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ESCOLA DE DOUTORAMENTO INTERNACIONAL
EN CIENCIAS DA SAÚDE DA USC

Yago
Leira Feijóo

Tese de doutoramento

Periodontal disease and cerebral atherosclerotic disease. Translational study

PD en Ciencias Odontolóxicas

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TESE DE DOUTORAMENTO

[PERIODONTAL DISEASE AND CEREBRAL ATHEROSCLEROTIC DISEASE. TRANSLATIONAL STUDY]

[Yago Leira Feijóo]

ESCOLA DE DOUTORAMENTO INTERNACIONAL
PROGRAMA DE DOUTORAMENTO EN CIENCIAS ODONTOLÓXICAS

SANTIAGO DE COMPOSTELA
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[Periodontal disease and cerebral atherosclerotic disease. Translational study]

D. Yago Leira Feijóo

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D. Juan Blanco Carrión

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Que el presente trabajo titulado **"ENFERMEDAD PERIODONTAL Y ENFERMEDAD CEREBRAL ATEROSCLERÓTICA. ESTUDIO TRASLACIONAL"** ha sido realizado bajo su dirección por el Licenciado en Odontología **YAGO LEIRA FEIJÓO**, reúne todos los requisitos para optar al Doctorado Internacional.

Los directores:

Prof. Dr. Juan Blanco Carrión Prof. Dr. José Castillo Sánchez

En Santiago de Compostela, 22 de mayo de 2018

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Prof. Dr. **JUAN BLANCO CARRIÓN**, Professor of Periodontology of the University of Santiago de Compostela and Prof. Dr. **JOSÉ CASTILLO SÁNCHEZ**, Scientific Director of the Health Research Institute of Santiago de Compostela (IDIS),

CERTIFY:

That the present research study entitled **"PERIODONTAL DISEASE AND CEREBRAL ATHEROSCLEROTIC DISEASE. TRANSLATIONAL STUDY"** has been carried out under their supervision by **YAGO LEIRA FEIJÓO,** graduated in Dentistry, fulfils the requirements for the International Doctorate.

Supervisors:

Prof. Dr. Juan Blanco Carrión Prof. Dr. José Castillo Sánchez

Santiago de Compostela, 22nd May 2018

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D.E.P.

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"Sólo la posibilidad teórica de que la enfermedad periodontal pueda predisponer a la aparición de un accidente cerebrovascular tiene una trascendencia alarmante para los millones de pacientes que la sufren".

“Las cosas buenas suceden a los que se dan prisa”.

Anais Nin

“A veces la vida te va a pegar en la cabeza con un ladrillo. Pero no pierdas la fe”.

Steve Jobs

“Confía en el tiempo, que suele dar dulces salidas a muchas amargas dificultades”.

Miguel de Cervantes Saavedra

“Cada fracaso enseña al hombre algo que necesitaba aprender”.

Charles Dickens

Periodontal disease and cerebral atherosclerotic disease. Translational study

RESUMO

Aínda que estableceuse unha asociación entre a enfermidade periodontal (PD) e o ictus isquémico de gran vaso, hai pouca evidencia sobre a relación entre a PD e infarto lacunar (LI), un subtipo de enfermidade cerebral de pequeno vaso que é responsable de aproximadamente o 25% dos casos de ictus isquémico. Polo tanto, o obxectivo deste estudo foi investigar se a PD está asociada co LI e de ser así, estudar os posibles mecanismos que podrían explicar esta asociación. No presente estudo, a PD asociouse positivamente co LI e cando está presente emerxeu como un dos principais factores que contribuiu a un estado sistémico pro-inflamatorio promovendo a disfunción endotelial con niveis elevados de IL-6, PTX3, sTWEAK, e $A\beta_{1\text{-}40}$ nos pacientes con LI. Ademáis, a PD moderada/avanzada activa foi un preditor independente de mal prognóstico nos pacientes con LI. Estes resultados foron corroborados nun estudo preclínico *in vivo* no modelo roedor no que a PD experimental inducida por lipopolisacárido de *Porphyromonas gingivalis* asociouse cunha resposta inflamatoria sistémica leve coa disrupción da función endotelial vascular.

PALABRAS CHAVE: enfermidade periodontal, infarto lacunar, inflamación, disfunción endotelial, enfermidade cerebral de pequeno vaso.

RESUMEN

Aunque se ha establecido una asociación entre enfermedad periodontal (PD) e ictus isquémico de gran vaso, existe poca evidencia sobre la relación entre PD e infarto lacunar (LI), un subtipo de enfermedad cerebral de pequeño vaso que es responsable de aproximadamente el 25% de los casos de ictus isquémico. Por lo tanto, el objetivo de nuestro trabajo fue investigar si la PD se asocia con LI y en caso de estarlo, estudiar posibles mecanismos que puedan explicar esta asociación. En el presente estudio la PD se asoció positivamente con el LI y, cuando está presente

emergió como uno de los principales factores que contribuyó a un estado sistémico pro-inflamatorio promoviendo la disfunción endotelial con niveles elevados de IL-6, PTX3, sTWEAK, and $A\beta_{1-40}$ en pacientes con LI. Además, la PD moderada/avanzada activa fue un predictor independiente de mal pronóstico en pacientes con LI. Estos hallazgos fueron corroborados en un estudio preclínico *in vivo* en el modelo roedor, en el cual la PD experimental inducida mediante lipopolisacárido de *Porphyromonas gingivalis* se asoció con una respuesta inflamatoria sistémica leve con disrupción de la función endotelial vascular.

PALABRAS CLAVE: enfermedad periodontal, infarto lacunar, inflamación, disfunción endotelial, enfermedad cerebral de pequeño vaso.

ABSTRACT

Although an association has been established between periodontal disease (PD) and large vessel ischemic stroke, little is known about the relationship between PD and lacunar infarct (LI), a subtype of cerebral small vessel disease that is responsible for almost 25% of the ischemic stroke cases. Our aim was, therefore, to investigate whether PD is linked with LI and if so, to study potential mechanisms underlying this association. In the present study we found that PD was positively associated with LI and, when present, emerged as one of the main contributors to an enhanced systemic inflammatory state promoting endothelial dysfunction with elevated levels of IL-6, PTX3, sTWEAK, and $A\beta_{1-40}$ in LI patients. Moreover, moderate to severe active PD was an independent predictor of poor functional outcome in LI patients. These findings were corroborated in a preclinical *in vivo* study in the rodent model, in which experimental PD induced with lipopolysaccharide from *Porphyromonas gingivalis* was associated with a mild systemic inflammatory response with disruption of the vascular endothelial function.

KEY WORDS: periodontal disease, lacunar infarct, inflammation, endothelial dysfunction, cerebral small vessel disease.

LIST OF PUBLICATIONS RELATED TO THIS THESIS

Leira Y, Blanco M, Blanco J, Castillo J. 2015. [Association between periodontal disease and cerebrovascular disease. A review of the literature]. Rev Neurol. 61(1):29–38.

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ABBREVIATIONS

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ABBREVIATIONS

Aa: Aggregatibacter actinomycetemcomitans
AAP: American Academy of Periodontology
Aβ: amyloid-beta
$A\beta_{1\text{-}40}$: amyloid-beta 1-40
$A\beta_{1\text{-}42}$: amyloid-beta 1-42
AD: Alzheimer´s disease
AIC: Akaike information criterion
ANOVA: one-way analysis of variance
APP: amyloid precursor protein
ApoE: apolipoprotein E
APRs: acute-phase reactants
ARIC: Atherosclerosis Risk in Communities
ARRIVE: Animal Research Reporting of In Vivo Experiments
BBB: blood-brain barrier
BMI: body mass index
BoP: bleeding on probing
CAL: clinical attachment level
CDC: Center for Disease Control and Prevention
CEJ: cemento-enamel junction
CI: confidence interval
CKD: chronic kidney disease
CMBs: cerebral microbleeds
CRP: C-reactive protein
CSVD: cerebral small vessel disease
CT: computed tomography
CV: coefficient of variation
CVD: cardiovascular disease
DALYs: disability-adjusted life years
DAMPs: damage associated molecular peptides
DMFT: decayed, missing and filled teeth index

ECs: endothelial cells
EDV: endothelium-dependent vasodilation
ELISA: enzyme-linked immunosorbent assay
eNOS: endothelial nitric oxide synthase
ET-1: endothelin-1
EcT: echo time
FA: flip angle
FimA: fimbriae A
FLAIR: fluid-attenuated inversion recovery
FMBS: full-mouth bleeding score
FMD: flow-mediated dilation
FMPS: full-mouth plaque score
Fn: Fusobacterium nucleatum
Fn14: fibroblast growth factor-inducible 14
GCF: gingival crevicular fluid
GI: gingival index
GR: gingival recession
HagA: hemagglutinin A
HDLs: high-density lipoproteins
HPA: hypothalamic-pituitary-adrenal axis
HR: hazard ratio
HSPs: heat-shock proteins
HSP60: heat-shock protein-60
IL-1A: Interleukin-1A
IL-1B: Interleukin-1B
IL-1β: Interleukin-1β
IL-6: Interleukin-6
IL-8: Interleukin-8
IL-10: Interleukin-10
IL-6R: cellular interleukin-6 receptor
ICAM-1: intercellular adhesion molecule-1
ICD: International Classification of Diseases
Ig-A: immunoglobulin-A
IKK: Iκβ kinase
IMT: intima-media thickness
LBP: LPS binding protein

LDLs: low-density lipoproteins
LI: lacunar infarct
LPS: lipopolysaccharide
μCT: micro-CT
MCAO: middle cerebral artery occlusion
MCP-1: monocyte chemotactic protein-1
mm^2: square millimetres
MMPs: matrix metalloproteinases
MMP-9: matrix metalloproteinase-9
MMP-2: matrix metalloproteinase-2
MRI: magnetic resonance imaging
mRS: modified Rankin scale
NA: number of averages
NF-κB: nuclear factor-κB
NHANES: National Health and Nutrition Examination Survey
NIH: National Health Insurance
NO: nitric oxide
NO_2: nitrous oxide
NSAIDs: non-steroidal anti-inflammatory drugs
O_2: oxygen
OPG: osteoprotegerin
OR: odds ratio
PAI-1: plasminogen-activator inhibitor-1
PD: periodontal disease/periodontitis
PESA: periodontal epithelial surface area
Pg: Porphyromonas gingivalis
PGE_2: prostaglandin E_2
Pi: Prevotella intermedia
PISA: periodontal inflamed surface area
PMNs: polymorphonuclear neutrophils
PPCs: periodontal profile classes
PPD: probing pocket depth
PTXs: pentraxins
PTX3: pentraxin 3
PVS: perivascular spaces
Qs: quartiles

r: Pearson´s correlation coefficient
RANKL: receptor activator of nuclear factor-κB ligand
ROC: receiver operating characteristic
ROIs: regions of interest
ROS: reactive oxygen species
RR: relative risk
RT: repetition time
SAA: serum amyloid protein A
sCD14: soluble CD14
SgI: signal intensity
SI: silent infarct
s-ICAM: soluble intercellular adhesion molecule
sIL-6R: soluble interleukin-6 receptor
SMC: smooth muscle cell
STROBE: Strengthening the Reporting of Observational Studies in Epidemiology
sTWEAK: soluble tumor necrosis factor-like weak inducer of apoptosis
SW: spectral bandwidth
T_1-w: T_1-weighted
T_2-w: T_2-weighted
Td: Treponema denticola
Tf: Tannerella forsythia
TF: tissue factor
TGs: triglycerides
TIA: transient ischemic attack
TLRs: toll-like receptors
TLR-2: toll-like receptor 2
TLR-4: toll-like receptor 4
TNF-α: tumor necrosis factor-α
TOAST: Trial of Org 10172 in Acute Stroke Treatment
tPA: tissue plasminogen-activator
TWEAK: tumor necrosis factor-like weak inducer of apoptosis
US: United States
VCAM-1: vascular cell adhesion molecule-1
WHO: World Health Organization
WMD: weighted mean difference

INTRODUCTION

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DE COMPOSTELA

INTRODUCTION

1. PERIODONTAL DISEASE

1.1. Definition

Periodontal disease or periodontitis (PD) is a chronic inflammatory condition, in which gums are infected by oral bacteria resulting in connective tissue breakdown, periodontal pocket formation, alveolar bone loss, and eventually tooth loss (Kinane et al. 2017). The first stage of chronic PD is plaque-induced gingivitis, defined as a reversible inflammation confined to the gingiva. Although both conditions are initiated and sustained by plaque accumulation, host factors (Genco and Borgnakke 2013) and genetics (Nares 2003) also play a role in the pathogenesis of the disease. Other rare forms of PD and much less prevalent might exist, such as acute necrotizing gingivitis (Dufty et al. 2017) and PD (Novak 1999) as well as aggressive PD (Catunda et al. 2018). Occasionally, another subgroup of PD could be observed, which refers to those forms of PD that are seen as a manifestation of systemic diseases (i.e., associated with hematologic or genetic disorders) (Deas et al. 2003) (Table 1).

PERIODONTAL CONDITION	Main characteristics
Gingivitis	• Gingival inflammation without loss of attachment and increased pocket depth (reversible) • Due to plaque accumulation
Chronic PD	• More common in adults, but can be observed in children • Slow to moderate rates of progression • Amount of plaque deposits consistent with periodontal severity destruction (frequent presence of subgingival calculus) • No discernible pattern of periodontal destruction

	• No familial aggregation of the cases • Based on the extent and severity it can be classified into: ✓ Mild, moderate or severe ✓ Localized and generalized
Acute necrotizing gingivitis and PD	• Rapid rates of progression • Gingival necrosis presenting as "punched-out papillae" in addition to gingival bleeding and pain • In certain cases, necrosis of periodontal ligament and alveolar bone can be observed • Associated with fusiform bacilli, spirochetes or viruses • Common in immunosuppressed hosts
Aggressive PD	Two forms: • Localized aggressive PD: ✓ More common in adolescents ✓ Rapid rates of progression ✓ Amount of plaque deposits not consistent with periodontal severity destruction (subgingival calculus usually absent) ✓ Periodontal destruction localized to permanent first molars and incisors ✓ Familial aggregation of the cases • Generalized aggressive PD: ✓ More common in people younger than 30 years of age, but can be observed in older patients ✓ Rapid rates of progression ✓ Amount of plaque deposits sometimes consistent with periodontal severity destruction (presence of subgingival calculus may or may not be) ✓ Periodontal destruction affects many teeth in addition to permanent first molars and incisors ✓ Familial aggregation of the cases
Syndromic chronic PD	• Hematologic disorders ✓ Acquired neutropenia ✓ Leukemias

	• Genetic disorders ✓ Down´s syndrome ✓ Chédiak-Higashi syndrome ✓ Papillon-Lefévre syndrome ✓ Marfan syndrome ✓ Ehlers-Danlos syndrome ✓ Others…

Table 1. Overview of the PDs classification (Armitage 1999).

Diagnosis of PD is based on severity and extent of clinical attachment level (CAL) and probing pocket depth (PPD). In 2007, the Centers for Disease Control and Prevention (CDC) in collaboration with the American Academy of Periodontology (AAP) developed case definitions for moderate and severe PD for use in epidemiological research (Page an Eke 2007). In addition, the same working group published in 2012 an updated paper, where they introduced the definition of mild PD cases (Eke et al. 2012) (Figure 1). However, the lack of a universal periodontal case definition has led both European and American experts in this field to publish a consensus paper in which they suggest that the case definitions developed by the CDC-AAP is the one that has to be used in periodontal epidemiological studies (Holfreter et al. 2015). This classification is based on linear measures (i.e., PPD and CAL) and, therefore, may not quantify the amount of inflamed periodontal tissue. Periodontal inflammation, which occurs locally but can disseminate systemically, is the biological basis for the plausibility of any potential association between PD and several other diseases. In this sense, any classification of PD as a potential risk factor of any systemic condition should quantify the inflammatory burden posed by PD. For this reason, a new classification was developed, namely the periodontal inflamed surface area (PISA), which is based not only on linear measures such as CAL and gingival recession (GR) but also on a sign of PD activity [i.e., bleeding on probing (BoP)]. Thus, PISA reflects the surface area of bleeding pocket epithelium in square millimetres (mm^2) (Nesse et al. 2008). Furthermore, recent evidence suggests that PISA could be a powerful tool to identify patients with active PD that were classified according to the CDC-AAP classification (Leira et al. 2018).

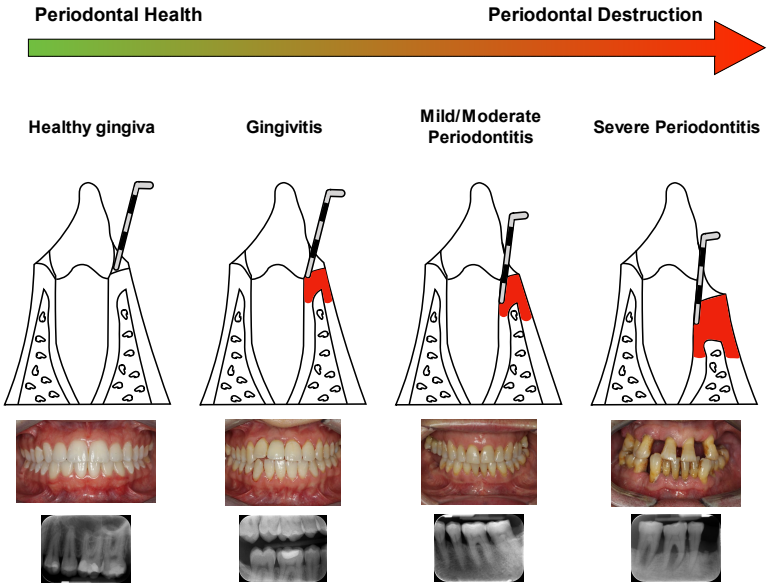

Figure 1. Schematics of healthy gingiva, gingivitis and PD.

1.2. Epidemiology

PD has an estimated prevalence ranging from 20% to 50% in the adult general population (Albandar and Rams 2002). In 2010, severe PD was the sixth-most prevalent condition, affecting 10.8% or 743 million people worldwide (Kassebaum et al. 2014). In terms of incidence, 701 cases per 100000 person-years were affected by this disease (Kassebaum et al. 2014). In Europe, whilst loss of alveolar bone and periodontal attachment is relatively common, the number of cases with severe PD is low (Sheiham and Netuveli 2002). Results from a population-based study namely the Study of Health in Pomerania (Germany) carried out in 4310 adults showed that the prevalence of CAL $\geq$ 3 mm was 89.7% and PPDs $\geq$ 4 mm were prevalent in 69.7% of subjects (Holtfreter et al. 2009). According to the CDC-AAP classification (Page an Eke 2007), 33.3% of people had moderate PD and only half of the whole population was free of PD or presented mild forms of PD (Holtfreter et al. 2009). Regarding elderly population, the

nationally representative Health 2000 Survey in Finland studied 784 subjects aged ≥ 65 years (Syrjälä et al. 2010). Of these, 28% had one to three teeth with PPD ≥ 4 mm and only 31% presented no teeth with deepened periodontal pockets (Syrjälä et al. 2010). In Spain, a cross-sectional study performed among 5130 workers showed that 28.3% and 10.1% of subjects had moderate (PPD = 4-5 mm) and severe (PPD ≥ 6 mm) periodontal pockets, respectively (Carasol et al. 2016). Gingival health was found only in 5.4% of the sample. In people aged ≥ 65 years, the percentage of employed adults with periodontal pockets increased dramatically up to 65.1%.

1.3. Pathogenesis

The *current model* of PD pathogenesis is based on a circular interrelation between the bacterial biofilm and the host response (Meyle and Chapple 2015). Unlike the *classical model* (Page and Kornman 1997) in which the periodontopathogens are responsible for inducing inflammation, advances in knowledge helped to understand that inflammation may also contributes to biofilm function and structure.

In the *classical model*, antigens, various other virulence factors [e.g., lipopolysaccharide (LPS)] and, occasionally, invading bacteria, comprise the microbial challenge, and the host responds with an immediate inflammatory and immune response, which can influence the microbial challenge. As a result, enhanced production of cytokines, prostanoids, and other inflammatory mediators [e.g., complement activation products and matrix metalloproteinases (MMPs)] occurs due to the host response. This state is perpetuated and mediates connective tissue breakdown and alveolar bone loss. These chained events are influenced by factors (i.e., genetic and environmental) and the sum of them depicts the clinical picture of PD. Additionally, disease severity and progression affects the nature and magnitude of the microbial challenge (Page and Kornman 1997).

In the *current model*, some new concepts were included (Figure 2). In order to maintain a healthy gingiva (health-promoting biofilm), a

symbiotic relationship should exist between the microbiota and the host response. Accordingly, the host can provide key nutrients through the gingival crevicular fluid (GCF), and the proteins and peptides that are released by microorganisms elicit a proportionate and resolving host response (Marsh 2003; Van Dyke 2008). In case of biofilm accumulation, some periodontopathogens such as *Fusobacterium nucleatum* (*Fn*) that are capable of sensing and influencing their own environment via chemical signals start to emerge and evoke a stronger host response leading to the onset of gingival inflammation and increase the supply of certain nutrients (e.g. heme), which are key to the proliferation of periodontopathogens such as *Porphyromonas gingivalis* (*Pg*) (Kolenbrander et al. 2002). This process is called "incipient dysbiosis" and in non-susceptible subjects it does not progress beyond gingivitis. On contrary, in susceptible individuals, an inappropriate and excessive response occurs in the host, in which a great number of cytokines, reactive oxygen species (ROS) and MMPs are produced and along with their antagonist produce periodontal tissue breakdown. The so-called damage associated molecular peptides (DAMPs) are released and the inflammatory response is enhanced. Due to poor innate inflammation resolving response, the periodontal inflammatory lesion becomes chronic. In addition, some viruses are capable of creating dysregulation in the immune system. When the chronic inflammatory state is established, a rich nutritional environment is created for sustaining dysbiosis due to healing process and inflammation leading to a pathogenic biofilm. The periodontal lesion is dominated by plasma cells and polymorphonuclear neutrophils (PMNs). The latter is responsible for dysregulating chemotactic and microbial processes and, as a result, failing to release pro-resolving lipid mediators. At this phase, because the natural process of inflammatory resolution is inactive, disruption of the biofilm is warranted to an extent in which health-promoting microorganisms can re-establish themselves and try to reduce the inflammatory process. As a result of the interrelation between the health-promoting biofilm and the host response, a balanced well-regulated inflammatory immune repertoire is restored. Nevertheless, a great variety exists with regards to the degree of biofilm reduction necessary to achieve in order to

establish symbiosis. On the one side, there are patients at a low risk of developing PD (disease-resistant patients). On the other side, high-risk patients are those who develop immediately PD, even if only mild plaque accumulation is present but is enough to trigger a destructive host response and, subsequently, periodontal tissue damage (Meyle and Chapple 2015).

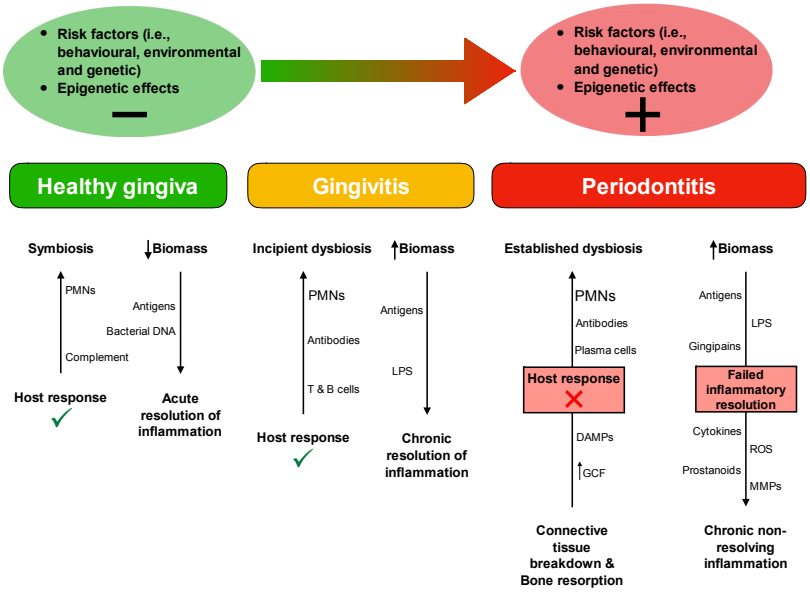

Figure 2. Current model of PD pathogenesis. Adapted from Meyle and Chapple (2015) and reproduced with permission from Wiley.

1.4. Risk factors

1.4.1. Aging

Epidemiologic studies showed more PD in older age groups as compared to younger groups (Grossi et al. 1994, 1995). Also, evidence demonstrated that plaque accumulation is more frequent and, as a result, more severe gingivitis could be observed in elderly people as compared to the younger ones, which suggest an age-related effect (Abdellatif and Burt 1987). However, PD could be more severe in the elderly due to

cumulative periodontal tissue destruction over a lifetime rather than an age-related issue affecting host susceptibility.

1.4.2. Gender

Male sex has been considered as one of the main risk factors for PD. Although being a man or a woman is genetically determined, no inherent difference between the two genders may exist in terms of susceptibility to PD (Genco and Borgnakke 2013). Nevertheless, gender-related lifestyle could be the responsible for the higher prevalence of PD in males in comparison to females in all groups of age, race/ethnic, and geographic locations (Grossi et al. 1994; Haas et al. 2012; Eke et al. 2012). Results from a nationally representative sample of non-institutionalized civilians in the United States (US) showed that men ha about 50% higher prevalence of PD (Eke et al. 2012). In fact, men had 180% more severe PD than women, with over half the men being affected (56.4%). In Spain, epidemiologic data demonstrated that males had more periodontal pockets compared with females (43.2% versus 31.6%) (Carasol et al. 2016).

1.4.3. Smoking

Since cigarette smoking was associated with acute necrotizing ulcerative gingivitis in the 50´s (Pindborg 1947), tobacco consumption has been studied as a risk factor for PD. Historically, the association between smoking and the presence of PD was controversial due to the fact that smokers had higher levels of plaque than non-smokers and, therefore, oral hygiene alone would account for the differences in the periodontal status between smokers and non-smokers. Nevertheless, it has been demonstrated that this statement was wrong because smoking was strongly associated with PD independent of plaque accumulation (Grossi et al. 1994, 1995). Currently, smoking is considered an important risk factor for PD. A meta-analysis of 2361 subjects showed that smoking was associated with the presence of PD, with an overall estimated odds ratio (OR) of 2.82 [95% confidence interval (CI): 2.36-3.39] (Papapanou 1996). The number of pack years of smoking was

positively correlated with the amount of attachment and bone loss, showing a clear dose-response (Grossi et al. 1994, 1995). Following non-surgical periodontal therapy, patients who smoked experienced less reduction in PPD than non-smokers (Labriola et al. 2005) and a causal relationship was found between poor periodontal wound healing and smoking habit (Heasman et al. 2006). If smoking cessation is implemented, periodontal patients will experience less progression of alveolar bone loss (Bolin et al. 1993) as well as a significant reduction in PPDs (Preshaw et al. 2005).

There are three hypotheses why cigarette smoking is detrimental to the periodontium. The first one is that smoking select for specific periodontopathogens [i.e., *Pg*, *Treponema denticola* (*Td*) and *Tannerella forsythia* (*Tf*)] leading to increased risk for development and progression of PD (Zambon et al. 1996). The second hypothesis suggests that smoking may result in peripheral vasoconstriction, probably associated with low doses of nicotine (Bergström et al. 2001; Morozumi et al. 2004). This fact leads to reduced gingival bleeding in smokers (Bergström et al. 2001) and less oxygen tension within the periodontal pocket and, thus, favours the overgrowth of *Pg* and *Td* (Genco and Borgnakke 2013). The third hypothesis is the impairment of neutrophil function by smoking via nicotine. This substance makes neutrophils more sensitive to bacterial challenge (Soder et al. 1999). Furthermore, nicotine inhibits the proliferation, chemotaxis, and attachment of fibroblasts from the periodontium leading to worse periodontal healing and regeneration (Cuff et al. 1989; James et al. 1999).

1.4.4. Diabetes

A two-year follow-up radiographic study among Pima Indians diagnosed with type 2 diabetes showed that poor glycaemic control could lead to both an increased risk for alveolar bone loss and more severe progression of PD than those without type 2 diabetes (Taylor et al. 1998). In addition, patients with a better control of the disease were less prone for bone-loss progression compared with patients who had

worse metabolic control (Taylor et al. 1998; Bandyopadhyay et al. 2010). What is more, it seems that diabetes precedes PD as it was observed an adjusted relative risk (RR) of incident PD of 2.6 (95% CI: 1.0-6.6) in patients with type 2 diabetes in comparison with non-diabetics (Nelson et al. 1990).

Pre-diabetes has also been associated with PD. In a case-control study, matched by age and sex, people with pre-diabetes (measured as impaired blood glucose levels) had moderate PD (Löesche et al. 2000). Cross-sectional data showed that the prevalence of subjects with alveolar bone loss ≥ 6 mm was significantly higher in those with impaired fasting glucose than among individuals without it (Zadik et al. 2010). Similarly, women with a history of gestational diabetes may be at a higher risk of having PD. In fact, results from the National Health and Nutrition Examination Survey (NHANES) carried out in the US population demonstrated that the prevalence of PD was significantly higher in women with a history of gestational diabetes compared to those without this condition (30.5% versus 4.8%) (Novak et al. 2006).

The biological mechanisms through which diabetes is associated with PD are mainly based on the inflammatory process. Inflammation is a central feature of both diseases. In periodontal tissues of diabetics, the inflammatory processes are up-regulated. Interleukin-1β (IL-1β) and prostaglandin E_2 (PGE_2) levels measures in GCF are higher in patients with diabetes than in non-diabetics with the same periodontal condition (Salvi et al. 1997a). Monocytes from patients with diabetes enhance the production of tumor necrosis factor-α (TNF-α), IL-1β and PGE_2 compared with those free of diabetes (Salvi et al. 1997a, 1997b; Engebretson et al. 2004). Additionally, elevated systemic inflammatory markers were found in diabetics (Dandona et al. 2004). There is another hypothesis suggesting that diabetes is responsible for a hyperreactive inflammatory response to the bacterial challenge and, thus, could enhance severity of PD. Accordingly, gingival mice tissues with experimental diabetes showed increased vascular permeability and impaired neutrophil chemotaxis, both of which can lead to worse

periodontal status given a similar bacterial challenge (Gyurko et al. 2006; Sima et al. 2010).

1.4.5. Obesity and metabolic syndrome

Several studies over the last decade showed a relationship between overweight/obesity and PD. A meta-analysis including mostly cross-sectional and case-control studies, demonstrated that the OR of having PD if an individual was either obese or overweight were 1.81 (95% CI: 1.42-2.30) and 1.27 (95% CI: 1.06-1.51), respectively, compared to those subjects with a normal body mass index (BMI) (Suvan et al. 2011). It has been hypothesized that overgrowth of periodonto-pathogens such as *Tf* could occur in individuals with a healthy periodontium who are obese, putting them at a higher risk for the onset and progression of PD (Haffajee and Sokransky 2009). It is well known that an elevated inflammatory response may be seen in obese individuals due to the production of numerous inflammation-related factors by adipose tissue. Data from 1221 adults, those in the highest quartile of BMI had the highest levels of TNF-α and soluble TNF-α receptors. Furthermore, a positive correlation was found between TNF-α levels with PD only in subjects included in the lowest quartile of BMI (Genco et al. 2005). Adipocytokines such as adiponectin or leptin measured in serum are elevated in patients with PD (Saito et al. 2008; Karthikeyan and Pradeep 2007). The latter, was suggested to be a pro-inflammatory mediator in the association between PD and acute myocardial infarct (Gundala et al. 2014) or chronic migraine (Leira et al. 2017a). Experimental data demonstrated that systemic low-grade inflammation along with elevated gene expression for TNF-α and C-reactive protein (CRP) in obese animals might lead obese rats to be more prone to develop PD (Endo et al. 2010). Additionally, it has been shown that after uncontrolled *Pg* infection in obese animals could predispose to increased alveolar bone loss via impaired immune response (Amar et al. 2007).

Regarding metabolic syndrome, a secondary analysis of the NHANES III showed that females that had 3 or more metabolic

syndrome components were at a 2-fold increased risk for developing PD (OR=2.1, 95% CI: 1.2-3.7). Moreover, it seems that abdominal obesity appeared to be the most significant contributor in this association (Andriankaja et al. 2010). This could be explained because there is an enhanced chronic systemic inflammatory response in subjects who have some metabolic syndrome components leading to impaired immunopathologic response to periodontopathogens and, as a result, leading to greater periodontal tissue destruction (Genco and Borgnakke 2013).

1.4.6. Osteoporosis, dietary calcium and vitamin D

A systematic review that included 35 studies showed an association between systemic osteoporosis and the presence of PD based on radiological parameters (e.g. alveolar bone loss or alveolar crest height). Nevertheless, when PD was assessed by clinical parameters (e.g., periodontal attachment loss), results were controversial (Martínez-Maestre et al. 2010). Results from the NHANES III revealed that subjects, especially women, with a low dietary calcium intake (< half of the recommended dietary allowance) had more severe PD (Nishida et al. 2000). In addition, a clinical trial demonstrated that individuals receiving periodontal maintenance therapy who took calcium and vitamin D supplements tend to have a better periodontal condition than those non-supplement takers (Miley et al. 2009).

1.4.7. Stress

A positive relationship was found between psychosocial stress status and PD. Accordingly, the more severe the stress in patients, the greater level of PD) (Peruzzo et al. 2007). A cross-sectional analysis showed that financial strain, which is a measure of chronic stress, was associated with a greater severity of PD measures either as clinical attachment loss or bone loss (Genco et al. 1999). However, subjects with the ability to cope with stressful/traumatic life events are less prone to develop more severe PD (Hugoson et al. 2002). Based on this, results

from a 2-year follow-up clinical trial demonstrated that patients with active coping behaviour had a milder disease levels than those with passive coping behaviour strategies (Wimmer et al. 2005). In terms of biological plausibility, higher cortisol levels were positively associated with more severe PD (Hilgert et al. 2006). Moreover, salivary cortisol was a predictor of periodontal attachment loss ≥ 5 mm (Rai et al. 2011; Rosania et al. 2009). On the other hand, stress can have a harmful effect on behaviour. As a result, subjects with stress may show poor oral hygiene, increased tobacco consumption, irregular dental check-ups as well as dietary changes, thus, worsening periodontal conditions.

1.4.8. Genetics

It is believed that some genes could modify PD. Genetics factors such as gene-gene interactions or gene-environmental interactions (i.e., epigenetic factors) may also be an important issue in the development of PD. It has been shown that familial aggregation of aggressive forms of PD is often high among certain families (40-50%) (Meng et al. 2001). A Brazilian study showed familial aggregation of PD in three generations of families (Rapp et al. 2011). Classical studies in twins showed that a substantial part of chronic PD might be attributed to genetics (Michalowicz et al. 1991, 2000). On contrary, a lack of correlation between both mono and dizygotic twins and clinical attachment loss and bone loss was found (Torres de Heens et al. 2010). Hence, in chronic forms of PD, the genetic background is not clear and remains controversial.

Regarding gene polymorphisms, up to date, there is no polymorphism that can be considered as a risk factor for PD. However, there is some interesting data in relation to IL-1 polymorphisms, in which it was found that IL-1A and IL-1B genetic variations were significant contributors to chronic PD, mainly in Caucasians (Karimbux et al. 2012).

Evidence from an emerging brand of periodontal research, so-called epigenetics, hypothesized that the methylation status of genes

affecting PGE_2 levels might be changed in periodontally-affected tissues, which suggests an epigenetic contribution to the inflammatory response posed by PD (Zhang et al. 2010).

1.5. Periodontal disease and atherosclerosis

1.5.1. Overview of periodontal disease as a chronic low-grade inflammatory condition

Besides the localized nature of PD, a plethora of systemic markers of this condition have been reported and speculated to contribute to systemic diseases (Loos 2005). In periodontal health, the epithelial barrier in the oral cavity along with the protective innate immune molecules inhibits periodontopathogens from entering into the periodontal tissues and the bloodstream. Hence, in a healthy gingiva, only small counts of bacteria (mostly facultative) enter into the circulation (Li et al. 2000). When the periodontal infection is present within the gingiva, it is hypothesized that the inflamed and ulcerated subgingival pocket epithelium provides an easy entrance for periodontal bacteria, many of which are gram-negative anaerobic. Bacteraemia may occur in PD, as periodontopathogens are capable of colonizing distant sites (Haraszthy et al. 2000). In addition, bacterial components such as LPS may also be disseminated into the bloodstream. These LPS together with bacterial antigens can trigger significant systemic inflammatory processes. Accordingly, white blood cells (e.g. PMNs) and acute-phase reactants (APRs) from endothelial cells (ECs) and hepatocytes may produce pro-inflammatory mediators. Moreover, locally produced pro-inflammatory molecules (i.e., IL-1β, TNF-α, IL-6, and PGE_2) are dumped into the systemic circulation and exert effects on distant organ systems (Moutsopoulos and Madianos 2006). Therefore, PD elicits a low-grade systemic inflammatory state.

1.5.2. Inflammatory mechanisms

1.5.2.1. Pro-inflammatory state

Several inflammatory biomarkers are present in elevated levels in the systemic circulation of periodontal patients than those without PD. It is hypothesized that pro-inflammatory cytokines and other markers are produced in the periodontal lesion (Preshaw and Taylor 2011). These mediators may be dumped into the bloodstream. If this happens and the biomarkers reach a level in which bioactivity can be preserved, they would impact tissues and distant organs. For example, they can affect the liver leading to an acute-phase response that would impact other organs. As a result, a number of chained events take place such as inflammatory changes in the endothelium, up-regulation of vascular adhesion molecules, promotion of cytokine production and, finally, initiation and acceleration of atheroma development (Schenkein and Loos 2013). As stated before, periodontal patients have frequent bacteremic episodes with LPS being present in the systemic circulation. Additionally, experimental data demonstrated that *Pg* infection could promote inflammatory responses in distant organs from the oral cavity (i.e., atheroma) (Gibson et al. 2006; Gibson and Genco 2007). A meta-analysis of 702 patients with PD and 902 non-PD subjects showed a statistically significant weighted mean difference (WMD) between groups of 1.65 mg/L (95% CI: 1.05-2.24) in serum/plasma CRP levels, which is an APR produced in the liver in response to, among other cytokines, IL-6 (Paraskevas et al. 2008). In addition, it has been shown that periodontal treatment might reduce the levels of CRP or IL-6 (D'Aiuto et al. 2013).

There is also evidence that MMPs, which play a pivotal role in both periodontal destruction (Meyle and Chapple 2015) and atherosclerotic plaque rupture, and can be induced by oral bacterial products (Hajishengallis et al. 2002). Gingipains, which are *Pg* proteases, can stimulate the production of MMPs as well as activate these markers when they are latent (Inamura et al. 2003). It has been demonstrated that high plasma MMP concentrations, especially MMP-9, in the acute

phase of a cerebral infarct is a strong predictor of hemorrhagic transformation in ischemic stroke through blood-brain barrier (BBB) disruption (Castellanos et al. 2003; Castillo et al. 2004).

1.5.2.2. Pro-thrombotic state

Increased fibrinogen is an indicator of systemic inflammation and it is considered to be a risk marker for atherosclerosis. Indeed, fibrinogen can increase blood viscosity and shear stress, thus, promoting EC activation and platelet aggregation. Moreover, this marker and its degradation products can be found in atheromas evoking cytokines production. It has been demonstrated that the number of periodontal pockets (> 15) was independently associated with the levels of fibrinogen (Schwahn et al. 2004) and, if PD is treated, a significant reduction in fibrinogen levels could be observed (Hussain Bokhari et al. 2009).

Other thrombotic marker, plasminogen-activator inhibitor-1 (PAI-1), decreases fibrinolysis by inhibiting the tissue plasminogen-activator (tPA). This property confers PAI-1 an important role in atherosclerosis. Levels of PAI-1 were increased in patients with severe PD, suggesting that when PD is present, a pro-thrombotic state could be found (Bizzarro et al. 2007).

1.5.2.3. Immune system activation

In some cases, periodontal patients show increased systemic antibody response to numerous periodontopathogens and some of them can induce cross-reactive antibodies that are capable of elevating the atherosclerosis risk or may accelerate the atherogenic process via endothelial inflammation enhancement, lipids uptake, or by means of blocking protective molecules that have anti-atherogenic effects. Therefore, periodontal bacteria can induce inflammatory responses via induction of immunity. It has been demonstrated that *Pg* expresses heat-shock proteins (HSPs) such as HSP60 (Lu and McBride 1994). In patients with PD, HSP60 stimulates inflammatory cytokines from

macrophages via toll-like receptors (TLRs) and anti-*Pg* HSP60 is increased compared to non-PD subjects (Ueki et al. 2002). *Pg* HSP60 contains both B- and T-cell epitopes cross-reactive with HSP60 (Choi et al. 2004). What is more, T-cell lines derived from atherosclerosis plaques are cross-reactive between human HSP and bacterial HSP60 (Ford et al. 2005). Antibody levels to bacterial HSP60 and human HSP60 were found to be higher in periodontal patients than periodontally healthy individuals, and HSP60-specific T-cells were detected in both the bloodstream and in some atherosclerotic patients (Yamazaki et al. 2004). These data would support the hypothesis that bacterial HSP could induce immune responses that might promote atheroma inflammation.

1.5.2.4. Increased cholesterol biosynthesis

Infection can modify serum lipids physiological properties. With regards to PD, plasmatic LPS together with acute-phase responses to systemic bacteria dissemination might promote enhanced cholesterol biosynthesis in the liver and it is transported as serum lipids capable of binding to LPS from oral bacteria. Based on this, periodontal infections both promote dyslipidemia and interact with serum lipids in order to potentiate their atherogenicity (Schenkein and Loos 2013).

While serum levels of inflammatory lipids such as high-density lipoproteins (HDLs) are decreased in periodontal patients (Nibali et al. 2007; Monteiro et al. 2009), low-density lipoproteins (LDLs) and triglycerides (TGs) are increased in these patients (Nibali et al. 2007; Monteiro et al. 2009). The latter lipid subforms, especially LDLs, can diffuse freely into the intimal layer of blood vessels and be recognizable by cellular receptors on phagocytes via either oxidative or proteolytic mechanisms. Subendothelial macrophages can become foam cells during the early stages of atheroma formation. Macrophage activation by release of inflammatory mediators may stimulate endothelial cells in order to release monocyte chemotactic protein-1 (MCP-1) and, thus, up-regulating cell-surface receptors that are involved in further monocyte recruitment into atherosclerotic lesions (Schenkein and Loos 2013).

However, periodontal therapy did not show a significant reduction on the levels of total cholesterol, LDLs or TGs (D'Aiuto et al. 2013).

1.5.3. Infectious mechanisms

Although the first report regarding host cells invasion by oral bacteria was carried out with *Aggregatibacter actinomycetemcomitans* (*Aa*) and carcinoma cells (Meyer et al. 1991), *Pg* has been extensively studied as a model of atherosclerosis organism. *Pg* invasion of host cells starts with adherence mediated by a great variety of cell-surface adhesins such as the major fimbriae (FimA) for ECs (Deshpande et al. 1998) and macrophages (Hajishengallis et al. 2006), gingipains (Amano 2010) or hemagglutinins (Kozarov et al. 1998). In macrophages, *Pg* internalizes through TLR-2 and β2integrin receptor complex signalling (Harokapakis et al. 2006). A relationship was found between elevated number of repeated domains in hemagglutinin A (HagA) and the invasive potential of *Pg* (Kozarov et al. 1998). Nevertheless, not only adhesins provide a mean by which *Pg* can invade ECs but also other periodontal bacteria such as *Fn* (Saito et al. 2008). It has been suggested that *Pg* induces monocyte migration enhancing the production of pro-inflammatory cytokines (Pollreisz et al. 2010). This periodontal bacterium is also capable of inducing pro-coagulant effects in human aortic ECs (Roth et al. 2006) leading to apoptosis of mononuclear cell adhesion to ECs (Roth et al. 2007a,b). Furthermore, it can be transmitted from one vascular cell to another as atherosclerosis progresses (Li et al 2008) and it can be reactivated after the internalization process (Rafferty et al. 2011).

A pathogenic model was proposed to elucidate why periodontal bacteria can be found in atheromas (Reyes et al. 2013). They suggest that oral bacteria derived from bacteraemia invade the endothelial layer and further spread into deeper tissue. Infected endothelium activation results in the release of pro-inflammatory chemokines such as MCP-1 within the lumen promoting blood monocytes and macrophages activation via their adhesion and diapedesis. Additionally, transmigrated leukocytes can contain viable bacteria. These pathogens can adhere to ECs, enter and

usurp the EC process for trafficking. At this point, these bacteria become uncultivable. However, their internalization via phagocytes or interactions with uninfected cells can reactivate them. Atheromas can grow due to macrophage-secreted growth factors-mediated smooth muscle cell (SMC) proliferation. Bacteria are also released upon host cell death, thus, re-infecting further cells.

1.5.4. Periodontal disease and endothelial dysfunction

The ECs maintain a balance between factors related to both vasodilation and vasoconstriction, and they are considered to play a significant role in the control of vascular tone (Endemann and Schiffrin 2004). When a disturbance in this balance occurs, an impaired endothelium-dependent vasodilation (EDV) is observed. It is believed that from ECs and macrophages and nitrogen species such as nitric oxide (NO) combine together restricting its bioactivity (Kvietys and Granger 2012). When ROS reacts with NO forms a potent oxidant namely peroxynitrite, which oxidizes a cofactor associated with the regulation of the levels of endothelial nitric oxide synthase (eNOS) within the ECs (Verma et al. 2000). This may block the vasodilatory action of NO. Furthermore, ROS up-regulate adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and MCP-1 (Endemann and Schiffrin 2004). In addition, endothelin-1 (ET-1) also reduces NO production (Münzel et al. 2008; Levine et al. 2012). As stated previously, PD elicits a state of low-grade systemic inflammation. If this systemic inflammatory state is sustained chronically, it might culminate in endothelial dysfunction through several mechanisms such as reduction of NO bioavailability, diminution of NO production or an increased inactivation of NO (Gurav 2014). For example, pro-inflammatory mediators expressed in PD (i.e., TNF-α and IL-6) reduce the production of eNOS in ECs, which is responsible for the production of NO (Huang and Vita 2006). Currently, one of the main non-invasive techniques to measure EDV is flow-mediated dilation (FMD) of the brachial artery. A meta-analysis showed that in periodontal patients (n=155), FMD was significantly lower than those without PD (n=136) (WMD=5.1%, 95% CI: 2.08-8.11)

(Orlandi et al. 2014). Furthermore, another quantitative analysis from three studies demonstrated that periodontal therapy significantly improved FMD (WMD=6.64%, 95% CI: 2.83-10.44) (Orlandi et al. 2014).

1.5.5. Periodontal disease and subclinical atherosclerosis

The intima-media wall thickness (IMT) of the carotid artery measured by B-Mode ultrasound is considered to be a useful non-invasive marker of preclinical atherosclerosis. A cross-sectional analysis from the Atherosclerosis Risk in Communities (ARIC) study showed that severe PD was associated with an IMT ≥ 1 mm (Beck et al. 2001). Moreover, a positive relationship was found between IMT and cumulative periodontal bacterial burden (Desvarieux et al. 2005). Results from a meta-analysis that included sixteen studies demonstrated that patients with PD have significantly higher carotid IMT values than periodontally healthy subjects (WMD=0.08 mm, 95% CI: 0.07-0.09) (Orlandi et al. 2014).

2. CEREBROVASCULAR DISEASE

2.1. Definition of stroke

According to the World Health Organization (WHO), stroke is defined as a rapidly developed clinical signs of focal (or global) disturbance of cerebral function, with symptoms lasting more than 24 hours or leading to death, with no apparent cause other than of vascular origin (Aho et al. 1980). In terms of pathophysiology, stroke can be divided into ischemic and hemorrhagic. Whilst ischemic stroke is caused by blood vessel obstruction, hemorrhagic stroke is the result of a blood vessel rupture or an abnormal vascular structure to the brain parenchyma or to the subarachnoid space. In both types of strokes, this cerebral blood flow interruption causes oxygen and nutrients deprivation to the surrounding cerebral tissues. Long-term deprivation may lead to both cell damage and death; thus, resulting in function loss

of the neural tissue that is clinically detectable in the form of motor dysfunction as well as typical neuroimaging findings are observed.

2.2. Epidemiology of stroke

Stroke is the second most common cause of mortality worldwide and it is the third leading cause of adult disability (Benjamin et al. 2018). Globally in 2013, there were almost 25.7 million stroke survivors (71% with ischemic stroke), 6.5 million deaths from stroke (51% from ischemic stroke), 113 million disability-adjusted life years (DALYs) due to stroke (58% due to ischemic stroke), and 10.3 million new strokes (67% ischemic) (Feigin et al. 2015).

In Spain, results from the IBERICTUS study showed that of the 2500 patients with a first episode of acute cerebrovascular disease, approximately 80% were ischemic strokes. There was an 11.6% of in-hospital mortality due to stroke. In addition, the unadjusted annual incidence rate of first-ever stroke was 187/100000 (95% CI: 180-194), being higher in men than for women (Díaz-Guzmán et al. 2012). Findings from another Spanish epidemiologic study demonstrated a 1-year mortality rate after stroke of 18.3% and almost 30% of patients have some kind of disability 1 year after stroke (Mar et al. 2015).

In terms of economics, data from the US revealed that the direct and indirect cost of stroke was $40.1 billion. The mean expense per patient for direct care for any type of service in the US was estimated at $6574. It is expected that between 2015 and 2035, the total direct medical stroke-related costs will be ranging from $36.7 billion to $94.3 billion, with much of the projected increase in costs arising from those patients 80 years (Benjamin et al. 2018).

2.3. Classification of stroke

As stated before, stroke can be divided into ischemic and hemorrhagic stroke. In turn, the latter can be classified in intracerebral hemorrhage and subarachnoid hemorrhage. Regarding cerebral ischemia,

two main groups exist that are brain infarction and transient ischemic attack (TIA). While brain infarct is defined as a qualitative or quantitative of the blood supply to a part of the brain with a neurologic dysfunction lasting > 24 hours due to cell death, TIA is a transient episode of neurologic dysfunction caused by ischemia (loss of blood supply), without acute infarction lasting < 24 hours.

From a clinical point of view, the most widely used classification for ischemic stroke is the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification system, which is based on the ischemic stroke etiology (Adams et al. 1993). The TOAST criteria includes the following categories:

- Large-artery atherosclerosis: these patients will have clinical and brain imaging findings of either significant (> 50%) stenosis or occlusion of a major brain artery or branch cortical artery, presumably due to atherosclerosis. Diagnostic studies should exclude potential sources of cardiogenic embolism.
- Cardioembolism: This category includes patients with arterial occlusions presumably due to an embolus arising in the heart (i.e., mechanical prosthetic valve, atrial fibrillation, atrial thrombus, sick sinus syndrome, recent myocardial infarction, left ventricular thrombus, dilated cardiomyopathy, akinetic left ventricular segment, atrial myxoma, and infective endocarditis). Potential large-artery atherosclerotic sources of thrombosis should be eliminated.
- **Small-artery occlusion:** Patients included in this category are those whose strokes are often labelled as lacunar infarcts (LIs) in other classifications (Bamford et al. 1987). The patient should have one of the traditional clinical lacunar syndromes and should not have evidence of cerebral cortical dysfunction. A history of diabetes mellitus or hypertension supports the clinical diagnosis. The patient should also have a normal computed tomography/magnetic resonance imaging (CT/MRI) examination or a relevant brain stem or subcortical hemispheric lesion with a diameter < 1.5 cm demonstrated. Potential cardiac

sources for embolism should be absent, and evaluation of the large extracranial arteries should not demonstrate a stenosis > 50% in an ipsilateral artery.

- Acute stroke of other determined etiology: This category includes patients with rare causes of stroke, such as non-atherosclerotic vasculopathies, hypercoagulable states, or hematologic disorders. These patients should have clinical and CT or MRI findings of an acute ischemic stroke, regardless of the size or location. Diagnostic studies should reveal one of these unusual causes of stroke. Cardiac sources of embolism and large-artery atherosclerosis should be excluded by other studies.
- Stroke of undetermined etiology: Some patients will have no likely etiology determined despite an extensive evaluation. In others, no cause is found but the evaluation was cursory. This category also includes patients with two or more potential causes of stroke so that the physician in unable to make a final diagnosis.

3. CEREBRAL SMALL VESSEL DISEASE

3.1. Definition

The term cerebral small vessel disease (CSVD) refers to a syndrome of clinical and imaging findings that are suggested to result from pathologies in perforating cerebral arterioles, capillaries and venules. CSVD is responsible for almost 45% of dementia, and accounts for about 20% of all stroke worldwide, 25% of ischemic strokes (or lacunar strokes), and about 20% of these cases present some level of disability (Pantoni 2010). CSVD includes small subcortical infarcts or LIs, silent infarcts (SIs), leukoaraiosis (white matter hyperintensities), prominent perivascular spaces (PVS) or Virchow-Robin spaces, and cerebral microbleeds (CMBs) (Figure 3). Normally, LIs cause acute stroke symptoms, while other CSVD lesions are clinically more insidious and, therefore, are referred to as “silent lesions”.

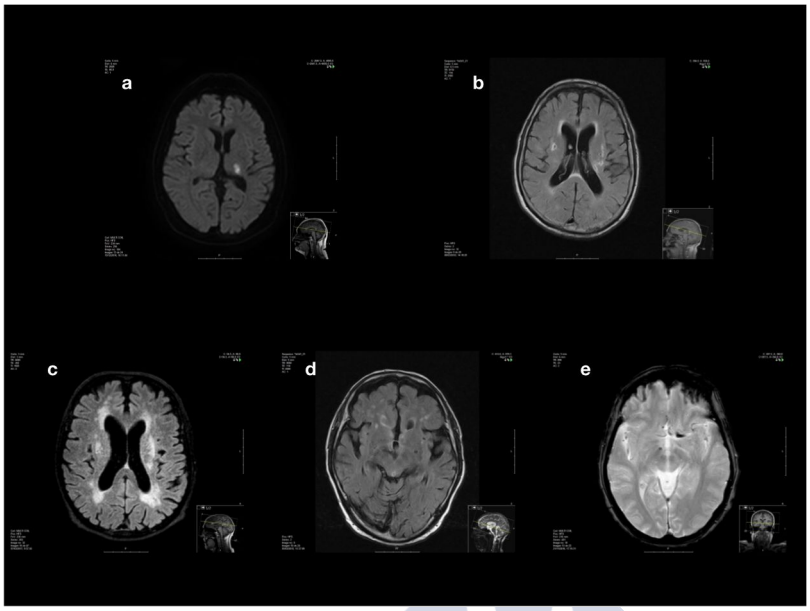

Figure 3. Neuroimaging examples of different types of CSVD. a: LI. b: SI. c: Leukoaraiosis. d: PVS. e: CMBs.

3.2. Subtypes

3.2.1. Lacunar infarct

Ischemic LI is defined as a stroke within a small deep perforating artery that is attributable to a recent small infarct (< 1.5 cm diameter) in the white matter, basal ganglia, pons or brainstem, and is consistent with a lacunar clinical syndrome (Wardlaw et al. 2013a).

The main causes of LIs are atheroma of parent arteries or perforating arterioles, embolism from the heart or carotid arteries, and lipohyalinosis or fibrinoid necrosis (Bailey et al. 2012). Atheroma in the middle cerebral artery is responsible for almost 20% of LI cases. Evidence regarding embolism as a common cause for LI is limited (< 10% of LI cases). Although the exact mechanism remains unclear, intrinsic CSVD such as lipohyalinosis (referred to hyaline deposition in the perforating arteries together with diffuse arteriopathy) is considered

as the most common cause of LI (Figure 4). In this sense, it has been suggested that endothelial dysfunction is present in the pathogenesis of atherothrombotic neurovascular disease such as LI (Blanco et al. 2005; Knottnerus et al. 2009). In a group of patients with different types of ischemic stroke, impaired FMD was more conspicuous in LI patients than those diagnosed with other types of ischemic stroke (Chen et al. 2006); and patients diagnosed with LI had a diminished FMD compared to healthy controls and to those with similar vascular risk factors (Pretnar-Oblak et al. 2006). In addition, it was found that patients with LI had significantly higher serum levels of ICAM-1 than controls (Hassan et al. 2003).

Risk factors for LI may include aging, diabetes mellitus or hypertension (Bezerra et al. 2012; Mast et al. 1995), and they are also considered to be predictors of poor outcome in these patients (Norrving 2003; Blanco et al. 2006). Moreover, there is an association between increased inflammatory response and early neurologic worsen in subjects with LI, thus, elevated concentrations of inflammatory markers such as IL-6 or TNF-α are related also to poor prognosis (Castellanos et al. 2002).

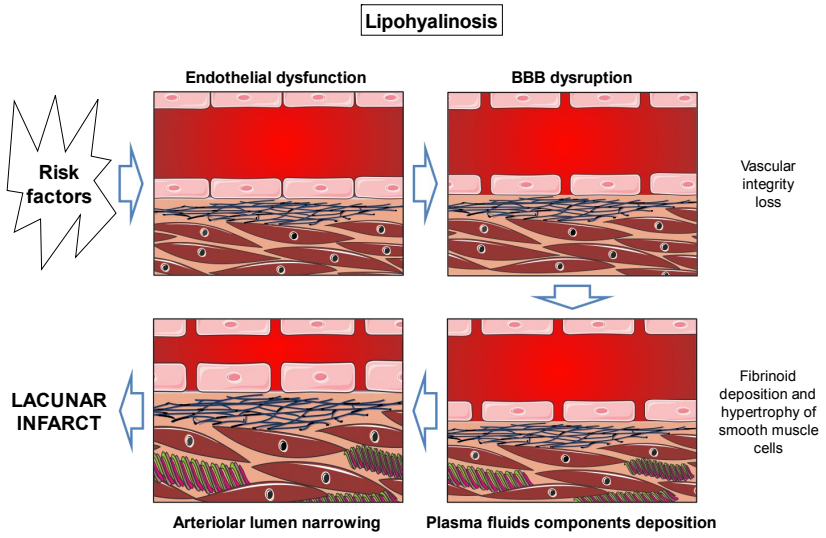

Figure 4. Schematics of LI physiopathology.

3.2.2. Silent infarcts

SIs of presumed vascular origin are round or ovoid, subcortical, fluid-filled cavities with a diameter ranging from 3 to 15 mm. SIs can occur without any prior symptoms, but can also be the result of a previous acute small subcortical infarct (i.e., LI) or hemorrhage (Wardlaw et al. 2013b). At fluid-attenuated inversion recovery (FLAIR) MRI, a SI is usually presented as a hypointense hole sometimes surrounded by a hyperintense rim. Although SIs might have lacked acute symptoms, when present in large numbers they are related to dementia, cognitive impairment and increased risk of stroke (Vermeer et al. 2003, 2007).

Results from the Cardiovascular Health Study showed that SIs are much more prevalent than LIs and they tend to be associated with ageing and high systolic blood pressure (Longstreth et al. 1998). In addition to these variables, basal carotid atheromatosis or low HDL were found to be associated with incident SIs (van Dijk et al. 2008; Gouw et al. 2008).

3.2.3. Leukoaraiosis (white matter hyperintensities)

Leukoaraiosis of presumed vascular origin are very common in older subjects and regarded as typical signs of CSVD. Symptoms of leukoaraiosis develop insidiously, such as cognitive impairment, dementia and depression (Pantoni 2010; Jiménez et al. 2008), but it increases near 3-fold the risk for developing stroke, 2-fold the risk for developing dementia and is also associated with a higher risk of overall death (Debette and Markus 2010). Leukoaraiosis are usually symmetrically and bilaterally distributed in the white matter. Examination by MRI shows these lesions as hyperintense in relation to the normal brain, and can be patchy or confluent depending on their severity.

Mechanisms explaining leukoaraiosis may include chronic partial ischemia secondary to diffuse atherosclerosis, hypotensive episodes

(e.g., cardiac arrhythmia or postural hypointension) (McQuinn and O´Leary 1987; Sulkava and Erkinjuntti 1987), breakage of the BBB, leakage of toxic fluids into the white matter (Pantoni et al. 1993), SIs (Conklin et al. 2014), and venous collagenosis (Moody et al. 1995).

Besides ageing, several studies showed an association between hypertension and leukoaraiosis (Liao et al. 1997; de Leeuw et al. 2002; Dufouil et al. 2001) and that effective hypertensive therapy was related with lower risk for developing this condition (de Leeuw et al. 2002; Dufouil et al. 2001). With regards to risk factors for progression of leukoaraiosis, again ageing and hypertension along with tobacco consumption were predictors of leukoaraiosis progression (van Dijk et al. 2008). Furthermore, ICAM-1 levels were associated with progression of leukoaraiosis, further supporting a potential causal role of endothelial activation in the pathogenesis of leukoaraiosis (Markus et al. 2005).

3.2.4. Prominent perivascular spaces or Virchow-Robin spaces

PVS are the extension of subarachnoid spaces that surround cerebral microvessels (Braffman et al. 1988). When enlarged, are commonly seen as hyperintense on T_2 MRI either punctuate or linear depending on how the image is situated with respect to the course of the vessel and with a diameter < 3 mm (Potter et al. 2015), but sometimes can be larger (Hernández-Mdel et al. 2013). Usually, PVS do not ha hyperintense rim on T_2-weighted (T_2-w) or FLAIR unless passing through a white matter hyperintensity area, which can help to discriminate between PVS and SIs.

It is suggested that more PVS could be associated with hypertension, cognitive decline and increased risk of dementia (Hernández-Mdel et al. 2013; Zhu et al. 2010; Maclullich et al. 2004). Although it has been hypothesized that impaired BBB or blockage of drainage of interstitial fluid might be responsible for enlarged PVS, the exact mechanisms underlying the physiopathology of prominent PVS remains unclear (Wardlaw et al. 2009; Weller et al. 2009).

3.2.5. Cerebral microbleeds

CMBs are small perivascular hemosiderin deposits (usually with macrophages), which presumably result from leakage through cerebral small vessels that can be visualized as small, rounded, homogeneous, and hypointense lesions on T_2-w (gradient-recalled echo) MRI or susceptibility-weighted imaging. Besides perivascular hemosiderin-laden macrophages, other pathological correlations with CMBs are old haematomas, intact erythrocytes, and microaneurysms (Shoamanesh et al. 2011). Lipofibrohyalinosis and amyloid angiopathy are the most common vascular findings in CMBs. Although most CMBs are asymptomatic, they can be associated with amyloid deposition due to its potential relationship with stroke and dementia (Cordonnier et al. 2007; Martínez-Ramirez et al. 2014).

Ageing and hypertension as well as diabetes are considered to be associated with both previous and incident cases of CMBs (Cordonnier et al. 2007, Poels et al. 2011).

4. PERIODONTAL DISEASE AS A RISK FACTOR FOR STROKE

There is plenty of evidence with regards to the association between PD and stroke (Table 2, 3, and 4). The vast majority of studies show a positive relationship between both diseases, but the lack of consensus regarding definitions of both diseases makes difficult to establish the accurate magnitude of the effect. Firstly, some studies include stroke as the outcome without discriminating between ischemic and hemorrhagic stroke (Morrison et al. 1999; Diouf et al. 2015; Lee et al. 2006; Beukers et al. 2017). This is a critical issue since hemorrhagic stroke has a different aetiology from cerebral ischemia and, thus, biologically is less feasible that PD could predispose to hemorrhage. In fact, when they are analysed separately, no association was found between PD and hemorrhagic stroke (Wu et al. 2000). Secondly, self-reported diagnosis of PD rather than clinical periodontal records can lead to bias when PD is defined (Howell et al. 2001; Joshipura et al. 2003; Yu et al. 2015). Similarly, stroke diagnosis should be based on CT/MRI examination and, several studies include self-reported measurements of stroke (Elter et al. 2003; Lee et al. 2006). As a result, meta-analyses available in this topic have similar

methodological problems (Janket et al. 2003; Khader et al. 2004; Sfyroeras et al. 2012; Lafon et al. 2014a). In order do avoid these issues, recently, a systematic review and meta-analysis was published with the aim to investigate the potential association between PD and ischemic stroke (Leira et al. 2017b). All the included studies (3 prospective cohort studies and 5 case-control studies) (Beck et al. 1999; Wu et al. 2000; Jimenez et al. 2009; Grau et al. 2004; Dörfer et al. 2004; Sim et al. 2008; Pradeep et al. 2010; Lafon et al. 2014b) defined PD with clinical measurements (i.e., CAL and PPD) and cerebral ischemia was based on reliable examinations (i.e., acute ischemic lesion on brain imaging and/or neurological deficit) and valid classifications such as the International Classification of Diseases (ICD) (Kokotailo and Hill 2005) and TOAST criteria (Adams et al. 1993). Results from a meta-analysis showed that, overall, patients with severe PD had 2.8-fold increased risk for developing ischemic stroke. If studies were analysed according to study design, the RR for cohort studies was 2.52 (95% CI: 1.77-3.58) and for case-control studies was slightly higher (RR=3.04; 95% CI: 1.10-8.43) (Figure 5). Therefore, it seems that subjects with severe PD are at a higher risk for having ischemic stroke.

First author, year	Type of study	Study population	Country	Stroke diagnosis	PD diagnosis	Follow-up (years)	Confounders adjusted	RR (95% CI)
Beck, 1996	Prospective	1,147 men	United States of America	Ischemic stroke: history and physical examination detecting sustained neurological consistent with cerebral thrombosis (ICD-8, 432-436)	Alveolar bone loss at interproximal tooth surfaces and worst clinical probing depth per tooth	25	Age, smoking, family history of heart disease, diastolic blood pressure, education (high school education or less) and type 2 diabetes	Whole mouth bone loss > 20%: 2.80 (1.45-5.48)
Morrison, 1999	Retrospective	10,120 subjects (35-84 years of age)	Canada	Fatal cerebrovascular disease (ICD-8, 430-438)	Obvious pockets and loose teeth	23	Age, serum total cholesterol, smoking status, diabetes, hypertensive status and province of residence	1.63 (0.72-3.67)
Wu, 2000	Prospective	9,962 subjects (25-74 years of age)	United States of America	Ischemic stroke (ICD-9, 433-434 and 436-438)	4 or more teeth with overt pockets or	10	Sex, age, race, education, poverty index, diabetes status,	Ischemic stroke (incident

				Hemorrhagic stroke (ICD-9, 430-432)	worse conditions		hypertension, smoking status, average alcohol use, BMI and serum cholesterol	events): 2.11 (1.30-3.42) Ischemic stroke (fatal events): 2.90 (1.49-5.62) Hemorrhagic stroke (incident events): 1.22 (0.53-2.83) Hemorrhagic stroke (fatal events): 1.12 (0.32-3.89)
Howell, 2001	Prospective	22,071 U.S. male physicians (40-84 years of age)	United States of America	Nonfatal stroke (typical neurological deficit, either sudden or rapid in onset, that lasted >24 h and was attributed to a cerebrovascular event)	Self-reported	12.3	Age, aspirin and beta-carotene treatment assignment, cigarette smoking, alcohol use, history of hypertension, BMI, reported history of diabetes, physical activity, parental history of myocardial infarct and angina	1.01 (0.81-1.27)
Joshipura, 2003	Prospective	41,380 male health professionals (40-75 years of age)	United States of America	Ischemic stroke (according to the National Survey of Stroke)	Self-reported	12	Age, smoking, alcohol consumptions, BMI, physical activity, family history of myocardial infarct, multivitamin supplement use, vitamin E use, history of hypertension, diabetes, hypercholesterole-mia and professions	1.33 (1.03-1.70)
Jimenez, 2009	Prospective	1,137 men	United States of America	Ischemic stroke: history and physical examination detecting sustained neurological consistent with	Radiographic alveolar bone loss and cumulative PPD	24	Age, BMI, HDL, total cholesterol, triglycerides, diagnosis of hypertension, mean systolic and diastolic blood, diabetes diagnosis,	Mean bone loss > 20%: 3.52 (1.59-7.81) Cumulative PPD > 30 mm:

				cerebral thrombosis (ICD-8, 432-436)			daily alcohol consumption, comprehensive smoking index, marital status, and baseline measures of education, occupation and income	1.07 (0.59-1.93)
Yu, 2015	Prospective	39,863 female healthcare professionals (≥ 45 years of age)	United States of America	Ischemic stroke: focal neurologic deficit of sudden onset that persisted > 24 h (clinical information, CT and MRI was used in order to distinguish hemorrhagic from ischemic events)	Self-reported	15.7	Age, race/ethnicity, BMI, education, smoking, diabetes, hypertension, hypercholesterolemia, family history of myocardial infarct and physical activities	Prevalent PD: 1.12 (0.91-1.37) Incident PD: 1.41 (1.02-1.95)
Sen, 2018	Prospective	6,736 dentates (45-64 years of age)	United States of America	Ischemic stroke (according to the National Survey of Stroke)	Periodontal profile classes (PPCs): PPC-A: periodontal health PPC-B: mild PD PPC-C: high gingival index score PPC-D: tooth loss PPC-E: posterior PD PPC-F: severe tooth loss PPC-G: severe PD	15	Age, sex, race/center, BMI, hypertension, diabetes mellitus, LDL, smoking, pack years, education	Incident ischemic stroke (overall): 2.06 (1.41-3.01) Cardioembolic subtype: 2.62 (1.22-5.63) Lacunar subtype: 1.34 (0.64-2.79) Thrombotic subtype: 2.18 (1.26-3.78)

Table 2. Summary of the most relevant cohort studies evaluating the association between PD and stroke.

First author, year	Study population	Country	Stroke diagnosis	PD diagnosis	Confounders adjusted	OR (95% CI)
Grau, 2004	303 hospitalized patients with acute cerebral ischemia, 300 population controls and 168 hospital controls (18-75 years of age)	Germany	Ischemic stroke (acute ischemic lesion on brain imaging and/or neurological deficits lasting >24 hours) or TIA (neurological deficit of <24 hours without new ischemic lesions). Furthermore, they used established criteria for etiologic stroke subtype analysis (atherothrombosis , cardioembolism, microangiopathy and cryptogenic)	Absence of PD: CAL ≤ 3 mm Mild PD: CAL 3 - ≤ 4.5 mm Moderate PD: CAL 4.5 - ≤ 6 mm Severe PD: CAL > 6 mm	Hypertension, diabetes, smoking (pack-years), previous stroke, father´s profession, dentist visits and number of teeth, stratified by sex and age	Mild PD: 1.48 (0.77-2.8) Moderate PD: 3.16 (1.52-6.6) Severe PD: 3.38 (1.35-8.4)
Dörfer, 2004	303 hospitalized patients with acute cerebral ischemia, 300 population controls (18-75 years of age)	Germany	Ischemic stroke (acute ischemic lesion on brain imaging and/or neurological deficits lasting >24 hours) or TIA (neurological deficit of <24 hours without new ischemic lesions)	Absence of PD: CAL ≤ 3 mm Mild PD: CAL 3 - ≤ 4.5 mm Moderate PD: CAL 4.5 - ≤ 6 mm Severe PD: CAL > 6 mm	Hypertension, diabetes, previous stroke, smoking, high lifetime alcohol consumption, atrial fibrillation, family history of stroke, and low childhood socioeconomic conditions, stratified by sex and age	Mild PD: 1.64 (0.73-4.39) Moderate PD: 4.82 (1.13-8.13) Severe PD: 7.38 (1.55-15.03)

Sim, 2008	265 non-fatal chronic strokes cases (118 ischemic cases, 143 hemorrhagic cases and 4 cases having both) and 214 non-stroke population controls (40-79 years of age)	Korea	Stroke (ischemic or hemorrhagic lesion using brain images taken by CT or MRI along with a comprehensive systemic examination)	Presence of deep PD: CAL ≥ 6 mm Mild PD: 0% - < 48.6% sites with CAL ≥ 5mm Moderate PD: 48.6% - < 73% sites with CAL ≥ 5mm Severe PD: ≥ 73% sites with CAL ≥ 5mm	Age, gender, daily toothbrushing ≥3, annual dental visit ≥1, number of missing teeth, duration of education ≥12 years, smoking, alcohol consumption, diabetes, hypertension, cardiac disease, BMI ≥25 kg/m^2, family history of hypertension, family history of diabetes, family history of cardiac disease, decayed, missing and filled teeth (DMFT) index and income ≥$1,000/month	Presence of deep PD: 3.97 (2.26-6.97) Moderate PD: 2.58 (1.39-4.81) Severe PD: 4.30 (2.27-8.16) Pooled for age subgroup (ischemic stroke): 4.28 (2.05-8.90)
Kim, 2010	118 patients diagnosed with hemorrhagic stroke and 214 healthy controls (40-79 years of age)	Korea	Hemorrhagic stroke (hemorrhagic brain lesions by CT and comprehensive systemic examinations)	Presence of deep PD: CAL ≥ 6 mm Mild PD: 0% - < 48.6% sites with CAL ≥ 5mm Moderate PD: 48.6% - < 73% sites with CAL ≥ 5mm Severe PD: ≥ 73% sites with CAL ≥ 5mm	Age, gender, frequency of toothbrushing, dentist visit, number of missing teeth, DMFT index, monthly income, education, smoking, alcohol consumption, hypertension, diabetes, cardiac	Presence of deep PD: 2.53 (1.14-5.61) Moderate PD: 1.88 (0.82-4.30) Severe PD: 1.72 (0.73-4.08)

					disease, BMI, family history of hypertension, family history of diabetes and family history of cardiac disease	
Pradeep, 2010	100 patients diagnosed with acute cerebral ischemia and 100 healthy controls (33-68 years of age)	India	Ischaemic stroke (acute ischemic lesion on brain imaging and/or neurological deficits lasting >24 hours) or TIA (neurological deficit of <24 hours without new ischemic lesions)	Absence/Mild PD: CAL ≤ 3mm Moderate PD: CAL > 3 - ≤ 4.5 mm Severe PD: CAL > 4.5 mm	Age, gender, diabetes, hypertension, cholesterol, smoking, alcohol, family history of stroke and education	Moderate PD: 1.5 (0.5-4.8) Severe PD: 2.4 (0.3-17.1)
Lafon, 2014	48 nonfatal ischemic stroke cases and 47 healthy controls	France	Ischemic stroke: rapid development of localized or global signs of brain dysfunction with symptoms lasting more than 24 h without any other apparent causes except those of vascular origin (ICD-10: I63. 0-9) and confirmed by MRI	Radiographic bone loss and % of sites with PPD > 5mm	Age, sex, school education level, tobacco consumption, diabetes, alcohol consumption, physical activity, hypercholesterolemia, DMFT>25, hypertension, coronary heart disease, CRP>5 mg/L and BMI>25	Bone loss > 20%: 1.05 (1.02-1.09) % of sites with PPD > 5 mm: 0.99 (0.92-1.08)
Diouf, 2015	100 patients diagnosed with stroke and 120 healthy controls	Senegal	Stroke diagnosis confirmed by cerebral scanner	CAL > 2 mm at least at two sites and PPD > 3 mm	Hypertension, physical activity and the interaction between PD and age	1.58 (1.10-3.02)

Leira, 2016	62 patients diagnosed with lacunar stroke and 60 healthy controls (58-71 years of age)	Spain	Lacunar stroke (presence of one of the characteristic clinical lacunar syndromes, neurological deficit lasting > 24 h, no evidence of cerebral cortical dysfunction, and a CT/MRI that was normal or showed a deep focal infarction in an appropriate location with a diameter ≤ 1.5 mm	Mild PD: ≥ 2 interproximal sites with CAL ≥ 3 mm and ≥ 2 interproximal sites with PPD ≥ 4 mm (not on the same tooth) or 1 site with PPD ≥ 5 mm Moderate PD: ≥ 2 interproximal sites with CAL ≥ 4 mm (not on the same tooth) or ≥ 2 interproximal sites with PPD ≥ 5 mm (also not on the same tooth) Severe PD: ≥ 2 interproximal sites with CAL ≥ 6 mm (not on the same tooth) and ≥ 1 interproximal sites with PPD ≥ 5 mm Total PD: mild + moderate + severe	Age, gender, hypertension, smoking habit, alcohol consumption, statin use and diabetes	Total PD: 4.05 (1.59-11.05) Severe PD: 3.53 (1.07-12.77)

Table 3. Summary of the most relevant case-control studies evaluating the association between PD and stroke.

First author, year	Study population	Country	Stroke diagnosis	PD diagnosis	Confounders adjusted	OR (95% CI)
Elter, 2003	6,436 subjects	United States of America	Self-reported stroke (ischemic or hemorrhagic) or TIA diagnosed by a physician, neurological deficits of rapid onset assessed by a structured interview	Extent of CAL 3+ mm (number of sites with CAL 3+ mm divided by the number of measured sites, times 100) Quartiles (Qs) of extent of CAL 3+ mm: -Q1: 0% - <6.5% -Q2: 6.5% - 15.4% -Q3: 15.4% - 31.4% -Q4: ≥ 31.4%	Age, sex, race/center, education, smoking status and intensity, hypertension, diabetes and prevalent coronary heart disease	Q2: 1.1 (0.9-1.5) Q3: 1.2 (0.9-1.5) Q4: 1.3 (1.02-1.7)
Lee, 2006	5,123 subjects	United States of America	Self-reported stroke	Cumulative PD (% of sites with CAL ≥ 2 mm and % of sites with CAL ≥ 3 mm)	Age and tobacco use	≥ 45% of sites with CAL ≥ 2 mm: 1.51 (0.96-2.38) ≥ 16.7% of sites with CAL ≥ 3 mm: 1.22 (0.78-1.91)
Beukers, 2017	60,174 subjects (>35 years of age)	The Netherlands	Stroke (medical health questionnaire of the patient)	≥ 1 diagnostic and treatment periodontal codes present	Age, sex, smoking, diabetes, hypertension, hypercholesterolemia and social economic status	1.55 (1.25-1.92)

Table 4. Summary of the most relevant cross-sectional studies evaluating the association between PD and stroke.

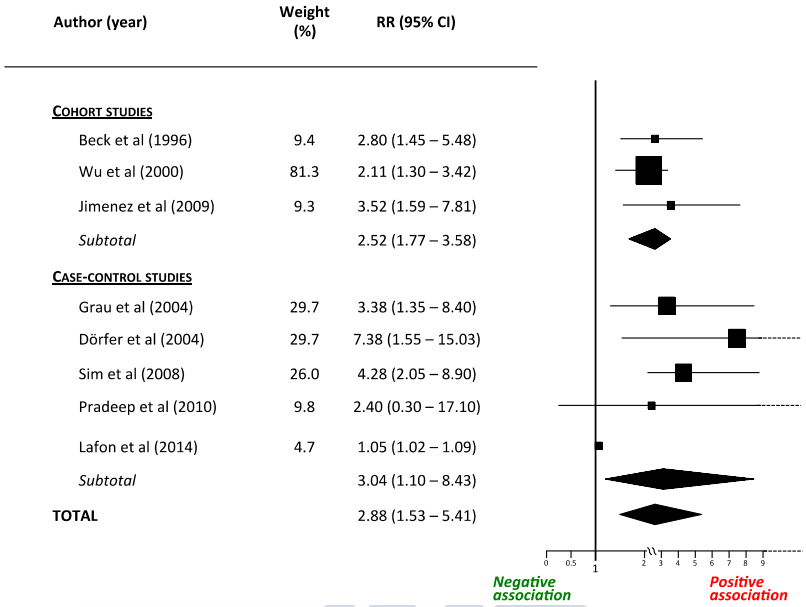

Figure 5. Meta-analyses of RRs for the association between severe PD and ischemic stroke. Adapted from Leira et al. (2017b) and reproduced with permission from Springer.

From a mechanistic point of view, similar pathways mentioned before (see section 1.5) may explain the association between PD and ischemic stroke including enhanced pro-inflammatory and pro-thrombotic state, innate immunity activation as well as promotion of dyslipidemia (Leira et al. 2015). It has been observed that patients with ischemic stroke that had more severe PD presented higher serum levels of IL-6 and s-ICAM as well as increased aortic arch atheroma plaque thickness and plaque calcification compared to those with less levels of PD (Sen et al. 2013, 2017). It was found that patients with a previous history of stroke were more often immunoglobulin (Ig)-A seropositive for *Pg* than those without a history of stroke. Furthermore, seropositive subjects were at double-risk for having a secondary stroke (Pussinen et al. 2004) and, systemic exposure to this periodontopathogen may predispose to incident cases of stroke (Pussinen et al. 2007). Additionally, serum antibody levels against another well-known periodontal pathogen [i.e., *Prevotella intermedia* (*Pi*)] were associated with ischemic atherothrombotic stroke through its correlation with

carotid artery atherosclerosis (Hosomi et al. 2012). Recently, sub-analysis from the ARIC study confirmed these results, in which it was concluded that PD is independently associated with incident ischemic stroke (Sen et al. 2018).

Regarding intervention studies, up to date, there is a lack of prospective clinical trials evaluating the effect of periodontal therapy in patients with stroke and surrogates markers of cerebral ischemia. However, results from retrospective analyses from the Taiwanese National Health Insurance (NIH) research database showed that dental prophylaxis and periodontal treatment was a protective factor for having incident ischemic stroke [Hazard ratio (HR)=0.79; 95% CI: 0.75-0.81 and HR=0.95; 95% CI: 0.91-0.99, respectively] (Lee et al. 2013). In addition, those subjects who received ≥ 1 tooth scaling within 2 years were less prone to develop a stroke (HR=0.81; 95% CI: 0.73-0.92) (Cheng et al. 2012).

5. BIOMARKERS

5.1. Systemic inflammation

5.1.1. Interleukin-6

IL-6 is a pluripotent cytokine with 26 kDa molecular mass, containing 185 amino acids and four-helix bundles structure, which is primarily derived from macrophages. This cytokine acts through its soluble receptor (sIL-6R) or its cellular receptor (IL-6R) (Jones et al. 2001). The main role of IL-6 is to be a pro-inflammatory cytokine considering that it can amplify inflammatory responses. Other roles of this cytokine include autoimmunity induction, differentiation of B and neuronal cells, APRs [e.g., CRP, serum amyloid protein A (SAA), haptoglobin, or fibrinogen] induction in hepatocytes, and induction of receptor activator of NF-κB ligand (RANKL) that will activate osteoclasts and bone resorption (Hirano et al. 1988; Ridker et al. 1997; Guo et al. 2011). Besides pro-inflammatory cells such as lymphocytes, macrophages, monocytes and neutrophils, other non-proinflammatory

cells are responsible for the production of IL-6 (i.e., fibroblasts, myocytes, osteoblasts, and ECs) (Matsuki et al. 1992).

This cytokine is considered to be an upstream regulator that plays a central role in propagating the downstream inflammatory response related to atherosclerosis. Indeed, IL-6 acts at different stages in the process of atherosclerosis (Hartman and Frishman 2014). In the development of an atherosclerotic plaque, IL-6 is largely responsible for coordinating the influx of inflammatory cells. ECs respond to the binding of a complex of IL-6 and sIL-6R by elaborating chemokines and increasing ICAM-1 expression, leading to leucocyte recruitment and transmigration (Romano et al. 1997). IL-6 stimulates the hypothalamic-pituitary-adrenal (HPA) axis through the peripheral synthesis of corticotropin-realising factor, thus, promoting pro-inflammatory effects (Späth-Schwalbe et al. 1994; Karalis et al. 1997). IL-6 activity upon naïve T-lymphocytes results in their differentiation into T-helper cells, which are able to continue propagation of the inflammatory cascade (Eddahri et al. 2009). In addition, IL-6 has pro-coagulant effects, primarily by tissue factor (TF) induction within monocytes (Neumann et al. 1997). The production of TF allows thrombus formation, and as a result, vascular event such as ischemic stroke (Rodríguez-Yáñez and Castillo 2008) at the site of atherosclerosis.

Inflammation is considered to play an important role in cerebral infarct deterioration (Castillo and Leira 2001). In patients with ischemic stroke, elevated levels of plasmatic IL-6 have been associated with the presence of early neurological deterioration (Castellanos et al. 2002), poor functional outcome (Blanco et al. 2006; Castellanos et al. 2002; Rodríguez-Yáñez et al. 2006), early CT ischemic changes (Rodríguez-Yáñez et al. 2008) as well as greater volume infarct, which is one of the main factors implicated in functional outcome of these patients (Leira et al. 2006). Results from a multicentre study demonstrated that elevated plasmatic levels of IL-6 in baseline increased the risk of new vascular disease event or death from vascular disease in patients with ischemic stroke, who were not anti-coagulated (Castillo et al. 2009). On

the other hand, lower levels of IL-6 are associated with ischemic tolerance in patients with ischemic stroke and a previous TIA, hence, with a good functional outcome (Castillo et al. 2003).

In PD, IL-6 seems to play a role in modulating the response to periodontal bacteria, leading to both local and systemic inflammation. An excessive IL-6 response may contribute to the development of a chronic inflammatory lesion, which can result in periodontal tissue breakdown and alveolar bone loss via MMPs and osteoclasts activity, T cells activation and amplification o the inflammatory cascade (Nibali et al. 2012). It has been shown that IL-6 levels were elevated both locally (i.e., GCF and saliva) and systemically (i.e., serum) in PD patients (Geivelis et al. 1993; Costa et al. 2010; Shimada et al. 2010). Moreover, periodontal treatment seems to reduce in the long-term serum IL-6 levels in severe periodontal patients (D'Aiuto et al. 2004; Shimada et al. 2010).

5.1.2. Interleukin-10

IL-10 modulates expression of cytokines, soluble mediators and cell surface molecules by cells of myeloid origin, with important consequences for their ability to activate and sustain immune and inflammatory responses (Moore et al. 2001). The effect of IL-10 on cytokine production and function of human macrophages are generally similar to those on monocytes, but less pronounced (Wilkes et al. 1995; Armstrong et al. 1996; Thomassen et al. 1996; Nicoid et al. 1995). The inhibitory effects of IL-10 on IL-1 and TNF production are crucial to its anti-inflammatory activities, because these cytokines often have synergistic activities on inflammatory pathways and processes, and amplify these responses by inducing secondary mediators such as chemokines and PGs (Niiro et al. 1994, 1995; Mertz et al. 1994). Furthermore, IL-10 downregulates the expression of TLR-4, which is the signal transducing receptor for bacterial LPS (Muzio et al. 2000).

Low or absent IL-10 leads to several changes in gene expression that ultimately results in deleterious vascular remodelling and impaired vascular relaxation in response to physiologic mediators, which would exacerbate secondary brain damage following acute brain injury (Garcia et al. 2017). Accordingly, patients with LIs and neurological deterioration had significantly lower IL-10 levels in plasma than those without worsening (Vila et al. 2003). In addition, plasmatic levels of IL-10 ≥ 30 pg/mL were independently associated with a good outcome in ischemic stroke patients who underwent systemic thrombolytic treatment with tPA (Rodríguez-Yáñez et al. 2013).

Due to its anti-inflammatory properties, IL-10 also plays an important role in PD (Sasaki et al. 2004). The lockout of PD may result in accelerating alveolar bone resorption and decreasing bone formation (Zhang et al. 2014). In fact, it has been demonstrated that IL-10 upregulated osteoprotegerin (OPG) expression but downregulated RANKL expression (Liu et al. 2006). Moreover, IL-10 may inhibits pro-inflammatory cytokines that are involved in osteoclasts proliferation, thus, inhibiting bone resorption (Boyle et al. 2003). On the other hand, IL-10 has the ability of promoting osteoblast differentiation eventually via pro-inflammatory cytokines downregulation (Dresner-Pollak et al. 2004).

5.2. Endothelial dysfunction

5.2.1 Pentraxin 3

Pentraxins (PTXs) belongs to the superfamily of APRs, which are divided into short and long PTXs. Short constituents include CRP and SAA, which are synthesized in the liver mostly upon IL-6 stimulation. On contrary, long constituent PTX3 is produced by neutrophils, fibroblasts, dendritic cells, macrophages, epithelial cells, and ECs, in response to pro-inflammatory signals such as bacterial products, TNF-α, and IL-1β and by TLR receptor engagement (Mantovani et al. 2008; Vilahur and Badimon 2015). Indeed, plasma PTX3 levels are associated with vascular endothelial dysfunction, atherosclerosis, inflammation, or

damage and reach the peak much earlier than CRP (Norata et al. 2010; Peri et al. 2000).

Evidence suggests that cardiovascular disease (CVD) (Fornai et al. 2016), chronic kidney disease (CKD) (Sjöberg et al. 2016), or increased risk of mortality after ischemic stroke (Ryu et al. 2012) are linked to higher PTX3 levels. With regards to PD, it has been hypothesized that PTX3 concentrations measured in GCF or serum could be a valuable diagnostic molecule for periodontal tissue destruction (Kathariya et al. 2013). In small-vessel vasculitides, PTX3 seems to be an indicator of disease activity, considering that endothelial cells from active skin lesions showed enhanced production of PTX3 (Fazzini et al. 2001).

One of the key features of atherosclerosis, which is cholesterol accumulation in the intima of the vessels, is related to an immune-inflammatory response leading to the recruitment and activation of different cellular types (i.e., macrophages, monocytes, PMNs and ECs). These cells are responsible for producing PTX3 in response to inflammatory stimuli commonly associated with the process of atherosclerosis (Bonacina et al. 2013; Fornai et al. 2016). In fact, a higher expression of PTX3 could be observed in human advanced atherosclerotic plaques (Rolph et al. 2002) and it has been suggested that PTX3 could be an indicator of carotid plaque vulnerability (Shindo et al. 2014) by binding the fibroblast growth factor 2, which play a role in the proliferation and migration of smooth muscle cells (Bassi et al. 2009). Experimental data demonstrated that PTX3 null/Apolipoprotein E (ApoE) null mice presented larger atherosclerotic lesions compared to those ApoE null only, and this fact was due to raised bone marrow monocytosis, elevated macrophage accumulation, and increased expression of adhesion molecules, chemokines, and cytokines in the vessel wall (Norata et al. 2009). PTX3 has also been studied as a potential therapeutic target against atherosclerotic progression. Based on this, PTX3 suppression was associated with a reduction in the inflammatory and apoptosis process that was mediated by the IKK/IκB/NF-κB pathway (Qiu et al. 2015). However, to date, it remains

unclear which are the mechanisms underlying the role of this protein in the onset and progression of atheromatosis (Casula et al. 2017).

PTX3 has also been studied as a contributor to vascular endothelial dysfunction. In the rodent model, exogenous administration of PTX3 significantly blunted NO production through the MMP-1 and P-selectin pathway leading to morphological alterations of ECs (Carrizzo et al. 2015). In addition, there is clinical evidence suggesting that plasma PTX3 is a more potent predictor of endothelial dysfunction compared to CRP (Yasunaga et al. 2014). Accordingly, an association was found between PTX3 levels and FMD, which is a direct measure of endothelial dysfunction. In patients with coronary artery disease (Yanusaga et al. 2014), CKD (Yilmaz et al. 2009a), or obstructive sleep apnea syndrome (Kanbay et al. 2015), PTX3 levels are negatively correlated with FMD. Therefore, giving insight of the potential role of this APR in endothelial impairment.

A study carried out in chronic periodontal patients has shown a positive correlation between periodontal clinical parameters such as CAL (a measure of prolonged exposure to PD) and PPD (an indicator of current PD) and PTX3 levels in general (Pradeep et al. 2011) and specific infection site (Pradeep et al. 2011; Fujita et al. 2012). Furthermore, cross-sectional clinical data showed that in subjects diagnosed with periodontitis, PTX3 concentrations in GCF were significantly elevated in periodontally affected sites compared to non-PD sites (Fujita et al. 2012). Regarding saliva samples of PTX3 levels, significant differences could only be seen at the subgroup level, namely generalized aggressive PD subgroup, when comparisons were made with controls (Gümüş et al. 2014) (Table 5).

First author, year	Study design	Diagnosis	Sample size	Age (mean)	PTX3 levels (ng/mL)	Method
Pradeep, 2011	Case-control (age- and sex-matched)	Chronic PD: Gingival Index (GI) >1; PPD ≥5 mm; CAL ≥3 mm; and radiographic evidence of bone loss	Patients: 15 Controls: 10	Patients: 33 Controls: 23	Patients: 3.07 ± 0.71 Controls: 1.60 ± 1.12	Serum (ELISA)
					Patients: 3.37 ± 1.45 Controls: 1.95 ± 0.91	GCF
Pradeep, 2012	Case-control (age- and sex-matched)	Chronic PD: GI >1; PPD ≥5 mm; CAL ≥3 mm; and radiographic evidence of bone loss. Cases were also diagnosed with CKD	Patients: 20 Controls: 20	Patients: 37 Controls: 30	Patients: 6.33 ± 2.74 Controls: 1.83 ± 0.75	Serum (ELISA)
Fujita, 2012	Cross-sectional	All patients were diagnosed with chronic PD. -Diseased sites: PPD ≥5 mm; CAL ≥3 mm and BoP -Healthy sites: PPD ≤3 mm and no BoP	50	59	Diseased sites: 0.64 ± 0.39 Control sites: 0.06 ± 0.10	GCF
Gümüş, 2014	Case-control (age-matched)	Subgroup generalized chronic PD: presence of ≥4 teeth in each jaw with a PPD ≥5 mm; CAL ≥4 mm; ≥50% bone loss in at least 2 quadrants; and BoP in >80% of proximal sites	Patients: 25 Controls: 22	Patients: 50 Controls: 47	Patients: 47.74 ± 28.16 Controls: 36.66 ± 23.37	Saliva
		Subgroup generalized aggressive PD: presence of at least 6 permanent teeth (including incisors and/or 1st molars) with at least one site with PPD and CAL ≥5 mm and 6 teeth other than 1st molars and incisors with similar PPD and CAL measurements; familial aggregation; and radiographic bone loss ≥30% of root length affecting ≥3 permanent teeth other than incisors and 1st molars	Patients: 25 Controls: 22	Patients: 36 Controls: 36	Patients: 51.95 ± 29.04 Controls: 29.85 ± 25.21	Saliva

Table 5. Summary of observational studies reporting PTX3 levels in patients with PD.

5.2.2. Soluble tumor necrosis factor-like weak inducer of apoptosis

Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) is a member of the TNF superfamily of cytokines that is synthesized as a type II transmembrane protein from which a soluble 17 kDa ligand factor with biological activity can be released [soluble TWEAK (sTWEAK)] (Chicheportiche et al. 1997). TWEAK is expressed in several tissues (i.e., heart, lung, and brain) (Wiley and Winkles 2003) and cells (i.e., ECs and SMCs). Different cellular responses are induced by sTWEAK such as cell proliferation, migration, differentiation, and angiogenesis (Lynch et al. 1999; Donohue et al. 2003; Desplat-Jégo et al. 2002; Tran et al. 2003). In addition, it is involved in the expression of pro-inflammatory mediators and adhesion molecules in ECs and astrocytes (Harada et al. 2002; Saas et al. 2000). TWEAK activity is mainly mediated via binding to fibroblast growth factor-inducible 14 (Fn14), which is a 14 kDa member of the TNF receptor family (Wiley et al. 2001) and is expressed by fibroblasts, ECs, epithelial cells, and tumor cells of non-lymphoid origin.

The interaction between TWEAK and Fn14 has been associated with endothelial dysfunction and the process of atherosclerosis by several mechanisms (Liu et al. 2017). TWEAK induces expression of adhesion molecules such as E-selectin and ICAM-1 on the cell surface of human umbilical vein ECs *in vitro* (Harada et al. 2002) and the TWEAK-Fn14 axis also induce MCP-1 and IL-8 secretion, which predominantly recruits monocytes and neutrophils (Blanco-Colio et al. 2007a). Vascular SMCs, which normally are in a contractile phenotype to maintain the vascular tone and diameter, in atherosclerosis is transformed into a pro-inflammatory phenotype that could be elicited through TWEAK/Fn14 interaction (Harada et al. 2002; Lynch et al. 1999). Furthermore, is has been demonstrated that in aortic SMCs, TWEAK was able to induce thrombotic molecules such as TF and PAI-1, which are involved in the process of thrombosis (Muñoz-García et al. 2011). TWEAK/Fn14 axis also showed some effects on monocytes and macrophages involved in the inflammatory process of atherosclerosis. Through Fn14 inhibition, the interaction TWEAK/

Fn14 may alter macrophage trafficking and increase lipid uptake of macrophages (Schapira et al. 2009). In addition, TWEAK was found to induce several pro-inflammatory mediators of atherogenesis such as IL-6, MCP-1 and IL-8 in activated monocytes (Kim et al. 2004) as well as able to enhance MMP-9 and MMP-2 activity in ApoE null mice (Sastre et al. 2014). Human studies showed that patients with CKD, sTWEAK levels were positively correlated with FMD (Yilmaz et al. 2009b, 2011) and associated with atheromatosis progression (Fernández-Laso et al. 2017). It was also found that in artery samples from patients with atherosclerosis, sTWEAK concentrations were negatively correlated with carotid IMT (Blanco-Colio et al. 2007b).

In cerebral ischemia, experimental data showed increased levels of TWEAK and Fn14 in the area surrounding the necrotic core, also known as ischemic penumbra region, after middle cerebral artery occlusion (MCAO) (Yepes et al. 2005). Moreover, intra-cerebroventicular injection of a soluble Fn14-Fc decoy receptor immediately after MCAO significantly reduced infarct volume as well as the apoptotic cell death in the ischemic penumbra region (Yepes et al. 2005). Results from an animal study showed that in response to ischemic signal during stroke, there was a release of TWEAK from astrocytes and its binds to Fn14 receptor activated the NF-κB pathway and also induced pro-inflammatory cytokines and MMP-9 leading to BBB disruption and increased permeability (Polavarapu et al. 2005). Additionally, administration of Fn14-Fc decoy receptor immediately or 1 hour after MCAO improved neurovascular permeability (Zhang et al. 2007). In humans, serum levels of TWEAK from ischemic stroke patients were significantly elevated compared to healthy controls (Inta et al. 2008). In brain tissues of patients who died due to ischemic stroke, the levels of Fn14 from infarct tissues were significantly higher than those brain tissues from the contralateral region without infarct (Inta et al. 2008).

Regarding PD, there is scarce literature of the potential role of TWEAK in the pathogenesis of the disease. However, it was found that in periodontally affected tissues, TWEAK and Fn14 were overexpressed compared to healthy periodontal tissues (Kataria et al.

2010) and that the TWEAK/Fn14 axis was responsible for the induction of IL-1β, ICAM-1 and VCAM-1 within human gingival fibroblasts (Hosokawa et al. 2006, 2012).

5.3. Amyloid-beta peptides

Amyloid-beta (Aβ) is a 38-to 43 amino-acid peptide, which is produced by proteolytic cleavage of amyloid precursor protein (APP). Plasma Aβ includes both $A\beta_{1-40}$ and $A\beta_{1-42}$. Whereas parenchymal Aβ deposition is considered to play a pivotal role in neuronal loss and cognitive impairment in Alzheimer´s disease (AD) (Hardy and Allsop 1991), progressive Aβ accumulation within the walls of cortical and leptomeningeal small arterioles is the pathological hallmark of cerebral amyloid angiopathy (Charidimou et al. 2012). Senile plaque amyloid is primarily comprised of $A\beta_{1-42}$, whilst amyloid of vascular origin consists of $A\beta_{1-40}$ species.

It has been hypothesized that exposure to $A\beta_{1-40}$, but not $A\beta_{1-42}$, produced a profound and selective alteration in the regulation of the cerebral circulation by ECs (Niwa et al. 2000a). Based on this, endothelial dysfunction by Aβ deposition has been related to LIs and leukoaraiosis (van Dijk et al. 2004; Gurol et a. 2006). Accordingly, diffuse-CSVD (i.e., LIs with leukoaraiosis) was independently associated with elevated plasma levels of $A\beta_{1-40}$ (Gomis et al. 2009).

It has been postulated that PD is involved in the synthesis and accumulation of Aβ in the brain. By means of positron emission tomography imaging techniques, PD was associated with Aβ deposition in brain areas known to be susceptible to AD (Kamer et al. 2015). Periodontally affected tissues from healthy patients also demonstrated overexpression of APP, thus, giving insight on the potential relationship between PD and Aβ accumulation (Kubota et al. 2014). Recently, it was found that serum $A\beta_{1-42}$ levels from patients diagnosed with cognitive impairment who had severe PD were significantly higher than those without cognitive decline or those with lower levels of PD (Gil-Montoya et al. 2017).

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GENERAL OBJECTIVES

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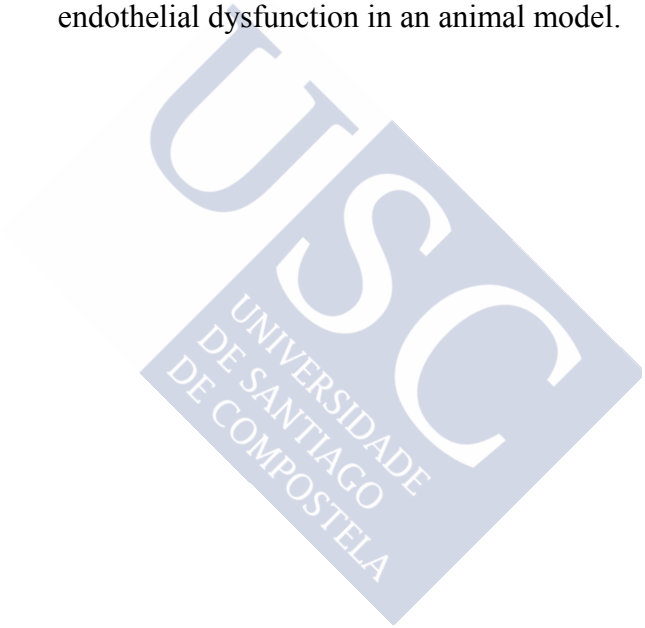
GENERAL OBJECTIVES

- To investigate the relationship between PD and LI.
- To delucidate potential mechanisms behind this association.
- To investigate whether PD could act as a chronic low-grade inflammatory systemic condition promoting vascular endothelial dysfunction in an animal model.



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CLINICAL STUDY

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CLINICAL STUDY

1. JUSTIFICATION

PD is among the ten most prevalent diseases affecting human beings. A growing body of evidence suggests that PD not only have local effects (i.e., gingiva) but also is capable of producing systemic side effects in distant organs (Loos 2005). In fact, it is speculated that the inflamed and ulcerated subgingival pocket epithelium forms an easy port of entry for periodontal bacteria. Bacteremia in PD has been reported after oral examination (Daly et al. 2001) and dental procedures (Heimdahl et al. 1990) and, interestingly, periodontopathogens have been identified in atheromatous plaques (Haraszthy et al. 2000). Additionally, bacterial components such as LPS may also be disseminated into the blood circulation (Geerts et al. 2002). These LPS together with bacterial antigens can trigger significant systemic inflammatory processes. Accordingly, white blood cells and acute-phase proteins from ECs and hepatocytes may produce pro-inflammatory mediators. Moreover, locally produced pro-inflammatory molecules may spill into the systemic circulation and exert effects on distant organ systems (Moutsopoulos and Madianos 2006). Based on this, PD has been regarded as a systemic inflammatory and endothelial vascular stressor that can act as an independent risk factor of large-vessel ischemic stroke (Leira et al. 2017c).

LI, a type of CSVD, is responsible for approximately 25% of the cases of ischemic stroke (Pantoni 2010). Atheromatous stenosis, thrombosis occlusion (Fisher 1979) or inflammation (Wardlaw 2005) have been suggested as the main causes of this disease. Although the exact mechanism remains unclear, intrinsic CSVD such as lipohyalinosis is considered as the most common cause of LI. Accordingly, endothelial dysfunction is present in the pathogenesis of

LI (Blanco et al. 2005; Knottnerus et al. 2009). Furthermore, there is an association between increased inflammatory response and early neurologic worsen in patients with LI showing elevated concentrations of pro-inflammatory markers such as IL-6 or TNF-α in those with a poor functional prognosis (Castellanos et al. 2002).

To date, little is known about the relationship between PD and small-vessel ischemic stroke (i.e., LI) with conflicting results (Leira et al. 2016; Sen et al. 2018). To the best of our knowledge, there is a lack of mechanistic studies investigating the potential role of PD as a source of systemic inflammation and endothelial dysfunction in patients with LI as well as if it could be a predictor of poor functional outcome in these patients.

Therefore, identifying conditions that could contribute to an enhanced systemic inflammatory state as well as promote endothelial dysfunction may have significant prognostic and treatment implications in patients with LI.

2. HYPOTHESIS

Our hypothesis is that PD is common in patients with LI and is associated with the presence of LI independently of well-known vascular risk factors. When PD is present in patients with LI, may result in an enhanced systemic inflammatory response with vascular dysfunction of the endothelium expressed by higher serum levels of both pro-inflammatory and endothelial dysfunction biomarkers. We also hypothesized that periodontal inflammation could be an independent predictor of poor functional prognosis in LI patients.

3. OBJECTIVES

3.1. Primary objective

- The main objective of this work is to investigate the association between PD and its clinical parameters and the presence of LI.

3.2. Secondary objectives

- To analyse possible physiopathological mechanisms through which PD could contribute to a higher risk for developing LI (i.e., systemic inflammation and endothelial dysfunction).
- To investigate whether periodontal inflammation predicts poor functional outcome in patients diagnosed with LI.

4. MATERIAL AND METHODS

4.1 Study design

A case-control study was carried out by the Periodontology Unit of the University of Santiago de Compostela in collaboration with the Stroke Unit of the University Clinical Hospital of Santiago de Compostela by following the STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) guidelines (Von Elm et al. 2008).

This research was performed in accordance with the Declaration of Helsinki of the World Medical Association (2008) and approved by the Ethics Committee of the Servizo Galego de Saúde (2016/399). Informed consent was obtained from each patient or their relatives after full explanation of the procedures.

4.2. Study population

4.2.1. Case group

Patients who had attended the Stroke Unit of the University Clinical Hospital of Santiago de Compostela between January 2014 and January 2015 were asked by telephone to participate in this study as cases. Cases were those with a diagnosis of LI based on the TOAST criteria (Adams et al. 1993) and they were included in the study if they fulfilled the following inclusion criteria: (i) >18 years of age; (ii) at least 15 teeth (excluding third molars); and (iii) written informed consent. Exclusion criteria were as follows: (i) patient who have received periodontal treatment in the previous 12 months; (ii) history of neurovascular and/or neuroinflammatory disease; (iii) systemic antibiotics, corticosteroids, and/or immunosuppressant therapy within 3 months prior to periodontal assessment; and (iv) chronic use of non-steroidal anti-inflammatory drugs (NSAIDs).

4.2.2. Control group

Healthy control subjects, matched by age and gender, were selected from the hospital database of the Service of Neurology. In order to include individuals without any neurological disorder, we reviewed 194 CT/MRI scans of subjects who were referred to the Service of Neurology with a suspicious diagnosis of certain neurological diseases such as non-specific headache, vestibular syndromes, brain tumours or altered level of consciousness between 2009-2013. Of these, 12 presented some subtype of asymptomatic CSVD [SI (n=4), leukoaraiosis (n=8)] and, thus, were excluded from the study. Therefore, 182 subjects free from any neurological disease were contacted by telephone and asked to participate. Inclusion and exclusion criteria were the same as for the case group. Control individuals were clinically examined and interviewed in parallel with patient recruitment.

4.3. Periodontal examination

The periodontal examination was performed by a single calibrated periodontist (YL). The calibration was completed before the start of the study in the Periodontology Unit of the Faculty of Odontology (University of Santiago de Compostela) using 10 non-study patients suffering from moderate or severe PD. Intra-examiner reliability was assessed by the intraclass correlation coefficients (for PPD, GR and CAL), which were 0.79, 0.87 and 0.79, respectively, demonstrating a high degree of reliability in the measurements (Leira et al. 2016). In the present study the following periodontal parameters were evaluated in all teeth (except 3º molars): (i) PPD, measured from the free gingival margin to the bottom of the sulcus; (ii) CAL, measured from the cemento-enamel junction to the bottom of the sulcus or pocket; (iii) GR, measured as the distance from the free gingival margin to the exposed cemento-enamel junction; (iv) full-mouth plaque score (FMPS), defined as the number of sites with detectable supragingival dental plaque divided by the total number of sites per mouth, multiplied by 100 (O'Leary et al. 1972); (v) full-mouth bleeding score (FMBS), defined as the number of sites with gingival bleeding on probing divided by the total number of sites per mouth, multiplied by 100 (Ainamo and Bay 1975); and (vi) the number of missing teeth (excluding 3º molars).

All measurements were recorded at six sites per tooth (mesio-buccal, disto-buccal, mid-buccal, mesio-lingual, disto-lingual, and mid-lingual), except for FMPS (four sites/tooth) using a sterile mouth mirror and with a calibrated University of North Carolina periodontal probe (UNC 15; Hu-Friedy, Chicago, IL, USA).

The presence of PD was defined according to the CDC-AAP consensus for epidemiologic studies (Eke et al. 2012; Holtfreter et al. 2015). Therefore, mild PD was defined as ≥2 interproximal sites with CAL ≥3 mm and ≥2 interproximal sites with PPD ≥4 mm (not on the same tooth) or 1 site with PPD ≥5 mm. Moderate PD was defined as ≥2 interproximal sites with CAL ≥4 mm (not on the same tooth) or ≥2

interproximal sites with PPD ≥5 mm, also not on the same tooth. Severe PD was defined as the presence of ≥2 interproximal sites with CAL ≥6 mm (not on the same tooth) and ≥1 interproximal site with PPD ≥5 mm. Total PD was the sum of mild, moderate, and severe PD.

In addition, a recently introduced measure of PD severity and activity, the PISA was calculated (Nesse et al. 2008). PISA reflects the surface area of bleeding pocket epithelium in mm^2. PISA was calculated with a Microsoft Excel spreadsheet in the following steps: (i) Mean CAL and GR for each particular tooth is calculated; (ii) Linear mean CAL and GR is translated into the periodontal epithelial surface area (PESA) for each specific tooth (Hujoel et al. 2001). The PESA for a particular tooth consists of the root surface area of that tooth measured in mm^2, which is covered with pocket epithelium; (iii) The PESA for a specific tooth is then multiplied by the proportion of sites around the tooth that was affected by BoP, resulting in the PISA for that particular tooth; and (iv) The sum of all individual PISAs around individual tooth is calculated, rendering the full-mouth PISA value in mm^2 of each participant.

4.4. Neurological examination

4.4.1. Neuroimaging examination

A CT/MRI scan was carried out in all cases at admission. MRI images were obtained on a 1.5 T system (1.5 Magneton Symphony, Siemens, Erlangen, Germany), with echo planar capabilities of 25 mT/m gradients and 300-350 μs rise times. The MRI protocol included T_1-w (TR/TE: 370/7.7 ms), T_2-w (TR/TE: 6020/113 ms), DP-w (TR/TE: 6020/113 ms) and FLAIR (TR/TE: 9000/114 ms) (Rodríguez et al. 2010). One neurologist who was blinded to the clinical data carried out the evaluation of CT/MRI (MR-Y). LI was diagnosed if the patient had one of the characteristic clinical lacunar syndromes, neurological deficit lasting >24 hours, no evidence of cerebral cortical dysfunction, and a CT/MRI that was normal or showed a deep focal infarction in an appropriate location with a diameter ≤15 mm. The

presence of a LI in the baseline CT in which the topography does not correspond with the present clinical syndrome was considered a SI. For the purpose of this study, the thalamus was included, along with the caudate nucleus, putamen, and globus pallidus, as "basal ganglia." Infarct volume was not calculated because of the unreliability of the CT in measuring small infarcts. Leukoaraiosis was defined as ill-defined hyperintensities ≥5 mm on both T_2 and FLAIR MRI images without prominent hypointensities on T_1-w MRI scans and as ill-defined and moderately hypodense areas of ≥5 mm on CT. Leukoaraiosis was classified according to the Fazekas criteria (Fazekas et al. 1991, 1993) using the modified Fazekas scale (Pantoni et al. 2002). This method yields two separate scores for subcortical and deep white matter lesions and periventricular lesions. The four-point Fazekas scale of increasing severity was used to classify each score. For the purpose of the study, the presence of leukoaraiosis was categorized with 0 indicating a patient without leukoaraiosis and 1 with leukoaraiosis.

Grade	Description
0	No white matter lesions
1	Lesion limited to frontal and/or occipital ventricle horn
2	Lateral ventricle bands
3a	Disperse periventricular and/or subcortical white matter lesions
3b	Confluent white matter and/or subcortical lesions
3c	Homogeneous white matter and/or subcortical lesions

Table 6. Modified Fazekas scale.

4.4.2. Ultrasound examination

The same explorer (SA), blinded to clinical data, performed the ultrasonographic study using high-resolution B-mode ultrasound [Aplio 50 (Toshiba aplio 50, MCM1754TSA, Rome, Italy) Toshiba SSA-700 (Toshiba Medical Systems Corporation, Otawara-SHI, Japan)] with a 7.5 MHz, linear-array transducer (Linear array transducer PLT-704AT, Toshiba, Tochigi, Japan; Phased array transducer PST-20CT, Toshiba, Tochigi, Japan) (Rodríguez et al. 2010). In brief, the image was focused on the posterior (far) wall of the left carotid artery. A minimum of four measurements of the common carotid far wall was taken 10 mm

proximal to the bifurcation, to derive the mean carotid intima-media thickness (IMT) (Raitakari et al. 2003). The presence of an atheroma plaque was evaluated in the common and internal carotid extracraneal arteries as well as the bifurcations according to standardised scanning and reading protocols (Touboul et al. 2007). Plaque was defined as a focal structure that encroaches into the arterial lumen at least 0.5 mm or 50% of the surrounding IMT value, or demonstrates a thickness >1.5 mm as measured from the media-adventitia interface to the intima-lumen interface. For the purpose of the study, the presence of carotid atheromatosis was categorized with 0 indicating a patient without carotid atheromatosis and 1 with it.

4.4.3. Outcome evaluation

Functional outcome was evaluated at 3 months using the modified Rankin scale (mRS) (UK TIA Study Group, 1988; van Swieten et al. 1988), and a poor outcome was defined as a mRS score >2.

Grade	Description
0	No symptoms at all
1	No significant disability despite symptoms: able to carry out all usual duties and activities
2	Slight disability: unable to carry out all previous activities but able to look after own affairs without assistance
3	Moderate disability: requiring some help, but able to walk without assistance
4	Moderately severe disability: unable to walk without assistance, and unable to attend to own bodily needs without assistance
5	Severe disability: bedridden, incontinent, and requiring constant nursing care and attention
6	Death

Table 7. Modified Rankin Scale scores.

4.5. Serum collection and laboratory tests

On admission for cases and the periodontal examination/interview day for controls, 2 mL of venous blood was collected from the antecubital fossa by venepuncture using a 20-gauge needle with a 2 mL syringe. Blood samples were allowed to clot at room temperature and

after 1 hour, serum was separated from blood by centrifugation (15 min. at 3000 g) and 0.5 mL of extracted serum was immediately transferred to 1.5 mL aliquots. Each aliquot was stored at –80ºC until the time of analysis. Serum levels of all biomarkers were measured by enzyme-linked immunosorbent assay (ELISA) technique following manufacturer instructions. IL-6 ELISA kit (Proteintech®, Manchester, United Kingdom) minimum assay sensitivity was 3.8 pg/ml with a intra-assay coefficient of variation (CV) of 5.0% and inter-assay CV of 6.4%; IL-10 ELISA kit (Proteintech®, Manchester, United Kingdom) minimum assay sensitivity was 0.5 pg/ml with a intra-assay coefficient of variation of 5.4% and inter-assay CV of 5.6%; PTX3 ELISA kit (Abnova™, Taipei City, Taiwan) minimum assay sensitivity was 10 pg/ml, with a intra-assay coefficient of variation of 6.9% and inter-assay CV of 7.0%; sTWEAK ELISA kit (Aviscera Bioscience®, Santa Clara, California, USA) minimum assay sensitivity was 10 pg/ml, with a intra-assay coefficient of variation of 5.0% and inter-assay CV of 2.3%; $A\beta_{1-40}$ ELISA kit (Elabscience®, Houston, Texas, USA) minimum assay sensitivity was 9.38 pg/ml, with a intra-assay coefficient of variation of 4.8% and inter-assay CV of 6.5%; and $A\beta_{1-42}$ ELISA kit (Elabscience®, Houston, Texas, USA) minimum assay sensitivity was 9.38 pg/ml, with a intra-assay coefficient of variation of 6.0% and inter-assay CV of 6.8%. Determinations were performed in the Clinical Neurosciences Research Laboratory.

4.6. Study variables

4.6.1. Demographic and clinical variables

- Age
- Gender
- Education level: low (< secondary school), medium (completed secondary school), and high (university studies)
- BMI: Standing body height was measured with the shoulders in a relaxed position and the arms hanging freely, using a commercial stadiometer. Thus, BMI was calculated as the weight divided by the square of height (kg/m^2)

4.6.2. Periodontal and dental variables

- FMPS (%)
- FMBS (%)
- Mean PPD (mm)
- Mean GR (mm)
- Mean CAL (mm)
- Mean PISA (mm^2)
- Number of sites with PPD ≥ 4 mm
- Number of sites with PPD ≥ 6 mm
- Number of sites with CAL ≥ 3 mm
- Number of sites with CAL ≥ 5 mm
- Number of present teeth
- Prevalence and severity of PD
- Last dental visit: within the last 12 months or less often
- Tooth brush frequency: < or ≥ 2 times/day
- Use of interdental care devices

4.6.3. Neurological variables

- LI location: hemispheric, basal ganglia, brainstem, and other locations
- Presence of SIs
- Presence of leukoaraiosis (white matter lesions)
- Presence of carotid atheromatosis
- mRS at admission
- mRS at 3 months

4.6.4. Vascular risk factors

- History of hypertension
- History of diabetes mellitus
- History of hypercholesterolemia
- Smoking habit: never, former or current smoker

- Alcohol consumption: considering “heavy drinkers” those who drink ≥ 60 g/day
- History of ischemic heart attack
- History of peripheral arterial disease

4.6.5. Use of medication

- Statins
- Antiaggregants
- Antihypertensives

4.6.6. Biomarkers

4.6.6.1. Systemic inflammation

- Serum levels of IL-6 (pg/mL)
- Serum levels of IL-10 (pg/mL)

4.6.6.2. Endothelial dysfunction

- Serum levels of PTX3 (pg/mL)
- Serum levels of sTWEAK (pg/mL)

4.6.6.3. Amyloid-beta peptides

- Serum levels of $A\beta_{1\text{-}40}$ (pg/mL)
- Serum levels of $A\beta_{1\text{-}42}$ (pg/mL)

4.7. Sample size calculation

The sample size calculation was performed using the Macro !NSize for PASW Statistics (http://www.metodo.uab.cat/macros.htm.). Based on a pilot study carried out by our group, to detect an expected OR of 4.20 in the association between PD and LI (Leira et al. 2016), assuming α-risk=0.05 and β-risk=0.10, a sample of 240 subjects was calculated (120 cases and 120 controls, 1:1 case:control).

4.8. Statistical analysis

All data analyses were performed with IBM SPSS Statistics 20.0 software for Mac (SPSS Inc., Chicago, IL, USA). Continuous normally distributed variables analysed with Kolmogorov-Smirnov test were reported as mean ± standard deviation, whereas continuous non-normally distributed variables were expressed as median [interquartile range]. Categorical variables were reported as percentages. Differences between two groups were assessed by independent *t* test (continuous normally distributed variables), *Mann-Whitney* test (continuous non-normally distributed variables) and X^2 test (categorical variables). One-way analysis of variance (*ANOVA*) was used to compare mean values between more than 2 groups. Additionally, Bonferroni post hoc tests for multiple comparisons between groups were used. Pearson´s correlation coefficient (r) was used to correlate PISA with significant biomarkers of poor functional outcome at 3 months.

Binary logistic regression models were performed to test potential associations between PD and its clinical parameters and lacunar infarct presence as well as with poor functional outcome at 3 months. The model selection procedure selected was stepwise regression with bidirectional elimination, i.e., a combination of forward selection and backward elimination. The criterion chosen for selection was the Akaike information criterion (AIC), so the model with the lower AIC was selected as the best in order to avoid collinearity effects (Leira et al. 2016). The selection procedure was replicated using the Bayesian information criterion and the covariates selected with the AIC. Multivariable linear regression analysis was used to test associations between PD and several biomarkers, also adjusted for significant variables.

To identify the best discriminant cut-off point of the mean PISA to identify poor outcome in patients with LI, a receiver operating characteristic (ROC) analysis was carried out.

All tests were performed at a significance level of $\alpha = 0.05$.

5. RESULTS

5.1. Study population

A total of 321 adults were asked to participate in this study. This population included 139 patients diagnosed with LI and 182 healthy controls. Of these, 19 cases and 25 controls were excluded for several reasons (Figure 6). The final study sample for the clinical study consisted of 120 cases and 157 controls. Regarding molecular analysis, data was obtained from 120 cases and 120 controls (Figure 6).

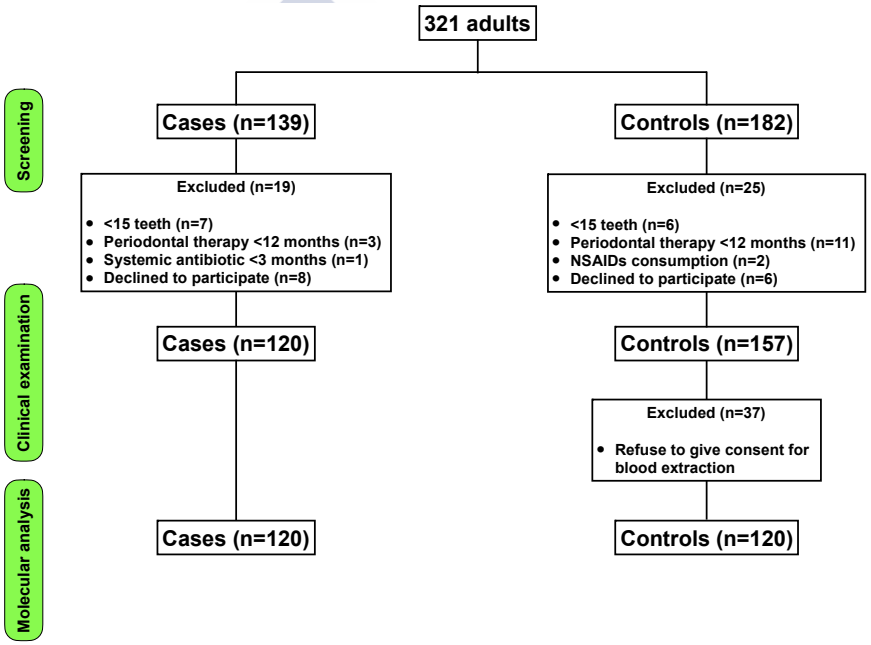

Figure 6. Flow chart of the clinical study.

5.2. Study groups – baseline characteristics

The number of patients with a history of hypertension, diabetes mellitus, hypercholesterolemia, ischemic heart disease, and peripheral arterial disease was significantly higher in cases than controls. As expected, patients with LI were taking significantly more medication than healthy controls (i.e., statins, antiaggregants, and antihypertensives (Table 8).

VARIABLES	Cases (n=120)	Controls (n=157)	*P*-value
Age (years)	66.4±9.9	65.4±9.9	0.391
Males, n (%)	82 (68.3)	108 (68.8)	0.935
BMI	26.9±3.4	26.7±4.7	0.726
Hypertension, n (%)	74 (61.7)	44 (28.0)	**<0.001**
Diabetes mellitus, n (%)	33 (27.5)	13 (8.3)	**<0.001**
Hypercholesterolemia, n (%)	55 (45.8)	28 (17.8)	**<0.001**
Ischemic heart disease, n (%)	22 (18.3)	4 (2.5)	**<0.001**
Peripheral arterial disease, n (%)	5 (4.2)	0 (0.0)	**0.010**
Smoking habit			0.075
-Never smoker, n (%)	82 (68.3)	126 (80.3)	
-Former smoker, n (%)	16 (13.3)	13 (8.3)	
-Current smoker, n (%)	22 (18.3)	18 (11.5)	
Alcohol consumption, n (%)	21 (17.5)	17 (10.9)	0.172
Medication			
-Statins, n (%)	56 (46.7)	28 (17.8)	**<0.001**
-Antiaggregants, n (%)	46 (38.3)	4 (2.5)	**<0.001**
-Antihypertensives, n (%)	70 (58.3)	44 (28.0)	**<0.001**
Education level			0.869
-High, n (%)	30 (25.0)	36 (22.9)	
-Medium, n (%)	52 (43.3)	67 (42.7)	
-Low, n (%)	38 (31.7)	54 (34.4)	

Table 8. Baseline characteristics.

5.3. Study groups – periodontal disease

LI cases and controls differed significantly in all periodontal parameters examined, including both cumulative measures of past PD (i.e., CAL), as well as measures of ongoing periodontal inflammatory activity (i.e., FMBS, PPD, and PISA) (Table 9). According to this, PD was present in 85 of 120 patients with LI (70.8%) and in 51 of 157 control subjects (30.8%) (Figure 7). Regarding PD severity, almost half of the periodontal patients with LI had severe PD compared to 7.8% in the control group (Figure 8). No differences were found between groups with regards to dental variables (Table 9).

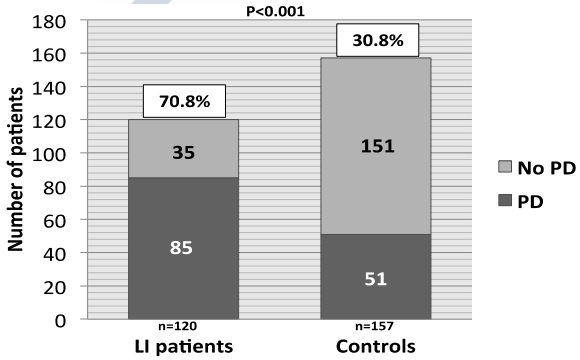

Figure 7. Prevalence of PD in subjects with and without LI.

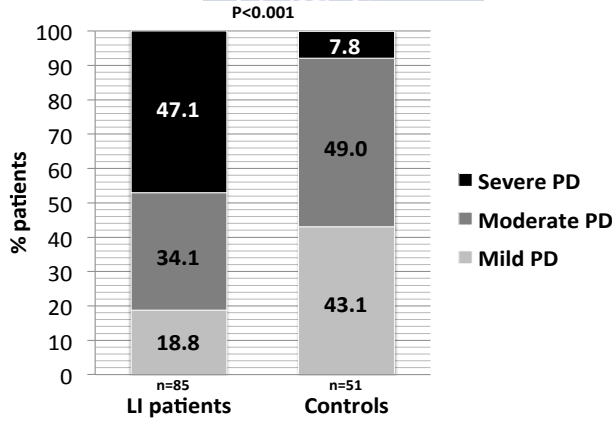

Figure 8. Percentage of patients according to PD severity in subjects with and without LI.

VARIABLES	Cases (n=120)	Controls (n=157)	*P*-value
FMPS (%)	54.8±17.5	27.0±12.0	**<0.001**
FMBS (%)	59.4±18.2	28.3±13.6	**<0.001**
PPD measures			
-Mean PPD (mm)	3.6±1.0	2.6±0.6	**<0.001**
-Number of sites/mouth PPD ≥4 mm	75.8±51.2	17.8±32.2	
-Number of sites/mouth PPD ≥6 mm	18.1±25.3	1.8±9.4	**<0.001**
GR (mm)	0.6±0.4	0.3±0.3	**<0.001**
CAL measures			
-Mean CAL (mm)	4.3±1.4	2.9±0.9	**<0.001**
-Number of sites/mouth CAL ≥3 mm	116.3±39.4	109.6±40.0	0.169
-Number of sites/mouth CAL ≥5 mm	63.7±50.1	17.7±30.8	**<0.001**
Number of present teeth	20.0±3.7	24.6±2.6	**<0.001**
PISA (mm^2)	1040.4±1145.8	193.2±357.1	**<0.001**
Last dental visit			0.152
-Within the last 12 months, n (%)	71 (59.2)	106 (67.5)	
-Less often, n (%)	49 (40.8)	51 (32.5)	
Tooth brush frequency			0.370
-<2 times/day	44 (36.7)	49 (31.2)	
-≥2 times7day	76 (63.3)	108 (68.8)	
Use of interdental care devices, n (%)	9 (7.5)	17 (10.8)	0.409

Table 9. Clinical periodontal parameters and dental variables.

Patients with LI who had PD showed significantly higher prevalence of leukoaraiosis and carotid atheromatosis than those without PD. Poor functional outcome at 3 months was found in 32.9% of LI patients with PD in comparison to 8.6% without PD (P=0.006) (Table 10).

VARIABLES	PD (n=85)	No PD (n=35)	*P*-value
Age (years)	67.5±10.0	63.8±9.5	0.061
Males, n (%)	60 (70.6)	22 (62.9)	0.408
BMI	26.9±3.7	27.1±2.3	0.734
Hypertension, n (%)	53 (62.4)	21 (60.0)	0.810
Diabetes mellitus, n (%)	24 (28.2)	9 (25.7)	0.779
Hypercholesterolemia, n (%)	36 (42.4)	19 (54.3)	0.233
Ischemic heart disease, n (%)	16 (18.8)	6 (17.1)	0.829
Peripheral arterial disease, n (%)	4 (4.7)	1 (2.9)	0.645
Smoking habit			0.900
-Never smoker, n (%)	58 (68.2)	24 (68.6)	
-Former smoker, n (%)	12 (14.1)	4 (11.4)	
-Current smoker, n (%)	15 (17.6)	7 (20.0)	
Alcohol consumption, n (%)	18 (21.1)	3 (8.6)	0.201
Medication			
-Statins, n (%)	36 (42.4)	20 (57.1)	0.140
-Antiaggregants, n (%)	30 (35.3)	16 (45.7)	0.286
-Antihypertensives, n (%)	49 (57.6)	21 (60.0)	0.812
Education level			0.894
-High, n (%)	21 (24.7)	9 (25.7)	
-Medium, n (%)	36 (42.4)	16 (45.7)	
-Low, n (%)	28 (32.9)	10 (28.6)	
Leukoaraiosis, n (%)	32 (37.6)	3 (8.6)	**0.001**
Carotid atheromatosis, n (%)	42 (49.4)	10 (28.6)	**0.036**
SIs, n (%)	36 (42.4)	11 (31.4)	0.265
mRS at admission	0.5±0.6	0.8±1.0	0.132
mRS at 3 months	1.6±1.2	1.0±0.9	**0.007**
Poor prognosis (mRS > 2), n (%)	28 (32.9)	3 (8.6)	**0.006**
LI location			0.799
-Hemispheric, n (%)	31 (36.5)	14 (40.0)	
-Basal ganglia, n (%)	43 (50.6)	18 (51.4)	
-Brainstem, n (%)	9 (10.6)	3 (8.6)	
-Other locations, n (%)	2 (2.4)	0 (0.0)	

Table 10. Characteristics of LI patients according to periodontal status.

5.4. Association between periodontal disease and its clinical parameters and the presence of lacunar infarct

After adjusting for age, gender, hypertension, diabetes mellitus, hypercholesterolemia, ischemic heart disease, smoking, and statins consumption in multiple logistic regression, among patients with PD, the odds for having LI was 3.3 (95% CI: 1.7-6.4) compared to those without PD (Figure 9). Likewise, severe PD was strongly associated

with the presence of LI (OR=9.8, 95% CI: 2.4-38.9; P<0.001), independently of the same confounding factors.

After adjusting for the most relevant clinical periodontal parameters, PISA was mildly associated with LI (OR=1.001, 95% CI: 1.001-1.002; P<0.001) (Figure 10).

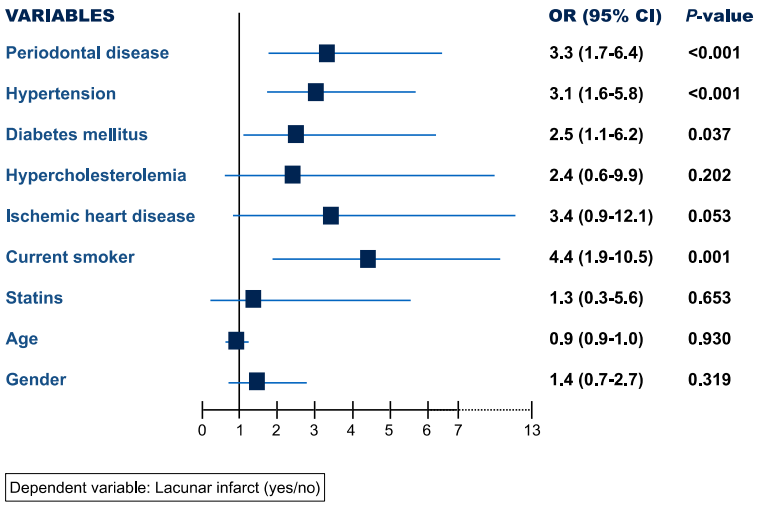

Figure 9. ORs and 95% CIs of PD calculated for the presence of LI. ORs are presented as adjusted values (blue squares) for age, gender, hypertension, diabetes mellitus, hypercholesterolemia, ischemic heart disease, smoking, and statins consumption.

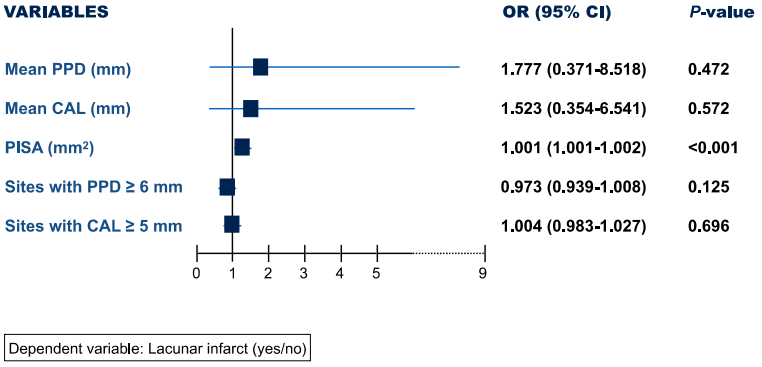

Figure 10. ORs and 95% CIs of clinical periodontal parameters calculated for the presence of LI. ORs are presented as adjusted values (blue squares) for mean PPD, mean CAL, PISA (mm^2), number of sites with PPD ≥ 6 mm, and number of sites with CAL ≥ 5 mm.

5.5. Molecular analysis

Patients with LI had significantly higher mean serum IL-6, PTX3, sTWEAK, and $A\beta_{1-40}$ levels than control subjects. On contrary, IL-10 serum concentrations were significantly lower in patients with LI compared to the control group. No differences were observed regarding $A\beta_{1-42}$ peptide (Table 11).

BIOMARKERS	Cases (n=120)	Controls (n=120)	*P*-value
Systemic inflammation			
-IL-6 (pg/mL)	17.9±6.0	5.4±1.1	**<0.001**
-IL-10 (pg/mL)	5.5±1.8	13.4±2.4	**<0.001**
Endothelial dysfunction			
-PTX3 (pg/mL)	1907.2±1295.7	557.0±296.3	**<0.001**
-sTWEAK (pg/mL)	197.9±93.6	27.6±17.3	**<0.001**
Aß peptides			
-$A\beta_{1-40}$ (pg/mL)	53.5±11.5	33.0±4.7	**<0.001**
-$A\beta_{1-42}$ (pg/mL)	50.5±15.2	48.6±9.9	0.253

Table 11. Serum levels of biomarkers in LI patients and controls.

5.5.1. IL-6 levels in sera according to periodontal status

Figure 11 depicts that the presence of PD was associated with higher mean IL-6 concentrations in both cases (21.0 vs. 10.2 pg/mL, P<0.001) and controls (6.6 vs. 4.8 pg/mL, P=0.002).

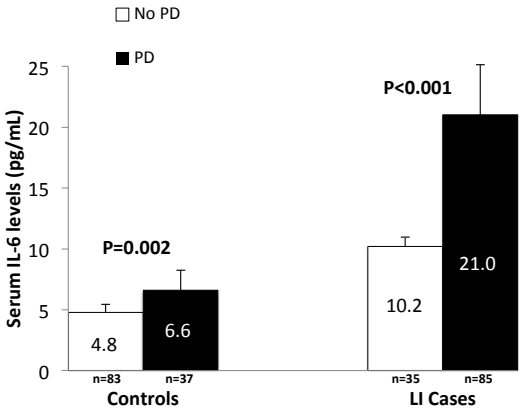

Figure 11. Serum IL-6 levels according to the presence or absence of PD.

5.5.2. IL-10 levels in sera according to periodontal status

As shown in Figure 12, the presence of PD was associated with lower mean IL-10 levels in the control group (11.9 vs. 14.0 pg/mL, P<0.001). No statistical differences were found in the LI group (5.7 vs. 5.0 pg/mL, P=0.740).

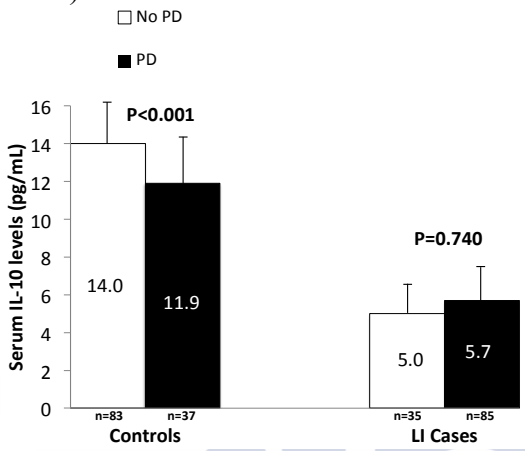

Figure 12. Serum IL-10 levels according to the presence or absence of PD.

5.5.3. PTX3 levels in sera according to periodontal status

A relationship was observed between the presence of PD and elevated levels of serum PTX3 in both groups. Among control subjects, this difference was modest and not significant. Within the cases, however, there was a highly significant increase in PTX3 levels associated with the presence of PD (2205.6 vs. 1182.6 pg/mL, P<0.001) (Figure 13).

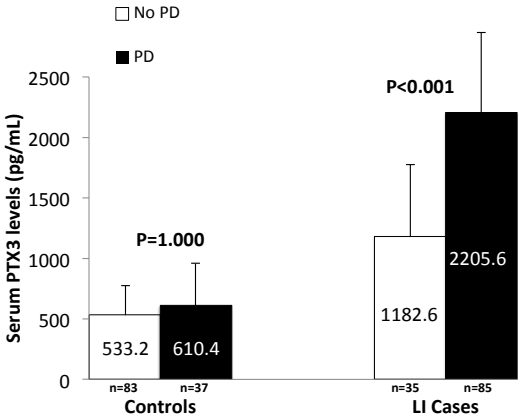

Figure 13. Serum PTX3 levels according to the presence or absence of PD.

5.5.4. sTWEAK levels in sera according to periodontal status

Similarly to IL-6, PD was associated with higher mean sTWEAK levels in both LI cases (240.7 vs. 40.7 pg/mL, P<0.001) and controls (45.6 vs. 19.5 pg/mL, P=0.029) (Figure 14).

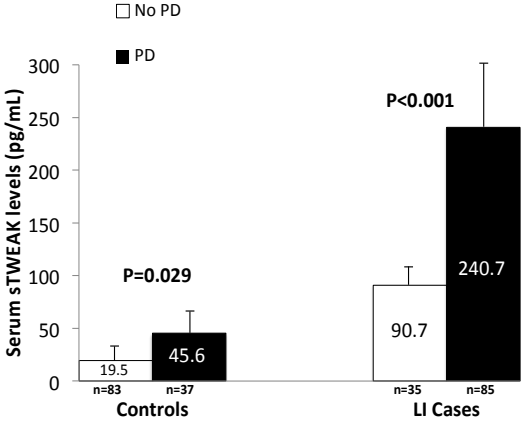

Figure 14. Serum sTWEAK levels according to the presence or absence of PD.

5.5.5. $A\beta_{1-40}$ levels in sera according to periodontal status

Figure 15 depicts that PD was associated with raised mean levels of $A\beta_{1-40}$ in both cases (58.7 vs. 41.0 pg/mL, P<0.001) and controls (36.6 vs. 31.4 pg/mL, P=0.001).

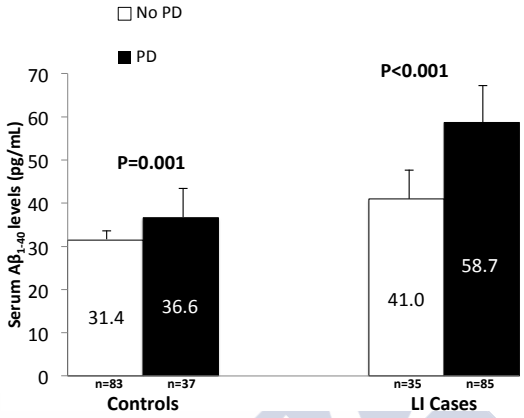

Figure 15. Serum $A\beta_{1-40}$ levels according to the presence or absence of PD.

5.5.6. $A\beta_{1-42}$ levels in sera according to periodontal status

No statistical differences were found in terms of $A\beta_{1-42}$ serum levels neither in the case group nor in the control group (Figure 16).

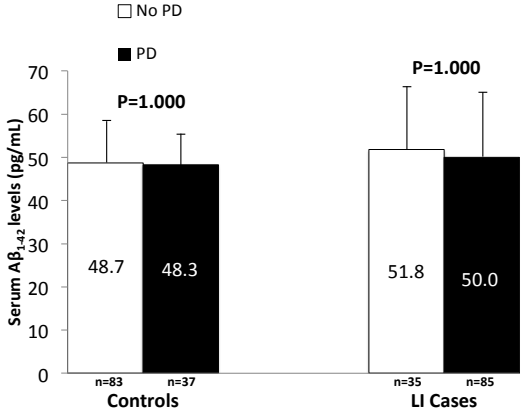

Figure 16. Serum $A\beta_{1-42}$ levels according to the presence or absence of PD.

5.6. Association between periodontal disease and periodontal inflamed surface area and elevated serum levels of biomarkers in lacunar infarct patients

5.6.1. IL-6

Mean serum IL-6 levels were significantly and positively associated with greater mean PISA (Model I: R^2=0.624, P<0.001). When PD exposure was examined as a categorical variable, multivariable linear regression analysis again indicated that PD in patients with LI was associated with significantly higher serum IL-6 concentrations (Model II: R^2=0.656, P<0.001) (Table 12).

	B	SE	PC	*P*-value
Model I (R^2=0.624)				
Age	0.080	0.043	0.177	0.065
Gender	-1.097	0.800	-0.131	0.173
Hypertension	-0.745	0.830	-0.086	0.372
Diabetes mellitus	0.483	0.872	0.053	0.581
Hypercholesterolemia	1.367	1.117	0.117	0.224
Ischemic heart disease	-0.580	0.997	-0.056	0.562
Peripheral arterial disease	0.944	1.833	0.050	0.608
Smoking habit	0.222	0.529	0.040	0.676
Statins	-0.652	1.179	-0.053	0.581
Leukoaraiosis	-0.163	0.831	-0.019	0.845
Carotid atheromatosis	0.006	0.771	0.001	0.994
PISA (mm^2)	0.004	0.000	0.791	**<0.001**
Model II (R^2=0.656)				
Age	-0.007	0.041	-0.017	0.862
Gender	-0.008	0.770	-0.001	0.992
Hypertension	-0.744	0.795	-0.090	0.351
Diabetes mellitus	0.561	0.835	0.065	0.503
Hypercholesterolemia	0.614	1.068	0.055	0.567
Ischemic heart disease	0.764	0.950	0.077	0.423
Peripheral arterial disease	-2.539	1.744	-0.139	0.148
Smoking habit	0.425	0.507	0.081	0.404
Statins	0.868	1.137	0.074	0.447
Leukoaraiosis	0.091	0.789	0.011	0.908
Carotid atheromatosis	-0.031	0.738	-0.004	0.966
PD	11.055	0.773	0.810	**<0.001**

Table 12. Multivariable linear regression models for IL-6 serum levels in LI patients.

5.6.2. PTX3

When examined as a continuous variable (i.e., PISA), multivariable regression analysis indicated that the presence of PD in patients with LI was associated with significantly higher mean PTX3 serum levels (Model I: R^2=0.260, P<0.001). In a model that included PD as a categorical variable and other confounding factors, PD was strongly associated with increased serum PTX3 levels (Model II: R^2=0.115, P<0.001) (Table 13).

	B	SE	PC	*P*-value
Model I (R^2=0.260)				
Age	21.073	12.856	0.129	0.104
Gender	-255.460	240.594	-0.084	0.291
Hypertension	-164.096	249.745	-0.052	0.513
Diabetes mellitus	216.444	262.332	0.065	0.411
Hypercholesterolemia	203.250	335.876	0.048	0.546
Ischemic heart disease	103.360	299.976	0.027	0.731
Peripheral arterial disease	-643.183	551.188	-0.092	0.246
Smoking habit	115.599	159.245	0.057	0.469
Statins	82.851	354.539	0.018	0.816
Leukoaraiosis	-457.492	249.888	-0.144	0.070
Carotid atheromatosis	142.866	231.941	0.049	0.539
PISA (mm^2)	0.609	0.096	0.502	**<0.001**
Model II (R^2=0.115)				
Age	10.830	14.076	0.074	0.443
Gender	-154.114	264.705	-0.056	0.562
Hypertension	-149.189	273.188	-0.053	0.586
Diabetes mellitus	246.879	286.878	0.083	0.391
Hypercholesterolemia	104.292	367.181	0.027	0.777
Ischemic heart disease	293.242	326.614	0.086	0.371
Peripheral arterial disease	-1105.987	599.569	-0.176	0.068
Smoking habit	132.839	174.300	0.073	0.448
Statins	200.735	390.968	0.050	0.609
Leukoaraiosis	-288.986	271.232	-0.102	0.289
Carotid atheromatosis	190.620	253.742	0.072	0.454
PD	1072.218	253.774	0.363	**<0.001**

Table 13. Multivariable linear regression models for PTX3 serum levels in LI patients.

5.6.3. sTWEAK

Mean sTWEAK levels in serum were significantly and positively associated with greater mean PISA (Model I: R^2=0.697, P<0.001). To be older, male as well as a previous history of hypercholesterolemia were also associated with increased serum levels of sTWEAK in LI patients. When PD exposure was examined as a categorical variable, multivariable linear regression analysis again indicated that PD in patients with LI was associated with significantly higher serum sTWEAK concentrations (Model II: R^2=0.527, P<0.001) (Table 14).

	B	SE	PC	*P*-value
Model I (R^2=0.697)				
Age	1.263	0.594	0.202	**0.036**
Gender	-25.424	11.178	-0.216	**0.025**
Hypertension	2.868	11.552	0.024	0.804
Diabetes mellitus	2.420	12.182	0.019	0.843
Hypercholesterolemia	32.471	15.521	0.199	**0.039**
Ischemic heart disease	-10.296	13.884	-0.072	0.460
Peripheral arterial disease	20.233	25.470	0.077	0.429
Smoking habit	8.280	7.359	0.109	0.263
Statins	-18.432	16.421	-0.108	0.264
Leukoaraiosis	10.351	11.554	0.087	0.372
Carotid atheromatosis	-18.675	10.717	-0.167	0.084
PISA (mm^2)	0.068	0.004	0.830	**<0.001**
Model II (R^2=0.527)				
Age	0.007	0.744	0.001	0.993
Gender	-11.654	14.044	-0.053	0.409
Hypertension	3.348	14.445	0.015	0.817
Diabetes mellitus	5.426	15.227	0.023	0.722
Hypercholesterolemia	20.759	19.396	0.068	0.287
Ischemic heart disease	11.515	17.286	0.042	0.507
Peripheral arterial disease	-33.204	31.666	-0.066	0.297
Smoking habit	10.965	9.207	0.075	0.236
Statins	-0.198	20.685	-0.001	0.992
Leukoaraiosis	21.767	14.330	0.096	0.132
Carotid atheromatosis	-16.336	13.401	-0.077	0.226
PD	149.082	14.099	0.670	**<0.001**

Table 14. Multivariable linear regression models for sTWEAK serum levels in LI patients.

5.6.4. $A\beta_{1-40}$

When examined as a continuous variable (i.e., PISA), multivariable regression analysis indicated that the presence of PD in patients with LI was associated with significantly higher mean $A\beta_{1-40}$ serum levels (Model I: R^2=0.653, P<0.001) along with a previous history of hypercholesterolemia. In a model that included PD as a categorical variable and other confounding factors, PD was strongly associated with increased serum $A\beta_{1-40}$ levels (Model II: R^2=0.467, P<0.001) (Table 15).

	B	SE	PC	*P*-value
Model I (R^2=0.653)				
Age	0.135	0.079	0.164	0.088
Gender	-2.478	1.474	-0.160	0.096
Hypertension	-1.209	1.530	-0.076	0.431
Diabetes mellitus	0.724	1.607	0.044	0.653
Hypercholesterolemia	4.133	2.058	0.191	**0.047**
Ischemic heart disease	-1.452	1.838	-0.076	0.431
Peripheral arterial disease	0.678	3.377	0.019	0.841
Smoking habit	0.367	0.976	0.036	0.707
Statins	-2.971	2.172	-0.131	0.174
Leukoaraiosis	0.876	1.531	0.055	0.568
Carotid atheromatosis	0.028	1.421	0.002	0.984
PISA (mm^2)	0.008	0.001	0.801	**<0.001**
Model II (R^2=0.467)				
Age	-0.014	0.098	-0.014	0.887
Gender	-0.808	1.837	-0.043	0.661
Hypertension	-1.097	1.896	-0.056	0.564
Diabetes mellitus	1.018	1.991	0.049	0.610
Hypercholesterolemia	2.762	2.548	0.104	0.281
Ischemic heart disease	1.096	2.266	0.047	0.630
Peripheral arterial disease	-5.705	4.160	-0.131	0.173
Smoking habit	0.666	1.209	0.053	0.583
Statins	-0.818	2.713	-0.029	0.764
Leukoaraiosis	2.356	1.882	0.120	0.213
Carotid atheromatosis	0.356	1.761	0.020	0.840
PD	17.273	1.844	0.671	**<0.001**

Table 15. Multivariable linear regression models for $A\beta_{1-40}$ serum levels in LI patients.

5.7. Predictors of poor prognosis in patients with lacunar infarct

5.7.1. Study groups – baseline characteristics

Of the 120 LI patients studied, 31 (25.8%) had a poor outcome at 3 months. Patients with poor outcome were significantly older and more likely to have a history of hypertension and diabetes mellitus. Accordingly, significantly more individuals in the poor outcome group were under hypertensive medication. Leukoaraiosis was present in 19 of 89 good outcome patients (21.3%) and in 16 of 31 patients with poor outcome (51.6%) (P=0.001). Furthermore, half of the poor outcome subjects showed SIs compared to a 33.7% in the good outcome group (P=0.038). No differences were observed between poor and good prognosis groups in relation to both demographic and vascular risk variables as well as LI location (Table 16).

VARIABLES	Good outcome (n=89)	Poor outcome (n=31)	*p*-value
Age (years)	65.5±10.6	69.1±7.3	**0.043**
Males, n (%)	65 (73.0)	17 (54.8)	0.061
BMI	27.4 [24.9-29.4]	27.0 [24.4-29.4]	0.854
Hypertension, n (%)	49 (55.1)	25 (80.6)	**0.012**
Diabetes mellitus, n (%)	19 (21.3)	14 (45.2)	**0.011**
Hypercholesterolemia, n (%)	38 (42.7)	17 (54.8)	0.243
Ischemic heart disease, n (%)	18 (20.2)	4 (12.9)	0.364
Peripheral arterial disease, n (%)	5 (5.6)	0 (0.0)	0.178
Smoking habit			0.641
-Never smoker, n (%)	59 (66.3)	23 (74.2)	
-Former smoker, n (%)	12 (13.5)	4 (12.9)	
-Current smoker, n (%)	18 (20.2)	4 (12.9)	
Alcohol consumption, n (%)	18 (20.2)	3 (9.7)	0.396
Medication			
-Statins, n (%)	38 (42.7)	18 (58.1)	0.140
-Antiaggregants, n (%)	33 (37.1)	13 (41.9)	0.632
-Antihypertensives, n (%)	46 (51.7)	24 (77.4)	**0.012**
Education level			0.477
-High, n (%)	20 (22.5)	10 (32.3)	
-Medium, n (%)	41 (46.1)	11 (35.5)	
-Low, n (%)	28 (31.5)	10 (32.3)	
Leukoaraiosis, n (%)	19 (21.3)	16 (51.6)	**0.001**
Carotid atheromatosis, n (%)	39 (43.8)	13 (41.9)	0.855
SIs, n (%)	30 (33.7)	17 (54.8)	**0.038**

LI location			0.878
-Hemispheric, n (%)	33 (37.1)	12 (38.7)	
-Basal ganglia, n (%)	46 (51.7)	15 (48.4)	
-Brainstem, n (%)	9 (10.1)	3 (9.7)	
-Other locations, n (%)	1 (1.1)	1 (3.2)	

Table 16. Baseline characteristics of LI patients according to functional outcome at 3 months.

5.7.2. Study groups – periodontal disease

The prevalence of PD in poor outcome subjects was significantly higher than those from the good outcome (90.3% vs. 64.0%, P=0.006) (Figure 17). There was an about 1.7-fold increase in the percentage of patients with poor outcome who suffered from severe PD compared with good outcome individuals (64.3% vs. 38.6, P=0.039) (Figure 18).

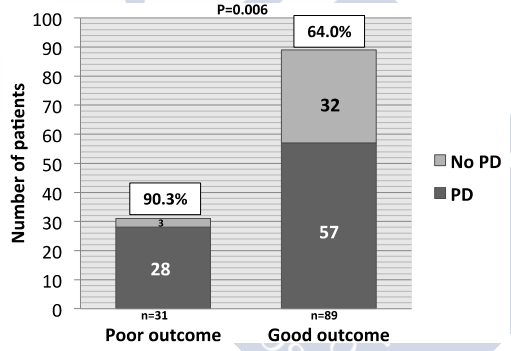

Figure 17. Prevalence of PD within LI patients according to functional outcome at 3 months.

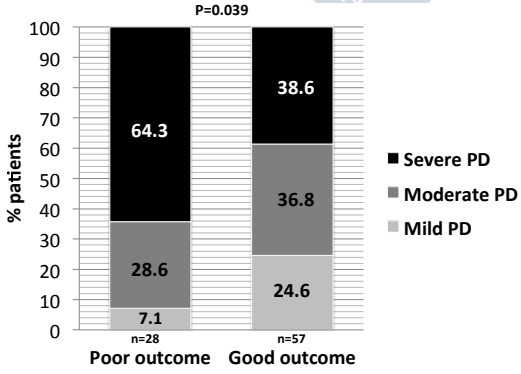

Figure 18. Severity of PD within LI patients according to functional outcome at 3 months.

Clinical periodontal parameters of current disease activity (i.e., FMBS, PPD, and PISA) were significantly elevated in patients with poor prognosis in comparison to good prognosis patients, whereas the main indicator of prolonged exposure to PD (i.e., CAL) did not differ between groups (Table 17).

VARIABLES	Good outcome (n=89)	Poor outcome (n=31)	*P*-value
FMPS (%)	54.7±17.0	54.9±19.3	0.951
FMBS (%)	57.5±19.0	65.1±14.7	**0.026**
PPD measures			
-Mean PPD (mm)	3.5±1.0	3.9±1.0	**0.046**
-Number of sites/mouth PPD ≥4 mm	69.6±49.1	93.7±53.6	**0.023**
-Number of sites/mouth PPD ≥6 mm	15.6±24.6	25.2±26.3	0.069
GR (mm)	0.6±0.5	0.6±0.4	0.542
CAL measures			
-Mean CAL (mm)	4.2±1.4	4.5±1.4	0.251
-Number of sites/mouth CAL ≥3 mm	114.5±39.3	121.3±39.9	0.414
-Number of sites/mouth CAL ≥5 mm	58.6±48.3	78.4±52.9	0.058
Number of present teeth	19.9±3.6	20.2±4.3	0.712
PISA (mm^2)	813.8±1041.5	1690.9±1198.6	**0.001**
Last dental visit			0.885
-Within the last 12 months, n (%)	53 (59.6)	18 (58.1)	
-Less often, n (%)	36 (40.4)	13 (41.9)	
Tooth brush frequency			0.480
-<2 times/day	31 (34.8)	13 (41.9)	
-≥2 times7day	58 (65.2)	18 (58.1)	
Use of interdental care devices, n (%)	5 (5.6)	4 (12.9)	0.185

Table 17. Clinical periodontal parameters and dental variables of LI patients according to functional outcome at 3 months.

5.7.3. Molecular analysis

Regarding serum biomarkers, the levels of IL-6, PTX3, sTWEAK, and $A\beta_{1\text{-}40}$ were significantly elevated in patients with poor outcome compared to those with good outcome (21.1 vs. 16.8 pg/mL, P<0.001; 2586.2 vs. 1670.7 pg/mL, P=0.007; 245.8 vs. 181.0 pg/mL, P=0.001; 60.2 vs. 51.2 pg/mL, P<0.001, respectively). No differences were observed in relation to IL-10 and $A\beta_{1\text{-}42}$ (Table 18).

BIOMARKERS	Good outcome (n=89)	Poor outcome (n=31)	*P*-value
Systemic inflammation			
-IL-6 (pg/mL)	16.8±5.9	21.1±5.2	**<0.001**
-IL-10 (pg/mL)	5.4±1.7	5.6±1.8	0.618
Endothelial dysfunction			
-PTX3 (pg/mL)	1670.7±1043.3	2586.2±1680.7	**0.007**
-sTWEAK (pg/mL)	181.0±88.8	245.8±91.4	**0.001**
Aß peptides			
-$A\beta_{1\text{-}40}$ (pg/mL)	51.2±11.0	60.2±10.7	**<0.001**
-$A\beta_{1\text{-}42}$ (pg/mL)	49.3±15.8	54.1±13.0	0.127

Table 18. Serum levels of biomarkers of LI patients according to functional outcome at 3 months.

5.7.4. Association between periodontal clinical parameters and poor outcome in patients with lacunar infarct

After adjusting for known poor prognosis factors, PISA and diabetes mellitus were the only predictors significantly associated with poor outcome in patients with LI (Table 19; Model I).

ROC analysis showed an area under the curve of 0.738 (95% CI: 0.632-0.844, P<0.001) (Figure 19), which suggest that a value ≥727 mm^2 predicted PISA association with poor outcome, with a sensitivity of 71% and a specificity of 70%. Therefore, after categorizing PISA (<727 mm^2 and ≥727 mm^2), this indicator of current PD activity along with a previous history of diabetes still reached statistical significance independently of both clinical (Table 19; Model II) and molecular variables (Table 19; Model III).

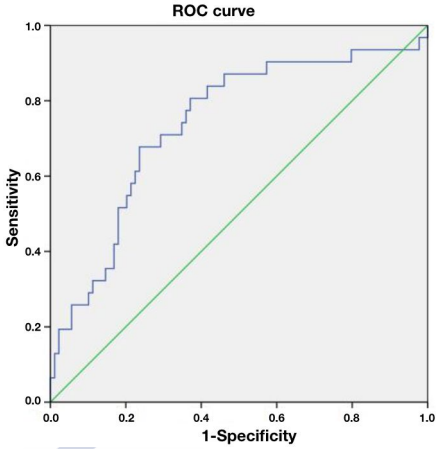

Figure 19. ROC analysis, in which poor outcome (mRS > 2) was the state variable and PISA the test variable.

Independent variables	OR	95% CI	*P*-value
Model I			
Age	1.010	0.953-1.071	0.726
Gender	2.424	0.826-7.113	0.107
Hypertension	2.267	0.702-7.323	0.171
Diabetes mellitus	2.814	1.002-7.903	**0.049**
Leukoaraiosis	1.816	0.640-5.155	0.262
SIs	1.789	0.668-4.792	0.248
FMBS	1.021	0.988-1.055	0.218
Mean PPD (mm)	0.992	0.575-1.711	0.976
PISA (mm^2)	1.001	1.000-1.001	**0.016**
Model II			
Age	1.014	0.956-1.077	0.639
Gender	2.202	0.712-6.810	0.171
Hypertension	2.597	0.754-8.954	0.131
Diabetes mellitus	3.430	1.136-10.354	**0.029**
Leukoaraiosis	1.879	0.637-5.544	0.253
SIs	1.537	0.556-4.254	0.407
FMBS	1.024	0.990-1.059	0.173
Mean PPD (mm)	0.871	0.489-1.552	0.639
PISA ≥ 727 mm^2	6.573	2.079-20.782	**0.001**
Model III			
IL-6 (pg/mL)	0.967	0.801-1.167	0.726
PTX3 (pg/mL)	1.000	1.000-1.001	0.262
sTWEAK (pg/mL)	0.996	0.984-1.009	0.559
$A\beta_{1-40}$ (pg/mL)	1.051	0.948-1.166	0.342
Diabetes mellitus	3.740	1.366-10.238	**0.010**
PISA ≥ 727 mm^2	5.189	1.070-25.157	**0.041**
Dependent variable: mRS > 2 (yes/no)			

Table 19. Logistic regression models for clinical and molecular predictors of poor outcome in LI patients.

5.7.5. Correlation between periodontal inflamed surface area and significant biomarkers in patients with poor outcome

A positive and strong correlation was found between PISA and IL-6 (r=0.738, P<0.001; Figure 20), PTX3 (r=0.468, P=0.008; Figure 21), sTWEAK (r=0.771, P<0.001; Figure 22), and $A\beta_{1\text{-}40}$ (r=0.745, P<0.001; Figure 23) in patients with poor prognosis.

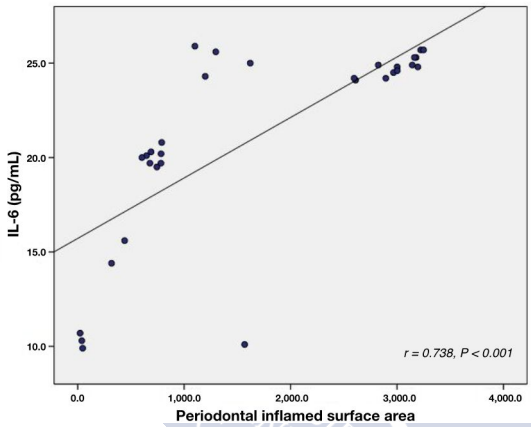

Figure 20. Correlation between PISA and IL-6 in patients with poor functional outcome.

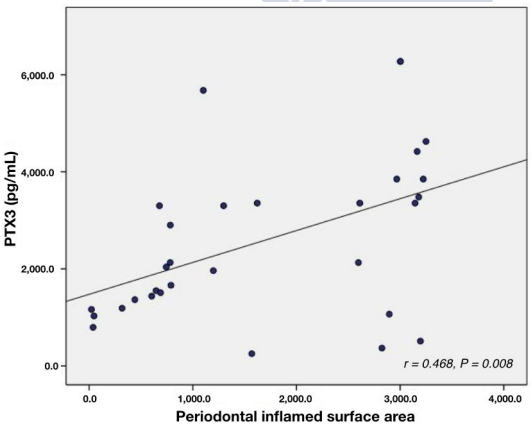

Figure 21. Correlation between PISA and PTX3 in patients with poor functional outcome.

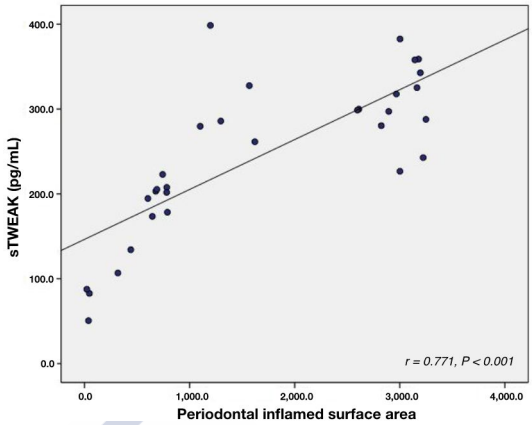

Figure 22. Correlation between PISA and sTWEAK in patients with poor functional outcome.

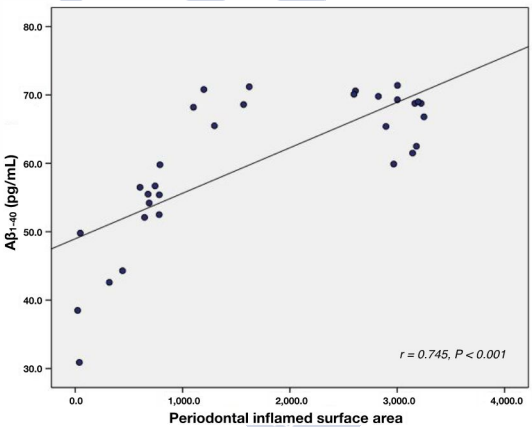

Figure 23. Correlation between PISA and $A\beta_{1-40}$ in patients with poor functional outcome.

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EXPERIMENTAL STUDY: complementary investigation

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EXPERIMENTAL STUDY: complementary investigation

1. JUSTIFICATION

Results from the clinical study showed that PD is prevalent in LI patients and when present it contributes to elevated serum levels of pro-inflammatory mediators and markers of endothelial dysfunction. Although this association was independent of other contributing factors, some conditions (i.e., ageing, hypertension or diabetes) as well as asymptomatic CSVD subtypes (i.e., leukoaraiosis) are also of great importance in the pathophysiology of LI. In order to investigate whether PD could be one of the main contributors to a low-grade chronic systemic inflammation state leading to endothelial dysfunction in LI, it makes reasonable to carry out a complementary experimental study using a PD-induced model in rats.

2. HYPOTHESIS

Based on the results obtained in the clinical study, we hypothesized that experimental PD in systemically healthy rats may evoke a mild acute inflammation effect (i.e., significant increase of IL-6 and PTX3 within the first week after PD induction) followed by dysfunction of the endothelium (i.e., significant increase of PTX3, sTWEAK and $A\beta_{1\text{-}40}$), thus, demonstrating that PD may be a significant contributor to an increased systemic pro-inflammatory state and endothelial dysfunction leading to a higher risk for developing LI.

3. OBJECTIVE

- To investigate in systemically healthy rats whether PD is associated with an enhanced inflammatory response and

vascular endothelial impairment measured by the same biomarkers analysed in the clinical study that were linked with an increased risk of LI.

4. MATERIAL AND METHODS

4.1. Experimental periodontitis model

4.1.1. *Porphyromonas gingivalis* lipopolysaccharide-induced periodontitis

Pg has been identified as a major aetiological factor in the pathogenesis of PD (van Winkelhoff et al. 2002). The LPS component of the cell wall of this bacterium and other gram-negative microorganisms is a significant inflammatory stimulus that triggers an innate immune response. Indeed, is responsible for the recruitment of PMNs, oedema, and vascular dilation in inflammatory periodontal tissue (Page and Schroeder 1981) as well as cytokines secretion, lytic enzyme production, and osteoclast activation (Jiang et al. 2002). Therefore, this model produces a histopathological aspect similar to that observed in established human PD, characterized by increased infiltration of leukocytes, higher levels of pro-inflammatory cytokines, collagen degradation, and alveolar bone loss (Graves et al. 2012).

There are other models of PD induction such as ligature and oral gavage models (Graves et al. 2008). Ligature model is indicated for situations in which an acute development of PD is required, as the thread around the tooth promotes rapid and severe bone destruction (de Molon et al. 2013). On the other hand, the oral gavage model is considered to be a chronic model of PD owing to the longer period of time required to evoke bone loss in comparison to other models (de Molon et al. 2013). The LPS model is considered to be a direct and easy method for the induction of controlled experimental PD because bone loss is localized, the stimulus is constant, and the alveolar bone destruction usually occurs within 7 days after the start of the bacterial LPS injections (Dumitrescu et al. 2004). This model is useful in

investigating the host-bacteria interaction and the activation of signalling pathways, in the studying of several pro-inflammatory and vascular function mediators, and in testing the performance of a specific cell or molecule in the pathogenesis process. However, the main limitation of the LPS model is the lack of bacterial colonization and the need for constant injections throughout the experiment (Table 20).

Hence, according to the purpose of the experimental study, it seems reasonable to use the *Pg*-LPS model for PD induction.

Model	Advantages	Disadvantages
Ligature	• Acute model for PD • Rapid and severe bone destruction • Bone loss occurs predictably over a period of 7 days	• Periodontal destruction may be aggravated by mechanical lesion • Host response decreases periodontal destruction • No significant gingival inflammation or bone loss in germ-free rats
LPS injection	• Accurate time of infection • More direct and controlled PD model • Greater experimental control over the pathogenic stimulus	• Lack of bacterial colonization • Need for constant injections throughout the experiment
Oral gavage	• Chronic model for PD • Easier and simple induction of PD • Establish a relationship between induction of PD and systemic conditions • Examines the impact of various components of the host responses	• Requirement of longer periods of time to produce bone loss • Host response decreases periodontal destruction

Table 20. Summary of advantages and disadvantages of the different rodent models of PD induction. Adapted from Graves et al. (2013).

4.1.2. Experimental design

An experimental study was carried out in the Clinical Neurosciences Research Laboratory of the University Clinical Hospital of Santiago de Compostela. The experimental protocol was approved by the Research Commission of the University Clinical Hospital of Santiago de Compostela. All experimental procedures were performed according to the Animal Care Committee European Union rules and the Spanish regulation (86/609/CEE, 2003/65/CE, 2010/63/EU, RD1201/2005 and RD53/2013). The Animal Research Reporting of In Vivo Experiments (ARRIVE) guidelines were followed in this experiment (Kilkenny et al. 2010).

Prior to PD induction, MRI and micro-CT (μCT) examination along with blood extraction (i.e., baseline) were carried out in all animals. In order to induce PD, *Pg*-LPS injections were performed on Mondays, Wednesdays, and Fridays during two weeks. Another MRI analysis together with a visual gingival analysis with a microscope was performed immediately before the second round of injections. After the last couple of injections, MRI and μCT scan were done to confirm periodontal tissue inflammation and alveolar bone loss. A set of blood extractions was carried out 1 day, 1, 2, and 3 weeks after the last couple of injections. Once all serum samples were stored, determinations of several biomarkers were done (Figure 24).

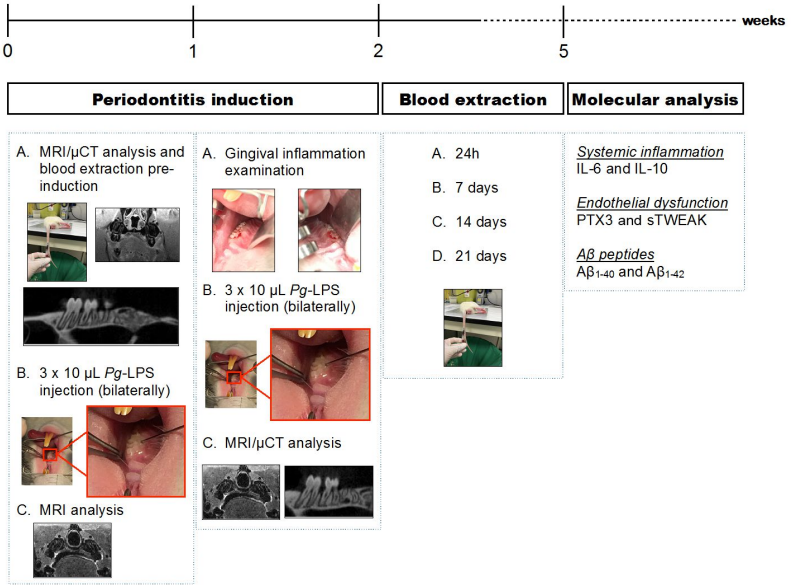

Figure 24. Experiment outline.

4.1.3. Animals and Anaesthesia

Six male Sprague-Dawley rats of 7 weeks of age and weighted between 300 and 350 g were used. Animals were housed individually, in stable environmental conditions (environmental temperature of 23ºC), relative humidity of 40% and a light-dark cycle of 12h, as well as free access to food and water.

Each animal was initially placed into an induction chamber attached to a sevoflurane anaesthetic vaporizer and anaesthesia was induced with 6% sevoflurane in a gas of 70% NO_2 and 30% O_2, followed by the application of a nose cone with 4% sevoflurane in the same proportion of the aforementioned gases to maintain anaesthesia during the experimental procedures. During surgery, all animals were subjected to temperature control, maintaining temperature at 37±0.5ºC by a thermostat-controlled electric pad (NeoBiotect, Spain) (Figure 25).

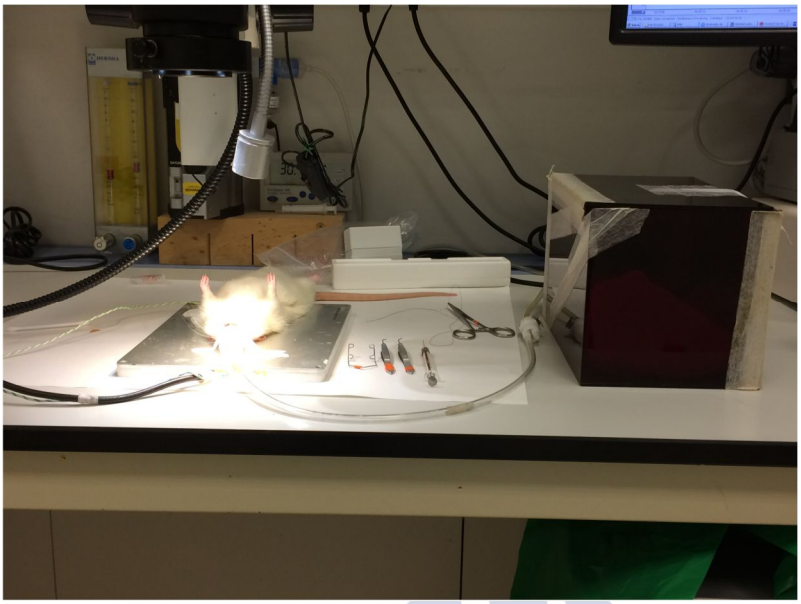

Figure 25. Experiment setting.

4.1.4. *Porphyromonas gingivalis* lipopolysaccharide preparation

Aliquots of 10 µL were prepared adding 1 mL of homogenized endotoxin-free water to 1 mg LPS from Pg (lyophilized). In total, 27 aliquots were prepared and stored at 4ºC, of which 24 were needed for the purpose of the study.

4.1.5. Periodontal induction procedure

The palatal gingiva between the first and second maxillary molars in both sides (i.e., right and left side) was injected with 10 µL of a saline solution containing 1 mg/mL LPS from *Pg* (tlrl-pglps, InvivoGen, San Diego, CA) using a Hamilton microsyringe (Agilent, Santa Clara, CA, USA) equipped with a blunted edge 30-gauge needle. This injection was followed by two additional injections at 48-hour intervals as described previously (Kador et al. 2011). This protocol was repeated the following week as recommended by Elburki and co-workers (Elburki et al. 2014). Therefore, PD induction lasted 14 days with a total

of 6 injections of *Pg*-LPS per side. In order to have a good access to perform the injections, the animals were placed facing up and the mouth was maintained open with a special microsurgical separator (Figure 26).

All experiments were performed by a single surgeon (YL).

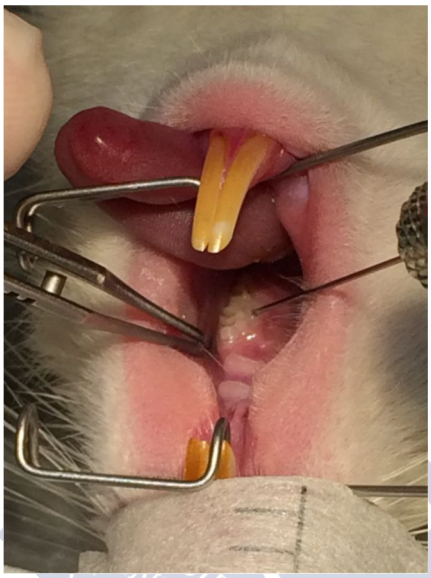

Figure 26. *Pg*-LPS injection.

4.2. MRI analysis

All animals were anesthetized by inhalation of 5% sevoflurane in a NO_2/O_2 mixture (70/30). Rectal temperature was maintained at 37±0.5ºC by using a feedback-controlled heating pad (Figure 27).

Figure 27. Animal preparation setting prior to MRI analysis.

4.2.1. MRI examination

All studies were conducted on a 9.4 T horizontal bore magnet (Bruker BioSpin, Ettlingen, Germany) with 440 mT/m gradients and a combination of a linear birdcage resonator (7 cm in diameter) for a signal transmission and a 2 x 2 surface coil array for signal detection (Figure 28).

Figure 28. MR unit.

4.2.2. MRI assessment

MR mouth localizer images in axial and coronal orientations were determined to then position accurately the slices of the molars to study, corresponding to RARE T2 sequence: with an echo time (EcT)=11.7 ms, repetition time (RT)=2s, rare factor=4, flip angle (FA)=180º, number of averages (NA)=1, spectral bandwidth (SW)=50 KHz, 10 slices of 1 mm, 25 x 25 mm^2 FOV (with saturation bands to suppress signal outside this FOV), a matrix size of 192 x 192 (isotropic in-plane resolution of 130 μm/pixel x 130 μm/pixel) and implemented without fat suppression option.

The progression of the periodontal lesions was determined from T_2-w) and T_1-w images acquired 0, 7 and 14 days after the onset of periodontal induction. Axial T_1-w were obtained with the use of RAREVTR sequence: with an EcT=8.44 ms, RT=12s, rare factor=4, FA=180º, NA=3, SW=50 KHz, 20 slices of 0.5 mm, 19.2 x 19.2 mm^2 FOV (with saturation bands to suppress signal outside this FOV), a matrix side of 192 x 192 (isotropic in-plane resolution of 100 μm/pixel x 100 μm/pixel) and implemented without fat suppression option. Axial T_2-w were obtained with the use of RARE T2 sequence: with an EcT=12.66 ms, RT=2s, rare factor=4, FA=114º, NA=6, SW=50 KHz, 20 slices of 0.5 mm, 30 x 30 mm^2 FOV (with saturation bands to suppress signal outside this FOV), a matrix side of 256 x 256 (isotropic in-plane resolution of 117 μm/pixel x 117 μm/pixel) and implemented without fat suppression option.

4.2.3. Data processing

Images were processed using ImageJ (Rasband WS, National Institutes of Health, Bethesda, MD, USA) on an independent computer workstation. From the T_1-w and T_2-w images, the signal intensity (SgI) of the palatal mucosa within the molar region was studied (first and second upper molars) (Figure 29). In order to focus accurately in the same plane of the mouth every week, planes were adjusted taking into account the localizer images (Figure 30). The regions of interest (ROIs)

were evaluated using the following equation and procedure: SgI=[(ROI mean)/(muscle ROI mean)]*100 (Lee et al. 2014). Location and dimension of the lesions were determined from T_1-w images as a relative palatal thickness.

All MRI analyses were performed by a single physicist (RI-R).

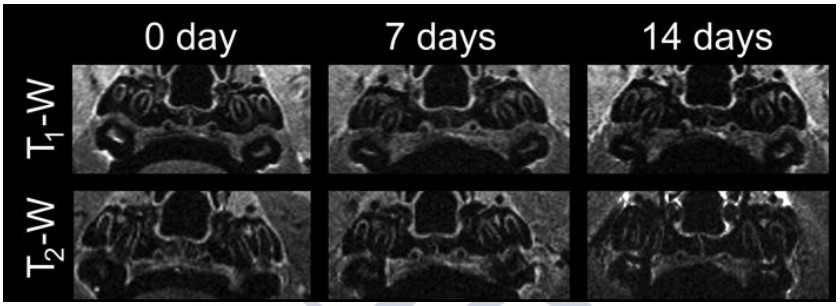

Figure 29. T_1-w and T_2-w images of periodontal inflammation evolution affecting the first upper molars.

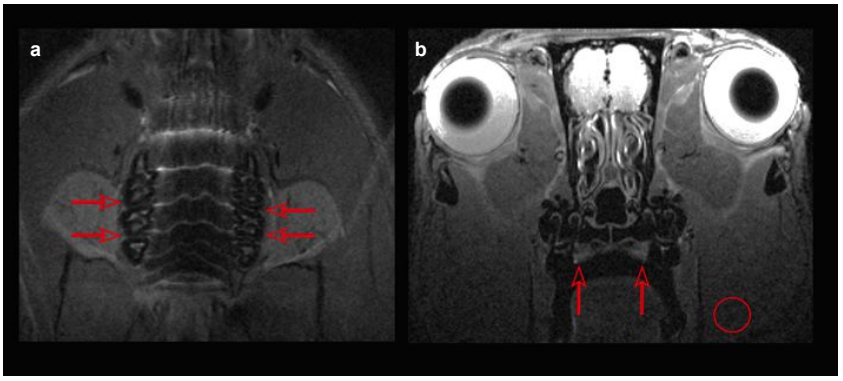

Figure 30. a: Coronal MR localizer image. In order to locate the same plane every week, red arrows indicate the planes of study. b: Representative axial T_2-w weighted image. Red arrows show the position of the molars evaluated. The location of the region of interest (red circle) was used to measure the muscle signal intensity.

4.3. µCT analysis

All animals were anesthetized by inhalation of 5% sevoflurane in a NO_2/O_2 mixture (70/30) using a bed centered in the head of the animal. Rectal temperature was maintained at 37±0.5ºC by using a feedback-controlled heating pad.

4.3.1. µCT examination

Maxillae were scanned using a µCT scanner (Bruker BioSpin, Woodbridge, Connecticut, USA) (Figure 31) with a voxel size of 0.045 mm (isotropic voxel) and X-ray energy of 45 kV and 400 µA. Each scan was conducted over a period of 35 min. at 0 and 14 days (Figure 31).

Figure 31. µCT unit.

4.3.2. Alveolar bone loss measurement

To quantify de amount of bone loss, the bone level was measured at the sagittal plane of both sides of each animal. Crossing the

interproximal contact point of the first and second molars crown, the distance between de cemento-enamel junction (CEJ) and the alveolar bone crest were measured for the distal surface of the first molar and the mesial surface of the second molar just below the contact point and 0.2 mm palatal to the contact point (Hiyari et al. 2015) (Figure 32).

All μCT analyses were performed by a single biologist (NG-L).

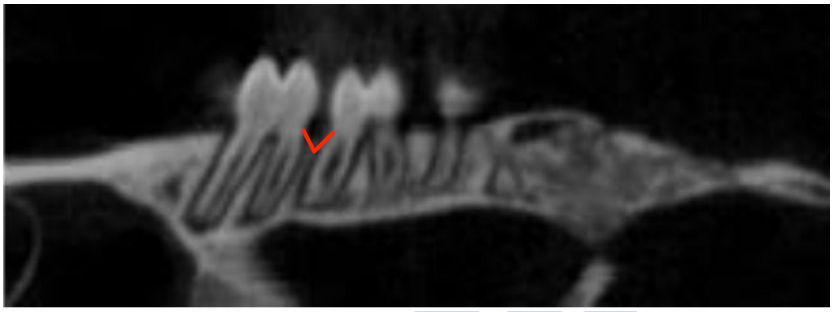

Figure 32. The distance from the CEJ to the alveolar crest was measured at the sagittal plane intersecting the interproximal molars. Red lines depict the measurement that was taken for distal of first molar and mesial of second molar.

4.4. Serum collection and laboratory tests

Prior to PD induction (baseline) and 24 h, 7, 14 and 21 days after the last couple of injections, 1800 μL of venous blood were collected from the tail of each animal by venepuncture using a 22-gauge needle with a 1 mL syringe (Figure 33). Blood samples were allowed to clot at room temperature and after 1 hour, serum was separated from blood by centrifugation (7 min. at 3000 g) and 700 μL of extracted serum was immediately transferred to 1.5 mL aliquots. Each aliquot was stored at –80ºC until the time of analysis. Serum levels of all biomarkers were measured by ELISA technique following manufacturer instructions. IL-6 ELISA kit (Picokine™, Boster Biological Technology, Pleasanton, California, USA) minimum assay sensitivity was 5.0 pg/ml with a intra-assay CV of 1.7%; IL-10 ELISA kit (Picokine™, Boster Biological Technology, Pleasanton, California, USA) minimum assay sensitivity was 4.0 pg/ml with a intra-assay CV of 7.4%; PTX3 ELISA kit (Fine

Test, Wuhan Fine Biotech, Wuhan, China) minimum assay sensitivity was 0.094 ng/ml, with a intra-assay CV of 1.5%; sTWEAK ELISA kit (Fine Test, Wuhan Fine Biotech, Wuhan, China) minimum assay sensitivity was 9.375 pg/ml, with a intra-assay CV of 4.4%; $A\beta_{1-40}$ ELISA kit (Fine Test, Wuhan Fine Biotech, Wuhan, China) minimum assay sensitivity was 46.875 pg/ml, with a intra-assay CV of 3.2%; and $A\beta_{1-42}$ ELISA kit (Fine Test, Wuhan Fine Biotech, Wuhan, China) minimum assay sensitivity was 9.375 pg/ml, with a intra-assay CV of 2.5%. Determinations were performed in the Clinical Neurosciences Research Laboratory.

Figure 33. Blood extraction from vein tail.

4.5. Euthanasia

After the last blood extraction and with the animal fully anaesthetised, sacrifice was performed by 2 mL intracardiac injection of potassium chloride.

4.6. Statistical analysis

All data analyses were performed with IBM SPSS Statistics 20.0 software for Mac (SPSS Inc., Chicago, IL, USA). Mean and standard deviation was calculated for continuous variables, after the method of Shapiro-Wilk was applied to confirm that the data were sampled from a normal distribution. Paired *t* test and analysis of variance for repeated measures were used to compare differences over time. Additionally, post hoc comparisons were carried out using Bonferroni corrections.

All tests were performed at a significance level of $\alpha = 0.05$.

5. RESULTS

5.1. Periodontal inflammation

Gingival inflammation was evident at day 7 of the experiment affecting the palatal side of the first and second molar region in both sides of the upper jaw (Figure 34).

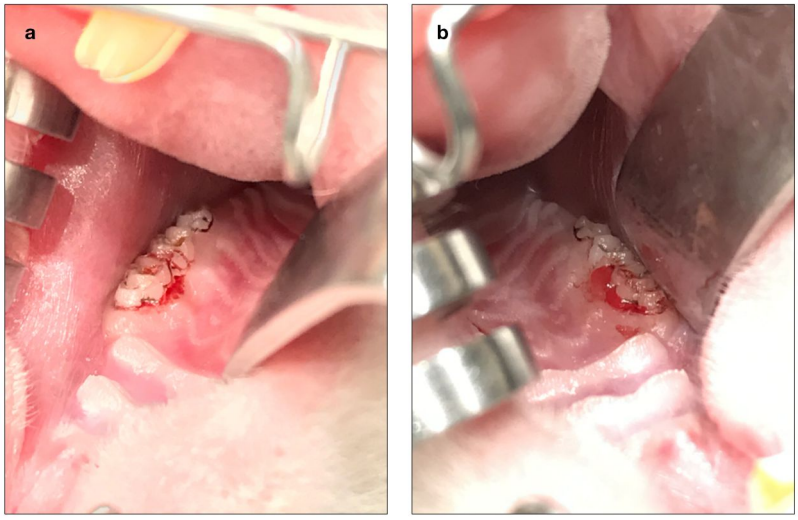

Figure 34. a: Gingival inflammation at 7 days (first upper left molar).
b: Gingival inflammation at 7 days (first upper right molar).

Periodontal inflammation was confirmed by means of MRI analysis. Differences in relative T_1 and T_2 signal intensities were found at 7 and 14 days after periodontal induction onset compared to baseline (Figure 35 and 36). When relative T_2-w signal intensity was analysed in the first and second upper right molar, statistical differences were observed between 7 and 14 days ($P<0.05$).

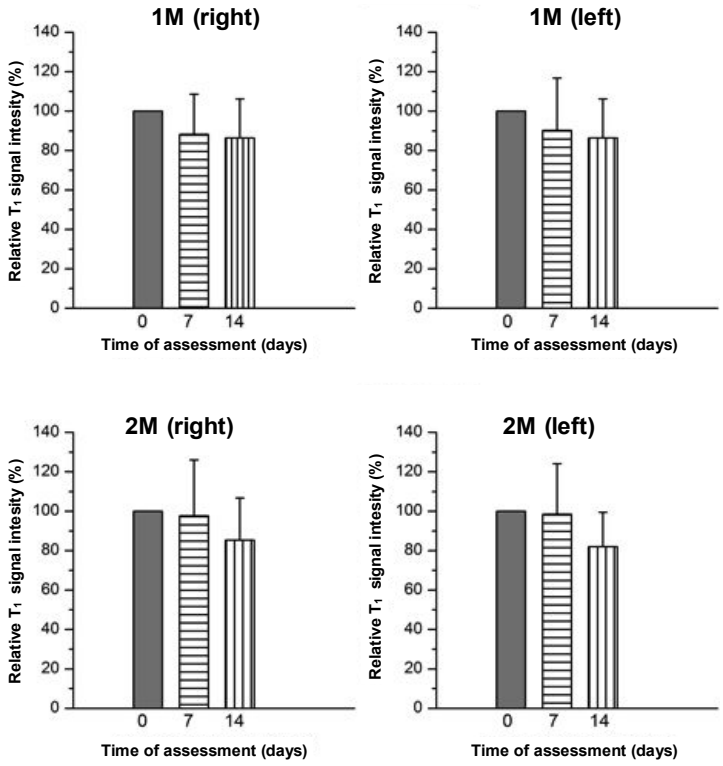

Figure 35. Relative T_1-w signal intensity.

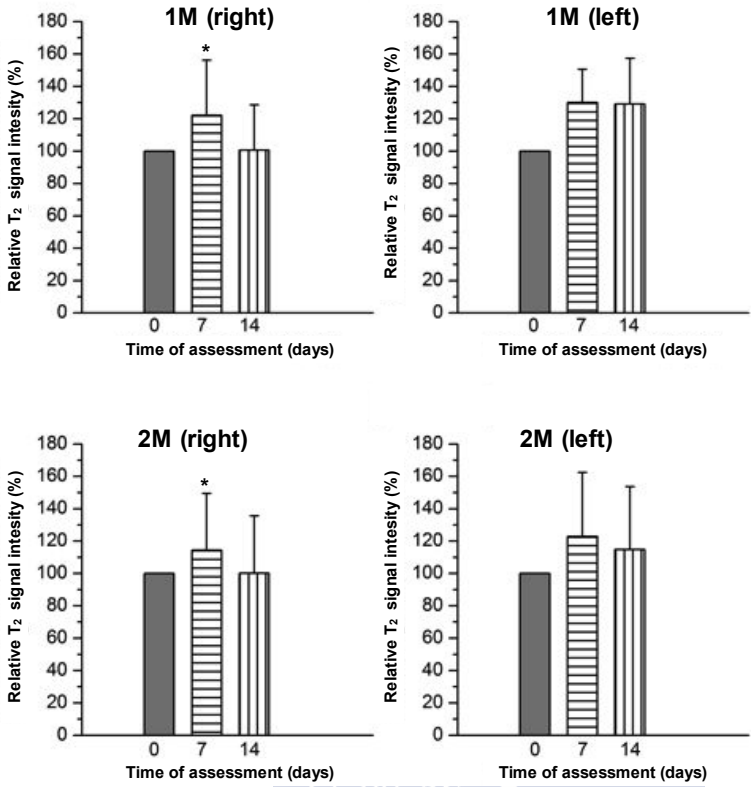

Figure 36. Relative T_2-w signal intensity. *7 vs. 14 days, P<0.05.

In terms of palatal thickness, which we considered as a surrogate measure of oedema, an increase in the relative percentage was found due to periodontal inflammation. In fact, statistically significant differences were observed for this measure in the second upper right molar between 7 days of periodontal induction and baseline (Figure 37).

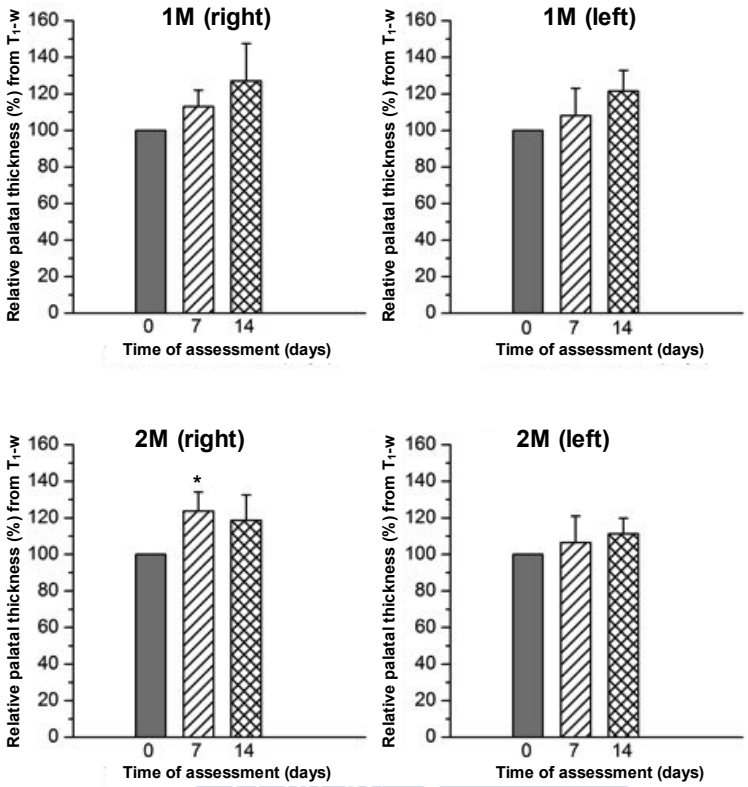

Figure 37. Relative palatal thickness (%) from T_1-w. *0 vs. 7 days, P<0.01.

5.2. Alveolar bone loss

The μCT analysis revealed statistical significant alveolar bone loss at the interproximal space after periodontal induction (i.e., 14 days) between the first and second maxillary molars bilaterally at the LPS-injected sites compared to baseline (Figure 38). Indeed, the distance between the CEJ and the bone crest was significantly greater at 14 days in both sides of the upper jaw compared to baseline measurements (Figure 39 and 40).

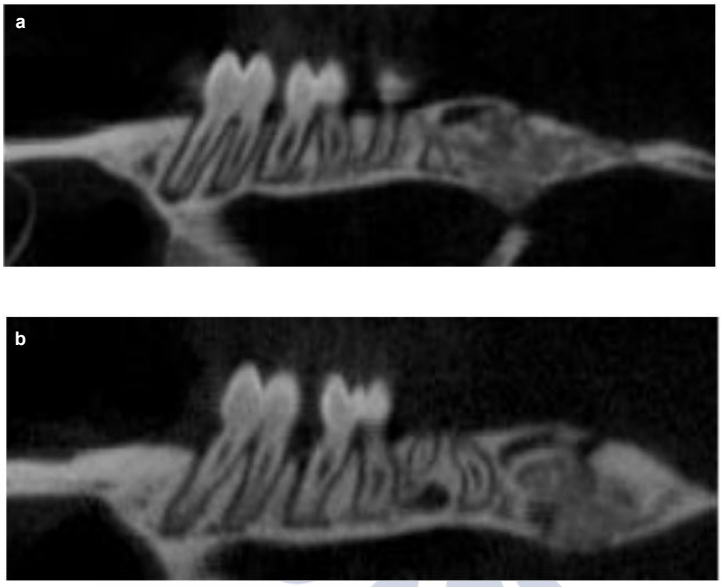

Figure 38. a: µCT image of upper molars at baseline. b: µCT image of upper molars at 14 days.

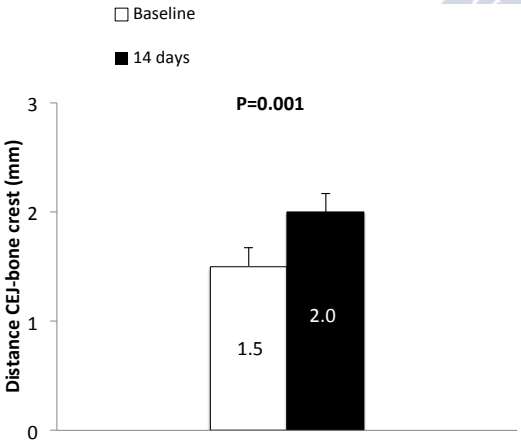

Figure 39. Alveolar bone loss in the upper right side.

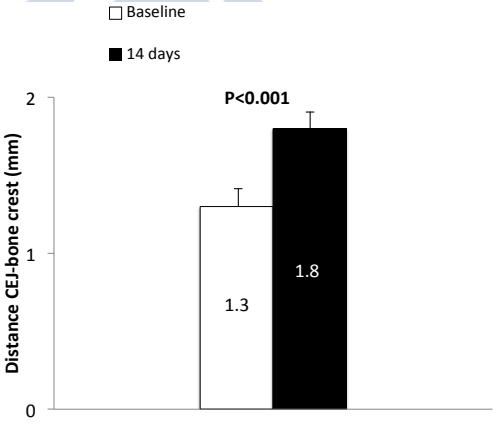

Figure 40. Alveolar bone loss in the upper left side.

5.3. Biomarkers

5.3.1. Systemic inflammation

A sharp increase was observed for IL-6 24 h after periodontal induction, which confirms its nature as an early inflammatory mediator. The levels of IL-6 decreased during the following 3 weeks but still were significantly higher compared to baseline (Figure 41).

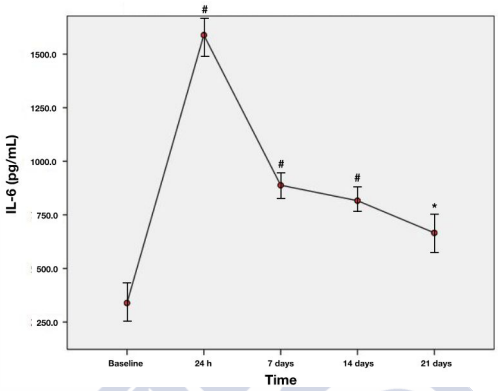

Figure 41. Changes in serum levels of IL-6 (pg/mL) at 24h, 7, 14, 21 days after periodontal induction. #P<0.001 compared to baseline, *P<0.05 compared to baseline.

On contrary, PD evoked a significant decrease in IL-10 serum levels at 24 h after the last LPS-injection, and the levels of this anti-inflammatory mediator continued to reduce up to 21 days (Figure 42).

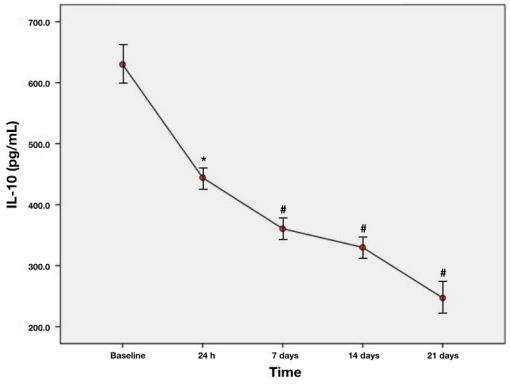

Figure 42. Changes in serum levels of IL-10 (pg/mL) at 24h, 7, 14, 21 days after periodontal induction. #P<0.001 compared to baseline, *P<0.05 compared to baseline.

5.3.2. Endothelial dysfunction

A mild acute significant increase in PTX3 levels at 24h following periodontal induction was observed, which was more pronounced as time was drawn on (Figure 43). Levels of sTWEAK were significantly elevated 1 week after the last LPS-injection compared to baseline and continued to increase in the following weeks (Figure 44).

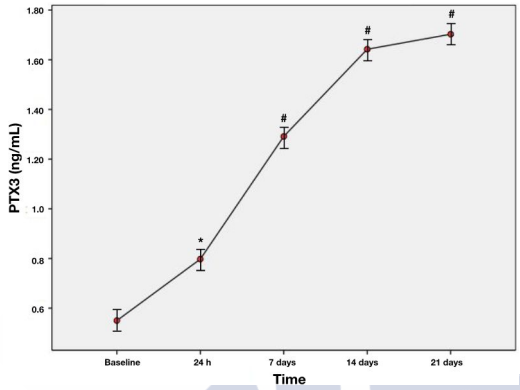

Figure 43. Changes in serum levels of PTX3 (ng/mL) at 24h, 7, 14, 21 days after periodontal induction. #P<0.001 compared to baseline, *P<0.05 compared to baseline.

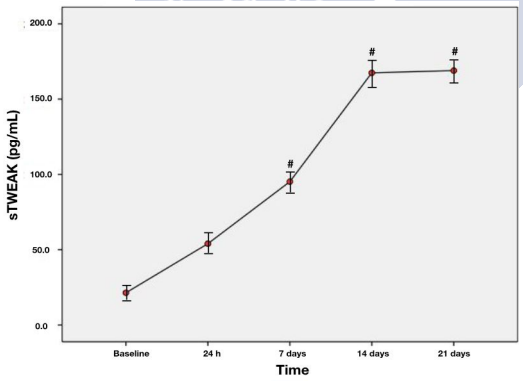

Figure 44. Changes in serum levels of sTWEAK (pg/mL) at 24h, 7, 14, 21 days after periodontal induction. #P<0.001 compared to baseline, *P<0.05 compared to baseline.

5.3.3. Aβ peptides

Similarly to PTX3, PD evoked a slight acute elevation of $A\beta_{1-40}$ serum levels that reached statistical significance compared to baseline and was maintained during the following two weeks. However, at day 21, a reduction in the levels of this biomarker was observed (Figure 45). Following experimental PD, $A\beta_{1-42}$ peptide levels peak much latter than the other endothelial dysfunction biomarkers (i.e., 21 days), confirming its nature of cognitive decline molecule (Figure 46).

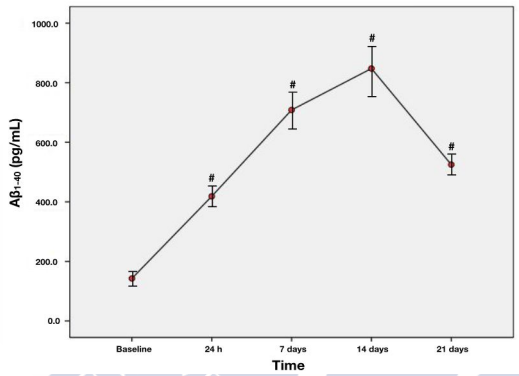

Figure 45. Changes in serum levels of $A\beta_{1-40}$ (pg/mL) at 24h, 7, 14, 21 days after periodontal induction. #P<0.001 compared to baseline, *P<0.05 compared to baseline.

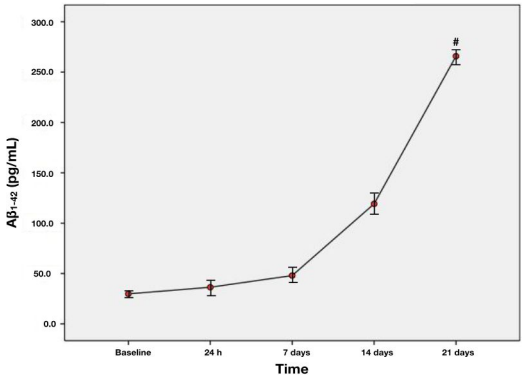

Figure 46. Changes in serum levels of $A\beta_{1-42}$ (pg/mL) at 24h, 7, 14, 21 days after periodontal induction. #P<0.001 compared to baseline, *P<0.05 compared to baseline.

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DISCUSSION

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DISCUSSION

In our study we have demonstrated that PD is very common and is present in almost three-quarters of our patients with LI. When present, PD is strongly associated with LI and appears to be a powerful and independent contributor to elevated levels of IL-6, PTX3, sTWEAK, and $A\beta_{1\text{-}40}$, thus, giving insight of PD as a potential source of systemic inflammation and vascular endothelial dysfunction in patients with LI. Moreover, we found that moderate to severe periodontal inflammation measured with the PISA method emerged as a significant predictor of poor functional outcome at 3 months in LI patients due to the potential enhanced systemic inflammatory state posed by PD and the consequent disruption of endothelial vascular function.

The prevalence of PD in our LI group (70.8%) was more than double compared to our control group without any neurological disorder (30.8%) and, accordingly was almost 2 times what is common in the Spanish general population (38.4%) (Carasol et al. 2016). Similar results have been previously reported in a case-control pilot study, in which it was found that PD was present in 69.4% of patients with LI in comparison with 31.7% of PD prevalence in healthy controls (Leira et al. 2016).

Epidemiological evidence has shown a link between PD and ischemic stroke. Recently, a meta-analysis of observational studies demonstrated that PD increased the risk of large vessel ischemic strokes by 2.5-fold in a combined analysis of three prospective studies (Leira et al. 2017b). Sub-analysis of case-control studies showed that the magnitude of the association was even higher (RR=3.04; 95% CI: 1.10-8.43) (Leira et al. 2017b). In the present study, we found a positive association between PD and the LI, independent of other well-known

risk factors for cerebrovascular diseases (OR=3.3; 95% CI=1.7-6.4, P<0.001). In addition, advanced forms of PD were also strongly associated with an increased risk of LI (OR=9.8; 95% CI: 2.4-38.9, P<0.001). Similarly to previous studies, history of hypertension (OR=3.1; 95% CI: 1.6-5.8, P<0.001 and diabetes mellitus (OR=2.5; 95% CI: 1.1-6.2, P=0.037) together with current smoking (OR=4.4; 95% CI: 1.9-10.5, P=0.001) were also significantly associated with the presence of LI (Bezerra et al. 2012).

Our results are in contrast with recent findings from the ARIC study, where authors found that PD was associated with both incident atherothrombotic and cardioembolic stroke but not with LI (Sen et al. 2018). There are several possible factors attributable to this difference. Firstly, periodontal case definition used in the ARIC study was based on periodontal profile classes rather than the one recommended in the last consensus for periodontal epidemiologic studies and the used in our study (Eke et al. 2012; Holtfreter et al. 2015). This fact, might have led to bias when a subject was classified as having PD and, therefore, to analyse the potential association between PD and LI. Secondly, no medication use was recorded in the ARIC study. There are certain types of drugs that could influence the periodontium. For instance, statins have an anti-inflammatory effect that was demonstrated to reduce gingival inflammation due to CRP and MMPs reduction as well as enhance bone regeneration by inhibiting osteoclast formation (Estanislau et al. 2015). Antiaggregants may lead to gingival bleeding and some anti-hypertensives could cause gingival overgrowth, hence, it would be of interest to record the use of them. In our study, the prevalence of patients with LI who consumed these drugs was significantly higher than those in the control group. In the logistic regression model, adjustment of statins was done in order to eliminate potential bias in the relationship between PD and LI. Because of the "collinearity phenomenon", the other medications were not adjusted in our analysis. Thirdly, the limited number of incident cases of LI makes difficult to state robust conclusions (n=61). In the present study, 120 LI patients were included and analysed in depth in terms of neuroimaging and ultrasound examination. Indeed, we found that patients with PD and

LI had significantly higher prevalence of leukoaraiosis and carotid atheromatosis compared to those LI patients without PD (37.6% vs. 8.6%, P=0.001; 49.4% vs. 28.6%, P=0.036; respectively). In this sense, findings from a Korean study in which 438 subjects free from dementia and stroke underwent dental examination showed that tooth loss, considered as the final stage of PD, was associated with the presence of leukoaraiosis (Minn et al. 2013). What is more, the greater teeth lost the greater risk for having leukoaraiosis (Minn et al. 2013). Regarding carotid atheromatosis, a large body of evidence suggests that PD is associated with increased carotid IMT, which is a measure of subclinical atherosclerosis (Beck et al. 2001; Hayashida et al. 2013; Cairo et al. 2009). Furthermore, LI patients might also present abnormal values of IMT (Nagai et al. 2002), hence, supporting our results that atheromatosis measured on carotid artery was more common in periodontal patients with LI than in periodontally healthy subjects diagnosed with LI.

Because PISA reflects the amount of periodontal inflamed tissue, it is believed to be an accurate method to assess both infectious and inflammatory burden posed by PD (Nesse et al. 2008; Leira et al. 2018). In fact, in our LI patients the mean estimated surface area of the periodontal ulcerated epithelium was 10.4 cm^2 compared to 1.9 cm^2 corresponding to the control group. Such a wound surface must be regarded as significant. It is speculated that the inflamed and ulcerated subgingival pocket epithelium forms an easy port of entry for periodontal bacteria either producing bacteremias or endotoxemias (Loos et al. 2005). Accordingly, our analysis provided evidence of a positive association between this continuous measure of PD activity (i.e., PISA) and LI (OR=1.001, 95% CI: 1.001-1.002, P<0.001). A previous study demonstrated that PPD was increased in LI, but failed to demonstrate a relationship between clinical periodontal parameters and the presence of LI (Taguchi et al. 2013). Similarly, although our patients with LI showed worse periodontal conditions in terms of past (i.e., CAL) and current PD (i.e., FMBS and PPD), none of the remaining clinical periodontal parameters were significantly associated with a higher risk of LI.

Besides the association study, we carried out a cross-sectional analysis examining potential biological pathways that could explain the relationship found between PD and LI. It has to be highlighted that, although we have examined the presence of PD at the time of LI, the natural history of PD suggests that this process was present long before the vascular acute event. The connective supporting tissue breakdown accompanied with alveolar bone loss and deep periodontal pocket formation is a prolonged process involving a chronic local gram-negative infection and a profound local inflammatory response. Without treating the disease, the breakdown of the oral-systemic barrier with ulceration of the pocket epithelium allows entry of local pro-inflammatory mediators and bacterial components into the peripheral blood circulation (Deliargyris et al. 2004). In our study, we observed higher IL-6 and PTX3 serum levels in the group of LI with PD, fact that it could be explained by the chronic underlying systemic inflammatory up-regulation posed by PD. Accordingly, in the animal study we found that both IL-6 and PTX3 act as acute-phase inflammatory mediators in the presence of PD that remained significantly elevated even three weeks after periodontal induction compared to basal levels of these biomarkers (prior to experimental PD).

Our results are in accordance with previous human and animal studies, in which it was demonstrated that PD might elicit an overexpression of these two pro-inflammatory with systemic consequences (Geivelis et al. 1993; Costa et al. 2010; Shimada et al. 2010; Pradeep et al. 2011; Brito et al. 2013; Keles et al. 2012). When multivariable linear regression analysis was performed, a continuous measure of current PD activity (i.e., PISA) was independently associated with increased serum IL-6 and PTX3 levels (R^2=0.624, P<0.001; R^2=0.260, P<0.001; respectively). Accordingly, PD emerged as the only significant contributor to elevated levels of IL-6 and PTX3 (R^2=0.656, P<0.001; R^2=0.115, P<0.001; respectively). Higher IL-6 levels in serum were associated with poor functional prognosis in LI patients (Blanco et al. 2006; Castellanos et al. 2002; Rodríguez-Yáñez et al. 2006). This could explain a significantly higher prevalence of our LI patients with poor outcome in the group of PD compared to those LI

patients without PD (32.9% vs. 8.6%, P=0.006). In order to confirm that PD elicit a systemic inflammatory response, we also measured an anti-inflammatory mediator (i.e., IL-10). Our results showed that controls had significantly higher serum levels of IL-10 compared to LI patients. This finding is in accordance with other study in which patients with LIs and neurological deterioration had lower IL-10 levels in plasma than those without worsening (Vila et al. 2003). Furthermore, patients with PD in the control group had significantly lower levels of IL-10 compared to those controls without PD, thus, demonstrating its role in terms of pro-inflammatory cytokines and alveolar bone resorption inhibition (Boyle et al. 2003). This observation was confirmed in the preclinical study, where we clearly observed that levels of this anti-inflammatory molecule decreased significantly from basal to the end of the experiment.

It is suggested that PD might elicit impaired EDV, the hallmark of endothelial dysfunction (Gurav et al. 2014). In the present study, elevated serum levels of vascular endothelial dysfunction biomarkers (i.e., PTX3, sTWEAK and $A\beta_{1\text{-}40}$) were found in periodontal patients with LI compared to those LI subjects without PD and non-LI individuals. Although PTX3 is an APR, it is produced by macrophages or ECs in response to an inflammatory stimulus related with atherosclerosis (Bonacina et al. 2013; Fornai et al. 2016) and is associated with diminished FMD, thus, being a more powerful predictor of endothelial dysfunction than CRP (Yasunaga et al. 2014). Within the LI group, mean PISA was significantly and positively associated with increased sTWEAK levels in serum (R^2=0.697, P<0.001). To be older, male as well as a previous history of hypercholesterolemia were also associated with elevated serum levels of sTWEAK in this group of patients. When PD exposure was examined as a categorical variable, emerged as the single strongest contributor to increased sTWEAK levels in serum (R^2=0.527, P<0.001). TWEAK is also induced by ECs or SMCs, and is involved in the expression of adhesion molecules in ECs and pro-inflammatory mediators (Harada et al. 2002; Saas et al. 2000). In periodontal tissues with PD, it appears to be overexpressed (Kataria et al. 2010). Animal models of MCAO showed increased levels

of TWEAK and Fn14 in the area surrounding the necrotic core (Yepes et al. 2005) and its ability to induce inflammatory mediators and MMP-9 leading to BBB disruption and increased permeability (Polavarapu et al. 2005). Therefore, it seems plausible that release of sTWEAK by PD could predispose to disruption of the BBB in patients with LI and, thus, leading to dysfunction of the endothelium. In our preclinical study, we found that sTWEAK reached the peak latter than PTX3, at 14 days after periodontal induction and increased levels were sustained until the end of the experiment. At 7 days, however, significant differences were observed in comparison to basal serum levels of sTWEAK.

Regarding Aβ peptides, while levels of serum $A\beta_{1-40}$ were significantly higher in LI patients than in controls, no differences were observed in $A\beta_{1-42}$ serum levels. Furthermore, patients who had PD presented significantly increased $A\beta_{1-40}$ levels compared to those with a healthy periodontium independent of the study group. Multivariable linear regression analysis again showed that both PISA and PD as a categorical variable were the main contributors to elevated levels of in serum together with hypercholesterolemia (R^2=0.653, P<0.001; R^2=0.467, P<0.001; respectively). Our animal model confirmed that PD was the main contributing factor to elevated serum levels of $A\beta_{1-40}$, showing statistical differences from basal at 24 h post-induction and were sustained until the end of the experiment. These findings are of great importance, due to the fact that it has been demonstrated that $A\beta_{1-40}$ has a physiopathological role in disrupting the endothelial vascular function in LI patients (Gomis et al. 2009). Endothelial dysfunction by Aβ deposition has been related to LIs and leukoaraiosis (van Dijk et al. 2004; Gurol et a. 2006). APP is cleaved by secretases to produce $A\beta_{1-40}$ and $A\beta_{1-42}$ among others. Preclinical data supports the hypothesis that $A\beta_{1-40}$ but not $A\beta_{1-42}$ causes cerebrovascular dysfunction mediated by ROS (Niwa et al. 2000a; 2000b). Surprisingly, it was at the end of our experiment (i.e., 21 days post-induction) when $A\beta_{1-42}$ serum levels showed a significant increase. It has been suggested that PD is involved in the synthesis and accumulation of Aβ in the brain (Kubota et al. 2014; Kamer et al. 2015). Serum $A\beta_{1-42}$ levels from patients diagnosed with cognitive impairment who had severe PD were significantly higher than

those without cognitive decline or those with lower levels of PD (Gil-Montoya et al. 2017). This fibrillogenic peptide $A\beta_{1-42}$ deposits early in senile amyloid plaques and is associated with AD. Because recent evidence suggests that PD could be associated with AD (Leira et al. 2017c), our experimental data might give some insight of the potential role of PD in the pathogenesis of AD and the cognitive decline process.

Recently, it has been proposed a pathogenic model in which periodontal infection could be associated with CSVD (Ihara and Yamamoto 2016). *Pg* was found in atherosclerotic plaques and has been linked with increased risk of ischemic stroke (Pussinen et al. 2007). Briefly, *Pg* adheres to and infects ECs not only to increase the expression of endothelial adhesion molecules and promote monocyte/macrophage infiltration but also to produce gingipains, which activate protease-activated receptors-1 and 4 on platelets to induce platelet aggregation (Lourbakos et al. 2001; Walter et al. 2004). On the other hand, *Pg* and its endotoxins may spill into the systemic circulation triggering systemic inflammatory processes (Moutsopoulos and Madianos 2006). Thus, infection from *Pg* could cause CSVD pathology through two mechanisms: a) directly producing vasculopathy with thrombotic occlusion leading to LI, or b) leading to BBB disruption through inflammation (Ihara and Yamamoto 2016). Based on our results, we confirm the latter hypothesis. Chronic increase of serum IL-6, PTX3, sTWEAK, and $A\beta_{1-40}$ concentrations in patients diagnosed with PD contributes to an enhanced systemic inflammatory state promoting endothelial dysfunction that produces BBB disruption and increased permeability, which in turn predisposes to arteriolar lumen narrowing leading to LI. Taken together all our data, PD seems to affect the brain not only causing inflammation but also disrupting the endothelial vascular function. The proximity between the oral cavity and brain may very well contribute to the pathogenesis of CSVD because of the ease in transmission of oral microbiota and its components as well as pro-inflammatory mediators via blood circulation to the basal location of the brain (Ihara and Yamamoto 2016).

In general, LIs have a good prognosis, as mortality is low and functional recovery is usually good (Petty et al. 2000; Fisher 1982). Nevertheless, in one third of patients suffering from LIs a certain grade of dependency could be seen as a result of a worse outcome (Clavier et al. 1994; Yokota et al. 2004; Samuelsson et al. 1996). Similarly to previous reports, in the present study 31 out of 120 patients with LI (25.8%) had a poor functional outcome at 3 months (Petty et al. 2000; Clavier et al. 1994; Yokota et al. 2004; Samuelsson et al. 1996; Roquer et al. 2004). Ageing, diabetes mellitus, hypertension, leukoaraiosis, and SIs on CT at admission have been recognised as potential predictors for poor prognosis (Samuelsson et al. 1996; Norrving 2003; De Jong et al. 2002; Blanco et al. 2006). In our sample, we found that patients with poor outcome were older and more likely to have a previous history of hypertension and diabetes mellitus. The prevalence of leukoaraiosis and SIs was also significantly higher in the poor outcome group compared to the good outcome group. Our group has reported that increased levels of serum pro-inflammatory molecules (i.e., IL-6 and TNF-α) could also be predictive factors of poor outcome in patients with LI (Blanco et al. 2006; Castellanos et al. 2002). As expected, we observed significantly elevated serum levels of IL-6, PTX3, sTWEAK, and $A\beta_{1\text{-}40}$ in poor functional outcome patients compared to those with good prognosis.

PD was more frequent in LI patients with poor prognosis than the subjects of the good outcome group (90.3% vs. 64.0%, P=0.006). While clinical periodontal parameters of current disease activity (i.e. FMBS, PPD, and PISA) were significantly elevated in poor outcome patients, the main indicator of prolonged exposure to PD (i.e., CAL) did not show statistical differences between the two groups. Importantly, our findings show that periodontal inflammation measured by the PISA method was significantly associated with poor outcome at 3 months. When PISA was categorized, a value ≥727 mm^2 (moderate to severe periodontal cases) (Leira et al. 2018) showed to be an independent powerful predictor of poor outcome (OR=6.5; 95% CI: 2.0-20.7, P=0.001). Nevertheless, history of diabetes mellitus also seems to predict poor functional prognosis in our LI patients (OR=3.4; 95% CI: 1.1-10.3, P=0.029). A previous study that included 230 ischemic stroke

patients showed that patients with advanced PD had greater neurological deficit on admission as well as poor functional outcome at 3 months compared to those without PD or with mild PD (Slowik et al. 2010). In PD it appears that the host overreacts to infectious stimuli by releasing increased amount of inflammatory molecules (i.e., IL-1, IL-6 and TNF-α) that may be disseminate to systemic circulation (Loos et al. 2000, 2005), which may contribute to pathogenesis of cerebral ischemia by accelerating brain atheroma plaques progression (Leira et al. 2015). Furthermore, it is suggested that systemic inflammation may be involved in early neurological deterioration in LI patients (Blanco et al. 2006; Castellanos et al. 2002). Because the PISA reflects the amount of periodontal inflamed tissue, our study showed that it could be an accurate method to assess both infectious and inflammatory burden posed by PD and, therefore, may predict worse outcome in LI patients with a diagnosed of moderate to severe PD. In our study, we found that PISA was as independent predictor of poor functional outcome after adjustment of pro-inflammatory and endothelial dysfunction biomarkers (OR=5.1; 95% CI: 1.0-25.1, P=0.041). Previously, it has been shown an association between high PISA values and HbA1c levels in diabetics (Nesse et al. 2009) as well as with decreased kidney function (Iwasaki et al. 2012). In accordance, our results demonstrate that PISA was independently related to worse functional prognosis. Moreover, the prevalence of severe PD was significantly higher in poor prognosis group compared with good outcome subjects (64.3% vs. 38.6%, P=0.039). Thus, a cut-off value of PISA $\geq$737 mm^2 (moderate to severe periodontal cases) could be a strong predictor of poor functional outcome in LI patients showing a sensitivity of 71% and a specificity of 70%.

The present findings could be explained if the link between increased periodontal inflammation and poor stroke outcome was indeed mediated by the inflammatory response and the consequently vascular dysfunction of the endothelium. We found that PISA was positively correlated with serum concentrations of IL-6 (r=0.738, P<0.001), PTX3 (r=0.468, P=0.008), sTWEAK (r=0.771, P<0.001), and $A\beta_{1\text{-}40}$ (r=0.745, P<0.001) in LI patients with poor functional

outcome. Therefore, it could be speculated that moderate to severe PD, when is active, predicts poor outcome at 3 months in LI, and this clinical scenario is mediated not only by enhanced systemic inflammation but also due to disruption of the vascular function of the endothelium posed by PD.

The role of infection in stroke prognosis has been studied before (Klimiec et al. 2016, 2018). Endotoxin (i.e., LPS derived form gram-negative bacteria such as periodontopathogens) activity increases during acute phase of ischemic stroke (Klimiec et al. 2016). The stimulation of TLR-4 by circulating endotoxin can potentially contribute to systemic inflammation by the release of pro-inflammatory mediators. In turn, systemic inflammation is associated with unfavourable outcome in stroke patients (Castellanos et al. 2002; Dziedzik et al. 2015). Additionally, circulating LPS can stimulate TLR-4 receptors in the brain and it has been shown that TLR-4 expressed on microglia, astrocytes and neurons participate in stroke-induced brain injury (Gesuete et al. 2014) and enhanced expression of TLR-4 on monocytes is associated with poor functional prognosis after stroke (Brea et al. 2011). Increased levels of key players in LPS signalling such as soluble CD14 (sCD14) and LPS binding protein (LBP) predicted a risk of death in ischemic stroke patients and together with elevated LPS activity were associated with post-stroke delirium (Klimiec et al. 2018). It was found that higher levels of PTX3 were independently associated with increased long-term mortality after ischemic stroke; thus, suggesting that this APR may be used as a powerful prognostic biomarker in patients with ischemic stroke (Ryu et al. 2012). Moreover, within LI patients, it has been demonstrated that IL-6, TNF-α and CRP were predictors of worse functional outcome (Blanco et al. 2006; Castellanos et al. 2002; Chei et al. 2011). This hypothesis is supported by experimental data suggesting a link between systemic inflammation and poor stroke outcome. Systemic inflammation induced by peripheral IL-1β challenge in the focal cerebral ischemia animal model of MCAO caused an alteration in the kinetics of BBB disruption through MMP-9 activity, hence, demonstrating a direct relationship between infection, systemic

inflammation and exacerbation of ischemic brain injury (McColl et al. 2008). Other experiment using the same animal model found that systemic IL-1β caused severe reduction in cerebral blood flow and increase in infarct volume. Restriction in cerebral blood flow together with activation of the cerebral vasculature and up-regulation of ET-1, which is a potent endogenous vasoconstrictor produced by ECs, in the ischemic penumbra. Additionally, blockage of ET-1 receptors reversed this hypoperfusion, reduced tissue damage, and improved functional outcome (Murray et al. 2014). These results, therefore, demonstrated that a systemic pro-inflammatory state might lead to persistent deficits in perfusion after reopening of an occluded cerebral vessel. Based on our data from the animal study in which experimental PD was associated with increased levels of IL-6 and PTX3, PD could be responsible for the aforementioned systemic pro-inflammatory state that might be involved in poor prognosis in LI patients.

In our study, sTWEAK also seems to be involved in the association between PD and poor prognosis in LI patients. It is suggested that the TWEAK-Fn14 axis within the neurovascular unit (i.e., EC-basement membrane-astrocyte interface) mediates the passage of inflammatory cells into the ischemic tissue leading to increased BBB permeability and cerebral oedema, which in turn might produce ischemic injury and neuronal death (Yepes 2013). In human studies, sTWEAK levels on admission were associated with an adverse short-term outcome in patients diagnosed with acute myocardial infarction (Chorianopoulos et al. 2010) and in CKD, its correlates with atheromatosis progression (Fernández-Laso et al. 2017), diminished kidney function and aggravation of the endothelial dysfunction (Yilmaz et al. 2009b). Our experimental results showed that sTWEAK was increased in rats with PD and gingival tissues of PD patients overexpress TWEAK (Kataria et al. 2010) that is responsible for inducing endothelial adhesion molecules such as VCAM-1 and ICAM-1 (Hosokawa et al. 2006), which are elevated in poor outcome stroke patients at 3 months (Castellanos et al. 2002; Richard et al. 2015). Regarding the latter CAM, besides its increase in LI patients with poor prognosis, an association was found between this molecule and early neurological

deterioration in these patients (Castellanos et al. 2002). Hence, it could be plausible that PD via sTWEAK chronic release could be related to poor functional outcome in our LI patients through endothelial vascular activation.

Ultimately, it has been proposed that APP and Aβ may also participate in ischemic brain damage. Even though AAP expression is increased in the post-ischemic brain (Banati et al. 1995), cerebral ischemia may facilitate cleavage of AAP into the toxic Aβ fragment (Yokota et al. 1996). These observations raise the possibility that ischemia leads to accumulation of Aβ, which in turn, could contribute to ischemic brain damage and poor outcome in stroke patients. Using the MCAO animal model, transgenic mice expressing AAP showed a more pronounced reduction of the cerebral blood flow than control animals and vasodilation in AAP trangenics was reduced by an 82%, thus, demonstrating that AAP overexpression increased the susceptibility of the brain to ischemic injury (Zhang et al. 1997). More experimental data demonstrated that circulating $A\beta_{1\text{-}40}$ is sufficient to induce cerebrovascular dysfunction (Park et al. 2013). $A\beta_{1\text{-}40}$ can cross the BBB (Zlokovic 2008) and administration of exogenous $A\beta_{1\text{-}40}$ into the circulation could conceivably enter the brain especially if the BBB is altered (Clifford et al. 2007). A pathogenic model has been proposed where LPS from gram-negative bacteria binds to TLR-4/CD14 receptors on peripheral monocytes/macrophages, neutrophils and on brain microglia (Zhan et al. 2018). TLR-4/CD14 activation by LPS leads to NF-κB mediated induction of cytokines in monocytes/ /neutrophils in blood and from microglia in brain. Since LPS does not enter normal brain when given alone (Banks and Erickson 2010; Banks et al. 2015), it is likely that other factors contribute to LPS entry into ageing brain including cerebral ischemia or hypoxia. Disrupted BBB might aid LPS to enter into the brain. Once LPS entered brain it would bind TLR-4/CD14 receptors on microglia that would activate NF-κB mediates increases of intracerebral cytokines. LPS induction of cytokines can increase APP and Aβ accumulation, which in turn can act on TLR-4 creating a positive feedback loop to increase Aβ (Zhan et al. 2018). LPS also acts on the BBB to decrease Aβ exit from brain (Banks

et al. 2015). As discussed before, it has been demonstrated that $A\beta_{1-40}$ has an important role in disrupting the endothelial vascular function (Gomis et al. 2009). Since levels of $A\beta_{1-40}$ were increased in our experimental PD, it could be plausible that $A\beta_{1-40}$-mediated PD might be associated with poor outcome in LI patients.

The present study has several limitations. The retrospective nature of our study design does not allow establishing a causal association between PD and LI, hence, based on our results PD could be considered as a risk indicator rather than a risk factor of LI. We did not measure brachial FMD, which is a validated surrogate of endothelial dysfunction and widely used. It would have been of interest to correlate our novel endothelial dysfunction biomarkers with FMD measures and to analyse if they are more specific to detect impaired vascular endothelium in LI patients with PD. However, it has been demonstrated that in presence of endothelial dysfunction, both abnormal PTX3 and sTWEAK serum levels are associated with impaired FMD (Yanusaga et al. 2014; Yilmaz et al. 2009b, 2011). Although BBB biomarkers (e.g., MMPs or cellular fibronectin) would also be of importance to analyse due to BBB disruption is present in the pathophysiology of LI, in our study we measured serum levels of sTWEAK and $A\beta_{1-40}$, as they are associated with increased permeability and disruption of the BBB. ApoE polymorphism could influence aggregation and clearance of Aβ and this was not registered in our study. Nevertheless, some controversy exists regarding the association between ApoE carriers and increased Aβ levels (van Dijk et al. 2004; Gurol et a. 2006). A word of caution is due in particular regarding the PISA method. The formulas that transform CAL and GR into surface area are based on mean values of both root surface area and root lengths, therefore, leading to bias when PISA is calculated (Nesse et al. 2008). Patients with gingival overgrowth due to antihypertensives may also influence PISA calculation, resulting in an underestimation of true PESA and thereby underestimate true PISA owing to the fact that the gingival margin is located above the CEJ (Nesse et al. 2008). The use of either antiaggregants or statins may also influenced PISA values because of either increased or reduced gingival bleeding, respectively.

Furthermore, smoking produces vasoconstriction (Bergström et al. 2001) and, therefore, could also lead to reduction in BoP and PISA values. In our study, however, we recorded the use of all these medications and they were included in our analysis, thus, avoiding bias. Despite all these shortcomings, PISA is considered to be a useful tool to use in periodontal medicine research because reflects the amount of periodontal inflamed tissue and, thus, can accurately measure the infectious and inflammatory burden posed by PD (Leira et al. 2018). Endotoxemia by LPS was discussed before as a plausible mechanism to link PD, systemic inflammation, endothelial dysfunction and poor outcome in LI. In our clinical study we did not include any potential measure of endotoxemia such as LPS activity or plasmatic levels of LBP. Nevertheless, in our animal study we induced PD by *Pg*-LPS injections and further TLR-4 activation, which mimics the underlying mechanism that could be observed in PD. Therefore, it seems reasonable to support our human results in the animal model that we have performed without measuring endotoxemia markers. It should also be noted that our complementary animal study has also some drawbacks. Even though the lack of a control group and a sham-operated group could be the major criticism, we considered the biomarker levels at basal of each animal as a control *per se* due to our purpose was to analyse the influence of PD in each biomarker. Finally, we have to be cautious when interpreting the results of the secondary study due to the low number of patients with poor functional outcome (n=31). Previous reports (Petty et al. 2000; Clavier et al. 1994; Yokota et al. 2004; Samuelsson et al. 1996; Roquer et al. 2004) showed that between 18-42% of LI patients presented poor functional outcome at 3 months. Accordingly, we observed worse prognosis in 25.8% of these patients, which was between the ranges proposed in the literature.

CONCLUSIONS

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CONCLUSIONS

1. PD is positively and independently associated with the presence of LI.
2. PISA, a clinical periodontal parameter of current active disease, is positively and independently associated with the presence of LI.
3. When PD is present in LI patients, results in an enhanced systemic inflammatory response promoting endothelial dysfunction with higher serum levels of IL-6, PTX3, sTWEAK, and $A\beta_{1\text{-}40}$.
4. Experimental PD confirms the role of PD as one of the main contributors to a pro-inflammatory state with disruption of the vascular endothelial function.
5. Moderate to severe active PD (i.e., PISA $\geq$ 727 mm^2) is an independent predictor of poor functional outcome at 3 months in patients with LI and this association is mediated not only by enhanced systemic inflammation but also due to disruption of the vascular function of the endothelium posed by PD.

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IMPLICATIONS FOR FUTURE RESEARCH

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- It is important to identify conditions that could contribute to an enhanced systemic inflammatory state as well as promote endothelial dysfunction in cerebrovascular diseases. In particular, PD seems to be one of these conditions and may have a significant prognostic implication in patients diagnosed with LI. However, studies with a large sample are warranted to confirm our results. Furthermore, if further longitudinal studies demonstrate that the association observed in this work is causal meaning PD included in the list of risk factors for developing LI, clinical trials should be performed to evaluate the potential benefit of periodontal therapy in patients with LI.

- In order to better understand the pathophysiological mechanisms underlying the association found in our work, preclinical *in vivo* studies are warranted using CSVD models (Hainsworth et al. 2012) to investigate how experimental PD could predispose to LI or leukoaraiosis.

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RESUMEN

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RESUMEN

La periodontitis o enfermedad periodontal y la enfermedad cerebrovascular son procesos muy prevalentes en personas de edad avanzada. Según una encuesta de salud oral realizada en España en 2010, el 10,4% de las personas de 65-74 años presenta bolsas periodontales profundas y el 13,7% de esa misma cohorte de edad sufre una pérdida de inserción clínica muy elevada (6-8 mm). En el caso de la enfermedad cerebrovascular, los estudios epidemiológicos sugieren una incidencia de 150 casos por 100.00 habitantes y año, sin contar los accidentes isquémicos transitorios, y una prevalencia de 4.000-8.000 casos por 100.000 habitantes. Las enfermedades cerebrovasculares constituyen un grave problema de salud pública en los países industrializados con una edad media de vida alta de la población. Es la primera causa de incapacidad en la edad adulta.

La periodontitis es una enfermedad inflamatoria crónica multifactorial producida por una infección bacteriana de los tejidos de soporte que rodean los dientes. Generalmente, el diagnóstico clínico de esta patología se basa en la aparición de bolsas periodontales y aumento de su profundidad, en la pérdida de inserción clínica, y en la presencia y extensión de pérdida ósea alveolar radiográfica.

La enfermedad cerebrovascular engloba en un sentido amplio a la isquemia (infartos cerebrales) y la hemorragia (hemorragia cerebral), aunque se puede dar una combinación de ambas lesiones (infartos hemorrágicos), lo que no es raro en el caso de infartos de origen embólico o venoso. El reparto proporcional de los diferentes tipos de enfermedades cerebrovasculares varía según los diferentes estudios. De forma general, se podría decir que los accidentes isquémicos transitorios incluyen el 16,4% de los casos, infartos aterotrombóticos (35%), infartos cardioembólicos (20%), infartos lacunares (18%),

hemorragias cerebrales (15,1%) e infartos de naturaleza no determinada (24%). Los distintos mecanismos fisiopatológicos implicados en cada entidad clínica les confieren unas características determinadas. De especial interés y singularidad es la microangiopatía cerebral o enfermedad cerebral del pequeño vaso implicada en la producción de infartos lacunares. Estos se definen como infartos que tienen lugar en pequeñas arteriolas perforantes profundas (< 15 mm) y son los causantes de aproximadamente un 25% de los ictus isquémicos. Las principales causas de los infartos lacunares son la presencia de placas de ateroma en las arteriolas perforantes o arterias parenterales, embolias procedentes del corazón o arterias carótidas, y la lipohialinosis o necrosis fibrinoide. El ateroma en la arteria cerebral media es responsable de casi el 20% de los casos de infarto lacunar. Con respecto a la embolia, la evidencia es limitada (<10% de los casos de infarto lacunar). Aunque no se conocen bien los mecanismos implicados en el origen de este tipo de ictus, la denominada enfermedad cerebral de pequeño vaso intrínseca como la lipohialinosis (referida a los depósitos hialinos en arterias perforantes junto con la arteriopatía difusa) se considera como la causa más común de los infartos lacunares. En este sentido, se ha sugerido que la disfunción endotelial podría estar presente en este tipo de enfermedad neurovascular.

Existe evidencia científica de que la periodontitis no sólo tiene efectos locales (en la encía) sino que también es capaz de producir efectos sistémicos en órganos distantes. De hecho, se especula que la bolsa ulcerada e inflamada proporciona un puerto de entrada fácil para bacterias periodontales. Se ha observado que en pacientes con periodontitis se puede producir bacteriemias tras procedimientos rutinarios dentales o tras un examen oral e interesantemente algunos periodontopatógenos han sido identificados en placas de ateroma. Además, componentes bacterianos como los lipopolisacáridos (LPS) puede también diseminarse al torrente sanguíneo. Estos LPS junto con los antígenos bacterianos puede provocar procesos inflamatorios significativos. De acuerdo con esto, componentes celulares sanguíneos y proteínas de fase aguda procedentes de células endoteliales y hepatocitos pueden producir mediadores inflamatorios. Además,

moléculas pro-inflamatorias producidas localmente pueden pasar a la circulación sistémica y llevar a cabo efectos nocivos en órganos distantes.

Por lo tanto, los objetivos generales del presente trabajo fueron:

- Investigar la asociación entre la periodontitis y la presencia de infarto lacunar.
- Estudiar posibles mecanismos fisiopatológicos (inflamación sistémica y disfunción endotelial) que podrían explicar dicha relación.
- Corroborar los resultados en un modelo experimental de periodontitis mediante inyección de LPS de una bacteria periodontal, la *Porphyromonas gingivalis*.

Estudio clínico-molecular

Nuestra hipotesis es que la periodontitis es prevalente en pacientes con infarto lacunartosis lacunar diagnosticados en la Unidad de Ictus ótesis es que la periodontitis es prevalente en pacientes con infarto lacunar y se asocia con la presencia de este subtipo de microangiopatía cerebral independientemente de otros factores de riesgo que ambas entidades comparten. Cuando la periodontitis está presente, puede resultar en una respuesta inflamatoria sistémica aumentada promoviendo la disrupción de la función endotelial vascular expresada con niveles séricos aumentados de biomarcadores de inflamación y disfunción endotelial. También sugerimos que la inflamación periodontal podría ser un predictor independiente de mal pronóstico funcional en pacientes con infarto lacunar.

El objetivo primario de este estudio fue investigar la asociación entre periodontitis y sus parámetros clínicos y la presencia de infarto lacunar.

Como objetivos secundarios:

- Analizar el papel de la inflamación sistémica y la disfunción del endotelio en este posible asociación.

- Investigar si la inflamación periodontal puede predecir un mal pronóstico en pacientes diagnosticados de infarto lacunar.

Para llevar a cabo los objetivos antes mencionados, se diseñó un estudio de casos y controles en el que se incluyeron 120 pacientes con infarto lacunar diagnosticados en la Unidad de Ictus del Hospital Clínico Universitario de Santiago y 157 controles sin enfermedades neurológicas aparentes tras la realización de un estudio de neuroimagen. A todos ellos se les realizó un examen periodontal completo (profundidad de bolsa periodontal, pérdida de inserción clínica, sangrado al sondaje, índice de placa, recesión gingival, dientes ausente así como el área de la superficie periodontal inflamada) y se registraron datos demográficos (edad, sexo nivel de educación), dentales (prevalencia de la periodontitis y gravedad de la misma, última visita dental, frecuencia de cepillado y uso del cepillo interdental) y médicos relevantes (historia previa de hipertensión, diabetes mellitus, hipercolesterolemia, cardiopatía isquémica, arteriopatía periférica obliterante, hábito tabáquico, consumo de alcohol, uso de medicación como antiagregantes, estatinas y antihipertensivos). Además, en los casos se obtuvieron variables de neuroimagen y neurosonológicas (localización del infarto lacunar, presencia de infartos silentes, leucoaraiosis y ateromatosis carotídea) así como el pronóstico a los tres meses según la escala de Rankin modificada. Además, a todos los casos y 120 controles se les realizó una extracción sanguínea para analizar los siguientes biomarcadores: inflamación sistémica [interleuquina (IL)-6, IL-10] y disfunción del endotelio [pentraxina (PTX)3, porción soluble del inductor débil de apoptosis de factor de necrosis tumoral (sTWEAK), beta amiloide $(\beta A)_{1\text{-}40}$ y $\beta A_{1\text{-}42}$].

Pacientes con infarto lacunar presentaron un peor estado periodontal, tanto en parámetros clínicos de enfermedad pasada (nivel de inserción clínico) como de enfermedad activa (sangrado gingival, bolsa periodontal y área de la superficie periodontal inflamada). De acuerdo a esto, la periodontitis estaba presente en 85 de 120 pacientes con infarto lacunar (70,8%) y en 51 de 157 en controles (30,8%). En

relación con la gravedad de la periodontitis, casi la mitad de los pacientes periodontales con infarto lacunar presentaron periodontitis avanzada comparado con el 7,8% en el grupo control. El número de pacientes con hipertensión, diabetes, hipercolesterolemia, cardiopatía isquémica y arteriopatía periférica obliterante fue significativamente mayor en los casos que en los controles. Como cabía de esperar, los pacientes con infarto lacunar tomaban más medicación que los controles. Aquellos pacientes con infarto lacunar que tenían periodontitis mostraron una mayor prevalencia de leucoaraiosis (lesiones de sustancia blanca asintomáticas) y ateromatosis carotídea que aquellos sin periodontitis. Además, se observó un mal pronóstico funcional a los 3 meses en el 32,9% de los pacientes con infarto lacunar y periodontitis en comparación con el 8,6% de los casos sin periodontitis (P=0,006). Tras el ajuste de edad, sexo, hipertensión, diabetes, hipercolesterolemia, tabaco, estatinas y enfermedad cardíaca, los pacientes con periodontitis, el riesgo de padecer un infarto lacunar fue de 3,3 (IC 95%: 1,7-6,4) comparado con aquellos sin periodontitis. Del mismo modo, formas avanzadas de periodontitis también se asociaron positiva e independientemente con la presencia de infarto lacunar (OR=9,8; IC 95%:2,4-38,9; P<0,001). Al analizar cuál de los parámetros periodontales podrían asociarse con infarto lacunar, sólo el método del área de la superficie periodontal inflamada mostró significación (OR=1,001, IC 95%:1,001-1,002; P<0,001).

Los pacientes con infarto lacunar mostraron niveles séricos significativamente más elevados de IL-6, PTX3, sTWEAK y βA_{1-40} que los controles. Por el contrario, los niveles séricos de IL-10 fueron más bajos en casos que en controles. Respecto a βA_{1-42} no hubo diferencias entre grupos. Cuando analizamos estos biomarcadores de acuerdo a la presencia o ausencia de periodontitis, la presencia de ésta se asoció positivamente con niveles séricos aumentados de IL-6, sTWEAK y βA_{1-40} en casos (21,0 vs. 10,2 pg/mL, P<0,001 / 240,7 vs. 40,7 pg/mL, P<0,001 / 58,7 vs. 41,0 pg/mL, P<0,001; respectivamente) y controles (6,6 vs. 4,8 pg/mL, P=0,002 / 45,6 vs. 19,5 pg/mL, P=0,029 / 36,6 vs. 31,4 pg/mL, P=0,001; respectivamente). En el caso de la PTX3, sólo hubo una asociación significativa en los casos (2205,6 vs. 1182,6

pg/mL, P<0,001). En cuanto a la IL-10, la asociación fue inversa, es decir, los controles con periodontitis mostraron niveles séricos de este marcador anti-inflamatorio significativamente inferiores a los del mismo grupo sin periodontitis (11,9 vs. 14,0 pg/mL, P<0,001). No se observaron diferencias entre ninguno de los subgrupos para la $\beta A_{1\text{-}42}$. El análisis de regresión lineal multivariante mostró que tanto la periodontitis tratada como variable categórica como una medida de actividad de la enfermedad periodontal (área de la superficie periodontal inflamada) contribuyeron de manera significativa a los niveles elevados de IL-6, sTWEAK, PTX3 y $\beta A_{1\text{-}40}$ en pacientes con infarto lacunar, independientemente de la edad, sexo, hipertensión, diabetes mellitus, hipercolesterolemia, cardiopatía isquémica, arteriopatía periférica obliterante, hábito tabáquico, consumo de estatinas, presencia de leucoaraiosis y ateromatosis carotídea.

De los 120 pacientes con infarto lacunar estudiados, 31 (25,8%) tuvieron un mal pronóstico funcional a los 3 meses. Estos pacientes eran de edad más avanzada y presentaron una mayor prevalencia de hipertensión y diabetes mellitus. Leucoaraiosis estaba presente en 19 de los 89 pacientes con buen pronóstico (21,3%) y en 16 de los 31 pacientes con mal pronóstico (51,6%) (P=0,001). Además, la mitad de estos pacientes mostraron infartos silentes comparados con el 33,7% del grupo con buen pronóstico (P=0,038). No se observaron diferencias para otras variables entre estos dos grupos. Respecto a la prevalencia de periodontitis, ésta fue significativamente mayor en el grupo de mal pronóstico comparado con el de buen pronóstico (90,3% vs. 64,0%, P=0,006). De igual manera, el % de periodontitis avanzada fue mayor en los pacientes con mal pronóstico respecto a los que tuvieron un buen pronóstico (64,3% vs. 38,6%, P=0,039). Los parámetros clínicos de actividad periodontal (sangrado al sondaje, bolsa periodontal y área de la superficie periodontal inflamada) estaban significativamente elevados en el grupo de pacientes con un mal pronóstico en comparación con aquellos con un buen pronóstico. Sin embargo, el principal indicador de enfermedad periodontal pasada (nivel de inserción clínico) no mostró diferencias estadísticamente significativas entre los dos grupos de pronóstico a los 3 meses.

Respecto las comparaciones entre los dos grupos de pronóstico de los diferentes biomarcadores analizados, los niveles séricos de IL-6 (21,1 vs. 16,8 pg/mL, P<0,001), sTWEAK (245,8 vs. 181,0 pg/mL, P=0,001), PTX3 (2586,2 vs. 1670,7 pg/mL, P=0,007) y βA_{1-40} (60,2 vs. 51,2 pg/mL, P<0,001) estaban significativamente aumentados en pacientes con un mal pronóstico en comparación con aquellos con buen pronóstico. Tras el ajuste de factores implicados en un mal pronóstico, el área de la superficie periodontal inflamada y la diabetes mellitus fueron predictores que se asociaron significativamente con un mal pronóstico en pacientes con infarto lacunar. El análisis de la curva COR mostró un are bajo la curva de o,738 (IC 95%: 0,632-0,844, P<0,001), la cual sugiere que un valor ≥727 mm^2 predijo una asociación entre el área de la superficie periodontal inflamada y un mal pronóstico, con una sensibilidad de 71% y una especificidad del 70%. Por lo tanto, tras categorizar el área de la superficie periodontal inflamada (<727 mm^2 y ≥727 mm^2), este indicador de enfermedad periodontal activa junto con la historia previa de diabetes mellitus siguieron siendo significativos independientemente de variables clínicas y moleculares. También se observó una correlación positiva entre el área de la superficie periodontal inflamada y la IL-6 (r=0,738, P<0,001), PTX3 (r=0,468, P=0,008), sTWEAK (r=0,771, P<0,001) y βA_{1-40} (r=0,745, P<0,001) en pacientes con infarto lacunar y un mal pronóstico a los 3 meses de evaluación funcional.

Estudio experimental

Los resultados del estudio clínico-molecular mostraron que la periodontitis es prevalente en pacientes diagnosticados con infarto lacunar y parece contribuir a niveles séricos elevados de mediadores inflamatorios así como de disfunción vascular del endotelio. Aunque esta asociación parece ser independiente, hay otros factores como la edad avanzada, hipertensión, diabetes así como la presencia de lesiones asintomáticas de sustancia blanca que también han mostrado ser relevantes en la fisiopatología del infarto lacunar y también se han relacionado significativamente con su presencia en nuestro estudio llevado a cabo en humanos. Para investigar si la periodontitis puede ser

uno de los principales factores que contribuyen a un estado de inflamación sistémico de bajo grado promoviendo la disrupción de la función vascular del endotelio en pacientes con infarto lacunar, parece razonable haber realizado un estudio experimental complementario utilizando el modelo de inyección de LPS de *Porphyromonas gingivalis* en ratas sistémicamente sanas. En base a todo ello, nuestra hipótesis es que la periodontitis experimental en ratas puede provocar un ligero efecto de inflamación aguda (con aumento significativo de IL-6 y PTX3 durante la primera semana tras la inducción de la periodontitis) seguido por la disfunción del endotelio (con aumento significativo de PTX3, sTWEAK y $\beta A_{1\text{-}40}$, por consiguiente, demostrando que la periodontitis puede ser un factor importante promoviendo un estado sistémico de inflamación asociado a la disfunción del endotelio pudiendo llevar a un riesgo aumentado para el desarrollo de un infarto lacunar. Por lo tanto, el objetivo de este estudio animal in vivo fue investigar en ratas sistémicamente sanas si la periodontitis se asocia con una respuesta sistémica inflamatoria aumentada junto con la disrupción de la función endotelial medidas mediante los mismo biomarcadores que se analizaron en el estudio clínico-molecular en humanos.

Para este experimento, se utilizaron 6 ratas macho Sprague-Dawley de 7 semanas de edad y con un peso entre 300 y 350 gramos. Antes de comenzar la inducción de la periodontitis, se llevó a cabo un análisis de los tejidos periodontales blandos y duros mediante micro tomografía axial computerizada (TC) e imagen por resonancia magnética (IRM) así como extracción sanguínea (basal) en todos los animales. Para inducir la periodontitis, se realizaron inyecciones de LPS de *Porphyromonas gingivalis* en la zona palatina entre el primer y segundo molar superior bilateralmente 3 días por semana con un día de separación cada una durante dos semanas. Se llevó a cabo un segundo estudio de imagen junto con una exploración visual a nivel gingival entre la primera y segunda semana para corroborar que se estaba produciendo una reacción inflamatoria en los tejidos periodontales característica de la periodontitis. Tras las últimas inyecciones, se hicieron otro micro TC y IRM para confirmar la pérdida ósea del hueso alveolar de la rata así como la inflamación de los tejidos de la encía. Se llevaron a cabo

extracciones seriada de sangre a las 24 horas post-inducción periodontal así como a la semana, 2 y 3 semanas tras la inducción de la periodontitis. Una vez que todas las muestras de suero estaban almacenadas, de realizaron las determinaciones de los biomarcadores mediante la técnica ELISA. Durante todos los procedimientos, los animales estaba completamente anestesiados. Una vez que el experimento se dio por finalizado, los animales fueron sacrificados mediante una inyección intracardiaca de 2 mL de cloruro potásico.

La inflamación gingival fue evidente al día 7 del experimento afectando a la parte palatina entre el primer y segundo molar superior en ambos lados de la arcada. Estos hallazgos clínicos fueron confirmados mediante el análisis por IRM. Se encontraron diferencias en la intensidades de la señal relativa en T_1 y T_2 a los 7 y 14 días tras la inducción periodontal comparado con las intensidades basales (previas a la inducción). De hecho, la intensidad relativa medida en T_2-w entre el primer y segundo molar superior derechos fue significativamente mayor a los 7 días en comparación con los 14 días ($P<0,05$). En relación al grosor del paladar, que se considera como una medida subrogada de edema, se observó un aumento en el porcentaje relativo debido a la inflamación periodontal. De hecho, se observaron diferencias estadísticamente significativas en cuanto al grosor palatino entre los 7 días y basal ($P<0,01$) en el segundo molar superior derecho.

El análisis realizado mediante micro TC reveló que a nivel interproximal (entre los primeros y segundos molares superiores), la pérdida ósea alveolar fue significativa. De acuerdo con estos hallazgos, la distancia entre la unión cemento-esmalte y la cresta ósea alveolar fue significativamente mayor a los 14 días en ambos lados de la arcada superior comparado con las mismas mediciones realizadas en basal. En el lado derecho los valores medios fueron 2,0 mm vs. 1,5 mm ($P=0,001$) y en el lado izquierdo de 1,8 mm vs. 1,3 mm ($P<0,001$).

Al analizar el comportamiento de los distintos marcadores durante el seguimiento del experimento, se observó un aumento agudo de la IL-6 a las 24 horas tras la inducción periodontal, lo cual confirma su

naturaleza como un mediador inflamatorio de acción temprana. Los niveles de IL-6 se redujeron durante las siguientes semanas pero siguieron manteniendo diferencias significativas en comparación con los niveles séricos en basal. Por el contrario, la periodontitis experimental produjo un descenso significativo de los niveles séricos de IL-10 a las 24 horas tras la última inyección de LPS, y los niveles de este mediador anti-inflamatorio continuaron reduciéndose hasta el final del experimento (21 días). La PTX3 mostró un aumento agudo significativo leve a las 24 horas, que fue más pronunciado a medida que iba avanzado el seguimiento del experimento. Los niveles séricos de sTWEAK se elevaron de manera significativa 1 semana después de la inducción periodontal comparado con basal. Este aumento continuó en las siguientes semanas hasta el final del experimento. En cuanto a los péptidos de βA, se observó un aumento agudo leve similar al de la PTX3 por parte del $\beta A_{1-40,}$ el cual alcanzó significación respecto a basal a la semana post-inducción periodontal. Este aumento de los niveles séricos de este péptido continuó hasta los 14 días, volviéndose a reducir a los 21 días tras la inducción experimental de la periodontitis. En el caso de $\beta A_{1-42,}$ su incremento alcanzó significación significativa a los 21 días de la inducción periodontal, lo que confirma su naturaleza de marcador de deterioro cognitivo más que de disfunción del endotelio.

Basándonos en los resultados obtenidos tanto en el estudio clínico-molecular en humanos como en el estudio experimental complementario, podemos concluir que la periodontitis se asocia con el infarto lacunar. Cuando la periodontitis está presente y activa en estos pacientes, puede producir un aumento del estado inflamatorio asociado con la disfunción del endotelio lo que puede llevar a que tengan un peor pronóstico funcional.

CONFLICT OF INTEREST STATEMENT

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CONFLICT OF INTEREST STATEMENT

Yago Leira Feijóo reported that there are not conflicts of interest in connection with this Doctoral Thesis.

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In the present study we found that periodontal disease (PD) was positively associated with lacunar infarct (LI) and, when present, emerged as one of the main contributors to an enhanced systemic inflamatory state promoting endothelial dysfunction with elevated levels of IL-6, PTX3, sTWEAK, and $A\beta_{1\text{-}40}$ in LI patients. Moreover, moderate to severe active PD was an independent predictor of poor functional outcome in LI patients. These findings were corroborated in a preclinical study, in which experimental PD induced with lipopolysaccharide from *Porphyromonas gingivalis* was associated with a mild systemic inflammatory response with disruption of the vascular endothelial function.