J Periodontal Implant Sci. 2021 Dec;51(6):409-421
https://doi.org/10.5051/jpis.2100180009
pISSN 2093-2278·eISSN 2093-2286

Research Article

Periodontal Science

Subgingival pathogens in chronic periodontitis patients affected by type 2 diabetes mellitus: a retrospective case-control study

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Received: Dec 28, 2020
Revised: Mar 20, 2021
Accepted: Apr 26, 2021

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Trial Registration
ClinicalTrials.gov Identifier: NCT03786133

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ABSTRACT

Purpose: The aim of this study was to compare the prevalence and bacterial load of 6 main periodontal pathogens between pairs of periodontal patients with and without type 2 diabetes mellitus. *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* genotypes were also investigated.
Methods: Twenty patients affected by chronic periodontitis and type 2 diabetes were retrospectively selected and matched to 20 patients without diabetes on the basis of the degree and severity of periodontal disease. Microbiological data of subgingival biofilms were analysed and compared for the examined pathogens: *A. actinomycetemcomitans*, *P. gingivalis*, *Prevotella intermedia*, *Treponema denticola*, *Fusobacterium nucleatum*, and *Tannerella forsythia*.
Results: The pairs were balanced in terms of demographic and clinical parameters, except for bleeding on probing and suppuration. In the microbiological test sites (4 for each patient), the mean probing pocket depth was 6.34±1.63 mm in patients with diabetes and 6.41±1.78 mm in patients without diabetes. No significant difference between pairs in the prevalence of *P. gingivalis* or the distribution of its genotypes was recorded. Patients with diabetes had a significantly greater amount of total bacterial load, *P. gingivalis*, *T. denticola*, *T. forsythia*, and *F. nucleatum* ($P<0.05$). Moreover, patients with diabetes had a higher number of sites with a greater cell count than patients without diabetes. When compared to the total bacterial load, only *T. forsythia* maintained its relative load in patients with diabetes ($P=0.001$).
Conclusions: This retrospective matched study supports the hypothesis that microbiological differences exist among periodontal patients with and without diabetes mellitus.

Trial Registration: ClinicalTrials.gov Identifier: NCT03786133

Keywords: Periodontitis; Type 2 diabetes; Tannerella forsythia; Microbiology

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Investigation: Marco Montevecchi, Leoluca Valeriani; Methodology: Marco Montevecchi, Gabriela Piana; Validation: Giovanni D'Alessandro; Writing - original draft: Marco Montevecchi, Leoluca Valeriani; Writing - review & editing: Maria Rosaria Gatto, Giovanni D'Alessandro, Gabriela Piana.

Conflict of Interest

No potential conflict of interest relevant to this article was reported.

INTRODUCTION

Diabetes and periodontitis are both chronic diseases with a major impact on society. Their incidence and socioeconomic implications require special efforts for prevention and management [1,2]. According to the International Diabetes Federation Diabetes Atlas, diabetes affects more than 425 million adults worldwide, and the trend is growing. Moreover, 1 person in 2 remains undiagnosed. Fewer data are available about the worldwide incidence of periodontitis; nonetheless, it is estimated to be the sixth most widespread disease in the world, affecting about 743 million people. [3]

During the last few years, it has been proven that a bidirectional association exists between hyperglycaemia and periodontitis. It has been shown that periodontitis may alter glycaemic control of type 2 diabetes, causing more severe complications, while patients with diabetes experience more advanced forms of periodontitis [4]. Several authors have investigated the mechanisms underlying this association, and one of the formulated hypotheses is that patients with diabetes may develop a specific and more aggressive subgingival biofilm [5].

This hypothesis is mainly supported by some differences in the periodontal microcirculation caused by diabetes, including compromised gingival neutrophil-mediated immune responses [6] and the presence of elevated glucose levels in the gingival crevicular fluid [7].

Periodontitis is characterised by gingival inflammation and bone loss as a result of an imbalance between the host immune response and pathogenic microorganisms. The bacterial biofilm is considered the initial agent and its pathogenicity is determined by qualitative and quantitative characteristics [8].

The seminal study of Socransky et al. [9], with its identification of microbiological complexes, represented the start of modern periodontal microbiology. Nevertheless, new discoveries about different genotypes among the main periodontal pathogens are further clarifying the pathogenic role of specific microorganisms. The JP2 clone of *Aggregatibacter actinomycetemcomitans* and the type II and IV Fim-A genotype of *Porphyromonas gingivalis* are most clearly associated with the progression of attachment loss [10,11].

Regarding the correlation between subgingival biofilm and type 2 diabetes mellitus, the current literature reports conflicting positions. Although a systematic review published in 2012 by Preshaw et al. [12] indicated that "there are probably subtle differences in microbial composition of the subgingival biofilm," a subsequent systematic review carried out in 2013 by Taylor et al. [13] did not support this hypothesis. In a recent update of the aforementioned review, the authors emphasised the need for further studies to substantiate this relationship [14].

In our opinion, these conflicting outcomes could be ascribed to the inadequate number of studies published on the topic and partially to epidemiological (insufficient sample size, lack of control for periodontal status) and microbiological limitations (considering prevalence without measuring the bacterial load) of the previous investigations.

Another issue relates to the different types of diabetes and periodontal disease. While several studies investigated the periodontal microbiota in patients with type 1 diabetes [15], fewer analyses have been carried out in patients with type 2 diabetes, and those studies have generally had small samples.

https://doi.org/10.5051/jpis.2100180009

This retrospective case-control study aimed to compare the prevalence and the bacterial load of 6 main periodontal pathogens between pairs with the same degree of chronic periodontitis, with or without type 2 diabetes mellitus. Differences in the genotypes of *P. gingivalis* and *A. actinomycetemcomitans* were also evaluated.

MATERIALS AND METHODS

Study setting and patients

The retrospective case-control study was carried out in Italy at the Division of Periodontology and Implantology of the Dental School of Alma Mater Studiorum – University of Bologna. The study protocol was previously approved by the Ethics Committee (Ethics Committee of Bologna-Imola reference number: CE 16044). This study was registered at ClinicalTrials.gov as NCT03786133.

Recruitment of the study sample was performed by analysing the medical records of adult patients with chronic periodontitis who visited the department between 2010 and 2016. Chronic periodontitis was diagnosed at the first visit according to the 1999 criteria of the American Academy of Periodontology [16] and their subsequent 2015 update [17].

The diagnosis of type 2 diabetes mellitus was self-reported by patients in their medical records and subsequently ascertained by the last blood test requested for the patient, along with a declaration from the patient's general practitioner. Patients without diabetes were ascertained by the results of the last blood test presenting normoglycaemia.

The inclusion criteria were the presence of at least 12 teeth (except for the third molar), age >18 years, and belonging to the Caucasian racial group.

Patients were excluded if they were pregnant or lactating, if they had history of systemic antibiotic use within 3 months prior to microbiological testing or anti-inflammatory therapy in the month before the visit, if they had any other systemic disease except diabetes mellitus (arthritis, ulcerative colitis, Crohn disease, osteoporosis or osteopenia, HIV infection, hematologic diseases, neoplastic diseases, cardiovascular diseases, or pathologies that could potentially interfere with the periodontitis and/or with diabetes), if they had mental disorders, and/or if they had received periodontal treatment in the 6 months before the microbiological test.

Individual matching on the basis of severity and extension of chronic periodontitis was performed by pairing a type 2 diabetes mellitus patient (T2DM) with a non-diabetes mellitus patient (NDM), but had the same severity and extent of periodontal disease. The extent of periodontal damage was defined by the percentage of sites with clinical attachment loss: ≤30% (localised) or >30% (generalised). The severity of periodontal damage was determined based on the degree of clinical attachment loss: 1–2 mm (mild), 3–4 mm (moderate), ≥5 mm (severe) [18]. The matching was performed by analysing the full-mouth periodontal records. The study design is shown in **Figure 1**.

Analysed variables

For each subject, the following data were collected: age, sex, smoking habits, metabolic control of diabetes, duration of diabetes, and number of missing teeth.

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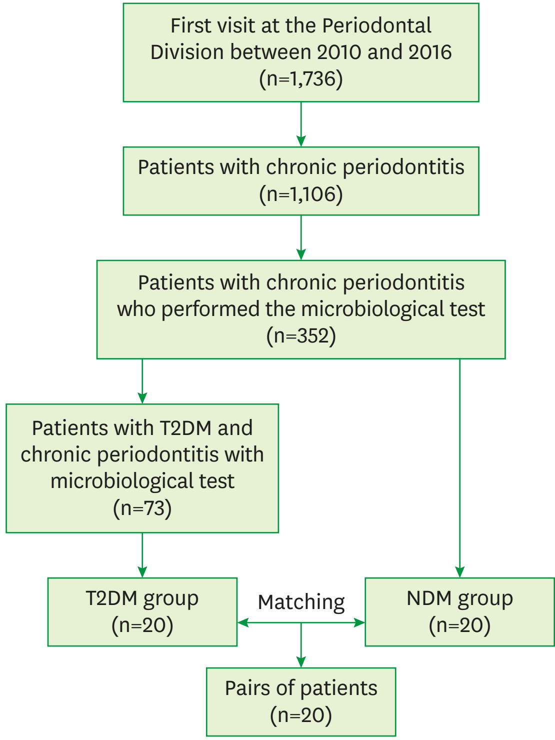

Figure 1. Study design.
T2DM: type 2 diabetes mellitus, NDM: non-diabetes mellitus.

For each subject, 4 periodontal sites were studied. Probing pocket depth (PPD), sites with bleeding on probing (BoP), sites with suppuration (Sup), and microbiological data were evaluated.

Patients were classified as non-smokers, those who smoked more or less than 10 cigarettes per day, and former smokers (at least 6 months of abstinence).

Microbiological sampling

The bacterial pathogens examined were *A. actinomycetemcomitans*, *P. gingivalis*, *Prevotella intermedia*, *Treponema denticola*, *Fusobacterium nucleatum*, and *Tannerella forsythia*. The bacterial load of each species, the total bacterial load at a single periodontal site, the *A. actinomycetemcomitans* and *P. gingivalis* genotypes, and the percentage of pathogens compared to the total load were analysed based on quantitative real-time polymerase chain reaction (PCR) with a sensitivity of 100 cells per type of pathogen. The threshold for the detection of the pathogen was dictated by the sensitivity of the test.

Microbiological tests were carried out in a standardised manner during the first appointment, before the beginning of periodontal therapy and right after the initial visit. As a rule of the division, at the initial visit, the clinician collects anamnestic data on health status (in case of eventual positivity for pathology, the last laboratory tests and a declaration from the patient's general practitioner are generally required) and performs a complete periodontal record (PPD, BoP, Sup, tooth mobility, clinical attachment level, periodontal phenotype, furcation involvement, etc.). Subsequently, a full-mouth X-ray periapical exam is performed only when necessary.

https://doi.org/10.5051/jpis.2100180009

The 4 microbiologically examined sampling sites were those with the highest value of PPD in each quadrant and were collected from both multi-rooted teeth and single-rooted teeth. All teeth with a poor prognosis in the short term and those that presented furcation defects, endodontic diseases, and/or extensive carious lesions were regularly excluded from microbiological tests. Oral hygiene habits were suspended on the day of the microbiological test and oral antiseptics were avoided for at least 10 hours before collection.

The collection and storage of the subgingival biofilm site-specific sample was performed according to a standardised method: isolation of the area from saliva through cotton rollers and an aspirator, careful removal of the supragingival plaque while avoiding trauma to the marginal tissues, insertion of a disposable sterile paper point at the bottom of the pocket for 10 seconds. The paper cone was then placed alone in a specific sterile tube, avoiding any form of contamination, and was analysed individually.

Plaque samples were sent to an external and independent laboratory for a proper examination (Biomolecular Diagnostic, Firenze, Italy). DNA extraction was performed using the QIACube HT® instrument (Qiagen GmbH, Hilden, Germany). The protocol recommended by the DNA extraction kit manufacturer was strictly followed. After extraction, the DNA was eluted in 150 μL of elution buffer, and subsequently the amount of DNA was measured with a spectrophotometer (Eppendorf BioSpectrometer, Hamburg, Germany). To evaluate in real time the amount of double-stranded DNA present after each synthesis cycle, 40 ng of target DNA was used for the detection of single bacterium using the real-time PCR methodology. The reaction mixtures contained a "reporter" fluorophore at the 5' end and a "quencher" molecule at the 3' end. These mixtures, together with pairs of specific primers (Applied Biosystems Inc., University Park, IL, USA) for each target, were placed in each tube with the QiAgility automatic dispenser (Qiagen GmbH).

Real-time PCR (Rotor-Gene Q; Qiagen GmbH) was performed for DNA amplification and detection. Primers and species-specific probes were designed using the Primer3 software (details about the primers and probes used are available on demand from the above-mentioned laboratory).

Positive samples in the screening for *A. actinomycetemcomitans* were then subjected to genotyping analysis for the identification of the 652 or JP2 strain through a melt curve analysis. *P. gingivalis* fimbriae differentiation was also carried out using specific primers for types FimA I, FimA II, and FimA IV.

Statistical analysis

The sample size was determined referring to the prevalence of *P. gingivalis* and *T. denticola*, as reported in a previous study [18]. The prevalence values estimated from Yuan et al. [18] were an average of prevalence at healthy and diseased sites in both patients with and without diabetes. Assuming for *P. gingivalis* a prevalence equal to 61% in patients without diabetes and 56% in patients with diabetes, with a margin of non-inferiority at 20%, at a level of significance of 5% with a power of 80%, at least 14 pairs of patients were required. Assuming for *T. denticola* a prevalence equal to 36% in patients without diabetes and 39% in patients with diabetes, with a non-inferiority margin of 10%, at a level of significance of 5% with a power of 80%, at least 18 pairs of patients were required.

 https://doi.org/10.5051/jpis.2100180009

The unit of statistical analysis was the pair of subjects, T2DM and NDM. Categorical data are described as proportions. Continuous data, after verifying their adaptation to a Gaussian distribution through the Shapiro-Wilk test, are described as median and interquartile range. Comparisons of bacterial loads among the 20 pairs of patients were performed by means of the Wilcoxon test, and comparisons of categorical data among the 20 pairs of patients were performed by means of the McNemar χ^2 test.

The level of alpha significance was set a priori at 0.05.

RESULTS

Twenty pairs of adults with chronic periodontitis and the presence or absence of type 2 diabetes were examined. The study sample was made up of 55% men in the T2DM group and 50% in the NDM group. Eleven pairs were concordant in terms of sex. There were 3 smokers among the patients with diabetes (1 smoked <10 cigarettes/day) and 5 (4 smoked <10 cigarettes/day) in patients without diabetes. The median age (interquartile range; IQR) (Wilcoxon test; P=0.211) and the mean probing depth±standard deviation (SD) (Wilcoxon test; P=0.706) were, respectively, 53 years (45–62 years) and 6.34±1.63 mm in patients with diabetes and 50 years (40–60 years) and 6.41±1.78 mm in patients without diabetes. The median number of missing teeth (IQR) was 4.5 (2–7) in T2DM patients and 1 (1–5) in NDM patients (Wilcoxon test: P=0.218). Demographic characteristics, smoking status, the number of missing teeth, and site-specific clinical parameters are reported by pairs in **Table 1**.

All patients with diabetes had the condition under metabolic control (haemoglobin A1c levels <7%) by metformin, insulin, and/or diet, and 65% of them had been diagnosed with diabetes for less than 15 years.

The prevalence of positive sites for each bacterial species in the pairs of patients is reported in **Table 2**. No significant difference was observed in the frequency of positive sites for the considered bacteria species between the pairs of subjects.

Table 3 and **Figure 2** report the absolute median cell count and comparison by sites among the pairs of patients. The median cell count of bacteria was always significantly higher in the T2DM group (P≤0.03), except for *P. intermedia* and *A. actinomycetemcomitans*, which

Table 1. Demographic characteristics, number of missing teeth, smoking habits, and site-specific clinical parameters in T2DM and NDM (pairs=20, sites=80)

Description	Number	*P* value
Pairs with more missing teeth in the T2DM patient	12	**0.359**[a)]
Pairs with more missing teeth in the NDM patient	7	
Pairs with a T2DM smoker and an NDM non-smoker	1	**0.375**[a)]
Pairs with a T2DM non-smoker and an NDM smoker	4	
Number of sites with PPD ≥5 mm in the T2DM patient and PPD <5 mm in the NDM patient	73	**0.598**[b)]
Number of sites with PPD ≥5 mm in the NDM patient and PPD <5 mm in the T2DM patient	71	
Number of sites with BoP present in the T2DM patient and absent in the NDM patient	30	**0.036**[b)]
Number of sites with BoP absent in the T2DM patient and present in the NDM patient	15	
Number of sites with Sup present in the T2DM patient and absent in the NDM patient	5	**0.001**[b)]
Number of sites with Sup absent in the T2DM patient and present in the NDM patient	25	

T2DM: type 2 diabetes mellitus, NDM: non-diabetes mellitus, PPD: probing pocket depth, BoP: bleeding on probing, Sup: suppuration on probing.
[a)]Binomial distribution; [b)] McNemar χ^2 test.

https://doi.org/10.5051/jpis.2100180009

Table 2. Prevalence of positive sites for the detection of the examined bacteria and comparison of the frequency of positive sites for bacterial species in the pairs of T2DM-NDM patients (pairs=20, sites=80)

Variable	Prevalence of positive sites			Pairs with more positive sites		
	T2DM patient	NDM patient	*P* value[a)]	T2DM patient	NDM patient	*P* value[a)]
Porphyromonas gingivalis	64 (80.0)	64 (80.0)	1.00	4 (20.0)	6 (30.0)	0.57
Treponema denticola	65 (81.3)	70 (87.5)	0.27	3 (15.0)	5 (25.0)	0.07
Tannerella forsythia	67 (83.8)	60 (75.0)	0.31	6 (30.0)	4 (20.0)	0.22
Prevotella intermedia	35 (43.8)	26 (32.5)	0.14	8 (40.0)	6 (30.0)	0.44
Fusobacterium nucleatum	74 (92.5)	76 (95.0)	0.51	3 (15.0)	7 (35.0)	0.20
Aggregatibacter actinomycetemcomitans	6 (7.5)	5 (6.3)	0.75	3 (15.0)	1 (5.0)	0.35

Values are presented as number (%).
T2DM: type 2 diabetes mellitus, NDM: non-diabetes mellitus.
[a)]The χ^2 test.

Table 3. Absolute median cell count and comparison by sites

Variable	T2DM	NDM	*P* value[a)]	Sites with cell count	
				T2DM>NDM (n=80)	T2DM<NDM (n=80)
Aggregatibacter actinomycetemcomitans	1.0×10^2 (1.0×10^2–1.0×10^2)	1.0×10^2 (1.0×10^2–1.0×10^2)	ND	9	2
Porphyromonas gingivalis	3.1×10^4 (7×10^2–2.3×10^5)	1.3×10^4 (1×10^2–1.8×10^5)	0.031	46	31
Tannerella forsythia	1.9×10^4 (8×10^2–3.6×10^5)	3×10^3 (1×10^2–3.1×10^4)	0.001	50	26
Prevotella intermedia	1.0×10^2 (1.0×10^2–7.4×10^3)	1.0×10^2 (1.0×10^2–1.3×10^3)	ND	28	20
Fusobacterium nucleatum	1.4×10^4 (2×10^3–8.2×10^4)	8×10^3 (2×10^3–2.0×10^4)	0.01	51	29
Treponema denticola	7.8×10^4 (2×10^3–5.1×10^5)	4.3×10^4 (3×10^3–3.1×10^5)	0.03	46	30
Total cell count	3×10^6 (1×10^6–2.1×10^7)	2×10^6 (1×10^6–3×10^6)	0.001	50	30

T2DM: type 2 diabetes mellitus, NDM: non-diabetes mellitus.
[a)]Wilcoxon test.

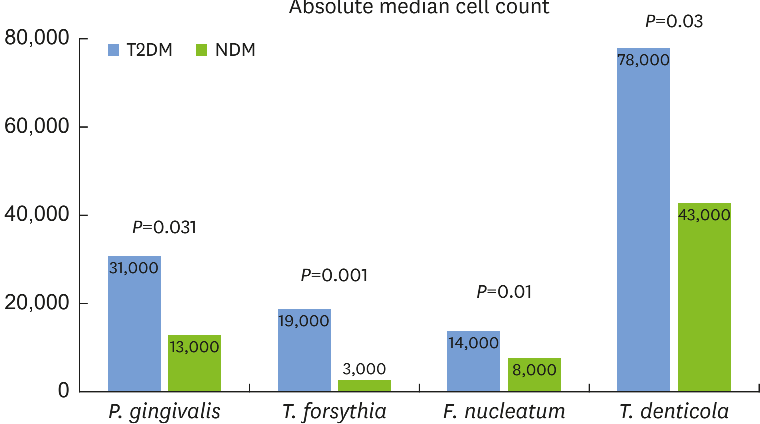

Figure 2. Bar graph showing the absolute median cell count in patients with diabetes and patients without diabetes. Only results exceeding the sensitivity of the test were reported.
T2DM: type 2 diabetes mellitus, NDM: non-diabetes mellitus.

have a median value equal to the sensitivity of the test in both groups, given their very low prevalence. The number of sites with a higher bacterial load was greater in the T2DM group than in the NDM group in all cases. A significant difference in the total cell count was observed between the pairs of patients. The number of pairs in which the bacteria count was higher in the patient with diabetes was greater than the number of pairs in which it was higher in the patient without diabetes.

Table 4 and **Figure 3** report the relative load of each bacterial species in the pairs of patients; considering the proportion of each species relative to the total cell count, a significant difference (*P*=0.001) was observed only for *T. forsythia*.

https://doi.org/10.5051/jpis.2100180009

Table 4. Percentage of each pathogen relative to the total cell count and comparison by sites

Variable	T2DM	NDM	P value[a)]	Sites with %	
				T2DM>NDM (n=80)	T2DM<NDM (n=80)
Aggregatibacter actinomycetemcomitans	0% (0%–0%)	0% (0%–0%)	ND	7	2
Porphyromonas gingivalis	1.5% (0.02%–7.6%)	0.9% (0.05%–7.6%)	0.941	38	39
Tannerella forsythia	1.1% (0.06%–3.1%)	0.3% (0.003%–0.8%)	0.001	52	24
Prevotella intermedia	0% (0%–0.2%)	0% (0%–0.04%)	ND	27	21
Fusobacterium nucleatum	0.46% (0.1%–1.4%)	0.32% (0.1%–0.99%)	0.270	42	38
Treponema denticola	2.7% (0.1%–10.5%)	2.9% (0.2%–12.6%)	0.389	37	39

T2DM: type 2 diabetes mellitus, NDM: non-diabetes mellitus.
a)Wilcoxon test.

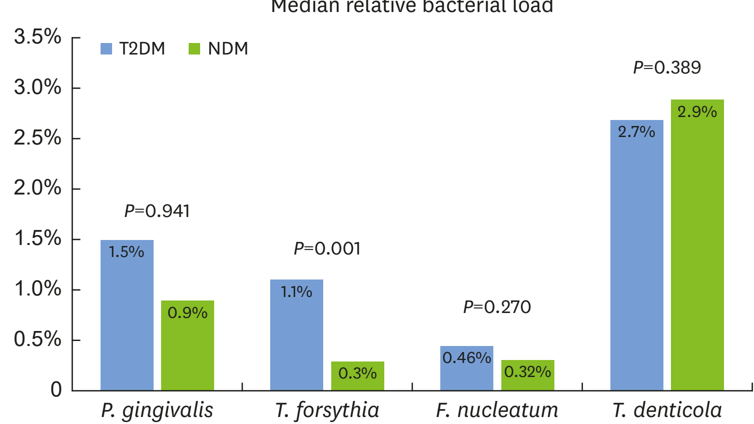

Figure 3. Bar graph showing the median relative bacterial load in patients with diabetes and patients without diabetes. Only results exceeding the sensitivity of the test were reported.
T2DM: type 2 diabetes mellitus, NDM: non-diabetes mellitus.

No significant difference between the T2DM and NDM pairs was observed in the distribution of *P. gingivalis* genotypes (type I, type II, and type IV) (McNemar χ^2 test, P=0.25).

DISCUSSION

Periodontal disease is a consequence of an altered balance among periodontal bacteria, host defences, and metabolism. Systemic pathologies including diabetes can affect this balance by worsening its clinical picture. [19] Many hypotheses have been postulated to explain the effects of diabetes on periodontal disease. Among them, the presence of different sub-marginal biofilms between individuals with and without diabetes has also been suggested, despite contradictory evidence. Some studies have reported significant differences in the bacterial composition of the dental plaque between individuals with and without type 2 diabetes [20-22], whereas others did not [18,23]. As clearly shown by a recent systematic review on this topic [24], several studies are characterised by a general high risk of bias, such as unclear sample size calculation, a lack of control for confounding factors, and/or a non-representativeness of the microbiological test sites.

Despite a limited sample size, the rigorous inclusion criteria and the standardised microbiological sampling used in this study support the relevance of the results. The present study was carried out before the publication and consequent adoption of the new periodontal classification [25]. Due to its retrospective design it was not possible to update the periodontal categorisation, especially for grading. However, no significant differences

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were detected in the number of missing teeth (P=0.218), the PPD mean values (P=0.706), and the number of sites with a PPD >5 mm between patients with and without diabetes (P=0.598). This observation suggests that the staging of the 2 groups was not meaningfully different. Obviously, however, the grading might be higher in the diabetes group, due to the presence of the condition.

In the present work, an analysis of the pathogen prevalence (i.e., detection frequencies) was carried out. In line with previous studies [13,26], no significant difference was detected between the 2 groups. These results were also confirmed by the analysis of pairs. The detected prevalence was, interestingly, very similar to that previously reported for a population of Italian periodontal patients [27].

Pathogen prevalence has been widely used in the past to describe the microorganism presence above certain thresholds (defined by the sensitivity of the test). This microbiological evaluation ought to be considered inaccurate today, since it refers to the mere absence/presence of the pathogen, without specifying its quantity. This concept was already emphasised by Yuan et al. [18] in 2001, further underscoring the insufficient sensitivity of culture tests, DNA probes, and immunological/immunofluorescence assays; instead, PCR is preferred for its precision.

As far as total bacterial load is concerned, according to Khader et al. [19] and Miranda et al. [28], patients with diabetes present a greater amount of subgingival biofilm. This is shown both by the difference in the total cell count and by the finding of the analysis of pairs that a greater number of sites in patients with diabetes had more bacteria than in patients without diabetes (right side of **Table 3**). Several hypotheses have been examined underlying this shift, potentially related to local immune dysfunction, cellular stress, and cytokine imbalance. Some factors, such as neutrophil adherence, chemotaxis, and phagocytosis, are often compromised in patients with diabetes, likely facilitating bacterial persistence and proliferation [29].

Focusing on the bacterial load of single pathogens, *T. forsythia* (P=0.001), *P. gingivalis* (P=0.031), *T. denticola* (P=0.03), and *F. nucleatum* (P=0.01) were quantitatively more represented in the T2DM group. Similar results emerged from a previous study of patients with adequate control of diabetes [30] and from the study of Li et al. [31], where the authors observed a higher quantity of *T. forsythia* and *T. denticola* in patients with diabetes than in those without diabetes.

Similar findings at species-level operational taxonomic units of *T. forsythia* were reported by Zhou et al. [22]. Conversely, the PCR results reported by Sardi et al. [32] and 16S rDNA sequencing reported by Casarin et al. [21] indicated that *T. forsythia* was more prevalent in NDM subjects.

Regarding *P. gingivalis*, most studies in the literature [18,22-24,31,32] detected no significant difference. However, contrary to the present results, Casarin et al. [21] reported a higher abundance of this species in their NDM subjects.

The bacterial load of *T. denticola* did not differ significantly between T2DM and NDM patients [18,21,22].

Field et al. [23] showed no significant difference in the distribution of *F. nucleatum* between patients with diabetes and patients without diabetes, while Casarin et al. [21] found that *F.*

https://doi.org/10.5051/jpis.2100180009

nucleatum was more present in patients with diabetes, and Joaquim et al. [26] found that it was more present in women with diabetes.

The source of the abovementioned discrepancies between our results and the published literature is unclear. Undoubtedly, differences in study designs and clinical/laboratory protocols might have played a crucial role in generating these differences [12]. Moreover, recent findings have suggested new individual characteristics that might influence microbiological factors, making comparisons between studies more difficult [33,34]. As these factors can potentially influence the study outcomes, they should not be ignored when comparing different studies. Furthermore, this consideration reinforces the value of strict matching between cases and controls.

In the present study, given that the sample size was determined using red complex bacteria, the reduced prevalence and bacterial load of *A. actinomycetemcomitans* and *P. intermedia* (lower than test sensitivity) may be explained by an insufficient sample size. Unlike *A. actinomycetemcomitans* (present overall at 11 sites), *P. intermedia* was detected several times (48 sites). It is interesting to note that despite working with a reduced number of sites and median values lower than the sensitivity of the test, the *P. intermedia* averages were quite different. This means that in the few sites containing *P. intermedia*, there were more bacteria in the T2DM patients than in the NDM patients. However, this finding must be confirmed by studies with larger sample sizes; therefore, it will not be discussed further.

Interestingly, when the pathogen load was assessed relative to the total bacterial load, no statistically significant differences between patients with and without diabetes were detected for *P. gingivalis*, *T. denticola*, and *F. nucleatum*. This indicates that the greater presence of these bacteria in the T2DM patients was probably a consequence of the overall increase in the total bacterial load.

Only *T. forsythia* was found to be more represented in T2DM patients in terms of the absolute and relative load, in agreement with Longo et al. [30].

Recent microbiological findings [35] can help explain this result. *T. forsythia*, which is associated with different forms of periodontal disease, is characterised by growth and commensalism with *F. nucleatum.* The latter, besides its general role within the subgingival biofilm, provides *T. forsythia* with N-acetyl muramic acid, one of the main components of the cell wall. *T. forsythia*, which does not synthesise this compound *de novo*, proliferates only in environments with abundant N-acetyl muramic acid or in co-culture with other species that supply it (such as *F. nucleatum*). [36]

In conditions of increased glucose, an asaccharolytic bacterium such as *T. forsythia* could exploit its greater availability both to replicate and to produce intermediate compounds, which are also useful for replication purposes. This results in energy savings in the form of ATP, which could be hypothesised to enhance the entrance of N-acetyl muramic acid into the bacterium, ultimately increasing peptidoglycan synthesis and replication of the bacterium.

The patients with diabetes included in this study had a substantial duration of diabetes and declared that they performed blood glucose tests on a daily basis. Given the retrospective nature of our study, it was not possible to confirm diabetes control by means of a laboratory analysis. Nonetheless, it is reasonable to hypothesise that the blood glucose values in patients with diabetes are on average higher and more variable over time than those of patients

without diabetes. Therefore, it can be speculated that the reproduction of *T. forsythia* may benefit from the glucose levels typical of diabetes, even if controlled, in the sub-gingival area.

It is interesting to notice that *T. forsythia*, in the presence of glucose, accumulates high levels of toxic methylglyoxal products [37] that may significantly contribute to periodontal damage in affected individuals. In light of these considerations, *T. forsythia* might seem to have played a relevant role in periodontal disease in the patients with diabetes. Further investigations of this specific possibility are consequently strongly suggested. Another aspect to be considered regarding *T. forsythia* is its association with the sites with suppuration. Periodontal abscess microbiota were demonstrated to be mainly composed of high levels of pathogenic species, including *F. nucleatum*, *P. gingivalis*, and *T. forsythia* [38]. Given that in our study, sites with suppuration were more frequent in the NDM group, it can be assumed that the gap between T2DM and NDM patients may be relatively high, especially for the aforementioned bacteria.

Smoking was not taken into consideration when pairing the patients; however, 17 of 20 the pairs were concordant in terms of smoking habits. Consequently, even if this is a potentially relevant factor that might impact the microbiological results, the studied samples were balanced, and no significant difference was observed between the members of the pairs (P=0.375).

Regarding the genotypes, the low frequency of *A. actinomycetemcomitans* permitted no genotypic identification, while no significant differences were found between the 2 groups for *P. gingivalis*. In accordance with our results, Davila-Perez et al. [39] in 2007 did not find any statistically significant differences, whereas the study of Makiura et al. [40] showed that the most prevalent type of fimbriae was type II.

Despite the retrospective design, the rigorous inclusion criteria of the present work allowed the comparison of 2 balanced groups. This aspect reinforces the obtained results, which support the hypothesis that periodontal patients with diabetes present subgingival microbial differences from patients without diabetes who have a similar degree of periodontal destruction. *T. forsythia,* in particular, appears to be strongly associated with this systemic condition. Prospective investigations on broader samples and with a wider range of bacteria are needed, aiming to augment the non-causal nature of the relationship observed in this study and the clinical relevance of these results.

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