TITLE

Periodontitis is associated with systemic inflammation and vascular endothelial dysfunction in lacunar infarct patients.

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Sentence summarizing the key finding of the study: Systemic inflammation and endothelial dysfunction could mediate the association between periodontitis and lacunar infarct.

ABSTRACT

Background: Periodontitis has been associated with lacunar infarct (LI), a type of cerebral small vessel disease. The objective of this study was to ascertain whether periodontitis is associated with increased circulating levels of systemic inflammation and endothelial dysfunction biomarkers in LI patients.

Methods: 120 patients with LI and 120 healthy controls underwent a full-mouth periodontal examination. The periodontal inflamed surface area (PISA) was calculated for each participant. Demographic, medical and neurological information were recorded from all of them. In addition, blood samples were collected in order to investigate differences in terms of interleukin (IL)-6, IL-10, pentraxin (PTX) 3, soluble fragment of tumor necrosis factor-like weak inducer of apoptosis (sTWEAK) and amyloid-beta (Aβ) peptides (i.e., $A\beta_{1-40}$, and $A\beta_{1-42}$) measured in serum.

Results: Periodontitis was independently associated with increased levels of IL-6 (R^2=0.656, P<0.001), PTX3 (R^2=0.115, P<0.001), sTWEAK (R^2=0.527, P<0.001), and $A\beta_{1-40}$ (R^2=0.467, P<0.001) in patients with LI. Within patients with poor outcome, PISA positively correlated with IL-6 (r=0.738, P<0.001), PTX3 (r=0.468, P=0.008), sTWEAK (r=0.771, P<0.001), and $A\beta_{1-40}$ (r=0.745, P<0.001).

Conclusions: Our data suggest a link between periodontitis, systemic inflammatory response, and disruption of the vascular endothelial function in LI patients. Experimental studies are needed to elucidate possible pathways through which periodontitis could lead to this systemic inflammatory state with impairment of the endothelial function in LI. Further longitudinal studies with large samples are warranted to confirm our findings.

KEY WORDS

Periodontitis; Lacunar stroke; Cerebral small vessel diseases; Inflammation; Endothelium.

INTRODUCTION

Lacunar infarct (LI), which is a small cerebral subcortical infarct, causes approximately 25% of the cases of ischemic stroke. [1] Atheroma in the middle cerebral artery is responsible for almost 20% of LI cases. [2] Evidence regarding embolism as a common cause for LI is limited (< 10% of LI cases). [2] Although the exact mechanism remains unclear, intrinsic cerebral small vessel disease such as lipohyalinosis (referred to hyaline deposition in the perforating arteries together with diffuse arteriopathy) is considered as the most common cause of LI. [2] It has been demonstrated that endothelial dysfunction by amyloid beta (Aβ) deposition has been related to LIs and leukoaraiosis (white matter hyperintensities). [3,4] In this sense, $A\beta_{1\text{-}40}$ was found to be involved in the disruption of the endothelial vascular function in LI patients. [5]

We have reported that high levels of serum inflammatory molecules [i.e., interleukin (IL)-6 and tumor necrosis factor (TNF)-α] could be predictive factors of poor outcome in patients with LIs leading to early neurologic worsening. [6,7] A member of the TNF superfamily, namely TNF-like weak inducer of apoptosis (TWEAK), was overexpressed after experimental cerebral ischemia in the area surrounding the necrotic core and in response to ischemic signal during experimental stroke, there is a release of this cytokine from astrocytes leading to blood-brain barrier disruption and increased permeability. [8,9] In humans, serum levels of TWEAK from ischemic stroke patients were significantly elevated compared to healthy controls. [10] Similarly, increased concentrations of pentraxin 3 (PTX3), an acute phase reactant (APR), are independently associated with poor prognosis and increased mortality in patients with ischemic stroke. [11] It has been proposed that amyloid precursor protein (APP) and Aβ could also participate in ischemic brain damage. Even though APP expression is increased in the post-ischemic brain, [12] cerebral ischemia may facilitate cleavage of APP into the toxic Aβ fragment. [13] 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.

In periodontitis, IL-6 plays an important role in modulating the response to periodontal bacteria, leading to both local and systemic inflammation. [14,15] There is a correlation between periodontal parameters and PTX3 levels systemically and site specific. [16,17] Saliva samples from periodontal patients also shown differences in levels of PTX3 compared to those without periodontal infection. [18] Periodontally affected tissues overexpress TWEAK and can induce endothelial activation molecules within human

gingival fibroblasts. [19,20] Recently, it has been postulated that periodontitis could be involved in the synthesis and accumulation of Aβ in the brain. [21] Moreover, levels of APP are increased in gingival tissues with periodontitis and severe periodontal patients with cognitive decline showed higher levels of $A\beta_{1-40}$ compared to those without periodontitis. [22,23]

Recently, our group found a significant association between periodontitis and LI. [24] However, the mechanisms underlying this relationship have not been yet elucidated. We hypothesize that in patients with LI, periodontitis my results in an enhanced systemic inflammatory response expressed by higher serum levels of both pro-inflammatory and endothelial dysfunction biomarkers. Hence, the aim of the study was to determine whether periodontitis is associated with higher serum levels of systemic inflammation and endothelial dysfunction markers in patients with LI.

MATERIALS AND METHODS

A cross-sectional 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. [25]

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 (ID:2016/399). Informed consent was obtained from each patient or their relatives after full explanation of the procedures.

Study population

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. From the 139 cases screened, 19 were excluded for the following reasons: < 15 teeth in the mouth (N=7), receive periodontal treatment in the previous 12 months (N=3), use of systemic antibiotic (N=1), decline to participate (N=8). Thus, 120 patients diagnosed with LI according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification [26] were included in the analysis. To include subjects without any neurological disease, 194 computed tomography/magnetic resonance imaging (CT/MRI) were reviewed from those referred to the Neurology Department with a suspicious diagnosis on non-confirmed neurological disorders such as non-specific headache, vestibular syndromes, brain tumors or altered level of consciousness between 2009 and 2013. We excluded 12

individuals due to the presence of cerebral small vessel disease asymptomatic subtypes: silent infarct (SI) (N=4) and leukoaraiosis (N=8). From the 182 controls, 25 were excluded for different reasons: < 15 teeth in the mouth (N=6), receive periodontal treatment in the previous 12 months (N=11), chronic use of non-steroidal anti-inflammatory drugs (N=2), and decline to participate (N=6). Therefore, 120 healthy controls without any type of cerebral small vessel disease matched by age and gender were finally included in the study. For both cases and controls demographic and medical information were obtained by means of a questionnaire.

Periodontal examination

The periodontal examination was performed by a single calibrated periodontist (YL). The calibration was recently reported. [24] Each case (20-30 days after LI) and control (maximum 1 month after CT/MRI) underwent a full-mouth periodontal examination, including probing depth (PD), clinical attachment level (CAL), gingival recession (Rec), full-mouth plaque score (FMPS), [27] and full-mouth bleeding score (FMBS) [28] as well as the number of missing teeth (excluding 3º molars). In addition, a measure of current PD activity, the periodontal inflamed surface area (PISA) was calculated based on CAL and Rec measurements combined with the presence of bleeding on probing. [29] Periodontitis and different grades of severity were defined according to the Centers for Disease Control and Prevention (CDC)-American Academy of Periodontology (AAP) consensus for epidemiologic studies. [30] Hence, mild periodontitis 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 PD ≥5 mm. Moderate periodontitis was defined as ≥2 interproximal sites with CAL ≥4 mm (not on the same tooth) or ≥2 interproximal sites with PD ≥5 mm, also not on the same tooth. Severe periodontitis was defined as the presence of ≥2 interproximal sites with CAL ≥6 mm (not on the same tooth) and ≥1 interproximal site with PD ≥5 mm. Total periodontitis was the sum of mild, moderate, and severe periodontitis.

Neurological examination

Neuroimaging and ultrasonographic evaluation was done at admission. A detailed description of the neurological examination protocol was published elsewhere. [24,31] In summary, LI was diagnosed when the patient had one of the clinical lacunar syndromes lasting >24 h, no evidence of cortical dysfunction, and a normal or deep focal infarction with a diameter ≤15 mm in an appropriate location visualized by CT/MRI. The presence of an LI in the baseline CT in which the topography did not correspond with

the present clinical syndrome was considered a silent infarct (i.e., asymptomatic brain infarcts). White matter hyperintensities (i.e., leukoaraiosis) was classified according to the Fazekas criteria [32,33] using the modified Fazekas scale. [34] The presence of an atheroma plaque was defined as a focal structure that encroaches into the arterial lumen at least 0.5 mm or 50% of the surrounding intima-media thickness (IMT) value, or demonstrates a thickness >1.5 mm as measured from the media-adventitia interface to the intima-lumen interface. [35] Poor functional outcome of LI patients at 3 months was defined as a value of the modified Rankin scale >2. [36,37]

Serum collection and laboratory test

On admission for cases and 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 [§] 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 [ǀ] 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 [¶] 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 [#] minimum assay sensitivity was 10 pg/ml, with a intra-assay coefficient of variation of 5.0% and inter-assay CV of 2.3%; $A\beta_{1\text{-}40}$ ELISA kit [**] 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 $A\beta_{1\text{-}42}$ ELISA kit [††] 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.

Statistical analysis

All data analyses were performed with statistics software [‡‡]. Continuous normally distributed variables analysed with Kolmogorov-Smirnov test were reported as mean ± standard deviation, whereas categorical variables were expressed as percentages. Differences between two groups were assessed by independent *t* test (continuous normally distributed variables) and X^2 test (categorical variables). Non-normally

distributed variables were reported as median [interquartile range] and compared applying Mann-Whitney U test. One-way analysis of variance (*ANOVA*) was used to compare mean values between more than two 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. Multivariable linear regression analysis was used to test associations between periodontitis and several biomarkers, adjusted for significant variables. Receiver operating curve (ROC) analysis was done to establish a cut-off point for PISA to predict poor outcome in LI patients. A logistic regression model was carried out to test the association between PISA and poor outcome adjusted for molecular and clinical variables.

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

RESULTS

Description of the groups

LI patients had more frequently a previous history of hypertension (61.7% vs. 33.3%, P<0.0001), diabetes (27.5% vs. 10.0%, P=0.001), hypercholesterolemia (45.8% vs. 18.3%, P<0.0001), ischemic heart disease (18.3% vs. 3.3%, P<0.0001) and peripheral artery disease (4.2% vs. 0.0%, P=0.02) compared to controls. As expected, they also took more medication (i.e., statins, antiaggregants and antihypertensives) than non-LI subjects (46.7% vs. 18.3%, 38.3% vs. 2.5%, 58.3% vs. 28.0%, all P<0.0001). No significant differences between groups were observed regarding age, gender, body mass index, smoking habit, alcohol consumption, and socioeconomic level (**Table 1**). Sub-analysis of LI patients according to periodontal status showed that those with periodontitis had a significantly higher prevalence of white matter hyperintensities (37.6% vs. 8.6%, P=0.001), increased IMT (49.4% vs. 28.6%, P=0.036) as well as a poor outcome at 3 months (32.9% vs. 8.6%, P=0.006) than LI patients without periodontitis.

Periodontal conditions

Periodontitis was present in 85 of 120 subjects with LI (70.8%) and in 37 of 120 controls (30.8%) (P<0.0001). Periodontal conditions were significantly worse in patients with LI than controls in terms of both past and current measures of periodontitis such as CAL (4.3±1.4 mm vs. 3.0±1.0 mm, P<0.0001), FMBS (59.4±18.2% vs. 29.7±15.0, P<0.0001), PD (3.6±1.0mm vs. 2.6±0.7, P<0.0001) and PISA

(1040.4±1145.8 mm^2 vs. 187.2±358.2 mm^2, P<0.0001) method in comparison to controls (**Table 1**).

Serum parameters in cases and controls

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 2**).

Patients with periodontitis had higher mean IL-6, sTWEAK, and $A\beta_{1-40}$ levels in both cases (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; respectively) and controls (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; respectively) compared to non-periodontal patients (**Figure 1A, 1D, and 1E**). As expected, **Figure 1B** showed that periodontal patients in the control group presented lower mean IL-10 levels than those without periodontitis (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). Among control subjects, difference in terms of PTX3 levels was modest and not significant. Within the cases, however, there was a highly significant increase in PTX3 levels within patients with periodontitis (2205.6 vs. 1182.6 pg/mL, P<0.001) (**Figure 1C**). 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 1F**).

Linear regression analyses

When examined as a continuous variable (i.e., PISA), multivariable regression models adjusted for age, gender, hypertension, diabetes, hypercholesterolemia, ischemic heart disease, peripheral arterial disease, smoking habit, statins, leukoaraiosis, and carotid atheromatosis indicated that PISA was associated with significantly higher mean serum levels of IL-6 (R^2=0.624, P<0.001) (**Supplementary Table 1, Model I**), PTX3 (R^2=0.260, P<0.001) (**Supplementary Table 2, Model I**), sTWEAK (R^2=0.697, P<0.001) (**Supplementary Table 3, Model I**), and $A\beta_{1-40}$ (R^2=0.653, P<0.001) (**Supplementary Table 4, Model I**) among LI patients. When periodontitis exposure was examined as a categorical variable, multivariable linear regression analysis adjusted for the same confounders again indicated that periodontitis in patients with LI was associated with significantly increased serum concentrations of IL-6 (R^2=0.656, P<0.001) (**Supplementary Table 1, Model II**), PTX3 (R^2=0.115, P<0.001) (**Supplementary Table 2, Model II**), sTWEAK (R^2=0.527, P<0.001) (**Supplementary**

Table 3, Model II), and $A\beta_{1-40}$ (R^2=0.467, P<0.001) (**Supplementary Table 4, Model II**).

Serum parameters in LI patients according to prognosis

Concentrations of IL-6, PTX3, sTWEAK, and $A\beta_{1-40}$ 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; 261.4 vs. 162.7 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 $A\beta_{1-42}$ (**Table 3**).

Correlations

A positive and strong correlation was found between PISA and IL-6 (r=0.738, P<0.001; **Figure 2A**), PTX3 (r=0.468, P=0.008; **Figure 2B**), sTWEAK (r=0.771, P<0.001; **Figure 2C**), and $A\beta_{1-40}$ (r=0.745, P<0.001; **Figure 2D**) in patients with poor prognosis.

Logistic regression analysis

After ROC analysis was performed to assess the optimal cut-off point to detect the presence of poor outcome, a logistic regression model revealed that a PISA value ≥ 727 mm^2 was independently associated with poor functional outcome at 3 months (OR=5.189; 95%CI: 1.070-25.157, P=0.041). Diabetes was also significantly associated with poor prognosis (OR=3.740; 95%CI:1.366-10.238, P=0.010) (**Supplementary Table 5**).

DISCUSSION

In the present study we aimed to investigate whether periodontitis could contribute to an inflammatory state promoting endothelial dysfunction in LI patients. We found that periodontitis was associated with increased serum levels of IL-6, PTX3, sTWEAK, and $A\beta_{1-40}$ in LI. In addition, this inflammatory state also mediated the relationship between cases with moderate to severe active periodontitis and poor functional outcome.

We observed that periodontitis and the PISA method were associated with elevated levels of IL-6 and PTX3 in LI patients, fact that it could be explained by the chronic underlying systemic inflammatory up-regulation posed by periodontitis. Our results are in accordance with previous human and animal studies, in which it was demonstrated that periodontitis might elicit an overexpression of these two pro-inflammatory with systemic consequences. [14,15,38,39] On contrary, we observed that IL-10 levels were significantly higher in those without the disease, thus, confirming its anti-inflammatory nature. Recently, it has been shown that PISA was positively correlated with PTX3

levels in patients with periodontitis without coronary artery disease (CAD) and IL-10 was inversely correlated with PD in patients with CAD. However, no association was found with IL-6. [40]

It has been suggested that periodontitis might be associated with endothelial dysfunction. [41] Although PTX3 is an APR, it is produced by macrophages or endothelial cells (ECs) in response to an inflammatory stimulus related with atherosclerosis [42,43] and is associated with diminished flow-mediated dilatation (FMD), thus, being a more powerful predictor of endothelial dysfunction than C-reactive protein. [44] TWEAK is also induced by ECs or smooth muscle cells, and is involved in the expression of adhesion molecules in ECs and pro-inflammatory mediators. [45,46] In periodontal tissues with periodontitis, it appears to be overexpressed. [19] Our results showed that increased levels of sTWEAK were strongly associated with periodontitis and PISA in LI. Animal models of middle cerebral artery occlusion showed increased levels of TWEAK and Fn14 in the area surrounding the necrotic core [8] and its ability to induce inflammatory mediators and matrix metalloproteinase (MMP)-9 leading to blood-brain barrier (BBB) disruption and increased permeability. [9] Therefore, it seems plausible that release of sTWEAK by periodontitis could predispose to disruption of the BBB in patients with LI and, therefore, being associated with dysfunction of the endothelium.

Regarding Aβ peptides, it has been suggested that periodontitis is involved in the synthesis and accumulation of Aβ in the brain [21,22] and endothelial dysfunction by Aβ deposition has been related to LIs and leukoaraiosis. [3,4] In our study, both PISA method and periodontitis as a categorical variable were associated with increased levels of $A\beta_{1-40}$ but no with $A\beta_{1-42}$ in LI patients. 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. [4] Similarly to our results, preclinical data supports the hypothesis that $A\beta_{1-40}$ but not $A\beta_{1-42}$ causes cerebrovascular dysfunction mediated by reactive oxygen species. [47,48]

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 independently associated with the presence of poor prognosis and was positively correlated with serum concentrations of IL-6, PTX3, sTWEAK, and $A\beta_{1-40}$ in LI patients with poor functional outcome. Therefore, it could be speculated that moderate to severe periodontitis (i.e., PISA $\geq$ 727 mm^2), [49] 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 periodontitis.

Our investigation has several strengths. Firstly, we were able to reject the null hypothesis (i.e., no association between periodontitis and increased levels of inflammatory and endothelial dysfunction markers in LI patients). As explained before, periodontitis although evokes a local inflammatory response could predispose to a chronic low-grade pro-inflammatory state affecting distant organs. In the present study, periodontitis was related with higher levels of markers that are associated with inflammation, vascular endothelial dysfunction and, ultimately, atherosclerosis. Thus, giving some insight on the potential role of periodontitis in the pathogenesis of LI. Secondly, patients with LI were diagnosed following a full protocol including clinical, neuroimaging and ultrasonographic information. In addition, prognosis at 3 months after stroke was also recorded. Thirdly, periodontitis was evaluated by means of a full-mouth periodontal chart and disease severity was established according to the CDC-AAP case definitions recommendations. [30]

However, we have to be cautious when interpreting the present results. There are limitations that are worth mentioning. Due to the cross-sectional nature of our investigation we cannot assess causality between periodontitis and the pathogenesis of LI. Therefore, other conditions well-known risk factors for developing LI such as hypertension or diabetes could also play an important role in the relationship between periodontitis and LI. We did not measure brachial FMD, which is considered as the hallmark of endothelial dysfunction. However, it has been demonstrated that in presence of endothelial dysfunction, both abnormal PTX3 and sTWEAK serum levels are associated with impaired FMD. [44,50] 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 $A\beta_{1\text{-}40}$, as they are associated with increased permeability and disruption of the BBB. Some factors could influence PISA values such as smoking or certain medications (i.e., antiaggregants, antihypertensives or statins). However, this information was recorded and adjusted on the linear regression models. One of the major questions in our study is whether increased inflammatory and endothelial dysfunction mediators in serum were the expression of a chronic low-grade systemic inflammatory state posed by periodontitis or originated as a result of brain ischemia itself. Although our logistic

regression model was adjusted for significant biomarkers, the potential role of cerebral ischemia in the serum levels of the biomarkers analysed in our study cannot be totally ruled out. We have to bear in mind that some conditions such as hypertension or diabetes are linked with systemic vascular inflammation and, therefore, could influence on circulating levels of the biomarkers analysed in our patients with LI. However, linear regression models from our analyses showed that periodontitis was an independent contributor to increased inflammation in LI patients. In addition, it has been shown that periodontal patients without co-morbidities overexpress circulating levels of pro-inflammatory markers such as IL-6 and PTX3,[15,16]. However, further studies including systemically healthy periodontal patients are needed to ascertain whether elevation of these pro-inflammatory molecules could be related specifically to periodontal inflammation or, indeed, act as mediators in the association between periodontitis and LI.

CONCLUSION

In conclusion, periodontitis is associated with an enhanced systemic inflammatory response promoting vascular endothelial dysfunction with higher serum levels of IL-6, PTX3, sTWEAK, and $A\beta_{1\text{-}40}$ in LI and in cases with active moderate to severe periodontitis this pro-inflammatory state was related with poor prognosis in LI patients. Further experimental studies are warranted to elucidate possible pathways through which periodontitis could lead to this systemic inflammatory state with impairment of the endothelial function in LI. Studies with large samples are also needed to confirm our results.

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CONFLICT OF INTEREST STATEMENT

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

REFERENCES

1. Bamford J, Sandercock P, Jones L, Warlow C. The natural history of lacunar infarction: the Oxfordshire Community Stroke Project. *Stroke* 1987;18:545–551.
2. Ahead of print. Regenhardt RW, Das AS, Lo EH, Caplan LR. Advances in understanding the pathophysiology of lacunar stroke: a review. [published online ahead of print Aug 27, 2018]. *JAMA Neurol*; doi:10.1001/jamaneurol.2018.1073.
3. van Dijk EJ, Prins ND, Vermeer SE, et al. Plasma amyloid beta, apolipoprotein E, lacunar infarcts, and white matter lesions. *Ann Neurol* 2004;55:570-575.
4. Gurol ME, Irizarry MC, Smith EE, et al. Plasma beta-amyloid and white matter lesions in AD, MCI, and cerebral amyloid angiopathy. *Neurology* 2006;66,23-29.
5. Gomis M, Sobrino T, Ois A, et al. Plasma beta-amyloid 1-40 is associated with diffuse small vessel disease subtype. *Stroke* 2009;40:3197-3200.
6. Blanco M, Castellanos M, Rodríguez-Yáñez M, et al. High blood pressure and inflammation are associated with poor prognosis in lacunar infarctions. *Cerebrovasc Dis* 2006;22:123-129.
7. Castellanos M, Castillo J, García MM, et al. Inflammation-mediated damage in progressing lacunar infarctions: a potential therapeutic target. *Stroke* 2002;33:982-987.
8. Yepes M, Brown SA, Moore EG, Smith EP, Lawrence DA, Winkles JA. A soluble Fn14-Fc decoy receptor reduces infarct volume in a murine model of cerebral ischemia. *Am J Pathol* 2005;166:511-520.
9. Polavarapu R, Gongora MC, Winkles JA, Yepes M. Tumor necrosis factor-like weak inducer of apoptosis increases the permeability of the neurovascular unit through nuclear factor-kappa B pathway activation. *J Neurosci* 2005;25:10094-10100.
10. Inta I, Frauenknecht K, Dörr H, et al. Induction of the cytokine TWEAK and its receptor Fn14 in ischemic stroke. *J Neurol Sci* 2008;275:117-120.
11. Ryu WS, Kim CK, Kim BJ, Kim C, Lee SH, Yoon BW. Pentraxin 3: a novel and independent prognostic marker in ischemic stroke. *Atherosclerosis* 2012;220:581-586.

12. Banati RB, Gehrmann J, Wiessner C, Hossmann KA, Kreutzberg GW. Glial expression of the beta-amyloid precursor protein (AAP) in global ischemia. J *Cereb Blood Flow Metab* 1995;15:647-654.
13. Yokota M, Saido TC, Tani E, Yamaura I, Minami N. Cytotoxic fragment of amyloid precursor protein accumulates in hippocampus after global forebrain ischemia. *J Cereb Blood Flow Metab* 1996;16;219-1223.
14. Geivelis M, Turner DW, Pederson ED, Lamberts BL. Measurements of interleukin-6 in gingival crevicular fluid from adults with destructive periodontal disease. *J Periodontol* 1993;64:980-983.
15. Shimada Y, Komatsu Y, Ikezawa-Suzuki I, Tai H, Sugita N, Yoshie H. The effect of periodontal treatment on serum leptin, interleukin-6, and C-reactive protein. *J Periodontol* 2010;81:1118-1123.
16. Pradeep AR, Kathariya R, Raghavendra NM, Sharma A. Levels of pentraxin-3 in gingival crevicular fluid and plasma in periodontal health and disease. *J Periodontol* 2011;82:734-741.
17. Fujita Y, Ito H, Sekino S, Numabe Y. Correlations between pentraxin 3 or cytokine levels in gingival crevicular fluid and clinical parameters of chronic periodontitis. *Odontology* 2012;100:215-221.
18. Gümüş P, Nizam N, Nalbanstoy A, Özçaka Ö, Buduneli N. Saliva and serum levels of pentraxin-3 and interleukin-1β in generalized aggressive or chronic periodontitis. *J Periodontol* 2014;85:e-40-46.
19. Kataria NG, Bartold PM, Dharmapatni AA, Atkins GJ, Holding CA, Haynes DR. Expression of tumor necrosis factor-like weak inducer of apoptosis (TWEAK) and its receptor, fibroblast growth factor-inducible 14 protein (Fn14), in healthy tissues and in tissues affected by periodontitis. *J Periodontal Res* 2010;45:564-573.
20. Hosokawa Y, Hosokawa I, Ozaki K, Nakae H, Matsuo T. Proinflammatory effects of tumour necrosis factor-like weak inducer of apoptosis (TWEAK) on human gingival fibroblasts. *Clin Exp Immunol* 2006;146:540-549.
21. Kamer AR, Pirraglia E, Tsui W, et al. Periodontal disease associates with higher brain amyloid load in normal elderly. *Neurobiol Aging* 2015;36:627-633.
22. Kubota T, Maruyama S, Abe D, et al. Amyloid beta (A4) precursor protein expression in human periodontitis-affected gingival tissues. *Arch Oral Biol* 2014;59:586-594.

23. Gil-Montoya JA, Barrios R, Santana S, et al. Association between periodontitis and amyloid β peptide in elderly people with and without cognitive impairment. *J Periodontol* 2017;88:1051-1058.
24. Leira Y, López-Dequidt I, Arias S, et al. Chronic periodontitis is associated with lacunar infarct: a case-control study. *Eur J Neurol* 2016;23:1572-1579.
25. Von Elm E, Altman DG, Egger M, Pocock SJ, Gøtzsche PC, Vandenbroucke JP.; STROBE Initiative. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement: guidelines for reporting observational studies. *J Clin Epidemiol* 2008;61:344-349.
26. Adams HP Jr, Bendixen BH, Kappelle LJ, et al. (1993). Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. TOAST. Trial of Org 10172 in Acute Stroke Treatment. *Stroke* 1993;24: 35-41.
27. O´Leary TJ, Drake RB, Naylor JE. The plaque control record. *J Periodontol* 1972;43:38.
28. Ainamo J, Bay I. Problems and proposals for recording gingivitis and plaque. *Int Dent J* 1975;25:229-235.
29. Nesse W, Abbas F, van der Ploeg I, Spijkervet FK, Dijkstra PU, Vissink A. Periodontal inflamed surface area: quantifying inflammatory burden. *J Clin Periodontol* 2008;35:668-673.
30. Eke PI, Page RC, Wei L, Thornton-Evans G, Genco RJ. (2012). Update of the case definitions for population-based surveillance of periodontitis. *J Periodontol* 2012;83:1449-1454.
31. Rodríguez I, Lema I, Blanco M, Rodríguez-Yáñez M, Leira R, Castillo J. Vascular retinal, neuroimaging and ultrasonographic markers of lacunar infarcts. *Int J Stroke* 2010;5:360-366.
32. Fazekas F, Kleinert R, Offenbacher H, et al. The morphologic correlate of incidental punctate white matter hyperintensities on MR images. *AJNR Am J Neuroradiol* 1991;12:915-921.
33. Fazekas F, Kleinert R, Offenbacher H, et al. Pathologic correlates of incidental MRI white matter signal hyperintensities. *Neurology* 1993;43:1683-1689.
34. Pantoni L, Simoni M, Pracucci G, Schmidt R, Barkhof F, Inzitari D; for the European Task Force on Age-Related White Matter Changes. Visual rating

scales for age-related white matter changes (leukoaraiosis): can heterogeneity be reduced? *Stroke* 2002;33:2827-2833.

35. Touboul PJ, Hennerici MG, Meairs S, et al. Mannheim carotid intima-media thickness consensus (2004-2006). An update on behalf of the Advisory Board of the 3rd and 4th Watching the Risk Symposium, 13th and 15th European Stroke Conferences, Mannheim, Germany, 2004, and Brussels, Belgium, 2006. *Cerebrovasc Dis* 2007;23:75-80.
36. United Kingdom transient ischaemic attack (UK-TIA) aspirin trial: interim results. UK-TIA Study Group. (1988). *Br Med J (Clin Res Ed)* 1988;296:316-320.
37. Van Swieten JC, Koudstaal PJ, Visser MC, Schouten HK, van Gijn J. Interobserver agreement for the assessment of handicap in stroke patients. *Stroke* 1988;19:604-607.
38. Brito LC, DalBó S, Striechen TM, et al. Experimental periodontitis promotes transient vascular inflammation and endothelial dysfunction. *Arch Oral Biol* 2013;58:1187-1198.
39. Keles GC, Balli U, Cetinkaya BO, et al. Biochemical analysis of pentraxin 3 and fibrinogen levels in experimental periodontitis model. *Mediators Inflamm* 2012;2012:1–7.
40. Temelli B, Yetkin Ay Z, Savaş HB, et al. Circulation levels of acute phase proteins pentraxin 3 and serum amyloid A in atherosclerosis have correlations with periodontal inflamed surface area. *J Appl Oral Sci* 2018;16:1-9.
41. Amar S, Gokce N, Morgan S, Loukideli M, Van Dyke TE, Vita JA. (2003). Periodontal disease is associated with brachial artery endothelial dysfunction and systemic inflammation. *Arterioscler Thromb Vasc Biol* 2003;23:1245-1249.
42. Bonacina F, Baragetti A, Catapano AL, Norata GD. Long pentraxin 3: experimental and clinical relevance in cardiovascular diseases. *Mediators Inflamm* 2013;2013:1–10.
43. Fornai F, Carrizzo A, Forte M, et al. The inflammatory protein Pentraxin 3 in cardiovascular disease. *Immun Ageing* 2016;13:1-9.
44. Yasunaga T, Ikeda S, Koga S, et al. Plasma pentraxin 3 is a more potent predictor of endothelial dysfunction than high-sensitive C-reactive protein. *Int Heart J* 2014;55:160-164.

45. Harada N, Nakayama M, Nakano H, Fukuchi Y, Yagita H, Okumura K. Pro-inflammatory effect of TWEAK/Fn14 interaction on human umbilical vein endothelial cells. *Biochem Biophys Res Commun* 2002;299:488-493.
46. Saas P, Boucraut J, Walker PR, et al. TWEAK stimulation of astrocytes and the proinflammatory consequences. *Glia* 2000; 32:102-107.
47. Niwa K, Carlson GA, Iadecola C. Exogenous A beta1-40 reproduces cerebrovascular alterations resulting form amyloid precursor protein overexpression in mice. *J Cereb Blood Flow Metab* 2000a;20:1659-1668.
48. Niwa K, Younkin L, Ebeling C, et al. Abeta 1-40-related reduction in functional hyperemia in mouse neocortex during somatosensory activation. *Proc Natl Acad Sci U S A* 200b;97:9735-9740.
49. Leira Y, Martín-Lancharro P, Blanco J. Periodontal inflamed surface area and periodontal case definition classification. *Acta Odontol Scand* 2018;76:195-198.
50. Yilmaz MI, Sonmez A, Ortiz A, et al. Soluble TWEAK and PTX3 in nondialysis CKD patients: Impact on endothelial dysfunction and cardiovascular outcomes. *Clin J Am Soc Nephrol* 2011;6:785-792.

Table 1. Baseline characteristics of participants.

VARIABLES	LI patients (n=120)	Controls (n=120)	*P*-value
Age (years)	66.4±9.9	64.9±10.0	0.225
Males, n (%)	82 (68.3)	86 (71.7)	0.573
BMI	26.9±3.4	27.5±4.2	0.235
Hypertension, n (%)	74 (61.7)	40 (33.3)	<0.0001
Diabetes mellitus, n (%)	33 (27.5)	12 (10.0)	0.001
Hypercholesterolemia, n (%)	55 (45.8)	22 (18.3)	<0.0001
Ischemic heart disease, n (%)	22 (18.3)	4 (3.3)	<0.0001
Peripheral arterial disease, n (%)	5 (4.2)	0 (0.0)	0.024
Current smoker, n (%)	22 (18.3)	12 (10.0)	0.056
Alcohol consumption, n (%)	9 (7.5)	6 (5.0)	0.224
Medication			
-Statins, n (%)	56 (46.7)	22 (18.3)	<0.0001
-Antiaggregants, n (%)	46 (38.3)	4 (3.3)	<0.0001
-Antihypertensives, n (%)	70 (58.3)	40 (33.3)	<0.0001
Low education level, n (%)	38 (31.7)	40 (33.3)	0.942
FMPS (%)	54.8±17.5	28.5±13.3	<0.0001
FMBS (%)	59.4±18.2	29.7±15.0	<0.0001
PD measures			
-Mean PD (mm)	3.6±1.0	2.6±0.7	<0.0001
-Number of sites/mouth PD ≥6 mm	18.1±25.3	2.3±10.7	<0.0001
Rec (mm)	0.6±0.4	0.4±0.3	<0.0001
CAL measures			
-Mean CAL (mm)	4.3±1.4	3.0±1.0	<0.0001
-Number of sites/mouth CAL ≥5 mm	21.0±34.2	63.7±50.1	<0.0001
Number of present teeth	20.0±3.7	25.3±2.1	<0.0001
PISA (mm^2)	1040.4±1145.8	187.7±358.2	<0.0001
Severity of periodontitis, n (%)			<0.0001
-Mild	16 (18.8)	15 (40.5)	
-Moderate	29 (34.1)	19 (51.4)	
-Severe	40 (47.1)	3 (8.1)	

LI: lacunar infarct; BMI: body mass index; FMPS: full-mouth plaque score; FMBS: full-mouth bleeding score; PD: pocket depth; Rec: gingival recession; CAL: clinical attachment level; PISA: periodontal inflamed surface area.

Table 2. Serum levels of biomarkers in LI patients and controls.

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
Aβ peptides			
-$A\beta_{1\text{-}40}$ (pg/mL)	53.5±11.5	33.0±4.7	<0.001
-$A\beta_{1\text{-}42}$ (pg/mL)	50.5±15.2	48.6±9.9	0.253

IL: interleukin; PTX: pentraxin; sTWEAK: soluble tumor necrosis factor-like weak inducer of apoptosis; Aβ: amyloid beta.

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

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)	5.2 [3.9-6.6]	5.4 [4.0-6.8]	0.634
Endothelial dysfunction			
-PTX3 (pg/mL)	1670.7±1043.3	2586.2±1680.7	0.007
-sTWEAK (pg/mL)	162.7 [95.0-273.1]	261.4 [194.6-317.7]	0.001
Aβ peptides			
-$A\beta_{1-40}$ (pg/mL)	51.2±11.0	60.2±10.7	<0.001
-$A\beta_{1-42}$ (pg/mL)	49.3±15.8	54.1±13.0	0.127

IL: interleukin; PTX: pentraxin; sTWEAK: soluble tumor necrosis factor-like weak inducer of apoptosis; Aβ: amyloid beta.

FIGURE LEGENDS

Figure 1. Serum levels of: **A)** IL-6 (pg/mL); **B)** IL-10 (pg/mL); **C)** PTX3 (pg/mL); **D)** sTWEAK (pg/mL); **E)** $A\beta_{1-40}$ (pg/mL); **F)** $A\beta_{1-42}$ (pg/mL) according to the presence or absence of PD.

Figure 2. Correlation between PISA and: **A)** IL-6; **B)** PTX3; **C)** sTWEAK; **D)** $A\beta_{1-40}$ in patients with poor functional outcome.

PRODUCT AND COMPANIES

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