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Levels of serum retinol binding protein 4 before and after non-surgical periodontal treatment in lean and obese subjects: an interventional study.

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Main Methodology:	Biochemistry

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author

The authors have addressed the concerns of the reviewers and can be accepted now.

Referee: 2

Comments to the Author

This study evaluated the serum levels of retinol binding protein 4 (RBP4) in obese and non-obese patients with chronic periodontitis before and after non-surgical periodontal therapy. The serum levels of this biomarker were also compared between non-obese and obese subjects with and without periodontitis. The authors revised several points of the paper. However, the main drawbacks of the paper remains: 1- RBP4 was evaluated only in serum; 2- RBP4 was assessed in a single short period of time after therapy (3 months); 3- the authors are overestimating their findings (please, notice that the association of RBP4 and periodontitis in the correlation analysis and regression model was weak); 4- the sample size calculation does not consider the main focus of the paper.

Response:

1. *RBP4 was evaluated only in serum.*

   Indeed, RBP4 was evaluated only in serum because we only obtained blood samples from the subjects, and so it is impossible to determine RBP4 in gingival crevicular fluid. However, we agree it would be very interesting to do this and we will consider it for futures studies.

2. *RBP4 was assessed in a single short period of time after therapy (3 months).*

   We evaluated the effect of periodontal treatment in a single period of time after periodontal therapy (3 months) in accordance with most of the studies published in the literature. Among the multitude of studies evaluating the response to non-surgical periodontal treatment in obese patients (Zuza et al. 2012, Al-Zahrani et al. 2012, Altay et al. 2013, Suvan et al. 2014, Öngöz-Dede et al. 2015, Bhardwaj et al. 2015, Bouaziz et al. 2015, Gonçalves et al. 2015, Gonçalves et al. 2015a, Balli et al. 2016, Balli et al. 2016a, Duzagac et el. 2016, Zuza et al. 2016), roughly 80% revaluate these parameters after short periods of time, most at 3 months (Zuza et al. 2012, Altay et al. 2013, Bhardwaj et al. 2015, Duzagac et el. 2016, Zuza et al. 2016), some of them at 2 months (Al-Zahrani et al. 2012, Suvan et al. 2014) and a few after just one month (Öngöz-Dede et al. 2015, Balli et al. 2016, Balli et al. 2016a), with very few studies re-evaluating the response to periodontal treatment in longer periods of time until 6 months (Bouaziz et al. 2015, Gonçalves et al. 2015) or until 12 months (Gonçalves et al. 2015a). In addition, when we designed the present study, we decided on the time point of 3 month post-therapy because this is an adequate period of time to evaluate periodontal healing after non-surgical periodontal treatment (Badersten et al. 1984).

   Based on our professional clinical experience, after 3-4 months of periodontal treatment, patients with chronic periodontitis show new deposits of plaque and calculus. Thus, studies evaluating the response to non-surgical periodontal treatment over long periods of time (6, 9 and 12 months) re-evaluate participants every 3 months, and periodontal maintenance therapy is applied at each appointment, including reinstruction of oral hygiene methods, professional plaque control and subgingival debridement of residual

pockets ≥4 mm presenting BOP. Thus, the benefit of longer-term follow-up is a cumulative effect of various periodontal therapies applied every 3 months.

In our study, during the re-evaluation appointment at 3 months after therapy we did not apply periodontal maintenance therapy, since our patients were referred to a periodontist for complete treatment and the corresponding follow up of their periodontal disease. The treatment of periodontal disease is not a therapy covered by the Spanish Public Health System and is not carried out as usual dental therapy in the University Hospital Dr. Peset, with the exception of this study, in which it was performed for research purposes by a dentist collaborator of our group.

However, taking into account the referee's suggestion, we have contacted all the participants with chronic periodontitis and have recruited 26 obese subjects who had not yet followed our recommendation to see a periodontist in order to avoid different periodontal treatment protocols at different time intervals. The results are shown in tables 1 and 2 of this letter: roughly 12 months after periodontal treatment RBP4 levels continued to decline when compared with baseline and 3 months, whereas clinical periodontal parameters 12 months after periodontal treatment remained stable with respect to 3 months, except plaque and calculus indexes, which increased since the initial treatment. In this new analysis, at about 12 months after periodontal treatment, the number of teeth with PD ≥4 mm did not correlate with serum RBP4 levels due to the reduced sample size (data not shown). Taking into account that a subpopulation of only 26 obese patients was revaluated, we considered that these data should not be included in the new version of the manuscript (unless Editor or referee consider it), because this was not the main objective of our study.

In the revised version of the manuscript we have clearly stated that the limitations of this study were to not evaluate RBP4 levels in gingival crevicular fluid and for longer periods of time. Indeed, we will consider this in future studies.

The text now reads: "To the best of our knowledge, this is the first study to demonstrate a significant decrease of serum RBP4 levels in obese and lean subjects after non-surgical periodontal treatment. However, there are several limitations that should be taken into consideration. RBP4 was evaluated only in serum and over a single, short period of time after periodontal therapy (3 months). Therefore, further longitudinal prospective studies that evaluate RBP4 levels in serum and gingival crevicular fluid over longer periods of time after non-surgical periodontal treatment are necessary to corroborate our findings. In addition, mechanistic studies involving larger patient samples and different ethnicities would help to determine the role of RBP4 in the pathogenesis and progression of periodontitis".

Table 1. Anthropometric parameters and biochemical variables in the study population with chronic periodontitis at baseline, 3 and 12 months after non-surgical periodontal treatment

	Obese with CP (n= 26)			
	Baseline	3 months	12 months	p value
BMI (kg/m2)	42.7 ± 6.3	42.2 ± 6.3	42.0 ± 6.8	0.188
SBP (mmHg)	137 ± 22	138 ± 17	136 ± 18	0.858
DBP (mmHg)	85 ± 15	89 ± 10	88 ± 10	0.253
Glucose(mg/dl)	95 ± 13	97± 13	97 ± 14	0.353
Insulin (µU/ml)	21.1 ± 12.7	20.9 ± 9.3	20.5 ± 9.5	0.977
HOMA-IR	4.98 ± 3.46	5.06 ± 2.480	5.00 ± 2.59	0.995
TC (mg/dl)	181 ± 36	190 ± 35	179 ± 35	0.122
HDLc (mg/dl)	42 ± 11	45 ± 13	43 ± 11	0.104
LDLc (mg/dl)	111 ± 30	116 ± 29	108 ± 30	0.191
TG (mg/dl)	138 (114, 171)	124 (107, 182)	136 (92, 193)	0.935
hsCRP (mg/l)	6.21 ± 4.71	6.40 ± 5.00	5.25 ± 4.59	0.178
RBP4 (mg/dl)	4.16 ± 1.24[a]	3.75 ± 1.09[b]	3.03 ± 0.65[c]	<0.001

Data are expressed as mean ± SD. Values of serum triglycerides were normalized using a log transformation. Values with different superscript letters were found to be significantly different when compared by one-way ANOVA of repeated measures followed by a Student-Newman-Keuls post hoc test.

CP: chronic periodontitis, BMI: body mass index, SBP: Systolic blood pressure, DBP: Diastolic blood pressure, TC: total cholesterol, HDLc: HDL cholesterol, LDLc: LDL cholesterol, TG: triglycerides, hsCRP: high sensitive C-reactive protein, RBP4: retinol-binding protein 4.

Table 2. Clinical periodontal parameters in the study population with chronic periodontitis at baseline, 3 and 12 months after non-surgical periodontal treatment

	Obese with CP (n= 26)			
	Baseline	3 months	12 months	p value
PD (mm)	2.99 ± 0.47[a]	2.83 ± 0.33[b]	2.84 ± 0.37[b]	0.010
CAL (mm)	3.05 ± 0.54[a]	2.90 ± 0.40[b]	2.92 ± 0.48[b]	0.028
Teeth PD ≥4 mm (n)	17.7 ± 5.4[a]	13.2 ± 5.7[b]	13.2 ± 6.5[b]	<0.001
Sites PD ≥4 mm (%)	29.6 ± 16.5[a]	18.5 ± 13.9[b]	19.0 ± 14.5[b]	<0.001
BOP (%)	29.0 ± 12.0[a]	15.9 ± 7.0[b]	18.8 ± 12.0[b]	<0.001
Plaque index (A.U)	1.05 ± 0.73[a]	0.74 ± 0.48[b]	0.91 ± 0.49[a,b]	0.021
Calculus index (A.U)	1.09 ± 0.61[a]	0.32 ± 0.24[b]	0.59 ± 0.39[c]	<0.001

Data are expressed as mean ± SD. Values with different superscript letters were significantly different when compared by one-way ANOVA of repeated measures followed by a Student-Newman-Keuls post hoc test.

CP: chronic periodontitis, PD: probing depth, CAL: clinical attachment loss, BOP: bleeding of probing.

3. *The authors are overestimating their findings (please, notice that the association of RBP4 and periodontitis in the correlation analysis and regression model was weak).*

   In accordance with the referee's comment, the association of RBP4 and periodontitis in the correlation analysis and regression model was weak when compared with periodontal parameters. To be honest, its involvement is not so strong as other periodontal parameters but it is the most potent predictor among analytical variables and the fact that the correlation is slight it is not an indicative of low significance. In fact, when we studied the Pearson's correlation between number of teeth PD ≥4 mm and anthropometric, biochemical and periodontal parameters, serum RBP4 levels, after TNFα levels, showed the highest correlation coefficient (r= 0.294, p=0.003), without taking into account the periodontal variables. It is obvious that periodontal parameters showed a high correlation coefficient among them, since many of them are calculated from PD. In addition, the multivariable regression model strengthen the involvement of RBP4 on the number of teeth PD ≥4 mm, in this case p-value was p=0.020 less significant than PD (p<0.001), but we have to consider that PD and number of teeth PD ≥4 mm are very close related variables. A standardized beta coefficient compares the strength of the effect of each individual independent variable to the dependent variable. The higher the absolute value of the beta coefficient, the stronger the effect which is expected in the case of PD and number of teeth PD ≥4 mm but the most important aspect of this analysis was the

appearance of RBP4 as predictor of dependent variable excluding others periodontal and biochemical parameters. Furthermore, we repeated the analysis excluding periodontal parameters and the only independent variable of the model was RBP4 as the most potent predictor of number of teeth PD ≥4 mm, although the model was weaker explaining the 8% of response variable (Table 3 of this letter).

Table 3. Stepwise multivariable regression model of number of teeth with PD ≥ 4 mm 3 months after non-surgical periodontal treatment, measured as a dependent variable.

Dependent variable	Independent variables	Unstandardized coefficients		Standardized coefficients	p-value
		B	SE	β	
n teeth PD ≥4 mm	RBP4	2.023	0.649	0.304	0.002
	Multiple R square adjusted			0.083	
		R		0.304	
		p		0.002	

Waist, WHR, SBP, DBP, HOMA-IR and TNFα were excluded from the model since they were not significant predictors (p>0.05).

Abbreviations: n PD ≥4 mm: number of teeth with probing depth ≥4 mm, RBP4: retinol-binding protein 4, WHR: waist-to-hip ratio, SBP: systolic blood pressure, DBP: diastolic blood pressure, HOMA-IR: homeostasis model assessment of insulin resistance, TNFα: tumour necrosis factor alpha.

4. The sample size calculation does not consider the main focus of the paper.

We apologise for the mistake in the previous version; in fact, we did not understand the requirement of the reviewer and we included the sample size calculation of the previous prevalence study with the objective of determining the association between obesity and periodontitis.

To perform the present study we considered a power of 90% in order to be able to detect differences among the groups in relation to the primary efficacy criterion (serum RBP4 levels variation) ≥0.8 mg/dl, assuming a common standard deviation of 1.0 mg/dl. These data were based on our previous study (Rocha et al. 2013), in which serum RBP4 levels were determined in an obese population with different degrees of insulin resistance. A sample size of thirty-three patients per group was required.

In addition, Kanoriya et al. (2016) published an article in which a total of 15-20 patients per group were evaluated in order to detect changes in RBP4 between lean and obese subjects with and without CP.

The text now reads: "A sample size of thirty-three patients per group was required in order to achieve a power of 90% to detect differences between the groups in relation to the primary efficacy criterion (serum RBP4 levels variation) of ≥0.8 mg/dl and assuming a common standard deviation of 1.0 mg/dl."

Other concerns

Abstract:

Aim: "to determine its involvement as a biomarker of periodontitis." This is not possible with the current experimental design. It is not clear in the aim that the levels of RBP4 were evaluated in serum.

Response: We have modified the aim in the abstract section according to the reviewer's comments. The text now reads: "Aim: Little is known about the role of RBP4 in periodontitis. Therefore, we aimed to evaluate serum RBP4 levels before and after periodontal therapy in lean and obese subjects with chronic periodontitis (CP) in order to determine its possible association with periodontitis."

Results: "Serum RBP4 levels were associated with … with higher levels in the CP groups than in subjects without CP in both lean and obese populations (3.06 vs 3.35 and 3.21 vs 3.74, respectively). This sentence is unclear.

Response: We have modified this sentence as follows: "Serum RBP4 levels were associated with…, showing patients with CP to have higher RBP4 levels than those without CP in both lean and obese populations (3.35 vs 3.06 and 3.74 vs 3.21, respectively).

"… except for the number of teeth with probing depth (PD) ≥4 mm…" Please, detail this exception.

Response: We have detailed this exception. The text now reads: "Following periodontal treatment, RBP4 and TNFα decreased and all periodontal parameters improved to the same extent in both groups, except for number of teeth with probing depth (PD) ≥4 mm, which improved to a less extent in obese than in lean subjects. In the multivariable regression model, number of teeth with PD ≥4 mm was independently associated with RBP4 (β=0.192)."

"Conclusion: RBP4 may be an important biomarker of chronic periodontitis." This reviewer does not agree with this statement. This study is too preliminary, RBP4 was evaluated only in serum…

Response: We agree with the referee when he/she says this is a preliminary study. The novelty of the study is the association of RBP4 in CP and the effect of periodontal treatment reducing this adipokine. So considering the definition of *biomarker*: *"A biologic feature that can be used to measure the presence or progress of disease or the effects of treatment"*, our results suggest a role of RBP4 as biomarker. Even although we determine RBP4 in serum, in medicine, biomarkers are often compounds isolated from serum, urine, or other fluids so we considered that it is not a limitation the fact that RBP4 was determined in serum but an advantage so it is easily determined and reliable.

Anyway, despite the evidence provided regarding the finding of a new biomarker for periodontitis, we have rewritten the conclusion in the abstract section. The text now reads: "Conclusion: RBP4 was associated with chronic periodontitis before and after non-surgical periodontal treatment."

Principal Findings:

"Serum RBP4 levels seem to be involved in an increased probability and extent of periodontitis" It is better "Serum RBP4 levels was associated with an increased probability and extent of periodontitis.

Response: In accordance with the referee, we have changed this sentence in the new version of the manuscript.

Methods

"We selected this periodontal treatment protocol based on growing evidence of its clinical efficacy (Matesanz-Pérez et al. 2013; Fang et al. 2016)".

Based on the number of residual pockets, are the authors sure that the therapy used was efficient?

Response: We applied a non-surgical periodontal treatment, which is considered the gold standard therapy in the management of chronic periodontitis, and whose efficacy has been established by multiple clinical trials (Akram et al. 2016).

We performed this therapy within a 24-hour period, in a single session, along with the use of antiseptics because this periodontal treatment protocol has been recommended as the first-choice treatment for adult chronic periodontitis, due to its modest additional clinical benefits with respect to quadrantwise therapy (Fang et al. 2016) and in order to reduce the number of visits and limit patient dropout from the study.

Based on our results, we can affirm that the periodontal treatment used was partially effective, as we observed a significant improvement in all clinical periodontal parameters in both lean and obese subjects. Moreover, 12 months after the periodontal treatment, we have observed that clinical periodontal parameters remain stable with respect to 3 months, except for plaque and calculus indexes, without supportive periodontal therapies since the initial treatment.

However, lean and obese subjects still retained several residual pockets (PD ≥4 mm) 3 months after non-surgical periodontal therapy, which may reflect the failure of this therapy in promoting a periodontal condition comparable to that of periodontally healthy patients. However, the persistence of residual periodontal pockets may account for a greater number of shallow pockets with PD=4 mm, as it will have reduced the number of deep pockets. In addition, as well as the development of periodontal disease, the effectiveness of periodontal treatment is negatively influenced by systemic, environmental and behavioural risk factors (such as systemic diseases, IL-1 polymorphisms, smoking, irregular or absent compliance with oral hygiene habits), which may be another explanation for the persistence of residual pockets.

Finally, based on our results, we determine that the periodontal treatment used was partially effective by improving all clinical periodontal parameters and promoting a reduction in serum TNFα and RBP4 levels, but did not achieve a periodontal condition comparable with that of the periodontally healthy patients.

The authors presented the mean values of waist and hip circumference. However, it seems that these measurements were not used in the diagnosis of obesity.

Response: No, these measurements were not used in the diagnosis of obesity. We defined obesity according to the criterion of the Spanish Society for the Study of Obesity, based on BMI. In fact, we included waist and hip circumference in the previous version of the manuscript (R1), following the reviewer's suggestion.

Salas-Salvadó, J., Rubio, M.A., Barbany, M. & Moreno, B. (Grupo Colaborativo de la SEEDO). (2007) SEEDO 2007 Consensus for the evaluation of overweight and obesity and the establishment of therapeutic intervention criteria. *Medicina Clínica* (Barc) **128**, 184–196.

The text now reads: "Patients were clustered into four groups based on their BMI and the diagnosis of chronic periodontitis (CP) as follows: lean without CP; lean with CP; obese without CP; and obese with CP. Obesity was diagnosed according to the criterion of the Spanish Society for the Study of Obesity when BMI ≥30 kg/m^2. Patients were defined as lean or normoweight when BMI ≤25 kg/m^2 (Salas-Salvadó et al. 2007)".

"which is in accordance with the high prevalence of periodontitis we had previously reported in our obese population (Martinez-Herrera et al. 2017)." The prevalence of periodontitis in the obese population previously reported is not adequate to calculate the sample size. The focus of study is the levels of RBP4. A primary variable based on this parameter should be used to justify the sample size.

Response: We apologise for the mistake in the previous revision. As we have mentioned above, we included the sample size calculation of the previous prevalence study with the objective of determining the association between obesity and periodontitis.

The text now reads: "A sample size of thirty-three patients per group was required in order to achieve a power of 90% to detect differences between the groups in relation to the primary efficacy criterion (serum RBP4 levels variation) of ≥0.8 mg/dl and assuming a common standard deviation of 1.0 mg/dl."

Results

A table showing clinical periodontal parameters for all groups at baseline should be included. Figure 1 only provides clinical data for the subjects with CP.

Response: Following the referee's suggestion we have included a table (Table 2 of the manuscript) showing clinical periodontal parameters for all groups at baseline.

In the results section we have described the differences among all groups in clinical periodontal parameters at baseline. The text now reads: "Clinical periodontal parameters for all groups at baseline are shown in Table 2. As expected, patients with CP had higher PD, CAL, number of teeth and percentage of sites with PD ≥4 mm, BOP, plaque index and calculus index than their counterparts without CP in both lean and obese populations. Obese subjects without CP showed higher PD, CAL, BOP, plaque and calculus than lean subjects without CP, with greater periodontal damage detected in the obese population than among lean subjects. Among the groups with CP, obese subjects displayed a higher number of teeth with PD ≥4 mm and higher plaque index than lean subjects."

Table 2. Clinical periodontal parameters in the study population at baseline.

	Lean		Obese		
	Without CP (n=64)	With CP (n=48)	Without CP (n=23)	With CP (n=96)	p-value

PD (mm)	2.37 ± 0.22	3.01 ± 0.48***	2.53 ± 0.18##	3.03 ± 0.45***	<0.001
CAL (mm)	2.39 ± 0.24	3.06 ± 0.57***	2.54 ± 0.18##	3.06 ± 0.49***	<0.001
Teeth PD ≥4 mm (n)	2.14 ± 1.27	15.81 ± 6.14***	2.04 ± 1.26	18.38 ± 6.18***¥	<0.001
Sites PD ≥4 mm (%)	1.9 ± 1.4	23.6 ± 17.7***	1.9 ± 1.5	29.1 ± 17.2***	<0.001
BOP (%)	7.8 ± 4.5	24.9 ± 15.3***	12.5 ± 7.2##	28.1 ± 13.4***	<0.001
Plaque index (A.U)	0.36 ± 0.28	0.90 ± 0.53***	0.67 ± 0.45##	1.12 ± 0.65***¥	<0.001
Calculus index (A.U)	0.44 ± 0.31	0.99 ± 0.48***	0.68 ± 0.37##	1.10 ± 0.58***	<0.001

Data are presented as mean ± SD and were compared by means of one-way ANOVA. ***p<0.001 compared with its respective control group without CP; ##p<0.01 when comparing lean *vs* obese without CP; ¥p<0.05 when comparing lean *vs* obese with CP.

Abbreviations: CP: chronic periodontitis, PD: probing depth, CAL: clinical attachment loss, teeth PD ≥4 mm: number of teeth with probing depth ≥4 mm, sites PD ≥4 mm: percentage of sites with PD ≥4 mm, BOP: bleeding of probing.

Discussion

"The findings of the present study suggest that serum RBP4 levels are associated with an increased presence of periodontitis,.." This sentence is unclear.

The discussion section is too speculative.

Response: We have rephrased this sentence as well as other speculative sentences. The text now reads:

"The findings of the present study suggest that serum RBP4 levels are associated with chronic periodontitis, as they are elevated in CP and decrease after non-surgical periodontal treatment".

"In addition, a weak but significant association was observed between number of teeth with PD ≥4 mm and RBP4, which suggests a possible role of this adipokine in the extent of periodontal disease. In light of these results, RBP4 could be considered an emerging inflammatory marker associated with chronic periodontitis".

"Since both obesity and periodontal disease have been associated with an increase in proinflammatory cytokines (Zimmermann et al. 2013) and given that RBP4 levels rise in obesity, diabetes and metabolic syndrome (Graham et al. 2006; Yang et al. 2005; Qi et al. 2007), it has been hypothesised that RBP4 may mediate the inflammatory response that links obesity and periodontitis".

"Overall, our results suggest a role for RBP4 in the inflammatory response associated with CP."

"Therefore, the reduction in the number of teeth with PD ≥4 mm after non-surgical periodontal treatment could suggest a decrease in the systemic inflammatory response. However, the association of RBP4 and number of teeth with PD ≥4 mm, although statistically significant, was weak and the results should be interpreted with caution."

In addition, we have also reformulated the conclusion of our study so that it is not so speculative. The text now reads: "Conclusion: To summarise, circulating RBP4 levels are associated with chronic periodontitis since their levels are higher in the presence of the disease and drop after non-surgical periodontal treatment."

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Levels of serum retinol binding protein 4 before and after non-surgical periodontal treatment in lean and obese subjects: an interventional study.

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Abstract

Aim: We aimed to evaluate serum RBP4 levels before and after periodontal therapy in lean and obese subjects with chronic periodontitis (CP) in order to determine its possible association with periodontitis.

Materials and Methods: This is an interventional study for which a total of 112 lean and 119 obese subjects were recruited. Patients with CP were evaluated before and after three months of non-surgical periodontal treatment. Periodontal, anthropometric, biochemical parameters and serum levels of TNFα, IL-6, hsCRP and RBP4 were assessed.

Results: Serum RBP4 levels were associated with an increased probability of periodontitis (OR= 1.60; 95% CI: 1.02-2.50), showing patients with CP to have higher RBP4 levels than those without CP in both lean and obese populations (3.35 vs 3.06 and 3.74 vs 3.21, respectively). Following periodontal treatment, RBP4 and TNFα decreased and all periodontal parameters improved to the same extent in both groups, except for number of teeth with probing depth (PD) ≥4 mm, which improved to a less extent in obese than in lean subjects. In the multivariable regression model, number of teeth with PD ≥4 mm was independently associated with RBP4 (β=0.192).

Conclusion: RBP4 was associated with chronic periodontitis before and after non-surgical periodontal treatment.

Clinical Relevance

Scientific rationale for the study: Serum retinol binding protein 4 (RBP4) levels have recently been linked to periodontitis, but the role of RBP4 in chronic periodontitis before and after non-surgical periodontal treatment is still unknown.

Principal findings: Serum RBP4 levels were associated with an increased probability and extent of periodontitis.

Practical implications: Serum RBP4 levels may represent an inflammatory marker of periodontitis detectable by a readily available analytical determination.

Introduction

Obesity is a highly prevalent condition characterised by the abnormal accumulation and storage of fat in adipose tissue (Boesing et al. 2009) and produced by an imbalance between energy intake and energy expenditure, which can be modified by genetic and environmental circumstances (Fadel et al. 2014). Obesity is associated with chronic, low-grade inflammation, which is involved, in turn, in the development of insulin resistance (IR) (Bullon et al. 2009) and is a major contributor to the development of hypertension, type 2 diabetes mellitus, arteriosclerosis, cardiovascular and cerebrovascular diseases (Pischon et al. 2007; Saito & Shimazaki 2007; Haffajee & Socransky 2009). In addition to these risk factors, obesity is also thought to increase the probability of periodontitis, a disease of the supporting structures of the teeth resulting from an interaction between pathogenic bacteria and the host immune response (Ylöstalo et al. 2008). In this sense, it has been suggested that IR plays a role in the pathogenesis of periodontitis (Genco et al. 2005). In fact, we have recently shown that periodontitis tends to be more extensive in obese patients with IR than in their respective counterparts without IR (Martinez-Herrera et al. 2017).

As an endocrine organ, adipose tissue is responsible for the synthesis and secretion of several adipokines, such as leptin, adiponectin and resistin and proinflammatory cytokines (mainly IL-6, IL-1 and TNFα), which affect the periodontal tissues and contribute to the pathophysiology of periodontitis (Ylöstalo et al. 2008).

More recently, it has been suggested that certain adipokines which are highly expressed in liver and visceral adipose tissue, such as chemerin, visfatin and

retinol binding protein 4 (RBP4), are implicated in insulin-resistant states (Marchetti et al. 2012).

RBP4 is a member of the lipocalin family that is secreted into the circulation while being bound to vitamin A and transthyretin. It has been shown that RBP4 is upregulated in insulin-resistant conditions associated with obesity and provokes IR by interfering with insulin receptor substrate 1 (Yang et al. 2005; Gerrits et al. 2012). In addition, it seems to be a predictor of cardiovascular disease (Alkharfy et al. 2012) and development of atherosclerosis, as it promotes atherogenic dyslipidaemia (Rocha et al. 2013) and is involved in the regulation of hypertension (Kraus et al. 2015). Recently, it has been established that RBP4 levels in gingival crevicular fluid and serum are positively correlated with body mass index (BMI) and periodontal parameters (Kanoriya et al. 2016). However, to date, no previous studies have evaluated RBP4 levels after non-surgical periodontal treatment.

In this context, research such as that reported here may provide valuable information about the association among RBP4 levels, obesity and periodontal disease. Therefore, the aim of this study was to determine serum RBP4 levels in obese and lean subjects with and without chronic periodontitis and to evaluate the effect of non-surgical periodontal treatment on RBP4 levels. In addition, we have explored the relationship between RBP4 levels and other clinical and periodontal parameters.

Materials and Methods

Study design

This was an interventional case-control study conducted at the University Hospital Dr. Peset (Valencia, Spain). The participants, all between the ages of 20 and 60 years old, were recruited at our hospital's Outpatient's Department of the Endocrinology and Nutrition and Stomatology Services.

Exclusion criteria were less than fourteen teeth, aggressive periodontitis, infectious or other inflammatory diseases, periodontal treatment in the last six months or antibiotics in the last three months, treatment with systemic anti-inflammatory drugs, pregnancy or lactation, secondary obesity (hypothyroidism, Cushing's syndrome), any medical condition requiring antibiotic treatment before the dental intervention, and diabetes mellitus according to ADA criteria (American Diabetes Association 2017).

This study – a human observational study structured according to STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) guidelines – was conducted in accordance with the Helsinki Declaration-based Ethical Principles for Medical Research Involving Human Subjects. All procedures were approved by our hospital's Ethics Committee, and written informed consent was obtained from all subjects.

Patients were clustered into four groups based on their BMI and the diagnosis of chronic periodontitis (CP) as follows: lean without CP; lean with CP; obese without CP; and obese with CP. Obesity was diagnosed according to the criterion of the Spanish Society for the Study of Obesity when BMI $\geq$30 kg/m^2. Patients were defined as lean or normoweight when BMI $\leq$25 kg/m^2 (Salas-Salvadó et al. 2007). CP was diagnosed when four or more teeth had one or more sites with

probing depth (PD) ≥4 mm and clinical attachment loss (CAL) ≥3 mm (Khader et al. 2009). All the participants diagnosed with periodontitis were submitted to non-surgical periodontal treatment consisting of oral hygiene instructions and full-mouth mechanical periodontal debridement with an ultrasonic device (Suprasson Newtron, Satelec, Acteon, Merignac, France) and manual curets (Hu-Friedy, Chicago, IL, USA) performed within a 24-hour period. Patients were instructed to use a chlorhexidine mouthwash (0.12%) for 60 seconds, twice per day, for 14 days. We selected this periodontal treatment protocol based on growing evidence of its clinical efficacy (Matesanz-Pérez et al. 2013; Fang et al. 2016).

Participants were interviewed about health-related characteristics and lifestyle, including current smoking habit (yes or no). Periodontal measurements, anthropometric data and biochemical determinations of blood samples were recorded at baseline and 3 months after treatment.

Clinical periodontal determinations

Periodontal assessments included measurements of PD, CAL, number of teeth and percentage of sites with PD ≥4 mm, gingival bleeding on probing (BOP), plaque index and calculus index, which were recorded used a manual periodontal probe PCP UNC-15 (Hu-Friedy, Chicago, IL, USA). We performed a full-mouth periodontal examination to measure PD, CAL and BOP at six sites per tooth for all teeth, excluding third molars. PD was measured from the gingival margin to the base of the clinical pocket, CAL was recorded as the distance from the cement-enamel junction to the base of the clinical pocket, and percentage of BOP was calculated by dividing the number of sites with bleeding on probing by the number of sites explored and multiplying this value by 100. We employed the

Silness and Löe simplified Plaque Index (1964) and the Greene and Vermillion simplified Calculus Index (1964) to assess and score the six representative Ramfjörd teeth.

The number of teeth and the percentage of sites with pathological periodontal pockets (PD ≥4 mm) determine the extent of periodontitis.

Anthropometric and biochemical determinations

Anthropometric measurements including weight (kg), height (m) and waist and hip circumferences (cm) were measured according to standardized methods. BMI was calculated as weight divided by the square of height (kg/m^2). Waist circumference was measured to the nearest 0.1 cm at the narrowest midpoint between the iliac crest and the lower margin of the ribcage using a measuring tape. Hip circumference was determined at the largest contour of the major trochanters, and waist-to-hip ratio (WHR) was calculated by dividing waist circumference by hip circumference.

Levels of glucose, total cholesterol (TC) and triglycerides (TG) in serum were determined by means of enzymatic methods. HDL cholesterol was recorded with a Beckman LX20 autoanalyzer (Beckman Coulter, La Brea, CA, USA) using a direct method. LDL cholesterol was calculated using Friedewald's formula. Insulin was determined by an immunochemiluminescence assay with an Immulite analyser (DPC, Los Angeles, CA, USA). IR was estimated by homeostasis model assessment of insulin resistance (HOMA-IR) index. High-sensitive C-reactive protein (hsCRP) was quantified by an immunonephelometric assay (Dade Behring BNII, Marburg, Germany). RBP4 concentrations were measured by nephelometry (Dade Behring, Marburg, Germany) and TNFα and IL-6 were determined with a Luminex 200 analyzer system (Austin, TX, USA).

Statistical analysis

A sample size of thirty-three patients per group was required in order to achieve a power of 90% to detect differences between the groups in relation to the primary efficacy criterion (serum RBP4 levels variation) of ≥0.8 mg/dl and assuming a common standard deviation of 1.0 mg/dl.

Statistical analysis was performed with SPSS software (SPSS Statistics Inc., Chicago, IL, USA). Continuous variables were expressed as mean and standard deviation (SD), or as median and 25^{th} and 75^{th} percentiles for parametric and non-parametric data, respectively. Analyses of binary logistic regression were conducted to estimate the probability of periodontitis, expressed as the odds ratio (OR). Qualitative data were expressed as percentages and a Chi-square test was employed to compare proportions. Data were compared with an ANOVA or Kruskal-Wallis, followed in each case by a post hoc test, and were adjusted by a univariate general linear model. Changes after periodontal treatment were evaluated with a paired Student's t-test or a Wilcoxon test, or using an unpaired Student's t-test or a Mann Whitney U-test. Pearson's correlation coefficients were employed to measure the strength of association between variables. The multivariable regression model was used to evaluate the relationship between two or more explanatory variables and a response variable. All the tests had a confidence interval of 95% and differences were considered significant when $p<0.05$.

Results

This study analysed a total of 231 subjects (64 men and 167 women) recruited between September 2015 and December 2016. The participants were divided into four groups according to BMI in lean and obese patients and diagnosed with or without CP: lean subjects without CP (n=64), lean patients with CP (n=48), obese subjects without CP (n=23) and obese patients with CP (n=96), which is in accordance with the high prevalence of periodontitis we had previously reported in our obese population (Martinez-Herrera et al. 2017).

Anthropometric and biochemical parameters of our population are shown in Table 1. As expected, obese subjects without CP had higher BMI, waist, WHR, SBP, DBP, insulin, HOMA-IR, LDLc, TG, TNFα and hsCRP and lower levels of HDLc than lean subjects without CP. These differences were also present in patients with CP, and a significant increase of RBP4 was observed in the obese population. Lean subjects with CP had higher BMI, waist, WHR, SBP, LDLc, TG, TNFα and RBP4 and lower levels of HDLc than lean subjects without CP. Obese patients with and without CP differed only with respect to TNFα and RBP4 levels. ANOVA revealed that differences in RBP4 among groups were maintained after adjustment for age and BMI (p=0.038). Total cholesterol and IL-6 did not differ significantly among the groups. Age and glucose were statistically significant, but differences were found between lean subjects without CP and obese subjects with CP. For smoking habit, 75% of subjects in both CP groups were non-smokers, and no differences were detected in the smoking rate among the four groups when compared by a Chi-square test (p=0.390).

When we submitted all the variables that differed significantly between individuals with and without CP to a binary logistic regression, we found BMI

(OR= 1.08; p<0.001; 95% CI: 1.04-1.13) and RBP4 (OR= 1.60; p=0.040; 95% CI: 1.02-2.50) to be significant predictors of an increased probability of periodontitis.

Clinical periodontal parameters for all groups at baseline are shown in Table 2. As expected, patients with CP had higher PD, CAL, number of teeth and percentage of sites with PD ≥4 mm, BOP, plaque index and calculus index than their counterparts without CP in both lean and obese populations. Obese subjects without CP showed higher PD, CAL, BOP, plaque and calculus than lean subjects without CP, with greater periodontal damage detected in the obese population than among lean subjects. Among the groups with CP, obese subjects displayed a higher number of teeth with PD ≥4 mm and higher plaque index than lean subjects. Thirty-seven patients with CP dropped out of the study, leaving a remaining 107 (33 lean and 74 obese) that underwent non-surgical periodontal treatment and were reviewed after 3 months.

The periodontal treatment significantly improved all the periodontal parameters to the same extent, with no differences observed between lean and obese patients, except for the number of teeth with PD ≥4 mm, (Figure 1C), in which case there was a 34.5% reduction in lean individuals and a 20.0% reduction in obese patients, representing statistically significant differences between the two groups (p=0.020). In line with this, the periodontal treatment was associated with a statistically significant decrease in RBP4 and TNFα serum levels in both populations (Table 3).

Since the reduction in the number of teeth with PD ≥4 mm was the only that showed statistical differences between lean and obese groups, we aimed to analyze its associations with the other parameters studied. The correlation

analysis demonstrated that, after periodontal treatment, the number of teeth with PD ≥4 mm correlated with waist, WHR, SBP, DBP, insulin, HOMA-IR, TNFα and RBP4 and with all periodontal variables (Table 4). In the multivariable regression model, the association of number of teeth with PD ≥4 mm with the correlated variables was evaluated as a potentially independent predictor using the stepwise method. The results showed that PD (β=0.785) and RBP4 (β=0.192) were independently associated with number of teeth with PD ≥4 mm, thus explaining 63% of the dependent variable (Table 5). Likewise, RBP4 levels correlated with waist, WHR, SBP, DBP, insulin, HOMA-IR, TG, TNFα, PD, CAL and percentage of sites with PD ≥4 mm (Table 4).

Discussion

The findings of the present study suggest that serum RBP4 levels are associated with chronic periodontitis, as they are elevated in CP and decrease after non-surgical periodontal treatment. This response seems to be associated with a significant decrease in TNFα levels. In addition, a weak but significant association was observed between number of teeth with PD ≥4 mm and RBP4, which suggests a possible role of this adipokine in the extent of periodontal disease. In light of these results, RBP4 could be considered an emerging inflammatory marker associated with chronic periodontitis.

Previous studies have suggested a multidirectional association between obesity and CP in which proinflammatory citokines and adipokines play a critical role, affecting periodontal tissue and directly contributing to the pathogenesis and extent of periodontitis (Genco et al. 2005; Marchetti et al. 2012). In a previous study carried out by our group (Martinez-Herrera et al. 2017), we have observed a high prevalence of periodontitis in our obese (80.9%) *vs* lean (41.2%) population. Moreover, we have determined that the risk of periodontitis in our population increased by 8% for every kg/m^2 of BMI, which is in line with previous reports (Ekuni et al. 2008). However, to date, no studies have explored the role of RBP4 in the probability of periodontitis developing. We have observed how said risk rose by 5% for every 0.1 mg/dl that RBP4 augmented. In line with this, RBP4 levels were higher in patients with periodontitis, whether lean or obese, and these differences remained after adjustment for age and BMI. Our results are in line with those of a previous study showing an association among RBP4, BMI and periodontal parameters in gingival crevicular fluid and serum levels (Kanoriya et al. 2016).

Since both obesity and periodontal disease have been associated with an increase in proinflammatory cytokines (Zimmermann et al. 2013) and given that RBP4 levels rise in obesity, diabetes and metabolic syndrome (Graham et al. 2006; Yang et al. 2005; Qi et al. 2007), it has been hypothesised that RBP4 may mediate the inflammatory response that links obesity and periodontitis. Indeed, it has been shown that RBP4 can contribute to the development of an inflammatory state in white adipose tissue through induction of JNK and TLR-4 in infiltrated macrophages due to an increase in the release of TNFα (Norseen et al. 2012). In the present study, we have observed elevated levels of RBP4 and TNFα in our population with periodontitis and a decrease in said levels after periodontal treatment, with a positive correlation between the two parameters. A significant drop in TNFα levels following periodontal treatment has been reported in previous studies (Altay et al. 2013; Zuza et al. 2011; Balli et al. 2016; Duzagac et al. 2016). Indeed, levels of TNFα and RBP4 have been interrelated in other pathologies; for example, in psoriasis, an autoimmune inflammatory disease in which treatment with antiTNFα has been found to reduce levels of RBP4 (Pina et al. 2016). Furthermore, previous studies have reported that interventions which ameliorate the inflammatory state, including exercise and weight loss in humans, can reduce serum RBP4 levels (Graham et al. 2006; Balagopal et al. 2007; Haider et al. 2007). In addition, a previous meta-analysis showed that RBP4 levels were higher in patients with polycystic ovary syndrome (PCOS) (Jia et al. 2014), a prevalent condition associated with IR and with a proinflammatory profile that leads to a rise in TNFα levels (Victor et al. 2011), which has also been associated with an increased prevalence and extent of periodontitis (Porwal et al. 2014).

Large epidemiologic studies have shown that RBP4 levels also correlate closely with hypertension, dyslipidemia and cardiovascular disease (Qi et al. 2007; Lambadiari et al. 2014; Meisinger et al. 2011; Alkharfy et al. 2012). In the present study, RBP4 was positively correlated with SBP, DBP and TG, as mentioned previously. However, we cannot attribute the alterations in RBP4 levels to a reduction in blood pressure or TG, since the latter two parameters were not modified following treatment. Overall, our results suggest a role for RBP4 in the inflammatory response associated with CP.

In the present study, all periodontal parameters improved significantly in lean and obese subjects after non-surgical periodontal treatment, with no differences detected between the two groups, with the exception of a reduction in the number of teeth with PD ≥4 mm, which was less pronounced in obese patients than in lean ones. This periodontal parameter represents the number of affected teeth with pathological periodontal pockets and was independently associated with RBP4 in the multivariable regression model, which may constitute a role for this adipokine in the extent of periodontitis. Therefore, the reduction in the number of teeth with PD ≥4 mm after non-surgical periodontal treatment could suggest a decrease in the systemic inflammatory response. However, the association of RBP4 and number of teeth with PD ≥4 mm, although statistically significant, was weak and the results should be interpreted with caution.

To the best of our knowledge, this is the first study to demonstrate a significant decrease of serum RBP4 levels in obese and lean subjects after non-surgical periodontal treatment. However, there are several limitations that should be taken into consideration. RBP4 was evaluated only in serum and over a single, short period of time after periodontal therapy (3 months). Therefore, further

longitudinal prospective studies that evaluate RBP4 levels in serum and gingival crevicular fluid over longer periods of time after non-surgical periodontal treatment are necessary to corroborate our findings. In addition, mechanistic studies involving larger patient samples and different ethnicities would help to determine the role of RBP4 in the pathogenesis and progression of periodontitis.

Conclusion

To summarise, circulating RBP4 levels are associated with chronic periodontitis since their levels are higher in the presence of the disease and drop after non-surgical periodontal treatment.

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Figure 1. Periodontal variables in the study population with chronic periodontitis.

Bar chart (as mean ± standard error) of patients at baseline (white bars) and 3 months after non-surgical periodontal treatment (grey bars). *p<0.05; **p<0.01; ***p<0.001 when data of lean and obese groups at baseline and 3 months were compared by a paired Student's t-test. #p<0.05 when lean and obese subjects were compared with an unpaired Student's t-test.

Abbreviations: PD: probing depth, CAL: clinical attachment loss, n teeth PD ≥4 mm: number of teeth with probing depth ≥4 mm, % sites PD ≥4 mm: percentage of sites with PD ≥4 mm, BOP: bleeding of probing.

Table 1. Anthropometric parameters and biochemical variables in the study population at baseline.

	Lean		Obese		
	Without CP	With CP	Without CP	With CP	p-value
n (% females)	64 (81)	48 (56)	23 (87)	96 (71)	0.010
Age (years)	36.8 ± 11.7	39.4 ± 8.3	42.2 ± 11.1	42.7 ± 10.2	0.007
BMI (kg/m^2)	21.8 ± 2.1	22.7 ± 1.9*	39.6 ± 7.0$^{###}$	42.5 ± 6.4$^{¥¥}$	<0.001
Waist (cm)	73.4 ± 9.6	83.4 ± 11.6***	117.1 ± 13.0$^{###}$	120.4 ± 15.7$^{¥¥¥}$	<0.001
WHR	0.75 ± 0.07	0.84 ± 0.09***	0.91 ± 0.09$^{###}$	0.90 ± 0.10$^{¥¥¥}$	<0.001
SBP (mmHg)	117 ± 14.9	123 ± 17.4*	127 ± 15.03$^{#}$	134 ± 17.3$^{¥¥}$	<0.001
DBP (mmHg)	73 ± 9.7	76 ± 11.2	79 ± 10.2$^{#}$	84 ± 11.4$^{¥¥¥}$	<0.001
Glucose (mg/dl)	87.6 ± 9.2	91.3 ± 11.9	92.5 ± 13.3	95.2 ± 11.8	0.001
Insulin (µU/ml)	6.8 ± 3.4	7.6 ± 3.4	15.9 ± 11$^{###}$	20 ± 14.2$^{¥¥¥}$	<0.001
HOMA-IR	1.48 ± 0.81	1.76 ± 0.96	3.78 ± 2.99$^{###}$	4.76 ± 3.69$^{¥¥¥}$	<0.001
TC (mg/dl)	182 ± 37	193 ± 31	193 ± 33	183 ± 32	0.211
HDLc (mg/dl)	61 ± 15	55 ± 12*	44 ± 9$^{###}$	42 ± 11$^{¥¥¥}$	<0.001
LDLc (mg/dl)	108 ± 27	119 ± 27*	126 ± 29$^{##}$	119 ± 24	0.009
TG (mg/dl)	61 (51, 73)	72 (60, 122)***	104 (73, 143)$^{###}$	123 (87, 167)$^{¥¥¥}$	<0.001
TNFα (pg/ ml)	7.72 ± 5.06	9.71 ± 4.36*	12.41 ± 6.06$^{##}$	17.57 ± 9.91**$^{¥¥¥}$	<0.001
IL-6 (pg/ml)	3.34 ± 2.78	4.06 ± 3.90	4.44 ± 2.71	3.72 ± 2.05	0.262
hsCRP (mg/l)	1.34 ± 2.26	2.20 ± 3.33	7.83 ± 8.27$^{###}$	8.42 ± 7.22$^{¥¥¥}$	<0.001
RBP4 (mg/dl)	3.06 ± 0.66	3.35 ± 0.70*	3.21 ± 0.75	3.74 ± 1.12*$^{¥¥}$	<0.001
Smoking (%) (n)	15.6 (10)	25.0 (12)	30.4 (7)	25.0 (24)	0.390

Data are presented as mean ± SD or percentage (n), except TG, which is represented as a median (25th and 75th percentiles). Data were compared by

means of one-way ANOVA and proportions were compared by a Chi-square test. *p<0.05, **p<0.01 and ***p<0.001 when compared with its respective control group without CP; $^{\#}$p<0.05, $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001 when comparing lean *vs* obese without CP; $^{¥¥}$p<0.01 and $^{¥¥¥}$p<0.001 when comparing lean *vs* obese with CP.

Abbreviations: CP: chronic periodontitis, BMI: body mass index, WHR: waist-to-hip ratio, SBP: systolic blood pressure, DBP: diastolic blood pressure, HOMA-IR: homeostasis model assessment of insulin resistance, TC: total cholesterol, LDLc: LDL cholesterol, HDLc: HDL cholesterol, TG: triglycerides, TNFα: tumor necrosis factor alpha, IL-6: interleukin-6, hsCRP: high sensitive C-reactive protein, RBP4: retinol-binding protein 4.

Table 2. Clinical periodontal parameters in the study population at baseline.

	Lean		Obese		p-value
	Without CP (n=64)	With CP (n=48)	Without CP (n=23)	With CP (n=96)	
PD (mm)	2.37 ± 0.22	3.01 ± 0.48***	2.53 ± 0.18##	3.03 ± 0.45***	<0.001
CAL (mm)	2.39 ± 0.24	3.06 ± 0.57***	2.54 ± 0.18##	3.06 ± 0.49***	<0.001
Teeth PD ≥4 mm (n)	2.14 ± 1.27	15.81 ± 6.14***	2.04 ± 1.26	18.38 ± 6.18***¥	<0.001
Sites PD ≥4 mm (%)	1.9 ± 1.4	23.6 ± 17.7***	1.9 ± 1.5	29.1 ± 17.2***	<0.001
BOP (%)	7.8 ± 4.5	24.9 ± 15.3***	12.5 ± 7.2##	28.1 ± 13.4***	<0.001
Plaque index (A.U)	0.36 ± 0.28	0.90 ± 0.53***	0.67 ± 0.45##	1.12 ± 0.65***¥	<0.001
Calculus index (A.U)	0.44 ± 0.31	0.99 ± 0.48***	0.68 ± 0.37##	1.10 ± 0.58***	<0.001

Data are presented as mean ± SD and were compared by means of one-way ANOVA ***p<0.001 compared with its respective control group without CP; ##p<0.01 when comparing lean *vs* obese without CP; ¥p<0.05 when comparing lean *vs* obese with CP.

Abbreviations: CP: chronic periodontitis, PD: probing depth, CAL: clinical attachment loss, teeth PD ≥4 mm: number of teeth with probing depth ≥4 mm, sites PD ≥4 mm: percentage of sites with PD ≥4 mm, BOP: bleeding of probing.

Table 3. Anthropometric parameters and biochemical variables in the study population with chronic periodontitis at baseline and 3 months after non-surgical periodontal treatment.

	Lean with CP (n= 33)		Obese with CP (n= 74)	
	Baseline	3 months	Baseline	3 months
BMI (kg/m^2)	22.4 ± 2.2	22.5 ± 2.3	41.6 ± 6.8	41.8 ± 6.8
Waist (cm)	83.2 ± 12.0	84.6 ± 12.5	119.2 ± 16.3	120.7 ± 15.3
WHR	0.85 ± 0.09	0.85 ± 0.08	0.90 ± 0.10	0.91 ± 0.10
SBP (mmHg)	121 ± 18.0	119 ± 17.6	134 ± 16.8	134 ± 16.9
DBP (mmHg)	74.0 ± 10.8	73.2 ± 10	84.2 ± 10.8	82.7 ± 10.8
Glucose(mg/dl)	91.5 ± 12.4	92.4 ± 11.4	95 ± 12	94.8 ± 12
Insulin (µU/ml)	8.4 ± 3.8	7.6 ± 4.2	20 ± 14.6	19.2 ± 11.3
HOMA-IR	1.98 ± 1.07	1.81 ± 1.20	4.73 ± 3.80	4.61 ± 3.17
TC (mg/dl)	195 ± 29	195 ± 28	182 ± 34	185 ± 40
HDLc (mg/dl)	55 ± 12	54 ± 11	42 ± 11	44 ± 12
LDLc (mg/dl)	121 ± 23	121 ± 24	121 ± 26	122 ± 31
TG (mg/dl)	68 (56, 128)	87 (66, 127)	128 (98, 167)	119 (98, 152)
TNFα (pg/ml)	10.80 ± 3.85	9.65 ± 4.89*	17.23 ± 9.86	13.90 ± 5.37*
IL-6 (pg/ml)	4.55 ± 4.15	4.59 ± 4.82	3.79 ± 2.04	3.38 ± 2.48
hsCRP (mg/l)	2.46 ± 3.80	1.68 ± 1.54	8.33 ± 7.78	8.57 ± 7.92
RBP4 (mg/dl)	3.20 ± 0.60	3.02 ± 0.67*	3.84 ± 1.06	3.46 ± 1.01***

Data are presented as mean ± standard deviation for all parametric data, except for TG, which showed a non-parametric distribution and is represented as a median (25th and 75th percentiles). *p<0.05; ***p<0.001 when data of lean and

obese groups at baseline and 3 months were compared by a paired Student's t-test for parametric data, or by a Wilcoxon test for TG data.

Abbreviations: CP: chronic periodontitis, BMI: body mass index, WHR: waist-to-hip ratio, SBP: systolic blood pressure, DBP: diastolic blood pressure, HOMA-IR: homeostasis model assessment of insulin resistance, TC: total cholesterol, LDLc: LDL cholesterol, HDLc: HDL cholesterol, TG: triglycerides, TNFα: tumor necrosis factor alpha, IL-6: interleukin-6, hsCRP: high sensitive C-reactive protein, RBP4: retinol-binding protein 4.

Table 4. Pearson's correlation coefficients between periodontal, anthropometric and biochemical parameters after non-surgical periodontal treatment.

	n PD ≥ 4 mm		RBP4	
	r	p	r	p
BMI	0.164	0.099	0.161	0.106
Waist	*0.277*	*0.005*	*0.317*	*0.001*
WHR	*0.275*	*0.005*	*0.344*	*<0.001*
SBP	*0.217*	*0.030*	*0.273*	*0.006*
DBP	*0.211*	*0.035*	*0.349*	*<0.001*
Glucose	0.027	0.789	0.077	0.441
Insulin	*0.265*	*0.007*	*0.265*	*0.007*
HOMA-IR	*0.274*	*0.006*	*0.263*	*0.008*
TC	-0.145	0.144	0.056	0.572
HDLc	-0.072	0.471	-0.027	0.787
LDLc	-0.185	0.073	-0.012	0.904
TG	0.167	0.092	*0.219*	*0.026*
TNFα	*0.303*	*0.018*	*0.389*	*0.002*
IL-6	0.118	0.366	-0.093	0.474
hsCRP	0.063	0.545	0.015	0.888
RBP4	*0.294*	*0.003*	------	------
PD	*0.751*	*<0.001*	*0.228*	*0.020*
CAL	*0.663*	*<0.001*	*0.252*	*0.010*
n PD ≥4 mm	------	------	*0.294*	*0.003*
% PD ≥4 mm	*0.924*	*<0.001*	*0.272*	*0.006*
BOP	*0.546*	*<0.001*	0.048	0.631
Plaque	*0.363*	*<0.001*	-0.055	0.579
Calculus	*0.201*	*0.042*	-0.006	0.952

Abbreviations: n PD ≥4 mm: number of teeth with probing depth ≥4 mm, RBP4: retinol-binding protein 4, BMI: body mass index, WHR: waist-to-hip ratio, SBP: systolic blood pressure, DBP: diastolic blood pressure, HOMA-IR: homeostasis

model assessment of insulin resistance, TC: total cholesterol, LDLc: LDL cholesterol, HDLc: HDL cholesterol, TG: triglycerides, TNFα: tumor necrosis factor alpha, IL-6: interleukin-6, hsCRP: high sensitive C-reactive protein, PD: probing depth, CAL: clinical attachment loss, % PD ≥4 mm: percentage of sites with PD ≥4 mm, BOP: bleeding of probing.

Table 5. Stepwise multivariable regression model of number of teeth with PD ≥ 4 mm after non-surgical periodontal treatment as a dependent variable.

Dependent variable	Independent variables	Unstandardized coefficients		Standardized coefficients	p-value
		B	SE	β	
n teeth PD ≥4 mm	PD	14.34	1.464	0.785	<0.001
	RBP4	1.18	0.491	0.192	0.020
	Multiple R square adjusted			0.628	
	R			0.801	
	p			<0.001	

Waist, WHR, SBP, DBP, HOMA-IR, TNFα, CAL, BOP, plaque and calculus were excluded from the model since they were not significant predictors (p>0.05).

Abbreviations: n PD ≥4 mm: number of teeth with probing depth ≥4 mm, PD: probing depth, RBP4: retinol-binding protein 4, WHR: waist-to-hip ratio, SBP: systolic blood pressure, DBP: diastolic blood pressure, HOMA-IR: homeostasis model assessment of insulin resistance, TNFα: tumor necrosis factor alpha, CAL: clinical attachment loss, BOP: bleeding on probing.

Levels of serum retinol binding protein 4 before and after non-surgical periodontal treatment in lean and obese subjects: an interventional study.

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Abstract

Aim: We aimed to evaluate serum RBP4 levels before and after periodontal therapy in lean and obese subjects with chronic periodontitis (CP) in order to determine its possible association with periodontitis.

Materials and Methods: This is an interventional study for which a total of 112 lean and 119 obese subjects were recruited. Patients with CP were evaluated before and after three months of non-surgical periodontal treatment. Periodontal, anthropometric, biochemical parameters and serum levels of TNFα, IL-6, hsCRP and RBP4 were assessed.

Results: Serum RBP4 levels were associated with an increased probability of periodontitis (OR= 1.60; 95% CI: 1.02-2.50), showing patients with CP to have higher RBP4 levels than those without CP in both lean and obese populations (3.35 vs 3.06 and 3.74 vs 3.21, respectively). Following periodontal treatment, RBP4 and TNFα decreased and all periodontal parameters improved to the same extent in both groups, except for number of teeth with probing depth (PD) ≥4 mm, which improved to a less extent in obese than in lean subjects. In the multivariable regression model, number of teeth with PD ≥4 mm was independently associated with RBP4 (β=0.192).

Conclusion: RBP4 was associated with chronic periodontitis before and after non-surgical periodontal treatment.

Clinical Relevance

Scientific rationale for the study: Serum retinol binding protein 4 (RBP4) levels have recently been linked to periodontitis, but the role of RBP4 in chronic periodontitis before and after non-surgical periodontal treatment is still unknown.

Principal findings: Serum RBP4 levels were associated with an increased probability and extent of periodontitis.

Practical implications: Serum RBP4 levels may represent an inflammatory marker of periodontitis detectable by a readily available analytical determination.

Introduction

Obesity is a highly prevalent condition characterised by the abnormal accumulation and storage of fat in adipose tissue (Boesing et al. 2009) and produced by an imbalance between energy intake and energy expenditure, which can be modified by genetic and environmental circumstances (Fadel et al. 2014). Obesity is associated with chronic, low-grade inflammation, which is involved, in turn, in the development of insulin resistance (IR) (Bullon et al. 2009) and is a major contributor to the development of hypertension, type 2 diabetes mellitus, arteriosclerosis, cardiovascular and cerebrovascular diseases (Pischon et al. 2007; Saito & Shimazaki 2007; Haffajee & Socransky 2009). In addition to these risk factors, obesity is also thought to increase the probability of periodontitis, a disease of the supporting structures of the teeth resulting from an interaction between pathogenic bacteria and the host immune response (Ylöstalo et al. 2008). In this sense, it has been suggested that IR plays a role in the pathogenesis of periodontitis (Genco et al. 2005). In fact, we have recently shown that periodontitis tends to be more extensive in obese patients with IR than in their respective counterparts without IR (Martinez-Herrera et al. 2017).

As an endocrine organ, adipose tissue is responsible for the synthesis and secretion of several adipokines, such as leptin, adiponectin and resistin and proinflammatory cytokines (mainly IL-6, IL-1 and TNFα), which affect the periodontal tissues and contribute to the pathophysiology of periodontitis (Ylöstalo et al. 2008).

More recently, it has been suggested that certain adipokines which are highly expressed in liver and visceral adipose tissue, such as chemerin, visfatin and

retinol binding protein 4 (RBP4), are implicated in insulin-resistant states (Marchetti et al. 2012).

RBP4 is a member of the lipocalin family that is secreted into the circulation while being bound to vitamin A and transthyretin. It has been shown that RBP4 is upregulated in insulin-resistant conditions associated with obesity and provokes IR by interfering with insulin receptor substrate 1 (Yang et al. 2005; Gerrits et al. 2012). In addition, it seems to be a predictor of cardiovascular disease (Alkharfy et al. 2012) and development of atherosclerosis, as it promotes atherogenic dyslipidaemia (Rocha et al. 2013) and is involved in the regulation of hypertension (Kraus et al. 2015). Recently, it has been established that RBP4 levels in gingival crevicular fluid and serum are positively correlated with body mass index (BMI) and periodontal parameters (Kanoriya et al. 2016). However, to date, no previous studies have evaluated RBP4 levels after non-surgical periodontal treatment.

In this context, research such as that reported here may provide valuable information about the association among RBP4 levels, obesity and periodontal disease. Therefore, the aim of this study was to determine serum RBP4 levels in obese and lean subjects with and without chronic periodontitis and to evaluate the effect of non-surgical periodontal treatment on RBP4 levels. In addition, we have explored the relationship between RBP4 levels and other clinical and periodontal parameters.

Materials and Methods

Study design

This was an interventional case-control study conducted at the University Hospital Dr. Peset (Valencia, Spain). The participants, all between the ages of 20 and 60 years old, were recruited at our hospital's Outpatient's Department of the Endocrinology and Nutrition and Stomatology Services.

Exclusion criteria were less than fourteen teeth, aggressive periodontitis, infectious or other inflammatory diseases, periodontal treatment in the last six months or antibiotics in the last three months, treatment with systemic anti-inflammatory drugs, pregnancy or lactation, secondary obesity (hypothyroidism, Cushing's syndrome), any medical condition requiring antibiotic treatment before the dental intervention, and diabetes mellitus according to ADA criteria (American Diabetes Association 2017).

This study – a human observational study structured according to STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) guidelines – was conducted in accordance with the Helsinki Declaration-based Ethical Principles for Medical Research Involving Human Subjects. All procedures were approved by our hospital's Ethics Committee, and written informed consent was obtained from all subjects.

Patients were clustered into four groups based on their BMI and the diagnosis of chronic periodontitis (CP) as follows: lean without CP; lean with CP; obese without CP; and obese with CP. Obesity was diagnosed according to the criterion of the Spanish Society for the Study of Obesity when BMI $\geq$30 kg/m^2. Patients were defined as lean or normoweight when BMI $\leq$25 kg/m^2 (Salas-Salvadó et al. 2007). CP was diagnosed when four or more teeth had one or more sites with

probing depth (PD) ≥4 mm and clinical attachment loss (CAL) ≥3 mm (Khader et al. 2009). All the participants diagnosed with periodontitis were submitted to non-surgical periodontal treatment consisting of oral hygiene instructions and full-mouth mechanical periodontal debridement with an ultrasonic device (Suprasson Newtron, Satelec, Acteon, Merignac, France) and manual curets (Hu-Friedy, Chicago, IL, USA) performed within a 24-hour period. Patients were instructed to use a chlorhexidine mouthwash (0.12%) for 60 seconds, twice per day, for 14 days. We selected this periodontal treatment protocol based on growing evidence of its clinical efficacy (Matesanz-Pérez et al. 2013; Fang et al. 2016).

Participants were interviewed about health-related characteristics and lifestyle, including current smoking habit (yes or no). Periodontal measurements, anthropometric data and biochemical determinations of blood samples were recorded at baseline and 3 months after treatment.

Clinical periodontal determinations

Periodontal assessments included measurements of PD, CAL, number of teeth and percentage of sites with PD ≥4 mm, gingival bleeding on probing (BOP), plaque index and calculus index, which were recorded used a manual periodontal probe PCP UNC-15 (Hu-Friedy, Chicago, IL, USA). We performed a full-mouth periodontal examination to measure PD, CAL and BOP at six sites per tooth for all teeth, excluding third molars. PD was measured from the gingival margin to the base of the clinical pocket, CAL was recorded as the distance from the cement-enamel junction to the base of the clinical pocket, and percentage of BOP was calculated by dividing the number of sites with bleeding on probing by the number of sites explored and multiplying this value by 100. We employed the

Silness and Löe simplified Plaque Index (1964) and the Greene and Vermillion simplified Calculus Index (1964) to assess and score the six representative Ramfjörd teeth.

The number of teeth and the percentage of sites with pathological periodontal pockets (PD ≥4 mm) determine the extent of periodontitis.

Anthropometric and biochemical determinations

Anthropometric measurements including weight (kg), height (m) and waist and hip circumferences (cm) were measured according to standardized methods. BMI was calculated as weight divided by the square of height (kg/m^2). Waist circumference was measured to the nearest 0.1 cm at the narrowest midpoint between the iliac crest and the lower margin of the ribcage using a measuring tape. Hip circumference was determined at the largest contour of the major trochanters, and waist-to-hip ratio (WHR) was calculated by dividing waist circumference by hip circumference.

Levels of glucose, total cholesterol (TC) and triglycerides (TG) in serum were determined by means of enzymatic methods. HDL cholesterol was recorded with a Beckman LX20 autoanalyzer (Beckman Coulter, La Brea, CA, USA) using a direct method. LDL cholesterol was calculated using Friedewald's formula. Insulin was determined by an immunochemiluminescence assay with an Immulite analyser (DPC, Los Angeles, CA, USA). IR was estimated by homeostasis model assessment of insulin resistance (HOMA-IR) index. High-sensitive C-reactive protein (hsCRP) was quantified by an immunonephelometric assay (Dade Behring BNII, Marburg, Germany). RBP4 concentrations were measured by nephelometry (Dade Behring, Marburg, Germany) and TNFα and IL-6 were determined with a Luminex 200 analyzer system (Austin, TX, USA).

Statistical analysis

A sample size of thirty-three patients per group was required in order to achieve a power of 90% to detect differences between the groups in relation to the primary efficacy criterion (serum RBP4 levels variation) of ≥0.8 mg/dl and assuming a common standard deviation of 1.0 mg/dl.

Statistical analysis was performed with SPSS software (SPSS Statistics Inc., Chicago, IL, USA). Continuous variables were expressed as mean and standard deviation (SD), or as median and 25^{th} and 75^{th} percentiles for parametric and non-parametric data, respectively. Analyses of binary logistic regression were conducted to estimate the probability of periodontitis, expressed as the odds ratio (OR). Qualitative data were expressed as percentages and a Chi-square test was employed to compare proportions. Data were compared with an ANOVA or Kruskal-Wallis, followed in each case by a post hoc test, and were adjusted by a univariate general linear model. Changes after periodontal treatment were evaluated with a paired Student's t-test or a Wilcoxon test, or using an unpaired Student's t-test or a Mann Whitney U-test. Pearson's correlation coefficients were employed to measure the strength of association between variables. The multivariable regression model was used to evaluate the relationship between two or more explanatory variables and a response variable. All the tests had a confidence interval of 95% and differences were considered significant when $p<0.05$.

Results

This study analysed a total of 231 subjects (64 men and 167 women) recruited between September 2015 and December 2016. The participants were divided into four groups according to BMI in lean and obese patients and diagnosed with or without CP: lean subjects without CP (n=64), lean patients with CP (n=48), obese subjects without CP (n=23) and obese patients with CP (n=96), which is in accordance with the high prevalence of periodontitis we had previously reported in our obese population (Martinez-Herrera et al. 2017).

Anthropometric and biochemical parameters of our population are shown in Table 1. As expected, obese subjects without CP had higher BMI, waist, WHR, SBP, DBP, insulin, HOMA-IR, LDLc, TG, TNFα and hsCRP and lower levels of HDLc than lean subjects without CP. These differences were also present in patients with CP, and a significant increase of RBP4 was observed in the obese population. Lean subjects with CP had higher BMI, waist, WHR, SBP, LDLc, TG, TNFα and RBP4 and lower levels of HDLc than lean subjects without CP. Obese patients with and without CP differed only with respect to TNFα and RBP4 levels. ANOVA revealed that differences in RBP4 among groups were maintained after adjustment for age and BMI (p=0.038). Total cholesterol and IL-6 did not differ significantly among the groups. Age and glucose were statistically significant, but differences were found between lean subjects without CP and obese subjects with CP. For smoking habit, 75% of subjects in both CP groups were non-smokers, and no differences were detected in the smoking rate among the four groups when compared by a Chi-square test (p=0.390).

When we submitted all the variables that differed significantly between individuals with and without CP to a binary logistic regression, we found BMI

(OR= 1.08; p<0.001; 95% CI: 1.04-1.13) and RBP4 (OR= 1.60; p=0.040; 95% CI: 1.02-2.50) to be significant predictors of an increased probability of periodontitis.

Clinical periodontal parameters for all groups at baseline are shown in Table 2. As expected, patients with CP had higher PD, CAL, number of teeth and percentage of sites with PD ≥4 mm, BOP, plaque index and calculus index than their counterparts without CP in both lean and obese populations. Obese subjects without CP showed higher PD, CAL, BOP, plaque and calculus than lean subjects without CP, with greater periodontal damage detected in the obese population than among lean subjects. Among the groups with CP, obese subjects displayed a higher number of teeth with PD ≥4 mm and higher plaque index than lean subjects. Thirty-seven patients with CP dropped out of the study, leaving a remaining 107 (33 lean and 74 obese) that underwent non-surgical periodontal treatment and were reviewed after 3 months.

The periodontal treatment significantly improved all the periodontal parameters to the same extent, with no differences observed between lean and obese patients, except for the number of teeth with PD ≥4 mm, (Figure 1C), in which case there was a 34.5% reduction in lean individuals and a 20.0% reduction in obese patients, representing statistically significant differences between the two groups (p=0.020). In line with this, the periodontal treatment was associated with a statistically significant decrease in RBP4 and TNFα serum levels in both populations (Table 3).

Since the reduction in the number of teeth with PD ≥4 mm was the only that showed statistical differences between lean and obese groups, we aimed to analyze its associations with the other parameters studied. The correlation

analysis demonstrated that, after periodontal treatment, the number of teeth with PD ≥4 mm correlated with waist, WHR, SBP, DBP, insulin, HOMA-IR, TNFα and RBP4 and with all periodontal variables (Table 4). In the multivariable regression model, the association of number of teeth with PD ≥4 mm with the correlated variables was evaluated as a potentially independent predictor using the stepwise method. The results showed that PD (β=0.785) and RBP4 (β=0.192) were independently associated with number of teeth with PD ≥4 mm, thus explaining 63% of the dependent variable (Table 5). Likewise, RBP4 levels correlated with waist, WHR, SBP, DBP, insulin, HOMA-IR, TG, TNFα, PD, CAL and percentage of sites with PD ≥4 mm (Table 4).

Discussion

The findings of the present study suggest that serum RBP4 levels are associated with chronic periodontitis, as they are elevated in CP and decrease after non-surgical periodontal treatment. This response seems to be associated with a significant decrease in TNFα levels. In addition, a weak but significant association was observed between number of teeth with PD ≥4 mm and RBP4, which suggests a possible role of this adipokine in the extent of periodontal disease. In light of these results, RBP4 could be considered an emerging inflammatory marker associated with chronic periodontitis.

Previous studies have suggested a multidirectional association between obesity and CP in which proinflammatory citokines and adipokines play a critical role, affecting periodontal tissue and directly contributing to the pathogenesis and extent of periodontitis (Genco et al. 2005; Marchetti et al. 2012). In a previous study carried out by our group (Martinez-Herrera et al. 2017), we have observed a high prevalence of periodontitis in our obese (80.9%) *vs* lean (41.2%) population. Moreover, we have determined that the risk of periodontitis in our population increased by 8% for every kg/m^2 of BMI, which is in line with previous reports (Ekuni et al. 2008). However, to date, no studies have explored the role of RBP4 in the probability of periodontitis developing. We have observed how said risk rose by 5% for every 0.1 mg/dl that RBP4 augmented. In line with this, RBP4 levels were higher in patients with periodontitis, whether lean or obese, and these differences remained after adjustment for age and BMI. Our results are in line with those of a previous study showing an association among RBP4, BMI and periodontal parameters in gingival crevicular fluid and serum levels (Kanoriya et al. 2016).

Since both obesity and periodontal disease have been associated with an increase in proinflammatory cytokines (Zimmermann et al. 2013) and given that RBP4 levels rise in obesity, diabetes and metabolic syndrome (Graham et al. 2006; Yang et al. 2005; Qi et al. 2007), it has been hypothesised that RBP4 may mediate the inflammatory response that links obesity and periodontitis. Indeed, it has been shown that RBP4 can contribute to the development of an inflammatory state in white adipose tissue through induction of JNK and TLR-4 in infiltrated macrophages due to an increase in the release of TNFα (Norseen et al. 2012). In the present study, we have observed elevated levels of RBP4 and TNFα in our population with periodontitis and a decrease in said levels after periodontal treatment, with a positive correlation between the two parameters. A significant drop in TNFα levels following periodontal treatment has been reported in previous studies (Altay et al. 2013; Zuza et al. 2011; Balli et al. 2016; Duzagac et al. 2016). Indeed, levels of TNFα and RBP4 have been interrelated in other pathologies; for example, in psoriasis, an autoimmune inflammatory disease in which treatment with antiTNFα has been found to reduce levels of RBP4 (Pina et al. 2016). Furthermore, previous studies have reported that interventions which ameliorate the inflammatory state, including exercise and weight loss in humans, can reduce serum RBP4 levels (Graham et al. 2006; Balagopal et al. 2007; Haider et al. 2007). In addition, a previous meta-analysis showed that RBP4 levels were higher in patients with polycystic ovary syndrome (PCOS) (Jia et al. 2014), a prevalent condition associated with IR and with a proinflammatory profile that leads to a rise in TNFα levels (Victor et al. 2011), which has also been associated with an increased prevalence and extent of periodontitis (Porwal et al. 2014).

Large epidemiologic studies have shown that RBP4 levels also correlate closely with hypertension, dyslipidemia and cardiovascular disease (Qi et al. 2007; Lambadiari et al. 2014; Meisinger et al. 2011; Alkharfy et al. 2012). In the present study, RBP4 was positively correlated with SBP, DBP and TG, as mentioned previously. However, we cannot attribute the alterations in RBP4 levels to a reduction in blood pressure or TG, since the latter two parameters were not modified following treatment. Overall, our results suggest a role for RBP4 in the inflammatory response associated with CP.

In the present study, all periodontal parameters improved significantly in lean and obese subjects after non-surgical periodontal treatment, with no differences detected between the two groups, with the exception of a reduction in the number of teeth with PD ≥4 mm, which was less pronounced in obese patients than in lean ones. This periodontal parameter represents the number of affected teeth with pathological periodontal pockets and was independently associated with RBP4 in the multivariable regression model, which may constitute a role for this adipokine in the extent of periodontitis. Therefore, the reduction in the number of teeth with PD ≥4 mm after non-surgical periodontal treatment could suggest a decrease in the systemic inflammatory response. However, the association of RBP4 and number of teeth with PD ≥4 mm, although statistically significant, was weak and the results should be interpreted with caution.

To the best of our knowledge, this is the first study to demonstrate a significant decrease of serum RBP4 levels in obese and lean subjects after non-surgical periodontal treatment. However, there are several limitations that should be taken into consideration. RBP4 was evaluated only in serum and over a single, short period of time after periodontal therapy (3 months). Therefore, further

longitudinal prospective studies that evaluate RBP4 levels in serum and gingival crevicular fluid over longer periods of time after non-surgical periodontal treatment are necessary to corroborate our findings. In addition, mechanistic studies involving larger patient samples and different ethnicities would help to determine the role of RBP4 in the pathogenesis and progression of periodontitis.

Conclusion

To summarise, circulating RBP4 levels are associated with chronic periodontitis since their levels are higher in the presence of the disease and drop after non-surgical periodontal treatment.

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Figure 1. Periodontal variables in the study population with chronic periodontitis.

Bar chart (as mean ± standard error) of patients at baseline (white bars) and 3 months after non-surgical periodontal treatment (grey bars). *p<0.05; **p<0.01; ***p<0.001 when data of lean and obese groups at baseline and 3 months were compared by a paired Student's t-test. #p<0.05 when lean and obese subjects were compared with an unpaired Student's t-test.

Abbreviations: PD: probing depth, CAL: clinical attachment loss, n teeth PD ≥4 mm: number of teeth with probing depth ≥4 mm, % sites PD ≥4 mm: percentage of sites with PD ≥4 mm, BOP: bleeding of probing.

Table 1. Anthropometric parameters and biochemical variables in the study population at baseline.

	Lean		Obese		
	Without CP	With CP	Without CP	With CP	p-value
n (% females)	64 (81)	48 (56)	23 (87)	96 (71)	0.010
Age (years)	36.8 ± 11.7	39.4 ± 8.3	42.2 ± 11.1	42.7 ± 10.2	0.007
BMI (kg/m^2)	21.8 ± 2.1	22.7 ± 1.9*	39.6 ± 7.0$^{###}$	42.5 ± 6.4$^{¥¥}$	<0.001
Waist (cm)	73.4 ± 9.6	83.4 ± 11.6***	117.1 ± 13.0$^{###}$	120.4 ± 15.7$^{¥¥¥}$	<0.001
WHR	0.75 ± 0.07	0.84 ± 0.09***	0.91 ± 0.09$^{###}$	0.90 ± 0.10$^{¥¥¥}$	<0.001
SBP (mmHg)	117 ± 14.9	123 ± 17.4*	127 ± 15.03$^{#}$	134 ± 17.3$^{¥¥}$	<0.001
DBP (mmHg)	73 ± 9.7	76 ± 11.2	79 ± 10.2$^{#}$	84 ± 11.4$^{¥¥¥}$	<0.001
Glucose (mg/dl)	87.6 ± 9.2	91.3 ± 11.9	92.5 ± 13.3	95.2 ± 11.8	0.001
Insulin (µU/ml)	6.8 ± 3.4	7.6 ± 3.4	15.9 ± 11$^{###}$	20 ± 14.2$^{¥¥¥}$	<0.001
HOMA-IR	1.48 ± 0.81	1.76 ± 0.96	3.78 ± 2.99$^{###}$	4.76 ± 3.69$^{¥¥¥}$	<0.001
TC (mg/dl)	182 ± 37	193 ± 31	193 ± 33	183 ± 32	0.211
HDLc (mg/dl)	61 ± 15	55 ± 12*	44 ± 9$^{###}$	42 ± 11$^{¥¥¥}$	<0.001
LDLc (mg/dl)	108 ± 27	119 ± 27*	126 ± 29$^{##}$	119 ± 24	0.009
TG (mg/dl)	61 (51, 73)	72 (60, 122)***	104 (73, 143)$^{###}$	123 (87, 167)$^{¥¥¥}$	<0.001
TNFα (pg/ ml)	7.72 ± 5.06	9.71 ± 4.36*	12.41 ± 6.06$^{##}$	17.57 ± 9.91**$^{¥¥¥}$	<0.001
IL-6 (pg/ml)	3.34 ± 2.78	4.06 ± 3.90	4.44 ± 2.71	3.72 ± 2.05	0.262
hsCRP (mg/l)	1.34 ± 2.26	2.20 ± 3.33	7.83 ± 8.27$^{###}$	8.42 ± 7.22$^{¥¥¥}$	<0.001
RBP4 (mg/dl)	3.06 ± 0.66	3.35 ± 0.70*	3.21 ± 0.75	3.74 ± 1.12*$^{¥¥}$	<0.001
Smoking (%) (n)	15.6 (10)	25.0 (12)	30.4 (7)	25.0 (24)	0.390

Data are presented as mean ± SD or percentage (n), except TG, which is represented as a median (25th and 75th percentiles). Data were compared by

means of one-way ANOVA and proportions were compared by a Chi-square test. *p<0.05, **p<0.01 and ***p<0.001 when compared with its respective control group without CP; $^{\#}$p<0.05, $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001 when comparing lean *vs* obese without CP; $^{¥¥}$p<0.01 and $^{¥¥¥}$p<0.001 when comparing lean *vs* obese with CP.

Abbreviations: CP: chronic periodontitis, BMI: body mass index, WHR: waist-to-hip ratio, SBP: systolic blood pressure, DBP: diastolic blood pressure, HOMA-IR: homeostasis model assessment of insulin resistance, TC: total cholesterol, LDLc: LDL cholesterol, HDLc: HDL cholesterol, TG: triglycerides, TNFα: tumor necrosis factor alpha, IL-6: interleukin-6, hsCRP: high sensitive C-reactive protein, RBP4: retinol-binding protein 4.

Table 2. Clinical periodontal parameters in the study population at baseline.

	Lean		Obese		p-value
	Without CP (n=64)	With CP (n=48)	Without CP (n=23)	With CP (n=96)	
PD (mm)	2.37 ± 0.22	3.01 ± 0.48***	2.53 ± 0.18##	3.03 ± 0.45***	<0.001
CAL (mm)	2.39 ± 0.24	3.06 ± 0.57***	2.54 ± 0.18##	3.06 ± 0.49***	<0.001
Teeth PD ≥4 mm (n)	2.14 ± 1.27	15.81 ± 6.14***	2.04 ± 1.26	18.38 ± 6.18***¥	<0.001
Sites PD ≥4 mm (%)	1.9 ± 1.4	23.6 ± 17.7***	1.9 ± 1.5	29.1 ± 17.2***	<0.001
BOP (%)	7.8 ± 4.5	24.9 ± 15.3***	12.5 ± 7.2##	28.1 ± 13.4***	<0.001
Plaque index (A.U)	0.36 ± 0.28	0.90 ± 0.53***	0.67 ± 0.45##	1.12 ± 0.65***¥	<0.001
Calculus index (A.U)	0.44 ± 0.31	0.99 ± 0.48***	0.68 ± 0.37##	1.10 ± 0.58***	<0.001

Data are presented as mean ± SD and were compared by means of one-way ANOVA ***p<0.001 compared with its respective control group without CP; ##p<0.01 when comparing lean *vs* obese without CP; ¥p<0.05 when comparing lean *vs* obese with CP.

Abbreviations: CP: chronic periodontitis, PD: probing depth, CAL: clinical attachment loss, teeth PD ≥4 mm: number of teeth with probing depth ≥4 mm, sites PD ≥4 mm: percentage of sites with PD ≥4 mm, BOP: bleeding of probing.

Table 3. Anthropometric parameters and biochemical variables in the study population with chronic periodontitis at baseline and 3 months after non-surgical periodontal treatment.

	Lean with CP (n= 33)		Obese with CP (n= 74)	
	Baseline	3 months	Baseline	3 months
BMI (kg/m^2)	22.4 ± 2.2	22.5 ± 2.3	41.6 ± 6.8	41.8 ± 6.8
Waist (cm)	83.2 ± 12.0	84.6 ± 12.5	119.2 ± 16.3	120.7 ± 15.3
WHR	0.85 ± 0.09	0.85 ± 0.08	0.90 ± 0.10	0.91 ± 0.10
SBP (mmHg)	121 ± 18.0	119 ± 17.6	134 ± 16.8	134 ± 16.9
DBP (mmHg)	74.0 ± 10.8	73.2 ± 10	84.2 ± 10.8	82.7 ± 10.8
Glucose(mg/dl)	91.5 ± 12.4	92.4 ± 11.4	95 ± 12	94.8 ± 12
Insulin (µU/ml)	8.4 ± 3.8	7.6 ± 4.2	20 ± 14.6	19.2 ± 11.3
HOMA-IR	1.98 ± 1.07	1.81 ± 1.20	4.73 ± 3.80	4.61 ± 3.17
TC (mg/dl)	195 ± 29	195 ± 28	182 ± 34	185 ± 40
HDLc (mg/dl)	55 ± 12	54 ± 11	42 ± 11	44 ± 12
LDLc (mg/dl)	121 ± 23	121 ± 24	121 ± 26	122 ± 31
TG (mg/dl)	68 (56, 128)	87 (66, 127)	128 (98, 167)	119 (98, 152)
TNFα (pg/ml)	10.80 ± 3.85	9.65 ± 4.89*	17.23 ± 9.86	13.90 ± 5.37*
IL-6 (pg/ml)	4.55 ± 4.15	4.59 ± 4.82	3.79 ± 2.04	3.38 ± 2.48
hsCRP (mg/l)	2.46 ± 3.80	1.68 ± 1.54	8.33 ± 7.78	8.57 ± 7.92
RBP4 (mg/dl)	3.20 ± 0.60	3.02 ± 0.67*	3.84 ± 1.06	3.46 ± 1.01***

Data are presented as mean ± standard deviation for all parametric data, except for TG, which showed a non-parametric distribution and is represented as a median (25th and 75th percentiles). *p<0.05; ***p<0.001 when data of lean and

obese groups at baseline and 3 months were compared by a paired Student's t-test for parametric data, or by a Wilcoxon test for TG data.

Abbreviations: CP: chronic periodontitis, BMI: body mass index, WHR: waist-to-hip ratio, SBP: systolic blood pressure, DBP: diastolic blood pressure, HOMA-IR: homeostasis model assessment of insulin resistance, TC: total cholesterol, LDLc: LDL cholesterol, HDLc: HDL cholesterol, TG: triglycerides, TNFα: tumor necrosis factor alpha, IL-6: interleukin-6, hsCRP: high sensitive C-reactive protein, RBP4: retinol-binding protein 4.

Table 4. Pearson's correlation coefficients between periodontal, anthropometric and biochemical parameters after non-surgical periodontal treatment.

	n PD ≥ 4 mm		RBP4	
	r	p	r	p
BMI	0.164	0.099	0.161	0.106
Waist	*0.277*	*0.005*	*0.317*	*0.001*
WHR	*0.275*	*0.005*	*0.344*	*<0.001*
SBP	*0.217*	*0.030*	*0.273*	*0.006*
DBP	*0.211*	*0.035*	*0.349*	*<0.001*
Glucose	0.027	0.789	0.077	0.441
Insulin	*0.265*	*0.007*	*0.265*	*0.007*
HOMA-IR	*0.274*	*0.006*	*0.263*	*0.008*
TC	-0.145	0.144	0.056	0.572
HDLc	-0.072	0.471	-0.027	0.787
LDLc	-0.185	0.073	-0.012	0.904
TG	0.167	0.092	*0.219*	*0.026*
TNFα	*0.303*	*0.018*	*0.389*	*0.002*
IL-6	0.118	0.366	-0.093	0.474
hsCRP	0.063	0.545	0.015	0.888
RBP4	*0.294*	*0.003*	------	------
PD	*0.751*	*<0.001*	*0.228*	*0.020*
CAL	*0.663*	*<0.001*	*0.252*	*0.010*
n PD ≥4 mm	------	------	*0.294*	*0.003*
% PD ≥4 mm	*0.924*	*<0.001*	*0.272*	*0.006*
BOP	*0.546*	*<0.001*	0.048	0.631
Plaque	*0.363*	*<0.001*	-0.055	0.579
Calculus	*0.201*	*0.042*	-0.006	0.952

Abbreviations: n PD ≥4 mm: number of teeth with probing depth ≥4 mm, RBP4: retinol-binding protein 4, BMI: body mass index, WHR: waist-to-hip ratio, SBP: systolic blood pressure, DBP: diastolic blood pressure, HOMA-IR: homeostasis

model assessment of insulin resistance, TC: total cholesterol, LDLc: LDL cholesterol, HDLc: HDL cholesterol, TG: triglycerides, TNFα: tumor necrosis factor alpha, IL-6: interleukin-6, hsCRP: high sensitive C-reactive protein, PD: probing depth, CAL: clinical attachment loss, % PD ≥4 mm: percentage of sites with PD ≥4 mm, BOP: bleeding of probing.

Table 5. Stepwise multivariable regression model of number of teeth with PD ≥ 4 mm after non-surgical periodontal treatment as a dependent variable.

Dependent variable	Independent variables	Unstandardized coefficients		Standardized coefficients	p-value
		B	SE	β	
n teeth PD ≥4 mm	PD	14.34	1.464	0.785	<0.001
	RBP4	1.18	0.491	0.192	0.020
	Multiple R square adjusted			0.628	
	R			0.801	
	p			<0.001	

Waist, WHR, SBP, DBP, HOMA-IR, TNFα, CAL, BOP, plaque and calculus were excluded from the model since they were not significant predictors ($p>0.05$).

Abbreviations: n PD ≥4 mm: number of teeth with probing depth ≥4 mm, PD: probing depth, RBP4: retinol-binding protein 4, WHR: waist-to-hip ratio, SBP: systolic blood pressure, DBP: diastolic blood pressure, HOMA-IR: homeostasis model assessment of insulin resistance, TNFα: tumor necrosis factor alpha, CAL: clinical attachment loss, BOP: bleeding on probing.

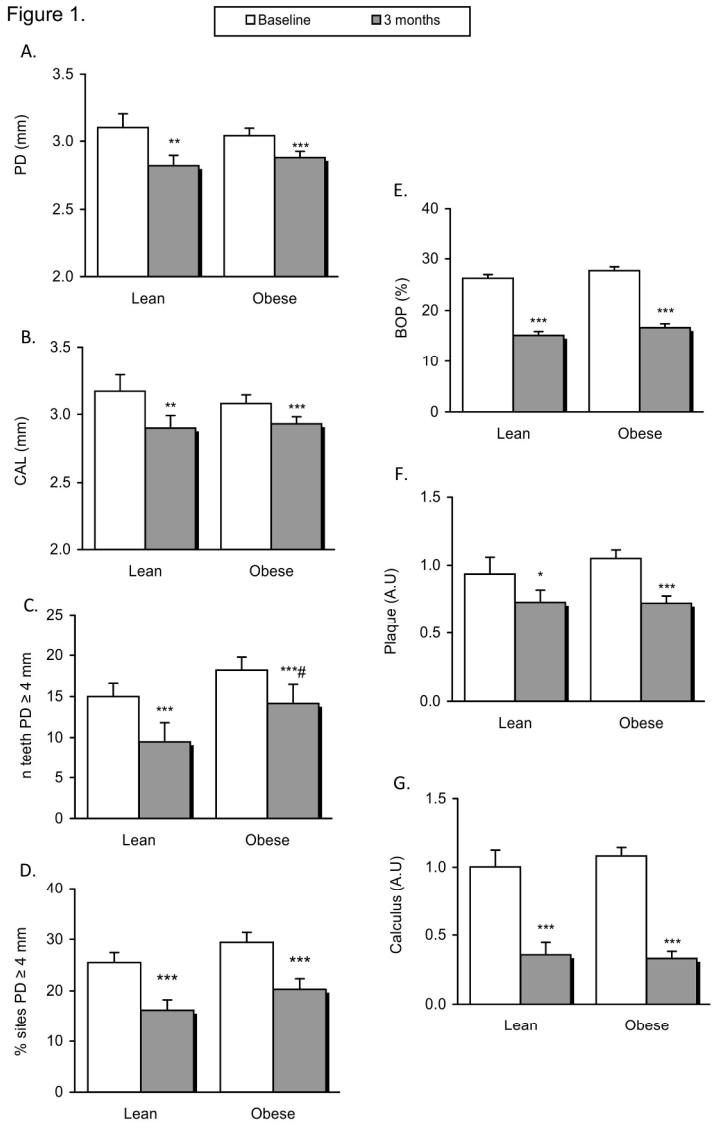

Figure 1. Periodontal variables in the study population with chronic periodontitis.

166x251mm (300 x 300 DPI)