TITLE "Do periodontal pathogens or associated virulence factors have a deleterious effect on the BBB, contributing to Alzheimer's Disease?"

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Running Title: The blood brain barrier and periodontitis.

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ABSTRACT

The central nervous system (CNS) is protected by a highly selective barrier, the blood brain barrier (BBB), that regulates the exchange and homeostasis of bloodborne molecules, excluding xenobiotics. This barrier forms the first line of defence by prohibiting pathogens from crossing to the CNS. Aging and chronic exposure of the BBB to pathogens renders it permeable, and this may give rise to pathology in the CNS such as Alzheimer's disease (AD). Researchers have linked pathogens associated with periodontitis to neuroinflammation and AD-like pathology *in vivo* and *in vitro*. Although the presence of periodontitis-associated bacteria has been linked to Alzheimer's disease in several clinical studies as DNA and virulence factors were confirmed in brain samples of human AD subjects, the mechanism by which the bacteria traverse to the brain and potentially influences neuropathology is unknown. In this review, we present current knowledge about the association between periodontitis and AD, the mechanism whereby periodontal pathogens might provoke neuroinflammation and how periodontal pathogens could affect the BBB. We suggest future studies, with emphasis on the use of human *in vitro* models of cells associated with the BBB to unravel the pathway of entry for these bacteria to the CNS and to reveal the molecular and cellular pathways involved in initiating the AD-like pathology. In conclusion, evidence demonstrate that bacteria associated with periodontitis and their virulence factors are capable of inflecting damage to the blood brain barrier and have a role in giving rise to pathology similar to that found in AD.

Keywords: Blood-Brain Barrier, Alzheimer's Disease, Periodontitis, Bacteria, Virulence Factors.

INTRODUCTION

Alzheimer's disease

Alzheimer's disease (AD) is a neurodegenerative disorder that affects the elderly population resulting in reduced function of the brain and cognitive impairment. Histopathologically, AD is characterised by the presence of insoluble extracellular amyloid-beta (Aβ) plaques and intracellular aggregates of hyperphosphorylated tau proteins (neurofibrillary tangles) [1]. For a long time, researchers have dwelled on the amyloid cascade hypothesis, which claimed that the accumulation of $A\beta_{42}$, a toxic polypeptide that forms aggregated amyloid plaques, was the cause of synaptic death, neuronal loss and cognitive impairment observed in AD [2]. Aβ plaques however, were also found in brain tissue of healthy subjects with no dementia, additionally there was no correlation observed between the load of Aβ and the severity of AD [3]. Subsequently, alternative hypotheses have arisen, including the inflammatory and the pathogen hypothesis.

The inflammatory AD hypothesis suggested that an inflammatory response in the brain parenchyma could perpetuate and provoke synaptic dysfunction and neurodegeneration [4, 5]. Inflammation has also been proposed to arise as a result of Aβ plaques, neurofibrillary tangles (NFTs) and a number of other related constituents [6]. Conversely, Aβ plaques and NFTs have also been considered as by-products of inflammation. It has been shown that an inflammatory response induces both astrocytes and microglial to produce pro-inflammatory cytokines; tumour necrosis factor-α (TNF- α), interleukin-1β (IL-1β), interleukin-6 (IL-6), as well as C-reactive protein (CRP) [7], both *in-vitro* and in AD tissues [8]. Additionally, *in-vitro* studies reported that pro-inflammatory cytokines, TNF- α, IL-1β and IL-6, can induce the production of $A\beta_{42}$ and tau protein phosphorylation [9, 10], which in turn stimulate microglial cells to produce a range of pro-inflammatory mediators [10]. Of relevance to the theme of this review, it has also been reported that systemic events created by injecting mice with lipopolysaccharide (LPS), results in an indirect increase in brain influx of Aβ and decrease in brain efflux of Aβ concomitant in the presence of elevated serum levels of IL-6, IL-10 and IL-13 [11].

The central tenet of the pathogen AD hypothesis is that viral, bacterial, or fungal pathogens could play a role in triggering AD pathology [12]. This hypothesis focuses on the fact that pathogens such as herpes simplex viruses (HSV) have been found in the brains of AD patients [13], and that Aβ accumulation may represent an anti-viral response, given some support by a recent study which reported that $A\beta_{42}$ shares a similar structure to that of antimicrobial peptides [14-16]. Whilst Aβ could be a defence mechanism that counters infectious agents, its long-term impact on brain tissue integrity could be harmful [17].

AD, the main cause of dementia, is projected to influence 115 million individuals all over the globe [1]. The estimated number of individuals with dementia in the UK in 2019 was 885,000 [18], and this figure is projected to rise to roughly 1.14 million in 2025 and about 2.1 million in 2051 [19]. During the next few decades, low- and middle-income countries are expected to witness the greatest increase of dementia cases compared to high-income countries due to the increasing number of older people [20, 21]. After taking into consideration the increasing number of older people in low- to middle-income countries driving the increased prevalence of world-wide dementia cases, the WHO found that there is an increased prevalence of dementia in these countries compared to age-standardised high income countries [22].

Periodontal disease

One of the most common chronic infectious states in the human body is periodontal disease (POD), prevalent in 20-50 % of the adult population [23]. Individuals from low-income countries face 1.8-fold increased risk of developing severe periodontitis compared to that of high-income individuals [24]. It is an inflammatory condition that affects the supporting tissues of teeth, namely the periodontium including the gingiva, surrounding bone and the collagen fibres of the suspensory periodontal ligament (PDL), that penetrate the cementum to anchor the tooth to the bone [25] Figure (1). The hallmark of POD is microbial dysbiosis leading to tissue loss that encompasses both soft and hard tissues of the periodontium [26], resulting in the formation of periodontal pockets and loss of attachment of the

junctional epithelium [27], and constitutes one of the main culprits of tooth loss in adults [28]. In the periodontal health, anaerobic gram-negative bacteria associated with periodontitis, make up a small proportion of the subgingival commensal bacteria. However, with the progression of the disease and the initial formation of pockets due to swelling of the gingiva, an increased number of anaerobic gram-negative bacteria starts to establish colonies in the pockets [27]. The headline microorganisms associated with POD are the anaerobic Gram-negative bacteria: *Porphyromonas Gingivalis (Pg), Tannerella Forsythia (Tf), Treponema denticola (Td)* and *Aggregatibacter actinomycetemcomitans (Aa),* and *Pg* is considered pivotal for disease development [29, 30]. *Pg* possesses numerous virulence factors capable of inducing damage to host cells and averting and subverting the host immune response. These include LPS, gingipains, fimbriae, lectins, proteases and capsule, the first three being the most virulent [31]. *Pg* releases LPS either after the bacterium is lysed or in the form of microvesicles produced from the membrane of the vital bacterium [32]. Gingipains are proteases secreted by *Pg* found in outer membrane vesicles (OMVs), and whilst they play a role in the interactivity between the host and *Pg* [33], their ability to subvert the host's immune system stems from their capability of degrading proteins of the complement system, such as C5 [34]. Fimbriae are prominent filamentous organelles that exist on the surface of a bacterium [35], that mediate the attachment of *Pg* to the host cells [36], and provoke the production of pro-inflammatory cytokines, IL-1β and IL-6, from macrophages [37]. The key issue in respect of the pathogen or inflammatory AD hypotheses, is that pro-inflammatory cytokines produced in the periodontal tissues, IL-1β, IL-6, TNF-a, chemokines IL-8, and the bacteria initiating and maintaining the inflammatory state, can access the systemic circulation through the ulcerated periodontal tissues and eventually interact with or even colonise distant organs [38]. This systemic invasion of bacteria and pro-inflammatory mediators constitute risk factors for various diseases such as cardiovascular disease, diabetes, atherosclerosis but of interest for this review AD [7, 38, 39].

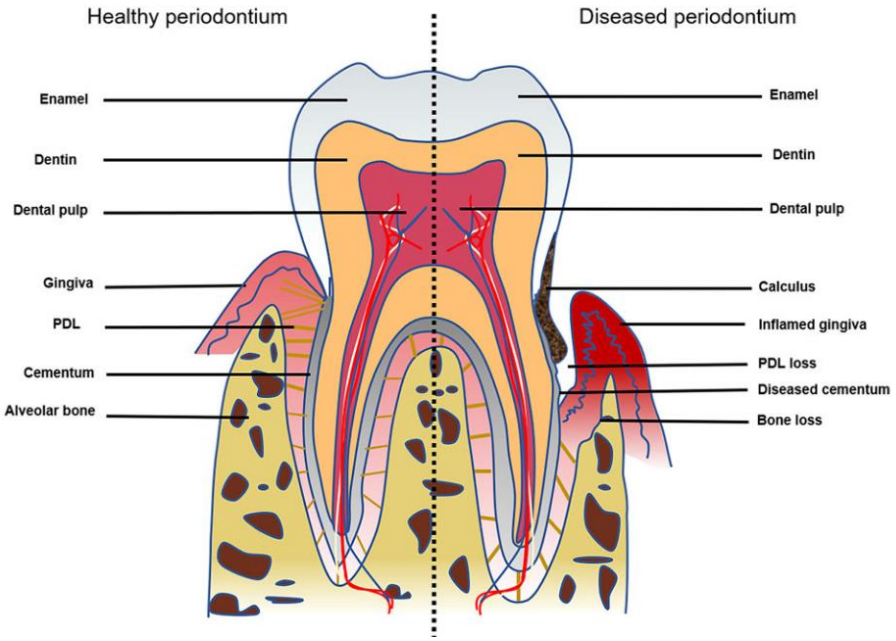

Figure 1. A diagram that compares the healthy periodontium with the diseased one. The accumulation of calculus, which is mineralized plaque renders the gingival tissue inflamed and could lead to loss of PDL and bone [40].

Alzheimer's disease and periodontal disease

When exploring the link between AD and POD, a number of human clinical studies have suggested that patients with periodontitis were more prone to developing dementia [41-47]. Poor cognitive performance, impaired memory and development of AD, has been associated with serum antibodies (IgG) to *Pg* [43, 48]. Stein *et al*., (2012) found that antibodies to *Pg* and *Td*, were elevated at baseline prior to the development of AD compared to participants who remained cognitively healthy [49]. Another study found patients receiving no periodontal treatment had a higher tendency to develop dementia compared to those who received treatment when comparing patients matched for gender, age, income, educational level and smoking [44].

The data from such studies should be interpreted with caution as the association between the two diseases, could be bi-directional [50]. AD might predispose to POD, as patients who suffer from cognitive decline could lack the facility to practice oral hygiene instructions, which may result in development of POD [49]. But the author's group support the concept that since periodontitis is a low-grade chronic source of systemic inflammation [51], it could promote neurodegenerative diseases like

AD, by a persistent release of systemic circulatory pro-inflammatory molecules or associated bacteria, with potential access to brain tissues.

A third factor to consider, that provides possible links between AD and POD is genetic susceptibility. A comparison of genome-wide association study (GWAS) datasets in human populations following pathogen exposure in early-life and from AD patients found that upregulated genes in the AD hippocampal transcriptome were related to immune and inflammatory processes upregulated during infection. Resistance to previous infections in early-life led to an immune mediated gain of function in APOE4, CR1, TREM2, which then links to deposition of Aβ and AD in later life [52], indicating chronic low grade infection and an antimicrobial immune response in the brain may result in neurodegeneration. Another mechanism by which POD could be linked to AD is by inducing dysbiosis in the gut microbiota. The alteration of gut microbiota has been found to be related to elevated Aβ in the CNS, impaired memory and spatial learning in APP/PS1 mouse model [53]. Similarly, the prolonged use of wide-spectrum antibiotics in an AD mouse model to alter the composition and diversity of the gut microbiota has led to an increase of the levels of peripheral cytokines, CCL11 and CXCL16, and of the levels of soluble Aβ, whilst Aβ plaques have decreased [54]. In addition, the use of clarithromycin and amoxicillin or metronidazole to treat Heliobacter pylori infection in humans could lead to neuropsychiatric disorders such as anxiety and delirium [55]. As for the role of POD in this regard, oral administration of *Pg* to C57BL/6 mice has led to a change in the composition of the gut microbiota and upregulation of IL-6 in the small intestine as well as compromising the gut barrier function [56]. In another study employing C57BL/6N mouse model, it has been reported that administrating *Pg* orally resulted in a significant increase of bacteroidales [57]. Furthermore, ligature-induced periodontitis in C57BL/6 J mice has led to developing periodontitis, glial activation and cognitive dysfunction as well as oral and gut microbiota dysbiosis. The gut microbiota dysbiosis that arose following POD was thought to lead to reduced expression of tight junction proteins, claudin-1, occludin and ZO-1, in the intestinal barrier in addition to a decrease in claudin-5, occludin, and ZO-1 in the BBB [58].

Numerous post-mortem studies have also explored the relationship between AD and POD. *Pg* LPS was detected in the brain tissues of AD patients whilst they were absent in post-mortem samples from aged-

matched non-AD subjects [59]. Dominy et al., (2019) reported the existence of gingipains antigens, proteases secreted by *Pg*, in higher loads in the brains of patients with AD cases, in comparison to brain tissues from non-AD patients. They also reported the presence of *Pg* DNA in the brain and CSF of AD patients [60]. Another study investigating whether *Treponema* invades the brain has shown that the majority of specimens (14 out of 16) from subjects with AD had *Treponema*, compared to a minority in controls, (4 out of 18) [61].

On a similar note, the DNA of *Td* and *Pg* was detected in the hippocampus of C57BL/6 mice 24 weeks post oral injection of either of these pathogens, and this provoked the production of $A\beta_{40}$ and $A\beta_{42}$ in the hippocampus with no difference between the pathogens [62]. Since *TD* belongs to the same genre taxonomy as *Treponema pallidum*, which is the pathogen involved in syphilis, it is no wonder that it is capable of translocating to the brain [63].

Before accessing brain parenchyma, pro-inflammatory mediators, virulence factors or microorganisms would have to cross or interact with the BBB, as a prelude to provoking inflammation-based changes.

Anatomical components of the neurovascular unit and blood brain barrier function

The BBB serves as a protector for the brain parenchyma, by being a highly selective interface that facilitates transport of nutrients into the brain whilst preventing access to pathogenic blood-borne agents [64]. The structural and functional relationship between the brain and blood vessels, known as the blood-brain barrier does not include just the brain endothelium barrier, it also incorporates components such as the neurons, perivascular astrocytes, pericytes, microglia and the extracellular matrix, collectively known as the neurovascular unit (NVU) [65] (Figure 2A). Pericytes are embedded in the basement membrane of the microvessels, where they envelop the outer surface of the vessels by establishing gap junctions with endothelial cells [66]. Pericytes play a key role by contributing to the stability of microvascular permeability and angiogenesis by producing growth factors and extracellular matrix [67]. In addition, they also possess phagocytotic features that help in the clearance of soluble small molecules using pinocytosis, such as dextran [68]. The end-feet of astrocytes, which enclose the capillary basement

membrane, facilitate an intimate connection between CNS neurons and the blood vessels of the NVU, in which astrocytes control blood flow after receiving signals from adjacent neurons [69]. The signalling mechanism involves neurons producing and releasing glutamate, inducing astrocytes to the release of either K^+ that serves as a vasodilator agent or ATP that results in vasoconstriction [70]. In addition, astrocytic end-feet establish an additional barrier known as the glia limitans by sheathing the blood vessels and the CNS surface [71]. Alongside the barrier function, astrocytes play a vital role in maintaining the integrity of the BBB in addition to regulating water homeostasis to and from the brain tissues [72, 73]. Microglia have also been demonstrated to play an important role in maintaining vascular integrity and modulating tight junction protein expression during inflammation [74]. Interestingly when in close proximity to the microvasculature, microglia were found to interact interdependently with NVU endothelial cells [75], such that activated endothelial cells have been shown to play a role in the activation of microglia [76], via release of chemokines [77]. Further, pro-inflammatory cytokines, IL-1β and TNF-α, produced by microglia were found to downregulate tight junction protein expression (ZO-1 and occludin), resulting in an increase of BBB permeability to sodium fluorescein [78, 79]. The interactions between the NVU and surrounding cells is further exemplified by work which demonstrates that microglia activated by LPS to the M1 phenotype, produce cytokines capable of inducing reactive astrocytosis to the A1 phenotype [80]. Microglia have also been shown to be highly responsive to pathogens [81], in contrast to astrocytes, which were not found to be activated following LPS treatment [82]. Similarly, microglia activated to the M2 phenotype, promoted A2 astrocytes reactivation through the production of anti-inflammatory cytokine IL-10 [83].

Further barrier properties are provided by the basement membrane, which consists of extracellular matrix proteins, laminin, nidogen, type IV collagen and heparan sulphate proteoglycans, produced by the endothelial cells, pericytes and astrocytes [84-86].

A key element in creating the barrier function is to maintain tight endothelial cell adherence. This is achieved by i) Tight junctions, formed by primary proteins (claudin, occludin, junctional adhesion molecules (JAMs) and accessory proteins of the zona occludens (ZO-1, ZO-2, ZO-3). They form intramembranous strands which compose a net-like meshwork surrounding the cells [87]. They adhere

the endothelial cells tightly together thus modulating the paracellular permeability, maintaining the integrity of the BBB and conferring high electrical resistance [88]; ii) Adherens junctions (AJs), connect adjacent endothelial cells via proteins of the cadherin superfamily, which are linked to the endothelial cytoskeleton by proteins of the cytoplasmic plaque, catenins [89] (Figure 2B).

The BBB is not an impassable barrier though. Small lipid soluble molecules travel across by passive diffusion, whilst other substances, such as IGF, glucose and vitamins, have to traverse the BBB via specific systems namely receptor-mediated transport, carrier mediated transport, efflux transport system and adsorptive endocytosis [90]. The carrier-mediated transporters could be active or passive [91] and can be located on the luminal and / or abluminal surfaces [92], designed to carry molecules from the circulation to the brain (uptake transporters) or in the opposite direction (efflux transporters) [93] (Figure 2C).

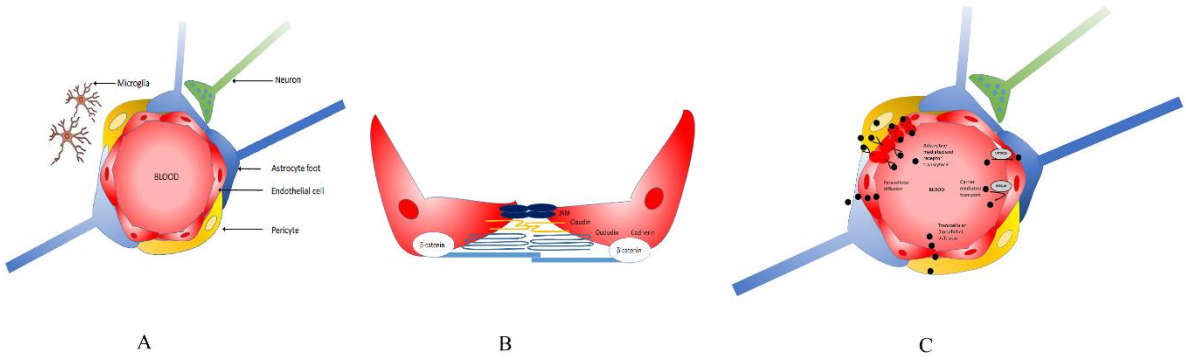

Figure 2. The blood-brain barrier is formed by (A) a network of cells (B) tight junction and adherens junction proteins form a physical barrier and (C) major transport pathways constitute the metabolic barrier.

What happens to the blood brain barrier function in Alzheimer's disease?

There is evidence that changes in the BBB integrity occur in Alzheimer's disease [94, 95]. From a therapeutic perspective, it would be helpful to fully understand the mechanism by which the disruption arises, how BBB integrity is repaired and how this integrity can be enhanced to prevent or reverse

resultant neuropathology at the early stages of disease. The question whether the dysfunction of the barrier happens because of the neuropathological changes or the latter emerges because of the former remains an important but difficult question to answer definitively [96].BBB disruption has been observed in early-stage AD patients compared to age-matched controls, where dynamic contrast enhanced MRI has shown leakage of contrast agents such as gadobutrol, that has been injected into the circulation, and clearly identified BBB located cerebral microbleeds [97]. In the healthy brain parenchyma there is an almost complete absence of neutrophils and lymphocytes as the endothelial tight junctions prohibit these cells from accessing the brain tissue [84]. The breakdown of the BBB has been reported using advanced dynamic contrast-enhanced MRI to commence in the hippocampus, which has a key role in learning and memory, and it becomes atrophic in AD despite possessing the highest barrier properties, during normal aging. This dysfunction of the BBB was greater in subjects with mild cognitive impairment (MCI) in comparison with age matched controls, and this breakdown of the BBB could contribute to cognitive impairment as the authors suggested [98]. Activated microglia and astrocytes have been shown to release pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α, and elevated levels of these molecules have been found to accompany AD lesions in brain tissues of AD [8], and these inflammatory molecules have the potential to increase the BBB permeability [64, 99-101]. Neuroinflammation has also been shown to upregulate endothelial leukocyte adhesion molecule expression, facilitating leukocyte transmigration into the CNS [102, 103]. The passage of such immune cells into the brain can result in the intracerebral production of pro-inflammatory cytokines, IL-17, IL-22, TNF-α and IFN-ϒ, which further compromises the integrity of the BBB by altering the expression of junctional proteins [104]. This can result in oedema, immune infiltration, ionic homeostasis disturbance and modified signalling and ultimately degradation of neurons [84]. A summary of other changes observed at the BBB in AD patients in shown in Table 1

Table 1. Changes at the blood brain barrier in AD.

Author (year)	Effect on BBB tissues	Model	Measure	AD stage
Yamada [105] Zlokovic (2005) [106] and Thomsen et al., (2017) [107]	BBB Basement membrane and Could be toxic to BBB Endothelial cells	Human	Aβ accumulation-cerebral amyloid angiopathy developed in more than 80 % AD patients	Post-mortem biopsies
Lepelletier et al., (2017) [108]	BBB Basement membrane	Human	Increased thickness and altered composition	Subclinical AD and AD patients
Yates et al., (2014) [109]	BBB Endothelium/ brain parenchyma	Human	Cerebral microbleeds and cortical siderosis	AD patients
Vogelsang et al (2018) [110]	BBB Transporters	Human brain derived circulating endothelial cells	Downregulation of GLUT-1	Blood collected from mild AD patients

Proposed mechanism linking periodontal disease, peripheral inflammation, and BBB disruption

The entry of pathogens and or associated virulence factors into brain parenchyma may be facilitated by disruption of the BBB in AD, possibly an early feature of the disease. Exploration of the possible mechanisms by which POD may induce changes at the BBB have been conducted by both *in vivo* and *in vitro* studies (Table 2). This evidence is supported by the knowledge that POD-associated bacteria, such as *Pg*, contribute to significant bacteraemia following dental procedures, such as root surface debridement [111], tooth brushing [112] and even chewing [111]. Could POD provide a chronic source of pathogens and thus a chronic assault on the integrity of the BBB?

Systemic cytokines, initiated by the chronic inflammatory changes associated with *Pg* infected periodontal pockets, could translocate to the brain either as a result of increased permeability of the BBB [113], or by using existing saturable transporters [114]. Peripheral TNF-α and IL-β have been shown to increase the permeability of the BBB by decreasing expression of occludin thus disrupting the integrity of tight junctions [113, 115], Figure 3A. After crossing the BBB, cytokines then have the potential to activate both endothelial and glial cells to produce other pro-inflammatory cytokines [116].

Support for this proposal can be found in studies such as Furutama et al., (2020) whereby POD was induced by placing ligatures on the second upper molars bilaterally of C57BL/6J WT mice. This led to an increase in serum IL-6, which in turn resulted in an increase of IL-1β in the hippocampus. This study also demonstrated that the mRNA expression and protein levels of claudin 5 were decreased. Moreover, the gap area between endothelial cells in the hippocampus was also increased, enhancing the permeability of the BBB but only for small molecules such as 3-KDa dextran [117]. A source of weakness in this study was that the authors did not confirm the development of periodontitis after placing the ligature.

Oxidative stress was reported to be the earliest event during AD as shown in a study that examined post-mortem AD brain tissues [118], and it has been shown to even occur before and lead to AD lesions [119]. In addition, throughout the inflammatory process, microglia and astrocytes not only produce cytokines, but also they produce NO^-, $O2^-$, H2O2 [120]. In a study looking at the oxidative stress arising following a *Pg* infection, four groups of mice were studied; i) transgenic mice that overexpress *TNF-α*, ii) wild-type mice, iii) *ApoE* knockout sham infected mice and iv) *ApoE* knockout *Pg* infected mice [121]. The *Pg* infected model showed the highest protein carbonyl content, a biomarker for oxidative stress, in the microvasculature of the hippocampus compared to the other groups, including the transgenic mice group. These findings suggested that oxidative protein damage caused by infection with *Pg* led to a compromise in BBB integrity in this model [121]. Moreover, *Pg* or endothelial microvesicles EMVs shed from human umbilical vein endothelial cells (HUVECs) following Pg infection were found to induce endothelial dysfunction, and this dysfunction was accompanied by oxidative stress [122]. Activating microglia by *Pg*LPS led to an increase in the production of reactive oxygen species, $O2^-$, and $O2^-$ was reported to compromise the BBB and increase its permeability [123], Figure 3C.

Table 2. In vitro and In vivo models investigating the effects of peripheral inflammation and/ or periodontal pathogen exposure on molecular and cellular components of the BBB

Author (year)	Model	Species	Mechanism	Findings
Basuroy et al., (2006) [113]	*In vitro* cerebral vascular endothelial cells	Porcine Murine	TNFα induced peripheral Inflammation	Decrease in occludin, increase BBB permeability
McCollet al., (2008) [115]	*In vivo* ischemic brain injury	Murine	IL-1β induced peripheral inflammation	Disruption to claudin-5 and collagen IV by MMP9
Furutama et al., (2020) [117]	*In vivo* ligature around molars to induce POD	Murine	POD induced peripheral and CNS inflammation IL-1β	Decrease in claudin-5, increase in endothelial permeability with POD
Rokad et al., (2017) [121]	*In vivo:* 1) TNF-α transgenic mice; 2) wild-type mice; 3) *ApoE* knockout sham infected mice and 4) *ApoE* knockout *Pg* infected	Murine	POD induced cerebral oxidative stress in hippocampal microvasculature	Tight junction proteins degradation, leading to a loss of functional BBB integrity with POD

Reviewing the effect of *Pg* reveals that it has also been shown to affect endothelial cells of the BBB directly, through the release of the proteolytic enzymes gingipains, which reduces the adhesion of human microvasculature endothelial cells (HMVEC), as well as inducing apoptosis [124], Figure 3B. Furthermore, gingipains have been shown capable of activating the blood clotting system producing thrombin, which has been shown to increase the permeability of the BBB *in vitro* [125, 126]. Thrombin has also been demonstrated to decrease the expression of ZO-1 and occludin in cultures of rat brain microvascular endothelial cells [127]. Additionally, *Pg* was found to adhere to HUVECs using E-selectin [128], a membrane protein used in the initial capture and recruitment of leukocytes in inflammatory responses [60, 129]. Khlgatian et al., demonstrated that *Pg* invaded (HUVECs) using

fimbriae and provoked the expression of cell adhesion molecules, capable of facilitating leukocyte transendothelial migration [130]. In human coronary artery endothelial (HCAE) cell cultures, it was observed that *Pg* invades the cells to inhibit phagosome formation and function and thus evade the attention of lysosomes [131]. Furthermore, gingipains may play a role in evading the immune response by breaking down receptors responsible for the detection of LPS by host cells [132], which could increase the level of *Pg* circulating the blood. *Pg* was also found to move from one vascular cell to another thus maintaining the inflammatory state [133]. *Pg* LPS was also shown to stimulate the expression of vascular cell adhesion molecule-1 (VCAM-1), in human aortic endothelial cells (HAECs) [134], giving further support to the possibility of leucocyte capture prior to transmigration. One interesting mechanism proposed is the "Trojan horse", Figure 3B. This is described as the paracellular pathway, whereby pathogens infect leukocytes and transmigrate the BBB by using infected leukocytes e.g. macrophages as a "Trojan horse" [135]. Here, bacteria either induce mutations in leukocytes so they possess the ability of translocating through the BBB endothelium , or they infect leukocytes that already possess this characteristic [136]. This might also be utilized by *Pg* to exploit macrophages to get further access to different systems [137].The potential for *Pg* to target endothelial cells of the NVU via tight junctions and leucocyte adhesion molecules raises the possibility of increasing the inflammatory traffic across the BBB.

Another mechanism by which POD could affect the BBB is by increasing the expression of receptors on endothelial cells, which in turn boost the influx of Aβ from the periphery into the CNS. Zeng et al., (2020) treated cultures of human temporal lobe microvessels (hCMEC/D3) with *Pg* and reported a Cat B dependent increase of the expression of the Receptor for Advanced Glycation End products (RAGE). RAGE was demonstrated to mediate the transportation of $A\beta_{42}$ by seeding hCMEC/D3 on transwell inserts in 24-well plate and measuring the fluorescent signal of $A\beta_{42}$ appearance in the basolateral side of the well [138]. The peripheral Aβ induced by intraperitoneal injections of *Pg* in mice was the central focus of a study done by Nie, Wu et al., (2019) whereby the expression of IL-1β, Cat B, $A\beta_{1\text{-}42}$, $A\beta_{3\text{-}42}$ and $A\beta PP_{770}$, the precursor of Aβ in the periphery, were found to be increased in the liver-resident macrophages following *Pg* infection [139]. $A\beta_{3\text{-}42}$ has been found to accumulate in greater quantities in the brain of AD patients [140], whilst remaining absent in healthy specimens [141]. An increase in the

expression of Cat B, $A\beta_{1\text{-}42,}$ $A\beta_{3\text{-}42}$ and $A\beta PP_{770}$ in the gingiva-resident macrophages was detected in samples of patients with POD [141]. The Zeng group also investigated the role of *Pg* in increasing the influx of $A\beta_{42}$ into the brain following *Pg* infection. They reported an increase of RAGE expression in endothelial cells, immunofluorescent staining, of C57BL/6J WT mice after intraperitoneal injections with Pg, as well as increased signal of $A\beta_{42}$ around the stained endothelial cells [138].

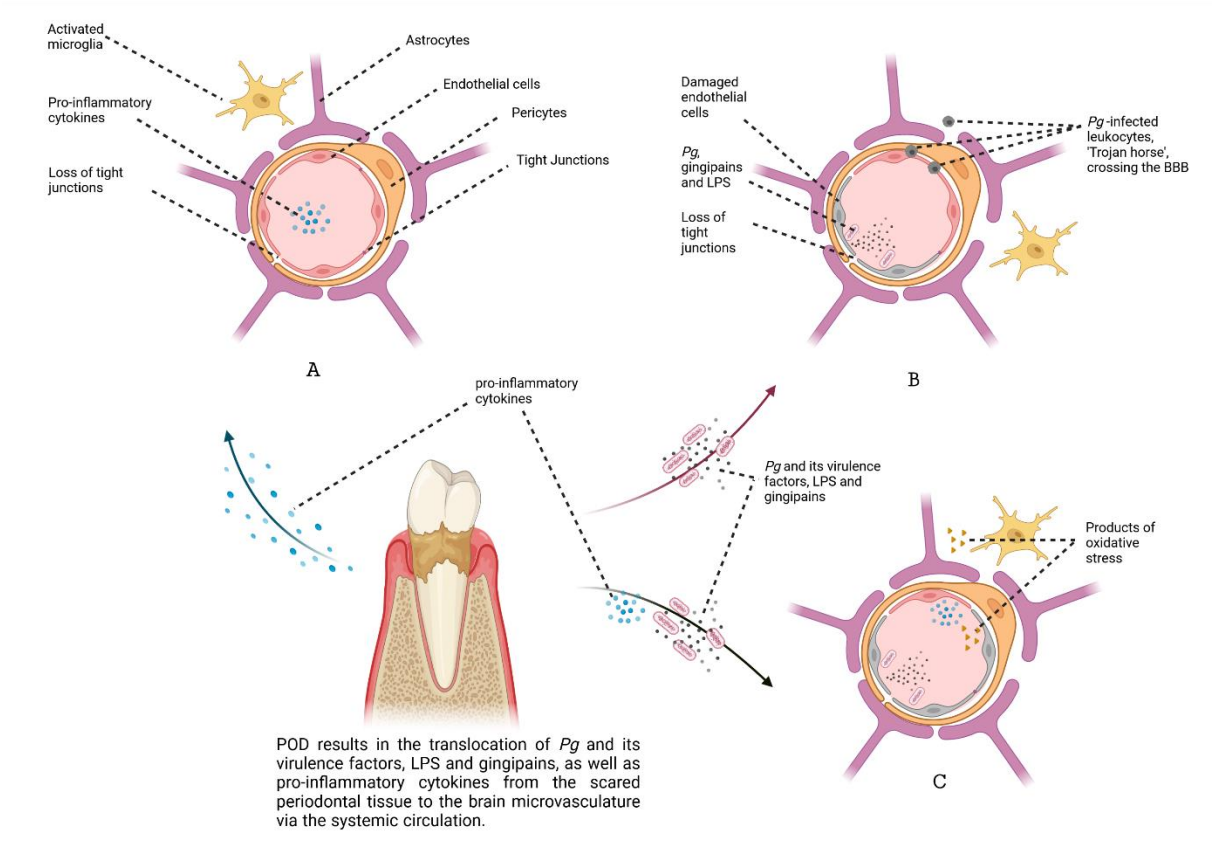

Figure 3. the translocation of (A) pro-inflammatory cytokines from the periodontal tissue to the brain microvasculature might lead to a loss of tight junctions and impair the BBB, which could activate microglia and astrocytes to produce inflammatory molecules. (B) Pg or its virulence factors, gingipains and LPS, can induce direct damage to endothelial cells and could lead to loss of tight junctions as well. Pg might also be able to infect leukocytes and use them as 'Trojan horse' to cross the BBB. (C) Pg, its virulence factors and pro-inflammatory cytokines produced because of POD can provoke the release of oxidative stress products from endothelial cells and microglia, which could compromise the function of the BBB. Created with BioRender.com

DISCUSSION

The BBB is maintained by the NVU with its cellular components, endothelial cells that adhere to each other by tight junction proteins, pericytes, neurons microglia and astrocytes, and extracellular components, basement membrane, to selectively filter the blood-borne substances and prevent

deleterious agents from entering the brain and inflecting any damage. Disruption of any component of the NVU can lead to an impairment of the barrier function and, in turn, harmful agents might cross to the brain. The BBB is disrupted following ischemic stroke, and this impairment takes place immediately after stroke and could last for several weeks [142]. About a third of patients develop dementia 3 years after the incidence of a stroke [143]. It is worth mentioning that TNF, IL-1β as well as IL-6 are increased in the blood and CSF of human subjects following the onset of a stroke [144]. IL-1β mRNA was found to be increased 42 times in the brain of mouse models 24 hours post stroke, and that of TNF reached a peak of 57.7 folds 12 hours following stroke [145]. On the basis that the overexpression of certain protein, heat shock protein 27, in endothelial cells of the BBB results in the protection of the barrier following stroke, which in turn protects the brain parenchyma [146], it could be concluded that the impairment of the BBB might lead to damaging the brain parenchyma not the other way around.
Researchers have been trying to elucidate the adverse effect of POD on components of the NVU that renders the BBB permeable, which can give rise to AD pathology or contribute to an existent one in the brain.

Clinical studies over the last two decades have provided a growing body of evidence associating POD with cognitive impairment and AD, but no causal link has been established yet. Supporting evidence comes from the detection of periodontal pathogen DNA and virulence factors in the post-mortem brain tissue of patients with AD [59-61]. In addition, correlations have emerged with upregulated genes in the AD hippocampal transcriptome overlapping with genes upregulated during infection, related to immune and inflammatory processes [52]. There are several mechanisms proposed for this association between AD and POD that to date have only been studied in *in vitro* or *in vivo* models: 1) the entry of periodontal pathogens across the BBB may be facilitated by disruption of barriers which occurs during aging and early AD; 2) periodontal pathogens such as *Pg* inflict damage to the CNS indirectly via peripheral inflammation, provoking systemic release of pro-inflammatory molecules which may compromise the integrity of the BBB and contribute to neuroinflammation and 3) periodontal pathogens cause direct damage to endothelial cells of the BBB through the release of virulence factors such as gingipains secreted by *Pg* 4) POD and the pathogens associated with it lead to a decrease of tight junction proteins

expression in the intestinal barrier and the BBB 5) periodontal pathogens yield an imbalance of the gut microbiota, which in turn results in increased levels of Aβ in the CNS 6) periodontal pathogens give rise to oxidative stress, the earliest event in AD, which compromises the BBB 7) Pg could infect and use leukocytes as 'Trojan horse' to cross the BBB and infiltrate the CNS.

Once *Pg* invades the endothelial cell, inhibits phagosomes and evades lysosomes, *Pg* can hijack the endosomal recycling pathway, spreading from one cell to another to propagate inflammation and potentially traverse to the CNS. Proinflammatory cytokines produced contribute to the loss of CNS barrier integrity and disruption of tight junctions which results in barriers becoming more permeable. Neuroinflammation causes the upregulation of leukocytes adhesion molecules expression, which allows leukocytes to transmigrate to the CNS, via the endothelial cells to assist the microglia [102]. The activated microglia and astrocytes then contribute to the developing neuropathology or aggravate an existent one.

However, caution in interpreting the evidence is required. All the mechanistic studies investigating the link between AD and POD have been conducted in *in vitro* non-human or *in vivo* models. Species differences exist between immune and inflammatory response to pathogens and neurotoxic Aβ plaques, and two-dimensional mono cell cultures do not reflect the complex signalling that exists between cells of the CNS barriers and basement membrane. For instance, Toll-like receptor 4 TLR 4, which is one of the receptors expressed by microglia and other cells in the body that allows the binding of LPS to the cells, is not expressed as highly in human microglia compared to rodents [147]. The findings of a recent study by Hayashi et al., (2019) did not support the previous research, as no effect on cognitive skills was observed following continuous intracerebroventricular injection of *Pg*LPS 2µg/day in 5xFAD mice [148]. They attributed this discrepancy to the low dose of *Pg*LPS compared to other studies that used 1mg/kg [149]. Results obtained from animal models could not be fully extrapolated to humans because of the various resilience levels exhibited by animals and humans to LPS. For example, the dosage of *Ec*LPS required to promote cytokine synthesis in mice was found to be 1000-10,000 fold of that which led to septic shock in human subjects, and this was attributed to cellular variations between human and mice [150].

Another limitation of current studies is the focus on one pathogen or virulence factor, rather than considering the dynamic and polymicrobial oral microbiome and how it reacts with the host microenvironment [151]. Using *Pg* LPS only and disregarding other virulence factors such as gingipains, (except for the study by Nonaka and Nakanishi 2020) might have yielded inaccurate results. To elaborate, as shown above, *Pg* LPS resulted in microglia secreting different cytokines such as TNF-α [152], but gingipains were found to cleave the soluble and membrane forms of TNF-α [153, 154]. IL-6 was shown to be produced by microglia after treating with PgLPS [152], but gingipains were reported to inactivate IL-6 as well [155]. Evidently, it is vital to test the effects of all the virulence factors produced by *Pg*.

It has been reported that immunostaining of cyclic adenoses monophosphate (cAMP), the second messenger involved in signal transduction pathways, was increased in the cortical microvasculature, especially the hippocampus, of tissues from AD patients compared to controls, and this upregulation was concomitant with increased staining of Aβ [156]. These findings indicate that cAMP might play a role in the accumulation of amyloid precursor protein (APP) intracellularly [156]. Moreover, higher concentrations of cAMP were found in the CSF of AD patients compared to controls [157].

However, it has been established that cAMP has numerous effects on the BBB based on its production. cAMP synthesis is mediated by the Adenylyl Cyclases (AC), which is a family of enzymes that have 10 isoforms, one of which is located in the cytoplasm and the rest are located in the membrane [158]. If the production of cAMP is mediated by transmembrane AC, then the resulted cAMP enhances the endothelial barrier functions. Conversely, the production of cAMP mediated by the soluble AC located in the cytoplasm leads to disruption of the barrier [159]. cAMP was found to increase the expression of tight junction protein claudin-5 in porcine endothelial cells [160]. Additionally, the fall of cAMP production in neurons due to chronic stress leads to compromised function of the BBB [161]. On the other hand, some bacteria can produce cytoplasmic AC, which in turn mediates the generation of cAMP that renders the endothelial cells permeable [162].

With regards to the mechanism of this messenger, high intracellular levels of cAMP result in the activation of protein kinase A (PKA) which in turn lead to the phosphorylation of cAMP-responsive element binding protein (CREB), and the latter provokes the synthesis of anti-inflammatory cytokines,

mediators that promote resolution of inflammation, and activation of macrophages into the M2 phenotype. Additionally, the activity of nuclear factor κB (NF- κB) is decreased, which decreases the production of pro-inflammatory cytokines. Activation of exchange protein directly activated by cAMP (Epac) also inhibits the release of pro-inflammatory cytokines [163]. Also, cAMP contributes to regulating the pro- and anti- inflammatory responses in immune cells [164]. The intracellular increase of cAMP in immune cells play a role in dampening the anti-inflammatory response by inhibition the production of several pro-inflammatory cytokines such as TNF-α and IL-12 [165, 166]. In addition, the elevation of intracellular cAMP in vascular endothelial cells curbs IL-6 receptor signalling [167]. Roflumilast, a cAMP elevator used for the treatment of peripheral inflammatory diseases, has led to a shift in the activation state of microglia towards the neuroprotective phenotype, M2 [168], and cAMP has inhibited the production of TNF-α from LPS-treated microglia [169].

Recognising that no *in vitro* model would ever be able to replicate the complexities of the *in vivo* brain microenvironment in humans; cell culture systems have advanced considerably and now some limitations can be addressed and *in vivo* extrapolations improved. For example, the use of primary derived human cells is often superior to immortalised cell-lines where phenotypic expression of key proteins is often diminished when compared to primary derived cell cultures. Also, three-dimensional, perfused multi-cellular cell cultures should be considered for future research when cell-cell signalling is such a pivotal part of the propagation of neuropathology. The presence of three-dimensional architecture, sheer stress and co-culture with multiple cell types improves the phenotypic expression of tight-junction proteins and transporters at the blood brain barrier. Three-dimensional cultures systems have been shown to have the potential to reproduce different characteristics of the brain physiology in AD disease [170].

It should be noted that there is evidence suggesting a link between POD and other neurodegenerative diseases such as Parkinson's disease (PD), and this evidence stems from several population-based studies. In a retrospective cohort study in Taiwan, POD was found to be accompanied with higher propensity to developing PD when compared with matched controls [171]. Similarly, Jeong et al., reported a weak correlation between POD and PD in a cohort study after adjusting for age, sex, smoking,

drinking, exercising, stroke and depression [172]. This association could be attributed to neuroinflammation, which is a prominent feature of PD [173], and as described in this review, POD has the potential to give rise to it. Moreover, peripheral inflammation is a contributor to PD pathology since the levels of IL-1β, IL-6, TNF-α are elevated in the periphery during the disease progression [174]. Furthermore, it has been suggested that dysbiosis of the gut microbiota plays a role in PD [175], and as aforementioned here, *Pg* can induce gut microbiota dysbiosis.

CONCLUSION

In summary, the pathogens and virulence factors associated with POD and/ or the pro-inflammatory cytokines produced following POD have been shown to potentially affect the BBB and contribute to loss of BBB integrity and disruption of tight junctions, which could be the key to pathogens gaining entry to the CNS. The pathogens possibly target the endothelial cells to propagate inflammation and recruit immune cells, developing neuropathology or aggravating an existent pathology. Research should ideally be conducted in a clinically relevant model, representative of the disease state to assess potential therapeutics for antimicrobial treatment of chronic periodontal disease, dampening of the chronic peripheral and neuroinflammatory state, promotion of clearance of neurotoxins. Ideally this would be conducted in a fully characterised human, multicellular model, representative of the CNS barriers and the associated macrophages.

CONFLICT OF INTEREST

The authors have no conflict of interest to report.

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