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Salivary and microbiological parameters of chronic periodontitis subjects with and without type 2 diabetes mellitus: a case-control study

Parâmetros salivares e microbiológicos em indivíduos com periodontite crônica com ou sem diabetes melito tipo 2: estudo caso-controle

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Resumo

Introdução: Diversos estudos têm investigado as diferenças dos parâmetros salivares e microbianos entre pacientes diabéticos e não diabéticos, contudo, diferenças específicas ainda não estão claras, principalmente devido aos efeitos de variáveis de confusão. **Objetivo**: O objetivo deste estudo caso-controle foi avaliar os parâmetros salivares e microbianos de indivíduos com doença periodontal crônica com ou sem diagnóstico de diabetes melito tipo 2. **Material e método**: Este estudo caso-controle incluiu 60 indivíduos com periodontite crônica, 30 diabéticos (casos) e 30 não diabéticos (controles), pareados pela severidade da doença periodontal, gênero e idade. Saliva total estimulada foi coletada de todos os voluntários para mensuração do pH salivar e fluxo salivar. Amostras bacterianas foram coletadas com pontas de papel absorvente dos sítios periodontais com maior profundidade de sondagem e perda de inserção clínica. A frequência de *A. actinomycetemcomitans*, *P. intermedia*, *P. gingivalis*, *T. forsythia* e *C. rectus* foi avaliada por PCR. Os dados foram analisados estatisticamente pelo teste *t de Student*, Mann -Whitney e Qui-quadrado ($p < 0,05$). **Resultado**: Diabéticos apresentaram maior nível de glicose salivar e menor fluxo salivar em comparação aos não diabéticos. *P. gingivalis* e *T. forsythia* foram os patógenos mais frequentes ($p < 0,05$). Frequência bacteriana não diferiu entre os casos e controles. **Conclusão**: A condição diabetes influenciou o fluxo e os níveis de glicose salivar. Dentro da mesma severidade da periodontite crônica, indivíduos diabéticos não mostraram maior frequência de patógenos periodontais em comparação aos seus controles.

Descritores: Saliva; bactérias; periodontite crônica; diabetes mellitus.

Abstract

Background: Several studies have investigated the differences in salivary parameters and microbial composition between diabetic and non-diabetic patients, however, specific differences are still not clear mainly due to the effects of confounder. **Aim**: The aim of this case-control study was to evaluate the salivary and microbial parameters of chronic periodontitis subjects with and without type 2 diabetes mellitus. **Material and method**: This case-control study included 60 chronic periodontitis subjects, 30 diabetics (case group) and 30 non-diabetics (control group), paired according to periodontitis severity, gender and age. Stimulated whole saliva was collected from all volunteers to measure the salivary pH and the salivary flow rate. Bacterial samples were collected with paper points from periodontal sites showing the deepest periodontal pocket depth associated with the highest clinical attachment loss. The frequency of *A. actinomycetemcomitans, P. intermedia, P. gingivalis, T. forsythia* and *C. rectus* was evaluated by PCR. Data was statistically analyzed by Student's t, Mann-Whitney and Chi-square ($p<0.05$). **Result**: Diabetic subjects showed higher salivary glucose levels and lower stimulated flow rates in comparison to non-diabetic controls. *P. gingivalis* and *T. forsythia* were the most frequent pathogens ($p<0.05$). Bacterial frequency did not differ between case and control groups. **Conclusion**: Diabetes status influenced salivary glucose levels and flow rate. Within the same severity of chronic periodontitis, diabetic subjects did not show higher frequency of periodontal pathogens in comparison to their paired controls.

Descriptors: Saliva; bacteria; chronic periodontitis; diabetes mellitus.

INTRODUCTION

Diabetes Mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from failure of insulin secretion, insulin action or both. Several pathogenic processes are involved in the development of diabetes. They range from autoimmune destruction of the beta-cells of the pancreas with consequent insulin deficiency to abnormalities that result in resistance to insulin action. The basis of abnormalities in carbohydrate, fat, and protein metabolism in diabetes is deficient action of insulin on target tissues[1]. The prevalence of type 2 diabetes worldwide, which is increasing rapidly, represents a significant burden to human health because of its numerous and often systemic complications. This challenge also affects Brazilian´s population[2-4]. The World Health Organization estimates that there are 220 million adults with diabetes in the world today and that in 2030 this number may double. Diabetes is responsible for 3.5% of all deaths caused by noncommunicable diseases, which represent 63% of all 57 million estimated global deaths[5].

The association between periodontal diseases and diabetes has been studied over the last decades and, today, there is enough scientific evidence to support the existence of a two-way relationship between them[6-9]. Diabetes increases the risk of incidence and severity for periodontitis[10]. This may be due to factors, such as vascular changes, neutrophil dysfunction, altered collagen synthesis, microbial factors and genetic predisposition[11]. The opposite relation can also be considered since the inflammatory response observed in periodontitis may affect insulin resistance[12].

Sbordone et al.[13] demonstrated in diabetic and non-diabetic patients a predominance of Gram-negative bacteria. However, microbial results are still controversial. According to Field et al.[14], *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans)*, *Fusobacterium nucleatum* (*F. nucleatum)* and *Porphyromonas gingivalis (P. gingivalis)* counts did not differ significantly between diabetics and non-diabetics. On the contrary, Sardi et al.[15] reported that diabetics had a higher prevalence of Candida *spp*. and a lower frequency of *Tannerella forsythia (T. Forsythia)*, when compared to non-diabetic subjects, Thus, additional studies are suggested for a better clarification of microbial behavior in diabetic patients.

Saliva is one important factor for oral health[16]. Unfortunately, diabetes seems to negatively impact salivary flow[17] which can partially explain the poorer oral status among diabetics.

Therefore, the aim of this case-control study was to evaluate the salivary parameters and microbiological profile of diagnosed chronic periodontitis subjects with and without type 2 diabetes mellitus.

MATERIAL AND METHOD

Participants included in the present study were recruited from two different areas, Taubaté-SP State and Porto Velho-RO State, Brazil. All subjects were screened from 2011 to 2012. The study had been previously approved by the Institutional Committee on Research Involving Human Subjects of the University of Taubate (protocol number 491-10). We established for this survey a non-probability convenience sample population.

Among 127 eligible subjects according to inclusion and exclusion criteria, 60 chronic periodontitis patients were allocated in two groups: 30 diabetics (case-group) and 30 non-diabetics (control-group), paired according to severity of chronic periodontitis, gender and age. All eligible patients were thoroughly informed of the nature, potential risks, and benefits of their participation in the study and signed an informed consent form. Detailed medical and dental data were collected. Physical examination, Body Mass Index (BMI), blood pressure and blood glycemic level were determined.

1. *Inclusion and Exclusion Criteria*

Inclusion criteria: ≥ 25 years old; both genders; ≥ 20 teeth (excluding third molars); diagnosis of generalized moderate chronic periodontitis based on the clinical and radiographic criteria[18]; and poorly controlled type 2 diabetes (fasting blood glucose level > 140mg/dL and 2-hour post-prandial blood glucose levels > 200mg/dL) or no diabetes. Diabetic subjects should have received this diagnosis at least 5 years and no longer than 10 years prior to the study. Subjects receiving insulin supplementation, diet regimen, or oral hypoglycemic agents were selected. The physician used additional clinical features such as polyuria, polydipsia and polyphagia to establish diabetes diagnosis. Among controls examinations of blood glucose levels were performed to confirm non-diabetic status.

Exclusion criteria: pregnancy; lactation; current smoking and smoking within the past 5 years; kidney problems or dialysis treatment; systemic diseases, except diabetes for the case group; treatment with any kind of medications that could alter the saliva flow (anti-depressants, anxiolytic, antihistamine, diuretics, and other); periodontal or antibiotic therapies in the previous 6 months, and use of mouthrinses containing antimicrobials agents in the preceding 2 months.

2. *Fasting Plasma Glucose and Glycated Hemoglobin Monitoring*

The laboratory performed the blood analyses of all patients, including fasting plasma glucose (FPG) and glycated hemoglobin (HbA1c) monitoring. FPG was measured using the glucose oxidase method (milligrams per deciliters), and HbA1c (percentage) was measured by high-performance liquid chromatography. Criteria for diagnosis of diabetes were HbA1c ≥ 6.5% and FPG ≥ 126 mg/dL (7.0 mmol/L). Fasting is defined as no calorie intake for at least 8 h.

3. *Assessment of Body Mass Index*

Height and weight (Welmy scale model 104-Santa Bárbara do Oeste-SP-Brazil) were taken by a trained staff member. BMI was calculated according to the International BMI Classification developed by the World Health Organization as weight divided by squared height (kg/m^2) to determine underweight (<18.5 kg/m^2),

normal weight (<25 kg/m^2), overweight (25 to 29.9 kg/m^2), and obesity: Class I (30 to 34.9 kg/m^2), Class II (35 to 39.9 kg/m^2) and Class III (> 40 kg/m^2).

4. *Clinical Examination*

One blinded, trained and calibrated examiner conducted all clinical measurements. Intra-examiner agreement was high (kappa = 0.84 for probing depth and 0.82 for clinical attachment level).

A complete periodontal examination was carried out. Measurements of pocket depth (PD), clinical attachment level (CAL), Plaque index (PI) and Gingival index (GI) – presence/absence[19] were obtained in six sites per tooth (mesio-buccal, buccal, disto-buccal, mesio-lingual, lingual and disto-lingual), excluding third molars, using a manual periodontal probe (PCPUNC 15 Hu-friedy Mfg Co Inc., Chicago, IL, USA). In addition, subjects underwent radiographic examination to evaluate the extent of periodontal bone resorption.

5. *Microbiological Examination*

Pooled subgingival samples were taken from five periodontal sites (> PD)[20]. Each selected tooth was isolated with sterile cotton rolls and the supragingival plaque was removed with sterile cotton pellets. A sterilized paper point (number 30) was carefully inserted to the depth of the periodontal pocket from the mesial dental aspect and kept in position for 60 s. The pooled subgingival samples were stored at –80 °C in microtubes containing 1mL of reduced Ringer's solution.

Presence of A.actinomycetemcomitans*, Prevotella intermedia (P. intermedia), P. gingivalis*, *T. forsythia* and *Campylobacter rectus (C. rectus)* was established by polymerase chain reaction (PCR) using specific primers [A.actinomycetemcomitans, sense, 5'-AAACCCATCTCTGAGTTCTTCTTC-3', and antisense, 5'-ATGCCAACTTGACGTTAAAT-3' (PCR product size 550 bp); *P. intermedia,* sense, 5'-TTTGTTGGGGAGTAAAGCGGG-3', and antisense, 5'-TCAACATCTCTGTATCCTGCGT-3' (575 bp); *P. gingivalis,* sense, 5'-AGGCAGCTTGCCATACTGCGG-3', and antisense, 5'-ACTGTTAGCAACTACCGATGT-3(404 bp); *T. forsythia,* sense, 5'-GCGTATGTAACCTGCCCGCA-3', and anti-sense, 5'-TGCTTCAGTGTCAGTTATACCT-3' (641 bp) and *C. rectus,* sense: 5'-TTTCGGAGCGTAAACTCCTTTTC-3', and antisense: 5'-TTTCTGCAAGCAGACACTCTT-3' (598 bp)] under standard conditions. The DNA was extracted using InstaGene Matrix (BioRad Laboratories, Hercules, CA, USA) and the PCR was performed in a Mastercycler Gradient (Eppendorf , Westbury, NY, USA) thermocycler as follows: one cycle 94 °C for 5 min., 35 cycles 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1min., and a final extension of 72 °C for 5 min. After electrophoresis in 1.5% agarose gel, DNA fragments were stained with SYBR Safet (Invitrogen, Carlsbad, CA, USA) and visualized by UV illumination. PCR products were compared with both positive and negative controls. A molecular weight marker (Ladder 100) was added in each set.

6. *Salivary Examination*

Stimulated saliva samples were collected from the volunteers between 09:00 and 11:00 hours to avoid circadian rhythm effects. No food or drink was permitted for 2 h before collection. During the sample collection, volunteers remained in a seated position, with their head tilted forward (approximately 45°). The procedure was accomplished in a quiet and well-ventilated room. Initially, volunteers were instructed to chew a Parafilms® (São Bernardo do Campo, SP-Brazil) block for 5 min. The examiner asked the individuals to spit out saliva each minute. The first 2 minutes. of collection were discarded, and so the analyses were performed using the last three collections[21]. Immediately after collection, the salivary pH was measured using a portable pH meter (Marconi P200, São Paulo, SP-Brazil). Its glass electrode was washed with deionized water and calibrated with buffer solutions pH 7.0 and 4.0.

Additionally, the salivary flow rate - defined as the total volume of saliva produced per unit time (mL/min.), whereas the volume of saliva (ml) was determined considering the difference between the weight (g) of the plastic tube before and after collection. The density of saliva was considered to be 1.0[22]. A stimulated salivary flow rate between 1.0 and 3.0mL/min. was considered normal, while values <0.7ml/min. indicated a reduced flow rate[23].

Subsequently, buffering capacity of saliva was performed by inoculating with a sterile disposable syringe, 1ml of total saliva in a bottle containing 3ml of 5mmol/l hydrochloric acid and a system-individual pH indicator (Dentobuff®, Orion Diagnostica, Helsinki, Finland). The bottle was vigorously shaken for 20 seconds and then was allowed to stand for 5 minutes, without the rubber cap for the elimination of carbon dioxide. The color that appeared in the bottle was visually compared with a graduated color scale in pH units, supplied by the manufacturer. The reading of the test was based on the following parameters: (i) very low buffering capacity or lower, pH 3.0 to 4.0, (ii) intermediate buffering capacity, pH 4.5 to 5.0, (iii) normal and good buffer capacity at a challenge of pH <5.5[24]. The glucose concentration was determined enzymatically in the saliva by the method Glucose Oxidase (Labtest, Lagoa Santa-MG-Brazil).

7. *Statistical Analysis*

An initial confirmatory statistical analysis was performed. At this phase all parameters measured to determine the paired population were evaluated. Subsequently, a comparative statistical analysis was done. BMI, body weight, salivary glucose levels and salivary flow rates were compared between diabetic and non-diabetic groups. In addition, frequency of the target bacterial species was compared intra- and inter-groups.

All statistical analyses were carried out using SPSSs 13.0 (IBM, Chicago-IL-USA) and Biostat 5.0 (Instituto Mamirauá, Tefé-AM-Brazil). Differences were considered significant when $p < 0.05$. Data was compared by Student's *t*, Mann-Whitney and Chi-square tests.

RESULT

The present case-control study included 60 subjects, males or females, 30 diabetics (case group), and 30 non-diabetics (control group), paired according to their clinical periodontal status, gender and age (Table 1). Therefore, as proposed by the study design, Table 2 shows that clinical periodontal Probing Depth (PD), Clinical Attachment Level (CAL), Plaque Index (PI) and Gingival Index (GI) did not differ between groups.

In addition, authors compared body weight and BMI between subjects in the control group and in the case group. Body weight and BMI were statistically similar ($p<0.05$). Also, diabetic and non-diabetic diagnoses were confirmed by the observed differences in blood glycemic levels (Table 3).

Salivary glucose levels and flow rates differed when case and control groups were compared (Table 4). Diabetes was associated with higher glucose levels and lower stimulated salivary flow rates. On the other hand, diabetes was not accompanied by differences in both salivary pH and buffering capacity.

Finally, frequency of the target bacterial species was compared intra- and inter-groups. Intra-group analysis demonstrated that *P. gingivalis* and *T. forsythia* were the most frequent pathogens in both case and control groups (Figure 1). Inter-groups comparative analysis did not show differences in the frequency of the searched periodontal pathogens (Figure 2).

Table 1. Distribution of study population by gender and age

	Systemic status [Type 2 Diabetes mellitus]		Total
	Diabetic Cases	Non-diabetic Controls	
Male	12	12	24
Female	18	18	36
Total	30	30	60
(Mean age ± SD years)	(49.23 ± 9.41)	(49.23 ± 9.41)	(49.23 ± 9.33)

SD – Standard Deviation.

Table 2. Mean values of the chronic periodontitis clinical parameters pocket depth (PD), clinical attachment level (CAL), plaque index (PI) and gingival index (GI) among diabetic and non-diabetic subjects

	PD Mean ± SD	CAL Mean ± SD	PI Mean ± SD	GI Mean ± SD
D	3.41 ± 0.64	3.81 ± 1.66	0.76 ± 0.24	0.48 ± 0.15
ND	3.61 ± 0.71	3.39 ± 1.72	0.71 ± 0.24	0.56 ± 0.20
p value	0.3011	0.1429	0.8948	0.8432

SD – Standard Deviation; Student's t Test and Mann Whitney Tests. D - Diabetic Cases. ND – Non-diabetic controls.

Table 3. Mean values of body weight, body mass index (BMI) and blood glycemic index, as related to diabetes status

	Weight Mean ± SD	Body Mass Index Mean ± SD	Blood Glycemic Index Mean ± SD
D	71.13 ± 12.38	27.48 ± 5.39	178.36 ± 73.01
ND	75.39 ± 22.38	28.46 ± 7.02	88.13 ± 11.05
p value	0.9328	0.9312	**0.0041***

SD – Standard Deviation; Student's t Test and Mann Whitney Tests. *statistically significant difference ($p< 0.05$). D - Diabetic Cases. ND – Non-diabetic controls.

Table 4. Mean values of salivary flow rates, pH, buffering capacity and salivary glucose levels in case and control groups

	Stimulated Saliva Flow Mean ± SD	pH Mean ± SD	Buffering Capacity Mean ± SD	Salivary Glucose Level Mean ± SD
D	0.94 ± 0.41	7.56 ± 0.40	5.31 ± 0.84	7.23 ± 2.19
ND	0.43 ± 0.32	7.45 ± 0.52	5.39 ± 1.01	1.98 ± 1.42
p value	**0.009***	0.7865	0.6978	**0.0231***

SD – Standard Deviation; Student's t and Mann Whitney Tests; * statistically significant difference ($p<0.05$); D - Diabetic Cases; ND – Non-diabetic controls.

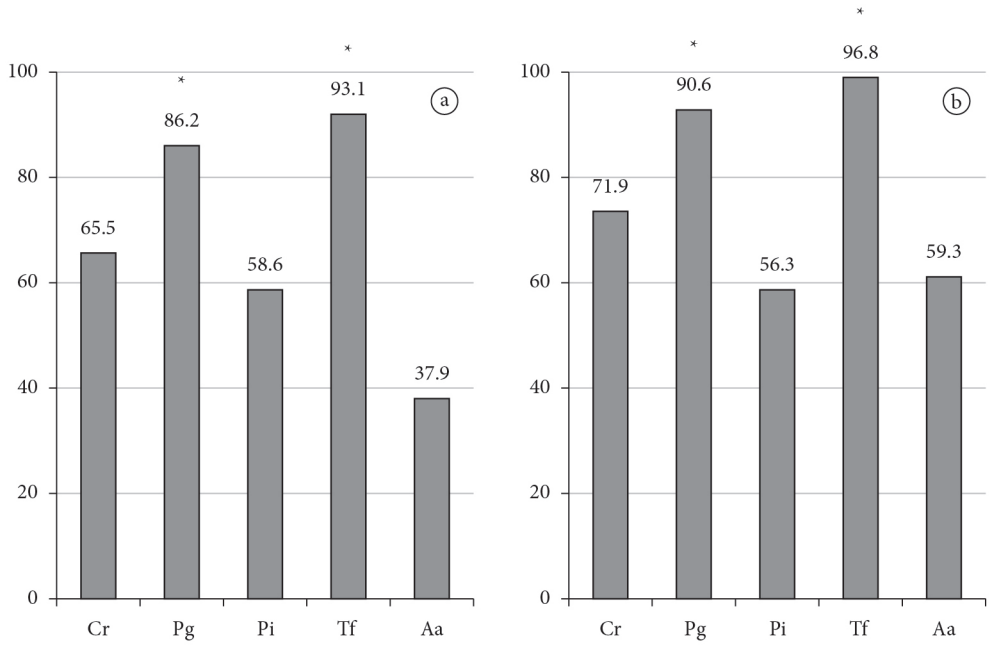

Figure 1. Frequency (%) of periodontal pathogens in diabetics (A – case group) and non- diabetics (B – control group) groups. Data from intra-group analysis. C.r – *C. rectus*; P.g – *P. gingivalis*; P.i – *P. intermedia*; T.f – *T. forsythia*; A.a – *A. actinomycetemcomitans*; Chi-Square Test ($p < 0.05$). *Statistically significant difference ($p<0.05$) – Chi-Square Test.

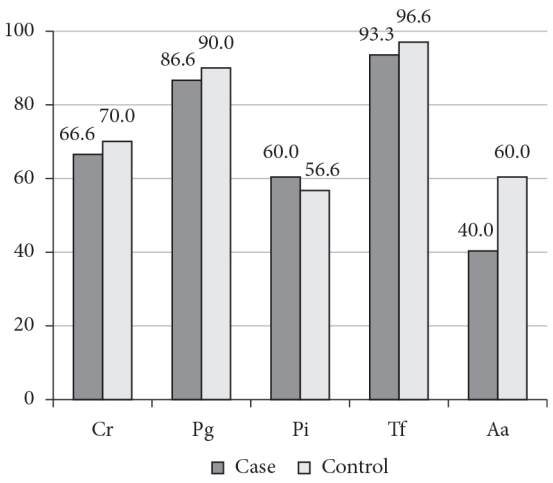

Figure 2. Frequency (%) of periodontal pathogens as related to systemic status of the individuals. Case indicates diabetic periodontally diseased subjects while control indicates the group composed of non-diabetic periodontally diseased subjects. Data from inter-group analysis. C.r – *C. rectus*; P.g – *P. gingivalis*; P.i – *P. intermedia*; T.f – *T. forsythia*; A.a – *A. actinomycetemcomitans*.

DISCUSSION

The increasing number of diabetic subjects worldwide brings up the discussion of diabetes management and the diseases associated comorbidities. Studies have supported a two-way relation between diabetes and periodontal status[6-9]. Key public strategies such as primary prevention and early diagnosis require deep knowledge related to risk factors and to the etiopathogenesis of the disease or diseases under analysis. Saliva has a straight risk relationship to oral diseases being a relevant auxiliary diagnostic body fluid. Also, many systemic diseases manifest themselves through salivary changes and diabetes seems to be one of them[17].

Although several studies have compared the composition of subgingival biofilm between diabetic and non-diabetic patients many aspects are still unclear[13-15]. Up to now literature is not enough to sustain whether a periodontal microbial difference exists or not. The existence of confounders can lead to misinterpretation. For this reason different study designs can help researchers to understand unanswered questions. Therefore, aiming at controlling the expected effects of some recognized modulators of periodontal microbiota – age, periodontal status – the present study used a matched population to offer a better understanding about the occurrence of target periodontal species in samples collected from diabetic subjects and their non-diabetic controls. In addition to limiting salivary/microbial and other systemic effects of diabetes a range of 5 years was used to determine the maximum duration of the disease. This came from the understanding that the longer a subject is exposed to diabetes the higher is the number of comorbidities, side effects and other health complications.

Combined with glycated hemoglobin, blood glucose levels are still one classical indicator of diabetes. Thus, changes in oral glucose levels have been investigated as an oral environmental change indicator among diabetics. After analyzing newly diagnosed type 2 diabetes subjects, Abikshyeet et al.[25] found significant correlations between salivary glucose and both blood glucose and blood glycated hemoglobin. In addition to glucose levels, salivary flow rate is also a theme of study. Lasisi, Fasanmade[17] reported differences related to salivary flow and glucose levels after comparing diabetic and non-diabetic subjects. On the other hand, Panchbhai et al.[26] only observed differences related to glucose levels. When these authors compared salivary flow rate among uncontrolled diabetes, controlled diabetes and healthy controls they failed to sustain a significant difference. In

the present study diabetic subjects showed higher levels of glucose in saliva and lower salivary flow rates. As one of the outcomes of a chronic reduced salivary flow, Borges et al.[27] found a prevalence of dry mouth of 25% among elderly Brazilian diabetic subjects and 46% of the subjects revealed hyposalivation in stimulated conditions.

Considering case and control groups *P. gingivalis* and *T. forsythia* were the most frequent species. Actually, both groups showed moderate to high frequency of all bacterial species, even for the pathogen *A. actinomycetemcomitans,* showing and approximate overall range from 40 to 90%. This observed frequency was compatible to other studies that evaluated chronic periodontitis subjects with no microbiological differences between case and control groups[14,28]. It seems that subgingival bacterial frequency was more dependent on local periodontal clinical status than on systemic diabetes status. In the present study the major factor that determined high bacterial frequency, was the periodontal status *per se*. Unexpectedly, neither the higher concentrations of glucose in saliva nor the lower flow rate did influence frequency of periodontal pathogens. Although Gram-negative bacteria are less glucolytic some species are able to uptake and use glucose as a substrate[29]. Despite the fact that other researchers also failed to report microbial differences between diabetics and non-diabetics[14,28], the present study design offered a better support for this lack of difference. Our findings can partially justify why antibiotics or antiseptics only offer diabetic subjects the same degree of benefits observed in systemic healthy controls.

The present study suggested that the greater severity of periodontal breakdown found among diabetic subjects is dictated by factors other than the bacterial component. Immunological factors are good candidates to sustain this pattern of greater severity. Therefore, efforts should be done to clarify the immunological component related to diabetes/periodontitis patients.

Diabetes status influenced salivary glucose levels and flow rate. Within the same severity of chronic periodontitis, diabetic subjects did not show higher frequency of periodontal pathogens in comparison to their paired controls.

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CONFLICTS OF INTERESTS

The authors declare no conflicts of interest.

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