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UNIVERSITÀ DEGLI STUDI DI PALERMO

DOCTORAL THESIS

"Effect of statin treatment on periodontitis. Relationship between periodontitis and plasma lipids"

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4. ABSTRACT

Background and objectives:

The scientific evidence shows a possible association between hyperlipidaemia and periodontitis, being the inflammation the common component between these two conditions. It is postulated that statins seem to have an effect at periodontal level by three different mechanisms. Firstly, the lipid lowering effect with a consequent improvement in the lipid level, could have an influence on the general inflammatory status, as well as on periodontitis. Secondly, a direct anti-inflammatory action on the periodontium independent of lipid-lowering and, thirdly, an anabolic effect on bone, reducing resorption and even stimulating bone regeneration. The presented thesis is divided in two parts. The first part consisted on a clinical study which aimed to determine whether simvastatin consumption and hyperlipidaemia were associated with a worse periodontal condition, non-specific inflammatory mediators and specific bone activity biomarkers. The second part of the study, was an *in vitro* study that aimed to assess the expression of differentiation, proliferation and cellular activity markers on a cellular population of osteoblast cell line treated with simvastatin.

Materials and Methods:

The clinical study consisted on a double-blind observational and analytical cross-sectional study. 73 patients were divided into three groups: 1) simvastatin-treated patients with hyperlipidemia (n = 29); 2) patients with hyperlipidemia treated by diet alone (n = 28); and 3) normolipidaemic patients (controls, n = 16). Periodontal clinical variables of all participants were gathered, a blood sample was drawn from each to determine the lipid profile (total cholesterol, triglycerides, low-density lipoprotein, and high-density lipoprotein), serum levels of acute phase reactants (C-reactive protein), erythrocyte

sedimentation rate, and bone metabolism markers (osteoprotegerin, osteocalcin, procollagen type I N-terminal propeptide, and C-terminal telopeptide of type I collagen). For the *in* vitro study, MG63 human osteosarcoma cells were cultured in the presence of simvastatin or solvent alone for 72 hours, and their proliferation was assessed by MTT assay. Cells from the culture were prepared for light, transmission and scanning electron microscopy studies. immunocytochemical was used to analyze the differentiation and proliferation markers Musashi-1, Ki-67, CD56 and CD44. All gathered data was analysed using descriptive, bivariate and multivariate methods to evaluate the association between exposure and effect, fitting the model by the different confounder variables.

Results:

Results from the clinical study showed that mean erythrocyte sedimentation rate was higher in the diet-treated patients with hyperlipidemia than in the normolipidaemic controls (P = 0.04). Serum osteoprotegerin concentrations were significantly higher in the simvastatin-treated patients with hyperlipidaemia than in the diet-treated patients with hyperlipidaemia (p=0.05). Multivariable linear regression analysis adjusted for age, sex, tobacco, and alcohol revealed that, compared with the normolipidaemic patients, the simvastatin-treated patients with hyperlipidemia showed a mean reduction of 0.8 mm (95% confidence interval = -1.5 to 0.0; p=0.05) in clinical attachment loss.

The *in vitro* results in the cultured MG63 showed that control cells had spheroid morphology with numerous secretion vesicles accumulated on the surface, observing no cytoplasmic projections with intercellular connections. However, cells cultured with simvastatin had a polygonal and spindle-shaped morphology, with cytoplasmic projections that interconnected cells. There were numerous microvilli-like filamentous projections on the surface with no defined pattern. At 72 hours of culture, CD56, Ki-67

and Musashi-1 expression were significantly reduced (p<0.001) in simvastatin-treated cells. CD44 expression was intense in both groups and was not affected by simvastatin treatment.

Conclusions:

The results of the clinical study showed that the intake of simvastatin is associated with increased serum osteoprotegerin concentrations, and this could have a protective effect against bone breakdown and periodontal attachment loss. The baseline systemic inflammatory state of patients with hyperlipidemia was indicated by their increased erythrocyte sedimentation rate. Regarding the *in vitro* results, MG63 cells cultured with simvastatin for 72 hours underwent morphological and surface changes. Simvastatin treatment exerts antiproliferative and differentiating effects on these cells as well as promoting recovery of cellular homeostasis.

Keywords: Cardiovascular risk, Hyperlipidaemia, Lipids, Lipoproteins, Periodontitis, Hydroxymethylglutaryl-CoA Reductase Inhibitors, Simvastatin, Inflammation, Osteoblasts.

5. RESUMEN

Introducción y objetivos:

La evidencia científica ha mostrado una posible asociación entre la hiperlipemia y la periodontitis debido al componente inflamatorio que estas dos condiciones tienen en común. Así mismo, se ha postulado como las estatinas parecen tener un efecto a nivel periodontal que estaría mediado por tres mecanismos distintos. En primer lugar, el efecto hipolipemiante conllevaría una mejora en el perfil lipídico y, por tanto, en el estado inflamatorio general del sujeto y en la periodontitis. En segundo lugar, un efecto anti-inflamatorio directo sobre el periodonto independiente del efecto hipolipemiante y, en tercer lugar, un efecto anabólico sobre el hueso, reduciendo la reabsorción y estimulando la regeneración ósea. La presente tesis se divide en dos partes. La primera parte consiste en un estudio clínico cuyo objetivo fue determinar si la hiperlipemia y el consumo de simvastatina estaban asociados a una peor condición periodontal, así como a la expresión de marcadores de inflamación inespecíficos y marcadores de actividad ósea. La segunda parte consistió en un estudio *in vitro* con el objetivo de comprobar el efecto de un tratamiento con simvastatina sobre marcadores de proliferación, diferenciación y actividad celular en una línea osteoblástica.

Material y métodos:

El estudio clínico consistió en un diseño transversal observacional y analítico. 73 pacientes fueron divididos en tres grupos: 1) pacientes hiperlipémicos tratados con simvastatina (n = 29); 2) pacientes hiperlipémicos tratados solo con dieta (n = 28); y 3) pacientes controles normolipémicos (n = 16). Se registraron variables clínicas periodontales en todos los participantes, así como muestras de sangre para determinación del perfil lipídico (colesterol total, triglicéridos, lipoproteínas de baja densidad y

lipoproteínas de alta densidad), niveles séricos de reactantes de fase aguda (proteína C reactiva), velocidad de sedimentación globular, y marcadores del metabolismo óseo (osteoprotegerina, osteocalcina, propéptido del procolágeno N-terminal tipo I y telopéptiodo C-terminal del colágeno tipo I). Para el estudio *in vitro,* células MG63 de osteosarcoma fueron cultivadas en presencia de simvastatina o sólo con el disolvente durante 72 horas. Se realizó una determinación de la proliferación mediante ensayo MTT. Tras el cultivo, las células fueron preparadas para estudio mediante microscopía óptica y electrónica de transmisión y barrido. Mediante técnica inmunocitoquímica se analizaron los marcadores de diferenciación y proliferación Musashi-1, Ki-67, CD56 y CD44. Todos los datos recogidos en ambos estudios fueron analizados de manera descriptiva y mediante técnicas bivariantes y multivariantes, para evaluar la asociación entre la exposición y el efecto, ajustando el modelo por las diferentes variables confundentes.

Resultados:

Los resultados del estudio clínico mostraron una velocidad de sedimentación globular mayor en los pacientes hiperlipémicos tratados solo con dieta frente a los controles normolipémicos (p=0.04). Las concentraciones de osteoprotegerina sérica fueron significativamente mayores en los pacientes hiperlipémicos tratados con simvastatina frente a los tratados solamente con dieta (p=0.05). El análisis de regresión multivariante ajustado por edad, sexo, consumo de tabaco y alcohol mostró que, comparado con los pacientes normolipémicos, los pacientes hiperlipémicos tratados con simvastatina mostraron una reducción de 0.8 mm (Intervalo de confianza 95%= -1.5 a 0.0; p=0.05) en los niveles de pérdida de inserción clínica.

Los resultados del estudio *in vitro* mostraron como las células MG63 control presentaron una morfología esferoide con numerosas vesículas en superficie, y no presentaban

proyecciones citoplasmáticas ni conexiones intercelulares. Por el contrario, las células MG63 cultivadas con simvastatina presentaron una morfología poligonal y fusiforme, con proyecciones citoplasmáticas interconectando células. Presentaban en superficie numerosas proyecciones filamentosas similares a *microvilli* sin un patrón definido. Tras 72 horas de cultivo, la expresión de CD56, Ki-67 y Musashi-1 se redujo de manera significativa ($p<0.001$) en las células tratadas con simvastatina. La expresión de CD44 fue intensa en ambos grupos y no se vio afectada por el tratamiento con simvastatina.

Conclusiones:

Los resultados del estudio clínico mostraron como el consume de simvastatina se asoció a unas concentraciones séricas superiores de osteoprotegerina. Esto podría implicar un efecto protector frente a la destrucción ósea y la pérdida de inserción periodontal. Los pacientes con hiperlipemia mostraron un estado inflamatorio basal reflejado en una mayor velocidad de sedimentación globular. Con respecto a los resultados *in vitro,* las células MG63 cultivadas en simvastatina durante 72 horas sufrieron cambios morfológicos y de superficie. El tratamiento con simvastatina ejerció efectos antiproliferativos y diferenciadores en estas células, promoviendo la recuperación de la homeostasis celular.

Palabras clave: Cardiovascular risk, Hyperlipidaemia, Lipids, Lipoproteins, Periodontitis, Hydroxymethylglutaryl-CoA Reductase Inhibitors, Simvastatin, Inflammation, Osteoblasts.

LIST OF PUBLICATIONS

The results of this doctoral thesis have been accepted for publication in indexed journal with impact factor, with the following references:

Magan-Fernandez A, Papay-Ramirez L, Tomas J, Marfil-Alvarez R, Rizzo M, Bravo M, et al. Association of simvastatin and hyperlipidemia with periodontal status and bone metabolism markers. J Periodontol. 2014;85(10):1408-15.

Mesa F, Magan-Fernandez A, Nikolic D, Marfil-Alvarez R, Nibali L, Rizzo M. Periodontitis, blood lipids and lipoproteins. Clinical Lipidology. 2014;9(2):261-76.

Magan-Fernandez A, Fernandez-Barbero JE, F OV, Ortiz R, Galindo-Moreno P, Mesa F. Simvastatin exerts antiproliferative and differentiating effects on MG63 osteoblast-like cells: Morphological and immunocytochemical study. J Periodontal Res. 2018;53(1):91-7.

INTRODUCTION

6. INTRODUCTION

Part of this section has been published as a comprehensive review with the following citation: Mesa F, Magan-Fernandez A, Nikolic D, Marfil-Alvarez R, Nibali L, Rizzo M. Periodontitis, blood lipids and lipoproteins. Clinical Lipidology. 2014;9(2):261-76.

6.1 PERIODONTITIS

6.1.1 Concept and Epidemiology

Periodontitis is an infectious and inflammatory disease that affects the tooth-supporting tissues, and exhibits a wide range of clinical, microbiological, and immunological manifestations. It is associated with and probably caused by a multifaceted dynamic interaction among specific subgingival microbes, host immune responses, hazardous environmental exposure, and genetic propensity (Slots, 2013). Periodontitis is considered as the main cause of tooth loss in people older than 40 years, being even more frequent that caries. A high prevalence of periodontitis has been reported, with more than 47% of adults (more than 60 million) in the USA affected and the prevalence still grows every year (Marcenes et al., 2013, Eke et al., 2015).

Clinical characteristics of periodontitis are: gingival inflammation (redness and swelling), bleeding on probing, reduced resistance from the periodontal tissues to probing and formation of a periodontal pocket, caused by the loss on insertion (destruction of connective tissue and alveolar bone). Chronic periodontitis is the consequence of an initial gingival inflammatory process, gingivitis. Gingivitis has a high prevalence during puberty and a maintained over the years can progress to periodontitis. Gingival recession,

tooth furcation exposure, tooth mobility, tooth migration, suppuration, pain and food packing are among the common clinical manifestations of periodontitis. Chronic inflammation causes the destruction of the connective tissue of the periodontal ligament, the resorption of the alveolar bone, causing mobility and eventually the loss of the tooth. The mean estimated alveolar bone destruction rate in millimeters is of 0.05-0.1 mm/year (Loe et al., 1992). The final consequence of periodontitis is the loss of the affected tooth after the complete loss of periodontal support.

Periodontal inflammation is characterized by a chronic inflammatory infiltrate of varying intensity. This infiltrate is mainly composed of lymphocytes, plasmatic cells and macrophages distributed in patches on the lamina propria, frequently surrounding vascular structures (Mesa et al., 2002).

Of all the species isolated in the oral cavity, only some species or clusters of these species has been shown to be pathogenic in periodontitis. These clusters, known as microbial complexes were characterized by Socransky et al. and given a colour code based on its pathogenic potential. Red complex, formed by *Tannerella forsythia, Porphyromonas gingivalis* and *Treponema denticola*, was associated to cases of greater severity (Socransky et al., 1998).

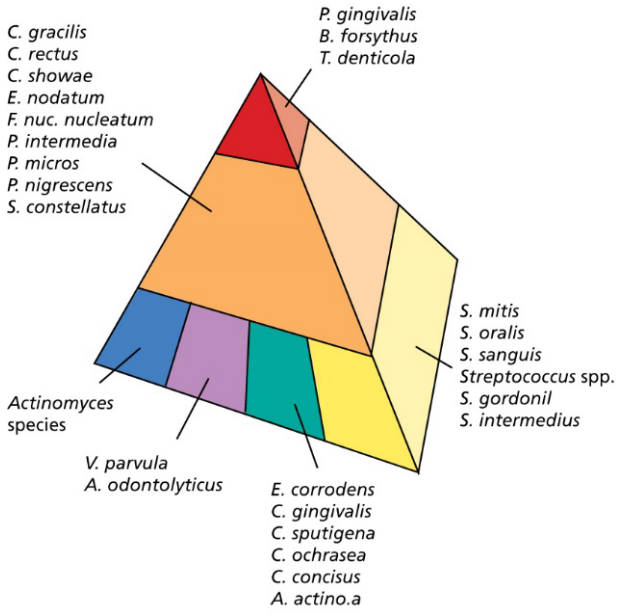

Figure 1. Periodontal microbial complexes (Socransky et al., 1998)

Periodontitis is initiated and maintained by the presence of a bacterial biofilm, but also the host defense mechanisms play an essential role in the pathogenesis and the individual susceptibility of the patient. A recent hypothesis about the pathogenesis of periodontitis states that the disease is initiated by a synergistic bacterial community, instead of specific pathogens. This community modulates the composition of the rest of the biofilm and can affect and be affected by the host response. This situation generates a situation of dysbiosis in the periodontal environment. According to this model, certain species of this community, called "Keystone pathogens" (Hajishengallis and Lamont, 2012), have essential roles in initiating and contributing to this dysbiosis. These species do not even require high levels of colonization in the biofilm. This hypothesis would explain the low counts (<0.01% of the total bacterial count) of a main periodontopathogen, such as *Porphyromonas gingivalis (P. gingivalis)*, that have been reported in periodontitis in previous studies (Torrungruang et al., 2015).

Periodontopathogens exert their main action through endotoxins like lipopolysaccharide (LPS), which activate membrane toll-like receptors of macrophages. This initiates an intracellular response that causes the synthesis of inflammatory cytokines like prostaglandin E2, interleukin-1ß or tumor necrosis factor α that cause tissue destruction by their action on epithelial cells, fibroblasts, macrophages, endothelial cells, mastocytes, eosinophils and neutrophils. These cells produce and release matrix metalloproteinases, that can cause the degradation of collagen and other components of extracellular matrix.

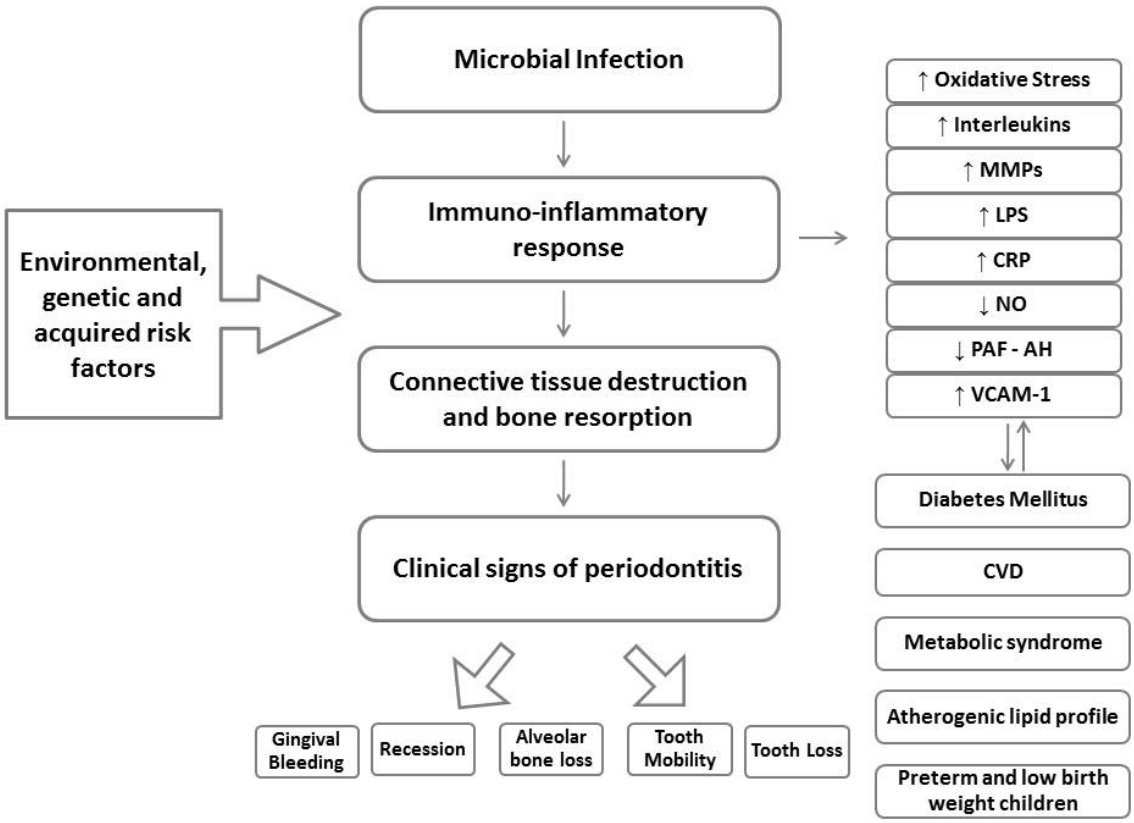

Figure 2 . Pathogenesis model of periodontitis

6.1.2 Risk factors

Since the onset of the inflammatory process and during all the evolution of the disease, a series of factors of diverse origin can intervene. These exert a significative effect in the severity and prognosis of periodontitis and can be modified or not by the patient.

6.1.2.1 Environmental Risk Factors

These factors are related to lifestyle habits of the patient. Smoking has been the most widely studied and strongly associated to periodontitis (AL-Harthi et al., 2018). A dose-dependent association has been described, affecting leucocyte function, phagocytosis and also promoting the colonization and growth of gram negative bacterial species (Leite et al., 2018). Psychosocial stress has also shown a significant association with periodontitis. The presence of stress has been even related to a poorer response to periodontal treatment. The proposed mechanism would be a dysregulation of the immune response of the host mediated by an upregulation of systemic corticosteroids due to stress (Mesa et al., 2014a).

6.1.2.2 Genetic Risk Factors

Genetic predisposition is also a relevant, since the host immune response plays a key role in the pathogenesis of periodontitis. Certain diseases and syndromes present also a periodontal affectation among their other signs, such as neutropenia o Papillon-Lefevre syndrome. It has been also described that certain cases of periodontitis present with a strong familial aggregation, showing that periodontitis predisposition has also an hereditary component (da Silva et al., 2017). Also, certain small variations of the DNA chain in the chromosome, known as polymorphisms, have also proven to cause a higher susceptibility to periodontitis. The polymorphism that has been more widely related to periodontitis is the interleukin-1 polymorphism, originally described by Kornman. Its

initial estimated prevalence was of 30% in adults with periodontitis and its presence was associated with a 18.9 times higher probability of developing a severe periodontal lesion (Kornman et al., 1997). An Odds ratio for tooth loss of 2.7 in patients positive for this polymorphism has been described, rising to 7.7 if the patient is also a smoker (Meisel et al., 2002).

6.1.2.3 Systemic Risk Factors: Diabetes

Several systemic diseases may affect periodontal health directly or the therapy may also have effects on the periodontium. Diabetes mellitus has been associated directly to periodontitis, and is also a primary metabolic condition with an elevated impact on cardiovascular (Rizzo et al., 2018). It has been reported that severe periodontal disease often coexists with severe diabetes mellitus and that diabetes is a risk factor for severe periodontal disease; thus, a "two-way" relationship is recognized (Borgnakke et al., 2013). This link is further strengthened by evidence of improvement in HbA1c levels of about 0.4-0.7% following periodontal treatment in patients with combined periodontitis and T2DM coming from different meta-analyses of RCTs (Teeuw et al., 2010, Simpson et al., 2015). A pathogenic model links inflammation to diabetes and periodontal infections, in which chronic systemic inflammation caused by periodontitis elevates the plasma concentrations of proinflammatory cytokines. This interacts and increases the generation of advanced glycation end-products and insulin resistance (Teeuw et al., 2010). Exposure to high-glucose levels can also affect cellular functions through increased reactive oxygen species activity (Clarkson et al., 2002).

6.2 THE FOCAL INFECTION THEORY AND THE CONCEPT OF PERIODONTAL MEDICINE

Initially it was thought that the main consequence of periodontitis was tooth loss, which was solved by prosthetic rehabilitation of the lost teeth. However, a link between periodontitis and other systemic pathologies began to be established later. Matilla et al. provided preliminary data evaluating the association between dental infection and acute myocardial infarction, justified because, according to these authors, 25% of patients with cardiovascular events do not present the traditional risk factors. In this case-control study, "dental infection" included caries lesions, periapical lesions, pericoronaritis, tooth loss and periodontal pockets, creating a dental index that could be adjusted in view of different confounding factors. The results demonstrated an association between dental infection and myocardial infarction, and the dental index was shown to be an independent predictor of this pathology (Mattila et al., 1989). The first study focusing on the relationship between periodontal state and cardiovascular morbidity/mortality rates, with a 14-year follow-up, was published four years later. The authors of this study concluded that patients with severe periodontitis at the onset of the study had a 25% higher risk of presenting a cardiovascular event than the persons without periodontitis (DeStefano et al., 1993).

The findings reported by Mattila et al. (Mattila et al., 1989) and Offenbacher et al. (Offenbacher et al., 1996), related for the first time periodontitis with myocardial infarction and preterm and/or low birthweight neonates, respectively. The latter introduces the term of "Periodontal Medicine" as a discipline focusing on the validation of the relationship between periodontitis and systemic diseases, and its biological plausibility in human studies and animal models (Offenbacher, 1996). These are

pathologies of great impact and social relevance, and the discovery brought back again the concept of focal infection (Pizzo et al., 2010). Since then, the research involving systemic implications of periodontitis has grown exponentially over the years, with epidemiological studies now relating it to at least 57 different systemic pathologies (Monsarrat et al., 2016).

As a result, the scientific community is accepting periodontitis as an infection focus from which oral bacteria, bacterial byproducts or inflammatory mediators can interact with other parts of the organism through blood dissemination. Periodontal pathogen components such as DNA, RNA or specific antigens from *P. gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Treponema denticola*, *Fusobacterium nucleatum* (*F. nucleatum*) or *Campylobacter rectus* have been isolated in different tissues outside their natural ecological niche, like atheroma plaques, placenta, amniotic sac, respiratory tract, pancreas, appendix or colon (Monsarrat et al., 2016, Chou et al., 2018). However, the presence of bacterial components cannot demonstrate with enough certainty if the bacterial species found in these tissues are dead or alive (Reyes et al., 2013, Kumar, 2017).

6.3 PERIODONTITIS AND CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD) is one of the leading worldwide causes of death, with 16.7 million deaths each year worldwide (Lockhart et al., 2012). Some papers suggest that periodontitis should now be considered a public health problem (Tonetti et al., 2013), due to its high prevalence and its consideration as a risk factor for CVD. Such is the volume of research supporting this association that the European Society of Cardiology includes

periodontitis as a disease that increases the risk of cardiovascular disease (Perk et al., 2012). Its section 3.7.6 reads: "Periodontitis is associated with endothelial dysfunction, atherosclerosis, and an increased risk of myocardial infarction and stroke. Confounding factors, however, such as low socio-economic status and cigarette smoking, probably play a significant role. Periodontitis can be considered a risk indicator for a generally decreased cardiovascular health status and its treatment is indicated as well as management of the underlying cardiovascular risk factor". The joint Workshop of the European Federation of Periodontology (EFP) and the American Academy of Periodontology (AAP) concluded in 2012 with regard to periodontitis and systemic diseases "There is a strong evidence that periodontitis imparts increased risk for future atherosclerotic cardiovascular disease" (Tonetti et al., 2013).

The American Heart Association concludes that periodontal disease is associated with vascular atherosclerosis, independently of known confounding factors; yet there is no evidence for a causal relation. That is, affirmations that imply a causal association between the two diseases are not justified. The group highlights as lines for future studies the need for uniform criteria in the measurement of periodontitis, and for controlled intervention studies that are well designed and involve treatment-response protocols of long duration (Lockhart et al., 2012). One of the last systematic reviews published is based on 12 studies, one of them by our research group (Cueto et al., 2005). In the authors´ opinion, the selected studies apply the most robust definition of periodontitis as a variable of exposure, defined by clinical attachment loss and alveolar bone loss with radiographic assessment, and atherosclerotic cardiovascular disease as the outcome variable (referring to atherosclerotic diseases of the heart and vasculature). Excepting one, the remainder shows a positive association between periodontitis or severity of periodontitis and the incidence of

atherosclerotic cardiovascular disease, regardless of the established cardiovascular risk factor. However, evidence for an association between periodontitis and peripheral arterial disease, or secondary cardiovascular events, is scarce (Dietrich et al., 2013).

18 systematic reviews and/or meta-analysis, mostly performed on observational studies, have addressed this topic since 2001 Since then, this evidence has been revised systematically several times (Hujoel, 2002, Scannapieco et al., 2003, Meurman et al., 2004, Beck et al., 2005a, Bahekar et al., 2007, Mustapha et al., 2007, Humphrey et al., 2008, Blaizot et al., 2009, Lockhart et al., 2012, Dietrich et al., 2013). Although the association between the two diseases has been confirmed, clinicians should be aware that not all systematic reviews of the periodontitis-CVD association have been conducted in a rigorous manner. Some of them exhibit significant structural and methodological variability (Kelly et al., 2013). The most recent meta-analysis, performed on 15 observational studies and including a total of 17,330 patients, showed that the presence of periodontal disease was associated with carotid atherosclerosis (OR: 1.27, 95% CI: 1.14-1.41), but the authors found substantial statistical heterogeneity (I^2=78.90%, p<0.0001). The inclusion of studies performed on different ethnicities may be one of the causes of this heterogeneity (Zeng et al., 2016).

Three recent observational studies of different designs should also be highlighted. The PAROKRANK study, one of the biggest epidemiologic studies performed to date, followed a case-control design in more than 1,600 Swedish patients. The results showed a significant increased risk for first acute myocardial infarction (AMI) among those diagnosed with periodontitis (OR 1.28, 95% CI 1.03-1.60) after adjusting for confounding variables (smoking habits, diabetes mellitus, years of education, and marital status). 43%

of the cases were diagnosed with periodontitis, verified by radiographic bone loss, and the risk for first AMI was near 30% higher than controls and independent of traditional risk factors (Ryden et al., 2016). This risk is on the range of 25-50% of CVD risk for periodontitis patients compared to healthy subjects reported by other authors in similar studies (Bartova et al., 2014). Beukers et al. reported an association of periodontitis with atherosclerotic disease independent of other confounders in a cross-sectional analysis of 60,174 participants, stratified by sex age and sex, in Dutch population (OR 1.59, 95% CI 1.39-1.81) (Beukers et al., 2017). The size of AMI is one of the determinants of its severity, as it indicates the degree of myocardial necrosis. The peak troponin I level in blood is used a marker of AMI size. In the study by Marfil-Alvarez et al., among 112 AMI patients that needed revascularization therapy (primary angioplasty), multiple mediated regression analysis showed higher levels of troponin I, and hence greater infarct size, in those patients diagnosed with periodontitis. According to their results, 65.18% of the AMI patients were diagnosed with periodontitis. This number was higher compared to previous studies, maybe attributable also to the higher severity that presented patients enrolled in this study compared to previous ones (Marfil-Alvarez et al., 2014).

As stated previously, the main consequence of periodontitis is tooth loss. Bahekar et al. studied the relationship between number of teeth and incidence of coronary heart disease. Their results showed that patients with less than 10 teeth presented a risk of 1.24 (95% CI 1.14-1.36, $p<0.0001$) for coronary heart disease compared to patients with more than 10 teeth (Bahekar et al., 2007). Also, another recent article showed that severe tooth loss, defined as more than 10 teeth, showed to be a predictor variable of cerebrovascular disease/silent cerebral infarction. Patients that lost more than 10 teeth showed an adjusted OR of 3.9 (95% CI 1.27-5.02, $p<0.001$) compared to patients with tooth loss between 0

and 5 teeth (Minn et al., 2013). A reduction in the number of posterior teeth was associated with a greater prevalence of atherosclerosis in a cross-sectional study on Japanese population (Tada et al., 2017). Hence, periodontitis acting as a chronic inflammatory process or as a cause of tooth loss, seems to be clearly associated with a greater prevalence or incidence of CVD events.

It has been shown that periodontitis can contribute to endothelial dysfunction (ED), a surrogate marker of atherosclerosis. It has been associated with lower values of flow-mediated dilation of the brachial artery, probably due to an increased thickening of the intima-media layer of the vessel. Periodontal bacteria, bacterial components or inflammatory mediators could cause a dysregulation in the endothelium and hence contribute to atheroma plaque formation (Moura et al., 2017, Punj et al., 2017). Only 8 articles to date have addressed the link between periodontitis and endothelial dysfunction (ED). ED was mostly assessed measuring flow-mediated dilation (FMD) of the brachial artery, considered the gold standard test. The results clearly conclude that periodontitis is associated with an impaired endothelial function (Amar et al., 2003, Holtfreter et al., 2013, Moura et al., 2017, Punj et al., 2017), and that periodontal treatment can improve it (Mercanoglu et al., 2004, Seinost et al., 2005, Elter et al., 2006, Blum et al., 2007). Desvarieux et al. reported for the first time that an improvement in the periodontal status, assessing it both clinically (probing pocket depth and attachment loss measurements at 6 sites per tooth using a UNC-15 periodontal probe) and microbiologically (quantitative assessment of 11 known periodontal pathogens by DNA-DNA checkerboard hybridization) was associated with less atherosclerosis progression (assessed by carotid intima-medial thickness (CIMT)). These findings were observed on a relatively short period (3-year median follow-up), supporting the hypothesis that faster atherosclerosis

progression could be the mechanistic model to explain previously published results associating periodontitis and CVD, and also emphasizing the possibility of primary periodontal care as a potential preventive measure for CVD (Desvarieux et al., 2013). It was also showed previously after a 5-year follow-up period that the diagnosis of periodontitis at the beginning of the study was associated with 4-times more probability of developing carotid atherosclerosis and 2.3-times for atherosclerosis progression if already diagnosed (Kiechl et al., 2001).

Although in the recent years there has been an increasing number of intervention studies, mainly focusing on the effect of periodontal treatment on endothelial function, the available evidence is still insufficient to establish a causal relationship. The multi-factorial nature of periodontitis and atherosclerosis, the chronic nature of both diseases, and other facts such as the identification of fatty streaks and foam cells in children/teenagers, and the limited sample sizes and short follow-up periods, make difficult nowadays to confirm a causal relationship, since some of the published results are contradictory.

6.3.1 Pathogenic mechanisms

To date, the relationship between periodontitis and cardiovascular disease has been explained by the action of periodontopathogenic bacteria in the process of atherogenesis. Two main pathways have been evoked in the relationship between the two: direct infection of the atheromatous plaque by periodontal bacteria; and the systemic inflammatory state that periodontal disease causes, which would contribute indirectly to atherosclerosis.

6.3.1.1 Bacterial infection

It has been documented that frequent bacteremia occur in patients with periodontitis, after daily regular oral hygiene procedures such as tooth brushing, that would implicate a chronic insult on the vasculature (Rivera et al., 2013). The role played by bacteria or their byproducts in the development of atherosclerosis has been widely demonstrated. Infectious processes have been shown to be an independent risk factor for endothelial dysfunction and the risk of atherosclerosis, being related with the presence of cardiovascular disease (Prasad et al., 2002). Furthermore, it is known that chronic infections intervene in the progression of atherosclerosis (Leinonen and Saikku, 2002). A prospective study with a five-year follow-up (n=825) analyzed the association of different chronic infections, among them periodontal infection, with atherosclerosis. The results show that all the chronic infections studied increased the risk of atherosclerosis significantly, especially among those patients who did not suffer from atherosclerosis at the onset of the study (OR = 4.08; p <0.0001), although the patients already affected also showed a higher risk for the progression of the atheromatous lesions (OR = 2.31; p <0.001) (Kiechl et al., 2001).

The scientific evidence available shows how periodontal bacterial species are directly involved in the process of atherogenesis (Spahr et al., 2006). Several studies have demonstrated the presence of periodontal pathogens in the atheroma plaque, by means of PCR or plate culture techniques (Elkaim et al., 2008, Chen et al., 2008, Gaetti-Jardim et al., 2009, Figuero et al., 2011). The most commonly identified periodontal pathogens are *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*, and species less often identified include *Tannerella forsythia*, *Treponema denticola*, *Prevotella intermedia*, *Prevotella nigrescens*, *Eikenella corrodens*, *Fusobacterium nucleatum* and *Campylobacter*

rectus. Most of these species have been found within macrophages or near apoptotic bodies (Chiu, 1999). The prevalence of each bacterial species varies considerably according to the different studies, without showing any pattern; but many plaques analyzed showed more than one bacterial species, leading to the hypothesis that the endothelial dysfunction is produced by the action of several bacterial species, not by the action of a single pathogen (Prasad et al., 2002). This global load of periodontal pathogens has been related in various studies of coronary disease with the "carotid intima-media thickness" (Spahr et al., 2006, Andriankaja et al., 2011).

The access of these pathogens to vascular tissue is explained by the production of bacteremia through the ulceration of the periodontal pocket, during habitual therapeutic procedures or even while brushing or chewing, in patients with gingivitis or periodontitis (Kinane et al., 2005, Forner et al., 2006). Other proposed mechanisms are the passage of bacteria through the cells of the periodontal pocket to the capillary system, by means of a transcellular mechanism (Takeuchi et al., 2011), or else due to phagocytosis by leukocytes, inside which the pathogens remain alive, avoiding lytic processes, then departing from these cells in a different zone of the organism (Carrion et al., 2012).

P. gingivalis is the most studied periodontal pathogen in terms of its interactions with cells from cardiovascular system. Chiu et al. published 19 years ago the first *in vivo* evidence of *P. gingivalis* in macrophages of human carotid atherosclerotic plaques by immunostaining (Chiu, 1999). A year later, Haraszthy et al. isolated in samples from 50 atheromatous plaques RNA from *A. actinomycetemcomitans*, *P. gingivalis, T. Forsythia* and *P. Intermedia*, 4 of the main periodontopathogens, and also *Chlamydia pneumoniae* and human cytomegalovirus, determined by 16S rRNA PCR. 22 of these plaques had at

least one periodontal species and 13 of them had 2 or more periodontopathogens (Haraszthy et al., 2000). Since then, several studies have demonstrated the presence of DNA, RNA or oral bacterial antigens in samples from human atheroma plaques. More recently, presence of 16S rRNA of *P. gingivalis, T. forsythia, T. denticola and P. nigrescens* was found both in samples from subgingival biofilm and atheromatous plaques (Mahalakshmi et al., 2017). These findings may be explained by the presence of bacterial compounds from dead species that arrive and infiltrate the atheroma plaque or the endothelial wall by blood dissemination. This fact may point to a more passive mechanism instead of active infection of vascular structures by these species. To our knowledge, only one study has successfully isolated alive species of *A. actinomycetemcomitans* and *P. gingivalis*, by culture of atheroma plaque samples with human endothelial cells from coronary arteries (Kozarov et al., 2005). In periodontitis, with an ulcerated sulcular epithelium, bacteria from the subgingival biofilm can easily go through the lamina propria, due to the lack of resistance of the tissue. They could also easily go through the endothelial cells of the subgingival inflamed vessels and access the blood stream. It is well documented that *A. actinomycetemcomitans*, *P. gingivalis* and *F. nucleatum* have the ability to invade epithelial, endothelial and immune cells, such as dendritic cells and monocytes (Cekici et al., 2014).

P. gingivalis has been isolated in circulating dendritic cells in patients with periodontitis and atheroma plaques (Carrion et al., 2012). This supports the hypothesis that periodontal pathogens could be phagocyted by immune cells and stay alive inside them, using them for dissemination through the blood flow and being able to arrive to the atheroma plaques. *P. gingivalis* has been shown to directly invade endothelial and smooth muscle cells, and survive inside them (Roth et al., 2006); according to animal models, this may accelerate the

process of atherogenesis (Gibson et al., 2004, Ford et al., 2005) and induce the apoptosis of endothelial cells (Roth et al., 2007). *A. actinomycetemcomitans* has also demonstrated an invasive capacity (Schenkein et al., 2000) and has been associated with an overexpression of MMP-9 and a pro-atherogenic profile, inducing the transformation of macrophages into "foam cells" (Tuomainen et al., 2008).

Another important contribution to research is the report of an altered lipid profile induced by bacteria of periodontal origin, with an increase in LDL and a decrease in the anti-atherogenic capacity of HDL (Pussinen et al., 2004c, Rizzo et al., 2012). *P. gingivalis* is capable of oxidizing the LDL (Bengtsson et al., 2008), which may also bond to circulating lipopolysaccharide (LPS) proceeding from the bacterial wall, forming LPS-LDL. These altered forms of LDL favor its deposit in the vascular wall, creating a pro-atherogenic and pro-inflammatory environment, and inducing the transformation of macrophages to "foam cells", by activating specific surface receptors (Morishita et al., 2013).

Nonetheless, only certain strains of periodontal pathogens have demonstrated an invasive capacity and a pro-coagulating effect (Takahashi et al., 2006). These effects are not observed when the action is due to a non-invasive mutant strain (Roth et al., 2006). In animal models it has been shown that mutant strains of *P. gingivalis*, genetically altered, displayed a non-invasive behavior, were less pro-atherogenic, and exerted a lesser pro-inflammatory effect than the wild-type strains (Gibson et al., 2004). Such results underline the decisive role of the bacterial strain in the invasion and survival of *P. gingivalis* within the cells of the vascular wall. For this reason, knowledge of how this bacterium interacts with the vascular endothelium may lead us to develop a therapy for lowering the risk of cardiac disease based on these mechanisms.

In light of the reviewed evidence, we propose an explicative model for the action of oral bacteria on atherogenesis. Some of the periodontal bacteria that gain access to the bloodstream may have the ability to invade the endothelial cells. This invasion gives rise to the activation of the endothelial cells, which express receptors of adhesion on surface (VCAM-1), and to their apoptosis, permitting the entry of microorganisms inside the vascular wall. This situation produces the arrival and activation of monocytes harboring live periodontal pathogens, previously phagocytized, which penetrate the vascular wall, where they are transformed into macrophages; these would suffer apoptosis due to the action of the bacteria, which would then be released to the medium. The oral bacteria in the medium could act upon the LDL, favoring its modification to oxidated LDL (oxLDL), which in turn would induce the transformation of macrophages into "foam cells" through the union to specific surface receptors of these cells, thus producing the release of pro-inflammatory cytokines ROS and MMPs.

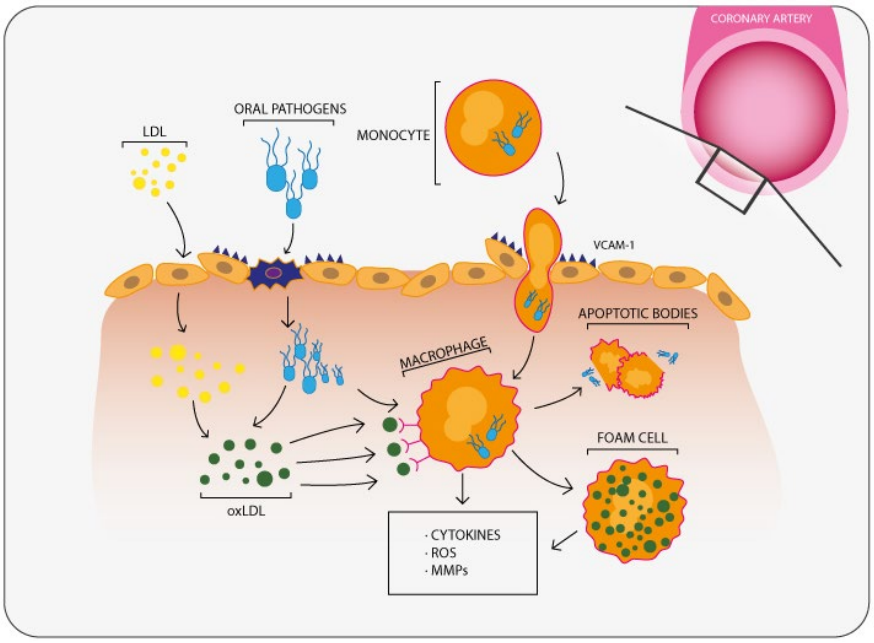

Figure 3. Model of the interaction between periodontal pathogens and the formation of atheromatous plaques (Mesa et al., 2014b)

The cell wall of anaerobic gram-negative bacteria is composed of peptidoglycans, polysaccharides, proteins, lipids, lipoproteins and lipopolysaccharide (LPS). LPS is a glycolipid endotoxin and the main component of the membrane of gram-negative bacteria, considered to be the most important surface antigen. It has a wide variety of functions that include maintaining the membrane structure, molecular mimetism, antibody inhibition, antigenic variations, immune system activation and mediation of the adherence to host cells. The exact mechanism of adherence of LPS is still unknown, but physicochemical interactions and lectin receptors on the cell surface have been postulated as possible mechanisms (Raetz and Whitfield, 2002). LPS-induced endotoxemia has been proposed as possible molecular mediator between periodontitis and CVD. In a recent study, 3 groups of patients were divided according to the grade of coronary stenosis assessed by coronary angiography (stenosis < 50%, stenosis ≥ 50% and acute coronary syndrome) and serum and salivary LPS levels were correlated with periodontitis and periodontal pathogens. Serum LPS were associated with periodontitis and periodontopathogen count and also with a greater risk for having a grade of stenosis ≥ 50%. The authors conclude that periodontitis is associated with a low-grade systemic inflammation that, together with other risk factors, would contribute to a higher risk for CVD (Liljestrand et al., 2017).

LPS is released by bacteria as a result of division or lysis and contacts with several host proteins and co-receptors, such as LPS binding protein (LBP), CD14, toll-like receptors (TLR) and MD-2. LBP needs to binded to LPS in order to allow CD14, expressed in the surface of macrophages, neutrophils and endothelial cells, to intervene and mediate in the transduction and recognition process, mediated by the TLR4/MD-2 complex (Lu et al., 2008). A genetic alteration of this receptor, conditioned by bacterial stimuli, may also play a role in the pathogenesis of atherosclerosis. Patients who survived to AMI have shown polymorphic forms in the allele (C (-260) T) of the promoter of the gene encoding CD14, compared to controls (Hubacek et al., 1999). Human umbilical veins infected with *P. gingivalis* or stimulated with LPS, have shown

exacerbated levels of oxidized LDL (ox-LDL) and tumor necrosis factor α (TNF-α), and inducing endothelial injury through increased endothelial cell death (Bugueno et al., 2016). Other mechanism that allow periodontal bacteria to invade endothelial cells is the higher surface expression of adhesion molecules by these cells, such as ICAM-1, VCAM-1, P-selectin, E-selectin and TLR-4 (Khlgatian et al., 2002).

Brain natriuretic peptide (BNP) is a 32-amino-acid peptide, released mainly from myocardial cells as response to stress on the ventricle wall. BNP is produced as a pro-hormone that is divided in two: the active BNP and the inactive N-terminal-pro-BNP. Elevated levels of BNP could act as a biomarker of atherosclerosis progression. Increased levels of microRNA from BNP have been found in coronary arteries with atherosclerotic lesions, and serum levels of BNP have been associated with coronary atherosclerosis burden measured by electron beam tomography (Wang et al., 2006). Plasma levels of N-terminal-pro-BNP increased as response to LPS and patients with periodontitis have higher serum N-terminal-pro-BNP levels compared to healthy individuals. In addition, N-terminal-pro-BNP levels increased as periodontitis severity progressed (Leira and Blanco, 2018).

Periodontal pathogens can produce toxins of different origin, such as proteases, adhesins, lectins and other molecules that regulate bacterial biofilm. These toxins can inhibit host immune response like the protease gingipain from *P. gingivalis*, which has the ability to degrade and/or inactivate different interleukins (1β, 6 and 8) by a process called "localized chemokine paralysis", or surface receptors from both immune (CD14 in monocytes) and non-immune cells (Intercellular Adhesion molecule 1 in epithelial cells and fibroblasts) (Tada et al., 2002, Tada et al., 2003). Platelets play a key role in the pathogenesis of thrombosis, and facilitate the adhesion of monocytes due to an interaction between CD40 receptors in both cells (Harding et al., 2004). Interestingly, increased number of circulating platelets have recently been reported in Korean patients with periodontitis compared with controls (Romandini et al., 2018). Another documented action of gingipain from *P. gingivalis* is the activation of protease-activated receptors type 1 and 4, highly expressed in the

platelet surface, inducing platelet aggregation in a similar way than thrombin. An increased platelet aggregation induced by *P. gingivalis* has been also observed in studies performed on platelet-rich plasma, which also supports this hypothesis (Li et al., 2008).

Cross-reactivity or molecular mimicry between bacterial components and the host have been described. The homology between human and bacterial heat shock proteins (HSPs) could activate atherosclerotic changes (Choi et al., 2004). This hypothesis states that the host immune system is not able to differentiate between self-HSPs and bacterial HSPs. Bacterial HSPs can be recognized as host HSPs and generate an autoimmune response that contributes to the progression of atherosclerosis. Endothelial cells express HSPs in atherosclerosis, and cross-reactive T-cells are present in the arteries and peripheral blood of patients with atherosclerosis (Ford et al., 2007). Cross-reactivity between HSP60 from *P. gingivalis* and human HSP60 from endothelial has been determined by specific antidobies. Also, T lymphocytes reactive against HSP60 from *P. gingivalis* have been found in peripheral blood from patients with atherosclerosis (Yamazaki et al., 2004). Results from a study published by our group, showed that untreated patients with mild periodontitis presented increased serum HSP60 and small, dense LDL concentrations, in comparison with controls matched by age and body mass index. Our findings indicated that atherogenic dyslipidemia and elevated circulating levels of HSP60 were associated with periodontitis (Rizzo et al., 2012).

6.3.1.2 Immunoinflammatory and thrombotic mechanisms

One of the best-known aspects of cardiovascular pathology is its association with the inflammatory response and with immunity-related mechanisms (Libby, 2002), a characteristic that it shares with periodontitis. Patients with periodontitis present an intense inflammatory infiltrate at the gingival connective tissue, with a predominance of plasma cells, monocytes/macrophages, and T and B lymphocytes.

The scientific literature documents a relationship between periodontitis and increased levels of systemic inflammatory mediators which, in turn, are related with cardiovascular pathology. One of the most studied markers is C Reactive Protein (CRP), considered as one of the best markers used in the prediction of cardiovascular risk (Blake and Ridker, 2001). It has been shown in observational, interventional studies and meta-analyses that periodontitis is associated with elevated local levels of CRP in the gingival crevicular fluid, as well as high systemic levels (Loos et al., 2000, Paraskevas et al., 2008, Buhlin et al., 2009, Yoshii et al., 2009, Nakajima et al., 2010). In contrast, other studies find no such association, including one study published by our research group; this may be due to the fact that the patients of our study presented mild periodontitis (Rizzo et al., 2012). Other markers found to be elevated in association with periodontitis are IL-6, (Loos et al., 2000, Buhlin et al., 2009, Nakajima et al., 2010), the plaque-activating factor (Chen et al., 2010) and MMPs (Tuomainen et al., 2008). These factors, when increased at a systemic level as a consequence of periodontitis, contribute to the development of atherosclerosis through their action as pro-inflammatory cytokines.

The presence of specific antibodies in serum against oral periodontopathogens can be an indication of exposure to bacteria or to bacterial by-products (Pussinen et al., 2004a, Beck et al., 2005b). One meta-analysis demonstrates that the systemic elevation of antibodies is closely associated with an increased risk of suffering an acute myocardial infarction (Mustapha et al., 2007) and that this raised level of antibodies constituted a stronger predictor of risk than CRP (Lund Haheim et al., 2008). In addition to antibodies produced against bacterial species (Pussinen et al., 2004b, Pussinen et al., 2005), there may also be antibodies produced in the face of other molecules, such as Heat-Shock Proteins (HSP), expressed by endothelial cells in situations of cellular stress. They would react by inducing

the formation of anti-HSP antibodies, related with atherosclerosis (Mayr et al., 2000) and with periodontitis (Rizzo et al., 2012, Leishman et al., 2012). Furthermore, the similarity between HSP and molecules of bacterial origin such as GroEL proteins, expressed by various periodontal pathogens, is known to produce cross-reactivity effects due to the phenomenon of "molecular mimicry", both molecules being found in the atheroma plaques and the subgingival plaque of patients with atherosclerosis and periodontitis (Tabeta et al., 2000, Ford et al., 2005). Other antibodies studied are anti-phosphorylcholine (anti-PC), anti-oxidized LDL (anti-oxLDL) and anti-cardiolipin (anti-CL) as potential mediating mechanisms between atherosclerosis and periodontal disease. Anti-cardiolipin (anti-CL) is an antibody related with the phenomenon of "auto-immune atherosclerosis", which occurs in patients with systemic lupus erythematosus. This antibody binds to the β2-glycoprotein (β2GP1), involved in the control of coagulation and the protection of the endothelial wall, and heightens cardiovascular risk. Periodontal pathogens can induce anti-CL activity via similarities in peptidic sequences (Chaston et al., 2014). Meanwhile, anti-PC IgG has been associated with periodontal attachment loss (Schenkein et al., 2004), binding to the phosphorylcholine of periodontal pathogens and to oxidized LDL, leading to phenomena of cross-reactivity (Schenkein et al., 2001). Other studies, however, attribute it a role in the protection against atherosclerosis (Scannapieco et al., 2003). Anti-oxLDL has also been found at the periodontal level (Schenkein et al., 2004), presenting a cross-reactivity with oxidized LDL and with the gingipain of *P. gingivalis* (Turunen et al., 2012).

Several coagulation factors likewise intervene significantly in the formation of atheroma plaque and thrombosis. For this reason, their expression in relation with periodontitis has also been studied. The levels of hemostatic markers undergo a significant decrease after the extraction of all the teeth of periodontal patients (Taylor et al., 2006), whereas the levels of

fibrinogen (Buhlin et al., 2009) and the expression of sP-selectin, associated with platelet activation (Papapanagiotou et al., 2009), were seen to be higher in patients with periodontitis when compared with healthy controls. In this context, expression of E-selectin was higher in patients with periodontitis independently whether those patients were smokers or not (Rezavandi et al., 2002), and this vascular cell adhesion molecule decreased significantly after periodontal therapy (Pischon et al., 2007). Further factors including Plasminogen-Activator Inhibitor 1 (PAI-1) or the von Willebrand factor (vWF) give contradictory results, and their relationship with periodontitis has not been elucidated to date (Bretz et al., 2005, Bizzarro et al., 2007).

6.3.2 Intervention studies

One key question about the relationship between periodontitis and cardiovascular disease is to determine whether periodontal treatment has some beneficial effects upon some of the markers of cardiovascular risk described above. The review by Ying Ouyang et al. concludes that the effect of periodontal therapy, according to the different studies, is variable and hardly determinant (Ying Ouyang et al., 2011). Moreover, Hujoel et al. affirm that treatment is unlikely to have any effect, given that the risk of cardiovascular pathology in edentulous patients shows no difference with respect to patients having teeth and periodontitis (Hujoel, 2002).

As mentioned earlier, CRP is the acute phase protein that is most frequently related with cardiovascular risk, and it is also widely associated with periodontitis, in numerous studies. For this reason, many publications and several meta-analyses have explored the effect of periodontal treatment on cardiovascular risk adopting CRP as the main marker. The meta-analyses show similar results, pointing to a significant reduction of CRP after periodontal

treatment (Paraskevas et al., 2008, Ioannidou et al., 2006, Freitas et al., 2012). Other acute-phase proteins studied are fibrinogen and serum amyloid A, giving controversial results. Both fibrinogen (Hussain Bokhari et al., 2009, Graziani et al., 2010) and serum amyloid A (Graziani et al., 2010) show insufficient evidence of possible effects resulting from periodontal treatment.

Endothelial function has been found to be altered in periodontal patients (Amar et al., 2003). Diverse studies have evaluated the effect of periodontal treatment on endothelial function, showing a positive effect upon this variable (Elter et al., 2006, Tonetti et al., 2007), measured by "flow-mediated dilation".

Studies assessing the effect of therapy on leukocyte count provide no clear conclusions: some show a reduced count (Lalla et al., 2007, Rastogi et al., 2012), while others report no significant differences (Marcaccini et al., 2009, Graziani et al., 2010).

The effects of periodontal treatment on IL-6, the tumor necrosis factor-alpha (TNFα), MMPs and tissue inhibitors of metalloproteinase (TIMP) present contradictory results in terms of their serum levels (Tonetti et al., 2007, Lalla et al., 2007, Marcaccini et al., 2010, Sun et al., 2011).

The phenomena of oxidative stress, and particularly reactive oxygen species (ROS), oxidated LDL (oxLDL) and other by-products of oxidative stress damage are reportedly pronounced in periodontal patients (Su et al., 2009). Periodontal treatment has been associated with decreased ROS and oxLDL (Tamaki et al., 2011, Montebugnoli et al., 2005), but this influence in oxidative stress was not observed in other studies (Lalla et al., 2007).

At present, knowledge regarding the effect of periodontal treatment on thrombotic and hemostatic markers is unclear, with diverse findings surrounding PAI-1 and the D-dimer (Montebugnoli et al., 2005, Lalla et al., 2007, Graziani et al., 2010), whereas the von Willebrand factor apparently plays no role whatsoever (Montebugnoli et al., 2005, Taylor et al., 2010).

The systemic inflammatory state generated by periodontitis was believed to affect endothelial function, possibly altering hemodynamics, implying an impact on blood pressure. Studies published on this topic do not demonstrate any significant decrease in arterial pressure as a result of periodontal treatment (Graziani et al., 2010).

The review by D'Aiuto et al., taking in 14 randomized clinical trials, concludes that the main findings about the effect of periodontal therapy involve CRP and the endothelial function, pointing to evidence of clear improvement after treatment. The validity of both these markers is questioned, however, as CRP and endothelial dysfunction may be subjected to several confounding factors. Instead, the consensual use of a biomarker such as "carotid intima-media thickness" is proposed, it being widely used in intervention studies on cardiovascular disease. Other results of the meta-analysis were a lack of effect or limited evidence after periodontal treatment upon the rest of the factors analyzed (D'Aiuto et al., 2013).

Still, the relationship between periodontitis and cardiovascular disease is far from clear. The real interaction at work is unknown, due to the presence of risk factors that are shared by the two pathologies (Mesa et al., 2008). Important questions also remain regarding the exact mechanisms that could establish an association, or the effect of periodontal treatment upon the prevention of cardiovascular disease. Findings published in the literature, while

demonstrating an association, do not allow researchers to establish a causal relation, since most consist of transversal studies.

6.4 PERIODONTITIS AND PLASMA LIPIDS

The first publication referring to the relationship between periodontitis and plasma lipids levels was published in the year 1999 by Ebersole et al., describing an association between periodontitis and the lipid profile in non-human primates (Ebersole et al., 1999). Since then, the relationship between periodontitis and lipid profile has been studied on several occasions, and a bidirectional relationship has even been established between the two pathologies (Fentoglu and Bozkurt, 2008). Lösche et al. described an association between high levels of TC, TG and LDL and the presence of periodontal disease in a study of 39 subjects (Losche et al., 2000). Later publications report similar results, in addition to observing a decrease in the levels of HDL (Katz et al., 2002, Nibali et al., 2007, Fentoglu et al., 2009, Ramirez-Tortosa et al., 2010). Rufail et al. determined higher levels of VLDL and small dense LDL in patients with aggressive periodontitis, as opposed to healthy subjects (Rufail et al., 2007). Our group in a recent research study has corroborated these results, reporting greater serum levels of small dense LDL in patients with mild periodontitis (Rizzo et al., 2012).

Meanwhile, other publications do not associate the presence of periodontal pockets with the levels of plasma lipids (Saxlin et al., 2008, Machado et al., 2005), not even when representing the lipid variables as TC/HDL and LDL/HDL ratios (Korhonen et al., 2011). More recently, the study by Almeida et al. reported no association between the presence

of hyperlipidemia —alone or in conjunction— with diabetes mellitus type 2, and periodontal disease (Almeida Abdo et al., 2013).

Schenkein et al., in a recent revision, affirms: “There is evidence from clinical studies that patients with periodontitis can demonstrate elevated levels of serum cholesterol as well as of LDL, small dense LDL, VLDL, and TGs, in concert with decreased levels of HDL, thus presenting with a more atherogenic lipid risk profile” (Schenkein and Loos, 2013).

6.4.1 Pathogenic mechanisms

The link between periodontitis and atherosclerosis has been described by two mechanisms, directly by the periodontal pathogens or indirectly by bacterial components or inflammatory mediators. A third mechanism that would also support this link, is the relationship between periodontitis and the lipid profile. Atherosclerosis has been classically defined as the passive accumulation of lipids in the coronary artery wall, and considered to be one of the main causes of CVD (Slocum et al., 2016). Nowadays an immunologic mechanism is considered instead to be responsible for its progression (Cole et al., 2010).

Different mechanisms have been proposed to relate the levels of serum lipids with periodontal disease. Iacopino et al. established that any situation generating a systemic pro-inflammatory state will be a potential agent of lipid imbalance, as in the case of periodontitis, in which cytokines including IL-1β and TNFα enhance the biosynthesis of cholesterol in the liver (Iacopino and Cutler, 2000). This fact is confirmed by Fentoglu et al., who described the association between IL-1β, IL-6 and TNFα levels and TC/HDL ratio (Fentoglu et al., 2011a).

On the other hand, the direct action of periodontal pathogens or their systemic by-products would also produce an immune-inflammatory response and changes in the lipid metabolism, leading to higher VLDL and LDL, as well as a decrease in the level and the anti-atherogenic potential of HDL (Pussinen et al., 2004c, Kallio et al., 2008, Maekawa et al., 2011). The LPS of *A. actinomycetemcomitans* was shown to influence the lipid profile, increasing LDL oxidation and the expression of receptors for LDL, involved in the control of plasma lipid levels (Morishita et al., 2013). The production of reactive oxygen species (ROS) as a consequence of periodontitis would contribute to an increase in the process of lipid peroxidation, generating a more atherogenic profile (Tamaki et al., 2011).

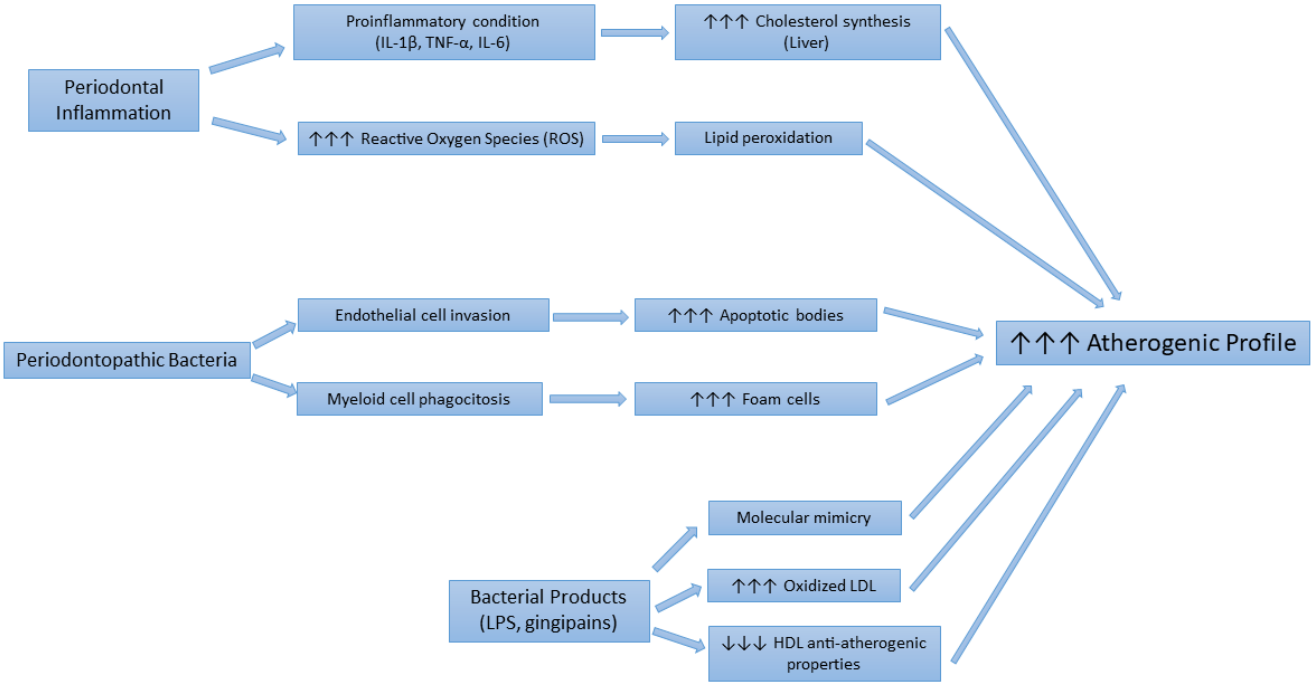

Figure 4. Model of the interaction between periodontitis and atherosclerosis

Small, dense low-density lipoproteins are an LDL subclass that have shown to be particularly atherogenic and have little affinity for the apoB/E receptor of hepatocytes, delaying their blood clearance. They also have a high affinity for vascular

glycosaminoglycans, which keeps them in contact with the vascular artery wall for a longer time (Gerber et al., 2017, Nikolic et al., 2013). Gingipain from *P. gingivalis* caused a selective proteolysis of apoB-100, a main component of LDL particles, and necessary for the binding of LDL to cell surface receptors, which is a crucial step for the promotion of atherosclerosis (Hashimoto et al., 2006).

Recently, a systematic review has been published investigating serum lipid levels (total cholesterol, triglycerides, LDL-cholesterol and HDL-cholesterol) in patients with and without periodontitis. The authors performed a meta-analysis with meta-regression of 19 studies, with a total of 2,104 participants. They found that the studies cannot provide consistent information, due to the great variability and the lack of consensus in the diagnosis and definition of periodontitis. Authors performed the analysis dividing the articles in those with secure diagnosis of periodontitis and those with insecure diagnosis of periodontitis. They conclude that periodontitis was significantly associated with a reduction of HDL-cholesterol and elevations of LDL-cholesterol and triglycerides. They state that periodontal inflammation may negatively affect serum lipid control, and also contributing to a higher risk for CVD. Lipid dysregulation would also increase the susceptibility to periodontitis, since dyslipidemia is associated with a state of systemic inflammation (Nepomuceno et al., 2017). This suggests a possible bi-directional relationship between dyslipidemia and periodontitis (Magan-Fernandez et al., 2014).

6.4.2 Intervention studies

In the past nine years, to our knowledge only nine publications focused on the quantification of serum lipid levels in patients with chronic periodontitis that underwent non-surgical periodontal treatment. Authors D'Aiuto et al., Oz et al. and Taylor et al.

report reduced values for total cholesterol and LDL in periodontal patients after their periodontal treatment (D'Aiuto et al., 2005, Oz et al., 2007, Taylor et al., 2010). Tamaki et al. found a decreased expression of oxLDL after periodontal treatment, which appears to indicate an effect upon oxidative stress (Tamaki et al., 2011). Other studies show an effect on HDL, pointing to a quantitative and qualitative improvement after treatment (Pussinen et al., 2004c, Acharya et al., 2010).

Notwithstanding, Higashi et al., Kamil et al. and Kallio et al. found no changes in lipid levels after periodontal treatment (Higashi et al., 2008, Kamil et al., 2011, Kallio et al., 2013). Finally, the recent meta-analysis of D'Aiuto et al. concludes that periodontal treatment has no influence on the lipid profile of an individual (D'Aiuto et al., 2013).

6.5 PERIODONTITIS AND LIPOPROTEINS

In favor of the existence the link between periodontitis and lipid metabolism is epidemiological evidence reporting significantly higher levels of TC, TG and ox-LDL in subjects with CHD and chronic periodontitis compared with those only with CHD (Tang et al., 2011). As mentioned above, LPS is the main virulence factor of the gram-negative periodontal pathogens, which induces inflammation and is considered as a pro-atherogenic molecule (Kallio et al., 2013). LPS is bound and cleared from the circulation by all lipoproteins: in healthy subjects LPS is usually bound to HDL, what is further linked to LPS neutralization, while in states when HDL level is low the majority of LPS is bound to very low density lipoproteins (VLDL) (Kallio et al., 2013). Kallio et al showed an association bwteeen LPS and pro-atherogenic lipoprotein particles (Kallio et al., 2008), that together with LDL induce lipid accumulation in macrophages and their

transformation into foam cells (Wiesner et al., 2010). It seems that periodontitis induces pro-atherogenic lipoprotein patterns and the association appears to be strongest for apoB-100-containing lipoproteins (Griffiths and Barbour, 2010), but details about the potential mechanisms and specific role of VLDL in this context have remained largely unknown. Moreover, *P. gingivalis* degrades apo B-100, the major protein component of LDL particles, which could explain the altered mobility of LDL rather than oxidation (Miyakawa et al., 2004). *P. gingivalis* can directly modify LDL into a form which facilitates its uptake into macrophages by a specific mechanism for directly aggregating LDL (Miyakawa et al., 2004). This is supported by fact that in subjects with periodontitis, in comparison to controls, sdLDL levels seem to be higher (Rizzo et al., 2012). Rufail et al. have shown increased TG in case of periodontitis as the result of increases in VLDL and a more robust increase in IDL particles, that appears to be more predictive of atherosclerosis progression than LDL, leading to increased CV risk of these subjects (Rufail et al., 2005). Even more, the authors suggest that neither total HDL levels nor the distribution of HDL subclasses is a major factor in the observed, increased CV risk. By contrary, Pussinen et al. (20) indicated that periodontal infections may alter the anti-atherogenic potency of HDL, indicating that HDL may affect the CV risk of these subjects in ways that are not related to the concentration of these particles (Rufail et al., 2005). Passoja et al. have reported that the association between periodontal inflammation and serum HDL existed independently of the microbial load and lipid-lowering therapy such as statins (Passoja et al., 2011). Interestingly, recent result indicates that nitric oxide (NO) production is reduced in periodontitis, especially in male population, with higher LDL and significant positive correlation of NO levels with HDL levels in whole population (Andrukhov et al., 2013). Also, it has been reported that HDL-C and fasting glucose are

importantly associated with periodontal disease especially in women, while in men it seems that high levels of TG were associated with lower odds of having periodontal disease (Andriankaja et al., 2010). In addition, anti-apolipoprotein A-1 (antiapoA-1) IgG autoantibodies have been proposed as a possible biomarker for CVD risk in periodontitis, as they are prevalent in these patients (especially younger) compared with controls and the best predictor of atherosclerosis burden (Wick et al., 2013).

An increase in oxLDL level is a known risk factor for CVD, and it seems to be associated with the progression of periodontitis, as described above. Additionally, *in vitro* study demonstrated that OxLDL increase production of the cytokine IL-8 in a human oral epithelial cell line (Ca9-22 cells), suggesting that lipoprotein metabolites could have a role in the inflammatory reaction in mucous membrane tissues, differently from that on endothelial cells blood vessels (Suzuki et al., 2010).

Serum proprotein convertase subtilisin/kexin 9 (PCSK9) concentrations might be associated with periodontal infection, as its level was significantly higher in patients with chronic periodontitis, who were otherwise healthy, when compared to healthy subjects (Miyazawa et al., 2012), independently by LDL-C levels. PCSK9 inhibition is a new strategy for lowering LDL-C levels (Banach et al., 2013), and it might have double effect in subjects with periodontitis.

Overall, subjects with periodontitis have an atherogenic lipoprotein profile and lower LDL-associated platelet-activating factor acetylhydrolase (PAF-AH) activity, hydrolyzed PAF and oxidized phospholipids that have pro-inflammatory and proatherogenic properties. These differences might explain the increased CVD risk of these subjects (Rufail et al., 2005) that has been discussed above.

6.5.1 Pathogenic Mechanisms

The increased number of LDL particles in subjects with periodontitis can be consequence of lower LDL-associated PAF-AH activity (i.e., PAF-AH activity per microgram of LDL protein) (Rizzo et al., 2009), that further may decrease the anti-atherogenic influence of PAF-AH, leading to increased CV risk, as PAF-AH activity is negatively associated with small LDL particles. It is known that sdLDL are more susceptible to oxidation than larger, more buoyant subspecies and oxidative modifications of LDL represent an early stage of atherosclerosis (Rizzo et al., 2012). However, it is more probably that oxidative stress and atherogenic dyslipidemia have a synergistic impact on atherosclerosis and cardiovascular diseases (Rizzo et al., 2009). In this context, lipoprotein-associated phospholipase A2 (Lp-PLA2), an enzyme that has been shown to be a risk factor of CVD, believed to be an independent CV risk factor, also involved in the degradation of the phospholipid mediator platelet-activating factor (PAF), has been significantly reduced by treatment of periodontitis (Losche et al., 2005). Interestingly, when *P. gingivalis* has been added to samples of whole human blood, after a short incubation period, LDL and HDL particles were isolated from the incubated blood samples, apoB-100 of LDL particles became degraded, i.e. LDL was proteolysed into distinct peptide fragments, and the LDL particles also became oxidatively modified (Oorni and Kovanen, 2008). Actually, the proteolytic enzymes of *P. gingivalis*, the arginine-specific bacterial cysteine protease, Arg-gingipain (gingipain R), is able to proteolyse apoB-100; this bacterial proteases can remain active in the whole blood. However, *P. gingivalis* microorganisms could also modify LDL within the arterial intima, not only in circulation, and this can be a novel biochemical mechanism of link between periodontitis and CVD (Oorni and Kovanen, 2008). *In vitro* studies showed that oxLDL increase the production of IL-8 and PGE2 via the NF-kB

pathway in human gingival epithelial cells, such as Ca9-22 and HOK cells (Nagahama et al., 2011, Sakiyama et al., 2010). Actually, the inhibitor of NF-kB completely suppressed the up-regulation of IL-8, while PGE2 production was partially suppressed, indicating that the oxLDL mediated increase in PGE2 production via COX-2 and PGES by several different pathways and also might be mediated by some scavenger receptors, since fucoidan and dextran sulfate blocked the action of OxLDL (Suzuki et al., 2010). One more proposed mechanism, leading as well to increased cytokine production in periodontitis, is that minimally modified LDL would stimulate dendritic cells (reside in arterial walls and accumulate in atherosclerotic lesions) and enhance the production of pro-inflammatory cytokines that promote atherogenic plaque development (Kikuchi et al., 2010). In addition, it has been shown in animal model that periodontal infection induces lower HDL-C, where *P. gingivalis* down regulates liver X receptors (LXRs), resulting in the suppression of an ATP-binding cassette A1-mediated HDL cholesterol generation (Miyazawa et al., 2012).

There is evidence suggesting that oral pathogens may increase expression of the HMG-CoA reductase, the enzyme which is the target of the widely used statin drugs (Griffiths and Barbour, 2010).

As we mentioned above, lipoproteins (especially HDL) are effective in binding and neutralizing LPS of Gram-negative bacteria, thereby limiting the expression of cytokines and lipid peroxidation, and have an antioxidant feature (Passoja et al., 2011). However, the protective role of HDL may be related not only to the level, but also to the quality of HDL (Khera et al., 2011). A causal link between the activation of the LPS pathway on innate immunity by periodontal microbiota and the occurrence of a high-fat diet has been

suggested in periodontal disease pathogenesis. This pathophysiological mechanism could be targeted by estrogens, which may thus represent a new therapeutic perspective to prevent or reduce periodontal occurrence inflammation (Blasco-Baque et al., 2012).

Data in the literature on the distribution of lipoprotein particles in periodontitis subjects are still sparse. However, on the basis available results, a relationship exists and should be explored more in the future by larger studies.

6.5.2 Intervention studies

Treatment of periodontitis might have beneficial effects on lipid metabolism and consequently decreases CHD risk (Tang et al., 2011). A recent study reported that the combination of the periodontal and antilipemic therapy provides beneficial effects on the metabolic and inflammatory control of hyperlipidemia via the decreases in the serum pro-inflammatory cytokines (Fentoglu et al., 2012). However, the potential roles of pro-inflammatory cytokines in the relationship between periodontitis and hyperlipidemia associated with infection and/or inflammation are still undetermined. Elucidating these roles could lead to new approaches in the control of both diseases (Fentoglu et al., 2012).

The results reported by Kallio et al (Kallio et al., 2013), of no significant effect of periodontal treatment on VLDL mass composition, LPS activity or apoE content can be consequence of a short follow-up (three months). Differences in the reported outcomes between different studies may also be influenced by heterogeneous ‘periodontal disease’ and ‘health’ definitions and by differences in laboratory methodologies. In addition, an earlier study showed that periodontal treatment had only minor effects on plasma LPS activity and distribution lipoprotein classes (Kallio et al., 2008). Periodontal treatment reduces the circulating LPS levels and thereby the levels of some pro-inflammatory

mediators that may lead to an increase in total cholesterol (Taylor et al., 2010). On the other hand, intensive periodontal therapy (subgingival mechanical debridement with an adjunctive local delivery of minocycline) significantly decreased total and LDL-C (D'Aiuto et al., 2005).

Interestingly, in subjects with type 2 diabetes mellitus, non-surgical periodontal treatment is associated with improved glycaemic control, but also TC, TG, LDL levels decreased, whereas these values increased slightly in the control group (Kiran et al., 2005). Of interest, levels of oxLDL in subjects with periodontitis decreased after non-surgical periodontal treatment (Tamaki et al., 2011), which positively correlated with a reduction in oxidative stress that indicate usefulness of periodontal treatment not only in improving periodontal health, but also maintaining cardiovascular health.

6.6 PERIODONTITIS AND STATINS

6.6.1 Statins: origin and mechanism of action

Statins are a pharmacological group of molecules inhibitors of the enzyme "Hydroxy-methyl-glutaryl-coenzyme A reductase" (HMG-CoA reductase). This enzyme is an essential step in the mevalonate pathway, the metabolic pathway for the hepatic synthesis of cholesterol and other isoprenoids (Stossel, 2008). Statins, especially simvastatin and atorvastatin, are among the most frequently prescribed pharmacological groups (Gu et al., 2014).

They were discovered in 1976 by Endo et al., when testing the ability of certain fungi-derived components to inhibit cholesterol synthesis. The first statin, known as compactin or mevastatin was produced by the fungus *Penicillium citrinium* (Endo et al., 1976). Its

use was discarded due to toxicity phenomena observed in animal studies. A new agent called lovastatin was developed some years after a got the FDA approval in 1987.

Statins began to gain relevance in cardiovascular disease prevention when the "Scandinavian Simvastatin Survival Group" reported in 1994 a reduction in LDL-cholesterol levels after therapy with statins, which also reduced significantly the incidence of cardiovascular events (Johannesson et al., 1997). This reduction on cardiovascular events was reported to be of 30% (Steinberg, 2007).

Statins inhibit in a competitive way the HMG-CoA reductase, responsible from the conversion of this component to mevalonate in the first steps of this metabolic pathway. Due to the inhibition of this pathway, the cholesterol synthesis is also inhibited and this causes a lipid-lowering effect at serum level. The most common secondary effect is the possibility of myalgias, and more rarely they can cause arthralgia, myopathies or rhabdomyolysis.

6.6.2 Pleiotropic effects

Besides their lipid-lowering action, they have been reported to exert pleiotropic effects independent of their main lipid-lowering action (Estanislau et al., 2015) and to possess anti-inflammatory, immunomodulatory, and antimicrobial properties that may be useful against infections associated with bone healing (Shah et al., 2015). These effects come from the other metabolite synthesis in which the mevalonate pathway is involved and are also inhibited with these drugs, having effects on other systemic processes (Liao, 2002). Examples of these processes are a reduced risk for dementia (Jick et al., 2000), or for breast cancer (Denoyelle et al., 2001).

An anti-inflammatory effect of statin has been shown through a reduction in the expression proinflammatory cytokines and mediators such as C-reactive protein or adhesion molecules related to inflammatory processes (Halcox and Deanfield, 2004, Sakoda et al., 2006). Statins can activate the AKT1/PI3K pathway, leading to the inhibition of HMG-CoA reductase, while statin-induced protein prenylation can have other important downstream effects, including angiogenesis (increased vascular-endothelial growth factor) and osteogenesis (increased Bone Morphogenetic Protein-2 (BMP-2) and reduced Receptor Activator of Nuclear Factor κB and its ligand (RANK-RANKL) (Dalcico et al., 2013).

Regarding the effect on bone, it was first reported by Mundy et al. in the year 1999. In a study performed on rodents, simvastatin and lovastatin exerted osteogenic effects when administered both orally and subcutaneously (Mundy et al., 1999). After that, statin intake failed to confirm a lower incidence of bone fractures in osteoporosis patients treated also with statins (Hatzigeorgiou and Jackson, 2005). Statin-treated patients were found to have higher serum osteoprotegerin levels (Magan-Fernandez et al., 2014) and manifested anti-resorption (osteoclast inhibition), and anti-inflammatory (decrease in IL-6, C-reactive protein, adhesion molecules, and reactive oxygen species) activities (Cai et al., 2015). The precise effects of statins can vary according to their type and concentration (Konstantinopoulos et al., 2007). Simvastatin and atorvastatin have shown to differentiate mesenchymal stem cells towards an osteoblastic cell line, and also inhibit bone resorption (Chuengsamarn et al., 2010, Pagkalos et al., 2010).

6.6.3 Potential applications of statins in periodontics

Statins may exert an impact at periodontal level by three different mechanisms. First, their hypolipidemic effect would lead to an indirect improvement of periodontal variables by

decreasing the lipid values, as described previously. Second, they also exert a direct anti-inflammatory action through a decrease in proinflammatory cytokines *via* inhibition of the mevalonate pathway (Sangwan et al., 2013, Fentoglu et al., 2011b, Suresh et al., 2013). Finally, they may have an effect on bone metabolism, although research results have been controversial (Mundy et al., 1999, Hatzigeorgiou and Jackson, 2005). Studies in rodents have shown that certain types of statin (simvastatin and atorvastatin) can stimulate embryonic stem cells towards osteogenic differentiation lines and can inhibit bone resorption (Pagkalos et al., 2010). This evidence leads some authors like Fentoglü et al. to consider that periodontal treatment combined with statins can contribute to greater hyperlipidemia control (Fentoglu and Bozkurt, 2008). As a result of this anti-resorptive effect, other studies have also reported a lower tooth loss with the intake of oral statins over time, as we as an improvement of periodontal parameters. The use of different types of locally administered statins as coadjuvants of scaling and root planing has reported to improve clinical periodontal parameters and recover bone crest height (Pradeep et al., 2016, Priyanka et al., 2017).

Results from implant studies in rodents found that simvastatin administration improved the bone contact ratio, bone density, and osseointegration (Du et al., 2009, Ayukawa et al., 2004), which was also found to be enhanced by topical fluvastatin application around implants (Moriyama et al., 2008). In contrast, other authors found no increase in bone density in statin-treated defects (Ezirganli et al., 2014), and a recent study reported that simvastatin loading of implant surfaces exerted significant effects for only two weeks (Nyan et al., 2014). Formation of new bone tissue was observed in calvaria of rats after the topical injection of fluvastatin using tricalcium phosphate as carrier (Jinno et al., 2009). However, high doses of local statin can cause inflammation when used for bone

regeneration, as reported by two studies in which simvastatin was applied to calvarial defects in rodents (Nyan et al., 2009, Ezirganli et al., 2014). Simvastatin was found to affect osteogenic differentiation in a murine model (Pagkalos et al., 2010), while an *in vitro* study showed that exposure to this drug slightly increased osteoblast expression of osteocalcin, osteoprotegerin, alkaline phosphatase, and other bone markers (Liu et al., 2012).

RATIONALE & HIPOTHESIS

7. RATIONALE & HIPOTHESIS

7.1 RATIONALE

Evidence to date shows an association between periodontitis and CVD. Because of that, the American Heart Association and the European Society of Cardiology have recently added periodontitis as one important risk factor in their guidelines. However, this evidence only points to an association and longitudinal studies are needed to achieve a deeper knowledge about a possible causal relationship between these two diseases. In view of that, further research needs to be encouraged in order to produce prospective studies focused on establishing a possible a real causal relationship between periodontitis and CVD. The increasing relevance of the role that periodontitis plays in CVD may make it necessary to integrate periodontal treatment and maintenance in the public health system, due to the great importance of CVD as the first cause of mortality in developed countries.

Shared risk factors such as genetic variants, socio-economic and behavioral factors are certainly in part responsible for the association between periodontitis and impaired lipid metabolism. It remains to be clarified a cause-effect interrelationship also exists, and therefore whether periodontitis can induce higher lipid levels or higher lipid levels imply periodontitis (Fentoglu and Bozkurt, 2008). Answer to these questions would explain if CVD prevention is feasible by means of a reduction in periodontal disease. However, it is clear that improved periodontal health may affect metabolic control of hyperlipidemia and could be considered as an adjunct to the standard measures of hyperlipidemic patient care (Fentoglu et al., 2010). Improved oral hygiene and non-surgical periodontal treatment are effective in decreasing oxLDL, which is positively associated with a

reduction in oxidative stress (Tamaki et al., 2011) and with effect on the inflammation markers, which enhance risk and contribute to the development of CVD (Losche et al., 2005). However, the underlying mechanisms remain unclear.

Finally, raising public consciousness about the importance of regular oral hygiene might *per se* decrease development of periodontitis and consequently CVD. In individuals suffering of periodontitis, affecting lipid metabolism might prevent and/or reduce CV risk, and periodontitis treatment might stabilize disturbed metabolism of lipids.

7.2 HIPOTHESIS

The hypothesis of the present study is that hyperlipidaemic patients tend to present greater prevalence of periodontitis and worse periodontal clinical variables. Statins play a protective role against periodontitis, reducing inflammation and limiting the resorption of alveolar bone. Also, statins may exert an anabolic effect on bone and may therefore have an impact on osteogenic differentiation and proliferation.

OBJECTIVES

8. OBJECTIVES

GENERAL OBJECTIVE

The objective of the present study was to determine whether simvastatin consumption and hyperlipidemia are associated with periodontal status and with specific bone activity biomarkers.

SPECIFIC OBJECTIVES

1. Evaluate the association between lipid profile and non-specific inflammation and periodontal clinical parameters between the patients of the study.
2. Evaluate the effect of the statin treatment on bone by measuring serum biomarkers of bone metabolism.
3. Determine the *in vitro* effects of simvastatin on the morphology and proliferation of the MG63 human tumor osteoblast cell line.
4. Determine the *in vitro* effects of simvastatin on differentiation factors of the MG63 human tumor osteoblast cell line.

MATERIALS & METHODS

9. MATERIALS & METHODS

The methodologies specified in this section are a summary of the procedures described in the published articles.

9.1 Article 1: Magan-Fernandez A, Papay-Ramirez L, Tomas J, Marfil-Alvarez R, Rizzo M, Bravo M, et al. Association of simvastatin and hyperlipidemia with periodontal status and bone metabolism markers. J Periodontol. 2014;85(10):1408-15.

9.1.1 Study Design

An observational cross-sectional and analytic study was performed in hyper-and normolipidemic patients under follow-up at the "Zaidin Sur" Healthcare Center (Granada, Spain) between March and June 2013. Three groups were selected: hyperlipidemic patients treated with simvastatin (S) (10-40 mg/day), hyperlipidemic patients treated by diet alone (D), and normolipidemic patients (C). Hyperlipidemia was defined as the presence of one or more altered values of the lipid profile (total cholesterol>200 mg/dL, triglycerides>150 mg/dL, LDL-C>130 mg/dL)19. The diagnosis of hyperlipidemia had to be made at least 3 months before the study: no distinction was drawn among hyperlipidemia types.

Inclusion criteria were: age>18 yrs, presence of ≥16 teeth (excluding third molars), and no receipt of periodontal treatment during the previous 6 months. Exclusion criteria were: pregnancy, breast-feeding, the presence of systemic disease with lipid/bone metabolism involvement or related to periodontal status or of neoplastic disease, antibiotic treatment during the previous 3 months, treatment with drugs that affect bone metabolism, and receipt of hormone therapy.

According to Sample Power 2.0 (SPSS Inc., Chicago, IL), 63 cases and 63 controls were necessary to detect a standardized difference of 0.5 according to Cohen's scale (1), in the quantitative study variables (biochemical, periodontal etc.), with a power of 80% and an alpha-error of 5%. With the sample size achieved in our study (80 cases and 78 controls) statistical power increased to 88%. Sample size was estimated following the general rule for the standardized difference in a given output variable (e.g., clinical attachment (CA) loss in mm, etc.) between the C group and the two hyperlipidemic groups. For ethical reasons, because the blood analyses were clinically justified in the hyperlipidemic groups but not in the C group, we did not follow the general rule of an approximately equal sample size in two groups being compared. We elected to obtain a given statistical power for an arbitrary sample size ratio of 2 (S and D) to 1 (C). The estimation was based, using the t-test for independent groups, on the detection of a standardized difference of 0.8 (large, according to Cohen's scale20). This gave a size of 15 patients (C) and 30 patients (S and D) for a power of 70% (beta=0.30), which is slightly lower than the usual 80% (to minimize the number of normolipidemic patients, for ethical reasons), and a significance level of two-sided alpha=0.05. Specific software¶ was used to estimate the sample size.

Informed consent was signed by all participants in the study, which was approved by the Research Ethics Committee of the Granada Health District (CEI Granada).

9.1.2 Variables of the study

9.1.2.1 Sociodemographic and periodontal variables

Data were gathered for all participants on age, gender, body mass index (BMI), and consumption of alcohol (grams/day) and tobacco (cigarettes/day). The periodontal examination was conducted by a researcher (A.M.) blinded to the group affiliation of the

subjects, using a periodontal probe# and determining the PD, CA loss in mm, and the gingival bleeding index (GI) 21 and plaque index (PI) 22 in percentages. Six sites per tooth were probed. The mean PD and CA loss values were calculated for each patient based on the highest PD and CA loss values measured from the six sites probed for each tooth. We also recorded the number of periodontal pockets of 4-6 mm, 7-9 mm, and >9 mm in each patient and the number of sites with CA loss of 3-5 mm, 6-8 mm, and >8 mm.

9.1.2.2 Biochemical variables

In the healthcare center, two blood samples were taken from each participant after 12 h fasting: a 2-ml sample with EDTA as anticoagulant to detect the erythrocyte sedimentation rate (ESR) in mm/h by Westergreen technique; and an 8.5-ml sample for serum extraction, which was stored at -40ºC for subsequent determination of the other biochemical variables. Total cholesterol, triglycerides, and HDL were determined by standard enzymatic-colorimetric methods 23-25 and LDL by the Friedewald formula. C-reactive protein (CRP) was determined with an immunoturbidimetric assay 26. The bone metabolism markers procollagen type I N-terminal propeptide (P1NP) and C-terminal telopeptide of type I collagen (CTX) were measured by electrochemiluminescence immunoassay27, 28. Serum osteoprotegerin (OPG) and osteocalcin (OCN) concentrations were quantified with an ELISA** 29, 30. Samples were analyzed by the Clinical Analysis Department of the San Cecilio University Hospital (Granada, Spain), which provided the reference values.

9.1.3 Statistical Analysis

A statistical software package†† was used for descriptive purposes and comparisons between groups (Table 1 and 2). Quantitative variables were expressed as mean±standard

deviation, and analyzed with ANOVA for global comparison. When Normality assumptions were violated (tested with Shapiro-Wilk's test), the descriptive results were expressed as median (IQR), and analyzed with Kruskal-Wallis test. For periodontal variables following the Normality assumptions, multivariable regression analyses (Table 3) were performed using specific software‡ ‡ (indicating simple random sampling [SRS] as design), to analyze the effect of group on periodontal variables after adjusting for subjects' age, sex, tobacco use and alcohol use. The statistical tests used are reported in table footnotes.

9.2 Article 2: Magán-Fernández A, O'Valle F, Abadía-Molina F, Muñoz R, Puga-Guil P, Mesa F. Characterization and comparison of neutrophil extracellular traps in gingival samples of periodontitis and gingivitis: A pilot study. Journal of Periodontal Research. 2018;0(0).

All procedures in this study were performed in accordance with the 1964 Helsinki declaration and its later amendments. The study was approved by the Ethics Committee of the School of Dentistry of the University of Granada (reference: FOD/UGR/08/2016).

9.2.1 Cell line

MG-63 human osteosarcoma cell lines were obtained from the University of Granada Scientific Instrumentation Centre (Spain). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% non-essential amino-acid solution in a humidified incubator at 37 ºC, using a standard mixture of 95% air and 5% CO2. Cultured cell monolayers were detached with a trypsin–

ethylenediaminetetraacetic acid (EDTA) solution (0.25%) and seeded into 24-well plates at a density of 15 × 103 cells per well or into T75 flasks (Nunc, Rochester, NY, USA) at a density of 500 × 103 cells per flask, depending on the experiment.

9.2.2 Materials

Simvastatin and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (Sigma-Aldrich LLC, St. Louis, MO, USA). Simvastatin was resuspended at a concentration of 20 mg/mL in DMSO and stored at -20 ºC.

9.2.3 Proliferation assay

After incubation for 24 h under culture conditions, cells in the 24-well plates were incubated with simvastatin (0.005-25 µM) for 72 h. The cytotoxicity of this treatment was then evaluated in triplicate by the 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) proliferation assay, adding 20 µL MTT solution in cell culture medium (5 mg/mL) to each well. After incubation for 4 h at this temperature, the culture medium was removed, and the resulting formazan crystals were dissolved in 200 µL DMSO. The optical density (OD) of the converted dye, which is proportional to the number of viable cells, was measured at 570 nm (with subtraction of background at 690 nm) using a Titertek multisKan colorimeter (Flow Laboratories, Irvine, CA, USA). The percentage of surviving cells was calculated as: [OD treated cells/OD control (untreated) cells)] × 100. The same procedure was applied to control cells.

9.2.4 Simvastatin culture

MG-63 cells were seeded in T75 flasks and grown in the presence of simvastatin for 72 h at a final concentration of 0.01 µM (the highest concentration with no effect on cell proliferation after 72 h of treatment) or in the presence of the same amount of DMSO as

control. Cells were then removed from the culture and prepared for microscopic and immunocytochemical studies.

9.2.5 Transmission Electron Microscopy study

Several pellets of treated and control MG63 cells were fixed in 2.5% glutaraldehyde solution and then postfixed in 1% OsO4 at 4 °C for 2 h, washed in distilled water, dehydrated in increasing concentrations of acetone, and embedded in Epon following a conventional protocol. Semithin sections were stained with toluidine blue solution. Ultrathin (~70 nm-thick) sections obtained using a Reichert Jung ULTRACUT ultramicrotome (Leica, Wetzlar, Germany) were stained with lead citrate and uranyl acetate and under a Libra 120 Plus TEM (Zeiss, Oberkochen, Germany).

9.2.6 Scanning electron microscopy study

Samples from both groups of cultured MG63 cells were gently removed and immediately immersed in a sodium cacodylate-buffered formaldehyde-glutaraldehyde fixative for 24 h at room temperature and post-fixed in 20% osmium tetroxide for 2 h. Samples were then dehydrated by serial transfer in ascending concentrations of acetone (50-100%) and infiltrated with liquid carbon dioxide before the critical drying point. Finally, samples were made electrically conductive by mounting them on aluminum slabs with a silver point, followed by sputter coating with gold/palladium to a thickness of approximately 250 A. After attachment of these specimens to an acrylic plate with glue tape, the plate was vertically divided into two pieces using a diamond disc with chisel and hammer, followed by examination of their cut surfaces with a Quanta 400 SEM (FEI, Hillsboro, OR, USA) at 5-10 kV.

9.2.7 Immunocytochemical analysis

After detachment from the plastic substrate, MG63 osteosarcoma cells from both groups were centrifuged at 1500 rpm for 3 min in phosphate buffer solution (PBS), washed, resuspended in PBS, and centrifuged at 11000 rpm for 30 s before embedding the resulting pellets in paraffin. Paraffin-embedded sections were dewaxed, hydrated, and heat-treated in 1 mm EDTA (pH 8) for antigenic unmasking in an antigen retrieval PT module (Thermo Fisher Scientific Inc., Waltham, MA) at 95 °C for 20 min. Sections were incubated for 16 h at 4 ºC with prediluted polyclonal antibody against Musashi-1 (mesenchymal stem cells, polyclonal, Sigma-Aldrich LLC, St. Louis, MO, USA) and Runx2 (runt related transcription factor 2, clone M-70) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) both at 1:100 dilution, and with prediluted monoclonal antibodies against Ki-67 (proliferative cells, clone SP-6), CD56 (osteoblast differentiation, clone 56C04), and CD44 (osteocyte cells, clone 156-3C11); sections were then incubated for 10 min at room temperature to analyze the cell differentiation and antiproliferative response. The immunocytochemical study was conducted using the micropolymer-peroxidase-based method (Master Polymer) with automatic immunostainer (Autostainer 480, Thermo Fisher Scientific Inc., Waltham, MA) followed by development with diaminobenzidine (Other monoclonal antibodies and reagents were obtained from Master Diagnóstica, Granada, Spain). Appropriate positive (tonsil) and negative (non-immune serum) controls were run concurrently. Hematoxylin was used for nuclear counterstaining. Results were expressed as percentages of positive cells for each antibody, counting 200 cells per high-magnification field (40x objective) in three independent experiments.

9.2.8 Statistical analysis

SPSS v. 20.0 (IBM SPSS, Armonk, NY, USA) was used for the statistical analysis. A non-parametric test (Mann-Whitney U-test) was applied to compare positive cells between control and treated cells. A $p<0.05$ was considered as statistically significant.

RESULTS

10. RESULTS

In this section, a summary of the results obtained in the published articles related to this thesis is detailed.

10.1 Article 1: Magan-Fernandez A, Papay-Ramirez L, Tomas J, Marfil-Alvarez R, Rizzo M, Bravo M, et al. Association of simvastatin and hyperlipidemia with periodontal status and bone metabolism markers. J Periodontol. 2014;85(10):1408-15.

The study included 73 participants: 16 normolipidaemic controls, 29 simvastatin-treated hyperlipidaemic patients, and 28 hyperlipidaemic patients treated by diet alone. Table 1 displays the values obtained for each socio-demographic and habit variable and the results of non-adjusted comparisons among the study groups. There were significant differences in age.

Table 1. Socio-demographic and habit characteristics by study group (n=73).

Variable	C (n=16)	S (n=29)	D (n=28)	p-value*			
				Global	C *vs* S	C *vs* D	S *vs* D
Sex, % males	50.0	62.1	35.7	0.138	0.639	0.543	0.085
Age (yrs), mean±sd	46.3±10.7	63.1±8.9	52.7±9.0	<0.001	<0.001	0.096	<0.001
Age (yrs), range	30-63	39-78	32-70				
BMI, mean±sd	27.5-5.6	27.6-3.7	27.9-5.8	0.965	>0.999	>0.999	>0.999
Tobacco (cig./day), %				0.130	0.051	0.207	0.339
0	62.5	89.7	78.6				
1-10	25.0	3.4	17.9				
11-30	12.5	6.9	3.6				
Alcohol (grams/day), %				0.848	0.648	0.917	0.625
0	81.3	75.9	78.6				
1-20	6.3	6.9	17.9				
21-84	12.5	17.2	3.6				

* For Global comparisons: Chi -quare (Sex), ANOVA (Age and BMI), and Kruskal-Wallis (Tobacco and Alcohol, with original values, i.e., with non-collapsing categories). For Paired comparisons: Corrected chi square (Sex), Bonferroni's method (Age and BMI), and Mann-Whitney Test (Tobacco and Alcohol).

Table 1. Socio-demographic and habit characteristics by study group

Periodontal and biochemical variables are shown in Table 2. The mean ESR was higher in the diet-treated hyperlipidaemic patients than in the normolipidaemic controls (p=0.037). Serum OPG concentrations were higher in the S group than in D group (p=0.049) and were similar between the D and C groups.

Table 2. Periodontal and Biochemical variables by study group (n=73).

Variable *	C (n=16)	S (n=29)	D (n=28)	p-value			
				Global	C vs S	C vs D	S vs D
Periodontal							
Average probing depth (mm.), mean±sd	2.3±1.0	2.0±0.9	2.3±0.9	0.523	0.990	>0.999	>0.999
Sites (n) with PD of 4-6 mm., median (IQR†)	1.5 (0-17.7)	0 (0-4)	1 (0-7.5)	0.582	0.352	0.626	0.466
Sites (n) with PD of 7-9 mm., median (IQR†)	0 (0-0)	0 (0-0)	0 (0-0)	0.073	0.287	0.019	0.161
Sites (n) with PD>9 mm., median (IQR†)	0	0	0	-	-	-	-
Average CA loss (mm.), mean±sd	3.0±1.6	2.7±1.2	2.6±1.1	0.724	>0.999	>0.999	>0.999
Sites (n) with CA loss of 3-5 mm., median (IQR†)	22.5 (0.7-36)	14 (7-41.5)	16 (3-22.5)	0.763	0.924	0.405	0.632
Sites (n) with CA loss of 6-8 mm., median (IQR†)	1 (0-12.2)	0 (0-5.5)	0 (0-2)	0.206	0.391	0.087	0.261
Sites (n) with CA loss>8 mm., median (IQR†)	0 (0-0.7)	0 (0-0)	0 (0-0)	0.272	0.373	0.106	0.418
Bleeding Index (% teeth), mean±sd	19±18	11±11	13±11	0.119	0.125	0.415	>0.999
Plaque Index (% surfaces), mean±sd	42±29	39±34	48±29	0.528	>0.999	>0.999	0.775
Biochemical							
Cholesterol (mg/dl), mean±sd	185±21	194±29	246±32	<0.001	0.992	<0.001	<0.001
TG (mg/dl), mean±sd	77±28	122±68	162±145	0.028	0.448	0.026	0.425
LDL (mg/dl), mean±sd‡	106±16	116±28	151±27	<0.001	0.674	<0.001	<0.001
HDL (mg/dl), mean±sd‡	63±12	57±11	65±21	0.193	0.748	>0.999	0.239
CRP (mg/l), median (IQR)	2.1 (0.4-5.5)	1.2 (0.4-2.8)	1.2 (0.7-3.2)	0.495	0.259	0.582	0.457
ESR (mm/h), median (IQR) §	5 (3.7-8)	7 (4-13)	9.5 (5-15)	0.119	0.139	0.037	0.532
OPG (pmol/L), mean±sd‖	3.5±1.4	4.5±1.9	3.5±1.1	0.033	0.160	>0.999	0.049
OCN (ng/mL), mean±sd‖	17±8	15±7	16±7	0.521	0.759	>0.999	>0.999
CTX (ng/mL), mean±sd‖	0.30±0.12	0.32±0.14	0.33±0.14	0.759	>0.999	>0.999	>0.999
P1NP (ng/mL), mean±sd‖	40±15	39±15	40±14	0.969	>0.999	>0.999	>0.999

* See text for a complete definition of acronyms.
† Interquartile range.
‡ Two missing values in S group.
§ Eight missing values (2 in C, 2 in S and 4 in D group).
‖Two missing values (1 in C and 1 in S group).
¶ For Global comparisons: ANOVA (for variables expressed with means) and Kruskal-Wallis (for variables expressed with medians); for Paired comparisons: Boferroni's method and Mann-Whitney Test, respectively.

Table 2. Periodontal and Biochemical variables by study group

We analyzed the associations (Pearson's correlations) of periodontal variables (PD, CA loss, GI and PI) with the lipid profile (cholesterol, TG, LDL and HDL). None of the correlations, which ranged between -0.13 and 0.18, were statistically significant (results not shown).

Multivariable linear regression analyses adjusted for sex, age, tobacco and alcohol (known confounding factors for periodontitis) were performed for the adjusted comparison of periodontal variables among groups. Results in table 3 show a mean reduction of 0.8 mm (p=0.050) in CA loss for the simvastatin-treated hyperlipidaemic patients in comparison to the normolipidaemic controls.

Table 3. Multivariable linear regression analysis for mean differences between Groups, adjusted for age, sex, tobacco and alcohol, on periodontal variables (n=73).

	S vs C			D vs C			S vs D		
Variable	β	(95%-CI)	p-value	β	(95%-CI)	p-value	β	(95%-CI)	p-value
Average Probing depth (mm.)	-0.4	(-1.1 to 0.3)	0.230	-0.0	(-0.6 to 0.6)	0.986	-0.4	(-0.9 to 0.1)	0.132
Average CA loss (mm.)	-0.8	(-1.5 to -0.0)	0.050	-0.3	(-1.1 to 0.4)	0.387	-0.4	(-1.1 to 0.2)	0.220
Bleeding Index (% teeth)	-6.1	(-17.4 to 5.2)	0.293	-5.1	(-14.9 to 4.8)	0.317	-1.1	(-8.1 to 6.0)	0.769
Plaque Index (% surfaces)	-4.1	(-25.2 to 17.0)	0.706	6.0	(-12.9 to 25.0)	0.533	-10.1	(-26.8 to 6.6)	0.239

Table 3. Multivariable linear regression analysis for mean differences between Groups, adjusted for age, sex, tobacco and alcohol, on periodontal variables

10.2 Article 2: Magán-Fernández A, O'Valle F, Abadía-Molina F, Muñoz R, Puga-Guil P, Mesa F. Characterization and comparison of neutrophil extracellular traps in gingival samples of periodontitis and gingivitis: A pilot study. Journal of Periodontal Research. 2018;0(0).

10.2.1 Cell viability test

After 72 h of exposure to simvastatin, the highest dose with no effect on cell proliferation was found to be 0.01 µM. At higher doses, the proliferative capacity of these cells began to decrease in comparison to control cells.

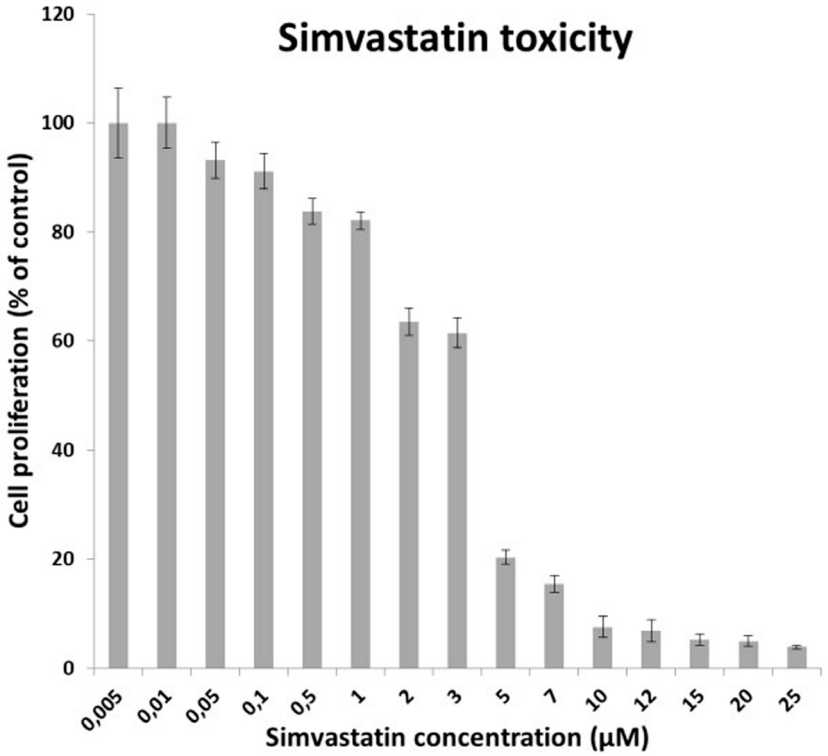

Figure 5. In vitro toxicity of simvastatin in MG63 cell line. Growth of MG63 cell was evaluated after 72 h of exposure to a wide range of simvastatin concentrations [0.005–25 µM] in comparison to control cells treated with solvent alone.

10.2.2 Electron microscopy

TEM study of cultured MG63 cells revealed a similar morphology to that reported by Fernández et al. (19). Control cells showed a clear low-density cytoplasm with an appreciable reduction in intracytoplasmic organelles, numerous unstructured free filaments in the cytoplasm, and many lysosomal bodies containing abundant phagocytic material. In contrast, simvastatin-treated MG63 cells showed a high density of cytoplasmic organelles, with a large number of mitochondria and numerous free ribosomes and lysosomal bodies. There was an appreciable increase in lipid vacuoles underlying the cell cytoplasmic membrane, which were grouped, fused, and intermixed with smaller vacuoles and structured vesicular invaginations. The cytoplasmic membrane contained numerous thin projections (see also SEM observations above).

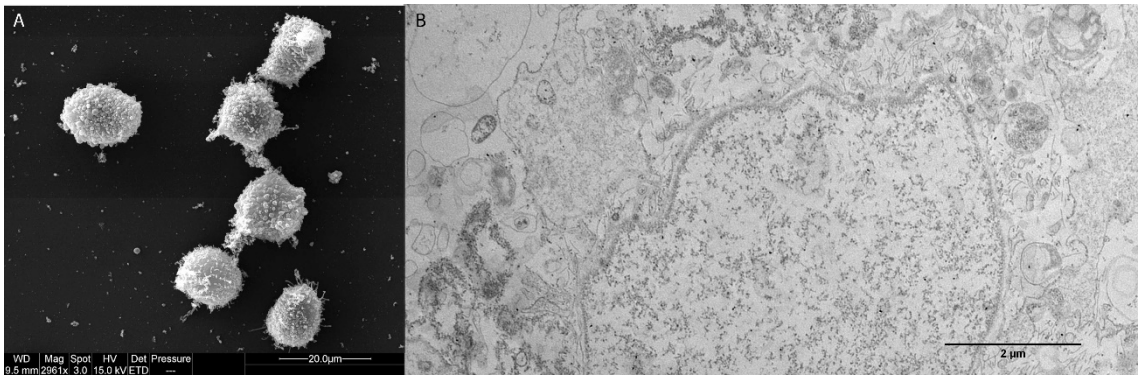

Figure 6. Ultrastructural study with SEM and TEM. A) Spheroid morphology and numerous secretion vesicle accumulations in control MG63 cells. No intercellular-connecting cytoplasmic projections were observed (scale bar in figure). B) Control cells treated with DMSO show a clear low-density cytoplasm with appreciable decrease in intracytoplasmic organelles, containing numerous free, unstructured filaments and a large number of lysosomal bodies incorporating abundant phagocytic material.

SEM study also showed the morphology of cultured MG63 cells to be similar to that reported by Fernández-Barbero et al. (Fernandez-Barbero et al., 2006). The morphology of control MG63 cells was spheroid, with a large accumulation of secretion vesicles on their surface but no cell-interconnecting cytoplasmic projections. Cells treated with simvastatin were spheroid, polygonal, or spindle-shaped, and the spindle-shaped cells had long cytoplasmic projections interconnecting cells. Numerous "microvilli-like" filamentous projections were also distributed over the whole surface of the cells, especially those with spheroid morphology, but they generally formed no defined pattern.

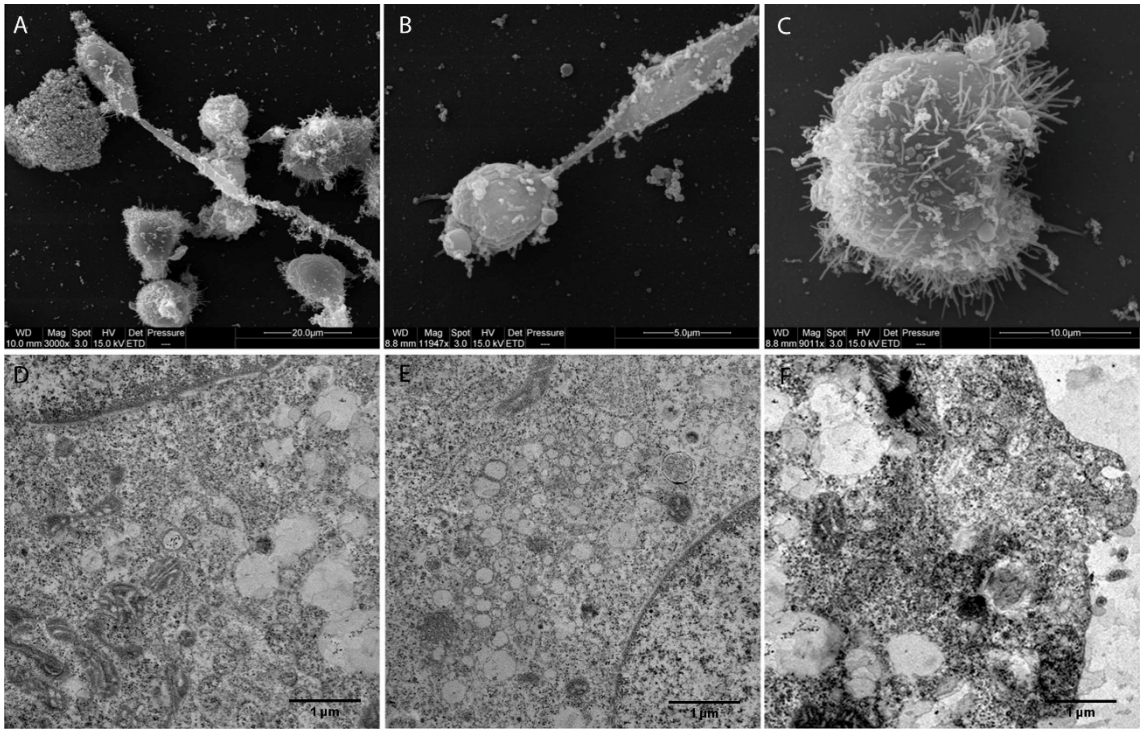

Figure 7. Ultrastructural study with SEM and TEM. A) Morphological heterogeneity (spheroid, polygonal, and spindle-shaped) of MG63 cells in cultures treated with simvastatin. B) Spindle-shaped cell with long projection that contacts spheroid cell in the simvastatin-treated culture. C) Spheroid cell with presence of numerous "microvilli-like" filamentous projections distributed homogeneously over its surface with no defined pattern (scale bar in figures). D) Simvastatin-treated MG63 cells showing dense cytoplasm with large number of mitochondria alongside free ribosomes and lysosomes. E) Significant increase in the accumulation of lipid vacuoles of varied size in the cytoplasm. F) Microphotograph showing organization of the "clathrin-type" subcellular microvesicular system.

10.2.3 Immunocytochemical results

Cytoplasmic membrane expression of CD44 was intense in all MG63 cells and was not modified by statin treatment. After 72 h of treatment, the CD56, Msi1, and Ki-67 expression of MG63 cells was significantly ($p<0.001$, Mann-Whitney U-test) decreased in comparison to control cells. No nuclear Runx2 expression was detected in MG63 cells.

Table 4. Percentage immunocytochemical marker expressions after 72 hours of culture.

	Control	Simvastatin	*p*-value*
CD44 (%)	99.6±0.78	100±0.0	NS
CD56 (%)	34.3±2.75	11.0±3.68	$p < 0.001$
Ki-67 (%)	87.6±4.86	33.0±5.31	$p < 0.001$
Msi1 (%)	97.6±1.69	16.6±3.31	$p < 0.001$
Runx2 (%)	0.5±0.54	0.5±0.54	NS

Values expressed as mean percentage± standard deviation of three independent experiments
*Control *vs*. Simvastatin, Mann-Whitney U-test. NS: Non-significant

Table 4. Percentage immunocytochemical marker expressions after 72 hours of culture

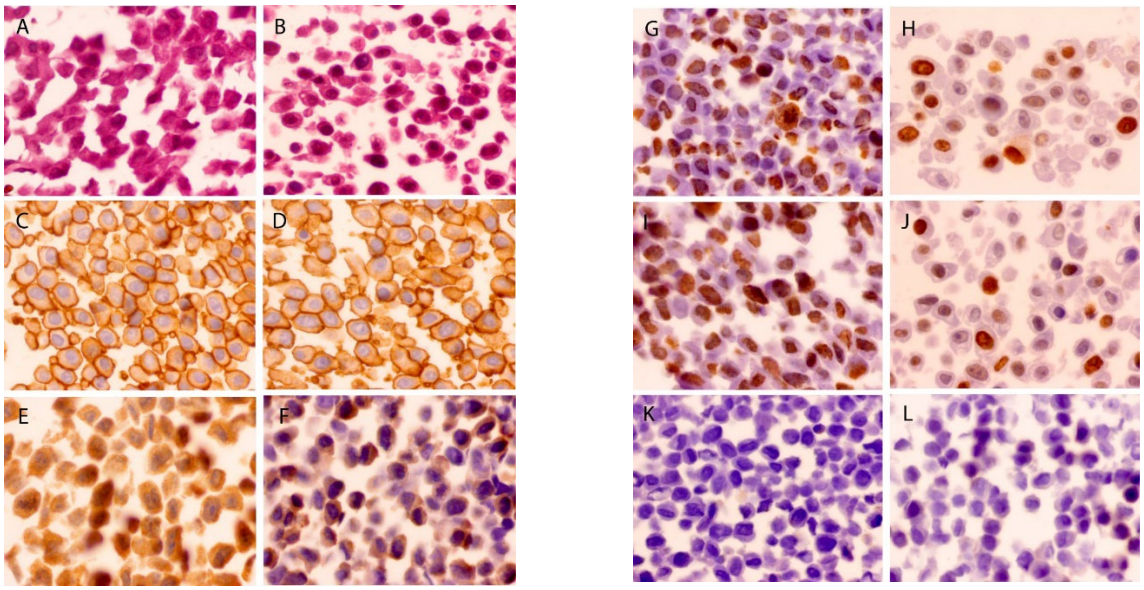

Figure 8. Morphological and immunocytochemical study. A and B) Increase in cytoplasmic vacuolization and apoptotic cells in simvastatin-treated MG63 cells. C and D) No modification of CD44 expression after incubation with simvastatin for 72 h; E and F) Reduction in CD56 expression; G and H) Reduction in nuclear expression of Ki-67 (cell proliferation); I and J) Reduction in nuclear expression of MSi-1; K and L) No induction of Runx2 expression (original magnification x40).

DISCUSSION

11. DISCUSSION

11.1 Article 1: Magan-Fernandez A, Papay-Ramirez L, Tomas J, Marfil-Alvarez R, Rizzo M, Bravo M, et al. Association of simvastatin and hyperlipidemia with periodontal status and bone metabolism markers. J Periodontol. 2014;85(10):1408-15.

In this study, serum OPG concentrations were higher in hyperlipidemic patients treated with simvastatin than in hyperlipidemic patients treated by dietary measures alone or in normolipidemic patients. The periodontal CA loss was lesser in simvastatin-treated hyperlipidemic patients than in normolipidemic patients. The periodontal status of study participants was evaluated according to their mean PD and CA loss values, also considering the number of periodontal pockets and the CA loss, both classified into three categories. This extensive evaluation of periodontal status was designed to facilitate detection of its possible association with hyperlipidemia and/or simvastatin. It has been reported that indexes such as the Community Periodontal Index (CPI) or Community Periodontal Index of Treatment Needs (CPITN) (Ainamo et al., 1982), used in previous studies on the relationship with hyperlipidemia, may provide data on prevalence and severity of periodontal conditions, but they should not be used for reporting levels of periodontal disease (Lewis et al., 1994).

The present results are in line with the findings of the only two published studies on the periodontal effects of statins in humans. Thus, Sangwan et al. (Sangwan et al., 2013) found that the periodontal status was similar between S and C groups but worse in the D group. Unlike in the present study, however, they considered mean PD values alone, which may underestimate the prevalence of periodontitis. Fajardo et al. (17), in a clinical

trial of normolipidemic periodontal patients treated with atorvastatin and habitual periodontal therapy or habitual periodontal therapy alone, found clinical and radiological improvements in the statin-treated group, including alveolar bone gain. However, their pilot study had a small sample (n=38) and a follow-up of only three months, too short to evaluate the effects of prolonged statin consumption, which is typical in hyperlipidemic patients. The duration of statin therapy in the treated patients in the present study ranged from 3 months to 132 months.

Simvastatin has been found to halt bone loss in animals with induced periodontitis, acting as an anti-inflammatory and bone formation promoter (Dalcico et al., 2013). Experimental studies have also found that its local application alone or in combination with bisphosphonates exerts positive effects on bone formation (Price et al., 2013). The direct relationship between statin consumption and OPG concentrations evidenced in the present study was also previously observed in vitro (Liu et al., 2012, Stein et al., 2011, Kaji et al., 2005). The improved CA loss values in our simvastatin-treated patients may be explained by their increased OPG concentrations. OPG would act by binding with receptor activator of nuclear factor κ B (RANK) and inhibiting the activation of this receptor by its ligand (RANKL), thereby activating osteoclastogenesis (Gori, 2000). The RANK/RANKL/OPG pathway is crucial for maintaining bone integrity, given the important role of this osteoclastogenesis control in bone resorption processes. We highlight that only lipophilic statins (e.g., simvastatin) have these effects on bone, because their lipophilia allows them to cross the cell membrane by passive diffusion mechanisms (Pagkalos et al., 2010). They also deliver benefits at bone level through their anti-inflammatory action against IL-6 and IL-8 cytokines (Sakoda et al., 2006) and through their angiogenic effect, being able to stimulate the vascular endothelial growth factor

(VEGF) (Maeda et al., 2003). The systemic administration of statins produces potent effects at periodontal level, and it was reported that their concentration in crevicular fluid is 10- to 100-fold higher than in serum (Sangwan et al., 2013), with an anti-inflammatory effect on gingival levels of IL-1β, according to Suresh et al. (Suresh et al., 2013). The prevalence of periodontitis among our statin-treated patients was low (n= 15), and a larger sample of treated patients might have revealed more favorable effects of the drug on the other periodontal variables studied. We found no effects of simvastatin on OCN, P1NP, and CTX levels, which may be because these markers do not participate in the action mechanism of this drug. Future studies should evaluate the effects of statins on other bone markers, such as bone morphogenetic proteins or VEGF.

Hyperlipidemia was defined by the presence of one or more elevated lipid profile values in the present study (see material and methods) and no association was found with the quantitative periodontal variables considered, as also reported by Rizzo et al. (Rizzo et al., 2012) and Saxlin et al. (Saxlin et al., 2008). However, other authors observed a relationship between hyperlipidemia and periodontitis. Fentoglu et al. (Fentoglu et al., 2009) associated hyperlipidemia with hyperactivity of white cells, alteration in polymorphonuclear leukocyte (PMN) function, and higher production of free radicals, indicating the action mechanisms that may be involved. Three studies with wide samples also reported on the association between hyperlipidemia and periodontitis. In a study of 10,590 subjects, Katz et al. (Katz et al., 2002) found the PD to be significantly associated with total cholesterol and LDL levels. Using data obtained from the Korean national health survey, Lee et al. (Lee et al., 2013)obtained an odds ratio (OR) of 1.2 for periodontitis in patients with hyperlipidemia in a sample of 18,210 subjects, identical to the OR found by Boland et al. (Boland et al., 2013) in a similar study of 2,475 patients.

The weak association found in these two studies may be attributable to their use of the CPI or CPITN index and WHO periodontal probe, which are designed to detect periodontal disease in large populations and may offer inadequate sensitivity for more precise studies, as previously stated. None of these three studies shed light on the mechanisms underlying the association between these diseases.

In the present study, the ESR was significantly higher (3.8 mm/h) in the D group than in the C group. This may reflect a baseline proinflammatory state attributable to the hyperlipidemia, given the capacity of this disease to increase the expression of IL-1β *via* PMN involvement (Cutler et al., 1999). The relationship between CRP levels and periodontitis is controversial. Whereas no association was observed in the present investigation or in the study by Rizzo et al. (Rizzo et al., 2012), a meta-analysis of 10 cross-sectional studies reported strong evidence of elevated plasma CRP levels in periodontitis patients versus controls (Paraskevas et al., 2008). There is a need for further investigations to quantify serum levels of IL-1β, IL-6, IL-8, and TNFα cytokines and establish the true proinflammatory role of hyperlipidemia.

11.2 Article 2: Magán-Fernández A, O'Valle F, Abadía-Molina F, Muñoz R, Puga-Guil P, Mesa F. Characterization and comparison of neutrophil extracellular traps in gingival samples of periodontitis and gingivitis: A pilot study. Journal of Periodontal Research. 2018;0(0).

Ultrastructural and immunocytochemical changes observed in this *in vitro* study suggest that simvastatin exerts differentiating and antiproliferative effects on the MG63 cell line.

Ultramicroscopic study of control MG63 cells revealed groups of cells with cytoplasmic elements characteristic of elevated cell activity and groups with high cytoplasmic destructuration, with a "cytoplasmic void" showing numerous destructured microfilaments and numerous lysosomal bodies containing cell material from different organelles. The abundant apoptotic phenomena observed in TEM images and the high expression of the proliferation marker Ki-67 indicate the presence of major stress in these cells. This cytoplasmic destructuration in MG63 cells was morphologically reverted by simvastatin treatment, which significantly reduced the number of cytoplasmic vacuoles containing cell material and increased the vesicular turnover at cytoplasm membrane level, with the emergence of structured vesicular invaginations and the acquisition of a normal cytoplasmic conformation. According to these results, simvastatin may act against cell stress, stabilizing osteogenic cell renewal processes. In the context of bone production, preservation, and remodeling, simvastatin may therefore function as a modulator of osteoclastogenesis/osteogenesis.

TEM findings of a large accumulation of lipid vacuoles in simvastatin-treated MG63 cells would result from statin-induced lipoprotein deprivation of the medium, which in the present case represents physiological normalization through recovery of an adequate lipid

cell metabolism. This interpretation is supported by SEM observations of numerous pinocytotic vesicles and abundant microvilli on the surface of treated cells. Simvastatin was previously reported to produce an accumulation of cytosolic lipid droplets in both non-malignant and malignant cells, which may contribute to their antiproliferative effects (Gbelcova et al., 2013). In contrast, Martinet et al. found little cytoplasm vacuolization of mesenchymal stem cells (MSCs) after treatment with 0.01 μM simvastatin (Martinet et al., 2008), which may be attributable to the difference in baseline differentiation between MSCs and MG63 cells, which have already acquired some degree of osteogenic differentiation. The previously documented absence of Runx2 expression suggests that the mechanism underlying osteogenic differentiation in this cell line is independent of the expression of this transcription factor (Lucero et al., 2013). These data indicate that the effectiveness of simvastatin may depend upon the initiation of osteogenic differentiation by target cells; in other words, the action of simvastatin may be dependent on the degree of differentiation.

Immunocytochemical analysis showed that simvastatin treatment had no significant effect on the CD44 expression of MG63 cells but significantly reduced their expression of CD56, Musashi-1, and Ki-67 (cell proliferation index). No Runx2 expression was detected in either treated or control cells. Given that CD56 and Musashi-1 expression is characteristic of stem cells and those with a low degree of differentiation (Kaneko et al., 2000, Gupta and Rosenberg, 2008), these results suggest that simvastatin treatment increases the differentiation degree of MG63 cells, which is consistent with the significant reduction in Ki-67 expression (proliferation-differentiation balance) (Stein et al., 1996). This mechanism may be dependent on the inhibition of geranylgeranyl pyrophosphate synthase or other upstream isoprenoids (Weivoda and Hohl, 2011). The response of

Musashi-1 can be modulated by environmental factors and can regulate physiological or pathological cell renewal (MacNicol et al., 2015). In vivo and in vitro studies have linked high CD56 expression to worse tumor cell behavior in comparison to low or negative expression (Ash et al., 2011). Regarding bone tissue, CD56-positive myeloma cells have been linked to the presence of lytic bone lesions (Ely and Knowles, 2002). Musashi-1 may play a possible role in osteogenic differentiation, downregulating the Wnt1 pathway and expression of the miR-148 family, because Musashi-1 knockdown was found to increase expression of these two pathways, which are involved in the osteogenic differentiation of stem cells (Hong et al., 2013). This reduction in Musashi-1 expression is also compatible with the osteoarticular tissue expression of Musashi-1, reported for the first time by our group, suggesting a possible involvement of this factor in tissue regeneration (O'Valle et al., 2015).

The effects of statins on bone metabolism were first reported by Mundy et al. in an *in vitro* animal model, which revealed higher BMP-2 expression in cultured osteoblast cells and increased medullar bone formation in rodents (Mundy et al., 1999). Later investigations related statin consumption to a higher bone density and lower incidence of fractures in osteoporosis patients, although a meta-analysis was unable to confirm this association (Hatzigeorgiou and Jackson, 2005). Statins were found to favor bone regeneration in an animal model of periodontitis, increasing BMP-2 and reducing RANK-RANKL (Dalcico et al., 2013), while a clinical study by our group observed higher osteoprotegerin levels in simvastatin-treated patients (Magan-Fernandez et al., 2014). Statin consumption was recently associated with lower tooth loss over time in a prospective epidemiologic study with 5-year follow-up in a European population (Meisel et al., 2014). Research is ongoing into the optimal statin concentrations to achieve anti-

inflammatory and bone anabolic effects in the local treatment of periodontal lesions; various carriers and drug delivery systems have been used, including the application of statins as bioactive agents on implant surfaces (Zhang et al., 2016, Yue et al., 2016, Kwon et al., 2015).

The proliferation and cell functions exhibited by osteosarcoma cells may not be fully representative of those in human primary osteoblasts, given reported differences between them (38). Nevertheless, the MG63 cell line has been widely used as an in vitro model for bone research, and findings on the proliferation of these cells have proven comparable to observations in primary human osteoblasts (39).

CONCLUSIONS

12. CONCLUSIONS

1. The lipid profile did not correlate with periodontal clinical parameters between patients with and without hyperlipidaemia. However, a baseline increased systemic inflammatory state in terms of erythrocyte sedimentation rate was shown by hyperlipidemic patients.
2. The intake of simvastatin was associated with increasing serum osteoprotegerin concentrations. This could have a protective effect against bone destruction and showed a reduced periodontal CA loss.
3. Simvastatin caused a reduction of proliferation in MG63 cells and also may cause a recovery of the normal morphological status of the cell.
4. Simvastatin treatment of osteogenic MG63 cells showed a better functional status, besides enhancing the differentiation of the cells compatible with an osteogenic change.

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13. REFERENCES

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APPENDICES

D. Miguel Ángel Calleja Hernández Secretario del Comité de Ética de la Investigación de Centro de Granada (CEI-GRANADA)

CERTIFICA

Que este Comité ha analizado la propuesta de D. Antonio Magán Fernández (UGR-Odontología) para que se realice el proyecto de investigación titulado: "Influencia de la hiperlipemia y el tratamiento con estatinas sobre la periodontitis" y considera que:

Se cumplen los requisitos necesarios de idoneidad del proyecto en relación con los objetivos del estudio.

La capacidad del investigador y los medios disponibles son apropiados para llevar a cabo el estudio.

Entendiendo que dicho estudio se ajusta a las normas éticas esenciales y criterios deontológicos que rigen en este centro.

Y que este Comité acepta que dicho estudio sea realizado por D. Antonio Magán Fernandez (como investigador principal en el mismo y colaboradores.

Lo que firmo en Granada a veintiocho de enero de dos mil trece.

CONSEJERÍA DE SALUD
Consejería de Salud
Granada
Comité de Ética de la Investigación
Servicio Andaluz de Salud

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HOJA INFORMATIVA PARA EL PACIENTE

Influencia de la hiperlipemia y el tratamiento con estatinas sobre la periodontitis

Antes de proceder a la firma del consentimiento informado, lea atentamente la información que a continuación se le facilita y realice las preguntas que considere oportunas.

Naturaleza

Usted va a participar en un estudio de investigación sobre enfermedad periodontal, comúnmente conocida como piorrea y niveles altos de colesterol, que cumple con las normas aprobadas en la declaración de Helsinki y cuyos objetivos son:

- Determinar la prevalencia de enfermedad periodontal en enfermos con patología del metabolismo graso.
- Valorar la mejora de las variables bioquímicas y orales con el consumo de estatinas, fármacos utilizados para el tratamiento del colesterol elevado.
- Valorar la asociación de sus valores de colesterol en sangre y la presencia de enfermedad periodontal.
- Valorar el efecto antiinflamatorio de las estatinas determinando marcadores de inflamación en sangre.
- Valorar el efecto de las estatinas sobre el hueso de soporte dental mediante la evaluación de proteínas del metabolismo óseo.

Para alcanzar esos objetivos, el investigador precisa:

1) Que conteste un cuestionario indicando sus datos personales y hábitos.

2) Que le realice un examen bucal consistente en revelar la placa bacteriana con reveladores orgánicos (para determinar higiene oral), y explorar la encía con una sonda periodontal para detectar lesiones de periodontitis.

3) Que me tomen una muestra de sangre para cuantificar:
    a. Proteína C reactiva (PCR).
    b. VSG (velocidad de sedimentación globular).
    c. Perfil lipídico: Colesterol total, triglicéridos, LDL y HDL.
    d. Citoquinas proinflamatorias.
    e. Proteínas del metabolismo óseo.

Importancia

Con la valoración de los parámetros analizados, se pretende conocer si unos valores elevados de colesterol en sangre conllevan un mayor riesgo de enfermedad periodontal, con la consecuente pérdida de dientes. Así mismo, también se pretende valorar el efecto de los fármacos para el colesterol sobre esta enfermedad que afecta a la encía y al hueso de soporte del diente.

Implicaciones para el donante/paciente

- La participación es totalmente voluntaria.
- El donante/paciente puede retirarse del estudio cuando así lo manifieste, sin dar explicaciones y sin que esto repercuta en sus cuidados médicos.
- Todos los datos carácter personal, obtenidos en este estudio son confidenciales y se tratarán conforme a la Ley Orgánica de Protección de Datos de Carácter Personal 15/99.
- La información obtenida se utilizará exclusivamente para los fines específicos de este estudio.

Riesgos de la investigación para el donante/paciente

En principio no existen riesgos añadidos a los propios de padecer unos niveles elevados de colesterol, dado que únicamente se realiza una acción de exploración y diagnóstico, sin efectos reales sobre el paciente. De hecho, la información que el paciente pueda obtener de su estado de salud oral o de sus niveles de colesterol puede contribuir a su concienciación para una mayor efectividad en su tratamiento.

En cuanto a las determinaciones bioquímicas a realizar, se harán a partir de una muestra de sangre obtenida de forma regular para un control rutinario de salud, y por ello, no existe riesgo añadido para el paciente.

Si requiere información adicional se puede poner en contacto con el investigador principal del proyecto en el teléfono: 616-633-825 o en el correo electrónico: amaganf@correo.ugr.es.

DOCUMENTO DE CONSENTIMIENTO INFORMADO

Influencia de la hiperlipemia y el tratamiento con estatinas sobre la periodontitis.

De conformidad con lo dispuesto en la legislación vigente:

- *Ley 14/1986, de 25 de abril, General de Sanidad*
- *Ley 2/1998, de 15 de junio, de Salud de Andalucía. (BOJA núm.74 de 4 de julio de 1998*
- *Ley 41/2002, de 14 de noviembre, básica reguladora de la autonomía del paciente y de derechos y obligaciones en materia de información y documentación clínica. (BOE núm.274 de 15 de noviembre de 2002).*

Yo D/Dña.....,
con DNI núm..... y mayor de edad

- He leído la hoja de información que se me ha entregado junto con este consentimiento.

- He podido hacer preguntas sobre el estudio "Influencia de la hiperlipemia y el tratamiento con estatinas sobre la periodontitis".

- He recibido suficiente información sobre el estudio "Influencia de la hiperlipemia y el tratamiento con estatinas sobre la periodontitis".

- He hablado con el odontólogo investigador D. ANTONIO MAGAN FERNANDEZ.

- Deseo ser informado/a de los resultados obtenidos y otros datos de carácter personal que se obtengan en el curso de la investigación, incluidos los descubrimientos inesperados que se puedan producir, siempre que esta información sea necesaria para evitar un grave perjuicio para mi salud o la de mis familiares biológicos.

Si No

- Se me ha informado que todos los datos obtenidos en este estudio serán confidenciales y se tratarán conforme establece la Ley Orgánica de Protección de Datos de Carácter Personal 15/99.

- Las muestras obtenidas en este estudio solo serán utilizadas para los fines específicos del mismo.

- Comprendo que mi participación es voluntaria y que presto libremente mi conformidad para participar en el estudio.

- Comprendo que puedo retirarme del estudio:

    1. Cuando quiera.
    2. Sin tener que dar explicaciones.
    3. Sin que esto repercuta en mis cuidados médicos.

Fecha Firma del paciente

Fecha Firma del Investigador y DNI

HOJA DE RECOGIDA DE DATOS

Influencia de la hiperlipemia y el tratamiento con estatinas sobre la periodontitis

Datos Personales

Apellidos:

Nombre:

Edad:

Sexo:

Raza:

Índice de Masa Corporal:

- Altura:
- Peso:

¿Bebedor?: SI NO

- Consumo de alcohol:

¿Fumador?: SI NO

- Consumo de tabaco (Cigarrillos/día):

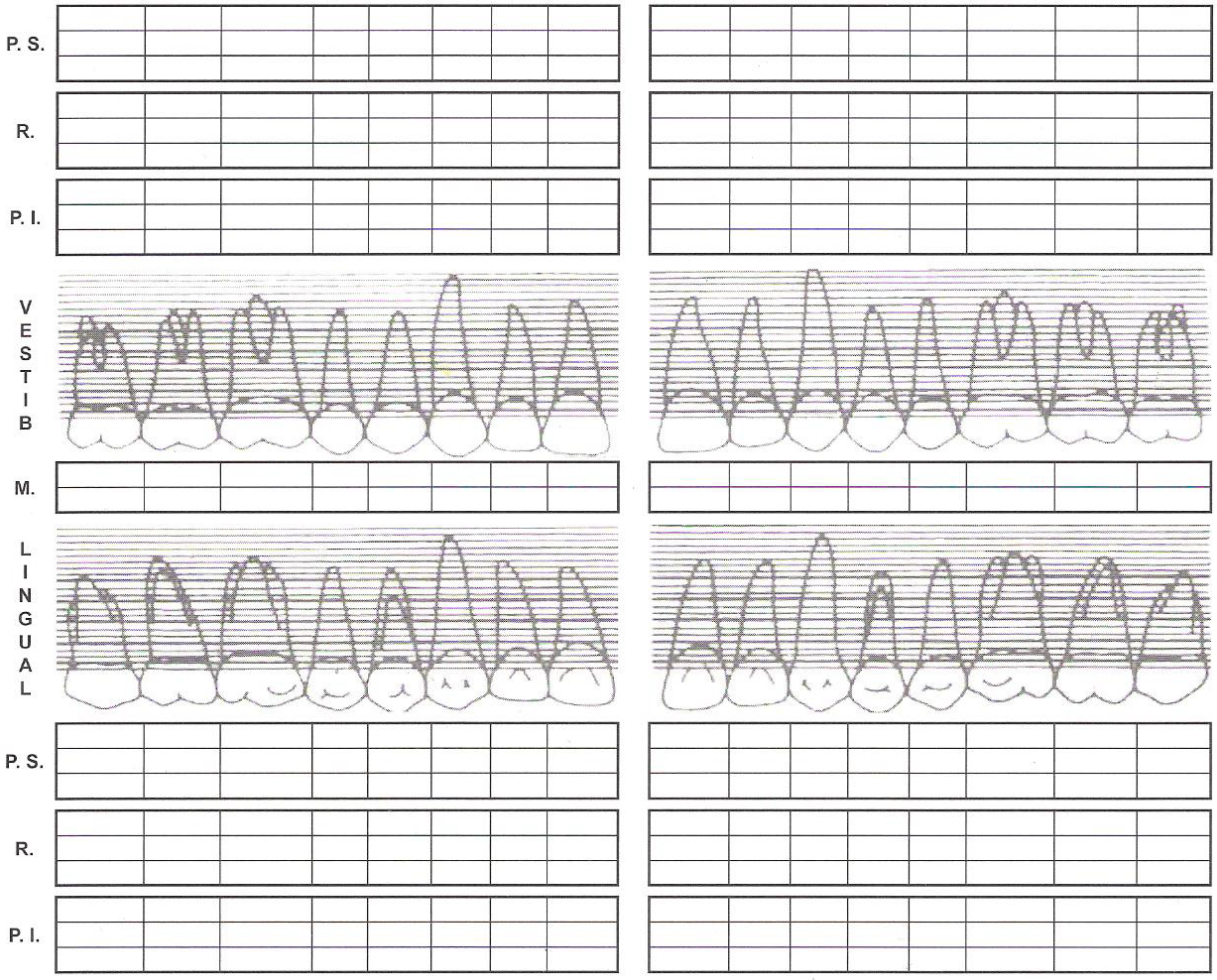
Estudios (el de mayor nivel):

Profesión:

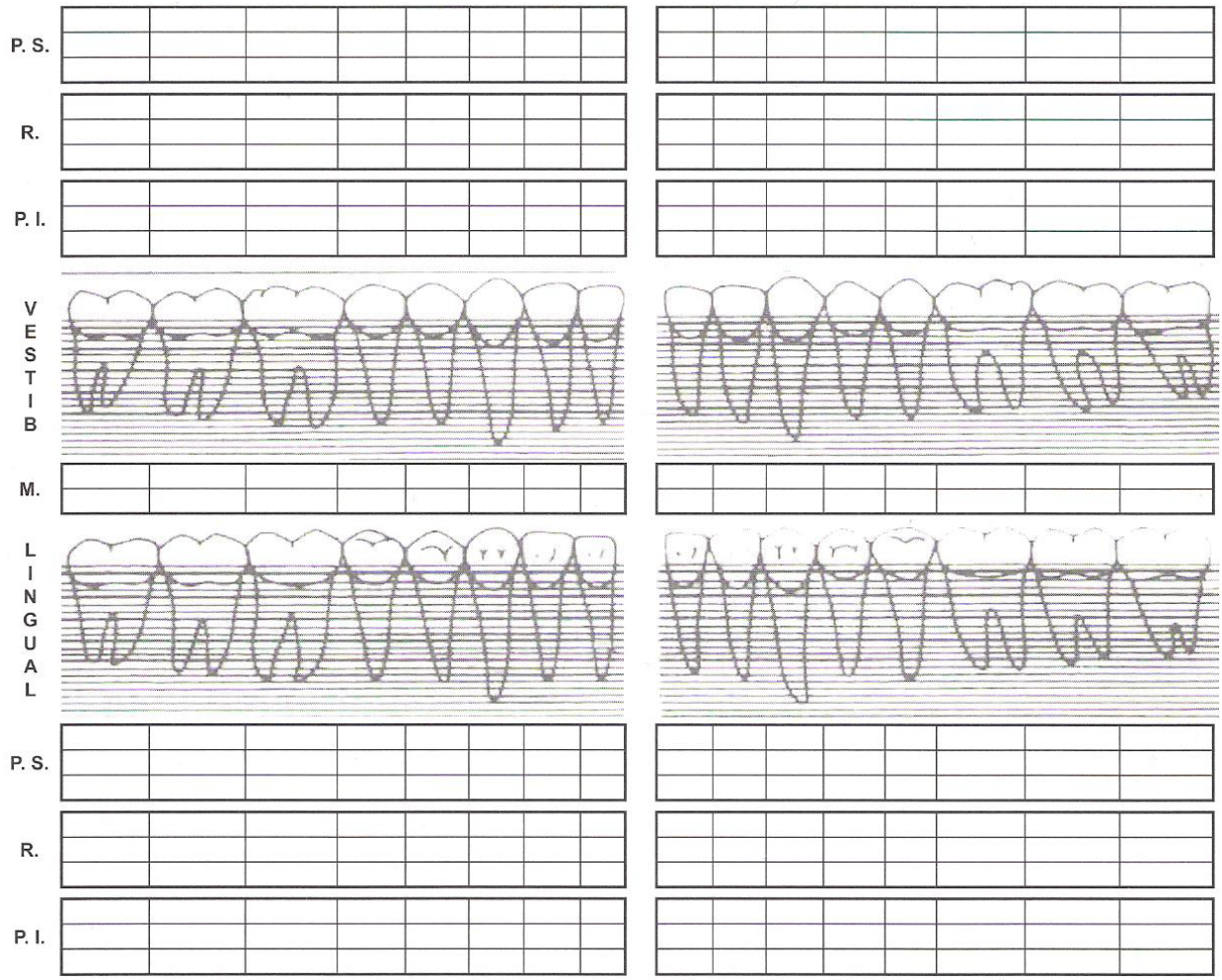
Estado civil:

Examen Periodontal

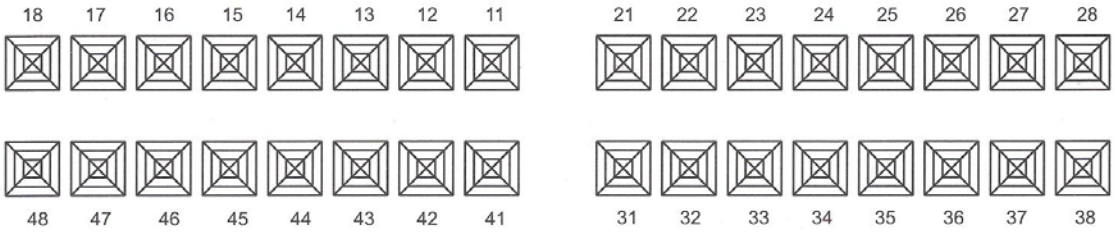
Arcada Superior:



Arcada Inferior:

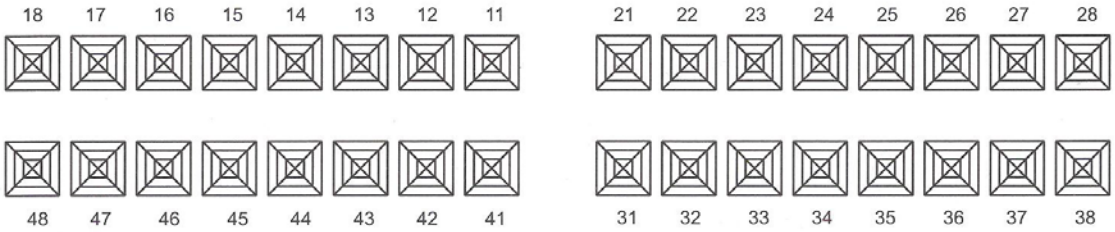


Índice de sangrado gingival



Valor total:

Índice de placa



Valor total:

Association of Simvastatin and Hyperlipidemia With Periodontal Status and Bone Metabolism Markers

Antonio Magán-Fernández,* Lara Papay-Ramírez,† Juan Tomás,‡ Rafael Marfil-Álvarez,* Manfredi Rizzo,§ Manuel Bravo,‖ and Francisco Mesa*

Background: The objective of this study is to determine whether simvastatin consumption and hyperlipidemia are associated with a worse periodontal condition and specific bone activity biomarkers.

Methods: This cross-sectional and analytic study includes 73 patients divided into three groups: 1) simvastatin-treated patients with hyperlipidemia (n = 29); 2) patients with hyperlipidemia treated by diet alone (n = 28); and 3) normolipidemic patients (controls, n = 16). The periodontal clinical variables of all participants were gathered, a blood sample was drawn from each to determine the lipid profile (total cholesterol, triglycerides, low-density lipoprotein, and high-density lipoprotein), serum levels of acute-phase reactants (C-reactive protein), erythrocyte sedimentation rate, and bone metabolism markers (osteoprotegerin [OPG], osteocalcin, procollagen type I N-terminal propeptide, and C-terminal telopeptide of type I collagen).

Results: The mean ESR was higher in the diet-treated patients with hyperlipidemia than in the normolipidemic controls ($P = 0.04$). Serum OPG concentrations were significantly higher in the simvastatin-treated patients with hyperlipidemia than in the diet-treated patients with hyperlipidemia ($P = 0.05$). Multivariable linear regression analysis adjusted for age, sex, tobacco, and alcohol revealed that, compared with the normolipidemic patients, the simvastatin-treated patients with hyperlipidemia showed a mean reduction of 0.8 mm (95% confidence interval = –1.5 to 0.0, $P = 0.05$) in clinical attachment loss.

Conclusions: Within the limits of this study, the findings suggest that the intake of simvastatin is associated with increasing serum OPG concentrations, and this could have a protective effect against bone breakdown and periodontal attachment loss. The baseline systemic inflammatory state of patients with hyperlipidemia is indicated by their increased erythrocyte sedimentation rate. *J Periodontol 2014;85:1408-1415.*

KEY WORDS

Lipid metabolism disorders; osteoprotegerin; periodontitis; simvastatin.

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Periodontitis is an infectious disease that affects the tooth-supporting tissues and exhibits a wide range of clinical, microbiologic, and immunologic manifestations. It is associated with and probably caused by a multifaceted dynamic interaction among specific infectious agents, host immune responses, hazardous environmental exposure, and genetic propensity.[1] The available scientific evidence appears to indicate an association between hyperlipidemia and periodontitis.[2,3] Researchers demonstrated the capacity of periodontal pathogens to alter the lipid profile, increasing low-density lipoprotein (LDL), very low-density lipoprotein, and total cholesterol levels and reducing high-density lipoprotein (HDL) levels.[4] It has also been reported that periodontal treatment improves the lipid profile.[5] Studies showed that periodontal pathogens affect the oxidative processes of different lipoproteins[6] and increase the expression of foam cell LDL receptor.[7] Conversely, an altered lipid profile has been associated with the expression of systemic proinflammatory cytokines[5,8] and heat shock proteins,[9] which affect the pathogenesis of periodontitis. Only two publications have linked periodontal probing depth (PD) with total cholesterol and LDL levels.[10,11]

Statins may exert an impact at the periodontal level by three different mechanisms. First, their hypolipidemic effect would lead to an indirect improvement of periodontal variables by decreasing the lipid values, as noted above. Second, they also exert a direct anti-inflammatory action through a decrease in proinflammatory cytokines via inhibition of the mevalonate pathway.[11-13] Finally, they may have an effect on bone metabolism, although research results have been controversial.[14,15] Studies in rodents have shown that certain types of statin (simvastatin and atorvastatin) can stimulate embryonic stem cells toward osteogenic differentiation lines and can inhibit bone resorption.[16] This evidence leads some authors, such as Fentoğlu et al.,[17] to consider that periodontal treatment combined with statins can contribute to greater hyperlipidemia control.

However, the available evidence is not yet sufficiently consistent to confirm the effect of statins on periodontal support tissues in humans, given that most evidence to date is based on animal or in vitro models, with only two clinical studies.[11,18] The objective of the present study is to determine whether simvastatin consumption and hyperlipidemia are associated with a worse periodontal condition and with specific bone activity biomarkers.

MATERIALS AND METHODS

An observational cross-sectional and analytic study was performed in normolipidemic patients and those with hyperlipidemia under follow-up at the Zaidín Sur Healthcare Center, Granada, Spain, from March 2013 to June 2013. Three groups were selected: 1) patients with hyperlipidemia treated with simvastatin (S) (10-40 mg/day); 2) patients with hyperlipidemia treated by diet alone (D); and 3) normolipidemic patients (C). Hyperlipidemia was defined as the presence of one or more altered values of the lipid profile (total cholesterol >200 mg/dL, triglycerides [TGs] >150 mg/dL, LDL cholesterol >130 mg/dL).[19] The diagnosis of hyperlipidemia had to be made at least 3 months before the study, and no distinction was drawn among hyperlipidemia types.

Inclusion criteria were as follows: 1) aged >18 years; 2) presence of ≥16 teeth (excluding third molars); and 3) no receipt of periodontal treatment during the previous 6 months. Exclusion criteria were as follows: 1) pregnancy; 2) breastfeeding; 3) the presence of systemic disease with lipid/bone metabolism involvement or related to periodontal status or neoplastic disease; 4) antibiotic treatment during the previous 3 months; 5) treatment with drugs that affect bone metabolism; and 6) receipt of hormone therapy.

Sample size was estimated following the general rule for the standardized difference in a given output variable (e.g., clinical attachment loss [AL] in millimeters) between the C group and the two hyperlipidemic groups. For ethical reasons, because the blood analyses were clinically justified in the hyperlipidemic groups but not in the C group, the general rule of an approximately equal sample size in two groups being compared was not followed. It was decided to obtain a given statistical power for an arbitrary sample size ratio of 2 (S and D groups) to 1 (C group). The estimation was based, using the *t* test for independent groups, on the detection of a standardized difference of 0.8 (large, according to Cohen's scale[20]). This gave a size of 15 patients (C group) and 30 patients (S and D groups) for a power of 70% ($\beta = 0.30$), which is slightly lower than the usual 80% (to minimize the number of normolipidemic patients for ethical reasons) and a significance level of two-sided $\alpha = 0.05$. Specific software¶ was used to estimate the sample size.

Informed consent was signed by all participants in the study, which was approved by the Research Ethics Committee of the Granada Health District.

Sociodemographic and Periodontal Variables

Data were gathered for all participants on age, sex, body mass index (BMI), and consumption of alcohol (grams per day) and tobacco (cigarettes per day). The periodontal examination was conducted

¶ Sample Power 2.0, IBM, Chicago, IL.

Table 1.

Sociodemographic and Habit Characteristics by Study Group (N = 73)

Variable	C (n = 16)	S (n = 29)	D (n = 28)	*P* Value*			
				Global	C versus S	C versus D	S versus D
Sex (% males)	50.0	62.1	35.7	0.14	0.64	0.54	0.09
Age (years, mean ± SD)	46.3 ± 10.7	63.1 ± 8.9	52.7 ± 9.0	<0.001	<0.001	0.10	<0.001
Age (years, range)	30 to 63	39 to 78	32 to 70				
BMI (mean ± SD)	27.5 ± 5.6	27.6 ± 3.7	27.9 ± 5.8	0.97	>0.99	>0.99	>0.99
Tobacco (cigarettes/day, %)				0.13	0.051	0.21	0.34
0	62.5	89.7	78.6				
1 to 10	25.0	3.4	17.9				
11 to 30	12.5	6.9	3.6				
Alcohol (g/day, %)				0.85	0.65	0.92	0.63
0	81.3	75.9	78.6				
1 to 20	6.3	6.9	17.9				
21 to 84	12.5	17.2	3.6				

* For global comparisons, χ^2 (sex), analysis of variance (age and BMI), and Kruskal–Wallis (tobacco and alcohol use, with original values, i.e., with non-collapsing categories) tests were used. For paired comparisons, corrected χ^2 (sex), Bonferroni method (age and BMI), and Mann–Whitney *U* test (tobacco and alcohol use) were used.

by a researcher (AM) masked to the group affiliation of the participants, using a periodontal probe# and determining the PD, AL (in millimeters), and the gingival bleeding (GI)[21] and plaque (PI) indices (in percentages).[22] Six sites per tooth were probed. The mean PD and AL values were calculated for each patient based on the highest PD and AL values measured from the six sites probed for each tooth. The number of periodontal pockets of 4 to 6, 7 to 9, and >9 mm in each patient and the number of sites with AL of 3 to 5, 6 to 8, and >8 mm were recorded.

Biochemical Variables

In the health care center, two blood samples were taken from each participant after 12 hours of fasting: a 2-mL sample with EDTA as anticoagulant to detect the erythrocyte sedimentation rate (ESR) in millimeters per hour by the Westergreen technique and an 8.5-mL sample for serum extraction, which was stored at –40°C for subsequent determination of the other biochemical variables. Total cholesterol, TGs, and HDL were determined by standard enzymatic–colorimetric methods[23-25] and LDL by the Friedewald formula. C-reactive protein (CRP) was determined with an immunoturbidimetric assay.[26] The bone metabolism markers procollagen type I N-terminal propeptide (PINP) and C-terminal telopeptide of type I collagen (CTX) were measured by electrochemiluminescence immunoassay.[27,28] Serum osteoprotegerin (OPG) and osteocalcin (OCN) concentrations were quantified with an enzyme-linked immunosorbent assay.**[29,30] Samples were analyzed by the Clinical Analysis Department of the San Cecilio University Hospital, Granada, Spain, which provided the reference values.

Statistical Analyses

A statistical software package†† was used for descriptive purposes and comparisons among groups (Tables 1 and 2). Quantitative variables were expressed as mean ± SD and analyzed with analysis of variance for global comparison. When normality assumptions were violated (tested with Shapiro–Wilk test), the descriptive results were expressed as median (interquartile range [IQR]) and analyzed with Kruskal–Wallis test. For periodontal variables following the normality assumptions, multivariable regression analyses (Table 3) were performed using specific software‡‡ (indicating simple random sampling as design) to analyze the effect of group on periodontal variables after adjusting for participants' age, sex, tobacco use, and alcohol use. The statistical tests used are reported in the table footnotes.

RESULTS

The study included 73 participants: 1) 16 in the C group; 2) 29 in the S group; and 3) 28 in the D group. Table 1 displays the values obtained for each sociodemographic and habit variable and

UNC no. 15 probe, Hu-Friedy, Chicago, IL.
** Immunodiagnostic Systems Limited, Boldon, U.K.
†† SPSS 20.0, IBM.
‡‡ SUDAAN 7.0, Research Triangle Institute, Research Triangle Park, NC.

Table 2.

Periodontal and Biochemical Variables by Study Group (N = 73)

Variable	C (n = 16)	S (n = 29)	D (n = 28)	*P* Value§			
				Global	C versus S	C versus D	S versus D
Periodontal							
Average PD (mm, mean ± SD)	2.3 ± 1.0	2.0 ± 0.9	2.3 ± 0.9	0.52	0.99	>0.99	>0.99
Sites (n) with PD 4 to 6 mm [median (IQR)]	1.5 (0 to 17.7)	0 (0 to 4)	1 (0 to 7.5)	0.58	0.35	0.63	0.47
Sites (n) with PD 7 to 9 mm [median (IQR)]	0 (0 to 0)	0 (0 to 0)	0 (0 to 0)	0.07	0.29	0.02	0.16
Sites (n) with PD >9 mm [median (IQR)]	0	0	0	—	—	—	—
Average AL (mm, mean ± SD)	3.0 ± 1.6	2.7 ± 1.2	2.6 ± 1.1	0.72	>0.99	>0.99	>0.99
Sites (n) with AL 3 to 5 mm [median (IQR)]	22.5 (0.7 to 36)	14 (7 to 41.5)	16 (3 to 22.5)	0.76	0.92	0.41	0.63
Sites (n) with AL 6 to 8 mm [median (IQR)]	1 (0 to 2.2)	0 (0 to 5.5)	0 (0 to 2)	0.21	0.39	0.09	0.26
Sites (n) with AL >8 mm [median (IQR)]	0 (0 to 0.7)	0 (0 to 0)	0 (0 to 0)	0.27	0.37	0.11	0.42
GI (% teeth, mean ± SD)	19 ± 18	11 ± 11	13 ± 11	0.12	0.13	0.42	>0.99
PI (% surfaces, mean ± SD)	42 ± 29	39 ± 34	48 ± 29	0.53	>0.99	>0.99	0.78
Biochemical							
Cholesterol (mg/dL, mean ± SD)	185 ± 21	194 ± 29	246 ± 32	<0.001	0.99	<0.001	<0.001
TG (mg/dL, mean ± SD)	77 ± 28	122 ± 68	162 ± 145	0.03	0.45	0.03	0.43
LDL (mg/dL, mean ± SD)*	106 ± 16	116 ± 28	151 ± 27	<0.001	0.67	<0.001	<0.001
HDL (mg/dL, mean ± SD)*	63 ± 12	57 ± 11	65 ± 21	0.19	0.75	>0.99	0.24
CRP [mg/L, median (IQR)]	2.1 (0.4 to 5.5)	1.2 (0.4 to 2.8)	1.2 (0.7 to 3.2)	0.50	0.26	0.58	0.46
ESR [mm/hour, median (IQR)]†	5 (3.7 to 8)	7 (4 to 13)	9.5 (5 to 15)	0.12	0.14	0.04	0.53
OPG (pmol/L, mean ± SD)‡	3.5 ± 1.4	4.5 ± 1.9	3.5 ± 1.1	0.03	0.16	>0.99	0.05
OCN (ng/mL, mean ± SD)‡	17 ± 8	15 ± 7	16 ± 7	0.52	0.76	>0.99	>0.99
CTX (ng/mL, mean ± SD)‡	0.30 ± 0.12	0.32 ± 0.14	0.33 ± 0.14	0.76	>0.99	>0.99	>0.99
PINP (ng/mL, mean ± SD)‡	40 ± 15	39 ± 15	40 ± 14	0.97	>0.99	>0.99	>0.99

IQR = interquartile range.
* Two missing values in S group.
† Eight missing values (two in the C group, two in the S group, and four in the D group).
‡ Two missing values (one in the C group and one in the S group).
§ For global comparisons, analysis of variance (for variables expressed with means) and Kruskal–Wallis test (for variables expressed with medians) were used. For paired comparisons, Bonferroni method and Mann–Whitney *U* test were used.

Table 3.

Multivariable Linear Regression Analysis for Mean Differences Among Groups, Adjusted for Age, Sex, Tobacco Use, and Alcohol Use, on Periodontal Variables (N = 73)

	S versus C			D versus C			S versus D		
Variable	β	(95% CI)	*P* Value	β	(95% CI)	*P* Value	β	(95% CI)	*P* Value
Average PD (mm)	–0.4	(–1.1 to 0.3)	0.23	–0.0	(–0.6 to 0.6)	0.99	–0.4	(–0.9 to 0.1)	0.13
Average AL (mm)	–0.8	(–1.5 to –0.0)	0.05	–0.3	(–1.1 to 0.4)	0.39	–0.4	(–1.1 to 0.2)	0.22
GI (% teeth)	–6.1	(–17.4 to 5.2)	0.29	–5.1	(–14.9 to 4.8)	0.32	–1.1	(–8.1 to 6.0)	0.77
PI (% surfaces)	–4.1	(–25.2 to 17.0)	0.71	6.0	(–12.9 to 25.0)	0.53	–10.1	(–26.8 to 6.6)	0.24

CI = confidence interval.

the results of non-adjusted comparisons among the study groups. There were significant differences in age. Periodontal and biochemical variables are shown in Table 2. The mean ESR was higher in the D group than in the C group ($P = 0.04$). Serum OPG concentrations were higher in the S group than in the D group ($P = 0.05$) and were similar between the D and C groups.

The associations (Pearson correlations) of periodontal variables (PD, AL, GI, and PI) with the lipid profile (cholesterol, TGs, LDL, and HDL) were analyzed. None of the correlations, which ranged from –0.13 to 0.18, were statistically significant (results not shown).

Multivariable linear regression analyses adjusted for sex, age, tobacco use, and alcohol use (known confounding factors for periodontitis) were performed for the adjusted comparison of periodontal variables among groups. Results in Table 3 show a mean reduction of 0.8 mm ($P = 0.05$) in AL for the S group compared with the C group.

DISCUSSION

In this study, serum OPG concentrations were higher in the S group than in the D or C groups. The periodontal AL was lesser in the S group than in the C group. The periodontal status of study participants was evaluated according to their mean PD and AL values, also taking into account the number of periodontal pockets and AL, both classified into three categories. This extensive evaluation of periodontal status was designed to facilitate detection of its possible association with hyperlipidemia and/or simvastatin. It has been reported that indices, such as the community periodontal index (CPI) or community periodontal index of treatment needs (CPITN),[31] used in previous studies on the relationship with hyperlipidemia, may provide data on the prevalence and severity of periodontal conditions, but they should not be used for reporting levels of periodontal disease.[32]

The present results are in line with the findings of the only two published studies on the periodontal effects of statins in humans. Thus, Sangwan et al.[11] found that the periodontal status was similar between the S and C groups but worse in the D group. However, unlike in the present study, they considered mean PD values alone, which may underestimate the prevalence of periodontitis. Fajardo et al.,[18] in a clinical trial of normolipidemic periodontal patients treated with atorvastatin and habitual periodontal therapy or habitual periodontal therapy alone, found clinical and radiologic improvements in the statin-treated group, including alveolar bone gain. However, their pilot study had a small sample (n = 38) and a follow-up of only 3 months, which is too short to evaluate the effects of prolonged statin consumption, which is typical in patients with hyperlipidemia. The duration of statin therapy in the treated patients in the present study ranges from 3 to 132 months.

Simvastatin has been found to halt bone loss in animals with induced periodontitis, acting as an anti-inflammatory and bone formation promoter.[33] Experimental studies also found that its local application alone or in combination with bisphosphonates exerts positive effects on bone formation.[34] The direct relationship between statin consumption and OPG concentrations evidenced in the present study was also observed previously in vitro.[35-37] The improved AL values in the S group in the present study may be explained by their increased OPG concentrations. OPG would act by binding with receptor activator of nuclear factor-kappa B (RANK) and inhibiting the activation of this receptor by its ligand (RANKL), thereby activating osteoclastogenesis.[38] The RANK/RANKL/OPG pathway is crucial for maintaining bone integrity, given the important role of this osteoclastogenesis control in bone resorption processes. It is highlighted that only lipophilic statins (e.g., simvastatin) have these effects on bone, because their lipophilia allows

them to cross the cell membrane by passive diffusion mechanisms.[16] They also deliver benefits at the bone level through their anti-inflammatory action against interleukin (IL)-6 and IL-8 cytokines[39] and through their angiogenic effect because they are able to stimulate the vascular endothelial growth factor (VEGF).[40] The systemic administration of statins produces potent effects at the periodontal level, and it was reported that their concentration in crevicular fluid is 10- to 100-fold higher than in serum,[11] with an anti-inflammatory effect on gingival levels of IL-1β, according to Suresh et al.[13] The prevalence of periodontitis among the statin-treated patients in the present study is low (n = 15), and a larger sample of treated patients might have revealed more favorable effects of the drug on the other periodontal variables studied. No effects of simvastatin on OCN, PINP, and CTX levels were found, which may be because these markers do not participate in the action mechanism of this drug. Future studies should evaluate the effects of statins on other bone markers, such as bone morphogenetic proteins or VEGF.

Hyperlipidemia is defined by the presence of one or more elevated lipid profile values in the present study (see Materials and Methods), and no association was found with the quantitative periodontal variables considered, as also reported by Rizzo et al.[9] and Saxlin et al.[41] However, other authors observed a relationship between hyperlipidemia and periodontitis. Fentoğlu et al.[42] associated hyperlipidemia with hyperactivity of white cells, alteration in polymorphonuclear leukocyte (PMN) function, and higher production of free radicals, indicating the action mechanisms that may be involved. Three studies with wide samples also reported on the association between hyperlipidemia and periodontitis. In a study of 10,590 individuals, Katz et al.[10] found PD to be significantly associated with total cholesterol and LDL levels. Using data obtained from the Korean national health survey, Lee et al.[2] obtained an odds ratio (OR) of 1.2 for periodontitis in patients with hyperlipidemia in a sample of 18,210 individuals, identical to the OR found by Boland et al.[3] in a similar study of 2,475 patients. The weak association found in these two studies may be attributable to their use of the CPI or CPITN and World Health Organization periodontal probe, which are designed to detect periodontal disease in large populations and may offer inadequate sensitivity for more precise studies, as stated previously. None of these three studies shed light on the mechanisms underlying the association between these diseases.

In the present study, the ESR was significantly higher (3.8 mm/hour) in the D group than in the C group. This may reflect a baseline proinflammatory state attributable to the hyperlipidemia, given the capacity of this disease to increase the expression of IL-1β via PMN involvement.[43] The relationship between CRP levels and periodontitis is controversial. Whereas no association was observed in the present investigation or in the study by Rizzo et al.,[9] a meta-analysis of 10 cross-sectional studies reported strong evidence of elevated plasma CRP levels in patients with periodontitis versus controls.[44] There is a need for additional investigations to quantify serum levels of IL-1β, IL-6, IL-8, and tumor necrosis factor-α cytokines and establish the true proinflammatory role of hyperlipidemia.

CONCLUSIONS

Within the limits of a cross-sectional study, the present findings suggest that the intake of simvastatin is associated with increasing serum OPG concentrations, and this could have a protective effect against bone breakdown and periodontal attachment loss. The baseline systemic inflammatory state of patients with hyperlipidemia is indicated by their increased erythrocyte sedimentation rate.

ACKNOWLEDGMENTS

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Review

Clinical Lipidology

Periodontitis, blood lipids and lipoproteins

Periodontitis, one of the most common chronic infections in adults, is characterized by the accumulation of dental plaque and infection by Gram-negative pathogenic bacteria, which further lead to the destruction of periodontal tissues. A relationship between chronic periodontitis and abnormalities in lipid and/or lipoprotein metabolism is not well understood yet. Periodontitis is associated with elevated proatherogenic plasma lipids, including small dense LDL, while oxidized LDL may act as an inflammatory stimulant in periodontitis. Periodontal pathogens may directly modify lipoprotein, including protective characteristics of HDL, and contribute to development of metabolic syndrome, Type 2 diabetes mellitus and coronary atherosclerosis. On the other hand, periodontal treatment is associated with modest HDL-C increases, LDL-C decreases and consequently lower cardiometabolic risk. Thus, oral check-ups and improved oral hygiene may reduce metabolic risk and antibodies against periodontal pathogens. Identification and monitoring of patients at high risk for periodontitis may help to prevent and/or reduce CV events.

Keywords: cardiovascular risk • inflammation • lipids • lipoproteins • periodontitis • sdLDL

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Periodontitis is a chronic oral infection caused by periodontopathogenic bacteria, and leads to loss of the supporting apparatus of the teeth. The production of inflammatory mediators, such as IL-8 and prostaglandin E2 (PGE2), is increased at the initiation, but remain increased during progression of periodontitis [1]. The pathogenesis of periodontitis is influenced by different risk factors. Oral pathogens invade periodontal tissues, generating an exacerbated immune-inflammatory response mediated by inherited risk factors such as polymorphisms and environmental factors such as smoking or systemic diseases, and finally leads to the destruction of the periodontal tissues around the tooth with typical clinical signs such as gingival bleeding, recession, bone loss, tooth mobility and eventually tooth loss [2]. This inflammatory process may be associated with the development of cardiovascular diseases (CVDs) (Figure 1). Mainly Gram-negative bacteria induce endotoxemia, and epidemiological and clinical studies indicate a link between periodontitis and CVD, such as acute myocardial infarction, coronary heart disease (CHD) and atherosclerosis [3–5]. The lipid levels (total cholesterol [TC] and triglycerides [TG]), as well as low-density lipoprotein-cholesterol (LDL-C) are increased in periodontitis. Higher prevalence of small dense LDL (sdLDL), an emerging cardiovascular risk factor [6,7] and lower circulating high-density lipoprotein-cholesterol (HDL-C) have been described in subjects with periodontitis compared with healthy subjects [8–10]. The relationship between low HDL level and the number of gingival pockets and gingival inflammation has been reported [11]. In addition, the ratio of TC to HDL (TC/HDL) was associated with changes in the gingival index in the hyperlipidemic subjects [12]. In addition, an association between the metabolic syndrome and periodontitis has been reported [13–17].

Interestingly, abdominal obesity among women, primarily large waist circumference,

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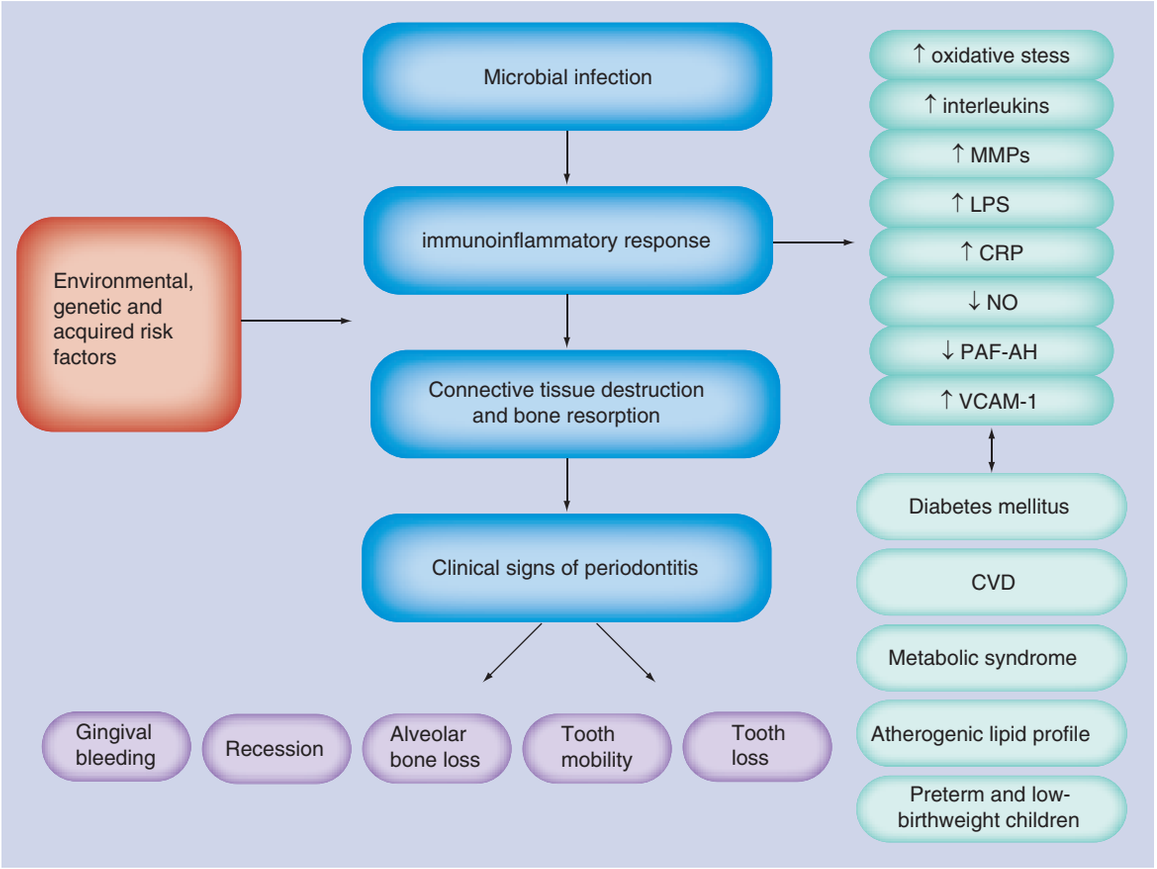

Figure 1. Model of periodontitis pathogenesis in humans.
CRP: C-reactive protein; LPS: Lipopolysaccharide; NO: Nitric oxide; PAF-AH: Platelet-activating factor acetylhydrolase; VCAM-1: Vascular cell adhesion protein 1.

has been reported as the most significant factor in association with periodontal disease [16]. However, a few studies have failed to show the association between lipids and periodontal infection [18,19].

It has been documented that the treatment of periodontitis has beneficial effects on markers of CVD, CHD, lipids and lipoproteins, including decreasing in levels of oxidized LDL (oxLDL) and oxidative stress [20]. However, the underlining mechanism of this relationship is still unclear. Anyhow, this correlation is important because periodontal disease might influence lipid concentrations and simultaneously CVD risk [11].

Periodontitis & cardiovascular risk

Background & epidemiological evidence

The first evidence of a relationship between periodontitis and CVD was published in 1989. Matilla *et al.* provided preliminary data evaluating the association between dental infection and acute myocardial infarction, justified because, according to these authors, 25% of patients with cardiovascular events do not present with the traditional risk factors. In this case–control study, 'dental infection' included caries lesions, periapical lesions, pericoronaritis, tooth loss and periodontal pockets, creating a dental index that could be adjusted in view of different confounding factors. The results demonstrated an association between dental infection and myocardial infarction, and the dental index was shown to be an independent predictor of this pathology [21]. The first study focusing on the relationship between periodontal state and cardiovascular morbidity/mortality rates, with a 14-year follow-up, was published 4 years later. The authors of this study concluded that patients with severe periodontitis at the onset of the study had a 25% higher risk of presenting with a cardiovascular event than the individuals without periodontitis [22].

Since then, this evidence has been reviewed systematically several times [5,23–31]. A recent report by a working group of the American Heart Association concludes that periodontal disease is associated with vascular atherosclerosis independently of known confounding factors, yet there is no evidence for a causal relation. That is, affirmations that imply a causal association between the two diseases are not justified. The group highlights as lines for future studies the need for

uniform criteria in the measurement of periodontitis, and for controlled intervention studies that are well designed and involve treatment–response protocols of long duration [30]. The last systematic review published [31] is based on 12 studies, one of them by our research group [32]. In the authors' opinion, the selected studies apply the most robust definition of periodontitis as a variable of exposure, defined by clinical attachment loss and alveolar bone loss with radiographic assessment, and atherosclerotic cardiovascular disease as the outcome variable (referring to atherosclerotic diseases of the heart and vasculature). Excepting one, the remainder shows a positive association between periodontitis or severity of periodontitis and the incidence of atherosclerotic cardiovascular disease, regardless of the established cardiovascular risk factor. However, evidence for an association between periodontitis and peripheral arterial disease, or secondary cardiovascular events, is scarce [31].

Such is the volume of research supporting this association that the European Society of Cardiology includes periodontitis as a disease that increases the risk of cardiovascular disease [33]. Its section 3.7.6 reads: "Periodontitis is associated with endothelial dysfunction, atherosclerosis, and an increased risk of myocardial infarction and stroke. Confounding factors, however, such as low socio-economic status and cigarette smoking, probably play a significant role. Periodontitis can be considered a risk indicator for a generally decreased cardiovascular health status and its treatment is indicated as well as management of the underlying cardiovascular risk factor". The joint Workshop of the European Federation of Periodontology (EFP) and the American Academy of Periodontology (AAP) concluded in 2012 with regard to periodontitis and systemic diseases "There is a strong evidence that periodontitis imparts increased risk for future atherosclerotic cardiovascular disease" [34].

Pathogenic mechanisms

To date, the relationship between periodontitis and CVD has been explained by the action of periodontopathogenic bacteria in the process of atherogenesis. Two main pathways have been evoked in the relationship between the two: direct infection of the atheromatous plaque by periodontal bacteria, and the systemic inflammatory state that periodontal disease causes, which would contribute indirectly to atherosclerosis.

Bacterial infection

The role played by bacteria or their by-products in the development of atherosclerosis has been widely demonstrated. Infectious processes have been shown to be an independent risk factor for endothelial dysfunction and the risk of atherosclerosis, being related to the presence of cardiovascular disease [35]. Furthermore, it is known that chronic infections intervene in the progression of atherosclerosis [36]. A prospective study with a 5-year follow-up (n = 825) analyzed the association of different chronic infections, among them periodontal infection, with atherosclerosis. The results show that all the chronic infections studied increased the risk of atherosclerosis significantly, especially among those patients who did not suffer from atherosclerosis at the onset of the study (odds ratio [OR]: 4.08; $p < 0.0001$), although the patients already affected also showed a higher risk for the progression of the atheromatous lesions (OR: 2.31; $p < 0.001$) [37].

The scientific evidence available shows how periodontal bacterial species are directly involved in the process of atherogenesis [38]. Several studies have demonstrated the presence of periodontal pathogens in the atheroma plaque by means of PCR or plate culture techniques [39–42]. The most commonly identified periodontal pathogens are *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*, and species less often identified include *Tannerella forsythia*, *Treponema denticola*, *Prevotella intermedia*, *Prevotella nigrescens*, *Eikenella corrodens*, *Fusobacterium nucleatum* and *Campylobacter rectus.* Most of these species have been found within macrophages or near apoptotic bodies [43]. The prevalence of each bacterial species varies considerably according to the different studies, without showing any pattern; but many plaques analyzed showed more than one bacterial species, leading to the hypothesis that the endothelial dysfunction is produced by the action of several bacterial species, not by the action of a single pathogen [35]. This global load of periodontal pathogens has been related in various studies of coronary disease to carotid intima-media thickness [38,44]. It has been shown that the severity of periodontitis influences the presence of carotid atheroma plaques ($p = 0.003$) [45], while by logistic regression analysis significant differences concerning age and periodontitis were seen in relation to the presence of atheroma plaques in the carotid intima.

The access of these pathogens to vascular tissue is explained by the production of bacteremia through the ulceration of the periodontal pocket during habitual therapeutic procedures, or even while brushing or chewing, in patients with gingivitis or periodontitis [46,47]. Other proposed mechanisms are the passage of bacteria through the cells of the periodontal pocket to the capillary system by means of a transcellular mechanism [48], or else due to phagocytosis by leukocytes, inside which the pathogens remain alive, avoiding lytic processes, then departing from these cells in a different zone of the organism [49].

P. gingivalis has been shown to directly invade endothelial and smooth muscle cells and survive inside them [50]; according to animal models, this may accelerate the process of atherogenesis [51,52] and induce the apoptosis of endothelial cells [53]. *A. actinomycetemcomitans* has also demonstrated an invasive capacity [54] and has been associated with an overexpression of matrix metalloproteinase (MMP)-9 and a proatherogenic profile, inducing the transformation of macrophages into 'foam cells' [55].

Another important contribution to research is the report of an altered lipid profile induced by bacteria of periodontal origin, with an increase in LDL and a decrease in the antiatherogenic capacity of HDL [10,56]. *P. gingivalis* is capable of oxidizing the LDL [57], which may also bond to circulating lipopolysaccharide (LPS) proceeding from the bacterial wall, forming LPS-LDL. These altered forms of LDL favor its deposit in the vascular wall, creating a proatherogenic and proinflammatory environment, and inducing the transformation of macrophages into foam cells by activating specific surface receptors [58].

Nonetheless, only certain strains of periodontal pathogens have demonstrated an invasive capacity and a procoagulating effect [59]. These effects are not observed when the action is due to a noninvasive mutant strain [50]. In animal models it has been shown that mutant strains of *P. gingivalis*, genetically altered, displayed a noninvasive behavior, were less pro-atherogenic and exerted a lesser proinflammatory effect than the wild-type strains [51]. Such results underline the decisive role of the bacterial strain in the invasion and survival of *P. gingivalis* within the cells of the vascular wall. For this reason, knowledge of how this bacterium interacts with the vascular endothelium may lead us to develop a therapy for lowering the risk of cardiac disease based on these mechanisms.

Model of the influence of periodontal bacteria on atherogenesis

In light of the reviewed evidence, we propose an explicative model for the action of oral bacteria on atherogenesis. Some of the periodontal bacteria that gain access to the bloodstream may have the ability to invade the endothelial cells. This invasion gives rise to activation of the endothelial cells, which express receptors of adhesion on the surface (vascular cell adhesion protein 1 [VCAM-1]) and causes their apoptosis, permitting the entry of microorganisms inside the vascular wall. This situation produces the arrival and activation of monocytes harboring live periodontal pathogens, previously phagocytized, which penetrate the vascular wall, where they are transformed into macrophages; these would suffer apoptosis due to the action of the bacteria, which would then be released to the medium. The oral bacteria in the medium could act upon the LDL, favoring its modification to oxLDL, which in turn would induce the transformation of macrophages into foam cells through the union to specific surface receptors of these cells, thus producing the release of proinflammatory cytokines, reactive oxygen species (ROS) and MMPs (Figures 2 & 3).

Immunoinflammatory & thrombotic mechanisms

One of the best-known aspects of cardiovascular pathology is its association with the inflammatory response and with immunity-related mechanisms [60], a characteristic that it shares with periodontitis. Patients with periodontitis present an intense inflammatory infiltrate at the gingival connective tissue, with a predominance of plasma cells, monocytes/macrophages, and T and B lymphocytes (Figure 2).

The scientific literature documents a relationship between periodontitis and increased levels of systemic inflammatory mediators, which, in turn, are related to cardiovascular pathology. One of the most studied markers is C-reactive protein (CRP), considered one of the best markers used in the prediction of cardiovascular risk [61]. It has been shown in observational and interventional studies, and meta-analyses that periodontitis is associated with elevated local levels of CRP in the gingival crevicular fluid, as well as high systemic levels [62–66]. In contrast, other studies found no such association, including one study published by our research group; this may be due to the fact that the patients in our study only presented with mild periodontitis [10]. Other markers found to be elevated in association with periodontitis are IL-6, [62,64,66], plaque-activating factor [67] and MMPs [55]. These factors, when increased at a systemic level as a consequence of periodontitis, contribute to the development of atherosclerosis through their action as proinflammatory cytokines.

The presence of specific antibodies in serum against oral periodontopathogens can be an indication of exposure to bacteria or to bacterial byproducts [68,69]. One meta-analysis demonstrates that the systemic elevation of antibodies is closely associated with an increased risk of suffering an acute myocardial infarction [28] and that this raised level of antibodies constituted a stronger predictor of risk than CRP [70]. In addition to antibodies produced against bacterial species [71,72], there may also be antibodies produced in response to other molecules, such as heat-shock protein (HSP), expressed by endothelial cells in situations of cellular stress. They would react by inducing the formation of anti-HSP antibodies related to atherosclerosis [73–75]

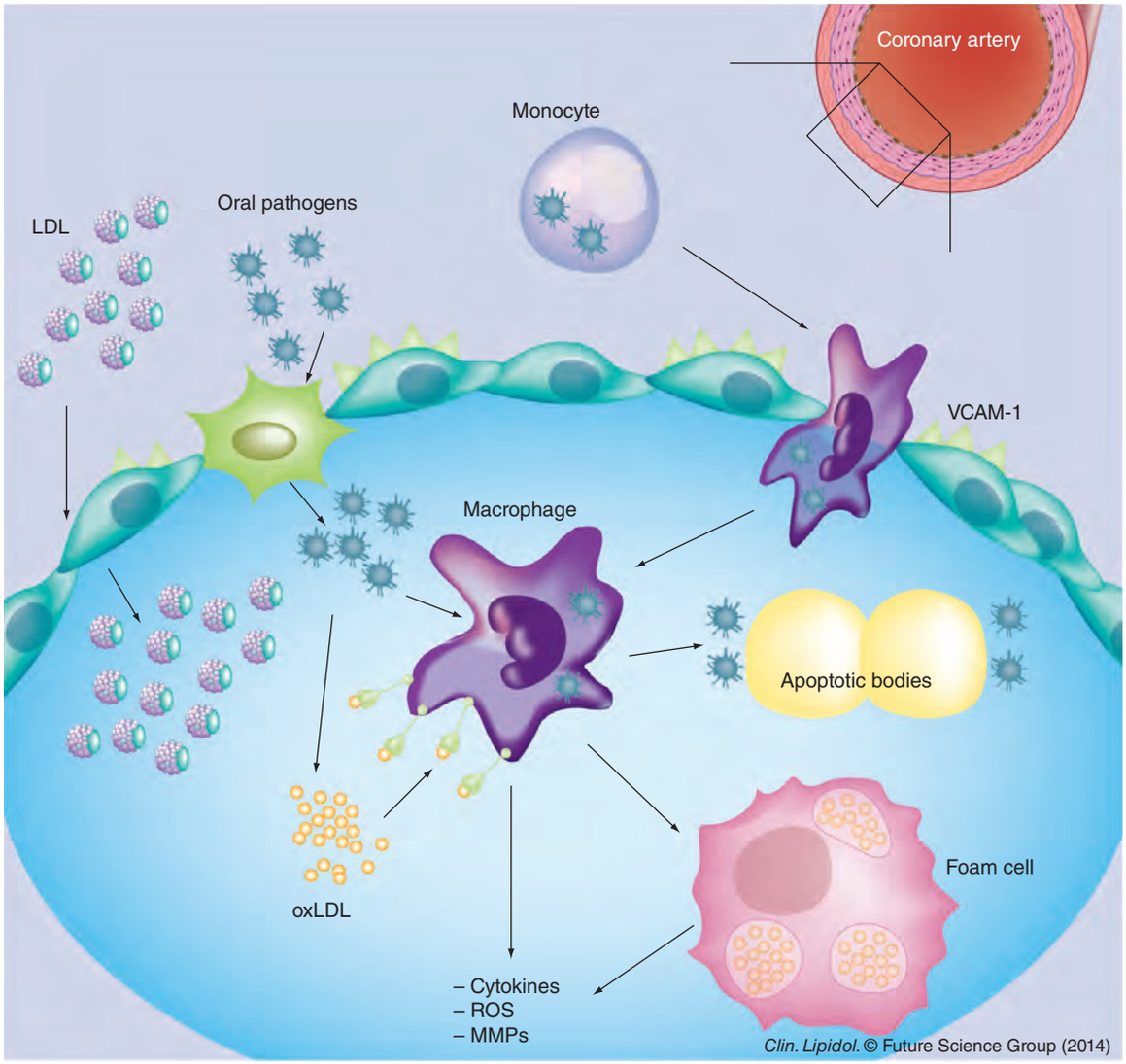

Figure 2. Linkage between periodontal pathogens and the formation of atheromatous plaques.
LDL: Low-density lipoprotein; MMP: Matrix metalloproteinase; NO: Nitric oxide; oxLDL: Oxidized low-density lipoprotein; ROS: Reactive oxygen species; VCAM-1: Vascular cell adhesion protein 1.

and periodontitis [10,76]. Furthermore, the similarity between HSP and molecules of bacterial origin such as GroEL proteins, expressed by various periodontal pathogens, is known to produce cross-reactivity effects due to the phenomenon of 'molecular mimicry', both molecules being found in the atheroma plaques and the subgingival plaque of patients with atherosclerosis and periodontitis [52,77]. Other antibodies studied include anti-phosphorylcholine (anti-PC), anti-oxidized LDL (anti-oxLDL) and anti-cardiolipin (anti-CL) as potential mediating mechanisms between atherosclerosis and periodontal disease. Anti-cardiolipin (anti-CL) is an antibody related to the phenomenon of 'autoimmune atherosclerosis', which occurs in patients with systemic lupus erythematosus. This antibody binds to the β2-glycoprotein (β2GP1), involved in the control of coagulation and the protection of the endothelial wall, and heightens cardiovascular risk. Periodontal pathogens can induce anti-CL activity via similarities in peptidic sequences [78]. Meanwhile, anti-PC IgG has been associated with periodontal attachment loss [79], binding to the phosphorylcholine of periodontal pathogens and to oxLDL, leading to phenomena of cross-reactivity [80]. Other studies, however, attribute it a role in the protection against atherosclerosis [24]. Anti-oxLDL has also been found at the periodontal level [79], presenting a cross-reactivity with oxLDL and with the gingipain of *P. gingivalis* [81].

Several coagulation factors likewise intervene significantly in the formation of atheroma plaque and thrombosis. For this reason, their expression in relation with periodontitis has also been studied. The levels of hemo-

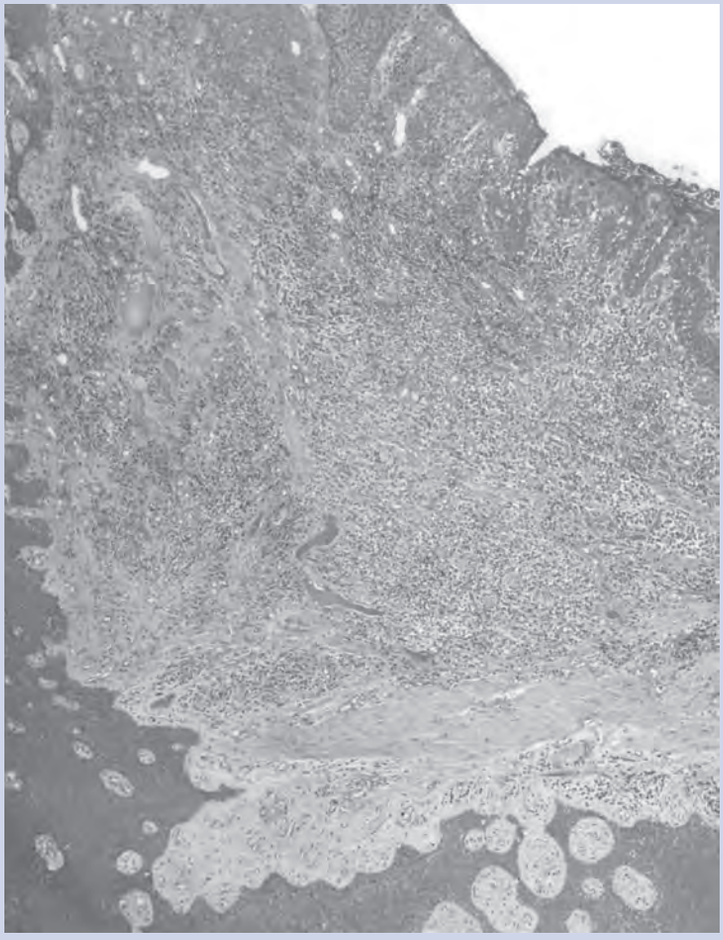

Figure 3. Patient with chronic adult periodontitis showing intense inflammatory infiltrate in almost the entire lamina propria.
Magnification: 4×.

static markers undergo a significant decrease after the extraction of all the teeth of periodontal patients [82], whereas the levels of fibrinogen [64] and the expression of sP-selectin, associated with platelet activation [83], were seen to be higher in patients with periodontitis when compared with healthy controls. In this context, expression of E-selectin was higher in patients with periodontitis independently of whether those patients were smokers or not [84], and this VCAM decreased significantly after periodontal therapy [85]. Further factors including plasminogen-activator inhibitor 1 (PAI-1) or von Willebrand factor (vWF) give contradictory results, and their relationship with periodontitis has not been elucidated to date [86,87].

Interventional studies

One key question about the relationship between periodontitis and cardiovascular disease is to determine whether periodontal treatment has some beneficial effects upon some of the markers of cardiovascular risk described above. The review by Ying Ouyang *et al.* concludes that the effect of periodontal therapy, according to the different studies, is variable and hardly determinant [88]. Moreover, Hujoel *et al.* affirm that treatment is unlikely to have any effect, given that the risk of cardiovascular pathology in edentulous patients shows no difference with respect to patients having teeth and periodontitis [23].

As mentioned earlier, CRP is the acute-phase protein that is most frequently related to cardiovascular risk, and it is also widely associated with periodontitis in numerous studies. For this reason, many publications and several meta-analyses have explored the effect of periodontal treatment on cardiovascular risk, adopting CRP as the main marker. The meta-analyses show similar results, pointing to a significant reduction of CRP after periodontal treatment [63,89,90]. Other acute-phase proteins studied include fibrinogen and serum amyloid A, giving controversial results. There is as yet insufficient evidence for changes in both fibrinogen [91,92] and serum amyloid A [92] following periodontal treatment.

Endothelial function has been found to be altered in periodontal patients [93]. Diverse studies have evaluated the effect of periodontal treatment on endothelial function, showing a positive effect upon this variable [94,95], measured by flow-mediated dilation.

Studies assessing the effect of therapy on leukocyte count provide no clear conclusions: some show a reduced count [96,97], while others report no significant differences [92,98].

The effects of periodontal treatment on IL-6, TNFα, MMPs and tissue inhibitors of metalloproteinase (TIMP) present contradictory results in terms of their serum levels [95,96,99,100].

The phenomena of oxidative stress, and particularly ROS, oxLDL and other by-products of oxidative stress damage, are reportedly pronounced in periodontal patients [101]. Periodontal treatment has been associated with decreased ROS and oxLDL [20,102], but this influence in oxidative stress was not observed in other studies [96].

At present, knowledge regarding the effect of periodontal treatment on thrombotic and hemostatic markers is unclear, with diverse findings surrounding PAI-1 and the D-dimer [92,96,102], whereas the von Willebrand factor apparently plays no role whatsoever [102,103].

The systemic inflammatory state generated by periodontitis was believed to affect endothelial function, possibly altering hemodynamics, implying an impact on blood pressure. Studies published on this topic do not demonstrate any significant decrease in arterial pressure as a result of periodontal treatment [92].

The recent review by D'Aiuto *et al.*, including 14 randomized clinical trials, concludes that the main findings about the effect of periodontal therapy involve CRP and endothelial function, pointing to evidence of clear improvement after treatment. The validity of both these markers is questioned, however, as CRP and endothelial dysfunction may be subjected to several confounding factors. Instead, the consensual use of a biomarker such as carotid intima-media thickness is proposed, it being widely used in intervention studies on cardiovascular disease. Other results of the meta-analysis were a lack of effect or limited evidence after periodontal treatment upon the rest of the factors analyzed [104].

Still, the relationship between periodontitis and cardiovascular disease is far from clear. The real interaction at work is unknown, due to the presence of risk factors that are shared by the two pathologies [105]. Important questions also remain regarding the exact mechanisms that could establish an association, or the effect of periodontal treatment upon the prevention of cardiovascular disease. Findings published in the literature, while demonstrating an association, do not allow researchers to establish a causal relationship, since most consist of transversal studies.

Periodontitis & plasma lipids

Background

The first publication making reference to the relationship between periodontitis and plasma lipids levels was published in the year 1999 by Ebersole *et al.*, describing an association between periodontitis and the lipid profile in nonhuman primates [106]. Since then, the relationship between periodontitis and lipid profile has been studied on several occasions, and a bidirectional relationship has even been established between the two pathologies [107]. Lösche *et al.* described an association between high levels of TC, TG and LDL, and the presence of periodontal disease in a study of 39 subjects [108]. Later publications report similar results, in addition to observing a decrease in the levels of HDL [8,109–111]. Rufail *et al.* determined higher levels of very low-density lipoprotein (VLDL) and small dense LDL in patients with aggressive periodontitis, as opposed to healthy subjects [112]. Our group in a recent research study has corroborated these results, reporting greater serum levels of small dense LDL in patients with mild periodontitis [10].

Meanwhile, other publications do not associate the presence of periodontal pockets with the levels of plasma lipids [18,113], not even when representing the lipid variables as TC/HDL and LDL/HDL ratios [114] and regardless of its intensity [113]. More recently, the study by Almeida *et al.* reported no association between the presence of hyperlipidemia – alone or in conjunction – with Type 2 diabetes mellitus, and periodontal disease [115].

Schenkein *et al.*, in a recent revision, stated that: "*There is evidence from clinical studies that patients with periodontitis can demonstrate elevated levels of serum cholesterol as well as of LDL, small dense LDL, VLDL, and TGs, in concert with decreased levels of HDL, thus presenting with a more atherogenic lipid risk profile*" [116]. It should be highlighted that several lipid-lowering agents, such as fibrates, are effective in managing atherogenic dyslipidemia [117,118].

Pathogenic mechanisms

Different mechanisms have been proposed to relate the levels of serum lipids with periodontal disease. Iacopino *et al.* established that any situation generating a systemic proinflammatory state will be a potential agent of lipid imbalance, as in the case of periodontitis, in which cytokines including IL-1β and TNFα enhance the biosynthesis of cholesterol in the liver [119]. This fact is confirmed by Fentoglu *et al.*, who described the association between IL-1β, IL-6 and TNFα levels and TC/HDL ratio [12].

On the other hand, the direct action of periodontal pathogens or their systemic by-products would also produce an immune-inflammatory response and changes in the lipid metabolism, leading to higher VLDL and LDL, as well as a decrease in the level and the antiatherogenic potential of HDL [9,56,120]. The LPS of *A. actinomycetemcomitans* was shown to influence the lipid profile, increasing LDL oxidation and the expression of receptors for LDL involved in the control of plasma lipid levels [58]. The production of ROS as a consequence of periodontitis would contribute to an increase in the process of lipid peroxidation, generating a more atherogenic profile [20].

Intervention studies

In the past 9 years, to our knowledge only nine publications focused on the quantification of serum lipid levels in patients with chronic periodontitis that underwent non-surgical periodontal treatment. The present review did not take into account those studies involving the periodontal treatment of patients presenting other pathologies associated with periodontitis, such as diabetes, given their effect as confounding factors.

Authors D'Aiuto *et al.*, Oz *et al.* and Taylor *et al.* report reduced values for total cholesterol and LDL in periodontal patients after their periodontal treatment [103,121,122]. Tamaki *et al.* found a decreased expression of oxLDL after periodontal treatment, which appears to

indicate an effect upon oxidative stress [20]. Other studies show an effect on HDL, pointing to a quantitative and qualitative improvement after treatment [56,123].

Notwithstanding, Higashi *et al.*, Kamil *et al.* and Kallio *et al.* found no changes in lipid levels after periodontal treatment [124–126]. Finally, the recent meta-analysis of D'Aiuto *et al.* concludes that periodontal treatment has no influence on the lipid profile of an individual [104].

Periodontitis & lipoproteins

Background & epidemiological evidence

In favor of the existence of a link between periodontitis and lipid metabolism is epidemiological evidence reporting significantly higher levels of TC, TG and oxLDL in subjects with CHD and chronic periodontitis compared with those only with CHD [127]. As mentioned above, LPS is the main virulence factor of the Gram-negative periodontal pathogens, which induces inflammation and is considered as a proatherogenic molecule [126]. LPS is bound and cleared from the circulation by all lipoproteins: in healthy subjects LPS is usually bound to HDL, which is further linked to LPS neutralization, while in states when HDL level is low the majority of LPS is bound to VLDL [126]. Kallio *et al.* showed an association between LPS and proatherogenic lipoprotein particles [120] that, together with LDL, induce lipid accumulation in macrophages and their transformation into foam cells [128]. It appears that periodontitis induces proatherogenic lipoprotein patterns and the association appears to be strongest for apoB-100-containing lipoproteins [129], but details about the potential mechanisms and specific role of VLDL in this context have remained largely unknown. Moreover, *P. gingivalis* degrades apo B-100, the major protein component of LDL particles, which could explain the altered mobility of LDL rather than oxidation [130]. *P. gingivalis* can directly modify LDL into a form that facilitates its uptake into macrophages by a specific mechanism for directly aggregating LDL [130]. This is supported by the fact that in subjects with periodontitis, in comparison to controls, sdLDL levels seem to be higher [10]. Rufail *et al.* have shown increased TG in cases of periodontitis as a result of increases in VLDL and a more robust increase in IDL particles, which appears to be more predictive of atherosclerosis progression than LDL, leading to increased CV risk in these subjects [131]. Furthermore, the authors suggest that neither total HDL levels nor the distribution of HDL subclasses is a major factor in the observed, increased CV risk. By contrary, Pussinen *et al.* indicated that periodontal infections may alter the antiatherogenic potency of HDL, indicating that HDL may affect the CV risk of these subjects in ways that are not related to the concentration of these particles [56]. Passoja *et al.* have reported that the association between periodontal inflammation and serum HDL existed independently of the microbial load and lipid-lowering therapy such as statins [132]. Interestingly, recent results indicate that nitric oxide (NO) production is reduced in periodontitis, especially in the male population, with higher LDL and significant positive correlation of NO levels with HDL levels in the whole population [133]. However, no significant correlation with periodontal disease severity was found. In addition, it has been reported that HDL-C and fasting glucose are importantly associated with periodontal disease, especially in women, while in men it seems that high levels of TG were associated with lower odds of having periodontal disease [16]. Furthermore, high blood glucose, low HDL-C and the prevalence of MS increase significantly with severity of periodontitis [134]. In addition, anti-apolipoprotein A-1 (antiapoA-1) IgG autoantibodies have been proposed as a possible biomarker for CVD risk in periodontitis, as they are prevalent in these patients (especially younger) compared with controls and the best predictor of atherosclerosis burden [135].

An increase in oxLDL level is a known risk factor for CVD, and it seems to be associated with the progression of periodontitis, as described above. Additionally, an *in vitro* study demonstrated that oxLDL increased production of the cytokine IL-8 in a human oral epithelial cell line (Ca9-22 cells), suggesting that lipoprotein metabolites could have a role in the inflammatory reaction in mucous membrane tissues, differently from that on endothelial cells blood vessels [136].

Serum proprotein convertase subtilisin/kexin 9 (PCSK9) concentrations might be associated with periodontal infection, as its level was significantly higher in patients with chronic periodontitis who were otherwise healthy when compared with healthy subjects [137], independently by LDL-C levels. PCSK9 inhibition is a new strategy for lowering LDL-C levels [138] and it might have a double effect in subjects with periodontitis.

Overall, subjects with periodontitis have an atherogenic lipoprotein profile and lower LDL-associated platelet-activating factor acetylhydrolase (PAF-AH) activity, hydrolyzed PAF, and oxidized phospholipids that have proinflammatory and proatherogenic properties. These differences might explain the increased CVD risk of these subjects [131] that has been discussed above. Although still debatable, it seems that severity and progression of periodontitis are associated with changes in serum components consistent with an acute-phase response, including lipids, lipoproteins and CRP [139].

Pathogenic mechanisms

The increased number of LDL particles in subjects with periodontitis can be a consequence of lower LDL-associated PAF-AH activity (i.e., PAF-AH activity per microgram of LDL protein) [140] that further may decrease the antiatherogenic influence of PAF-AH, leading to increased CV risk, as PAF-AH activity is negatively associated with small LDL particles. It is known that sdLDL are more susceptible to oxidation than larger, more buoyant subspecies and oxidative modifications of LDL represent an early stage of atherosclerosis [10]. However, it is more probable that oxidative stress and atherogenic dyslipidemia have a synergistic impact on atherosclerosis and cardiovascular diseases [140]. In this context, lipoprotein-associated phospholipase A_2 (Lp-PLA_2), an enzyme that has been shown to be a risk factor for CVD and is believed to be an independent CV risk factor that is also involved in the degradation of the phospholipid mediator platelet-activating factor (PAF), has been significantly reduced by treatment of periodontitis [141]. Interestingly, when *P. gingivalis* has been added to samples of whole human blood, after a short incubation period LDL and HDL particles were isolated from the incubated blood samples and apoB-100 of LDL particles became degraded, that is, LDL was proteolyzed into distinct peptide fragments, and the LDL particles also became oxidatively modified [142]. Actually, the proteolytic enzymes of *P. gingivalis*, the arginine-specific bacterial cysteine protease, Arg-gingipain (gingipain R), is able to proteolyze apoB-100; this bacterial protease can remain active in the whole blood. However, *P. gingivalis* microorganisms could also modify LDL within the arterial intima, not only in the circulation, and this can be a novel biochemical mechanism that links periodontitis and CVD [142]. *In vitro* studies have shown that oxLDL increases the production of IL-8 and PGE2 via the NF-κB pathway in human gingival epithelial cells, such as Ca9-22 and HOK cells [143,144]. Actually, the inhibitor of NF-κB completely suppressed the upregulation of IL-8, while PGE_2 production was partially suppressed, indicating that the oxLDL-mediated increase in PGE_2 production via COX-2 and PGES by several different pathways and also might be mediated by some scavenger receptors, since fucoidan and dextran sulfate blocked the action of oxLDL [136]. One more proposed mechanism, leading as well to increased cytokine production in periodontitis, is that minimally modified LDL would stimulate dendritic cells (which reside in the arterial walls and accumulate in atherosclerotic lesions) and enhance the production of proinflammatory cytokines that promote atherogenic plaque development [145]. In addition, it has been shown in animal models that periodontal infection induces lower HDL-C, whereas *P. gingivalis* downregulates liver X receptors (LXRs), resulting in the suppression of an ATP-binding cassette A1-mediated HDL-cholesterol generation [137].

There is evidence suggesting that oral pathogens may increase expression of the HMG-CoA reductase, the enzyme that is the target of the widely used statin drugs [129].

As we mentioned above, lipoproteins (especially HDL) are effective in binding and neutralizing LPS of Gram-negative bacteria, thereby limiting the expression of cytokines and lipid peroxidation, and have an antioxidant feature [132]. However, the protective role of HDL may be related not only to the level, but also to the quality of HDL [146]. A causal link between the activation of the LPS pathway on innate immunity by periodontal microbiota and the occurrence of a high-fat diet has been suggested in periodontal disease pathogenesis. This pathophysiological mechanism could be targeted by estrogens, which may thus represent a new therapeutic perspective to prevent or reduce periodontal occurrence inflammation [147].

Data in the literature on the distribution of lipoprotein particles in periodontitis subjects are still sparse. However, on the basis available results, a relationship exists and should be explored more in the future by larger studies.

Effects of periodontal treatment

Treatment of periodontitis might have beneficial effects on lipid metabolism and consequently decreases CHD risk [127]. A recent study reported that the combination of periodontal and anti-lipemic therapy provides beneficial effects on the metabolic and inflammatory control of hyperlipidemia via decreases in serum pro-inflammatory cytokines [148]. However, the potential roles of proinflammatory cytokines in the relationship between periodontitis and hyperlipidemia associated with infection and/or inflammation are still undetermined. Elucidating these roles could lead to new approaches in the control of both diseases [148].

The results reported by Kallio *et al.* [126] showing no significant effect of periodontal treatment on VLDL mass composition, LPS activity or apoE content can be a consequence of a short follow-up (3 months). Differences in the reported outcomes between different studies may also be influenced by heterogeneous 'periodontal disease' and 'health' definitions, and by differences in laboratory methodologies. In addition, an earlier study showed that periodontal treatment had only minor effects on plasma LPS activity and distribution lipoprotein classes [120]. Periodontal treatment reduces the circulating LPS levels and thereby the levels of some pro-inflammatory mediators that may lead to an increase in total cholesterol [103]. On

the other hand, intensive periodontal therapy (subgingival mechanical debridement with an adjunctive local delivery of minocycline) significantly decreased total and LDL-C [121].

Interestingly, in subjects with Type 2 diabetes mellitus, nonsurgical periodontal treatment is associated with improved glycaemic control, but also TC, TG and LDL levels decreased, whereas these values increased slightly in the control group [149]. Of interest, levels of oxLDL in subjects with periodontitis decreased after nonsurgical periodontal treatment [20], which positively correlated with a reduction in oxidative stress that indicate the usefulness of periodontal treatment not only in improving periodontal health, but also maintaining cardiovascular health.

Finally, a noninvasive measure of antipathogen antibodies (which correlate with CVD) has been proposed as a standard component of the physical examination in the future [141]. This strategy might facilitate the identification of subjects at risk of losing their teeth to periodontal disease, but will also help to prevent or reduce CVD or atherosclerosis [129].

Conclusion

Subjects affected by periodontitis appear to have a typical pattern-B lipoprotein profile: increased plasma triglycerides (VLDL and IDL), low plasma levels of HDL and a predominance of the small LDLs [10,131]. The higher concentration of small LDLs, subclasses vulnerable to oxidation, together with the lower activity of the enzyme PAF-AH, lead to atherogenic effects and enhanced CV risk [131].

Shared risk factors such as genetic variants, socioeconomic and behavioral factors are certainly in part responsible for the association between periodontitis and impaired lipid metabolism. It remains to be clarified whether a cause–effect inter-relationship also exists, and therefore whether periodontitis can induce higher lipid levels or whether higher lipid levels imply periodontitis [107]. The answer to these questions would explain whether CVD prevention is feasible by means of a reduction in periodontal disease. However, it is clear that improved periodontal health may affect metabolic control of hyperlipidemia and could be considered as an adjunct to the standard measures of hyperlipidemic patient care [150]. Improved oral hygiene and nonsurgical periodontal treatment are effective in decreasing oxLDL, which is positively associated with a reduction in oxidative stress [20] and with an effect on inflammation markers, which enhance risk and contribute to the development of CVD [141]. However, the underlying mechanisms remain unclear.

Future perspective

Evidence to date shows an association between periodontitis and CVD. Because of that, the American Heart Association and the European Society of Cardiology have recently added periodontitis as one important risk factor in their guidelines. However, this evidence only points to an association and longitudinal studies are needed to achieve a deeper knowledge about a possible causal relationship between these two diseases. In view of that, further research needs to be encouraged in order to produce prospective studies focused on establishing a possible a real causal relationship between periodontitis and CVD.

The increasing relevance of the role that periodontitis plays in CVD may make it necessary to integrate periodontal treatment and maintenance in the public health system due to the great importance of CVD as the first cause of mortality in developed countries.

Finally, raising public consciousness about the importance of regular oral hygiene might *per se* decrease the development of periodontitis and consequently CVD. In individuals suffering from periodontitis, affecting lipid metabolism might prevent and/or reduce CV risk, and periodontitis treatment might stabilize the disturbed metabolism of lipids.

Executive summary

Periodontitis & cardiovascular risk

- Periodontitis is associated with increased inflammatory markers, abnormalities in lipid and lipoprotein metabolism and oxidative stress. This is in turn linked with an increase in the risk of developing cardiovascular disease.

Periodontitis & lipoproteins

- Higher levels of total cholesterol, pattern-B lipoprotein profile: increased plasma triglycerides (very low-density lipoproteins and intermediate-density lipoproteins), low plasma levels of high-density lipoproteins, with a predominance of the small low-density lipoproteins (LDLs), as well as increased oxidized-LDLs, are characteristics of patients with periodontitis.

Effects of periodontal treatment on lipoproteins

- Periodontal treatment is associated with modest high-density lipoprotein-cholesterol increases and decreased LDL-cholesterol, which may consequently lower the risk of cardiovascular disease events.

Conclusion

- Careful management of patients with periodontitis may help to prevent and/or reduce cardiovascular disease events in these patients. Oral check-ups should be added to regular systemic assessments in usual clinical practice.

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• **Interesting article suggesting that improved periodontal health may influence metabolic control of hyperlipidemia.**

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ORIGINAL ARTICLE

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Simvastatin exerts antiproliferative and differentiating effects on MG63 osteoblast-like cells: Morphological and immunocytochemical study

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Background and Objective: Current evidence suggests that statins exert an anabolic effect on bone and may therefore impact on osteogenic differentiation and proliferation. These effects can be useful for their use in guided bone regeneration. The objective of this study was to determine the in vitro effects of simvastatin on the differentiation and proliferation of MG63 human osteoblast tumor cells.

Material and Methods: MG63 human osteosarcoma cells were cultured in the presence of simvastatin or solvent alone for 72 hours, and their proliferation was assessed by MTT assay. Cells from the culture were prepared for light, transmission and scanning electron microscopy studies. immunocytochemical was used to analyze the differentiation and proliferation markers Musashi-1, Ki-67, CD56 and CD44.

Results: Cultured MG63 control cells showed spheroid morphology with numerous secretion vesicles accumulated on the surface, observing no cytoplasmic projections with intercellular connections. However, cells cultured with simvastatin had a polygonal and spindle-shaped morphology, with cytoplasmic projections that interconnected cells. There were numerous microvilli-like filamentous projections on the surface with no defined pattern. At 72 hours of culture, CD56, Ki-67 and Musashi-1 expression was significantly reduced ($P < .001$) in simvastatin-treated cells. CD44 expression was intense in both groups and was not affected by simvastatin treatment.

Conclusion: MG63 cells cultured with simvastatin for 72 hours undergo morphological and surface changes. Simvastatin treatment exerts antiproliferative and differentiating effects on these cells as well as promoting recovery of cellular homeostasis.

KEYWORDS
cell differentiation, cell proliferation, electron microscopy, immunohistochemistry, osteoblasts, Simvastatin

1 | INTRODUCTION

Statins are hypolipidemic drugs that inhibit hydroxy-methyl-glutaryl co-enzyme A reductase of the mevalonate pathway, which is involved in hepatic cholesterol synthesis, and they are responsible for the production of steroid and non-steroid isoprenoids.[1] Statins, particularly simvastatin and atorvastatin, are among the most frequently prescribed pharmacological groups.[2] Besides their lipid-lowering action, they have been reported to exert pleiotropic effects[3] and to possess anti-inflammatory, immunomodulatory and antimicrobial properties

that may be useful against infections associated with bone healing.[4] Statins can activate the AKT1/PI3K pathway, leading to the inhibition of hydroxy-methyl-glutaryl co-enzyme A reductase, while statin-induced protein prenylation can have other important downstream effects, including angiogenesis (increased vascular-endothelial growth factor) and osteogenesis (increased bone morphogenetic protein-2 (BMP-2)) and reduced receptor activator of nuclear factor κB and its ligand (RANK-RANKL).[5] Statin-treated patients were found to have higher serum osteoprotegerin levels[6] and manifested anti-resorption (osteoclast inhibition), and anti-inflammatory (decrease in interleukin-6, C-reactive protein, adhesion molecules and reactive oxygen species) activities.[7] The precise effects of statins can vary according to their type and concentration.[8] The use of local statins as coadjuvants alongside scaling and root planing were reported to improve clinical periodontal parameters and recover bone crest height.[9] Experimental studies of implants in rodents found that simvastatin administration improved the bone contact ratio, bone density and osseointegration,[10,11] which was also found to be enhanced by topical fluvastatin application around implants.[12] In contrast, other authors found no increase in bone density in statin-treated defects,[13] and a recent study reported that simvastatin loading of implant surfaces exerted significant effects for only 2 weeks.[14] Formation of new bone tissue was observed in calvaria of rats after the topical injection of fluvastatin using tricalcium phosphate as carrier.[15] However, high doses of local statin can cause inflammation when used for bone regeneration, as reported by two studies in which simvastatin was applied to calvarial defects in rodents.[13,16] Simvastatin was found to affect osteogenic differentiation in a murine model,[17] while an in vitro study showed that exposure to this drug slightly increased osteoblast expression of osteocalcin, osteoprotegerin, alkaline phosphatase and other bone markers.[18]

The objective of the present study was to determine the in vitro effects of simvastatin on the differentiation and proliferation of the MG63 human tumor osteoblast cell line.

2 | MATERIAL AND METHODS

All procedures in this study were performed in accordance with the 1964 Helsinki declaration and its later amendments. The study was approved by the Ethics Committee of the School of Dentistry of the University of Granada (reference: FOD/UGR/08/2016).

2.1 | Cell line

MG-63 human osteosarcoma cell lines were obtained from the University of Granada Scientific Instrumentation Centre (Spain). Cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and 1% non-essential amino acid solution in a humidified incubator at 37°C, using a standard mixture of 95% air and 5% CO_2. Cultured cell monolayers were detached with a trypsin-ethylenediaminetetraacetic acid solution (.25%) and seeded into 24-well plates at a density of 15×10^3 cells per well or into T75 flasks (Nunc, Rochester, NY, USA) at a density of 500×10^3 cells per flask, depending on the experiment.

2.2 | Materials

Simvastatin and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich LLC (St. Louis, MO, USA). Simvastatin was resuspended at a concentration of 20 mg/mL in DMSO and stored at −20°C.

2.3 | Proliferation assay

After incubation for 24 hours under culture conditions, cells in the 24-well plates were incubated with simvastatin (.005-25 μmol/L) for 72 hours. The cytotoxicity of this treatment was then evaluated in triplicate by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) proliferation assay, adding 20 μL MTT solution in cell culture medium (5 mg/mL) to each well. After incubation for 4 hours at this temperature, the culture medium was removed and the resulting formazan crystals were dissolved in 200 μL DMSO. The optical density (OD) of the converted dye, which is proportional to the number of viable cells, was measured at 570 nm (with subtraction of background at 690 nm) using a Titertek multisKan colorimeter (Flow Laboratories, Irvine, CA, USA). The percentage of surviving cells was calculated as: (OD treated cells/OD control [untreated] cells)×100. The same procedure was applied to control cells.

2.4 | Simvastatin culture

MG-63 cells were seeded in T75 flasks and grown in the presence of simvastatin for 72 hours at a final concentration of .01 μmol/L (the highest concentration with no effect on cell proliferation after 72 hours of treatment) or in the presence of the same amount of DMSO as control. Cells were then removed from the culture and prepared for microscopic and immunocytochemical studies.

2.5 | Transmission electron microscopy study

Several pellets of treated and control MG63 cells were fixed in 2.5% glutaraldehyde solution and then postfixed in 1% OsO_4 at 4°C for 2 hours, washed in distilled water, dehydrated in increasing concentrations of acetone and embedded in Epon following a conventional protocol. Semithin sections were stained with toluidine blue solution. Ultrathin (~70 nm thick) sections obtained using a Reichert Jung ULTRACUT ultramicrotome (Leica, Wetzlar, Germany) were stained with lead citrate and uranyl acetate and under a Libra 120 Plus transmission electron microscopy (TEM; Zeiss, Oberkochen, Germany).

2.6 | Scanning electron microscopy study

Samples from both groups of cultured MG63 cells were gently removed and immediately immersed in a sodium cacodylate-buffered

formaldehyde-glutaraldehyde fixative for 24 hours at room temperature and post-fixed in 20% osmium tetroxide for 2 hours. Samples were then dehydrated by serial transfer in ascending concentrations of acetone (50%-100%) and infiltrated with liquid carbon dioxide before the critical drying point. Finally, samples were made electrically conductive by mounting them on aluminum slabs with a silver point, followed by sputter coating with gold/palladium to a thickness of approximately 250 Å. After attachment of these specimens to an acrylic plate with glue tape, the plate was vertically divided into two pieces using a diamond disc with chisel and hammer, followed by examination of their cut surfaces with a Quanta 400 SEM (FEI, Hillsboro, OR, USA) at 5-10 kV.

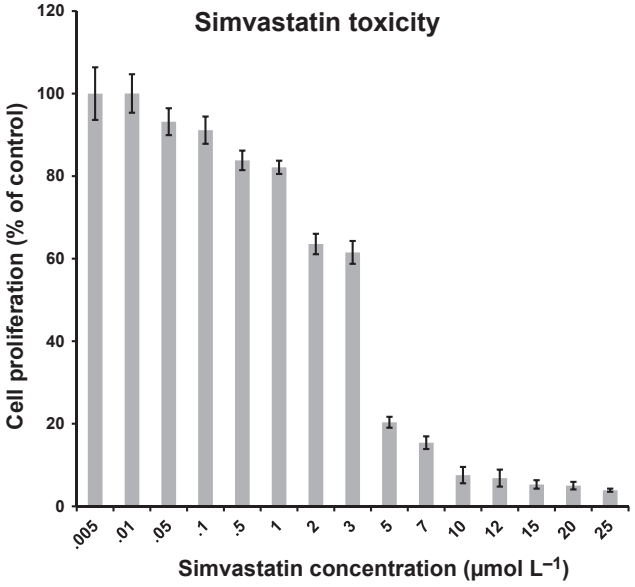

FIGURE 1 In vitro toxicity of simvastatin in MG63 cell line. Growth of MG63 cell was evaluated after 72 h of exposure to a wide range of simvastatin concentrations (.005-25 μmol/L) in comparison to control cells treated with solvent alone. Data expressed as the mean value±SD of triplicate cultures

2.7 | Immunocytochemical analysis

After detachment from the plastic substrate, MG63 osteosarcoma cells from both groups were centrifuged at 302 *g* for 3 minutes in phosphate buffer solution, washed, resuspended in phosphate-buffered saline and centrifuged at 7393 *g* for 30 seconds before embedding the resulting pellets in paraffin. Paraffin-embedded sections were dewaxed, hydrated and heat-treated in 1 mm ethylenediaminetetraacetic acid (pH 8) for antigenic unmasking in an antigen retrieval PT module (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 95°C for 20 minutes. Sections were incubated for 16 hours at 4°C with prediluted polyclonal antibody against Musashi-1 (mesenchymal stem cells, polyclonal; Sigma-Aldrich LLC) and runt related transcription factor 2 (Runx2, clone M-70) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) both at 1:100 dilution, and with prediluted monoclonal antibodies against Ki-67 (proliferative cells, clone SP-6), CD56 (osteoblast differentiation, clone 56C04) and CD44 (osteocyte cells, clone 156-3C11); sections were then incubated for 10 minutes at room temperature to analyze the cell differentiation and antiproliferative response. The immunocytochemical study was conducted using the micropolymer-peroxidase-based method (Master Polymer) with automatic immunostainer (Autostainer 480; Thermo Fisher Scientific Inc., Waltham, MA, USA) followed by development with diaminobenzidine (other monoclonal antibodies and reagents were obtained from Master Diagnóstica, Granada, Spain). Appropriate positive (tonsil) and negative (non-immune serum) controls were run concurrently. Hematoxylin was used for nuclear counterstaining. Results were expressed as percentages of positive cells for each antibody, counting 200 cells per high-magnification field (40× objective) in three independent experiments.

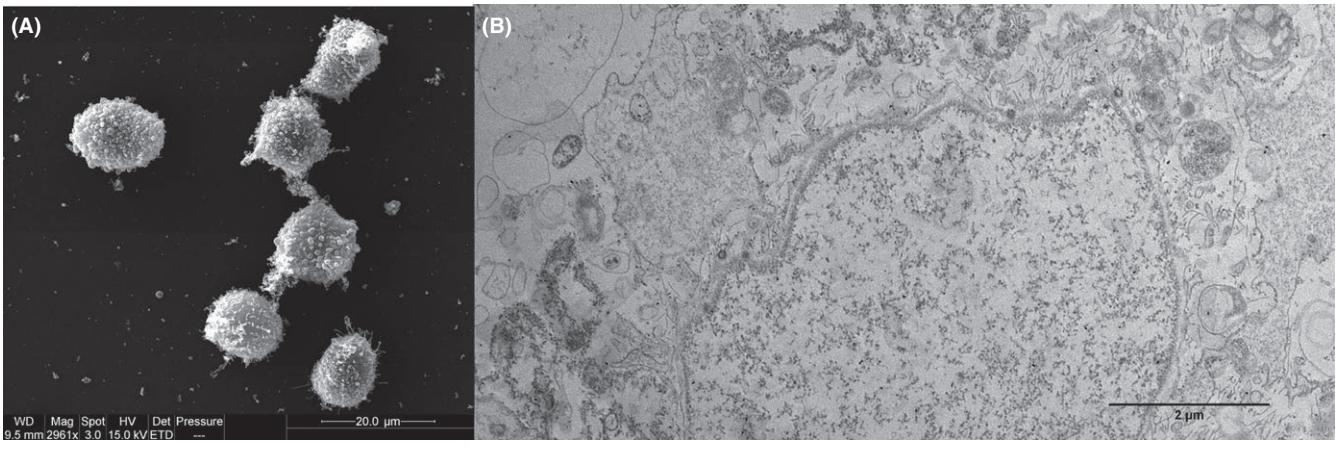

FIGURE 2 Ultrastructural study with scanning and transmission electron microscopy. (A) Spheroid morphology and numerous secretion vesicle accumulations in control MG63 cells. No intercellular-connecting cytoplasmic projections were observed (scale bar in figure). (B) Control cells treated with dimethyl sulfoxide show a clear low-density cytoplasm with appreciable decrease in intracytoplasmic organelles, containing numerous free, unstructured filaments and a large number of lysosomal bodies incorporating abundant phagocytic material

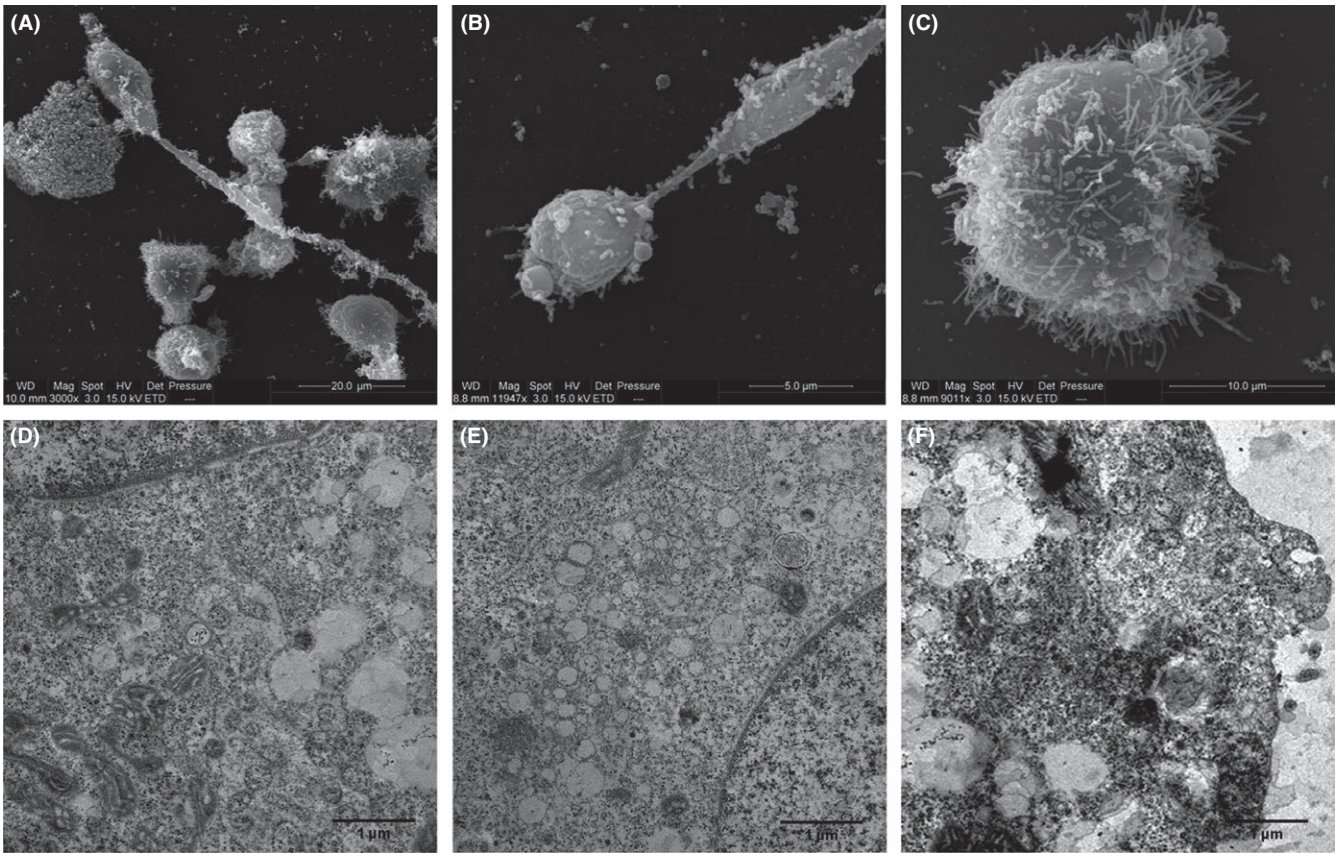

FIGURE 3 Ultrastructural study with scanning and transmission electron microscopy. (A) Morphological heterogeneity (spheroid, polygonal and spindle-shaped) of MG63 cells in cultures treated with simvastatin. (B) Spindle-shaped cell with long projection that makes contact with spheroid cell in the simvastatin-treated culture. (C) Spheroid cell with presence of numerous "microvilli-like" filamentous projections distributed homogeneously over its surface with no defined pattern (scale bar in figures). (D) Simvastatin-treated MG63 cells showing dense cytoplasm with large amount of mitochondria alongside free ribosomes and lysosomes. (E) Significant increase in the accumulation of lipid vacuoles of varied size in the cytoplasm. (F) Microphotograph showing organization of the "clathrin-type" subcellular microvesicular system

2.8 | Statistical analysis

SPSS v. 20.0 (IBM SPSS, Armonk, NY, USA) was used for the statistical analysis. A non-parametric test (Mann-Whitney *U*-test) was applied to compare positive cells between control and treated cells. $P < .05$ was considered significant.

3 | RESULTS

3.1 | Cell viability test

After 72 hours of exposure to simvastatin, the highest dose with no effect on cell proliferation was .01 µmol/L. At higher doses, the proliferative capacity of these cells began to decrease in comparison to control cells (Figure 1).

3.2 | Electron microscopy

TEM study of cultured MG63 cells revealed a similar morphology to that reported by Fernández-Barbero et al.[19] Control cells showed a clear low-density cytoplasm with an appreciable reduction in intracytoplasmic organelles, numerous unstructured free filaments in the cytoplasm and many lysosomal bodies containing abundant phagocytic material (Figure 2). In contrast, simvastatin-treated MG63 cells showed a high density of cytoplasmic organelles, with a large amount of mitochondria and numerous free ribosomes and lysosomal bodies (Figure 3). There was an appreciable increase in lipid vacuoles underlying the cell cytoplasmic membrane, which were grouped, fused and intermixed with smaller vacuoles (Figure 3) and structured vesicular invaginations. The cytoplasmic membrane contained numerous thin projections (see also SEM observations above).

SEM study also showed the morphology of cultured MG63 cells to be similar to that reported by Fernández-Barbero et al.[19] The morphology of control MG63 cells was spheroid, with a large accumulation of secretion vesicles on their surface but no cell-interconnecting cytoplasmic projections (Figure 2). Cells treated with simvastatin were spheroid, polygonal or spindle-shaped (Figure 3), and the spindle-shaped cells had long cytoplasmic projections interconnecting cells (Figure 3). Numerous "microvilli-like" filamentous projections were also distributed over the whole surface of the cells, particularly those with spheroid morphology, but they generally formed no defined pattern (Figure 3).

TABLE 1 Percentage immunocytochemical marker expressions after 72 h of culture

	Control	Simvastatin	*P*-value
CD44 (%)	99.6 ± .78	100 ± .0	NS
CD56 (%)	34.3 ± 2.75	11.0 ± 3.68	<.001*
Ki-67 (%)	87.6 ± 4.86	33.0 ± 5.31	<.001*
Msi1 (%)	97.6 ± 1.69	16.6 ± 3.31	<.001*
Runx2 (%)	.5 ± .54	.5 ± .54	NS

NS, non-significant.Values expressed as mean percentage±SD of three independent experiments.
*Control vs simvastatin, Mann-Whitney *U*-test.

3.3 | Immunocytochemical results

Cytoplasmic membrane expression of CD44 was intense in all MG63 cells and was not modified by statin treatment. After 72 hours of treatment, the CD56, Msi1 and Ki-67 expression of MG63 cells was significantly ($P < .001$, Mann-Whitney *U*-test) decreased in comparison to control cells (Table 1, Figure 4). No nuclear Runx2 expression was detected in MG63 cells.

4 | DISCUSSION

Ultrastructural and immunocytochemical changes observed in this in vitro study suggest that simvastatin exerts differentiating and antiproliferative effects on the MG63 cell line.

Ultramicroscopic study of control MG63 cells revealed groups of cells with cytoplasmic elements characteristic of elevated cell activity and groups with high cytoplasmic destructuration, with a "cytoplasmic void" showing numerous destructured microfilaments and numerous lysosomal bodies containing cell material from different organelles. The abundant apoptotic phenomena observed in TEM images and the high expression of the proliferation marker Ki-67 indicate the presence of major stress in these cells. This cytoplasmic destructuration in MG63 cells was morphologically reverted by simvastatin treatment, which significantly reduced the number of cytoplasmic vacuoles containing cell material and increased the vesicular turnover at cytoplasm membrane level, with the emergence of structured vesicular invaginations and the acquisition of a normal cytoplasmic conformation. According to these results, simvastatin may act against cell stress, stabilizing osteogenic cell renewal processes. In the context of bone production, preservation and remodeling, simvastatin may therefore function as a modulator of osteoclastogenesis/osteogenesis.

TEM findings of a large accumulation of lipid vacuoles in simvastatin-treated MG63 cells would result from statin-induced lipoprotein deprivation of the medium, which in the present case represents physiological normalization through recovery of an adequate lipid cell metabolism. This interpretation is supported by SEM observations of numerous pinocytotic vesicles and abundant microvilli on the surface of treated cells. Simvastatin was previously reported to produce an accumulation of cytosolic lipid droplets in both non-malignant and malignant cells, which may contribute to their antiproliferative effects.[20] In contrast, Martinet et al found little cytoplasm vacuolization of mesenchymal stem cells after treatment with .01 μmol/L

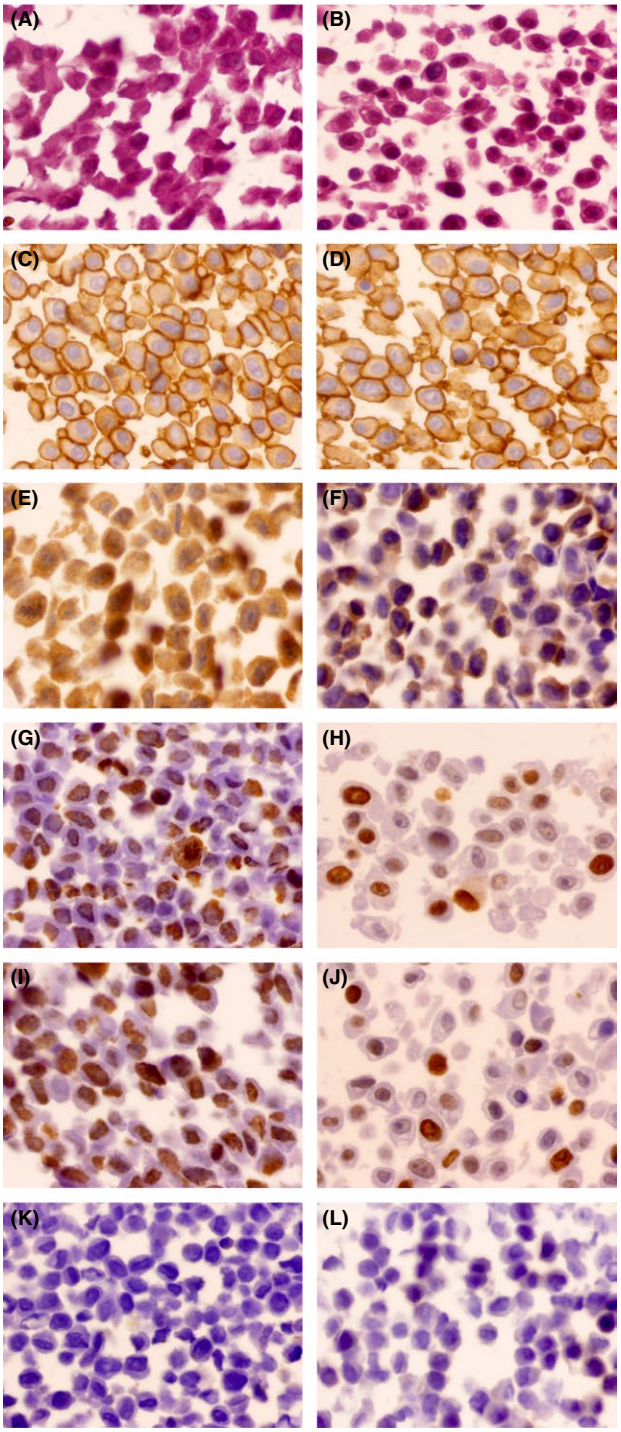

FIGURE 4 Morphological and immunocytochemical study. (A) and (B) Increase in cytoplasmic vacuolization and apoptotic cells in simvastatin-treated MG63 cells. (C) and (D) No modification of CD44 expression after incubation with simvastatin for 72 h; (E) and (F) reduction in CD56 expression; (G) and (H) reduction in nuclear expression of Ki-67 (cell proliferation); (I) and (J) reduction in nuclear expression of MSi-1; (K) and (L) no induction of Runx2 expression (original magnification ×40)

simvastatin,[21] which may be attributable to the difference in baseline differentiation between mesenchymal stem cells and MG63 cells, which have already acquired some degree of osteogenic differentiation. The previously documented absence of Runx2 expression suggests that the mechanism underlying osteogenic differentiation in this cell line is independent of the expression of this transcription factor.[22] These data indicate that the effectiveness of simvastatin may depend upon the initiation of osteogenic differentiation by target cells; in other words, the action of simvastatin may be dependent on the degree of differentiation.

Immunocytochemical analysis showed that simvastatin treatment had no significant effect on the CD44 expression of MG63 cells but significantly reduced their expression of CD56, Musashi-1 and Ki-67 (cell proliferation index). No Runx2 expression was detected in either treated or control cells. Given that CD56 and Musashi-1 expression is characteristic of stem cells and those with a low degree of differentiation,[23,24] these results suggest that simvastatin treatment increases the differentiation degree of MG63 cells, which is consistent with the significant reduction in Ki-67 expression (proliferation-differentiation balance).[25] This mechanism may be dependent on the inhibition of geranylgeranyl pyrophosphate synthase or other upstream isoprenoids.[26] The response of Musashi-1 can be modulated by environmental factors and can regulate physiological or pathological cell renewal.[27] In vivo and in vitro studies have linked high CD56 expression to worse tumor cell behavior in comparison to low or negative expression.[28] Regarding bone tissue, CD56-positive myeloma cells have been linked to the presence of lytic bone lesions.[29] Musashi-1 may play a possible role in osteogenic differentiation, downregulating the Wnt1 pathway and expression of the miR-148 family, because Musashi-1 knockdown was found to increase expression of these two pathways, which are involved in the osteogenic differentiation of stem cells.[30] This reduction in Musashi-1 expression is also compatible with the osteoarticular tissue expression of Musashi-1, reported for the first time by our group, suggesting a possible involvement of this factor in tissue regeneration.[31]

The effects of statins on bone metabolism were first reported by Mundy et al in an in vitro animal model, which revealed higher BMP-2 expression in cultured osteoblast cells and increased medullar bone formation in rodents.[32] Later investigations related statin consumption to a higher bone density and lower incidence of fractures in patients with osteoporosis, although a meta-analysis was unable to confirm this association.[33] Statins were found to favor bone regeneration in an animal model of periodontitis, increasing BMP-2 and reducing RANK-RANKL,[5] while a clinical study by our group observed higher osteoprotegerin levels in simvastatin-treated patients.[6] Statin consumption was recently associated with lower tooth loss over time in a prospective epidemiologic study with 5 year follow-up in a European population.[34] Research is ongoing into the optimal statin concentrations to achieve anti-inflammatory and bone anabolic effects in the local treatment of periodontal lesions; various carriers and drug delivery systems have been used, including the application of statins as bioactive agents on implant surfaces.[35-37]

The proliferation and cell functions exhibited by osteosarcoma cells may not be fully representative of those in human primary osteoblasts, given reported differences between them.[38] Nevertheless, the MG63 cell line has been widely used as an in vitro model for bone research, and findings on the proliferation of these cells have proven comparable to observations in primary human osteoblasts.[39]

Finally, the immunocytochemical and ultramicroscopic results obtained suggest that simvastatin treatment of osteogenic cells may provide a pathway for recovery of the normal morphological and functional status of bone tissue, besides enhancing the differentiation degree of the cells and reducing their proliferation. Further research is warranted to explore the therapeutic potential of this novel approach in periodontal bone preservation and remodeling.

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CONFLICT OF INTEREST

The authors report no conflicts of interest related to this study.

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