The effect of experimental periodontitis, experimental diabetes and their combination on the serum levels of adiponectin, leptin, IL-6, IL-18, MCP-1, RANTES and sICAM-1 in rats

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Abstract

Background: The aim of this study was to assess ligature-induced periodontitis, streptozotocin-induced diabetes and their combination on serum levels of adiponectin, leptin, interleukin-6 (IL-6), interleukin-18 (IL-18), monocyte chemoattractant protein-1 (MCP-1), regulated on activation, normal T cell expressed and secreted (RANTES) and soluble intercellular adhesion molecule-1 (sICAM-1).

Materials and Methods: Forty-seven Wistar rats were studied: experimental periodontitis (13), experimental diabetes (10), experimental diabetes and experimental periodontitis (12) and health (12, controls). Diabetes was induced by streptozotocin injection on day 1. Periodontitis was induced by ligation on day 16. Serum levels of adiponectin, leptin, IL-6, IL-18, MCP-1, RANTES and sICAM-1 were assessed on days 16 (baseline) and 77 (final) by multiplex immunoassay.

Results: Periodontitis significantly increased adiponectin and reduced IL-18. Diabetes significantly reduced leptin. The combination of diabetres and periodontitis: (a) significantly reduced leptin and IL-18 and significantly increased IL-6 than control, (b) showed stronger significance in leptin reduction than diabetes (c) reduced adiponectin and leptin than periodontitis.

Conclusions: Periodontitis induced changes indicative of systemic inflammation. The combination of T1 diabetes and periodontitis induced systemic inflammation and serum changes of biomarkers involved in the esthablishment and progression of T1 diabetes. Their combination might affect the systemic inflammation generated by T1 diabetes.

Keywords: periodontitis, type 1 diabetes mellitus, streptozotocin diabetes, rats, biomarkers.

Introduction

In diabetes, impaired insulin secretion and/or deficient insulin activity lead to chronic hyperglycemia (American Diabetes Association, 2014). In diabetes, chronic hyperglycemia affects the periodontal tissues (Pontes Andersen *et al.*, 2007) through altered cellular immunity, microangiopathy and formation of advanced glycation end products. There is a two-way relationship between diabetes and periodontitis (Chapple *et al.*, 2013). Most studies have explored the relationship between type 2 (T2) diabetes and periodontitis. The pathophysiology of T2 diabetes is however different to that of the immune-mediated type 1 (T1) diabetes. There are limited data

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on the relationship of T1 diabetes, which results from a cellular mediated autoimmune destruction of the b-cells of the pancreas (American Diabetes Association, 2014), to periodontitis. For T1 diabetic patients, there are insufficient data on the association of periodontitis with poorer glycaemic control and on the effect of periodontal treatment on glycaemic control (Sanz *et al.*, 2018). There is evidence that patients with both periodontitis and T1 diabetes have significantly more renal complications (Sanz *et al.*, 2018).

Biologic markers (or biomarkers) are implicated in host immune responses. The local levels of biomarkers of inflammation (or inflammation biomarkers) might be altered in periodontitis as compared to health (Zekeridou *et al.*, 2019). Periodontitis might induce systemic inflammation to a certain degree (Buduneli and Kinane, 2011), which might alter the serum levels of the inflammation biomarkers (Zekeridou *et al.*, 2019). In T1 diabetes, the changes in the serum levels of certain biomarkers might differ between recently diagnosed and older cases (Dogan *et al.*, 2006). In chronic hyperglycemia, the accumulation of advanced glycation end products leads to the release of inflammation biomarkers, which act topically and affect other biomarkers (Takeda *et al.*, 2006). Several biomarkers are being explored in relation to either diabetes or periodontitis or both, such as adiponectin, leptin, interleukins, tumor necrosis factor-α (TNF-α), chemokines, glycogen-like peptide-1(GLP-1) and cell adhesion molecules (Harms *et al.*, 2015; Zhu *et al.*, 2017; Chen *et al.*, 2017; Pal China *et al.*, 2018, Dokumacioglu *et al.*, 2018; Duarte *et al.*, 2019, Kurtdede *et al.*, 2019).

The limited data available on the relationship between T1 diabetes and periodontitis led the authors to examine whether the systemic burden of periodontitis is aggravated in the presence of T1 diabetes and whether the systemic burden of T1 diabetes is altered by periodontitis. Studies in humans would present limitations in design, such as variations in severity of periodontal tissue loss, glucose levels, duration of T1 diabetes and diabetes complications. Moreover, most of the T1 diabetes patients would receive medication for diabetes, unless very recently diagnosed. An animal study was therefore selected to overcome some of the possible limitations of such a study in humans. There is insufficient evidence regarding the combination of experimental periodontitis and experimental T1 diabetes in terms of serum levels of biomarkers implicated in diabetes, inflammation and osseous metabolism.

Among all animal models, experimentally induced periodontitis and experimentally induced diabetes have been mostly studied in rodents (Pontes Andersen *et al.*, 2007). Ligating molars and sustaining ligation around them for relatively long time induces periodontitis in rats (Holzhausen *et al.*, 2004; Pepelassi *et al.*, 2012). Intravenous injection of 45 mg/kg streptozotocin induces diabetes in rats (Holzhausen *et al.*, 2004; Pepelassi *et al.*, 2012; Xynogala *et al.*, 2012). The streptozotocin-induced diabetes in rats is T1 diabetes (Mealey and Oates, 2006). An earlier study in the same rat sample showed histometrically that the combination of streptozotocin-induced diabetes and ligature-induced periodontitis led to non-significantly greater alveolar bone loss than ligature-induced periodontitis alone (Pepelassi *et al.*, 2012). Moreover, that study showed that the addition of experimental periodontitis to experimental diabetes did not affect the serum glucose levels (Pepelassi *et al.*, 2012).

The authors examined the following hypotheses: (1) experimental periodontitis, experimental diabetes and their combination affects the circulating biomarkers and (2) the impact of the combination of experimental diabetes and experimental periodontitis on the circulating biomarkers is different than that of each disease alone.

The aim of this study in rats was to assess the effect of ligature-induced periodontitis, streptozotocin-induced diabetes and their combination on the serum levels of adiponectin, leptin, interleukin-6 (IL-6), interleukin-18 (IL-18), monocyte chemoattractant protein-1 (MCP-1), regulated on activation, normal T cell expressed and secreted (RANTES) and soluble intercellular adhesion molecule-1 (sICAM-1).

Material and methods

Sixty adult male Wistar rats (225-250 g) were used. The animals were randomly classified into four groups of 15 each: (1) experimental T1 diabetes (D), (2) experimental periodontitis (P), (3) experimental T1 diabetes and experimental periodontitis (DP) and (4) systemically and periodontally healthy rats (healthy controls, C). The study was designed as a randomized, parallel-arm animal 77 day experiment. The study was conducted in accordance with guidelines approved by the Council of the American Psychological Society (1980) and the European Communities Council Directive of 24 November 1996 (86/609/EEC). The study was approved by the National and Kapodistrian University of Athens Ethics and Research Committee and by the Veterinary Directorate of the Prefecture of Athens.

Animal origin, initial evaluation, housing conditions, diabetes induction, body weight assessment, glucose assessment, ligation, post-ligation monitoring, histologic assessment of the alveolar bone loss and sacrifice conditions have been analyzed in an earlier study involving the same rat sample (Pepelassi *et al.*, 2012). In brief, T1 diabetes was induced by intravenous injection of streptozotocin (45 mg/kg) in the tail vein. Two rat groups (D, DP) were subjected to streptozotocin injection on day 1 of the study. Diabetes was successfully induced if the serum glucose levels were >300 mg/dl up to day 5 after the streptozotocin injection, as assessed with a

glucometer. Periodontitis was induced at the maxillary right second molar by intrasulcular placement and ligation (ligation) of 4/0 silk suture. Two rat groups were ligated (P, DP), whereas C and D groups were not ligated. Ligation was performed on day 16 and remained in place for 61 days. Serum glucose levels were assessed on days 1 to 5, 16, 23, 30, 37, 44, 51, 58, 65, 72 and 77 (15 glucose assessments per animal). Housing and breeding conditions were kept standard for the whole study.

Venous blood samples (3.5 ml each) were collected from the tail vein of each animal on days 16 and 77 (2 blood samples per animal) for assessment of the serum levels of adiponectin, leptin, IL-6, IL-18, RANTES, MCP-1, and sICAM-1. Blood sampling was performed early in the morning, under an overnight fasting state. Serum separator tubes were used and samples were allowed to clot for two hours at room temperature. Then, samples were centrifuged at 3000 rpm for 15 minutes. Serum was removed, aliquoted and stored in Eppendorf tubes at -80^{o} C. For each animal, serum levels of adiponectin, leptin, IL-6, IL-18, RANTES, MCP-1 and sICAM-1 were assessed on days 16 (baseline values) and 77 (final values). On day 77 (end of the study) all rats were sacrificed.

Serum samples were analyzed by using multiplex immunoassay and read with Luminex 100 (Multiplexed Biomarker Immunoassays for Luminex® Instrumentation/ xMAP® Technology-Luminex Corporation, Austin, USA). Adiponectin levels were determined with MillipexTM Map Kit (Millipore Corporation, Billerica, MA, USA, Rat CVD Panel 3, # RCVD3-89K). The levels of leptin, IL-6, IL-18, RANTES and MCP-1 were determined with MillipexTM Map Kit (Millipore Corporation, Billerica, MA, USA, Rat Cytokine/Chemokine, RCYTO-80K, RCYTO-80K-PMX). sICAM-1 levels were determined with MillipexTM Map Kit (Millipore Corporation, Billerica, MA, USA, Rat CVD Hormone Panel 2, # RCVD2-89K). The animal was the unit of measurement for the biomarker values.

Statistical analysis

Mean values and standard deviations (SD) or median values and 1 and 3 quartiles (Q1-Q3) (when the normality assumption was not met) were calculated for the levels of adiponectin, leptin, IL-6, IL-18, RANTES, MCP-1 and sICAM-1 at two time points (day 16-baseline, day 77-final). One-way analysis of variance (ANOVA) or the non-parametric Kruskall-Wallis test was used for comparison of these measurements among the four groups (C, D, P, DP) at each time point (baseline, final). Two-by-two comparisons were assessed using the t-test or Mann-Whitney test (when the normality assumption was not met). Comparisons within groups between different time points were examined using the t-test for paired data or non-parametric Wilcoxon matched pairs signed ranks tests. Bonferroni correction was used to adjust p-values for multiple comparisons. All analyses were performed using the statistical package Stata 9.0 (Stata, College Station, TX, USA). The level of statistical significance was set at 5% ($p = 0.05$).

Results

Among the 60 rats initially included in the study, 47 rats survived. The number of animals that survived were 10, 13, 12 and 12, rats for each of the D, P, DP and C groups, respectively. The survival rate did not significantly differ among rat groups. Periodontitis was induced in all ligated rats. Ligation of maxillary second molar with 4/0 silk suture ligature for 61 days was successful in inducing alveolar bone loss in Wistar rats and therefore successful in inducing periodontitis (Pepelassi *et al*., 2012). T1 diabetes was induced in all streptozotocin-injected rats. The intravenous injection of 45 mg/kg streptozotocin was successful in inducing T1 diabetes in Wistar rats (Pepelassi *et al*., 2012). For all biomarkers studied and for each animal group, median values at day 16 (baseline values) and 77 (final values) were calculated. At baseline, there were statistically significant differences among groups for adiponectin, IL-6 and sICAM-1. At the final evaluation, there were statistically significant differences among groups for adiponectin, leptin, IL-6 and IL-18. Specifically, adiponectin was statistically significantly higher for P than C, D and DP. Leptin was statistically significantly higher for C than D and DP as well as for P than D and DP. IL-6 was statistically significantly higher for DP than C. IL-18 was statistically significantly lower for C than P and DP (Table 1).

Comparison between final and baseline values for each biomarker studied and for each group showed the following (Table 2). Statistically significant differences between final and baseline values were found for adiponectin in P, D and DP, for leptin in C and D, for IL-6 in C, for IL-18 in C and P and for RANTES in D. Marginal statistically significant difference ($p=0.05$) between final and baseline values were found for sICAM-1 in DP (Table 2). Comparison of the change in values between the final and baseline evaluation for all biomarkers studied among the four animal groups is shown in Table 3 and Figure 1. Statistically significant differences in level change among groups were found for adiponectin (between D and P as well as between D and DP), leptin (between C and DP as well as between D and P), IL-18 (between P and DP) and RANTES (between C and D) (Table 3).

Discussion

Periodontitis significantly increased serum adiponectin, significantly reduced serum IL-18 and non-significantly increased serum leptin as compared to control. T1 diabetes significantly reduced serum leptin and non-significantly reduced serum adiponectin as compared to control. Both adiponectin and leptin were significantly lower in T1 diabetes than periodontitis. The combination

Table 1. Comparison of the final (at day 77) values of adiponectin, leptin, IL-6, IL-18, RANTES, MPC-1 and sICAM-1 among animal groups by using Kruskal-Wallis and Mann-Whitney test.

Agent	Animal group				F-test	P-value
(pg/ml)	C n=12	D n=10	P n=13	DP n=12		
Adiponectin §	11.0 (7.2-17.1) [a]	8.5 (5.0-11.1) [b]	20.6 (17.9-22.4) [a,b,c]	8.9 (7.0-121.0) [c]	21.00	0.0001
Leptin	5,172.9 (3,879-8,931.6) [d,e]	1.395,1 (693,9-1.909,6) [f,e]	6,050.7 (2,423.9-6,623.9) [f,g]	1,096.2 (814.9-2,611.5) [d,g]	19.26	0.0002
IL-6	24.4 (24.4-102.1) [h]	251.1 (24.4-503.0)	575.1 (216.0-1,370.6)	567.1(164.6-2,198.5) [h]	9.67	0.02
IL-18	61.8 (48.1-72.8) [i,j]	53.0 (45.4-89.3)	123.4 (81.1-222.1) [i]	207.3 (113.9-432.1) [j]	16.24	0.001
RANTES	7,286.7 (6,662.2-16,553.9)	5,200.7 (4,096.4-6,021.1)	7,401.7 (4,170-13,729.3)	8,630.9 (5,178.5-14,086.3)	4.97	0.17
MCP-1	39.3 (24.4-81.4)	81.4 (24.4-208.4)	24.4 (24.4-24.4)	24.4 (24.4-149.0)	1.83	0.61
sICAM-1	13,491.0 (2.061-105,475.3)	2,222 (2,170.5-2,400.6)	4,774.2 (4,148.8-5,056.8)	6,074.7 (4,098.4-7,133.9)	6.21	0.10

[a, b, c, d, e, f, g, h, i, j] statistically significant difference between groups using Mann-Whitney test with Bonferroni correction.
C: control, D: diabetes, P: periodontitis, DP: diabetes and periodontitis.
§ μg/ml

of T1 diabetes and periodontitis as compared to control significantly affected leptin, IL-6 and IL-18, by reducing leptin and IL-18 and increasing IL-6. The co-existence of T1 diabetes and periodontitis led to stronger significance in leptin reduction as compared to that induced by T1 diabetes alone. The combination of T1diabetes and periodontitis as compared to periodontitis reduced adiponectin and leptin.

The present findings that streptozotocin-induced T1 diabetes significantly reduced serum leptin as compared to health are in agreement with other reported findings in rats (Havel *et al.,* 1998; Sindelar *et al.,* 1999; Soliman *et al*., 2001; Gülen and Dinçer, 2007) and mice (Coe and McCabe, 2007). The present study and the studies by Havel *et al.* (1998), Sindelar *et al.* (1999), Soliman *et al.* (2001) and Gülen and Dinçer (2007) share common experimental design in the animal model and experimental diabetes induction methods. The similarities in study design strengthen the significance of agreement in leptin reduction between these studies. The reduction in serum leptin has been reported to start soon after the induction of streptozotocin-induced diabetes (Havel *et al*., 1998).

Serum leptin levels were similar for dogs with newly diagnosed and naturally occurring T1 diabetes as compared to healthy dogs (Kim *et al*., 2015). This relationship persisted even in the absence of concurrent disease, such as acute pancreatitis, chronic kidney disease or hyperadrenocorticism, as compared to healthy dogs (Kim *et al*., 2015). Significantly elevated serum leptin levels have been reported in dogs with newly diagnosed and naturally occurring T1 diabetes with concurrent disease as compared to healthy dogs. Among dogs with newly diagnosed and naturally occurring T1 diabetes, the presence of concurrent diseases significantly increased serum leptin levels as compared to their absence (Kim *et al*., 2015). Therefore, it has been suggested that the leptin increase was probably a result of concurrent disorders rather than an effect of chronic hyperglycemia as a result of diabetes. It seems that in newly diagnosed and naturally occurring T1 diabetes in dogs the presence of concurrent disease affects serum leptin levels as compared to their absence (Kim *et al*., 2015).

In the present study, there were no significant indications that diabetes reduced adiponectin. Serum adiponectin levels were significantly reduced in dogs with newly diagnosed and naturally occurring T1 diabetes as compared to healthy dogs (Kim *et al*., 2015).

In children with newly diagnosed T1 diabetes, serum leptin levels were significantly lower before the initiation of insulin treatment as compared to 5 days after the initiation of insulin treatment (Soliman *et al*., 2002). In insulin treated T1 diabetic children, serum leptin levels were significantly higher as compared to healthy controls (Soliman *et al*., 2002).

The present impact of experimental periodontitis either alone or combined with experimental T1 diabetes on serum adiponectin and leptin cannot be directly compared to previous findings in animals due to paucity of studies. Impact of periodontitis on serum adiponectin and leptin levels was found in a recent systematic review and meta-analysis in humans presenting body mass index (BMI) <30, where adiponectin was reduced and leptin was increased in periodontitis as compared to health (Zhu *et al*., 2017).

Table 2. Comparison between the final and baseline values of adiponectin, leptin, IL-6, IL-18, RANTES, MPC-1 and sICAM-1 in each animal group by Wilcoxon matched pairs signed ranks tests.

Agent	Animal group			
	C	D	P	DP
(pg/ml)	n=12	n=10	n=13	n=12
Adiponectin $_b$ [§]	15.4(8.9-23.2)	16.5(15.3-17.3)	17.7(15.1-21.4)	7.7(5.8-11.2)
Adiponectin $_f$ [§]	11.0(7.2-17.1)	8.5(5.0-11.1)	20.6(17.9-22.4)	8.9(7.0-12,1)
p-value	0.39	0.02	0.009	0.02
Leptin $_b$	3,423.4(1,442.5-5,400.8)	600.5(381.5-1,178.5)	6,795.5(1,151.9-9,390.9)	865.4(675.1-2,516.6)
Leptin $_f$	5,172.9(3,879-8,931.6)	1,395.1(693.9-1,909.6)	6,050.7(2,423.9-6,623.9)	1,096.2(8,14.9-2,611.5)
p-value	0.005	0.01	0.58	0.61
IL-6 $_b$	78.1(45.9-255.4)	1,129.2(85.0-2,851.9)	533.3(306.6-2,449.8)	641.9(306.6-2.022.6)
IL-6 $_f$	24.4(23.9-102.1)	251.1(24.4-503.0)	575.1(216.0-1.307.6)	567.1(164.6-2,198.5)
p-value	0.045	0.17	0.80	0.39
IL-18 $_b$	122.7(101.5-199.4)	194.3(154.2-234.7)	309.0(240.2-377.1)	203.1(141.7-401,7)
IL-18 $_f$	61.8(48.1-72.8)	53.0(45.4-89.3)	123.4(81.1-222.1)	207.3(113.9-432.1)
p-value	0.002	0.07	0.002	0.48
RANTES $_b$	5,792.9(4,501.1-8,379.2)	7,482.2(6,761.0-10,942.3)	5,742.3(3,634.7-10,917.5)	7,078.3(3,928.2-10,954.7)
RANTES $_f$	7,286.7(6,662.2-16,553.9)	5,200.7(4,096.4-6,021.1)	7,401.7(4,170-13,729.3)	8,630.9(5,178.5-14,086.3)
p-value	0.05	0.04	0.94	0.31
MCP-1 $_b$	76.5(24.4-143.7)	242.2(196.4-258.1)	64.5(24.4-170.6)	64.5(24.4-338.1)
MCP-1 $_f$	39.3(24.4-81.4)	81.4(24.4-208.4)	24.4(24.4-24.4)	24.4(24.4-149.0)
p-value	0.93	0.06	1.00	0.19
sICAM-1 $_b$	2,222(2,092.7-2,234.0)	2,264.8(2,222-139,964.6)	4,667.3(2,946.2-4,889.1)	6,474.7(5,746.0-7,502.4)
sICAM-1 $_f$	13,491.0(2,061-105,475.3)	2,222(2,170.5-2,400.6)	4,774.2(4,148.8-5,056.8)	6,074.7(4.098.4-7,133.9)
p-value	0.24	0.22	0.81	0.05

$_b$Baseline values (at day 16).
$_f$Final values (at day 77).
C: control, D: diabetes, P: periodontitis, DP: diabetes and periodontitis.
[§]μg/ml

Table 3. Comparison of the changes (between final and baseline evaluation) in adiponectin, leptin, IL-6, IL-18, RANTES, MPC-1 and sICAM-1 among animal groups by using Kruskal-Wallis and Mann-Whitney test.

Biomarkers	Animal group				F-test	P-value
(pg/ml)	C n=12	D n=10	P n=13	DP n=12		
ΔAdiponectin[§]	-4.1(-10.5, -2.9)	-9.4(-11.9, -1.8) [a, b]	4.1(1.3, 6.6) [b]	1.2(0.3, 3.1) [a]	15.68	0.001
ΔLeptin	2,782.4 (676.7, 4,836.5) [c]	753.8 (244.9, 1,398.6) [d]	-232.9 (-4,888.2, 2,918.6) [d]	108.8 (-381.0, 451.8) [c]	8.49	0.04
ΔIL-6	-42.0 (-106.1, -10.0)	-166.1 (-2,838.1, 10.6)	77.2 (-2,036.8, 498.2)	195.7 (-476.6, 1.180.3)	2.86	0.41
ΔIL-18	-71.8 (-128.4, -34.2)	-113.5 (-176.8, -88.1)	-177.7 (-295.1, -103.5) [e]	-19.9 (-143.0, 97.7) [e]	9.34	0.03
ΔRANTES	3,500.3 (-503.1, 10,138.4) [f]	-1,567.2 (-3,800.1, -938.9) [f]	-302.4 (-3,599.1, 3,199.2)	668.8 (-881.1, 8,897.2)	8.58	0.04
ΔMCP-1	0.1(-88.7, 9.9)	-44.0(-189.8, 0)	0(-120.8, 57.8)	0(-38.7, 0)	4.23	0.24
ΔsICAM-1	11,720.5 (-317.9, 103,317.2)	-68.5 (-50,079.4, 53.1)	170.3 (-775.1, 1,592.7)	-397.8 (-1.329.6, -73.7)	7.00	0.07

[a, b, c, d, e, f] statistically significant difference between groups using Mann-Whitney test with Bonferroni correction.
Δ : change between final (day 77) and baseline (day 16) evaluation
C: control, D: diabetes, P: periodontitis, DP: diabetes and periodontitis
[§] μg/ml

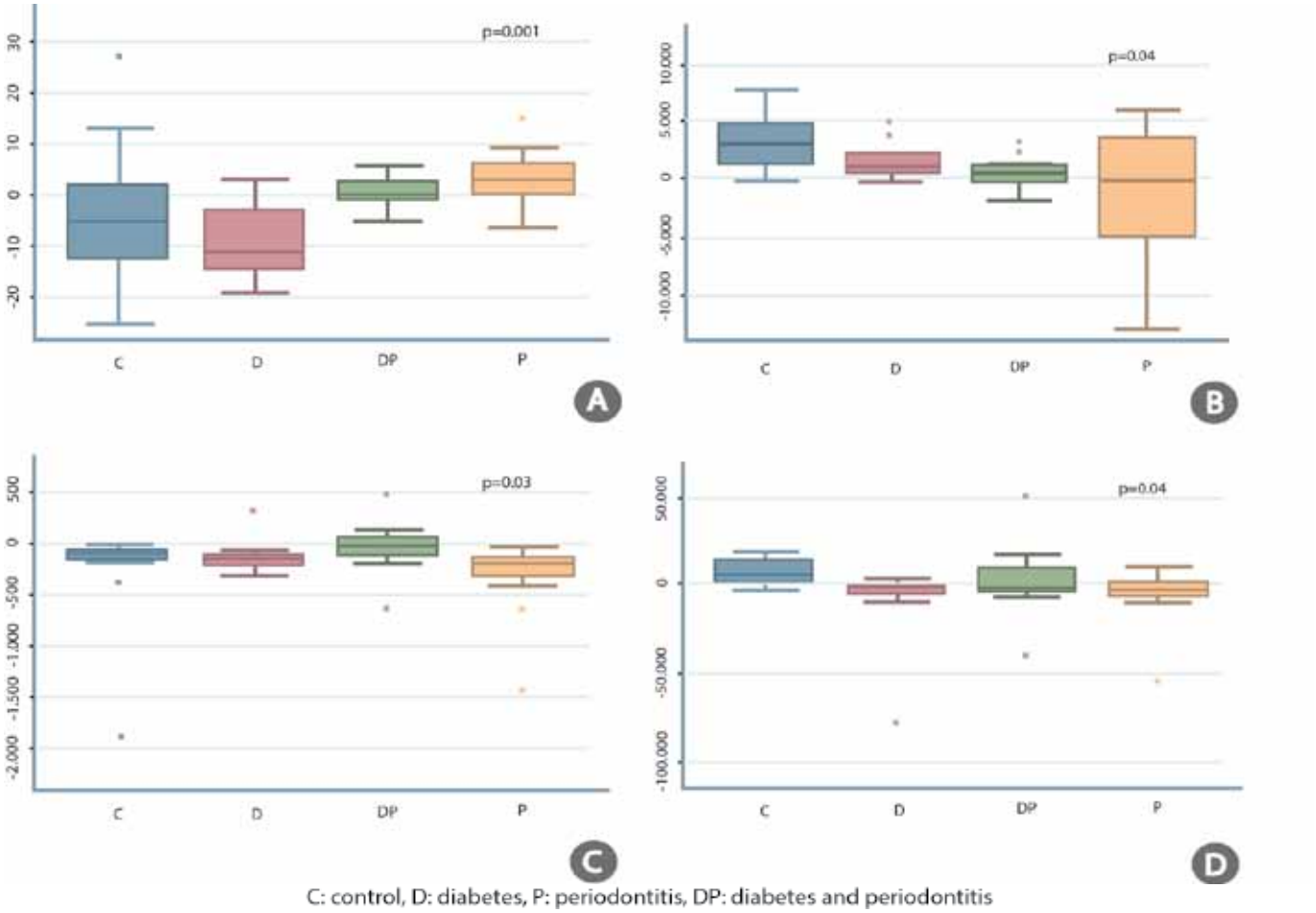

Figure 1 (A, B, C, D). Box plots displaying the distribution of changes (between final and baseline evaluation) in biomarker levels by animal group and comparison of biomarker level changes among animal groups. 1A: adiponectin. 1B: leptin. 1C: IL-18. 1D:RANTES.

Adiponectin and leptin seem to be related to periodontal disease through roles in the inflammation and osseous metabolism. Adiponectin mediates anti-inflammatory effects by blocking NF-κB activation and reducing cytokines (Deschner *et al.*, 2014). Moreover, it might have proinflammatory effect (Bas *et al.*, 2014). It functions in cell proliferation, differentiation, and regeneration (Deschner *et al.*, 2014). It is involved in bone formation both directly and indirectly. The various types of adiponectin present differential binding to receptors (adipoR1 and adipoR2) with cell-specific receptor variants in bone. A beneficial role of adipoR1 in bone metabolism has been suggested. The downstream signaling of adipoR1 in osteoblasts involves stimulation of oxidative phosphorylation leading to increased differentiation. At the same time, adiponectin suppresses the ratio of the receptor activator of nuclear factor κ-B ligand (RANKL) to osteoprotegerin (OPG) in osteoblasts, which suppresses osteoclastogenesis (Pal China *et al.*, 2018). Leptin, which is a proinflammatory cytokine, modulates the function of immunocytes leading to the release of other inflammation mediators. In the periodontal tissues, leptin increases the synthesis of proinflammatory and proteolytic molecules, whereas adiponectin downregulates the production of such mediators (Deschner *et al.*, 2014). Leptin and adiponectin are produced in periodontal tissues and are regulated by periodontopathogenic bacteria (Deschner *et al.*, 2014). Leptin is also involved in osseous metabolism both directly and indirectly. It acts on osteoblasts directly, by enhancing proliferation and differentiation and inhibiting apoptosis (Gordeladze *et al.*, 2002). It inhibits RANKL expression and stimulates osteoprotegerin expression (Burguera *et al.*, 2001). It affects the osseous metabolism indirectly through the endocrine and central nervous system. Through the central nervous system, leptin induces bone loss and inhibits osteogenesis (Takeda *et al.*, 2002). Leptin causes insulin secretion and thereafter both glucose and insulin enhance insulin secretion (Sarath and Rajkumar, 2011). In T1 diabetes, where pancreatic cells do not produce insulin, leptin does not affect insulin levels. The present findings on lower serum leptin levels in T1 diabetes than health might be partly attributed to insufficient insulin levels. Leptin secretion is not enhanced by insulin in T1 diabetes, since insulin is either not produced or produced at very low levels. The secretion of leptin is enhanced by glucose as well, which is elevated in T1 diabetes. It seems that both low insulin and high glucose concentration contributed to the reduced leptin levels in diabetes in the present study.

The present findings regarding serum IL-6 levels for periodontitis and health are in agreement with findings by Luo *et al.* (2014) in Sprague-Dawley rats with ligature-induced periodontitis. However, they are in disagreement with findings by Lu *et al.* (2016) in Wistar rats with ligature-induced periodontitis and with findings by Kurtdede *et al.* (2019) in dogs with naturally occurring moderate to severe periodontitis, who reported significantly elevated serum IL-6 levels in periodontitis than health. Similar serum IL-6 levels for periodontitis and health have been reported in humans (de Queiroz *et al.*, 2008) as well. Serum IL-6 is a potent marker of the inflammatory response. It seems that in the present study the local inflammation generated by localized periodontitis did not induce serum changes in IL-6 to the extent to significantly elevate the IL-6 levels.

The present findings on similar serum IL-6 levels for T1 diabetes and health are in disagreement with previous findings in animals (Jain *et al.*, 2009; Tirgan *et al.*, 2012; Kim *et al.*, 2015; Li *et al.*, 2018; Dokumacioglu *et al.*, 2018). Specifically, serum IL-6 levels were significantly elevated in rats with streptozotocin-induced diabetes as compared to healthy rats (Jain *et al.*, 2009; Tirgan *et al.*, 2012; Li *et al.*, 2018; Dokumacioglu *et al.*, 2018) and in dogs with newly diagnosed and naturally occurring T1 diabetes as compared to healthy dogs (Kim *et al.*, 2015). However, in the present study IL-6 was non-significantly higher in diabetes than in health at 2.5 months after the induction of diabetes. At the early diabetes stages (at 16 days), IL-6 levels were significantly higher in only one of the two diabetic groups as compared to healthy controls. Therefore, the present findings indicate that diabetes may increase serum IL-6 both at early and later diabetes stages. The IL-6 increase was higher at early rather than later diabetes stages and implies activation of the systemic inflammatory process in streptozotocin-induced diabetes, particularly during the initial diabetes stages. This IL-6 increase occurring soon after T1 diabetes induction might be indicative of ongoing destruction of the beta-pancreatic cells. Similarly, Dogan *et al.* (2006) had found in humans that serum IL-6 level change was greater for recently diagnosed T1 diabetics receiving insulin as compared to diabetics with older diagnosis and insulin treatment for longer time.

The present findings on absence of significant T1 diabetes effect on serum IL-18 are in disagreement with findings by Rothe *et al.* (1997), who found elevated levels in mice with experimental T1 diabetes, with findings by Vatandost *et al.* (2012), who found elevated levels in Wistar rats with streptozotocin-induced T1 diabetes and with findings by Kim *et al.* (2015), who found elevated levels in dogs with newly diagnosed and naturally occurring T1 diabetes. Elevated serum IL-18 at early T1 diabetes stages has also been reported in humans (Nicoletti *et al.*, 2001) as well. IL-18 is implicated in the pathogenesis of inflammatory diseases and T1 diabetes (Harms *et al.*, 2015). IL-18 is involved in the autoimmune destruction of pancreatic β-cells leading to T1 diabetes (Oikawa *et al.*, 2003; Harms *et al.*, 2015). It enhances the proliferation of Th1 cells in vitro and the differentiation of Th cells into Th1 cells and an increase of Th1 over Th2 cells. An increase in activities related to Th1 cells has been found during the destruction of the pancreatic islets in T1 diabetic mice and in mice sensitive to developing T1 diabetes (Harms *et al.*, 2015). Thus it seems that IL-18 enhances immune reactions related to Th1 cells at early diabetes stages (Oikawa *et al.*, 2003). The non-significant IL-18 serum level increase found at the early T1 diabetes stages in the present study might be partly attributed to the aforementioned relation between IL-18 and Th-dependent immune response at the early T1 diabetes stages.

In periodontitis, the balance between the immune reactions related to Th1 cells and those related to Th2 cells is important. IL-18 enhances immune reactions related to both Th1 and Th2 cells (Orozco *et al.*, 2006). Specifically, IL-18 enhances the production of cytokines binding to Th1 cells, such as IL-2, granulocyte-macrophage colony-stimulating factor and IFN-γ, enhances the production of cytokines binding to Th2 cells, such as IL-3, IL-4 and IL-5, enhances the production of IL-1β and has synergistic effect to IL-12 (Orozco *et al.*, 2006). In the present experimental periodontitis model, synergistic and antagonistic effects among the mediators of inflammation may have resulted in the non-significant IL-18 increase. Thus ligation-induced localized periodontitis did not cause systemic inflammation to the extent needed to significantly increase serum IL-18. An effect of periodontitis on serum IL-18 levels has been reported in humans (Sánchez-Hernández *et al.* 2011) as well.

T1 diabetes either alone or combined with perodontitis affected leptin, IL-6 and IL-18. The changes of these three biomarkers reached significance when the two diseases were combined revealing synergistic changes. Taken together, these findings imply that the presence of periodontitis in T1 diabetes affected the biomarker profile. Therefore, there are indications that the combination of T1 diabetes and periodontitis might affect the systemic inflammation induced by T1 diabetes.

Periodontitis did not significantly affect serum RANTES levels in the present study. In an earlier study, at 12 weeks of experimental periodontal infection with a polybacterial consortium of 4 well-characterized periodontal pathogens, the infected mice demonstrated increased RANTES levels as compared to sham-infected mice (Chukkapalli *et al.*, 2015). Increased RANTES levels have been found in periodontitis patients (de Queiroz *et al.*, 2008). RANTES is locally produced in inflammation, recruits inflammatory cells and enhances the release of other host response mediators. It is an important mediator of the host response in periodontitis (Cekici *et al.*, 2014), which acts mainly locally. The induction of localized periodontitis, might partly explain the present absence of higher RANTES levels in periodontitis than health.

In this study there were non-significant indications that in early T1 diabetes stages serum RANTES levels were higher for T1 diabetes than health. Serum RANTES was elevated in children recently diagnosed with T1 diabetes as compared to children with increased risk to develop T1 diabetes (Stechova *et al*., 2007).

In T1 diabetes, the sustained hyperglycemia for two more months significantly reduced RANTES, since final levels were significantly lower than baseline. The findings on significant RANTES reduction in T1 diabetes, as a result of sustained hyperglycemia for longer, cannot be compared to previous findings, since the possible effect of the sustained hyperglycemia on serum RANTES has not been assessed.

The present findings on absence of effect of T1 diabetes on serum MCP-1 levels disagree with previous findings on higher MCP-1 levels in rats with streptozotocin-induced T1diabetes as compared to healthy rats (Jain *et al*., 2009; Chen *et al*., 2017). The production of advanced glycation end products and reactive oxygen species, as a result of chronic hyperglycemia, leads to the release of MCP-1. MCP-1 stimulates monocytes and macrophages and helps the recruitment of memory T cells and natural killer cells to sites of inflammation (Yap *et al*., 2017). Moreover, it plays an important role in the progression of diabetes complications (Yap *et al*., 2017). Absence of a significant effect of T1 diabetes on serum sICAM-1 levels, which was found in this study, has been repoted previously in humans (Doğruel *et al*. 2001).

The local inflammation generated by the ligature-induced periodontitis around one tooth led to changes in the serum levels of a limited number of the selected inflammation biomarkers. In general, serum of periodontitis patients exhibited lower protein concentrations of cytokines than gingival tissues and gingival crevicular fluid (Duarte *et al*., 2019). In the future, it would be interesting to study generalized experimental periodontitis. Comparison between localized and generalized experimental periodontitis might reveal differences in serum levels due to the different levels of local inflammation. Comparison between the serum and local levels of the biomarkers in experimental periodontitis might help to elucidate the link between the local and systemic inflammation. In this study, the levels of the biomarkers were assessed 16 and 77 days after the streptozotocin injection. In future studies, the evaluation of the biomarker levels at the day of study initiation, that is prior to streptozotocin injection, might be added. This would offer the possibility to compare the biomarkers levels at three time points within each diabetic group, specifically prior to streptozotocin injection, soon after the streptozotocin injection and at a later diabetes stage. Assessing the possible effect of diabetes regulation on the serum biomarkers levels would be challenging. It seems that the localized nature of the experimental periodontitis, the relatively small sample size, the wide variation in values and the use of serum might had hidden findings and relationships that otherwise might had proved to be significant. Moreover, findings should be interpreted with caution since periodontitis and T1 diabetes were experimentally induced (not naturally occurring) and they were tested in animals (not in humans).

Conclusions

Within its limits, this study demonstrated the following. The local inflammation generated by localized periodontitis induced changes indicative of systemic inflammation. The combination of T1 diabetes and periodontitis induced systemic inflammation and serum changes of biomarkers involved in the esthablishment and progression of T1 diabetes. The combination of T1 diabetes and periodontitis might affect the systemic inflammation induced by T1 diabetes.

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