Molecular inflammation and oxidative stress are shared mechanisms involved in both myocardial infarction and periodontitis.

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Abstract

Background and Objective. Our aims were to improve the understanding of the pathogenic relationship between cardiovascular diseases and periodontitis and to generate new perspectives in the prevention and treatment of acute myocardial infarction (AMI) and periodontitis. The present study evaluates possible differences in inflammation, oxidative stress and autophagy markers among subject suffering AMI, periodontitis or both, to explore possible common pathogenic mechanisms.

Material and Methods. A total of 260 subjects were enrolled in the study, 106 subjects that survived to a first AMI (AMI group) and 154 subjects had no cardiac events in their clinical record (control group). A questionnaire was used to assess age, height, weight, blood pressure and heart rate. The clinical probing depth, clinical attachment loss, number of remaining teeth and average number of sites with bleeding on probing were assessed. Lipid peroxidation and protein levels of phosphorylated AMP-activated protein kinase (p-AMPK) and microtubule-associated proteins 1A/1B-light chain 3-II (LC3-II) were determined in isolated peripheral blood mononuclear cells by thiobarbituric acid reactive substances (TBARS) assay and western-blot, respectively. Plasma levels of interleukin-1β were determined using a commercial ELISA kit. All the obtained variables were compared between subjects suffering an AMI with or without periodontitis and control subject periodontal healthy or with periodontitis.

Results. A higher proportion of subjects suffering AMI+periodontitis than only AMI (without periodontitis) was found. Higher levels of TBARS were found in subjects with periodontitis than in subjects without periodontitis in both AMI and control subjects. Positive correlations between IL-1β levels and TBARS and between IL-1β levels and LC3-II were found only in control subjects.

Conclusions. Results from the present study are consistent with the suggestion of periodontitis as a potential risk factor for AMI. Periodontitis association with circulating lipid peroxides in both AMI and control subjects were found. The absence of differences in IL-1β levels between AMI subjects (only AMI *vs*. AMI+periodontitis) suggests that oxidative stress could be the main pathogenic link between AMI and periodontitis.

1. INTRODUCTION

According to the World Health Organization (WHO), 17.9 million people die each year from cardiovascular diseases (CVDs), an estimated 31% of all deaths worldwide.[1] This implies that CVDs are the first cause of death globally with heart attacks and strokes producing 85% of all CVD deaths.[1] Besides associated mortality, the remained high incidence of atherosclerotic CVDs also entails an important direct and indirect economic burden over the world.[2] At public health level, the most cost-effective way to reduce the prevalence of a disease is via preventive measures.[3] In this sense, most CVDs can be prevented by addressing behavioral risk factors such as tobacco use, unhealthy diet and obesity.[1] Therefore, people with CVDs, but also people at high cardiovascular risk, need early detection and management. Classic risk factors explaining the occurrence of CVDs include hypertension, diabetes and hyperlipidemia.[1] However, in some cases of CVD, the underlying reasons remain unknown. In addition, there are some inconsistencies regarding the importance of risk factors for the different CVDs,[4] but the reasons are mostly unknown yet. Strategies to improve the prevention of CVDs require a better understanding of the pathogenic mechanisms and the impact of nontraditional risk factors.

Increasing evidence suggests periodontitis as a potential risk factor for increased morbidity or mortality of several systemic conditions, including coronary atherosclerotic complications such as angina pectoris and acute myocardial infarction (AMI).[5] Periodontitis, the advanced form of periodontal disease,[6] is considered an inflammatory disease characterized by an abnormal host response to oral bacteria affecting approximately 10-15% of the population. Thus, periodontitis is the sixth most prevalent human disease globally.[7] The inflammatory cascade is particularly important in the atherosclerotic process and in adverse cardiovascular events such as AMI and sudden cardiac death.[8] On the other hand, different inflammatory factors released during

periodontal inflammation[7] can reach blood and promote systemic and arterial inflammation complicating the early atherosclerotic processes and advancing the atherosclerotic plaque development and the risk for plaque instability[9] which can lead to atherothrombotic cardiovascular events such as myocardial infarction.[10] Indirectly, pro-inflammatory cytokines and chemokines also could cause oxidative stress by recruiting polymorphonuclear leukocytes[7], that produce reactive oxygen species (ROS),[11,12] and increasing mitochondrial production of ROS.[13] Actually, overwhelming evidence indicates that oxidative stress plays a central role in the periodontal tissue and alveolar bone destruction.[11,1415-16] Likewise, abnormal peripheral blood mononuclear cells (PBMCs) performance observed in periodontitis subjects also may promote oxidative stress[17] since an elevated production of ROS or inflammatory mediators at periodontal tissues could contribute to systemic oxidative stress resulting in oxidative damage at other areas.

Periodontitis can be not only a risk factor for CVDs, but also a condition modifying other primary risk factors associated with CVDs. In this sense, a state of systemic inflammation as consequence of the periodontal inflammation would cause the stiffness of large arteries and increases the pulse wave velocity contributing to the pathogenesis of hypertension.[18-22] Likewise; serum pro-inflammatory cytokines may have a vital role in the association between periodontitis and dyslipidemia[23] favouring the underlying sub-intimal lipid accumulation. Furthermore, it could also be possible that periodontal disease and CVDs share the same risk factors and pathogenic mechanisms. It is proposed that the aggravation of hyperlipidemic state is also linked with periodontal inflammation by the up-regulation of serum and gingival crevicular fluid pro-inflammatory cytokines.[23] Lastly, oxidative stress could be also a factor that facilitates both, diseases onset and/or progression by evoking immune responses resulting in a

higher expression of pro-inflammatory cytokines[24] or driving NLRP3 inflammasome activation[2]. Furthermore, oxidized-LDLs are also able to induce secretion of IL-1β by macrophages via ROS-dependent NLRP3 inflammasome activation[26] linking dyslipidemia, oxidative stress and inflammatory diseases.

Finally, autophagy that has shown to be crucial for the maintenance of intracellular homeostasis and development in most cells including cells of cardiovascular origin (cardiomyocytes, endothelial cells, and arterial smooth muscle cells) and periodontal cells has been related to oxidative stress and inflammation.[27-28] Indeed, autophagic or mitophagic defects have been associated with an increased propensity of laboratory animals to spontaneously develop specific cardiovascular disorders, including multiple forms of cardiomyopathy.[29] Likewise, an elevated autophagosome production has been evidenced by image, protein levels and gene expression analyses in periodontal ligament stem cells from periodontitis patients[30]

Achieving a better understanding of the pathogenic relationship between CVDs and periodontitis is a primary goal in the research for the prevention and treatment of atherosclerotic CVDs. Our aim was to improve the understanding of the pathogenic relationship between CVDs and periodontitis exploring possible differences in inflammation, oxidative stress, and autophagy markers. This aim would allow us to generate new perspectives in the prevention and treatment of AMI and periodontitis. The present study compares between AMI subjects (only AMI or AMI+periodontitis) and control subjects (healthy or only periodontitis). The AMI subjects are subjects who have survived to a first AMI, whereas the control subjects have no cardiac events in their clinical record. Clinical parameters and molecular markers of pathogenic mechanisms involved in AMI and/or periodontitis were evaluated.

2. MATERIAL AND METHODS

2.1. Patients

Male subjects over thirty five years of age, who have not experienced a prior cardiac event and who had no significant coronary disease attending the Dental School of the University of Sevilla (Control) without clinical records of previous cardiac events or diabetes diagnosis and male subjects attending and receiving treatment at the cardiovascular rehabilitation service of the University Hospital "Virgen Macarena", Sevilla, Spain, who had experienced and survived to a previous AMI were asked to participate in the study. Informed consent was obtained from each subject and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a prior approval by the Human Research Committee of Sevilla University.

The sample size was determined for the comparison of a numerical variable between four groups defined by the combination of two dichotomous variables: cardiovascular (yes / no) and periodontitis (yes / no). Data regarding the variances of the means (0.01, 0.27 and 0.07) and the common variance of the groups (0.4) were obtained from a similar published study. Setting the values of α at 5% and powers at 90% (for factor A) and 99% (for factor B and for interaction) we need between 43 patients in each of the 4 groups.

2.2. Clinical examination

2.2.1. General data

Demographic characteristics (age, height and weight), medical record (blood pressure, heart rate, waist circumference and fasting blood glucose (FBG) were recorded and body mass index (BMI) was calculated from standardized measurements of weight and height (weight in kg divided by height in square meters).

2.2.2. Oral examination

The following periodontal parameters were recorded: clinical probing depth (PD), clinical attachment loss (CAL), number of remaining teeth, percentage of sites with bleeding on probing (BOP). All periodontal measures were assessed by the same examiner, whose assessments of the first ten subjects were calibrated for reproducibility before the study; an intra-subject correlation coefficient of 92% was found for PD.

2.2.4 Periodontitis definitions

Periodontitis was defined as the presence of ≥2 interproximal sites with CAL ≥6 mm in different teeth and ≥1 interproximal site with PD ≥5 mm.[32] The severity and extent of periodontal disease were also expressed as a percentage of sites with attachment loss of 4 mm or more, and subjects were categorized as follows: 0% = no periodontitis, >0%–33% = mild periodontitis, >33%–67% = moderate periodontitis, and >67% = severe periodontitis.[33] Therefore, the Control and AMI groups were subcategorized according to these definitions.

2.3. Blood sample collection

Venous blood samples (5 ml) were collected into glass tubes containing K_3-EDTA (Venoject, Terumo, Leuven, Belgium) using the venipuncture procedure. The samples were collected after a 12-h fasting period. All samples were immediately centrifuged at 1000 x *g* for 15 min at 4°C, and plasma was divided into aliquots of 250 µl for immediate analysis or stored at -80°C. PBMCs were isolated and concentrated by isopycnic centrifugation using Histopaque-1119 and Histopaque-1077 (Sigma Chemical Co., St. Louis, MO, USA).

2.4. Lipid peroxidation

Lipid peroxidation in PBMCs was estimated with a thiobarbituric acid reactive substances (TBARS) assay. This assay is performed measuring the levels of malondialdehyde (MDA). The levels of TBARS were determined using a method based on a reaction with thiobarbituric acid (TBA). The reaction was performed at 90°C for 15 min at pH 2-3. The sample was incorporated into two volumes of cold 10% (w/v) trichloroacetic acid to precipitate proteins. This precipitate was pelleted by centrifugation anA total of 260 subjects were enrolled in the study, 106 subjects had no previous AMI history (control group) and 154 subjects had suffered AMI previously (AMI group).d an aliquot of the supernatant was reacted with an equal volume of 0.67% (w/v) TBA in a boiling water bath for 10 min. After cooling, the absorbance was read at 532 nm. The results were expressed as nmol MDA per mg of protein.

2.5. Serum interleukin-1β levels

Interleukin (IL)-1β levels were determine in plasma samples using a commercial ELISA kit (GenWay, San Diego CA, USA) according to the manufacturer's instructions.

2.6. Western blotting

Proteins isolated from PBMC were assayed by western blotting to determine protein levels of phosphorylated AMP-activated protein kinase (p-AMPK) and microtubule-associated proteins 1A/1B-light chain 3-II (LC3-II) as previously reported [34,35] using primary antibodies against p-AMPK, LC3-II and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, Dallas, TX) in a final dilution of 1:1000.

2.7. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics software version 22. Data were represented as means and standard deviations for continuous variables and as percentages for qualitative variables. Pairwise comparisons of continuous variables were performed using Student's *t*-test for normally distributed data or Mann-Whitney U-test for non-normally distributed data. Furthermore, comparisons among more than two groups were performed using analysis of variance (ANOVA) or the non-parametric Kruskal-Wallis test for normally and non-normally distribute data, respectively. Qualitative variables were compared using the Chi-square test of independence.

3. RESULTS

3.1 General data

A total of 260 subjects were enrolled in the study, 106 subjects had no previous AMI history (control group) and 154 subjects had suffered AMI previously (AMI group). These two groups were further divided according to the oral health examination results into subjects with periodontitis (only periodontitis or AMI+periodontitis) or periodontally healthy subjects (healthy or only AMI). Therefore, a total of four groups were established: healthy (n=57); only periodontitis (n=49); only AMI (n=58), and AMI+periodontitis (n=96). Table 1 shows the general data of the sample population. Control subjects presented higher height and systolic blood pressure and lower pulse than AMI subjects. For diastolic blood pressure, these differences were maintained between groups without cardiac events and the AMI+periodontitis group. However, no differences in weight among the four groups were found. BMI, waist circumference and FBG levels in healthy subjects were lower compared to both groups of AMI subjects. Control subjects with periodontitis also showed lower waist circumferences and FBG levels than those found in both groups of AMI subjects. Healthy subjects were slightly younger than AMI+periodontitis subjects.

3.2 Oral examination results

Figure 1 represents the percentage of subjects found in the control group (healthy and only periodontitis) and in the AMI group (only AMI and AMI+periodontitis) separated according to periodontitis severity. The percentage of control subjects without any level of periodontitis was higher than the percentage of AMI subjects without periodontitis. Control subjects with only mild or only severe periodontitis (without AMI) was lower than the percentage of subjects with mild periodontitis+AMI or severe

periodontitis+AMI, respectively. Figure 2 shows mean values and SD of characteristics assessed by the oral examinations (number of teeth, PD, CAL, and BOP). The mean values of PD, CAL and BOP were higher in subjects with periodontitis compared to those periodontally healthy regardless if they had suffered a cardiac event or not. However, differences in the number of teeth were only present between the healthy group and the AMI+periodontitis group, showing the latter a lower value.

3.3 Levels of p-AMPK, LC3-II, MDA and interleukin-1β

Figure 3 shows the protein expression levels of p-AMPK and LC3-II and the levels of MDA in PBMCs, it also shows the serum levels of IL-1β. The levels of MDA determined by TBARS assay were higher in both groups suffering periodontitis, regardless if the subjects have had the cardiac condition or not. Healthy controls showed lower IL-1β serum levels compared with the other three groups. The highest levels of AMPK were found in the healthy control subjects followed by only periodontitis subjects and only AMI subjects, whereas the lowest values were found for AMI+periodontitis subjects. Finally, LC3-II protein levels were the highest for only periodontitis subjects and the lowest for only AMI subjects.

3.4 Correlation between continuous variables

The Pearson's bivariate correlation coefficients between oral and molecular parameters in control subjects and AMI subjects are shown in Table 2. Statistically significant correlations between PD and the four molecular parameters were found in both groups. In AMI subjects, the values of the Pearson's correlations coefficient between PD

and the molecular parameters were positive indicating a direct relationship. However, an inverse correlation was found between AMPK and PD in control subjects.

4. DISCUSSION

The present study aimed to improve our understanding of the pathogenic relationship between AMI and periodontitis and to generate new perspectives in the prevention and treatment of atherosclerotic CVDs and periodontal diseases. For these purposes, subjects with or without periodontitis in a group of subjects that have survived to a first AMI and another group of subjects with no cardiac events in their clinical record were compared evaluating both clinical parameters and molecular markers of common pathogenic mechanisms. To date, many epidemiological studies on the relationship between periodontal health status and atherosclerotic CVDs have been conducted, their results ranged from determinations of no causative relationship to strong connections between the two conditions.[36] Regardless periodontitis diagnosis, patients suffering an AMI present higher values of BMI, waist circumference, systolic blood pressure and FBG levels compared with periodontal healthy patients without cardiac event records. This is expected since hypertension, impaired glucose tolerance and obesity are components included in metabolic syndrome definition,[37] which increases risk for coronary heart diseases.[38] Still, in the present study, a higher proportion of AMI+periodontitis subjects compared to those subjects without periodontitis was found. This difference is also present in the control group, but its magnitude is lower. There are several reasons that could explain the absence of significant findings in some previous studies. Notwithstanding, a recent systematic review indicates that most studies published in the literature supports a modest but consistent association between periodontal disease and

overall CVDs, atherosclerosis and AMI.[39] Results from the present study would support the suggestion of periodontitis as a potential risk factor for AMI.

Despite the reported epidemiological associations between periodontal status and cardiovascular health, periodontitis can be not only a risk factor for CVDs, but also a condition modifying other primary risk factors that could potentially influence the occurrence and course of CVDs.[40] Furthermore it could also be possible that periodontal disease and CVDs have some risk factors in common. Both periodontal diseases and atherosclerotic CVDs are considered inflammatory diseases. At periodontal level, an abnormal host cell response against bacterial pathogens featured by an excessive and continuous production of inflammatory mediators has been proposed as a crucial player in the progression of the disease and tissue destruction. Concerning CVDs, coronary, but also aortic atherosclerotic plaque formation and progression is driven by macrophage infiltration. In many infective and inflammatory diseases, an important pathogenic role has been attributed to pro-inflammatory cytokines promoting cell adhesion, permeability and apoptosis as part of the inflammatory response by interacting with specific receptors on various cell types.[41] Critical events occurring during periodontal disease have been considered a consequence of higher concentrations of different pro-inflammatory cytokines. This fact has been evidenced by the high levels of this type of cytokines found in diseased periodontal tissues[42,43] or gingival crevicular fluid (GCF) of periodontitis patients.[42,44,45] Likewise, persistent increases in cytokines have been associated with vascular dysfunction and vascular disease such as atherosclerosis and hypertension.[41] In particular, IL-1β is one of the central mediators in the cytokine network implicated in inflammation control and regulation.[27,43] Some researchers have suggested that periodontal disease is an independent contributor to systemic inflammation.[5,46] This is based in the fact that common clinical markers of systemic inflammation (C-reactive

protein, IL-6, haptoglobin, and fibrinogen) have been reported to be higher in periodontal patients with AMI than in only AMI patients.[5] However, clinical data on IL-1β levels are scarce. In the present study, no differences concerning IL-1β between AMI subject with or without periodontitis were found. These results are consistent with those found in a previous study conducted in patients with periodontitis, coronary heart disease, or both diseases.[42] In that study, authors reported higher serum IL-1β levels in all groups of patients compared to healthy controls, but no differences in serum IL-1β levels were found among the three pathological conditions studied.[42]

Oxidative stress has been involved in the pathogenesis of coronary atherosclerotic complications and some of its risk factors.[47,48] Likewise, different studies have shown that periodontitis is related to excessive reactive oxygen species (ROS) production or elevated oxidative damage in periodontal tissue,[17] GCF[49] or gingival blood.[50,51] A possible origin for increased oxidative stress has been evidenced studying fibroblasts obtained from periodontitis patients where bacterial lipopolysaccharide led to increased oxidative stress and mitochondrial dysfunction by a decrease in mitochondrial protein expression, mitochondrial mass, and mitochondrial membrane potential.[17] In the present study, periodontitis has been associated with higher concentrations of the circulating lipid peroxidation end product MDA in only periodontitis and in AMI+periodontitis subjects. In turn, elevated circulating markers of oxidative damage in periodontitis subjects have also been reported in previous studies.[49,50,52] This systemic effect of periodontitis has been attributed to the diffusion of ROS produced in the periodontal lesion into the blood stream.[53] A lower citrate synthase activity and high levels of ROS production have also been reported in PBMCs from periodontitis patients, suggesting that this condition could lead to mitochondrial dysfunction and ROS overproduction in these cells.[17] Moreover, the increase in circulating oxidative stress biomarkers, as occurs with diabetes mellitus

and inappropriate nutrition, could damage periodontal tissues.[53,56] Because the present study and most previous studies are cross-sectional, it is still not clear whether the increase of oxidative stress is the cause or the consequence of periodontitis.

In the present study, a positive correlation between lipid peroxidation and IL-1β levels was found in control subjects. This correlation can be explained because polymorphonuclear cells, whose activity and number is increased by high levels of pro-inflammatory cytokines, produce ROS via the respiratory burst mechanism as part of the defense response to infection. Moreover, these cytokines also interact with mitochondria to increase ROS production.[44] Furthermore; oxidative stress is one of the factors that could explain the pathophysiological mechanism of inflammatory conditions occurring in CVD and periodontitis. ROS can evoke immune responses through redox-sensitive gene transcription factors such as NF-κB resulting in the expression of pro-inflammatory cytokines. In addition, oxidative metabolism dictates the inflammatory status of macrophages, effects that may be upstream of inflammasome activity and IL-1β release.[57,58] Abnormal PBMC performance observed in periodontitis subjects may promote oxidative stress and alter cytokine homeostasis.[17] Therefore, an elevated production of ROS at periodontal tissues could result in oxidative damage and IL-1β release. However, the relationship between TBARS and IL-1β was no statistically significant in AMI subjects. This result could be a consequence of IL-1β production during the AMI[48] and, thus, it would not reflect the levels prior to the cardiac event.

Alterations in autophagy or autophagy-dependent mechanisms have also been suggested in the pathogenesis of inflammatory disorders. An elevated autophagosome production has been evidenced by image, protein levels and gene expression analyses in periodontal ligament stem cells from periodontitis patients.[59] Here, LC3-II levels in PBMC were used to evaluate this process. LC3-II is a conjugated form of LC3 protein

used as membrane marker for autophagosome and autophagolysosomes.[60] This marker in the present study would suggest that autophagy initiation is higher in only periodontitis subjects than in AMI+periodontitis subjects. Decreased levels of both LC3 gene expression and LC3-II protein have been reported in the peripheral leucocytes of patients with coronary artery disease. This fact indicates that autophagosome formation is decreased, supporting a positive relationship between this process and atherosclerosis at coronary artery level.[61] Importantly, in another case-control study, beclin-1 and Atg7 gene expression suggested that autophagy initiation was lower in subjects with acute coronary syndrome than in those with stable angina pectoris and in control groups, emphasizing the importance of autophagy for atherosclerotic plaque stability.[44] In the present study, LC3-II levels were lower in subjects that have suffered AMI than in those without cardiac event history, regardless periodontitis diagnosis. This suggests that maintaining a higher rate of autophagy initiation could contribute to decrease AMI risk. On the other hand, it seems that circulating levels of this marker can be altered by periodontal condition.

Some studies have suggested that IL-1β induces autophagy with beneficial effects against oxidative stress in other cell types.[27,29] The involvement of IL-1β in autophagy in PBMC would be supported by the correlation found in the present study between IL-1β and LC3-II levels in groups without AMI history (control subjects). Importantly, no significant correlation was found between the same markers when an AMI had occurred. Autophagy also can increase in response to the accumulation of oxidatively damaged cell structures. Moreover, some redox-sensitive pathways also result in autophagy.[24] In turn, this process can protect cells against oxidative damage, regulating ROS generation and scavenging. Autophagy has also been correlated with mitochondrial production of ROS in PBMC obtained from periodontitis patients in a previous study. Concomitantly, a

significantly positive correlation between mitochondrial ROS and autophagy makers was also observed.[62] The values of LC3-II levels and TBARS obtained in our study show a correlation in the same sense, but only in AMI subjects. Therefore, ROS levels or oxidative damage would be the main stimulus triggering autophagy in this group.

With regard to AMPK, the information available from the literature is rather scarce in relation to periodontitis. In the present study, PBMC levels of the more active form of AMPK (p-AMPK) are lower in subjects with periodontitis in the absence of AMI than in healthy subjects. Regarding cardiovascular system, this marker has been reported to be decreased in patients with acute coronary syndrome compared to control patients.[44] In our study, differences were found between AMI subjects (only AMI *vs.* AMI+periodontitis). Interestingly, in the AMI group, AMPK levels are inversely correlated with TBARS, which would be in consistency with the role of AMPK in the mechanisms of activation of the antioxidant defense. However, no correlation between AMPK and IL-1β was noted, despite of some suggested roles for AMPK-dependent pathways in the NLRP3 inflammasome regulation.[63]

Several limitations of the study need to be addressed. One of the major strengths of the present study was the well-defined control and test groups. Many studies are prone to misclassify periodontal status by using measures to assess periodontitis known to underestimate the true extent and severity of periodontitis, including indices based on non-probing assessment and questionnaires, as well as assessments of partial-mouth probing.[64] In the present study full-mouth recordings were used to define periodontitis and its severity. The latter makes possible to analyze if periodontitis prevalence is similar for different degrees of the disease. However, the association magnitude and significance may be influenced by the small sample size when the severity is high. Furthermore, the CVD-affected group consisted of a well-defined group of patients with coronary heart

disease, as all test subjects had suffered an AMI. However, control subjects were not recruited at the same hospital as the AMI subjects. Unfortunately, as consequence of only including subjects that have survived to a first AMI, sample size was more reduced respect to subjects suffering AMIs in general population. In addition, this study only included subjects who were referred to a periodontal clinic in the control group leading to overrepresentation of periodontal disease compared with the general population. Other important limitation is the absence of female subjects.

5. CONCLUSIONS

Results from the present study are consistent with the suggestion that periodontitis is a risk factor for AMI. Periodontitis association with peroxidative lipid damage in both control and AMI subjects suggests that oxidative stress could be a common pathogenic factor between both diseases. In addition, a positive correlation between lipid peroxidation and IL-1β levels was found in control subjects which could be a consequence of ROS production by polymorphonuclear cell stimulated by proinflammatory cytokines. However, no differences in IL-1β levels were found between AMI subjects. Still, current IL-1β levels could be different from levels prior to the cardiac event since such cytokine is also produced during AMI. Because our study and most of previous studies are cross-sectional, it is not clear whether the increase in oxidative stress is the cause or the consequence of periodontitis.

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CONFLICT OF INTEREST

The authors declare no potential conflicts of interest related to this article.

REFERENCES

1. A66_R10-en.pdf,(n.d.). http://apps.who.int/gb/ebwha/pdf_files/WHA66/A66_R10-en.pdf?ua=1 (accessed December 5, 2018).
2. Murray CJ, Lopez AD. Alternative projections of mortality and disability by cause 1990–2020: Global Burden of Disease Study. *The Lancet.* 1997;349:1498-1504.
3. Ford ES, Ajani UA, Croft JA, et al. Explaining the decrease in US deaths from coronary disease, 1980–2000. *N Engl J Med.* 2007;356:2388-2398.
4. Webster R, Heeley E. Perceptions of risk: understanding cardiovascular disease. *Risk Manag Healthc Policy*. 2010;3:49-60.
5. Moutsopoulos NM, Madianos PN. Low-grade inflammation in chronic infectious diseases: paradigm of periodontal infections *Ann NY Acad Sci.* 2006;1088:251-264.
6. Kassebaum NJ, Bernabé E, Dahiya M, Bhandari B, Murray CJL, Marcenes W. Global burden of severe periodontitis in 1990-2010: a systematic review and meta-regression. *J Dent Res.* 2014;93:1045-1053.
7. Cekici A, Kantarci A, Hasturk H, Van Dyke TE. Inflammatory and immune pathways in the pathogenesis of periodontal disease. *Periodontol 2000*. 2014;64:57-80.
8. Shrivastava AK, Singh HV, Raizada A, Singh SK. C-reactive protein, inflammation and coronary heart disease. *Egypt Heart J.* 2015;67:89-97.
9. Schenkein HA, Loos BG. Inflammatory Mechanisms Linking Periodontal Diseases to Cardiovascular Diseases. J Clin Periodontol. 2013; 40(014):S51–S69.
10. Kizildag A, Arabaci T, Dogan GE. Relationship between periodontitis and cardiovascular diseases: A literature review. *World J Stomatol.* 2014;3(1):1-9.
11. Kurita-Ochiai T, Jia R, Cai Y, Yamaguchi Y, Yamamoto M. Periodontal Disease-Induced Atherosclerosis and Oxidative Stress. *Antioxidants (Basel)*. 2015;4(3):577–590.

12. Giannopoulou C, Krause KH, Muller F. The NADPH oxidase NOX2 plays a role in periodontal pathologies. *Semin Immunopathol*. 2008;30:273–278.
13. Yang D, Elner SG, Bian Z-M, Till GO, Petty HR, Elnera VM. Pro-inflammatory Cytokines Increase Reactive Oxygen Species through Mitochondria and NADPH Oxidase in Cultured RPE Cells. *Exp Eye Res*. 2007;85(4):462–472.
14. Pourova J, Kottova M, Voprsalova M, Pour M. Reactive oxygen and nitrogen species in normal physiological processes. *Acta Physiol*. 2010;198:15–35.
15. Katsuragi H, Ohtake M, Kurasawa I, Saito K. Intracellular production and extracellular release of oxygen radicals by PMNs and oxidative stress on PMNs during phagocytosis of periodontopathic bacteria. *Odontology*. 2003;91:13–18.
16. Sculley DV, Langley-Evans SC. Salivary antioxidants and periodontal disease status. *Proc Nutr Soc*. 2002;61:137–143.
17. Bullon P, Cordero MD, Quiles JL, Morillo JM, Ramirez-Tortosa MC, Battino M. Mitochondrial dysfunction promoted by Porphyromonas gingivalis lipopolysaccharide as a possible link between cardiovascular disease and periodontitis. *Free Radic Biol Med*. 2011;50(10):1336-43.
18. Franek E, Napora M, Blach A, Budlewski T, Gozdowski D, Jedynasty K, et al. Blood pressure and left ventricular mass in subjects with type 2 diabetes and gingivitis or chronic periodontitis. *J Clin Periodontol*. 2010;37:875–80.
19. Amar S, Gokce N, Morgan S, Loukideli M, Van Dyke TE, Vita JA. Periodontal disease is associated with brachial artery endothelial dysfunction and systemic inflammation. Arterioscler *Thromb Vasc Biol*. 2003;23:1245–9. 59.
20. Vlachopoulos C, Dima I, Aznaouridis K, Vasiliadou C, Ioakeimidis N, Aggeli C, et al. Acute systemic inflammation increases arterial stiffness and decreases wave reflections in healthy individuals. *Circulation*. 2005;112:2193–200.

21. Tsioufis C, Kasiakogias A, Thomopoulos C, Stefanadis C. Periodontitis and blood pressure: The concept of dental hypertension. *Atherosclerosis.* 2011;219:1–9.
22. Gurav AN. The association of periodontitis and metabolic syndrome. *Dent Res J.* 2014;11(1):1–10.
23. Fentoglu O, Bozkurt FY. The Bi-Directional Relationship between Periodontal Disease and Hyperlipidemia. *Eur J Dent.* 2008 2:142–146.
24. Liu C, Mo L, Niu Y, Li X, Zhou X, Xu. The role of reactive oxygen species and autophagy in periodontitis and their potential Linkage. *Front Physiol.* 2017;8:439.
25. Martinon F. Signaling by ROS drives inflammasome activation. *Eur J Immunol.* 2010;40(3):616-9.
26. An Y, Liu W, Xue P, Zhang Y, Wang Q, Jin Y. Increased autophagy is required to protect periodontal ligament stem cells from apoptosis in inflammatory microenvironment. *J Clin Periodontol*. 2016;43:618-625.
27. Joosten LA, Netea MG, Dinarello CA. Interleukin-1β in innate inflammation, autophagy and immunity. *Semin Immunol*. 2013;25:416-424.
28. Ansari MY, Khan MN, Haqqi TM. IL-1β induces low levels of autophagy which clears dysfunctional mitochondria and inhibits ROS, MMP-13 and ADAMTS5 expression in OA chondrocytes. *Osteoarthritis Cartilage*. 2016;24:S135-S136.
29. Griffin ST, Parcon PA, Balasubramaniam M, et al. Il-1β influences autophagy by mediating upregulation of parkin and parkin neddylation in cell culture and animal models, and mimics the pattern seen in ad brain. *Alzheimers Dement J Alzheimers Assoc*. 2018;14:P737-P738.
30. Liu W, Yin Y, Zhou Z, He M, Dai Y. OxLDL-induced IL-1 beta secretion promoting foam cells formation was mainly via CD36 mediated ROS production leading to NLRP3 inflammasome activation. *Inflamm Res*. 2014;63(1):33-43.

31. Bravo-San Pedro JM, Kroemer G, Galluzzi L. Autophagy and mitophagy in Cardiovascular Disease. Circulation Res. 2017;120:1812–1824.
32. Page RC, Eke PI. Case definitions for use in population-based surveillance of periodontitis. *J Periodontol.* 2007;78(7 Suppl):1387-99.
33. Arbes SJ, Jr, Slade GD, Beck JD. Association between extent of periodontal attachment loss and self-reported history of heart attack: an analysis of NHANES III data. *J Dent Res.* 1999;78:1777-1782.
34. Cheng J, Qiao L, Xu L, et al. Lower AMP-activated protein kinase level is associated with the vulnerability of coronary atherosclerotic plaques by attenuating the expression of monocyte autophagy. *Coron Artery Dis.* 2015;26:322-327.
35. Bullon P, Marin-Aguilar F, Roman-Malo L. AMPK/mitochondria in metabolic diseases. In: Cordero MD, Viollet B, eds. *AMP-Activated Protein Kinase*. AG
36. Friedewald VE, Kornman KS, Beck JD, et al. American Journal of Cardiology, Journal of Periodontology, The American Journal of Cardiology and Journal of Periodontology editors' consensus: periodontitis and atherosclerotic cardiovascular disease. *J Periodontol.* 2009;80:1021-1032.
37. Reaven GM. The metabolic syndrome: Requiescat in pace. Clin Chem. 2005;51:931–8.
38. Bray GA, Champagne CM. Obesity and the Metabolic Syndrome: Implications for dietetics practitioners. J Am Diet Assoc. 2004;104:86–9.
39. Xu S, Song M, Xiong Y, Liu X, He Y, Qin Z. The association between periodontal disease and the risk of myocardial infarction: a pooled analysis of observational studies. *BMC Cardiovasc Disord*. 2017;17:50.
40. Górska R, Dembowska E, Konopka TP, Wysokińska-Miszczuk J, Pietruska M, Ganowicz E. Correlation between the state of periodontal tissues and selected risk

factors for periodontitis and myocardial infarction. *Adv Clin Exp Med*. 2017;26:505-514.

41. Sprague AH, Khalil RA. Inflammatory cytokines in vascular dysfunction and vascular disease. *Biochem Pharmacol*. 2009;78:539-552.
42. Zhu H, Lin X, Zheng P, Chen H. Inflammatory cytokine levels in patients with periodontitis and/or coronary heart disease. *Int J Clin Exp Pathol*. 2015;8:2214-220.
43. Okada H, Murakami S. Cytokine Expression in periodontal health and disease. *Crit Rev Oral Biol Med*. 1998;9:248-266.
44. Orozco A, Gemmell E, Bickel M, Seymour GJ. Interleukin-1 β, interleukin-12 and interleukin-18 levels in gingival fluid and serum of patients with gingivitis and periodontitis. *Oral Microbiol Immunol*. 2006;21:256-260.
45. Toldo S, Mezzaroma E, Tassell BWV, et al. Interleukin-1β blockade improves cardiac remodelling after myocardial infarction without interrupting the inflammasome in the mouse. *Exp Physiol*. 2013;98:734-745.
46. Dinarello CA. A clinical perspective of IL-1β as the gatekeeper of inflammation. *Eur J Immunol*. 2011;41:1203-1217.
47. He F, Zuo L. Redox roles of reactive oxygen species in cardiovascular diseases. *Int J Mol Sci*. 2015;16:27770-27780.
48. Paneni F, Cañestro CD, Libby P, Lüscher TF, Camici GG. The Aging Cardiovascular System: Understanding it at the cellular and clinical levels. *J Am Coll Cardiol*. 2017;69:1952-1967.
49. Akalin FA, Baltacioğlu E, Alver A, Karabulut E. Lipid peroxidation levels and total oxidant status in serum, saliva and gingival crevicular fluid in patients with chronic periodontitis. *J Clin Periodontol*. 2007;34:558-565.

50. Baltacioğlu E, Akalin FA, Alver A, Değer O, Karabulut E. Protein carbonyl levels in serum and gingival crevicular fluid in patients with chronic periodontitis. *Arch Oral Biol*. 2008;53:716-722.

51. Konopka T, Król K, Kopeć W, Gerber H. Total antioxidant status and 8-hydroxy-2′-deoxyguanosine levels in gingival and peripheral blood of periodontitis patients. *Arch Immunol Ther Exp*. 2007;55:417-425.

52. Panjamurthy K, Manoharan S, Ramachandran CR. Lipid peroxidation and antioxidant status in patients with periodontitis. *Cell Mol Biol Lett*. 2005;10:255-264.

53. Tomofuji T, Irie K, Sanbe T, et al. Periodontitis and increase in circulating oxidative stress. *Jpn Dent Sci Rev*. 2009;45:46-51.

54. Graves DT, Kayal RA. Diabetic complications and dysregulated innate immunity. *Front Biosci J Virtual Libr*. 2008;13:1227-1239.

55. Irie K, Tomofuji T, Tamaki N, et al. Effects of ethanol consumption on periodontal inflammation in rats. *J Dent Res*. 2008;87:456-460.

56. Sanbe T, Tomofuji T, Ekuni D, Azuma T, Tamaki N, Yamamoto T. Oral administration of vitamin C prevents alveolar bone resorption induced by high dietary cholesterol in rats. *J Periodontol*. 2007;78:2165-2170.

57. Nukaga J, Kobayashi M, Shinki S, et al. Regulatory effects of interleukin-1β and prostaglandin E2 on expression of receptor activator of nuclear factor-κB Ligand in human periodontal ligament cells. *J. Periodontol*. 2004;75:249-259.

58. van de Veerdonk FL, Smeekens SP, Joosten LAB, et al. Reactive oxygen species-independent activation of the IL-1beta inflammasome in cells from patients with chronic granulomatous disease. *Proc Natl Acad Sci USA*. 2010;107:3030-3033.

59. An Y, Liu W, Xue P, Zhang Y, Wang Q, Jin Y. Increased autophagy is required to protect periodontal ligament stem cells from apoptosis in inflammatory microenvironment. *J Clin Periodontol*. 2016;43:618-625.

60. Klionsky DJ, Baehrecke EH, Brumell JH, et al. A comprehensive glossary of autophagy-related molecules and processes (2nd edition). *Autophagy*. 2011;7:1273-1294.

61. Wu G, Wei G, Huang J, Pang S, Liu L, Yan B. Decreased gene expression of LC3 in peripheral leucocytes of patients with coronary artery disease. *Eur J Clin Invest*. 2011;41:958-963.

62. Bullon P, Cordero MD, Quiles JL, et al. Autophagy in periodontitis patients and gingival fibroblasts: unraveling the link between chronic diseases and inflammation. *BMC Med*. 2012;10:122.

63. Cordero MD, Williams MR, Ryffel B. AMP-Activated Protein Kinase Regulation of the NLRP3 Inflammasome during Aging. *Trends Endocrinol Metab*. 2018;29:8-17.

64. Baelum V, Papapanou PN. CPITN and the epidemiology of periodontal disease. *Community Dent Oral Epidemiol*. 1996;24:367-368.

FIGURE LEGENDS

Figure 1. Periodontitis classification according to cardiac condition. AMI: acute myocardial infarction. Data are represented as % of the sample population. * statistically significant differences ($P<0.05$).

Figure 2. Oral examination according to the group distribution. AMI: acute myocardial infarction; BOP: bleeding on probing; CAL: clinical attachment level; PD: probing

pocket depth. PERIO: periodontitis. Results are represented as mean ± SD. For each parameter, bars not sharing superscript letters are significantly different ($P<0.05$).

Figure 3. Peripheral blood mononuclear cells lipid peroxidation, serum interleukin-1beta (IL-1β) and heart protein levels of 5' adenosine monophosphate-activated protein kinase (AMPK) and microtubule-associated protein 1A/1B-light chain II (LC3-II) in the experimental groups. AMI: acute myocardial infarction. PERIO: periodontitis. Results are represented as mean ± SD. For each parameter, bars not sharing superscript letters are significantly different ($P<0.05$).

Table 1. General data of the sample population.

	Healthy (n=57)	Periodontitis (n=49)	AMI (n=58)	AMI + periodontitis (n=96)
Age (years)	49.80±12.18[a]	55.86±11.62[ab]	54.84±11.625[ab]	56.81±9.56[b]
Height (cm)	175.00±7.34[b]	174.00±6.24[b]	169.16±6.20[a]	168.86±6.524[a]
Weight (kg)	84.58±11.53	87.48±16.86	87.97±13.77	84.89±14.38
BMI (kg/m^2)	27.71±3.76[a]	28.64±4.44[ab]	30.77±4.78[b]	29.71±4.34[b]
Waist circunferente (cm)	98.61±13.01[a]	98.99±16.55[a]	105.44±10.88[b]	121.12±59.17[b]
Systolic blood pressure (mm Hg)	143.7±23.8[b]	155.3±20.6[b]	115.7±16.3[a]	121.0±16.3[a]
Diastolic blood pressure (mm Hg)	83.4±12.0[b]	85.7±10.1b[b]	70.7±8.5[a]	74.2±9.8[ab]
Fasting blood glucose (mg/dL)	95.69±25.52[a]	97.14±19.57[a]	108.59±33.77[b]	106.54±15.29[b]
Heart rate (beats/min)	65.2±9.2[a]	69.0±12.0[a]	75.0±13.6[b]	75.7±14.4[b]

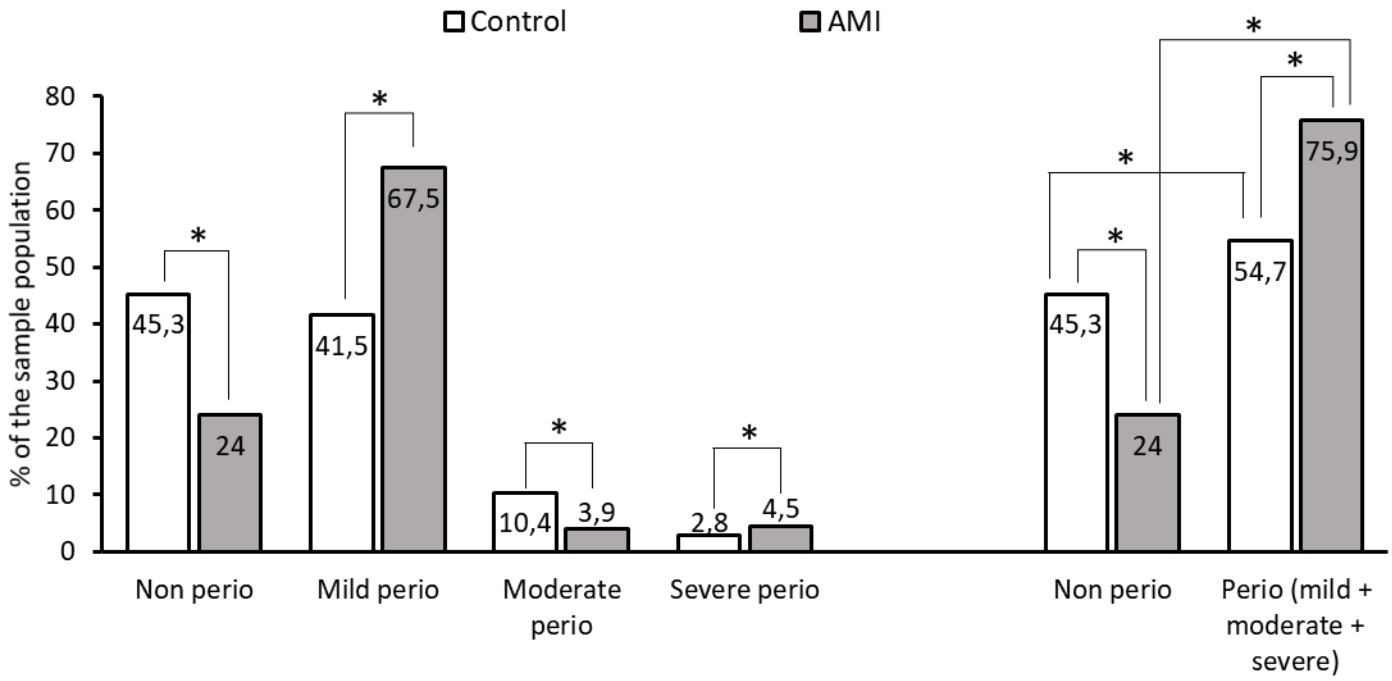
AMI: acute myocardial infarction BMI: body mass index. Data are expressed as mean ± standard deviation. For each variable, means not sharing superscript letters are statistically different (P<0.05).

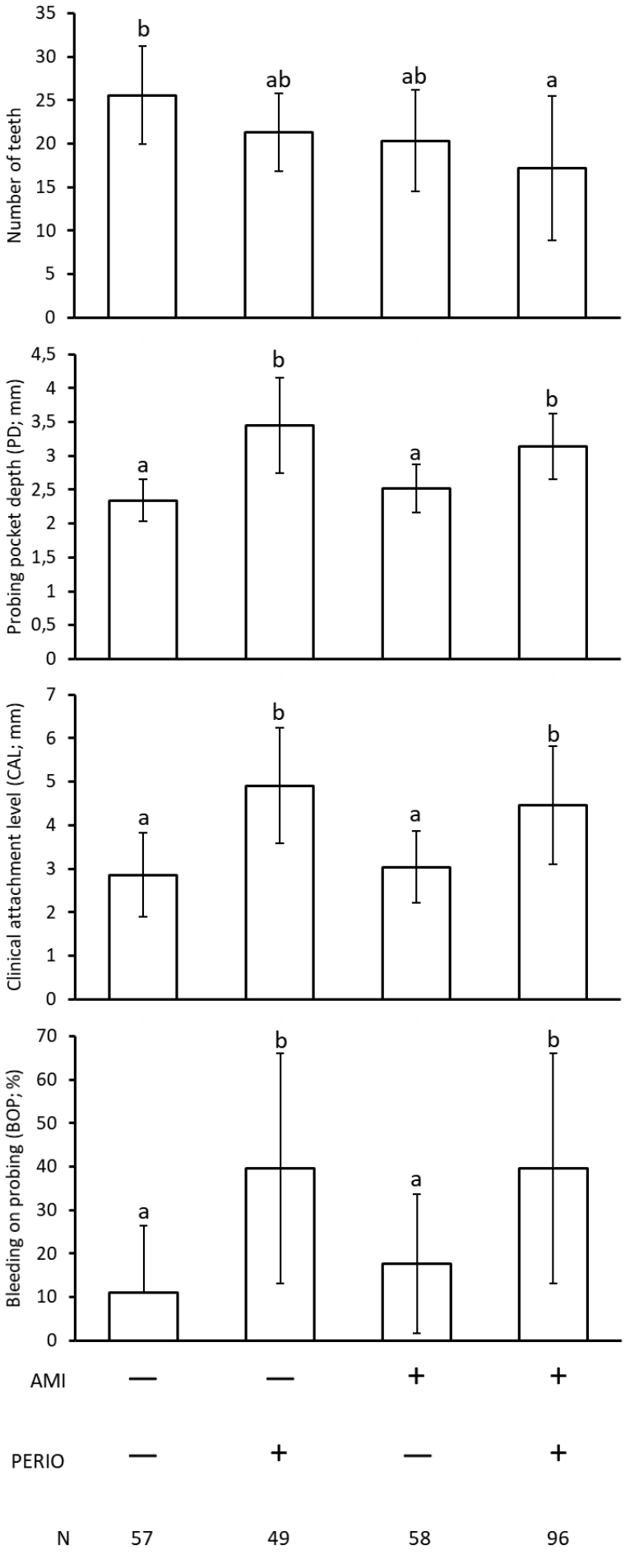
Table 2. Pearson's correlation coefficients (r) between periodontal and molecular parameters.

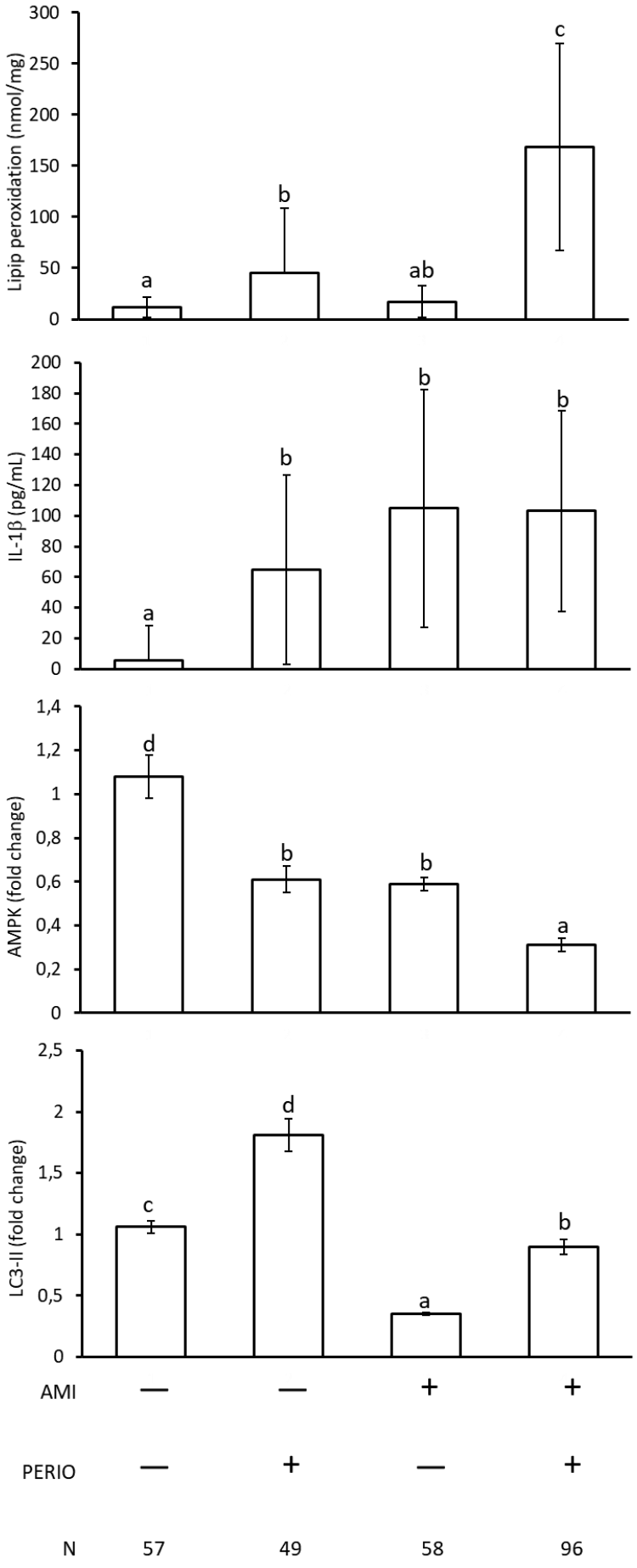
	No. of teeth	PD	CAL	BOP	TBARS	IL-β
			Control subjects			
TBARS	-0.100	0.208*	0.279*	0.292*	1	0.327*
IL-1β	-0.076	0.872*	0.427	0.4449*	0.327*	1
AMPK	-0.007	-0.807*	-0.898*	-0.382	-0.216	-0.362
LC3-II	0.056	0.872*	0.427	0.375	-0.002	0.512*
			AMI subjects			
TBARS	-0.055	0.476*	-0.488*	0.317*	1	-0.048
IL-1β	-0.027	0.476*	-0.058	0.011	-0.048	1
AMPK	0.345	0.828*	-0.725*	-0.461*	-0.725*	0.070
LC3-II	-0.296	0.807*	0.649*	0.574*	0.725*	-0.004

AMI: acute myocardial infarction; AMPK: 5' adenosine monophosphate-activated protein kinase; BOP: bleeding on probing; CAL: clinical attachment level; IL-1β: interleukin 1β; LC3-II: Microtubule-associated proteins 1A/1B-light chain 3-II; PD: probing pocket depth.

*Statistically significant differences ($P<0.05$).


Control
AMI
% of the sample population
80
70
60
50
40
30
20
10
0
45,3
24
41,5
67,5
10,4
3,9
2,8
4,5
45,3
24
54,7
75,9
*
Non perio
Mild perio
Moderate perio
Severe perio
Non perio
Perio (mild + moderate + severe)


Number of teeth
35
30
25
20
15
10
5
0
b
ab
ab
a
Probing pocket depth (PD; mm)
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
a
b
a
b
Clinical attachment level (CAL; mm)
7
6
5
4
3
2
1
0
a
b
a
b
Bleeding on probing (BOP; %)
70
60
50
40
30
20
10
0
a
b
a
b
AMI
—
—
+
+
PERIO
—
+
—
+
N
57
49
58
96


Lipip peroxidation (nmol/mg)
300
250
200
150
100
50
0
a
b
ab
c
IL-1β (pg/mL)
200
180
160
140
120
100
80
60
40
20
0
a
b
b
b
AMPK (fold change)
1,4
1,2
1
0,8
0,6
0,4
0,2
0
d
b
b
a
LC3-II (fold change)
2,5
2
1,5
1
0,5
0
c
d
a
b
AMI — — + +
PERIO — + — +
N 57 49 58 96