Evaluation of recurrence of periodontal disease after treatment in obese and normal weight patients: two-year follow-up

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One-sentence summary: Obesity does not seem to promote a negative effect for the recurrence of the periodontitis.

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Abstract

Background: Obesity may represent a chronic low-grade inflammation, but there is a lack of long-term longitudinal studies. The aim of this longitudinal study was to evaluate the recurrence of periodontal disease in obese and normal weight patients submitted to scaling and root planning.

Methods: The study included 22 patients who had received periodontal treatment two years previously, 13 obese and nine non-obese. The patients were evaluated for anthropometric measurements of body mass index, waist circumference, waist-hip ratio, and fat percentage through bioimpedance. The following periodontal parameters were recorded: visible plaque index (VPI), gingival bleeding index (GBI), probing depth (PD), clinical attachment level (CAL) and bleeding on probing (BOP). The immunological evaluation analyzed the proinflammatory cytokines interleukin 1-

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1902/jper.10519.

beta (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor-alpha (TNF-α) in the gingival crevicular fluid (GCF).

Results: Obese and normal weight patients did not differ in relation to the periodontal parameters of VPI, GBI, PD, CAL, or POB two years after completion of the periodontal therapy. Sites with periodontitis in obese individuals showed higher levels of IL-6 and TNF-α in the gingival fluid ($p < 0.05$).

Conclusion: Obese and normal weight individuals had similar periodontal behaviors, with low recurrence of the disease; however, obesity was related to increased inflammatory activity in gingival fluid, which may become a risk indicator for future greater recurrence of periodontal disease in the presence of inadequate plaque control.

KEYWORDS

body weight, gingival crevicular fluid, inflammation, obesity, periodontal diseases

INTRODUCTION

Studies in rats[1-3] and in humans[4-10] have highlighted obesity as a potential risk indicator for the onset and progression of periodontitis. Obesity has been associated with systemic and inflammatory immunological alterations, characterized by changes in the levels of leptin, adiponectin, resistin, and visfatin, as well as tumor necrosis factor-alpha (TNF-α), interleukin 6 (IL-6), monocyte chemoattractant protein 1, and interleukin 1 receptor antagonist.[11] Obesity has also been related to increased alveolar bone loss and a more pronounced periodontal inflammatory response, which may reflect in the clinical severity of periodontitis.[3]

Studies evaluating the response to periodontal treatment in obese and non-obese individuals are scarce.[12] Considering that obesity may represent a systemic exacerbation of the inflammatory process through a chronic low-grade inflammation,[11,13,14] an important issue would be

the verification of the long-term clinical response in obese and normal weight patients undergoing scaling and root planning (SRP). A systematic review and meta-analysis showed that obesity seems not exert a modifier effect on periodontal healing, once obese and non-obese individuals revealed similar improvement of clinical conditions after SRP, but the authors emphasized that interventional prospective case-controls with long-term follow up are required for further investigations.[15] A prospective study with five years follow-up was conducted in Japanese individuals to evaluate whether body mass index was related to the development of periodontal disease[16]; however, despite the long term follow-up it was not a interventional study.

A pioneer study from our research team in a three month short- term follow-up demonstrated an improvement in the clinical periodontal condition and a decrease in the levels of circulating pro-inflammatory cytokines in obese and normal weight individuals; however, higher levels of IL-6 and TNF-α remained in the serum of obese individuals.[13] These results were confirmed by other studies.[17,18] Similarly, the levels of TNF-α, IL-6, resistin, adiponectin, and leptin in the gingival fluid and serum of obese and non-obese periodontitis patients, showed that TNF-α and resistin presented a significant increase in obese patients at baseline, 3, 6, and 12 months post-SRP treatment.[8] Based on the assumption that obese individuals remained with higher levels of some circulating proinflammatory cytokines three months post- SRP treatment in our pioneer study (Zuza et al.)13, the present study aimed to perform a two-year follow-up to verify the periodontal clinical parameters and the levels of proinflammatory cytokines in the gingival crevicular fluid at sites with and without periodontitis in these previously treated obese and normal weight subjects to evaluate the disease recurrence.

MATERIAL AND METHODS

The methodology was described and reproduced considering the previous work published by our research group.[13] This study was approved by the human subjects ethics board of the University Center of Educational Foundation of Barretos, UNIFEB (protocol 001/2012) and was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2013. The volunteers were verbally informed and signed a consent form, attesting to their voluntary participation in the research.

This prospective clinical study aimed to follow up 52 patients (27 obese and 25 non-obese) with generalized periodontitis who had received non-surgical periodontal treatment by scaling and root planning approximately two years before at UNIFEB.[13] Attempts were made to schedule patients on mobile or fixed phones, following a pattern, with at least five attempts to contact each person. When telephone contact was not possible, a registered letter was sent to the address noted on the clinical record; however, only 13 obese and nine non-obese patients returned for reevaluation (Figure 1).

In this long-term longitudinal study, new measurements of body and periodontal parameters were performed two years after beginning the study (baseline). During this period, oral hygiene instruction (OHI) and supragingival prophylaxis were performed one year after baseline. The study design can be visualized in figure 2.

All patients presented generalized periodontitis initially and some sites with periodontitis remained with periodontitis after periodontal treatment. It was verified a reduction in moderate and severe dregree with periodontitis, but some cases the periodontitis was controlled, but not eradicated. [13]

Calibration and reproducibility

A single investigator (EMB) was trained and calibrated for the evaluation of the initial periodontal clinical parameters, as described in a previous study.[13] The reevaluations of periodontal

clinical parameters and anthropometric measurements were performed by the same examiner (EMB).

Body parameters

Individuals were considered obese when they presented a Body Mass Index (BMI) ≥ 30 kg/m^2. The obese condition was confirmed by measurement of the Waist-Hip Ratio - WHR (≥0.85 for women and ≥0.9 for men),[19] Waist Circumference - WC (> 88cm for women and> 102cm for men),[20] and Bioimpedance or Bioelectrical Impedance, which measures the percentage of Body Fat - BF (≥ 35% for women and ≥ 25% for men).[21] Patients were considered normal weight if they presented a BMI between 18.5 and 24.9 kg/m^2 and WHR, WC, and BF measurements lower than the reference values for obese patients. WHR, BF and WC were used just as complementary exams for the diagnosis of obesity so they grouped men and women within the same group regardless the different values existing for gender.

For the calculation of BMI, the formula of weight (kg) divided by the square of height (m^2)[20] was used. Weight was measured on a common scale, with variation per kilogram, evaluated by INMETRO[*]. Height was measured using a metallic ruler, with a variation of 0.5cm, fixed to a steel support. Volunteers were measured and weighed without shoes.

Waist circumference (WC) was measured using a tape measure graduated in centimeters at the height of the umbilical scar. Measurements of hip circumference were also performed with a measuring tape graduated in centimeters. Accurate assessment of the fat mass and lean mass of the individuals was performed using the bioelectrical impedance method (or bioimpedance). Bioimpedance was measured with an apparatus[5], following the manufacturer's guidelines[†]. Data

[*]National Institute of Metrology, Standardization and Industrial Quality, Rio de Janeiro, Brazil.

[†]Omron Healthcare Co, Ltd, Tokyo, Japan.

related to weight, height, age, sex, and physical activity were input into the device prior to its application. Patients then stood with their legs apart and their arms at a 90-degree position relative to the trunk so that the low intensity electrical current could travel through the entire body.

The association of methods, such as the measurements of BMI, WC, WHR, and Bioimpedance, aimed to certify the obesity condition, since the use of a single parameter, such as BMI, does not enable differentiation between fat and muscle fibers[5].

Periodontal parameters

The periodontal clinical examination was performed using a PCPUNC-15 millimeter probe[‡], flat mirror, under direct light and a dry field. The data were recorded in specific clinical records.

The visible plaque index (VPI) was evaluated after drying with air jets, with the presence of microbial deposits visible to the naked eye in the cervical third, on four surfaces (mesial, distal, vestibular, and lingual) of all teeth present. The registry was considered dichotomous as present or absent VPI.[22] In this way, the percentage of sites with visible plaque was calculated considering the number of surfaces with plaque, divided by the total number of surfaces × 100. The result was expressed as a percentage of surfaces with visible plaque.

The gingival bleeding index (GBI) was evaluated using the periodontal probe, inserted slightly into the gingival sulcus, at an angle of approximately 45º, moving from the distal to mesial surface of all teeth, on four surfaces (distal, buccal, mesial, and lingual/palatine). The GBI record was considered dichotomous as present or absent.[22] The percentage of sites with marginal bleeding was calculated considering the number of surfaces with marginal bleeding, divided by the number of total surfaces × 100.

[‡] Hu-Friedy®, Chicago, IL, USA.

The probing depth (PD) was considered as the distance in millimeters between the free gingival margin and the fundus of the gingival sulcus or periodontal pocket.[23] The clinical attachment level (CAL) was considered as the distance in millimeters, between the cement enamel junction (CEJ) and the fundus of the periodontal pocket. Bleeding on probing (BOP) was verified after clinical probing, being considered as present or absent.[23] Periodontal probing was performed on all teeth at six sites per tooth in the distobuccal, buccal, mesiobuccal, distal-lingual, lingual, and mesio-lingual regions.

Sites were divided into three subgroups according to the depth of PD and CAL parameters, which were categorized as: shallow (0 to 3 mm); mild-to-moderate (4 to 6 mm); and deep (≥ 7 mm).

Evaluation of gingival crevicular fluid

Individuals were divided in four groups for the gingival crevicular fluid (GCF) evaluation at the 2 years period, being considered the periodontal site as unit of analysis. Cytokines levels of IL-6, TNF-α, and IL-1β were evaluated in healthy and diseased sites, which was categorized for analysis in subjects with normal weight or obesity. This analysis was performed only in the present study, two years after treatment. The molecular expression in the GCF was not approached at short term because in the initial study systemic analysis was prioritized.[13]

The methodology for GCF collection was adapted from Figueredo et al.[24] In both groups, obese and normal weight, five sites with PD ≥ 5 mm and BOP (test) and five sites with PD ≤ 3 mm without BOP (control) were collected. When the patient did not present five sites in either category, the samples were collected at the same sites, with a three-minute interval for the renewal of the GCF.

The sites were isolated with cotton rolls and dried gently with a jet of air. The supragingival plaque was carefully removed with gauze and the GCF was collected with #30 absorbent paper tips[§]. The cones were inserted into the periodontal pocket and/ or sulcus until light resistance and held for 30 seconds. Samples visibly contaminated with blood were discarded. Samples from sites of the same category (with or without periodontal disease) were collected in the same *Eppendorfs* as a *"pool"* according to the experimental group (obese or normal weight) and stored in a freezer at -20°C. Subsequently, 100 uL of sterile phosphate-buffered saline (PBS) was added to the tubes containing the absorbent paper cones, vortexed, and centrifuged at 1500 rpm for 10 minutes. The supernatant was collected and the concentrations of interleukin 1 beta (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor-alpha (TNF-α) were evaluated using ELISA kits according to the manufacturer's guidelines[**]. The samples were diluted 1:1 with an assay diluent kit. The final value in pg/ml was normalized (divided) by the weight of each sample (of the absorbent cones) shortly after collection.

Sample size calculation

The sample size for intergroup comparisons was calculated with a priori type of power analysis by a Mann-Whitney test for independent means of the clinical attachment level (CAL) ≥ 4 mm based on data from our previous study.[13] The mean and standard deviation (SD) differences before and 3 months post-therapy for obese and normal weight patients were calculated [obese, CAL initial – final = 23.5% (SD initial - final: 4.7); normal weight, CAL initial – final = 19.5% (SD initial - final: 2.6). The sample size was estimated to be 14 patients per group (total sample of 28; one tail) with a statistical power of 80% and error a as 0.05 (effect size d= 1,026849).

[§]Tanari®, Manacapuru, Brazil.

[**]eBioscience®, San Diego, CA, USA.

Statistical analysis

The experimental data were submitted to parametric or non-parametric statistical analysis using the Shapiro-Wilk test, with the computer program (BioEstat 5.0, Belém, Brazil). The Wilcoxon test was used for non-parametric dependent data and the paired t-test for parametric data for comparison between the initial period and after two years anthropometric measurements for each group, with no comparison between groups. The ANOVA was used, followed by the Tukey test, to compare periods (initial, 90 days, and two years) and groups (normal weight and obese) in relation to periodontal parameters. The levels of proinflammatory cytokines IL-6, IL-1β, and TNF-α in the GCF of normal weight and obese patients were also compared with ANOVA, followed by the Tukey test. Data are presented as mean and standard deviation. The level of significance was set at 5%.

RESULTS

Of the nine normal weight patients, two were men and seven women, with a mean age of 44.3 ± 7.4 years. In the obese group, three men and ten women returned, with a mean age of 46.2 ± 7.3 years.

Body parameters

The anthropometric measures of the patients in the initial study and after two years of follow-up can be observed in Table 1 for normal weight and obese subjects. It was verified that in the normal weight group there was a significant increase in BMI and WC. The obese group presented increased BMI, WC, WHR, and body fat, and the obesity condition was maintained two years later when compared to the initial study.

Periodontal parameters

The periodontal treatment significantly reduced the VPI in the normal weight group (from 73.7 ± 14.1 to 8.5 ± 2.0) and in the obese group (from 60.8 ± 13.55 to 13.3 ± 6.4) after 90 days. When comparing the 90-day period with the two-year follow-up, there was an increase in VPI in both the normal (61.8 ± 11.1) and obese groups (45.6 ± 16.0) ($P < 0.05$). The comparison between the groups did not demonstrate a statistically significant difference between the analysis periods, showing that the groups behaved in a similar way ($p > 0.05$) (Figure 3A).

The GBI (Figure 3B) and BOP (Figure 3C) presented high initial levels in both the normal weight (GBI: 28.3 ± 21.1, BOP: 51.2 ± 3.7) and obese groups (GBI: 23, 1 ± 8.05, BOP: 43.7 ± 12.7), with a significant reduction at 90 days post-treatment in the non-obese (GBI: 3.8 ± 2.5; BOP: 8.4 ± 3.7) and obese groups (GBI*:* 3.1 ± 2.7; BOP*:* 7.7 ± 1.89) ($p<0.05$), which was maintained up 2 years in the non-obese (GBI: 5.6 ± 3,25; BOP: 10.0 ± 3.3) and obese patients (GBI: 3.8 ± 1.9; BOP: 11.6 ± 6.9) ($P > 0.05$).

In table 2 it can be seen that in the intragroup evaluation the PD and CAL ≤ 3 mm (shallow sites) presented significant increases 90 days post-treatment in relation to the initial period ($p <0.05$), and remained unchanged after two years ($p > 0.05$) and without significant differences between the normal weight and obese groups ($p > 0.05$). With regard to PD and CAL 4-6 mm (mild-to-mederate sites), PD and CAL ≥ 7 mm (deep sites), in the intragroup evaluation, it was found that there was a statistically significant reduction 90 days after treatment in relation to the initial period ($p < 0.05$), which remained stable after two years ($p > 0.05$). The evaluation between groups for these parameters did not demonstrate statistically significant differences ($p > 0.05$).

Evaluation of gingival crevicular fluid

In each study group, obese and normal weight, cytokines levels of IL-6, TNF-α, and IL-1β were evaluated in sites with periodontal disease (PD) and without PD (Figure 4 A-C). This analysis was performed only in the present study, two years after treatment.

In the sites with PD, higher levels of IL-6 were observed in obese (25.9 ± 5.5 pg/ml) than normal weight patients (9.1 ± 2.4 pg/ml, $p<0.05$). The sites without PD presented similar values in obese and normal weight groups, 0.03 ± 0.07 pg/ml and 0.03 ± 0.05 pg/ml respectively (Figure 4A).

Figure 4B shows the mean values of TNF-α in the obese group with PD (5.9 ± 2.1 pg/ml) and without PD (0.9 ± 0.4 pg/ml). For the normal weight group, values of 1.2 ± 1.0 pg/ml were observed at sites with PD and 0.5 ± 0.4 pg/ml at sites without PD. It was found that patients with obesity and PD presented higher levels of these cytokines when compared to the other conditions ($p <0.05$). Obese patients without PD and normal weight patients with or without PD did not differ significantly from each other ($p> 0.05$).

In the obese group and normal weight group with DP, high levels of IL-1β were observed, with no statistically significant difference between groups ($p> 0.05$). In the obese group, IL-1β levels at sites with PD and without PD were 213.3 ± 66.7 pg/ml and 8.0 ± 2.2 pg/ml, respectively. In the normal weight group, the values were 170.6 ± 81.7 pg/ml for sites with PD and 2.4 ± 1.2 pg/ml for those without DP (Figure 4C).

DISCUSSION

In the present evaluation, after two years there were alterations in the anthropometric parameters in some patients, however these changes did not compromise the results of the

study. For the normal weight group, although there were changes in BMI and WC measurements, these remained in the range considered adequate for normal weight.

For the obese group, there were changes in BMI, WHR, WC, and BF, characterizing an increase in the intensity of obesity. Thus, all patients in this group remained obese in the long term, with no weight loss. This fact is important for the interpretation of the results, since weight loss could improve the inflammatory condition caused by obesity, interfering in the circulating levels of IL-6, TNF-alpha, and C-reactive protein.[25] Moreover, weight loss has been observed to be able to improve the response to periodontal treatment in previously obese patients.[26] In both groups, obese and normal weight, the parameters of GBI, BoP, PD ≤ 3 mm, PD 4-6 mm, PD ≥ 7 mm, CAL ≤ 3 mm, CAL 4-6 mm, and CAL ≥ 7 mm remained stable over time. On the other hand, the VPI returned to high values, compatible with the initial values, demonstrating the importance of a regular maintenance program,[27] since these patients were recalled for maintenance only after one year of the end of periodontal treatment. It is emphasized that plaque accumulation is directly related to gingival inflammation,[28] demonstrating the importance of plaque control and a maintenance program to prevent recurrence of the disease.[27]

From the available studies, the most of them showed that both obese and normal weight patients presented a similar periodontal response, with improvement in periodontal clinical parameters after non-surgical therapy,[7,8,13,17,18] which corroborates with the findings of the present study. Studies evaluating the response to periodontal treatment in obese patients showed follow-up periods ranging from 2 to 12 months.[7,8,13,17,18,29] To our knowledge, this is the first study to evaluate the response to periodontal therapy after 24 months.

In the present study, the proinflammatory cytokines IL-6, TNF-α, and IL-1β were evaluated in the GCF of sites with and without periodontal disease, in patients with obesity and normal weight. The results showed higher concentrations of IL-6 and TNF-α in the GCF of sites with periodontal disease in obese compared to normal weight individuals ($p < 0.05$). On the other hand, levels of IL-1β in the GCF were elevated at sites with periodontal disease in both obese and non-obese patients ($P > 0.05$). Other authors have demonstrated higher levels of IL-6 and TNF-α in the GCF of obese patients compared to patients with normal weight.[8] These results are relevant as they may lead to a greater future recurrence of periodontal disease in the long term, together with negligence in plaque control.

In addition, other results have shown elevated levels of TNF-α in the GCF of young individuals with BMI ≥ 40 kg/m^2, suggesting that local TNF-α levels may be affected by obesity through a systemic effect.[30] Elevated levels of proinflammatory cytokines such as IL-6 and TNF were found in the blood of obese individuals.[13,31] This fact can be explained as adipose tissue is able to release high concentrations of cytokines and other inflammatory factors, exacerbating systemic inflammation.[11,31,32,33,34]

As a limitation of the present study, we consider the small sample size, which demonstrates the difficulty of conducting longitudinal studies. The low return rate may be due to the lack of a regular maintenance program, which would help raise the awareness and motivation of these patients regarding the importance of ongoing follow-up. Due to the small sample size, it was not possible to make inferences on serum proinflammatory cytokine levels, as performed in our previous short-term study.[13] Only the GCF evaluation was accomplished in healthy and diseases sites in subjects with normal weight or obesity. Other long-term longitudinal studies with a larger number of participants should be performed to generate additional scientific

evidence. Additionally, 25 and 27 people per group are considered at the start point, however at the end, groups were composed of 13 and 9 respectively, so the effect size, power and significance could be harmed.

CONCLUSION

It was concluded that obese and normal weight individuals had similar periodontal behaviors, with low recurrence of the disease; however, obesity was related to increased inflammatory activity in gingival fluid, which may become a risk indicator for future greater recurrence of periodontal disease in the presence of inadequate plaque control.

Conflicts of Interest:

The authors declare that no competing financial interests exist.

Authors contribution:

Elizangela C. Zuza: substantial contributions to conception and design of the study and drafting the article or revising it critically for important intellectual contente.

Juliana R. Pires: substantial contributions to conception and design of the study.

Ana Alice de Almeida: substantial contributions to acquisition of data.

Benedicto E. C. Toledo: drafting the article or revising it critically for important intellectual contente.

Morgana R. Guimarães and Carlos Rossa Junior: substantial contributions to analysis, or interpretation of data.

Eliane M. Barroso: substantial contributions to acquisition of data.

ACKNOWLEDGMENTS

We would like to thank the National Council for Scientific and Technological Development (CNPq) for the assistance provided to carry out this research (process 476390-2007-9), Brasília, Brazil.

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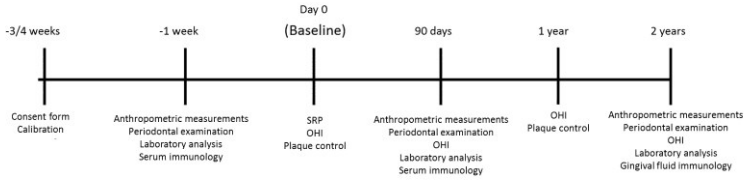

FIGURE 1 Design of the study

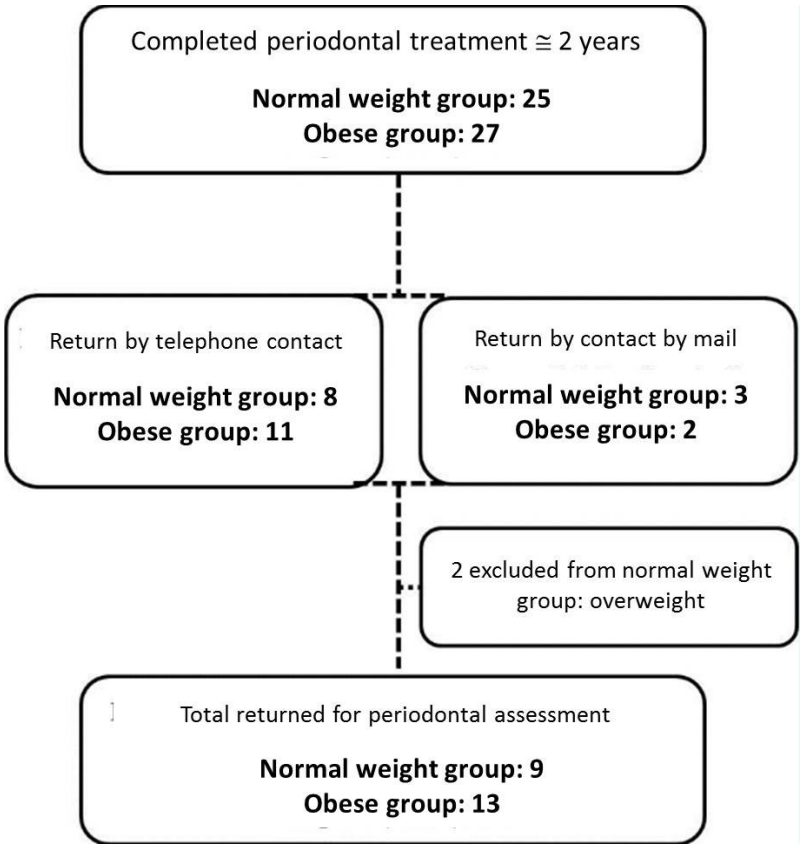

FIGURE 2 Flowchart of the study

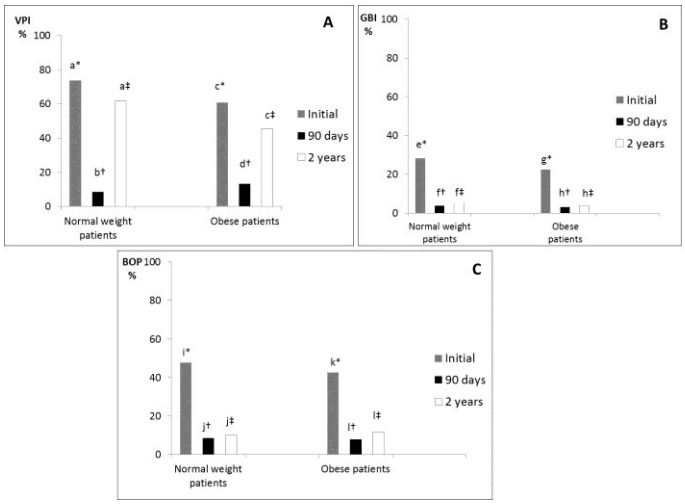

FIGURE 3 A) Graphical representation of visible plaque index (%VPI); **B)** Graphical representation of gingival bleeding index (%GBI); **C)** Graphical representation of bleeding on probing (%BOP). [a,b,c,d...] Different letters indicate statistically significant differences by mean among the initial, 90 days and 2 year periods within the same group (Anova followed by Tukey, $P<0.05$); [*,†,‡] Different symbols show statistically significant differences by mean between groups in the same periods: initial × initial, 90 days × 90 days, 2 years × 2 years (Anova followed by Tukey, $P<0.05$).

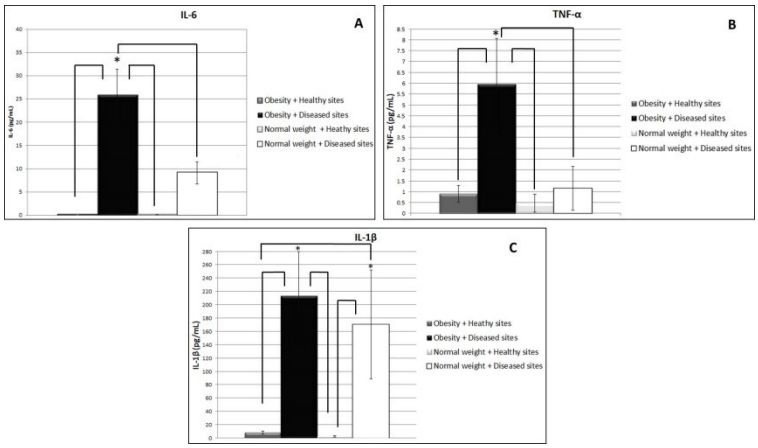

FIGURE 4 A) IL-6 levels (pg/mL) at sites without and with periodontal disease in obese and normal weight patients.* Statistically Significant difference when compared to the other groups (by Tukey, $P<0.05$); **B)** TNF-α levels (pg/mL) at sites without and with periodontal disease in obese and normal weight patients.*Statistically significant difference when compared to the other groups (Anova followed by Tukey, $P<0.05$); **C)** IL1-β levels (pg/mL) at sites without and with periodontal disease in obese and normal weight patients. *Significant difference when compared to obese group + healthy sites and normal weight group + healthy sites (Anova followed by Tukey, $P <0.05$). There was no significant difference between obesity + diseased sites and Normal Weight + diseased sites (Anova one way followed by Tukey, $P> 0.05$).

TABLE 1 Anthropometric measurements and glycemic data

Parameters	**Normal Weight Group (N=9)**			**Obese Group (N=13)**		
	Initial	2 years	P-value	Initial	2 years	P-value
Body mass index (kg/m^2)	23.2 ± 2.0	25.7 ± 2.2	0.0015[†,*]	33.5 ± 2.9	36.4 ± 4.8	<0.0001[‡,*]
Waist circumference (cm)	82.8 ± 7.4	87.8 ± 6.85	0.0049[†,*]	101.6 ± 6.2	109 ± 5.5	0.0004[‡,*]
Waist-hip ratio	0.78 ± 0.06	0.82 ± 0.07	0.5067[‡]	0.90 ± 0.04	0.92 ± 0.04	0.0482[†,*]
Body fat (%)	28.5 ± 3.9	29.1 ± 2.75	0.6246[†]	41.9 ± 1.4	44.5 ± 2.2	<0.0001[‡,*]
Glycemia (mg/dL)	84.3 ± 9.5	90.1 ± 6.6	0.0221[†,*]	89.8 ± 6.0	89.9 ± 5.9	0.85[‡]

[†]Paired t-test for parametric dependent data and [‡]Wilcoxon test for non-parametric dependent data;

[*]P <0.05 indicates a statistically significant difference between the initial period and after two years follow-up.

TABLE 2. Periodontal parameters probing depth (PD) and clinical attachment level (CAL) of the patients included in the study

Periodontal Parameters (%)	**Normal Weight Group (N=9)**			**Obese Group (N=13)**		
	Initial	90 days	2 years	Initial	90 days	2 years
PD ≤ 3 mm	55.7 ± 12.8 a,*	86.7 ± 6.1 b,†	84.9 ± 6.9 b,‡	67.8 ± 5.7 c,*	86.3 ± 4.25 d,†	82.2 ± 7.5 d,‡
PD 4-6 mm	32.7 ± 7.8 a,§	13.8 ± 4.1 b,‖	14.2 ± 5.3 b,¶	29.1 ± 6.1 c,§	10.6 ± 3.6 d,‖	13.8 ± 4.6 d,¶
PD ≥ 7 mm	7.5 ± 4.4 a,#	0.26 ± 0.48 b,**	0.4 ± 0.69 b,††	5.8 ± 3.5 c,#	0.98 ± 1.8 d,**	1.98 ± 1.6 d,††
CAL ≤ 3 mm	55.4 ±6.3 a,*	70.8 ± 7.2 b,†	69.6 ± 7.7 b,‡	64.2 ± 6.1 c,*	76.4 ± 4.2 d,†	71.35 ± 4.8 d,‡
CAL 4-6 mm	41.4 ± 5.0 a,§	26.4 ± 6.75 b,‖	24.5 ± 5.0 b,¶	34.7 ± 5.0 c,§	21.8 ± 3.9 d,‖	23 ± 5.7 d,¶
CAL ≥ 7 mm	7.8 ± 2.8 a,#	2.4 ± 2.1 b,**	3.3 ± 2.2 b,††	7.2 ± 2.9 c,#	2.74 ± 1.9 d, **	3.65 ± 3.1 d,††

[a,b,c,d]Different letters in the rows indicate statistically significant differences among periods (initial × 90 days × 2 years) within the same group (ANOVA followed by Tukey test, P <0.05);

[*,†,‡,§,‖,¶,#]Similar symbols in the same periods (initial × initial, 90 days × 90 days, 2 years × 2 years) do not indicate statistically significant differences between groups (ANOVA followed by Tukey test, P >0.05).

[††]G*Power, Düsseldorf, Germany.