Thus, several individuals who were preselected during a clinical examination did not present an altered level of HbA1c. As a result, they were excluded from the study. Another important factor that enhanced the difficulty of the process in including subjects with type-2 DM was the obligation that they must be free of any other systemic disease, as described in the exclusion criteria. Most of the individuals with type-2 DM presented some associated disease, such as hypertension, dyslipidemia and other conditions that caused them to be excluded from this analysis. These conditions result in a lengthy selection process (i.e. approximately 30 mo), especially with respect to individuals with type-2 DM, and a reduced number of individuals were included. We believe that the restricted inclusion criteria are an important step in studies that compare subjects with and without DM. In this vein, the results, although unedited, should be considered with caution and future studies ought to attempt to enroll a larger and different population in order to confirm our results.

The second point that should be considered as a study limitation is the fact that DM individuals presented a higher mean plaque index and older age. The older age could result in the worst-case periodontal condition, as it could reflect the accumulation of damage sustained by the periodontium over time (42). Accordingly, subjects with type-2 diabetes who are older than individuals who do not have type-2 DM, could be affected by this 'time-to-disease' process although, in the present study, clinical parameters were not significantly different between individuals with or without type-2 DM. Regarding plaque index, DM subjects presented a statistically higher accumulation of biofilm than Non-DM individuals. The role of biofilm as an etiologic agent of periodontal disease and the direct relationship between plaque accumulation and disease occurrence are well established details. So, the fact that the DM subjects presented a higher biofilm index could represent a different aspect of periodontitis development. However, in contrast, the bleeding index did not corroborate the plaque index. Although they presented a higher plaque index, individuals with type-2 DM had a lower bleeding index value. This unexpected result should be considered in future studies carried out to identify the possible significance of this difference in microbiological terms, although all pockets from which biofilm was harvested bled while being probed.

Therefore, within the limits of the present study, it could be concluded that subjects with uncontrolled type-2 diabetes showed differences in subgingival biodiversity in comparison with Non-DM subjects.

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References

1. Mealey BL, Rose LF. Diabetes mellitus and inflammatory periodontal diseases. *Curr Opin Endocrinol Diabetes Obes* 2008;**15:**135–141.
2. Ribeiro FV, de Mendonça AC, Santos VR, Bastos MF, Figueiredo LC, Duarte PM. Cytokines and bone-related factors in systemically healthy patients with chronic periodontitis and patients with type 2 diabetes and chronic periodontitis. *J Periodontol* 2011;**82:**1187–1196. doi: 10.1902/jop.2011.100643
3. Santos VR, Ribeiro FV, Lima JA, Napimoga MH, Bastos MF, Duarte PM. Cytokine levels in sites of chronic periodontitis of poorly controlled and well-controlled type 2 diabetic subjects. *J Clin Periodontol* 2010;**37:**1049–1058.
4. Duarte PM, de Oliveira MC, Tambeli CH, Parada CA, Casati MZ, Nociti FH Jr. Overexpression of interleukin-1beta and interleukin-6 may play an important role in periodontal breakdown in type 2 diabetic patients. *J Periodontal Res* 2007;**42:** 377–381.
5. Katz PP, Wirthlin MR Jr, Szpunar SM, Selby JV, Sepe SJ, Showstack JA. Epidemiology and prevention of periodontal disease in individuals with diabetes. *Diabetes Care* 1991;**14:**375–385.
6. Yuan K, Chang CJ, Hsu PC, Sun HS, Tseng CC, Wang JR. Detection of putative periodontal pathogens in non-insulin-dependent diabetes mellitus and non-diabetes mellitus by polymerase chain reaction. *J Periodontal Res* 2001;**36:** 18–24.
7. Tervonen T, Oliver RC, Wol LF, Bereuter J, Anderson LA, Aeppli DM. Prevalence of periodontal pathogens with varying

metabolic control of diabetes mellitus. *J Clin Periodontol* 1994;**21:**375–379.
8. Hintao J, Teanpaisan R, Chongsuvivatwong V, Ratarasan C, Dahlen G. The microbiological profiles of saliva, supragingival and subgingival plaque and dental caries in adults with and without type 2 diabetes mellitus. *Oral Microbiol Immunol* 2007;**22:**175–181.
9. Ficara AJ, Levin MP, Grower MF, Kramer GD. A comparison of the glucose and protein content of gingival fluid from diabetics and nondiabetics. *J Periodontal Res* 1975;**10:**171–175.
10. Mashimo PA, Yamamoto Y, Slots J, Park BH, Genco RJ. The periodontal microbiota of juvenile diabetics: culture, immunofluorescense and serum antibody studies. *J Periodontol* 1983;**54:**415–420.
11. Campus G, Salem A, Uzzau S, Baldoni E, Tonolo G. Diabetes and periodontal disease: a case-control study. *J Periodontol* 2005;**76:**418–425.
12. da Cruz GA, de Toledo S, Sallum EA *et al*. Clinical and laboratory evaluations of non-surgical periodontal treatment in subjects with diabetes mellitus. *J Periodontol* 2008;**79:**1150–1157.
13. Sardi JC, Duque C, Camargo GA, Hofling JF, Gonçalves RB. Periodontal conditions and prevalence of putative periodontopathogens and *Candida* spp. in insulin-dependent type 2 diabetic and non-diabetic patients with chronic periodontitis–a pilot study. *Arch Oral Biol* 2011;**56:**1098–1105 [Epub ahead of print].
14. Armitage GC. Development of a classification system for periodontal diseases and conditions. *Ann Periodontol* 1999;**4:**1–6.
15. American Diabetes Association. Executive summary: standards of medical care in diabetes-2009. *Diabetes Care* 2009;**32:** S6–S12.
16. Ainamo J, Bay I. Problems and proposals for recording gingivitis and plaque. *Int Dent J* 1975;**25:**229–235.
17. Mühlemann HR, Son S. Gingival sulcus bleeding–a leading symptom in initial gingivitis. *Helv Odontol Acta* 1972;**15:** 107–113.
18. Casarin RC, Ribeiro Edel P, Mariano FS, Nociti FH Jr, Casati MZ, Gonçalves RB. Levels of *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis*, inflammatory cytokines and species-specific immunoglobulin G in generalized aggressive and chronic periodontitis. *J Periodontal Res* 2010;**45:**635–642.
19. Lane DJ. S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, eds. *Nucleic Acid Techniques in Bacterial Systematics*. Chichester: John Wiley & Sons, 1991:115–175.
20. Jukes TH, Cantor CR. Evolution of protein molecules. In: Munro HN, ed. *Mammalian Protein Metabolism*. New York: Academic Press, 1969:21–132.
21. Chen T, Yu WH, Izard J, Baranova OV, Lakshmanan A, Dewhirst FE. The Human Oral Microbiome Database: a web accessible resource for investigating oral microbe taxonomic and genomic information. *Database* 2010; Article ID baq013, doi: 10.1093/database/baq013. Online open access: http://database.oxfordjournals.org/cgi/content/full/2010/0/baq013.
22. Shchipkova AY, Nagaraja HN, Kumar PS. Subgingival microbial profiles of smokers with periodontitis. *J Dent Res* 2010;**89:**1247–1253.
23. Sandholm L, Swanljung O, Rytomaa I, Kaprio EA, Maenpaa J. Morphotypes of the subgingival microbiota in diabetic adolescents in Finland. *J Periodontol* 1989;**60:**526–528.
24. Casarin RC, Duarte PM, Santos VR *et al*. Influence of glycemic control on Epstein-Bar and Cytomegalovirus infection in periodontal pocket of type 2 diabetic subjects. *Arch Oral Biol* 2010;**55:**902–906.
25. Makiura N, Ojima M, Kou Y *et al*. Relationship of *Porphyromonas gingivalis* with glycemic level in patients with type 2 diabetes following periodontal treatment. *Oral Microbiol Immunol* 2008;**23:**348–351.
26. Aly FZ, Blackwell CC, Mackenzie DA, Weir DM, Clarke BF. Factors influencing oral carriage of yeasts among individuals with diabetes mellitus. *Epidemiol Infect* 1992;**109:**507–518.
27. Nowakowska D, Kurnatowska A, Stray-Pedersen B, Wilczyński J. Species distribution and influence of glycemic control on fungal infections in pregnant women with diabetes. *J Infect* 2004;**48:**339–346.
28. Kumar PS, Griffen AL, Moeschberger ML, Leys EJ. Identification of candidate periodontal pathogens and beneficial species by quantitative 16S clonal analysis. *J Clin Microbiol* 2005;**43:**3944–3955.
29. Paster BJ, Boches SK, Galvin JL *et al*. Bacterial diversity in human subgingival plaque. *J Bacteriol* 2001;**183:**3770–3783.
30. Ciantar M, Gilthorpe MS, Hurel SJ, Newman HN, Wilson M, Spratt DA. *Capnocytophaga* spp. in periodontitis patients manifesting diabetes mellitus. *J Periodontol* 2005;**76:**194–203.
31. Spratt DA, Greenman J, Schaffer AG. *Capnocytophaga gingivalis*: effects of glucose concentration on growth and hydrolytic enzyme production. *Microbiology* 1996;**142:**2161–2164.
32. Kumar PS, Griffen AL, Barton JA, Paster BJ, Moeschberger ML, Leys EJ. New bacterial species associated with chronic periodontitis. *J Dent Res* 2003;**82:** 338–344.
33. Ximénez-Fyvie LA, Haffajee AD, Socransky SS. Microbial composition of supra- and subgingival plaque in subjects with adult periodontitis. *J Clin Periodontol* 2000;**27:**722–732.
34. Suda R, Lai CH, Yang HW, Hasegawa K. *Eikenella corrodens* in subgingival plaque: relationship to age and periodontal condition. *J Periodontol* 2002;**73:**886–891.
35. Müller HP, Heinecke A, Borneff M, Knopf A, Kiencke C, Pohl S. Microbial ecology of *Actinobacillus actinomycetemcomitans, Eikenella corrodens* and *Capnocytophaga* spp. in adult periodontitis. *J Periodontal Res* 1997;**32:**530–542.
36. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. *J Clin Periodontol* 1998;**25:**134–144.
37. Diaz PI, Zilm PS, Rogers AH. *Fusobacterium nucleatum* supports the growth of *Porphyromonas gingivalis* in oxygenated and carbon-dioxide-depleted environments. *Microbiology* 2002;**148:**467–472.
38. Brinig MM, Lepp PW, Ouverney CC, Armitage GC, Relman DA. Prevalence of bacteria of division TM7 in human subgingival plaque and their association with disease. *Appl Environ Microbiol* 2003;**69:** 1687–1694.
39. Bab IA, Sela MN, Ginsburg I, Dishon T. Inflammatory lesions and bone resorption induced in the rat periodontium by lipoteichoic acid of *Streptococcus mutans*. *Inflammation* 1979;**3:**345–358.
40. Keller JF, Carrouel F, Colomb E *et al*. Toll-like receptor 2 activation by lipoteichoic acid induces differential production of pro-inflammatory cytokines in human odontoblasts, dental pulp fibroblasts and immature dendritic cells. *Immunobiology* 2010;**215:**53–59.
41. Ebersole JL, Holt SC, Hansard R, Novak MJ. Microbiologic and immunologic characteristics of periodontal disease in Hispanic americans with type 2 diabetes. *J Periodontol* 2008;**79:**637–646.
42. Susanto H, Nesse W. Periodontitis prevalence and severity in Indonesians with type 2 diabetes. *J Periodontol* 2010;**82:** 550–557.