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Periodontopathogens, Candida spp. and immunological aspects in type 2 diabetes mellitus patients with chronic periodontitis

Gabriela Alessandra Da Cruz Galhardo Camargo[1], Natalia Linhares Coutinho Silva[2], Ana Luísa Palhares de Miranda[2], Jorge Luiz Mendonça Tributino[3], Natália Helena Colombo[4], Cristiane Duque[4]

[1]Department of Periodontology, Fluminense Federal University, Nova Friburgo, Rio de Janeiro, Brazil. Doutor Sylvio Henrique Braune, 22, Centro, 28625-650, Nova Friburgo, Rio de Janeiro, Brazil. Phone number 55- 22-981496066

[2]LASSBio, Faculty of Pharmacy, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil. Carlos Chagas Filho, 373, CCS, Bloco BSS, sala 22, 21941-902, Rio de Janeiro, Brazil. Phone number 55-21-25626503

[3]Institute of Biomedical Sciences, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil. Carlos Chagas Filho 373, CCS, Bloco J sala J01-029, Ilha do Fundão, 21941-902, Rio de Janeiro, Brazil. Phone number 55-21-22805694

[4]Department of Pediatric Dentistry and Public Health, Araçatuba Dental School, Univ Estadual Paulista (UNESP), Araçatuba, Brazil. José Bonifácio, 1193, Vila Mendonça, 16015050, Araçatuba, São Paulo, Brazil. Phone number 55- 18-36363315

Abstract

This study aimed to evaluate clinical, microbiological and immunological parameters in type 2 diabetes mellitus (DM) in comparison with normoglycemic patients (NDM). Glycemic and lipid profiles and periodontal clinical status were determined for thirty-three patients (17 DM and 16 NDM). The presence of periodontopathogens and species of Candida in subgingival sites were determined by polymerase chain reaction and immunological parameters by ELISA assays. All glycemic and clinical parameters evaluated were higher in the DM group, with statistical difference for fasting glucose, glycated-hemoglobin, and periodontal parameters. Lipid profile (except triglycerides), levels of TNF-α and myeloperoxidase and the prevalence of the tested microorganisms were similar between the groups, except for *Candida albicans* and *Candida glabrata*, which was higher in the DM group. In conclusion, although microbiological and immunological parameters were similar in the DM and NDM groups, periodontitis and the levels of some species of *Candida* were more severe in DM patients.

Keywords: Diabetes mellitus, periodontal disease, *Candida* spp., TNF-α, myeloperoxidase.

Introduction

Type 2 diabetes mellitus (DM) is a metabolic disease characterized by insulin resistance and relative or absolute insulin deficiency[1]. This type of diabetes represents 85-90% of the diabetic group and is associated with lifestyle factors, mainly obesity and lack of physical activity, and genetic susceptibility. Blood glucose levels can be controlled with dietary changes and body fat reduction[2]. The prevalence of diabetes mellitus has increased significantly and it is estimated that by the year 2030 the world population of people with diabetes will be around 552 million, and in Brazil will reach 19 million[3].

Patients with chronic hyperglycemia have increased susceptibility to opportunistic infections, as well as oral infections[4]. Periodontitis is the most common chronic oral

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Correspondence to:
Profa. Dra. Cristiane Duque
Department of Pediatric Dentistry and Public Health, Araçatuba Dental School, Univ Estadual Paulista (UNESP), Araçatuba, Brazil. José Bonifácio, 1193, Vila Mendonça, 16015050, Araçatuba, São Paulo, Brazil. Phone number 55- 18-36363315
e-mail: cduque@foa.unesp.br, cristianeduque@yahoo.com.br

infection, after dental caries, that causes tooth loss in adults and has been considered the sixth most common complication of diabetes mellitus[3]. Periodontal disease is more prevalent and more severe in diabetes mellitus than in normoglycemic patients and periodontal infection may impair glycemic control, since it is able to activate a systemic inflammatory response[4]. Thus, investigators have considered the mechanism of both diseases to be bidirectional, suggesting that one influences the control of the other[5].

There are conflicting data regarding whether DM individuals with chronic periodontitis present an altered subgingival microbiota compared with nondiabetic patients. Ebersole et al.[6] reported that periodontitis sites in DM individuals showed a higher frequency of *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis* and *Campylobacter* spp. when compared with nondiabetic patients. Field et al.[7] quantified the subgingival levels of *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum* and *Porphyromonas gingivalis* in patients with periodontitis and found no significant differences between the subgingival microbiota of DM patients compared with non-diabetic subjects. A report demonstrated that the periodontal pockets of DM patients harbored a higher prevalence of *Candida* spp., mainly *C. albicans* and *C. dubliniensis*, compared with periodontal sites from non-diabetic patients[8]. The same study reported a lower prevalence of *Tannerella forsythia* in periodontitis sites of DM patients compared with non-diabetic patients[8].

The presence of bacteria is an essential primary factor for the onset of periodontal disease, however, considering the presence of this alone is not sufficient to predict the onset or severity of periodontal disease, it is also necessary to consider the susceptibility of the host which contributes significantly to the appearance of pathological changes in the periodontium; an imbalance between host and microorganisms that may be caused by local or systemic factors, such as diabetes mellitus, is required[8].

Studies have pointed out that tissue destruction in periodontal disease results from the interaction of bacteria and their products (such as lipopolysaccharide - LPS) with macrophage activation, triggering the local secretion of inflammatory mediators, especially tumor necrosis factor - α (TNF-α), interleukin - 1β (IL-1β), prostaglandin - E2 (PGE2) and interleukin - 6 (IL-6)[9,10]. These mediators are responsible for periodontal breakdown, leading to the clinical signs and symptoms of periodontal disease[10]. In diabetes mellitus, these mechanisms seem to be accomplished through a low resistance to infection, exaggerated inflammatory response and a deficiency in healing, justifying the severity of periodontal disease in these patients[4].

In periodontal disease, neutrophils degranulation can release myeloperoxidase (MPO), among other inflammatory mediators, and MPO has been appointed as a promising marker of periodontal disease activity11. In systemically healthy patients, MPO levels were higher in periodontitis sites compared to gingivitis and healthy sites[12]. Gonçalves et al.[13] analyzed MPO activity in the gingival crevicular fluid of DM with inadequate metabolic control and non-diabetic patients, both with chronic periodontitis, and observed that MPO activity was lower in DM compared to the control group before and after periodontal treatment. Furthermore, after the periodontal therapy the MPO activity was decreased in both groups[13].

The aim of this study was to evaluate clinical parameters, the presence of putative periodontopathogenic bacteria and *Candida* spp. and the levels of TNF-α and MPO in subgingival sites of non-diabetic and type 2 diabetes mellitus patients with chronic periodontitis.

Material and methods

Thirty-three patients (17 with type 2 diabetes mellitus (DM) aged 53.41 + 9.48, 16 non-diabetes mellitus patients (NDM) aged 47.87 ± 10.37 were selected to participate in this study. All subjects were recruited from the Department of Periodontology, School of Dentistry, Fluminense Federal University, Nova Friburgo, Rio de Janeiro State, Brazil, over a period of 6 months between 2011 and 2012. The study protocol was approved (protocol number: CAAE – 0434.0.258.000-11) by the Ethics Committee of the School of Medicine, Fluminense Federal University. Prior to participation, the purpose and procedures of the study were fully explained to all patients, who consequently gave written informed consent in accordance with the Helsinki Declaration. Medical and dental histories were taken and patients received clinical evaluation at prescreening visits. The inclusion criteria were: presence of advanced periodontitis defined by bleeding on probing in sites where probing depth was ≥ 5 mm in a minimum of two teeth in different arches; radiographic bone loss ranging from 30% to 50%[14,15]. The exclusion criteria were: patients submitted to periodontal treatment in the last 6 months; patients with systemic diseases; osteoporosis; pregnant lactating females; use of immune suppressive medication, phenytoin, cyclosporine, calcium channel blockers or any use of antibiotics or nonsteroidal anti-inflammatory drugs in the previous 3 months; and any medical conditions requiring immunotherapy or a diagnosis of HIV+ or AIDS, which could interfere with the periodontium status.

Glycemic and lipid profile parameters

A specialized professional collected blood samples from the peripherical vein (cubital fossa) of the individuals who had fasted overnight. Samples were collected in vacuum collection tubes and sent to the Raul Sertã Hospital Laboratory at Nova Friburgo/RJ for clinical analysis of the following glycemic and lipid parameters: fasting glucose levels (FGL), glycosylated hemoglobin (HbA1c), triglycerides (TRG), High–Density Lipoprotein (HDL) and Low-Density Lipoprotein (LDL), using specific kits (Gold Analisa, Belo Horizonte/MG).

Clinical examination

An experienced periodontist evaluated the clinical parameters and selected two unirradicular teeth for the protocol procedure. Each selected tooth was measured according to the following periodontal parameters: Plaque index (PI), bleeding on probe (BOP), pocket probing depth (PPD), gingival recession

(GR), clinical attachment level (CAL) using a periodontal probe PCP15 (PCP-UNC15, Hu-Friedy, Chicago, IL); six sites (mesio-buccal, mediobuccal, disto-buccal, mesio-lingual, medio-lingual, disto-lingual)[16] were recorded. Two sites with probing depth (PPD) > 5mm were selected for microbiological and immunological analysis. After clinical measurements, the supragingival biofilm was removed with sterile gauze. Gingival crevicular samples were taken from the 4 sites with the deepest PPD (≥ 5mm) in each patient, using a sterile paper point from the deepest pocket for 30s. Pooled biofilms from each site were separated in two microtubes containing Tris -EDTA buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 7.5) and were stored at -20° C. The samples were analyzed microbiologically using PCR.

Microbiological Assessment - PCR assays

DNA was extracted using a protocol originally described by Sardi et al.[8], and quantified in a spectrophotometer at 260 nm (Genesys 10UV, Rochester, NY, USA), in order to obtain a standard concentration of 100 ng/mL and stored at −20°C for subsequent PCR reactions. Briefly, samples were submitted to a lysing solution (extraction buffer and proteinase K) and then purified using chloroform: isoamil-alcohol, followed by DNA precipitation with isopropanol and 70% ethanol. The DNA was ressuspended in TE buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 7.5, with 10 µg/mL RNAse). Microbial molecular identification was carried out through PCR with specific primers for *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Prevotella intermedia*, *Campylobacter rectus*, *Candida albicans*, *Candida glabrata*, *Candida tropicalis* and *Candida dubliniensis* (Table 1). PCR amplification was performed with a TGradient 96 PCR system (TX96, Amplitherm, USA) under thermal conditions specific for each pair of primers. The PCR products were separated by electrophoresis in 2% agarose gels and Tris-borate-EDTA running buffer (pH 8.0). The molecular mass ladder (100 bp DNA ladder, Gibco, Grand Island, NY, USA) was included for running in the agarose gel. The DNA was stained with 0.1µL of Sybr Safe/mL (Invitrogen, CA, USA) and visualized under UV illumination (Pharmacia LKB-MacroVue, San Gabriel, CA, USA). Photographs of the images were taken (Image Mater – LISCAP, VDS, Pharmacia Biotech Piscataway, NJ, USA) and analyzed.

Table 1 - Primers sequences for PCR assays.

Species (Reference)	Sequences (5´-3´)	Amplicon length(bp)	Number of accession GenBank
A. actinomycetemcomitans (Ashimoto et al.[17])	For: AAA CCC ATC TCT GAC TTC TTC TTC Rev: ATG CCA ACT TGA CGT TA AT	557	NC_014629.1
P. gingivalis (Benkirane et al.[42])	For: AAT CGT AAC GGG CGA CAC AC Rev: GGG TTG CTC CTT CAT CAT AC	593	NC_002950.2
T. forsythia (Slots et al.[43])	For: GCG TAT GTA ACC TGC CCG CA Rev: TGC TTC AGT GTC AGT TAT ACC T	641	NC_016610.1 JAEZ01000014.1
P. intermedia (Ashimoto et al.[17])	For: TTT GTT GGG GAG TAA AGC GGG Rev: TAC ACA TCT CTG TAT CCT GCG T	575	NC_017861.1
C. rectus (Ashimoto et al.[17])	For: TTTCGGAGCGTAAACTCCTTTTC Rev: TTTCTGCAAGCAGACACTCTT	595	ACFU01000050.1
C. albicans (Sardi et al.[8])	For:ACTGCTCAAACCATCTCTGG Rev: CACAAGGCAAATGAAGGAAT	452	AJIQ01000008.1
C. glabrata (Sardi et al.[8])	For: GGAGATAGACTGGGCGTTAT Rev: GTTGTTCAATGGCTTTCTTC	314	XP_448539.1
C. tropicalis (Sardi et al.[8])	For: CACCCAAACAATTACCAAGT Rev: TGCAAACTCTTTACCTGGAT	253	NW_003020040.1
C. dubliniensis (Donnelly et al.[44])	For: GTATTTGTCGTTCCCCTTTC Rev: GTGTTGTGTGCACTAACGTC	288	NC_012860.1

Tumor necrose factor-α (TNF-α) and Myeloperoxidase (MPO) level measurement

Gingival crevicular fluid (GCF) was sampled 1 week after clinical examination, by a researcher blinded to the clinical parameters. GCF samples were taken from two different sites labeled as 1 or 2, in both sites the deepest PPD was ≥ 5 mm and BOP was chosen for sampling the same patient. The sites were isolated with sterile cotton rolls and dried with an air syringe to eliminate the possibility of contamination with saliva. GCF samples were obtained by placing a calibrated, volumetric microcapillary pipette with an internal diameter of 1.1 mm and a capacity of 5 µL. Sites that did not express the appropriate volume of fluid and micropipettes, which were contaminated with blood and saliva, were not included in the study[14]. The GCF was immediately placed into separate tubes containing 250 µL phosphate-buffered saline. The samples were stored at -20°C and analyzed by a single, blinded examiner using commercial kits of TNF-α and MPO enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA).

Statistical Analysis

Required sample size was determined by OpenEpi (OpenEpi, Version 3.03a, 2015) and was calculated to detect a 0.05 difference between BOP (NDM) and BOP (DM) with a power level of 84%.

The samples size was based on previous studies in the literature[18,19] using data related to differences in mean and standard deviation values, determining a minimum of 13 patients with type 2 diabetes mellitus required to detect significant differences in clinical and laboratorial variables between groups. The primary efficacy variables were whole-mouth mean BOP (NDM) and BOP (DM). Statistical tests were performed using the software SPSS, version 17.0 (Chicago, IL) to compare differences between the DM and NDM groups. Age and gender were compared between the groups using the Student's t-test. The Mann Whitney U test was performed to compare clinical and immunological parameters (PI, BOP, PPD, GR, CAL, Fasting glucose and HbA1c, LDL, HDL, TRG, TNF-α and MPO). Microbiological analysis of periodontopathogenic bacteria was performed using chi-square tests. Spearman's rho tests were applied to explore correlations between clinical, microbiological and immunological parameters, according to the presence of diabetes mellitus. Statistical significance for all variables was defined at the 5% level.

Results

Study subjects

No significant differences were observed between the DM and NDM groups, considering age (DM: 53.41 ± 9.48 and NDM: 47.87 ± 10.37, p = 0.751) or gender (DM: Male 52.9%, Female 47.55 and NDM: Male 37.5%, Female 62.5%, p = 0.292). The mean duration of diabetes was 7.76 ± 7.61 years ranging from 1 to 30 years.

Glycemic and lipid profiles

Descriptive statistics with means/medians/quartiles of the glycemic and lipid variables are presented in Table 2. Fasting glucose levels and HbA1c were statistically higher in DM compared to NDM showing glycemic differences between the groups. DM patients had a poorer glycemic control. Positive correlations were found between HbA1c and BOP in diabetic patients (Spearman's rho, 0.502, p = 0.04). LDL and HDL levels were similar between the groups and remained within the normal values. TRG values were statistically higher (Mann Whitney test, p = 0.03) in diabetic compared to non-diabetic patients. TRG was also positively correlated with GR (Spearman's rho, 0.568, p = 0.017) and CAL (Spearman's rho, 0.516, p = 0.034).

Periodontal parameters

PI and BOP indices were evaluated on all tooth surfaces and determined for the whole mouth (%). PPD, GR and CAL were calculated in millimeters. Means/medians/quartiles of the clinical parameters recorded for both groups are summarized in Table 2. Diabetic patients presented significantly higher values for PD, GR and CAL compared to the control group. The other clinical parameters did not differ significantly between the groups. For both groups, positive correlations (0.491-0.910) were observed between PPD, GR and CAL values, showing that these parameters are intrinsically associated with the periodontal status (Spearman's rho, p < 0.05).

Table 2 - Summary of clinical and laboratorial parameters and proinflammatory mediators levels for DM and NDM groups. Data were expressed in medians (inter-quartiles).

			DM	NDM	p value
Clinical parameters		PI (%)**	69.84 (51.78-91.7)	61.94 (43.45-77.94)	0.363
		BOP (%)	46.66 (35.2-65.74)	51.83 (24.25-68.34)	0.958
		PPD (mm)	5.5 (5.24-5.65)	5.19 (5-5.29)	0.045*
		GR (mm)	1.81 (1.33-3.11)	0 (0-2.28)	0.045*
		CAL (mm)	6.1 (5.5-7.39)	5.2 (5-5,8)	0.014*
Laboratorial parameters	Glycemic profile	FGL (mg/dL)	131 (94-172)	89 (78.75-97.25)	0.006*
		HbA1c (%)	7.6 (6.85-8.3)	4.7 (4.5-5)	0.000*
	Lipid profile	LDL (mg/dL)	125 (107-139)	93 (87.5-136.0)	0.165
		HDL(mg/dL)	44 (38-48)	47.5 (40.75-55.5)	0.102
		TGR (mg/dL)	146 (89-261)	74 (54.5-154)	0.033*
Proinflammatory mediators levels		TNF-α (pg/mL)	1.03 (0.76-1.22)	1.18 (0.9-1.49)	0.068
		MPO (pg/mL)	0.028 (0.01-0.07)	0.06 (0.026-0.079)	0.382

* Statistically significant difference between DM and NDM groups (Mann Whitney *U*-test, p <0.05).
**Plaque index (PI), bleeding on probe (BOP), pocket probing depth (PPD), gingival recession (GR), clinical attachment level (CAL), fasting glucose levels (FGL), glycosylated hemoglobin (HbA1c), triglycerides (TRG), High –Density Lipoprotein (HDL) and Low-Density Lipoprotein (LDL).

Microbiological analysis

Figure 1 shows the prevalence of *A. actinomycetemcomitans* (Aa), *P. gingivalis* (Pg), *T. forsythia* (Tf), *P. intermedia* (Pi), *C. rectus* (Cr), *C. albicans* (Ca), *C. glabrata* (Cg), *C. tropicalis* (Ct) and *C. dubliniensis* (Cd) in the DM and NDM groups. A significant difference between the groups was observed only for *C. albicans* and *C. glabrata*. The prevalence of putative periodontopathogens (Aa, Pg, Tf, Pi and Cr) was similar between DM and NDM patients. Twenty-four of the 33 patients (17 DM and 7 NDM) were colonized by *C. albicans* and twenty (13 DM and 7 NDM) by *C. glabrata*. All patients harbored *C. dubliniensis* and none presented *C. tropicalis*. The percentage of sites harboring *C. albicans*, *T. forsythensis* and *P. gingivalis* simultaneously was statistically higher in DM compared to NDM patients (Figure 2). The association of four or five periodontopathogens and *C. albicans* or *C. glabrata* was observed only in DM patients (Figures 2 and 3).

Immunological analysis

Levels of TNF-α and MPO measured in gingival crevicular fluid are presented in Table 2. No statistically significant differences were found for either proinflammatory marker between the DM and NDM groups. No correlations were verified between these biological markers and clinical, glycemic, lipid or microbiological profiles (Spearman's rho correlation, p > 0.05).

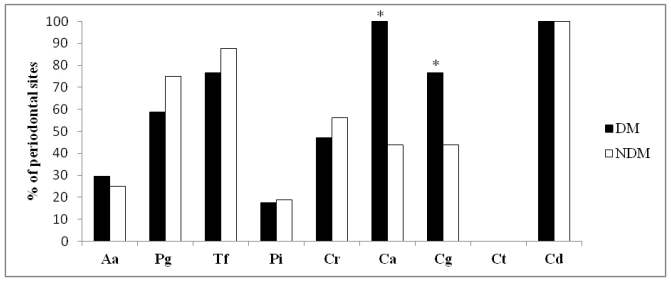

Fig. 1 - Frequency of periodontal sites harboring *Aggregatibacter actinomycetemcomitans* (Aa), *Porphyromonas gingivalis* (Pg), *Tannerella forsythia* (Tf), *Prevotella intermedia* (Pi), *Campylobacter rectus* (Cr), *Candida albicans* (Ca), *Candida glabrata* (Cg), *Candida tropicalis* (Ct) and *Candida dubliniensis* (Cd) in the DM and NDM groups.
*Statistically significant differences were found between the groups for Ca and Cg, according to $\chi 2$ tests (test, $p < 0.05$).

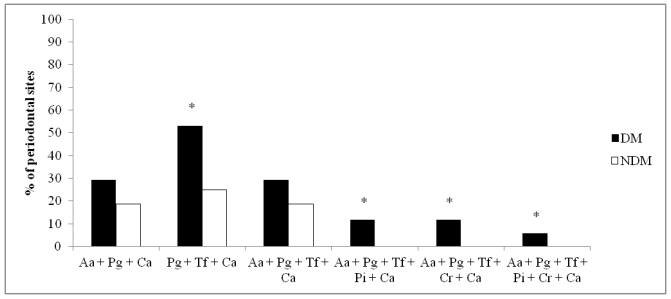

Fig. 2 - Frequency of periodontal sites (%) harboring combinations of putative periodontopathogens with *Candida albicans* (Ca) in the DM and NDM groups. *Statistically significant differences between the DM and NDM groups, according to $\chi 2$ tests ($p < 0.05$). *Aggregatibacter actinomycetemcomitans* (Aa), *Porphyromonas gingivalis* (Pg), *Tannerella forsythia* (Tf), *Prevotella intermedia*, *Campylobacter rectus* (Cr).

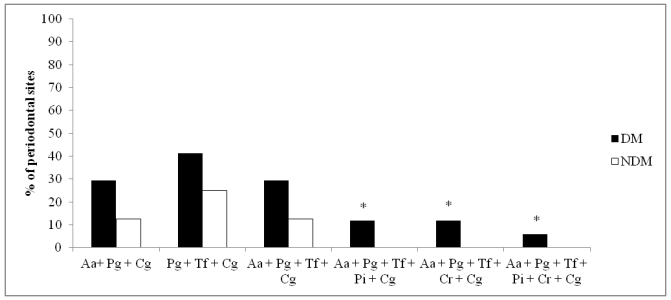

Fig. 3 - Frequency of periodontal sites (%) harboring combinations of putative periodontopathogens with *Candida glabrata* (Cg) in the DM and NDM groups. *Statistically significant differences between the DM and NDM groups, according to $\chi 2$ tests ($p < 0.05$). *Aggregatibacter actinomycetemcomitans* (Aa), *Porphyromonas gingivalis* (Pg), *Tannerella forsythia* (Tf), *Prevotella intermedia*, *Campylobacter rectus* (Cr).

Discussion

Although the majority of the published studies are cross-sectional, providing a limited possibility of a causal-effect relationship[10], substantial evidence has indicated diabetes mellitus (DM) as a risk factor for periodontal disease (PD)[3]. The results of the present study confirmed that patients with type 2 diabetes mellitus (DM) presented significantly higher values of PPD, GR and CAL compared to the control group and consequently greater severity of PD. The 2009-meta-analysis, investigated whether or not diabetes is a risk factor for periodontitis and included papers published between 1980 and 2007. Twenty-seven cross-sectional studies were included and detected more periodontal disease in diabetic compared to non-diabetic subjects. Although methodological flaws were found, the results showed a significant association between CAL (difference 1.00, 95% CI 0.15-1.84), PPD (difference 0.46, 95% CI 0.01-0.91) and DM, confirming that DM is an important risk factor for periodontitis[5].

Glycemic profile is usually measured in studies with diabetics to evaluate the influence of these parameters on the prevalence, extent and severity of periodontitis. Generally, well-controlled diabetes with glycemic parameters within the normal values seems to have little effect on the risk of periodontitis, however, the contrary is observed in poorly controlled patients. In the current study, fasting glucose levels and HbA1c were statistically higher in DM compared to NDM patients. Our glycemic results are consistent with important systematic reviews that investigated associations between diabetes, glycemic control and complications, showing that PD was more prevalent among individuals with poorer glycemic control[5,20]. Both studies supported the evidence that diabetes has an adverse effect on periodontal status and PD has an adverse effect on glycemic control. Due to the cross-sectional design of the present study, it is not possible to state that periodontal disease had an effect on glycemic control. This evidence could be provided by treatment and longitudinal studies. In poorly controlled diabetes, there is a nonspecific glycation of lipids and proteins that forms reactive oxygen species (ROS). In the case of DM, ROS are considered as a major risk for developing micro and macrovascular complications. They participate in the formation of advanced glycated end products (AGE) that induce crosslinkation processes in the structure of proteins, such as collagen, modifying blood vessel structure. After binding to their specific receptors (RAGE), they lead to cytokine production and proinflammatory effects[21]. All these factors can exacerbate the severity of periodontal disease, besides contributing to the development of systemic complications such as retinopathy, nephropathy, neuropathy and other diseases[4].

Another occurrence concomitant to diabetes mellitus is dyslipidemia, defined as a high blood concentration of lipids, especially LDL and triglycerides (TRG). Studies have indicated an association between elevation in blood lipoproteins and alterations in periodontal disease[2,22]. However, other clinical trials failed to identify this relationship[22,23]. In the current study, both groups with periodontal disease, independent of the presence of diabetes, presented normal values of LDL. However, DM patients had high levels of TRG, above normal values (> 150 mg/dL), and were positively correlated with two important periodontal parameters, GR and CAL. Our results are in agreement with Tu et al.[24] who evaluated associations between lipid parameters and periodontitis and found a positive correlation for TRG and C-reactive protein (CRP). Controversially, Sora et al.[25] evaluated the relationship of metabolic syndrome (five cardiovascular risk factors: abdominal obesity, hypertension, reduced HDL, elevated TRG and elevated

fasting glucose or diabetes), and these factors individually, with the extent of severe periodontitis among patients with DM from Gullah. They found a relationship between metabolic syndrome and periodontitis in this population; however, no results from the multivariable binomial regression demonstrated a relationship between the isolated components of metabolic syndrome, including HDL and TRG, and periodontitis in these individuals with diabetes. The authors explained that more than one risk factor linked to diabetes is necessary to increase the extent of periodontitis and commented that their results may not apply to other populations with a different genetic and socio-cultural background. However, Almeida Abdo et al.[26] found no associations between dyslipidemia and periodontal disease. They observed that diabetes, age and smoking had positive correlations with periodontitis (CAL $\geq$ 3 and CAL $\geq$ 5).

Periodontal health is dependent on a balance between the bacteria harboring the subgingival biofilm and the host response to them. Environmental changes may modify the bacterial challenge or host immune response, as occurs in diabetes patients[7]. The prevalence of putative periodontal bacteria in DM compared to NDM has been previously investigated by some authors[7,8,27,28]. The bacterial species belonging to the "red and orange complexes" are the most frequently studied and there is no consensus whether specific periodontal pathogens can harbor subgingival sites in subjects with DM when compared with NDM. In the present study, the frequency of periodontal sites harboring *A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythia*, *P. intermedia* and *C. rectus* (Cr) was statistically similar between the groups. Our results corroborate with those found by Field et al.[7], Sardi et al.[8] and Yuan et al.[28] and differ from other studies[29-32]. We focused on subgingival sites with periodontal disease, specifically deep pockets, for both groups, also reported by the majority of these studies. Contrarily, the study conducted by Aemaimanan et al.[30] found high levels of T. forsythia in healthy sites and *P. gingivalis* and *T. forsythia* in gingivitis sites and a higher quantity of *P. gingivalis* only in periodontal sites of poorly controlled DM patients compared to the NDM group. Recent studies have indicated a high prevalence of *T. forsythia* in DM patients with periodontitis and their possible role in the severity of periodontitis[29,30]. Li et al.[31] also demonstrated not only higher levels of *T. forsythia* but also *T. denticola*, however lower levels of *P. intermedia* in the subgingival plaque of chinese patients with type 2 diabetes mellitus. Zhou et al.[32] concluded that subjects with healthy periodontium harbored different genera (abundance of *Prevotella*, *Pseudomonas* and *Tannerella*) compared to patients with periodontitis (abundance in *Actinobacteria*, *Proteobacteria* and *Bacteriodetes*), and in both groups, the authors detected that diabetic and nondiabetic subjects harbored bacteria at several taxonomic levels with significant different prevalence or abundance. These discrepancies in the results could be related to the differences in the lifestyle of the populations, which interferes directly in the composition of the subgingival microbiota[33].

Diabetic patients are known for their predisposition to oral infections caused by *Candida* spp., particularly those with poor glycemic control. This condition may occur concomitantly with a higher incidence and greater severity of periodontitis[34]. In the current study, species of *Candida* were detected from deep pockets in both groups with chronic periodontitis, regardless of the presence of diabetes. However, two of these species, *C. albicans* and *C. glabrata*, were more frequently found in DM patients. *Candida* spp. have also been identified in periodontal sites by other investigators[8,35,36], but only the study developed by Melton et al.[36] demonstrated higher levels of *C. albicans* (53%) followed by *C. glabrata* (20%), similar to those obtained in the present study. *C. albicans* has been described as the most prevalent and pathogenic species of the genus[34] and plays a role in immune evasion and adherence to epithelium, causing inflammatory reactions[37] and may be detrimental to the periodontal environment. *C. glabrata* has emerged as an important opportunist pathogen in oropharyngeal candidiasis, after *C. albicans*. The frequency of *C. glabrata* infections increased up to 50% in some populations, due to immunosuppressive therapies and broad spectrum antibiotics[37]. A significant correlation between poor glycemic control and periodontal *Candida* colonization has been reported[8,34,35] and is in agreement with our results. Glucose level concentration in gingival crevicular fluid is known to be associated with blood glucose level[9] and could partly explain the proliferation of *Candida* in periodontal sites[35].

Another suggested effect of poorly controlled glycemic status in DM is the aggregation of local cytokine response[9]. In the current study, TNF-alpha was chosen for its intrinsic relationship with the severity of periodontal disease, but this study aimed to evaluate if this cytokine could be more elevated in diabetic patients due to their glycemic status. However, there was no difference between the groups. The relevance of the cytokine profile in subjects with DM and chronic periodontitis has been explored and the levels of these inflammatory markers seem not to differ from normoglycemic patients with a similar periodontal condition[9,38]. Javed et al.[38] evaluated databases from 1988 to 2011 and concluded that gingival crevicular fluid cytokine profile, including TNF-alpha, in patients with or without DM seems to be related to the severity of periodontal inflammation and the diabetes is secondary. Besides, the action of many of the biomarkers is short-lived and is likely to vary based upon stimulatory molecules, leading to a limited local response at affected sites. After activation of downstream biomarkers and/or local cell activation, many biomarkers, such as IL-6 and TNF-alpha, are degraded quickly[39]. The myeloperoxidase (MPO) activity was also evaluated in the present study. MPO is one of the peroxidase systems responsible for immunological defense in saliva and gingival crevicular fluid. Studies have demonstrated an increase in the MPO level in systemically healthy patients with periodontitis[40]. For diabetic subjects, two studies showed different results, with greater MPO activity[13,41]. In our study, there was no statistical difference in the MPO levels obtained from gingival crevicular fluid between diabetic and non-diabetic individuals, both with periodontal disease. The presence of diabetes did not influence the level of this proinflammatory marker, in agreement with Tenovuo et al.[41]. Controversially, Gonçalves et al.[13] found lower MPO activity in diabetic patients with chronic periodontitis, before and after periodontal treatment. There are methodological differences between the present study and the above-mentioned investigations. The first one[41] analyzed

saliva samples from patients with different types of diabetes, not exclusively DM and the last one[13] evaluated enzyme activity, not its concentration, as in the present study. These methodological differences make it difficult to compare the present study with the other investigations.

Within the limitations of this study, mainly the small sample size, it may be concluded that although chronic periodontitis was clinically more severe, the level of TNF–α, MPO and the prevalence of putative periodontal pathogens were not different in DM patients compared to non-diabetic individuals. An interesting fact was the high frequency of *C. albicans* and *C. glabrata*, which seem to have a preferential capacity for colonizing the periodontal pockets of diabetic patients. More longitudinal studies are necessary to confirm the influence of the species of *Candida* on the progression of periodontal disease.

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