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Masteroppgave

A Relationship between *Porphyromonas gingivalis* and Alzheimer's disease

Can *Porphyromonas gingivalis* cross the BBB?

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Abstract

Background: *Porphyromonas gingivalis*, a keystone pathogen implicated in periodontal disease, has been associated with neurological disorders, such as Alzheimer's disease. They are both chronic diseases, and they share several risk factors that could explain an association between them. Several pathways have been suggested where *P.gingivalis* ultimately could lead to inflammation in the brain, and therefore Alzheimer's. In addition, there are mechanisms which *P.gingivalis* could use to cross the blood brain barrier (BBB) directly.

Objective: To perform a literature search to find an association between periodontal health and Alzheimer's disease. In addition, an experimental study that aims to find if different strains of P.gingivalis can cross the BBB hCMEC/D3 *in vitro* model, and if there is a temporal relationship to this.

Methods: The penetration ability of different *P.gingivalis* strains on BBB *in vitro* model hCMEC/D3 was investigated at two different timepoints. The hCMEC/D3 were infected with each their own strain prior to transmission electron microscopy, which was used to take images to determine the penetration degree after 2 hours and after 6 hours. The images were subsequently analysed, and data was plotted on Microsoft Excel 2016 to calculate mean, perform ANOVA two-way test.

Results: The different *P.gingivalis* strains, A7A1, ATCC33277, W83, 195P63 had different penetration ability on the hCMEC/D3 BBB model, at both timepoints. The study did not yield statistical significance, but there is a quantitative difference in the mean of each of the strains' penetration ability.

Conclusion: These findings underscore how factors such as strains, time, virulence factors change the penetration ability and therefore the integrity of the BBB. *P.gingivalis* is able to cross the BBB, but the efficiency of it depends on bacterial characteristics.

Keywords: Alzheimer's disease; *Porphyromonas gingivalis*; Periodontitis; blood-brain barrier; NVU, Astrocytes, Pericytes, OMVs; gingipains ; *in vitro* BBB model; APOE, microglia, tau, amyloid-beta, nervus trigeminus, virulence factors.

Sammendrag

Bakgrunn: *Porphyromonas gingivalis*, et periodontalt patogen, har blitt assosiert med nevrologiske sykdommer, som Alzheimers sykdom. Begge er kroniske sykdommer, og begge deler mange risikofaktorer som kan være med på å forklare en assosiasjon mellom dem. Flere mulige veier har blitt foreslått hvor *P.gingivalis* kan føre til en inflammasjon i hjernen, som over tid kan gi opphav til Alzheimers sykdom. I tillegg, er det spesifikke mekanismer som *P.gingivalis* bruker for å krysse blod hjernebarrieren direkte.

Målsetting: Å gjøre en litteraturstudie for å undersøke om det er en assosiasjon mellom periodontal helse og Alzheimers sykdom. I tillegg det ble utført et *in vitro* pilot eksperiment for å undersøke om ulike ulike stammer av *P.gingivalis* kan krysse blod hjerne barrieren (hCMEC/D3 *in vitro* modell), og avdekke hvem av de som eventuelt krysser ved ulike tidspunkter.

Metoder: Det ble undersøkt evnen til å krysse blod hjernebarrieren (*in vitro* model hCMEC/D3) med ulike stammer av *P.gingivalis* på to ulike tidspunkter. Hvert *in vitro* forsøk ble infisert av en spesifikk stamme, deretter støpt inn før det ble utført transmisjon elektronmikroskopi (TEM) for å kunne ta bilder. Penetrasjonsevne ble målt etter 2 og 6 timer. Elektronmikroskopiske bilder av dette materialet ble deretter analysert. Data ble overført til Microsoft Excel 2016 for å kunne regne ut gjennomsnitt, standardavvik og for å kunne utføre en ANOVA to veis test.

Resultater: De ulike *P.gingivalis* stammene A7A1, ATCC33277, W83, 195P63 hadde ulike penetrasjonsgrader på begge tidspunkt. Selv om studien ikke ga noen statistisk signifikante resultater i penetrasjonsgrad, viste det en kvantitativ forskjell mellom strains.

Konklusjoner: Funnene tyder på at virulence faktorer og tid er det som kan påvirke bakterienes evne til å krysse blod hjernebarriaren. Penetrasjons effektiviteten var stamme-avhengig, og dette kan skyldes ulike egenskaper ved stammene.

Nøkkelord: Alzheimer’s disease; *Porphyromonas gingivalis*; Periodontitis; blood-brain barrier; NVU, Astrocytes, Pericytes, OMVs; gingipains ; *in vitro* BBB model; APOE, microglia, tau, amyloid-beta, nervus trigeminus, virulence factors.

Acknowledgements

I am pleased to present this Master's thesis, "The Relationship between P.gingivalis and Alzheimer's disease: Can *P.gingivalis* cross the BBB?"

Many have contributed to this work, to whom I wish to express my gratitude. Firstly and foremost, to my main supervisor, Professor Dr. Linda Hildegard Bergersen, group leader of Brain and Muscle Energy group, at the faculty of Dentistry in the University of Oslo. Thank you for your guidance and continuous support over the years throughout this project, it would not have been possible without you. In addition, I would like to express my gratitude to Postdoc Ingrid Åmellem for providing the hCMEC/D3 cells used for the experiment, and Associate Professor Morten Enersen for the continuous support throughout this project. I am also deeply thankful for senior engineer Anne Karin Kristoffersen, and chief engineer Yiqing Cai for providing the cell cultures and the Transmission Electron Microscopic images. I would also like to thank PhD candidate Imen Belhaj for always being available and helping me through this project. Finally, I want to thank the members of the Institute of Oral Biology at the University of Oslo for always being available for questions and guidance.

Thank you once again for your unwavering support and encouragement during these years.

Table of Contents

List of Abbreviations

Abbreviation	Meaning
AB	Amyloid-beta
AB42	amyloid-beta 42
AD	Alzheimer's disease
AGE	advanced glycation end product
AJs	adherens junctions
An	a.naesundii
APOE	apolipoprotein E
APP	amyloid precursor protein
BBB	blood brain barrier
BM	basement membrane
Caveolin-1	Cav-1
ECs	Endothelial cells
EM	electron microscopy
EOAD	early onset alzheimer's disease
ERK	extracellular signal regulated kinase
GSK-3Beta	glycogen synthase3 beta
hCMEC/D3	human cerebral microvascular endothelial cells/D3
IgG	immunoglobulin G
IL 10	interleukin 10
IL 1β	Interleukin-β
IL 6	interleukin-6
IL 8	interleukin-8
iPSC	induced pluripotent stem cells
JAMs	junctional adhesion molecules
LOAD	late onset alzheimer's disease
LPS	Lipopolysaccharide

LRR	Leucin rich receptor
MAPK	microtubule affinity regulation kinases
MMP-9	matrix metalloproteinase 9
NTFs	neurofibrillary tangles
NVUs	Neurovascular unit
OMVs	outer membrane vesicles
PC	pericytes
PD	Periodontal disease
PG	Porphyromonas gingivalis, P.gingivalis
PHF	paired helical filaments
PI3K	phosphoinositide 2 kinase
PKA	protein kinase A
SF	straight filaments
TEER	transendothelial electrical resistance
TEM	transmission electron microscopy
TJs	tight junctions
TLR4	toll-like receptor 4
TNF-a	tumour necrosis factor alpha
WT	wild type
ZO-1	Zonula occludens 1

Chapter 1: Introduction of the Master's Thesis

Alzheimer's disease (AD) is a neurodegenerative disorder. In recent years, scientific evidence has suggested a potential association between periodontal disease and the development of Alzheimer's disease. *Porphyromonas gingivalis*, a keystone pathogen of periodontal disease, could be an important pathogen in Alzheimer's progression. It is thought that *P.gingivalis* could cross the blood brain-barrier (BBB) and possibly exacerbate AD within the brain. This Master's thesis consists of two parts; A literary search which investigates the intricate relationship between *Porphyromonas gingivalis* and AD, and an experimental study which aims to answer: Can *P.gingivalis* cross the BBB?

Firstly, it was conducted a thorough literature search studying the possible intricate relationship between *P.gingivalis* (PG), the BBB and Alzheimer's disease. Part of the research was to find associations between PG and AD, any underlying mechanisms in which *P.gingivalis* can cross the BBB, and if once breached the BBB if *P.gingivalis* could lead to AD. Once a broad comprehension of the subject was established, articles were included or excluded based on relevance, quality, and reliability. The database used to find articles was Pubmed, using the keywords; Alzheimer's disease; *Porphyromonas gingivalis*; Periodontitis; blood-brain barrier; NVU, Astrocytes, Pericytes, OMVs; gingipains ; *in vitro* BBB model; APOE, microglia, tau, amyloid-beta, nervus trigeminus, virulence factors, and the connector AND to combine search terms. Part One ends with a discussion, where interconnections are explored and possible links are suggested between Alzheimer's disease and periodontal disease.

Once the literature search was completed, an experimental framework was designed to investigate if *P.gingivalis* was able to cross the BBB. It was decided to perform an *in vitro* BBB model with hCMEC/D3 cells, and these were infected with four different strains of *P.gingivalis*; W83, ATCC33277, 195P63, A7A1. Each of the strains infected the BBB for 2 and 6 hours *in vitro*, and after the cells were embedded, transmission electron microscopy (TEM) was performed to take images and investigate the permeability index. Once the images were analysed, quantitative and statistical analysis were performed using Microsoft Excel 2016. The methods, materials and results are thoroughly presented. Part Two ends with a discussion where the results are interpreted.

In conclusion, this Master's thesis explores a link between *Porphyromonas gingivalis* and Alzheimer's disease through a literature search and an experimental *in vitro* pilot study. My main aim is to contribute scientifically to the exciting field of research in the years to come. In the next chapter we will start with Part One, the literary search, where periodontal disease and its pathology is presented.

PART ONE

Chapter 2: Periodontal Disease

General information

Periodontal diseases are characterised by an infection in the periodontium, a tissue composed of the gingiva, the periodontal ligament, teeth and alveolar bone. (Armitage, 1999; Tonetti et al., 2018). Periodontal diseases can be divided into different categories such as gingivitis, and marginal periodontitis. Gingivitis is inflammation caused by the bacteria present in the biofilm and plaque, and it clinically presents often as red, swollen and bleeding gums (Rathee and Jain, 2023). Periodontitis, is a chronic irreversible inflammatory condition, which is caused by excessive accumulation of dental plaque localised by the gingival margins. There is a equilibrium disturbance, a dysbiosis within the subgingival microbiome, and it triggers an inflammation in the periodontium (Harding et al., 2017b) Over time there will be a loss of periodontal ligaments, the alveolar bone will migrate apically, and ultimately it will lead to tooth loss (Armitage, 1999; Kanagasingam et al., 2020; Leira et al., 2017, Poole et al., 2013; Singhrao and Olsen., 2019; Tonetti et al., 2018).

There are several forms of periodontitis, where marginal periodontitis varies from mild, moderate and severe forms. According to Eke et al. (2015) 46% had periodontitis in the USA, and 8.9% of them had severe periodontitis. However, in another article they suggest that the severe form makes up 15-20% (Leira et al., 2017). When diagnosing these forms, it is based on clinical and radiographic examinations, which determine the degree of periodontal attachment and alveolar bone loss. (Tonetti et al., 2018).

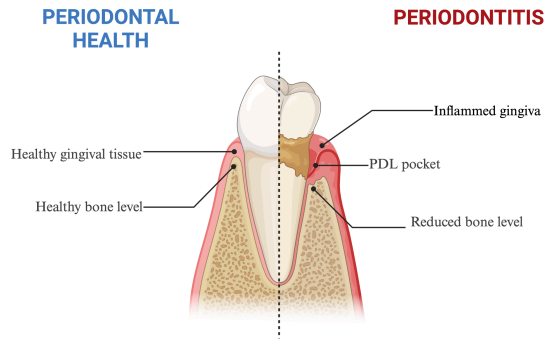

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Fig.1
This is an illustration inspired from Biorender. It compares how a tooth looks when it has periodontal health, and when there is periodontitis. In healthy teeth the gingiva is pink, tight around the tooth and there is no marginal bone loss. Patients with periodontitis will show clinical signs such as an edematous gingiva, deep PDL pockets and marginal bone loss caused by the local infection (Marsh et al., 2016, Tonetti et al., 2018)

The risk factors

Periodontitis is a multifactorial disease and there are several risk factors interacting with one another. Some of the factors that can affect the development of PD are age, ethnicity, gender, culture, socioeconomic status and they can have interactions with one another (Darby, 2022; Könönen et al., 2019). In addition, we can broadly classify them into modifiable and non modifiable risk factors, which are demonstrated in Fig. 2. (Armitage,1999; Tonetti et al, 2018; Singhrao and Olsen, 2019; Van dyke and Sheilesh, 2005). However, it is important to note that age is not always categorised as a risk factor, as there is loss of alveolar bone with age that is not pathological (Van Dyke and Sheilesh, 2005).

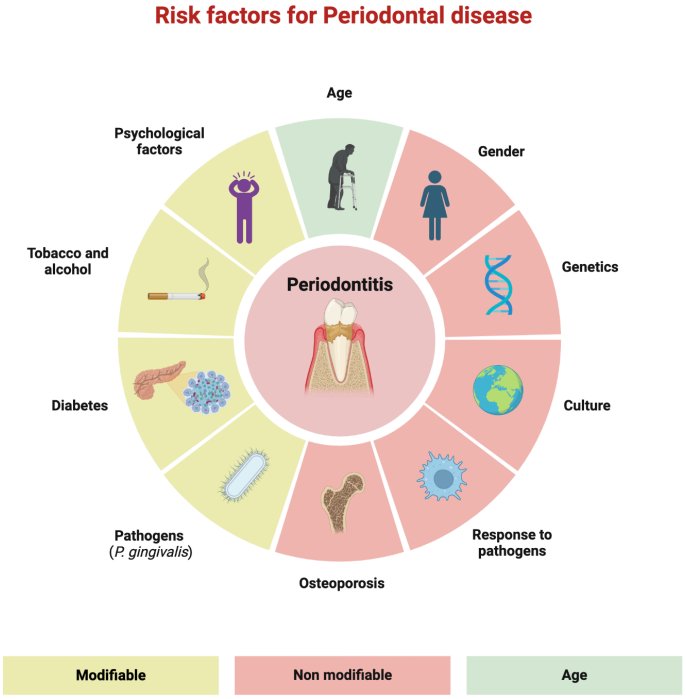

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Fig. 2

Risk factors are categorised into modifiable and non-modifiable risk factors. The modifiable risk factors are smoking, alcohol consumption, diabetes mellitus, psychological factors such as stress and the pathogens present in the biofilm. The non-modifiable risk factors are genetics, the host response to the pathogens, and osteoporosis. In addition, while not pathological age causes marginal bone loss (Van Dyke and Sheilesh, 2005).

The Oral Biofilm

Within the oral cavity we find a sticky biofilm that forms on the surfaces of teeth, gingival crevices and periodontal pockets. This is a polymicrobial ecosystem, otherwise known as plaque, and when is not regularly removed through oral hygiene practices it is what triggers periodontal disease (Marsh, 2006; Marsh et al., 2016).

From the moment teeth are erupted into the oral cavity, the surfaces are coated by a film of molecules mainly composed of proteins and glycoproteins which derive from the saliva (Marsh et al., 2016). Once an extracellular polymeric structure is present, motile bacteria are able to find and bind themselves to it via chemotaxis. This first colonisation can only be done by certain streptococci as they are reliant on epithelial cells, and then a pellicle is formed (Whittaker et al., 1996). As the biofilm develops it attracts other bacteria by the secretion of substances, a polymer matrix is formed, and it develops until a microbial homeostasis is achieved. This is a dynamic state, where the species are exchanging synergistic and antagonistic interactions (Marsh, 1994).

When the biofilm is not regularly removed, this biofilm changes physiology, and the phenotype will change with the changes in environment (Marsh, 2006). When certain pathogens are present, they can incite a systemic inflammation by producing proinflammatory cytokines, chemokines, other mediators, and when the immune cells are activated it causes an exaggerated immune system response, which ultimately causes bone loss (Kinane et al., 2017; Larsen and Fiehn, 2017; Poole et al., 2013).

The Red Complex

When searching for the aetiology of periodontitis, there have been identified several microorganisms which are associated with the development of the disease. It is observed a gradual change from gram-positive to gram-negative, and from aerobic to anaerobic as we sink further into the periodontal pocket. This will change the phenotype of the biofilm, which affects their metabolism and the end products produced in the deeper parts of the periodontal pocket (Marsh, 2006; Marsh et al., 2016)

Depending on the composition of the bacteria, they have been divided into groups such as the yellow, purple and orange complex among others **(**Socransky et al., 1998; Marsh et al., 2016). The red complex develops in the later stages of biofilm development, and is associated with the epithelial lining of the deepest pockets (Marsh et al., 2016; Nayak et al., 2018). The red

complex is a specific interaction between *Treponema denticola, Tannarella forsythia*, and ultimately *Porphyromonas gingivalis*. These are a group of commensal opportunists, and they will disrupt the balance from homeostasis to dysbiosis upon infection (Harding, 2017b). For this research, it has been decided to focus on *Porphyromonas gingivalis, P.gingivalis*, as it has been identified as a possible keystone pathogen associated with the development of Alzheimer's disease.

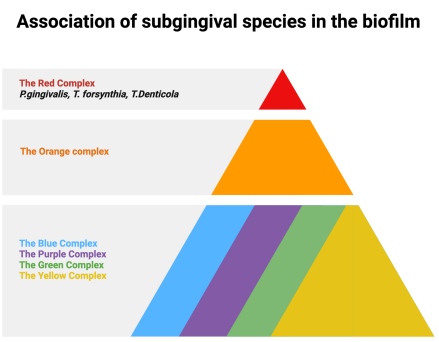

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Fig.3
Illustration adapted from Socransky (1998). Bacteria in the oral biofilm aggregates into different complexes, which means that certain pathogens. Purple, yellow and green are frequently found and associated in healthy gingiva. However orange complexes are found in deeper pockets are precede the Red complex, which is composed of *P.gingivalis, T.denticola,* and *T.forsythia* (Marsh et al., 2016; Socransky et al., 1998)

Porphyromonas gingivalis

Porphyromonas gingivalis is a keystone pathogen in periodontal disease. It is a nonmotile anaerobic bacterium, which means it thrives in environments where oxygen is scarce, such as the periodontal pockets, which is a symptom of periodontal disease (Bostanci and Belibasakis, 2012). Since it is nonmotile, it depends on the buffer created by the saliva to bind to teeth surfaces or the gingival crevices, and is therefore called a secondary coloniser, as they bind to other bacteria such as actinomyces, and mitis groups, as well as *streptococci gordonii* and *P.intermedia* (Bodet et al., 2007; How et al., 2016). The way in which *P.gingivalis* binds itself to the biofilm is by using major fimbriae, Mfa1 to bind to streptococci, and Leucin Rich Repear (LRR) to bind other microorganisms (Hasegawa and Nagano, 2021). Since they inhabit deep pockets, they are reliant in the fermentation of amino acids instead of carbohydrates, making them asaccharaolytic (Bostanci and Belibasakis, 2012). In order to stay alive, in addition to amino acids they require heme, vitamin K and hemin to evolve and grow, all of which are found in periodontal pockets (Ali et al., 1996).

In the following section the different virulence factors of *Porphyromonas gingivalis* will be presented.

Virulence factors

Virulence is an organism's ability to infiltrate and establish an infection within the host. These virulence factors are molecules that enable colonisation on a cellular level, which can be categorised as secretory, cytosolic or membrane associated (Sharma et al., 2016). *Porphyromonas gingivalis* have several of them such as the "capsule, outer membrane, its associated LPS, fimbriae, proteinases, and selected enzymes" (Singhrao et al., p.3, 2015), all of which are required to colonise the host. This factors will be presented closer each of them;

The Capsule

In order for bacteria to become established in the oral cavity, they must adhere to mucosal surfaces, which happens with the help of the capsule (Yoshimura et al., 2009). The capsule is composed by a polysaccharide layer which envelopes the cell, and thereby protects it against external influences. This capsule also enables the bacteria to adhere via receptors and adhesins more efficiently to the mucosal surfaces such as the gingiva and the teeth (Yoshimura et al., 2009; How et al., 2016). Another article revealed that a capsule affects the initial adhesion to the epithelial cells in the periodontal pockets (Dierickx et al. 2003)
There are several different strains of *Porphyromonas gingivalis*, some which are encapsulated and therefore more virulent, whereas others are not. According to Laine et al. (1997) the ability of inoculation depended on the presence of the capsule, where encapsulated bacteria would cause infection after inoculation, while the non-encapsulated strains were killed by immune cells like macrophages and dendritic cells in localised abscesses (Laine et al., 1997). However in an article by Irshad et al. (2012), they found that the bacteria with capsules may invade less efficiently gingival fibroblasts compared to bacteria without capsules.

Outer and Inner Membrane

Porphyromonas gingivalis consists of two cell membranes inside that capsule, the outer and inner membrane (Bos et al., 2007). The outer membrane, otherwise known as an asymmetrical layer, consists of two leaflets or layers, made up of lipopolysaccharides (LPS) with a peptidoglycan layer between them. The inner membrane is made up of a phospholipid layer with proteins (Bos et al., 2007; Nikaido, 2003). The function of these two membranes within the capsule is to create a selective barrier, where only certain molecules are able to

cross into the cell via the portin proteins (Nikaido, 2003). The host recognizes the outer membrane as an antigen, which means it activates the immune system by activating immune cells like T-helper cells in patients with aggressive periodontitis, which will lead to higher concentrations of cytokines such as IL-1B and IL-6 compared to healthy individuals (How et al., 2016)

Fimbrae

Fimbrae are slender protein-based that extend outward from the outer membrane of the bacterial (How et al., 2016). These proteinaceous appendages facilitate the "invasion of host cells, biofilm formation, cell motility, and transport of proteins and DNA across cell membranes." (Enersen, p. 2, 2013). *P.gingivalis* expresses two variations of this appendage, the Major and the Mfa1 fimbriae. The Major consists of fibrillin, a subunit protein encoded by the FimA gene, while Mfa1 fimbriae constitutes of an Mfa protein (How et al., 2016). These structures "likely influence the development of periodontal diseases" (Enersen, p.1, 2013).

LPS

Anchored to the cell wall, lies the LPS, which is major component in Gram-negative bacteria.What is interesting about the LPS in *P.gingivalis* is that the innate host defence system does not recognize it as effectively as other Gram-negative species, for example *Escherichia coli* (Liu et al., 2008). The LPS is usually composed by a lipid A, an inner- and outer core and an O antigen, however, in *P.gingivalis* the structure is slightly different, which is demonstrated in the figure.4 (Schromm et al, 2000). Lipid A works as an antigen, and it activates the TLR pathway, either TLR2 or TLR4, which in turn activates the production of pro-inflammatory cytokines, TNF-alpha, and IL-6. Over time it could cause alveolar bone loss (Papadopoulos et al, 2013; Holden et al., 2014). According to Kato et al (2014) LPS has the ability to inhibit osteoblastic differentiation in addition to its mineralization in the periodontal ligament, and thereby affects the regeneration ability of periodontal tissue (Kato et al.,2014). In another article, Lipid A's ability to induce inflammatory responses disrupts bone remodelling (Herath et al. 2011). At the same time LPS can inhibit the secretion of interleukin-8 (IL-8) by gingival epithelial cell, by preventing the activation of inflammatory cells (Yoshimura et al., 1997, Darveau et al.,1998), which will allow the accumulation of periodontal bacteria within the gingival crevice.

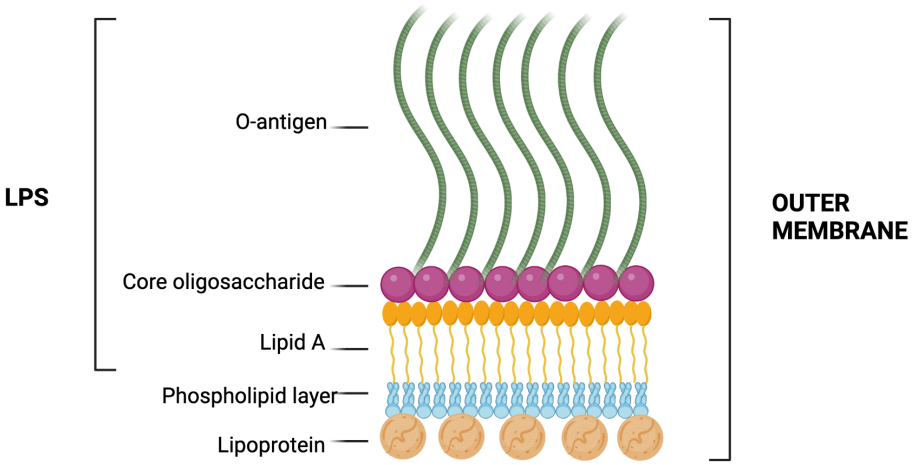

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Fig.4 Illustration adapted from How et al. (2016)
The outer membrane of the bacteria is composed by the LPS, the phospholipid layer and lipoprotein. LPS is one of *P.gingivalis* virulence factors and is composed by three structures Lipid A, oligosaccharide and O-antigen. This will activate the inflammatory system within the host. (How et al., 2016)

Gingipains

Gingipains are trypsin-like cysteine proteinases, which cleave the C-terminal after arginine or lysine residues, they are respectively called gingipain R (RgpA and RgpB), and K (Kgp) (Potempa et al., 2003). Gingipain R has the ability to degrade extracellular matrix components, such as integrin, fibronectin binding, cytokine, immunoglobulin and complement factors (Curtis et al., 2001). Gingpain K, cleaves by the C terminal where lysine is present in peptides. When this amino acid is cleaved, many proteins will be degraded that are vital for the connective tissue and plasma. These proteases cleave for instance hemopexin, transferrin, haemoglobin and haptoglobin (Sroka et al., 2001). Subsequently once substances like fibrinogen and heme are degraded, it will partially inhibit the blood's ability to coagulate, leading to increased bleeding in the periodontal pockets, which increases proliferation of *P.gingivalis* in those deep pockets (Sroka et al., 2001). Additionally, *P.gingivalis* will activate the kallikrein cascade, which induces vascular permeability. (Sroka et al., 2001).
Further information of gingipains, and the relationship between periodontitis and Alzheimers will be given in the following chapters.

Summary

Porphyromonas gingivalis, a bacterium associated with periodontal disease, has received attention not only for its impact on oral health but for its potential link to neurological disorders (Borsa et al., 2021; Leira et al., 2017). Understanding the mechanisms by which *P.gingivalis* may contribute to the development or exacerbation of Alzheimer's could shed light on new avenues for prevention and treatment strategies (Leira et al., 2017)

Chapter 3: Alzheimer's Disease

General information

Alzheimer's disease is a progressive neurological disorder, and the leading cause of dementia in the elderly (World Health Organization, 2023). This neurodegeneration causes "cognitive impairment, memory loss, psycho-behavioural disturbances, and language disability" (Leira et al., p.2, 2017). Alzheimer's poses significant challenges for patients, caregivers and healthcare systems. There are different types of AD, and they can be classified as familial or sporadic types, where the latter makes up 95% of all the cases (Singhrao and Olsen, 2019). Recent research has shed light on the potential association between chronic periodontal infection and the development of neuropathological changes reminiscent of Alzheimer's disease (AD). In addition, age and the loss of up to nine teeth might be two factors that correlate sporadic AD with chronic periodontitis (Singhrao and Olsen, 2019; Stein et al., 2007).

The neuropathological hallmark features of Alzheimer's disease include the accumulation of beta-amyloid plaques (Aβ) and tau protein tangles in the brain. Aβ plaques, which are formed from the aggregation of amyloid-beta peptides, disrupt neuronal function and contribute to synaptic dysfunction and neurotoxicity. (Singhrao and Olsen, 2019; Olsen, 2021) The tau protein tangles are accumulated hyperphosphorylated tau protein, which causes neuronal damage and eventually cell death. These pathological changes result in widespread neurodegeneration, particularly affecting regions crucial for memory and cognitive function, such as the hippocampus and cerebral cortex. (Singhrao and Olsen, 2019; Olsen, 2021). In order to diagnose AD there must be signs of cognitive deficiency, as well as the presence of intraneuronal neurofibrillary tangles (NFTs), and extraneuronal amyloid (Aβ) plaques in the brain (Dugger and Dickson, 2017; Hyman et al., 2012; Kanagasingam, 2020).

Ageing causes many changes within the central nervous system, such as brain size, cognition and vasculature (Peters, 2006). After the age of 40, the brain loses around 5% of its weight, with a rapid decline after 70 years. (Svennerholm et al., 1997; Scahill et al., 2003). Macroscopically it has been observed a widespread cortical atrophy, for example in the gyri, as well as an enlargement of sulcal spaces (DeTure and Dickson, 2019). All of which is potentiated even further when pathology like AD is involved. From the age of 65 it is difficult to differentiate and distinguish which symptoms are caused by age or late onset AD (LOAD), making it harder to diagnose (Guerreiro et al., 2015; Hou et al., 2019).

Risk factors of AD

Alzheimer's disease is affected by several risk factors. Some of them are demographic which include age, gender, race, ethnicity or social class (Armstrong, 2019). In addition, some factors such as the exposure to the metal aluminium, or vascular infection and traumatic brain injuries could affect the development of AD (Armstrong, 2019). Furthermore, genetics also influence and are associated as possible risk factors for early onset familial AD (EOAD). These alleles include Presenilin type 1 and 2, amyloid precursor protein (APP) and apolipoprotein (Armstrong, 2019; Khanahmadi et al., 2015; Van Cauwenberghe et al., 2016).

Alzheimer's diseases pathogenesis

There are several factors which affect the pathogenesis of Alzheimer's disease. For example, the dysfunction of mitochondria could generate free radicals within the cell, and ultimately lead to AD (Dhapola et al., 2021). In the following illustration, Figure 5, inspired by Dhapola et al. (2021), it demonstrates different factors that could affect the pathogenesis of Alzheimer's are presented. In this master's thesis, we shall concentrate on the formation of Aβ plaques, and tau hyperphosphorylation and its relationship with *P.gingivalis*.

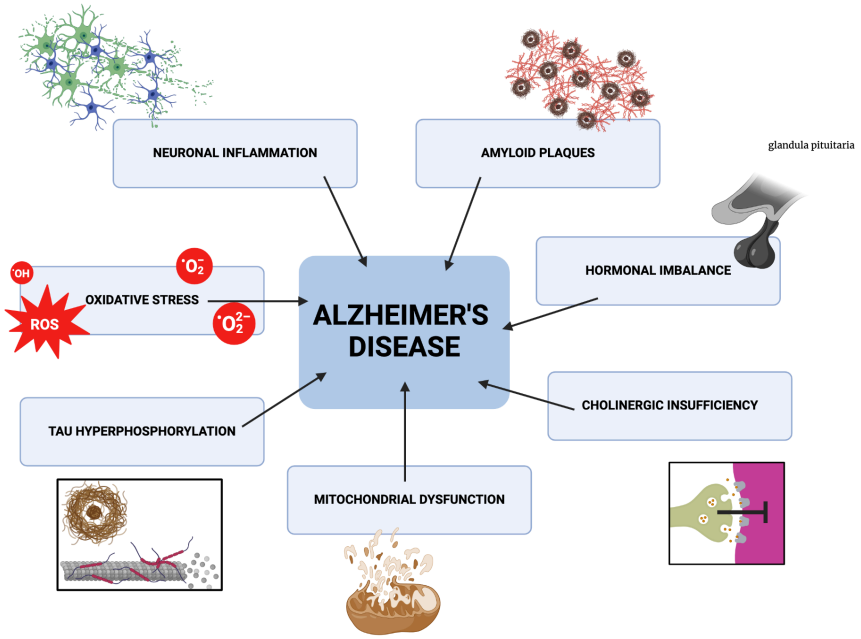

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Fig. 5 There are seven main causes for the development of AD (1) amyloid plaques (2) hormonal imbalance (3) cholinergic insufficiency (4) mitochondrial dysfunction (5) tau hyperphosphorylation (6) oxidative stress (7) neuronal inflammation (Dhapola et al., 2021)

Mechanism and pathophysiology

Several hypotheses have been presented to explain this multifactorial disorder. In this literary search we shall focus on the amyloid and tau hypothesis, as research has found connections between *Porphyromonas gingivalis* and these pathways.

Amyloid cascade hypothesis

The amyloid beta hypothesis is characterised by abnormal accumulation of amyloid β (Aβ) plaques in the central nervous system, which is the brain and the brainstem (Hardy and Higgings, 1992). Amyloid β is a 4kDa fragment of the amyloid precursor protein (APP), which is produced physiologically by different neurons (Blennow et al., 2006). However, it has been found that the AD brain accumulates an Aβ concentration equivalent to seven years worth of amyloid β production, not only this, but of all the variants there is an increased concentration of Aβ42 (Karran et al., 2011). Through a process which is described later, plaques of Aβ are accumulated, and they act like a pathological trigger which harms the neurons, and that will lead to the formation of NFTs, neuronal dysfunction and ultimately apoptosis, which is cell death (Hardy and Higgings, 1992; Selkoe, 1999). This accumulation has been observed in the ageing brain, and has become a pathological hallmark for AD, alongside with tau neurofibrillary tangles (NFTs) (Cras et al., 1991; Hardy and Selkoe, 2002; Jack et al., 2018, Selkoe and Hardy, 2016). The amyloid cascade is characterised by the accumulation, aggregation and misfolding of Aβ within the brain (Sikanyika et al., 2019).

Plaque formation

Amyloid plaques are formed by peptides, Aβ peptides (Masters et al., 1985). These will polymerise into polymorphic oligomers, in turn, these will cause a proinflammatory response where microglia are activated, and cytokines synthesised and released (Selkoe, 1994). The first step on plaque formation is the cleavage of amyloid precursor protein (APP). This protein is a type I membrane glycoprotein, and it can be processed by two different pathways, the non-amyloidogenic and the amyloidogenic pathway. The first one mentioned is cleaved by β-secretases and y-secretases which results in a soluble protein, and the latter pathway APP is cleaved by α-secretase (Blennow et al.,2006; Kojro et al., 2001; Liu et al, 2013). In the amyloidogenic pathway β-secretase cleaves the APP generating a C-terminal fragment (C99), and the soluble Aβ peptide protein is subsequently cleaved by y-secretase, which makes it insoluble (Selkoe and Schenk, 2003). Other enzymes that cleave APP are BACE, and BACE2 (Dingwall, 2001). This causes an imbalance between the production and the

clearance of Aβ peptide because the peptides will spontaneously aggregate into oligomers in plaques which are found in the endosomes, and the Golgi apparatus of neurons, this phenomenon is known as Aβ dyshomeostasis (Hook et al., 2008; Singhrao and Olsen, 2019). This soluble/insoluble Aβ contributes to the formation of insoluble plaques in the AD brain. (Cataldo et al., 1997; Hook et al, 2008). These findings are supported by experiments which have demonstrated that the cleaving enzyme BACE1 has a higher affinity for mutant forms of AβPP in comparison to wild type APP (wtAPP) (Blennow et al.,2006; Liu et al., 2013).

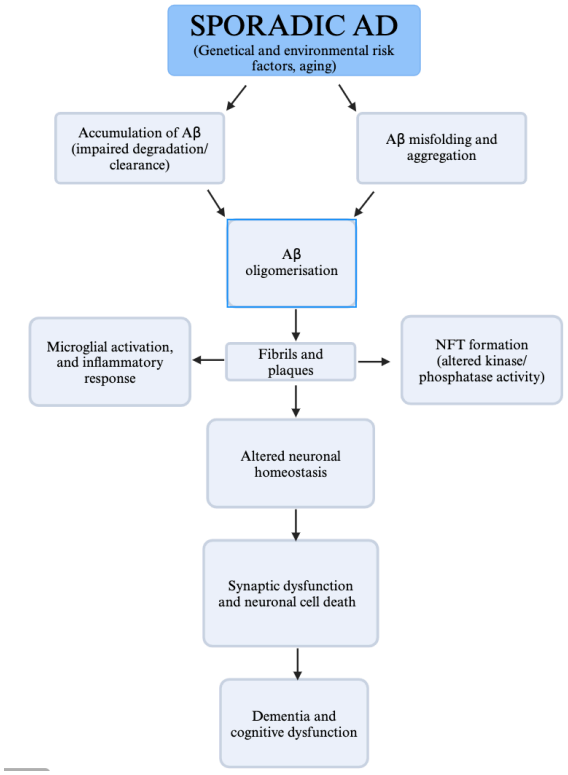

Fig.6 This illustration is inspired by Sikanyika et al. (2019) Sporadic Alzheimer's disease is caused by two main principles: the accumulation, and the misfolding and aggregation of Aβ. When Aβ is not cleared due to impairment of degradation, it causes Aβ oligomerization, which will subsequently lead to the precipitation of fibrils and plaques. In turn, this will lead to the formation of NFT's, microglial activation, and dyshomestasis of the neurons. When there is dysheomestasis, the cells die and over time it will lead to dementia and cognitive dysfunction (Sikanyika et al., 2019)

Aβ and *P.gingivalis*

The amyloid hypothesis explains how Aβ plaques could be synthesised, but the effect that the *Porhyromonas gingivalis* on AD hallmarks will be presented in this section. The pathways in which *P. gingivalis* (PG) could enter will be discussed later on, Poole et al. (2013) suggested that the bacterium could either directly affect the brain or though the effects of its virulence factors such as gingipains and lipopolysaccharide (LPS) . Which effects does *P.gingivalis* have on Aβ?

A study by Ilievski et al. (2018) they performed a series of experiments comparing the effects on Aβ and tau in mice infected with PG compared to control groups. They demonstrated that there was an extracellular increase of Aβ42 concentration in mice hippocampus and frontal cortex in those who were infected, but there was no change in the control groups. In addition, it was found astrocytes had a higher intracellular level of Aβ42 compared to the controls. In a study by Wu et al., (2017) they demonstrated that chronic exposure to LPS of PG lead to an increase of cathepsin β, which is an enzyme that cleaves AβPP, in both microglia and neurons, and it increased the production of IL-1β in the hippocampus. They also found that the presence of *Porphyromonas gingivalis* LPS lead to an accumulation of Aβ within neurons (Wu et al., 2017). In addition, it has been found that gingipains, which are proteolytic enzymes, have the ability to cleave AβPP in a similar manner to cathepsin B, and it might contribute to an increase of Aβ in the central nervous system (Hook et al., 2009; Wu et al., 2017). Other articles suggest that the peripheral increase of Aβ, found in the periodontium, might contribute to the total Aβ in the AD brain (Leira et al, 2019; Gil-Montoya et al., 2016; Nie et al., 2019).

Tau hypothesis

The other AD hallmark is the hyperphosphorylation of tau proteins. The tau hypothesis is a key pathological process characterised by the formation of neurofibrillary tangles (NFTs), which are neurofibrillary lesions that accumulate within neurons (DeTure and Dickson, 2019; Trojanowski and Mattson, 2003)**.**

Tau proteins, which come in at least six isoforms, are vital to the stabilisation of neuronal microtubules. They have a carboxy terminal (C-terminal), and an amino terminal (N-terminal) part (Brandt et al., 1995; Goedert et al., 1989; Lee et al.,1989). When there is insufficient amounts of tau, it will lead to a disturbance in the structure of the cytoskeleton and changes the morphology of the neurons, all of which affects synaptic dysfunction, neurodegeneration and the transportation along the axon (Braak et al., 1994; Combs et al., 2019).

Hyperphosphorylation of tau is partially caused by the potential involvement of glycogen synthase kinase-3 beta (GSK-3B) in this process, where it could phosphorylate tau residues at Ser396, Thr231 (Hanger et al., 2007). Hyperpshohorylated tau polymerised into paired helical filaments (PHF), and straight filaments (SF), otherwise known as neurofibrillary tangles (Köpke et al., 1993). The formation of tau oligomers and neurofibrillary tangled could lead to neuronal dysfunction (Takashima, 2016).

Recent studies have implicated *Porphyromonas gingivalis* in the phosphorylation of tau protein (Dominy et al, 2019; Haditch et al., 2020). Dominy et al. (2019) demonstrates that *P.gingivalis* can hydrolyze tau protein, thereby contributing to this modification. Iievski et al (2018), found neuropathological lesions resembling those found in AD, in mice that had marginal chronic periodontitis. Haditch et al. (2020) found that following *P.gingivalis* infection, tau was phosphorylated in certain sites like T231.

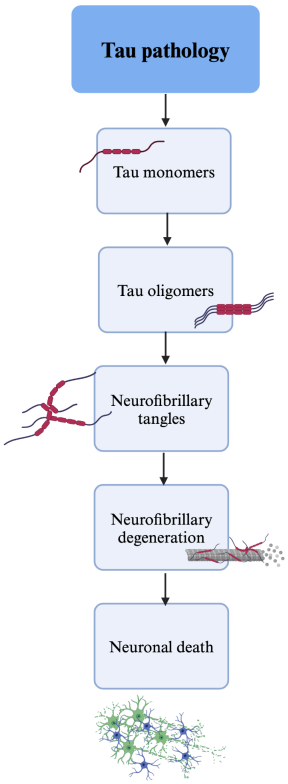

Fig. 7 We observe the formation of NTFs, and how it ultimately leads to neuronal death. The tau assembles into monomers, oligomers and they organise themselves into tangles. Once the tangles are formed, it will lead to the degeneration of neurofibrillary. This compromises the cell structure and integrity and lead to neuronal death (Takashima, 2016)

Tau and *P.gingivalis*

Dominy et al., (2019) demonstrated how the presence of gingipains led to a degradation of tau, this is due to its proteolytic abilities. However Haditsch et al., (2020) suggested that hyperphosphorylated tau had greater resistance against gingipains, and even considered it being protective against gingipains. In this study, using induced pluripotent stem cells (iPSC), and when infected with *P.gingivalis*, it led to increased phosphorylation at Thr231 of the tau protein. Their hypothesis is that this bacterium compromises the structure and the transport of neurons within the brain, and the loss of synapses (Haditsch et al., 2020).
Furthermore, the serotype K1 strain of *P.gingivalis* (W83) was found to induce phosphorylation of tau at Ser 396 in wild-type mice in vivo (Ilievski et al, 2018), which had been previously evaluated by Hanger et al. (2007). In addition, GSK-B may mediate the

phosphorylation of brain tau through immune responses mediated by *P.gingivalis*, observed in studies by (Ilievski et al 2018; Haditsch et al, 2020). If GSK-B and gingipains have similar cleavage activities, this would further support the possibility that gingipains is able to phosphorylate tau. Not only this but gingipains might be able to co-localize microtubules and paired helical filaments (PHFs), constituting neurofibrillary tangles (NFTs) in AD brains (Dominy et al., 2019). In this study it was found that, N and C termini that tau can be hydrolyzed by these proteases. These fragments by the N and C terminus, contain "VWIINK" and "VGIVYK" which binds to microtubule binding domains, and supports microtubule filaments (Dominy et al., 2019). Cleaving on these sites, would cause a severe disruption of microtubules, and then the formation of NFT lesions (Dominy et al., 2019; Barbier et al., 2019). Furthermore, Dominy et al. (2019) concludes that the presence of *P.gingivalis* could affect the balance of tau inflammation, phosphorylation and the formation of NFT lesions, all of which play a role in AD pathophysiology.

All of these findings support a potential contribution *Porphyromonas gingivalis*, the periodontal pathogen, has to the development of Alzheimer's disease. This could have important significance for the development of therapeutic interventions.

APOE ε4 and *P.gingivalis*

Human APOE ε4 protein, a 34kDa glycoprotein, is encoded by the APOE gene located on chromosome 19q13.2, and is recognized as the single most significant genetic risk factor for sporadic Alzheimer's Disease (AD), also known as late-onset Alzheimer's disease (LOAD) (Corder et al., 1993; Pires and Rego 2023; Poirier et al., 1993; Safieh et al., 2019). In other words APOE ε4 is a susceptibility gene inheritance (Saunders et al.,1993). Furthermore, the APOE ε4 allele is present in 40-65% of all AD patients (Pires and Rego, 2023). A meta analysis demonstrated that when a patient carries this allele, and has a diabetes diagnosis additionally they have a 250% higher risk of developing dementia, that the patients carrying APOE ε4 alone with 35% risk (Li et al., 2020). The presence and the incidence of APOE ε4, causes deteriorating memory (Poole et al., 2013). This protein is synthesised by astrocytes for the protection of neurons, and is a cholesterol transporting protein (Poole et al., 2013).

By using neuroimaging and cerebrospinal fluid (CSF) different studies have associated ApoE ε4 with the deposition of Aβ in the brain (Jack et al., 2015; Lim et al., 2012; Morris et al., 2010; Sunderland et al., 2004). It has been demonstrated that patients carrying this allele has a

increased risk of accumulating Aβ, Aβ-plaques in the cerebral cortex, suggesting that the allele has an effect on Aβ metabolism (Kok et al., 2009; Polvikoski et al., 1995; Nyarko et al., 2018; Schmechel et al., 1993). It is thought that this allele alters Aβ deposition by affecting the metabolism and the aggregation, and it is possible that age might exacerbate this effect when the gene is present (Lim et al., 2017; Lim et al., 2018; Verghese et al., 2011). Furthermore, in vitro and mouse models have revealed that ApoE modulates the activity of y-secretase and therefore the downstream production of Aβ (Irizarry et al., 2004). In other studies using embryonic stem cells and induced pluripotent stem cell (iPSC), they demonstrated that the allele has the ability to regulate the transcription of amyloid precursor protein (APP), and therefore AB production. It was observed a higher Aβ production and secretion in the iPSC derived neurons in the ones carrying ApoE e4 compared the APOE e3 (Huang et al., 2017; Lin et al., 2018; Wang et al., 2018). ApoE have different alleles, and while APOE e4 is associated with higher risk ApoE e2 does not only lower the risk of neurodegeneration, but it could be neuroprotective (Corder et al., 1994; Suri et al., 2013).

Alzheimer's disease and microglia

It is thought that the progression of Alzheimer's disease might be linked to general chronic inflammation (Chen et al., 2017; Sparks Stein et al., 2012). Microglia are the brain's immune cells, and they work as if they were macrophages, and they regulate the development and maintenance of neurons (Rock et al., 2004; Colonna et al., 2017). Additionally, they are the brain's main defence (Ginhoux et al., 2010). These cells are derived from haematopoietic precursors that migrate from the yolk sac into the CNS parenchyma (Ginhoux et al., 2010). There are two phenotypes of this cell, M1 which has "pro-inflammatory and neurotoxic responses, while the M2 phenotype mostly mediates anti-inflammatory and neuroprotective functions" (Tang and Le, 2015).When activated in the M1 pathway it will release proinflammatory cytokines such as TNF-α and interleukin-1β, whereas in the M2 pathway it will be involved in tissue repair, release chemokines, activation neurotrophic pathways and phagocytosis damaged neurons (Tang and Le, 2015)

Microglia response to Amyloid-β and Tau

Microglia might play a role in the regulation of Aβ metabolism and clearance, and since they are the brain's macrophages they have a role of internalisation and engulfing Aβ (Lee and Landreth, 2010). It is the grade of the inflammatory response that can affect the state of microglia, and then regulate the Aβ levels (Lee and Landreth, 2010). Supporting this

hypothesis, extracellular deposition of Aβ42 was detected in the parenchyma of the brain from experimental groups, whereas controls showed no such deposition (Ilievski et al., 2018). If periodontal disease increases a general inflammatory response, which activates the microglia within the brain this could mean that chronic periodontal infection could contribute to one of the major AD hallmarks.

Activated microglia is observed in close proximity to NFTs in AD patients (Sheffield et al., 2000), where they can degrade tau particles, but also propagate the spread of tau pathology (Bolós et al., 2016; Asai et al., 2015). Studies have shown the presence of hyperphosphorylated tau in aged dystrophic microglia. Impairment of clearance mechanisms upon ageing, can lead to intracellular accumulation of pathological tau, and promote microglia dystrophy (Asai et al., 2015). NFT formation is accelerated by microglial inflammation (Asai et al., 2015; Bhaskar et al., 2010).

Microglia and *Porphyromonas gingivalis*

Within the pia mater and the arachnoid mater, two membranes that surround the brain, there are leptomeningeal cells. These cells contribute to the formation of the blood-brain barrier, and are involved in the central nervous system homeostasis. (Wu et al., 2005; Wu et al., 2006). These leptomeningeal cells are able to transduce inflammatory signals to microglia, which are immune cells found in the central nervous system (Liu et al., 2013) It is thought that through the leptomeningeal cells *P.gingivalis* might affect microglia directly and indirectly, in this section we will describe how the bacterium could affect the brains immunity cells.

A study by Liu et al. (2013), suggested that leptomeninges when activated by the proinflammatory mediators produced by chronic periodontitis, transduced inflammatory signals to the microglia. They found that the presence of TNF-α and IL-1β had increased in these leptomeningeal cells after being exposed to LPS from *P.gingivalis*, not only this, but they had increased the mRNA levels within microglia (Liu et al., 2013). When Cunningham et al. (2005) administered LPS directly into the peritoneum it was observed the activation of microglial cells, and these findings were when ultrapure *P.gingvalis* activated the microglia in an 18h *in vitro* test by Memedovski et al. (2020). The inflammatory process that has been induced by LPS, had priorly been associated with the loss of cognitive function, where learning and memory was impaired when rats were administered LPS (Tanaka et al., 2006). Several articles have suggested that LPS mediated neuroinflammation is caused by the

toll-like receptor (TLR4) signalling pathway, and it leads to cognitive dysfunction (Cunningham et al., 2005). Ilievski et al., (2018) demonstrated that the DNA of PG could be found within the microglia after oral administrations of *P. gingivalis* in wild-type. In their study, gingipains were identified within microglia in the capillaries of the thalamus in mice after a mono- *P.gingivalis* infection. Additionally, in those infected it was observed microgliosis, astrogliosis and significantly elevated levels of proinflammatory cytokines such as IL-6, TNF-α and IL-1β compared to control groups. They concluded that due to the neurodegenerative changes in the experimental groups, there was a potential link between chronic periodontal disease and neurodegeneration (Ilievski et al. 2018).
Furthermore, Wu et al. (2017) used mice models to demonstrate an age-dependent response. Aged mice had higher expressions of inflammatory molecules, such as interleukin-1 beta (IL-1β) and interleukin-10 (IL-10) mRNA in the cortex compared to adult mice. In addition, those that received a peripheral injection with LPS had elevated IL-1β and microglia in aged mice (Wu et al., 2017). This molecule, IL-1β, is a pro-inflammatory cytokine that has been implicated in synaptic loss, and could possibly lead to a decline in cognitive abilities (Bellinger et al., 1993; Mishra et al., 2012; Hong et al., 2016). Additionally, IL-1β indirectly stimulates amyloid-beta (Aβ) cleavage by cathepsin β, an enzyme involved in Aβ production, through its cognate receptor IL-1R on neurons (Wu et al., 2017).

Summary

Alzheimer's is characterised by the presence of tau and Aβ, and recent research has tried to discover the relationship between AD and *P.gingivalis* through different mechanisms. In the next section an association between AD and PD will be presented.

Chapter 4: An Association Between AD and PD

Alzheimer's disease and periodontitis are two distinct medical conditions that affect different parts of the body. Alzheimer's disease primarily impacts the brain, while periodontitis affects the oral cavity and surrounding tissues. However, there are some similarities and potential connections between these two conditions, and several studies in recent years have been trying to uncover this hypothetical relationship (Borsa et al., 2021; Leira et al., 2017).
In this chapter it will be discussed the possible associations, mechanisms between the two conditions, and what systematic reviews and other literature have written regarding this relationship.
Here are some similarities and potential connections between these two conditions;

1. Activation of the immune system: periodontal disease is characterised by the presence of local inflammation in the periodontium (Armitage, 1999; Borsa et al., 2021). It responds to a bacterial infection in the periodontal pockets which leads to proinflammatory molecules to enter the systemic blood stream. Inflammation can be seen in the AD affected areas of the brain (Akiyama, 2000).
2. Risk factors: there are many shared risk factors such as age, genetics, and lifestyle factors. The main difference is that in periodontitis the main causative factors is microorganisms (Gil-Montoya et al., 2016)
3. Systemic impact: both conditions affect systemic health. Periodontitis has been linked to cardiovascular disease, cancer and diabetes, while Alzheimer's disease has other health implications (Liccardo et al., 2020;Hajishengallis, 2014).
4. Bacterial connection: this may be the link between the two pathologies. It has been hypothesised that oral bacteria in the oral cavity enters and spreads through the bloodstream and potentially reaches the brain, where it triggers or exacerbates inflammation (Lei et al 2023; Li et al., 2024; Nonaka et al. 2022, Pritchard et al. 2022)

It is important to note that this relationship remains hypothetical, it is an area of ongoing research and the exact nature of the connection, if any, remains not understood. More research is needed to establish causality, and to understand possible mechanisms at play.

Exploring the Association Between Alzheimer's and Periodontitis

Genetics

In a study by Jin et al. (2021) they investigated whether there were shared molecular mechanisms between Alzheimer's Disease and Periodontitis by a transcriptomic analysis. They did this by downloading gene expression datasets from Gene Expression Omnibus (GEO) database, and overlapping them with the AD related genes downloaded from the DisGeNET database. Then with a Boruta algorithm they were able to identify how these genes crosstalk. Their results were that C4A; C4B CXCL12, FCGR3A, IL-1β and MMP are possible molecular genes between periodontitis and AD (Jin et al., 2021).

Comorbidities and environmental factors

The main comorbidities between Alzheimer's disease and periodontal disease are diabetes mellitus, which is a major risk factor, and atherosclerosis (Kanagasingam et al., 2020). There are several mechanisms overlapping between diabetes mellitus and AD, some of which mitochondrial dysfunction, oxidative stress and inflammation (Pugazhenthi et al., 2017). This could be a link between two diseases, and further exacerbating their pathogenesis. An indirect relationship between PD and cognitive function worth mentioning, is through cardiovascular health. Episodes such as strokes, that can be caused by atherosclerosis, have a direct effect on cognitive function, which could lead to alterations in oral health, and ultimately leading to PD (Sanz et al, 2020). Ultimately, both these diseases are affected by environmental factors such as smoking, low socioeconomic status, poor nutrition, sedentary lifestyle (Kanagasingam et al, 2020).

A bidirectional relationship

There is a potential bidirectional link between AD and PD, where people who are suffering from cognitive deficiencies that comes from AD are susceptible for oral diseases, such as periodontitis, and people who already have a periodontitis diagnosis has an increased risk of Alzheimer's disease (Liccardo et al., 2020; Nascimento et al., 2019). It has been found that those with poor oral hygiene, and neglected daily tooth brushing had a higher risk of developing dementia. Those who never brushed had a 20-35% increased risk compared with those who brushed everyday, and those that never flossed had an increased risk of 30% of developing dementia (Paganini-Hill et al., 2011). There are several studies demonstrating a

link between dementia, periodontitis and tooth loss (Kangasingam et al., 2020; Singhrao and Olsen, 2019; Stein et al., 2007).

Inadequate oral hygiene increases the number of anaerobic microorganisms in dental plaque, and promotes proliferation of the pathogens and the release of toxic factors (Liccardo et al., 2020). As mentioned earlier, *Porphyromonas gingivalis* would locally recruit neutrophils to the periodontal pocket and release molecules such as reactive oxygen species, that attack the host tissue and enhance the pathogenesis of periodontitis (Liccardo et al, 2020; Hajishengallis, 2014). These pathogens and their corresponding virulence factors would enter the bloodstream, and lead to a systemic inflammation (Liccardo et al., 2020; Poole et al., 2013). Once the systemic inflammation is established it could activate microglia or release proinflammatory molecules, which would compromise the integrity of the BBB. Once compromised, periodontal pathogens could enter and further exacerbate neurodegeneration (Harding et al., 2017a; Holmes et al., 2013; Perry et al., 2007; Pritchard et al., 2017).
This direction has been supported by several factors. Firstly it has been found that AD patients have higher concentrations of LPS, the membrane component i Gram-negative bacterium, when compared with healthy control. In addition, as mentioned previously injections of LPS could active microglia which induces the releases of interleukins and TNF-alpha, and it has also been found that the presence of cytokines and chemokines might be linked with the accumulation of Aβ42. (Díaz-Zúñiga et al. 2019; Godbout et al., 2005). In another article it was demonstrated that LPS from PG had the ability to bind in glial cells, and if this is colocalized within Aβ plaques, this could ultimately lead to neurodegeneration as extracellular formation of Aβ42 led to neurodegeneration in wild mice (Ilievksy et al., 2018). Finally, Kamer et al. (2009) identifies that patients with AD had elevated cytokine levels, and serum antibodies of *T. forsythia, A.actinomycetemcomitans and P.gingivalis*. Supporting these findings, Dominy et al. (2019) demonstrated that *P.gingivalis* with their virulence factors were detected exclusively in AD brains compared to healthy control groups.

The other direction of this bidirectional pathway is that those already diagnosed with Alzheimer's disease and other dementias will struggle to maintain ideal oral health, increasing the risk to develop periodontitis when the plaque is not removed through brushing daily (Delwel et al., 2017; Foley et al., 2017; Leira et al.,2017). Martande et al. (2014) found that patients with dementia had worse periodontal health compared to healthy controls. Furthermore in a systematic review by Foley et al. (2017) and in a comprehensive review by Delwel et al. (2017) they supported these findings where patients with dementia had worse

oral health, and scored higher in parameters such as bleeding on probing, periodontal pockets (Delwel et al., 2017; Foley et al., 2017).

In a trial emulation they divided into a periodontally treated cohort consisting of 177 patients, and another cohort of 409 untreated patients. After an oral examination, and other tests such as MRI and statistical analysis they found that there was a positive effect on AD-related atrophy, were periodontal treatment could benefit the progress of Alzheimer's (Schwahn et al., 2021). These findings suggest that not only there could be an association between PD and AD, but treating the periodontium could possibly be used as a preventative measure for developing Alzheimer's.

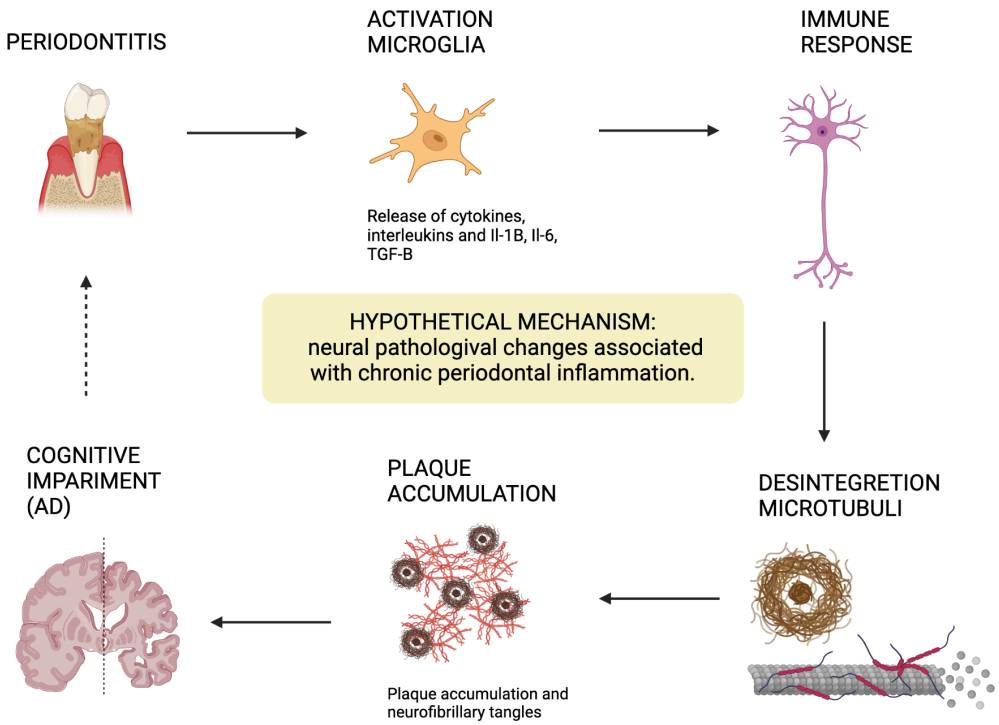

Fig. 8 Periodontitis leads to the activation of microglia, which will in turn release cytokines and other proinflammatory molecules. This will lead to an inflammatory response within the brain, and it will lead to the disintegration of microtubuli and plaque accumulation (Dziedzic, 2022)

Systematic Reviews and Meta-Analysis on the Association

There are several systematic reviews that explore the association between Periodontitis and Alzheimer's disease alongside other dementias. In this section, we will mention different systematic reviews and studies with their findings.

In a Systematic Review by Borsa et al. (2021), they included five out of 802 articles in a qualitative analysis, which lead to the conclusion that there is a promising connection between periodontal diseases and AD. Three of those articles were case-control studies (Noble et al., 2014; Panzarella et al., 2020; Syrjälä et al., 2012), one was a cohort study (Ide et al., 2016) and one was an observational cross-sectional study (Beydoun et al., 2020), and it was found contradictory evidence. In the study by Panzarella et al. (2020), it was found higher bacterial load of periodontal pathogens in AD brains, and Noble et al. (2014) demonstrated that patients with a higher concentration of *A.naeslundii* serum immunoglobulin G (IgG) had a greater risk of developing AD (Panzarella et al., 2020; Noble et al., 2014). Beydoun et al. (2020) found that *Porphyromonas gingivalis*, alongside other periodontal pathogens, had an increased AD mortality risk for patients over 65 years of age. However, Ide et al. (2016) did not find an association between serum levels of anti-P.gingivalis antibodies, and the grade of cognitive decline (Ide et al., 2016). All of the articles concluded that more investigation and experiments were needed to disclose the possible relationship, that that as of today there is not sufficient evidence to fully understand these interactions.

In a different systematic review with Meta-analysis by Leira et al. (2017) they included 5 studies out of 550 articles and abstracts, 2 of which were cross-sectional (Martande et al., 2014; Syrjälä et al., 2012), 2 case-control (De Souza Rolim et al., 2013; Gil-Montoya et al., 2015) and 1 longitudinal study (Ship et al., 1994). In this review they found a strong association between PD and AD, however they concluded that the severity of the periodontitis is a detrimental factor that must be taken into account. It was the severe forms of periodontitis that lead to a significant association with AD with (OR 2.98, 95% CI 1.58-5.62). However, we must note that there was no consensus on PD criteria (Leira et al., 2017). They concluded in the same way as Borsa, that there are few studies and measurements to really determine the association between periodontitis and Alzheimer's disease. They suggested that more observational studies were needed, and mentioned that while ethically incorrect a retrospect cohort design would be efficient (Leira et al., 2017). These findings were further supported by a systematic literature search by Said-Sadier et al. (2023), where six cohort

studies, three cross sectional suited and two-case control studies were included. Instead of a meta-analysis, they made a qualitative synthesis. Their results showed that patients with chronic periodontitis for at least eight years, had a higher risk of developing a cognitive impairment and dementia. (Said-Sadier et al., 2023).

Contradictorily, in a systematic review and Meta-Analysis of clinical studies by Dziedzic (2022) concluded that there is no direct evidence of a causal link between periodontitis and age-related cognitive impairment. It included seventeen clinical studies, including fourteen cohorts, one cross-sectional, and two case-control studies, in this systematic review. According to the article there is weak evidence that there is a positive association between periodontitis and a higher risk of dementia or Alzheimer's disease. However, Dziedzic (2022) mentions that a bidirectional relationship may be plausible.

In conclusion there are conflicting and contradictory conclusions in these systematic reviews. Some of them found associations between periodontitis and Alzheimer's disease, while others did not find them. They acknowledge the limitations of their studies, and they agree that with the few studies, and measurements that are available more studies are required to determine whether there is an association.

Ways of Inflammation

There are several ways that have been proposed in which periodontal pathogens like *P.gingivalis* could prompt neurodegeneration and Alzheimer's disease. As of today, three main pathological interactions that have been proposed are (1) A systemic inflammation affects the central nervous system due to chronic peripheral inflammation, host response, and increased proinflammatory cytokine levels (Liccardo et al., 2020) (2) direct invasion of periodontal pathogens and their products into the cerebral region via the circulatory system, triggering a cascade of inflammatory responses within brain tissue (Dziedzic, 2022). (3) An infection of the trigeminal nerve that innervates the periodontal ligament (Cook et al., 2013; Goto et al., 2020; Kanagasingam et al., 2020).

The Induction of a Systemic Immune Response

Chronic oral inflammation, caused by periodontitis, could possibly lead to a systemic overactivity that contributes to neurodegeneration (Poole et al., 2013). Leira et al. (2017) suggested that periodontal-derived cytokines could lead to an increase in brain cytokine

levels. There are many hypothesis on how this could be achieved including; endothelial malfunction, oxidative stress, periopathogens bacteriemia and their mediators, the association between serum IgG antibodies and elevated proinflammatory cytokine level, C-reactive protein, and TNF-α in individuals with cognitive decline (Dziedzic, 2022; Noble et al., 2014). All of these molecules, including the antigens,will interact with the immune system and trigger an inflammatory response (Ding et al., 2018; Singhrao and Olsen, 2019). That includes the production of cytokines, interleukins and their increased level in the circulatory system. The development of AD is caused by neurodegeneration, which can be a response of systemic inflammation cascades such as the microglial activation and release of proinflammatory molecules (Liccardo et al., 2020). This immune response may cause the aggregation of beta-amyloid (Aβ) and hyperphosphorylated tau proteins to accumulate in cerebral plaques and create neurofibrillary tangles (Kangasingam et al., 2020).

Trigeminal infection

The periodontal ligament is innervated by the trigeminal nerve, which is the fifth brain nerve, and all three of its branches joining at the trigeminal ganglia (Huff et al., 2022). This ganglion is located adjacent to the locus coeruleus in the brain stem, the pons anatomical areas, which are where emotions, behaviour and stress are partially controlled (Cook et al., 2013; Goto et al., 2020; Kanagasingam et al., 2020). It is also the areas of the brain that are early involved in AD development, and it has been observed in tau pathology (Braak and Braak, 1991; Braak et al., 2011). In a study it was performed surgical extractions of molars in triple transgenic AD mice (3xTg-AD), and the release of cytotoxic Aβ, and neurodegeneration in the locus coeruleus was observed (Goto et al, 2020). It has been also suggested that infection of peripheral nerves could accelerate neuroinflammation (Leira et al, 2017). A hypothesis proposed is that after *P.gingivalis* and its virulence factors infect the trigeminal nerve, it would change the homeostasis in locus coeruleus and potentially change an individual's personality (Cook et al., 2013).

A direct invasion of the periodontal pathogens

In recent years it has been found that a leaky blood brain barrier (BBB), with compromised structure, could lead to a direct infection of the brain. In recent years it has been explored if periodontal pathogens, like *Porphyromonas gingivalis*, are able to directly cross into the brain (Leira et al., 2017; Nonaka et al., 2022; Pritchard et al., 2022; Lei et al., 2023; Li et al., 2024). But how can *P.gingivalis* cross the BBB? These are mechanisms discussed in the following chapters.

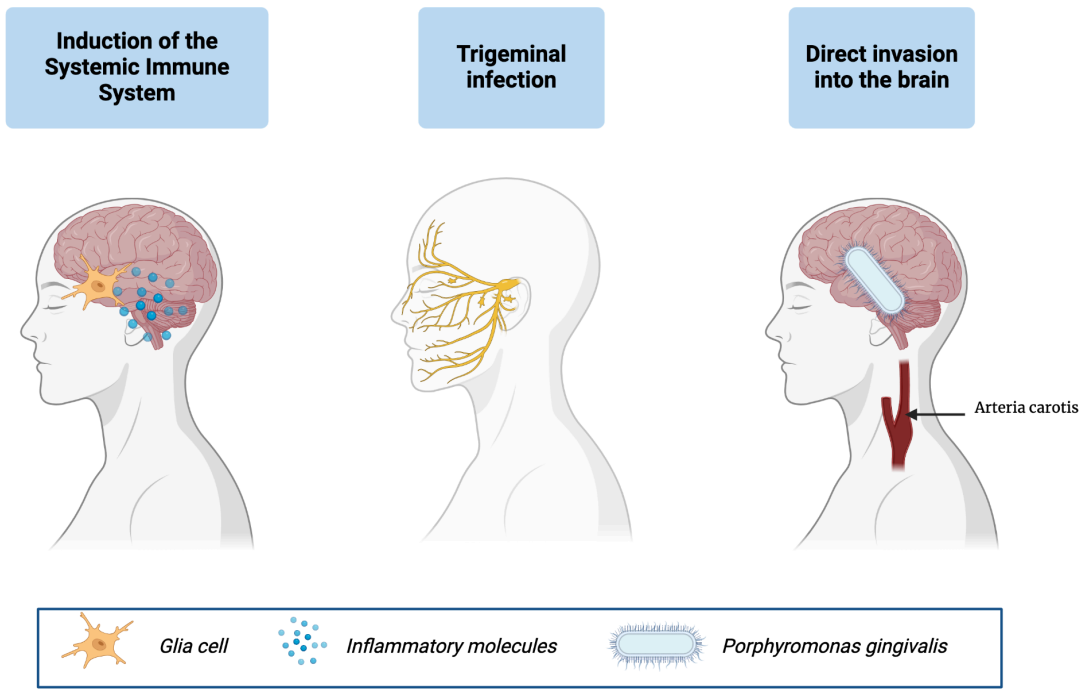

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Fig.9 This illustration demonstrates three possible ways in which *P.gingivalis*, like other periodontal pathogens, could potentially exacerbate neuron degeneration. (1) A systemic inflammation will affect the brain's microenvironment, leading to a local inflammation within the brain. (2) Periodontal pathogens infect the trigeminal nerve, and the inflammation propagates from the nerve into the trigeminal ganglion and ultimately the brainstem where locus coeruleus is located. (3) *Porphyromonas gingivalis* is able to cross a dysfunctional BBB, and infect the brain directly.

Chapter 5: THE BBB

The Importance of the BBB

The blood-brain barrier (BBB) is one of three physical barriers that protects the brain and its microenvironment. The other two are the arachnoid epithelium and the choroid plexus epithelium, which separates the circulatory blood from the cerebrospinal fluid and plays a role in protecting the brain (Abbott et al., 2006). The BBB is composed of a unit of cells that encircle the microvessels of the brain, otherwise known as the NVU, and it is essential for the protection from endogenous and exogenous substances that could harm the brain (Zenaro et al., 2017; Zhao et al., 2022). The NVU regulates the efflux and influx of ions and molecules such as oxygen and nutrients to ensure that the brain has a normal function (Abbott, 2010; Saunders et al., 2012; Keaney and Campbell, 2015). This structure will become dysfunctional in patients that have AD (Zenaro et al., 2017).

There are several factors that can affect the permeability of the BBB, some of them are age, gender, temperature and physical exercise (Zhao et al., 2022). It has been also suggested that genetics could be a factor that affects the structure of the BBB, mainly the ApoE gene. It has been demonstrated that ApoE knockout mice are at risk of the developing AD, and it was observed accelerated BBB breakdown, and the degeneration of capillary pericytes (Methia et al., 2001; Montagne et al., 2020; Pritchard et al., 2022). This dysfunction was associated with the presence of matrix metalloproteinase-9 (MMP-9), where the degradation of tight junctions (TJs) and basement proteins were observed (Sweeney et al., 2019).

This barrier must remain intact, otherwise a dysfunction could lead to pathological aspects such as “(1) the leakage of circulating substances from the plasma into the CNS; (2) the modulation of transporters leading to an inadequate nutrient supply, the accumulation of toxins in the CNS, or the entry of compounds that are normally excluded; and (3) the altered expression and/or secretion of proteins by NVU cells, which can promote inflammation, oxidative stress and neuronal damage” (Zenaro et al.,p.43, 2017).

In conclusion, this dynamic structure is responsible for maintaining the homeostasis of the brain and a dysregulation of the NVU could potentially lead to the breakdown of the BBB (Daneman and Prat, 2015; Keaney and Campbell, 2015; Zhao et al., 2022).

The Structure of the BBB

The BBB is made up by a collection of cells that form the neurovascular unit (NVU), which are endothelial cells (ECs), vascular myocytes, the pericytes (PCs), astrocytes, a basement membrane, microglial cells and neurons (Abbot et al., 2010; Daneman and Prat, 2015; Hawkins and Davis, 2005; Iadecola, 2004; Pritchard et al., 2022; Zlokovic, 2005; Zhao et al., 2022). The capillaries that make up the BBB are about 400 miles long, therefore becoming the primary entrypoint for pathogens in the systemic circulation (Cipolla, 2009; Pritchard et al., 2022). These endothelial cells, which regulate the transportation of substances across the CNS, are bound to each other by proteins such as tight junctions, junctional and adherence proteins, which are indispensable for its integrity (Zhao et al., 2022). Transcytosis across this membrane is severely limited by the shared basement membrane, the lack of fenestrae and the proteins that bind the cells to one another (Pritchard et al., 2022).

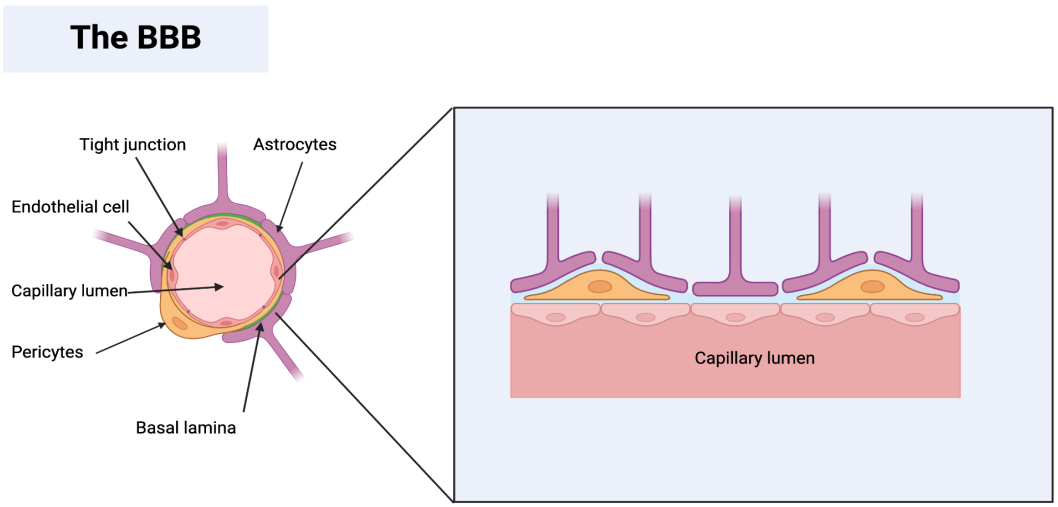

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Fig. 10 Schematic representation of a cerebral capillary with a cross-sectional view. These are composed by astrocyte endfeet surrounding and supporting the inner structures of the capillaries. Within this layer we can find pericytes which with the basal lamina enclose the capillary lumen. In the innermost layer we can find vascular endothelial cells who are linked to one another through tight junctions (TJs) (Zenaro et al, 2017).

Endothelial cells (ECs)

The core element of the BBB are the brain microvessel endothelial cells (BMECs), and it works as a selective barrier which regulates the microenvironment (Abbot et al., 2006; Zenaro et al., 2017). These cells are polarised by proteins such as TJs which creates an luminal (apical) and abluminal (basolateral) compartments, which facilitates transportation of substances across the BBB and determines the permeability for different types of molecules. (Abbot et al., 2006; Daneman and Prat, 2015; Zhao et al., 2022).
The functions of BMECs, were classified by Zenaro et al. (2017) into three different categories: (1) A paracellular diffusion barrier; it allows only small hydrophilic substances to cross the membrane between the ECs (Brightman and Reese, 1969; Pappenheimer et al., 1951; Zenaro et al., 2017) (2) A communication interface between the central nervous system and the periphery (Ransohoff and Engelhardt, 2012; Zenaro et al., 2017) (3) BBB transportation regulation of the influx/efflux of macromolecules (Loscher and Potschka, 2005; Saunders et al., 2013; Xiao and Gan, 2013; Zenaro et al., 2017).

Junctional complexes

These endothelial cells, ECs, are tightly bound to each other by junctional complexes that consist of tight junctions (TJs), and adherens junctions (AJs). Junctional complexes are composed by transmembrane proteins and molecules such as occludin, claudins and junctional adhesion molecules (JAMs) (Bauer et al., 2010; Chiba et al., 2008; Zenaro et al., 2017). In recent studies it has been suggested that *P.gingivalis* might be able to degrade the tight junctions, which is why they will be described further (Nonaka et al., 2022; Pritchard et al., 2022).

The main function of tight junctions is to prevent the diffusion of molecules, and large proteins across the BBB, and by creating a paracellular space it allows the BBB to have an extremely selective transportation (Zlokovic, 2008; Tietz and Engelhardt, 2015; Zenaro et al., 2017). This complex is made up of claudins and occludin.
Out of the 25 variants of claudins, which are the backbone of tight junctions, only four types are expressed in the brain, these are claudin 1,3,5,12 and their difference affects the function and structure of the TJs (Liebner et al., 2000; Morita et al., 1999; Nitta et al., 2003; Tsukita and Furuse, 1999; Wolburg et al., 2003; Zenaro et al., 2017). Occludin is a transmembrane, and its function is to bind the cytoplasmic zone occludens (ZO), proteins types 1 and 2 of neighbouring cells to each other (Furuse et al., 1993; Schneeberger and Lynch, 2004; Zenaro

et al., 2017). ZO1- is a large phosphoprotein, and it is when it is bound to occludins and claudins that the tight junctions are made (Fanning et al., 1998; Pritchard et al., 2022). Newer research has demonstrated that through OMVs from *P.gingivalis* the integrity of the TJs could be affected, and therefore the BBB structure. (Tornavaca et al., 2015; Pritchard et al., 2022). Adherens junctions are composed of JAMS, VE-cadherin and other smaller molecules and its is proposed that neutrophils might exploit this in order to migrate across the BBB and into the brain (Zenaro et al., 2017).

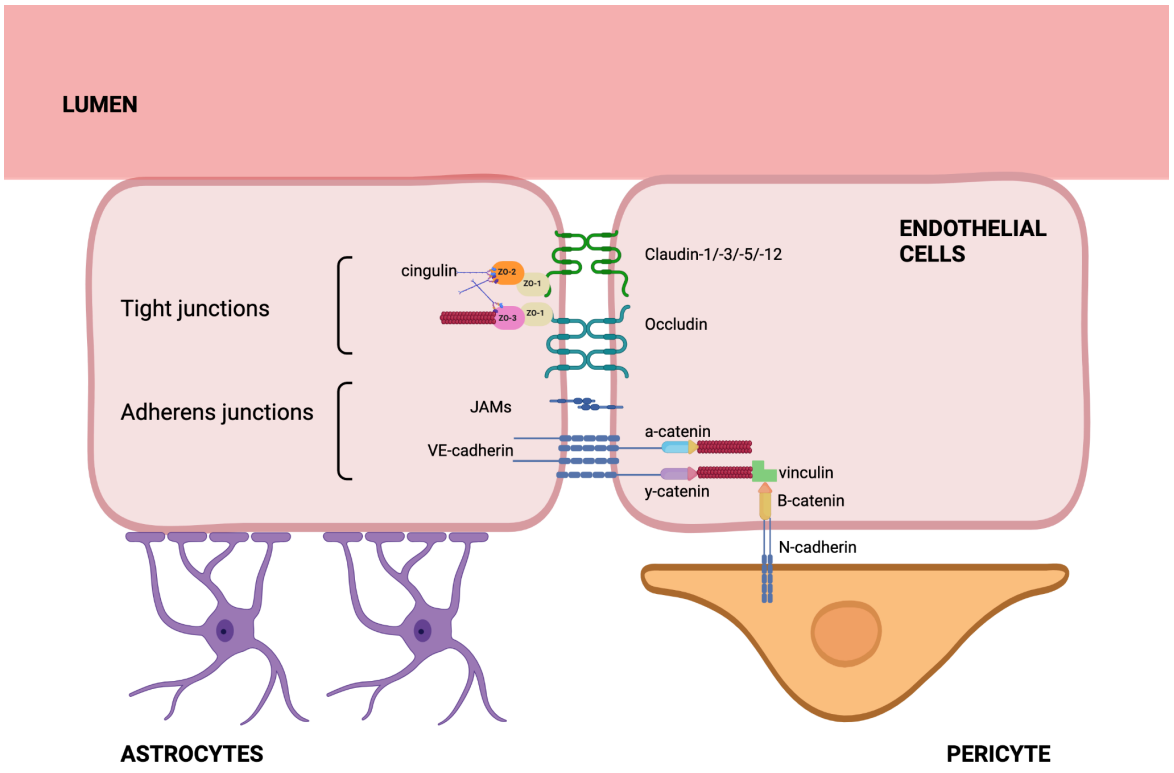

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Fig. 11 Schematic representation of how vascular endothelial cells are bound to each other through tight junctions and adherens junctions. The tight junctions are composed of four different types of claudin, occludin, cingulin, and three different types of zone occludens (ZO-1), (ZO-2), (ZO-3). Adherens junctions are composed by JAMs, VE-cadherin and other molecules such as a-catenin, y-catenin and vinculin. This creates an luminal, where the systemic blood circulates, and an abluminal side. The other cells such as astrocytes and pericytes bins to the endothelial cells and creates the NVU (Daneman and Prat, 2015; Keaney and Campbell, 2015; Zenaro et al., 2017)

Astrocytes

Astrocytes, which are a subtype of glial cells make up for the majority of glial cells in the central nervous system (CNS). Astrocytes are separated by two activated states: the pro-inflammatory phenotype (A1) the anti-inflammatory phenotype (A2), which determines whether it destroys or repairs the NVU (Liu et al., 2020). These cells do not conduct electrical signals like neurons (Wei and Morrison, 2023). Their appearance is star-like, where they use their foot processes to communicate with surrounding cells such as BMECs or other neurons. Their endothelial feet encircle the abluminal side of cerebral capillaries, and this is the way in which they interact with the BBB (Keaney and Campbell, 2015). They have other fundamental roles in order to stabilise and regulate the BBB such as neuroprotection, maintaining a homeostatic balance in neurons, and controlling the ion concentrations intra- and extracellularly. In addition, it is responsible for controlling the concentrations of neurotransmitters and therefore the modulation of synaptic transmission (Keaney and Campbell, 2015; Rodríguez-Arellano et al., 2016; Wei and Morrison, 2023). They also release molecules such as phospholipid transporter molecule Apolipoprotein E in order to maintain barrier tightness (Keaney and Campbell 2015).

Pericytes

CNS pericytes (PCs) are cells that have derived from the neural crest, unlike other PCs which we find elsewhere and are derived from the mesoderm (Majesky, 2007). The pericytes are mural cells embedded in the basement membrane located abluminal to the ECs (Bhattacharya et al., 2020; Daneman et al., 2010). It is in the BBB where we find the highest concentration of pericytes, their function is to control the capillary diameter of the endothelial cells, and therefore the blood that flows into the brain (Winkler et.al, 2011). This ability is possible because the elongated processes contain contractile proteins, and these that extend into the abluminal surface, and they regulate the diameter of the capillary (Peppiatt et al. 2006; Hall et al. 2014). Additionally, pericytes have other functions such as tissue reparation, the regulation of angiogenesis, general maintenance, and the deposition of extracellular matrix in (Armulik et al., 2010; Armulik et al., 2011; Daneman et al., 2010).

Basement Membrane (BM)

The vascular tube, which is also called the vascular glia limitans perivascularis, is a acellular component of the NVU which is synthesised by different cell types (Del Zoppo et al., 2006; Sorokin, 2010). It consists of an inner, and an outer parenchymal basement membrane (BM)

(Del Zoppo et al. 2006; Sorokin, 2010). These two membranes are synthesised by different cells, while the parenchymal BM is secreted by astrocytic cells, the vascular BM is composed by the matrix of ECs and PCs. (Daneman and Prat., 2015; Seeger et al., 2019). The disruption of BMs structure, and thereby the BBB dysfunction is often caused by matrix metalloproteinases, which lead to an leukocyte infiltration observed in neurological disorders (Daneman, 2015). This could potentially lead to Alzheimer's disease and other neurological disorders.

Chapter 6: *Porphyromonas gingivalis* and the BBB

While the field remains rather new there are some pathways described as possible routes for *Porphyromonas gingivalis* to penetrate directly the blood-brain barrier (BBB), and could further support the hypothesis that oral bacteria could have a role in neurological degeneration such as Alzheimer's disease (AD).

The potential mechanisms are:

- Mfsd2a pathway/Caveolin-1 transcytosis pathway (Lei et al., 2023; Li et al., 2024)
- OMVs degradation of tight junctions (Nonaka et al., 2022; Pritchard et al., 2022)

Mechanisms for crossing the BBB

Mfsd2a/Cav-1 transcytosis pathway

The study by Lei et al. (2023) aimed to investigate the correlation between *Porphyromonas gingivalis* and the blood-brain barrier (BBB), suggesting a potential link between oral pathogens and neurological conditions, specifically Alzheimer's disease. Although the precise mechanism by which *P.gingivalis* crosses the BBB remains unknown, this research proposes the regulation of the Mfsd2a/Cav-1 transcytosis pathway as a plausible mechanism (Lei, 2023)

Transcytosis is the process by which extracellular substances are transported across cells, the very first step is called endocytosis, where extracellular material is taken up into vacuoles within the cell (Hu et al., 2019; Niu et al., 2020). There are several pathways that mediate endocytosis, and these are dependent on proteins such as clathrin and caveolae, whose function is to facilitate the internalisation of the bacteria within epithelial cells or phagocytes (Andreone et al., 2017; Fillipini and D'Alessio, 2020; Hu et al., 2019; Niu et al., 2020). Caveolae are the organelles that contain the absorbed substances enveloped, these are flask-shaped and are also involved in other functions such as signal transduction (Fillipini and D'Alessio, 2020; Andreone et al., 2017). One of the main components of this organelle is Caveolin-1 (Cav-1), and this protein could affect the ability of *Porphyromonas gingivalis* to be internalised within the cells (Busija et al., 2017; Tamai et al., 2005). Which is why Lei (2023) proposed a caveolae/Cav-1 mediated transcytosis.

Caveolae/Cav-1 transcytosis

Lei (2023) infected rats with an intravenous injection on their tails with *Porphyrmonas gingivalis* and Evans blue dye to demonstrate if infection led to an increased permeability of the BBB. After infection, the brain of the rats had higher levels of albumin concentration in the cerebral cortex and hippocampus, and the infiltration of Evans blue was much more prominent in the infected than in the control group (Lei, 2023). Following these results, they added *P.gingivalis* to an BMCECs BBB in vitro model and they found that the expression of Cav-1 protein had increased significantly by 1.9-fold after infection. They also detected *P.gingivalis* in the microvessels and brain parenchyma, suggesting that the bacterium could cross the BBB into the brain. Notably, they found that the expression of occludin protein, which is associated with BBB integrity, is not affected by *P.gingivalis* infection in hippocampus and cortex tissues (Lei, 2023). Furthermore, it was found that Cav-1 was able to bind through a hydrogen bond to RgpA, one of the virulence factors of *P.gingvialis*. They also found that P.gingivalis significantly inhibits the expression of Mfsd2a, and the overexpression of this protein counteracted the effects of the bacterium. Moreover, knockout Mfsd2a mice did have augmented transcytosis but did not affect the tight junction (TJ) structures (Lei, 2023)

In this study by Lei (2023) they also found that the intensity of the infection determined which tissues and to what extent it was affected. A low intensity bacteremia of P.gingivalis primarily affected the hippocampus tissues, however if it was a high-intensity it also impacted the cortical brain. However, they suggested that a low intensity infection could lead to effects of the cortex if the tissues were exposed long enough (Lei, 2023). They also suggested that the flora from the intestinal and respiratory systems might disrupt the BBB, thereby enabling transcellular or extracellular transport (Lei, 2023).

In another study, after intravenous injection of *P.gingivalis* it was found in cortical and hippocampal neurons significant vacuolar degeneration, altered cell arrangement and nuclear pyknosis. In the groups infected with P.gingivalis, LPS and gingipains it was found a vasodilation in the cerebral cortex, while no such findings were found in the control group. Additionally, those infected with *P.gingivalis* LPS demonstrated nuclear pyknosis and vacuolar degeneration (Li, 2024).

The Ddx3x/Mfsd2a/Cav-1 regulatory axis

In a new study by Li (2024), it was found a significantly decreased expression of Mfsd2a in the hippocampus and cortex subsequent to an intravenous injection of *P.gingivalis* and gingipains (Li et al., 2024). It was found that the effect of *P.gingivalis* on Mfsd2a was time-dependent, where the mRNA expression was decreased the longer the BMECs were exposed to the bacteria. More specifically, it was found that it was the concentration of RgpA that led to a significant reduction in the expression of Mfs2da, while *P.gingivalis* LPS had no significant effect. RpgA had the ability to modify Ddx3x methylation and expression, thereby reducing Mfsd2a expression. Subsequent to RgpA treatment they found demethylation of Ddx3x protein at Arg363-Arg376 using QE high-resolution mass spectrometry. After using a computer simulation they found that RgpA protein was able to bind at 306 sites in the Ddx3x protein, and make strong hydrogen bonds between them. To further demonstrate this relationship upon Ddx3x overexpression, an increased Mfsd2a mRNA expression, and it reversed the effect of RpgA significantly. (Li, 2024)

This study concluded that gingipains impaired learning and memory in C57BL/6 mice. They tested their escaping abilities in control groups compared with the gingipain group, and it was found a significant inhibition in their learning ability. However they did not find a significant difference in the *P.gingivalis* LPS, compared with the control group. Gingipains increased blood-brain barrier (BBB) permeability *in vivo* and *in vitro*, where albumin leaked into the hippocampus and cerebral cortex (Li, 2024)

These findings suggest that *P. gingivalis* enhances BBB permeability through the Mfsd2a/Cav-1 transcytosis pathway. Caveolae and Cav-1 play crucial roles in facilitating bacterial entry into BMECs, with the interaction between Cav-1 and P. gingivalis-gingipain supporting their involvement in BBB breach. The inhibition of Mfsd2a expression by *P. gingivalis* highlights its regulatory role in transcytosis and BBB permeability. The potential reversal of *P. gingivalis*-induced effects by Mfsd2a overexpression emphasises its potential as a therapeutic target for preventing neurological impairments associated with *P. gingivalis* infection.

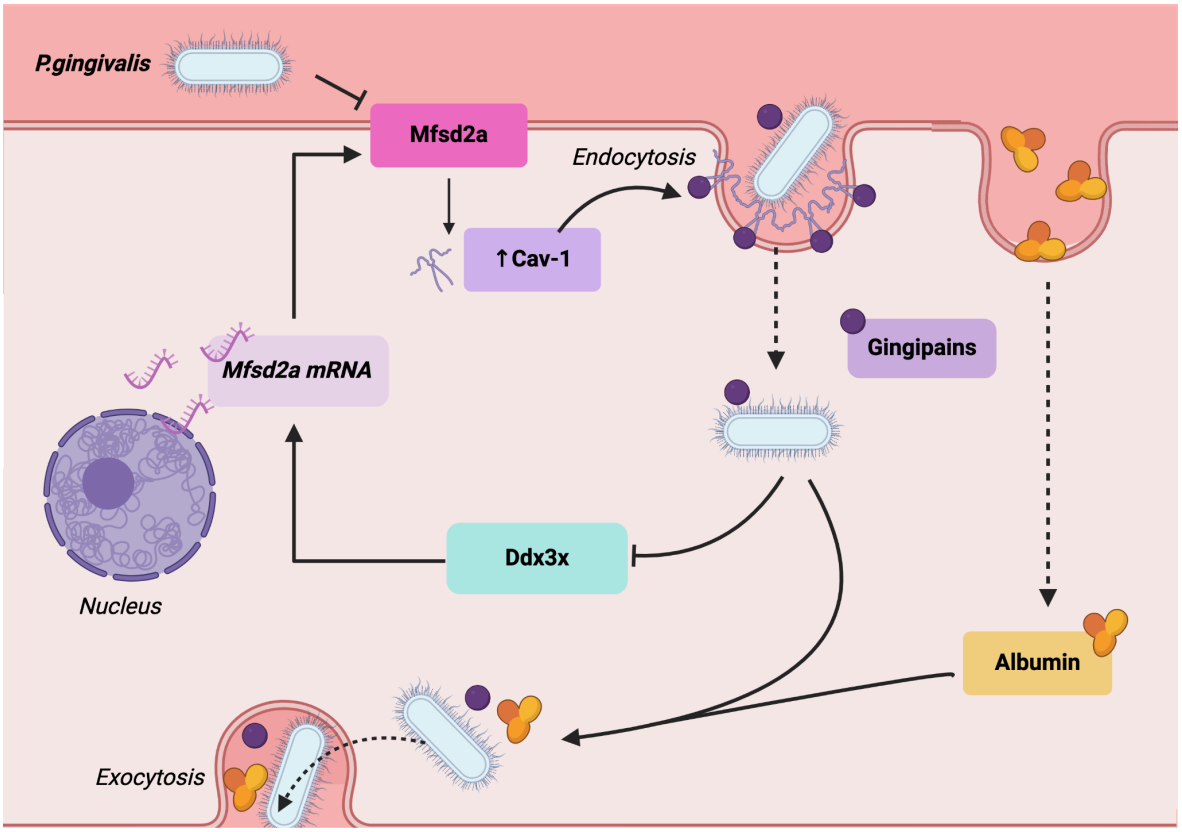

Fig. 12 This illustration is inspired by the work of Lei (2023) and Li (2024). It demonstrates how P.gingivalis from the systemic circulation reaches the brain. Once here it increases the Caveolin/Cav-1 through the inhibition of Mfsd2a protein. This fill create vacuoles, caveolin intracellularly were P.gingivalis, gingipains albumin and other extracellular molecules are brought into the cell. Once inside the cell P.gingivalis has the ability to demethylate Ddx3x, which lead to less Mfsd2a in the endothelial cell (Lei et al., 2023; Li et al., 2024)

OMVs degradation of tight junctions

There are studies that suggest that gingipains are able to break down epithelial transmembrane proteins, some of them are occludin, E-cadherin and β-integrin. If the tight junctions are compromised, the structure of the BBB would be disrupted and bacteria alongside other pathogens could enter the brain (Kanagasingam et al., 2020; Nonaka et al., 2022; Pritchard, 2022)

A study conducted by Nonaka et al. (2022) demonstrated that gingipains had the ability to increase the permeability of hCMEC/D3 cell monolayers, which are human cerebral microvascular endothelial cells. It was thought that the gingipains could degrade proteins vital

to the architecture of tight junctions such as Zonula occludens-1 (ZO-1) and occludin, and thereby increase the permeability of the BBB (Nonaka et al., 2022).
In this study it was used three different strains of *Porphyromonas gingivalis* to infect the hCMEC/D3 monolayer; wild-type (WT), KDP129 and KDP136. The latter strains are Kgp deficient, and both Kgp and Rgp deficient respectively. The results were similar when infected with the WT and the culture supernatant, where it was observed a significant reduction of ZO-1 and occludin levels in the monolayer. However, when infected with the bacteria and culture supernatant of the other strains, it was not observed a significant reduction in the ZO-1 proteins levels, and the concentration of occludin remained at a high compared to the WT. These findings suggest that Kgp and Rgp are involved in the degradation of tight junctions (TJs) in hCMEC/D3 cells (Nonaka et al., 2022). In order to support these findings the cultures were added specific gingipain inhibitors, KYT1 and KYT36, which inhibit Rgp and Kgp respectively. After the addition of these inhibitors, the degradation was impaired, the concentration of the proteins remained high, suggesting that gingipains have the ability to degrade ZO-1 and occludin.
It must also be taken into account that ZO-1 is an intracellular protein, and occludin is a type II transmembrane protein with no lysine cleavage sites for Kgp, further strengthening the hypothesis that cleavage must happen intracellularly (Nonaka et al., 2022). However, in another study using immunohistochemistry, it was found that *P.gingivalis* and gingipains did not reduce the expression of occludin in the hippocampus and cerebral cortex of the mouse brain (Li et al., 2024).

Degradation of Occludin and ZO-1

In the study they suggested that occludin was mainly degraded by gingipain Kgp, one of *Porphyromonas gingivalis* virulence factors. Nonaka et al. (2022) identified the localization of gingipains in the hCMEC/D3 cells by using antibodies, where RgpA and/or Kgp were observed in the cytoplasm, and the nuclei of the monolayer, suggesting an intracellular localization (Nonaka et al., 2022). A similar intracellular localization of *P.gingivalis* and its virulence factors was detected in microglia, astrocytes and neurons in the hippocampus of mice after being orally infected with *P.gingivalis* (Ilievski et al., 2018).
However, the degradation of ZO-1 showed some variation. In the experiments conducted by infection suggested that it was gingipain Rgp responsible for degradation of ZO-1, however in the culture supernatant it suggested Kgp had this function. Then, in *in vitro* assays it was suggested that both Kgp and Rgp contributed to the degradation of ZO-1, but since occludin

did not have lysine residues extracellularly it could not explain how Kgp cleaved on the protein (Nonaka, 2022). This leads us to a question, how could Kgp cleave if there are no extracellular lysine domains for them to cleave?

The OMVs

Subsequently, Nonaka (2022) performed a series of experiments including in vitro assays and immunohistochemical analysis, strengthening the hypothesis that gingipains could degrade these proteins (Nonaka, 2022). Similarly to other Gram-negative bacteria, *Porphyromonas gingivalis* releases outer membrane vesicles (OMVs), which transport virulence factors. The OMVs are produced by components of *P. gingivalis* from its outer membrane, including LPS, muramic acid, a capsule, fimbriae. These OMVs carry gingipains which are internalised. (Pritchard et al., 2022). In these studies it was observed that when OMVs deliver gingipains to cells, it lead to a significant protein degradation of ZO-1 and occludin in hCMEC/D3 cells (Nonaka, 2022). These findings were supported by a different study where it was observed a disruption of ZO-1 in a hBMEC monolayer model when infected with *P.gingivalis* OMVs (Pritchard et al., 2022).

The study by Pritchard et al (2022) focused on the movement of lipopolysaccharide (LPS) across the barrier, in the presence of OMVs (Pritchard et al., 2022). At first, it was studied the relationship between the concentration of OMVs taken up by endocytosis, and the response in the BBB model. Unconjugated LPS was added into this model, and a drop in transendothelial electrical resistance (TEER) was observed. When the concentrations were lowered, the TEER reduction was temporary and had a partial or complete recovery in BBB structure. This could be attributed to the neighbouring cell's ability to overcome apoptosis, which could mean that the cells themselves or the tight junctions that bind them have the ability to recover when the cells are exposed to lower levels of endotoxins. However, models that were infected with higher concentrations of LPS and OMVs lead to an irreversible disruption, and when unable to recover, it leads to apoptosis or pyroptosis (Pritchard et al., 2022). Not only this, but when applied unconjugated LPS and OMVs it was observed FICT-dextran in the basolateral compartment, implying that there was an increase in paracellular flow, which could mean a widening of paracellular gaps compared to control groups (Pritchard et al., 2022). The virulence factor that affected TEER the most were OMVs, and showed less recovery, and when conjugated with FITC it did cross the BBB (Pritchard et al, 2022).

In conclusion, the study by Pritchard et al (2022) demonstrated that OMVs, unconjugated LPS and FITC conjugated LPS affected the integrity *in vitro* BBB model, furthermore it was observed a ZO-1 disruption (Pritchard et al., 2022).

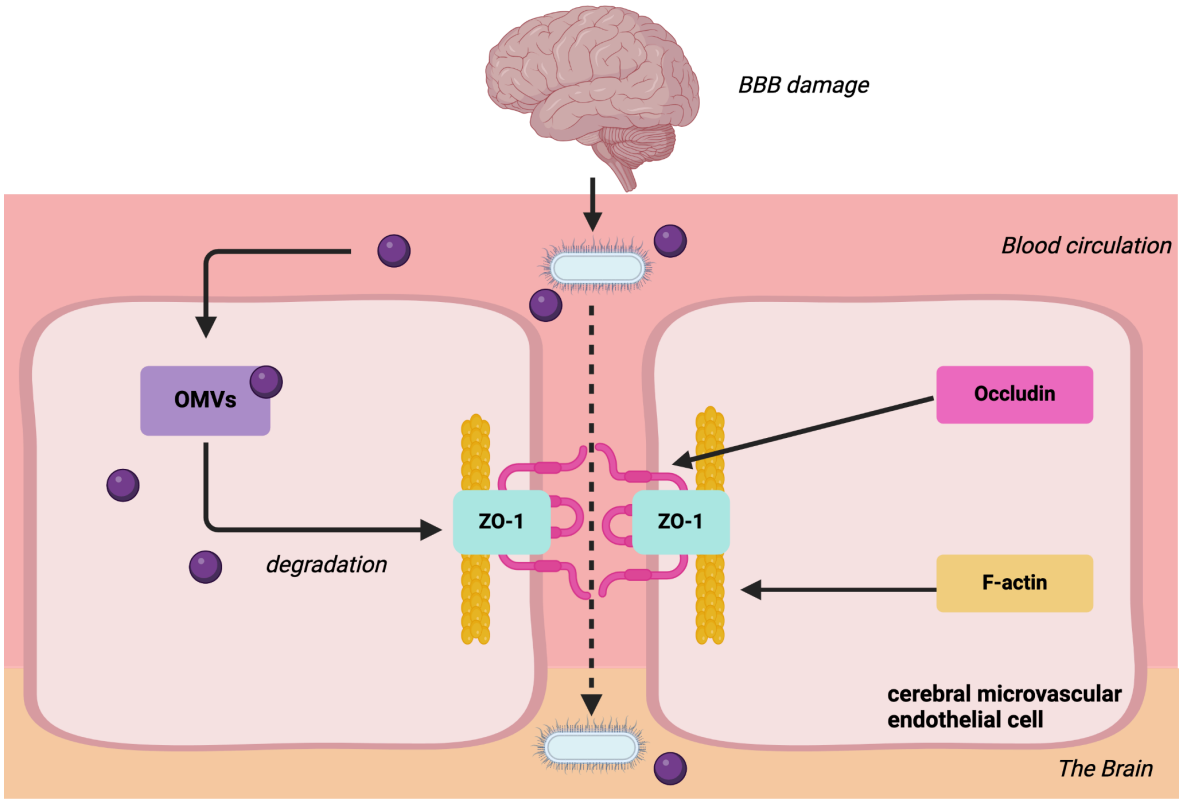

Fig. 13

Porphyromonas gingivalis is brought via the systemic blood circulation into a leaky brain. *P.gingivalis* and OMVs, which carry gingipains and LPS, could enter cerebral endothelial cells. Once intracellularly they could degrade proteins such as occludin, ZO-1, and F-actin which bind the endothelial to one another. This would further facilitate transport of *P.gingivalis* into the brain (Nonaka et al., 2022; Pritchard et al., 2022)

Chapter 7: Methodology of Literature Search

For this master's thesis a systematic approach was used in order to conduct a comprehensive literature search about the intricate relationship between *Porphyromonas gingivalis* and Alzheimer's disease. The following steps outline the methodology employed:

Firstly, keywords and search terms were identified and selected through brainstorming prior to the literature search, choosing the main themes, and through discussions with the supervisor. As a broader understanding was established and articles were read, new keywords were identified and subsequently included.

The keywords included; Alzheimer's disease; *Porphyromonas gingivalis*; Periodontitis; blood-brain barrier; NVU, Astrocytes, Pericytes, OMVs; gingipains ; *in vitro* BBB model; APOE, microglia, tau, amyloid-beta, nervus trigeminus, virulence factors.

In order for precise articles to be selected it was used in addition to the keywords, the Boolean operator (AND) to create search strings, this helped to narrow down the results of the search in the database used, Pubmed. This database has an extensive coverage of articles about the subject, and most of them are available to the public. If articles were not available, they were accessed through The University Library:oria, which gave access to many electronic journals and interesting articles.
However the search was still too broad, and in order to narrow down the results, filters on Pubmed were used. Some examples are data range, document type, and publication time. To ensure that relevant literature was chosen, a three-stage screening process was implemented based on criteria determined prior to the literature search. Some of this criteria was accuracy, clarity and credibility. The first step of this process was to screen abstracts against this criteria, if they did not align with the criteria the articles were excluded. For the second step introductions and discussions were carefully evaluated and if they were relevant for the last step their methods and results were studied. The data that was extracted from these articles were annotated down and organised into the appropriate sections and categories. Once sufficient information was gathered it was written summaries of each section, which would be reviewed and rewritten as new information was gathered.

Chapter 8: Discussion of the Literature Search

Alzheimer's disease (AD) is the most common form of dementia and its rapidly increasing in prevalence world wide, by 2050 it is expected that 152 million patients will be suffering from dementia (World Health Organization, 2017). As dementia, AD, increases in our society we must acknowledge the emotional, economical and structural challenges it presents. Which is why it is imperative that we broaden our understanding of the disease, try to find preventative measures and hopefully a cure.

There are several articles that investigate if there is an association between Alzheimer's disease and periodontal disease (Borsa et al., 2021; Dziedzic, 2022; Leira et al., 2017; Said-Sadier, 2023). It was found a higher bacterial load of periodontal pathogens in the AD brain compared to controls, and that the severity of the periodontal disease could affect AD development (Leira et al., 2017; Panzarella, 2022). In addition, there are several genes and risk factors such as diabetes mellitus and atherosclerosis, which have been identified which could indicate a relationship between the diseases (Jin et al., 2021; Kanagasingam et al., 2020).
However it is important to note that association is not a synonym to cause, and even if these articles checked all of Bradford Hill's (1965) criteria on the principles of association, it is not sufficient to determine a causative relationship. Which is why, as all the systemic reviews mentioned, we need to investigate further a possible relationship. In light of these findings it could be that periodontitis has a positive effect on Alzheimer's or that they have a mutual positive effect, supporting the bidirectional hypothesis. However we must not forget that confounding is possible, and that there is a third unknown factor that could affect the pathology and the development between the two diseases simultaneously. This leads us to the question, what is the relationship between these two diseases?

A plausible answer is the bidirectional relationship, as suggested by Dziedzic (2022). It is thought that in one of the directions patients with poor plaque control would develop periodontitis over time, which in addition to lead to marginal bone loss, triggers a systemic inflammatory reaction where pro-inflammatory molecules are synthesised (Liccardo et al., 2020). These molecules, once in the systemic circulatory system, are able to reach the brain's circulatory system. Microglia, which are the brain's immune cells, can be activated by these molecules and will in turn activate a local inflammatory response within the brain (Colonna et al., 2017; Rock et al., 2004). Once the microglia are activated it is possible that it degrades

tau proteins and regulates Aβ levels as well as its deposition (Asai et al., 2015; Bolos et al., 2016; Lee and Landreth, 2010; Ilievski, 2018). One the neuropathological hallmarks, Aβ plaques and tau tangles are present in the brain, it could possibly lead neurodegeneration overall in the hippocampus and cerebral cortex with cognitive deficiencies as the end result (Dugger and Dickson, 2017; Hyman, 2012; Kanagasingam et al., 2020; Olsen, 2021; Singhrao and Olsen, 2019). The other direction is that those with already cognitive deficiencies, due to memory or motor skills, would struggle to maintain ideal oral hygiene increasing the risk of developing periodontitis (Delwel et al., 2017; Foley et al., 2017). However there could also be a third option, that there is a simultaneous development of AD and PD due to a confounding third factor. , where early memory loss could lead to patients forgetting or struggling with brushing their teeth, and the circle just potentiates itself. While these are hypotheses on how Alzheimer and periodontal disease are interconnected, it does not explain fully how periodontal pathogens can cause this. Which leads us to the next question, which pathways can *P.gingivali*s exploit to affect AD?

As suggested previously, there are three possible pathways in which *P.gingivalis* could trigger an inflammation reaction within the brain (1) Induction of the systemic immune system (2) Trigeminal infection (3) Direct invasion of periodontal pathogens.

The bidirectional relationships build on the hypothesis that a systemic activation of the immune system will lead to an overactivity that contributes to neurodegeneration (Poole et al., 2013). If leptomeningeal cells could transduce inflammatory signals from the systemic circulation into the brain by activating microglia, this could explain how this neurodegeneration could be triggered (Liu et al., 2013; Poole et al., 2013). Once the microglia are activated, and proinflammatory molecules are released it would activate an inflammatory cascade within the brain (Liccardo et al., 2020). It has also been previously described how microglia could play a role in amyloid-β and tau pathology, which are AD hallmarks, as it leads to the accelerated aggregation of hyperphosphorylated tau and Aβ in the brain after being activated due to infection (Asai et al., 2015; Bhasakar et al., 2010; Ilievski et al., 2018; Kanagasingam et al., 2020; Sheng et al., 2003). This communication of the systemic environment into the brain via leptomeningeal cells could be a potential pathway in which *Porphyromonas gingivalis* directly and indirectly affects AD pathology.

The second suggested pathway is the involvement of the trigeminal nerve, which innervates the periodontium, and are the branches of the trigeminal ganglia (Huff et al., 2022). It is

thought that since this ganglion is an anatomical neighbour of the locus coeruleus, the pons, and the brain stem, any changes in homeostasis within the nerve could alter this areas, which are responsible for emotions and the patients conduct (Cook et al, 2013; Goto et al., 2020; Kanagasingam et al., 2020). If *P.gingivalis* could alter the nerves homeostasis it could explain how a person's personality would alter over time.

The last pathway is direct invasion into the brain. In order for this to be possible the bacteria would have to cross the BBB which is a barrier that envelops the brain. The BBB's most important role is to prevent dangerous substances from affecting the brain's microenvironment, and which is why it is built up by the NVU, a complex system of cells that work together (Zenaro et al., 2017). This leads us to the next question, which mechanisms does *P.gingivalis* exploit to cross the BBB?

As of today the two mechanisms proposed that enabled the bacterium to cross the BBB are (1) the Mfsd2a/Caveolin-1 transcytosis pathway (2) OMV degradation of tight junctions. The Mfsd2a is inhibited by the presence of *P.gingivalis*, and it lead to an increased presence of caveolae-like structures. The bacterium was internalised by caveolae, and it was found that the concentration of Cav-1 had increased after infection (Lei et al., 2023). Once inside, the bacteria or its virulence factors modified the methylation of Ddx3x reducing Mfsd2a expression. All of these findings conclude that gingipains impaired learning and memory, and there was an increased permeability of the BBB facilitating the transport of substances like albumin into the brain (Li et al., 2024).
Porphyromonas gingivalis has the ability to destroy the tight junctions that bind endothelial cells to each other. It has been found that gingipains, Kgp and Rgp had the ability to degrade ZO-1 and occludin to various degrees (Nonaka et al., 2022). However, what challenges these findings was to identify in which way these gingipains were able to destroy these intracellular proteins, as there is no extracellular cleaving site for them to bind on. This led to the conclusion that for these virulence factors to work, they must enter the endothelial cells first, and they would have to degrade the proteins intracellularly (Nonaka, 2022). This mechanism was found by Pritchard (2022) where it has been suggested that OMVs with virulence factors like LPS or gingipains would be taken up by the cell, and then have an intracellular effect. It was found that the higher the concentration of LPS and OMVs, the cell had reduced ability to repair itself leading to apoptosis, and increased paracellular flow (Pritchard, 2022).
While further investigation is needed these four articles propose mechanisms which *Porphyromonas gingivalis* exploits in order to be able to cross the BBB. However, we must

remember that *P.gingivalis* more often that not co-exists with *Tannerella forsythia* and *Treponema denticola* making the Red Complex (Marsh and Martins, 2016).
Different species could have different virulence and characteristics enabling them to exploit other mechanisms, it is also possible that a conglomerate of pathogens might have a more effective infection where they potentiate each other's effect. Within each species there are different strains all of which have different virulence degrees, meaning that perhaps patients carrying specific types of strains could be more at risk of pathogens crossing the BBB, and perhaps trigger AD. It could be that the degradation of BBB is caused by an intricate coordination of mechanisms each step enabled by a specific bacterium.

During a lifetime individuals are probably exposed to low grade inflammation due to plaque accumulation. All of the experiments conducted are during low term exposure, but what happens in the brain when we are exposed to low grade inflammation over decades? How does this affect the integrity of the BBB?
Patients with suboptimal oral hygiene causes a local inflammation and recruitment of cells like neutrophils, which will subsequently enter the systemic circulatory system, and cause systemic inflammation (Liccardo et al., 2020; Poole et al., 2013). It has been demonstrated by Paganini-Hill (2011) that patients that did not brush sufficiently had an 20-35% increased risk of developing dementia, compared to individuals that brushed everyday. This local inflammation could become a low grade systemic inflammation, which in time could affect the structure and the integrity of the BBB. Once this layer is weakened, pathogens could hypothetically enter, or have an facilitated infection into the brain.

In conclusion, we are faced with a societal challenge where individuals live longer but the prevalence of dementia overall increases with 10 million new cases every year (World Health Organization, 2017). As of today there are no efficient medicines which cures Alzhmeimer's, which is why preventative measures are vital to ensure that quality of life is maintained as we live longer. In Norway in 2023 only 20,7% of the elderly and patients with long-term illness that received home care were under the observation of the public dental health sector (Statistisk sentralbyrå, 2023). This is negligence of arguably one of the most vulnerable patient groups, and with a higher risk of developing Alzheimer's and periodontitis alongside other comorbidities. In my opinion, by prioritising oral health this could perhaps be used as a preventative measure, and to ensure a better quality of life for the patients in nursing homes and patients receiving home care. Prevention of the disease could relieve some of the emotional and economical burden and will increase the quality of life for the patients.

In this part of the Master's thesis we have studied the relationship between periodontal disease and Alzheimer's disease through the keystone pathogen, *Porphyromonas gingivalis*. In the following section of the Master's thesis, Part Two, we will present the experiment, and hopefully answer our question: Can *P.gingivalis* cross the BBB?

PART TWO

Chapter 9: The Experiment

In this chapter, the experimental study that was performed will be presented, and answer the core question: Can *P.gingivalis* cross the BBB?

There are four main steps of the experiment (1) Preparing a cell culture of hCMEC/D3 cells, which will function as the BBB (2) Infect the BBB with the strains of *P.gingivalis*, preparing them for transmission electron microscopy (TEM) (3) Take images with TEM to determine permeability ability (4) analyse the images and perform statistical tests such as ANOVA two way. Firstly, the strains used and the methods of the experiment will be presented followed by the results and a discussion.

The strains

The different strains of *Porphyromonas gingivalis* have different anatomical structure, and therefore also virulence factors. The information about the strains is limited, but some factors are known and they might explain their penetration ability.

W83

This particular strain has a thicker capsule than other variants, and they produce less leukocytes than other strains of *P.gingivalis* (Katz et al. 1996). In another study they demonstrated that the LPS, one of the capsules structures, could be altered by the deletion of an element near the 5' end of K-antigen genes (Bainbridge et al., 2015). This strain also has less fimbrae on its surface than other strains, type IV FimA, which are necessary to adhere to the host's tissue (Amano et al., 2004). Finally, it was found that it has similarly to ATCC33277 OmpA-like protein, RagA and RagB (Imai et al., 2005)

ATCC33277

This strain has the ability to adhere more effectively to host tissues and other microbes due to its presence to a large number of fimbrae type 1 (Amano et al., 2005). This could facilitate adheration to the hCMEC/D3 cells.

A7A1

It had been found that strain A7A1 causes higher levels of serum IgG antibodies when the patient has periodontitis, but there is no significant increase in healthy patients or gingivitis (Chen, Siddiqui and Olsen, 2017; Ebersole et al., 2020). The virulence factors identified are a capsule type K3, and fimbrillin type II (Aduse-Opoku et al., 2006; Laine et al., 1998; Nakano et al., 2004)

195P63

As of today there is not much information available about the characteristics of this strain.

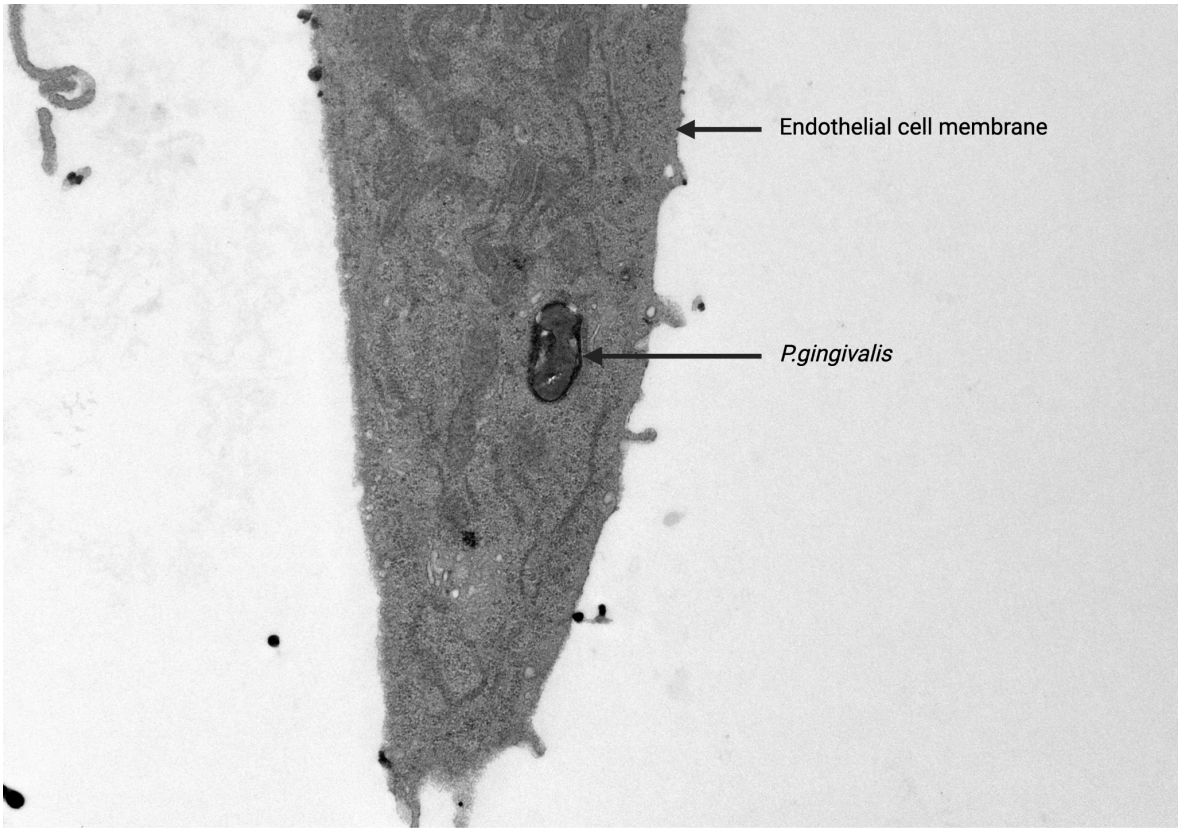

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Fig. 14 The transmission electron microscopy image (TEM) shows how *P.gingivalis* strain A7A1 is enclosed within the human cerebral microvascular endothelial cells/D3 (hCMEC/D3).

Chapter 10: Materials and Methods

In this chapter, the experimental procedures and methodologies which were employed in the study will be presented. From cell culture techniques, fixation protocols and electron microscopy. For the experiment a cell culture was prepared using a hCMEC/D3, which were subsequently prepared for electron microscopy by infecting them, and finally the images were taken using TEM. For the experiments protocols from Bergersen Laboratory were used, in addition to the protocol from Nature "Immunogold quantification of amino acids and proteins in complex subcellular compartments" by Bergersen and Storm-Mathisen (2008). My aim is to provide a comprehensive understanding of the experimental framework guiding our research.

Cell Culture hCMEC/D3 protocol

The protocol utilised in this study was developed by Bergersen Laboratory.

Materials: Cell Culture hCMEC/D3 protocol

- EndoGRO(™)- MV Complete Media Kir (Cat.No. SCME004), supplemented with 1 ng/mL FGF-2 (not in the kit) (10,56 µl)
- Collagen type I
- Flask 5-10 mL
- Incubator
- Refrigerator
- Water bath
- Ethanol 70%
- Pipette 1 or 2mL
- Conical tube 15mL
- Pipette 10mL
- Cryopreservative (DMSO)
- Accumax(Cat. No. SCR006)
- Trypsin EDTA (Cat. No. SM-2003-C)
- Plate
- hCMEC/D3 media

Methods: Cell Culture hCMEC/D3 protocol

Preparing EndoGro(™) Basal Medium 475mL:

- EndoGRO-LS Supplement, 0.2% (1.0 mL)
- rh EGF, 5 ng/mL (0.5 mL)
- L-Glutamine, 10 mM (25 mL)
- Hydrocortisone Hemisuccinate, 1.0 µg/mL (0.5 mL)
- Heparin Sulfate, 0.75 U/mL (0.5 mL)
- Ascorbic Acid, 50 µg/mL (0.5 mL)
- FBS, 5% (25 mL)
- Supplemented with 1 ng/mL FGF-2 (not in the kit) (10,56 µl)

Coating of flasks:

- Step 1: Thaw the collagen type I from the (Cat.No. SCME004)
- Step 2: Dilute 1 mL of collagen type I with 19 mL 1X PBS. This is to be mixed gently.
- Step 3: Coat flask with 1:20 diluted collagen type I solution. The flasks must be stored at 2-8°C for 5-6 days.
- Step 4: Use 5-10mL of the diluted solution for the T75 flasks, while 15-25mL for the T225 flask. This are to be incubated in for one hour in 37°C
- Step 5: Aspirate the solution before the cells, hCMEC/D3, are planted.

Thawing of Cells:

Do not thaw the cells until EndoGro(™) Basal Medium mentioned above is at hand.

- Step 6: Remove the vial of hCMEC/D3 cells from liquid nitrogen and incubate in a 37°C water bath. Closely monitor until the cells are completely thawed. Maximum cell viability is dependent on the rapid and complete thawing of frozen cells. Do not vortex the cells.
- Step 7: As soon as the cells are completely thawed, disinfect the outside of the vial with 70% ethanol. Proceed immediately to the next step.
- Step 8: In a laminar flow hood, use a 1 or 2 mL pipette to transfer the cells to a sterile 15 mL conical tube. Be careful not to introduce any bubbles during the transfer process.

- Step 9: Using a 10 mL pipette, slowly add dropwise 9ml of hCEMC/D3 Medium (pre-warmed to 37°C) to the 15 mL conical tube. IMPORTANT: Do not add the whole volume of media at once to the cells. This may result in decreased cell viability due to osmotic shock.
- Step 10: Gently mix the cell suspension by slow pipeting up and down twice. Be careful to not introduce any bubbles.
- Step 11: Centrifuge the tube at 300 x g for 2-3 minutes to pellet the cells.
- Step 12: Decant as much of the supernatant as possible. Steps 5-8 are necessary to remove residual cryopreservative (DMSO).
- Step 13: Resuspend the cells in a total volume of 10 -12 mL hCMEC/D3 medium (pre-warmed to 37°C).
- Step 14: Plate the cell mixture onto a pre-coated T75 tissue culture flask (See section on ECM Coating of Flasks).
- Step 15: Incubate the cells at 37°C in a 5% CO2 humidified incubator.
- Step 16: The next day, exchange the medium with fresh hCMEC/D3 Medium pre-warmed to 37°C. Exchange with fresh medium every two to three days thereafter.
- Step 17: When the cells are approximately 80% confluent (3-4 days after plating cells at the density they can be dissociated with Accumax™ (Cat. No. SCR006) or trypsin-EDTA (Cat. No. SM-2003-C) and passaged or alternatively frozen for later use.

Subculturing of Cells

- Step 18: Carefully remove the medium from the T75 tissue culture flask containing the confluent layer of hCMEC/D3 cells.
- Step 19: Apply 3-5 mL of Accumax™ or trypsin-EDTA solution and incubate in a 37°C incubator for 3-5 minutes.
- Step 20: Inspect the plate and ensure the complete detachment of cells by gently tapping the side of the plate with the palm of your hand.
- Step 21: Add 8 mL of hCMEC/D3 medium (pre-warmed to 37°C) to the plate.
- Step 22: Gently rotate the plate to mix the cell suspension. Transfer the dissociated cells to a 15 mL conical tube.
- Step 23: Centrifuge the tube at 300 x g for 3-5 minutes to pellet the cells.
- Step 24: Discard the supernatant.

- Step 25: Apply 2 mL of hCMEC/D3 media (pre-warmed to 37°C) to the conical tube and resuspend the cells thoroughly.
- Step 25: Count the number of cells using a hemocytometer.
- Step 26: Plate the cells to the desired density (typical split ratio is 1:3 to 1:6)
- Step 27: Cryopreservation of Cells; hCMEC/D3 cells can be frozen in hCMEC/D3 Medium with 10% DMSO using a Nalgene slow freeze Mr. Frosty container.

Preparation of the Cells for TEM (2 and 6 hours)

The protocol utilised in this study was developed by Bergersen Laboratory.

Materials: Preparation of Cells for TEM (2 and 6 hours)

- Cell culture (hCMEC/D3)
- 10 25 cm^3 flaks
- Collagen I solution 10(5) cells/mL
- Strains; A7A1-28, ATCC32377, W83, 195P63
- PBS, phosphate buffer saline
    - OD 600UL
- Incubator
- Glutaraldehyde 2,5%
- Tubes
- Centrifugation

Method: Preparation of Cells for TEM (2 and 6 hours)

- Have at hand 10 flasks in total of 25 cm^3, one flask per strain for each time point.
- Step 1: Coat the flasks with collagen I solution in the same way as the wells, to each flask add 5mL of cells with a concentration of (10^5 celler/mL)
    - The cells are approximately 80% confluent upon addition of bacteria
    - Added strains; A7A1-28, ATCC32277, W83 and 195PG3
- Step 2: Resuspend the bacteria with 1,3mL PBS, measure OD (600uL), OD; 0,8, and add 200uL to each flask with 2 mL medium
- Step 3: Wash gently the bacteria after 2 hours with 1x PBS (phosphate buffer saline)
- Step 4: Incubate for 6 hours 5 of the flasks with a fresh medium and washed with 1x PBS before fixation

- Step 5: The cells (5 flaks) to be harvested after 2 hours are fixed immediately after the first wash
- Step 6: The cells are fixed (2,5% glutaraldehyde in PBS) and then scraped lightly into a tube, which is centrifuged.

Transmission electron microscopy (TEM)

This part of the experiment is based on the protocol from Nature “Immunogold quantification of amino acids and proteins in complex subcellular compartments” by Bergersen and Storm-Mathisen (2008).

Materials: Transmission electron microscopy (TEM)

Reagents:

- Pentobarbital. Caution:Toxic
- 25% (wt/vol) glutaraldehyde (GA; Electron Microscopic Sciences, cat.no. 16210). Caution: Toxic (irritant, allergen, carcinogen).
- Paraformaldehyde (PFA; Electron Microscopic Sciences, cat.no. 19219). Caution: Toxic.
- Dextran 70 (Mw 70000; Sigma-Aldrich,cat.no. 31390)
- Lowicryl HM20 (Polysicences)
- Sodium azide (Sigma- Aldrich, cat.no. 71290)
- Lead citrate (Electron Microscopic Sciences, cat.no. 17800)
- Uranyl acetate (Sigma-Aldrich, cat.no. 73943)
- Parafilm (pechiney Plastic Packing)

Equipment:

- Peristaltic pump (Watson-Marlow U/D 323; Watson-Marlow Bredel)
- Cryofixation unit (Reichert KF80, Reichert)
- Cryo Substitution unit (Reichert)
- Ultramicrotome (Reichert)
- Diamond knife for ultrathin sectioning (Diatome)
- Nickel mesh grids (300-600 mesh) (300 mesh square; Electron Microscopic Sciences, cat.no. G300-Ni)
- Tweezers (Dumont tweezers; Electron Microscopic Sciences, cat.no. 72800-D)

- Electron microscope (Philips CM10 or Teknai 12) with software (AnalySIS, Olympus) for image acquisition.

Reagent Setup:

- 0.1M sodium phosphate buffer (PBS)
- 0.1 M sodium phosphate buffer (PBS)
- Fixative: Prepare freshly a 500 ml solution of 4% (wt/vol) PFA and 0.1% (vol/vol) GA or a 1% (wt/vol) PFA and 2.5% (vol/vol) GA solution in PB.
- 2% (vol/vol) H2O2
- 0.05 M Tris buffer
- 0.3% (wt/vol) or 0.9% (wt/vol) NaCl
- Tris-buffered saline Triton (TBST)
- Blocking solution: TBST with 2% (wt/vol) HSA
- Sodium borohydride/Gly solution
- Contrast-enhancing agents: 1% (wt/vol) uranyl acetate and 0.3% (wt/vol) lead citrate solution in UFWater
- Sodium ethanolate: Saturated solution of 100 g NaOH in 700 ml absolute ethanol

Methods: Transmission Electron Microscopy (TEM)

Embedding procedure in Lowicryl HM20

Lowicryl HM20 is a water-insoluble acrylic-based resin that polymerizes at low temperatures catalysed by UV light. The freeze- substitution embedding protocol:

- Quickly freeze the tissue in liquid propane (-170°C) to avoid ice crystal formation.
- Dehydrate the specimens and treat them with uranyl acetate (1.5% dissolved in anhydrous methanol) to enhance tissue contrast (45°C, 30 h).
- Infiltrate the tissue with the resin for 24 h.
- Initiate the first phase of UV light 'curing' at 45°C for 24 h, followed by UV irradiation at 0°C for 35 h.

Ultrathin Sectioning

Cut ultrathin sections on an ultramicrotome with a diamond knife at 70–100 nm.

- Select ultrathin sections with a bright gold colour.
- Place the ultrathin sections on nickel mesh grids (routinely 300 mesh).
- It is suggested to leave the grids at room temperature

Image acquisition

Take electron micrographs with a Tecnai 12 electron microscope, usually at a magnification of x460,000. The micrographs are stored as TIFF files.

Quantitative EM Micrographs

The images are analysed on the computer where data was stored on Excel spreadsheet. ANOVA two-way test was performed. In order to asses the effects of the strains, an experimental design involving a two-way analysis of variance (ANOVA) was used.
To maintain consistency and eliminate bias, the operator was blinded while counting specimens, not only this but the operator was blinded into which strains were used for the experiment, thereby did not know which were control groups or the different characteristics of the strains.

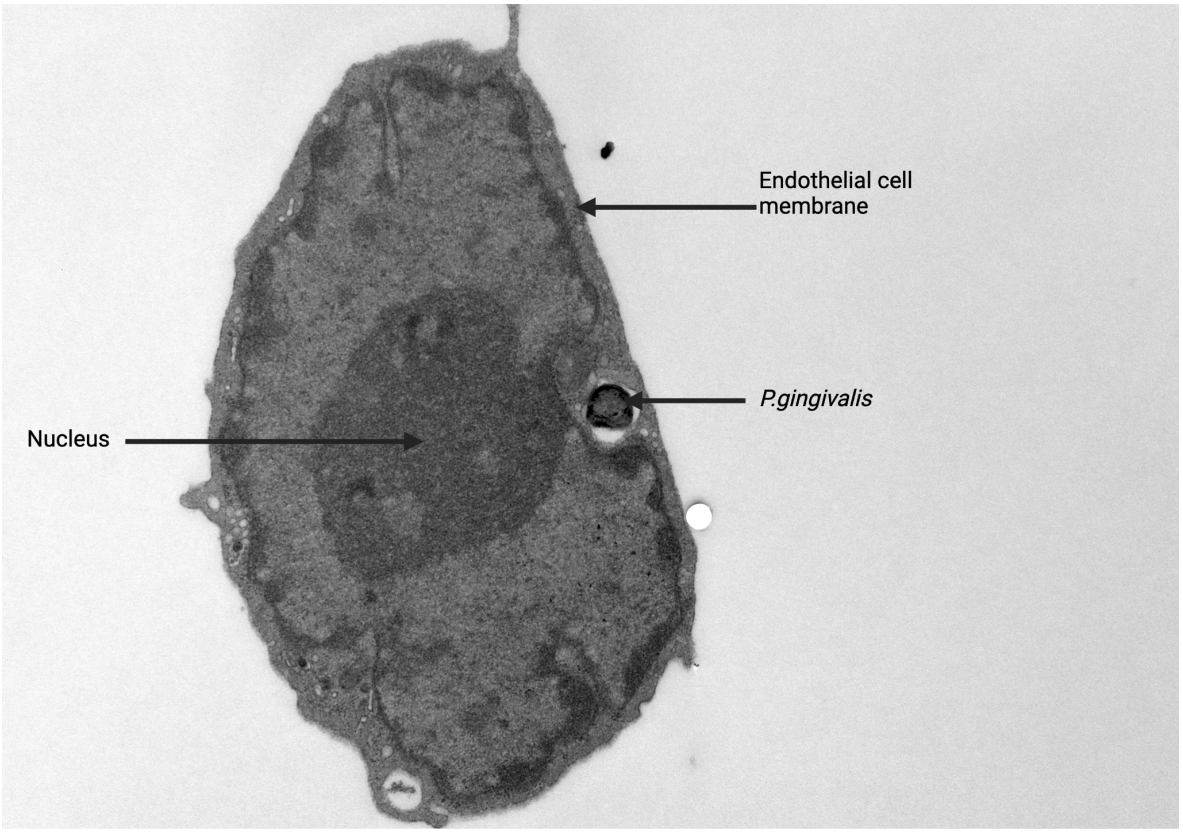

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Fig. 15 Electron microscopy image of hCMEC/D3 cell infected with *Porphyromonas gingivalis*, strain A7A1. The hCMEC/D3 cell has a clearly defined membrane and nucleus. After two hours of infection we observe internalised *P.gingivalis* enclosed by a single-layer membrane.

Analysing *P.gingivalis*; The Criteria

In order to properly analyse the pictures we selected six criteria that had to be present when counting the cells, these are;

1) *P.gingivalis* is localised outside the cell
2) *P.gingivalis* invades the cell
3) *P.gingivalis* inside epithelial cells without endocytic vacuoles
4) *P.gingivalis* encapsulated by single membrane structure
5) *P.gingivalis* encapsulated by double-membrane structures
6) Deformed partial vacuoles

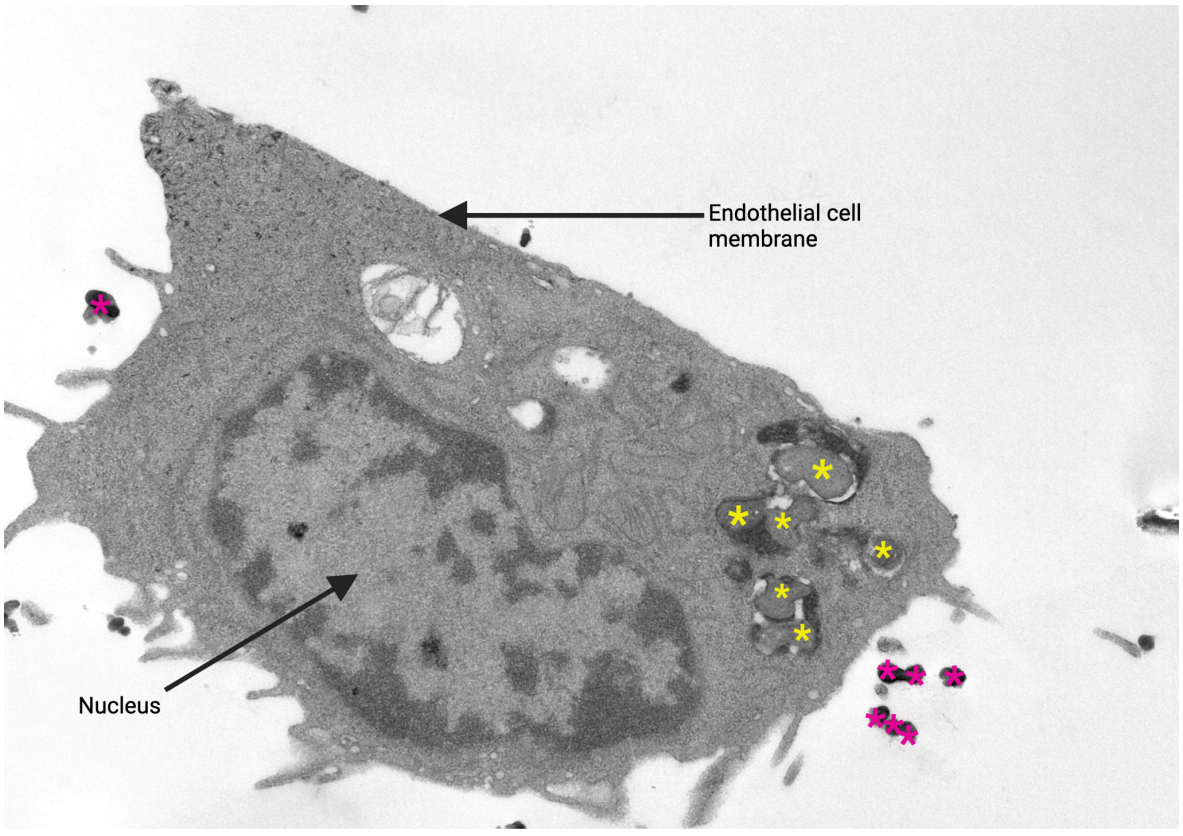

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Fig. 16 *Porphyromonas gingivalis* successfully invading the hCMEC/D3 in vitro model. The asterisk symbol (*) in yellow demonstrates six different *P.gingivalis* bacteria of the same strain, A7A1, encapsulated by the endothelial cell. The asterisk symbol (*) in pink demonstrates the same strain outside of the cell.

Chapter 11: Results

The penetration ability of the four strains, A7A1, ATCC33277, W83, 195P63 across the BBB hCMEC/D3 model was investigated at two distinct time points: hour 2 and hour 6. Images were taken with transmission electron microscopy (TEM), data was allotted on Microsoft Excel 2016 and subsequently analysed with ANOVA. While it demonstrated no significant P-value under (0,05), it demonstrated a quantitative difference in the penetration ability between the strains at both timepoints.

It was forty samples of each strain, at each time point that were analysed quantitatively, and the data was allotted in Microsoft Excel 2016. After the 40 samples were analysed, using Microsoft Excel 2016 the mean bacteria that was able to penetrate the hCMEC/D3 cells was calculated for each strain. A graph was created using those means, where the x-axis represents the different strains, and the y-axis describes the mean that was able to penetrate at both timepoints. While the blue bars represent each strain at hour 2, the pink ones represent at the experiment end 6 hours.

After two hours the strain A7A1 demonstrated to be most effective penetrating the hCMEC/D3 cells with a mean of 0,4 (SD=0,81), narrowly followed by 195P63 with a mean of 0,375 (SD=0,90). The last two strains ATCC33277 and W83 shared a mean of 0,175 (SD=0,45) at hour 2, and had therefore the lowest penetration ability of the four strains

After six hours the penetration ability changed noticeably, and ATCC33277 went from the lowest penetration index to have the most efficient invading abilities on hCMEC/D3 with a mean of 1,025 (SD=1,1). It was closely followed by 195P63 with a mean of 0,85 (SD=2,2). W83 had a mean penetration of 0,75 (SD=1,2) at 6h. The strain with the lowest penetrating ability over time was A7A1, with a mean of 0,575 (SD=1,1)

After the graph an ANOVA two way test With Replication using Microsoft Excel 2016. The P-value was 0,00006 at sample level, 0,781 at column-level, and 0,312 at interaction level. While the results did not yield statistical significance, we can observe in the graph a quantitative difference in the ability of penetration when comparing the strains, and comparing the two timepoints. Demonstrating that is not just bacteria characteristics, but temporal factors that determine penetration ability.

Overall, these findings underscore the dynamic nature of BBB penetration by different strains over time. While some strains consistently demonstrate higher penetration abilities, others exhibit variability in their efficacy, suggesting potential temporal and strain-specific factors influencing BBB permeability. Further exploration of these factors is warranted to elucidate the mechanisms underlying strain-dependent BBB penetration and its implications for therapeutic interventions targeting the central nervous system.

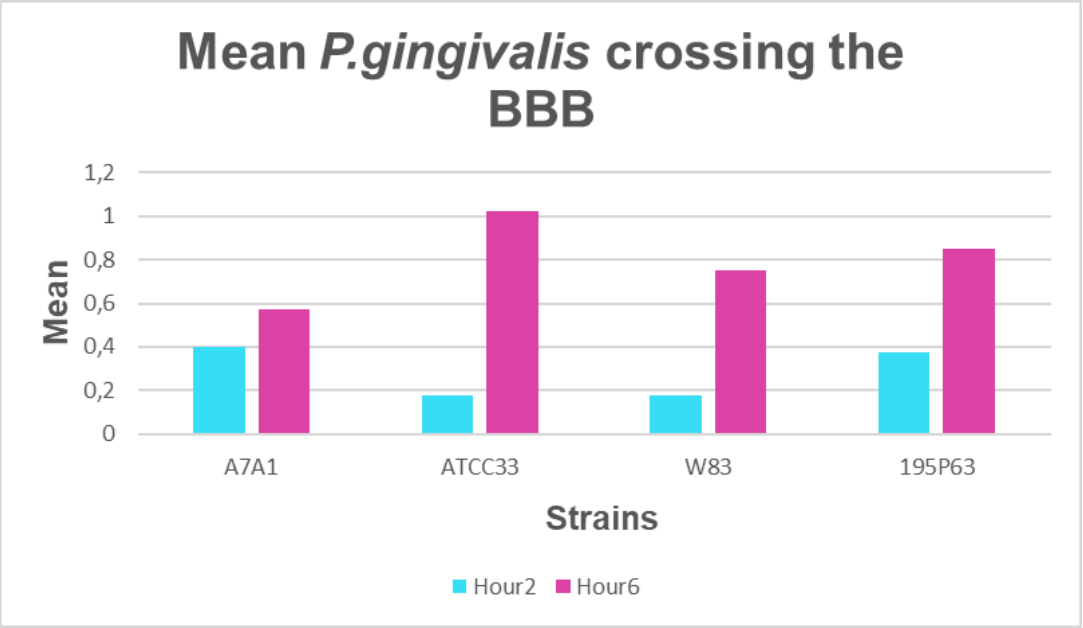

Fig. 17

Different *P.gingivalis* strains were placed on an *in vitro* BBB model of hCMEC/D3 cells, and were incubated for 2h or 6h. The strains are, A7A1, ATCC33277 (ATCC33), W83, 195P63. With the electron microscopy images were taken, and then further analysed quantitatively using Excel 2016. While it demonstrated no significant P-value under (0,05), it demonstrated a quantitative difference in the penetration ability between the strains and both timepoints. An ANOVA two-way test was conducted and the results were P-values of 0,00006 at sample level, 0,781 at column-level, and 0,312 at interaction level.

After calculating the mean with Excel 2016 of 40 samples of each strain, at each time point, a graph was plotted. The x-axis stands for the four different strains used for the experiment, and the y-axis describes the mean bacteria that was able to cross at each timepoint. The blue bars show the mean of 2 hours, while the pink ones for 6 hours.

At hour two the strain A7A1 demonstrated to be most effective penetrating the hCMEC/D3 cells with a mean of 0,4, narrowly followed by 195P63 with a mean of 0,375. The last two strains ATCC33277 and W83 had an identical mean of 0,175 at hour 2, and had therefore the lowest penetration ability of the four strains.

At hour six the penetration ability changed noticeably, and ATCC33277 went from the lowest penetration index to have the most efficient invading abilities on hCMEC/D3 with a mean of 1,025. It was closely followed by 195P63 with a mean of 0,85. W83 had a mean penetration of 0,75 at 6h. The strain with the lowest penetrating ability over time was A7A1, with a mean of 0,575. These results demonstrate that strains and their virulence factors have important effects on its ability to enter hCMEC/D3 cells. Not only this, but there is a temporal factor where more bacteria are able to penetrate the cell the longer the experiment is driven.

Chapter 12: Discussion of the Experiment

In this chapter, the experiment will be presented and discussed further and the question "Can *P.gingivalis* cross the BBB?" will be answered.

Discussion of the Experiment design

This experiment was an *in vitro* assay that aimed to investigate if *P.gingivalis* was able to cross the BBB. For the study, hCMEC/D3 cells were used, which are brain microvascular endothelial cells, and they were infected with four different strains of *P.gingivalis*; A7A1, ATCC33277, W83, 195P63. In order to measure how many bacteria were able to cross, and penetrate these hCMEC/D3 cells, TEM was used to take images which were subsequently analysed quantitatively.

In order to avoid bias, it was not known which types of strains were used in the experiments, and these were chosen by the supervisor. In addition, it was not known which strains corresponded with which at the two time points hour 2 and hour 6. It was not until every sample was analysed, that it was revealed which ones corresponded in order to be able to perform statistical analysis on Microsoft Excel 2016. Once the ANOVA two way test was performed, the strains of *Porphyromonas gingivalis* were revealed. This ensured that the process of data collection was conducted objectively and without influence from prior knowledge of the experimental groups. The raw data were stored securely and are available upon request.

Limitations of the Experiment

In vitro blood-brain barrier (BBB) models, are very good experimental models used by many studies, but they do have some limitations (Pritchard et al., 2022; Nonaka et al. 2022).This model does not replicate exactly the human BBB, as it is only composed of hCMEC/D3 cells and have none of the other NVU components, like astrocytes or pericytes (Zenaro et al., 2017). Since it lacks these intricate dynamic interactions, and does not demonstrate the structural and functional properties of the BBB, we cannot predict the same results in a living patient with a BBB.
However, BBB *in vitro* models do offer several advantages when conducting the experiments. The conditions such as pH and temperature can be meticulously controlled, and several cells like bacteria can be added and changed allowing a wide range of research. In addition, it

reduces the need for experimentation on animals, thereby aligning with ethical considerations. Other experimental designs that could be considered for the future are microfluidic BBB models, *in vivo* animal models and *ex vivo* brain slice models.

Furthermore, while 40 samples for each strain at each time point is enough for a pilot study, the experiment should be run multiple times with a larger sample size. In order to get more results, avoid false positives it is imperative that future research includes more samples, perhaps more strains and shorter intervals per time point like every half an hour or hour. This way, the efficiency of the different strains will be more clear, and perhaps yield statistical significance.

Future directions

While this pilot study presented valuable insights into *P.gingivalis* ability to cross the BBB, there are several approaches and areas that have to be investigated further in order to expand and validate these findings. In the following paragraphs directions for future experiments will be outlined:

As a pilot study, few strains were used and few samples for each strain. Future research should increase the sample size, as well as the number of types of strains. It is also necessary to replicate the study multiple times to support these initial finds, and discover more data. When the number of replications is increased it will strengthen the evidence, and enhance the study's reliability and validity. In addition, it would reduce any false results. In addition, more time points should be selected with shorter intervals between them, for example each half an hour or hour in a longer time frame, even months. This will further reveal and support the temporal hypothesis proposed.

While the pilot study demonstrated interesting results regarding the strains' different ability to penetrate the hCMEC/D3 cells, we still have to investigate further the mechanisms which the bacteria uses to enter the cell. Future research should try to uncover these mechanisms in order to explain how *P.gingivalis* crosses the human BBB.

Discussion of the Results

This study demonstrated that while *P.gingivalis* can cross the BBB, the penetration ability changes between different strains, and at the two time points the BBB was exposed to infection. This leads us to another question, what determines a strains ability of penetration?

ATCC33277 had the highest penetration ability after six hours, and this could possibly be attributed to the fact that they have a large number of fimbrae type 1 (Amano et al., 2005). Fimbrae is what facilitates a microbe to adhere to each other and to the host's tissue, and allows penetration (Enersen et al., 2013). In my opinion, this efficient infection could be attributed to the presence of this particular fimbrae, which would permit the bacterium to bind to the endothelial cells more successfully than the other strains.

While A7A1 was an efficient invader in short term exposure, its ability decreased the longer the experiment was run. From being the most efficient, to having the lowest penetration mean of 0,575 at hour 6. This could be explained by its particular virulence factors, a fimbrillin type II and a K3 capsule (Aduse-Opoku, 2006; Laine et al., 1998; Nanakon, 2004). Perhaps the fimbrillin type III is less efficient that the type 1 on endothelial cells, or that the capsule causes a less efficient invasion.

W83 has a thicker capsule when compared with the other strains, and sometimes capsulated bacteria might invade less efficiently (Irshad et al., 2012; Katz et al., 1996). It could be that the presence of the capsule makes them more resilient against phagocytosis, which could explain why it did not penetrate the hCMEC/D3-cell compared with ATCC33277 or 196P63.

195P63 is not a strain extensively studied, but it is most probable that it shares similar virulence factors as the others. Perhaps what allows them to have the second most effective penetration at both timepoints is the presence of fimbrae or other virulence factor that facilitates colonisation. Further research is needed to identify this bacterium's characteristics.

It is not fully documented how all the virulence factors differ within the strains of *P.gingivalis*, but we know they have some similar fundamental qualities. Virulence as mentioned earlier, is the ability to infiltrate a host, which is colonisation (Sharma et al., 2017). *P.gingivalis* have a lot of different virulence factors such as the "capsule, outer membrane, its associated LPS, fimbriae, proteinases and selected enzymes" (Singhrao et al., p.3, 2015). All

of which could participate in the penetration of the BBB, from fimbrae's ability to adhere to the host, the LPS antigen abilities, or the gingipains ability to degrade tight junctions (Enersen et al., 2013; How et al., 2016; Nonaka et al., 2022).
Furthermore, we must take into account that time is an important factor affecting the grade of infection. The longer the BMCEs cells were exposed to *P.gingivalis,* the more bacteria were able to enter them. This suggests that virulence is not the only factor determining penetration ability, but time as well. ATCC33277 is a strain that perhaps needed more time to establish infection, but once it established the infection it was much greater. However A7A1 could be one that infiltrates the cells quite quickly, but progression stagnates with time in comparison to other strains.
In Norway the life expectancy for women is 84,35 years and for men 80,92 years (Haug, 2023) Therefore exposure will not be limited to hours but decades, allowing bacteria longer time to cross the BBB and affect its structure. In my opinion, patients with uncontrolled periodontitis would be exposed to continuous infection over time and it could possibly lead to weakened BBB with age.

In conclusion, while *P.gingivalis* can infiltrate hCMEC/D3 cells, the mechanisms behind it are not fully understood. Recent studies have suggested different mechanisms of infiltration, but further studies are needed to support these findings. Which leads us to the next question, even if *P.gingivalis* could infiltrate the BBB, would it cause Alzheimer's disease?

Chapter 14: Conclusion

This Master's thesis offers a broader insight into the relationship between periodontal diseases and Alzheimer's through a systematic literature search and a pilot experimental study. Our findings bring us closer to our question mentioned at page 11; Can *P.gingivalis* cross the BBB?

The literature search provided an overview about the current knowledge there is about the relationship between periodontal diseases, and Alzheimer's disease through the pathogen *Porphyromonas gingivalis*. It was an extensive research of several articles over decades of knowledge, and new groundbreaking articles that are exposing the hidden mechanisms in which *P.gingivalis* can cross the BBB. Several pathways have been suggested, and pathogen specific mechanisms in which *P.gingivalis* can weaken the BBB.

The pilot experimental study answered the second question, *Porphyromonas gingivalis* is able to cross the blood–brain barrier. Through meticulous experimentation, I demonstrated variations in BBB permeability among different *P.gingivalis* strains, highlighting the importance of strain-specific factors in mediating bacterial entry into the central nervous system. Furthermore, the observations of temporal fluctuations in BBB permeability suggest dynamic interactions between *P.gingivalis* and the BBB over time, implicating that time could be a factor in the pathophysiology of AD. However, it must be acknowledged the limitations of the study, as we wish to increase sample size and more detailed intervals in future studies.

Overall, this study contributes to our understanding of the intricate interplay between periodontal pathogens such as *P.gingivalis* and neurodegenerative diseases like AD. It provided valuable insight for future research as we broaden our knowledge on Alzheimer's disease.

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