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TITLE: Periodontitis and Systemic Markers of Neurodegeneration. A case-control study.

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ABSTRACT

Aim: To investigate whether periodontitis is associated with amyloid beta (Aβ) peptides and if systemic inflammation could act as a potential mediator of this link.

Material and Methods: A case-control study was designed including 75 patients with periodontitis (cases) and 75 age-balanced and gender-matched participants without periodontitis (controls). Full-mouth periodontal evaluation was performed in all participants. Demographic, clinical and behaviour data were also recorded. Fasting blood samples were collected and serum levels of interleukin 6 (IL-6), high sensitivity C-reactive protein (hs-CRP), $A\beta_{1-40}$ and $A\beta_{1-42}$ were determined.

Results: Cases showed higher levels of IL-6 (8.7±3.2 vs. 4.8±0.5 pg/mL), hs-CRP (3.3±1.2 vs. 0.9±0.7 mg/L), $A\beta_{1-40}$ (37.3±6.0 vs. 30.3±1.8 pg/mL) and $A\beta_{1-42}$ (54.5±10.6 vs. 36.5±10.0 pg/mL) when compared to controls (all $p<0.001$). Diagnosis of periodontitis was statistically significantly associated with circulating $A\beta_{1-40}$ (β $coefficient_{adjusted}$=6.9, 95%CI: 5.4-8.3; $p<0.001$) and $A\beta_{1-42}$ (β $coefficient_{adjusted}$ =17.8, 95%CI: 14.4-21.3; $p<0.001$). Mediation analysis confirmed hs-CRP and IL-6 as mediators of this association.

Conclusions: Periodontitis is associated with increased peripheral levels of Aβ. These findings could be explained by enhanced systemic inflammation that can be seen in patients with periodontitis.

CLINICAL RELEVANCE

Scientific rationale for study: Peripheral inflammation is associated with high load of Aβ peptides in Alzheimer´s disease. Periodontitis increases the risk of having cognitive decline; however, little is known on the potential role of periodontitis as a contributor to increased systemic concentrations of Aβ peptides in otherwise healthy individuals.

Principal findings: Circulating Aβ peptides are elevated in periodontitis. A link between diagnosis of periodontitis and Aβ was observed and enhanced systemic inflammation could explain this relationship.

Practical implications: Our results suggest that periodontitis via systemic inflammation could contribute to increased peripheral levels of Aβ peptides, which are key elements in the pathogenesis of Alzheimer´s disease.

INTRODUCTION

Amyloid plaques contain small peptides of different lengths (from 39 to 43 amino acids), the so-called Aβ peptides, which are the result of sequential proteolytic cleavage of the amyloid precursor protein (AAP) (Murphy & Levine 3rd, 2010). There are two main types of Aβ peptides that are $A\beta_{1-40}$ and $A\beta_{1-42}$. While $A\beta_{1-40}$ is abundant and less neurotoxic, $A\beta_{1-42}$ is less abundant and severely neurotoxic as well as more prone to aggregate to amyloid plaques (Tiwari et al., 2019). Extracellular deposits of these Aβ peptides in brain tissue are considered as one of the key hallmarks of Alzheimer's disease (AD), which is the most common form of age-related neurodegenerative disease (Magalingam et al., 2018). It has been shown that high levels of chronic inflammation measured by for instance acute phase reactants [i.e., C-reactive protein (CRP)] or pro-inflammatory cytokines such as interleukin 6 (IL-6) can be toxic or further stimulate Aβ production, aggregation and toxicity in the brain (Perry 2004).

In the last decades, human periodontitis and its ultimate sequel (i.e., tooth loss) have been associated with cognitive decline/impairment in patients with AD (Ide et al., 2016, Takeuchi et al., 2017, Holmer et al., 2018). A recent meta-analysis of observational studies showed that subjects diagnosed with periodontitis have a 1.6 fold-increased risk of developing AD (Leira et al., 2017). We recently demonstrated that bacterial endotoxin (i.e., lipopolysaccharide) from *Porphyromonas gingivalis* (*P. gingivalis*), the keystone pathogen of periodontitis, is capable of inducing systemic increase of Aβ peptides (i.e., $A\beta_{1-40}$ and $A\beta_{1-42}$) and this elevation correlated to alveolar bone loss in an animal model (Leira et al., 2019a). *P. gingivalis* and its toxic products can colonize areas from the brain resulting in enhanced neuroinflammation and overproduction of $A\beta_{1-42}$, which is part of amyloid plaques present in AD patients (Dominy et al., 2019). Tissues from otherwise healthy individuals but suffering from periodontitis showed overexpression of APP, which produces Aβ peptides (Kubota et al., 2014). Periodontitis was also associated with high $A\beta_{1-42}$ concentrations in individuals with cognitive decline (Gil-Montoya et al., 2017). On the other hand, high levels of periodontal inflammation were recently related to systemic increase of $A\beta_{1-40}$ in patients with a subtype of cerebral small vessel disease that is closely linked to vascular dementia and this association was mediated by enhanced systemic inflammation due to periodontitis (Leira et al., 2019b).

IL-6 is responsible for the regulation of the acute-phase response produced by the host after

infection. CRP is one of the main acute-phase reactants and is primarily synthesized by IL-6 on the liver (Sproston & Ashworth, 2018). During inflammation, there is a linear correlation between increasing levels of IL-6 and CRP (Sproston & Ashworth, 2018). Therefore, both molecules are widely used to assess the presence and severity of low-grade inflammation (Sproston & Ashworth, 2018). Patients with untreated periodontitis show increased of circulating levels of CRP or IL-6, which reflects higher levels of inflammation beyond the oral cavity in this population (Loos et al., 2000).

Nevertheless, to the best of our knowledge, no human studies have investigated the effect of periodontitis on peripheral Aβ peptides in systemically healthy individuals. Our hypothesis was that patients with periodontitis have higher serum levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ and that systemic inflammation could mediate this increase. Hence, the aim of the study was two-fold. The primary objective was to investigate whether there is an association between diagnosis of periodontitis and Aβ peptides. As a secondary objective we tested if systemic inflammation could be a mediator of this relationship.

MATERIALS AND METHODS

Study design and participants

For this age-balanced and gender-matched case-control study, 150 participants otherwise healthy were recruited from the Faculty of Odontology of Santiago de Compostela (Spain) during the period comprised between January 2014 and April 2017. Cases consisted of 75 patients diagnosed with periodontitis (Eke et al., 2012) among referrals to the Periodontology Unit (University of Santiago de Compostela, Spain) for diagnosis and treatment of periodontitis. Seventy-five controls were defined as those without any clinical/radiographic signs of periodontal disease (including gingivitis and periodontitis) and/or history of this disease. These participants were identified among friends of the patients with periodontitis (n=29) or from a research registry of participants from previous studies carried out by our research group (n=46) (Leira et al., 2018; Leira et al., 2019b; Ameijeira et al., 2019).

Exclusion criteria were as follows: (i) <18 years of age; (ii) <15 teeth (excluding third molars and retained roots); (iii) periodontal treatment in the last year; 3) evident neurological diseases confirmed clinically and/or by Computed Tomography/Magnetic Resonance Imaging (e.g. neuroinflammatory, neurovascular or neurodegenerative conditions); (iv) concomitant medical conditions (e.g. diabetes, cardiovascular diseases, hypertension or hypercholesterolemia) or active

infectious/inflammatory diseases (e.g. HIV, hepatitis, tuberculosis, rheumatoid arthritis, allergies or asthma); (v) pregnant or lactating females; (vi) malignancy; (vii) treatment with systemic antibiotics, corticosteroids and/or immunosuppressive agents within 3 months prior to periodontal examination (**Supplemental Figure**).

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).Written informed consent was obtained from each participant or their relatives after full explanation of the periodontal examination and blood sample collection. The study was performed following the STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) guidelines (Von Elm et al., 2008).

Periodontal assessment and demographic information

A full-mouth periodontal examination was performed in all participants by a calibrated periodontist (YL) (Leira et al., 2018; Leira et al., 2019b; Ameijeira et al., 2019). The following parameters were measured in all teeth (except third molars): probing pocket depth (PPD), clinical attachment level (CAL), full-mouth plaque score (FMPS), and full-mouth bleeding score (FMBS) (Ainamo & Bay, 1975). Measurements were recorded at 6 sites per tooth (mesiobuccal, distobuccal, midbuccal, mesiolingual, distolingual, and midlingual) using a calibrated University of North Carolina periodontal probe (UNC 15; Hu-Friedy, Chicago, IL, USA). Slight 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 PPD ≥5 mm. Moderate periodontitis was defined as ≥2 interproximal sites with CAL of ≥4 mm (not on the same tooth) or ≥2 interproximal sites with PPD of ≥5 mm, also not on the same tooth. Severe periodontitis was defined as the presence of ≥2 interproximal sites with CAL of ≥6 mm (not on the same tooth) and ≥1 interproximal sites with PPD of ≥5 mm. Total periodontitis was the sum of slight, moderate, and severe periodontitis (Eke et al., 2012).

In addition, the periodontal inflamed surface area (PISA) was calculated for all participants. PISA reflects the surface area of bleeding pocket epithelium in mm^2. As previously described (Leira et al., 2018; Leira et al., 2019b, Leira et al, 2019c), PISA was calculated using a Microsoft Excel spreadsheet, in the following steps:(i) mean CAL and gingival recession for each particular tooth is calculated;(ii) linear mean CAL and gingival recession 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 bleeding on probing, resulting in the PISA for that particular tooth; and (iv) the sum of all individual PISAs around individual teeth is calculated, rendering the full-mouth PISA value in mm^2 for each participant (Nesse et al., 2008).

For both groups, smoking status was evaluated by a questionnaire. Body mass index (BMI) was calculated for all participants using the formula weight/$height^2$ (kg/m^2).

Sample collection and laboratory tests

Fasting blood samples were obtained in the morning at the same time as the periodontal assessment and interview. Briefly, 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 by centrifugation (15 minutes at 3000 g) and 0.5 mL of extracted serum was immediately transferred to 1.5-mL aliquots. Each aliquot was stored at −80°C until required for analysis. Serum levels of hs-CRP were measured using an immunodiagnostic IMMULITE® 2000 Systems (Siemens Healthcare Diagnostics, Malvern, PA, USA); minimum assay sensitivity was 0.2 mg/L. Serum levels of IL-6 and Aβ peptides were measured by enzyme-linked immunosorbent assay (ELISA) technique following the manufacturer's instructions IL-6 ELISA kit (Proteintech™, Manchester, UK) minimum assay sensitivity was 3.8 pg/mL; $A\beta_{1\text{-}40}$ ELISA kit (Elabscience®, Houston, TX) minimum assay sensitivity was 9.38 pg/mL with an intra-assay coefficient of variation (CV) of 4.6% and inter-assay CV of 6.5%; and $A\beta_{1\text{-}42}$ ELISA kit (Elabscience®, Houston, TX) minimum assay sensitivity was 9.38 pg/mL with an intra-assay coefficient of variation (CV) of 6.0% and inter-assay CV of 6.8%.

Determinations were performed in an independent laboratory blinded to clinical data. Clinical investigators were unaware of the laboratory results until the study had ended. hs-CRP was determined in the Central Laboratory of the Clinical University Hospital of Santiago de Compostela whereas Aβ peptides and IL-6 were determined in the Clinical Neurosciences Research Laboratory of the same hospital.

Statistical analysis

For this observational study, a sample size of 69 patients per group (case vs. controls) (1:1 ratio) was sufficient to detect a 5 pg/mL difference in serum $A\beta_{1\text{-}40}$ between study groups, with a standard deviation of 9 pg/mL and assuming α-value=0.05 and statistical power of 90% (Leira et al., 2019b).

Mean values ± standard deviation (SD) and median [P_{25}, P_{75}] were calculated for normally and non-normally distributed continuous variables, respectively. Statistical tests used to compare continuous data were *independent t* test or *Mann-Whitney U* test. Categorical variables were reported as percentages and compared by X^2 test. Non-parametric correlation analyses between biomarkers and clinical periodontal parameters were performed using *Spearman's rank correlation coefficient*.

Linear regression models adjusted for confounders (i.e., age, gender, BMI and smoking) were created to test association between periodontitis and Aβ peptides as well as with $A\beta_{42:40}$ ratio. Mediation analysis was carried out to investigate whether systemic inflammation (measured by hs-CRP and IL-6) could explain the potential association between periodontitis and Aβ peptides as well as with $A\beta_{42:40}$ ratio. In order to so, the PROCESS macro for SPSS (Hayes 2013) was used. Each analytical step was adjusted for confounders previously named. Mediation analysis tests if the association between a predictor/exposure (X= periodontitis) and an outcome (Y=Aβ peptides and $A\beta_{42:40}$ ratio) is mediated through a mediator (M=systemic inflammatory markers). Estimates with 95% confidence intervals were calculated using ordinary least square regression for each step. The indirect effect of the predictor on the outcome mediated via the mediator (also named mediated effect) was considered significant if the corresponding 95% bootstrap confidence interval did not include zero. This approach is the one recommended by Hayes due to it does not assume a normal distribution of the sample (Hayes 2013). The percentage of the effect of periodontitis on Aβ peptides and $A\beta_{42:40}$ ratio mediated by markers of systemic inflammation was also calculated using the following formula: (mediated effect/total effect)*100.

All tests were carried out at a significance level of α=0.05 using IBM SPSS Statistics (version 24.0).

RESULTS

Demographic, clinical and periodontal data

Table 1 depicts participant's characteristics and demographic factors. Cases and controls were balanced for age and matched for gender. No statistically significant differences were observed in terms of BMI and smoking status.

Cases had greater periodontal inflammation when compared to controls (**Table 1**). Both measures of active gingival inflammation (PPD, FMBS and PISA) and historic periodontal tissue attachment loss (CAL) were significantly higher in cases than in controls. Also, the level of

bacterial plaque accumulation was greater in those with periodontitis than in controls. On average, cases had one fewer tooth present in the mouth compared to those without periodontitis (**Table 1**). Within periodontal patients, 23 (30.7%) had slight periodontitis, 30 (40.0%) presented moderate periodontitis and 22 (29.3%) had severe periodontitis.

Biomarkers

Cases presented statistically significantly higher serum levels of IL-6, hs-CRP, $A\beta_{1-40}$ and $A\beta_{1-42}$ compared to controls (all $p<0.001$) (**Table 2**).

Serum levels of IL-6 (12.4±3.6 ng/mL vs. 7.9±0.9 ng/mL, $p<0.001$ and vs. 6.3±0.8 ng/mL, $p<0.001$) (**Figure 1A**), hs-CRP (4.9±0.9 mg/L vs. 3.1±0.2 mg/L, $p<0.001$ and vs. 2.2±0.3, $p<0.001$) (**Figure 1B**), $A\beta_{1-40}$ (43.9±4.9 ng/mL vs. 36.1±4.0 ng/mL, $p<0.001$ and vs. 32.6±2.9 ng/mL, $p<0.001$) (**Figure 1C**) and $A\beta_{1-42}$ (66.2±6.1 ng/mL vs. 54.5±5.4 ng/mL, $p<0.001$ and vs. 43.3±5.8 ng/mL, $p<0.001$) (**Figure 1D**) were statistically significantly elevated in severe periodontal patients in comparison to those cases with moderate or slight forms of periodontitis. Similarly, the moderate group presented higher levels of IL-6 (7.9±0.9 ng/mL vs. 6.3±0.8 ng/mL, $p=0.001$) (**Figure 1A**), hs-CRP (3.1±0.2 mg/L vs. 2.2±0.3, $p<0.001$) (**Figure 1B**), $A\beta_{1-40}$ (36.1±4.0 ng/mL vs. 32.6±2.9 ng/mL, $p=0.015$) (**Figure 1C**) and $A\beta_{1-42}$ (54.5±5.4 ng/mL vs. 43.3±5.8 ng/mL, $p=0.005$) (**Figure 1D**) than slight periodontal patients. When levels of each biomarkers were compared between each grade of severity of periodontitis and the group of participants with a healthy periodontium, statistically significant differences were also found [for IL-6: healthy (4.8±0.5 ng/mL) vs. (slight (6.3±0.8 ng/mL, $p<0.001$), moderate (7.9±0.9 ng/mL, $p<0.001$) and severe (12.4±3.6 ng/mL, $p<0.001$) (**Figure 1A)**; for hs-CRP: healthy (0.9±0.7 mg/L) vs. (slight (2.2±0.3, $p<0.001$), moderate (3.1±0.2 mg/L, $p<0.001$) and severe (4.9±0.9 mg/L, $p<0.001$) (**Figure 1B)**, for $A\beta_{1-40}$: healthy (30.3±1.8 ng/mL) vs. (slight (32.6±2.9 ng/mL, $p=0.015$), moderate (36.1±4.0 ng/mL, $p<0.001$) and severe (43.9±4.9 ng/mL, $p<0.001$) (**Figure 1C)**; for $A\beta_{1-42}$: healthy (36.5±10.0 ng/mL) vs. (slight (43.3±5.8 ng/mL, $p=0.005$), moderate (54.5±5.4 ng/mL, $p<0.001$) and severe (66.2±6.1 ng/mL, $p<0.001$) (**Figure 1D)**.

$A\beta_{42:40}$ ratio was higher in cases than controls (**Table 2**). Compared to periodontally healthy subjects (1.2±0.3 ng/mL), greater $A\beta_{42:40}$ ratio was observed in severe (1.5±0.1, $p<0.001$) and moderate cases (1.5±0.2, $p<0.001$) but not for slight periodontal individuals (1.3±0.2, $p=0.507$). No statistically significant differences were found between different grades of severity of periodontitis (data not shown).

Correlation between biomarkers and clinical periodontal variables

Strong positive correlations were found between clinical periodontal parameters (PPD, CAL, FMBS, FMPS and PISA) and IL-6, hs-CRP, $A\beta_{1-40}$ and $A\beta_{1-42}$ as well as $A\beta_{42:40}$ ratio (**Table 3**). IL-6 correlated positively to $A\beta_{140}$ and $A\beta_{1-42}$ ($r=0.250$, $p<0.001$ and $r=0.735$, $p<0.001$; respectively) (**Figure 2A and 2B**). Similarly, hs-CRP strongly correlated with both $A\beta_{1-40}$ ($r=0.656$, $p<0.001$) and $A\beta_{1-42}$ ($r=0.702$, $p<0.001$) (**Figure 2C and 2D**). Statistically significant correlations were also found for $A\beta_{42:40}$ ratio (IL-6: $r=0.453$, $p<0.001$ and hs-CRP: $r=0.401$, $p<0.001$).

Association between periodontitis and Aβ peptides and mediation analysis

Diagnosis of periodontitis was statistically significantly associated with circulating $A\beta_{1-40}$ (β $coefficient_{adjusted}=6.9$, 95%CI: 5.4-8.3; $p<0.001$) and $A\beta_{1-42}$ (β $coefficient_{adjusted}=17.8$, 95%CI: 14.4-21.3; $p<0.001$) as well as with $A\beta_{42:40}$ ratio (β $coefficient_{adjusted}=0.2$, 95%CI: 0.1-0.3; $p<0.001$).

Mediation analysis showed a statistically significant effect of both markers of systemic inflammation (hs-CRP and IL-6) mediating the association between periodontitis and both Aβ peptides (**Table 4**). Periodontitis was statistically significantly associated with increased levels of systemic inflammation (all $p<0.001$). Systemic inflammatory markers were also statistically significantly related to higher levels of Aβ peptides (all $p<0.001$). The indirect (mediated) effect of periodontitis on Aβ peptides mediated through hs-CRP and IL-6 was statistically significant (95%CI did not include 0). The percentage mediated in the association between periodontitis and Aβ peptides through hs-CRP and IL-6 ranged from 38% to 82% (**Table 4**). Similar findings were observed when continuous measures of periodontitis were used as exposure, for example, PPD and CAL (data not shown). No significant mediation effect was found when $A\beta_{42:40}$ ratio was used as an outcome (**Table 4**).

DISCUSSION

In the present age-balanced and gender-matched case-control study, periodontitis was associated with increased peripheral levels of Aβ peptides and this relationship was mediated by systemic inflammation.

The link between periodontitis and systemic inflammation is well documented (Loos et al., 2000; Amar et al., 2003; Paraskevas et al.; 2008). Further, periodontal treatment reduces pro-inflammatory markers measured in peripheral blood (D'Aiuto et al., 2004). In the present study, we confirmed that periodontitis was associated with elevated serum levels of hs-CRP and IL-6 and

this increase was linked to severity of periodontitis. Previous observational studies from different cohorts demonstrated increased level of systemic inflammation (measured by circulating CRP concentrations) in patients with periodontitis (Slade et al., 2000; Noack et al., 2001; Linden et al., 2008; Pitiphat et al., 2008; Ardila & Guzmán, 2015). In the present study, clinical parameters of current/active periodontal inflammation (PPD, FMBS and PISA) and history of periodontal attachment loss (CAL) were positively correlated with both markers of systemic inflammation.

Aβ deposits play an important role in the pathogenesis of AD, as they are the main component of senile plaques in brain grey matter (Murphy & Levine 3rd, 2010). Extracellular aggregation of Aβ plaques is associated with widespread neuronal atrophy resulting in gradual neuronal death and memory loss. The most relevant finding in the present study is the overexpression of Aβ peptides ($A\beta_{140}$ and $A\beta_{1\text{-}42}$) in periodontitis subjects with good general health. Systemic levels of Aβ peptides were significantly elevated in patients with periodontitis compared to controls. Also, clinical periodontal parameters positively correlated to increased Aβ peptides concentrations. Previously, it has been reported that in cognitively normal healthy elderly measures of periodontitis such as CAL were associated with high brain amyloid accumulation assessed by positron emission tomography (Kamer et al., 2015). The present results are in line with a recent animal experiment carried out by our group, which has shown that bacterial endotoxin from *P. gingivalis* evoked a raise in peripheral Aβ peptides in systemically healthy rats during 21 days of follow-up (Leira et al., 2019a). Other experiments have also demonstrated that chronic exposure of *P. gingivalis* and its toxins is capable of inducing brain colonization and induction of $A\beta_{1\text{-}42}$, microglia-mediated neuroinflammation as well as learning and memory impairment (Wu et al., 2017; Dominy et al. 2019), thus, supporting the hypothesis that a chronic peripheral inflammatory condition such as periodontitis could be involved in the onset/progression of AD. In addition to neurotoxicity, $A\beta_{1\text{-}40}$ can also contribute to cerebral vascular pathology and endothelial dysfunction when deposited in cerebral microvessels (Niwa et al., 2000a; Niwa et al., 2000b; Iadecola et al., 2009). Indeed, we recently showed that active periodontal inflammation was associated with increased systemic levels of $A\beta_{1\text{-}40}$ in patients diagnosed with a subtype of cerebral small vessel disease (lacunar infarcts) that could cause vascular dementia (Leira et al., 2019b).

Different mechanisms may explain why periodontitis subjects had higher circulating levels of Aβ peptides. On the one hand, a direct production of Aβ could occur in periodontitis. It has been shown that gingival tissues with periodontitis are able to express specific genes related to Aβ production such as APP mRNA (Kubota et al., 2014). On the other hand, an indirect pathway by

which Aβ is produced due to periodontitis could be also speculated. Evidence suggests that systemic inflammation can produce Aβ in the systemic circulation and promotes its accumulation in the brain (Perry 2004). Our results showed a positive correlation between systemic inflammatory markers (hs-CRP and IL-6) and Aβ peptides, therefore, explaining the association between periodontitis, systemic inflammation and Aβ overexpression (Wang et al., 2019). In addition, mediation analysis showed that markers of systemic inflammation (hs-CRP and IL-6) acted as mediators in the association between periodontitis and Aβ peptides, therefore, increased systemic inflammation could be a plausible biological explanation of our findings. However, more research is needed in this area to elucidate the exact mechanisms behind this relationship because when the $A\beta_{42:40}$ ratio was used as an outcome no significant mediation effect was observed by inflammation perhaps because inflammatory markers were not associated with $A\beta_{42:40}$ ratio. For instance, whether systemic Aβ peptides in patients with periodontitis are able to cross the blood-brain barrier and accumulate on the brain or whether systemic inflammatory markers can actually induce neuroinflammation in these patients and therefore lead to neuronal damage.

Some limitations of the present study should be addressed. Firstly, the retrospective design of the study does not allow us to test causality on the association between periodontitis and circulating Aβ peptides. Secondly, participants were in good systemic health but this was confirmed by means of a self-reported questionnaire and some of them might presented with poor metabolic control (including high blood pressure, poor glycaemic control, dyslipidemia…) and undiagnosed hypertension, diabetes or hypercholesterolemia as well as high BMI which could have influenced levels of both systemic inflammation and Aβ peptides (Shah et al., 2012; de Miguel et al., 2015; Marsland et al., 2010; Razay et al., 2007; Ellulu et al., 2017; Lee et al. 2008). Thirdly, some of the controls were friends of cases. This fact could have led to selection bias as they could be more motivated and have higher response rates compared to general population controls that do not know the case. Also these people might be more sociable and extrovert as they are willing to participate in the study compared to less sociable and introverted subjects (Wacholder et al., 1992). Therefore, potentially this type of controls could show better oral and general health as well as less systemic inflammation than the general population. Although a full cognitive assessment including specific cognitive tests was not done in these participants, none of them showed clinical cognitive dysfunction (confirmed by subjective cognitive examination recording slowness of thought, inappropriateness and mood) (Kipps & Hodges, 2005) or neuroimaging findings potentially associated with any evident form of dementia (e.g., brain damage, cortical/subcortical

atrophy, hydrocephaly and cerebral small vessel disease). Future studies including cognitive healthy subjects confirmed with robust cognitive tests are needed to confirm our results. Another limitation to be considered was the use of traditional ELISA technique to measure blood Aβ peptides. New ultrasensitive technologies (i.e., immunoaffinity-based assays) now available in the market such as immunomagnetic reduction (IMR) and single molecule array (SIMOA) must be considered in future studies to overcome the challenges of detection encountered using ELISA-based technique. Both IMR and SIMOA although promising, still need validation in different populations by means of multicentre studies (Lue et al., 2017). Lastly, readers should interpret the present results with caution. AD pathogenesis is very complex. The amyloid hypothesis might not explain all cases of AD. In fact, cognitively normal individuals can have Aβ deposits and also AD patients can present very few Aβ deposits (Edison et al., 2007; Li et al. 2008). Distribution and extension of senile plaques is sometimes similar in patients with dementia compared to subjects with preserved cognitive function (Davis et al., 1999; Fagan et al., 2009). Furthermore, Aβ peptides accumulation alone represents an extremely early event in the amyloid cascade leading to neurodegeneration (Ricciarelli et al., 2017). Further studies including AD patients with and without periodontitis are needed to demonstrate whether periodontitis can feature as an early therapeutic target for AD.

In summary, it can be concluded that periodontitis is associated with high serum levels of Aβ peptides. This finding could be due to enhanced systemic inflammation observed in patients with periodontitis. Further longitudinal evidence from cohort studies using different populations is warranted to confirm our preliminary results. Future research investigating the potential role of periodontitis in the neurodegenerative process is needed.

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FIGURE LEGENDS

Figure 1. Serum levels of biomarkers according to severity of periodontitis [slight (N=23), moderate (N=30) and severe (N=22)]: **A)** IL-6; **B)** hs-CRP; **C)** $A\beta_{1-40}$; **D)** $A\beta_{1-42}$.

Figure 2. Spearman's correlation coefficient for systemic inflammatory markers and Aβ peptides: **A)** IL-6 and $A\beta_{1-40}$; **B)** IL-6 and $A\beta_{1-42}$; **C)** hs-CRP and $A\beta_{1-40}$; **D)** hs-CRP and $A\beta_{1-42}$.

Variables	Periodontitis (n=75)	No Periodontitis (n=75)	p-value
Age (years)	44.8±10.3	44.7±12.2	0.983
Gender, Male, n (%)	49 (65.3)	49 (65.3)	1.000
BMI (kg/m^2)	25.5 [12.9, 29.4]	23.4 [21.0, 27.4]	0.079
Smoking status, n (%)			0.252
-Current	11 (14.7)	7 (9.3)	
-Former	8 (10.7)	4 (5.3)	
-Never	56 (74.7)	64 (85.3)	
Periodontal clinical parameters			
-PPD (mm)	3.2±0.9	2.0±0.4	**<0.001**
-CAL (mm)	3.5±1.0	2.1±0.5	**<0.001**
-FMBS (%)	57.8±23.4	23.8±8.8	**<0.001**
-FMPS (%)	66.2±17.3	27.9±12.1	**<0.001**
-PISA (mm^2)	610.8 [320.9, 1139.0]	26.5 [17.5, 43.8]	**<0.001**
Teeth present, n	26.2±1.8	27.0±0.7	**0.017**

Table 1. Baseline characteristics.

BMI: body mass index; PPD: probing pocket depth; CAL: clinical attachment level; FMBS: full-mouth bleeding score; FMPS: full-mouth plaque score; PISA: periodontal inflamed surface area.

Variables	Periodontitis (n=75)	No Periodontitis (n=75)	p-value
Systemic inflammation			
-IL-6 (pg/mL)	8.7±3.2	4.8±0.5	**<0.001**
-hs-CRP (mg/L)	3.3±1.2	0.9±0.7	**<0.001**
Aβ peptides			
-$A\beta_{1-40}$ (pg/mL)	37.3±6.0	30.3±1.8	**<0.001**
-$A\beta_{1-42}$ (pg/mL)	54.5±10.6	36.5±10.0	**<0.001**
$A\beta_{42:40}$ (pg/mL)	1.5±0.2	1.2±0.3	**<0.001**

Table 2. Biochemical parameters.

IL-6: interleukin 6; hs-CRP: high sensitivity C-reactive protein; $A\beta_{1-40}$ and $A\beta_{1-42}$: amyloid beta 1-40 and 1-42; $A\beta_{42:40}$: amyloid beta 1-40 and 1-42 ratio.

Table 3. Spearman's correlation coefficient for clinical periodontal parameters and biomarkers.

	PPD (mm)	CAL (mm)	FMBS (%)	FMPS (%)	PISA (mm^2)
IL-6 (pg/mL)	0.702	0.692	0.680	0.636	0.821
p-value	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
hs-CRP (mg/L)	0.730	0.723	0.764	0.701	0.895
p-value	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
$A\beta_{1-40}$ (pg/mL)	0.625	0.640	0.610	0.529	0.692
p-value	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
$A\beta_{1-42}$ (pg/mL)	0.636	0.661	0.621	0.502	0.740
p-value	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
$A\beta_{42:40}$ (pg/mL)	0.360	0.381	0.330	0.300	0.429
p-value	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**

PPD: probing pocket depth; CAL: clinical attachment level; FMBS: full-mouth bleeding score; FMPS: full-mouth plaque score; PISA: periodontal inflamed surface area; IL-6: interleukin 6; hs-CRP: high sensitivity C-reactive protein; $A\beta_{1-40}$ and $A\beta_{1-42}$: amyloid beta 1-40 and 1-42; $A\beta_{42:40}$: amyloid beta 1-40 and 1-42 ratio.

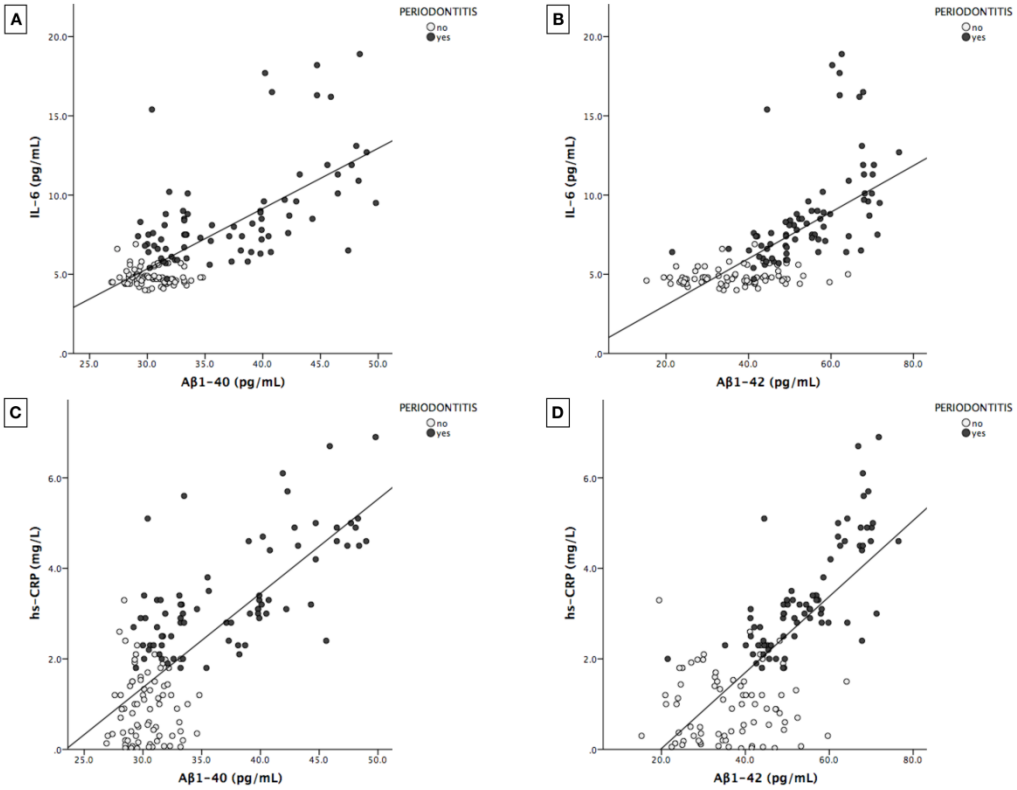
Table 4. Mediation of systemic inflammation for the association between periodontitis and Aβ peptides as well as with $A\beta_{42:40}$ ratio.

Model A (exposure: periodontitis / outcome: $A\beta_{1-40}$)	**Mediator: hs-CRP**			
Effect	**Estimate**	**SE**	**p-value**	**95% CI**
a (exposure → mediator)	2.40	0.17	**<0.001**	2.07-2.74
b (mediator → outcome)	2.34	0.31	**<0.001**	1.72-2.96
c (total effect)	6.86	0.75	**<0.001**	5.38-8.34
c' (direct effect)	1.23	0.99	0.22	-0.74-3.18
ab (mediated effect)	5.63	1.04	-	3.58-7.62
ab/c (hs-CRP percentage mediated) = 82%				
Model B (exposure: periodontitis / outcome: $A\beta_{1-40}$)	**Mediator: IL-6**			
Effect	**Estimate**	**SE**	**p-value**	**95% CI**
a (exposure → mediator)	3.81	0.38	**<0.001**	3.05-4.56
b (mediator → outcome)	0.98	0.14	**<0.001**	0.70-1.26
c (total effect)	6.86	0.75	**<0.001**	5.38-8.34
c' (direct effect)	3.14	0.85	**<0.001**	1.47-4.81
ab (mediated effect)	3.72	0.75	-	2.31-5.23
ab/c (IL-6 percentage mediated) = 54%				
Model C (exposure: periodontitis / outcome: $A\beta_{1-42}$)	**Mediator: hs-CRP**			
Effect	**Estimate**	**SE**	**p-value**	**95% CI**
a (exposure → mediator)	2.40	0.17	**<0.001**	2.07-2.74
b (mediator → outcome)	4.56	0.76	**<0.001**	3.05-6.07
c (total effect)	17.84	1.73	**<0.001**	14.42-21.26
c' (direct effect)	6.87	2.41	**<0.001**	2.12-11.63
ab (mediated effect)	10.97	2.09	-	6.79-14.95
ab/c (hs-CRP percentage mediated) = 61%				
Model D (exposure: periodontitis / outcome: $A\beta_{1-42}$)	**Mediator: IL-6**			
Effect	**Estimate**	**SE**	**p-value**	**95% CI**
a (exposure → mediator)	3.81	0.38	**<0.001**	1.59-6.85
b (mediator → outcome)	1.80	0.35	**<0.001**	1.11-2.48
c (total effect)	17.84	1.73	**<0.001**	14.42-21.26
c' (direct effect)	11.00	2.07	**<0.001**	6.92-15.09
ab (mediated effect)	6.84	1.36	-	4.56-9.76

ab/c (IL-6 percentage mediated) = 38%				
Model E (exposure: periodontitis/ outcome $A\beta_{42:40}$)	**Mediator: hs-CRP**			
Effect	**Estimate**	**SE**	**p-value**	**95% CI**
a (exposure → mediator)	2.40	0.16	**<0.001**	2.06-2.73
b (mediator → outcome)	0.03	0.02	0.21	-0.01-0.07
c (total effect)	0.25	0.05	**<0.001**	0.35-0.78
c' (direct effect)	0.17	0.07	**0.02**	0.02-0.33
ab (mediated effect)	0.07	0.05	**-**	-0.03-0.17
ab/c (IL-6 percentage mediated) = No mediation				
Model F (exposure: periodontitis/ outcome $A\beta_{42:40}$)	**Mediator: IL-6**			
Effect	**Estimate**	**SE**	**p-value**	**95% CI**
a (exposure → mediator)	3.80	0.38	**<0.001**	3.04-4.56
b (mediator → outcome)	0.01	0.01	0.30	-0.01-0.03
c (total effect)	0.25	0.05	**<0.001**	0.35-0.78
c' (direct effect)	0.21	0.06	**0.001**	0.08-0.33
ab (mediated effect)	0.04	0.02	**-**	-0.00-0.09
ab/c (IL-6 percentage mediated) = No mediation				

All models are adjusted for age, gender, body mass index and smoking.

IL-6: interleukin 6; hs-CRP: high sensitivity C-reactive protein; $A\beta_{1-40}$ and $A\beta_{1-42}$: amyloid beta 1-40 and 1-42; $A\beta_{42:40}$: amyloid beta 1-40 and 1-42 ratio.



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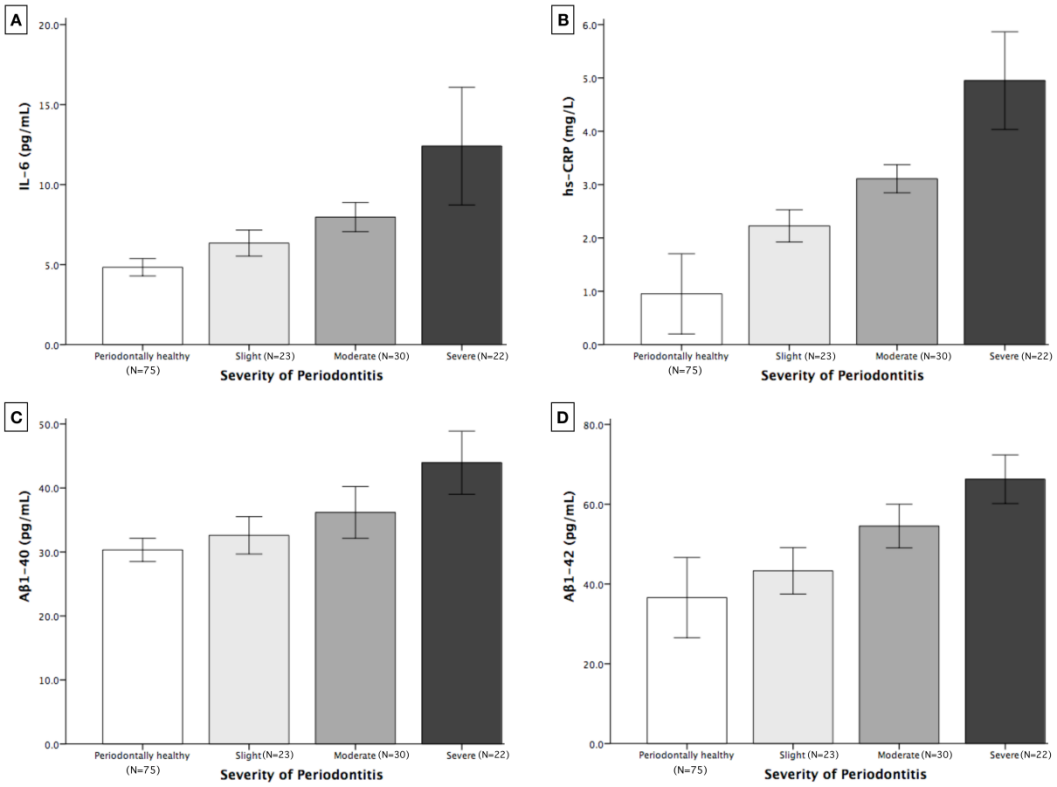

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