INSTITUTO UNIVERSITÁRIO EGAS MONIZ

MESTRADO INTEGRADO EM MEDICINA DENTÁRIA

ASSOCIATION BETWEEN PERIODONTITIS AND ALPHA-SYNUCLEIN: A CASE-CONTROL STUDY

Trabalho submetido por
Daniel José Ribeiro da Cruz
para a obtenção do grau de Mestre em Medicina Dentária

outubro de 2021

INSTITUTO UNIVERSITÁRIO EGAS MONIZ

MESTRADO INTEGRADO EM MEDICINA DENTÁRIA

ASSOCIATION BETWEEN PERIODONTITIS AND ALPHA-SYNUCLEIN: A CASE-CONTROL STUDY

Trabalho submetido por
Daniel José Ribeiro da Cruz
para a obtenção do grau de Mestre em Medicina Dentária

Trabalho orientado por
Prof. Doutor José João Baltazar Mendes

e coorientado por
Mestre João Tiago da Silva Botelho

outubro de 2021

“We keep moving forward, opening up new doors and doing new things, because we're curious... and curiosity keeps leading us down new paths”

Walt Disney

AGRADECIMENTOS

Em primeiro lugar, quero agradecer ao meu orientador, o Prof. Doutor José João Mendes pela confiança e oportunidade para integrar a equipa P2D - Doença de Parkinson e Doença Periodontal. Em seguida, agradecer a disponibilidade, a partilha de conhecimentos e experiência durante este percurso.

Ao meu coorientador, Mestre João Botelho agradeço a entrega para este projeto, a partilha de conhecimento e criatividade na resolução de problemas. Agradeço também a motivação e rigor científico sem os quais este trabalho não seria possível.

Ao Prof. Doutor Luís Proença quero agradecer pela ajuda prestada na organização e estrutura da recolha de dados bem como durante todo o processo de análise estatística.

À Prof.ª Doutora Vanessa Machado deixo o meu mais sincero agradecimento pela determinação, perseverança e pelo auxílio prestado para concluir este projeto.

À Mestre Patrícia Lyra agradeço toda a entrega, disponibilidade e profissionalismo que partilhou comigo durante o trabalho de campo. Agradeço também toda a ajuda e dicas de quem já passou pela mesma situação e soube antecipar as minhas dificuldades.

Às técnicas de laboratório Susana e Natacha, agradeço a ajuda na preparação das amostras salivares.

Ao Prof. Doutor Hugo Miranda agradeço a disponibilidade para a análise laboratorial das amostras salivares.

À minha colega de box e grande amiga Inês tenho a agradecer o carinho, entrega, tempo e confiança para estar sempre do meu lado como ninguém. Agradeço-te por teres estado lá quando mais precisei. À Patrão, que foi acolhida pela box 7 e que tornou o 5º ano tão fantástico agradeço a coragem de entrar neste grupo louco e toda ajuda nestes anos.

Às minhas amigas muito especiais, Joana, Madalena e Mariana, agradeço a vossa disponibilidade e presença durante estes 5 anos.

Ao Instituto Universitário Egas Moniz agradeço a oportunidade de integrar esta casa que fica marcada no coração. Aqui cresci, errei, falhei e sobretudo superei as minhas expectativas.

Por último agradeço aos meus pais que tornaram todo este percurso possível. Sempre estiveram incondicionalmente ao meu lado e disponíveis para qualquer situação.

ABSTRACT

Background: Periodontitis is an inflammatory disease of the periodontium that has been associated with neurodegenerative diseases through the inflammatory burden of this pathology. The core of this association is often based upon molecules that play an important neural role. As such, alpha-synuclein (α-syn) is a protein located in pre-synaptic terminals and synucleinopathies may have a link with periodontitis, however salivary α-syn has never been assessed according to periodontal status.

Aims: To study the association between periodontal status and periodontal inflamed surface area with salivary levels of α-syn through a pilot sample.

Materials and methods: Patients attending a triage appointment at Egas Moniz Dental Clinic were enrolled in this observational study. A sample of non-stimulated saliva was collected following a full-mouth periodontal examination and circumferential probing. Furthermore, mean periodontal epithelium surface area (PESA) and periodontal inflamed surface area (PISA) were calculated. Salivary samples were quantified for protein concentration (cut-off 1.00mg/mL). Next, α-syn was quantified following enzyme-linked immunosorbent assay.

Results: From an initial sample of 66 patients, 47 participants were enrolled and sorted according to the periodontal status. Age and smoking habits were variables significantly related with periodontal status. PISA showed a moderate correlation with salivary levels of α-syn. After filtering by gender, PISA showed different salivary α-syn curves.

Conclusion: PISA is correlated with moderate strength with salivary levels of α-syn. Considering this study limitations, further work is necessary with a sample increase, a prospective follow-up and an intervention design.

Keywords: α-synuclein; neurodegenerative disease; periodontitis; periodontal disease.

RESUMO

Contexto: A periodontite é uma doença inflamatória do periodonto, que tem sido associada com doenças neurodegenerativas, através da carga inflamatória despoletada por esta patologia. A base desta associação está, muitas vezes, concentrada em moléculas com um papel nervoso importante. Neste sentido, a alfa-sinucleína (α-sinucleína) é uma proteína localizada nos terminais pré-sinápticos e as sinucleinopatias têm sido relacionadas com a periodontite, contudo tal nunca foi estudado no que diz respeito à α-sinucleína salivar.

Objetivo: Investigar a associação entre o estado periodontal e a área de inflamação de superfície periodontal com os níveis salivares de α-sinucleína numa amostra consecutiva piloto.

Materiais e métodos: Pacientes de triagem da Clínica Dentária Egas Moniz foram incluídos neste estudo observacional. A saliva não estimulada foi recolhida e, de seguida, realizámos uma análise periodontal através de examinação total e sondagem circunferencial. Foram quantificados os valores médios de área de superfície periodontal epitelial (PESA) e inflamada (PISA). As amostras salivares foram quantificadas para a concentração proteica (*cut-off* de 1.00 mg/mL). De seguida, a α-sinucleína foi quantificada através de método *Enzyme-Linked Immunosorbent Assay.*

Resultados: De uma amostra inicial de 66 pacientes, 47 participantes foram incluídos divididos de acordo com o estado periodontal. A idade e o ser fumador foram fatores significativamente relacionados com o estado periodontal. A PISA apresentou uma correlação significativa de nível moderado com os níveis de α-sinucleína salivar. Quando separados por sexo, as curvas de relação de PISA com a α-sinucleína salivar apresentaram padrões distintos.

Conclusão: A PISA está correlacionada com força moderada com os níveis salivares de α-sinucleína. Considerando as limitações deste estudo, será necessário um aumento da amostra, um acompanhamento prospetivo e um *design* de intervenção.

Palavras-chave: α-sinucleína; doença neurodegenerativa; periodontite; doença periodontal.

TABLE OF CONTENTS

I. INTRODUCTION 13
1. Periodontitis 13
1.1. Epidemiology 13
1.2. Prevalence and Incidence 13
1.3. Etiology 14
1.4. Pathogenesis 15
1.5. Clinical features 17
1.6. Periodontal Classification and Inflammation Measurement 19
1.7. Inflammatory markers 20
2. Alpha-synuclein 21
2.1. Structural properties and function of α-syn 21
2.2. α-syn post-translational modifications 23
2.3. α-syn and the oral cavity 24
2.4. Disease implication and related conditions 25
2.5. Potential biomarker for neurodegenerative diseases 26
3. Periodontitis and α-syn interplay 26
4. Aims 28
III. MATERIALS AND METHODS 29
3.1. Ethical considerations 29
3.2. Study type and setting 29
3.4. Study sample 29
3.5. Eligibility Criteria 29
3.6. Collection of variables of interest 30
3.6.1. Saliva collection 30
3.6.2. Periodontal examination 31
3.7. A-syn quantification 32

3.8. Statistical analysis 33
IV. RESULTS 35
V. DISCUSSION 39
5.1. Strengths and Limitations 40
VI. CONCLUSION 43
6.1. Future perspectives 43
VII. REFERENCES 45

LIST OF FIGURES

Figure 1: Diagram representing plaque formation and development to trigger periodontitis 15

Figure 2: Periodontitis progression patterns 17

Figure 3: Periodontitis is associated with other inflammatory diseases 19

Figure 4: α-syn gene sequence showing the repeating motif "KTKEGV", a hydrophobic central domain (NAC) and a C-terminal 22

Figure 5: Proposed link between periodontitis and Parkinson's Disease. 27

Figure 6: Saliva collection schematic process 31

Figure 7: Schematic representation of α-syn quantification. 33

Figure 8: Participant's flowchart 35

Figure 9: Scatterplot displaying the overall correlation between α-syn levels and PISA 37

Figure 10: Scatterplot displaying the correlation between α-syn levels and PISA according to gender 37

LIST OF TABLES

Table 1: Participant's characteristics stratified according to periodontal disease 36

Table 2: Periodontal clinical characteristics stratified according to periodontal disease. 36

Table 3: α-syn levels stratified according to periodontal disease. 36

LIST OF ABBREVIATIONS

AD – Alzheimer's Disease

AGEs - Advanced Glycation End Products

Aβ - Amyloid Beta

BAD - Bcl-2-Associated Death Promoter

BCA - Bicinchoninic Acid

BoP - Bleeding on Probing

CAL – Clinical Attachment Loss

CEDOC - Centro de Estudos de Doenças Crónicas

CK-1 - Casein Kinase-1

CK-2 - Casein Kinase-2

CSF - Cerebrospinal Fluid

CSP-α - Cysteine String Protein-alpha

DJ-1 - Protein deglycase-1

DLB - Dementia with Lewys Bodies

E1 - Ubiquitin-Activating Enzyme 1

E2 - Conjugating Enzyme 2

E3 – Convertase Enzyme 3

ELISA - Enzyme-Linked Immunosorbent Assay

EMDC – Egas Moniz Dental Clinic

ERK - Extracellular Regulated Kinases

GCF – Gingival Crevicular Fluid

IL-1 – Interleukin-1

IL-11 – Interleukin-11

IL-12 – Interleukin-12

IL-18 - Interleukin-18

IL-1Ra - Interleukin-1 receptor antagonist

IL-1α – Interleukin 1-alpha

IL-1β - Interleukin 1-beta

IL-33 - Interleukin-33

IL-6 – Interleukin-6

IL-7 – Interleukin-7

IL-8 – Interleukin-8

LB - Lewy Bodies

MAC - Membrane Attack Complex

MSA - Multiple System Atrophy

n – Number of cases

NR4A2 - Nuclear Receptor Subfamily 4 Group A Member 2

OPG – Osteoprotegerin

PD - Parkinson's Disease

PINK1 - PTEN Induced Kinase-1

PKC - Protein Kinase C

PLD2 - Phospholipase-D2

PTM - Post-Translational Modifications

RANKL - Receptor Activator of Nuclear Factor Kappa-B Ligand

RBC - Red Blood Cells

SD – Standard deviation

STROBE - Strengthening the Reporting of Observational Studies in Epidemiology

SUMO - Small Ubiquitin-like Modifiers

TNF-α - Tumor Necrosis Factor-alpha

UCH-L1 - Ubiquitin Carboxy-terminal Hydrolase L1

α-sinucleína - Alfa-sinucleína

α-syn - Alpha-synuclein

I. INTRODUCTION

1. Periodontitis

1.1. Epidemiology

Periodontitis is a chronic inflammatory disease that affects the supporting tissues of teeth (Darveau, 2010). Clinically, periodontitis is characterized by deep pockets around teeth, loss of connective tissue and alveolar bone destruction (Pihlstrom et al., 2005). This condition is caused by an active and virulent dysbiotic plaque and an immune response from a susceptible host (Pihlstrom et al., 2005).

Quality of life of individuals suffering from periodontal disease is significantly impaired (Buset et al., 2016). Periodontal patients encounter several setbacks upon enduring the destructive effect of periodontitis (Borges et al., 2013). Masticatory performance is negatively affected when the periodontium is destructed. However, periodontal treatment is capable of restoring the lost quality of life within one week (Shanbhag et al., 2012) and up to 3 months (Botelho et al., 2020) following nonsurgical periodontal treatment.

Periodontitis has a significant burden on worldwide economy (Botelho et al., 2021; Listl et al., 2015). Data from 2015 suggests that periodontal disease, tooth loss and caries have incurred in a loss of over 144 billion dollars worldwide (Listl et al., 2015). Moreover, a recent study regarding the impact periodontitis has in the United States and European economy showed that over 150 billion dollars and euros, respectively, were loss due to periodontal disease alone (Botelho et al., 2021).

1.2. Prevalence and Incidence

Briefly, periodontitis can be classified in generalized or localized and according to severity between mild, moderate, and severe (Wiebe & Putnins, 2000). According to data recorded from 2010, severe periodontitis was predicted to affect 10,8% of all population (Kassebaum et al., 2014). Incidence wise, severe periodontitis was forecasted to target 701 individuals per 100 000 person-years (Kassebaum et al., 2014). Overall, periodontitis in the United States when taken individuals older than 30 shows prevalence values of 47,2% (Eke et al., 2012). Furthermore, periodontitis seems to have a bigger impact in lower educated individuals (Eke et al., 2012).

Periodontitis appears to have an “age effect”, meaning it worsens as the individual age increases (Kassebaum et al., 2014). This appears to be caused by the condition’s cumulative effect along with prolonged exposure to risk factors (Lang & Lindhe, 2015). As such, periodontitis steadily increases after 30 years of age. Furthermore, ethnic groups' prevalence appears to be different as well, while Hispanics have the highest prevalence, non-Hispanic whites have the lowest (Eke et al., 2015). Finally, socioeconomic conditions and decreased education also showed an increase in periodontitis prevalence (Eke et al., 2015).

1.3. Etiology

Periodontitis is mostly preceded by a reversible inflammatory reaction of the gingiva, also known as gingivitis (Albandar & Rams, 2002). Gingivitis is caused by poor oral hygiene which in turns results in accumulation of supragingival plaque, leading to its inflammation. Despite this, periodontitis requires a host susceptibility as explained in figure 2 (Pihlstrom et al., 2005).

Plaque formation plays an important role in the periodontitis onset. As such, plaque genesis follows a six-step process. First, adsorption occurs which is a pellicle that covers the teeth consisting of mostly proteins, phosphoproteins and glycoproteins (Al-Hashimi & Levine, 1989). A reversible adhesion follows and is characterized by long-range low attraction forces to the teeth (Lang & Lindhe, 2015; Tatakis & Kumar, 2005). Afterwards, this attraction becomes stronger and turns to be a permanent adhesion consisting of covalent and hydrogen bonds (Lang & Lindhe, 2015). It is at this moment that secondary colonizers begin to attach to the primary colonizers increasing the plaque’s diversity and functional aggregation (Tatakis & Kumar, 2005). The plaque continues to mature and grow to a point where a change in the environment occurs, from an aerobic state to a predominantly facultative anaerobic. Finally, bacteria may detach from their original location and colonize other areas of the mouth, starting this process again (Lang & Lindhe, 2015).

Thus, a constant cycle of plaque formation followed by its removal is compatible with a healthy periodontium (Lang & Lindhe, 2015). However, when this does not happen the plaque undergoes an environmental change (microbial shift) developing a nonspecific pathogenicity illustrated in figure 1. In the supragingival area the environment changes,

once again, from a facultative to an obligate anaerobic. Therefore, it produces a new nutritional supply that favors proteolytic bacterias such as *Porphyromonas gingivalis*, *Treponema denticola* and *bacteroides forsythus* (*Tannerella forsythia*) (Nishihara & Koseki, 2004).

Despite its highly plaque induced reaction, periodontitis appears to have a genetic factor associated with it. Gene mutations that affect the host's inflammatory response seem to be the most likely to influence periodontitis progression (Pihlstrom et al., 2005).

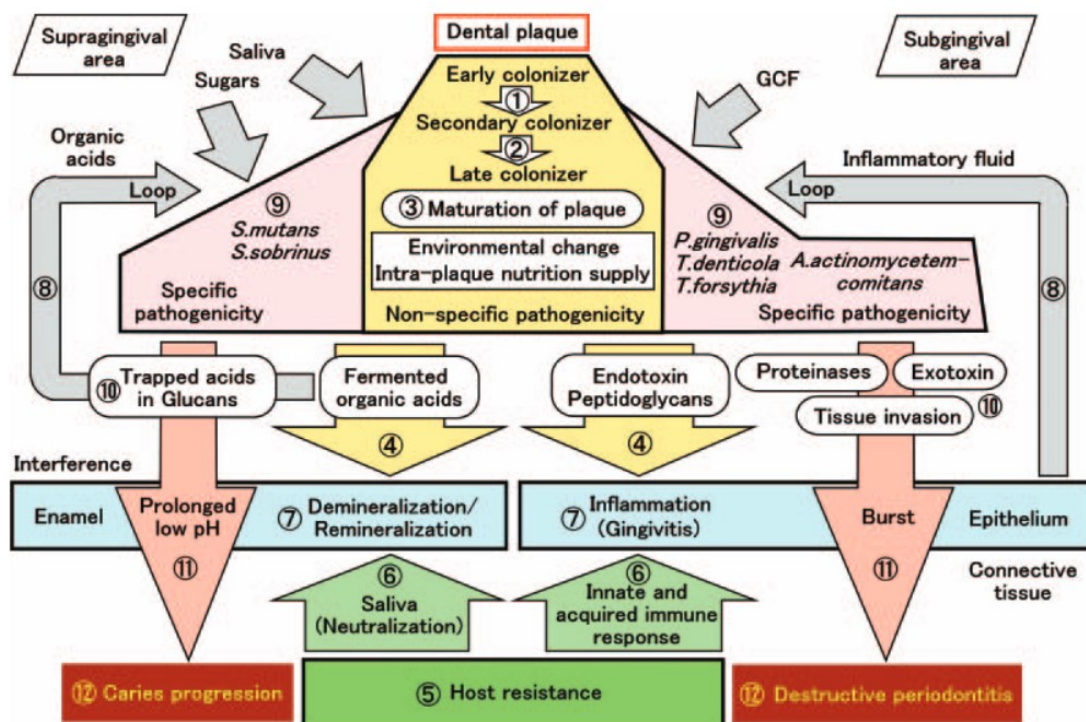


Figure 1: Diagram representing plaque formation and development to trigger periodontitis. *(by Nishihara et al. 2014, reprinted with the permission from John Wiley & Sons - Books).*

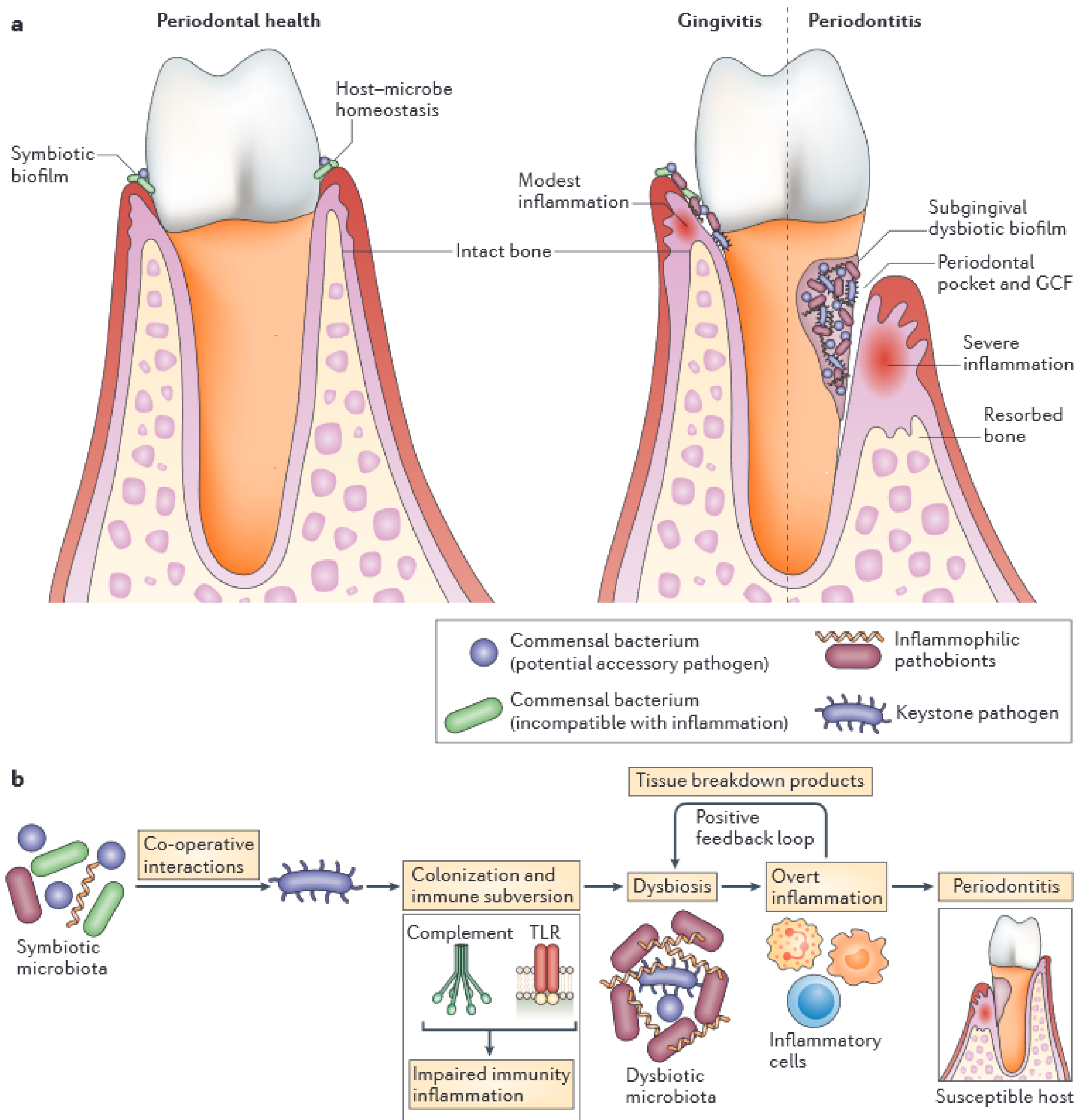
1.4. Pathogenesis

In a gingivitis state, the immune system recruits certain cells to the site by producing cytokines and chemokines. If gingivitis is not treated, and the lesion can prevail, a chronic inflammation is established. This triggers an innate immune response that will lead to an adaptive response of the immunity system (Cekici et al., 2014).

First, the complement cascade is activated through the alternative pathway. In this pathway a lipopolysaccharide or a zymosan may trigger its onset (Cekici et al., 2014). In

the plasma, there's a significant amount of C3 that can be spontaneously hydrolyzed to form C3b. This allows factor B to bind to C3b which in turn allows factor D to cleave C3bB into C3bBb - C3 convertase. This convertase is unstable and requires Factor P to bind and stabilize it. C3 convertase is now ready to cleave C3 into C3a (which initiates an inflammatory response) and C3b (that coats the bacteria's surface). C5 may now be cleaved in C5a and C5b. C5b binds to C6 to C9 proteins leading to formation of the Membrane Attack Complex (MAC) (Janeway et al., 2001). In periodontitis, a cysteinyl proteinase (Gingipain) from *Porphyromonas gingivalis* can cleave C3 and C5, hence protecting the bacteria from the MAC (Popadiak et al., 2007). This releases a potent chemotactic factor and induces leukocytic infiltration, possibly explaining the fast onset of the inflammatory response associated with periodontitis (Wingrove et al., 1992).

Bone resorption happens in periodontitis when there are enough inflammatory mediators to trigger this resorption along with an inflammatory state that extends to the alveolar bone (Cochran, 2008). Thus, proinflammatory cytokines concentration – Interleukin-1 (IL-1), Interleukin-6 (IL-6), Interleukin-7 (IL-7), Interleukin-11 (IL-11) and Tumor Necrosis Factor-alpha (TNF-α) - are extremely important to trigger this effect (Cochran, 2008). In physiologic conditions there is a balance between Receptor activator of nuclear factor kappa-B ligand (RANKL) and Osteoprotegerin (OPG) ratio. When this ratio increases, whether by an increase in RANKL or a decrease in OPG, bone resorption occurs. Therefore, IL-1, IL-6, IL-7 and TNF-α tip the balance of osteoclastogenesis by increasing RANKL expression as well as decreasing OPG expression (Nakashima et al., 2000).



GCF - Gingival Crevicular Fluid

Figure 2: Periodontitis progression patterns. **(a)** Periodontitis follows a progression pattern from an inflammation state of the gingiva with pocketing ≤3mm, however, it may reach depths of ≥4 mm and incur in bone loss. (**b**) Upon meeting a susceptible host, oral pathogens act together as a polymicrobial community to induce bone destruction, causing a microbial dysbiosis through subversion of the immune system and leading to over-inflammation (by Hajishengallis & Chavakis (2021), reprinted with permission from Springer Nature Customer Service Centre GmbH).

1.5. Clinical features

In a state of gingival health, the oral tissues show a coral pink color with a smooth and regular texture (Lang & Lindhe, 2015). In normal conditions the biologic space extends from the junctional epithelium, with 0,97 mm of depth, to the connective tissue with 1,07

mm of depth. Therefore, the biological width is 2,04 mm (Gargiulo et al., 1961). When gingivitis starts developing there is a change in the tissues, starting with erythema, edema, gingival bleeding after brushing and loss of stippling (Highfield, 2009). When gingivitis is allowed to progress and there is a host susceptibility it may transform into periodontitis. In this case the hallmark of periodontitis is clinical attachment loss (CAL) (Papapanou & Susin, 2017). This loss may occur due to gingival migration towards the apical portion of the teeth or due to a destruction of alveolar bone which both increase probing depth. Another marker that can be used to determine the disease's activity is the existence of hemorrhage or suppuration upon probing. If there is not, it is likely to assume that the disease is not active (Papapanou & Susin, 2017).

Despite periodontitis targeting the oral cavity it is believed that it affects other systemic diseases, mainly inflammatory conditions such as hypertension (Aguilera et al., 2020), diabetes *mellitus* (Borgnakke et al., 2013), rheumatoid arthritis (Hussain et al., 2020), polycystic ovarian syndrome (Machado et al., 2020) or Alzheimer's disease (AD) (Leira et al., 2017). As such, periodontitis has a great impact on many inflammatory diseases and in general health. Recently, a retrospective cohort study of 18 years has shown that periodontitis significantly increases comorbidities along with a higher mortality rate (Zhao et al., 2019). Several mechanisms have been proposed to enlighten periodontal bacteria dissemination to other tissues. However, a conclusion has not been established yet (Figure 3).

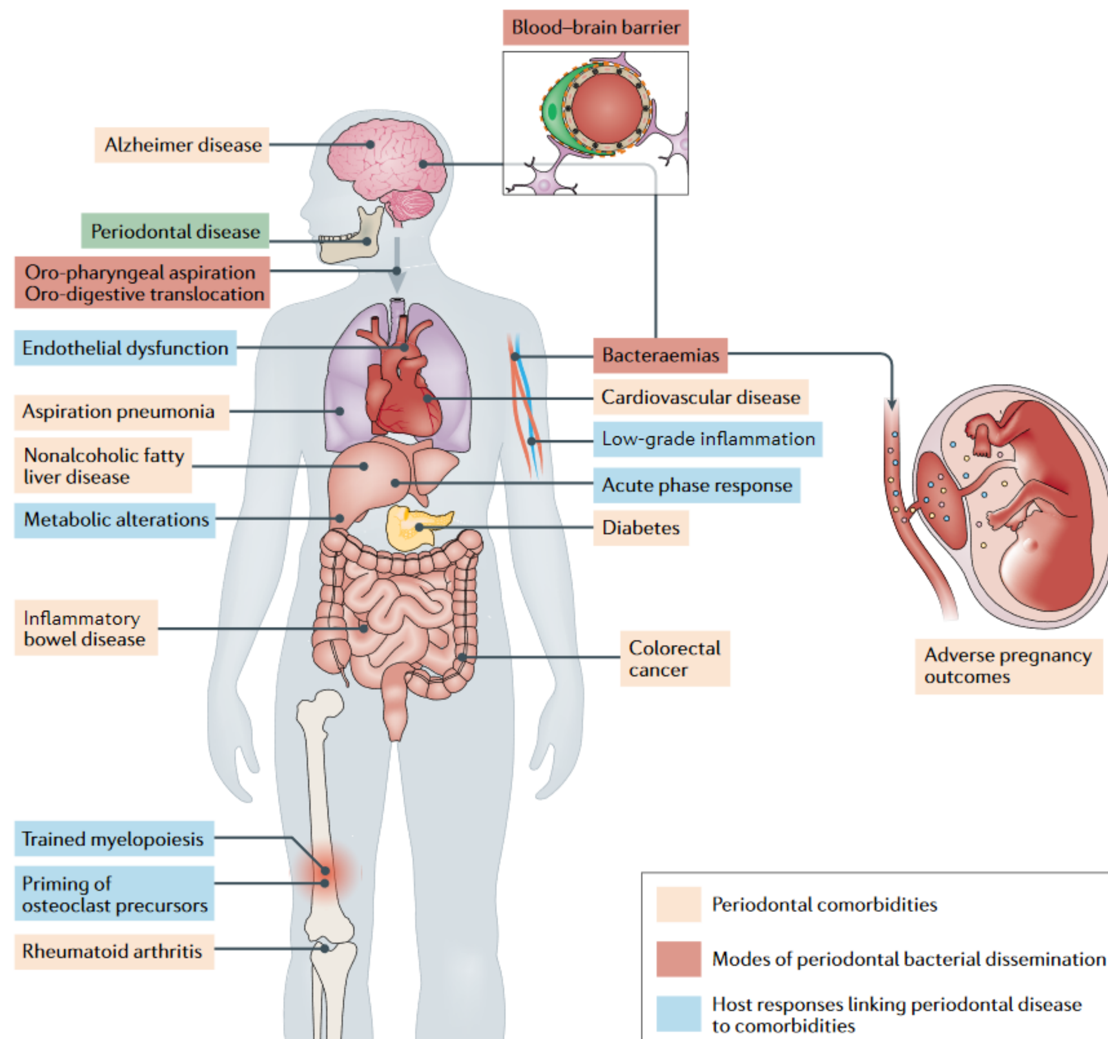


Figure 3: Periodontitis is associated with other inflammatory diseases. Periodontal disease has a primary inflammatory machinery; hence it can trigger bacteremia and systemic inflammation. A possible explanation suggested by the authors is through hematogenous dissemination or oro-pharyngeal and oro-digestive routes to trigger a distant inflammation (by Hajishengallis & Chavakis (2021), reprinted with permission from Springer Nature Customer Service Centre GmbH).

1.6. Periodontal Classification and Inflammation Measurement

When classifying periodontitis first it is important to distinguish its form, whether it is a necrotizing periodontitis, a manifestation of systemic disease or a periodontitis (Tonetti et al., 2018). Clinically periodontitis is characterized by a CAL in the interdental space higher or equal of two or more non-adjacent teeth; or if there is a CAL higher or equal to

3 mm in buccal or lingual with pocketing depth of more than 3 mm in two or more teeth. CAL cannot be due to non-periodontal causes (Tonetti et al., 2018).

Upon clinical diagnosis periodontitis is categorized by stage, which indicates the severity and complexity of treatment, and grading that reflects the disease progression rate (Tonetti et al., 2018).

Another important measure for understanding periodontitis impact is periodontal epithelium surface area (PESA) (Nesse et al., 2008). Through PESA we can quantify the root surface area that is covered by epithelium (Leira et al., 2017). However, one shortcoming of this classification is that it does not quantify periodontal tissue inflammation. Thus, periodontal inflamed surface area (PISA) classification exists (Nesse et al., 2008). This classification aims to measure the bleeding area of a tooth pocket, therefore considering the inflammatory burden of periodontitis (Nesse et al., 2008). This method proves to be worthy as periodontal inflammation seems to be the link to several diseases (Leira et al., 2017). Despite this, PISA has some weaknesses too: for example, it cannot differentiate from a pseudo-pocket or a gingival overgrowth. PISA appears to be way more effective when assessing risk factor for other diseases as it calculates inflammation areas (Nesse et al., 2008).

1.7. Inflammatory markers

Tissue destruction is triggered by several pathways that induce an increase in production of proinflammatory cytokines (Birkedal-Hansen, 1993). These cytokines are interleukin 1 (IL-1) which is a group formed by three forms namely Interleukin 1-alpha (IL-1α), Interleukin 1-beta (IL-1β) and Interleukin-1 receptor antagonist (IL-1Ra) (Mantovani et al., 2019). The first two have an agonistic action and the last, as the name suggests it, has an antagonist effect and are associated with periodontitis susceptibility (Lavu et al., 2015). Furthermore, IL-1β seems to play a role in the pathogenesis of periodontal disease (Pan et al., 2019). Interleukin-18 (IL-18) and Interleukin-33 (IL-33) are part of IL-1 family and are responsible for periodontitis destructive action through alveolar bone resorption (Köseoğlu et al., 2015; Yoshinaka et al., 2014). Furthermore, in chronic periodontitis IL-18 seems to be increased and can be lowered to healthy values through non-surgical periodontal treatment (Campos et al., 2012).

Secondly, IL-6 promotes the production of inflammatory proteins, hence it controls and manages the inflammatory response. When IL-6 activity is allowed to carry on, acute inflammation turns to a chronic state (Gabay, 2006). IL-6 plays an important role in periodontitis chronic inflammation. Increased values were noted in patients with severe periodontitis whilst not having any other pathology (Loos et al., 2000). Furthermore, IL-6 expression is induced in the starting and acute phase of periodontitis (Ebersole et al., 2014; Pan, 2019).

Interleukin-8 (IL-8) is a cytokine that targets neutrophil activation in inflammatory areas. Recent studies have revealed that its levels may be increased in gingival crevicular fluid (GCF). This is explained because cytokines originate from the GCF instead of salivary glands (Finoti et al., 2017; Ruhl et al., 2004).

Lastly, TNF-α is a cytokine released by macrophages that induces an increased bone loss by increasing osteoclast production (Graves et al., 2001). Moreover, in chronic periodontitis TNF-α is elevated in GCF and serum (Madureira et al., 2018 & Górska et al., 2003).

2. Alpha-synuclein

2.1. Structural properties and function of α-syn

Alpha-synuclein (α-syn) is part of a three-way synuclein family of proteins, including beta-synuclein and gamma-synuclein (Lavedan et al., 1998). From a structural standpoint, α-syn is a naturally unfolded protein (Weinreb et al., 1996) of a 140-aminoacid chain with 14.5 kDa (Jakes et al., 1994). This protein has been found in several human tissues such as the nervous system, blood and heart (Jakes et al., 1994). Notwithstanding, is mostly expressed in the brain as it is located at the presynaptic nerve terminals (Jakes et al., 1994) representing 0,1% of all brain proteins (Tofaris & Spillantini, 2005). The structure of a-syn is divided in three regions: 1) N-terminal that repeats the sequence "KTKEGV"; 2) a hydrophobic central domain (NAC); and, 3) a C-terminal which is highly disordered and negatively charged (Figure 4) (Jakes et al., 1994).

There are strong similarities between α-syn 11-mer located at the N-terminal sequence and the α-helix lipid binding domain in apolipoproteins (George et al., 1995). Thus, it strengthens the hypothesis that α-syn plays a role in synaptosomal membrane (Maroteaux & Scheller, 1991). Furthermore, α-syn acts as a Phospholipase-D2 (PLD2) inhibitor at

the membrane surface which is responsible for vesicular transport (Jenco et al., 1998). Additionally, α-syn appears to regulate dopamine release following a study in knockout mice where an electrical stimulation was induced to evaluate synaptic overflow (Abeliovich et al., 2000).

A family of proteins that act as chaperones assisting in kinase stability (Tzivion et al., 1998) and are present in the brain are the 14-3-3 family (Baxter et al., 2002). Furthermore, α-syn can bind to the 14-3-3 family and has a toxic effect when overexpressed (Ostrerova et al., 1999). Also, α-syn can bind to 14-3-3 ligands such as extracellular regulated kinases (ERK), Bcl-2-associated death promoter (BAD) and Protein Kinase C (PKC) (Ostrerova et al., 1999). Thus, α-syn accumulation could play a role in cell viability but further research is required (Ostrerova et al., 1999).

However, α-syn appears to have a protective role on nerve terminals. When Cysteine String Protein-alpha (CSP-α) (Zinsmaier, 2010) is artificially deleted and α-syn is present, it prevents neurodegeneration that would be expected to occur (Chandra et al., 2005). On the other hand, if α-syn is not present upon CSP-α artificially deficiency, it triggers lethal neurodegeneration leading to mice death (Chandra et al., 2005).

Despite being detected mostly in the brain, α-syn is also present in the blood, mostly (99%) in red blood cells (RBC) however its function remains unknown (Barbour et al., 2008). Furthermore, α-syn was detected in other body fluids such as cerebrospinal fluid (CSF) its origin remains unknown (El-Agnaf et al., 2003).

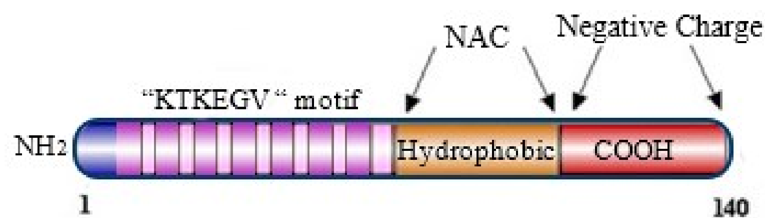


Figure 4: α-syn gene sequence showing the repeating motif "KTKEGV", a hydrophobic central domain (NAC) and a C-terminal (Tofaris & Spillatini (2005), adapted with permission from John Wiley & Sons - Books).

2.2. α-syn post-translational modifications

Post-translational modifications (PTMs) provide proteins a greater source of diversity and extend their possible functions (Duan, 2015). Molecularly, PTMs are responsible for altering the structure, conformation, size and charge of proteins (Clark et al., 2005). As a protein, α-syn undergoes this type of changes and diversity shifts that can be important to understand this protein physiology and pathological function (Schmid et al., 2013). Firstly, α-syn undergoes phosphorylation at the C-terminal domain, primarily at Ser-129 (Okochi et al., 2000). However, a secondary phosphorylation process occurs at residue 87 (Okochi et al., 2000). Casein kinase-1 (CK-1) and casein kinase-2 (CK-2) recognize the motif required to phosphorylate the serine residue 129 which are always flanked by acidic residues (Okochi et al., 2000). This PTM is a key factor in understanding α-syn pathological function because phosphorylated α-syn has been detected in Lewy Bodies (LB) inclusions (Anderson et al., 2006). Furthermore, upon phosphorylation at Ser-87, α-syn capability to bind to lipid membranes is reduced (Paleologou et al., 2010). Possibly since this PTM destabilizes the helical conformation of α-syn which in turn reduces lipid-binding affinity near the phosphorylation site (Paleologou et al., 2010).

Upon exposure to nitrating agents, nitration can occur forming a dityrosine by oxidation of tyrosine residues (Souza et al., 2000). Thus, it forms an extremely stable nitrated α-syn due to the covalent cross-linking connection bond (Souza et al., 2000).

Another PTM that can occur is ubiquitination which is a modification that targets protein function at proteasome degradation by ubiquitin (Pickart, 2001). Three enzymes are required to undergo ubiquitination: ubiquitin-activating enzyme (E1) that's responsible for the nucleophilic attack; a conjugating enzyme (E2) that binds to E1; a ligase (E3) that acts as an intermediate to transfer ubiquitin to lysine residues (Hershko et al., 1983).

At the N-terminal region, α-syn goes through acetylation (Anderson et al., 2006). During this process, α-syn shows an increase in lipid membrane binding capability due to it's α-helical propensity (Bartels et al., 2014). Furthermore, an in vitro study showed this modification to protect against toxicity and aggregation of α-syn by decreasing sirtuin 2 levels – an enzyme responsible for modulating aggregation and toxicity of α-syn (Oliveira et al., 2017).

After binding of small ubiquitin-like modifiers (SUMO) to lysine residues α-syn suffers a process called sumoylation (Geiss-Friedlander & Melchior, 2007). In α-syn,

sumoylation appears to decrease aggregation and thus, toxicity by preventing α-syn deconjugation that would lead to cell death (Krumova et al., 2011).

Lastly, $COOH^-$ and NH^+ groups can react in a way that leads to irrevocable modifications, originating the Advanced Glycation End Products (AGEs) (Brownlee et al., 1984). Regarding α-syn glycation, it appears to occur at the N-terminal region (Miranda et al., 2017). Glycation appears to affect α-syn extensively: it greatly reduces α-syn ubiquitination, increases oligomerization and induces loss of neuronal cells (Miranda et al., 2017).

2.3. α-syn and the oral cavity

The oral cavity presents itself as an easily and noninvasive way to collect samples for the study of potential biomarkers (Malamud, 2011; Yeh et al., 2010). Saliva can be collected without expensive medical setting and instruments (Yeh et al., 2010). Furthermore, it has several benefits besides the easy accessibility. For example, α-syn collection can be faulty when contaminated by blood even if just small amounts (Cao et al., 2019). Thus, the mouth seems to be an opportunity to collect a free of blood sample for analysis (Cao et al., 2019). Saliva sample handling is also less complex than other body fluids (e.g., blood) and the volume for analysis is considered small although it varies between analytes studied (Greabu, 2015).
Beyond its increased cerebral presence, α-syn was reported to be detected in salivary samples (Devic et al., 2011). How α-syn is transported into saliva is still unknown, yet a possible thesis is the production of α-syn through the nervous system of salivary glands (Devic et al., 2011). Alternatively, Devic et al. (2011) also proposed that salivary α-syn may be the derived from serum or CSF.

Al-Nimer et al. (2014), investigated how α-syn levels changed when comparing a sample of 20 healthy controls and 20 patients with Parkinson's disease (PD). Its conclusion was that salivary α-syn was significantly decreased in PD patients when compared to healthy controls (Al-Nimer et al., 2014). Furthermore, a larger cohort study in which a sample of saliva from 60 patients with diagnosed PD and 40 healthy controls was collected and found promising results. In this study, salivary α-syn_{total} was decreased in PD patients whilst α-$syn_{oligomeric}$ levels were increased (Vivacqua et al., 2016). This reduction is possibly due an early intracellular aggregation of α-syn in the brain in early stages of PD

patients (Cersosimo et al., 2012). However, Kang et al. (2016) work did not corroborate the results mentioned previously as it showed there were no statistical difference between α-syn$_{oligomeric}$ in PD patients and healthy controls (Kang et al., 2016). Thus, current studies are contradictory on salivary α-syn potential as a diagnostic marker (Goldman et al., 2018). It is important in further research to establish a homogenous analysis of saliva samples as it could be a reason for such disparities in results (Kang et al., 2016).

2.4. Disease implication and related conditions

Completely understanding α-syn function remains a challenge to date (Bendor et al., 2013). However, it is clear that it plays some role in neurodegenerative diseases such as PD, multiple system atrophy (MSA) and dementia with lewys bodies (DLB) (Bendor et al., 2013).

PD consists of a degeneration in dopaminergic neurons in the nigrostriatal pathway of the substantia nigra pars compacta (Miller et al., 2021) . This leads to a decrease of dopamine in the brain (Greenamyre & Hastings, 2004; Kouli et al., 2018). Other neurons seem to also be affected resulting in its heterogeneous characterization (Kouli et al., 2018). Furthermore, an accumulation of LB is present in PD consisting of pathological protein aggregates mostly of ɑ-syn (Miller et al., 2021). Therefore, dopaminergic neuron degeneration along with the presence of LB are considered the two major hallmarks for PD (Rizek et al., 2016).

MSA consists of a gliosis process in nigrostriatal and olivopontocerebellar structures along with cell loss. Contrarily to PD, MSA leads to an accumulation of fibrillar α-syn in oligodendrocytes forming glial cytoplasmic inclusions (Papp et al., 1989).

Lastly, α-syn plays a similar role in DLB and PD. Upon an autopsy, it is not possible to distinguish between the two conditions (McKeith, 2007). However, on a clinical standpoint, DLB patients present themselves with visuoperceptual, executive and attentional deterioration (McKeith, 2007). Furthermore, a decrease in attention and alertness are often present and severe (McKeith, 2007).

2.5. Potential biomarker for neurodegenerative diseases

Biomarkers are currently seen as elements with likely future importance for neurodegenerative diseases onset and progression, as a definitive diagnosis of PD or AD is currently only possible via postmortem autopsy (Weller, 2018; Samii et al., 2004). Therefore, markers of neurodegenerative diseases have gain particular interest for diagnosis, monitor disease's progression or to understand the response to a certain therapeutic intervention (Emamzadeh & Surguchov, 2018). Regarding α-syn related conditions, PD does not present a pathognomonic marker but rather an association of hallmarks with still uncertain consistency (Delenclos et al., 2016).

The most widely studied PD biomarkers are α-syn, Parkin, ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), PTEN Induced Kinase-1 (PINK1), Protein deglycase-1 (DJ-1) and Nuclear Receptor Subfamily 4 Group A Member 2 (NR4A2) genes on a genetic scope (Rachakonda et al., 2004). Furthermore, biochemical markers exist such as loss of dopamine transporters (Parkinson Study Group, 2000), accumulation of LB and biomarkers in blood and CSF such as α-syn (Miranda et al., 2017).

Each biomarker can be used to diagnose the disease in a certain stage (Rachakonda et al., 2004). A combination of several biomarkers may be key to help assist in diagnosing neurodegenerative diseases, but it remains a complementary source to clinic observation (Rachakonda et al., 2004).

3. Periodontitis and α-syn interplay

Despite the need for more studies to establish a clear association between neurodegenerative diseases and periodontitis, it is important to formulate hypotheses that may lead to this likely relationship. Therefore, several mechanisms have been proposed, including the interaction between the oral-gut axis leading to bacteria translocation from the oral cavity to the gut (Hajishengallis & Chavakis, 2021). This translocation occurs after a bacterial invasion of the periodontal pocket and dissemination to the bloodstream (Figure 5) (Kaur et al., 2016). In this case, *Porphyromonas gingivalis* disrupts the endothelial barrier increasing endothelial permeability and inducing proinflammatory cytokines, increased leukocyte extravasation and platelet aggregation (Hajishengallis & Chavakis, 2021). Another hypothesis concerns the activation of microglia cells (through bacterial production of IL-1β, Interleukin-12 (IL-12), TNF-α) and which consequently

triggers a neuroinflammatory process (Pasqualetti et al., 2015). If this condition persists it can lead to a neurodegeneration effect that persists as long as the chronic inflammatory situation continues (Liu & Hong, 2003).

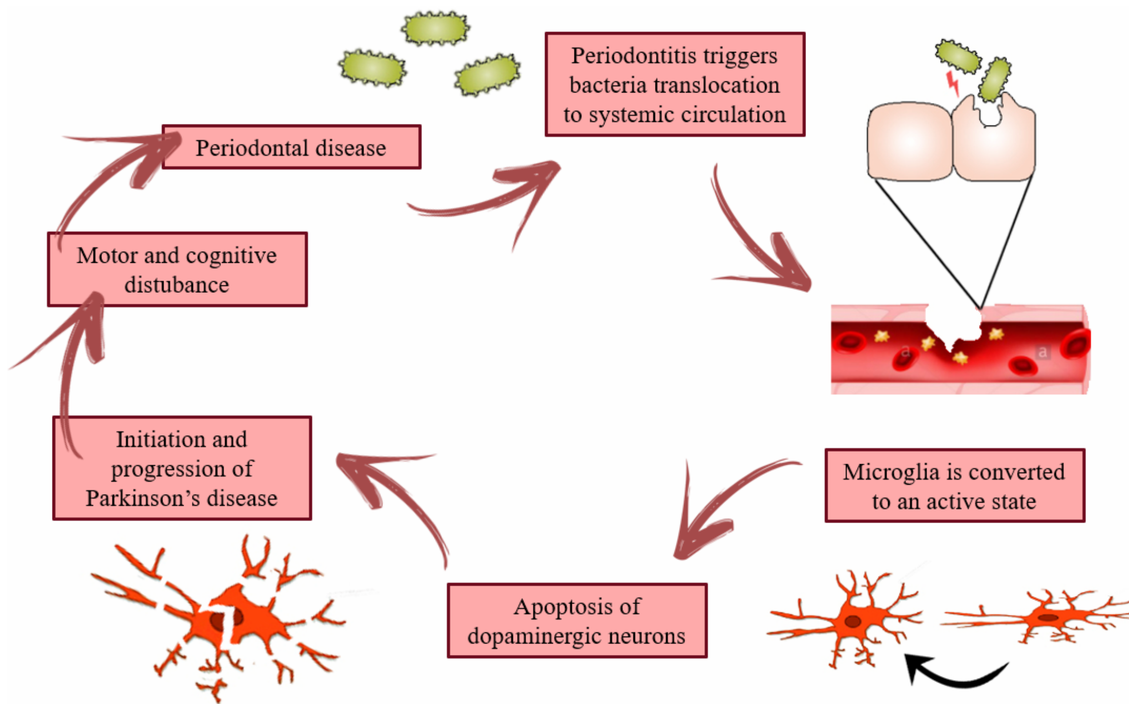


Figure 5: Proposed link between Periodontitis and Parkinson's Disease. Periodontitis bacteria translocation triggers a conversion process of the microglia into an active state. Thus, leading to necrosis or apoptosis of dopaminergic neurons and triggering PD's onset or aggravation. (by Kaur et al. 2016, adapted with permission from John Wiley & Sons - Books).

Beyond any reasonable hypothesis, there is the express need for basic research regarding associated molecules and clinical measures. For this reason, exploring whether a-syn levels may correlate with the periodontal status becomes imperative.

4. Aims

This study aims to explore the association between periodontitis and salivary α-syn. Thus, the following PECO questions was established: "In adult individuals, is periodontitis associated with salivary levels of α-syn?"

- P (Population): Adult individuals
- E (Exposure): Periodontitis
- C (Comparison): Patients with healthy periodontium
- O (Outcome): Salivary levels of α-syn

III. MATERIALS AND METHODS

3.1. Ethical considerations

The present study was approved by the Egas Moniz Ethics Committee (IRB number 939). All participants provided a signed informed consent prior to the beginning of the study. This research follows The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement (Elm et al., 2007).

3.2. Study type and setting

At the Egas Moniz Dental Clinic (EMDC), first-time incoming patients were invited to participate. After explaining the purpose of the study, a questionnaire was delivered, followed by collection of unstimulated saliva and periodontal examination. The questionnaire comprised sociodemographic (age and sex) and smoking habits data. The variable 'smoking habits' was categorized as: non-smoker (0); former smoker (1); or active smoker (2).

Salivary samples were prepared (see section 3.6.1) and then analyzed at the DysbrainD Laboratory from the Centro de Estudos de Doenças Crónicas (CEDOC), NOVA Medical School (see section 3.7).

3.4. Study sample

Patients' inclusion and samples collection began in March 2021 until July 2021.

3.5. Eligibility Criteria

In this study a number of inclusion criteria were set as follows: 18 years old or older; and, willing to participate in the study. Our exclusion criteria were patients:

1. Prior history of periodontal treatment in the past 12 months;
2. Systemic antibiotic treatment in the past 6 months prior to periodontal examination;
3. Taking corticosteroids and/or immunosuppressive agents in the last 3 months prior to periodontal examination;

4. Pregnant women or undergoing lactating phases (Mascarenhas, 2003);
5. Patients with salivary protein concentration < 1.00 mg/mL.

3.6. Collection of variables of interest

Patients were invited following the triage appointment. Study design was explained, informed consent was signed and the questionnaire applied. Following this, unstimulated saliva samples were collected following a validated approach described elsewhere (Kang et al., 2016). These samples were all taken between 8.00 and 11.00 a.m. in order to target confounding possibly from the circadian rhythm (Kang et al., 2016). Furthermore, all samples were collected before periodontal probing to reduce the possibility of blood contamination due to bleeding on probing (BoP).

3.6.1. Saliva collection

Before saliva collection the patient was asked about a certain type of behavior that may hinder saliva collection results. As such, a spreadsheet was filled that had information about alcohol or coffee consumption along with smoking in the last 12 hours. Furthermore, high activity practice was also registered along with taking high sugar or acidic food. It was also asked if the participant had not eaten or brushed his teeth in the last 60 minutes. Lastly, the oral cavity was examined for any oral disease.

After gathering this information, saliva collection took place accordingly. The patient was asked to rinse his mouth in order to remove food residues and wait 10 minutes to prevent diluting the saliva sample. The participant was then instructed to allow saliva to pool underneath the tongue and resist the urge to swallow it. The participant was also instructed to avoid blowing or spitting to avoid bubbles. Afterwards, it was instructed to lean forward and allow saliva to drip into the collection vial for 2 minutes or until a volume of 2mL was achieved. If any blood contamination was visible in the saliva sample, it was discarded and the process repeated.

Saliva samples were treated according to Kang et al. (2016) protocol. All samples were refrigerated with ice immediately after collection and a Protease Inhibitor Cocktail (100 µL/ 1 mL of whole saliva, Cat#P2714, Sigma Aldrich, St. Louis, MO, USA) was added. Next, the samples were vortexed twice. First at a low speed for 2.600 x g for 15 minutes

and then at a high speed for 15.000 x g for 15 minutes at 4ºC (Kang et al., 2016). Lastly, the samples were aliquoted in 60uL eppendorfs and stored at -80ºC until shipped for analysis to DysbrainD Laboratory from CEDOC, NOVA Medical School. Saliva collection is explained through an illustrative scheme in figure 6.

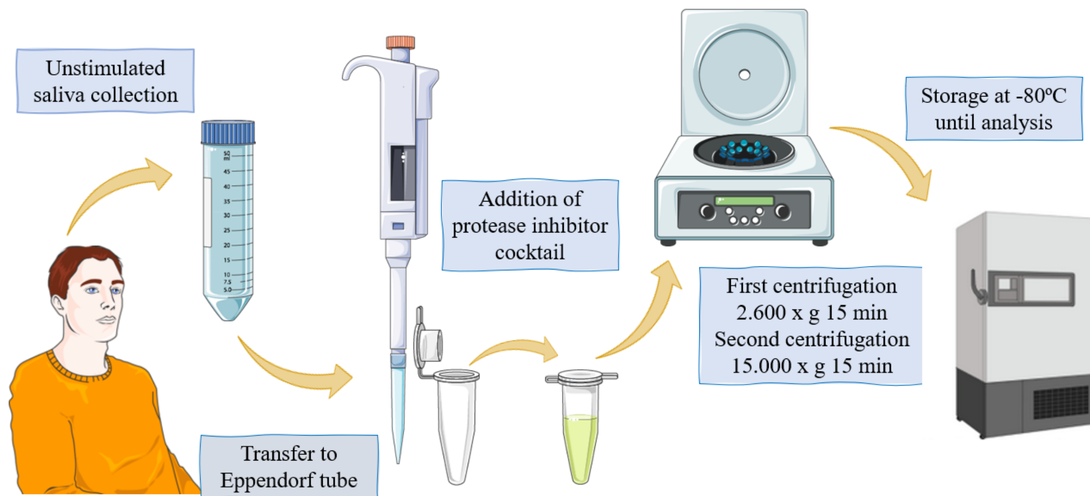


Figure 6: Saliva collection schematic process. Saliva was collected through unstimulated process and immediately placed on ice. Afterwards it was transferred to Eppendorf's with the help of a micropipette. Next a protein inhibition cocktail (100 µL/ 1 mL of whole saliva, Cat#P2714, Sigma Aldrich, St. Louis, MO, USA) was added and centrifuged at 2.600 x g 15min followed by 15.000 x g 15 min. Lastly the samples were stored until analysis at -80ºC. Original image.

3.6.2. Periodontal examination

Periodontal analysis consisted of a full-mouth examination performed by two previously calibrated examiners (J.B. and P.L.) (Botelho et al., 2019). Full-mouth periodontal examination has been proven to be more effective and more accurate than using other partial-mouth diagnostic strategies (Machado et al., 2018).

As such, probing depth, BoP, gingival margin level was evaluated in six locations per tooth (mesiobuccal, buccal, distobuccal, mesiolingual, lingual and distolingual) with a North Carolina periodontal probe (CP-15, Hu-Friedy; Chicago, Illanois, USA). The CP-15 probe followed the gingival sulcus through each tooth in a continuous motion to determine probing depth. BoP was assessed after visualization of hemorrhage upon completion of each quadrant probing. Further evaluation was performed regarding plaque index, gingival index in 4 locations per tooth (mesiobuccal, buccal, distobuccal and lingual). Next, when present, furcation anomalies were measured with a 2N color-coded

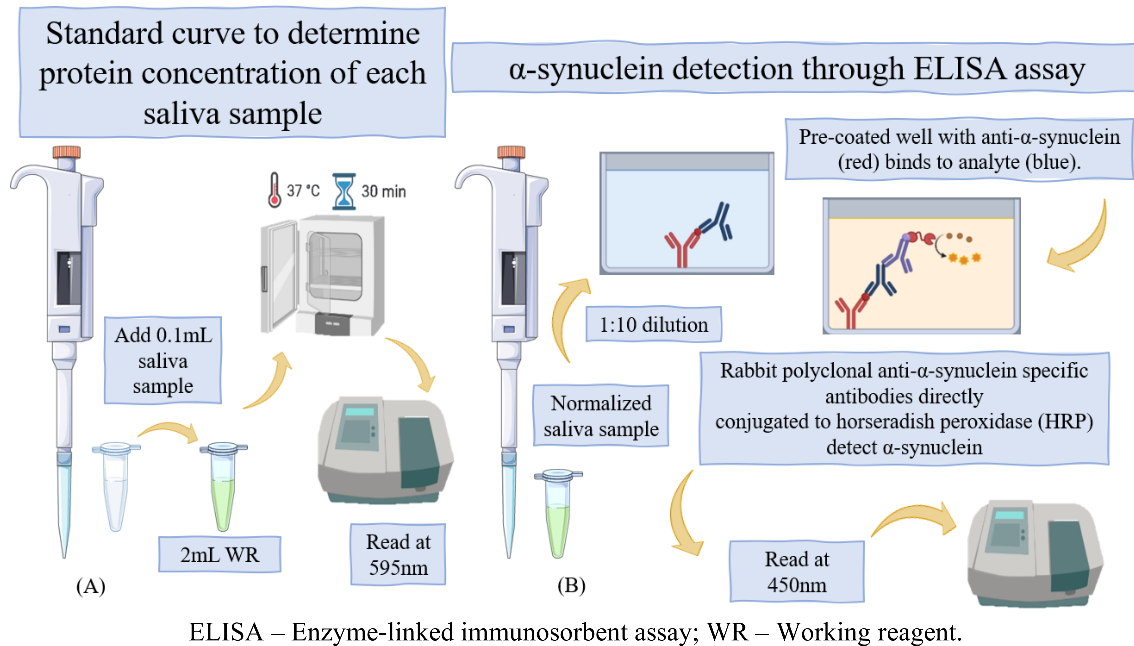
Nabers Probe (Hu-Friedy; Chicago, Illanois, USA) in molars (upper and lower) and upper premolars. Finally, tooth mobility was evaluated. Lastly, periodontitis cases were defined according to the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. As such, CAL ≥ 2mm nonadjacent teeth or buccal or oral CAL ≥ 3mm with pocketing > 3mm is detectable at ≥ 2 teeth were classified as Periodontal Diseases.

Also, PISA and PESA were estimated in Microsoft Excel spreadsheet, for each tooth in all subjects. The final sum accounted for all values from each present tooth. PISA and PESA are area surfaces measured in squared millimeters (mm^2) (Hujoel et al., 2001; Nesse et al., 2008).

3.7. A-syn quantification

Prior to the quantification of salivary α-syn levels, a standard bicinchoninic acid (BCA) protein assay was performed to measure the protein concentration. Samples evidencing less than 1.00 mg/mL were excluded from the enzyme-linked immunosorbent assay (ELISA) test, due to the risk of biased results.

Salivary α-syn levels were then measure through ELISA. Previously to analysis, salivary total protein was normalized through BCA Protein Assay Kit (Thermofisher Scientific, UK) by diluting saliva samples in 2mL of working reagent and incubating for 30minutes at 30ºC. Next, samples were read at 595nm through spectrophotometric measurement and a standard curve was elaborated. This was performed in order to avoid bias in salivary secretion rate and ensure the same amount of salivary protein was submitted to analysis. Furthermore, normalized samples were diluted in 1:10 following fabricant's instructions. To determine salivary α-syn levels we used anti-alpha synuclein quantitative ELISA kit (SensoLyte 55550). Finally, total α-syn concentration was assessed through spectrophotometric measurement at 450nm in an appropriate microplate reader. A summary of α-syn quantification is represented in figure 7.



ELISA – Enzyme-linked immunosorbent assay; WR – Working reagent.

Figure 7: Schematic representation of α-syn quantification. (A). First, saliva samples were normalized according to the manufacturer's protocol: 0.1mL saliva was mixed with 2mL of working reagent (WR) and incubated at 37ºC for 30 minutes. Next absorbance values were read through spectrophotometer analysis and elaborated a standard protein concentration curve. **(B).** Afterwards, diluted 1:10 normalized saliva samples were added to ELISA well's which were pre-coated with anti-α-synuclein monoclonal antibodies (red) to which α-syn (blue) will bind. Next rabbit polyclonal anti-α-synuclein specific antibodies directly conjugated to HRP detect α-synuclein. Lastly, it can be read in spectrophotometer at 495nm. Original image.

3.8. Statistical analysis

Data was uploaded into Microsoft Excel. A previously prepared sheet was used with an appropriate algorithm to assess the periodontal case definition. Descriptive measures are described as mean ± Standard deviation (SD) for continuous variables, and number of cases (n) and percentage (%) for categorical variables. As normality and homoscedasticity were not confirmed, Mann–Whitney was used to compare continuous variables. For categorical variables we compared baseline variables according to the periodontal status using Chi-square test. The correlation between PISA and PESA with α-syn were analyzed using Spearman's test. Additionally, to investigate the trend in PISA levels according to α-syn values in patients and according to gender, we explored a graph using scatterplots from ggplot2 package for R (version 4.0), and the tendency was computed and fitted via 'geom_smooth'. A significance level of 5% was set in all inferential analyses.

IV. RESULTS

From a preliminary sample of 109 patients referred to EMDC for a triage appointment, 42 patients were excluded after applying the eligibility criteria (Figure 6). As such, 66 patients underwent saliva collection. However, 19 of these participants could not reach a minimum required salivary protein concentration (1.00 mg/mL or higher) and were, therefore, excluded. Lastly, our final sample had a total of 47 participants.

Figure 8: Participant's flowchart.

Of the 47 patients analyzed, 27 had periodontitis of which 48,1% (n=13) were females, whilst the remaining 51.9% (n=14) were males. On the other hand, from the healthy participants (n=20) 45% were females (n=9) whereas 55% were males (n=11). Gender was not found to be statistically significantly when associated with periodontitis (p>0,001) With regard to age, patients with periodontal disease had a mean age of 53.4 years while healthy controls had 36.1 years. Age was a significant variable related to the periodontal status (p<0,001). Regarding smoking habits, there were 30 non-smoker individuals of which 48,1% (n=13) had periodontitis and 85% (n=17) were healthy. When concerning former smokers (n=10), 29,6% (n=8) had periodontitis whereas 10% (n=2) were disease free. Lastly, from 7 active smokers, 22.2% (n=6) had periodontal disease and 5% (n=1) were healthy. As such, we found a positive association between non-smoker and periodontal disease (p<0,001) whilst no association was found between former or active smokers (p>0,001) (Table 1).

Table 1: Participant's characteristics stratified according to periodontal disease

Variable	Periodontitis (n=27)	Healthy (n=20)	p-value	Total (n=47)
Age, mean (SD)	53.4 (18.0)	36.1 (17.1)	<0.001	
Gender, n (%)				
Female	13 (48.1)	9 (45.0)	0.694	22 (46.8)
Male	14 (51.9)	11 (55.0)		25 (53.2)
Smoking habits, n (%)				
Non-smoker	13 (48.1)	17 (85.0)	<0.001	30 (63.8)
Former smoker	8 (29.6)	2 (10.0)		10 (21.3)
Active smoker	6 (22.2)	1 (5.0)		7 (14.9)

n – number of participants; SD – Standard deviation.

In regard to periodontal characteristics, periodontal patients expectedly showed significantly higher levels of BoP, missing teeth, mean probing pocket depth and mean CAL than healthy participants ($p<0.001$). Lastly, PISA values did exhibit differences between periodontitis and non-periodontitis patients (Table 2).

Table 2: Periodontal clinical characteristics stratified according to periodontal disease.

	Periodontitis (n=27)	Healthy (n=20)	p-value
BoP, mean (SD) (%)	8.8 (12.6)	1.8 (3.7)	<0.001
Missing teeth, mean (SD)	8.4 (6.5)	2.3 (5.4)	<0.001
Mean PPD, mean (SD) (mm)	2.0 (0.6)	1.7 (0.3)	<0.001
Mean CAL, mean (SD) (mm)	0.2 (0.2)	0.1 (0.1)	<0.001
PESA, mean (SD) (mm^2)	198.0 (95.5)	211.0 (64.0)	0.473
PISA, mean (SD) (mm^2)	26.6 (37.6)	2.8 (6.7)	0.029

BoP- Bleeding on probing; CAL – Clinical attachment loss; n - number of participants; PESA – Periodontal epithelium surface area; PISA – Periodontal inflamed surface area; PPD – Probing pocket depth; SD – Standard deviation.

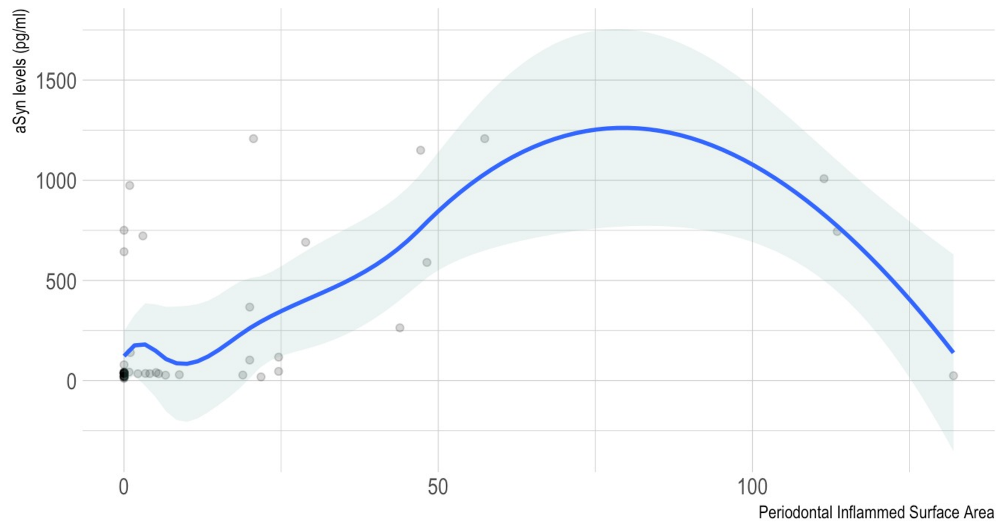
Table 3: α-syn levels stratified according to periodontal disease.

	Periodontitis (n=27)	Healthy (n=20)	p-value
α-syn levels (pg/mL), mean (SD)	340.5 (403.5)	125.0 (298.2)	0.514
Saliva protein concentration (mg/mL), mean (SD)	2.2 (1.2)	1.8 (1.4)	0.387

α-syn – alpha-synuclein; SD – Standard deviation; n – number of participants.

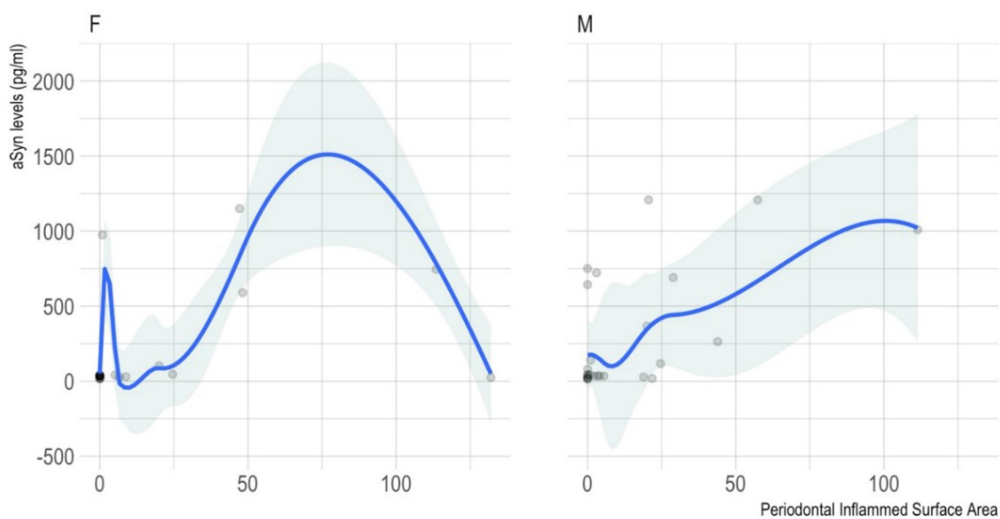
In regard to α-syn levels in periodontitis and healthy controls we did not find a statistically significant difference (p=0,514). Likewise, protein concentration was not statistically different between the two variables (p=0,387) (Table 3).

To understand the link between salivary α-syn levels and PISA values, we inspected the correlation of both variables (Figure 9) and according to gender (Figure 10). In the first scenario we found a moderate correlation (rho=0.442, p=0.005). When exploring this link graphically (Figure 10), two different curve trends were found between males and females.



α-synuclein – alpha-synuclein; PISA – Periodontal inflamed surface area.

Figure 9: Scatterplot displaying the overall correlation between α-syn levels and PISA.



α-synuclein – alpha-synuclein; F – Female participants; M – Male participants; PISA – Periodontal inflamed surface area.

Figure 10: Scatterplot displaying the correlation between α-syn levels and PISA according to gender.

V. DISCUSSION

We aimed to enlighten a possible relationship between periodontitis and salivary levels of α-syn when compared to healthy controls. To our understanding, this is the first study to evaluate a possible relationship between periodontal disease and salivary α-syn. Our study had varying degrees of periodontal disease from patients with mild, moderate and severe periodontitis (Wiebe & Putnins, 2000).

Periodontal disease is implicated in several conditions, mainly inflammatory diseases like hypertension (Aguilera et al., 2020), diabetes *mellitus* (Borgnakke et al., 2013) and rheumatoid arthritis (Hussain et al., 2020). Thus, it is important to understand how periodontitis could influence α-syn levels, a protein associated with neural inflammation (Pasqualetti et al., 2015).

The screened population sample age is higher in periodontitis compared to healthy controls, and these results align with previous results from a large representative study, held in the same geographic location as the EMDC (Botelho et al., 2019). Overall, the periodontal status did not exhibit an association with salivary levels of α-syn. However, when further exploring in detail some more comprehensive periodontal measures, we found a correlation between α-syn salivary levels and PISA. This could be an early sign of some relationship between periodontal inflammation and α-syn alterations, although more research is needed to confirm such results.

There are no previous studies that investigated α-syn and periodontal inflammation so comparing our results becomes a challenge. Despite lacking field work about periodontitis and α-syn, there are results from other neural markers and periodontal disease. According to the work of Ilievski et al. (2018), mice induced with periodontitis showed increased levels of amyloid beta (Aβ) – 42 which is linked to neural diseases like AD. Furthermore, mice with induced periodontitis showed increased levels of IL-6, IL-1β and TNF-α, proinflammatory cytokines, when compared to controls. Recently, Leira et al. (2020), found an association between periodontal disease and Aβ peptides that was mediated by systemic inflammation (Leira et al., 2020).

In regards to salivary α-syn, there are studies that aimed to understand its relationship with neurodegenerative diseases. Vivacqua et al. (2019), studied α-syn for the differential diagnosis of PD and found that total salivary α-syn was lower than in healthy controls. However, Al-Nimer et al. (2014) reported higher levels of α-syn in PD patients compared

to controls and Kang et al. (2016), did not find any difference in salivary levels of α-syn in PD and healthy controls.

A possible explanation for higher levels of α-syn could be through the nervous system of salivary glands (Devic et al., 2011). Furthermore, an autopsy-based work revealed the presence of α-syn pathology in salivary glands (Tredici et al., 2010). Despite lacking studies regarding salivary α-syn, it is suspected that this protein triggers an inflammatory response that can accelerate neuronal degeneration in diseases like PD (Hall et al., 2018). Thus, it is important to understand how periodontal inflammation can increase α-syn levels and how it reacts to periodontal treatment.

Ultimately, we showed that there is a correlation between periodontal inflammation and salivary levels of α-syn. These results raise several questions that must be answered in future studies. In the future, it becomes imperative to increment the number of participants studied, with a more detailed medical status and implementing periodontal treatment to see whether α-syn levels change across the periodontal therapy program.

5.1. Strengths and Limitations

Our study had several strengths such as the sample was collected randomly via direct approach at the EMDC first appointment before any dental treatment was executed, hence decreasing any possible confounding regarding periodontal status. Furthermore, we employed a full-mouth series probe screening protocol that is easily replicable to allow possible future comparison studies. Additionally, we assessed periodontal disease through a full mouth periodontal examination because it is more effective than other partial-mouth diagnostic strategies (Machado et al., 2018).

However, this work has some shortcomings worth mentioning. This study took place under extreme circumstances caused by the worldwide pandemic. Hence, our sample was significantly lower than what we would expect, thus a follow-up research is needed in the near future. This will allow us to validate the data assessed through this investigation and solidify the presented results. Furthermore, due to sample collection restrictions we were not able to exclude concomitant systemic diseases like diabetes and cardiovascular diseases which are known to hinder results due to oxidative stress and α-syn misfolding (Vivacqua et al., 2016). Additionally, ELISA analysis may underestimate the real salivary α-syn concentration, due to the antibodies used in the assay that are aimed at linear

epitopes of α-syn and fail to detect aggregated forms of α-syn (Vivacqua et al., 2019). A possible alternative is the quantification of blood samples. Lastly, our observation work design lacks the possibility to establish causality.

VI. CONCLUSION

In this preliminary clinical study, periodontal status presented some degree of association with α-syn salivary levels. A moderate correlation between α-syn levels and PISA was observed. Further research is paramount to validate our results and study whether periodontal treatment might exert some effect on α-syn salivary levels.

6.1. Future perspectives

It is important to replicate this study to achieve a larger sample and verify the results obtained. Moreover, it is important that other studies focus on patients without any diagnosed disease that could hinder results. An effort should be made to standardize saliva collection as well as sample processing. Furthermore, periodontal examination should follow a similar path with a special focus on full mouth records for a complete and correct classification.

VII. REFERENCES

Abeliovich, A., Schmitz, Y., Fariñas, I., Choi-Lundberg, D., Ho, W.-H., Castillo, P. E., Shinsky, N., Verdugo, J. M. G., Armanini, M., Ryan, A., Hynes, M., Phillips, H., Sulzer, D., & Rosenthal, A. (2000). Mice lacking α-synuclein display functional deficits in the nigrostriatal dopamine system. *Neuron*, *25*(1), 239–252. https:/doi.org/10.1016/s0896-6273(00)80886-7.

Aguilera, E. M., Suvan, J., Buti, J., Czesnikiewicz-Guzik, M., Ribeiro, A. B., Orlandi, M., Guzik, T. J., Hingorani, A. D., Nart, J., & D'Aiuto, F. (2020). Periodontitis is associated with hypertension: A systematic review and meta-analysis. *Cardiovascular Research*, *116*(1), 28–39. https://doi.org/10.1093/cvr/cvz201.

Albandar, J. M., & Rams, T. E. (2002). Global epidemiology of periodontal diseases: An overview. *Periodontology 2000*, *29*(1), 7–10. https://doi.org/10.1034/j.1600-0757.2002.290101.x.

Al-Hashimi, I., & Levine, M. J. (1989). Characterization of in vivo salivary-derived enamel pellicle. *Archives of Oral Biology*, *34*(4), 289–295. https://doi.org/10.1016/0003-9969(89)90070-8.

Al-Nimer, M. S., Mshatat, S. F., & Abdulla, H. I. (2014). Saliva α-synuclein and a high extinction coefficient protein: A novel approach in assessment biomarkers of Parkinson's disease. *North American Journal of Medical Sciences, 6*(12), 633. https://dx.doi.org/10.4103%2F1947-2714.147980.

Anderson, J. P., Walker, D. E., Goldstein, J. M., Laat, R., Banducci, K., Caccavello, R. J., Barbour, R., Huang, J., Kling, K., Lee, M., Diep, L., Keim, P. S., Shen, X., Chataway, T., Schlossmacher, M. G., Seubert, P., Schenk, D., Sinha, S., Gai, W. P., & Chilcote, T. J. (2006). Phosphorylation of Ser-129 is the dominant pathological modification of alpha-synuclein in familial and sporadic Lewy body disease. *The*

Journal of Biological Chemistry, *281*(40), 29739–29752. https://doi.org/10.1074/jbc.m600933200.

Barbour, R., Kling, K., Anderson, J. P., Banducci, K., Cole, T., Diep, L., Fox, M., Goldstein, J. M., Soriano, F., Seubert, P., & Chilcote, T. J. (2008). Red blood cells are the major source of alpha-synuclein in blood. *Neuro-Degenerative Diseases*, *5*(2), 55–59. https://doi.org/10.1159/000112832.

Bartels, T., Kim, N. C., Luth, E. S., & Selkoe, D. J. (2014). N-alpha-acetylation of α-synuclein increases its helical folding propensity, GM1 binding specificity and resistance to aggregation. *PloS One*, *9*(7), 1-10. https://doi.org/10.1371/journal.pone.0103727.

Baxter, H. C., Fraser, J. R., Liu, W. G., Forster, J. L., Clokie, S., Steinacker, P., Otto, M., Bahn, E., Wiltfang, J., & Aitken, A. (2002). Specific 14-3-3 isoform detection and immunolocalization in prion diseases. *Biochemical Society Transactions*, *30*(4), 387–391. https://doi.org/10.1042/bst0300387.

Bendor, J. T., Logan, T. P., & Edwards, R. H. (2013). The function of α-synuclein. *Neuron*, *79*(6), 1044–1066. https://doi.org/10.1016/j.neuron.2013.09.004.

Birkedal-Hansen, H. (1993). Role of cytokines and inflammatory mediators in tissue destruction. *Journal of Periodontal Research*, *28*(6). https://doi.org/10.1111/j.1600-0765.1993.tb02113.x.

Borges, T. D. F., Regalo, S. C., Taba, M., Siéssere, S., Mestriner, W., & Semprini, M. (2013). Changes in masticatory performance and quality of life in individuals with chronic periodontitis. *Journal of periodontology, 84*(3), 325-331. https://doi.org/10.1902/jop.2012.120069.

Borgnakke, W. S., Ylostalo, P. V., Taylor, G. W., & Genco, R. J. (2013). Effect of periodontal disease on diabetes: Systematic review of epidemiologic observational

evidence. *Journal of Periodontology*, *84*(4), S135–S152. https://doi.org/10.1902/jop.2013.1340013.

Botelho, J., Machado, V., Leira, Y., Proença, L., Chambrone, L., & Mendes, J. J. (2021). Economic burden of periodontitis in the United States of America and Europe–an updated estimation. *Journal of Periodontology.* https://doi.org/10.1002/jper.21-0111.

Botelho, J., Machado, V., Proença, L., Alves, R., Cavacas, M. A., Amaro, L., & Mendes, J. J. (2019). Study of periodontal health in Almada-Seixal (SoPHiAS): A cross-sectional study in the Lisbon metropolitan area. *Scientific Reports*, *9*(1), 1-10. https://doi.org/10.1038/s41598-019-52116-6.

Botelho, J., Machado, V., Proença, L., Bellini, D. H., Chambrone, L., Alcoforado, G., & Mendes, J. J. (2020). The impact of nonsurgical periodontal treatment on oral health-related quality of life: A systematic review and meta-analysis. *Clinical Oral Investigations, 24(*2), 585-596. https://doi.org/10.1007/s00784-019-03188-1.

Brownlee, M., Vlassara, H., & Cerami, A. (1984). Nonenzymatic glycosylation and the pathogenesis of diabetic complications. *Annals of Internal Medicine*, *101*(4), 527–537. https://doi.org/10.7326/0003-4819-101-4-527.

Buset, S. L., Walter, C., Friedmann, A., Weiger, R., Borgnakke, W. S., & Zitzmann, N. U. (2016). Are periodontal diseases really silent? A systematic review of their effect on quality of life. *Journal of Clinical Periodontology, 43*(4), 333-344. https://doi.org/10.1111/jcpe.12517.

Campos, B. O. D., Fischer, R. G., Gustafsson, A., & Figueredo, C. M. D. S. (2012). Effectiveness of non-surgical treatment to reduce IL-18 levels in the gingival crevicular fluid of patients with periodontal disease. *Brazilian Dental Journal, 23*, 428-432. https://doi.org/10.1590/s0103-64402012000400020.

Cao, Z., Wu, Y., Liu, G., Jiang, Y., Wang, X., Wang, Z., & Feng, T. (2019). α-synuclein in salivary extracellular vesicles as a potential biomarker of Parkinson's disease. *Neuroscience Letters, 696*, 114-120. https://doi.org/10.1016/j.neulet.2018.12.030.

Cekici, A., Kantarci, A., Hasturk, H., & Dyke, T. E. V. (2014). Inflammatory and immune pathways in the pathogenesis of periodontal disease. *Periodontology 2000*, *64*(1), 57–80. https://doi.org/10.1111/prd.12002.

Cersosimo, M. G., & Benarroch, E. E. (2012). Pathological correlates of gastrointestinal dysfunction in Parkinson's disease. *Neurobiology of disease, 46(*3), 559-564. https://doi.org/10.1016/j.nbd.2011.10.014.

Chandra, S., Gallardo, G., Fernández-Chacón, R., Schlüter, O. M., & Südhof, T. C. (2005). Alpha-synuclein cooperates with CSPalpha in preventing neurodegeneration. *Cell*, *123*(3), 383–396. https://doi.org/10.1016/j.cell.2005.09.028.

Clark, R. S. B., Bayir, H., & Jenkins, L. W. (2005). Posttranslational protein modifications. *Critical Care Medicine*, *33*(12), S407–S409. https://doi.org/10.1097/01.ccm.0000191712.96336.51.

Cochran, D. L. (2008). Inflammation and bone loss in periodontal disease. *Journal of Periodontology*, *79*(8), 1569–1576. https://doi.org/10.1902/jop.2008.080233.

Darveau, R. P. (2010). Periodontitis: A polymicrobial disruption of host homeostasis. *Nature Reviews. Microbiology*, *8*(7), 481–490. https://doi.org/10.1038/nrmicro2337.

Dayer, J. M., Feige, U., Edwards, C. K., & Burger, D. (2001). Anti–interleukin-1 therapy in rheumatic diseases. *Current Opinion in Rheumatology*, *13*(3), 170–176. https://doi.org/10.1097/00002281-200105000-00004.

Delenclos, M., Jones, D. R., McLean, P. J., & Uitti, R. J. (2016). Biomarkers in

Parkinson's disease: Advances and strategies. *Parkinsonism & Related Disorders*, *22*(1), 106–110. https://doi.org/10.1016/j.parkreldis.2015.09.048.

Devic, I., Hwang, H., Edgar, J. S., Izutsu, K., Presland, R., Pan, C., Goodlett, D. R., Wang, Y., Armaly, J., Tumas, V., Zabetian, C. P., Leverenz, J. B., Shi, M., & Zhang, J. (2011). Salivary α-synuclein and DJ-1: Potential biomarkers for Parkinson's disease. *Brain: A Journal of Neurology*, *134*(7), 1-5. https://doi.org/10.1093/brain/awr015.

Duan, G. D. W. (2015). The roles of post-translational modifications in the context of protein interaction networks. *PLoS Computational Biology*, *11*(2), 1-23. https://doi.org/10.1371/journal.pcbi.1004049.

Dudley, D. J. (1997). Pre-term labor: An intra-uterine inflammatory response syndrome? *Journal of Reproductive Immunology*, *36*(1-2), 93–109. https://doi.org/10.1016/s0165-0378(97)00065-x.

Ebersole, J. L., Kirakodu, S., Novak, M. J., Stromberg, A. J., Shen, S., Orraca, L., Gonzalez-Martinez, J., Burgos, A., & Gonzalez, O. A. (2014). Cytokine gene expression profiles during initiation, progression and resolution of periodontitis. *Journal of Clinical Periodontology,* *41*(9), 853-861. https://doi.org/10.1111/jcpe.12286.

Eke, P. I., Dye, B. A., Wei, L., Slade, G. D., Thornton-Evans, G. O., Borgnakke, W. S., Taylor, G. W., Page, R. C., Beck, J. D., & Genco, R. J. (2015). Update on prevalence of periodontitis in adults in the United States: NHANES 2009 to 2012. *Journal of Periodontology*, *86*(5), 611–622. https://doi.org/10.1902/jop.2015.140520.

Eke, P. I., Dye, B. A., Wei, L., Thornton-Evans, G. O., & Genco, R. J. (2012). Prevalence of periodontitis in adults in the United States: 2009 and 2010. *Journal of Dental Research, 91*(10), 914-920. https://doi.org/10.1177/0022034512457373.

El-Agnaf, O. M. A., Salem, S. A., Paleologou, K. E., Cooper, L. J., Fullwood, N. J.,

Gibson, M. J., Curran, M. D., Court, J. A., Mann, D. M. A., Ikeda, S. I., Cookson, M. R., Hardy, J., & Allsop, D. (2003). Alpha-synuclein implicated in Parkinson's disease is present in extracellular biological fluids, including human plasma. *FASEB Journal, 17*(13), 1-16. https://doi.org/10.1096/fj.03-0098fje.

Elm, E. V., Altman, D. G., Egger, M., Pocock, S. J., Gøtzsche, P. C., & Vandenbroucke, J. P. (2007). The strengthening the reporting of observational studies in epidemiology (STROBE) statement: Guidelines for reporting observational studies. *The Lancet*, *370*(9596), 1453–1457. https://doi.org/10.1136/bmj.39335.541782.AD.

Emamzadeh, F. N., & Surguchov, A. (2018). Parkinson's disease: Biomarkers, treatment, and risk factors. *Frontiers in Neuroscience*, *12, 1-14*. https://doi.org/10.3389/fnins.2018.00612.

Finoti, L. S., Nepomuceno, R., Pigossi, S. C., Corbi, S. C., Secolin, R., & Scarel-Caminaga, R. M. (2017). Association between interleukin-8 levels and chronic periodontal disease: A PRISMA-compliant systematic review and meta-analysis. *Medicine*, *96*(22), 1-8. https://doi.org/10.1097/md.0000000000006932.

Gabay, C. (2006). Interleukin-6 and chronic inflammation. *Arthritis Research & Therapy*, *8*(2), 1-6. https://doi.org/10.1186/ar1917.

Gargiulo, A. W., Wentz, F. M., & Orban, B. (1961). Dimensions and relations of the dentogingival junction in humans. *Journal of Periodontology*, *32*(3), 261–267. https://doi.org/10.1902/jop.1961.32.3.261.

Geiss-Friedlander, R., & Melchior, F. (2007). Concepts in sumoylation: A decade on. *Nature Reviews Molecular Cell Biology*, *8*(12), 947–956. https://doi.org/10.1038/nrm2293.

Gemmell, E., & Seymour, G. J. (1998). Cytokine profiles of cells extracted from humans with periodontal diseases. *Journal of Dental Research*, *77*(1), 16–26.

https://doi.org/10.1177/00220345980770010101.

George, J. M., Jin, H., Woods, W. S., & Clayton, D. F. (1995). Characterization of a novel protein regulated during the critical period for song learning in the zebra finch. *Neuron*, *15*(2), 361–372. https://doi.org/10.1016/0896-6273(95)90040-3.

Goldman, J. G., Andrews, H., Amara, A., Naito, A., Alcalay, R. N., Shaw, L. M., Taylor, P., Xie, T., Tuite, P., Henchcliffe, C., Hogarth, P., Frank, S., Saint-Hilaire, M. H., Frasier, M., Arnedo, V., Reimer, A. N., Sutherland, M., Swanson-Fischer, C., Gwinn, K., & Kang, U. J. (2018). Cerebrospinal fluid, plasma, and saliva in the BioFIND study: Relationships among biomarkers and Parkinson's disease features. *Movement Disorders, 33*(2), 282–288. https://doi.org/10.1002/mds.27232.

Górska, R., Gregorek, H., Kowalski, J., Laskus-Perendyk, A., Syczewska, M., & Madaliński, K. (2003). Relationship between clinical parameters and cytokine profiles in inflamed gingival tissue and serum samples from patients with chronic periodontitis. *Journal of Clinical Periodontology, 30*(12), 1046-1052. https://doi.org/10.1046/j.0303-6979.2003.00425.x.

Graves, D. T., Oskoui, M., Volejnikova, S., Naguib, G., Cai, S., Desta, T., Kakouras, A., & Jiang, Y. (2001). Tumor necrosis factor modulates fibroblast apoptosis, PMN recruitment, and osteoclast formation in response to P. gingivalis infection. *Journal of Dental Research*, *80*(10), 1875–1879. https://doi.org/10.1177/00220345010800100301.

Greabu, M., & Calenic, B. (2015). Saliva: A diagnosis fluid for oral and general diseases. *Organic Chemistry: Current Research, 4*(1), 1-4. https://doi.org/10.4172/2161-0401.1000131.

Greenamyre, J. T., & Hastings, T. G. (2004). Biomedicine. Parkinson's divergent causes, convergent mechanisms. *Science*, *304*(5674), 1120–1122.

http://dx.doi.org/10.1126/science.1098966.

Hajishengallis, G., & Chavakis, T. (2021). Local and systemic mechanisms linking periodontal disease and inflammatory comorbidities. *Nature Reviews Immunology 21*, 426-440. https://doi.org/10.1038/s41577-020-00488-6.

Hall, S., Janelidze, S., Surova, Y., Widner, H., Zetterberg, H., & Hansson, O. (2018). Cerebrospinal fluid concentrations of inflammatory markers in Parkinson's disease and atypical parkinsonian disorders. *Scientific reports, 8*(1), 1-9. https://doi.org/10.1038/s41598-018-31517-z.

Hershko, A., Heller, H., Elias, S., & Ciechanover, A. (1983). Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown. *Journal of Biological Chemistry*, *258*(13), 8206–8214. https://doi.org/10.1016/s0021-9258(20)82050-x.

Highfield, J. (2009). Diagnosis and classification of periodontal disease. *Australian Dental Journal*, *54*(1), S11–S26. https://doi.org/10.1111/j.1834-7819.2009.01140.x.

Hujoel, P. P., White, B. A., Garcia, R. I., & Listgarten, M. A. (2001). The dentogingival epithelial surface area revisited. *Journal of Periodontal Research, 36*(1), 48-55. https://doi.org/10.1034/j.1600-0765.2001.00011.x.

Hussain, S. B., Botelho, J., Machado, V., Zehra, S. A., Mendes, J. J., Ciurtin, C., Orlandi, M., & D'Aiuto, F. (2020). Is there a bidirectional association between rheumatoid arthritis and periodontitis? A systematic review and meta-analysis. *Seminars in Arthritis and Rheumatism*, *50*(3), 414–422. https://doi.org/10.1016/j.semarthrit.2020.01.009

Ilievski, V., Zuchowska, P. K., Green, S. J., Toth, P. T., Ragozzino, M. E., Le, K., Aljewari, H. W., O'Brien-Simpson, N., Reynolds, E., & Watanabe, K. (2018). Chronic oral application of a periodontal pathogen results in brain inflammation,

neurodegeneration and amyloid beta production in wild type mice. *PLoS One, 13*(10), 1-24.

Jakes, R., Spillantini, M. G., & Goedert, M. (1994). Identification of two distinct synucleins from human brain. *FEBS Letters*, *345*(1), 27–32. https://doi.org/10.1016/0014-5793(94)00395-5.

Janeway, C. A., Travers, P., Walport, M., & Shlomchik, M. J. (2001). The complement system and innate immunity. In *Immunobiology: The Immune System in Health and Disease. (5th ed)*. Garland Science. https://doi.org/10.5860/choice.32-3876.

Jenco, J. M., Rawlingson, A., Daniels, B., & Morris, A. J. (1998). Regulation of phospholipase D2: Selective inhibition of mammalian phospholipase D isoenzymes by alpha- and beta-synucleins. *Biochemistry*, *37*(14), 4901–4909. https://doi.org/10.1021/bi972776r.

Kang, W., Chen, W., Yang, Q., Zhang, L., Zhang, L., Wang, X., Dong, F., Zhao, Y., Chen, S., Quinn, T. J., Zhang, J., Chen, S., & Liu, J. (2016). Salivary total α-synuclein, oligomeric α-synuclein and SNCA variants in Parkinson's disease patients. *Scientific Reports*, *6*(1), 1-8. https://dx.doi.org/10.1038%2Fsrep28143.

Kassebaum, N. J., Bernabé, E., Dahiya, M., Bhandari, B., Murray, C. J. L., & Marcenes, W. (2014). Global burden of severe periodontitis in 1990-2010: A systematic review and meta-regression. *Journal of Dental Research, 93*(11), 1045-1053. https://doi.org/10.1177/0022034514552491.

Kaur, T., Uppoor, A., & Naik, D. (2016). Parkinson's disease and periodontitis - the missing link? A review. *Gerodontology*, *33*(4), 434–438. https://doi.org/10.1111/ger.12188.

Köseoğlu, S., Hatipoğlu, M., Sağlam, M., Enhoş, Ş., & Esen, H. H. (2015). Interleukin-33 could play an important role in the pathogenesis of periodontitis. *Journal of*

Periodontal Research, 50(4), 525-534. https://doi.org/10.1111/jre.12235.

Kouli, A., Torsney, K. M., & Kuan, W. L. (2018). Parkinson's disease: Etiology, neuropathology, and pathogenesis. In *Parkinson's disease: Pathogenesis and Clinical Aspects*. Codon Publications. https://doi.org/10.15586/codonpublications.parkinsonsdisease.2018.ind.

Krumova, P., Meulmeester, E., Garrido, M., Tirard, M., Hsiao, H. H., Bossis, G., Urlaub, H., Zweckstetter, M., Kügler, S., Melchior, F., Bähr, M., & Weishaupt, J. H. (2011). Sumoylation inhibits α-synuclein aggregation and toxicity. *The Journal of Cell Biology*, *194*(1), 49–60. https://doi.org/10.1083/jcb.201010117.

Lang, N. P., & Lindhe, J. (2015). *Clinical Periodontology and Implant Dentistry*. (6th ed.). John Wiley & Sons. https://doi.org/10.1097/id.0000000000000679.

Lavedan, C., Leroy, E., Dehejia, A., Buchholtz, S., Dutra, A., Nussbaum, R. L., & Polymeropoulos, M. H. (1998). Identification, localization and characterization of the human gamma-synuclein gene. *Human Genetics*, *103*(1), 106–112. https://doi.org/10.1007/s004390050792.

Lavu, V., Venkatesan, V., Lakkakula, B. V. K. S., Venugopal, P., Paul, S. F. D., & Rao, S. R. (2015). Polymorphic regions in the interleukin-1 gene and susceptibility to chronic periodontitis: a genetic association study. *Genetic Testing and Molecular Biomarkers, 19*(4), 175-181. https://doi.org/10.1089/gtmb.2014.0275.

Leira, Y., Domínguez, C., Seoane, J., Seoane-Romero, J., Pías-Peleteiro, J. M., Takkouche, B., Blanco, J., & Aldrey, J. M. (2017). Is periodontal disease associated with Alzheimer's disease? A systematic review with meta-analysis. *Neuroepidemiology*, *48*(1-2), 21–31. https://doi.org/10.1159/000458411.

Leira, Y., Carballo, Á., Orlandi, M., Aldrey, J. M., Pías-Peleteiro, J. M., Moreno, F., Vásquez-Vásquez, L., Campos, F., D'Aiuto, F., Castillo, J., Sobrino, T., & Blanco,

J. (2020). Periodontitis and systemic markers of neurodegeneration: A case–control study. *Journal of Clinical Periodontology, 47*(5), 561-571.

Listl, S., Galloway, J., Mossey, P. A., & Marcenes, W. (2015). Global economic impact of dental diseases. *Journal of Dental Research, 94*(10), 1355-1361. https://doi.org/10.1177/0022034515602879.

Liu, B., & Hong, J. S. (2003). Role of microglia in inflammation-mediated neurodegenerative diseases: Mechanisms and strategies for therapeutic intervention. *The Journal of Pharmacology and Experimental Therapeutics*, *304*(1), 1–7. http://dx.doi.org/10.1124/jpet.102.035048.

Loos, B. G., Craandijk, J., Hoek, F. J., Wertheim-van, P. M., & Velden, U. V. D. (2000). Elevation of systemic markers related to cardiovascular diseases in the peripheral blood of periodontitis patients. *Journal of Periodontology*, *71*(10), 1528–1534. https://doi.org/10.1902/jop.2000.71.10.1528.

Machado, V., Botelho, J., Mascarenhas, P., Cavacas, M. A., Alves, R., & Mendes, J. J. (2018). Partial recording protocols performance on the assessment of periodontitis severity and extent: bias magnitudes, sensibility, and specificity. *Revista Portugesa de Estomatologia, 59*(3), 145–153. http://dx.doi.org/10.24873/j.rpemd.2018.11.239.

Machado, V., Escalda, C., Proença, L., Mendes, J. J., & Botelho, J. (2020). Is there a bidirectional association between polycystic ovarian syndrome and periodontitis? A systematic review and meta-analysis. *Journal of Clinical Medicine*, *9*(6), 1-16. https://doi.org/10.3390/jcm9061961.

Madureira, D. F., Lima, I. L. D. A., Costa, G. C., Lages, E. M. B., Martins, C. C., & Silva, T. A. (2018). Tumor necrosis factor-alpha in gingival crevicular fluid as a diagnostic marker for periodontal diseases: A systematic review. *Journal of Evidence Based Dental Practice, 18*(4), 315-331. https://doi.org/10.1016/j.jebdp.2018.04.001.

Malamud, D. (2011). Saliva as a diagnostic fluid. *Dental Clinics of North America, 55*(1), 159–178. https://doi.org/10.1016/j.cden.2010.08.004.

Mantovani, A., Dinarello, C. A., Molgora, M., & Garlanda, C. (2019). Interleukin-1 and related cytokines in the regulation of inflammation and immunity. *Immunity, 50*(4), 778-795. https://doi.org/10.1016/j.immuni.2019.03.012.

Maroteaux, L., & Scheller, R. H. (1991). The rat brain synucleins; family of proteins transiently associated with neuronal membrane. *Molecular Brain Research*, *11*(3-4), 335–343. https://doi.org/10.1016/0169-328x(91)90043-w.

Mascarenhas, P., Gapski, R., Al-Shammari, K., & Wang, H. L. (2003). Influence of sex hormones on the periodontium. *Journal of Clinical Periodontology, 30(*8), 671-681. https://doi.org/10.1034/J.1600-051X.2003.00055.X.

McKeith, I. (2007). Dementia with Lewy bodies. *Handbook of Clinical Neurology*, *84*(3), 531–548. https://doi.org/10.1016/S0072-9752(07)84060-7.

Miller, K. M., Mercado, N. M., & Sortwell, C. E. (2021). Synucleinopathy-associated pathogenesis in Parkinson's disease and the potential for brain-derived neurotrophic factor. *Npj Parkinson's Disease*, *7*(1), 1–9. https://doi.org/10.1038/s41531-021-00179-6.

Miranda, H. V., Cássio, R., Correia-Guedes, L., Gomes, M. A., Chegão, A., Miranda, E., Soares, T., Coelho, M., Rosa, M. M., Ferreira, J. J., & Outeiro, T. F. (2017). Posttranslational modifications of blood-derived alpha-synuclein as biochemical markers for Parkinson's disease. *Scientific Reports*, *7*(1), 1–11. https://doi.org/10.1038/s41598-017-14175-5.

Miranda, H., Szego, É. M., Oliveira, L. M. A., Breda, C., Darendelioglu, E., Oliveira, R. M., Ferreira, D. G., Gomes, M. A., Rott, R., Oliveira, M., Munari, F., Enguita, F. J., Simões, T., Rodrigues, E. F., Heinrich, M., Martins, I. C., Zamolo, I., Riess, O.,

Cordeiro, C., Ponces-Freire, A., Lashuel, H. A., Santos, N. C., Lopes, L. V., Xiang, W., Jovin, T. M., Penque, D., Engelender, S., Zweckstetter, M., Klucken, J., Giorgini, F., Quintas, A., & Outeiro, T. F. (2017). Glycation potentiates α-synuclein-associated neurodegeneration in synucleinopathies. *Brain*, *140*(5), 1399–1419. https://doi.org/10.1093/brain/awx056.

Nakashima, T., Kobayashi, Y., Yamasaki, S., Kawakami, A., Eguchi, K., Sasaki, H., & Sakai, H. (2000). Protein expression and functional difference of membrane-bound and soluble receptor activator of NF-κB ligand: Modulation of the expression by osteotropic factors and cytokines. *Biochemical and Biophysical Research Communications*, *275*(3), 768–775. https://doi.org/10.1006/bbrc.2000.3379.

Nesse, W., Abbas, F., Ploeg, I. V. D., Spijkervet, F. K. L., Dijkstra, P. U., & Vissink, A. (2008). Periodontal inflamed surface area: Quantifying inflammatory burden. *Journal of Clinical Periodontology, 35*(8), 668-673. https://doi.org/10.1111/j.1600-051x.2008.01249.x.

Nishihara, T., & Koseki, T. (2004). Microbial etiology of periodontitis. *Periodontology 2000*, *36*(1), 14–26. https://doi.org/10.1111/j.1600-0757.2004.03671.x.

Okochi, M., Walter, J., Koyama, A., Nakajo, S., Baba, M., Iwatsubo, T., Meijer, L., Kahle, P. J., & Haass, C. (2000). Constitutive phosphorylation of the Parkinson's disease associated α-synuclein. *The Journal of Biological Chemistry*, *275*(1), 390–397. https://doi.org/10.1074/jbc.275.1.390.

Oliveira, R. M., Miranda, H. V., Francelle, L., Pinho, R., Szegö, É. M., Martinho, R., Munari, F., Lázaro, D. F., Moniot, S., Guerreiro, P., Fonseca, L., Marijanovic, Z., Antas, P., Gerhardt, E., Enguita, F. J., Fauvet, B., Penque, D., Pais, T. F., Tong, Q., Becker, S., Kugler, S., Lashuel, H. A., Steegborn, C., Zweckstetter, M. & Outeiro, T. F. (2017). The mechanism of sirtuin 2–mediated exacerbation of alpha-synuclein

toxicity in models of Parkinson disease. *PLoS Biology*, *15*(3). https://doi.org/10.1371/journal.pbio.2000374.

Ostrerova, N., Petrucelli, L., Farrer, M., Mehta, N., Choi, P., Hardy, J., & Wolozin, B. (1999). α-synuclein shares physical and functional homology with 14-3-3 proteins. *The Journal of Neuroscience*, *19*(14), 5782–5791. https://doi.org/10.1523/jneurosci.19-14-05782.1999.

Paleologou, K. E., Oueslati, A., Shakked, G., Rospigliosi, C. C., Kim, H. Y., Lamberto, G. R., Fernandez, C. O., Schmid, A., Chegini, F., Gai, W. P., Chiappe, D., Moniatte, M., Schneider, B. L., Aebischer, P., Eliezer, D., Zweckstetter, M., Masliah, E., & Lashuel, H. A. (2010). Phosphorylation at S87 is enhanced in synucleinopathies, inhibits alpha-synuclein oligomerization, and influences synuclein-membrane interactions. *The Journal of Neuroscience*, *30*(9), 3184–3198. https://doi.org/10.1523/jneurosci.5922-09.2010.

Pan, W., Wang, Q., & Chen, Q. (2019). The cytokine network involved in the host immune response to periodontitis. *International Journal of Oral Science, 11*(3), 1-13. https://doi.org/10.1038/s41368-019-0064-z.

Papapanou, P. N., & Susin, C. (2017). Periodontitis epidemiology: Is periodontitis under-recognized, over-diagnosed, or both? *Periodontology 2000*, *75*(1), 45–51. https://doi.org/10.1111/prd.12200.

Papp, M. I., Kahn, J. E., & Lantos, P. L. (1989). Glial cytoplasmic inclusions in the CNS of patients with multiple system atrophy (striatonigral degeneration, olivopontocerebellar atrophy and Shy-Drager syndrome). *Journal of the Neurological Sciences*, *94*(1-3), 79–100. https://doi.org/10.1016/0022-510x(89)90219-0.

Parkinson Study Group. (2000). *A multicenter assessment of dopamine transporter*

imaging with DOPASCAN/SPECT in parkinsonism. PSG.

Pasqualetti, G., Brooks, D. J., & Edison, P. (2015). The role of neuroinflammation in dementias. *Current Neurology and Neuroscience Reports*, *15*(4), 17. https://doi.org/10.1007/s11910-015-0531-7.

Pickart, C. M. (2001). Mechanisms underlying ubiquitination. *Annual Review of Biochemistry*, *70*, 503–533. https://doi.org/10.1146/annurev.biochem.70.1.503.

Pihlstrom, B. L., Michalowicz, B. S., & Johnson, N. W. (2005). Periodontal diseases. *The Lancet*, *366*(9499), 1809–1820. https://doi.org/10.1016/s0140-6736(05)67728-8.

Popadiak, K., Potempa, J., Riesbeck, K., & Blom, A. M. (2007). Biphasic effect of gingipains from porphyromonas gingivalis on the human complement system. *The Journal of Immunology*, *178*(11), 7242–7250. https://doi.org/10.4049/jimmunol.178.11.7242.

Rachakonda, V., Pan, T. H., & Le, W. D. (2004). Biomarkers of neurodegenerative disorders: How good are they? *Cell Research*, *14*(5), 349–358. https://doi.org/10.1038/sj.cr.7290235.

Rizek, P., Kumar, N., & Jog, M. S. (2016). An update on the diagnosis and treatment of Parkinson disease. *Canadian Medical Association Journal*, *188*(16), 1157-1165. https://dx.doi.org/10.1503%2Fcmaj.151179.

Ruhl, S., Hamberger, S., Betz, R., Sukkar, T., Schmalz, G., Seymour, R. A., Hiller, K. A., & Thomason, J. M. (2004). Salivary proteins and cytokines in drug-induced gingival overgrowth. *Journal of Dental Research*, *83*(4), 322–326. https://doi.org/10.1177%2F154405910408300410.

Samii, A., Nutt, J. G., & Ransom, B. R. (2004). Parkinson's disease. *The Lancet*, *363*(9423), 1783–1793. https://doi.org/10.1016/s0140-6736(04)16305-8.

Schmid, A. W., Fauvet, B., Moniatte, M., & Lashuel, H. A. (2013). Alpha-synuclein post-

translational modifications as potential biomarkers for Parkinson disease and other synucleinopathies. *Molecular & Cellular Proteomics*, *12*(12), 3543. https://doi.org/10.1074/mcp.r113.032730.

Shanbhag, S., Dahiya, M., & Croucher, R. (2012). The impact of periodontal therapy on oral health-related quality of life in adults: A systematic review. *Journal of Clinical Periodontology, 39*(8), 725-735. https://doi.org/10.1111/j.1600-051x.2012.01910.x.

Souza, J. M., Giasson, B. I., Chen, Q., Lee, V. M., & Ischiropoulos, H. (2000). Dityrosine cross-linking promotes formation of stable alpha-synuclein polymers. *The Journal of Biological Chemistry*, *275*(24), 18344–18349. https://doi.org/10.1074/jbc.m000206200.

Tatakis, D. N., & Kumar, P. S. (2005). Etiology and pathogenesis of periodontal diseases. *Dental Clinics of North America*, *49*(3), 491–516. https://doi.org/10.1016/j.cden.2005.03.001.

Tofaris, G. K., & Spillantini, M. G. (2005). Alpha-synuclein dysfunction in Lewy body diseases. *Movement Disorders*, *20*(12), S37–S44. https://doi.org/10.1002/mds.20538.

Tonetti, M. S., Greenwell, H., & Kornman, K. S. (2018). Staging and grading of periodontitis: Framework and proposal of a new classification and case definition. *Journal of Periodontology*, *89*(1), S159–S172. https://doi.org/10.1002/jper.18-0006.

Tredici, K., Hawkes, C. H., Ghebremedhin, E., & Braak, H. (2010). Lewy pathology in the submandibular gland of individuals with incidental Lewy body disease and sporadic Parkinson's disease. *Acta Neuropathologica, 119*(6), 703-713. https://dx.doi.org/10.1007/s00401-010-0665-2.

Tzivion, G., Luo, Z., & Avruch, J. (1998). A dimeric 14-3-3 protein is an essential cofactor for Raf kinase activity. *Nature*, *394*, 88–92. https://doi.org/10.1038/27938.

Vivacqua, G., Latorre, A., Suppa, A., Nardi, M., Pietracupa, S., Mancinelli, R., Fabbrini, G., Colosimo, C., Gaudito, E., & Berardelli, A. (2016). Abnormal salivary total and oligomeric alpha-synuclein in Parkinson's disease. *PloS one, 11*(3). https://doi.org/10.1371/journal.pone.0151156.

Vivacqua, G., Suppa, A., Mancinelli, R., Belvisi, D., Fabbrini, A., Costanzo, M., Formica, A., Onori, P., Fabbrini, G., & Berardelli, A. (2019). Salivary alpha-synuclein in the diagnosis of Parkinson's disease and Progressive Supranuclear Palsy. *Parkinsonism & Related Disorders, 63*, 143-148. https://doi.org/10.1016/j.parkreldis.2019.02.014.

Weinreb, P. H., Zhen, W., Poon, A. W., Conway, K. A., & Lansbury, P. T. (1996). NACP, a protein implicated in Alzheimer's disease and learning, is natively unfolded. *Biochemistry*, *35*(43), 13709–13715. https://doi.org/10.1021/bi961799n.

Weller, A. B. (2018). Current understanding of Alzheimer's disease diagnosis and treatment. *F1000Research*, *7,* 1-9. https://doi.org/10.12688/f1000research.14506.1.

Wiebe, C. B., & Putnins, E. E. (2000). The periodontal disease classification system of the American Academy of Periodontology: An update. *Journal of Canadian Dental Association, 66*(11), 594-599. https://pubmed.ncbi.nlm.nih.gov/11253351/.

Wingrove, J. A., DiScipio, R. G., Chen, Z., Potempa, J., Travis, J., & Hugli, T. E. (1992). Activation of complement components C3 and C5 by a cysteine proteinase (gingipain-1) from Porphyromonas (Bacteroides) gingivalis. *The Journal of Biological Chemistry*, *267*(26), 18902–18907. https://linkinghub.elsevier.com/retrieve/pii/S0021-9258(19)37046-2.

Yeh, C. K., Christodoulides, N. J., Floriano, P. N., Miller, C. S., Ebersole, J. L., Weigum, S. E., McDevitt, J., & Redding, S. W. (2010). Current development of saliva/oral fluid-based diagnostics. *Texas Dental Journal, 127*(7), 651–661. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3742318/pdf/nihms496804.pdf.

Yoshinaka, K., Shoji, N., Nishioka, T., Sugawara, Y., Hoshino, T., Sugawara, S., & Sasano, T. (2014). Increased interleukin-18 in the gingival tissues evokes chronic periodontitis after bacterial infection. *The Tohoku Journal of Experimental Medicine, 232*(3), 215-222. https://doi.org/10.1620/tjem.232.215.

Zhao, D., Zhen, Z., Pelekos, G., Yiu, K. H., & Jin, L. (2019). Periodontal disease increases the risk for onset of systemic comorbidities in dental hospital attendees: An 18-year retrospective cohort study. *Journal of Periodontology*, *90*(3), 225–233. https://doi.org/10.1002/jper.18-0224.

Zinsmaier, K. E. (2010). Cysteine-string protein's neuroprotective role. *Journal of Neurogenetics*, *24*(3), 120–132. https://doi.org/10.3109/01677063.2010.489625.

VIII. APPENDICES

Appendix 1

Comissão de Ética EGAS MONIZ

Proc. Interno nº 939

Ex.mo Senhor
Daniel José da Cruz

Monte de Caparica, 25 de fevereiro de 2021.

Ex.mo Senhor,

Em resposta ao Pedido de Parecer que submeteu à apreciação da Comissão de Ética da Egas Moniz, com o tema denominado **"Associação da periodontite com os níveis salivares de marcadores inflamatórios e alfa-sinucleina: estudo de caso controlo"**, foi aprovado por unanimidade.

Com os melhores cumprimentos,

A Presidente da Comissão de Ética da Egas Moniz

Profª. Doutora Maria Fernanda de Mesquita

EGAS MONIZ – COOPERATIVA DE ENSINO SUPERIOR, CRL
Campus Universitário – Quinta da Granja – Monte de Caparica
2829-511 Caparica

Appendix 2

Consentimento Informado

Código | IMP:EM.PE.17_03

Monte de Caparica, ____de ________________ de 20__

Exmo.(a) Sr.(a),

No âmbito do Mestrado Integrado em Medicina Dentária na Unidade Curricular de Orientação Tutorial de Projeto Final do Instituto Universitário Egas Moniz, sob a orientação do Prof. Doutor José João Baltazar Mendes, solicita-se autorização para a participação no estudo "Associação da Periodontite com os níveis salivares de marcadores inflamatórios e alfa-sinucleína: estudo de caso-controlo". O objetivo deste estudo é o de avaliar a alteração dos níveis de algumas proteínas da saliva e o estado dos tecidos que estão ao redor dos dentes.

O estado de saúde das gengivas pode causar uma inflamação geral. No entanto, não existem informações sobre se ter esta inflamação (que denominamos por periodontite – inflamação dos tecidos à volta do dente) altera algumas proteínas da saliva.

Neste estudo observacional, a população-alvo do estudo são: indivíduos com idade igual ou superior a 18 anos; com mais de 15 dentes presentes em boca, sem doenças previamente diagnosticadas; não ter realizado tratamento periodontal no último ano; não ter tomado antibióticos, corticosteróides e/ou agentes imunossupressores nos últimos três meses; em mulheres, não estar grávida ou em fase lactante.

Nesta consulta será efetuado:

1) Um questionário em relação a idade, sexo, nacionalidade, hábitos de higiene oral como o número de escovagens diárias, o uso de fio dentário e a última consulta no médico dentista;

2) Recolha de informações sobre a altura, peso;

3) Avaliação do estado periodontal através de uma avaliação não invasiva das gengivas.

4) Recolha de saliva, com um algodão debaixo da sua língua durante 2 minutos, sendo um procedimento rápido e sem desconforto.

Consentimento Informado

Código | IMP:EM.PE.17_03

Este estudo pode trazer benefícios tais como estabelecer uma relação entre a inflamação das gengivas com algumas proteínas salivares. A sua participação neste estudo é voluntária. Tem o direito de recusar a sua participação no estudo a qualquer momento, sem prejuízos para si. O estudo mereceu parecer favorável da Comissão de Ética Egas Moniz. Os contactos serão feitos em ambiente de privacidade clínico, ao abrigo do Código Deontológico e de Conduta Ética da Ordem dos Médicos Dentistas. No caso de aceitar participar neste projeto de investigação, ser-lhe-á atribuído um código de anonimização e os dados serão armazenados numa base de dados offline, com acesso por password. Todos os dados serão eliminados 3 anos após a finalização do projeto. Para além disto, caso deseje participar no estudo, poderá também dirigir-se à Clínica Universitária Egas Moniz para usufruir de uma consulta de Medicina Dentária Preventiva para uma destartarização e aplicação de uma moldeira de flúor gratuitamente.

(Riscar o que não interessa)

ACEITO/NÃO ACEITO participar neste estudo, confirmando que fui esclarecido sobre as condições do mesmo e que não tenho dúvidas.

____

(Assinatura do participante ou, no caso de menores, do pai/mãe ou tutor legal)

Appendix 3

4																
5		Prótese desadaptada?	Sim ☐	Não ☐												
6		Pilar de prótese	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
7		Implant	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
8		CAL	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
9	V	Calculus	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐
10	V	Plaque	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐
11	V	GI	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐
12	V	BoP	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐
13	V	Rec	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
14	V	PD														
15			17	16	15	14	13	12	11	21	22	23	24	25	26	27
16	P	PD														
17	P	Rec	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
18	P	BoP	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐
19	P	GI	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐
20	P	Plaque	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐
21	P	Calculus	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐
22		CAL	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
23		MOB														
24		FURCA														
25																
26		Average CAL	0	0	0	0	0	0	0	0	0	0	0	0	0	0

28		Prótese desadaptada?	Sim ☐	Não ☐												
29		Pilar de prótese	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
30		Implant	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
31		CAL	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
32	L	Calculus	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐
33	L	Plaque	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐
34	L	GI	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐
35	L	BoP	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐
36	L	Rec	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
37	L	PD														
38			47	46	45	44	43	42	41	31	32	33	34	35	36	37
39	V	PD														
40	V	Rec	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
41	V	BoP	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐
42	V	GI	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐
43	V	Plaque	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐
44	V	Calculus	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐	☐☐☐
45		CAL	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
46		MOB														
47		FURCA														
48																
49		Average CAL	0	0	0	0	0	0	0	0	0	0	0	0	0	0