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Diabetes mellitus-associated periodontitis: differences between type 1 and type 2 diabetes mellitus

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Background and Objective: Although many studies have appeared about diabetes mellitus-associated periodontitis, few have compared periodontitis inflammatory markers between type 1 (T1DM) and type 2 diabetes mellitus (T2DM), and information regarding this issue is scarce and contradictory. We evaluated the levels of plasma C-reactive protein and of interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) in gingival crevicular fluid in two groups of subjects affected by T1DM and T2DM, in order to identify possible differences between the two classes in the inflammatory mechanisms of diabetes mellitus-associated periodontitis.

Material and Methods: Plasma C-reactive protein and gingival crevicular fluid IL-1β, IL-6 and TNF-α were measured in periodontitis patients affected by type 1 (P-T1DM, $n = 24$) and type 2 diabetes mellitus (P-T2DM, $n = 24$).

Results: Gingival crevicular fluid levels of IL-1β and TNF-α in P-T1DM subjects were significantly higher than in P-T2DM subjects. In P-T1DM subjects, we found significant negative correlations between the duration of diabetes mellitus and IL-1β and between the duration of diabetes mellitus and TNF-α.

Conclusion: This study shows that IL-1β and TNF-α levels in periodontitis patients with T1DM are affected by the duration of diabetes mellitus.

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Diabetes mellitus (DM), the most common human endocrine disease, is a metabolic disorder characterized by chronic hyperglycaemia and is caused by the interaction of environmental factors, such as obesity, sedentary lifestyle and high-calorie food intake, and genetic susceptibility (1). The two main categories of diabetes mellitus are type 1 (T1DM) and type 2 DM (T2DM). Type 1 DM results from the autoimmune destruction of pancreatic islet cells, eventually leading to the loss of insulin production, and is usually diagnosed in children and young adults, though some studies have reported that 15–30% of all such cases occurs after 30 years of age (1); in contrast, the onset of T2DM is usually in adulthood, and it is characterized by an increase in insulin resistance associated with a varying inability of pancreatic β-cells to secrete sufficient amounts of insulin to compensate (1).

Besides representing a risk factor for numerous organ complications (2), DM is an important and independent risk factor for development of gingivitis and periodontal disease (1). During the inflammatory process of periodontitis, four molecules play a fundamental role: C-reactive protein (CRP), interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α). C-Reactive protein is a plasmatic protein involved in the systemic inflammatory response and has been associated with alveolar bone loss (3). Interleukin-1β also plays a pivotal role in chronic inflammation, and elevated levels of this molecule have been detected in gingival crevicular fluid sites

of recent bone and attachment loss in periodontitis patients (4). Interleukin-6 synergizes with IL-1β to enhance the recruitment and formation of osteoclasts, which induce bone resorption (5,6). Tumour necrosis factor-α is involved in upregulating the production of collagenase, prostaglandin E_2, chemokines, cytokines and cell adhesion molecules, as well as stimulating bone resorption-related factors (7,8).

Several experimental studies have investigated the mechanisms underlying the interaction between periodontal disease and DM (1); in particular, Salvi *et al*. (9,10), report that individuals with DM react with an abnormally higher release of IL-1β and TNF-α than do nondiabetic subjects to the same bacterial burden. Moreover, our group has also recently provided insight on the same subject by demonstrating that the expression of gingival vascular endothelial growth factor in subjects with periodontitis is greater in those with DM than in those who were systemically healthy (11).

Although many studies have appeared about diabetes mellitus-associated periodontal disease, few have compared periodontal disease inflammatory markers between T1DM and T2DM, and information regarding this issue is scarce and contradictory (11,12). On the basis of this assumption, the present study is designed to evaluate plasma CRP levels and gingival crevicular fluid levels of the cytokines IL-1β, IL-6 and TNF-α in periodontitis subjects with T1DM and T2DM in order to identify any differences between T1DM and T2DM in the inflammatory mechanisms of diabetes mellitus-associated periodontal disease. To do this, we examined diabetic subjects affected by the same degree of periodontal disease so as to eliminate the bias given by the effect of periodontal parameters on inflammatory markers (10,13–15) and to evaluate the effect on the latter of other factors in the two classes of subjects.

Material and methods

The study was carried out between May 2004 and September 2009. In order to recruit 24 periodontitis patients with T1DM (P-T1DM) and 24 periodontitis patients with T2DM (P-T2DM) a consecutive series of 180 T1DM and 243 T2DM subjects attending the Division of Endocrinology, Department of Clinical Medicine and Applied Biotechnologies of the Polytechnic University of Marche were screened; among these, 20 T1DM and 95 T2DM subjects did not give their consent to participate, while another 4 T1DM and 41 T2DM subjects did not comply with the screening criteria; among the remaining 156 T1DM and 107 T2DM subjects subsequently assessed in the Division of Periodontology, Department of Clinical and Dental Sciences and Institute of Pathology of the Polytechnic University of Marche, Ancona, Italy, 132 T1DM and 83 T2DM subjects did not present inclusion criteria.

We used the following inclusion criteria: (i) diagnosis of T1DM or T2DM (16) at least 12 mo prior to the study; (ii) presence of at least 20 teeth; (iii) age > 35 years; and (iv) diagnosis of generalized, severe, chronic periodontitis defined by the presence of more than 30% of measured sites with > 5 mm clinical attachment loss (17,18); this diagnosis was made by evaluating whether the periodontal destruction was commensurate with plaque levels or other local contributing etiological factors; all participants showed bone loss > 50% in all teeth.

Exclusion criteria were as follows: (i) presence of any important disease except for DM; (ii) being a smoker; (iii) having taken antibiotics, corticosteroids or nonsteroidal anti-inflammatory drugs within the 6 mo prior to treatment; and (iv) having undergone periodontal treatment within the previous 2 years.

All P-T1DM subjects were on insulin therapy; three of the P-T2DM subjects were following a suitable dietetic regime, three were on insulin therapy, 10 were being treated with a combination of insulin and oral hypoglycaemic agents, and eight subjects were on therapy only with oral hypoglycaemic agents. Oral hypoglycaemic agents and/or insulin were taken in stable doses. Anti-hypertensive drugs were taken by three P-T1DM and twelve P-T2DM subjects. No other drug was taken.

The duration of DM was defined as the interval between the diagnosis of DM and the starting date of the study.

This study was carried out in compliance with an informed protocol approved by the Ethics Committee of the Polytechnic University of Marche, Ancona, Italy. The research objectives were explained to the patients, who then signed an informed consent form.

Determination of periodontal status

All participants were evaluated clinically and radiographically. The following clinical and periodontal parameters were assessed by the same examiner (S.D.A.), who was blind to group assignment: (i) plaque index (19); (ii) gingival index (19); (iii) sulcus bleeding index (20); (iv) probing depth, measured to the nearest millimetre from the gingival margin to the bottom of the pocket; and (v) clinical attachment loss, measured from the cemento-enamel junction to the bottom of the pocket.

The probing depth and clinical attachment loss were assessed at six sites around each tooth (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual and disto-lingual locations) for the whole mouth excluding third molars.

The same type of calibrated periodontal probe (15 mm, probe tip diameter = 0.5 mm; University of North Carolina; UNC-15, Hu Friedy®, Chicago, IL, USA) was used for all measurements.

Periapical radiographs were performed by the same investigator (S.D.A.) using the long-cone paralleling technique with the same radiographic equipment, film, exposure and development conditions. Callipers were used for the measurements, which were expressed in millimetres and corrected to account for the magnification factor of the equipment used. Bone loss was expressed as a fraction of the total root length at two sites (mesial and distal) for every tooth.

Collection of gingival crevicular fluid

Gingival crevicular fluid samples were collected from six maxillary anterior

sites of each patient with >5 mm probing depth [maxillary teeth were selected for sampling in order to reduce the possibility of contamination with saliva, a procedure already used by other authors (21,22)]. After isolating the sampling teeth with cotton rolls and drying them gently (supragingival plaque was removed if present), a standard paper strip (Periopaper, IDE Interstate, Amityville, NY, USA) was inserted into the sulcus to the depth where mild resistance was felt and was left *in situ* for 30 s; strips contaminated with blood were excluded. After collection of gingival crevicular fluid, the strips were immediately taken to a calibrated Periotron 8000 (Proflow Inc., Amityville, NY, USA) to determine gingival crevicular fluid volume and placed in sterile Eppendorf tubes containing a buffer with 10 mM NaH_2PO_4 and 150 mM NaCl (pH 7.2), followed by mixing and centrifugation at 800*g* for 10 min, as performed in other studies (23). The gingival crevicular fluid samples were stored at −80°C until subsequent analysis.

Assays of cytokines in gingival crevicular fluid

Gingival crevicular fluid samples were thawed at room temperature. The supernatants were collected and used to determine IL-1β, IL-6 and TNF-α by ultrasensitive solid-phase ELISAs (Biosource International, Camarillo, CA, USA); assay sensitivity was 0.00001 ng/mL for IL-1β, 0.00010 ng/mL for IL-6 and 0.00009 ng/mL for TNF-α. Analyses were performed according to the manufacturer's protocol. All ELISA determinations were performed in triplicate. Results were calculated using the standard curves created in each assay. For each gingival crevicular fluid sample, cytokine concentration (in nanograms per millilitre) was calculated from the volume of gingival crevicular fluid measured with a calibrated Periotron, according to the following formula: cytokine concentration (in ng/mL) = total cytokine (in ng)/volume (in mL). The mean concentrations obtained from all gingival crevicular fluid samples represented the levels of IL-1β, IL-6 and TNF-α for each subject.

Plasma assays

Fasting blood samples were collected. Plasma glucose was assayed on a Hitachi 911 analyser (Hitachi, Tokyo, Japan) using the glucose oxidase method (intra- and interbatch coefficient of variation <4%). Glycated haemoglobin (HbA_{1c}) was measured in whole blood using ion exchange high-performance liquid chromatography with the Bio-Rad Variant Haemoglobin Testing System (Bio-Rad Laboratories, Hercules, CA, USA). Plasma high-sensitive C-reactive protein (hs-CRP) concentrations were measured by a particle-enhanced immunoturbidimetric assay (Roche Diagnostic, Mannheim, Germany) using anti-CRP mouse monoclonal antibodies coupled to latex microparticles and expressed in milligrams per litre.

Statistical analysis

The Shapiro–Wilk test was applied to verify the normal distribution of the continuous variables. Continuous variables were expressed as means ± SD if normally distributed and as medians (range) if not normally distributed. Comparisons between the two groups were made with Student's unpaired *t*-test or the Mann–Whitney *U*-test. Pearson (or Spearman) correlations were performed. Significance was set at $p < 0.05$. Statistical analyses were performed using the SPSS 16 package (SPSS Inc., Chicago, IL, USA).

Results

Figure 1 shows the full mouth means of the periodontal parameters, none of which differed significantly between the two groups.

Table 1 shows the clinical characteristics of the studied subjects. The two groups differed in duration of DM (found to be significantly longer in P-T1DM subjects than in those with P-T2DM) and age (found to be significantly lower in P-T1DM subjects than in P-T2DM subjects).

The P-T1DM subjects had significantly higher gingival crevicular fluid IL-1β and TNF-α levels than the P-T2DM subjects (Table 2).

Table 3 shows the correlations between some clinical parameters and inflammatory markers in the two groups of subjects. In P-T1DM subjects, we found significant negative correlations both between the duration of DM and gingival crevicular fluid IL-1β and between the duration of DM and gingival crevicular fluid TNF-α; no other significant correlation was found.

Discussion

Many studies have examined the inflammatory process underlying diabetes mellitus-associated periodontal disease (1); however, few have investigated differences in the inflammatory mechanisms of diabetes mellitus-associated periodontal disease between T1DM and T2DM (11,12). To gain greater insight into this issue, we evaluated plasma hs-CRP and gingival crevicular fluid IL-1β, IL-6 and TNF-α levels in patients affected by generalized, severe, chronic periodontitis with T1DM and T2DM. Our choice to select patients with the same degrees of periodontal disease was due to the need to eliminate the bias produced by the effect of periodontal disease severity on inflammatory markers (10,13–15) and therefore to evaluate which other factors could condition inflammation in the two classes of diabetic subjects.

Unexpectedly, we found higher levels of gingival crevicular fluid IL-1β and TNF-α in P-T1DM than in P-T2DM. Given this result, we carried out correlations between the inflammatory markers and some clinical parameters, i.e. age, duration of DM, body mass index and HbA_{1c} (Table 3), which, on the basis of data present in the literature, could in theory influence the gingival crevicular fluid cytokine and plasma hs-CRP levels. In fact, age is an element that influences many factors forming the basis of the interrelationship between DM and periodontal disease (24); length of DM conditions the severity of periodontal disease (25); body mass index and HbA_{1c} were seen to be positively

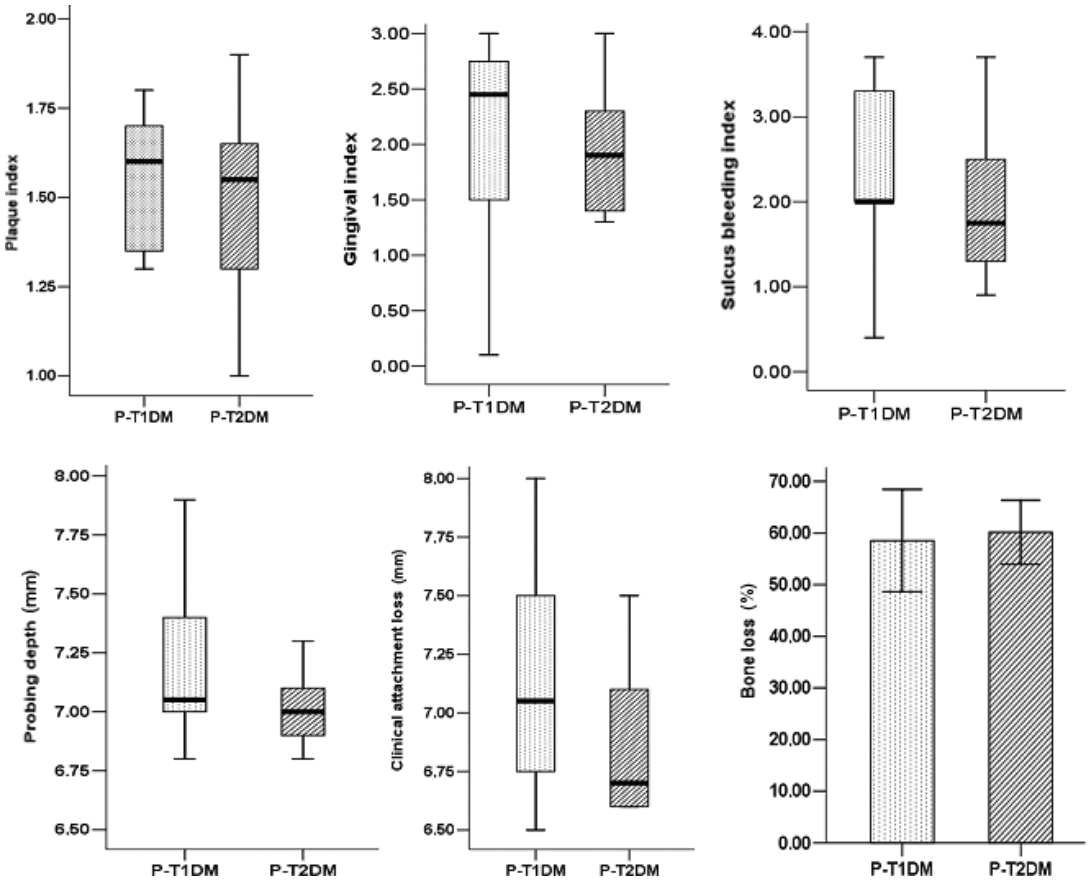

Fig. 1. Full mouth means of periodontal parameters of the subjects studied; the variables are represented as bar graphs if normally distributed and as box plots if not normally distributed. Statistical comparison between the two groups was carried out for each variable; no significant difference between the two groups was found. For bar graphs, the bars represent the means and error bars correspond to SD; for box plots, the horizontal lines represent the medians, the boxes indicate the interquartile range and the whiskers show the data range.

Table 1. Clinical parameters of diabetic subjects

	P-T1DM	P-T2DM	Statistical significance
Age (years)	43.5 (36–50)	63.5 (49–79)	$p < 0.001$
Duration of diabetes mellitus (mo)	72 (20–120)	35 (13–120)	$p = 0.032$
Body mass index (kg/m^2)	30.33 ± 4.98	31.95 ± 5.32	NS
Fasting glucose (mg/dL)	91.5 (86–123)	94 (85–129)	NS
Glycated haemoglobin (%)	8.28 ± 0.82	7.79 ± 0.95	NS

Continuous variables are expressed as means ± SD if normally distributed, and as medians (range) if not normally distributed. Statistical comparison between the two groups: NS, not statistically significant. Abbreviations: P-T1DM, periodontitis patients with type 1 diabetes mellitus; and P-T2DM, periodontitis patients with type 2 diabetes mellitus.

related to some proinflammatory cytokines in the gingival crevicular fluid (26,27); and age, duration of DM, body mass index and HbA_{1c} were also found to be positively correlated with the serum CRP (28–30), which is a well-known predictor of cardiovascular risk and is usually found to be elevated in periodontal disease (30). In our patients, we found a significant and negative correlation in P-T1DM subjects both between the duration of T1DM and gingival crevicular fluid IL-1β and between the duration of T1DM and gingival crevicular fluid TNF-α (Table 3); a possible explanation of this could lie in the existence of a proinflammatory imbalance in the early phases of T1DM, as demonstrated by the fact that a greater lipopolysaccharide-stimulated production of interleukin-1 was found in newly diagnosed T1DM subjects compared with the long-standing T1DM subjects (31). Furthermore, higher plasma levels of IL-1β have been found in T1DM subjects with recent onset of the disease than in those with a longer duration of the disease (32). Given that the levels of the inflammatory mediators in gingival crevicular fluid can be conditioned by plasma levels of the same mediators (1), one could hypothesize that in P-T1DM subjects with a more recent diagnosis of T1DM the high levels of gingival crevicular fluid IL-1β and TNF-α could be due to the high plasma levels of these two cytokines, which are deeply involved in the early stage of T1DM development (33–35). In contrast, there were no correlations

Table 2. Levels of plasma high-sensitive C-reactive protein (hs-CRP), gingival crevicular fluid interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α)

	P-T1DM	P-T2DM	Statistical significance
IL-1β (ng/mL)	75.25 ± 5.82	57.08 ± 6.48	$p < 0.001$
IL-6 (ng/mL)	16 (15.1–24.97)	16 (10.12–60.1)	NS
TNF-α (ng/mL)	30.5 (20.2–36.05)	22 (3.5–32.1)	$p < 0.001$
hs-CRP (mg/L)	3.1 (1–4)	2.8 (1–3.9)	NS

Continuous variables are expressed as means ± SD if normally distributed, and as medians (range) if not normally distributed. Statistical comparison between the two groups: NS, not statistically significant.

Table 3. Correlations between clinical parameters and inflammatory markers in the two groups of subjects

	Age (years)	Duration of diabetes mellitus (mo)	Body mass index (kg/m^2)	Glycated haemoglobin (%)
P-T1DM				
IL-1β (ng/mL)	$r = -0.276$ NS	$r = -0.564$ $p = 0.004$	$r = 0.263$ NS	$r = 0.184$ NS
IL-6 (ng/mL)	$r = -0.258$ NS	$r = 0.048$ NS	$r = 0.065$ NS	$r = 0.391$ $p = 0.06$
TNF-α (ng/mL)	$r = 0.201$ NS	$r = -0.446$ $p = 0.029$	$r = 0.281$ NS	$r = -0.281$ NS
hs-CRP (mg/L)	$r = -0.044$ NS	$r = -0.215$ NS	$r = 0.144$ NS	$r = -0.199$ NS
P-T2DM				
IL-1β (ng/mL)	$r = 0.029$ NS	$r = 0.260$ NS	$r = -0.204$ NS	$r = 0.388$ $p = 0.06$
IL-6 (ng/mL)	$r = 0.145$ NS	$r = 0.058$ NS	$r = -0.139$ NS	$r = -0.087$ NS
TNF-α (ng/mL)	$r = 0.301$ NS	$r = 0.202$ NS	$r = 0.298$ NS	$r = -0.098$ NS
hs-CRP (mg/L)	$r = 0.129$ NS	$r = 0.053$ NS	$r = -0.103$ NS	$r = 0.055$ NS

between DM duration and gingival crevicular fluid IL-1β or TNF-α in P-T2DM patients (Table 3), probably because of the different physiopathological mechanisms relevant for the development of T2DM, which are mainly linked to insulin resistance (1). Clearly, even though our hypotheses are theoretically sustained, they need to be validated by specific studies to evaluate the relationships between inflammatory mediators in the gingival crevicular fluid and duration of DM.

It is also worth noting that our data showed positive but not fully significant correlations between HbA_{1c} and gingival crevicular fluid IL-6 in P-T1DM and between HbA_{1c} and gingival crevicular fluid IL-1β in P-T2DM (Table 3). These results are not surprising, given that hyperglycaemia causes the accumulation of advanced glycation end-products (36). Interaction of advanced glycation end-products with their monocyte receptors increases oxidative cellular stress and activates the transcription nuclear factor-κB, which alters the monocyte/macrophage phenotype, resulting in increased production of proinflammatory cytokines (37,38). We believe that in our data this correlation did not reach full statistical significance owing to the low number of subjects studied.

Some limitations need to be acknowledged. In our study, collection of gingival crevicular fluid was made only for the anterior teeth; given that the volume of gingival crevicular fluid is not homogeneous among all types of teeth independently of the clinical periodontal status (39,40), our findings need to be confirmed by studies which also include gingival crevicular fluid sampling of the posterior teeth. Also, theoretically, it cannot be completely excluded that P-T1DM subjects, given the severity of periodontal disease at such a young age, could have a form of aggressive periodontal disease. Regarding this aspect, the following characteristics of our sample must be taken into consideration: (i) our subjects were over 35 years of age; (ii) there was a consistency between periodontal destruction and amount of local contributing etiological factors such as plaque levels; (iii) patients affected by periodontal disease were not systemically healthy but suffering from DM; and (iv) no familial aggregation for periodontal disease existed (data not shown). The simultaneous presence of all these elements makes the presence of a form of aggressive periodontal disease very improbable (41–43).

In conclusion, our study shows, for the first time, that gingival crevicular fluid IL-1β and TNF-α levels in P-\T1DM patients are affected by the length of DM and are higher in cases of recent onset of the disease; this finding could constitute a physiopathological basis for the association between T1DM and an increased risk for periodontal destruction in children under 10 years of age (44) and further highlights the necessity of treating periodontitis during the early stages of T1DM development.

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