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Possibility of a vaccine?

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Monografia orientada pelo Professor Doutor António José Leitão das Neves Almeida, Professor Catedrático

Mestrado Integrado em Ciências Farmacêuticas

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Trabalho Final de Mestrado Integrado em Ciências Farmacêuticas apresentado à Universidade de Lisboa através da Faculdade de Farmácia

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Resumo

A doença de Alzheimer é a forma mais comum de demência sendo caracterizada por disfunção cognitiva progressiva e acumulação cerebral de placas β-amiloide e neurofibrilhas tau. Mais de um século após o primeiro caso, as razões subjacentes à neurodegeneração permanecem por esclarecer dificultando a pesquisa de novos alvos terapêuticos e a aprovação de terapias dirigidas. As mutações autossómicas dominantes nos genes *APP, PSEN1* e *PSEN2* esclarecem alguns diagnósticos. No entanto, casos familiares de Alzheimer são raros sendo a doença de Alzheimer esporádica responsável pela maioria dos casos da patologia. Diferentes teorias tentam explicar a etiologia esporádica da doença de Alzheimer. Recentemente, algumas equipas de investigadores propuseram uma etiologia bacteriológica da doença.

A cavidade oral hospeda uma variedade de microrganismos comensais. Durante a infância, o microbioma oral rapidamente se torna mais complexo. Depois disso, permanece relativamente estável durante a idade adulta. No entanto, alterações transitórias ou crónicas alteram o microbioma oral e favorecem a disbiose. A periodontite e outras patologias orais levam ao desenvolvimento de bactérias Gram-negativas. A *Porphyromonas gingivalis*, em particular, ganhou especial destaque pela sua possível ligação entre a periodontite e a doença de Alzheimer.

Sinais indicativos da presença de *Porphyromonas gingivalis* foram identificados na análise *postmortem* do cérebro de doentes com doença de Alzheimer. Estudos in vitro e in vitro confirmam o eixo boca-cérebro e resultados recentes sugerem um possível mecanismo de invasão e patogénese. Após invasão da corrente sanguínea, os fatores de virulência da bactéria atingem o sistema nervoso onde interagem com diferentes tipos celulares. Através de um mecanismo mediado pela interleucina 1β, a *Porphyromonas gingivalis* mostrou ser capaz de induzir o desenvolvimento dos achados histopatológicos da doença de Alzheimer.

Após décadas de retrocessos na pesquisa de novos alvos, terapias e abordagens preventivas, a etiologia bacteriológica traz esperança para o desenvolvimento de novas metodologias. Embora a imunização com antigénios de *Porphyromonas gingivalis* possa vir a ser uma estratégia contra a demência, estudos mais aprofundados são ainda necessários.

Palavras-chave: Doença de Alzheimer, microbioma oral, doenças periodontais, *Porphyromonas gingivalis*, vacina

Abstract

Alzheimer’s Disease is the most common form of dementia and is marked by the progressive cognitive disfunction and the cerebral accumulation of amyloid-β and tau fibrils. More than a century after the first case report, the reasons behind the neurodegeneration remain unclear hampering the research for new therapeutic targets and the approval of targeted therapies. Autosomal dominant mutations in the *APP*, *PSEN1* and *PSEN2* genes account for a small percentage of the diagnosis. However, familial cases of Alzheimer's are rare and sporadic Alzheimer’s Disease is responsible for most cases of the disease. Different theories try to explain sporadic Alzheimer’s Disease aetiology. Recently, some research teams purposed a bacteriological aetiology of the disease.

The oral cavity is home to a variety of commensal microorganisms. During early infancy, the oral microbiome rapidly becomes more complex. Afterwards, it remains relatively stable throughout adulthood. However, transitory or chronic changes alter the oral microbiome and promote dysbiosis. Periodontitis and other oral pathologies favour Gram-negative bacteria. *Porphyromonas gingivalis*, in particular, has gained interest as a possible link between periodontal diseases and Alzheimer’s Disease.

Porphyromonas gingivalis hallmarks have been identified in the *postmortem* brain analysis of Alzheimer’s Disease patients. In-vitro and in-vitro studies confirm the mouth-brain axis and recent papers have suggested a possible mechanism of invasion and pathogenesis. After escaping through the bloodstream, the bacteria's virulence factors reach the central nervous system and interact with the different cell types. *Porphyromonas gingivalis*, through an interleukin 1β mediated mechanism, was shown to induce the development of the classical histopathological findings of Alzheimer's disease.

After decades of draw backs in the research of new targets, therapies and preventive approaches, the bacteriological aetiology brings hope for the development of new methods. Although the immunization with *Porphyromonas gingivalis* antigens presents as an exciting strategy against dementia, further research is still needed.

Key words: Alzheimer’s Disease, oral microbiome, periodontal diseases, *Porphyromonas gingivalis*, vaccine

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Abbreviations

ACE	Angiotensin-Converting Enzyme
AD	Alzheimer's Disease
APC	Antigen Presenting Cell
ApoE	Apolipoprotein E
APP	Amyloid Precursor Protein
Aβ	β-amyloid Protein
BBB	Blood-Brain Barrier
BCSFB	Blood-Cerebrospinal Fluid Barrier
CatB	Cathepsin B
CD	Cluster of Differentiation
CDC	Centers for Disease Control and Prevention
CDT	Cytolethal Distending Toxin
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
DC	Dendritic Cells
FAD	Familial Alzheimer's Disease
IDE	Insulin-degrading enzyme
Ig	Immunoglobulin
IL	Interleukin
IL-1R	Interleukin 1 receptor
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex
NEP	Neprilysin
NF-κB	Nuclear Factor κB
OMV	Outer Membrane Vesicle
PAMP	Pathogen-Associated Molecular Patterns
PCR	Polymerase Chain Reaction
PD	Periodontal Disease
Pg	*Porphyromonas gingivalis*
PgLPS	*Porphyromonas gingavalis* lipopolysaccharide
PLGA	Poly (Lactic-co-Glycolic Acid)

PP2A	Protein Phosphatase 2A
PSEN	Presenilin
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SAD	Sporadic Alzheimer's Disease
TCR	T-Cell receptor
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
USA	United States of America

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1. Introduction

Alzheimer's Disease (AD) was first described as a new disorder in 1907 using clinical observation and *postmortem* silver impregnation of the nervous system. In his original case report, Alois Alzheimer stated that the 51-years-old patient was suffering from memory loss and delusional symptoms that oscillated between stronger and weaker periods. The *postmortem* microscopic analysis of the brain showed neuritic amyloid plaques as well as neurofibrillary tangles (1). This description was used throughout Europe and the United States of America (USA) to diagnose identical cases. However, it was only in 1910 the term *Alzheimer's Disease* was coined by A. Alzheimer's mentor (2).

From a single case report over a century ago to thousands of cases today, AD became the leading cause of dementia and neurodegeneration across the globe. Nonetheless, the numbers will continue to expand as the average life expectancy increases. During a seven-year period, the numbers have grown from one case every seven seconds to one case every four seconds (3).

Data from Portugal is scanty. Whilst numbers may vary between authors, they all show a pattern of growing prevalence as we follow the age groups (3–5). The 2019's European Union's Health Programme estimates that 1.88% live with dementia in Portugal. Surprisingly, the European yearbook reveals that males are the most affected gender in younger ages, but the trend flips when we consider the total population (6).

The key symptom of AD is memory loss, but dementia can result from similar-looking neurodegenerative diseases. Previously, these diseases were wrongfully diagnosed as AD due to a lack of knowledge. Moreover, only suspected patients presenting different signs and symptoms of dementia are carefully evaluated by clinicians with the help of modern brain imaging techniques. Nonetheless, *postmortem* histopathological features of AD can be present in asymptomatic patients (7,8). This fact led to a new classification of AD, published by the National Institute of Aging in 2012 (7). Nowadays, AD is considered as a spectrum of three stages raging from an early, preclinical stage, to a final stage with symptoms of dementia (7).

The diagnosis can be further categorized by dividing AD into two groups depending on the etiology. The first group refers to the hereditary 'Familial' Alzheimer's Disease (FAD) forms while the Sporadic Alzheimer's Disease (SAD) falls under the second (non-hereditary) group (9).

Patients with FAD have mutations in selected genes and usually develop AD earlier in life. Besides that, clinical presentation, neuropathology and neuroimaging are similar to patients suffering from SAD (10). Thus far three genes have been identified linked to autosomal-dominant FAD: amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*) and presenilin 2 (*PSEN2*) all resulting in the increased production of β-amyloid protein (Aβ) (9,10).

Aβ results from the two-step proteolytic cleavage of APP by β-secretase and γ-secretase. It is important to note, however, that β-secretase competes with α-secretase for the substrate. When α-secretase is responsible for the first proteolytic cleavage, γ-secretase action results in a fragment with neurotrophic and neuroprotective properties. The α-secretase is the preferred pathway under normal circumstances. That said, only when APP production is increased, Aβ accumulates and forms the classical aggregates (as revised in (9)).

Mutations in the *APP* gene, in chromosome 21, were the first to be discovered by different laboratories (11–13). These mutations are responsible for increasing the APP affinity to β-Secretase as well as changing the cellular compartment where cleavage occurs, increasing the Aβ production (14). Patients with APP mutations usually present symptoms of dementia in their 50s. Further, cerebral hemorrhage may be present due to extensive amyloid angiopathy (10). The APP's mutations investigation was followed by discoveries in the *PSEN1* gene, in chromosome 14 (15,16), and the *PSEN2* gene, in chromosome 1 (17–19). The encoded proteins are part of the catalytic core of γ-secretase but the mutations effects and the pathophysiological mechanism remain unclear (14).

On the other hand, not much is known about the SAD etiology (9). Although SAD cannot be predicted by autosomal-dominant mutations, various polymorphisms increase the likelihood of late-onset AD. The most significant is the *APOE* ε4 allele increasing the risk of developing the disease (20). The apolipoprotein E (apoE) ε4 is one of the three isoforms of the apoE, encoded by the *APOE* gene. ApoE may interact with both Aβ and tau proteins but the underlying mechanism requires further research (20,21).

Besides the genetic-related AD, other theories try to explain SAD. A popular one, *The amyloid hypothesis,* is supported by the fact that amyloid plaques, a characteristic neuropathological finding in AD, are rich in Aβ. This theory, originally proposed in 1992 (22) was backed up by the discovery of FAD cases directly linked to genes associated with APP and its cleavage (23). In addition, it was observed that high concentrations of Aβ correlate with negative cognitive capacity making this the most acceptable theory for several years (24). Despite this, older

patients with cognitive capable brains showed an accumulation of Aβ and the therapeutical reduction of Aβ in AD patients using immunotherapy did not exhibit the expected results (25). Thus, *The amyloid hypothesis* fell under major criticism and other theories arose.

The mitochondrial cascade hypothesis (26) tries to explain the tangle formation and the relationship between AD and aging. In 2004, Swerdlow and Khan proposed that mitochondrial aging due to the accumulation of mutations and the increase in reactive oxygen species (ROS) are the basis for the histopathologic characteristics of AD. Firstly, ROS can alter proteins decreasing their solubility and, in turn, insoluble β-sheet conformations are favoured. This is also true for the Aβ formed from APP leading to its aggregation in amyloid plaques. Moreover, ROS are responsible for the neuronal loss by promoting the cell intrinsic apoptosis pathway. Lastly, ROS accumulation also explains the neurofibrillary tangles seen in *postmortem* microscopic analysis. As neurons are being lost by apoptosis, those that maintain some proliferative ability re-enter the mitotic cycle replacing the lost ones. However, cell cycle arrest during the G2-M phase results in tau hyperphosphorylation and tangle formation. These neurons do not complete the normal cell cycle and lose viability due to mitochondrial dysfunction that induced the re-entry in the mitotic cycle.

More recently, the identification of polymorphisms in inflammation-related genes and its correlation with late-onset AD (27), as well as the observation of inflammatory mediators in brains and plasma of AD patients (28), gave rise to the *Inflammation hypothesis.* In this model (29), when inflammatory stress is present, healthy aged neurons with axonal varicosities can lose their ability to extrude them. Thereafter, varicosities grow in number and become full of APP, reaching a swollen state. When its 'breaking point' is reached, axonal leakage occurs with APP being liberated. However, APP is not correctly processed by APP-specific secretases resulting in Aβ aggregation to form senile plaques. At the same time, tau becomes hyperphosphorylated and, when amyloid plaques begin to form, caspase activation leads to formation of neurofibrillary tangles. Additionally, in this scenario of leaking axons and liberation of debris that cannot be properly cleared, microglia becomes hyperactivated. As such, it increases the production of inflammatory proteins triggering a neuroinflammation process.

Infections capable of promoting low-grade chronic inflammation are one of the biggest sources of inflammatory stress. Among the most common pathogens are oral bacteria that can activate the immune system and trigger the cascade of events described above (30). That said, the bacteriological etiology of AD, particularly *Porphyromonas gingivalis*' role, has gained the

interest of different research teams. In the last years, *P. gingivalis* has been identified in brain tissue of AD patients (31) and, recently, it was proven to induce memory impairment and age-dependent neuroinflammation in mice (32).

This represents a valuable opportunity for research since the development of a preventive approach is an exciting strategy that can prove as a significant adjunct to current AD therapies.

2. Oral microbiome

The oral cavity hosts a small ecosystem of commensal and symbiotic microorganisms consisting of not only bacteria but also fungi, parasites and archaea. These microorganisms have evolved with the human species over millions of years and contribute to normal human physiology (33).

The discovery of the highly conserved 16S-RNA and the advances in biomolecular technology allowed for the identification of oral bacteria by genus and species (33). These studies led to the conclusion that, when dysbiosis is present, the oral microbiome can contribute to disease development.

2.1. Physiological and microbiome changes during aging

Throughout the embryonic phase, an intact amniotic membrane prevents bacterial colonization. As such, prior to birth, the human body is sterile. However, during birth and the following hours, the body becomes exposed to the surrounding environment allowing the acquisition of bacteria (34).

During the first months of life, the only environment for bacterial colonization in the mouth is the oral mucosa (35). In this predentate phase, the limitation in the surfaces available for bacterial adherence restricts the variety of bacteria able to colonize the oral cavity (36). Curiously, the microbiome is highly diverse between peers. Despite the differences, a core microbiome can be identified accounting for 45% of the total microbiome. The core microbiome is mainly composed of *Streptococcus, Gemella* and *Granulicatella,* belonging to the Firmicutes phylum, and *Veillonella,* belonging to the Irmicutes phylum (35).

The basic microflora built during the newborn phase rapidly becomes more complex during early childhood. of the first deciduous teeth allows the emergence of new surfaces - hard tooth and gingival sulcus. Furthermore, as the newborn begins its solid dietary habits, it increases the diversity of bacteria in the oral (36,37). At around 6 months old, the *Firmicutes* relative prevalence starts to decrease as other families begin to develop. *Proteobacteria, Actinobacteria, Bacteroidetes* and *Fusobacteria* increase the alpha diversity. In contrast, beta diversity decreases with age (38,39). A study by Mason, in 2018, showed that predentate infants

demonstrate significantly higher levels of gram-positive facultative and lower levels of gram-negative facultative species than dentate individuals (35).

After the two waves of bacterial acquisition, the oral microbiome remains relatively stable during adulthood (40,41). However, changes in geographic locations, diet, fluctuation in the salivary flow, and long-term use of antibiotics can induce transitory changes (42). On the other hand, chronic changes in the microbiome mainly reflect oral and systemic health status. Locally, the most relevant diseases include dental caries, endodontic infections, and periodontal diseases (PD) (43). In general, these diseases promote the microbial imbalance, known as dysbiosis. During this process, the human body faces a decrease in the beneficial symbionts and the consequent increase in the pathogenic microorganisms (43).

Dental caries are described as a process of demineralization and remineralization. It can be the result of frequent ingestion of carbohydrates, poor dental hygiene or reduction of the salivary flow that buffers the pH. In this scenario, oral pH gradually becomes more acidic which, in turn, alters the microbiome by favoring acidogenic and acid-tolerant bacteria (44). The most acidophilic strains of the non-mutants *streptococci*, mutants *streptococci*, *Actinomyces, lactobacilli,* and *Bifidobacterium* prevail (41,44,45).

Progression of dental caries leads to infection and possible necrosis of the pulp (43). This is the description of a primary endodontic infection, where the infection and inflammation precede the microbial invasion. Here, the most prevalent bacteria species are from the Bacteroides, *Porphyromonas, Prevotella, Fusobacterium, Treponema, Peptostreptococcos, Eubacterium*, and *Camphylobacter* genus. When endodontic infection is secondary, also known as post-treatment infection, the bacteria detected are resistant to harsher environments. *Enterococci, Streptococci, Lactobacilli, Actinomyces* and *Candida* are found in this situation (46,47). *Enterococci* presence is interesting to analyse because, under normal circumstances, it is not present in the oral cavity (43). Their source is most likely pasteurized cheese and lack of dental hygiene (48).

Lastly, PD comprise the inflammatory conditions progressively affecting the gingiva, alveolar bone, and periodontal ligament (49). In these diseases, the healthy microbiome is lost, and Gram-negative anaerobes grow in prevalence (43,49,50). *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*), *Porphyromonas gingivalis* (*P. gingivalis*), *Tannerella forsythia (T. forsythia), Treponema denticola (T. denticola),*

Campylobacter gracilis (C. gracilis), Eubacterium nodatum (E. nodatum) and *Prevotella intermedia (P. intermedia)* were observed in subjects with localized aggressive periodontitis (51). Periodontitis and the associated dysbiosis can have a negative impact not only in oral health but also on overall health status, promoting low-grade chronic inflammation (49).

As individuals age, these oral diseases become more prevalent and, when left untreated, lead to the loss of teeth (52). Besides mouth changes from oral diseases, elderlies also go through different physiological changes in the oral cavity (52). In the table below, normal aging and pathological states are differentiated.

Table 1 Physiological and pathological processes in the aging mouth. Adapted from (52).

	Physiological aging process	**Pathological states**
Dentition	Fracture lines Incisal edges are chipped Change in color	Caries Loss of significant tooth structure
Periodontium	Limited attachment loss, observed as recession on buccal surfaces	Extensive alveolar bone loss Tooth mobility
Oral mucosa	Adequate barrier function Wound healing slightly delayed	Thinning mucosa Dysplastic change
Salivary flow	Reduction of salivary production, excretion, and composition, but still adequate	Altered by medications and diseases
Temporomandibular joints	No discomfort	Pain Inability to properly masticate
Masticatory function	Reduced, but still adequate	Inability to properly masticate

The oral microbiome reflects these differences in aging. Gazdeck observed that edentulous individuals have a lower alpha diversity than dentate individuals, presumably from loss of microbiota present in the tooth surfaces (53). These observations are in line with the Singh's data, who further noted that non-healthy aging shows an increase in *Streptococcus*, *Veillonella*, *Rothia* while *Neisseria* was increased in the healthy aged individuals (54).

2.2. Connecting Periodontitis to Alzheimer's Disease

Periodontitis is an inflammatory-infectious disease. This means that different bacteria are the basis for its manifestation but inflammatory processes are crucial for disease progression. On

the other hand, AD can have a variety of etiological sources since different mechanisms can be causing or exacerbating the disease.

Establishing a link between oral disease and neurodegenerative diseases such as AD can be troublesome. To do so, researchers first demonstrated the association between periodontitis and AD. Different epidemiological studies, in humans, have suggested that tooth loss and periodontitis have a positive correlation with dementia and memory loss (55,56). Other studies showed the interdependence between periodontitis and Aβ levels (57). However, these studies fail to prove that periodontitis is the cause of the neurological findings. In fact, AD patients have considerably lower dental hygiene due to disease progression (58). That said, the question to be asked is "Is periodontitis causing AD or is AD causing periodontitis?"

To untangle the periodontitis – Alzheimer – periodontitis paradox it is important to unravel the cause and establish a common risk factor. Nowadays, bacteria seem to be the most plausible element linking the two diseases.

2.2.1. *Aggregatibacter actinomycetemcomitans*

A. actinomycetemcomitans is a facultative anaerobic Gram-negative bacterium with seven serotypes, from a to g. The serotypes are defined by antigenicity of the O-polysaccharide in *A. actinomycetemcomitans* lipopolysaccharide (LPS), one of the virulence factors. Other virulence factors include the leukotoxin, a cytolethal distending toxin (CDT), and outer membrane vesicles (OMVs) (59). Together, these properties contribute to the most aggressive forms of periodontitis (60). Although its link with periodontal diseases has been recognized, its relation to AD is not yet fully understood. Some researchers are trying to decipher the *A. actinomycetemcomitans-periodontitis-Alzheimer* triad and the answer may be laying in the LPS.

In brain cell cultures, LPS from serotype b of *A. actinomycetemcomitans* induces the expression of pro-inflammatory proteins in microglia and the hippocampus (61). These results demonstrate that LPS from serotype b is neuroimmunogenic but data showing that *A. actinomycetemcomitans* or its LPS can reach the brain is still lacking.

2.2.2. *Porphyromonas gingivalis*

P. gingivalis is a keystone Gram-negative anaerobic bacterium found in most patients with periodontitis. That means that, even in low abundance, *P. gingivalis* can promote dysbiosis. Through its gingipains, *P. gingivalis* can modulate the complement cascade activation, reduce bacterial clearance, and alter the composition of the local microbiota (62).

Outside the oral cavity, *postmortem* evidence proved that individuals with dementia had a significantly higher presence of LPS (63), DNA (31) and gingipains (31) from *P. gingivalis*. Moreover, *P. gingivalis* DNA was recently identified in saliva and Cerebrospinal Fluid (CSF) of patients with moderate cognitive decline, diagnosed with probable AD (31).

After observational and experimental studies showed an association between AD and periodontitis, *P. gingivalis* gained interest as a probable cause.

2.2.3. *Spirochetes*

Spirochetes can be transmitted to humans in a multitude of ways and cause different diseases depending on the species in question.

Treponema pallidum is sexually transmitted to humans and is usually responsible for syphilis (64). Nonetheless, Noguchi and Moore (65) demonstrated that *T. pallidum* could reach the brain and cause a disease known as neurosyphilis. These patients show signs of progressive dementia, cortical atrophy and local amyloidosis.

Borrelia burgdorferi (*B. burgdorferi*) enters the body when humans get bitten by an infected tick. As result, patients get diagnosed with Lyme disease when erythema migrans appear (64). If left untreated, Lyme disease can progress to dementia (66).

The historical association between spirochetes and dementia raised the question if oral spirochetes could cross the blood-brain barrier (BBB) and contribute to AD. In the human mouth, different species of *Treponema* can be found when dysbiosis is present (64). In 2002, Riviere (67) made breakthroughs. Using PCR and immunohistochemical assays he proved that oral *Treponemas* could reach the brain in AD patients.

3. From mouth to brain: Is there a link?

Periodontitis is a chronic oral disease caused by dysbiosis, promoting Gram-negative bacteria development. Alzheimer's Disease, on the other hand, is an irreversible, neurodegenerative brain disorder that results in memory loss, dementia and death. At first sight, periodontitis and AD are two very different diseases. As seen, they may nonetheless share common risk factors that influence its onset and progression.

Although bacteria can be the basis for both periodontitis and AD, proving the trilogy Bacteria – Periodontitis – Alzheimer's Disease needs more than just a correlation between the three. The mechanisms of how oral bacteria can surpass the blood-brain barrier, dribble the immune system and reach the nervous system, interacting with both neurons and microglia are essential to understand the causation link.

3.1. Bacterial entrance in the Central Nervous System

3.1.1. Barriers of the Central Nervous System

In normal conditions, the Central Nervous System (CNS) is inaccessible for external agents. The physical and chemical protection is made possible by the cranium structure, meninges, the CSF and the BBB (68).

The meninges comprise three layers surrounding the brain and spinal cord. The closest to the skull is called the dura mater and it is where blood circulates. Right after comes the arachnoid and, lastly, the pia mater envelopes the brain and spinal cord (68).

The CFS is secreted by the choroid plexuses and its main function is to maintain the chemical homeostasis of the brain (69). Its compartment is located between the arachnoid and pia mater bathing both the subarachnoid space and the ventricles. As such, the CSF is in close contact with the peripheral blood circulating in the dura mater (68). To prevent contamination, there is a blood-cerebrospinal fluid barrier (BCSFB). This barrier is composed of the arachnoid, the choroid plexuses and the pial microvessels (69).

Moreover, the vessels surrounding the CNS also have unique properties that allow the separation between the brain microcirculation and the peripheral circulation. The BBB is composed of three different cell types. The endothelial cells line the blood vessels. These

endothelial cells show different properties from those found in other vessels. Parallel to other endothelial cells, those in the BBB have receptors and transporter channels. However, these cells lack fenestrae and are connected through adherent and tight junctions to form a barrier. Together, these characteristics allow for the selective diffusion of small neutral molecules and blockage of big or positively charged molecules (68). The structure of the BBB is supported by a star-shaped cell, the astrocytes. Besides functioning as a support system, astrocytes also recruit microglia when infections are detected. Lastly, pericytes are multi-function cells in close contact with the endothelial cells. They help support the microvessels and influence permeability (70).

3.1.2. Sites and mechanism of invasion

Despite all the existing barriers, several pathogens can gain access to the CNS. To do so, pathogens maneuver the brain barriers and penetrate the CNS through blood circulation (hematogenous dissemination) or cranial nerves (intracranial retrograde dissemination).

The great exposure of the nasal cavity to the external environment makes it a direct portal to the brain. The cilia of olfactory primary neurons line and penetrate the nasal mucosa. From there, neurons project axons to the olfactory bulb within the CNS. As such, microbes travel by axonal transport and access the CNS. Moreover, pathogens can be transported within the perineural space and reach the subarachnoid space (68,71). Likewise, pathogens in the nasal cavity can also use the trigeminal nerve. The trigeminal nerve innervates three zones - ophthalmic, maxillary, and mandibular - having both sensory and motor functions. The ophthalmic and maxillary branches project axons to the olfactory epithelia providing a similar route to the CNS. However, as the trigeminal nerve is the largest cranial nerve, infections in ophthalmic structures, facial skin and oral cavity can theoretically use it as a via to the brain (68).

On the other hand, the hematogenous dissemination of microbial pathogens postulates that microbes can invade the blood and reach the brain where they, by disruption of the BBB or BCSFB, penetrate the CNS (68).

For BBB transport through the transcellular route pathogens need to adhere to the apical side of the endothelial cell, penetrate it and leave through the basolateral side of the endothelium (68). Differently, in the paracellular pathway microbes cross the BBB between two cells. As

such, the adherent junctions and tight junctions that connect the endothelial cells must be disrupted, altering the permeability (68,72). Different mediators can play a role during the opening of the endothelial barrier. Neurotrophic pathogens can secrete proteases that degrade the tight junction. Moreover, cytokines like interleukins, interferons and tumour necrosis factors (TNF) are commonly elevated during inflammatory states and induce BBB hyperpermeability (72). These facts help pathogens reach the brain after infection. The paracellular, contrarily to the transcellular pathway, is exclusively passive. It is driven by a gradient and requires no interplay between the endothelial cells and the pathogen. That said, the transcellular crossing is more selective as microbes need to have a set of electrochemical properties or ligands allowing the interaction (72).

In contrast, pathogens can also cross the BBB and enter the CNS by indirect transfer. After infection of a peripheral organ, outside the CNS, phagocytes are recruited to combat the microorganisms. However, after the internalization of the pathogen, the microbe exploits the phagocyte capacities and promotes its migration to the brain through the bloodstream (73). When the phagocyte arrives at the brain, it transmigrates with the masked microbe inside. This pathway is especially important for intracellular pathogens who are capable of surviving and replicate within white cells (68).

3.1.3. Translocation of oral bacteria to the brain

The studies described in section *2.2.* showed that oral periodontal bacteria could reach the brain and had a positive correlation with AD. To test the hypothesis of periodontitis being the cause of neurodegeneration, different animal models were used. Despite the differences, the study design was similar across labs. Mice were divided into two groups one being administered with oral bacteria and the other serving as a control. Together, these studies showed that oral bacteria could leave the original infection site and reach the brain. After colonization of CNS, the bacteria induced inflammation (74,75), cognitive disfunction (76) and elevation of Aβ levels (74).

Thus, periodontal bacteria can indeed reach the brain and promote Alzheimer-like features. Although the underlying mechanism by which the bacteria or its virulence factors transmigrate was not elucidated in the papers, some hypotheses have been made.

Gram-negative bacteria can manipulate the host immune response and deliver virulence factors in OMVs. That said, OMVs serve as a mechanism for host-pathogen interaction. In *A. actinomycetemcomitans,* OMVs can transport small molecules including LPS (59) and leukotoxin (77). Moreover, a recent *in vivo* study showed that OMVs can cross the blood-brain barrier (BBB) and transport extracellular RNAs (78). Although not confirmed, one can hypothesize that *A. actinomycetemcomitans* LPS can reach the brain carried by OMVs, enter it by a lipoprotein-mediated transport mechanism (79) and induce an inflammatory response that contributes to AD pathoetiology. Singhrao and Olsen (80) also pose the OMV theory as the most probable pathway for *P. gengivalis* translocation.

For *spirochetes,* Riviere (67) purposed a neurological pathway as *Treponema* species commonly found in the oral cavity were detected in the trigeminal ganglia of *postmortem* AD patients. Although these bacteria were also found in controls, the extent and diversity of the infection were significantly higher in AD patients.

3.2. Evading the immune system

3.2.1. Immunosurveillance of the CNS

The physical and chemical protection of the CNS, described above, is extended to its immune system. In peripheral organs, dendritic cells (DC) sense an immunogen, internalize and process it into small peptides that are presented in the major histocompatibility complex (MHC). After DC present the antigen to T-cells, through its T-cell receptor (TCR), the lymphocyte becomes activated. Activated T-cells travel to the infected tissue and participate in the pathogen elimination by recruiting other monocytes and releasing antibodies (81). Contrarily, the lack of parenchymal DC in the CNS prevents T-cells to be called upon when immunogens are injected into CNS parenchyma (82). Additionally, the BBB properties restrain inflammatory proteins to enter the brain. Lastly, the parenchymal environment suppresses inflammation by featuring anti-inflammatory cytokines. Together, these three characteristics grant the CNS immune privilege (81).

This immune privilege situation can, at first sight, seem risky. However, it is in fact a way of protecting parenchymal cells. Outside a lab experience, humans are not exposed to bacterial lysates following a non-traumatic micro-injection into the CNS. That is to say that, before a pathogen reaches the CNS, it triggers an innate and adaptative immune response in its initial

site of infection. Therefore, it would be unnecessary to initiate a second cascade of events leading to T cell activation and uptake to the CNS. Moreover, exacerbated inflammatory reactions damage neurons and glial cells. In fact, high concentrations of TNF-α results in neuronal death (81).

As such, the CNS is in constant patrol by specialized immune cells to rapidly gain control of microorganisms and prevent the need for inflammatory responses and, consequently, parenchymal damage (83). Curiously, immune surveillance is not equal across all brain regions. In regions where BBB tight junctions are reduced, the CNS is at higher risk of exposure to pathogens. As such, immune cells tend to accumulate in these areas so sampling is more frequent (83).

In the parenchyma, microglia act as a specialized macrophage. Microglia enter the CNS during the embryonic phase and are entrapped by the BBB. Thus, peripheral macrophages cannot surpass the BBB and differentiate into microglia. Instead, microglia expand through the proliferation of already existing progenitor cells (82).

During immune-resting times, microglia are kept in a quiescent state. In this state, microglia present less dendritic ramifications and slower movements. Thus, its main job is to patrol and sample the environment. If dead cells are recognized, microglia can clean the debris in the CNS by phagocytosis. However, during an infectious attack, they serve as first-line defenders secreting cytokines (83). Although microglia do not fit into the more classical DC definition, they have been shown to have weak antigen-presenting activity. That said, contrary to what was first believed, microglia can chemoattract other immune cells that sit around the parenchyma (81).

Outside the parenchyma, the immune cell population is vaster and more diverse. While in the choroid plexus we can find DC and macrophages; in the meninges and perivascular space, only macrophages have been identified. However, both DC and macrophages have antigen-presenting features. After an immunogen is recognized, this antigen-presenting capacity is enhanced. To do so, macrophages express higher levels of MHC II, adhesion and co-stimulating molecules. As such, they are capable of presenting immunogens to patrolling T cells (82).

In the healthy brain, T cells are only featured in the CSF. This is composed mainly of T cells, particularly CD4+ naïve T cells. Nonetheless, some central and effector memory T cells can be identified. Effector memory T cells do not express lymph-node-homing receptors but, instead, express inflamed-tissues-homing receptors. Thus, these lymphocytes can rapidly start their

effector function without further differentiation. On the other hand, central memory T cells maintain naïve properties and need to migrate to lymph nodes where they become re-activated (82).

During immune surveillance, T cells translocate from peripheral blood across the choroid plexus and enter the CSF gaining access to the subarachnoid space (83). As the interstitial fluid of the CNS drains into CSF, T cells can encounter meningeal macrophages in the subarachnoid space. There, their role is to sample the CSF, looking for immunogens. If no signal is recognized, T cells exit the subarachnoid space travel through the nasal mucosa to the secondary lymphoid organs. However, if T cell receptors recognize a ligand, immune cells are recruited to the CNS (81).

3.2.2. *P. gingivalis* bullets: The importance of virulence factors

The modern definition of "virulence factors" describes them as a set of molecules that enable microorganisms to establish and maintain as a species within a host. For that matter, virulence factors help with the colonization, immunoevasion, immunosuppression and nutrition of a pathogen.

P. gingivalis is, as described, a Gram-negative anaerobic bacterium associated with PD and Alzheimer's Disease. As such, its virulence factors are here briefly outlined according to Holt review (84).

Capsule

The capsule is the most exterior layer of *P. gingivalis* and is seen, in transmission electron microscopy, as a thick layer surrounding the microorganism. However, some strains appear to be lacking such components.

The chemical composition of the capsule differs from strain to strain but all have shown to be a mix of polysaccharides. Therefore, encapsulated *P. gingivalis* has higher hydrophobicity. Moreover, as this layer of sugars covers the outer membrane, the LPS is masked and its ability to activate the complement cascade is dismissed. Together, these reasons allow the encapsulated strains to have a decreased induction of polymorphonuclear cells and, consequently, be more resistant to phagocytosis.

Outer membrane

P. gingivalis cell wall, like other Gram-negative bacteria, is a multilayer consisting of an outer membrane, in-between peptidoglycan and an inner cytoplasmic membrane. However, the virulence potential of bacteria lies in the outer membrane. There, it is possible to identify several different structures. Namely, the LPS, lipoproteins, transport proteins and fimbriae.

The LPS is an amphipathic large molecule consisting of an O-polysaccharide, exposed to the exterior environment, and a lipid-A, connected to the lipidic portion of the membrane leaflet. Moreover, the O-polysaccharide has immunobiological activity while the lipid-A contains the endotoxicity. Nonetheless, it is important to note that, in *P.gingivalis*, the lipid-A induces negligible pyrogenicity and endotoxicity. The low endotoxic activity allows *P.gingivalis* to grow and colonize in the host tissue without being detected. However, it is still able to stimulate interleukins and induce an inflammatory response that contributes to its pathogenicity.

Fimbriae constitute a variety of adhesive pili involved in the attachment to host cells and other bacteria. Besides its biofilm capability, *P.gingivalis* fimbriae stimulate the antibody and cell-mediated response.

Gingipains

Gingipains are part of a cysteine proteinase family that cleave upstream to arginine or lysine residues.

After being formed in the cytoplasmatic region, gingipains can regulate their movement to the exterior. To do this, they form pores across the outer membrane from which they can pass. Once out and exposed to the exterior, gingipains can serve their purpose and facilitate the evasion of host defense mechanisms.

The mechanisms of virulence include erosion of periodontal tissues, induction of vascular permeability, disruption of tight-junction connections and inhibition of polymorphonuclear killing ability.

Hemin-binding protein

As the name suggests, the hemin-binding protein plays a role during heme acquisition and facilitates the bacterial interaction with red blood cells.

As PD progress, the bacterium becomes confined to the periodontal pocket. As such, the hemin-binding protein is crucial for the *P.gingivalis* attainment of heme, an absolute growth requirement.

Outer membrane vesicles

From the outer membrane, Gram-negative bacteria can produce outer membrane vesicles. As already described, these vesicles serve as a transport mechanism and can carry all the outer membrane-associated virulence factors, like the LPS. Moreover, the gingipains can be transported through the OMVs and participate in toxin delivery.

3.2.3. Interaction between *P. gingivalis* and brain cells

P. gingivalis and its virulence factors have been extensively studied to access the bacterial role in neuroinflammation and neurodegeneration. Recent data, showed in detail in table 2, unravel a possible molecular mechanism involving *P. gingivalis* LPS (PgLPS) to promote Aβ accumulation.

In microglia, PgLPS interacts with Toll-like receptor (TLR) 2 to activate the NF-κB pathway. NF-κB targets genes involved in the inflammation progress, pro-IL-1β being one of those. Cathepsin B, a lysosomal cysteine protease, cleaves pro-IL-1β and mature IL-1β can now leave microglial cells and interact with IL-1R in neurons. In neurons, IL-1β activates once again the NF-κB pathway to induce the production of APP. Cathepsin B cleaves the APP into Aβ (85).

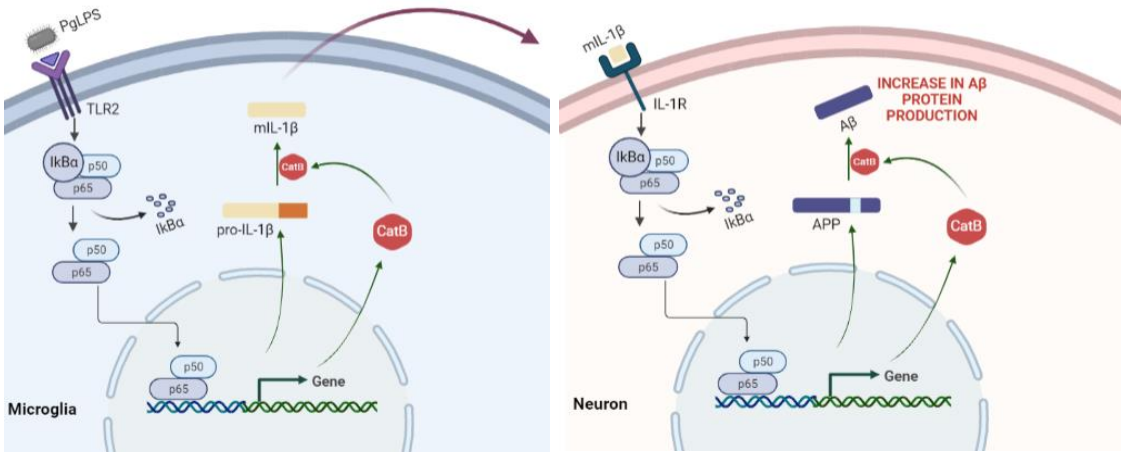

Figure 1 Aβ increase in the CNS during *P. gingivalis* infection
PgLPS interacts with microglial TLR2 promoting mIL-1β production. Interaction of IL-1β with its receptor in neurons results in increase expression of Aβ.

Table 2 In vivo and in-vitro studies exploring the of *P.gingivalis* role during Alzheimer-Disease neuroinflammation

Strain	Animal Model	Cell Model	Results	Conclusion	Ref
ATCC 33277	**Wild Sprague-Dawley** (n=40) G1 (n=10): 10^8 CFU/200 µL I.V per rat 3x/week 4 weeks G2 (n=10): 200 µL PBS per rat 3x/week 4 weeks G3 (n=10): 10^8 CFU/200 µL I.V per rat 3x/week 12 weeks G4 (n=10): 200 µL PBS per rat 3x/week 12 weeks	**Hippocampal neuronal cell line HT22** G1: pretreated with PP2A promoter D-erythrosphingosine for 2h and incubated with 10 ng IL-1β/mL for 12h G2: incubated with 10 ng IL-1β/mL for 12h G3: incubated with D-erythrosphingosine for 12h	**In-vivo results** > Tau phosphorylation was increased and activated astrocytes were detected after 12 weeks of infection > Hippocampal IL-1β, TNF-α, and IL-6 increased in a time-dependent manner > P.gingivalis and its gingipains were not detected in the hippocampus **In-vitro results** > IL-1β increased tau phosphorylation > P. gingivalis inhibited the activity of PP2A in the hippocampus	P.gingivalis can promote the secretion of inflammatory cytokines in the circulatory system, particularly IL-1β. In the mice hippocampus, IL-1β seems to induce tau hyperphosphorylation by inhibiting PP2A, the main tau phosphatase, activity.	(87)
ATCC 33277	**Twelve-month-old female mice** G1 (n=10): 10^8 CFU/100 µL intraperitoneally per mouse 3x/week 3 weeks G2 (n=10): 100 µL PBS per rat 3x/week 3 weeks	**RAW264.7 cells (mouse macrophage cell line)** Refer to article for more details	**In-vivo results** > Infection increased the mRNA levels of TLR2, IL-1β, APP and CatB in the mice liver but did not alter expression of Aβ degrading enzymes (NEP, IDE and ACE) > Liver macrophages shifted to a pro-inflammatory phenotype **In-vitro results** > Increased expression of IL-1β, TLR2, CatB and Aβ > A CatB-specific inhibitor and a NF-κB-specific inhibitor decreased the production of IL-1β	P. gingivalis infection induces IL-1β production in liver macrophages through the activation of TLR2/NF-κB signaling. NF-κB dependent CatB is required for both cleaving pro-IL-1β to mature-IL-1 β and processing APP to induce the accumulation of peripheral Aβ that could enter the brain.	(86)
Not Applicable	G1 (n=6): Wild-type 2 months old mice G2 (n=6): Wil-type 12 months old mice G3 (n=6): 2 months old CatB -/- G4 (n=6): 12 months old CatB -/- 1mg PgLPS/kg/day intraperitoneally for 5 weeks	**Immortalized microglial cell line MG6.** Primary culture obtained from wild-type and CatB -/- mice **Hippocampal neurons were isolated from wild-type mice** Refer to article for more details	**In-vivo results** In middle aged mice exposed to PgLPS for 5 weeks > CatB is increased and CatB dependent learning and memory were impaired. > CatB-dependent IL-1β, TLR2, TLR4 and Aβ are increased **In-vitro results** Exposition to PgLPS results in > Increased the production of IL-1β and TLR2. This was inhibited with TLR2 antibodies and CatB and NF-κB-specific inhibitors > Induces the CatB-dependent microglia activation required for intracellular Ab accumulation in hippocampal neurons	Chronic systemic exposure to PgLPS initiates intracellular accumulation of Aβ dependent on microglia inflammation in middle-aged mice.	(85)

Outside the CNS, a similar pathway has been proposed to occur in liver macrophages. Although far from neurons, liver macrophages live for approximately two to three weeks before dying from apoptosis. Afterward, Aβ produced in response to PgLPS is liberated into the systemic circulation and could reach the brain. It is important to note, however, that $A\beta_{3\text{-}42}$ showed a significantly higher production than $A\beta_{1\text{-}42}$. This is especially crucial if we consider that $A\beta_{3\text{-}42}$ shows a higher aggregation propensity (86).

As such, the interaction between PgLPS and macrophages in the CNS and periphery can induce the production of Aβ. Although Aβ is thought to be a key protein during AD pathology, some findings (88) suggest it may have a dual protective/damaging role. In-vivo studies showed that Aβ has antimicrobial activity. In the original report, it is proposed that oligomerization of Aβ mediates the protective activity (88).

P. gingivalis, through an IL-1β mediated pathway, is also responsible for the hyperphosphorylation of tau protein (87). In-vitro, *P. gingivalis* was able to inhibit the activity of protein phosphatase 2A (PP2A) in the hippocampus. PP2A is a serine/threonine phosphatase and it is known as the predominant phosphatase in the human brain. PP2A down-regulation correlates with tau hyperphosphorylation. It is now thought that *P. gingivalis* promotes IL-1β production and that the cytokine increase leads to tau hyperphosphorylation by inhibition of PP2A (87). Moreover, *P. gingivalis* gingipains are recognized as being able to fragment tau protein (31). As tau fragmentation induces the formation of insoluble and hyperphosphorylated aggregates (31), gingipains may contribute to the latter pathway or provide a parallel process to the formation of tau fibrils.

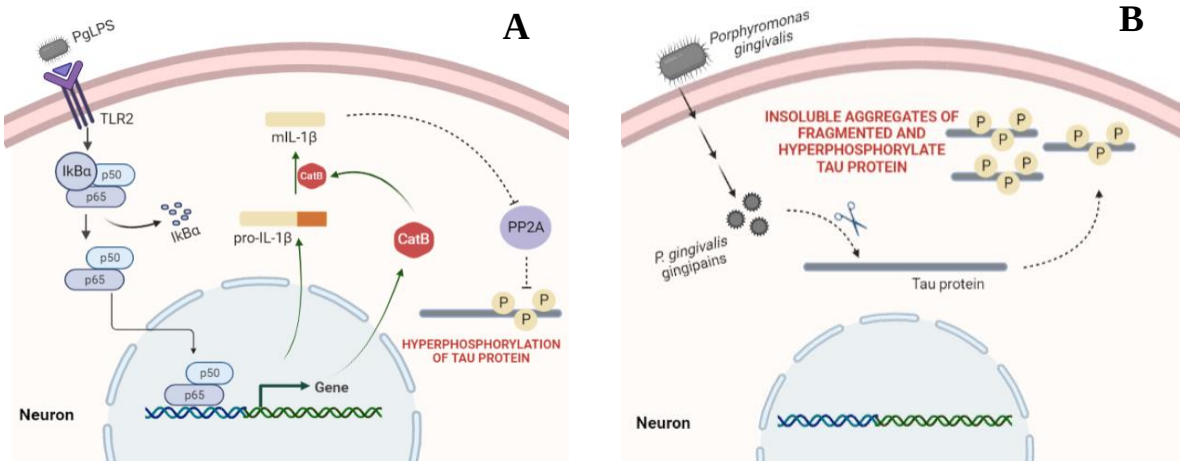

Figure 2 Hyperphosphorylation of tau protein during *P. gingivalis* infection
Different hypothesis try to explain the molecular mechanism behind the hyperphosphorylation of tau protein during *P. gingivalis* infection. (A) IL-1β mediated mechanism. (B) Gingipains mediated mechanism.

Lastly, it is important to underline a possible bias of these analyses. Both studies (86,87) used *P. gingivalis* ATCC 33277 and, although the molecular pathway may be similar across strains, Díaz-Zúñiga (89) already showed that AD pathology is serotype dependent. Even between capsular strains, tau phosphorylation, neuroinflammation and memory were differently impaired.

4. Cutting the link with a prophylactic vaccine

According to the Centers for Disease Control and Prevention (CDC), Alzheimer's Disease is responsible for more than 120 000 deaths in the USA alone. During the last decade, these numbers have gone up and AD is becoming a more common cause of death (90).

However, the public health burden of the disease does not stop in its mortality rates. Before dying from AD, patients usually survive for about 4 to 8 years. As the disease progress, patients become dependent increasing the burden of the disease (90).

The current treatment available for mild to moderate AD consists of Acetylcholinesterase inhibitors – donepezil, rivastigmine and galantamine. For moderate to severe cases, memantine, an N-methyl-D-aspartate receptor inhibiter, can be added or given as monotherapy (91). None of these therapeutical strategies act in the core neuronal findings. To target Aβ plaques and tau fibrillary tangles, some antibodies have been tested but without satisfactory results up to date. Active and passive immunotherapy has also been tested as a prophylactic strategy against AD but the immunization approach, clinical trial design, and patient recruitment are drawing back the process (92).

After years without understanding SAD etiology, the bacterial hypothesis presents as a strong etiologic candidate to the cause and/or exacerbation of the disease. The recognition of an infectious etiologic factor is crucial to the development of novel therapeutical targets and, most importantly, prophylactic approaches.

The main goal of designing a prophylactic vaccine is to reduce the incidence of the disease and its public health burden. However, this challenge brings presumed barriers that are here discussed alongside plausible strategies to overcome the obstacles.

4.1. Experimental design of a vaccine

4.1.1. Whole-cell immunization vs *P. gingivalis*-specific antigens

The first vaccines used in history used whole-cell immunization to prevent viral diseases. Thus, this well-established technology was the first to be tested against *P. gingivalis* infection (93).

Whole-cell immunization includes attenuated live vaccines and inactivated vaccines. For *P. gingivalis* both strategies were tested using heat-killed and formalin-killed cells but the results

were not satisfactory. Although mouse vaccination could elicit the production of *P. gingivalis*-specific antibodies in the serum, complete protection was not induced (93).

As whole-cell vaccines failed to demonstrate the desired outcome and as technology evolved, research turned its eyes into immunization with *P. gingivalis*-specific antigens (93). Several structures, theoretically all the virulence factors that have been described in *P. gingivalis*, can be useful to produce a vaccine target or adjuvant (94).

As seen, the PgLPS is a major component during AD development. Thus, it would make sense to develop an LPS-based vaccine to prevent the disease. This hypothesis, however, has not been tested yet. Nonetheless, results from periodontitis studies show that immunization with LPS does not induce a protective immune response nor serum immunoglobulin (Ig) G (93,95).

Capsule-based vaccines present an interesting strategy as capsular strains of *P. gingivalis* are more resistant to phagocytosis (84). However, not all strains have this component (84) and, thus, these vaccines would not prevent the disease in all patients. Epidemiological studies in PD patients found that capsular strains were present in almost 50% (93). No similar study was found using AD patients.

Purely capsule-based vaccines have been tested only once. Subcutaneous administration of whole capsule polysaccharide resulted in the elevation of the IgG and IgM titers (93,95).

On the other hand, fimbriae vaccines have been extensively studied. Results depended on delivery mode. Systemic delivery - subcutaneous or intramuscular - resulted in serum IgM and IgG antibodies. Mucosal delivery - gastrointestinal and intranasal - stimulated both systemic and mucosal immunity, as seen by serum antibodies and salivary IgA (93,95).

However, conjugates between capsular polysaccharide and fimbria protein were more effective than vaccines composed of capsule or fimbriae alone (93,95).

From a PD prevention perspective, gingipains present as an important approach as they are involved in the destruction of gingival tissue and alveolar bone loss (94). As such, gingipains-based vaccines have been tested in different animal models using different systemic delivery systems. In small mammals, like mouse and rat models, these vaccines effectively resulted in higher serum IgG and salivary Ig. Moreover, alveolar bone loss was prevented. Subcutaneous immunization of a macaque model verified these results (93,95). Gingipains role in *P. gingivalis* translocation to the CNS and its interaction with brain cells is not fully established yet but it may play a role in the development of tau filaments (31). These positive outcomes in the

prevention of PD may not translate into the prevention of AD but present as an interesting starting point.

Hemagglutinin is not a dominant surface component nor an immunodominant antigen (93). Nonetheless, it mediates bacterial agglutination to erythrocytes and can interact with TLR4 (94). Based on its potential as a virulence factor, hemagglutinin-based vaccines were tested. Subcutaneous administration of a hemagglutinin-only vaccine resulted in no specific salivary IgA antibodies while serum antibodies varied (IgM was only slightly increased and IgG was actively enhanced). However, intranasal administration with a monophosphoryl lipid A conjugate improved the results (93,95).

Lastly, immunization with outer membrane proteins specific antigens were researched using different mouse models in different delivery systems. Although results had slight differences depending on the outer membrane protein used and administration route, this strategy induced serum and salivary antibodies and reduced periodontal lesion size (93,95).

Briefly, these results show that vaccine conjugates are more effective in PD prevention (93,95) and this hypothesis should be carefully evaluated during AD vaccine development.

As the bacterial role in AD development is only now being unraveled, the outcomes used during *P. gingivalis* vaccine studies have been serum antibodies titers and periodontitis prevention. As such, the review of studies described here does not represent the real potential of *P. gingivalis* vaccines in the prevention of AD but could be an important beginning point.

For AD prophylactic prevention additional studies are necessary. Ideally, these studies should contemplate in-vitro experiences using both strategies - whole cell immunization vs *P. gingivalis*-specific antigens – and further in-vivo studies should be carried out with the strategies that showed the best outcomes.

4.1.2. Mucosal vaccine for Alzheimer's Disease

The vast majority of microorganisms first come into contact with the human body through mucosal surfaces. If we consider *P. gingivalis* role in Alzheimer´s Disease, the oral cavity is the mucosal surface on which we should focus.

The oral epithelium is associated with dendritic and Langerhans cells. Both cells have the ability to recognize pathogen-associated molecular patterns (PAMPs) through TLRs. The migration of

these cells to mucosal lymphoid foci initiates a cascade of events that result in either immune activation or immune tolerance depending on microenvironmental signals (96). Immune tolerance is important for commensal bacteria homeostasis and to prevent exaggerated immune reactions to ingested food (97). Another important factor in oral mucosal immunity is the presence of IgA in the salivary fluid that protects the oral cavity from bacterial colonization. IgA antibodies induce phagocytosis and activate the complement cascade pathway (96).

Collective, these local mechanisms prevent microorganism's entry and suggest an important role for mucosal vaccines (97).

Although the majority of vaccines are administered through intramuscular or subcutaneous injection, studies show that mucosal immunity is enhanced by mucosal routes of administration (97). These comprise intranasal, oral, gastrointestinal, intravaginal and intrarectal administration (97). Gastrointestinal administration is here referred to vaccines that, after oral delivery, are absorbed in the gastrointestinal tract and reach M cells in the gut. In contrast, oral administration refers to vaccines that target the oral cavity which includes the buccal and sublingual mucosa and the tonsils.

After mucosal vaccination, antigen-presenting cells (APC) recognize the antigens and introduce them to T cells. During this priming phase, APC induce the expression of homing receptors that guide lymphocytes to mucosal surfaces. However, these receptors can recognize ligands expressed in different locations since they are often expressed in a multitude of mucosal tissues. As such, mucosal vaccination can induce immune responses not only on the side of administration but in distal locations as well. Important of note is that intravaginal and intrarectal administration did not show this property (98).

For *P. gingivalis* immunization, intranasal and gastrointestinal vaccines have been tested and the results showed successful systemic and mucosal immunity (93,95). The oral cavity was underutilized but it presents some compelling characteristics (97). A comparison between the three routes of administration is shown below, in table 3.

Briefly, alternative routes of administration are less invasive increasing population compliance but the bioavailability is variable. Degradation of the antigen after administration or poor antigen uptake through the epithelium may explain the variability (97). As such, vaccines need to be formulated with different delivery systems that improve vaccine immunogenicity (99).

Table 3 Advantages and disadvantages of different mucosal administration routes

	Advantages	Disadvantages
Gastrointestinal	> Easy handling > Higher compliance > Low cost of production > Induces mucosal and systemic immunity	> Degradation of the vaccine at the gastrointestinal tract > Safety concerns due to live attenuated formulations > Low bioavailability
Intranasal	> Higher compliance > Highly vascularized mucosal surface > Practical for mass vaccination > Induces mucosal and systemic immunity	> Risk of neurological adverse reactions > May exacerbate nasal or respiratory inflammation > Variable efficacy
Oral cavity	> Easily accessible > Minimally invasive > Favorable characteristics (compared to the gastrointestinal tract) > Induces mucosal and systemic immunity	> Lack of appropriate dosage forms > Physical barriers (epithelium and salivary flow and composition) > Predisposition to immune tolerance

One of the most popular strategies to enhance vaccine efficacy for mucosal immunization is entrapping the antigen in a particle (99). Enclosing the antigen not only protects against degradation but also allows it to become more similar to the original pathogen. Thus, particles are better taken up by APCs (100). Particles can have a variety of different compositions from liposomes to polymers (99).

Polymeric particles, including chitosan and poly (lactic-co-glycolic acid) (PLGA), enable slow release of the antigen, prolonging the exposure (99). These characteristics allow polymeric vaccines to be given in a single dose. Moreover, the mucoadhesiveness of chitosan makes it ideal for mucosal delivery as it enhances APC uptake (99). Lipidic particles are also used as drug carriers. For intranasal immunization, liposomes stimulate the mucosal and systemic immune response (99).

Regardless of composition, particles need to conform to a set of characteristics to be taken up by APCs. Particle size, shape and functionalization are among the most important properties. Particle-based mucosal vaccination via the respiratory tract is more effective with positive charged, earthworm-like particles with sizes up to 500 nm (101). If oral administration is the preferred route for gastrointestinal absorption, particles should have a size ranging from 100 to 500 nm. However, the authors do not agree on other surface properties (102).

The nanoscale, used in particles, can also be applied to gels. Nanogels are prepared from polymers with a three-dimensional crosslinked network (103). Nanogels have been used as both drug and vaccine delivery systems through intranasal administration (103). As vaccine carriers, nanogels must gain access to the nasal epithelial layer without being entrapped by the mucus. For effective delivery, nanogel encapsulated antigens must have a size of 50nm to freely circulate across the mucus (103).

Particles and nanogels, however, are not suitable for vaccination in the oral cavity. The nanoparticles used for intranasal and gastrointestinal delivery are usually formulated in aqueous suspension which does not allow for an adequate residence time in the mouth (97). Formulations with higher viscosity or mucoadhesion could, theoretically, surpass this barrier but results did not corroborate the theory (97). Technologies that increase residence time, such as mucoadhesive gels or tablets, do not increase immunological response due to an inability in crossing the epithelial barrier in the oral cavity. As such, antigens are not effectively presented to DC that reside deeper in the mucosa (97).

Creighton and Woodrow proposed that microneedle-based vaccines might be an effective delivery system to overcome physiological barriers in oral cavity vaccination (97). Microneedles can be classified into four different types, according to delivery technology. For vaccine delivery, hollow and dissolving microneedles have been the preferred method (104). Hollow microneedles function similarly to traditionally looking syringes but the needles are shorter in size. The hollow space allows the microneedle to be filled with liquid formulations that are then injected through the skin. Contrarily, dissolving microneedles are made of water-soluble materials that dissolve and release the antigen after being pushed through the mucosa (104).

A hollow microneedle vaccine loaded with nanoparticles is technically feasible. Combining the advantages of nanoparticles with microneedles as a delivery system theoretically allows an effective antigen delivery that resembles the natural infection.

5. Conclusion and future perspectives

Alzheimer's Disease is the leading cause of dementia worldwide and the sixth most common cause of death in the USA. As such, prophylactic strategies against AD have been the focus of investigation for many years. Thus far, the negative outcomes with monoclonal antibodies against Aβ and tau proteins have cut short the possibility of an immunotherapy-based prophylactic vaccine.

The *postmortem* analysis of AD patients' brains and the consequent identification of different oral bacteria are the basis for the bacteriological etiology hypothesis of Alzheimer's disease. *P. gingivalis,* in particular, is now recognized as a probable risk factor contributing to the establishment or worsening of the disease. Although the molecular pathophysiological mechanism remains unknown, *P. gingivalis* participates in both Aβ aggregation and tau hyperphosphorylation through an IL-1β mediated pathway.

The relationship between *P. gingivalis* infection and Alzheimer's Disease brings back the hope for the development of a more ''traditionally-looking'' vaccine that ameliorates or prevents neurodegeneration.

Studies in periodontitis prevention have already evaluated *P. gingivalis* immunization. However, periodontitis results might not apply to AD. As such, vaccine design should contemplate carefully choosing the best antigen and antigen delivery system for an effective mucosal immune response. Although other mucosal routes have proven to be effective in producing salivary antibodies, the oral mucosa presents as the most natural administration route for the vaccine. Specialized delivery systems, such as microneedles, might be needed to successfully deliver *P. gingivalis* antigens to the oral mucosa.

The question of who and when should receive a vaccine remains unclear. Epidemiological studies and validation of predictive biomarkers will help in the selection.

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