

INSTITUTO UNIVERSITÁRIO EGAS MONIZ

DOUTORAMENTO EM CIÊNCIAS BIOMÉDICAS

THE IMPACT OF PERIODONTAL INFECTION AND ITS TREATMENT ON ALPHA-SYNUCLEIN LEVELS AND INFLAMMATION

Trabalho submetido por
Patrícia Soares Lyra
para a obtenção do grau de Doutor em Ciências Biomédicas

Trabalho orientado por
Prof. Doutor José João Baltazar Mendes

março de 2025

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e coorientado por
Prof. Doutor João Tiago da Silva Botelho
Prof. Doutor Kallol Ray Chaudhuri

março de 2025

DEDICATÓRIA

Aos meus pais, Fátima e George

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O documento de aprovação pela Comissão de Ética da Egas Moniz encontra-se no Anexo 1.

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RESUMO

A periodontite - doença inflamatória crónica dos tecidos de suporte dos dentes, que pode culminar na perda dentária - é uma relevante fonte de inflamação e apresenta um potencial impacto negativo na saúde sistémica. Por um lado, a infeção periodontal não tratada pode resultar na disseminação de bactérias periodontopatogénicas e dos seus produtos para tecidos distantes com implicações patológicas. Por outro lado, levanta-se a hipótese de que a periodontite possa induzir uma carga inflamatória a nível sistémico, através da disseminação sanguínea de mediadores inflamatórios. Este estado de inflamação crónica a nível periférico sustenta a associação da infeção periodontal a várias patologias extraorais de carácter inflamatório. São elas, a diabetes mellitus, doenças cardiovasculares, cancro, artrite reumatoide, desfechos adversos da gravidez ou até mesmo condições neurodegenerativas, como a doença de Alzheimer e, mais recentemente, a doença de Parkinson.

De facto, existe uma clara associação entre a periodontite e as α -sinucleinopatias - um grupo de doenças neurodegenerativas caracterizadas pela agregação intracelular anormal da α -sinucleína. Este é o caso da doença de Parkinson, devido às limitações motoras e cognitivas que lhe são inerentes e ao consequente impacto na saúde oral dos pacientes. Até à data, os mecanismos biológicos e patológicos da proteína α -sinucleína (α -Syn) não são inteiramente conhecidos e a influência de fatores ambientais, tais como inflamação crónica a nível sistémico ou os distúrbios disbióticos na microflora comensal intestinal e/ou periodontal, não podem ser descartados como parte dos importantes mecanismos multifactoriais envolvidos no início e na progressão da doença de Parkinson. Neste contexto, existe também um potencial para a prevenção indireta e/ou gestão clínica das α -sinucleinopatias, através do tratamento de patologias periféricas crónicas, inflamatórias e disbióticas, como é o caso da periodontite. Em suma: a evidência que relaciona a doença de Parkinson e a periodontite permanece inconclusiva, em consequência da falta de estudos de intervenção centrados em vias biológicas relevantes.

No contexto atual, de envelhecimento da população, existe uma preocupação real com a crescente prevalência de doenças neurodegenerativas, cujo risco geralmente aumenta com a idade; bem como com o impacto sistémico deletério da periodontite – a doença inflamatória crónica não transmissível mais comum a nível mundial. Neste sentido, este estudo teve por objetivo investigar se o padrão crónico da periodontite pode ser responsável por estados inflamatórios crónicos com efeitos desconhecidos na doença de Parkinson e noutras α -sinucleinopatias. Adicionalmente, o objetivo passou por ajudar a estabelecer bases para um potencial mecanismo biológico que associa a infeção periodontal (e o seu tratamento) à doença de Parkinson, através do seu biomarcador principal, a α -Syn, o que nunca foi investigado.

Para este efeito, iniciámos este projeto de investigação com uma extensa revisão da literatura disponível até à data sobre as vias mecanísticas que envolvem a α -Syn e a inflamação, assim como o potencial papel da disbiose microbiana e da mitigação inflamatória sistémica na α -Syn, tal como apresentado no **Artigo I (Capítulo 3)**. De um modo geral, a microbiota e a inflamação parecem ter um papel significativo na patogénese das α -sinucleinopatias. Para além da disbiose intestinal, a disbiose do microbioma oral deve ser considerada na patologia da α -sinucleína. Finalmente, a gestão terapêutica de patologias inflamatórias crónicas, como a periodontite, pode produzir resultados benéficos para o desfecho clínico das α -sinucleinopatias.

Adicionalmente, a publicação do **Artigo II (Capítulo 4)** permitiu observar a carga sistémica da periodontite em doentes de Parkinson, comparando os níveis de proteína C-reativa de alta sensibilidade (hs-CRP) de casos de periodontite auto-reportada com os de doentes periodontalmente saudáveis numa população com doença de Parkinson, utilizando dados disponíveis na base de dados americana National Health and Nutrition Examination Survey (NHANES) e seguindo os critérios de elegibilidade. Esta investigação permitiu reforçar a hipótese de os pacientes com doença de Parkinson com periodontite apresentarem uma elevada carga inflamatória sistémica, que se pode traduzir em níveis mais elevados de hs-CRP circulante. Este estudo estabeleceu as bases para uma pesquisa mais aprofundada sobre a carga inflamatória sistémica associada à periodontite e ao seu tratamento, utilizando os níveis de α -Syn em pessoas sem

comprometimento neurológico, o que fizemos subsequentemente no **Artigo IV (Capítulo 6)**.

Com o intuito de otimizar a estratégia para a identificação de pacientes, no terceiro estudo, **Artigo III (Capítulo 5)**, adaptámos e validámos transculturalmente uma estratégia de auto-relato para prever a prevalência da periodontite numa população portuguesa. Os nossos resultados mostraram que as medidas de auto-relato da periodontite, combinadas com fatores de risco (como o tabagismo e a diabetes mellitus), apresentaram validade preditiva para a periodontite e a sua forma grave na população portuguesa, utilizando as definições de caso de 2018 e 2012.

Por último, no **Artigo IV (Capítulo 6)**, os níveis salivares e sanguíneos basais de α -Syn, interleucina (IL)-6 e IL1- β foram analisados de acordo com o estado periodontal e 30 dias após o tratamento periodontal não cirúrgico. Não foi detetada uma associação linear entre a saliva e os níveis plasmáticos de α -Syn na avaliação inicial, indicando que a biodisponibilidade de ambos os marcadores parece funcionar independentemente. Além disso, o diagnóstico periodontal não pareceu alterar os níveis basais de α -Syn em ambos os fluidos biológicos. Ao analisar os efeitos da terapia periodontal não cirúrgica nos níveis de α -Syn, os nossos resultados não mostram variações significativas no plasma. Todavia, os níveis de α -Syn salivar exibiram uma diminuição após 30 dias, especialmente em pacientes com periodontite.

A presente tese confirmou que o tratamento periodontal não cirúrgico parece ter efeito nos níveis salivares de α -Syn. Estes resultados apresentam relevância clínica quando se utiliza a α -Syn salivar como biomarcador no diagnóstico e na gestão clínica de α -sinucleinopatias, sendo a terapia periodontal um fator confundente se realizada pelo menos até um mês antes da análise. No entanto, estes resultados preliminares requerem confirmação adicional em futuros estudos prospetivos e de maior dimensão.

Palavras-chave: periodontite, carga inflamatória, tratamento periodontal, α -sinucleína, α -sinucleinopatias, biomarcadores.

ABSTRACT

Periodontitis - a chronic inflammatory disease of tooth-supporting tissues, that can lead to tooth loss - is a major source of inflammation and can have a disruptive effect on systemic health. On the one hand, an untreated periodontal infection may result in the dissemination of periodontal bacteria and their products to distant tissues with pathological implications; and on the other hand, there is the hypothesis of a periodontitis-induced systemic inflammatory burden, through the haematological dissemination of inflammatory mediators. The resulting state of peripheral chronic inflammation supports the association of periodontal infection with several extra-oral inflammation-driven pathologies, namely diabetes mellitus, cardiovascular disease, cancer, rheumatoid arthritis, adverse pregnancy outcomes or even neurodegenerative conditions such as Alzheimer's disease (AD) and, more recently, Parkinson's disease (PD).

In fact, there is a clear association between periodontitis and α -synucleinopathies - a group of neurodegenerative disorders characterised by abnormal intracellular aggregation of α -synuclein (α -Syn) - such as PD, due to the inherent motor and cognitive impairments and the consequent impact on the oral health of PD patients. To date, the biological and pathological mechanisms of protein α -Syn remain unclear, and the contributing effect of environmental influences such as systemic chronic inflammation and dysbiotic disturbances in commensal gut and/or periodontal microflora are not discarded as one of the key multifactorial mechanisms of PD onset and progression. In this context, there is also a potential for the indirect prevention and/or management of α -synucleinopathies through the treatment of peripheral chronic, inflammatory, and dysbiotic pathologies, such as periodontitis. All in all, the evidence linking PD and periodontitis remains inconclusive due to the lack of intervention studies focusing on relevant biological pathways.

In today's context of an ageing population, there is real concern about the growing prevalence of neurodegenerative diseases, whose risk usually increases with age; as well for the deleterious systemic impact of periodontitis – the most common chronic inflammatory non-communicable illness worldwide. To this end, this study aimed to investigate whether the chronic pattern of periodontitis may

be responsible for persistent inflammatory states with unknown consequences in PD and other α -synucleinopathies. In addition, the aim was to help establish the basis for a potential biological mechanism linking periodontal infection (and its treatment) with PD through its keystone marker, α -Syn, which has never been investigated.

For this purpose, we initiated this research project with an extensive review of the available evidence to date on the mechanistic pathways of α -Syn to inflammation, as well as the potential role of microbial dysbiosis and systemic inflammatory mitigation on α -Syn, as presented in **Paper I (Chapter 3)**. Overall, microbiota and inflammation seem to have a significant role in the pathogenesis of α -synucleinopathies. Alongside intestinal dysbiosis, oral microbiota dysbiosis ought to be considered in α -Syn pathology. Finally, the therapeutic management of chronic inflammatory pathologies, such as periodontitis, may yield beneficial results for the clinical outcome of α -synucleinopathies.

Furthermore, the publication of **Paper II (Chapter 4)** allowed for the observation of the systemic burden of periodontitis in PD patients by comparing the high-sensitivity C-reactive protein (hs-CRP) levels of self-reported periodontitis cases with periodontally healthy counterparts in a PD population, using available data from the American database National Health and Nutrition Examination Survey (NHANES) and following eligibility criteria. Through this study, we were able to strengthen the existing hypothesis that PD patients with periodontitis carry a systemic inflammatory burden, which can be translated into higher circulating hs-CRP levels. This study provided a reasonable basis for further investigation of the systemic inflammatory burden associated with periodontitis and its treatment using α -Syn levels in people otherwise free of neurological impairment, which we subsequently did in **Paper IV (Chapter 6)**.

In order to achieve the most effective strategies for patient identification, in our third study, **Paper III (Chapter 5)**, we cross-culturally adapted and validated a self-report strategy for predicting periodontitis prevalence in a Portuguese population. Our results showed that self-report measures of periodontitis combined with risk factors (such as smoking status and diabetes mellitus) had predictive validity for periodontitis and its severe form in the Portuguese population, using both the 2018 and 2012 case definitions.

Finally, in **Paper IV (Chapter 6)**, baseline salivary and blood levels of α -Syn, Interleukin (IL)-6 and IL1- β were analyzed according to the periodontal status and 30 days following standard periodontal therapy. We found no linear association between the saliva and plasma α -Syn levels at baseline, indicating that the bioavailability of both markers seems to function independently. Also, periodontal diagnosis does not seem to alter baseline α -Syn levels in both biological fluids. When analysing the effects of non-surgical periodontal therapy (NSPT) on α -Syn levels, our results show no significant variations in plasma, but salivary α -Syn levels showed a decrease at the 30-day of follow-up, especially in periodontitis patients.

The present thesis confirmed that NSPT appears to have an effect on salivary α -Syn levels. These results present clinical relevance when using salivary α -Syn as a biomarker in the in diagnosis or clinical management of α -synucleinopathies, with periodontal therapy being a confounding factor if performed at least up to one month prior to the analysis. However, these preliminary findings require further confirmation in future larger and prospective studies.

Keywords: Periodontitis, inflammatory burden, periodontal treatment, α -synuclein, α -synucleinopathies, biomarkers.

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ABBREVIATIONS LIST

MGJ | Mucogingival junction

AG | Attached gingiva

FG | Free gingiva

GCF | Gingival crevicular fluid

CEJ | Cementoenamel junction

LP | Lamina propria

OGE | Oral gingival epithelium

OSE | Oral sulcular epithelium

OJE | Oral junctional epithelium

BOP | Bleeding on probing

CAL | Clinical attachment loss

PPD | Periodontal probing depth

CDC | Centers for Disease Control and Prevention

AAP | American Academy of Periodontology

NHANES | National Health and Nutrition Examination Survey

IL | Interleukin

TNF | Tumor necrosis factor

CRP | C-reactive protein

AD | Alzheimer's disease

PD | Parkinson's disease

SRP | Scaling and root planing

NSPT | Non-surgical periodontal therapy

α -Syn | Alpha-synuclein

SNCA | α -Syn gene

CSF | Cerebrospinal fluid

GI | Gastrointestinal

CNS | Central nervous system

REM | Rapid eye movement

BBB | Blood-brain barrier

NLRP3 | Inflammasome cytosolic nod-like receptor protein 3

RANTES | Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted

ENS | Enteric nervous system

ROS | Reactive oxygen species

hs-CRP | High-sensitivity C-reactive protein

SDG | Sustainable development goals

PISA | Periodontal inflamed surface area

ELISA | Enzyme-linked immunosorbent assay

STROBE | Strengthening the Reporting of Observational Studies in Epidemiology

CHAPTER 1

1. General Introduction

1.1. Periodontal Health and Disease

1.1.1. The Periodontium and Periodontal Health

The periodontium is a dynamic and functional system that comprises all supporting structures of the teeth consisting in four main tissues: the gingiva, the periodontal ligament, the root cementum and the alveolar bone proper (Figure 1) (Hassell, 1993; Lang et al., 2021).

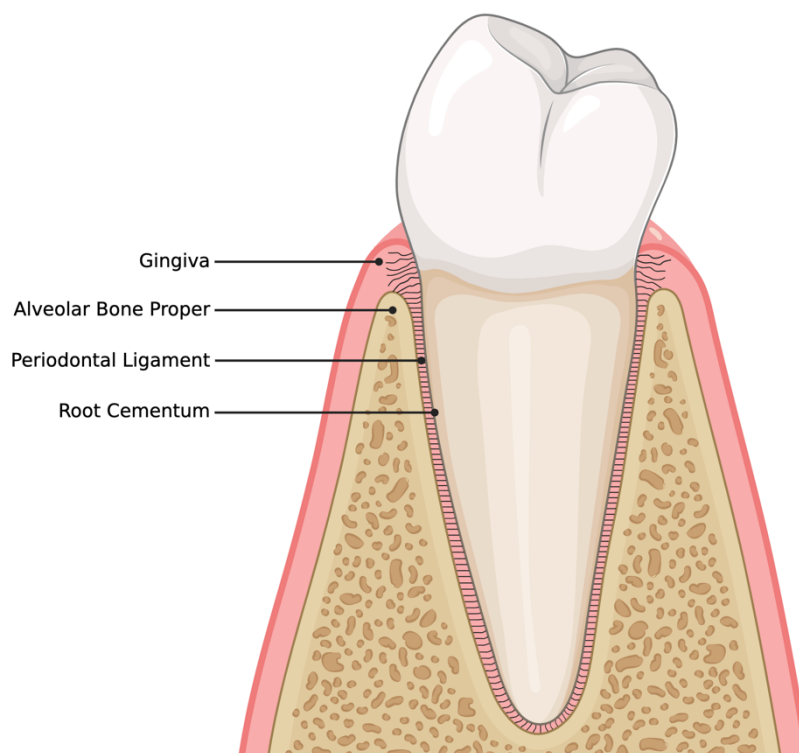


Figure 6 | **Periodontium's composition** (original image).

Macroscopically, the gingiva (part of the masticatory mucosa) is the coral pink keratinized tissue, often showing minor depressions or “stippling”, that covers the alveolar process and surrounds the cervical surface of the teeth. It extends apically and is separated by the mucogingival junction (MGJ) from the non-keratinized dark red lining oral mucosa that culminates in the oral vestibule (Figure 2) (Hassell, 1993; Lang et al., 2021). The attached gingiva (AG) extends coronally from the mucogingival line and is firmly anchored to the underlying

periosteum by collagen fibers (Figure 2). The free gingiva (FG), on the other hand, surrounds (but is not connected to) each tooth (Figure 2) and its inner surface forms the gingival sulcus, which contains gingival crevicular fluid (GCF), a serous physiologic exudate containing oral bacteria, tissue-breakdown products inflammatory mediators, enzymes, and blood cells, that can transform to an inflammatory exudate when disease is clinically present. Indeed, GCF has been the subject of extensive study as a source of biomarkers, with the potential for use as a diagnostic and prognostic tool for periodontal diseases (Barros et al., 2016). When healthy, all interdental spaces are filled with gingival papillae (Hassell, 1993).

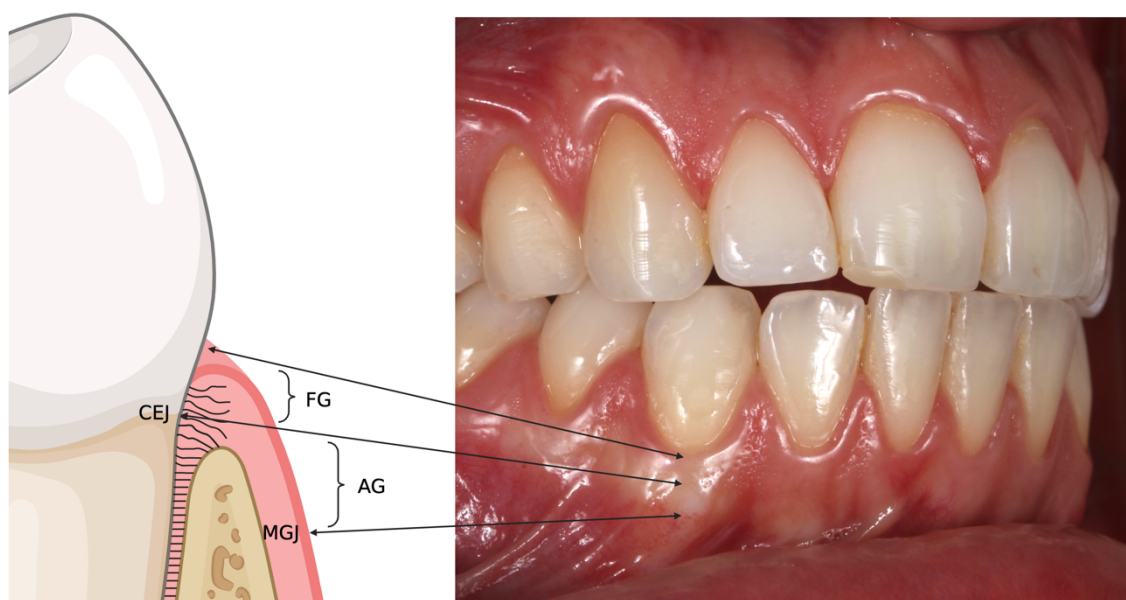


Figure 7 | **Macroscopic anatomy of the periodontium.** FG, free gingiva; CEJ, cemento-enamel junction; AG, attached gingiva; MGJ, mucogingival junction (original image).

In terms of microstructural anatomy, the gingiva consists of an epithelial layer and an underlying connective tissue layer, the *lamina propria* (LP)(Figure 3) (Lang et al., 2021). The external layer of both attached and free marginal gingiva is composed by the oral gingival epithelium (OGE), facing the oral cavity; the oral sulcular epithelium (OSE) is the extension of the OGE into the lateral wall of the gingival sulcus; the oral junctional epithelium (OJE) is the apical limit of the OSE and provides the attachment to hard tooth surfaces (enamel or cementum) (Figure 3) (Hassell, 1993).

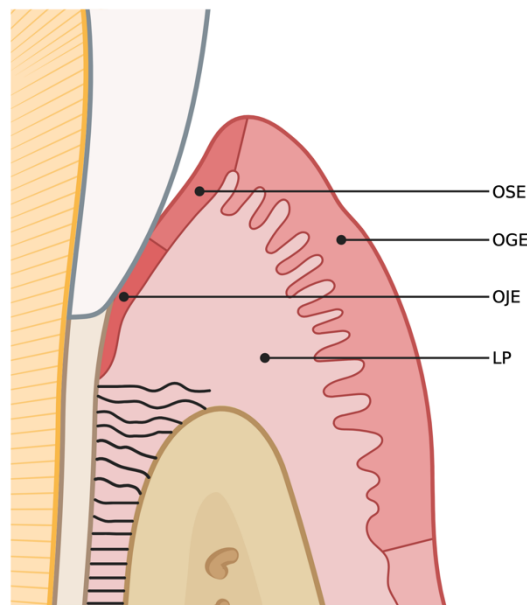


Figure 8 | **Microstructural anatomy of the periodontium.** OSE, oral sulcular epithelium; OGE, oral gingival epithelium; OJE, oral junctional epithelium; LP, lamina propria (original image).

The periodontal ligament is a highly vascularized and innervated connective tissue, rich in collagen fibres, continuous with the LP of the gingiva. The periodontal ligament surrounds the tooth roots and connects the root cementum – a specialized mineralized tissue that covers the root surfaces and is characterized by continuous deposition throughout life - to the wall of the alveolar socket (Lang et al., 2021).

The alveolar process extends from the basal bone of the maxilla and mandible, forms and supports the tooth sockets, and surrounds the tooth from the apex to approximately 1 millimeter apical to the cemento-enamel junction (CEJ). The alveolar process consists of the alveolar bone (cancellous, spongy or trabecular bone) and the alveolar bone proper (cortical bone or lamina dura). The latter covers the alveolus of the tooth and is continuous with the alveolar bone (Lang et al., 2021).

The periodontium is designed to support and protect. The periodontal tissues surrounding the teeth not only anchor the teeth to the alveolar bone, resist masticatory forces and maintain the surface integrity of the masticatory mucosa, but also act as a physical barrier, providing the primary peripheral defense against microbial infection, as part of the innate immune system. These functions

rely on the defensive architecture of the periodontium, which allows the circulation of defensive cells through tissue compartments without disrupting basic tissue integrity (Schroeder & Listgarten, 1997; Şenel, 2021).

Indeed, gingival tissues contain increased numbers of inflammatory cell infiltrates harbored in intercellular spaces with high turnover: neutrophils, lymphocytes, macrophages and other plasma cells. These defense cells provide an effective barrier to bacterial invasion and promote tissue regeneration and repair (Schroeder & Listgarten, 1997). The surface of the OJE is in fact highly permeable, allowing fluid and cells to circulate into the oral cavity. This provides an effective defense mechanism in response to constant stimuli (Bosshardt, 2017).

Periodontal Health vs. Disease:

Clinically, periodontal health can be defined by the absence of inflammation associated with gingivitis or periodontitis, which not only predates disease development but can also be restored after successful disease management. Therefore, the concept of periodontal health has recently been defined as either an intact periodontium (whether untouched or clinically well preserved) or a reduced periodontium (resulting from stabilised or controlled periodontal disease) (Lang & Bartold, 2018).

It is essential to consider a few key determinants of clinical periodontal health and disease when evaluating a patient's periodontal status and managing periodontal disease. In particular, the microbiological determinants, that comprise the composition of supragingival plaque and subgingival biofilm. The host-dependent determinants, that encompass local predisposing factors – including dental restorations, tooth anatomy, tooth position and crowding – and systemic modifying factors – such as host immune function, systemic health and genetics. And additionally, the environmental determinants or modifying factors, such as smoking, medications, stress and nutrition. In this regard, factors that facilitate the accumulation of dental plaque, such as tooth anatomy or position and the presence of restorations, are designated as predisposing factors. Moreover, modifying factors exert an influence on an individual's response to subgingival

plaque accumulation, which can include smoking, systemic conditions and medications (Lang & Bartold, 2018).

Even when dental plaque biofilm levels are minimised, clinically healthy gingiva remains in a constant state of low-grade defence. Indeed, an inflammatory infiltrate is perpetually present within gingival tissues as part of a physiological immune surveillance mechanism. This is due to the existence of a balanced equilibrium between commensal organisms and antigenic challenges within the oral cavity, which are compatible with clinical health (Murakami et al., 2018; Schroeder & Listgarten, 1997).

Nevertheless, when dental plaque accumulates over days or weeks without disruption or removal from the teeth, the development of gingivitis occurs due to a loss of symbiosis between the biofilm and the host's immune and inflammatory response. Gingivitis is typically plaque-induced, however, it can also result from endogenous hormonal shifts, pharmacological agents, systemic disorders, and malnourishment. The clinical features of gingivitis include a trigger of high bacterial plaque burden, clinical signs and symptoms of inflammation – such as redness, heat, swelling and pain - in the gingival tissue, reversibility of the inflammation by disrupting/removing the biofilm and ultimately the stability of attachment levels on the periodontium (Murakami et al., 2018).

Furthermore, the accumulation of plaque is accelerated at sites of gingival inflammation (Murakami et al., 2018). Therefore, the cumulative inflammatory response that occurs as consequence of gingival inflammation can result in the onset of periodontitis, which is due to a shift in microbial composition and the overpowering of several pathogenic agents, which in turn causes heightened host-driven tissue damage, thinning and ulcerating the OJE (Lang & Bartold, 2018). Following the onset of periodontitis, key bacterial species within the subgingival dental plaque can invade the ulcerated OJE and lead to tissue damage and periodontal attachment loss, causing the breakdown of periodontal ligament fibres and the destruction of the underlying connective tissue and alveolar bone. This deepening of the usually shallow gingival sulcus results in the formation of a periodontal pocket, a defining feature of periodontal disease, and the OJE converts into “pocket epithelium” as it migrates apically (Figure 4) (Bosshardt, 2017; Donos, 2018; Nibali, 2018).

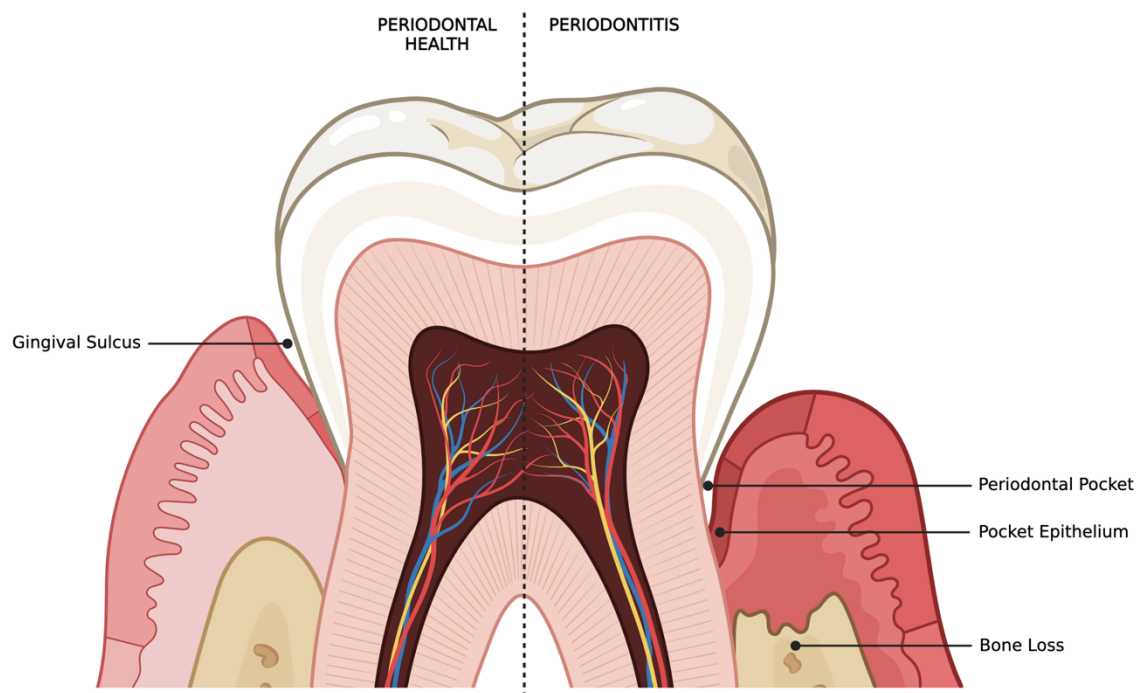


Figure 9 | **The effects of periodontitis on the periodontium** (original image).

Consequently, gingival inflammation is a prerequisite for the subsequent development of periodontitis and progressive attachment loss around teeth, although insufficient by its own (Genco & Borgnakke, 2013). Nevertheless, the management of gingivitis represents a fundamental preventive strategy for the establishment and recurrence of periodontitis (Murakami et al., 2018).

1.1.2. Periodontitis

Periodontitis is a chronic, infection-driven, inflammatory disease of the periodontium (Papapanou et al., 2018; Tonetti et al., 2018a). This disease is multifactorial and driven by the complex interaction between microbial dysbiosis of subgingival biofilms and the host's immune and inflammatory responses (Könönen et al., 2019). Moreover, an individual's susceptibility or resistance to disease initiation and rate of progression is dependent on the interplay of individual environmental and/or behavioural and genetic risk factors (Genco & Borgnakke, 2013). In fact, as abovementioned, habits such as smoking, alcohol consumption, dietary regimens, obesity, diabetes mellitus and oral hygiene

practices, are good examples of modifying risk factors to be considered and adjusted for the management of periodontitis (Genco & Borgnakke, 2013).

Local Clinical Features:

Periodontitis is clinically characterized by the chronic inflammation of the gingival tissues, which include bleeding on probing (BOP) - which is currently accepted as a proxy of tissue inflammation and consistent with bad oral hygiene habits (Tonetti et al., 2018b) - and periodontal pocket formation as its hallmark features (Donos, 2018).

Periodontitis is a progressive disease. Therefore, if untreated or inadequately treated, periodontitis results in the irreversible loss of periodontal support – clinical attachment loss (CAL) - through the permanent destruction of the periodontal ligament and alveolar bone (which can be detected radiographically), leading to gum recession, root furcation involvement, tooth mobility, pain and discomfort and ultimately tooth loss (Darveau, 2010; Haffajee & Socransky, 1986; Newman et al., 2018; Papapanou et al., 2018; Tonetti et al., 2018a).

The clinical features of periodontitis, particularly tooth loss and the resulting masticatory dysfunction, can contribute to oral frailty and disability, which in turn has an impact on an individual's quality of life (Ferreira et al., 2017), nutrition (Tonetti et al., 2017), aesthetic confidence, and general well-being (Buset et al., 2016; Ferreira et al., 2017). Furthermore, periodontitis has been linked to a decreased life expectancy (Romandini et al., 2021; Zhao et al., 2019).

Furthermore, periodontitis is characterised by periods of active and inactive phases throughout the course of the disease. In fact, in its initial stages, periodontitis may exhibit pronounced and widespread clinical manifestations, while subsequently alternating with periods of inactivity or periods of acute exacerbation (Goodson et al., 1982; Socransky et al., 1984).

Epidemiology and Economic Burden:

Periodontitis is not only considered the sixth-most prevalent condition globally but is also the most common chronic inflammatory non-communicable illness

worldwide (Kassebaum et al., 2014) and therefore constituting a public health problem (Peres et al., 2019).

In fact, periodontitis affects more than 50% of the adult population globally and, being a chronic condition, periodontitis diagnosis and management has been estimated to cause a loss of \$154.06B in the United States and €158.64B in Europe, in 2018 (Botelho, Machado, Leira, et al., 2021; Tonetti et al., 2017).

In the Portuguese context, the estimated prevalence of periodontitis in the adult Portuguese population is quite high, with reports of 59,9% in the South Lisbon Metropolitan Area (Botelho et al., 2019) and 48.6% in Northern Portugal (Relvas et al., 2022). Nevertheless, an extensive and updated investigation at a national level is necessary.

Furthermore, periodontitis is a progressive (but not linear) age-dependent condition that typically emerges in early adulthood and develops dynamically over time, while predominantly affecting men (Ebersole et al., 2016; Paul I. Eke et al., 2015, 2016; Kassebaum et al., 2014). Alongside the immunological deficiencies associated with aging, the high prevalence of periodontitis among the elderly is also significantly due to the prolonged exposure to specific environmental/behavioural risk factors (Borrell & Papapanou, 2005; Ebersole et al., 2016; Neely et al., 2001). For this reason, aging and age-dependent systemic conditions have been consistently linked to periodontitis (Nascimento et al., 2019).

Diagnosis:

The conventional method for diagnosing periodontitis is through a clinically conducted periodontal examination, which includes a comprehensive full-mouth assessment of periodontal probing depth (PPD), CAL, and BOP. This assessment involves six sites per tooth and is conducted alongside radiographic examination, in accordance with the European Federation of Periodontology/American Academy of Periodontology Classification of 2018 (Tonetti et al., 2018a). Despite being the gold standard for identifying periodontitis cases, this procedure is time-consuming and requires the involvement of

calibrated professionals, specialized equipment and aseptic strategies, inherent to a chair-side dental consultation (Papapanou et al., 2018).

Most recently, self-reported tools have been proposed for periodontitis screening, especially in large scale epidemiological settings, as an efficient and cost-effective surveillance tool (Dietrich et al., 2007). Indeed, a brief self-report questionnaire was developed and validated by the Centers for Disease Control and Prevention (CDC) in collaboration with the American Academy of Periodontology (AAP) and incorporated into the American database National Health and Nutrition Examination Survey (NHANES) (P. I. Eke et al., 2013). Subsequently, this questionnaire has been cross-culturally adapted and validated in numerous other populations (Carra et al., 2018; Cyrino et al., 2011; Iwasaki et al., 2021; Montero et al., 2020; Saka-Herrán et al., 2019; Verhulst et al., 2019). Additionally, self-report strategies have been similarly employed in other medical contexts (Bowlin et al., 1993; Martin et al., 2000; Verhoeff et al., 2021).

1.1.3. Periodontal Infection and Inflammation

Local inflammation:

Inflammation occurs as a result of the establishment of a bacterial infection. It is considered a physiological response in acute setups where inflammation is rapidly triggered and of short duration, whereas it can be considered pathological when there is no resolution of the aetiological factor and the infectious clinical setting becomes persistent and provokes a chronic dysregulation of inflammatory and immune pathways in susceptible individuals, as in the case of periodontitis. The latter involves both the innate and acquired immunity (Cekici et al., 2014).

As formerly mentioned, the periodontium is a well-adapted defence system, particularly the OJE, where a unique seal between the root surface and the gingiva protects the underlying tissues from constant exposure to oral microbes and their by-products (Bosshardt & Lang, 2004). However, as periodontitis involves a low-grade state of chronic microbial challenge and inflammation, this barrier can be disrupted leading to apical migration of OJE through the root surface, collagen destruction and, ultimately, periodontal pocket formation. Inevitably, an inflammatory cell infiltrate will settle in the subjacent tissues, as

resident cells such as keratinocytes, fibroblasts, and dendritic cells produce proinflammatory cytokines – interleukin (IL)-1, IL-6, IL-23, tumor necrosis factor (TNF)- α , chemokines - IL-8 - and antimicrobial peptides that recruit neutrophils and other non-resident immune cells (macrophages, lymphocytes, plasma cells, and mast cells) to the site of infection by chemoattractant gradients (Figure 5) (Benakanakere & Kinane, 2011; Cekici et al., 2014).

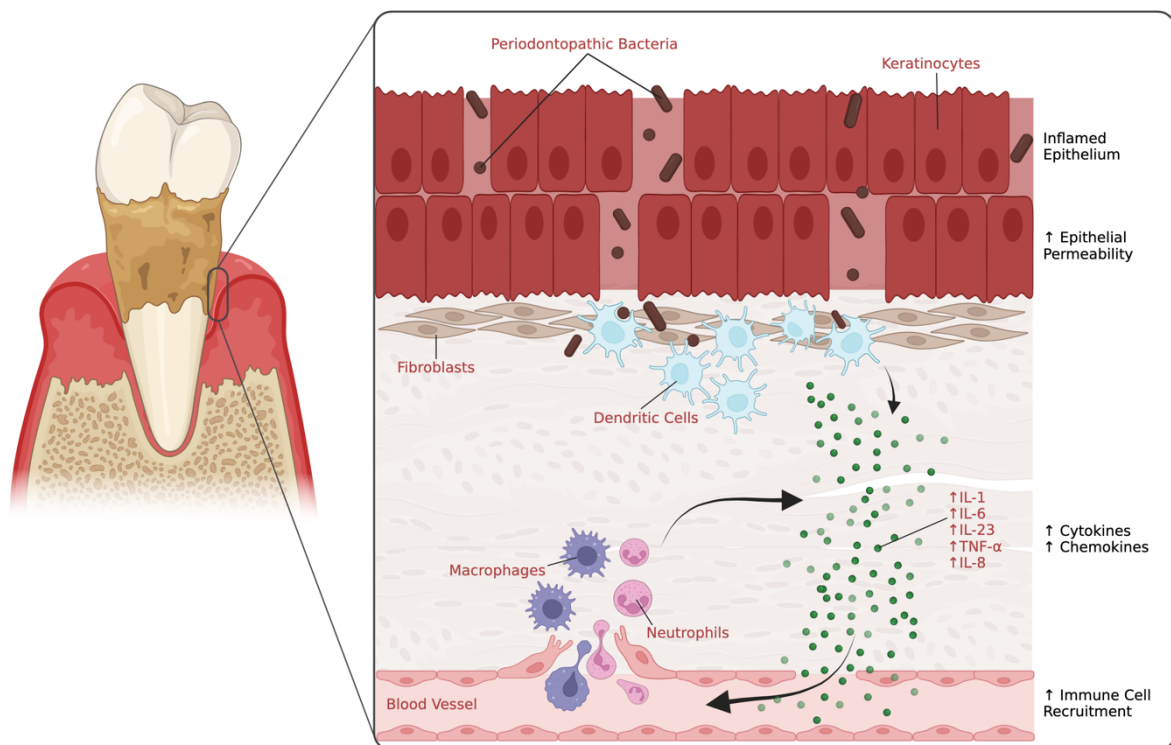


Figure 10 | **Local inflammation mechanisms of periodontitis** (original image).

Also, in the local inflammation mechanisms of periodontitis, the formation of new tissue by resident cells (keratinocytes, fibroblasts, osteoblasts) is inhibited, while the degradation of tissue by neutrophils, macrophages and osteoclasts is promoted (Könönen et al., 2019).

Systemic inflammation and influence on Systemic Health:

Periodontitis represents a substantial source of inflammation. On the one hand, an active and untreated periodontal infection may result in the potential dissemination of periodontal bacteria and their products (such as gingipains from

Porphyromonas gingivalis) to extra-oral regions, through multiple routes, including hematogenous (Loos, 2005), oropharyngeal (Mammen et al., 2020), and orodigestive routes (Arimatsu et al., 2014) (Figure 6). Similarly, local inflammatory mediators mobilised into periodontal tissues may disseminate into the bloodstream and cause a low-grade systemic inflammatory burden (Figure 6) (Hajishengallis & Chavakis, 2021; Zhao et al., 2019).

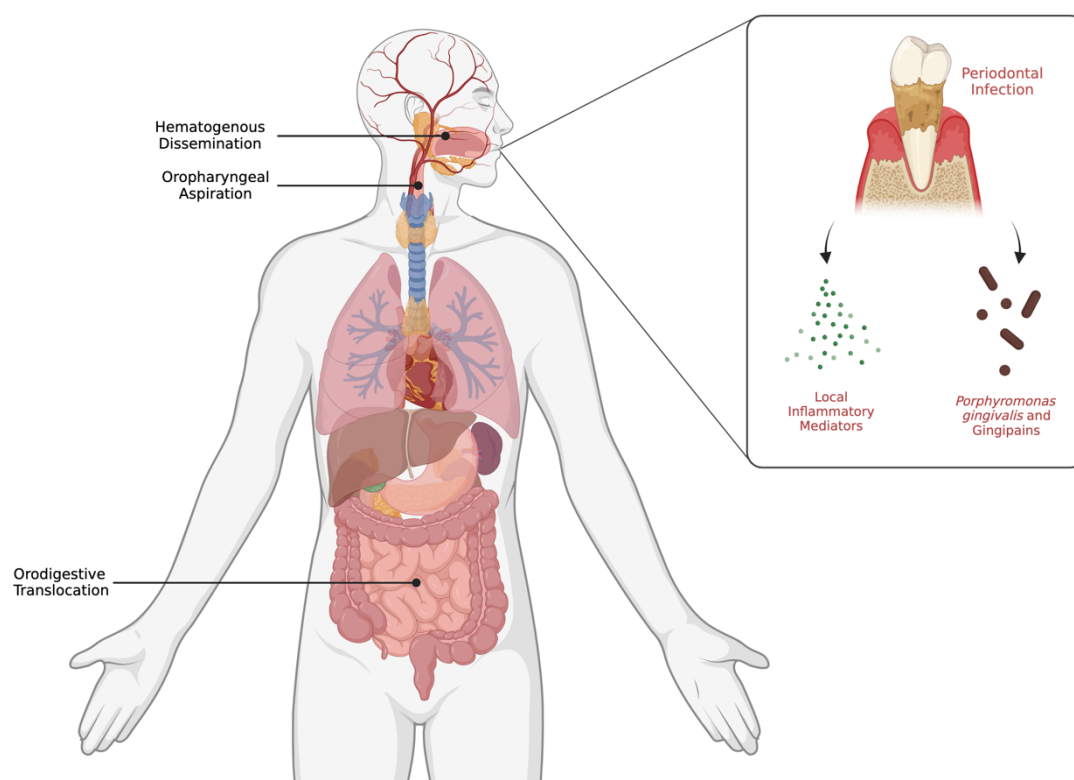


Figure 6 | **Pathways to periodontal induced systemic inflammatory burden** (original image).

Such scenarios may give rise to peripheral chronic inflammation, thereby triggering a cascade of inflammatory biomarkers, such as IL-1, IL-6, IL-1 β , C-reactive protein (CRP), TNF- α , fibrinogen and neutrophils (Ebersole et al., 2018; Hajishengallis & Chavakis, 2021), which can lead to the occurrence or aggravate extra-oral inflammatory pathologies.

In consequence, periodontitis has been associated with a range of chronic inflammation-driven diseases that might ultimately lead to premature death, including: diabetes mellitus, cardiovascular diseases (Sanz, Marco del Castillo, et al., 2020; Tonetti & Van Dyke, 2013), adverse pregnancy outcomes (Sanz &

Kornman, 2013), breast or colon cancer, rheumatoid arthritis (Al-Katma et al., 2007; Potempa et al., 2017) or even cognitive function and neurodegenerative conditions such as Alzheimer's disease (AD) and Parkinson's Disease (PD) (Botelho, Leira, et al., 2021; Dominy et al., 2019; Hajishengallis & Chavakis, 2021; A. Li et al., 2021; Lyra et al., 2020).

1.1.4. Periodontal Treatment

The most recent clinical practice guidelines on periodontal therapy include different treatment phases: (1) Patient behaviour modification, involving instructions on adequate hygiene practices and risk factor management to remove supragingival dental biofilm; (2) Medical subgingival instrumentation, encompassing mechanical debridement, scaling and root planing (SRP), potentially accompanied by chemical adjuvants to reduce or eliminate subgingival dental biofilm; (3) Surgical interventions, applied in cases where non-surgical periodontal therapy (NSPT) provided an insufficient response, aimed at regenerating or resecting complex lesions; and (4) Supportive periodontal care, focused on maintaining periodontal stability at regular intervals according to the patient's individual needs (Sanz, Herrera, et al., 2020). Ultimately, the key to long-term treatment success is the patient's daily oral hygiene habits and ongoing professional periodontal therapy (Axelsson et al., 2004).

In cases of deep residual periodontal pockets after NSPT, especially in more severe or aggressive periodontitis cases, adjunctive therapies such as antimicrobials, probiotics, lasers, anti-inflammatory drugs, antioxidant micronutrients and host modulators have been proposed (Graziani et al., 2017; Rabelo et al., 2015).

The aim of NSPT is to achieve significant clinical improvements by changing the composition of the subgingival microbial profile, reducing infection, controlling inflammation, halting tissue destruction, preventing disease progression and restoring periodontal health (Teughels et al., 2020). However, the recovery of lost tissue is limited and depends on the local characteristics of the defect and the systemic health status of the patient (Könönen et al., 2019).

Furthermore, NSPT has demonstrated improvements not only in the oral health-related quality of life of periodontitis patients (Botelho, Machado, et al., 2020) but also indications of a positive impact on general health. Not only can NSPT result in a reduction in serum levels of systemic inflammatory markers, but also seem to have a clinical positive impact in the context of atherosclerosis, cardiovascular disease, and diabetes mellitus (Bokhari et al., 2013; Botelho et al., 2022; D'Aiuto et al., 2013; Teeuw et al., 2014; X. Wang et al., 2014).

1.2. Alpha-synuclein and Related Conditions

1.2.1. Alpha-synuclein

Alpha-synuclein (α -Syn) is a small 140-amino acid protein, which is mainly believed to exist in a monomeric unstructured native form (Mor et al., 2016; Snead & Eliezer, 2014). Encoded by the α -Syn gene (SNCA) on the long arm of human chromosome 4 (Polymeropoulos et al., 1997), α -Syn is amply expressed in several body cells, such as in erythrocytes and immune cells (Ait Wahmane et al., 2020), but particularly in neural cells at the presynaptic nerve terminals of the central nervous system (Kramer & Schulz-Schaeffer, 2007). The protein α -Syn displays a dynamic structural flexibility and conformational plasticity, displaying a range of alterations in its conformation and existing primarily in soluble (unstructured/disordered) forms and eventually in membrane-bound (partially structured/ordered) forms. Indeed, upon binding to lipidic membranes, α -Syn adopts highly ordered helical conformations (Fusco et al., 2018). Therefore, at a cellular level, α -Syn can be found in the cytoplasm or connected to several membrane targets, with expression in various organelles (plasma membrane, nucleus, mitochondria, endoplasmic reticulum, golgi apparatus and the endolysosomal system) (Devi & Anandatheerthavarada, 2010; Goers et al., 2003; Gosavi et al., 2002; Guardia-Laguarta et al., 2014; W. W. Li et al., 2007; Mavroei & Xilouri, 2021; McLean et al., 2000; Pinho et al., 2019; Snead & Eliezer, 2014).

Extracellular α -Syn has been detected in various bodily fluids and peripheral tissues, including cerebrospinal fluid (CSF), saliva, serum, plasma, urine, and tears. Furthermore, it has been identified in salivary glands and multiple organs

within the gastrointestinal (GI) tract (Bougea et al., 2019; Chang et al., 2020; Kang et al., 2016; Magalhães & Lashuel, 2022; Malek et al., 2014; Vivacqua et al., 2016). Interestingly, there seems to be a bi-directional movement of α -Syn between the blood and the brain (Ganguly et al., 2021).

Nevertheless, there is still no consensus regarding the fundamental physiological characteristics of α -Syn, including its structure, conformation, function, and aggregation mechanisms (Selkoe et al., 2014; Snead & Eliezer, 2014).

Alpha-synuclein and Health:

In physiological conditions, α -Syn is believed to play a role in membrane-associated processes at the presynaptic level, such as the regulation of synaptic vesicle trafficking, maintenance and plasticity (Snead & Eliezer, 2014). Specifically, α -Syn is involved in the processes of vesicle priming/docking, fusion and dilation (Figure 7) (Logan et al., 2017; Mor et al., 2016).

Furthermore, α -Syn is involved in molecular chaperone activity, facilitating the release and reuptake of neurotransmitters such as dopamine, which enables synaptic function (Brás et al., 2020; Calo et al., 2016; Mor et al., 2016). Additionally, α -Syn serves as a molecular chaperone regarding the assembly and recycling of synaptic vesicles of the SNARE complex during the aging process (Figure 7) (Burré et al., 2010; Fusco et al., 2018).

Moreover, α -Syn has been demonstrated to play a crucial part in the differentiation of B lymphocytes and the regulation of T cell function (Xiao et al., 2014). In this capacity, it can either facilitate the progression of disease pathology, as observed in PD, or serve to protect against proinflammatory responses or infections (Ait Wahmane et al., 2020; Lesteberg & Beckham, 2019). Additionally, α -Syn might participate in the mitigation of oxidative stress in mitochondria (Fusco et al., 2018).

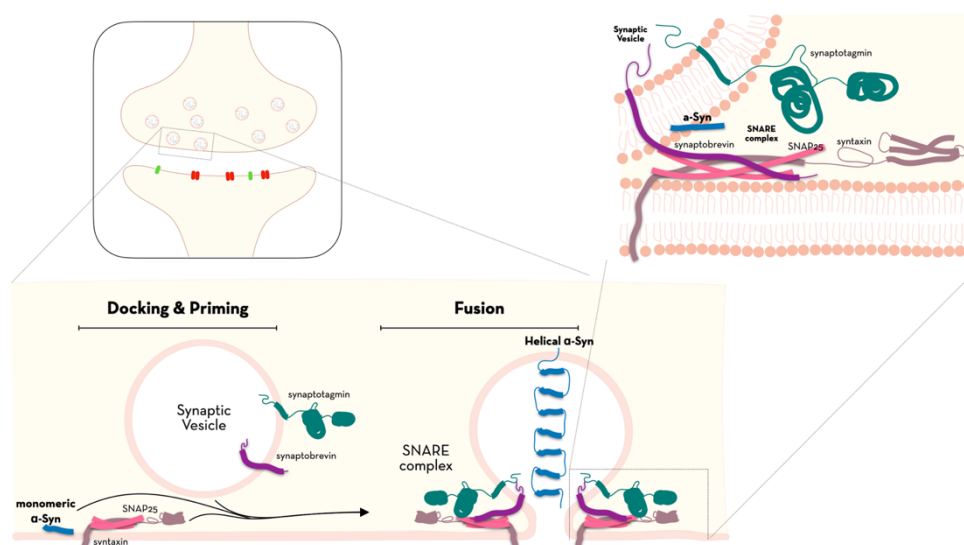


Figure 7 | **α -Syn's role in vesicle priming, fusion and dilation of exocytotic fusion pores and in the promotion of SNARE-complex assembly** (original image).

Alpha-synuclein and Disease:

The dynamic transition from water-soluble to membrane-bound states and the consequent shift in α -Syn's three-dimensional forms is of great importance for understanding its biological behaviour, both in health and in pathology (Fusco et al., 2018).

Even though α -Syn can assume different compact aggregation-resistant conformations, under stress conditions α -Syn monomers can undergo post-translational modifications that can lead to overexpression and elevated intracellular levels, thereby promoting aggregation and the formation of toxic α -Syn oligomers and protofibrils, which do not occur under homeostatic and physiological conditions (Figure 8) (Brás et al., 2020; Cardinale et al., 2021; Ingelsson, 2016; Ip et al., 2017; Lashuel et al., 2013; Mor et al., 2016).

It is proposed that the α -Syn toxic species have the capacity to interact with cell function through specific intracellular pathways, disrupting organelle function and thereby directly fueling neuronal cytotoxicity and synaptotoxicity (Figure 8) (Forloni, 2023). This in turn can impact the distribution and activation of synaptic proteins, preventing neurotransmitter exocytosis and the overall neuronal synaptic communication (Cardinale et al., 2021; Poewe et al., 2017). In fact, α -

Syn dysfunction might result in dysregulation of dopamine synthesis, transport and storage (C. Wang et al., 2021).

Also, α -Syn aggregates have a roll in the activation, recruitment and function of various glial cells (La Vitola et al., 2021). Ultimately, this could result in of α -Syn-mediated neurodegeneration and neuroinflammation (Figure 8) (Hijaz & Volpicelli-Daley, 2020; Peelaerts et al., 2015).

Additionally, the central spread and deposition of α -Syn pathology seems to occur through prion-like mechanisms throughout various brain sites, especially dopaminergic neurons in the substantia nigra pars compacta (the core neurodegeneration region in PD) (Brundin & Melki, 2017; Del Tredici & Braak, 2016; Frost & Diamond, 2010; Lema Tomé et al., 2013). However, the precise mechanism by which α -Syn accumulates and spreads through the central nervous system (CNS) remains unclear. Nevertheless, it is thought to be the result of a complex interplay between multiple factors, including the natural ageing process, environmental influences (such as the dysbiosis of gut commensal microflora), exposure to toxins and genetic mutations (Fitzgerald et al., 2019; Lázaro et al., 2014).

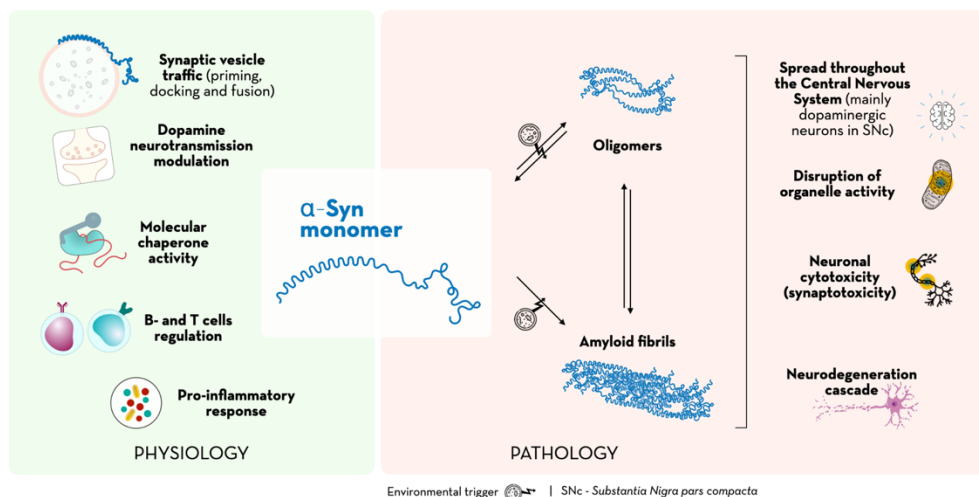


Figure 8 | α -Syn's physiological and pathological paths (original image).

1.2.2. Alpha-synuclein Related Conditions

α -Syn has been linked to a number of neurodegenerative disorders, collectively known as α -synucleinopathies (Galvin et al., 2001). These include PD, a subset

of AD, Dementia with Lewy Bodies and Multiple System Atrophy (Cardinale et al., 2021; Fitzgerald et al., 2019; Galvin et al., 1999; Grazia Spillantini et al., 1998; Lewis et al., 2010; Spillantini et al., 1998).

The α -synucleinopathies constitute a heterogeneous group of neurodegenerative disorders, with distinct clinical and pathological phenotypes, even though they share a common pathological lesion: the formation of α -Syn aggregates in selectively vulnerable populations of neurons and glia cells, which may ultimately result in neuronal dysfunction and death (Galvin et al., 2001; Peng et al., 2018).

Parkinson's Disease:

PD constitutes a progressive neurodegenerative condition affecting the CNS (Kalia & Lang, 2015). Its hallmark pathological features include: 1) the loss of dopaminergic neurons in the midbrain region, especially targeting the substantia nigra pars compacta (Figure 9); 2) and the presence of intraneuronal Lewy bodies and Lewy neurites in the surviving neurons, spreading to other regions of the SNC with a prion-like mechanism (Brundin & Melki, 2017; Cardinale et al., 2021; Frost & Diamond, 2010; Lema Tomé et al., 2013; Obeso et al., 2010; Poewe et al., 2017). Lewy bodies are abnormal cytoplasmic protein deposits primarily composed of aggregated α -Syn fibrils, while Lewy neurites are found in the axonal processes and dendrites (Cardinale et al., 2021; Obeso et al., 2010; Poewe et al., 2017).

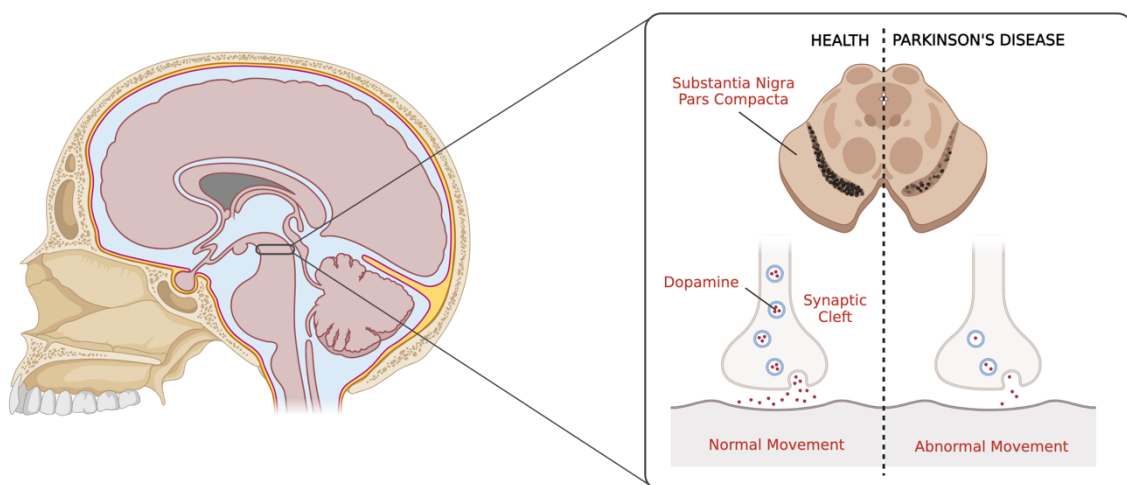


Figure 9 | **Dopaminergic neuronal function at the Substantia Nigra Pars Compacta in health and disease** (original image).

It has been proposed that the onset and progression of PD may be multifactorial and linked to a range of key mechanisms, including α -Syn-induced neuronal dysfunction, neuroinflammation, mitochondrial dysfunction and oxidative stress, systemic chronic inflammation, and even gut and/or periodontal dysbiosis (Heintz-Buschart et al., 2018; Poewe et al., 2017; Qin et al., 2016; Tysnes & Storstein, 2017; van Vuuren et al., 2021). The influence of genetic (in familial PD forms) and its combination with environmental and idiopathic factors (in sporadic forms, the majority of PD cases) seems most likely, even though the aetiology of PD remains uncertain (Baba et al., 2006; Ball et al., 2019; Emamzadeh & Surguchov, 2018).

Clinical Features:

PD is recognized as a heterogeneous condition, not only due to its pathological features, genetic predispositions and exposures, but also due to the diversity of its clinical phenotypes (Foltynie et al., 2002; Wüllner et al., 2023).

PD is typically clinically identified by key motor symptoms, including resting tremor, muscular rigidity, bradykinesia, and postural and gait dysfunction. Additionally, there are notable non-motor characteristics, the majority of which are present during the prodromal phase and are believed to manifest up to 30 years prior to the clinical detection of PD. These include olfactory dysfunction, cognitive decline, psychiatric disturbances, sleep abnormalities (rapid eye movement [REM]-sleep behavior disorder), autonomic dysfunction (such as difficulty swallowing and constipation), pain and fatigue (Chaudhuri et al., 2006; Jankovic, 2008; Kalia & Lang, 2015).

Furthermore, the pharmacological therapy employed in PD, namely dopaminergic drugs, has been linked to onset additional clinical complications, thereby representing a significant therapeutic limitation (Picconi et al., 2018).

Consequently, both PD natural progression and treatment impact patient daily life activities, such as oral hygiene practices (Lyra et al., 2020), and significantly impair patient overall quality of life (Martinez-Martin et al., 2011; Poewe et al., 2017).

Epidemiology and Economic Burden:

PD is not only the most common synucleinopathy (Cardinale et al., 2021), but also one of the most prevalent and rapidly growing neurodegenerative diseases (Chaudhuri et al., 2024; Dorsey & Bloem, 2018; Pringsheim et al., 2014). Indeed, PD is considered an upcoming non-infectious pandemic and represents the fastest growing and most significant medical and social burden of our time (Dorsey & Bloem, 2018).

Global estimates suggest that 6.2-6.9 million people had PD in 2015 (Feigin et al., 2017). With demographic shifts in most countries as the population is ageing, the prevalence of PD is expected to double in the coming decades, although these numbers are likely to be underestimates (Dorsey & Bloem, 2018; Feigin et al., 2017; Pringsheim et al., 2014; Rossi et al., 2018). There has also been a trend towards decreasing smoking rates, which may also support the projection of a future PD pandemic, as smoking is associated with a 50% reduced risk of PD (Mappin-Kasirer et al., 2020).

In addition, PD tends to affect 1.5-2 times more men than women (Wooten et al., 2004). This may be explained by a neuroprotective effect of oestrogen in women, which increases striatal dopamine levels and results in a later age of onset in women and a typically milder clinical phenotype with slower disease progression rates (Haaxma et al., 2007; Wooten et al., 2004).

On a global scale, the risk of PD increases dramatically with age (Pringsheim et al., 2014). Although cases of PD can vary in onset age, the most common age of onset is 65-70 years (Tysnes & Storstein, 2017).

Geographical location also appears to influence the prevalence of PD, as it appears to be lower in Asia compared to North America, Europe and Australia. This may be due to differences in genetic or environmental susceptibility in these regions (Pringsheim et al., 2014).

Diagnosis:

PD has been clinically diagnosed by identifying its key motor signs including tremor, rigidity, bradykinesia and postural instability (Gelb et al., 1999; Reichmann, 2010). Additional reliable clinical features, such as asymmetric onset (Melamed & Poewe, 2012) and the patient's response to dopaminergic drugs, can also be considered to improve diagnostic accuracy (de Lau & Breteler, 2006; Larsen et al., 1994).

However, the definitive diagnosis of PD can only be confirmed by post-mortem evaluation at autopsy, so the clinical diagnosis remains speculative and usually depends on various follow-up examinations, with misdiagnosis still a common problem as non-classical disease presentations can occur (Larsen et al., 1994; Litvan et al., 2003).

Therefore, the use of a PD-specific biomarker may be a solution to these diagnostic difficulties. In this context, α -Syn has emerged as a promising biomarker for PD due to its critical role in disease pathogenesis and its presence in several bodily fluids and peripheral tissues (Atik et al., 2016; Ganguly et al., 2021). In fact, α -Syn can be detected in the CSF, saliva, serum, urine and GI tract of patients with PD (Bougea et al., 2019; Kang et al., 2016; Miranda et al., 2017; Vivacqua et al., 2016).

1.2.3. Alpha-synucleinopathies and Inflammation

Local inflammation:

Inflammation influences the pathogenesis of nearly all neurodegenerative disorders, and specifically, α -Syn pathology and neuroinflammation play a crucial role in the pathogenesis of α -synucleinopathies, including PD (Cardinale et al., 2021; Lai et al., 2022; Lim et al., 2016).

In fact, neuron-released toxic α -Syn not only are directly responsible for neuronal dysfunction and pathological spreading, but also stand as a key component of neuronal inflammatory patterns, triggering inflammatory responses in resident glial cells, that usually provide support to neurons in the nervous system (Forloni, 2023; Lai et al., 2022; Lim et al., 2016). This complex pathological environment

modulates microglial activation and astrocyte hyperactivity/dysfunction (Forloni, 2023; Gelders et al., 2018; Hirsch et al., 2012; Lai et al., 2022).

Microglia, the principal innate immune glia cells of the CNS - which are in many ways similar in macrophages due to diverse phenotypes and qualities - are integral to maintaining homeostasis, clearing cellular debris or toxins (including toxic α -Syn), and supplying neurotrophic factors (nerve growth factors) (Colonna & Butovsky, 2017). In fact, microglial cells present both neuroprotective and neuroinflammatory properties, however, their degradation properties are likely to diminish in aged individuals and pathologic states (Lim et al., 2016). However, chronic activation of microglia by α -Syn aggregates induces an inflammatory response that can contribute to sustained neuroinflammation and neuronal injury, and the subsequent progression of neurodegenerative diseases (Forloni, 2023).

Astrocytes, the most abundant star-shaped glial cells in brain tissue, are critically involved in maintaining neuronal function and homeostasis. They perform several essential physiological roles, including the maintenance of the blood-brain barrier (BBB), regulating water and ion balance, participating in synaptic activity, and providing metabolic, nutritional, energetic, and structural support to neurons. Additionally, astrocytes contribute to neurorepair mechanisms in response to injury or damage (Khakh & Sofroniew, 2015; Lai et al., 2022; C. Wang et al., 2021). In pathological conditions, toxic α -Syn species can be incorporated in the cytoplasm of astrocytes, which are then able to spread α -Syn pathology via cell-to-cell transmission throughout the brain (Brundin & Melki, 2017; Frost & Diamond, 2010; Lai et al., 2022; C. Wang et al., 2021). Ultimately, astrocytes contribution to neurodegeneration is two-fold: α -Syn-induced astrocyte, that impairs homeostatic essential roles; and astrocyte reactivity that leads to the production of deleterious factors and contribute to neurotoxicity (Forloni, 2023).

Apart from the activation of innate CNS glia cells, the recruitment of peripheral immune cells (peripheral lymphocyte infiltration, in particular T cells and natural killer cells) has also been observed in the brain of individuals with α -synucleinopathies, which might contribute to neuronal dysfunction (Brochard et al., 2009; Cardinale et al., 2021; Lai et al., 2022; Lim et al., 2016).

Moreover, the inflammatory response triggered by aggregated α -Syn can also lead to an increase in gene expression of proinflammatory mediators or cytokines, which are a component of sustained inflammation and progressive α -Syn pathology (Lai et al., 2022; Lim et al., 2016). Consequently, there is an in situ increase of IL-1, IL-2, IL-6, IL-1 β and inflammasome cytosolic nod-like receptor protein 3 (NLRP3), in the CSF and brains of patients with PD, particularly in the substantia nigra pars compacta (Cardinale et al., 2021; Gustot et al., 2015; Kannarkat et al., 2013; La Vitola et al., 2021; Rydbirk et al., 2017).

The precise manner in which inflammation contributes to the selective vulnerability of neurons in α -synucleinopathies such as PD remains unclear, as well as its potential role in disease initiation and/or progression. Nevertheless, the hypothesis that genetic predisposition in conjunction with environmental exposures may ultimately result in selective nigral dopaminergic degeneration appears to be the most probable (Lai et al., 2022; Lim et al., 2016).

Systemic inflammation:

With respect to the systemic inflammatory burden, inflammation observed in α -synucleinopathies, it is notable that variations in the serum levels of proinflammatory cytokines have also been reported in patients with PD when compared to controls, namely a significant increase TNF- α , IL-1 β , IL-2, IL-6, IL-10 and, NLRP3, CRP, and Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted (RANTES). Nevertheless, such variations should be interpreted with caution, as other external confounding factors (medical conditions, medication regimens, etc.) may be present (Chatterjee et al., 2020; Fan et al., 2020; Lai et al., 2022; Qin et al., 2016; Usenko et al., 2020).

Furthermore, altered lymphocytic patterns were observed in the peripheral blood of patients with PD, including increased monocyte and natural killer cell activation, although not all T cells (Earls & Lee, 2020; Lai et al., 2022; Niwa et al., 2012; C. Sun et al., 2019; Wijeyekoon et al., 2018).

Additionally, in regards to systemic involvement, the bidirectional communication between the GI tract and the CNS in neurodegenerative disorders must be taken into account (Forloni, 2023).

It is known that disturbances in the gut–brain axis have been found to be associated with PD's non-motor features, such as the GI dysfunction that usually manifests decades prior to PD clinical diagnosis (Klann et al., 2022; Perez-Pardo et al., 2018). Reports of neuronal and immune dysfunction in the enteric nervous system (ENS) and major alterations of the gut microbiota seem to be key players in this gut–brain axis and the early PD pathology, where α -Syn appears to have a fundamental role (Forloni, 2023; Ryman et al., 2023). In fact, α -Syn pathology is hypothesised to occur in the ENS and then propagate to the CNS via the vagus nerve, as α -Syn toxic species were found in the myenteric and submucosal plexus of the ENS, the vagal nerve and even the olfactory nucleus in early staging of PD (Braak et al., 2006; Forloni, 2023; Stevenson et al., 2020). The hypothesis of a peripheral start of the α -synucleinopathies can co-exist with the CNS hallmark α -Syn pathology and propagation (Farrow et al., 2022; Forloni, 2023).

All in all, α -Syn propagation and deposition is constantly associated with α -Syn-induced inflammation, and in turn, inflammation promotes the cerebral propagation and accumulation of α -Syn pathology (T. K. Kim et al., 2022; Lee et al., 2010), such as intestinal inflammation (Forloni, 2023; Kishimoto et al., 2019).

1.3. Periodontal infection in alpha-synuclein homeostasis

The link between periodontitis and α -synucleinopathies such as PD has been consistently attributed to PD's motor and cognitive challenges. As expected, the progression of PD impacts the patient's capacity to perform self-care routines, such as regular oral hygiene practices, and can frequently result in the delay or inability to attend dental appointments. It is therefore evident that oral health can typically decline as a consequence of the emergence of dental caries and periodontitis (Auffret et al., 2021; Einarsdóttir et al., 2009; Hanaoka & Kashihara, 2009; Martimbianco et al., 2021; Nakayama et al., 2004; Ribeiro et al., 2017; Schwarz et al., 2006; van Stiphout et al., 2018; Zlotnik et al., 2015). In fact, we have recently demonstrated a high periodontitis prevalence in PD patients (Lyra et al., 2020).

Also, periodontitis exerts a disruptive impact on systemic health. This may be due to the bacterial spread of periodontopathogenic bacteria and their products, or

due to periodontitis-induced inflammatory burden (Armingohar et al., 2014; Hajishengallis & Chavakis, 2021; Könönen et al., 2019; Meurman & Bascones-Martinez, 2021; Mougeot et al., 2017; Zhao et al., 2019).

On the one hand the presence of key periodontal pathogens in distant tissues, coupled with inflammatory responses, may indicate that oral microbial migration may precipitate peripheral reactions, which are frequently linked to the pathological processes in systemic diseases. This lends further support to the association between periodontal infection and several conditions, namely neurodegenerative conditions, as previously reported in the context of cognitive function (Botelho, Leira, et al., 2021; A. Li et al., 2021), dementia (Leira et al., 2017), AD (Dominy et al., 2019) and, most recently, PD (C.-K. Chen et al., 2017; Lyra et al., 2020).

Indeed, *Porphyromonas gingivalis*, the keystone pathogen of periodontitis, and gingipains, its toxic proteases, have been identified in the brain of AD patients (Dominy et al., 2019). Furthermore, there have been reports of an increase in the deposition of beta amyloid (the precursor protein of AD) in the brains of periodontitis-induced mice models for AD (Ishida et al., 2017). In the same way, periodontitis infection might have the capacity to interfere with α -Syn levels, in the context of α -synucleinopathies. Although evidence in this regard is still sparse, this hypothesis cannot be discarded, as gingipains have also been shown to accumulate in dopaminergic neurons of the substantia nigra in humans, specifically interacting with α -Syn (Ermini et al., 2023).

Moreover, the available evidence suggests that there is an elevated risk of developing PD following a periodontitis infection (C.-K. Chen et al., 2017). Indeed, there is evidence that disruptions in the commensal microbiota may be a driving force in α -synucleinopathies. This is firmly established within the scope of the microbiota-gut-brain axis, in which changes in the gut microbiota and their products might contribute to the misfolding and abnormal aggregation of α -Syn in the ENS and the establishment of local inflammatory processes. Subsequently, the transportation of these substances via projections of the vagus nerve and autonomic enteric fibres to the CNS results in the loss of dopaminergic neurons and a microglial inflammatory response, which is a key factor in the pathogenesis

of α -synucleinopathies (Fitzgerald et al., 2019; Jo et al., 2022; Stolzenberg et al., 2017).

Together with gut microbiota, the oral cavity and its microbiota are an integral part of the GI tract, and periodontal infection also appears to serve as a significant gateway for bacterial translocation in PD (van Vuuren et al., 2021). Additionally, PD patients appear to present differences regarding the level of certain species of gut and oral microbiome when compared to healthy counterparts, with higher abundance of *Lactobacillus* and opportunistic pathogens (Jo et al., 2022).

Conversely, it has been hypothesized that systemic inflammation caused by periodontitis may lead to the development of chronic neuroinflammation and, subsequently, interfere with the pathogenesis of PD. Indeed, an active periodontal infection appears to facilitate the secretion of pro-inflammatory cytokines, including IL-1, IL-6, TNF- α , and reactive oxygen species (ROS), contributing to the increased likelihood of developing PD (C.-K. Chen et al., 2017; Furutama et al., 2020).

Therefore, the potential for the indirect prevention of α -synucleinopathies, including PD, and other neurodegenerative diseases, through the management of chronic, inflammatory, and dysbiotic pathologies in the periphery is a novel and transformative proposition (Fitzgerald et al., 2019; La Vitola et al., 2021). Indeed, the modulation of overall cytokine levels has demonstrated to improve the motor function of individuals with PD (Manocha et al., 2017). Thus, it appears to be crucial to provide patients with a global health assessment, addressing not only gut inflammation, but also patients' oral condition. This should encompass not only the improvement of patients' oral hygiene practices but also the delivery of specialized medical care (C. K. Chen et al., 2018).

In conclusion, the evidence linking PD and periodontitis remains inconclusive due to the lack of intervention studies focusing on relevant biological pathways. The current evidence base remains reliant on bioinformatic genomic analysis (Botelho, Mascarenhas, et al., 2020; Hu et al., 2022), which is insufficient to draw robust conclusions regarding the link between the two conditions. Therefore, we aim to contribute to establishing the basis for a potential biological mechanism linking periodontal infection and PD through its keystone marker α -Syn, which

has never been investigated. Also, the chronic pattern of periodontitis may be responsible for sustained inflammatory states with unknown consequences in PD and other α -synucleinopathies.

CHAPTER 2

2. Aims

To date, research has had its focus on diagnosed neurodegeneration, but it is still unclear how the inflammatory burden of periodontal infection and its treatment impacts α -Syn homeostasis.

To meet this gap of knowledge, this project presented a two-fold aim:

- To assess baseline salivary and blood levels of inflammatory markers of interest (IL-6 and IL1- β) and α -Syn according to the periodontal status. Also, to deliver periodontal therapy in periodontitis patients and analyse these salivary and blood markers of interest up to 30 days after treatment.
- To explore the systemic repercussions of the periodontitis infection in PD patients, we will compare the levels of high-sensitivity C-reactive protein (hs-CRP) of self-reported periodontitis cases with healthy ones, amongst PD patients, using available data from the NHANES.

With these aims, we expect to clarify the physiological behaviour of these markers of interest in salivary and blood levels according to the periodontal status, as well as the effect of periodontal treatment on them, particularly on α -Syn. Also, to further contribute to the “neuro-perio” field of study, specifically the impact of periodontitis-induced inflammation roles in the pathophysiology of PD. This will be crucial in future research studying periodontitis and α -Syn-related conditions, such as PD.

Framed within the Sustainable Development Goals (SDG) proposed by the United Nations General Assembly (2015), this research project was designed to follow the pledge to ensure healthy lives and wellbeing (SDG 3 - Good health and wellbeing), to ensure quality education (SDG 4 - Quality education) and to establish long-lasting partnerships (SDG 17 - Partnerships for the goals).

CHAPTER 3

3. Revisiting Alpha-Synuclein Pathways to Inflammation

This chapter was based on the published work:

Paper I – Revisiting Alpha-Synuclein Pathways to Inflammation

To revisit α -Syn pathways to inflammation, with the aim of clarifying and updating the available evidence to date, an original review article was successfully published in the International Journal of Molecular Sciences (April 12, 2023).

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Review

Revisiting Alpha-Synuclein Pathways to Inflammation

Patrícia Lyra ^{1,2,*}, Vanessa Machado ^{1,2} , Silvia Rota ^{3,4} , Kallol Ray Chaudhuri ^{3,4} , João Botelho ^{1,2} and José João Mendes ^{1,2}

- ¹ Egas Moniz Center for Interdisciplinary Research (CiiEM), Egas Moniz School of Health and Science, Caparica, 2829-511 Almada, Portugal; vmachado@egasmoniz.edu.pt (V.M.); jbotelho@egasmoniz.edu.pt (J.B.); jmendes@egasmoniz.edu.pt (J.J.M.)
 - ² Evidence-Based Hub, CiiEM, Egas Moniz—Cooperativa de Ensino Superior, Caparica, 2829-511 Almada, Portugal
 - ³ Department of Basic & Clinical Neuroscience, Institute of Psychiatry, Psychology & Neuroscience, King's College London, London WC2R 2LS, UK; silvia.rota@kcl.ac.uk (S.R.); ray.chaudhuri@kcl.ac.uk (K.R.C.)
 - ⁴ Parkinson's Foundation Center of Excellence, King's College Hospital, London SE5 9RS, UK
- * Correspondence: plyra@egasmoniz.edu.pt

Abstract: Alpha-synuclein (α -Syn) is a short presynaptic protein with an active role on synaptic vesicle traffic and the neurotransmitter release and reuptake cycle. The α -Syn pathology intertwines with the formation of Lewy Bodies (multiprotein intraneuronal aggregations), which, combined with inflammatory events, define various α -synucleinopathies, such as Parkinson's Disease (PD). In this review, we summarize the current knowledge on α -Syn mechanistic pathways to inflammation, as well as the eventual role of microbial dysbiosis on α -Syn. Furthermore, we explore the possible influence of inflammatory mitigation on α -Syn. In conclusion, and given the rising burden of neurodegenerative disorders, it is pressing to clarify the pathophysiological processes underlying α -synucleinopathies, in order to consider the mitigation of existing low-grade chronic inflammatory states as a potential pathway toward the management and prevention of such conditions, with the aim of starting to search for concrete clinical recommendations in this particular population.

Keywords: alpha-synuclein; inflammation; neuroinflammation; synucleinopathies; Parkinson's disease



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1. Introduction

Alpha-synuclein (α -Syn) is a monomer that is expressed at different levels, as it seems to be present not only in nerves—at the presynaptic nerve terminals of both the Central Nervous System (CNS) and the Peripheral Nervous System (PNS) [1]—but also at the erythrocytes and immune cells level [2]. Intracellularly, α -Syn can be found in membranes and in the cytoplasm, as well as in several organelles, such as the nucleus, mitochondria, endoplasmic reticulum, golgi apparatus, and the endolysosomal system, even though its role on these organelles still remains unclear [3–11]. This small soluble monomer consists of 140 amino acids encoded by the α -Syn gene on the long arm of human chromosome 4 [12]. Under physiological conditions, α -Syn is thought to be involved in the regulation of synaptic vesicle traffic, particularly neurotransmitter release and reuptake [13,14]. Specifically, α -Syn acts in vesicle priming, fusion, and dilation of exocytotic fusion pores [15]—as illustrated in Figure 1—increasing the local release of calcium (Ca^{2+}), which is critical for ATP-induced exocytosis [16] and modulates the dopamine transporter (DAT1), regulating dopamine neurotransmission [17]. Furthermore, α -Syn's multimeric membrane-bound state presents molecular chaperone activity, essential to sustaining normal SNARE-complex formation during aging (demonstrated in Figure 1), as it promotes the folding of synaptic fusion components called Soluble NSF Attachment Protein REceptors (SNAREs) at the presynaptic plasma membrane in conjunction with cysteine string protein-alpha/DnaJ Heat Shock Protein Family (Hsp40) Member C5 (DNAJC5) [18]. Furthermore, in terms

of its immunomodulatory function, α -Syn seems to be involved in the development of B lymphocytes and in the regulation of T cells [19], playing different roles in either promoting disease pathogeny (as is the case of PD) and in protecting against proinflammatory responses or infections [2,20].

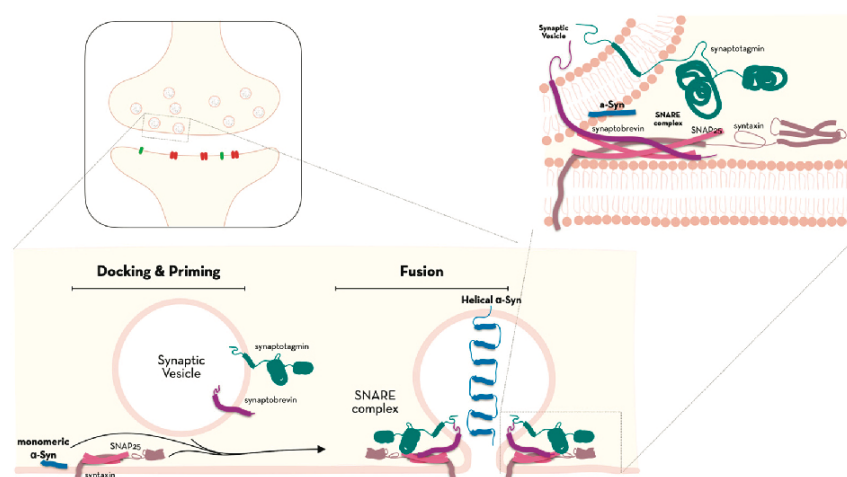


Figure 1. α -Syn's role in vesicle priming, fusion, and dilation of exocytotic fusion pores and in the promotion of SNARE-complex assembly.

There is a dynamic balance between the different three-dimensional forms that α -Syn can undertake, varying from the monomeric and oligomeric forms to the formation of fibrils, which does not easily occur in a homeostatic environment [21]. Evidence shows, however, that under pathological conditions (in which an environmental trigger such as the settlement of a virus or bacterial infections have been hypothesized), α -Syn monomers can undergo post-translational modifications that cause overexpression and an increase in intracellular levels, fostering its aggregation and development of toxic α -Syn oligomers and protofibrils [13,22–24], which subsequently spread throughout the CNS.

Indeed, the gastrointestinal tract or even exposed olfactory neurons have been suggested as possible entry routes for these pathogens [20]. The α -Syn toxic species act through cell-autonomous mechanisms in addition to non-cell-autonomous ones [25]. The former may disrupt organelle activity (by increasing oxidative stress or by impairing the ubiquitin-proteasome machinery and mitochondrial function). This organelle disruption may be responsible for neuronal cytotoxicity, while the latter may be responsible for inducing synaptotoxicity and affecting the distribution and activation of synaptic proteins, preventing neurotransmitter exocytosis and neuronal synaptic communication [22,26] and possibly the recruitment and activation of glial cells (resident immunocompetent cells of the CNS) [27]. This could ultimately lead to the neurodegeneration cascade [28–30]. In addition, the perforation of the neuronal plasma membrane is enabled by extracellular α -Syn oligomers, increasing its conductance and the influx of Ca^{2+} ions and glucose, partially supporting the synaptotoxicity observed in α -synucleinopathies [31]. Figure 2 represents the physiological and pathological paths of α -Syn.

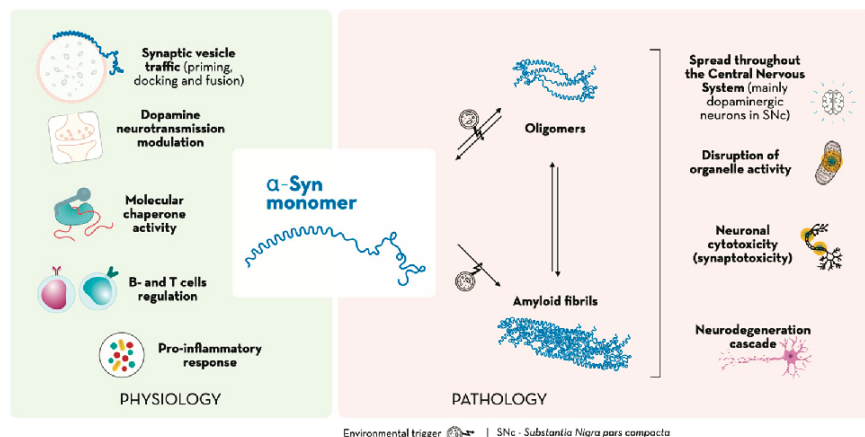


Figure 2. α -Syn's physiological and pathological paths.

The transmission of α -Syn pathology spans throughout various brain regions, especially dopaminergic neurons in the substantia nigra pars compacta (the core site of neurodegeneration in PD), although the full extent of its impact remains largely unknown [32]. Furthermore, α -Syn can be detected as a biomarker in PD patients' cerebrospinal fluid (CSF), saliva, serum, urine, and gastrointestinal tract [33–36]. Several studies have also attempted to measure α -Syn in blood cells and plasma, but so far, the results have been inconsistent [37].

Indeed, PD is considered to be the most common synucleinopathy [22], and represents the fastest growing and most significant medical and social burden of our time [38]. The α -Syn gene was the first gene to be associated with PD, and the knowledge that a single missense/point mutation or duplications of the gene cause Parkinsonism, especially the autosomal dominant form, supports the strong association between α -Syn and PD [12,39,40]. Nevertheless, it is noteworthy that non-genetic (environmental and idiopathic) factors appear to be responsible for the vast majority of PD cases [41] and have led to the consideration of the CRISPR/Cas9-mediated technology far beyond in this regard [42,43]. In terms of its pathological features, PD is characterized by the loss of dopaminergic neurons in the midbrain region of the substantia nigra pars compacta, resulting in a marked decrease in dopamine levels in the synaptic terminals, as well as the presence of intraneuronal dense inclusions of Lewy bodies (aggregations of misfolded α -syn, ubiquitin, complement proteins, and cytoplasmic structural proteins) in different regions of the brain and body (substantia nigra pars compacta, cerebral cortex, dorsal nucleus of the vagus nerve, sympathetic ganglia, and myenteric plexus of the gut) [22,44–46]. Overall, PD is a highly systemic and multifactorial neurodegenerative disease whose motor and non-motor symptoms (NMSs) have a major impact on patients' quality of life. [26,45,47,48]. Even though PD is only diagnosed upon the detection of its progressive cardinal motor symptoms (resting tremor, muscular rigidity, and bradykinesia), often together with a variety of NMSs [49,50], a prodromal phase is believed to occur up to 30 years prior to clinical diagnosis, which includes signs such as hyposmia (olfactory dysfunction), sleep abnormalities (Rapid eye movement (REM)-sleep behavior disorder), cardiac sympathetic denervation, constipation, depression, and pain [41,51,52]. During this early prodromal phase of PD, synaptic alterations occur prior to neuronal death, namely the accumulation of toxic α -Syn in the presynaptic terminals, affecting neurotransmitter release [22].

In fact, PD presents a multifactorial etiology that remains unclear thus far, even though it seems to be dependent both on environmental and genetic factors, while encompassing several biological mechanisms such as α -Syn pathology, mitochondrial dysfunction, oxidative stress, synaptic plasticity, neuroinflammation, chronic systemic inflammation (translated in the dysregulation of circulating inflammatory cytokines), and even gut and

periodontal dysbiosis [53,54]. Even though α -Syn dysfunction is a prominent key component of PD and other α -synucleinopathies, such as dementia with Lewy bodies (DLB) and multiple system atrophy (MSA), the reason it accumulates is still an active area of research [45]. To that end, the intent of this review is to revisit α -Syn pathways to inflammation, aiming to clarify and update the available evidence to date, as well as to promote further research in the hopes of ultimately establishing future clinical recommendations.

2. α -Syn and Inflammation

Recent evidence from both in vitro and in vivo models report on the major role neuroinflammation plays on the pathophysiology of neurodegenerative disorders, including PD, as it is pivotal factor between PD's genetic predisposition and environmental exposures [55–59]. In fact, the use of non-steroidal anti-inflammatory drugs appears to reduce the risk of developing PD [60]. It is known that α -Syn accumulation stands as a key component of these neuronal inflammatory patterns, in particular, by modulating microglial function and upregulating the inflammatory cascade [61]. In detail, toxic α -Syn species that are translocated intracellularly can trigger microglia hyperactivity, activate astrocytes, increase the gene expression of proinflammatory factors, and summon peripheral immune cells to the surroundings of the pre-apoptotic and apoptotic dopaminergic neurons on the CNS, all of which might induce neuronal dysfunction [22,62,63].

Therefore, alongside peripheral lymphocyte infiltration, there is an in situ increase in inflammatory mediators such as interleukin (IL)-1 β and inflammasome cytosolic nod-like receptor protein 3 (NLRP3) in PD patients' CSF and brains [64,65]. The chronic overexpression of IL-1 β at the substantia nigra pars compacta level of mice culminates in dopaminergic degeneration through glial activation and motor deficits [27]. The same inflammatory burden was systematically reported in animal studies, with reports of NLRP3 activation in the serum of PD mice, and in human trials with elevated peripheral blood levels of NLRP3, interleukin (IL)-6, tumor necrosis factor- α (TNF- α), IL-1 β , IL-2, IL-10, C-Reactive Protein (CRP), and Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted (RANTES) in PD patients [53,66,67]. This evidence points out NLRP3 and IL-1 β as major inflammatory candidates with serological diagnostic potential in PD [66,68]. On the one hand, NLRP3 can be activated by toxic α -Syn peripherally and released in human monocytes and microglial cells [67,69,70], amplifying the inflammatory response [71], while also secreting IL-1 β from peripheral blood mononuclear cells and microglia [67,69,70]. On the other hand, a rare NLRP3 polymorphism is associated with decreased PD risk [72]. In sum, inflammogens IL-1 β and NLRP3 might be involved in PD neurodegeneration, onset, and progression [72,73]; however, further research on the α -syn–inflammasome relationship is warranted.

Furthermore, the IL-6 inflammogen is known for its role in neuropathology and is thought to present both pro-inflammatory and anti-inflammatory function in PD, with 2.3-fold higher concentration levels at the periphery in PD patients when compared to controls [74].

3. Microbial Dysbiosis and α -Syn

The microbiota—which consists of thousands of bacterial, viral, and fungal species that inhabit different parts of the human body—plays a critical role in human health, not only through its barrier function against pathogens, but also through its regulatory role of the immune system as well as its impact on other important functions, such as the regulation of movement [75]. The human gut microbiota in particular has been the focus of intense research. This microbiota is shaped by lifetime determinants (such as diet, disease history, age, or genetic heritage) [76,77] and produces a variety of molecules, some of which can enter the bloodstream and affect overall systemic health [78]. In a recent cross-sectional study, gut microbiota and plasma metabolites of 8583 participants of the population-based Swedish CARDioPulmonary bioImage study, with ages comprised between 50 to 64 years, were studied [79]. Using metagenomics and ultra-high-performance

liquid chromatography combined with mass spectrometry, Dekkers et al. [79] found that 58% of the individual variance of plasma metabolites were explained by a specific gut microbiome. Similar results were found by Diener et al. [80], by analyzing 930 blood metabolites against genetics and gut microbiome variation in 1569 individuals. Overall, 69% of the found associations were the result of sole microbiome interactions, 15% due to genetic reasons, and 16% a hybrid genomic–microbiome interaction [80].

Therefore, disruptions on microbiota present the potential to lead to broad immune dysfunction with possible neuronal consequences [45]. Thus, a dysbiotic phenomenon of the commensal microflora may be a precursor of diseases, especially inflammatory ones, as is the case of irritable bowel syndrome, periodontitis, liver disease, rheumatoid arthritis, obesity, diabetes, or even neurological disorders, as is the case of depression, anxiety, and PD [45,81,82].

The endotoxin lipopolysaccharide (LPS), being the Gram-negative bacteria's main membrane component that hampers phagocytosis by immune system cells, stands out as one of the major bacterial defense mechanisms [45]. Recent evidence based on α -Syn-induced mice exposed to LPS (intraperitoneally injected) reported cognitive deficits and enhanced dopaminergic neuronal loss, as α -Syn adopted its fibrillar form, which suggests that LPS-induced neuroinflammation and the PD-related genetic background interact synergically [27,83]. Therefore, bacterial exposure may be a driving force in α -synucleinopathies [84,85]. LPS also induces the expression of chemokines [86–89].

3.1. Gut Microbiota and α -Syn

Recent lines of research have been exploring the potential mechanisms by which changes in the gut microbiota and their products (such as LPS and intestinal-mucosa-derived inflammatory factors) might contribute to the misfolding and abnormal aggregation of α -Syn in the enteric nervous system (ENS), and upon transportation via projections of the vagus nerve and autonomic enteric fibers, in the CNS [43,77,78]. The transport of toxic α -Syn species through the microbiota–gut–brain axis ultimately results in the loss of dopaminergic neurons and causes a microglial inflammatory response that is in the pathogenesis of α -synucleinopathies [83]. In fact, PD patients appear to present differences regarding the level of certain species of gut bacteria when compared to healthy counterparts [84–87]. Therefore, it is crucial to highlight the microbiota–gut–brain axis, as it represents a complex and interdependent network between the ENS, the gut microbiota, the immune system, and the brain and provides key insights into how intestinal alterations might affect distant organs, such as the brain [88]. In fact, the gastrointestinal tract's communication network with the CNS includes pathways such as the systemic circulation of hormones, inflammatory cytokines, and microbial products, as well as the autonomic nervous system through the vagus nerve [52]. Interestingly enough, of the non-motor features that comprise the prodromal phase of PD—which include gastrointestinal, olfactory dysfunction, autonomic dysregulation, fatigue, sleep disorders, and mood disturbances—the early constipation and gastrointestinal inflammation support the involvement of the microbiota–gut–brain axis [87–91]. However, even though the gut microbiota's role in neurodegeneration has started to be explored, research on the involvement of oral microbiota on such mechanisms is still due [87,92].

In a recently conducted study, Shi et al. [90] explored whether mucosal microbiota in PD patients would correlate with changes in intestinal mucosal α -Syn. To this end, nineteen PD patients were compared to healthy counterparts for duodenal and sigmoid mucosal samples with next-generation metagenomic sequencing. Overall, the results showed that oligomer α -Syn in the sigmoid mucosa is transferred from the epithelial intestinal wall to the cytoplasm, acinar lumen, and stroma. Furthermore, the intestinal mucosal microbiota composition changed with the increase in the relative abundance of pro-inflammation inducing bacteria in the duodenal mucosa [90]. Beyond the unequivocal potential for diagnosis of PD using such samples, these results show that intestinal microbiota may have a role in the levels of α -Syn at the intestinal mucosa.

3.2. Oral Microbiota and α -Syn

There is a clear clinical association between periodontitis and PD that has been centered on both the progressive installment of motor disturbances and cognitive decline, which implicate on the patient's self-care ability and compromises oral hygiene, as well as fewer dental attendances [54], which ultimately precipitate oral diseases [91–93]. However, the extent of the existing evidence regarding a concrete α -Syn or other crosstalk biomarkers linking periodontitis and PD is sparse and still relies on a bioinformatic genomic analysis [94,95].

Periodontitis knowingly disturbs systemic health, either through its inflammatory burden or bacterial blood dissemination [96–99]. In particular, an association between periodontal inflammation and neurodegenerative conditions has been reported in studies regarding cognitive function [100,101], dementia [102], and very recently PD [103]. The hypothesis in which an active periodontal infection promotes the secretion of pro-inflammatory cytokines such as interleukin (IL)-1, IL-6, TNF- α , and reactive oxygen species (ROS) and might increase the risk of PD has been proposed [104], alongside the fact that periodontal infection constitutes a new entryway for bacterial translocation in PD [54]. In addition, not only were *Porphyromonas gingivalis* and its toxic proteases (gingipains) identified in the brain of Alzheimer's disease (AD) patients [55], but an increase in the deposition of beta amyloid has also been reported in the brain of periodontitis-induced mice models for AD [105]. The presence of these key periodontal pathogens in distant tissues, and their association with inflammation, may suggest that the migration of these microorganisms might cause local inflammatory reactions, and are often related to pathological mechanisms of major neurological diseases.

We have recently demonstrated a higher prevalence of periodontitis in PD patients [103] and possible systemic repercussions with the elevation of circulating white blood cell counts [106] and c-reactive protein [107]. In fact, systemic inflammation caused by periodontitis has been hypothesized to develop chronic neuroinflammation and ultimately interfere with PD pathogenesis [104]. The chronic pattern of periodontitis may be responsible for sustained local and systemic inflammatory states with unknown consequences in PD, perhaps suggesting the involvement of a microbiota–mouth–brain axis, and these results may pinpoint mechanistic clues for future research on the biological mechanisms and paths. In addition, immunologically different traits and patterns mediated by oral microbiota might interfere with the pro-inflammatory state in PD, with the individual as well as horizontal and vertical inheritance having a conceivable role.

The first study to investigate the oral microbiota in PD was based on the hypothesis that this disease is often characterized by neuropathological changes in olfactory and gastrointestinal tissues. Therefore, Pereira et al. compared oral and nasal samples from PD patients with controls [108]. Gene sequencing data revealed a different oral and nasal microbiota in the abundance of individual bacterial taxa, but without significant differences in PD patients. Despite the lack of apparent differences, the authors outlined the potential importance of tracking gut microbiota for potential clinical purposes. Later, Rozas et al. [109] conducted a similar cross-sectional case–control study, examining the oral microbiota from hard and soft tissues of PD patients (and matched healthy controls). The bacterial identification results showed significant differences in soft tissue diversity with a higher abundance of opportunistic oral pathogens in PD. When potential confounders were examined, the presence of dysphagia, drooling, and salivary pH emerged as the most influential. Taken together, these findings revealed novel microbiota differences and clinical signs that could explain them.

Years later, and with new data pointing to the importance of diet in the pathophysiology of PD, Zapala et al. [110] compared the dietary preferences and oral microbiota profile of PD patients with healthy matched controls. Gene sequencing showed that the oral microbiota in PD differed from the controls, with a lower abundance of *Proteobacteria*, *Pastescibacteria*, and *Tenericutes*. On the other hand, a high relative abundance of *Prevotella*, *Streptococcus*, and *Lactobacillus* was found. In addition, dietary patterns showed correla-

tions with microbial taxa, suggesting a possible role of diet in the oral bacterial profile of PD patients.

In addition, a recent exploratory study examined the composition of oral microbiota (saliva and subgingival samples) and the level of oral inflammation (by periodontal and dental examination and quantification of gingival crevicular fluid levels of IL-1 β , IL-6, IL-1 receptor antagonist, interferon- γ , and TNF- α) [111]. Both in saliva and subgingival plaque, the composition of plaque showed different patterns between PD and control subjects [111]. There was a higher abundance of *Streptococcus mutans*, *Kingella oralis*, *Actinomyces AFQC_s*, *Veillonella AFUJ_s*, *Scardovia*, *Lactobacillaceae*, *Negativicutes*, and *Firmicutes*. On the contrary, there was less abundance of *Treponema KE332528_s*, *Lachnospiraceae AM420052_s*, and *phylum SR1* [111]. Even though there were no differences between dental and periodontal statuses among PD and control patients, there was a higher level of IL-1 β and IL-1 receptor antagonists in the gingival crevicular fluid of people with PD, showing a different inflammatory pattern on the periodontal apparatus.

The apparent specific oral microbiota profiling in PD raised other questions of major importance regarding the pathophysiological features of this movement disorder. Particularly, Zheng et al. [112] collected oral mucosa samples using a cytological brush from people with PD and age-matched controls. Immunofluorescence analysis revealed increased α -Syn, pS129, and oligomeric α -Syn levels in oral mucosa cells of PD patients. While α -Syn species were distributed intracellularly, pS129 was mainly located in the cytoplasm, and oligomeric α -Syn in the nucleus and perinuclear cytoplasm. In addition, the oral mucosa α -Syn and oligomeric α -Syn levels of participants with PD significantly correlated with the clinical staging of PD, assessed with the Hoehn–Yahr scales.

At that point, several studies were able to identify a distinct gut microbial composition in PD. Jo et al. [113] furthered the research on the microbiome by studying the functional alteration of the microbiome in PD. The taxonomic oral and gut microbiome profile significantly differed between PD patients and healthy controls, with a higher abundance of *Lactobacillus* and opportunistic pathogens [113]. Functional analysis revealed a down-regulation of microbial glutamate and arginine biosynthesis gene markers and an up-regulation of antibiotic resistance gene markers in PD patients compared to healthy controls [113].

At this stage, research has identified changes in the oral microbiota of PD patients, with reductions in some bacterial species and increases in others, but without consistent patterns that may be explained by multiple factors. To this date, we expect the number of studies investigating this particular issue to increase. This body of knowledge may contribute to new frontiers in our understanding of how the oral microbiota, which is an integral part of the gut microbiota, plays a role in PD. On the one hand, these microbial changes can potentially serve as a non-invasive diagnostic tool for PD. By analyzing the genetic material of the oral microbiota, researchers can identify specific bacterial species or gene pathways associated with PD. This information could be used to develop diagnostic tests that can detect the disease earlier and more accurately than current methods. On the other hand, gene sequencing of the oral microbiota could also lead to the development of new treatments for PD. Researchers could identify specific bacterial species or gene pathways that contribute to the disease and target them with probiotics or other therapies. These treatments could potentially slow or even halt the progression of PD. While this is still highly speculative, several lines of evidence point to the key role of the gut microbiota in the CNS, the so-called gut–brain axis, and this should be a central focus of research in the coming years.

4. Inflammatory Mitigation and α -Syn

Therefore, and in alignment with recent evidence in which there is a bidirectional communication system amongst the microbiota, the immune, and the nervous systems in PD, the possibility that disease settlement and progression could be stopped indirectly before reaching the brain is revolutionary [45]. In fact, controlling overall cytokine levels

significantly improves PD's motor function [114]. In an animal study using a PD mice model, Manocha et al. explored how neuroinflammation in PD could impact cytokine changes, neuron loss, gliosis, and behavioral dysfunction [114], and then mice had their immune response modulated by a calcineurin/NFAT inhibitor. This evokes the urgency for patients' global health evaluation, correct clinical diagnosis, and redirection to specialists in order to manage these chronic inflammatory pathologies with the further goal of preventing α -synucleinopathies such as PD and other neurodegenerative diseases [27].

It appears to be crucial to address gut inflammation, particularly in cases of bowel inflammation or periodontal infection. Recent evidence indicates that having poor periodontal health is linked to a higher likelihood of developing PD [104]. Consequently, it is not only essential to encourage patients to improve their oral hygiene practices, but it is also vital to provide them with specialized and ongoing medical care, such as dental scaling, which is the current gold-standard of the non-surgical treatment for periodontitis [115]. By doing so, it may be possible to achieve a protective effect against PD.

Moreover, there seems to be evidence to suggest that various treatments aimed at modulating the gut microbiota can have beneficial effects on PD. For example, fecal microbiota transplantation (FMT) has been shown to have therapeutic potential by restoring the gut microbiota of PD patients and improving their clinical motor and non-motor symptoms, including gastrointestinal symptoms such as constipation [116]. Similarly, pre-, post-, and probiotic therapies have been found to not only affect the clinical scores and some metabolic parameters (such as hs-CRP and insulin metabolism) in human studies, but also to significantly reduce motor impairments related to gait pattern, overall balance, and coordination of movement in a PD mouse model [117–123]. Additionally, therapeutic interventions in which the administration of antibiotics is involved have been found to present regulatory effects on dopaminergic neurotoxicity in the brain [124–127]. Furthermore, vagotomy procedures have been associated with a decrease in the risk of developing PD, while also potentially providing neuroprotective effects against PD pathology, which warrants further exploration [128,129].

5. Conclusions

To this date, neurodegenerative conditions (such as α -synucleinopathies and PD in particular) are the leading cause of life-limiting disability worldwide. The effects of the microbiota on the misfolding mechanisms of α -Syn in the ENS, the CNS, and in inducing chronic inflammatory states seem to play a key role in the pathogenesis of α -synucleinopathies, even though they are still unclear thus far. In addition, the role of the oral microbiota—which is an integral part of the gastrointestinal tract—as well as its dysbiosis, also needs to be considered in the establishment of the microbiota–gut–brain axis. Therefore, it is urgent to clarify the existing evidence on α -Syn pathways to inflammation, in the hopes of contributing to the establishment of clear pathological processes, as well as to consider the mitigation of these low-grade chronic inflammatory states, as a potential pathway toward the management and prevention of such conditions. Ultimately, the goal is to establish future clinical and robust recommendations with significant impact in this current aging population.

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CHAPTER 4

4. Self-reported periodontitis and C-reactive protein in Parkinson's disease: a cross-sectional study of two American cohorts

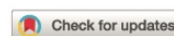
This chapter was based on the published work:

Paper II – Self-reported periodontitis and C-reactive protein in Parkinson's disease: a cross-sectional study of two American cohorts

In fulfilment of the second aim of this project – to explore the systemic repercussions of the periodontitis infection in PD patients, we will compare the levels of hs-CRP of self-reported periodontitis cases with healthy ones, amongst PD patients, using available data from the NHANES – an original research was conducted and successfully published in the npj Parkinson's Disease (April 13, 2022).

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ARTICLE OPEN



Self-reported periodontitis and C-reactive protein in Parkinson's disease: a cross-sectional study of two American cohorts

Patrícia Lyra^{1,2}, João Botelho^{1,2}, Vanessa Machado^{1,2}, Sílvia Rota^{3,4}, Ryan Walker⁵, Juliet Staunton⁵, Luís Proença^{2,6}, Kallol Ray Chaudhuri^{3,4} and José João Mendes^{1,2}

Periodontitis triggers systemic repercussions, such as elevated levels of high-sensitive C-reactive protein (hs-CRP). This has never been studied within Parkinson's Disease (PD). The aim of this study is to compare hs-CRP levels of self-reported periodontitis cases versus cases without periodontitis in PD patients. Data from the National Health and Nutrition Examination Survey (2015–2016 and 2017–2018 waves) were analyzed. PD cases were identified through medication regimens and periodontitis cases through a validated self-report questionnaire. 51 participants were included (24 females, 27 males, with mean age of 62.96 (14.71)). While the self-reported periodontitis group presented elevated levels of circulating hs-CRP (5.36 vs. 1.99 mg/L, $p = 0.031$), the self-reported without periodontitis group presented higher lymphocyte levels (29.35 vs. 28.03%, $p = 0.007$). Blood levels of hs-CRP were significantly higher in PD cases with self-reported periodontitis. Apart from the lymphocyte levels, there were no other significant differences according to the self-reported periodontal status. Future studies shall explore this association using clinical measures.

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INTRODUCTION

Parkinson's Disease (PD) is the fastest growing neurodegenerative movement disorder, affecting around 10 million people worldwide^{1,2}. This chronic, progressive and degenerative condition of both the peripheral and central nervous systems^{3,4} is clinically heterogeneous, with various motor and non-motor clinical features⁵. It has been hypothesized that the onset and progression of PD, unclear thus far, is dependent on the conjugation of different key factors such as neuroinflammation, alpha-synuclein induced neuronal dysfunction (through intracellular aggregation into Lewy bodies, which stand as the pathological hallmark of PD), systemic chronic inflammation (translated in the dysregulation of circulating inflammatory cytokines) and even gut and periodontal dysbiosis^{6,7}.

In the advanced stages, beyond the debilitating and interfering impact of motor and non-motor symptoms (NMS) on everyday-life activities, PD also has a major detrimental effect on patients' overall quality of life⁸. Oral health is no exception and may be deteriorated in PD resulting from impaired oral hygiene and lack of oral care^{9–17}.

Among the possible oral conditions that may arise from inadequate oral care is periodontitis, a chronic, infectious, and inflammatory condition characterized by the destruction of the periodontium¹⁸. The physiopathology of periodontitis involves dental plaque dysbiosis and an uncontrolled immune response attacking the periodontal tissues¹⁹. Even though a clinical periodontal diagnosis is a gold standard, the self-report of periodontitis is an interesting epidemiological strategy that has been successfully developed and validated^{20–22}. As an example, a recent prospective cohort study analyzed self-reported periodontitis relationship with female fecundability²³, showing the potential of this self-reported measure in epidemiological scenarios.

The mutual link between PD and periodontitis has been studied recently. On the one hand, fine motor impairments and cognitive decline in PD patients compromise oral hygiene habits and general oral health status^{9–16,24}. On the other hand, evidence has surged on bacterial inflammagens—including major virulence factors of key periodontal pathogens such as *Porphyromonas gingivalis*, like lipopolysaccharide (LPS) and gingipains—fueling a systemic inflammatory state that might be involved on the development of PD^{4,25}. Furthermore, periodontitis was associated with a leukocytosis state in PD patients²⁶. Also, higher blood levels of amyloid beta were found in periodontitis, mediated by inflammatory markers such as IL-6 and high-sensitive C-reactive protein (CRP)²⁷. In fact, CRP is a widely evaluated non-specific biomarker in the clinical context, not only in the diagnosis and monitoring of acute inflammatory and infectious events but also in the management and prediction of chronic inflammatory conditions, such as cardiovascular and neurodegenerative diseases^{28,29}. There are also increased levels of pro-inflammatory cytokines in PD, including CRP⁶. However, hs-CRP levels have never been studied in PD cases according to their periodontal status, and this may provide useful information in the PD-periodontitis link regarding its systemic inflammatory burden.

Hence, we aimed to compare the hs-CRP levels of individuals with PD, according to their self-reported periodontal status.

RESULTS

Population

From the 19,225 evaluated participants of the 2015–2016 and 2017–2018 NHANES waves, 119 reported medication regimens

¹Clinical Research Unit (CRU), Centro de Investigação Interdisciplinar Egas Moniz (CiiEM), Egas Moniz—Cooperativa de Ensino Superior, Almada, Portugal. ²Evidence-Based Hub, CRU, CiiEM, Egas Moniz—Cooperativa de Ensino Superior, Almada, Portugal. ³King's College London, Department of Basic & Clinical Neuroscience, Institute of Psychiatry, Psychology & Neuroscience, King's College London, London, UK. ⁴Parkinson's Foundation Center of Excellence, King's College Hospital, London, UK. ⁵King's College Hospital NHS Foundation Trust, London, UK. ⁶Quantitative Methods for Health Research (MQHS), CiiEM, Egas Moniz—Cooperativa de Ensino Superior, Almada, Portugal.

[✉]email: patricialyra10@gmail.com

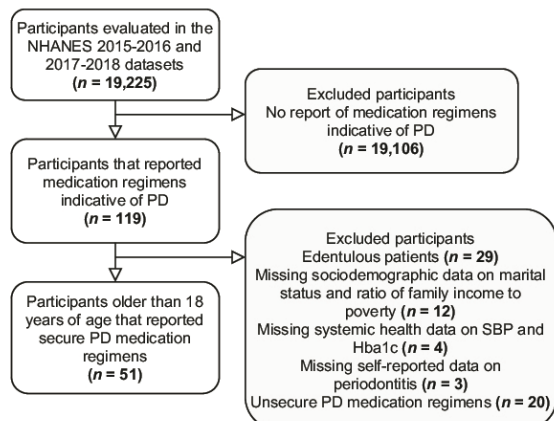


Fig. 1 Flowchart of patient inclusion. Data describes the steps involved in the inclusion of the final sample with the number of patients and respective reasons.

indicative of PD, and 51 adults were included for analysis with the detailed reasons for exclusion presented in the flowchart of Fig. 1.

The sample consisted of 24 females (47.06%) and 27 males (52.94%), with a mean group age of ~63 years (Table 1). Most participants were non-Hispanic whites (60.78%), reported an educational level higher than high school (58.82%), and were non-smokers (54.90%). However, only one statistically significant association was found between the self-report of periodontitis and sociodemographic data, namely the marital status ($p = 0.025$). The “no periodontitis” group presented a higher number of singles (28.57%), and the “periodontitis” group presented the majority of married/living with partner status (65.22%).

Regarding general health status, this group of PD patients presented a mean value of 2.55 total chronic medical conditions, 25.49% suffered from diabetes mellitus, and 66.67% from hypertension, although cases were evenly distributed (Table 1). Also, the sample presented approximately a mean value of 7.47 missing teeth. All in all, no statistically significant differences were found on general health covariates according to the self-report of periodontitis.

Blood and biochemical parameters

The levels of biochemical parameters and complete blood count with 5-part differential were analyzed in order to assess the systemic status of these participants according to the self-reported periodontal status (Table 2).

Overall, statistically significant differences were found for hs-CRP levels ($p = 0.031$) and lymphocyte percentage ($p = 0.007$). The “periodontitis” group presented higher mean levels of hs-CRP (5.36 vs. 1.99 mg/L) when compared to the “without periodontitis” group, while the “without periodontitis” group presented a higher mean percentage of lymphocytes when compared to the “periodontitis” group (29.35 vs. 28.03%).

In order to explore potential confounding variables on the hs-CRP values, we observed that PD patients with diabetes mellitus ($p = 0.130$), hypertension ($p = 0.844$), coronary heart disease ($p = 0.405$), emphysema ($p = 0.365$), asthma ($p = 0.184$), hepatic conditions ($p = 0.888$) or cancer ($p = 0.354$) had non-significant differences in serum levels of this marker. Similarly, active smokers did not present significant differences in serum levels of hs-CRP ($p = 0.343$).

DISCUSSION

The results of the present study showed that self-reported periodontitis is associated with higher circulating levels of hs-CRP in PD patients. No differences were found on white cells, red cells, and platelets according to the self-reported periodontal status of PD individuals, except for the lymphocyte percentage.

With this study, we ultimately aimed to strengthen the existing hypothesis that PD patients with periodontitis carry a systemic inflammatory burden, which can be translated into higher hs-CRP levels²⁷. In fact, hs-CRP is one of the circulatory inflammatory markers previously known to be aggravated in patients with the periodontal disease when compared to healthy control groups^{30–32}. Therefore, our results align and corroborate the previous lines of evidence in this regard³². Furthermore, to explore the hypothesis that the concomitant presence of other systemic conditions may interfere with the high hs-CRP levels in PD patients, we compared hs-CRP levels in PD patients with and without diabetes mellitus ($p = 0.130$), hypertension ($p = 0.844$), coronary heart disease ($p = 0.405$), emphysema ($p = 0.365$), asthma ($p = 0.184$), hepatic conditions ($p = 0.888$) or cancer ($p = 0.354$). No statistically significant differences were found, which indicates that the presence of other systemic diseases was not a confounding factor to the elevated hs-CRP levels in PD patients. Likewise, an active-smoker status was also not a confounding factor to high serum levels of hs-CRP ($p = 0.343$).

The clinical relevance of elevated CRP is worth discussing. This serum biomarker is mostly produced hepatically, triggered by acute and/or chronic inflammatory events²⁹. In the past years, CRP has been shown to play a key role in the management of inflammatory diseases such as cardiovascular diseases³³, neurodegenerative diseases (such as Alzheimer’s Disease (AD) and PD)^{28,34} or even periodontitis²⁷. In addition to activating the complement classical pathway, CRP also binds to several tissues and membranes propelling the inflammatory reaction through cytokines and nuclear antigens. In what PD concerns, this may be of importance because systemic and cerebral inflammation is increasingly cited in its pathophysiological basis regarded as a syndrome by many³⁵. Besides, neuroinflammation has been shown to be an important contributor to the pathogenesis of the Parkinsonian process and may aggravate the process of nigral neurodegeneration in animal models of PD³⁶. Furthermore, in the periodontitis-PD link, and besides the established effects PD impairments cause in oral health that may ultimately lead to the development of periodontitis^{9–16,24}, the infectious nature of periodontitis may have implications on gut microbiota³⁷ which is known to be abnormal in PD³⁶.

Self-reported periodontitis is a validated, efficient, and accepted measure of periodontitis cases, with higher validity upon a combination of several self-report questions^{20–22}. In fact, the self-report strategy has been previously validated in other contexts, such as to identify cases of hypertension, diabetes mellitus, hypercholesterolemia³⁸, risk factors for cardiovascular disease³⁹, and even bruxism in PD patients⁴⁰. All in all, self-report enables larger scale epidemiologic studies and low-cost surveillance of symptoms, risk factors, and diseases of interest²⁰.

However, even though the periodontitis-leukocytosis link is well established—especially given the infectious nature of periodontitis whose effects summons WBC to the lesioned site^{26,41,42}—the self-reported “periodontitis” group presented slightly lower lymphocyte percentage when compared to the “without periodontitis” group. This can be explained through the fact that elevated levels of WBC would be more probable upon clinical diagnosis of periodontitis cases. Therefore, as the clinical periodontal diagnosis is far more preferable and reliable, the use of self-reported measures of periodontitis stands as a limitation in this study, even though this method has been previously validated^{20–22} and provides a cost-effective means of great-scale

Table 1. General characteristics of PD patients according to the self-report of periodontitis.

Variables	Self-reported		p-value	Overall (n = 51)
	Without Periodontitis (n = 28)	Periodontitis (n = 23)		
Age (years), mean (SD)	65.36 (14.79)	60.04 (14.39)	0.133	62.96 (14.71)
Females, n (%)	14 (50.0)	10 (43.48)	0.855	24 (47.06)
Ethnicity, n (%)				
Mexican American	1 (3.57)	1 (4.35)	0.352	2 (3.92)
Other Hispanic	1 (3.57)	4 (17.39)		5 (9.80)
Non-Hispanic white	17 (60.71)	14 (60.87)		31 (60.78)
Non-Hispanic black	7 (25.00)	2 (8.70)		9 (17.65)
Other Race—including multi-racial	2 (7.14)	2 (8.70)		4 (7.84)
Educational Level, n (%)				
<High school	8 (28.57)	5 (21.74)	0.852	13 (25.49)
High school	5 (17.86)	3 (13.04)		8 (15.69)
>High school	15 (53.57)	15 (65.22)		30 (58.82)
Marital Status, n (%)				
Single	8 (28.57)	3 (13.04)	0.025	11 (21.57)
Married/Living with partner	14 (50.00)	15 (65.22)		29 (56.86)
Divorced/Separated/ Widowed	6 (21.43)	5 (21.74)		11 (21.57)
FI/PR, mean (SD)	2.62 (1.71)	1.92 (1.46)	0.221	2.31 (2.08)
Smoking status, n (%)				
Non-smokers	14 (50.00)	14 (60.87)	0.552	28 (54.90)
Former smokers	4 (14.29)	4 (17.39)		8 (15.69)
Active smokers	10 (35.71)	5 (21.74)		15 (29.41)
Chronic medical conditions, mean (SD)	2.75 (2.25)	5.91 (1.87)	0.406	2.55 (2.08)
Diabetes, n (%)	5 (17.86)	8 (34.78)	0.279	13 (25.49)
Hba1c, mean (SD)	6.03 (1.00)	5.91 (1.24)	0.064	5.98 (1.10)
Hypertension, n (%)	19 (67.86)	15 (65.22)	1.000	34 (66.67)
SBP, mean (SD)	141.95 (25.29)	129.86 (24.07)	0.173	136.50 (25.24)
DBP, mean (SD)	77.36 (12.39)	70.15 (8.88)	0.176	74.11 (11.43)
Missing teeth, mean (SD)	6.82 (6.60)	8.26 (6.66)	0.537	7.47 (6.60)

DBP Diastolic Blood Pressure, FI/PR Family income/poverty ratio, Hba1c Hemoglobin A1C level, n number of cases, SBP Systolic Blood Pressure, SD Standard Deviation.
*Mann–Whitney test for continuous variables and Chi-square test for categorical variables.

monitoring of oral health²⁰. Furthermore, disease severity and activity could not be appraised through a self-report method, which is also fully reliable on the patient's knowledge of the disease and full awareness of a previous clinical diagnosis. Thus, the possibility of unmeasured confounding through this method of identification of periodontitis cases cannot be discarded²³.

Additionally, the sample size may be considered limited and thus a shortcoming of this study. Perhaps, this might be because we are trying to signpost an underappreciated condition of difficult diagnosis⁵. Despite the small sample number, the collected data is of clinical significance, and we hope to pave the way for future larger studies on this condition worldwide (for instance using the MDS non-motor study group network). Also, the secondary study design based on the available NHANES data has been previously applied and accepted in several recent studies, including the PD-case selection method employed^{26,43}. Nonetheless, due to the used PD-case selection method, data on disease duration was not available, which would have been relevant to evaluate disease staging. Furthermore, the observational nature of the study impairs the conclusion of causality, thus robust evidence has been reported regarding periodontitis increasing circulating levels of CRP and hs-CRP⁴².

All in all, the present study evaluates the association between self-reported periodontitis and hs-CRP levels in PD patients.

Hence, future research should continue to focus on the systemic repercussions of the periodontitis infection in PD patients, in the hopes of potentially clarifying the causality of the PD-periodontitis link. Furthermore, future research including in-depth clinical measures of periodontitis (such as periodontal pocket depth and clinical attachment loss), will provide further confirmation on the association with circulating systemic inflammatory surrogates.

METHODS

Study design

In this secondary study, data was extracted and further analyzed from the National Health and Nutrition Examination Survey (NHANES), a representative and stratified multistage health-related survey conducted on non-institutionalized U.S. citizens. The STrengthening the Reporting of OBservational studies in Epidemiology (STROBE) guideline was followed (Table S1, Supplementary Materials)⁴⁴.

Setting, participants, and study size

Data from the NHANES 2015–2016 and 2017–2018 databases were used for the present study. Our analysis deemed the following inclusion criteria: 18 years of age or older; and undertaking secure PD medication regimens. Edentulous patients, missing data (on sociodemographic and/or systemic health information), and unsecure PD medication regimens as previously defined (Cabergoline, Orphenadrine, and Pramipexole)²⁶ were excluded.

Variables	Self-reported		p-value*	Overall (n = 51)
	Without periodontitis (n = 28)	Periodontitis (n = 23)		
Biochemical parameters, mean (SD)				
hs-CRP (mg/L)	1.99 (2.03)	5.36 (6.37)	0.031	3.51 (4.82)
Total Cholesterol (mmol/L)	4.83 (0.82)	4.58 (1.17)	0.389	4.71 (1.01)
HDL-Cholesterol (mmol/L)	1.56 (0.56)	1.35 (0.39)	0.515	1.46 (0.50)
Hematologic parameters, mean (SD)				
WBC count (10 ⁹ /L)	6.88 (2.23)	7.89 (2.24)	0.103	7.33 (2.31)
Lymphocyte percent (%)	29.35 (11.83)	28.03 (8.75)	0.007	28.76 (10.68)
Monocyte percent (%)	8.81 (2.64)	8.31 (2)	0.850	8.58 (2.41)
Segmented neutrophils percent (%)	58.16 (12.21)	60.46 (9.05)	0.596	59.20 (11.07)
Eosinophils percent (%)	2.87 (1.88)	2.44 (1.21)	0.513	2.68 (1.64)
Basophils percent (%)	0.95 (0.4)	0.87 (0.29)	0.682	0.91 (0.36)
Lymphocytes (10 ⁹ /L)	1.93 (0.77)	2.17 (0.76)	0.214	2.04 (0.78)
Monocytes (10 ⁹ /L)	0.57 (0.19)	0.64 (0.2)	0.946	0.60 (0.20)
Segmented neutrophils (10 ⁹ /L)	4.12 (1.86)	4.83 (1.77)	0.142	4.44 (1.87)
Eosinophils (10 ⁹ /L)	0.2 (0.15)	0.2 (0.11)	0.696	0.20 (0.13)
Basophils (10 ⁹ /L)	0.07 (0.05)	0.07 (0.05)	0.886	0.07 (0.05)
RBC count (10 ¹² /L)	4.7 (0.4)	4.65 (0.59)	0.643	4.68 (0.50)
Hemoglobin (g/dL)	13.91 (1.42)	13.5 (1.67)	0.872	13.73 (1.57)
Hematocrit (%)	41.92 (3.77)	40.59 (4.42)	0.344	41.32 (4.17)
Mean cell volume (fL)	89.29 (5.11)	88.01 (9.34)	0.400	88.71 (7.43)
Mean cell hemoglobin (pg)	29.62 (2.29)	29.28 (3.76)	0.985	29.47 (3.07)
Mean Cell Hgb Conc. (g/dL)	33.15 (0.95)	33.2 (1.28)	0.519	33.17 (1.13)
RDC width (%)	14.28 (1.06)	15.08 (1.55)	0.052	14.64 (1.37)
Platelet count (1000 cells/uL)	248.46 (87.7)	219.04 (77.86)	0.195	235.20 (85.52)
MPV (fL)	8.22 (0.77)	8.33 (0.86)	0.726	8.27 (0.82)
* Mann-Whitney test. WBC White Blood Cells, RBC Red Blood Cells, MCV Mean Cell Volume, MCH Mean Cell Hemoglobin, MCHC Mean Cell Hemoglobin Concentration, RCD Red Cell Distribution, MPV Mean Platelet Volume.				

Detailed information on sampling, design, and medical records are displayed at www.cdc.gov/nchs/nhanes.htm (accessed in April 2021). Health-related data-collection protocols from the NHANES 2015–2016 and 2017–2018 datasets underwent revision and approval by the Centers for Disease Control (CDC) and Prevention National Increase for Health Statistics Research (NCHS) Ethics Review Board, Atlanta USA, and all study participants provided written informed consent⁴⁵.

Variables and data measurement

PD definition. PD cases were identified in the NHANES database through the report of specific PD medications, according to a previous study²⁶. Hence, the reported use of Bzotropine, Carbidopa, Levodopa, Ropinirole, Methyldopa, Entacapone, and Amantadine were considered PD medications indicative of PD, therefore validating a PD case^{46,47}. Cabergoline, Orphenadrine, and Pramipexole all present other known clinical applications apart from PD—Cabergoline is used to treat high levels of prolactin hormone⁴⁸, Orphenadrine is used to treat muscle spasms in musculoskeletal conditions⁴⁹ and Pramipexole is also used to treat restless legs syndrome (RLS)⁵⁰—and therefore were considered unsecure medications for the selection of PD cases.

Periodontitis definition. Periodontitis cases were pinpointed through a positive self-report on either one of the following oral health-related (OHR) questions, all regarding the moment when the survey was applied: “Do you think you might have gum disease?”, “Ever had treatment for gum disease?” and “Ever been told of bone loss around teeth?”. This method of self-reporting periodontitis has been previously validated and is indicative of a periodontitis case^{20–22}.

Demographic characteristics. Age, gender, ethnicity, level of education, marital status, family income to poverty ratio, and smoking status were the self-reported sociodemographic variables collected and analyzed from NHANES datasets.

Regarding ethnicity, “Mexican American”, “Other Hispanic”, “Non-Hispanic White”, “Non-Hispanic Black” and “Other race—including multi-racial” were the used designations, as indicated in the NHANES self-reported questionnaires⁴³.

The level of education in individuals aged over 20 was categorized as follows: “<high school” (including <9th grade, 9–11th grade, and 12th grade with no diploma), “high school” (including high school grad/GED or equivalent) and “>high school” (including some college or AA degree and college graduate or above)⁵¹.

Concerning patients’ marital status, the used definition included “single” (never married), “married/living with a partner”, and “divorced/separated/widowed”⁵².

With regards to the reported family income to poverty ratio, a continuous score from 0 to 5 was given: “0” corresponding to no income, “5” corresponding to an income 5 or more times above the federal poverty threshold⁵³.

At last, smoking status was defined as “active smokers” (reporting a consumption of ≥100 cigarettes during their lifetime and still currently smoking), “former smokers” (reporting smoking ≥100 cigarettes during their lifetime and presently ceased smoking) and non-smokers (reporting having smoked <100 cigarettes during their lifetimes)²⁶.

Health characteristics. The systemic health status of the included participants was overall characterized through a sum of chronic medical conditions—asthma, psoriasis, gout, congestive heart failure, coronary

heart disease, angina, heart attack, stroke, emphysema, thyroid, bronchitis, liver, and cancer—which was statistically considered a continuous variable. Furthermore, Diabetes Mellitus (DM) was separately defined through self-report information and confirmed with glycated hemoglobin levels (HbA1c)⁵⁴. Levels of HbA1c > 8% were considered uncontrolled DM cases⁵⁵. Also, high blood pressure cases were defined from previous self-reports of medical-informed hypertension and were further confirmed with systolic and diastolic blood pressure levels (>140 mmHg and >90 mmHg, respectively)⁵⁶.

Blood and biochemical parameters. Serum fractions of hs-CRP (mg/L), HDL-cholesterol (mg/dL), and total cholesterol were analyzed from blood specimens of the NHANES database^{57,58}.

Also, complete blood count with 5-part differential data was gathered, and information on white Blood Cell (WBC) count (10⁹/L), percentage of Lymphocyte (%), percentage of Monocyte (%), percentage of Segmented Neutrophils (%), percentage of Eosinophils (%), percentage of Basophils (%), Lymphocyte (10⁹/L), Monocyte (10⁹/L), Segmented neutrophils (10⁹/L), Eosinophils (10⁹/L), Basophils (10⁹/L), Red Blood Cell (RBC) count (10¹²/L), Hemoglobin (g/dL), Hematocrit (%), Mean Cell Volume (MCV) (fL), Mean Cell Hemoglobin (MCH) (pg), Mean Cell Hemoglobin Concentration (MCHC) (g/dL), Red Cell Distribution (RCD) width (%), Platelet count (1000 cells/uL) and Mean Platelet Volume (MPV) (fL) was analyzed.

Blood collection occurred ~3 weeks following interviews, as detailed in <https://www.cdc.gov/nchs/nhanes/continuousnhanes/manuals.aspx?BeginYear=2015> (accessed in April 2021).

Data management, analysis, and statistical methods

Data analysis of the 2015–2016 and 2017–2018 NHANES datasets was conducted through IBM SPSS Statistics version 26.0.0.0 for Macintosh (Armonk, New York, IBM Corp.). Data were uploaded via SAS Universal Viewer and handled with Microsoft Excel. Continuous variables are reported through mean ± standard deviation (SD), while the number of cases (*n*) and percentage (%) represent categorical variables distribution among group categories. Upon assessment of data non-normality and homoscedasticity, Mann–Whitney test was applied for comparison of continuous variables. Chi-square test was used to evaluate association between the categorical variables. A 5% significance level was used in all inferential analyses.

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

DATA AVAILABILITY

The analyzed data that supports the findings of this study are available in the NHANES database, a publicly accessible repository that does not issue DOIs, www.cdc.gov/nchs/nhanes.htm. The used datasets can be located under the “Survey Data and Documentation” tab, followed by the “NHANES 2015–2016” and “NHANES 2017–2018” databases tabs. “Demographics Data”, “Examination Data”, “Laboratory Data” and “Questionnaire Data” were consulted.

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AUTHOR CONTRIBUTIONS

Conceptualization, P.L., J.B., and V.M.; methodology, P.L., J.B. and V.M.; validation, J.B., V.M., S.R., L.P., and K.R.C.; formal analysis, P.L., J.B., S.R., and K.R.C.; investigation, P.L., J.B. and V.M.; resources J.B. and V.M.; data curation, P.L. and J.B.; writing—original draft preparation, all authors; writing—review and editing, all authors; visualization, P.L. and J.B.; supervision, K.R.C. and J.J.M.; project administration, J.J.M.; All authors have read and agreed to the published version of the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to Patrícia Lyra.

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CHAPTER 5

5. Self-Reported Measures of Periodontitis in a Portuguese Population: A Validation Study

This chapter was based on the published work:

Paper III – Self-Reported Measures of Periodontitis in a Portuguese Population:
A Validation Study

The self-report strategy seems to be a promising tool in epidemiological settings. This original research article was developed to validate a self-reported strategy in predicting periodontitis in a Portuguese population and was successfully published in the Journal of Personalized Medicine (August 14, 2022).

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Article

Self-Reported Measures of Periodontitis in a Portuguese Population: A Validation Study

Vanessa Machado ^{1,2,*}, Patrícia Lyra ^{1,†}, Catarina Santos ¹, Luís Proença ^{1,2,3}, José João Mendes ^{1,2}
and João Botelho ^{1,2}

- ¹ Clinical Research Unit, Centro de Investigação Interdisciplinar Egas Moniz (CiiEM),
Egas Moniz—Cooperativa de Ensino Superior, 2829-511 Almada, Portugal
² Evidence-Based Hub, CiiEM, Egas Moniz—Cooperativa de Ensino Superior, 2829-511 Almada, Portugal
³ Quantitative Methods for Health Research, CiiEM, Egas Moniz—Cooperativa de Ensino Superior,
2829-511 Almada, Portugal
* Correspondence: vmachado@egasmoniz.edu.pt
† These authors contributed equally to this work.

Abstract: Self-reported questionnaires have been developed and validated in multiple populations as useful tools to estimate the prevalence of periodontitis in epidemiological settings. This study aimed to explore the accuracy of self-reporting for predicting the prevalence of periodontitis in a Portuguese population. The questionnaires were given to patients at a university clinic. Thirteen self-reported questions on periodontal health were gathered in a patient-reported questionnaire. Then, self-reporting responses were validated using full-mouth periodontal examination as a comparison. Multivariable logistic regression was used to analyze sensitivity, specificity, accuracy, precision, and area under the curve-receiver operator characteristic (AUC-ROC). Self-reported answers from 103 participants (58 females and 45 males) were included. Self-reported gum health, loose teeth, tooth appearance, and use of dental floss were associated with different definitions of severe periodontitis. The self-reported questions on “having gum disease,” combined with “having gum treatment” and “having lost bone” were the items with higher performance for the 2018 case definition and the 2012 case definition, as well as for each respective severity staging. Categorization of tooth loss was only valuable for the prediction of periodontitis cases according to the 2012 case definition and its severe stage. Multiple self-reporting set-ups showed elevated performance levels for predicting periodontitis in Portuguese patients. These results may pave the way for future epidemiological surveillance programs using self-reporting approaches.

Keywords: oral health surveys; periodontitis; periodontal disease; surveillance; precision; self-reported measures



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1. Introduction

Periodontal diseases, including gingivitis and periodontitis, are the sixth-most prevalent conditions contributing to the global burden of chronic non-communicable illnesses [1]. Beyond its prevalence, affecting more than 50% of the adult population worldwide [1], periodontal disease has been shown to have implications for quality of life [2] and nutrition [3], aesthetic confidence, and general well-being, varying with the increasing grading and severity of periodontitis [2,4]. In addition to its social impact, periodontitis is robustly linked with systemic disease and inflammatory burden [5].

Clinically-based periodontal examinations, including full-mouth periodontal probing depth (PD), clinical attachment loss (CAL), and bleeding on probing (BOP), are the gold standard for periodontitis diagnosis [6]. These clinical measures are not complex to obtain but are time-consuming, require trained professionals, specialized equipment, and infection control strategies. Facing these challenges, the Centers for Disease Control and Prevention (CDC) proposed a self-reported strategy to screen periodontitis that has been successfully

validated. Particularly in screening systems and epidemiological research, it becomes highly relevant to explore such non-clinical and self-reported approaches to make periodontal conditions surveillance more cost-effective [7].

The eight-item questionnaire proposed in a collaboration between CDC and the American Academy of Periodontology (AAP) was validated and implemented in the National Health and Nutrition Examination Survey (NHANES) [8]. This tool is a valid instrument to screen the prevalence of periodontitis in American dentate adults [9–11]. This CDC/AAP questionnaire has been successfully cross-culturally adapted and validated for Spanish [12,13], French [14], Brazilian [15], Dutch [16] and Japanese [17] populations, with discriminative capability for detecting individuals with periodontitis. Nonetheless, this epidemiological tool has never been validated in Portugal, a country with high prevalence and burden of periodontitis.

The present study aimed to cross-culturally adapt and validate a periodontitis self-reported questionnaire based on the European Federation of Periodontology (EFP)/AAP and CDC/AAP tool together with other questions with predictive performance in a Portuguese population. To our view, assessing the validity of translations of the self-reported questions to Portuguese and attesting its predictive capacity will be an important addition to the literature on the surveillance of periodontitis.

2. Materials and Methods

This study is reported following the Transparent Reporting of a multivariable prediction model for Individual Prognosis or Diagnosis (TRIPOD) guidelines for reporting predictive models [18]. This study received approval by the institutional review board (Egas Moniz Ethics Committee n°. 595) and we obtained signed informed consent from all participants. This study was developed in compliance with the World Medical Association Helsinki Declaration (2013).

2.1. Study Design and Participants

First-time patients seeking dental care at the Egas Moniz Dental Clinic (EMDC), located in Almada (Portugal), were invited to participate in the study. Patients were deemed eligible if they met the following inclusion criteria: aged at least 18 years old; being able to read and understand Portuguese; agreeing to participate in the study; completing the questionnaire; and receiving a complete periodontal examination. As exclusion criteria, patients having less than two teeth or who had been pre-diagnosed with a severe or terminal disease (for instance, advanced heart failure, end-stage kidney disease, or advanced-stage cancer) [17] were not included.

Sample size calculation was conducted in R (version 4.0.0) using the ‘pwr’ package. Considering a prevalence of periodontitis of 59.9%, observed in a previous large epidemiological study undertaken in the Almada-Seixal region (Portugal) [19], we estimated a minimum required sample of 101 patients, considering a 5% significance level and 80% power.

2.2. Self-Reported Questions

The questionnaire included a total of thirteen self-reported questions, with eight of them based on the original CDC/AAP [8]. The remaining five questions were selected from a group of past studies that presented good performance results [13,17] (Table 1).

2.3. Study Design and Participants

The questionnaire collected information regarding age, sex, smoking status, education level, and toothbrushing per day. Information regarding data management for the logistic regression analysis can be found in Section 2.5 (Statistical analysis).

Table 1. Original and Portuguese versions of the self-reported periodontal screening questionnaire.

Original Question	Translated Question in Portuguese	Question Variable	Reference
Do you think you might have gum disease? Yes No Don't know Refused	Acha que pode ter doença das gengivas? Sim Não Não Sei Recuso-me a responder	Gum disease	Eke et al. (2013) [8]
Overall, how would you rate the health of your teeth and gums? Excellent / Very good / Good / Fair / Poor / Don't know / Refused	No geral, como classificaria a saúde de seus dentes e gengivas? Excelente Muito boa Boa Razoável Fraca Não Sei Recuso-me a responder	Teeth/gum health	Eke et al. (2013) [8]
Have you ever had treatment for gum disease, such as scaling and root planning, sometimes called "deep" cleaning? Yes No Don't know Refused	Alguma vez recebeu tratamento para a doença das gengivas tal como alisamento ou raspagem radicular, por vezes chamada limpeza profunda? Sim Não Não Sei Recuso-me a responder	Had gum treatment	Eke et al. (2013) [8]
Have you ever had any teeth become loose on their own, without an injury? Yes No Don't know Refused	Já teve algum dente que começou a abanar por si só, sem nenhum trauma ou lesão? Sim Não Não Sei Recuso-me a responder	Loose tooth	Eke et al. (2013) [8]
Have you ever been told by a dental professional that you lost bone around your teeth? Yes No Don't know Refused	Já algum profissional dentário lhe disse que tinha perdido osso ao redor dos dentes? Sim Não Não Sei Recuso-me a responder	Lost bone	Eke et al. (2013) [8]
During the past 3 months have you noticed a tooth that doesn't look right? Yes No Don't know Refused	Nos últimos 3 meses, reparou se algum dente não tenha estado bem? Sim Não Não Sei Recuso-me a responder	Tooth does not look right	Eke et al. (2013) [8]
Aside from brushing your teeth with a toothbrush, in the last 7 days, how many times did you use dental floss or any other device to clean between your teeth? _____ Number Refused	Além de escovar os dentes com escova de dentes, nos últimos sete dias, quantas vezes usou fio dentário ou qualquer outro dispositivo para limpar a região entre os dentes? _____ Número Recuso-me a responder	Floss use	Eke et al. (2013) [8]
Do your gums usually bleed either when brushing or chewing? Yes No Don't know Refused	Sangra habitualmente das gengivas sem escovar ou mastigar? Sim Não Não Sei Recuso-me a responder	Gum bleeding	Saka-Herrán et al. (2020) [13]
During the past three months, have you had bleeding gums? Never Hardly ever Sometimes Fairly often Very often	Nos últimos 3 meses, sangrou das gengivas? Nunca Quase nunca Às vezes Com bastante frequência Muitas vezes	Gum bleeding last 3 months	Iwasaki et al. (2021) [17]
Have you lost teeth in recent years because of mobility? Yes No Don't know Refused	Perdeu algum dente nos últimos anos por ter começado a abanar? Sim Não Não Sei Recuso-me a responder	Loose teeth loss	Saka-Herrán et al. (2020) [13]
Have you felt pain in your gums during the last months? Yes No Don't know Refused	Nos últimos meses, doeram-lhe as suas gengivas? Sim Não Não Sei Recuso-me a responder	Gum pain	Saka-Herrán et al. (2020) [13]
In the past years have you noticed that your teeth are longer or that you have receding gums? Yes No Don't know Refused	Nos últimos meses, notou que os seus dentes estão maiores e as gengivas mais retraídas do que o normal? Sim Não Não Sei Recuso-me a responder	Gum retraction	Saka-Herrán et al. (2020) [13]
In the last years have you noticed that you see the roots of several of your teeth? Yes No Don't know Refused	Nos últimos meses, notou que se vêem as raízes dos seus dentes? Sim Não Não Sei Recuso-me a responder	Roots visible	Saka-Herrán et al. (2020) [13]

2.4. Periodontal Examination and Periodontitis Case Definition

Following the self-reported questionnaire, patients underwent periodontal examination by trained and calibrated examiners (kappa values > 0.8) via a periodontal probe (PCP 12, Hu-Friedy Chicago, IL, USA). The examiners were blinded to the questionnaire responses. A circumferential, six sites per tooth examination protocol was performed, excluding third molars, implants, and retained roots. PD, CAL and gingival margin re-

cession (REC) were registered to the nearest mm. BOP was also recorded. Tooth loss was registered as well, accounting for the total number of teeth present. Data was uploaded to a Microsoft Excel database and used to compute periodontal case definitions using algorithms as previously described [20,21].

Cases were diagnosed as periodontitis and according to each staging based on the EFP/AAP 2018 case definition and stage III/IV form (accepted as severe stage) [6]:

- Periodontitis case: Interdental CAL at ≥ 2 non-adjacent teeth, or buccal/oral CAL ≥ 3 mm with pocketing > 3 mm at ≥ 2 teeth;
- Severe periodontitis: interdental CAL at site of greatest loss of ≥ 5 mm; or, radiographic bone loss extending to mid-third of root and beyond.

To explore the suitability of self-reported measures to a wide variety of periodontitis case definitions we also defined patients according to The CDC/AAP 2012 [22]:

- Periodontitis (using the mild definition as cutoff): ≥ 2 interproximal sites with CAL ≥ 3 mm, and ≥ 2 interproximal sites with PPD ≥ 4 mm (not on same tooth) or one site with PD ≥ 5 mm;
- Severe periodontitis: ≥ 2 interproximal sites with CAL ≥ 6 mm (not on the same tooth) and ≥ 1 interproximal site with PPD ≥ 5 mm.

2.5. Statistical Analysis

All analyses were conducted in R (version. 4.0.0), using the package ‘cvAUC’. Age was dichotomized to <45 (0) and ≥ 45 years old (1) according to a previous study report [19]. Smoking status was dichotomized into no active smoking (0) (including non-smokers and former smokers) or active smoking (1). Education was categorized as: elementary school (0) or middle school/higher education (1). Tooth loss was obtained from the periodontal examination (Section 2.4) and was categorized as 0, 1–5 or ≥ 6 tooth loss (for logistic regression this was dichotomized as <6 teeth lost (0) or ≥ 6 (1)).

Multivariable logistic regression analyses were used to predict the periodontal outcomes, according to the different case definitions that were tested. Crude and adjusted odds ratio (OR) were determined within these procedures.

Four sets of predictor variables were tested against these periodontal outcomes using multivariable binary logistic regression procedures: (a) the thirteen oral health self-reported questions; (b) demographic and risk factors (age, sex, educational level, smoking status, diabetes, and tooth loss); (c) combined self-reported oral health questions and demographic/risk factors; and (d) the selection of the best significant subset of predictor variables using the method of all possible equations. The significance level was set at 5%.

The predictive validity of these questions for the different disease definitions was assessed by the area under the curve-receiver operator characteristic (AUC/ROC) analysis. Sensitivity, specificity, accuracy, precision, and the Akaike Information Criterion (AIC) were evaluated.

3. Results

3.1. Participants’ Characteristics

All participants were recruited between November 2021 and April 2022. In this population subset, 61.2% and 68.9% were diagnosed with periodontitis, according to the EFP/AAP 2018 and CDC/AAP 2012 periodontitis case definitions, respectively. The mean age of the total sample was 50.6 (± 16.0) years, with the most severe periodontitis being diagnosed in older participants: 59.2 (± 11.5) and 58.3 (± 11.7). Those participants with severe periodontitis were mainly males (56.4% and 55.4%), presenting lower education levels (41.0% and 42.9%), predominantly non-smokers (51.3% and 48.2%), and had six or more teeth missing (69.2% and 67.9%), according to EFP/AAP 2018 and CDC/AAP 2012 case definitions, respectively (Table 2).

Table 2. Socio-demographic characteristics of the participants.

Variable	Total (n = 103)	EFP/AAP 2018 Periodontitis (n = 63)	EFP/AAP 2018 Severe Periodontitis (n = 39)	CDC/AAP 2012 Periodontitis (n = 71)	CDC/AAP 2012 Severe Periodontitis (n = 56)
Age	50.6 (16.0)	56.3 (13.2)	59.2 (11.5)	54.7 (13.9)	58.3 (11.7)
Sex					
Female	58 (56.3)	31 (49.2)	17 (43.6)	38 (53.5)	25 (44.6)
Male	45 (43.7)	32 (50.8)	22 (56.4)	33 (46.5)	31 (55.4)
Education level					
Elementary	32 (31.1)	27 (42.9)	16 (41.0)	27 (38.0)	24 (42.9)
Middle	39 (37.9)	20 (31.7)	12 (30.8)	25 (35.2)	18 (32.1)
Higher	32 (31.1)	16 (25.4)	11 (28.2)	19 (26.8)	14 (25.0)
Smoking habits					
Non-smoker	63 (61.2)	33 (52.4)	20 (51.3)	38 (53.5)	27 (48.2)
Former smoker	13 (12.6)	11 (17.5)	7 (17.9)	12 (16.9)	11 (19.6)
Current smoker	27 (26.2)	19 (30.2)	12 (30.8)	21 (29.6)	18 (32.1)
Tooth loss					
0	21 (20.4)	4 (6.3.0)	0 (0.0)	5 (7.0)	2 (3.6)
1–5	36 (35.0)	19 (30.2)	12 (30.8)	24 (33.8)	16 (28.6)
≥6	46 (44.7)	40 (63.5)	27 (69.2)	42 (59.2)	38 (67.9)
Toothbrushing per day					
1	14 (13.6)	12 (19.0)	5 (12.8)	12 (16.9)	11 (19.6)
2	57 (55.3)	34 (54.0)	20 (51.3)	38 (53.5)	30 (53.6)
3 or more	32 (31.1)	17 (27.0)	14 (35.9)	21 (29.6)	15 (26.8)

Categorical variables are expressed as n (%), and continuous variables are expressed as mean (SD), within each periodontal condition category. n—number of participants.

3.2. Predictive Ability of the Self-Reported Questions

The overall response distribution to the thirteen questions depicted a scarce percentage of ‘Refused/Don’t know’ responses, and a very substantial response rate in all questions (Supplementary Table S1). Each self-reported question showed significant association results with the different periodontitis definitions used (EFP/AAP 2018 and CDC/AAP 2012) (Table 3). A positive response to the question on ‘Gum disease’ (Q1) (54.4), was highly associated with having severe periodontitis according to EFP/AAP 2018; those patients unanimously (100%) indicated a positive response. Contrariwise, the questions about ‘Floss use’, ‘Gum bleeding last 3 months’, and ‘Gum bleeding’ were not associated with periodontitis (Table 3). The strongest crude associations were found for ‘Gum disease’, ‘Loose tooth’, ‘Lost bone’ and ‘Gum retraction’ in participants with severe periodontitis according to EFP/AAP 2018.

In multivariate modeling (Tables 4 and 5), reduced models outperformed the prediction of periodontitis and severe periodontitis according to both the EFP/AAP definition and the CDC/AAP definition. All reduced models showed an AUC over 0.80, suggesting excellent discrimination potential for periodontitis (0.86), according to the EFP/AAP definition, and periodontitis (0.86) and its severe form (0.80) in the CDC/AAP definition. For severe periodontitis according to the EFP/AAP 2018 periodontal case definition, the prediction value was deemed acceptable (0.71). All reduced models included the question regarding recognizing having ‘Gum disease’ (Q1). Furthermore, ‘Had Gum Treatment’ (Q3), ‘Loose tooth’ (Q4), ‘Lost bone’ (Q5), and ‘Loose Tooth Loss’ as a risk factors were also significant questions.

Table 3. Adjusted odds ratio (OR) for each of the four periodontitis outcomes.

Question	Adjusted OR (Standard Error) ^a			
	EFP/AAP 2018 Periodontitis (n = 63)	EFP/AAP 2018 Severe Periodontitis (n = 39)	CDC/AAP 2012 Periodontitis (n = 71)	CDC/AAP 2012 Severe Periodontitis (n = 56)
Gum disease	53.0 (8.5–330.9) ***	4.3 (1.8–10.1) ***	30.0 (5.8–156.0) ***	14.7 (3.7–58.1) ***
Teeth/gum health	7.7 (0.1–508.0)	-	109.8 (10.7–1126.8) ***	-
Had gum treatment	12.4 (2.4–63.2) **	1.9 (0.8–4.5) **	20.3 (2.4–171.8) **	6.2 (1.8–21.6) **
Loose tooth	13.6 (2.7–69.2) **	6.9 (2.8–17.1) ***	8.4 (2.4–30.1) *	5.6 (1.7–18.0) **
Lost bone	12.4 (2.9–54.1) ***	7.1 (2.9–17.4) ***	-	18.3 (4.3–77.9) ***
Tooth does not look right	4.4 (1.4–13.8) **	5.2 (2.1–12.8)	0.7 (0.3–1.7) *	2.9 (1.0–8.2) *
Floss use	1.3 (0.4–3.7)	0.7 (0.3–1.5)	1.8 (1.1–3.1)	1.9 (0.6–5.7)
Gum bleeding	6.2 (1.8–21.4) **	1.5 (0.7–3.3)	2.7 (0.9–7.7)	2.7 (0.9–8.1)
Gum bleeding last 3 months	6.0 (0.3–107.9)	2.9 (1.0–8.3)	-	2.6 (0.6–11.8)
Loose tooth loss	31.5 (1.8–561.8) *	3.9 (1.5–10.5) **	11.0 (1.1–111.9) *	7.3 (1.2–44.6) *
Gum pain	6.3 (1.4–27.9) *	1.2 (0.5–3.1)	2.8 (0.8–9.5)	2.3 (0.7–7.9)
Gum retraction	3.0 (0.9–9.9)	4.6 (1.9–11.0) ***	12.5 (2.5–63.3) **	3.7 (1.1–11.9) *
Roots visible	2.7 (0.8–9.2)	4.1 (1.7–10.0) **	9.3 (1.8–47.7) **	2.6 (0.8–8.4)

^a Multivariable logistic regression adjusted for age, sex, education, smoking habits, tooth loss, and employment status. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 4. Logistic regression models for the periodontitis EFP/AAP case definition of 2018 and respective performance analysis.

Question	EFP/AAP 2018 Periodontitis (Models)				EFP/AAP 2018 Severe Periodontitis (Models)			
	1	2	3	4	1	2	3	4
Gum disease	X		X	X	X		X	X
Teeth/gum health	X		X		X		X	
Had gum treatment	X		X	X	X		X	
Loose tooth	X		X		X		X	
Lost bone	X		X	X	X		X	X
Tooth does not look right	X		X		X		X	
Floss use	X		X		X		X	
Gum bleeding	X		X		X		X	
Gum bleeding last 3 months	X		X		X		X	
Loose tooth loss	X		X		X		X	
Gum pain	X		X		X		X	
Gum retraction	X		X		X		X	
Roots visible	X		X		X		X	
Age		X	X			X	X	
Sex		X	X			X	X	
Education		X	X			X	X	
Employment status		X	X			X	X	
Smoking habits		X	X			X	X	
AUC	0.58	0.49	0.50	0.86	0.55	0.50	0.50	0.71
Sensitivity (%)	100.0	0.98	100.0	88.9	100.0	100.0	100.0	79.5
Specificity (%)	15.4	0.0	0.0	82.5	9.4	0.0	0.0	62.5
Accuracy (%)	67.6	61.2	60.2	86.4	43.7	37.9	37.9	68.9
Precision (%)	65.6	61.2	60.8	88.9	40.2	37.9	37.9	66.0
AIC	78.3	113.1	75.8	87.0	116.2	119.8	130.1	117.8

AAP, American Academy of Periodontology; AIC, Akaike Information Criterion; AUC, Area Under the Curve; EFP, European Federation of Periodontology; P, Periodontitis; SP, Severe Periodontitis.

Table 5. Logistic regression models for the periodontitis CDC/AAP case definition of 2012 and respective performance analysis.

Question	EFP/AAP 2018 Periodontitis (Models)				EFP/AAP 2018 Severe Periodontitis (Models)			
	1	2	3	4	1	2	3	4
Gum disease	X		X	X	X		X	X
Teeth/gum health	X		X		X		X	
Had gum treatment	X		X	X	X		X	X
Loose tooth	X		X		X		X	
Lost bone	X		X		X		X	
Tooth does not look right	X		X		X		X	
Floss use	X		X		X		X	
Gum bleeding	X		X		X		X	
Gum bleeding last 3 months	X		X		X		X	
Loose tooth loss	X		X		X		X	
Gum pain	X		X		X		X	
Gum retraction	X		X		X		X	
Roots visible	X		X		X		X	
Age		X	X			X	X	
Sex		X	X			X	X	
Education		X	X			X	X	
Employment status		X	X			X	X	
Smoking habits		X	X			X	X	
Tooth Loss		X	X	X				X
AUC	0.57	0.49	0.50	0.86	0.56	0.50	0.49	0.80
Sensitivity (%)	98.6	98.4	100.0	96.8	100.0	100.0	98.2	96.8
Specificity (%)	15.6	0.0	0.0	75.0	12.8	0.0	0.0	75.0
Accuracy (%)	72.8	60.2	68.9	88.3	60.2	54.4	53.4	88.3
Precision (%)	72.2	60.8	68.9	85.9	57.7	54.4	53.9	85.9

AAP, American Academy of Periodontology; AIC, Akaike Information Criterion; AUC, Area Under the Curve; CDC, Centers for Disease Control and Prevention; P, Periodontitis; SP, Severe Periodontitis.

4. Discussion

In this study, the validity of a 13-item self-reported questionnaire for periodontitis, translated to Portuguese, was assessed. We tested the performance of this set of questions in predicting cases of both periodontitis and severe periodontitis in two accepted case definitions. This questionnaire included eight items of the original CDC/AAP questionnaire [8] and five items from other similar studies [13,17]. Our results show that the predictive values of the reduced models were 86% in both the EFP/AAP 2018 and the CDC/AAP 2012 case definitions, while for severe periodontitis the reduced models ranged between 71% and 80% for the EFP/AAP 2018 and the CDC/AAP 2012, respectively. In line with previous studies using similar methodology, these results may provide potential for periodontal surveillance and screening, but might not be useful for etiological studies [12].

The self-report questionnaire for periodontitis not only is a relevant alternative strategy to the clinical diagnosis, with good validity and a reliability to screen individuals with periodontitis, but it also requires fewer resources [8,11,23]. This method enables large scale epidemiological studies and facilitates the challenging diagnosis of periodontitis at the populational level [17]. In fact, self-report as a tool has been previously validated in other medical contexts as well [24–26]. Therefore, we believe this questionnaire is a particularly important tool for tracking and managing public health measures for oral health, particularly in Portugal.

In the Portuguese context, oral health care is mostly centralized in private practices using an out-of-pocket system and dental services are generally lacking in the National Health Service [27]. This limits the population's access to professional dental services and is felt more keenly by deprived socioeconomic and disadvantaged people. Nonetheless, a system of “dental vouchers” was created in 2008 to promote free oral health care in target popu-

lations (children, adolescents, and vulnerable groups such as pregnant women, patients with Human Immunodeficiency Virus, and elders with low-socioeconomic status) [28]. This panorama might explain the high prevalence of periodontal disease in the Portuguese population, as reported recently by Botelho et al. [19], and previously [29], although the latter did not follow the gold standard full mouth periodontal examination, compromising the sensibility and specificity of the results [30]. The burden of periodontal disease in Portugal supports the need for a comprehensive national oral program, the implementation of simple but accurate screening tools for oral diseases (such as the self-report method) and the promotion of awareness campaigns for oral health.

Our results using multivariable prediction models, including the self-reported perception of gum disease and previous gum treatment with the number of teeth lost characteristics, performed well in predicting periodontitis cases (AUC = 0.86), for both periodontal classifications. Specifically in severe periodontitis, the gum disease and lost bone self-reported information had the highest predictive ability (AUC = 0.71 and 0.80 for EFP/AAP 2018 and CDC/AAP 2012, respectively). Comparing our findings with previous studies, the multivariable prediction model for severe periodontitis had an AUC of 0.83 [8], 0.82 [14], 0.75 [12], 0.86 [13], 0.82 [16] in the American (NHANES), French, Dutch and Spanish (both the Di@bet.es Study participants and in the Catalan adult subjects) populations, respectively. The slightly different predictive capacity could be attributed to population characteristics or periodontal case definition. For example, Carra et al. [14] used a periodontal screening score while Iwasaki et al. [17] used the CDC/AAP 2012 periodontal case definition.

From a surveillance point of view, the abundance of new strategies and tools seems to guide us towards a possible combination of technological strategies that will be very powerful in the preventive diagnosis of periodontitis. Combined with self-report questionnaires that are applicable in any setting (i.e., at any sort of health service or even at home), we will be able to create tree health and risk assessments along with 1.0 and 2.0 software tools [31,32]. We foresee that a set of interactions with patients may be carried out prior to any clinical diagnosis, based only on self-report and complementary diagnostic tests (radiographs, blood and/or salivary analyses), by using artificial intelligence empowered strategies [33]. This set of questions could constitute a real evolution in periodontal diagnosis and treatment towards a holistic, more personalized process, and one more focused on the individual needs of patients. In addition, it will allow a closer monitoring of patients treated and under maintenance programs, strengthening the prevention of periodontitis recurrence and its risk factors.

Strengths and Limitations

The translation to Portuguese was performed by experts, following a rigorous but clear linguistic strategy, in order to keep the Portuguese version an equivalent measure of the original English questionnaire, while maintaining good content-related validity. This sample was a consecutive pool of newly incoming patients at the university dental clinic, although its location may have contributed to limit the generalizability of our results to the entire Portuguese population. In addition, we have employed widely used periodontitis case definitions and its severity staging, enhancing the importance of such results, and empowering its clinical validity in future screening/surveillance.

Furthermore, the accuracy and heterogeneity of results are influenced by other determinants that are intrinsically related to the populations studied, such as health literacy, access, socioeconomic status, or awareness of dental care services [12].

5. Conclusions

Self-report measures of periodontitis combined with risk factors showed predictive validity towards periodontitis and its severe form in the Portuguese population, using the 2018 and 2012 case definitions.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/jpm12081315/s1>, Supplementary Table S1. Responses to questions stratified by periodontitis status according to the EFP/AAP 2018 and CDC/AAP 2012 case definitions and respective severe staging.

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CHAPTER 6

6. Non-Surgical Periodontal Therapy's Influence on Alpha-Synuclein and Inflammatory Marker Levels: A Pilot Study

This chapter was based on the published work:

Paper IV – Non-Surgical Periodontal Therapy's Influence on Alpha-Synuclein and Inflammatory Marker Levels: A Pilot Study

In fulfilment of the first aim of this project – to assess baseline salivary and blood levels of inflammatory markers of interest (IL-6 and IL1- β) and α -Syn according to the periodontal status; also, to deliver periodontal therapy in periodontitis patients and analyse these salivary and blood markers of interest up to 30 days after treatment – an original research was conducted and successfully published in the Journal of Clinical Medicine (June 19, 2024).

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Article

Non-Surgical Periodontal Therapy's Influence on Alpha-Synuclein and Inflammatory Marker Levels: A Pilot Study

Patrícia Lyra ¹, João Botelho ¹, Silvia Rota ^{2,3}, Karolina Poplawska-Domaszewicz ^{3,4}, Vanessa Machado ¹, Daniela Guerreiro ¹, Luís Proença ¹, Helena Barroso ¹, José João Mendes ^{1,*} and Kallol Ray Chaudhuri ^{2,3}

- ¹ Egas Moniz Center for Interdisciplinary Research, Egas Moniz School of Health and Science, 2829-511 Almada, Portugal; plyra@egasmoniz.edu.pt (P.L.); jbotelho@egasmoniz.edu.pt (J.B.); vmachado@egasmoniz.edu.pt (V.M.); dguerreiro@egasmoniz.edu.pt (D.G.); lproenca@egasmoniz.edu.pt (L.P.); mhbarroso@egasmoniz.edu.pt (H.B.)
 - ² Department of Basic & Clinical Neuroscience, Institute of Psychiatry, Psychology & Neuroscience, King's College London, London WC2R 2LS, UK; silvia.rota@kcl.ac.uk (S.R.); ray.chaudhuri@kcl.ac.uk (K.R.C.)
 - ³ Parkinson's Foundation Center of Excellence, King's College Hospital, London SE5 9RS, UK; karolina.poplawska@usk.poznan.pl
 - ⁴ Department of Neurology, Poznan University of Medical Sciences, 60-355 Poznan, Poland
- * Correspondence: jmendes@egasmoniz.edu.pt



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Abstract: Background: Microbial dysbiosis may contribute to alpha-synuclein (α -Syn) homeostasis disruption, yet the burden of inflammatory periodontal infection and its treatment have never been studied in this regard. We aimed to compare the cytokine and α -Syn levels in the saliva and blood of patients with periodontitis who underwent non-surgical periodontal therapy (NSPT) and those of their healthy counterparts. **Methods:** Periodontal examination and saliva and blood sample collection were carried out in incoming patients at a university clinic. The periodontitis group (PG) received NSPT. The sample collection and periodontal observation were repeated 30 days after. IL-6, IL-1 β and total α -Syn were quantified using immunoassay methods. The periodontal inflamed surface area (PISA) was calculated as a proxy for periodontal inflammation. **Results:** Eleven participants formed the PG, and there were fifteen healthy controls (HC). At baseline, no correlation between salivary and plasma α -Syn was found. The salivary α -Syn levels revealed a tendency to decrease 30 days after, particularly in the PD cases. The variation in PISA and α -Syn showed significant correlation. Salivary α -Syn correlated negatively with salivary IL-6 levels at both timepoints in the total sample ($\rho = -0.394$ and $\rho = -0.451$) and in the HC ($\rho = -0.632$ and $\rho = -0.561$). Variations in plasma IL-6 and α -Syn were negatively correlated ($\rho = -0.518$) in the healthy participants. Baseline plasma IL-1 β negatively correlated with plasmatic α -Syn at 30 days in the HC ($\rho = -0.581$). **Conclusions:** Salivary and plasma α -Syn bioavailability operate independently, and periodontal diagnosis was not a confounding factor. Salivary α -Syn levels were significantly affected by NSPT, contrary to plasma levels. These results should be confirmed in future larger and prospective studies.

Keywords: alpha-synuclein; cytokines; periodontitis; periodontal disease; oral health; periodontal treatment; inflammatory burden

1. Introduction

Alpha-synuclein (α -Syn) is a short-sized protein involved in synaptic vesicle biology and neurotransmitter traffic [1], also found in blood and immune cells [2,3]. In α -Syn homeostasis disruption, toxic species form through protein aggregation into oligomers or protofibrils [4], spreading throughout the central nervous system (CNS), which might cause neurodegeneration [5,6]. Extracellularly, α -Syn can be found in body fluids or peripheral tissues, such as cerebrospinal fluid (CSF), saliva, serum, plasma, urine, tears, the salivary glands and various organs of the gastrointestinal tract [2,7–11]. Therefore, it has

been reported as a promising biomarker in Parkinson's disease (PD), the most common synucleinopathy [12]. The reason it accumulates is not fully known but is thought to be multifactorial, including aging, environmental influences (such as the dysbiosis of the gut commensal microflora), exposure to toxins and genetic mutations [13–15].

A significant source of inflammation is the globally prevalent periodontitis, an integral part of the gastrointestinal tract [16,17]. This condition affects the supporting tissues of the teeth due to the accumulation of dental biofilm, imbalanced microbiota and an immunological response [18]. If untreated, periodontal bacteria and their products spread and cause inflammation in extra-oral sites or the spillover of local inflammatory mediators from periodontal tissues into the bloodstream [19]. This triggers an inflammatory cascade, including high levels of IL-1, IL-6, C-reactive protein (CRP), TNF- α , fibrinogen and circulating neutrophils [19,20]. In addition to this low-grade inflammation, the resulting bacteremia is accompanied by toxic products, such as gingipains (from *Porphyromonas gingivalis*), which have been implicated in the pathology of several diseases, namely breast and colon cancer and rheumatoid arthritis, and in the brains of Alzheimer's and PD patients [19,21–23].

We recently reviewed how microbial-dysbiosis-related inflammation may play a role in α -Syn pathways [3]. Mice exposed to lipopolysaccharide (LPS) exhibited α -Syn adopting a fibrillar form in the brain tissue, with cognitive deficits and enhanced dopaminergic neuronal loss, suggesting LPS-induced neuroinflammation and the periodontitis-related genetic background interact synergically [24,25]. Therefore, disruptions in commensal microbiota may be a driving force in α -synucleinopathies [15,26], as firmly established within the microbiota–gut–brain axis [26,27]. Gingipains, for example, have been shown to accumulate in dopaminergic neurons of the substantia nigra in humans, specifically interacting with α -Syn [28]. Nevertheless, research focuses on diagnosed neurodegeneration, but it is still unclear how the inflammatory burden of periodontal infection and its treatment impact synuclein homeostasis. This is relevant because it may unveil clues on pathogenesis and synergistic interactions, as α -Syn is a protein with physiological functions, novel insights into the gut–brain axis and even a role as a biomarker of periodontal health.

With this study, we aimed to assess the baseline salivary and blood levels of inflammatory markers of interest (IL-6 and IL1- β) and α -Syn in a case–control study. Additionally, we delivered non-surgical periodontal therapy (NSPT) in periodontitis patients and compared the salivary and blood surrogate variation at a 30-day follow-up with their healthy counterparts.

2. Materials and Methods

2.1. Study Setting, Eligibility Criteria and Sample Size

This study was a case–control study adjusted for sex at a 1:1 ratio. Incoming patients observed at the Egas Moniz Dental Clinic (EMDC) were included in consecutive sampling according to their periodontal status.

To be included, adult patients had to sign informed consent. The exclusion criteria were as follows: NSPT performed in the last year; antibiotic therapy, corticosteroids and immunosuppressive agents in the last 3 months; pregnant or lactating women; non-periodontal active inflammatory processes of the oral cavity.

The study was conducted in compliance with the relevant laws and institutional guidelines and was approved in January 2022 by the Egas Moniz Ethics Committee (Institutional Review Board, protocol 824). All the participants gave informed written consent to the study procedures, and the study was carried out in observance of the Declaration of Helsinki, as revised in 2013. This study is reported as per the STrengthening the Reporting of OBservational studies in Epidemiology (STROBE) guidelines (Supplementary Table S13) [29].

Considering the lack of studies comparing the impact of NSPT on α -Syn to provide a basis for sample size calculation, we conducted a preliminary pilot study with a sample

size of 15 patients per group. This sample size was based on previous studies with a similar design [30,31].

2.2. Demographics Characteristics

Data were collected through a self-reported questionnaire on sociodemographic and behavioral aspects, including age, sex, educational level, marital status, smoking habits, oral care and hygiene-related behaviors (last dental visit, toothbrushing frequency, toothbrush type and interproximal cleaning) and diabetes mellitus (DM).

We categorically registered the education levels according to the 2011 International Standard Classification of Education (ISCED-2011) [32]: elementary (ISCED 1–2 levels), middle (ISCED 3–4 levels), higher (ISCED 5–8 levels). Marital status was categorized as Single, Married/Living with Partner or Divorced/Separated/Widowed.

Smoking status was defined as non-smoker (category 0), former smoker (category 1) or active smoker (category 2), following a previous methodology [21]. Current smokers were those who had smoked ≥ 100 cigarettes during their lifetime and were still active smokers. Former smokers were defined as those who had smoked ≥ 100 cigarettes during their lifetime but had stopped. Those who had smoked < 100 cigarettes during their lifetimes were categorized as never smokers.

DM cases were based on the patients' auto-reports and then confirmed by medication regimens.

2.3. Periodontal Clinical Examination and Diagnosis

A full-mouth periodontal examination was performed at EMDC and during the 30-day follow-up consultations post-NSPT. All fully erupted teeth were examined using a manual periodontal North Carolina probe (Hu-Friedy; Chicago, IL, USA), and the number of missing teeth was recorded. Pocket depth (PD) was measured as the distance from the free gingival margin to the bottom of the pocket. Recessions (REC) represented the distance from the cemento-enamel junction (CEJ) to the free gingival margin (a negative sign was assigned if the gingival margin was located coronally to the CEJ). Interdental clinical attachment loss (CAL) was calculated as the algebraic sum of the REC and PD measurements for each site. All the measurements were rounded to the lowest whole millimeter. Furcation involvement (FI) was assessed using a 2N probe (Hu-Friedy; Chicago, IL, USA), and tooth mobility was also appraised.

A periodontitis case implies interdental CAL ≥ 2 mm of non-adjacent teeth or buccal or oral CAL ≥ 3 mm, with PD > 3 mm detectable at ≥ 2 teeth [33]. The periodontitis staging was defined according to severity and extent. Regarding severity, it was categorized as mild (stage 1) if interdental CAL at the site of the greatest loss was 1–2 mm, moderate (stage 2) if it was 3–4 mm and severe (stage 3 and stage 4) if it was ≥ 5 mm. Additionally, the extent was described as localized ($< 30\%$ of teeth involved), generalized ($\geq 30\%$ of teeth involved) or a molar/incisor pattern.

The periodontal inflamed surface area (PISA) was calculated as a proxy for periodontal inflammation. For each tooth, we computed the PISA as a continuous variable. The PISA comprised the surface area of the bleeding pocket epithelium [34,35].

2.4. Saliva Collection Protocol

Unstimulated saliva collection took place before a periodontal diagnosis at the EMDC and before clinical observation during the 30-day follow-up consultations post-NSPT. The minimum collection volume of unstimulated saliva was 5 mL [36].

Following saliva collection, the samples were stored at -80°C . Upon analysis, after thawing, the samples were centrifuged at $9.300\times g$ for 5 min, and the supernatants were divided into 0.5 mL aliquots to be analyzed.

2.5. Blood Collection Protocol

The blood sample collection took place before a periodontal diagnosis at the EMDC and before clinical observation during the 30-day follow-up consultations post-NSPT. Blood collection of 2.7 mL of venous blood was performed from the antecubital fossa using venipuncture, using a Safety-Multifly® 21G needle with an 80 mm tube (SARSTEDT REF 85.1638.205) and Ethylenediaminetetraacetic acid-treated tubes (S-Monovette® 2.7 mL K3E, 1.6 mg EDTA/mL) (SARSTEDT REF 05.1167.001) [7].

Following blood collection, the plasma samples underwent refrigerated centrifugation for 15 min at $1.500 \times g$. The resulting plasma supernatants were transferred into clean and low-residue polypropylene tubes. The samples were stored at -80°C before examination.

2.6. Periodontal Treatment

Periodontitis cases underwent NSPT, performed in one or two sessions, according to the severity and extent of periodontitis. Subgingival instrumentation was performed through debridement, scaling and root planing. No adjuvant chemical therapy was prescribed. Patients requiring additional periodontal (surgical) treatment and re-evaluations were redirected to the periodontology department of the EMDC for continued treatment [37].

2.7. Salivary and Blood Circulating Markers

Quantification of the salivary and blood levels of total α -synuclein was performed using the Human SNC α (Synuclein Alpha) ELISA Kit (REF E-EL-H0983-96T; Elabscience, Houston, TX, USA). As this study aims to investigate the physiological α -Syn levels in a population without a neurological diagnosis, we opted to quantify the total α -Syn levels, as oligomeric forms are mostly associated with PD and we did not want to discard the monomeric protein forms [10]. The levels of the inflammatory markers of interest (IL-1 β and IL-6) were also measured by means of immunoassay kits—the High Sensitive ELISA Kit for Interleukin 6 (IL6) (REF HEA079Hu-96T; Cloud-Clone Corp., Houston, TX, USA) and the Human IL-1 β (Interleukin 1 Beta) ELISA Kit (REF E-EL-H0149-96T; Elabscience, Houston, TX, USA). The assays were carried out in accordance with the manufacturer's instructions.

2.8. Data Management and Statistical Analysis

Data analyses were performed using IBM SPSS Statistics v.29. The data were submitted for descriptive and inferential analyses. The association between categorical variables was checked using appropriate inferential statistical methodologies (chi-squared and Fisher's exact test). Inferential comparison of the biomarker levels between groups was performed using Student's *t*-test (data's adequacy to normality was previously checked). The correlation between the inflammatory marker levels was assessed using Spearman's rank correlation coefficient. A 5% significance level ($p \leq 0.05$) was established in the inferential analyses.

3. Results

3.1. Participants/Sample Description/Population

A total of 54 new patients at the EMDC were invited to participate in the study (Figure 1), and 24 participants were excluded either because they refused to participate or did not meet the eligibility criteria or due to difficulties in sample collection. Thus, 30 participants were initially enrolled; however, 4 patients were lost mid-study and did not complete the programmed follow-up consultation. After clinical periodontal diagnosis, 11 patients formed the PG, while 15 formed the HC. Within the PG, there were five cases of stage II periodontitis (two of grade A and three of grade B), five cases of stage III periodontitis (two of grade A and three of grade B) and one case of stage IV periodontitis (grade C).

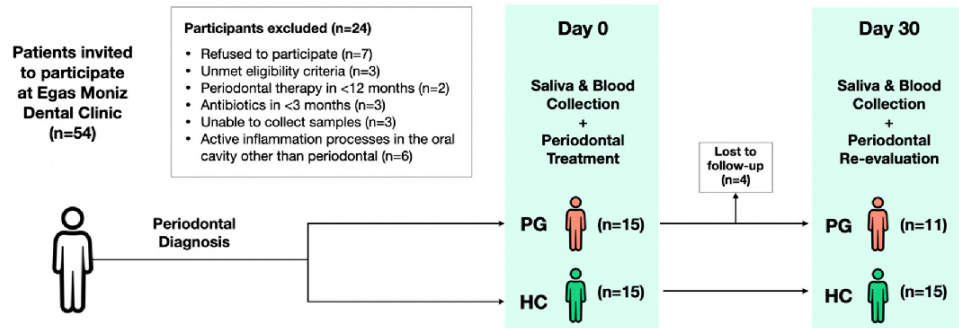


Figure 1. Flowchart of participants. Abbreviations: n, number of participants; PG, periodontitis group; HC, healthy controls.

The participants' sociodemographic and oral health behavior data are detailed in Table 1. The final sample presented a majority of women (53.8%). The periodontitis participants were significantly older than their healthy counterparts ($p = 0.024$). Most of the participants reported a middle educational level (57.7%) and being married/living with a partner (46.2%), and half reported non-smoking habits (50.0%). Regarding oral care habits, 61.5% of the participants reported their last dental visit within the last six months, brushing their teeth twice or more a day (96.2%) and mostly using a manual toothbrush (84.6%). The interproximal cleaning habits significantly differed between the case group and the HC, with the PG having a lower proportion of reported interproximal hygiene habits ($p = 0.005$). Only three patients (11.5% from total) reported a diagnosis of diabetes mellitus.

Table 1. Participants' sociodemographic and oral health behavioral data.

Variable	Total (n = 26)	HC (n = 15)	PG (n = 11)	p-Value *
Age, mean (SD) (Min–Max)	50.1 (17.8) (19–77)	43.5 (18.6) (19–70)	59.1 (12.3) (40–77)	0.024 *
Sex, % (n)				0.391
Female	53.8 (14)	46.7 (7)	63.6 (7)	
Male	46.2 (12)	53.3 (8)	36.4 (4)	
Educational Level, % (n)				-
Elementary	15.4 (4)	13.3 (2)	18.2 (2)	
Middle	57.7 (15)	60.0 (9)	54.5 (6)	
Higher	26.9 (7)	26.7 (4)	27.3 (3)	
Marital Status, % (n)				-
Single	34.6 (9)	40.0 (6)	27.3 (3)	
Married/Living with Partner	46.2 (12)	53.3 (8)	36.4 (4)	
Divorced/Separated/Widowed	19.2 (5)	6.7 (1)	36.4 (4)	
Smoking Habits, % (n)				-
Non-Smoker	50.0 (13)	53.3 (8)	45.5 (5)	
Former Smoker	26.9 (7)	26.7 (4)	27.3 (3)	
Active Smoker	23.1 (6)	20.0 (3)	27.3 (3)	
Last Dental Visit, % (n)				-
<6 Months	61.5 (16)	66.7 (10)	54.5 (6)	
6–12 Months	15.4 (4)	13.3 (2)	18.2 (2)	
>12 Months	23.1 (6)	20.0 (3)	27.3 (3)	
Toothbrushing, % (n)				-
Once a Day	3.8 (1)	0.0 (0)	9.1 (1)	
Twice or More a Day	96.2 (25)	100.0 (15)	90.9 (10)	
Toothbrush Type, % (n)				0.279
Manual	84.6 (22)	93.3 (14)	72.7 (8)	
Electric	15.4 (4)	6.7 (1)	27.3 (3)	

Table 1. Cont.

Variable	Total (n = 26)	HC (n = 15)	PG (n = 11)	p-Value *
Interproximal Cleaning, % (n)				
No	76.9 (20)	73.3 (11)	81.8 (9)	0.005 *
Often/Yes	23.1 (6)	26.7 (4)	18.2 (2)	
Diabetes Mellitus, % (n)				
Yes	11.5 (3)	0.0 (0)	27.3 (3)	-
No	88.5 (23)	100.0 (15)	72.7 (8)	

* Chi-square/Fisher's exact test, except for "age", where Student's *t*-test was used. Significant *p*-values (*p* < 0.05) denoted with an asterisk (*). Abbreviations: SD, standard deviation; *n*, number of participants; Min, minimum; Max, maximum.

3.2. Plasma and Saliva Levels before and after Treatment

Our results seem to indicate no dependent association between the saliva and plasma α -Syn levels at baseline (Figure 2). Furthermore, throughout the time course of the study, no significant variations were seen in plasma α -Syn levels in either group (Figure 3). However, salivary α -Syn levels revealed a decrease by the 30-day mark, especially in the PG, not only in the mean levels but also in a shortening in the distribution of values (Figure 3). Accordingly, following periodontal treatment, the higher the reduction in the periodontal inflamed surface area (PISA), the more likely a higher negative variation in α -Syn levels was compared to the baseline (Figure 4).

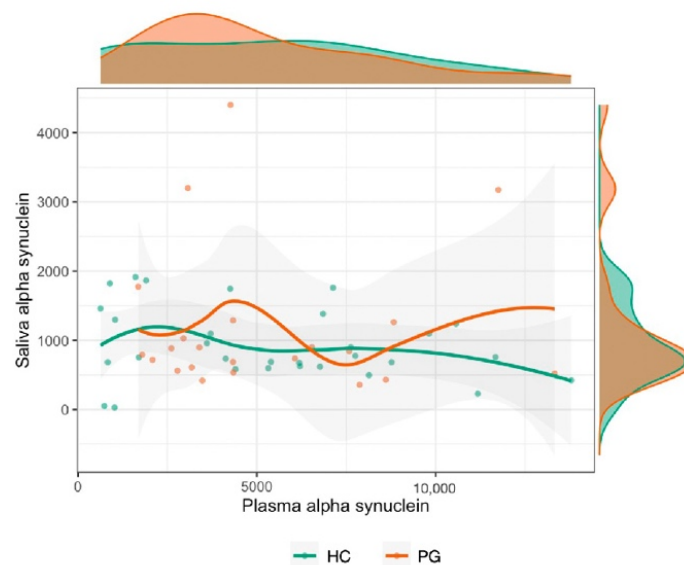


Figure 2. Relationship between baseline blood and salivary α -synuclein levels with respective distribution. The flattened, almost linear plot inside the graph seems to indicate no relationship between saliva and plasma α -Syn levels at baseline. Outside the plot, the distribution of baseline α -Syn plasma levels in periodontal cases is mostly concentrated to the left (top section), and baseline salivary levels are mostly concentrated to the right (lateral section). Abbreviations: PG, periodontitis group; HC, healthy controls.

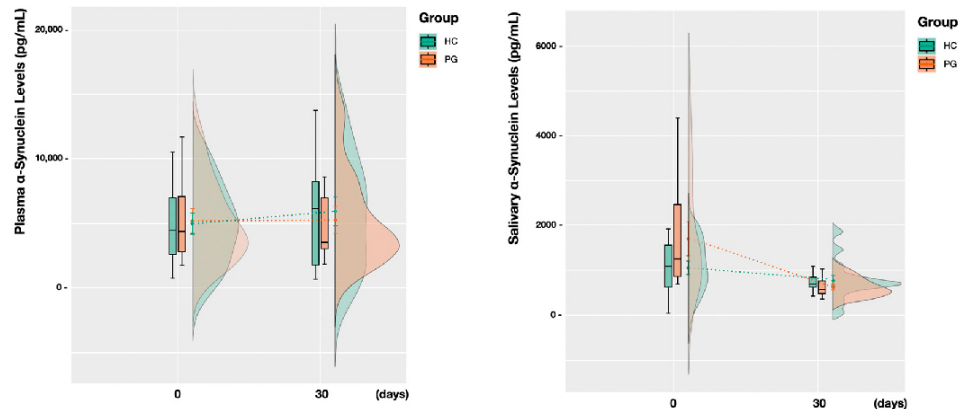


Figure 3. Plasma and salivary α -synuclein levels at baseline and follow-up. Regarding plasma α -Syn levels, both the average values and distribution of values at baseline and at the 30-day follow-up remained very similar between groups. However, salivary α -Syn levels revealed a decrease in mean levels (closer to the HC) and a shortening in the distribution of values. Abbreviations: PG, periodontitis group; HC, healthy controls.

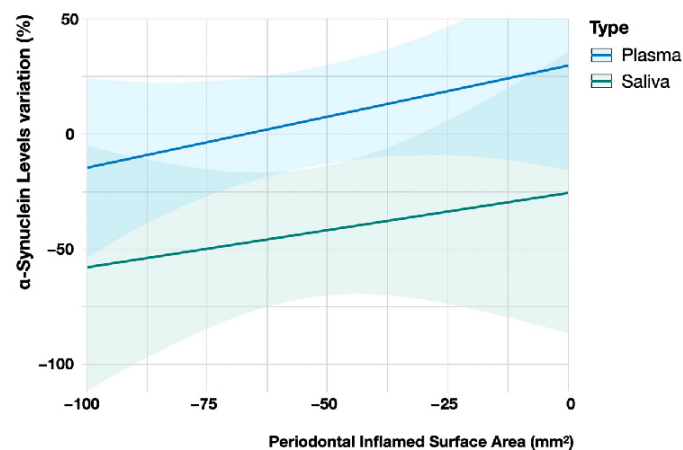


Figure 4. Salivary and plasma alpha-synuclein variation vs. periodontal inflamed surface area variation after periodontal therapy. Comparing baseline with follow-up values, we observed a higher negative α -Syn variation in saliva than in plasma (Figure 3). The higher the reduction in periodontal inflamed surface area, the more likely finding a higher negative variation in α -synuclein levels compared to the baseline was (as was previously supported in Figure 3). When the reduction in PISA was complete (that is, equal to -100%), a greater effect on α -Syn was noticed.

We found no differences in the α -Syn levels in either group at baseline or follow-up for either saliva or blood (Table 2). Regarding the IL-6 analysis, no differences were found in the mean salivary levels of IL-6 between groups at baseline or follow-up. However, significant differences between the PG and HC were found regarding the IL-6 plasma levels, indicating that the PG presented higher mean IL-6 values at baseline ($p = 0.008$) and follow-up ($p = 0.034$). When analyzing the time-dependent behavior (from T0 to T30), a significant difference was found for the IL1- β salivary level mean difference ($p = 0.012$) when comparing both groups. This was reflected by an increase in the PG and a decrease for the HC.

Table 2. Biomarker levels (plasma and salivary) before (T0) and after treatment (T30).

Variable	Total (n = 26)	HC (n = 15)	PG (n = 11)	p-Value *
α-synuclein (pg/mL), mean (SD)				
Salivary, T0	1365.1 (964.4)	1090.4 (566.3)	1739.6 (1268.3)	0.090
Plasma, T0	5067.2 (3091.9)	4977.9 (3155.6)	5189.1 (3151.1)	0.867
Salivary, T30	711.0 (369.6)	772.9 (449.3)	626.7 (212.9)	0.329
Plasma, T30	5643.1 (3883.3)	5929.5 (4267.5)	5252.6 (3451.5)	0.670
Salivary, (Diff., T30-T0)	−654.0 (1021.6)	−317.5 (452.1)	−1112.8 (1386.3)	0.093
Plasma, (Diff., T30-T0)	575.9 (1948.8)	951.6 (1844.3)	63.6 (2057.3)	0.259
IL-6 (pg/mL), mean (SD)				
Salivary, T0	6.0 (7.8)	4.4 (6.2)	8.1 (9.4)	0.240
Plasma, T0	6.3 (3.7)	4.7 (3.8)	8.4 (2.2)	0.008 *
Salivary, T30	6.4 (7.8)	6.2 (9.2)	6.6 (5.6)	0.908
Plasma, T30	4.7 (3.0)	3.6 (3.0)	6.1 (2.5)	0.034 *
Salivary, (Diff., T30-T0)	0.4 (7.2)	1.8 (8.4)	−1.5 (5.1)	0.256
Plasma, (Diff., T30-T0)	−1.6 (2.0)	−1.1 (1.7)	−2.3 (2.4)	0.137
IL1-β (pg/mL), mean (SD)				
Salivary, T0	462.3 (318.4)	502.2 (358.4)	407.8 (260.7)	0.466
Plasma, T0	162.6 (80.5)	151.2 (89.5)	178.0 (67.2)	0.412
Salivary, T30	468.0 (385.4)	421.3 (398.6)	531.6 (375.8)	0.482
Plasma, T30	84.4 (52.0)	77.6 (45.9)	93.7 (60.3)	0.447
Salivary, (Diff., T30-T0)	−5.7 (212.1)	−80.9 (169.3)	123.8 (213.8)	0.012 *
Plasma, (Diff., T30-T0)	−78.2 (52.3)	−73.6 (51.3)	−84.4 (55.7)	0.614

* Student's *t*-test. Significant *p*-values (*p* < 0.05) denoted with an asterisk (*). Abbreviations: SD, standard deviation; *n*, number of participants; Diff, difference; T0, baseline; T30, follow-up; α-Syn, alpha-synuclein; IL-6, interleukin 6; IL1-β, interleukin 1-β.

3.3. Correlation Analysis of Markers

Correlations between the salivary and plasma markers both in the overall sample and according to periodontal status are detailed in the Supplementary File. Salivary α-Syn showed a significant negative correlation with salivary IL-6 in the overall sample ($\rho = -0.394$ and $\rho = -0.451$) and in the HC ($\rho = -0.632$ and $\rho = -0.561$), both at baseline and 30 days after NSPT (Supplementary Tables S1, S2, S7 and S8). Furthermore, salivary IL-6 showed a significant negative correlation with salivary IL1-β in the overall sample ($\rho = -0.755$ and $\rho = -0.535$) and in both groups—HC ($\rho = -0.718$ and $\rho = -0.518$) and PG ($\rho = -0.664$ and $\rho = -0.818$)—at baseline and 30 days after NSPT, respectively (Supplementary Tables S1, S2, S4, S5, S7 and S8). There was also a significant negative correlation between IL-6 plasma levels and IL1-β plasma levels ($\rho = -0.643$) and α-Syn plasma levels ($\rho = -0.518$) in the HC (Supplementary Table S9). When analyzing the marker levels at baseline versus at 30-day follow-up, our results show a significant negative correlation between salivary α-Syn at baseline and salivary IL-6 at 30-day follow-up ($\rho = -0.492$) and vice versa ($\rho = -0.562$ **) in the total sample (Supplementary Table S10), as well as in the HC ($\rho = -0.729$ and $\rho = -0.657$) (Supplementary Table S12). In addition, salivary IL-6 at baseline significantly negative correlates with salivary IL1-β at the 30-day follow-up in the overall sample ($\rho = -0.478$ and $\rho = -0.675$) and in both the PG ($\rho = -0.664$ and $\rho = -0.764$) and the HC ($\rho = -0.518$ and $\rho = -0.532$) (Supplementary Tables S10–S12). Finally, plasmatic IL1-β at baseline shows a significant negative correlation with plasmatic α-Syn at 30-day follow-up in the HC ($\rho = -0.581$) (Supplementary Table S12).

4. Discussion

The salivary and blood levels of α-Syn and inflammatory markers of interest (IL-6 and IL1-β) were assessed according to periodontal status at baseline and 30 days following NSPT for periodontitis cases. To the best of our knowledge, this is the first time the monthly variation in α-Syn levels has been described in a group of patients with periodontitis. To

date, there has been no previous mention of the monthly variation in α -Syn in a population without any kind of neurological diagnosis, and therefore this is also a novel report regarding the HC.

Our results show no linear association between the saliva and plasma α -Syn levels at baseline (Figure 2), indicating that the bioavailability of both markers seems to function independently. Also, the similar behavioral tendency of both groups at baseline seems to indicate that a periodontal diagnosis does not influence the overall variations in the α -Syn plasma and salivary levels.

When it comes to analyzing the effects of NSPT on α -Syn levels, our results show that throughout the time course of the study, no significant variations were seen in the plasma α -Syn levels (Figure 3). However, the salivary α -Syn levels revealed a decrease by the 30-day mark, especially in the PG, not only in the mean levels but also in a shortening in the distribution of values (Figure 4). In other words, comparing the baseline with the follow-up values, we observed a higher negative α -Syn variation in the saliva than in the plasma after periodontal treatment. In fact, the higher the reduction in the PISA, the more likely we were to find a higher negative variation in the α -Syn levels compared to the baseline (Figure 4). Ultimately, when the reduction in the PISA was complete, a greater effect on salivary α -Syn was noticed.

Therefore, NSPT in the periodontitis cases seem to be a confounding factor in the evaluation of α -Syn salivary levels. This shows the clinical implications of our results, which present NSPT as a confounding factor in the medical diagnosis using biomarkers, specifically salivary α -Syn. In fact, recent evidence points to salivary α -Syn as a diagnostic biomarker in α -synucleinopathies, and this is a subject of increasing interest nowadays [8–10]. These results indicate that recent NSPT seems to have a local effect in the oral cavity and must be taken into consideration by health professionals when analyzing α -Syn salivary levels for diagnosis purposes. NSPT with scaling may possibly lead to some local inflammation, which may have implications for salivary α -Syn values.

The characteristics of our periodontitis group align with the literature, as the periodontitis patients were significantly older than their healthy counterparts ($p = 0.024$) and performed lower levels of interproximal hygiene ($p = 0.005$) [38,39]. Also, the periodontitis group presented significantly higher baseline ($p = 0.008$) and follow-up ($p = 0.034$) IL-6 plasma values, corroborating with numerous studies reporting that markers of inflammatory response are elevated in subjects suffering from periodontitis, as periodontitis is an inflammatory disease that awakes the immune response with a cascade of inflammatory markers that are not limited to the diseased sites [40]. Furthermore, a significant difference was found for the IL-1 β salivary level mean difference ($p = 0.012$) when comparing the overtime variation (from T0 to T30) in both groups. Our results showed an increase in the PG and a decrease in the HC. The literature supports that when analyzing a large number of blood inflammatory markers in response to periodontal treatment, great heterogeneity regarding the systemic response and inflammatory markers behavior has been reported [41]. Also, a recent study analyzing blood samples reported a variation in the number of peaks after periodontal treatment, with some of them increasing immediately after 1 month and decreasing again at 3 months and vice versa [42]. However, when it comes to saliva samples, a previous meta-analysis showed no statistically significant decrease or alterations in the levels of IL-1 β and other inflammatory markers after NSPT between treatment and healthy control groups [43], suggesting the need for cautious interpretation of the present results.

The small size of our sample is the main limitation of this study, and therefore the results should be interpreted with caution, as there are inherent biases in case-control studies. The correlations between salivary and plasma markers according to periodontal status must also be considered carefully due to the small sample size, although it reveals clues to be clarified in future studies. Also, other systemic inflammatory diseases may have been present at the same time. However, this case-control study had a 10-month inclusion period and followed the strict STROBE guidelines [29], as detailed in Supplementary

Table S13. Although this is not an ecological study per se, it observes the normal behavior of a group of people, and even the clinical intervention is a routine treatment for periodontitis.

With the increase in life expectancy over recent decades and the growing number of patients with synucleinopathies [12], it is crucial to gain a better understanding of the behavior of α -Syn and other inflammatory markers to develop novel clinical strategies to alter disease progression or even prevent disease onset.

5. Conclusions

Salivary and plasma α -Syn levels showed no association with periodontal diagnosis. NSPT seems to affect salivary α -Syn levels but not the plasma. NSPT may be a confounding factor when using salivary α -Syn as a biomarker. Future research should confirm these preliminary data using larger and longer prospective studies.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/jcm13123586/s1>. Table S1. Correlation between biomarker levels of the overall sample at baseline (T0); Table S2. Correlation between biomarker levels of the overall sample at the 30-day follow-up (T30); Table S3. Correlation between the variation (Δ) in biomarker levels of the overall sample (T30-T0); Table S4. Correlation between biomarker levels of the periodontitis group at baseline (T0); Table S5. Correlation between biomarker levels of the periodontitis group at 30-day follow-up (T30); Table S6. Correlation between the variation (Δ) in biomarker levels of the periodontitis group (T30-T0); Table S7. Correlation between biomarker levels of the healthy control group at baseline (T0); Table S8. Correlation between biomarker levels of the healthy control group at 30-day follow-up (T30); Table S9. Correlation between the variation (Δ) in biomarker levels of the healthy control group (T30-T0); Table S10. Correlation between biomarker levels of the overall sample at baseline (T0) and at 30-day follow-up (T30); Table S11. Correlation between biomarker levels of the periodontitis group at baseline (T0) and at 30-day follow-up (T30); Table S12. Correlation between biomarker levels of the control group at baseline (T0) and at 30-day follow-up (T30); Table S13. STROBE statement—checklist of items that should be included in reports of case-control studies.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Egas Moniz Ethics Committee (Process n°. 824, 17 January 2022).

Informed Consent Statement: Informed consent was obtained from all the subjects involved in the study.

Data Availability Statement: The original contributions presented in the study are included in the article/supplementary material; further inquiries can be directed to the corresponding author.

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CHAPTER 7

7. General Discussion, Concluding Remarks and Future Directions

7.1. General Discussion

Our main goal was to investigate the physiological behaviour of α -Syn according to the periodontal status and following periodontal treatment if needed. To our knowledge, this study describes the monthly variation of peripheral α -Syn levels in people living with and without periodontitis who have not been diagnosed with neurological disorders. With this we aimed to contribute to the understanding of the interplay between the periodontium homeostasis and possibly α -Syn-related conditions. Overall, salivary α -Syn levels were shown to be significantly influenced by NSPT, contrary to plasma levels. Additionally, salivary and plasma α -Syn bioavailability appear to operate independently, despite of periodontal diagnosis. Thus, when using salivary α -Syn as a biomarker in α -synucleinopathies diagnosis and management, NSPT may be a confounding factor at least if performed up to one month prior.

It was deemed imperative to initiate this research project by comprehensively reviewing the available evidence to date on α -Syn mechanistic pathways to inflammation, as well as the potential role of microbial dysbiosis and systemic inflammatory mitigation on α -Syn, as presented in Chapter 3 (**Paper I**). The project was subsequently designed with a two-fold aim, the first of which was regarded as the central study objective, only to be addressed upon the project's conclusion.

The secondary aim was answered beforehand with the publication of **Paper II** (Chapter 4), where periodontitis systemic burden was observed in PD patients, by comparing the levels of hs-CRP of self-reported periodontitis cases with periodontally healthy counterparts, amongst a PD population, using available data from the American database NHANES. To date, there is no such database available for the Portuguese context. We found that self-reported periodontitis is associated with higher circulating levels of hs-CRP in PD patients. Oddly, the group that self-reported no periodontitis presented higher lymphocyte levels, which could be due to the limitations of the self-reported strategy. Through this study, we were able to strengthen the existing hypothesis in which PD patients with periodontitis carried a systemic inflammatory burden, which can be translated into higher hs-CRP levels. In fact, our results align with previous studies who reported enhanced systemic inflammation in periodontitis patients,

with the elevation of peripheral levels of hs-CRP and IL-6 when compared to controls (Leira et al., 2020; Marcaccini et al., 2009; Noack et al., 2001; Vanessa Machado et al., 2021). In fact, hs-CRP is known to be triggered by acute and/or chronic inflammatory events (Windgassen et al., 2011) and has been shown to play a key role in the management of various inflammatory diseases besides periodontitis, namely neurodegenerative diseases like AD (Song et al., 2015) and PD (Ying-yi Luan & Yong-ming Yao, 2018). This study established a reasonable foundation to further investigate the systemic inflammatory burden associated with periodontitis and α -Syn levels in people otherwise free from neurological impairment, which we did subsequently in **Paper IV** (Chapter 6).

A self-reported strategy was employed for the selection of periodontitis cases in **Paper II** (Chapter 4), as it is a validated, efficient, and accepted surveillance method for periodontitis, presenting higher validity upon a combination of several self-report questions (Blicher et al., 2005; P. I. Eke et al., 2013; Heaton et al., 2017). This self-report strategy had never been validated for periodontitis in a Portuguese population, but was in other populations (Cyrino et al., 2011; Dietrich et al., 2007; Iwasaki et al., 2021; Montero et al., 2020; Saka-Herrán et al., 2019; Verhulst et al., 2019). The same approach was employed in other noncommunicable diseases, including the identification of cases of hypertension, diabetes mellitus, hypercholesterolemia (Martin et al., 2000), risk factors for cardiovascular disease (Bowlin et al., 1993) and even bruxism in PD patients (Verhoeff et al., 2021). Therefore, as the self-report strategy seems to be a promising cost-effective tool in epidemiological settings, particularly in cases where screening patients is key, such as but not restricted to the inclusion of patients for clinical studies. The success of **Paper IV** (Chapter 6), therefore, hinged on adopting the most effective strategies for patient identification. For this reason, we cross-culturally adapted and validated a self-reported strategy in predicting periodontitis prevalence to a Portuguese population on our third study, **Paper III** (Chapter 5). Our results showed that self-report measures of periodontitis combined with risk factors (such as smoking status and diabetes mellitus) reported predictive validity towards periodontitis and its severe form in the Portuguese population. In the evaluated set of questions, the self-reported perception of gum disease and previous gum treatment, along with the number

of teeth lost, performed well in predicting periodontitis cases for both periodontal classifications employed: the 2018 EFP/AAP classification (Tonetti et al., 2018a) and the 2012 CDC/AAP classification (Paul I. Eke et al., 2012). In particular, the self-reported information regarding the presence of gum disease and the loss of bone had the highest predictive ability in cases of severe periodontitis. These results reinforce the utility of this strategy for the surveillance of a prevalent and frequently undiagnosed disease such as periodontitis.

Finally, the first and main aim of this PhD project was met lastly, in **Paper IV** (Chapter 6), where baseline salivary and blood levels of α -Syn, IL-6 and IL1- β were analyzed according to the periodontal status and 30 days following standard periodontal therapy. We found no linear association between the saliva and plasma α -Syn levels at baseline, indicating that the bioavailability of both markers seems to function independently. Also, periodontal diagnosis does not seem to alter baseline α -Syn levels in both biological fluids. When analysing the effects of NSPT on α -Syn levels, our results show no significant variations in plasma, but salivary α -Syn levels showed a decrease at the 30-day of follow-up, especially in periodontitis patients. Previous studies have reported a decrease in total α -Syn levels in the saliva of PD patients, but not considering periodontal disease (Al-Nimer et al., 2014; Devic et al., 2011; Vivacqua et al., 2016). In fact, the reduction of the periodontal inflamed surface area (PISA) following periodontal treatment also showed greater variation of salivary α -Syn levels. These results present real clinical impact as NSPT performed in periodontitis patients seems to be a disrupting factor in the reliability of α -Syn salivary levels, often used as a biomarker for α -synucleinopathies (Bougea et al., 2019; Kang et al., 2016; Vivacqua et al., 2016). As such, this ought to be taken into consideration by health professionals. In regard to the evaluated cytokines, IL-6 plasma levels were higher in periodontitis patients both at baseline and follow-up, which is aligned with reports of local and peripheral elevated inflammatory markers in periodontitis patients (Marcaccini et al., 2009; X. J. Sun et al., 2009). When analysing the time-dependent behaviour of IL1- β (from baseline to the 30-day follow-up), its salivary mean difference reflected an increase in the periodontitis group and a decrease for the healthy controls. However, these results must be interpreted with caution, as there are reports of great heterogeneity regarding the systemic

response after periodontal treatment (Behle et al., 2009; J. Y. Kim & Kim, 2020; Zekeridou et al., 2019).

There are some limitations that are worthy of mention in relation to the various papers that make up this thesis. Regarding **Paper II** (Chapter 4), patients who self-reported periodontitis presented slightly lower lymphocyte percentage when compared to their healthy counterparts, even though the periodontitis-leukocytosis link is well established (Botelho, Lyra, et al., 2020; Botelho, Machado, Hussain, et al., 2021; Kumar et al., 2014). Perhaps such results would be more likely if a formal clinical diagnosis of periodontitis cases were performed. As further discussed in **Paper III** (Chapter 5), full-mouth PPD, CAL and BOP are the gold standard for periodontitis diagnosis (Tonetti et al., 2018a), which constitute a more reliable clinical diagnosis than the use of self-reported measures. However, even though these clinical measures are not complex to obtain, they are inherently time-consuming, require trained professionals, specialized equipment, and infection control strategies. Furthermore, self-reported strategies have been widely used and validated and allow for cost-effective large-scale monitoring of oral health (Blicher et al., 2005; P. I. Eke et al., 2013; Heaton et al., 2017). However, they remain fully reliant on the patient's knowledge of the disease and of previous clinical diagnosis, and disease severity and activity cannot be appraised. Also, the observational nature of the study in **Paper II** (Chapter 4) impairs the conclusion of causality, although results align with previous reports (Botelho, Machado, Hussain, et al., 2021). Similarly, even though the results of **Paper III** (Chapter 5) may provide potential for periodontal surveillance and screening, they might not be useful for etiological studies.

In both **Paper II** (Chapter 4) and **Paper IV** (Chapter 6), the sample size may be considered limited and thus a shortcoming. Regarding the first case, the small sample probably can be explained with the characteristically underdiagnosis of PD cases (Larsen et al., 1994; Litvan et al., 2003). Furthermore, due to study design constraints in **Paper IV** (Chapter 6), the need for a follow-up consultation 30 days after patient inclusion led to a significant drop-out rate, which highly affected sample size. Nevertheless, in both cases, the collected data remains of clinical significance. In the case of **Paper III** (Chapter 5), the sample consisted of a consecutive pool of newly incoming patients at the university dental clinic,

although geographic location and the reliant socio-economic context of the resident community may have contributed to limit the generalizability of our results to the entire Portuguese population. Besides socioeconomic status, health literacy, health access, or awareness of dental care services are determinants that also influence the accuracy and heterogeneity of results, being intrinsically related to the populations studied.

As a case-control study, **Paper IV** (Chapter 6) faces the inherent bias of this type of study, namely the possibility of co-existence of other un-reported systemic inflammatory diseases, which could potentially affect our findings regarding the biomarkers evaluated. Also, we initially planned on analyzing NLRP3 alongside IL-6 and IL1- β as inflammatory markers of interest, knowing its role on neuroinflammation and its interaction with α -Syn toxic species (Chatterjee et al., 2020; Fan et al., 2020), but were forced to abandon the quantification of salivary and blood NLRP3 levels due to laboratory limitations regarding enzyme-linked immunosorbent assay (ELISA) testing. Also, α -Syn measurements in biological fluids can be challenging due to variability of results in different studies (Ganguly et al., 2021). One possible cause could be attributed to the possible contamination of plasma samples with lysed red blood cells. Also, the variations could be attributed to the differences in quantifications protocol applied among studies. Furthermore, we know that α -Syn exists in various forms (monomeric, oligomeric and post translationally modified forms), which can add to the complexity of measurement of this protein, and that can also be seen as a limitation because it is not known how the quantification method in a particular assay can cross-react with other forms of α -Syn (Ganguly et al., 2021). In order to somehow diminish this possibility, we choose to evaluate total α -Syn through immunoassay methods.

Nevertheless, there are notable strengths among the conducted studies. When it comes to **Paper II** (Chapter 4), we tested the hypothesis that the concomitant presence of other systemic conditions might have interfered with the high levels of hs-CRP in PD patients, by comparing hs-CRP levels in PD patients with and without diabetes mellitus, hypertension, coronary heart disease, emphysema, asthma, hepatic conditions or cancer. Results indicated that other systemic diseases were not confounding factors to the elevated hs-CRP levels in PD

patients, neither was active tabagism. Also, in alignment with the conducted work in **Paper III** (Chapter 5), we strongly believe that despite the limitations of the self-report strategies, they are a great epidemiologic strategy to enable larger scale studies and low-cost surveillance of symptoms, risk factors and diseases, in this case facilitating the challenging diagnosis of periodontitis at the populational level (Blicher et al., 2005; P. I. Eke et al., 2013; Heaton et al., 2017; Iwasaki et al., 2021). Furthermore, we employed the two widely used periodontitis case definitions and its severity staging – the EFP/AAP 2018 case definition (Tonetti et al., 2018a) and the CDC/AAP 2012 (Paul I. Eke et al., 2012) – which strengthens the clinical validity of our results in future screenings.

Additionally, regarding **Paper IV** (Chapter 6), even though we ended up with a small sample size, the conducted case–control study had a 10-month inclusion period and followed the strict Strengthening The Reporting Of Observational Studies In Epidemiology (STROBE) guidelines (Von Elm et al., 2007). The easy accessibility of saliva and blood makes the sampled bodily fluids a great option for the monitorization of biomarker levels of disease for diagnostic purposes. As previously stated, the primary novelty of this study is that it is the first to describe the monthly variation of peripheral α -Syn levels in a group of periodontitis patients, as well as in a group of individuals without a diagnosis of neurological disorders. Despite not being an ecological study *per se*, it observes the normal behaviour of a group of individuals, and even the performed clinical intervention is within routine treatment for periodontitis.

7.2. Concluding Remarks

- **Paper I** – Microbiota and inflammation appear to play an important role in the pathogenesis of α -synucleinopathies. In addition to intestinal dysbiosis, dysbiosis of the oral microbiota should be seriously considered in α -Syn pathology. Finally, the therapeutic management of systemic chronic inflammatory pathologies, like periodontitis, may be beneficial for the clinical outcome of α -synucleinopathies.

- **Paper II** - Blood levels of hs-CRP were significantly higher in PD cases with self-reported periodontitis. However, the group that did not self-report periodontitis presented higher lymphocyte levels.
- **Paper III** - Self-report measures of periodontitis combined with risk factors reported predictive validity towards periodontitis and its severe form in the Portuguese population, using the 2018 and 2012 case definitions.
- **Paper IV** - Salivary and plasma α -Syn levels showed no association with periodontal diagnosis. NSPT seems to affect salivary α -Syn levels but not the plasma. Ultimately, NSPT may be a confounding factor when using salivary α -Syn as a biomarker.

Overall, with this thesis, we concluded that:

- PD patients that self-reported periodontitis presented elevated blood levels of hs-CRP, when compared with healthy counterparts.
- Self-report measures of periodontitis reported predictive validity towards periodontitis in the Portuguese population.
- NSPT seems to affect salivary α -Syn levels. Ultimately, NSPT is a confounding factor in medical diagnosis using biomarkers, specifically salivary α -Syn.

7.3. Future Directions

The results of the present thesis bring new insights into the physiological behavior of α -Syn in the periphery, as well as the impact of periodontal treatment in salivary α -Syn levels. These results present clinical value when using salivary α -Syn as a biomarker in α -synucleinopathies, with NSPT being a confounding factor if performed at least up to one month prior to the analysis. However, these results ought to be confirmed in future larger and prospective studies.

In light of the increase in life expectancy over recent decades and the growing prevalence of neurodegenerative disorders, especially α -synucleinopathies,

there is a pressing need to gain a deeper understanding of the underlying pathophysiological processes associated with α -synucleinopathies, namely the behaviour of α -Syn and other inflammatory markers. This knowledge has the potential to steer the development of novel clinical strategies to mitigate existing low-grade chronic inflammatory states, such as periodontitis. Such strategies may, in turn, positively affect α -synucleinopathies, by altering disease progression or even prevent disease onset. The goal is to launch a search for future clinical recommendations that can be implemented.

Moreover, future research should continue to focus on the systemic repercussions of periodontitis infection in patients with PD, with the aim of potentially clarifying the causality of the PD-periodontitis link. Furthermore, future research should include in-depth formal clinical measures of periodontitis (such as periodontal pocket depth and clinical attachment loss) in order to provide further confirmation of the association with circulating systemic inflammatory surrogates. Also, a comprehensive examination of the oral microbiota and microbial alterations in individuals with PD might reveal some promise as a non-invasive diagnostic approach for PD. Furthermore, gene sequencing of the oral microbiota may facilitate the development of innovative therapeutic strategies.

Additionally, from an epidemiological surveillance perspective, the emergence of novel and innovative strategies and tools, such as the implementation of self-report questionnaires, appears to hold considerable promise for both the early detection and the management of chronic, under-valued diseases like periodontitis. This has the potential to subsequently direct patients to seek formal clinical follow-up. The self-report questionnaires are a valuable tool due to their applicability in any setting (clinical or at home) and cost-effectiveness. We foresee that that a series of patient interactions may be undertaken before a clinical diagnosis is reached. These will rely exclusively on self-reporting tools and additional diagnostic tests (radiographs, blood and/or salivary analysis), coupled with artificial intelligence-powered approaches.

CHAPTER 8

8. References

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CHAPTER 9

9. Annexes

Annex 1. Ethical Clearance from Egas Moniz Ethics Committee.



Comissão de Ética EGAS MONIZ

Proc. Interno nº 1040

Ex.ma Senhora
Patrícia Lyra

Monte de Caparica, 27 de janeiro de 2022.

Ex.ma Senhora,

Em resposta ao Pedido de Parecer que submeteu à apreciação da Comissão de Ética da Egas Moniz, com o tema denominado **“The impact of periodontal infection and its treatment on alfa-synuclein levels and inflammation”**, foi aprovado por unanimidade.

A Presidente da Comissão de Ética da Egas Moniz


Prof.ª Doutora Maria Fernanda de Mesquita

Annex 2. Data Gathering Clearance.



Recolha de Dados

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Curso / Laboratório Associado / Clínica Dentária Egas Moniz / Outro	Doutoramento em Ciências Biomédicas	Ano Letivo	2021/2022
Unidade Curricular / Serviço/Outro	N/A	Ano / Semestre	N/A
Estudante(s) / Docente / Profissional/Investigador	Patrícia Lyra (Egas Moniz) João Botelho (Egas Moniz) Vanessa Machado (Egas Moniz) José João Mendes (Egas Moniz) Luís Proença (Egas Moniz) Helena Barroso (Egas Moniz) Ana Delgado (Egas Moniz) Kallol Ray Chaudhuri (King's College London) Silvia Rota (King's College London)		
Docente Responsável / Investigador Responsável	José João Baltazar Mendes (Docente Responsável) Patrícia Lyra (Investigador Responsável)		
Contactos	email jmendes@egasmoniz.edu.pt patricialyra10@gmail.com	Telefone	+351 212 946 700 +351 924281244
Local de recolha de dados	Clínica Dentária Egas Moniz		
Morada	Campus Universitário, Quinta da Granja, 2829-511 Monte de Caparica, Almada		
Período de recolha	Novembro de 2021 - Junho de 2025		
Área Científica do projeto	Ciências Dentárias		
Título do projeto	<i>The impact of periodontal infection and its treatment on alpha-synuclein levels and inflammation</i>		
Objetivo(s) da investigação	1. To assess the baseline salivary levels of inflammatory markers of interest (NLRP3, IL-6 and IL1 beta) and alpha-synuclein according to the periodontal status; 2. To explore the clinical implications of periodontal therapy in these markers of interest (NLRP3, IL-6, IL1 beta alpha-synuclein); 3. To assess the baseline blood levels of the same parameters, as well as the impact of periodontal therapy; 4. To compare the levels of hs-CRP of self-reported periodontitis cases with self-reported healthy periodontium cases amongst PD patients. With these aims, we expect to clarify the physiological salivary and blood levels of these markers of interest according to the periodontal status and the effect of periodontal treatment on them, particularly on α-synuclein. This will be key in future research studying periodontitis and α-synuclein-related conditions, as is Parkinson's Disease.		



Recolha de Dados

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Resumo do projeto

We intent to further explore the “neuro-perio” field of study, specifically the impact of periodontitis-induced inflammation and of the periodontal pathogens’ roles in the pathophysiology of Parkinson’s Disease.

This is a case-control study adjusted for age, gender and periodontal status, with an estimated preliminary sample of 40 patients per group (case vs. control, ratio 1:1; case group - patients with periodontitis; control group - patients without periodontitis).

Adults with 10 or more teeth present were included (excluding root remnants and third molars). Exclusion criteria will be as follows: nonsurgical periodontal treatment performed in the last year; antibiotic therapy, corticosteroids and immunosuppressive agents in the last 3 months; pregnant or lactating women.

PHASE A - Assessment of the baseline salivary levels of inflammatory markers of interest and α -synuclein according to the periodontal status

1. Inclusion of a random sample of participants at the Triage Department of the Egas Moniz Dental Clinic (estimated preliminary sample of 40 participants per group).
2. Saliva collection and periodontal diagnosis (with the periodontal diagnosis, we expect to divide participants according to their periodontal status).
3. Quantification of salivary baseline levels of inflammatory markers and α -synuclein (both in healthy and periodontitis cases).

PHASE B - Evaluation of the clinical implications of periodontal therapy in the salivary levels of inflammatory markers of interest and α -synuclein in periodontitis patients (included in PHASE A)

4. Non-surgical treatment of periodontitis cases.
5. Periodontal reassessment and recollection of saliva at the 7-day and 30-day follow-up consultations (both case and control groups).
6. Quantification of salivary levels of inflammatory markers and α -synuclein after non-surgical periodontal treatment.

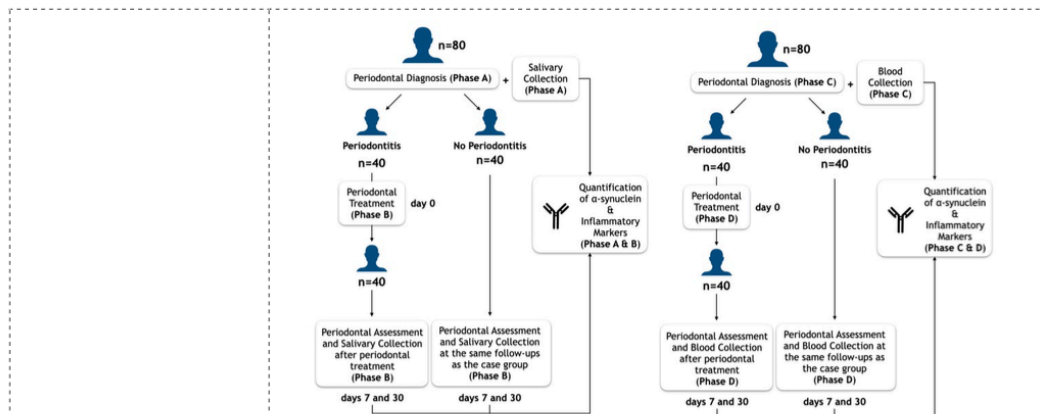
PHASE C - Assessment of blood levels of the inflammatory markers of interest and α -synuclein according to the periodontal status

7. Inclusion of a random sample of participants at the Triage Department of the Egas Moniz Dental Clinic (estimated preliminary sample of 40 participants per group).
8. Blood sample collection and periodontal diagnosis (with the periodontal diagnosis, we expect to divide participants according to their periodontal status).
9. Quantification of baseline blood levels of inflammatory markers and α -synuclein (both in healthy and periodontitis cases).

PHASE D - Evaluation of the clinical implications of periodontal therapy in the blood levels of inflammatory markers of interest and α -synuclein (included in PHASE C)

10. Non-surgical treatment of periodontitis cases.
11. Periodontal reassessment and recollection of blood at the 7-day and 30-day follow-up consultations (both case and control groups).

Quantification of blood levels of inflammatory markers and α -synuclein after non-surgical periodontal treatment.



PHASE E - Comparison of hs-CRP levels of self-reported periodontitis cases with self-reported healthy periodontium cases amongst a group of PD patients

1. Identification of PD cases in the National Health and Nutrition Examination Survey (NHANES) database through specific medication regimens.
2. Profiling of the included PD patients according to self-report of periodontal status.
3. Evaluation and comparison of blood and biochemical parameters according to the self-reported periodontal status.

Metodologia de recolha de dados

Saliva Collection Protocol (Kang et al., 2016)

Unstimulated saliva collection will take place before periodontal diagnosis at the Triage Department of the Egas Moniz Dental Clinic and before clinical observation during the 7-day and 30-day follow-up consultations post nonsurgical periodontal treatment. The minimum collection volume of unstimulated saliva will be of 1.5 ml. All samples should be collected between 9 a.m. and 11 a.m., to avoid interference of the circadian cycle.

Factors to consider before sample collection: to avoid foods with high sugar, acidity, or caffeine immediately before sample collection; to document the consumption of alcohol, caffeine, nicotine, and prescription/over-the-counter medications within the prior 12 hours; to document intense physical activity and the presence of oral disease, injury or inflammation; not to brush teeth or eat a major meal within 60 minutes of sample collection; to rinse mouth with water to remove food residues at least 10 minutes prior to saliva collection.

Following saliva collection, the whole sample should be immediately refrigerated on ice. A Protease Inhibitor Cocktail (100 µl/1 ml of whole saliva, Cat#P2714, Sigma Aldrich, St. Louis, MO, USA) will be added to the sample to minimize protein degradation and the sample will be vortexed repeatedly. The first centrifugation will be at low speed (2,600 rpm×g for 15 minutes at 4°C), followed by a second centrifugation at a high speed (15,000 rpm×g for 15 minutes at 4°C), to remove particles. Finally, the supernatant will be divided into 0.5 ml aliquots, and then stored at -20°C before analysis.

Periodontal Diagnosis Protocol (according to the European Federation of Periodontology/American Academy of Periodontology Classification of 2018)(Tonetti et al., 2018)

A full-mouth periodontal examination will be performed at the Triage Department of the Egas Moniz Dental Clinic and during the 7-day and 30-day follow-up consultations post nonsurgical periodontal treatment (duration of approximately 30 minutes). All fully erupted teeth (excluding third molars,



Recolha de Dados

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implants and retained roots) will be examined through a manual periodontal North Carolina probe (Hu-Friedy; Chicago, Illinois, USA) and the number of missing teeth will be recorded. Pocket depth (PD) will be measured as the distance from the free gingival margin to the bottom of the pocket. Recessions (REC) will represent the distance from the cemento-enamel junction (CEJ) to the free gingival margin (a negative sign will be assigned if the gingival margin is located coronally to the CEJ). Interdental clinical attachment loss (CAL) will be calculated as the algebraic sum of REC and PD measurements for each site. All measurements will be rounded to the lowest whole millimeter. Furcation involvement (FI) will be assessed using a 2N probe (Hu-Friedy; Chicago, Illinois, USA) and tooth mobility will be also appraised.

Periodontitis Case Definition (Tonetti et al., 2018)

A periodontitis case implies interdental CAL ≥ 2 mm of non-adjacent teeth, or Buccal or Oral CAL ≥ 3 mm, with PD > 3 mm detectable at ≥ 2 teeth.

Periodontitis staging will be defined according to severity and extent. Regarding severity, it can be categorized as mild (stage 1) if interdental CAL at the site of greatest loss is of 1-2 mm, moderate (stage 2) if it is of 3-4 mm and severe (stage 3 and stage 4) if it is of ≥ 5 mm. Additionally, the extent will be described as localized ($<30\%$ of teeth involved), generalized ($\geq 30\%$ of teeth involved) or molar/incisor pattern.

Quantification of Salivary/Blood Baseline Levels of Inflammatory Markers and α -synuclein in Healthy and Periodontitis Cases Protocol

The quantification of salivary/blood levels will take place at three different stages: after the initial periodontal diagnosis (of the case and control groups); at the 7-day follow-up consultation after nonsurgical periodontal treatment; and at the 30-day follow-up consultation after nonsurgical periodontal treatment.

The quantification of salivary levels of total and oligomeric α -synuclein will be performed through the use of anti- α -Synuclein Quantitative ELISA Kit (SensaLyte 55550) and Human Alpha Synuclein oligomer ELISA Kit (MyBioSource, MBS730762), respectively (Vivacqua et al., 2016). Levels of the inflammatory markers of interest (IL-1 β , IL-6 and NLRP3) will also be measured by means of immunoassay kits (abcam®) (Karpenko et al., 2018).

Non-surgical Treatment of Periodontitis Cases Protocol (Sanz et al., 2020; Suvan et al., 2020)

Periodontitis cases will be provided nonsurgical periodontal treatment. Initial periodontal treatment will be performed in two or four sessions, according to the severity and extent of periodontitis. Subgingival instrumentation will be performed through debridement, scaling and root planning. No adjuvant chemical therapy will be prescribed. Seven days and thirty days after nonsurgical periodontal treatment, the periodontal status will be reassessed. Patients requiring additional periodontal (surgical) treatment, evaluation of all clinical parameters will be performed upon completion of the treatment.

Blood Collection Protocol (Chang et al., 2020)

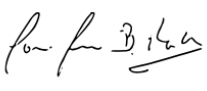
The blood sample collection will take place before periodontal diagnosis at the Triage Department of the Egas Moniz Dental Clinic and before clinical observation during the 7-day and 30-day follow-up consultations post nonsurgical periodontal treatment. Blood should be collected in the morning and patients should be fasting. Blood collection of 2 ml of venous blood will be performed from the antecubital fossa by venipuncture, using a 20-gauge needle with a 2 ml syringe.

Following blood collection: Plasma samples will derive from Ethylenediaminetetraacetic acid-treated tubes (EDTA), while serum samples will result from leaving blood samples undisturbed at room temperature for 15-30 minutes. Plasma or serum samples will undergo refrigerated centrifugation for 15 min at 1,500g. The resulting serum or plasma supernatants will be transferred



Recolha de Dados

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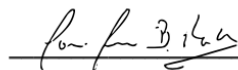
into clean and low residue polypropylene tubes. Samples will be stored at -80°C before examination.	
Assinatura do docente responsável / Investigador responsável interno ou externo	
Data	19/11/2021

Eu, Docente Responsável / Investigador Responsável, confirmo que as Declarações de Autorização necessárias para o estudo foram entregues:

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