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Relação entre os níveis de ácido lipoteicóico e perfil de citocinas no ambiente subgengival de indivíduos diabéticos: estudo de caso controle

Relationship between cytokine pattern and lipoteichoic acid in the subgingival environment of diabetics and non-diabetics subjects: a case-control study

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Trabalho de Conclusão de Curso apresentado à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtencão do título de Cirurgião Dentista.

Undergraduate final work presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Dental Surgeon.

Orientador: Prof. Dr. Renato Corrêa Viana Casarin;
Coorientador: Thiago Perez Rangel

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RESUMO

A Diabetes Mellitus (DM) é uma doença multifatorial de origem metabólica, que tem entre seus efeitos sistêmicos, a interdependência com a doença periodontal. Ambas são doenças crônicas altamente prevalentes e interrelacionadas, tendo uma relação bidirecional. A hiperglicemia resulta em alterações do sistema imune que podem exacerbar a doença periodontal induzida por bactérias; por outro lado, a infecção periodontal pode complicar a gravidade do diabetes e o controle metabólico glicêmico. Essa dupla via sugere que o controle da infecção periodontal é essencial para o manejo do diabetes mellitus, assim como o controle glicêmico é importante para a prevenção e controle da doença periodontal. Como já demonstrado por Kumar et al. (2006), ocorre uma mudança da microbiota oral em pacientes periodontais com DM: número maior de bactérias gram positivas, sugerindo um papel maior dessas bactérias na progressão da doença e ao fracasso dos tratamentos. O principal fator de virulência das bactérias gram-positivas é o ácido lipoteicóico (LTA), uma endotoxina bacteriana capaz de causar uma exacerbação da resposta inflamatória. Então, sugere-se um papel importante das bactérias gram-positivas no processo inflamatório, e consequente impacto que pode causar nos pacientes diabéticos. O objetivo do presente estudo foi avaliar os níveis de LTA de bactérias gram-positivas e sua relação com o perfil de citocinas no ambiente subgengival de indivíduos diabéticos e normoglicêmicos. Desta maneira, para as análises do presente estudo, foram selecionadas 30 pacientes, divididos em dois grupos - 15 indivíduos diabéticos com periodontite crônica generalizada e 15 normoglicêmicos com periodontite crônica generalizada (grupo controle). Cada indivíduo foi clinicamente avaliado quanto à sua condição periodontal e foram coletadas amostras do fluido gengival crevicular de cada participante e, posteriormente, realizadas as análises dos níveis de LTA, através do General Endotoxin ELISA. Como resultado, os diabéticos apresentaram maiores níveis de LTA no ambiente subgengival do que os normoglicêmicos ($p < 0,05$). Em relação aos níveis de citocinas, os indivíduos com DM apresentaram níveis mais elevados de IL-10, IL-1ß e MMP- 2 ($p < 0,05$). Assim, a maior presença de fatores de virulência e o comportamento celular alterado promoveram um aumento não apenas nos marcadores pró-inflamatórios, mas também nos anti-inflamatórios, tentando equilibrar a defesa do hospedeiro. No entanto, o LTA se correlacionou negativamente

com a IL-10, somado aos seus níveis mais altos na DM, esse equilíbrio esperado tornou-se uma resposta enfraquecida e induziu uma destruição exacerbada.

Palavras-chave: Periodontite. Diabetes Mellitus. Fator de virulência.

ABSTRACT

Diabetes Mellitus (DM) is a multifactorial disease of metabolic origin, which has among its systemic effects, interdependence with periodontal disease. Both are highly prevalent and interrelated chronic diseases, having a bidirectional relationship. Hyperglycemia results in changes in the immune system that can exacerbate bacteria-induced periodontal disease; on the other hand, periodontal infection can complicate the severity of diabetes and glycemic metabolic control. This double route suggests that the control of periodontal infection is essential for the management of diabetes mellitus, just as glycemic control is important for the prevention and control of periodontal disease. As already demonstrated by Kumar et al. (2006), there is a change in the oral microbiota in periodontal patients with DM: a greater number of gram positive bacteria, suggesting a greater role of these bacteria in the progression of the disease and the failure of treatments. The main virulence factor of gram-positive bacteria is lipoteichoic acid (ATL), a bacterial endotoxin capable of causing an exacerbation of the inflammatory response. So, an important role of gram-positive bacteria in the inflammatory process is suggested, and the consequent impact it can have on diabetic patients. The objective of the present study was to evaluate the levels of ATL of gram-positive bacteria and their relationship with the cytokine profile in the subgingival environment of diabetic and normoglycemic individuals. Thus, for the analysis of the present study, 30 patients were selected, divided into two groups - 15 diabetic individuals with generalized chronic periodontitis and 15 normo glycemic individuals with generalized chronic periodontitis (control group). Each individual was clinically assessed for their periodontal condition and samples of crevicular gingival fluid were collected from each participant and, subsequently, analyzes of ATL levels were performed using the General Endotoxin ELISA Kit. As a result, diabetics had higher levels of ATL in the subgingival environment than normoglycemic ones ($p < 0.05$). Regarding cytokine levels, individuals with DM had higher levels of IL-10, IL-1ß and MMP-2 ($p < 0.05$). Therefore, the greater presence of virulence factors and altered cellular behavior promoted an increase not only in pro-inflammatory markers, but also in anti-inflammatory ones, trying to balance the host's defense. However, the LTA correlated negatively with IL-10, added to its higher levels in DM, this expected balance became a weakened response and induced an exacerbated destruction.

Key words: Periodontitis. Diabetes Mellitus. Virulence factor

SUMÁRIO

1 INTRODUÇÃO

A Diabetes Mellitus (DM) é definida como um grupo de doenças de origem metabólicas. A hiperglicemia resultante da falha na secreção ou na ação da insulina. É dividido em dois tipos principais, tipo I: relacionado à destruição autoimune das células pancreáticas e, tipo II: relacionado à alteração na produção e resistência celular à insulina. Independente do grau de desenvolvimento do país, a DM consiste em um importante e crescente problema de saúde mundial. No Brasil, os dados mais recentes apontam números superiores a dezesseis milhões de diagnósticos, o que caracteriza um aumento na incidência em mais de sessenta por cento nos últimos dez anos. Entre os efeitos sistêmicos da DM, a medicina inclui a periodontite como uma importante ocorrência dessa doença.

A doença periodontal e a diabetes mellitus são doenças crônicas altamente prevalentes e inter-relacionadas, tendo uma relação bidirecional. A hiperglicemia do diabetes resulta em alterações do sistema imune que podem exacerbar a doença periodontal induzida por bactérias; por outro lado, a infecção periodontal pode complicar a gravidade do diabetes e o controle metabólico glicêmico. Essa dupla via sugere que o controle da infecção periodontal é essencial para o manejo do diabetes mellitus, assim como o controle glicêmico é importante para a prevenção e controle da doença periodontal.

A influência da hiperglicemia sobre a doença periodontal, se dá por diferentes vias. Na presença da DM, ocorre um acúmulo dos produtos finais da glicação avançada (AGEs), no plasma e tecidos. Os AGEs têm a capacidade de se ligar a receptores de membranas das células (RAGE – receptor de produtos finais glicosilados), os quais estão presentes em células endoteliais, monócitos/macrófagos, células do sistema nervoso e também células musculares. A interação entre AGEs e RAGE, aumenta o estre sse oxidativo celular, o que resulta em maior produção e secreção de citocinas pró - inflamatórias, associadas à diferenciação e atividade de osteoclastos, causando maior reabsorção óssea e à produção de metaloproteases da matriz (MMPs). Contudo, outra via de relação entre o avanço da periodontite e a presença da DM, é a alteração microbiológica no ambiente subgengival.

Kumar et al. (2006) mostraram que ocorre uma mudança da microbiota oral em pacientes periodontais com DM, gerando fatores locais específicos na bolsa periodontal, criando uma constituição microbiana diferenciada. Esse achado é

corroborado por diferentes autores (Zhou et al, 2013, Demmer et al, 2015, Joaquim et al, 2017). Contudo, um ponto importante é a mudança de pontos específicos na microbiota. Casarin et al, (2013) observaram uma microbiota subgengival diferente entre pacientes com DM e periodontite. Enretanto, assim como recentemente mostrado por Ganesan et al. (2017), indivíduos com DM apresentaram um grande número de bactérias gram - positivas em pacientes diabéticos, sugerindo um papel maior dessas bactérias na progressão da doença e ao fracasso dos tratamentos. No entanto, a maioria dos estudos sobre gram-positivas são relativas ao seu envolvimento em processos cariosos e contaminação pulpar, associadas a lesões perirradiculares, e ao insucesso do tratamento endodôntico, não havendo na literatura estudos focados na relação desse tipo de microrganismos com a periodontite.

O principal fator de virulência das bactérias gram-positivas é o ácido lipoteicóico (LTA), uma endotoxina bacteriana que é liberado após lise. Estes se ligam a receptores como toll-like 2 (TLR2), que são expressos nas células hospedeiras do periodonto. A ativação de TLR2 dependente de LTA em células dendríticas imaturas, segundo Keller et al. (2009), leva à produção significativa de TNF-alfa e IL-1beta, exacerbando a resposta inflamatória. Também foi sugerido o papel do LTA, associado à persistência de lesões, ativando macrófagos e o sistema complemento do hospedeiro, levando a autólise tecidual.

Assim, sugere-se um papel importante das bactérias gram-positivas no processo inflamatório, e consequente impacto que pode causar nos pacientes diabéticos. O conhecimento da relação entre microrganismos e seus constituintes, em especial o LTA, e o padrão de citocinas liberadas nesses individuos, torna-se de ainda maior importância. O presente projeto de iniciação científica integra-se a dissertação de mestrado intitulada “Diabetes Mellitus altera a produção de citocinas próinflamatórias mediadas por lipopolissacarídeos e ácidos lipoteicóicos nos tecidos periodontais”, em que se avaliou tanto os níveis de LTA e LPS presentes nas bolsas periodontais e sua relação com o perfil de citocinas e MMPs no ambiente subgengival de pacientes diabéticos em comparação com pacientes normoglicêmicos.

2 ARTIGO: SUBGINGIVAL ENDOTOXIN AND LIPOTEICHOIC ACID MODULATE CYTOKINE PRODUCTION IN DIABETIC SUBJECTS: A Case-control Study

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Diabetes mellitus alters cytokine/protease levels in gingival crevicular fluid, what could be associated to increased LTA/LPS levels.

Abstract

Background: Periodontal disease and diabetes mellitus (DM) are highly prevalent and interrelated diseases, resulting in altered host response microbiota. Thus, this study aimed to evaluate the impact of DM on local levels of lipopolysaccharide (LPS) and lipoteichoic acid (LTA) and their relationship with cytokines and matrix metalloproteinases' (MMPs) profile.

Methods: This case–control study included diabetic (n = 15) and non-diabetic (n = 15) subjects presenting Stage 3-4, Grade C, Periodontitis. Gingival crevicular fluid (GCF) was collected, and LPS and LTA levels were analyzed by enzyme-linked immunosorbent assay (ELISA), while IFN-γ, IL-10, IL-17, IL-1β, IL-4, MMP-2, and MMP-9 were measured by LUMINEX/MAGpix. Mann–Whitney and Spearman's correlation tests were used to compared and to correlate variables ($p < 0.05$).

Results: Higher levels of LTA, LPS, IL-10, IL-1β, and MMP-2 ($p < 0.05$) and lower levels of IL-17 were found in the DM group ($p < 0.05$). Non-diabetic subjects presented higher LPS, IFN-γ, IL-17, and MMP-2 levels and lower IL-10 concentration ($p < 0.05$). No significant correlation was seen between LPS and cytokine profile in non-diabetic. Local levels of LTA were positively correlated with IL-17 and MMP-2 and negatively with IL-10.

Conclusion: LTA and LPS drove the inflammatory profile through the modulation of cytokines and MMPs in a different manner in DM and non-diabetic subjects.

Key Words: Diabetes Mellitus. Cytokines. Periodontitis. Virulence Factors.

Introduction

Diabetes mellitus (DM) is defined as a group of diseases of metabolic origin resulting from the failure of secretion or action of insulin. It is divided into two main types: type I—related to the autoimmune destruction of pancreatic cells; and type II—related to alteration in production and cellular resistance to insulin (Mealey & Oates, 2006). Regardless of the degree of development of the country, DM is a significant and growing global health problem. In Brazil, the most re- cent data point to over sixteen million diagnoses, which has led to an increase in the incidence of more than sixty percent in the last ten years World Health Organization, 2016). Listed as one of the most associated conditions with DM is periodontal disease.

Epidemiological studies have shown that periodontitis is associ- ated with an increase in incident DM, having a bidirectional relation- ship (Preshaw et al., 2012). Hyperglycemia results in changes in the immune system that may exacerbate periodontal disease induced by bacteria; on the other hand, the periodontal infection can jeopar- dize glycemic metabolic control (Gurav, 2016; Llambés et al., 2015; Preshaw et al., 2012). This two-sided pathway suggests that the con- trol of periodontal infection is essential for the management of dia- betes mellitus, as glycemic control is essential for the prevention and control of periodontal disease (Preshaw et al., (2012). This patho- logical cycle is associated not only with alteration of host response but also with an altered microbiota (Casarin et al., 2013; Ganesan et al., 2017; Longo et al., 2018).

Casarin et al., (2013) showed that a change of the subgingival mi- crobiota occurs in periodontal patients with DM, generating specific local factors in the periodontal pocket and creating a differentiated microbial constitution compared with non-diabetic periodontal pa- tients. This finding is corroborated by different authors that showed higher levels of well- recognized pathogens, *T. forsythia*, *P. gingivalis*, and *F. nucleatum* (Demmer et al., 2015; Joaquim et al., 2018; Kumar et al., 2006; Yang et al., 2016; Zhou et al., 2013). Interestingly, al- though the common periodontal disease-associated species have been described as anaerobic gram negatives, several studies have shown that the diabetic-associated microbiome has a higher number of gram-positive bacteria (Casarin et al., 2013; Kumar et al., 2006). It suggests the possibility of a greater role for these bacteria in disease progression and treatment failure. *Selenomonas spp*, *Gemella spp,* and *Capnocytophaga spp* are examples of gram-positive species detected in higher prevalence or levels in subgingival environment of DM sub- jects (Casarin et al., 2013). It is possible that these

bacteria could lead to inflammatory response if virulence factors can be detected in them. The main virulence factor of gram-positive bacteria is lipote- ichoic acid (LTA), a bacterial endotoxin that is released after lysis (Ginsburg, 2002; Klukowska et al.,2017). These bind to receptors such as toll-like 2 (TLR2), which are expressed in the host cells of the periodontium. Activation of LTA-dependent TLR2 in immature dendritic cells (Keller et al., 2010) leads to significant IL-1beta pro- duction, exacerbating the inflammatory response. It has also been suggested that LTA could be associated with the persistence of lesions by activating macrophages and the complement system of the host, leading to tissue autolysis (Endo et al., 2013). On the other hand, in gram-negative bacteria, the virulence factor is lipopolysac- charides (LPS), an external membrane component. Host receptors TLR2 and TLR4 can recognize LPS triggering a response that, when unbalanced or excessive, can destroy periodontal tissue (Yoshioka et al., 2008). Not surprisingly, robust evidence has shown a different cytokine pattern in DM (Sima & Van Dyke, 2016).

Since the subgingival environment in the periodontal disease has a large concentration of gram-positive and gram-negative bacteria, the presence of these proteases could alter the progression of peri- odontal disease. This pathological pattern may still be worsened in individuals with systemic conditions that alter the pattern of cellular response, such as DM. However, the LPS and also LTA levels in the subgingival environment and their impact on locally released cyto- kines remain still unknown.

Thus, the objective of the study was to evaluate the levels of LTA and LPS presented in the periodontal pockets in diabetic patients and their relationship with the cytokine and MMP profile in the sub- gingival environment of diabetic individuals compared to non-dia- betic subjects.

Material and Methods

Study Design

This study was a case–control trial, conformed to the STROBE (strengthening the reporting of observational studies in epidemi- ology) guidelines, comparing DM and non-diabetic subjects and was approved by the Institutional Review Board (IRB) of Piracicaba Dental School (CAAE 89303418.8.0000.5418). Recruitment was made from June 2017 to September 2018, and consent was ob- tained before the collection of data and samples. Subjects were allocated to each group according to inclusion criteria:

DM group (n = 15)—Diagnosis of type 2 diabetes mellitus for at least two years and glycated hemoglobin (HbA1c) higher than 7%, characterizing a Grade C disease (Papapanou et al., 2018); have at least 20 teeth; presence of generalized Grade C, Stage ¾ periodon- titis (Papapanou et al., 2018) (≥10 periodontal pockets with probing pocket depths of >4 mm and marginal alveolar bone loss of >30%); and age over 35 years.

Non-Diabetic group (n = 15)—Systemic health; have at least 20 teeth; presence of generalized Grade C, Stage ¾ periodonti- tis (Papapanou et al., 2018) (≥10 periodontal pockets with probing pocket depths of >4 mm and marginal alveolar bone loss of >30%); and age over 35 years.

The exclusion criteria were as follows: (a) Presence of other types of periodontal disease; (b) Be on a diet or nutritional monitoring; (c) Pregnant and lactating women; (d) Smokers; (e) Be in orthodontic treatment; (f) Have completed periodontal treatment in the last year; (g) Used antimicrobial mouthwashes in the last 30 days; and 8. Use of medications that alter the course of periodontal disease.

Clinical Evaluation

After recruitment, all patients were instructed on the causes and con- sequences of periodontal disease, as well as on preventive techniques, including the technique of brushing and flossing. Clinical param- eters were collected, including plaque index, gingival index, pocket depth, bleeding on probing, and clinical attachment level (Casarin et al., 2010). All parameters were evaluated by a calibrated examiner (intra-class correlation of 87% for CAL). After sample collection, pa- tients were treated with Full Mouth Ultrasonic Disinfection protocol and included in supportive therapy (Cirano et al., 2012).

Cytokine/protease profile

From each individual, gingival crevicular fluid (GCF) was collected from 4 deep pockets (PPD > 7mm), one per quadrant. After re- moval of the supragingival biofilm, the teeth were washed, and the area was isolated using cotton rolls and gently dried with air jets. The GFC was collected through the insertion of filter paper strips‡ into the periodontal pocket for 30 s (Casarin et al., 2010). Two paper strips[1] were used, at the same time, per site, to ob- tain an adequate volume of GCF and immediately frozen on −20˚C freezer in dried tube. All paper strips were pooled per patient in the same tube. For analysis of the local cytokine profile, GCF sam- ples were pooled from each patient and analyzed for the detec- tion of IL-10, IL-1beta, IL-17, IL-4 IFN-γ, MMP-2, and MMP-9 by Luminex/MAGpix technology[2], using commercially available kits[3] following manufacturer's instructions. Briefly, samples were di- luted at PBS + Tween5% buffer, vortexed for 30seconds, prior to analysis. After gentle centrifugation, 25 µL of supernatants was placed in 96-well plates along with immunomarked beads spe- cific for each cytokine/protease. After beads incubation, second- ary antibodies and substrate were provided and plates readed at MAGpix platform. The sample's concentration was estimated from the standard curve using a five-parameter polynomial equa- tion using Xponent® software (Millipore, Corporation). The mean concentration of each marker was calculated using the individual as a statistical unit and expressed as pg/ml. Moreover, in order to correct any samples' volume differences, total protein of each sample was determined using a standard Bradford reaction. For analysis, besides individuals' values, cytokine was grouped accord- ing to biological function (Pro-inflammatory ones (PRO) = sum of IL-1beta, IL-17 and IFN-γ; Anti-inflammatory ones (ANTI) = sum of IL-10 and IL-4) and ratios between them were also done.

LPS and LTA analysis

At the same sites and after GCF collection, after at least 30 sec- onds of waiting time, two apyrogenic paper points[4] were inserted in periodontal pockets for 30 seconds and immediately frozen on −20˚C freezer. These samples were used for LPS and LTA analysis. LPS levels were analyzed using the General Endotoxin ELISA Kit[5] (0.01-1.00 EU/mL), according to instructions from the manufacturer. Briefly, after plate and reagents' preparation, standard solution, supplied by the manufacturer, as well as blanks and 50
µL of sam- ples were added to wells. Then, the primary antibody was inserted and the plate incubated for 1 hour at 37°C. After incubation, the plate was washed using

the manufacturer's wash buffer, and then a secondary antibody added. The plate was incubated for 45 min at 37°C, washed and the substrate and stop solution added to each well. LPS levels will be analyzed through an ELISA reader[6] at 450 nm.

LTA levels were analyzed using the human LTA ELISA Kit[e] (20 to 0.312 ng/ml) according to the manufacturer's instruction. The ELISA plate was conditioned with the LTA monoclonal antibody supplied by the kit manufacturer, and the standard, control and 50

µL sample solutions inserted. They were incubated for 60 min at 37°C, after which it was washed and a substrate was added, thus allowing the plate to read. The plate was read, and LTA levels were analyzed through an ELISA reader[f] at 450 nm. As previously described, total protein was determined using a Bradford reaction[7], and this concentration was used to adjustment.

Statistical analysis

This transversal study, presenting as primary variable the levels of LTA, set a sample size based on a previous study (Sarda et al., 2016), once this is the first one to analyze this virulence factor in periodon- tal pockets. For all analyses, the subject was considered the experi- mental unit and all comparisons were made by a blinded statistician. Firstly, the cytokine, MMP, LPS, and LTA levels in each group were compared by Student's *t* or Mann– Whitney test, depending on the normality evidenced by the Shapiro–Wilk test. A Spearman correla- tion tests were used to assess the relationship between LPS and LTA levels and locally released cytokines levels. All analyses considered a significance level of 5%.

Results

Demographic and clinical data

From June 2017 to September 2018, a total of 1134 subjects re- ferred to the Graduated Clinic of Piracicaba Dental School were examined. From this total, 15 type-2 diabetics and 15 non-diabetic patients were included in the study. Table 1 displays the demo- graphic and clinical data from both groups. Only on fasting plasma glucose, a statistical difference ($p > 0.05$) was seen. Confirming the hyperglycemic poorly controlled status of DM group, the HbA1c lev- els were 7.3 ± 0.7 (ranging from 7.0 to 8.7).

LTA, LPS, and cytokines/protease levels

Diabetic subjects presented higher levels of LPS and LTA in the sub- gingival environment than non-diabetic ones ($p < 0.05$) (Figure 1). Regarding cytokine levels, DM subjects presented higher levels of IL-10, IL-1ß, and MMP-2 ($p < 0.05$). The IL-1ß/IL-10, and PRO/ANTI
were higher in DM subjects than in non-diabetics ($p < 0.05$). No dif- ference between groups was noted regarding other cytokines/ratios (Table 2).

Correlation between virulence factors and GFC profile

At Table 3, it could be noted that LPS was not significantly corre- lated to any cytokine/protease in non-diabetic subjects ($p > 0.05$), while LTA directly and significantly correlated with IL-17, as well as IL-17/IL-10 ratio and MMP-2 levels, while negatively correlated with IL-10 levels ($p < 0.05$). On the other hand, in DM subjects, LPS was directly correlated to IFN-γ, IL-17, IL-1β/IL-10 ratio, PRO/ANTI ratio, and MMP-2, while negatively correlated with IL-10 ($p < 0.05$). LTA presented a direct and significant correlation with IL- 17 and MMP-2 levels, while it was negatively correlated with IL-10 levels, despite the glycemic status ($p < 0.05$).

TABLE 1 Demographical and clinical parameters of subjects included in the study

	DM group (n = 15)	Non-diabetic group (n = 15)
Age (years ± SD)	45.1 ± 4.3	42.2 ± 3.8
Gender (n female)	11	12
Fasting plasma glucose (dg/mL ± SD)	157.5 ± 64.0*	93.5 ± 7.4
HbA1c (%±SD)	7.3 ± 0.7	–
Plaque index—PI (%±SD)	34.2 ± 14.1	38.5 ± 16.9
Gingival index—GI (%±SD)	8.9 ± 2.0	10.5 ± 2.6
Full Mouth Probing Depth—FMPD (mm ± SD)	2.7 ± 0.1	2.2 ± 0.1
Full Mouth Clinical Attachment Level—FMCAL (mm ± SD)	3.36 ± 0.8	2.3 ± 0.3
Bleeding on probing—BoP (%±SD)	35.9 ± 11.7	25.5 ± 12.3
Percentage of sites with PD > 5mm and BoP	10.3 ± 7.3	14.9 ± 5.4
Probing depth of sampling sites (mm ± SD)	7.6 ± 1.0	7.1 ± 0.3

Abbreviations: dg, decigram; SD, standard deviation.
*Indicates statistical difference between groups (Student's *t* test, $p > 0.05$).

TA B L E 2 GFC levels (mean (standard error)) of cytokines, MMPs (pg/mL), and total protein (ug/mL) in DM and non-diabetic subjects.

	IFN-γ	IL-10	IL-17	IL-1β	IL-4	MMP-2	MMP-9	Total protein
DM	12.28 (3.87)	0.94 (0.16)*	2.22 (0.47)*	551.29 (157.59)*	1.52 (0.76)	71152.01 (20181.21)*	171415.12 (53053.87)	0.18 (0.12)
Non-diabetic	19.69 (4.73)	0.43 (0.08)	7.08 (1.94)	106.23 (38.30)	1.80 (0.78)	19002.96 (9098.23)	172679.69 (33131.18)	0.17 (0.12)

*indicates a statistical difference between groups (Student's t and Mann–Whitney tests, $p < 0.05$).

TABLE 3 Correlation (r(p)) between cytokines/proteases (pg/mL) and LPS/LTA levels in diabetic (DM) and non-diabetic subjects

	DM		Non-Diabetic	
	LPS	LTA	LPS	LTA
IFN-γ	0.538 (0.04)	0.459 (0.09)	0.042 (0.88)	0.397 (0.138)
IL-10	−0.798 (<0.001)	−0.731 (0.003)	−0.323 (0.25)	−0.615 (0.01)
IL-17	0.820 (<0.0001)	0.740 (0.003)	0.231 (0.42)	0.572 (0.025)
IL-1β	−0.112 (0.69)	−0.340 (0.22)	0.169 (0.55)	0.125 (0.65)
IL-4	0.288 (0.30)	0.430 (0.12)	−0.094 (0.738)	0.087 (0.74)
MMP-2	0.880 (<0.0001)	0.644 (0.01)	0.202 (0.47)	0.736 (0.001)
MMP-9	−0.020 (0.92)	−0.156 (0.583)	−0.059 (0.83)	0.408 (0.127)
IL-1β/IL-10	0.539 (0.046)	−0.042 (0.886)	0.423 (0.132)	0.397 (0.149)
IL-17/IL-10	0.360 (0.206)	−0.062 (0.834)	−0.003 (0.992)	0.666 (0.007)
PRO/ANTI	0.551 (0.041)	−0.015 (0.958)	0.385 (0.174)	0.360 (0.187)

Note: Gray color indicates statistically significant correlation (Spearman's correlation test, $p < 0.05$).

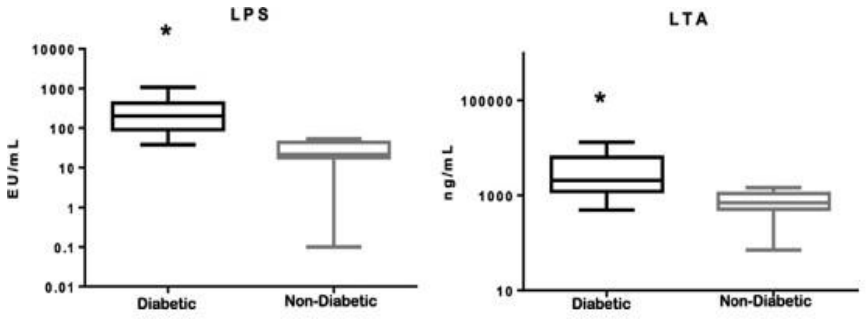

FIGURE 1 The concentration of LPS (EU/mL) and LTA (ng/mL) in subgingival samples of DM and non-diabetic subjects.*indicates a statistical difference between groups (Mann–Whitney test, $p < 0.05$)

Discussion

Diabetes mellitus is a metabolic disease, considered as a risk factor of periodontitis. Several studies tried to clarify how this systemic con- dition affects periodontal homeostasis, leading to a higher degree of destruction and inflammation. Within several pathogenic aspects cited by literature, changes in the microbiota have been described as an essential aspect and some authors suggested not only an in- crease in gram-negative pathogens colonization but also a higher level of gram positives. Since both types of bacteria present a mem- brane virulence factor (LPS and LTA, respectively), the present study evaluated the LPS and LTA levels in the subgingival environment of DM and non- diabetics and its associations with the cytokine profile. Results showed that although LPS and LTA were augmented in DM, the local release of cytokines and MMPs was differently affected in non-diabetic and DM subjects, indicating a possible different role of each virulence factor in systemically affected subjects. Considering the bidirectional relationship (periodontitis affecting DM control), understand how this interaction occurs is essential.

In 2013, our group suggested a possible role of gram positive in a hyperglycemic pocket, once *Gemella, Eikenella, Selenomonas, Actinomyces*, and *Streptococcus* genera—all of which gram-posi- tive—were detected at higher levels in deep pockets of DM than in non-DM subjects (Casarin et al., 2013). Recently, Longo et al., (2018), also identified enrichment in facultative gram positives in DM, cor- roborating other studies (Casarin et al., 2013; Demmer et al., 2015; Joaquim et al., 2018; Yang et al., 2016; Zhou et al., 2013). This higher number of gram positives could explain the higher level of LTA in DM than in non-diabetic subjects observed in the present study. To the best of our knowledge, this is the first study assessing LTA levels in periodontal pockets.

LTA is a potent cell stimulator, promoting the release of various molecules (Kang et al., 2016). The binding of LTA to TLR2 induces the release of IL-1β and TNF-alpha by human monocytes (Hessle et al., 2005), IL-18 by macrophages (Hara et al., 2018), induced β-cat- enin pathway activation (and NF-κB activity) and pro-inflammatory cytokine expression by epithelial cells (Jang et al., 2015), IL-6 and TNF-alpha by blood cells (Koch et al., 2014). Interestingly, this is the first *in vivo* study trying to correlate LTA levels and its potential as a local host response modulator. The results showed that both populations, DM and Non-diabetics, presented a similar correlation between LTA and the cytokine profile in periodontal pockets—the higher LTA level, the higher MMP-2 and IL-17 and lower IL-10 release in the subgingival environment.

Ahn et al., (2018) in a mice model, showed that LTA isolated from *Lactobacillus plantarum* reduced IL-10 production. In the same way, Volz et al., (2018), identified a reduction in IL-10 release by dendritic cells after LTA stimulation. However, the authors highlighted that this reduction only occurs when cells were stimulated by *S. aureus*-LTA and not *S. epidermidis*-LTA, suggesting IL-10 regulation could depend on the species of bacteria. Considered as an anti-inflammatory cy- tokine, IL-10 can down-regulate the synthesis of pro-inflammatory cytokines and chemokines such as IL-1, IL-6, TNF-α, and nitric oxide (Houri-Haddad et al., 2007; Mosser & Zhang, 2008), regulating the host homeostasis. Therefore, IL-10 has also been considered an es- sential regulator of bone homeostasis in homeostatic and inflam- matory conditions (Al-Rasheed et al., 2003; Carmody et al., 2002). Another interesting finding is the fact that IL-10 down-regulate gelatinase (MMP2) and type IV collagenase (MMP-9) production (Mosser & Zhang, 2008). It collaborates to understand a higher pro- duction of MMP-2 and the positive and robust association between LTA.

MMP-2, also named gelatinase A, has played a significant role in matrix destruction during periodontal disease (Franco et al., 2017). In the present study, besides LTA, also LPS was correlated to higher MMP2 release, and DM subjects showed higher levels than non-dia- betics. MMPs have been proposed as master regulators of inflamma- tion, through proteolysis of chemokines, growth factors, receptors, and their binding proteins (Franco et al., 2017). MMP also acts as an intracellular multifunctional protein, resulting in pro- or anti- inflam- matory functions, leading to either tissue homeostasis or pathology (Franco et al., 2017; Van Lint et al., 2005). In DM subjects, higher lev- els of MMP-2 have been described in DM population, also associated with systemic adverse effects, like retinopathy, nephropathy, and cardiovascular disease (Kostov et al., 2016; Kowluru, 2010; Peeters et al., 2015; Thrailkill et al., 2007). In an esophageal cancer cell cul- ture, MMP-2 was induced by IL-17A, through ROS/NF-κB/MMP-2/9 signaling pathway activation (Song & Yang, 2017). In the present study, IL-17 showed different levels in DM and non-DM subjects, which may suggest a modulating role of LTA in DM and non-diabetic subjects.

Regarding IL-17, the present study showed a positive correla- tion with LTA in DM and non-diabetic ones. Although no difference between groups has been observed, IL-17 has been suggested as an important marker in periodontal destruction in DM subjects (Graves et al., 2018; López del Valle et al., 2015; Silva et al., 2012), regulating other inflammatory features (Silva et al., 2012). This relationship between LTA and

IL-17 also could be affected in an- other way. Recently, an animal study demonstrates that diabetes increased the pathogenicity of the oral microbiome. When it was transferred to normal germ-free hosts, it increased the osteoclas- togenesis and periodontal bone loss regulated by IL-17 (Graves et al., 2018). The authors also discuss that hyperglycemia could modify bacterial behavior and virulence—for example, stimulation of LTA production by them, which creates a vicious cycle for the disease. This possible pathway should be better explored in future studies. Moreover, other virulence factors also could be founded in gram-positive, as lipoproteins, what has been proved to be ca- pable of inducing inflammation and could interfere in disease pro- gression (Kim et al., 2017). In summary, LTA is a crucial feature of periodontitis destruction, showing a potential line of research to understand the periodontal disease on DM patients. The present study is, to the best of our knowledge, the first to evaluate LTA levels in periodontal pockets, and future studies should consider its possible role in the pathogenesis of periodontitis.

Meanwhile, one of the most striking results of the present study is the dissimilarity between the LPS-cytokine relationship observed in DM and non-diabetic. While in non-diabetic LPS lev- els was not significantly correlated to anyone cytokine/protease, in diabetics, it was. Only in DM subjects, LPS was positively cor-

related to IL-17, MMP-2, IFN-γ, IL-1ß/IL-10, and PRO/ANTI ratios. Moreover, it was negatively associated with IL-10. Additionally, diabetic subjects presented a higher level of LPS in subgingival sam- ples than non- diabetic. LPS is a well-known and studied virulence factor from gram negatives that have been historically associated with the release of pro-inflammatory cytokines and reduction in anti-inflammatory ones (Ding & Jin, 2014; Jiang et al., 2018). However, in the local release of cytokines, there was a different result in DM and non-DM subjects. This differential modulation could be attributed to changes in cell behavior after a long period of hyperglycemia. An interesting and recent study in monkeys eval- uated the different pathways activated during periodontal disease development in hyperglycemia condition. The authors showed an increase in advanced glycation end products (AGES), beta-defen-sin and also in IL-17 in DM monkeys compared to non-DM ones. They suggest that hyperglycemic conditions might lead to the de- struction of periodontal tissues by accelerating the inflammatory response and weakening the defense system in periodontal tissues (Jiang et al., 2018). This conclusion corroborates with Kumar and collaborators (Kumar et al., 2020) who suggest different interac- tion between cytokine and microbiota in diabetic patients. Altered cell function could be the

explanation why several studies. As ob- served in the present study, diabetic condition specially in poorly controlled subjects (levels of HbA1c > 7%) induces to a local dis- similarity in cytokine production and increased risk for periodontal destruction (Genco & Borgnakke, 2000; Jiang et al., 2018; Kumar et al., 2020; Papapanou et al., 2018; Santos et al., 2010).

The imbalance in DM host response became clear when analyz- ing the axis IL-10- IL- 1ß. A contradictory result is a negative correla- tion of LPS (and also LTA) with IL-10, although in DM, a higher level of IL-10 and LPS/LTA was seen. When looking for results, although as increased IL-10 could bring the idea of a more anti-inflammatory en- vironment, the ratio IL-1ß/IL-10 and also PRO/ANTI ratio were higher in DM, showing the hyper- inflamed local condition, what corrobo- rates another study (Acharya et al., 2017). Thus, the higher presence of virulence factors and altered cell behavior promoted an increase not only in pro-inflammatory but also in anti-inflammatory markers, trying to “equilibrate” the host defense. However, based on the fact that LPS/LTA negatively correlated to IL-10 (Ahn et al., 2018; Volz et al., 2018)—summed to their higher levels in DM—this expected equilibrium became a weaken response and induces an exacerbated destruction.

Future studies should correlate these events with a larger num- ber of participants, given the limited number of individuals in this research. Moreover, the non-diabetic subjects were confirmed only by fasting plasma glucose, once they have never been diagnosed be- fore. So, HbA1c testing also in healthy population could be done in future studies. Beside these limitations, our data for the first time show the possibility of gram-positive bacteria playing a active role on periodontal disease and bring a new light on the periodontal disease on DM subjects.

Conclusion

Diabetic patients had a higher local level of LTA and LPS than non- diabetics, resulting in a disequilibrium on the host response. LTA and LPS may have the capacity to drive the inflammatory profile through the modulation of cytokines and MMPs in a different manner in DM and non-diabetic subjects.

PEER RE VIE W

The peer review history for this article is available at https://publo ns.com/publon/10.1111/odi.13661.

ENDNOTES

[1] Periopaper, Oraflow.

[2] MAGpix.

[3] HCYTOMAG-60K and HMMPMAG, Merck, Darmstadt, Germany.

[4] Tanari, Manaus, AM.

[5] My BioSource, San Diego, CA

[6] ELISA.

[7] BioRad, Hercules, CA.

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3 CONCLUSÃO

Os diabéticos apresentaram maiores níveis de LTA no ambiente subgengival do que os normoglicêmicos ($p<0,05$). Em relação aos níveis de citocinas, os indivíduos com DM apresentaram níveis mais elevados de IL-10, IL-1ß e MMP- 2 ($p <0,05$). Assim, a maior presença de fatores de virulência e o comportamento celular alterado podem estar relacionados a um aumento não apenas nos marcadores pró-inflamatórios, mas também alterações naqueles anti-inflamatórios, tentando equilibrar a defesa do hospedeiro. No entanto, o LTA se correlacionou negativamente com a IL-10 e, somado aos seus níveis mais altos na DM, esse equilíbrio esperado tornou-se uma resposta enfraquecida, induzindo a uma destruição exacerbada.

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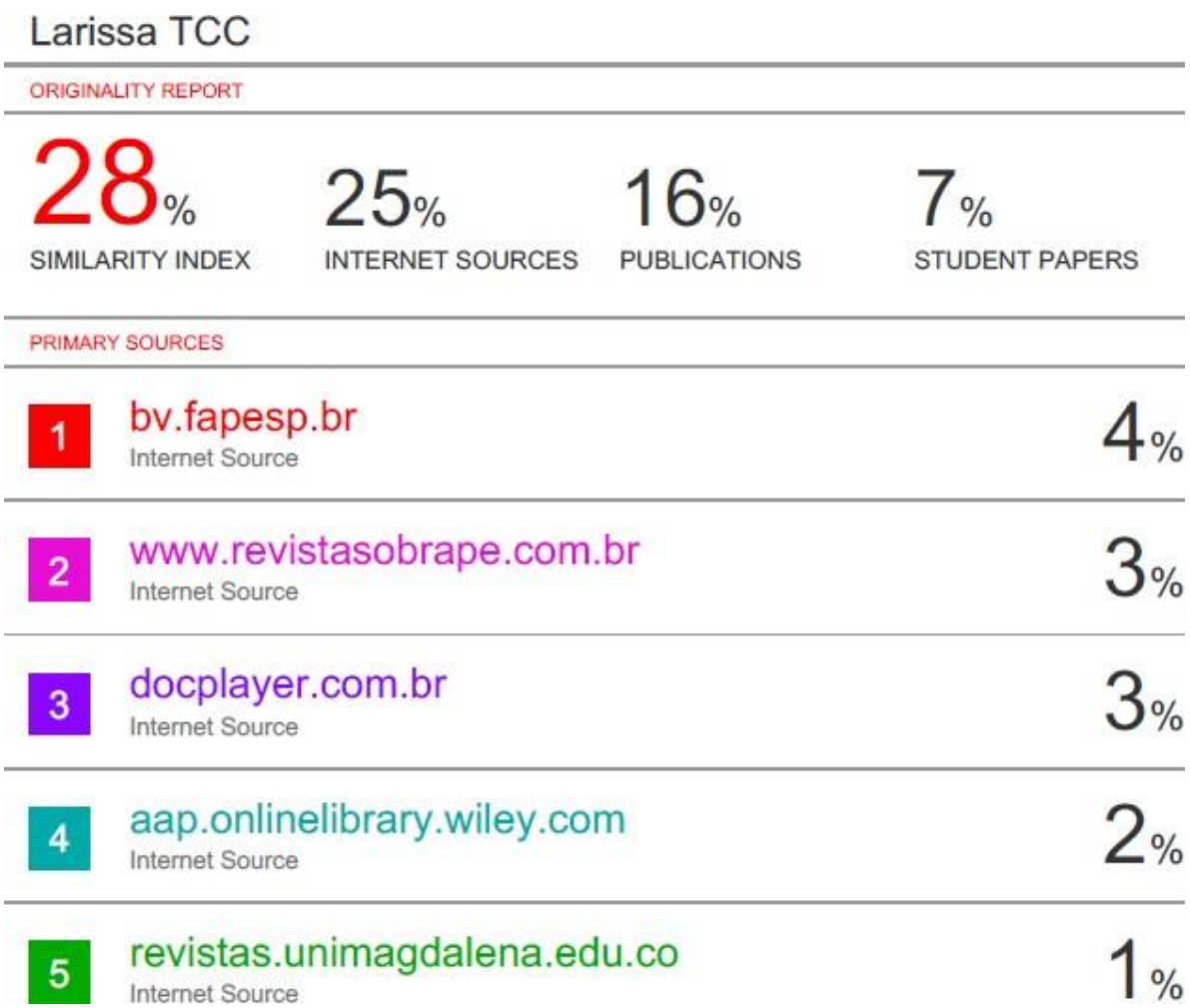
Anexo 1 – Verificacao de originalidade e prevenção de plagio



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Anexo 2 – Comitê de Ética em Pesquisa

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa "**Relação entre o perfil de citocinas e níveis de lipopolissacarídeos e ácido lipoteicóico no ambiente subgengival de indivíduos diabéticos e não diabéticos**", CAAE **89303418.8.0000.5418**, dos pesquisadores **Thiago Perez Rangel, Lara de Anchieta Caponi, Larissa Cristina Spirito Pena** e **Renato Corrêa Vianna Casarin**, satisfaz as exigências das resoluções específicas sobre ética em pesquisa com seres humanos do Conselho Nacional de Saúde – Ministério da Saúde e foi aprovado por este comitê em 22/08/2018.

The Research Ethics Committee of the Piracicaba Dental School of the University of Campinas (FOP-UNICAMP) certifies that research project "**Relationship between cytokine pattern and lipopolysaccharides and lipoteichoic acid in the subgingival environment of diabetics and non-diabetics subjects**", CAAE **89303418.8.0000.5418**, of the researcher's **Thiago Perez Rangel, Lara de Anchieta Caponi, Larissa Cristina Spirito Pena** and **Renato Corrêa Vianna Casarin**, meets the requirements of the specific resolutions on ethics in research with human beings of the National Health Council - Ministry of Health, and was approved by this committee on 22nd of August of 2018.

Profa. Fernanda Miori Pascon
Vice Coordenador
CEP/FOP/UNICAMP

Prof. Jacks Jorge Junior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo e a lista de autores aparecem como fornecidos pelos pesquisadores, sem qualquer edição.
Notice: The title and the list of researchers of the project appears as provided by the authors, without editing.

Anexo 3 – Iniciação Científica

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FUNDAÇÃO DE AMPARO À PESQUISA DO ESTADO DE SÃO PAULO
TERMO DE OUTORGA E ACEITAÇÃO DE BOLSAS NO PAÍS
PROCESSO 2018/12208-9

Pelo presente instrumento, a Fundação de Amparo à Pesquisa do Estado de São Paulo, com sede na Rua Pio XI, nº 1500, Alto da Lapa, São Paulo, Capital, inscrita no CNPJ/MF sob o nº 43.828.151/0001-45, doravante denominada OUTORGANTE, por meio de seu Conselho Técnico-Administrativo, nos termos do Artigo 14, letra "b", da Lei Estadual nº 5.918, de 18 de outubro de 1960, concede ao(s) OUTORGADO(S), a seguir qualificado(s), Bolsa para a realização do Projeto de Pesquisa a seguir especificado, nas instalações e com o apoio da INSTITUIÇÃO SEDE, de acordo com as especificações, cláusulas e condições descritas a seguir e nos Anexos, que passam a ser parte integrante deste Termo.	
1.OUTORGADOS	
1.1 BOLSISTA:	Larissa Cristina Spirito Pena CPF: 402.612.348-03 RG: 496723157-SSP/SP
1.2 ORIENTADOR/SUPERVISOR:	Renato Corrêa Viana Casarin CPF: 223.852.218-29 RG: 33965711X-SSP/SP
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3.Instituição Sede:	Faculdade de Odontologia de Piracicaba/FOP Universidade Estadual de Campinas/UNICAMP
4.Projeto de Pesquisa:	Relação entre os níveis de ácido lipoteicóico e perfil de citocinas no ambiente subgengival de indivíduos diabéticos: estudo de caso controle.
5.Linha de Fomento:	Programas Regulares / Bolsas / No País / Iniciação Científica
6.Área/Subárea:	Odontologia Periodontia
7.Coordenação:	Saúde II
8.Período da vigência:	01/03/2019 a 29/02/2020
9.Relatórios Científicos:	10/08/2019, 10/03/2020
10.Prestações de Contas:	10/08/2019, 10/03/2020

Anexo 4 – Artigo Publicado

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ORIGINAL ARTICLE

ORAL DISEASES WILEY

Subgingival endotoxin and lipoteichoic acid modulate cytokine production in diabetic subjects: A Case–control Study

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Abstract

Background: Periodontal disease and diabetes mellitus (DM) are highly prevalent and interrelated diseases, resulting in altered host response microbiota. Thus, this study aimed to evaluate the impact of DM on local levels of lipopolysaccharide (LPS) and lipoteichoic acid (LTA) and their relationship with cytokines and matrix metalloproteinases' (MMPs) profile.

Methods: This case–control study included diabetic (n = 15) and non-diabetic (n = 15) subjects presenting Stage 3-4, Grade C, Periodontitis. Gingival crevicular fluid (GCF) was collected, and LPS and LTA levels were analyzed by enzyme-linked immunosorbent assay (ELISA), while IFN-γ, IL-10, IL-17, IL-1β, IL-4, MMP-2, and MMP-9 were measured by LUMINEX/MAGpix. Mann–Whitney and Spearman's correlation tests were used to compared and to correlate variables ($p < 0.05$).

Results: Higher levels of LTA, LPS, IL-10, IL-1β, and MMP-2 ($p < 0.05$) and lower levels of IL-17 were found in the DM group ($p < 0.05$). Non-diabetic subjects presented higher LPS, IFN-γ, IL-17, and MMP-2 levels and lower IL-10 concentration ($p < 0.05$). No significant correlation was seen between LPS and cytokine profile in non-diabetic. Local levels of LTA were positively correlated with IL-17 and MMP-2 and negatively with IL-10.

Conclusion: LTA and LPS drove the inflammatory profile through the modulation of cytokines and MMPs in a different manner in DM and non-diabetic subjects.