

Central Lancashire Online Knowledge (CLoK)

Title	An Investigation into the Effects of Outer Membrane Vesicles and Lipopolysaccharide of Porphyromonas gingivalis on Blood-Brain Barrier Integrity, Permeability, and Disruption of Scaffolding Proteins in a Human in vitro Model
Туре	Article
URL	https://clok.uclan.ac.uk/id/eprint/40482/
DOI	https://doi.org/10.3233/jad-215054
Date	2022
Citation	Pritchard, Anna, Fabian, Zsolt, Lawrence, Clare Louise, Morton, Glyn, Crean, Stjohn and Alder, Jane Elizabeth (2022) An Investigation into the Effects of Outer Membrane Vesicles and Lipopolysaccharide of Porphyromonas gingivalis on Blood-Brain Barrier Integrity, Permeability, and Disruption of Scaffolding Proteins in a Human in vitro Model. Journal of Alzheimer's Disease. pp. 1-22. ISSN 1387-2877
Creators	Pritchard, Anna, Fabian, Zsolt, Lawrence, Clare Louise, Morton, Glyn, Crean, Stjohn and Alder, Jane Elizabeth

It is advisable to refer to the publisher's version if you intend to cite from the work. https://doi.org/10.3233/jad-215054

For information about Research at UCLan please go to http://www.uclan.ac.uk/research/

All outputs in CLoK are protected by Intellectual Property Rights law, including Copyright law. Copyright, IPR and Moral Rights for the works on this site are retained by the individual authors and/or other copyright owners. Terms and conditions for use of this material are defined in the <u>http://clok.uclan.ac.uk/policies/</u>

1	An investigation into the effects of outer membrane vesicles and
2	Lipopolysaccharide (LPS) of <i>Porphyromonas gingivalis</i> on blood
3	brain barrier integrity, permeability and disruption of scaffolding
4	proteins in a human in vitro model.
5	
6	Anna Barlach Pritchard ^a , Zsolt Fabian ^b , Clare L Lawrence ^c , Glyn Morton ^d ,
7	StJohn Crean ^a and Jane E Alder ^c
8	^a Brain and Behaviour Centre, Faculty of Clinical and Biomedical Sciences,
9	School of Dentistry, University of Central Lancashire, Preston, UK.
10	^b School of Medicine, University of Central Lancashire, Preston, UK
11	^c Brain and Behaviour Centre, Faculty of Clinical and Biomedical Sciences,
12	School of Pharmacy and Biomedical Sciences, University of Central
13	Lancashire, Preston, UK.
14	^d School of Forensic and Investigative Science, University of Central
15	Lancashire, Preston, UK
16	*Correspondence to Anna Barlach Pritchard, Brain and Behaviour Centre,
17	Faculty of Clinical and Biomedical Sciences, School of Dentistry, University of
18	Central Lancashire, Preston, UK. Tel.: +44 0 1772 895906; E-mail:
19	abpritchard@uclan.ac.uk

22 <u>ABSTRACT</u>

23	The journey and effects of gum disease key pathogens such as Porphyromonas
24	gingivalis (P.gingivalis) and its virulence factors to and on the central nervous system
25	is of great interest with respect to development of therapeutics and preventative
26	strategies. The role of chronic infections and associated inflammation is important as
27	both are known to weaken the first line of defence for the brain; the blood brain
28	barrier (BBB). The focus of this study is to utilise an established human in vitro BBB
29	model to evaluate the effects of <i>P.gingivalis</i> virulence factors Lipopolysaccharide
30	(LPS) and outer membrane vesicles (OMVs) on a primary-derived human model
31	representing the neuro vascular unit of the BBB. Changes to the integrity of the BBB
32	after application of <i>P.gingivalis</i> LPS and OMVs were investigated and correlated with
33	transport of LPS . Additionally, the effect of <i>P.gingivalis</i> LPS and OMVs on human
34	brain microvascular endothelial cells in monolayer was evaluated using
35	immunofluorescence microscopy. The integrity of the BBB model was weakened by
36	application of <i>P.gingivalis</i> LPS and OMVs, as measured by a decrease in electrical
37	resistance (TEER) and a recovery deficit was seen in comparison to the controls.
38	Application of <i>P.gingivalis</i> OMVs to a monoculture of human brain microvascular
39	endothelial cells (HBMEC) showed disruption of the tight junction zona occludens
40	protein (ZO-1) compared to controls. These findings show that the integrity of
41	healthy cells and potentially their tight junctions of the human BBB could be
42	weakened by association with <i>P.gingivalis</i> virulence factors LPS and OMVs containing
43	proteolytic enzymes (gingipains).

44 <u>Keywords</u>

45 Blood brain barrier, HBMEC, Alzheimer's Disease, micro biome, Periodontal disease,
46 *in vitro* BBB model.

48 <u>BACKGROUND</u>

49	The concept of a microbial cause or risk factor for neurodegenerating disease such
50	as sporadic Alzheimer's Disease has gathered momentum in the past decade. Why
51	some individuals develop sporadic Alzheimer's Disease (AD) while others are
52	resistant remains unresolved. There is currently no effective treatment for AD or a
53	way of slowing or stopping the rate of neurodegeneration. What has emerged is a
54	body of evidence over the past decade which supports a possible microbial role in
55	the development of neurodegeneration, which includes epidemiological data, post-
56	mortem and experimental studies. The evidence suggests that microbials potentially
57	linked to AD may be viral, bacterial or fungal in origin [1,2,3,4]. It is known that there
58	are multiple risk factors for developing sporadic AD, the most significant being
59	advanced age [4]. However, the possibility of a microbial role as a causative factor
60	for AD, opens new possibilities for the discovery of novel preventative measures and
61	therapeutic targets. Pivotal to the discovery of such novel drug targets is the need to
62	create research models that are representative of human physiology and disease
63	state.
64	AD is a chronic neurodegenerative condition which is known to develop over
65	decades, displaying histological hallmarks of extracellular amyloid- β (A β) plaques
66	and hyper phosphorylated intracellular Tau tangles within the brain parenchyma [5].

AD can also be considered as a chronic inflammatory disease and has been linked to
inflammatory events [6] such as traumatic brain injury or vascular disease, with
subsequent activation of the immune system and release of appropriate
inflammatory mediators purposely to protect the brain. If an individual predisposed
either through age, genetics, illness or lifestyle habits (smoking, diet, exercise) [7],

72	develops a persistent inflammatory trigger, then this can establish a chronic tissue
73	reaction with devastating toxic effects at a cellular level [8] and even initiating a path
74	to recognised AD pathology [6]. Sporadic AD presents late in life in individuals who
75	may not have an overt history of an acute inflammatory event, but the pathological
76	end point is the same [8]. The search for a trigger of raised levels of pro-
77	inflammatory mediators and oxidative stress, identified in AD patients goes on.
78	The detection of multiple microbials in post-mortem brains from AD individuals and
79	the notion that A β can behave as an anti-microbial peptide [9,10] raises the
80	possibility that an external [chronic] assault on brain tissues, for example infection
81	by the oral periodontal disease bacteria Porphyromonas gingivalis (P.gingivalis),
82	could cumulatively damage brain tissue and even lead to low level
83	neurodegenerative changes decades before a clinical diagnosis of AD presents [8].
84	Periodontal disease (PD) is a chronic infection caused by bacteria in the gums around
85	teeth represented by microbial dysbiosis and tissue destruction [11,12]. PD has been
86	linked to other organ specific disease such as Alzheimer's Disease, atherosclerosis,
87	and diabetes mellitus [13,14]. The progression of PD in humans is determined by
88	microbiological, environmental and genetic factors (multiple polymorphisms) [15]. It
89	can go undiagnosed for years and even if oral hygiene measures are improved, there
90	is a potential of daily bacteraemia (s) of periodontal pathogens when chewing foods
91	or cleaning teeth [16]. It is this chronic bacterial load in the circulation which is
92	proposed to contribute to a systemic chronic inflammatory state.
93	P.gingivalis, an anaerobic Gram-negative coccobacillus, is a key pathogen of PD and
94	has numerous mechanisms which can affect the surroundings tissues including i)
95	endotoxin or lipopolysaccharide (LPS) mainly located on the outer membrane and ii)
96	via release of outer membrane vesicles (OMVs) by the Type IX secretion system. The

97 working hypothesis the authors propose is that a chronic assault on the BBB from 98 circulating periodontal pathogens and/or their associated virulence factors could 99 lead to disruption of the integrity of the barrier (either by increased permeability or 100 reduction in clearance). Whilst there is some evidence to support the concept it 101 remains incompletely evidenced in humans and P.gingivalis cells have not yet been 102 found in the brain of AD patients or test animals[13]. However, human post-mortem 103 studies have found evidence of *P.gingivalis* DNA and virulence factors, LPS and 104 proteases secreted by *P.gingivalis* (gingipains), in the brains of AD individuals 105 [17,18]. In vivo animal studies, investigating the administration of P.gingivalis 106 associated virulence factors have shown that these substances travel to and settle in 107 the animal's brain [19,20]. Illievski et al., (2018) [20] showed that infection of mice 108 with *P.gingivalis induced* neuroinflammation and appeared to induce the deposition 109 of intracerebral A β protein, drawing similarities to the human AD pathology. It 110 remains unclear however, whether the reason for the A β protein seen in the brain of the test animals was due to a direct cerebral invasion of intact *P.gingivalis*, its 111 virulence factors such as gingipains or an indirect effect from the inflammatory 112 mediators of systemic infection [20]. The authors suggest two pathways for 113 114 P.gingivalis inducing neurodegenerative changes. Either i) P.gingivalis can access the 115 brain directly or ii) the bacteria can orchestrate the neurological changes from a 116 distant site of infection, i.e., the periodontal pockets in the oral cavity. The key 117 question remains if or how P.gingivalis and its virulence factors access brain tissue, how do they cross the BBB? 118

119 P.GINGIVALIS AND ASSOCIATED VIRULENCE FACTORS

120 The virulence or invasive ability of a *P.gingivalis* strain can be classified according to 121 the expression of fimbriae, capsule, LPS and gingipains release [21]. These virulence

122 factors originate from the mother cell but are disseminated wider by release of 123 OMVs. As an example, the non-capsulated laboratory strain FDC 381 has been 124 shown to invade carcinoma cells 10³ times more than other *P.gingivalis* strains [21], 125 though FDC 381 is classed as a less virulent type causing only mild localised 126 abscesses [22]. These findings highlight two key aspects of *P.gingivalis* behaviour 127 which have significance for pathological development, the ability of the bacterium to 128 i) invade tissues and ii) modulate the subsequent immune response of the host. Furthermore, a variance between different invasive abilities of OMVs from 129 130 *P.gingivalis* strains has been attributed to the expression of long fimbriae (not FimA) 131 and the gingipain adhesive domains in the outer membrane [23]. OMVs of P.gingivalis are highly enriched in the proteolytic gingipains (RgpA/B and Kgp) and 132 133 the nano sized spheres also incorporate high concentrations of LPS and fimbriae [24] 134 which are sustainable in human tissues such as the brain [25]. *P.gingivalis* OMVs, 135 containing a high concentration of enzymes, are considered to have both harmful 136 and beneficial roles, enabling *P.gingivalis* to regulate its microenvironment [26]. 137 Internalised OMVs are associated with cell degradation [27] and induction of an 138 innate immune response with a greater intensity than initiated by the bacteria itself. 139 Gingipains are also believed to help *P.gingivalis* evade the reach of the immune 140 system, making *P.gingivalis* so successful in establishing a chronic diseased state 141 [28]. 142 P.gingivalis has also been shown to dysregulate dendritic cells by disturbing their 143 ability toward autophagy and apoptosis [29] endowing this pathogen with an 144 exceptional ability for self-preservation. Labelled P.gingivalis OMVs have also been 145 shown to be taken up by cortical microglial cells in mice, 24 -48 hours after 146 peripheral injection highlighting the potential reach of this virulence factor. It has

147 even been suggested that OMVs may act as a decoy to the host immune system,

148	diverting attention and thus protecting the mother cell from elimination [30],
149	forming another element of <i>P.gingivalis'</i> immune evasive strategy. The size and
150	proteolytic capacity of the OMVs makes the spread into tissues easier than for the
151	intact bacteria and OMVs are more likely to survive transport to remote organs.
152	LPS from the outer membrane of Gram-negative bacteria is a powerful pro-
153	inflammatory pathogen associated molecular pattern (PAMP) and previous studies
154	support the capabilities of oral bacterial LPS as an inducer of peripheral
155	inflammatory responses and as an initiating factor in intracerebral inflammatory
156	activity [17,20]. The LPS of <i>P.gingivalis</i> has been extensively studied in relation to its
157	pathogenicity, is found in both a soluble and membrane bound form and binds to
158	TLR4 enhanced by sCD14 [31] activating pro-inflammatory pathways.

159 HUMAN BLOOD BRAIN BARRIER

160 The cells of the blood brain barrier, also described as the neuro vascular unit (NVU), 161 comprise of endothelial cells, pericytes, astrocytes and neurons [32]. The 400 miles of capillaries in the human brain [33] makes this the largest potential entry point for 162 163 pathogens to the CNS, but its intimate integrity affords a significant barrier. This 164 integrity arises from; endothelial cell i) intercellular tight junctions displaying high 165 electrical resistance, limiting any transcytosis compared to peripheral endothelial 166 cells [34], ii) lack of fenestrae (transcellular pores) and iii) shared basement 167 membrane with pericytes with reduced pinocytic activity. Human in vitro BBB 168 models are used to investigate both disease and drug interactions of this interface 169 providing a very valuable tool to assess permeability, transport and transendothelial 170 electrical resistance (TEER), as well as expression of proteins [35]. The benefits of 171 using a human primary-derived cell-based model are numerous and combinations of

172 cells of the NVU have been validated and standardised, for a comprehensive review173 see [35].

- 174 The integrity of the BBB is reduced naturally as we age [36] and multiple neurological conditions have been associated with a "leaky" BBB [34], including AD and 175 176 Parkinson's disease, implying the importance its role in brain homeostasis and risk of age-related neurodegeneration [13]. Many of these neurological conditions also 177 178 present with a raised level of systemic pro-inflammatory cytokines which are also 179 thought capable of contributing to weakening in the barrier's integument [37,38]. 180 Studies in mouse apolipoprotein E (ApoE) knock-out models [39] and humans who 181 express the E4 isoform of (APOE4), the most prevalent predisposed genetic risk 182 factor for AD, also show accelerated breakdown of the BBB structure and 183 degeneration of brain capillary pericytes required for barrier integrity [40].
- 184
- 185

186 CNS PERMEATION BY P.GINGIVALIS AND ASSOCIATED VIRULENCE FACTORS

187	The journey for <i>P.gingivalis</i> and associated virulence factors, from the periodontal
188	pocket to the central nervous system (CNS) has been suggested to follow a number
189	of possible routes, such as tracking along the trigeminal or olfactory nerves [41], by
190	being internalised by peripheral immune cells and subsequently transferring to the
191	CNS or finally arriving in the systemic circulation at the BBB or the blood
192	cerebrospinal fluid barrier (BCSFB) [42].
193	Both Gram negative and positive bacteria can cross at the BBB and BCSFB interfaces
194	to the CNS by transcytosis. Bacteria such as Neisseria.meningitidis are able to open
195	endothelial intercellular junctions to cross the CNS barriers in acute infection [42]

196and *P.gingivalis* gingipains have been shown to degrade the epithelial JAM-1 protein197[43] and induce cell adhesion molecule cleavage and apoptosis in human198microvascular endothelial cells [44]. *P.gingivalis* has also been demonstrated to199induce apoptosis and tight junction disruption in cultured human lung epithelial cells200[45]. It is not clear however, whether these findings were caused by the bacteria or201its virulence factors.

- 202
- 203 <u>NEUROINFLAMMATION</u>

204 If *P.gingivalis* was shown to induce damage to the BBB of an individual, some time 205 before any neuroinflammation becomes clinically detectable, then the authors 206 propose that bacteria or associated virulence factors must be capable of damaging 207 the BBB enough to trigger a change either to its integrity allowing an influx of 208 inflammagens and/or weakening the barrier's normal clearance strategies. If the 209 initial causal factor for the pro-inflammatory state is not resolved, then any 210 subsequent effects of chronic oxidative stress on the NVU cells can lead to loss of 211 redox balance, alterations in numbers and differentiation of T-cells subpopulations 212 and subsequent loss of regulation of the neuroinflammatory response [46]. Animal 213 studies for example have demonstrated that LPS can incite oxidative stress, 214 activation of glial cells and tight junction degradation in the NVU and surrounding 215 cells [47,48]. 216 Though much research has been undertaken to understand the events at the BBB in

217diseased individuals [47,48,49] and the effects of *P.gingivalis* and its virulence218factors on tissues, very little is known about what effect this bacterium and its219virulence factors exert on the cells of the blood-brain interface especially in the pre-220clinical stages.

221		The authors thus pose the question, could a persistent level of <i>P.gingivalis</i> virulence
222		factors in the circulation, renewed daily by chewing or tooth brushing [16] be
223		sufficient to cause damage to the BBB in an otherwise healthy state?
224		The aim of this study was to investigate how the <i>P.gingivalis</i> virulence factors LPS
225		and OMVs affected primary human cells in an <i>in vitro</i> BBB in the absence of the
226		bacterial mother cell. This was assessed by investigating i) BBB integrity by
227		transendothelial electrical resistance across the barrier, ii) how barrier permeability
228		to fluorescent labelled LPS and dextrans was altered by the presence of <i>P.gingivalis</i>
229		LPS and OMVs and iii) how LPS and OMVs interacted with the human brain
230		microvascular endothelial cells.
231		
232	<u>METHODS</u>	
233	<u>BBB MODEL</u>	
234		Primary-derived cell lines of human brain microvascular endothelial cells (HBMECs)
235		(Neuromics USA) at passage 3, human brain vascular pericytes (HBVP) (ScienCell,
236		USA) at passage 3 and human astrocytes (HA) cells (ScienCell, USA) at passage 3
237		were grown in flasks pre-coated with either AlphaBioCoat solution (Neuromics, USA)
238		or Poly-L-Lysine 10 mg/ml (Sciencell, USA). Cells were cultured in complete
239		endothelial cell growth basal medium (EBM) (Lonza, Switzerland), complete pericyte
240		medium (PM) (Sciencell, USA) and astrocyte basal medium (ABM) (Lonza,
241		Switzerland) with addition of human serum (Life Science Group, UK). The cells were
242		grown to a confluency of 85-90% in humidified incubator at 37 $^\circ$ C, 5 % CO $_2$. The
243		media was changed in all culture vessels every 48 hours until 50% confluent after

this point every 24 hours. Trypsin (TrypLeexpress, Gibco, Thermofisher, USA) and

245 Hank's Balanced Salt Solution (Gibco, Thermosfisher, USA) were used for passaging 246 cells. All observations of the cells were carried out under an inverted light 247 microscope (Leica DMIL light microscope from Leica Microsystems GmbH, Germany). 248 The viability and counting of the cells were assessed by using 1:1 Trypan Blue 0.4% (Sigma-Aldrich) and a haemocytometer [50,51]. Transwell[™] multiple well plate with 249 permeable polycarbonate membrane inserts (6.5 mm, 8.0 µm pore) (Corning, Fisher, 250 251 UK) were coated with fibronectin (Sigma, UK) The HA and HBVP were seeded on the 252 basolateral side of the insert and the HBMEC on the apical side [50]. The cells were 253 maintained with medium of equal volume of PM, ABM and EBM (Lonza, Switzerland 254 and Sciencell, USA) in a 37 °C humidified incubator under 5 % CO2.

255 BBB INTEGRITY

256	After 4 days the integrity of the barrier model was tested by measuring the trans-
257	endothelial electrical resistance (TEER) with an EVOM-2 instrument (WPI, UK). As the
258	barrier becomes established the TEER value rise expressed in Ohm/cm ² [52]. The
259	triculture <i>in-vitro</i> BBB model was considered ready for testing when the TEER values
260	reached an average of 260 Ohm/cm ² [50,53]. The triculture barrier integrity were
261	also assessed by application of FITC dextran 3 -5 kD, which was added to the apical
262	compartment of the inserts and incubated for specific time periods according to the
263	test protocols in the 37 $^\circ C$ humidified incubator under 5 $\%$ CO2. At the specified time
264	points in the test protocol samples were removed from the basolateral
265	compartment and measured in a GENios Pro plate reader (Tecan, Austria) at 490 nm
266	excitation and 520 nm emission (gain 40, 22°C). Standard curves were produced
267	from standard solutions of FITC 3-5 kDa and FITC-conjugated LPS in the range 0.04 to
268	100 μg/ ml.

269 <u>CULTURE OF BACTERIA</u>

270 P.gingivalis ATCC-BAA-1703 (strain FDC 381) was purchased from LGC limited (UK) in 271 freeze dried vials. The bacteria were cultured according to the supplier's 272 instructions. Briefly the P.gingivalis FDC 381 were cultured in ATCC medium 2722, 273 supplemented tryptic soy broth (TSB) (TSB 3%, Yeast extract 0.5%, L-cystein 274 hydrochloride 0.05%, Hemin (5 mcg/ml) with K₂HPO₄, Vitamin K1 (1mcg/ml) (Sigma 275 Aldrich, UK)) on TSA with 5% sheep blood (Thermo Scientific, UK) and FAA agar with 276 7% horse blood Neomycin (75mg/l) (E and O, UK). All cultures were incubated at 277 37°C in an anaerobic chamber (Bactron, USA) using an anaerobic gas mixture of 5% 278 H_2 , 5% CO₂ and 90% N₂. The cultures were grown for 3 days. The optical density (OD) of the broth cultures were measured daily and selected for use when between OD₆₅₅ 279 280 0.1 and 1 [54]. The cultures were Gram stained and imaged for quality control daily 281 to ensure monoculture samples utilising a Gram staining kit (Merck, UK) and inverted 282 light microscope using a X40 and X100 objective (Leica DMIL, Germany) and a Nicon 283 DS-L4 camera and software.

284 ISOLATION OF OUTER MEMBRANE VESICLES OF P.GINGIVALIS

285 The outer membrane vesicles of *P.gingivalis* FDC 381 were isolated following the 286 protocol used by Seyama et al. (2020)[54]. The bacterial culture in TSB was centrifuged at 2800 ×g for 15 min at 4 °C to separate the vesicles from the bacterial 287 288 cells. The supernatant was passed through a 0.2 µm syringe filter (Millipore, UK) and 289 then concentrated to under 1 mL by using an Ultra-15 Centrifugal Filter for the 290 nominal molecular weight limit (NMWL) 100K (Sigma-Aldrich, UK). The concentrate 291 was mixed with total exosome isolation reagent for culture (Life technologies, UK) 292 and this was incubated at 4 °C overnight. The samples were centrifuged at 10,000 ×g 293 for 60 min at 4 °C. The vesicles were eluted in 100 µL X1PBS. The TSB without 294 bacteria was treated by the same method as a negative control. The diameter of the

295outer membrane vesicles was measured and mono-dispersity ensured using a296Zetasizer (Malvern Zetasizer Nano, Panalytical Instalment Ltd., UK) (55) and the297concentrations of the samples was measured on the NanoDrop Spectrophotometer298(280 nm) (Nanodrop 2000, Thermo Scientific, UK).

299

300 BBB RESPONSE TO P.GINGIVALIS VIRULENCE FACTORS

301 The *in vitro* BBB model response to virulence factors of *P.gingivalis* was tested by 302 incubations with various concentrations (0.1 μ g/ml, 0.3 μ g/ml, 1 μ g/ml, 10 μ g/ml, 50 303 µg/ml and 100 µg/ml) of *P.gingivalis* LPS (Invivogen, France) and *P.gingivalis* outer 304 membrane vesicles (OMV) and 1 μ g/ml, 10 μ g/ml, 50 μ g/ml and 100 μ g/ml of 305 P.gingivalis LPS-FITC conjugate (Nanocs, USA). An experiment was also conducted with application of *P.gingivalis* LPS-FITC conjugate (Nanocs, USA) in combination 306 with OMVs at 10 µg/ml. Control wells included no treatment (cell and media alone) 307 308 and a cell blank (fibronectin insert, no cells). All measurements were made from 309 each well in five times (TEER) or three times (Permeability, Papp), each plate had 310 three wells (intraassay variability check). The test samples were diluted in complete 311 medium of equal measures of EBM, PM and ABM. The test samples were placed in 312 the apical compartment of the transwell and the TEER was measured at set time 313 points (0.5,1, 2, 4, 24, 48 and 72 hours). At each time point the TEER values of the 314 wells were measured 5 times and triplicate samples were collected from the 315 basolateral compartment.

316 APPARENT PERMEABILITY OF THE BLOOD BRAIN BARRIER TO P.GINGIVALIS VIRULENCE FACTORS

317Samples from the basolateral side of the BBB model were measured in the GENios318Pro plate reader (Tecan, Austria) at 490 nm excitation and 520 nm emission (gain 40,

319		22°C)[52], to quantify the appearance of fluorescent labelled LPS or FITC-dextran.
320		The appearance from the apical to the basolateral compartment was calculated from
321		standard curves of known concentrations of both FITC- dextran (3-5 kDa)(Sigma-
322		Aldrich, UK) and the <i>P.gingivalis</i> LPS-FITC conjugate (Nanocs, USA) and the data
323		from the standard curves were used to calculate the permeability (Papp) values in
324		each experiment as shown in Equation 1.
325		$P_{app} = \left(\frac{V}{A \times C_0}\right) \times \left(\frac{dQ}{dt}\right) \text{Equation 1}$
326		where:
327		V = Volume of basolateral compartment (V= 0.6 cm ³)
328		A = surface area of the polycarbonate membrane (0.3 cm^2)
329		C_0 = Initial concentration of the <i>P.gingivalis</i> LPS-FITC conjugate or FITC-Dextran in the
330		apical well
331		dQ = concentration of <i>P.gingivalis</i> LPS-FITC conjugate or FITC-Dextran collected from
332		the basolateral part (µg/ ml) (passing across the cell layer to basolateral side).
333		dt = Change in time (sec)
334		
335		
336	<u>HUMAN IL6 ELI</u>	<u>ISA</u>
337		As a quality assurance measure to check all virulence factors (LPS, LPS-FITC
338		conjugate and OMVs) were capable of producing an inflammatory response, all test
339		reagents were evaluated for inflammasome activity by incubation (100 μ g/ml) with
340		HBVP for 4 hours in triplicate and the spent cell culture media was assayed for

341 human IL-6 release using a commercially available enzyme-linked immunosorbent 342 assay (ELISA) kit (Sigma-Aldrich, USA). Test samples (spent media), controls (sample diluent buffer) and human IL-6 standards (100 µL) were added to the precoated 96 343 well ELISA plate. This was incubated at 4 °C for 24 hours with gentle shaking. After 344 345 removal of the samples the wells washed with X1 wash buffer four times. 346 Biotinylated detection antibody was added and incubated for 1 hour at room 347 temperature with gentle shaking. The wells were washed four times with X1 wash 348 buffer and HRP- streptavidin solution was incubated for 45 minutes at room 349 temperature with gentle shaking. After washing four times with X1 wash buffer, ELISA colorimetric TMB reagent was added. This was incubated for 30 minutes at 350 351 room temperature covered to exclude light while gently shaking. Then stop solution 352 was added and the absorbance was measured in the 96-well plate immediately at 353 450 nm (Tecan, Austria). A standard curve was produced, the concentration of IL-6 in 354 the samples was calculated.

355 IMMUNOFLUORESCENT IMAGING OF P.GINGIVALIS VIRULENCE FACTORS INTERACTIONS WITH TIGHT

356 JUNCTION PROTEINS OF HBMEC CELLS

357	HBMECs (Neuromics, USA) were seeded at a density of 250000 cells/ml in black,
358	tissue culture treated 24-well μ -plates (IBIDI at Thistle scientific, UK) and grown in
359	EBM (Lonza, Switzerland) in a 37 °C humidified incubator under 5 % CO $_2$ for 8 days.
360	The cells were tested for viability with Trypan Blue (Sigma-Aldrich, UK) and by visual
361	daily inspection. On day 7 the cells were treated with 0.1 $\mu\text{g}/\text{ml}$ and 0.3 $\mu\text{g}/\text{ml}$ of
362	unconjugated <i>P.gingivalis</i> LPS (Invivogen, France) or <i>P.gingivalis</i> outer membrane
363	vesicles (OMV) diluted in EBM (Lonza, Switzerland) and incubated for 24 hours. After
364	this incubation period the cells were fixed for IF protocol described below.

365 After incubation with the test samples the cells were washed in x1 PBS and fixed 366 with 4% formaldehyde, washed and permeabilised with x1 PBS and 0.1% Triton-X 367 (Sigma- Aldrich, UK) and blocked with 20 % normal goat serum (Stratech, UK) in 1x PBS with 0.1% Triton-X for 60 minutes. The cells were incubated with the primary 368 369 antibody ZO-1 (D6L1E) Rabbit mAb (1:400) (Cell signalling, NL) at 4°C for 12 hours 370 and shaking and the secondary antibody Cy™5 AffiniPure Goat Anti-Rabbit IgG 371 (1:800) (Jacsonimmuno, USA) for an hour at 4°C. The cells were counter stained with 372 DAPI(1:3500) (Stratech, UK) and imaged in a Zeiss Cell Observer system featuring the 373 Zeiss definite focus, Colibri LED illumination and AxioVision 4 digital image 374 processing software (Carl Zeiss Microscopy, Germany) detecting the signal for DAPI at ex:358 nm em:463 nm and Cy5 ex:646 nm em:664 nm. The images were viewed 375 376 and processed using Zen 2.3 Lite software.

377 <u>STATISTICAL ANALYSIS</u>

378The TEER and the permeability (Papp) data obtained from the *in vitro* BBB model was379tested for homogeneity of variances and normality using the Shapiro-Wilk test.380Difference between treatment groups was analysed using an ANOVA with Dunnett's381post-hoc analysis or an independent t-test for comparison between independent382experiments. The analysis was performed using the Statistical Package SPSS Version38326 and 27 (IBM, USA). Statistical significance was defined when (*) P < 0.05, and</td>384highly significant when (**) P < 0.01 and (***) P < 0.001.</td>

385

386 <u>RESULTS</u>

387 BASELINE VARIABILITY AND MODELLING TEER CHANGES IN THE BBB MODEL

388 The baseline of the experimental model and optimisation was carried out to 389 establish whether the primary cells and the 3-layer model were suitable for the planned experiments and not affected by the presence of the tracer compound. 390 Prior to the start of the experiment, TEER values were consistent from day 7, with a 391 typical variation of \pm 10 Ω / cm² over a 4 hour time period. Upon addition of either 392 unconjugated *P.gingivalis* LPS (test wells), media (blank wells) or FITC (control wells) 393 394 to the apical side of the BBB, there was a small initial dip in TEER at the start of the 395 experiment that was attributable to movement artefact and slight disturbance of the BBB in all wells, typically this was $\pm 25 \Omega/cm^2$ and recovery to baseline was observed 396 in the control wells within 2 hours. The response of TEER in test wells upon addition 397 398 of unconjugated LPS often showed a lower drop in TEER and that did not always 399 recover to pre-baseline TEER. To determine whether the change in TEER was 400 significantly different from baseline variation or movement artefacts, the pattern of 401 response was modelled and the magnitude ($\Delta TEER$), recovery time and rate of 402 change were defined as shown in Figure 1 and were then used for statistical 403 comparison between the control and test wells at different LPS concentrations.

404





Figure 1. Modelling the magnitude and rate of change in TEER after application of

test sample to the in vitro BBB model

408

409 <u>CONFIRMATION OF INFLAMMATORY RESPONSE OF VIRULENCE FACTORS TESTED, MEASURED BY</u>
 410 <u>HUMAN IL6 ELISA</u>

- 411 In order to show that the virulence factors applied to the BBB model (unconjugated 412 P.gingivalis LPS, FITC P.gingivalis LPS and P.gingivalis OMVs) had a biological 413 response all samples were tested for induction of IL6 in HBPC prior to use. The inflammatory response of HBPC cells following exposure to unconjugated 414 P.gingivalis LPS, FITC P.gingivalis LPS conjugated and P.gingivalis OMVs (all 100 415 416 μ g/ml) after 4 hours of co-incubation, negative controls were media only. A standard 417 curve was prepared and used to determine the concentrations of IL6 secreted by the 418 cells in the test wells. This test was repeated every time a new conjugate reagent was used for the first time in triplicates. The results showed an elevated level of IL6 419 420 in the test samples compared to controls (data not shown). These results were seen as a positive control of the virulence activity in the samples tested. 421
- 422

423 EFFECT OF UNCONJUGATED P.GINGIVALIS LPS ON THE BBB INTEGRITY

424The *in vitro* model was tested with un conjugated *P.gingivalis* LPS to assess the425barrier response to this virulence factor. Application of unconjugated *P.gingivalis*.426LPS to the BBB caused a significant decrease in TEER for 0.3 µg/ml ($P \le 0.05$), 10427µg/ml ($P \le 0.05$) and 100 µg/ml ($P \le 0.05$) when compared the magnitude of change428to the control well, where FITC alone or media alone were administered (Figure 2A).429The magnitude of recovery of TEER values determined as the maximum TEER430measured during the recovery phase. For all wells treated with unconjugated

431P.gingivalis LPS there was still a deficit in recovery of the BBB integrity compared to432the pre-incubation phase, as indicated by the deficit in TEER 72 hours post433incubation relative to the baseline at time zero. The magnitude of deficit in TEER was434significantly greater in the test wells where unconjugated P.gingivalis LPS was435applied at 0.3 μ g/ml (P \leq 0.05), 10 μ g/ml (P \leq 0.05) and highly significant with 100436 μ g/ml (P \leq 0.01) compared to the control wells where FITC-alone was applied to the437BBB (Figure 2B).





- 447 The integrity of the *in vitro* BBB model was also assessed by testing the wells with
- 448 FITC-Dextran 3-5 kD as a marker of tight junction permeability. After incubation with
- 449 unconjugated *P.gingivalis*. LPS or media (blank) for set time points. FITC-Dextran 3-5
- 450 kD was added to the wells and the fluorescent appearance of FITC-dextran on the
- 451 basolateral side of the BBB was measured. It was observed that the FITC-Dextran

452	appeared earlier in the wells with 10 and 100 $\mu g/ml$ of unconjugated P.gingivalis LPS
453	following pre-incubation however this was not statistically significant relative to the
454	blank wells and no significant concentration dependent effect in <i>P.gingivalis</i> LPS
455	treatment related to FITC-dextran appearance was observed for the complete test
456	period. The percentage appearance of FITC-dextran appeared to increase following
457	longer exposure (24-72 hours) to <i>P.gingivalis LPS</i> , as shown in Figure 3C-E.





Figure 3. Shows the percentage of FITC-dextran (3-5 kDa) permeating through the in vitro BBB after incubation with increasing concentrations of unconjugated P.g. LPS (0-100 μ g/mL) for 1 h (A); 4 h (B); 24 h (C); 48 h (D); 72 h (E) and all exposure times compared together (F). Each data point represents mean ± SD from three wells and two experimental repeats (n=6).

466The apparent permeability (Papp) of FITC-dextran (100 µg/ mL) after incubation for46730 minutes was calculated for three time points (60, 120 and 240 minutes) as468described previously. Final Papp values at 60 min, 120 min and 240 min were found469to be 1.04×10^{-8} +/- 2.3×10^{-8} cm/s, 8.7×10^{-8} +/- 1.7×10^{-7} cm/s and 4.8×10^{-8} +/- 4.7470 $\times 10^{-8}$ cm/s.

471 EFFECT OF FITC-CONJUGATED P.GINGIVALIS LPS ON THE BBB INTEGRITY

To investigate potential transport across the in vitro BBB model a FITC labelled P.gingivalis LPS was applied to the established model and appearance of the conjugate was measured with the models integrity. It was shown that there were no significant differences in the magnitude of TEER response between the wells with application of all concentrations of FITC-P.gingivalis LPS conjugate and the control (FITC alone), however a decrease in TEER was observed in all wells after application of 1,10,50 and 100 μ g/ml (Figure 4A). These wells did not appear to recover as well compared to controls (Figure 4B).

TEER (Ω / cm²) TEER (Ω / cm²) Blank Blank [LPS] (μ g/mL) [LPS] (μ g/mL)

В

А

Figure 4 Changes in BBB integrity measured by calculating the magnitude of decrease in TEER in response to application of conjugated FITC-P.gingivalis LPS (A) and the magnitude of deficit in recovery of TEER 72 hours post application of conjugated FITC-P.gingivalis LPS relative to initial baseline TEER (B).No statistical significance of response was measured using an ANOVA with Dunnett's post-hoc relative to the

486 487	control (administration of FITC alone). Data represents mean \pm SD from three wells and two experimental repeats (n=6).
488	
489	The appearance of the FITC <i>P.gingivalis</i> LPS was greatest in the highest
490	concentration (100µg/ml) at 1 and 4 hours (Figure 5A and 5B) and an increase of
491	percentage appearance was observed with the concentrations 10 and 50 $\mu\text{g}/\text{ml}$ as
492	the experiment progressed at 24 and 48 hours (Figure 5C and 5D). The percentage
493	appearance of the FITC <i>P.gingivalis</i> LPS in the basolateral compartment of the model
494	did not exceed 5% during the duration of the experiments. A drop in the TEER values
495	were seen to correlate with the appearance of the conjugate in all the wells of the
496	higher concentrations (50 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$ LPS) (data not shown).
497	All the wells were tested with FITC-Dextran at the end of each experiment to assess
498	the final integrity of the barrier (data not shown).
499	



507 where *P<0.05 and **P <0.01. Data represents mean ± SD from three wells and two 508 experimental repeats (n=6).

509 EFFECT OF P.GINGIVALIS OMVS ON THE BBB INTEGRITY

510The application of *P.gingivalis* OMVs showed similar patterns to the LPS study. The511magnitude of decrease in TEER observed in response to treatment with OMVs was512significantly different from the control group for the 0.1 μ L/ mL (*P*<0.01); 0.3 μ L/ mL513(*P*<0.05); 50 μ L/ mL (*P*<0.05) and 100 μ L/ mL (*P*<0.01) (Figure 6A). This decrease in</td>514TEER did not recover to pre-treatment baseline for the wells treated with 50 and 100515 μ L/ mL *P.g* OMVs as the magnitude of deficit was highly significantly different to the516control group (*P*<0.001), as shown in Figure 6B.</td>





Figure 6 Changes in BBB integrity measured by calculating the magnitude of decrease in TEER in response to application of P.gingivalis OMVs (A) and the magnitude of deficit in recovery of TEER 72 h post application of conjugated P.gingivalis OMVs relative to initial baseline TEER (B). Statistical significance of response was measured using an ANOVA with Dunnett's post-hoc relative to the control (administration of FITC alone) where *P<0.05, **P <0.01and and ***P <0.001. Data represents mean ± SD from three wells and two experimental repeats (n=6).

527	Figure 7 shows the appearance of FITC-Dextran permeation following incubation of
528	the in vitro BBB with increasing concentrations of P.gingivalis. OMV exposed for
529	varying durations. Similar to the unconjugated LPS, the effect of OMV treatment on
530	the extent of FITC-dextran permeation was fairly constant after the 12 hour
531	exposure, but this time the permeation did appear to increase as the concentration
532	of OMV increased (Figure 7C-E).
533	
534	
535	
536	



vitro BBB model after application of P.gingivalis OMVs, percentage appearance relative to the stock FITC-dextran administered to the basolateral side after 1 h (A); 4 h (B); 24 h (C); 48 h (D); 72 (E) and a comparison of all time points (E). Statistical significance of response was measured using an ANOVA with Dunnett's post-hoc relative to the control (administration of FITC alone) where *P<0.05 and **P <0.01. Data represents mean ± SD from three wells and two experimental repeats (n=6).

547 EFFECT OF P.GINGIVALIS OMV AND LPS ON THE BBB INTEGRITY

548	The in vitro BBB model was tested with FITC conjugated P.gingivalis LPS in the
549	presence of a constant concentration of OMV to assess the effect the OMVs could
550	potentially have on the appearance of the conjugated LPS in the basolateral
551	compartment. The concentration of OMV was chosen (10 μ g/ml) as this was the
552	lowest which showed an effect in the BBB models integrity in the OMV only
553	experiment previously (Figure 7D). The controls for the combined experiment were
554	media only and OMV (10 $\mu\text{g/ml})$ only. The data was compared to the previous
555	experiments with FITC <i>P.gingivalis</i> LPS only (Figure 5). The application of <i>P.gingivalis</i>
556	LPS-FITC conjugate in conjunction with 10 μ g/ml <i>P.gingivalis</i> OMVs showed a similar
557	pattern in terms of response in the BBB model as seen in the previous experiments.
558	The TEER responses in these experiments showed a significant difference in the
559	100µg/mL FITC <i>P.gingivalis</i> LPS conjugate with larger magnitudes of change in TEER
560	compared to the controls (P<0.05) (Figure 8A). The higher the concentration of FITC
561	P.gingivalis LPS with OMV, a reduced recovery was observed, although this was only
562	significant in the highest concentration of 50 μ g/mL and highly significant in the
563	100µg/mL FITC P.gingivalis LPS with 10 µg/ml OMVs ((P<0.05 and (P<0.001)) (figure
564	8B). An increase in the permeability was seen especially after 24 hours where some
565	of the increases were 5-fold compared to the experiment with <i>P.gingivalis</i> LPS-FITC
566	conjugate application only, though this increase was not significant (Figures 5 and
567	9C).







Figure 9 Percentage appearance of FITC P.gingivalis LPS conjugate on the basolateral side of the in vitro BBB model relative to the stock administered to the apical side after 1 h (A); 4 h (B); 24 h (C); 48 h (D); 72 (E) and a comparison of all time points (E). Statistical significance of response was measured using an ANOVA with Dunnett's post-hoc relative to the control (administration of FITC alone) where *P<0.05 and **P <0.01. Data represents mean ± SD from three wells and two experimental repeats (n=6).

588 <u>ASSESSMENT OF VIRULENCE FACTORS INTERACTIONS WITH HBMEC MONOLAYERS MEASURED BY</u>

589 <u>IMMUNOFLUORESCENCE MICROSCOPY</u>

590	The experiments of application of virulence factors to the BBB model indicated a
591	potential disruption of the barrier. These findings lead to the experiment with
592	application of i) unconjugated P.gingivalis LPS (0.1 and 0.3 $\mu\text{g/ml})$ and ii) OMVs (0.1
593	and 0.3 $\mu g/ml)$ to HBMEC cells in monolayer with an aim to determine how the
594	observed BBB model disruption happened, all the experiments were repeated 3
595	times independently. The HBMEC cells were chosen for this experiment as these are
596	the first cells coming into contact with the virulence factors in the <i>in vitro</i> model. The
597	negative control cells (no virulence factors applied) showed the expected position of
598	the ZO-1 protein at the cell-cell junctions and the signal of the ZO-1 protein
599	appeared clear and well organised in these controls (Figure 10B and 10C). The
600	concentrations for these experiments were based on observations after application
601	of virulence factors of higher concentrations in the optimisation stages showed the
602	HBMEC cells viability were consistently acceptable at these concentrations. All the
603	test wells and controls were imaged using the same exposure times and all post
604	exposure modifications were carried out to the same level with the Zen software.
605	The HBMEC monolayer with application of <i>P.gingivalis</i> LPS showed no noticeable
606	effect on the ZO-1 signal (Figure 10 D-F) (only 0.3 $\mu g/ml$ shown) and similar
607	observations were made in the wells with 0.1 μ g/ml <i>P.gingivalis</i> OMV application
608	(Figure 10 G-I) compared to the untreated controls (media only). The wells with
609	application of 0.3 μ g/ml P.gingivalis OMVs showed a more diffused signal from the
610	ZO-1 proteins compared to untreated controls, which could appear as a reduction in
611	the signal (Figure 10 J-L) though a change in the signal from the application of 0.3
612	μ g/ml P.gingivalis OMVs were seen in all 3 repeat experiments it was not clear if the
613	change was a displacement of the ZO-1 protein or reduced numbers as the

- 614 experiment here did not quantify the protein. All experiments were conducted with 615 controls both for treatments, antibodies and counterstains and all showed the 616 correct results.
- 617
- 618
- 619
- 620

622

623

624



Figure 10. Immunofluorescent study of application of P.gingivalis virulence factors to HBMEC cells. HBMEC cells in monolayer were treated with EBM (A-C), 0.3 μg/ml P.gingivalis LPS (D-F), 0.1 μg/ml P.gingivalis OMVs (G-I) or 0.3 μg/ml P.gingivalis

- 625OMVs (J-L) for 24 hours. Panels A, D, G and J show the nuclei stained with DAPI and626detected at 358 nm (blue). Panels B, E, H and K show Cy5 signal detected at 646 nm627(red) detecting the primary ZO-1 (D6L1E) Rabbit mAb. Panels C, F,I and L represent628the composite pictures. Images were taken with 60x objective oil lens with the same629exposure times. Scale bar 20 μm. (For interpretation of the reference to colour in this630figure legend, the reader is referred to the article).
- 631
- 632
- 633 <u>DISCUSSION</u>

634	In this study we investigated the effect of <i>P.gingivalis</i> virulence factors on the cells in
635	a human in vitro BBB model. Our observations regarding the movement of
636	P.gingivalis LPS across to the CNS side of the model (Figure 5) and the enhancement
637	of LPS appearance in the basolateral compartment in the presence of OMVs (Figure
638	9), supports findings from animal and other <i>in vitro</i> studies [17,19,20,27], though the
639	methodology used in this study has not, to our knowledge been previously
640	published. Designing robust human studies of chronic bacterial interaction at the
641	BBB is challenging and making a link to sporadic AD must be carried out cautiously
642	for numerous reasons, such as the latent period before AD pathological changes are
643	first identified in brain tissues, and time to clinical symptomatology development
644	both in the arena of multiple other risk factors associated with AD development. In
645	vitro BBB models have been widely used for decades to investigate drug transport
646	and individual disease processes, including A eta clearance mechanisms in relation to
647	AD [49,56]. Most BBB model studies investigating bacterial related interactions have
648	focused on acute events [57,58].
649	The <i>in vitro</i> BBB model utilised in this study was developed by Kumar et al.

650 (2014)[50] and subsequently validated for investigation of drug transportation across

651 the BBB . Although the main protocols for the model were established, some 652 adjustments had to be made for it to be meaningful in our investigation. It was vital 653 to establish continuous barrier integrity or function and to ensure the model was 654 suitable for the duration of testing and not affected by the application of FITC tracer 655 compounds. The concentrations of virulence factors applied were deduced from 656 previous studies [7,59] and by optimising the test protocols for which both high and low doses were included. Starting with low concentrations, the virulence factors 657 658 assessment evidenced whether there was a concentration dependent relationship 659 with the endotoxin and OMVs which would indicate the resilience of the BBB model 660 to these virulence factors. After application of unconjugated *P.gingivalis* LPS, a clear pattern emerged (Figure 1), of an initial drop in TEER values including the controls, 661 662 but it was observed that the drop in the wells which had endotoxin applied were 663 both greater in values and failed to recover as well as those in the control wells 664 indicating a lasting measurable effect in the BBB model barrier function. It was 665 concluded that the initial small drop in TEER values in the control wells (average of 25 ohms/cm²) would need to be regarded as an artefact and test results within these 666 667 ranges were reviewed taking this into consideration by ensuring appropriate blank 668 (media only) and zero controls (FITC only) were included in every experiment where 669 appropriate. This artifact was not observed by Kumar et al. (2014) because the 670 protocols utilised here included continuously measuring of the TEER levels 671 throughout the experiments, whereas Kumar et al. used TEER as a quality control of 672 the barrier function before and after the experimental drug transport protocols. 673 This artifact can be explained as following the application of the Evom electrodes or 674 the media change, a short-lived disturbance could have occurred in the conductance 675 across the BBB model. This initial drop recovered in all the control wells and as the 676 protocol continued for 72 hours, a clear distinction between the test and control

677 wells was facilitated (data not shown).

678 A significant drop in TEER values was seen in wells tested with the concentrations of 679 unconjugated *P.gingivalis* LPS (0.3, 10, 100µg/ml) (Figure 2A) and the recovery of 680 TEER in these wells were also significantly less compared to controls (in 100 µg/ml 681 highly significant, *P* <0.01) (Figure 2B). Furthermore, a significant drop in TEER was 682 observed after application of *P.gingivalis* OMVs (0.3 and 50 μ g/ml) (*P* <0.05) which 683 were highly significant after application of 0.1 and 100μ g/ml (*P* < 0.01) (Figure 6A). 684 The recovery in the wells with 50 and $100\mu g/ml$ had a highly significant (P <0.001) 685 deficit compared to the controls (Figure 6B), indicating that these virulence factors 686 affected the in vitro BBB model in a prolonged and negative fashion. However, at 687 lower concentrations (0.1,0.3 and $1 \mu g/ml$) some of the TEER value reductions were 688 temporary followed by partial or complete recovery (Figure 6B). The authors suggest 689 this may have arisen as a result of i) an initial apoptosis event subsequently 690 overcome by the surviving neighbouring cells expanding to repair the damaged area 691 or ii) an initial disruption of the tight junction complexes followed by a reparatory 692 upregulation event. These observations are important as they indicate the cells of 693 the BBB model have an ability to recover if the endotoxin is applied at a low level. 694 Applied to a human clinical scenario this means that after a low level, low frequency 695 endotoxin contact with the BBB, the NVU cells appear to retain the ability to 696 preserve the barrier's integrity. Clinically this could correlate with the adoption of an 697 improvement in oral hygiene or if systemic risk factors for PD, such as diabetes, were eliminated or reduced. 698

699The wells demonstrating less ability to recover their TEER values, could indicate that700the cells in those wells were unable to survive or expand, or the LPS and OMV could701have influenced the continuity of the cell layer either by causing pyroptosis [38],702apoptosis or irreversible tight junction disruption [43,60]. It was recorded that there

- were reductions in TEER readings in the wells receiving FITC *P.gingivalis* LPS
 conjugate in media compared to controls and that these TEER levels also did not
 recover as well, though none of these TEER value differences were statistically
 significant compared to the controls (Figure 4A and
- 707 4B).

708 The appearance of FITC dextran in the basolateral compartment gave an indication 709 of disruption to the barrier integrity after unconjugated *P.gingivalis* LPS and OMV 710 application at various concentrations. Average Papp values were then calculated at 60 min, 120 min and 240 min and were found to be 1.04×10^{-8} cm/s, 8.7 x 10^{-8} cm/s 711 and 4.8 x 10⁻⁸ cm/s. In permeability assays for drug transportation poor permeability 712 is indicated by Papp values of $0 - 1.4 \times 10^{-6}$ cm/s and high permeability by values in the 713 range of 5 x 10⁻⁵ - 9 x 10⁻⁵ cm/s. The low calculated Papp values in our study suggest 714 715 that the Papp for the tracer compound FITC-dextran were low indicating the BBB 716 model retained its overall barrier function for the first two hours, though allowing 717 enough permeation to measure a difference between test wells (Figure 3). The most 718 significant effect in the models TEER measurements were observed in the wells with concentrations of unconjugated LPS and OMV at 0.3 µg/ml, 10 µg/ml, 50 µg/ml and 719 720 100 µg/ml and contextualised to a theoretical physiological condition, the lowest of 721 these values, $0.3 \mu g/mL$ is most clinically applicable [7,59].

722The changes in the BBB model cells after application of the FITC-*P.gingivalis* LPS in723conjunction with 10 μg/ml of *P.gingivalis* OMV showed a significant difference in the724magnitude of change in the TEER values and a significant deficit in recovery of the725values (highly significant in 100 μg/ml FITC-*P.gingivalis* LPS) which, highlighted the726potency of the OMVs containing gingipains pr (Figure 8).

727 The TEER measurements of an in vitro BBB model reflect the ionic conduction 728 paracellularly in the cell layers, whereas the percentage appearance of a tracer 729 compound in the BLC represents paracellular waterflow associated with increased 730 pore size at the tight junctions [52]. Transcellular ion transport function and 731 paracellular permeability of solute transport are differentially regulated [52], where 732 the factors affecting perfusion of a molecule across the BBB is size, shape and 733 lipophilicity. TEER is a valuable assessment of the in vitro BBB integrity as it is easy to 734 quantify and if carried out with care, non-invasive. It is however important to be 735 aware of the limitations of TEER measurements, where variations can occur due to 736 factors such as medium content, temperature and the passage numbers in the cell 737 lines at the time of measurements [52]. As the protocols in this study were 738 performed under the same conditions using the same equipment and cell passage 739 numbers, some of the potential variables could be excluded. As an example, the 740 EVOM probe was calibrated in the same manner before each measurement and five 741 readings were taken from the individual wells with each experiment, providing the 742 authors with confidence in the longitudinal magnitude of change and TEER endpoint results 743 744 The biphasic TEER pattern observed in all the virulence factor application protocols 745 (data not shown) could potentially also be explained by the exponential growth of 746 the cells in the wells or a cell response to the applied reagents such as an 747 upregulation in the cells, making the TEER appear to recover with time. This would 748 suggest that any disruptions to the BBB cells which could be measurable by TEER, 749 would have to counteract this progression in cell density and tight junction 750 maturation or would otherwise go undetected. It is most likely that the recovery of 751 the BBB observed in the wells, throughout our experiments, reflects an increased 752 number of cells in the BBB as there was no other significant increases in permeability

recorded for the remainder of the test period when further endotoxin was applied,
or the concentrations of LPS added were not high enough to induce measurable
changes to the percentage appearance in the BLC (Figures 3 and 5).

756Previous validation studies of *in vitro* BBB models like ours have shown that a TEER757(read) value in the range of 120 – 130 ohms/cm² is enough for transport studies [61].758When setting up the BBB model for testing transport and permeability in this project759the aim was to achieve values of TEER (read) – TEER (blank) ≥ 260 ohms/cm² [61],

760 this was achieved in all the

761 protocols.

762 A tracer compound used in an *in-vitro* BBB model can potentially interfere with the 763 test reagents and affect the integrity of the barrier [52]. This issue was addressed by 764 using test wells with FITC-dextran only throughout the study. Fluorophore and dye 765 tracer compounds are not always sensitive enough to show subtle changes in barrier model permeability [52] which is a weakness of this type of study and bias can be 766 767 introduced if the sensitivity in the measuring equipment is not high enough to pick 768 up the compound at small levels. The FITC dextran molecule used here has been 769 shown to cross the BBB model via intercellular diffusion [62] and any increase in 770 intercellular channels would allow greater amounts to pass into the BLC. This study 771 demonstrated that the appearance of FITC dextran into the BLC occurred early 772 (between 1 and 4 hours) after the initial application of unconjugated *P.gingivalis* LPS, 773 particularly with the testing of the higher concentrations of LPS, but subsequent 774 applications failed to demonstrate any clear correlation between the concentration 775 of applied endotoxin and the percentage appearance measured in the BLC. This 776 implies that the higher concentrations of LPS were able to induce an increase in 777 paracellular flow, possibly by increasing paracellular gaps, at initial application

778 compared to controls (Figure 3). However, further increases were not demonstrated 779 by additional applications implying a finite capacity for paracellular flow increase. 780 These findings were supported by the Papp calculations of the FITC dextran 781 throughout the protocols and the percentage appearance values which also 782 remained low after application of unconjugated LPS. The levels of FITC-dextran 783 appearance seen throughout the unconjugated LPS experiments were persistently 784 low with the maximum appearance at 0.7% (Figure 3) which is encouraging in terms 785 of demonstrating the quality of the barrier model [50]. In comparison the 786 percentage appearance of FITC dextran seen in the OMV alone experiment were 787 higher with the maximum percentage appearance being 15-fold higher than the LPS 788 alone (Figure 7). The maximum percentage appearance of the FITC LPS conjugate in 789 the BLC was 5% during the experiments (Figure 5) and a small increase to 8% 790 maximum percentage appearance was observed when 10 μ g/ml was added to the 791 FITC-LPS conjugate (Figure 9). The increased percentage appearance in the OMV 792 alone (Figure 7) could be explained by the presence of the proteolytic enzymes or 793 gingipains within the OMVs which could also explain the increased permeability of the FITC *P.gingivalis* LPS in the presence of 10 µg/ml of OMV (Figure 9). The enzymes 794 795 could create greater gaps between the barriers cell layers allowing greater perfusion 796 of the molecules to the BLC. The differences in percentage appearance between 797 the FITC *P.gingivalis* LPS and the FITC dextran molecules (Figures 3 and 5), where the 798 percentage detected in the BLC of FITC P.gingivalis LPS was approximately 5 fold 799 larger than FITC dextran appearance after unconjugated LPS, can be explained as a 800 potential difference in the virulence between the two compounds (conjugated and 801 unconjugated LPS) inflicting different effects on the pore sizes within the barrier, or 802 variances in the size, shape and/or lipophilicity between FITC dextran and FITC LPS 803 molecules could attribute to this observation. The P.gingivalis LPS could also affect

805 though the LPS product used for both reagents originated from the same source. 806 The immune response of the *P.gingivalis* LPS, *P.gingivalis* LPS FITC conjugate and 807 P.gingivalis OMV utilised in the experiments was examined by an IL-6 ELISA after 808 application for 4 hours to HBPC. IL6 is a proinflammatory cytokine and the induction 809 of this cytokine would give an indication of the biological activity of the virulence 810 factors utilised in this study, as the presence of LPS would induce TNF- α pathways in 811 the HBPC to release IL6 [63]. The response and induction of IL-6 from all reagents 812 measured in the cell media, were of a similar quantity and levels were increased 813 compared to controls (media only)(data not shown).

the cells of the BBB model differently at receptor level after being conjugated , even

804

814 The unconjugated *P.gingivalis* LPS used in this study was the standard preparation 815 (defined by preparation by supplier) which has been noted to induce a stronger 816 inflammatory response than the pure version (both supplied by Invivogen) possibly as a result of impurities of lipoproteins in the standard preparation, activating TLR2 817 818 as well as TLR4 [64]. Furthermore, the standard P.gingivalis LPS has also been found 819 to show a stronger inflammatory response in human periodontal stem cells after 24 820 hours than the pure version (64). In our BBB protocols we saw an increased response 821 at 24 hours with both *P.gingivalis* LPS and OMVs which indicates that the induced 822 response could be more complex than a simple apoptosis of the cells and involve 823 inflammatory pathways affecting the integrity of the barrier. The OMVs applied in 824 this study were extracted from a culture of the laboratory strain *P.gingivalis* FDC 381 825 which is classed as a less virulent *P.gingivalis* strain but has a high ability to be 826 internalized in human cells [65]. This non-capsular strain has been shown to be a 827 strong immune stimulant, (even activating TLR2) a property attributed to an intact 828 fimB allele but with a less gingipain activity [66]. It is possible that the difference in 829 appearance of the *P.gingivalis* LPS FITC conjugate on the CNS side of the model with

and without the presence of the OMVs would have been more significant if a less
virulent *P.gingivalis* LPS product had been used such as the purified product
mentioned previously.

833 The *in vitro* BBB model has limitations such as the delicate nature of working with 834 primary-derived cell lines and the measurements derived from this study do not 835 divulge much information about a cellular level activity. There is an increased 836 consensus that to gain more accurate knowledge of AD, microbial pathology human 837 models need to be developed, as pathological and inflammatory pathways in 838 rodents significantly differ from humans [6, 31] especially in relation to molecules 839 such as LPS [38]. The expression in the author's human BBB model cells is closer to 840 the in vivo state than a murine model and there is potential in our model to gain 841 further cellular level information. The role of microglial cells in neurodegeneration is 842 undisputed and expansion of this model to include human microglial cells could broaden the applications for this type of protocol. In addition, also BBB organoids 843 844 could potentially be applied to this type of study [67].

845 There is evidence to suggest that *P.gingivalis* may not need to enter the brain to 846 cause neuroinflammation [38]. Even healthy humans have been shown to have low 847 levels of LPS in their blood [68], but this is found to be elevated in AD and PD 848 patients [69,70,71]. AD patients have been found to have 2-3 times as much LPS in 849 the brains as healthy individuals [72]. LPS is released from the bacteria either when 850 it is degraded or when outer membrane vesicles are released [38] and the GI 851 microbiome has been shown to be the main contributor to systemic presence. LPS 852 has been suggested as an intermediary between bacteria and the CNS at low levels 853 (physiological) conditions [73] in rodents, and a lipo-protein transport mechanism to 854 the CNS has been suggested, but it is not yet known exactly how LPS enters the brain

in humans. It is possible that transport mechanisms are responsible for the
appearance of the LPS on the BLC (CNS) side of the model used in this study and this
topic warrants further investigation, OMVs are known to have the ability to be
internalised by human cells which is another avenue to explore.

This study however has demonstrated that the integrity of a BBB model is reduced by the presence of *P.gingivalis* virulence factors seen by a measurable reduction in TEER levels and making the barrier more permeable and the subsequent increased appearance of LPS in the BLC (CNS side of BBB).

863 Bacterial LPS has been found to change the permeability of the BBB at high doses 864 [74] as seen in sepsis causing significant CNS disability. *P.gingivalis* LPS has the 865 potential to cause neuroinflammation via the blood directly acting at the BBB, by 866 inducing pro-inflammatory cytokines, initiating pro inflammatory pathways in the tissues of the neuro vascular unit and activating microglial cells without entering the 867 brain [38]. In addition, this study has indicated that *P.gingivalis* LPS also has the 868 869 potential to cross the BBB as seen in the FITC *P.gingivalis* LPS experiments, 870 potentially explaining how systemic circulating LPS could induce neuroinflammation. 871 The subsequent immunological response to LPS is well documented (for a review see 872 44) and the toxicity of an endotoxin is determined by how the host reacts to it [75]. 873 Both immunological activation and tolerance [76,77] can explain how chronic exposure to even medium and low levels of *P.gingivalis* LPS could lead to 874 875 neurodegeneration by induction of pro-inflammatory pathways and activation of 876 microglia and it is plausible that a low concentration of *P.gingivalis* virulence factors 877 can induce damage to the NVU cells [78,79]. Here we have shown, in a BBB model, 878 that whole bacteria do not need to be present as *P.gingivalis* LPS and OMVs have the 879 armoury to induce alteration of the barrier integrity providing access to the CNS 880 tissues. A question remains about how much damage, over what period of time is

882

required, before the balance is tipped towards a path where the BBB cells cannot recover?

883 The aim of applying *P. gingivalis* LPS and OMVs to a HBMEC monolayer was to 884 investigate whether the changes observed in the BBB model protocols could be seen 885 at a cellular level and to determine how these changes occurred. The negative 886 control cells (no virulence factors applied) showed the expected position of the ZO-1 887 protein at the cell-cell junctions [80] with the signal of the ZO-1 protein appearing 888 well organised (Figure 10B and 10C). The HBMEC monolayer with application of 889 P.gingivalis LPS showed no noticeable effect on the ZO-1 signal (Figure 10 D-F) (only 890 0.3 μ g/ml shown) and similar observations were made in the wells with 0.1 μ g/ml 891 *P.gingivalis* OMV application (Figure 10 G-I) compared to the untreated controls 892 (media only). The wells with application of 0.3 μ g/ml *P.gingivalis* OMVs showed a 893 more diffused signal from the ZO-1 proteins compared to untreated controls, which could appear as a reduction in the signal (Figure 10 J-L). All the test wells and 894 895 controls were imaged using the same exposure times and all post exposure 896 modifications were carried out to the same level with the Zen software. Though a 897 change in the Cy5 signal after the application of 0.3 μ g/ml *P.gingivalis* OMVs were 898 seen in all 3 repeat experiments compared to untreated controls, it was not clear if 899 the change was a displacement of the ZO-1 protein or reduced numbers as the 900 experiment here did not quantify the protein. ZO-1 is a tight junction adaptor 901 protein connecting the actin skeleton to tight junctions such as claudin and the 902 binding of Zo-1 to actin is essential for regulation of permeability in epithelial cells 903 [81]. Tornavaca et al. (2015)[82] showed that in primary endothelial cells ZO-1 is a 904 central regulator of tight junctions depending on the strictly endothelial specific 905 adhesion molecule vascular endothelial (VE) -cadherin. These endothelial junctions 906 were found to influence the spatial actomyosin organization, cell-cell tension and

907 migration across the endothelium, but also angiogenesis and barrier formation. This 908 study used human dermal microvascular endothelial cells (HDMEC-c) and not 909 HBMECs, but this places ZO-1 central to the development, integrity and maintenance 910 of the BBB and if *P.gingivalis* OMVs are able to disrupt the functionality of ZO-1 this 911 could have devastating consequences to the integrity of the blood brain interface. 912 ZO-1 is a large phosphoprotein and post-translational alterations such as 913 phosphorylation would lead to ZO-1 dissociation from the tight junction complex 914 [83], which could potentially be how *P.gingivalis* virulence factors affect this protein 915 and explain the TEER and diffusion changes observed in our study, where notably the 916 application of *P.gingivalis* OMVs had the greatest effect on both measurements. 917 Multiple studies have investigated the effect of *P.gingivalis* LPS and OMVs on cells, 918 both human and animal, but not on cells of the human BBB. It was clear from our 919 study that the *P.gingivalis* LPS both unconjugated and FITC conjugated and OMVs did 920 affect the cells of this in vitro BBB model and caused barrier integrity changes. There 921 is potential to investigate more nuanced changes in the cells by using the protocol 922 described here and applying further protocols which could also potentially reveal 923 changes at the lower concentration of LPS level which the TEER and percent 924 appearance methods did not have the sensitivity to reveal.

925 <u>CONCLUSION</u>

926The two virulence factors of *P.gingivalis* (LPS and OMVs) were seen to induce927changes in the human *in vitro* BBB model cells. Unconjugated *P.gingivalis* LPS, FITC928conjugated *P.gingivalis* LPS with 10 μg/ml of OMV and OMVs alone had a significant929effect on the integrity of the *in vitro* BBB model which were measurable by TEER930showing a significantly greater magnitude of change after application and a931significant deficit in recovery of the models TEER values. These changes were also

932 observed after application of virulence factors at a physiological relevant level (0.3 933 µg/ml). The application of *P. gingivalis* OMVs resulted in the most significant 934 magnitude of change in the TEER values of the barrier and the most significant 935 deficit of recovery. These experiments also confirmed that endotoxin from 936 *P.gingivalis* conjugated with FITC was able to cross the barrier model and that the 937 percentage of LPS conjugate appearing on the CNS side of the model were increased 938 by the presence of *P.gingivalis* OMVs. The ZO-1 proteins in a HBMEC monolayer 939 model showed disruption after contact with P.gingivalis OMVs compared to 940 controls.

941 Further investigations at cellular level are warranted to contribute to the knowledge 942 pool of how endotoxin from periodontal disease could have an influence on 943 neuroinflammatory states and potentially contribute to or exacerbate neurodegeneration. If these observations are applied to the human BBB, then 944 945 infection with P.gingivalis and their inflammagens LPS and OMVs could cause 946 damage to an otherwise healthy and non-predisposed individual. It is tempting to 947 attribute the link between sporadic Alzheimer's Disease and periodontal disease to 948 the effect of this multi-talented pathogen, when the chronicity of the inflammatory 949 state seen in established periodontal disease could be the main culprit. This 950 highlights not only the need for good oral hygiene, but also the importance of 951 diagnosis and optimal management of dental patients presenting with unstable 952 periodontal disease. Until there is a therapeutic remedy which can protect the BBB 953 from chronic inflammation, prevention remains the key.

954ACKNOWLEDGEMENTSAP acknowledges funding from the Faculty of Dental Surgery,955Royal College of Surgeons England pump priming grant and the kind donation from956The Norah and Fred Roberts Memorial Trust.

957 <u>CONFLICT OF INTEREST</u>

958 The authors declare no conflict of interest.

959

960 <u>Supplementary information:</u>



961

962 P.gingivalis FDC 381 Gram stain and on FAA Neomycin (E&O, UK) shows
963 coccobacillus at x100 and Black stain colonies of the strain with haemolytic halos on
964 NEOFAA agar.

965

966 <u>References</u>

Alonso R, Pisa D, Marina AI, Morato E, Rábano A, Carrasco L. Fungal infection in patients with
 Alzheimer's disease. J Alzheimers Dis. 2014;41(1):301-11. doi: 10.3233/JAD-132681. PMID:
 24614898.

Miklossy J. Bacterial Amyloid and DNA are Important Constituents of Senile Plaques: Further
 Evidence of the Spirochetal and Biofilm Nature of Senile Plaques. J Alzheimers Dis. 2016 Jun
 13;53(4):1459-73. doi: 10.3233/JAD-160451. PMID: 27314530; PMCID: PMC4981904.

3. Itzhaki RF, Lin WR, Shang D, Wilcock GK, Faragher B, Jamieson GA. Herpes simplex virus type
1 in brain and risk of Alzheimer's disease. Lancet. 1997 Jan 25;349(9047):241-4. doi: 10.1016/S01406736(96)10149-5. PMID: 9014911.

Pritchard AB, Crean S, Olsen I, Singhrao SK. Periodontitis, Microbiomes and their Role in
 Alzheimer's Disease. Front Aging Neurosci. 2017 Oct 24;9:336. doi: 10.3389/fnagi.2017.00336. PMID:
 29114218; PMCID: PMC5660720.

Braak H, Braak E. Morphologie des Morbus Alzheimer [Morphology of Alzheimer disease].
 Fortschr Med. 1990 Nov 20;108(33):621-4. German. Erratum in: Fortschr Med 1991 Mar
 20;109(8):186. PMID: 2289729.

- Fulop T, Tripathi S, Rodrigues S, Desroches M, Bunt T, Eiser A, Bernier F, Beauregard PB,
 Barron AE, Khalil A, Plotka A, Hirokawa K, Larbi A, Bocti C, Laurent B, Frost EH, Witkowski JM.
 Targeting Impaired Antimicrobial Immunity in the Brain for the Treatment of Alzheimer's Disease.
 Neuropsychiatr Dis Treat. 2021 May 4;17:1311-1339. doi: 10.2147/NDT.S264910. PMID: 33976546;
 PMCID: PMC8106529.
- 987 7. Guo, T., Zhang, D., Zeng, Y. et al. Molecular and cellular mechanisms underlying the
 988 pathogenesis of Alzheimer's disease. Mol Neurodegeneration 15, 40 (2020).
 989 https://doi.org/10.1186/s13024-020-00391-7
- Sevenich Lisa, Brain-Resident Microglia and Blood-Borne Macrophages Orchestrate Central
 Nervous System Inflammation in Neurodegenerative Disorders and Brain Cancer, Frontiers in
 Immunology, Vol. 9, 2018, p 697, https://www.frontiersin.org/article/10.3389/fimmu.2018.00697,
 DOI=10.3389/fimmu.2018.00697, ISSN=1664-3224
- 9949.Soscia SJ, Kirby JE, Washicosky KJ, et al. The Alzheimer's disease-associated amyloid beta-995protein is an antimicrobial peptide. PLoS One. 2010;5(3):e9505. doi:10.1371/journal.pone.0009505
- Sumar DK, Choi SH, Washicosky KJ, et al. Amyloid-β peptide protects against microbial
 infection in mouse and worm models of Alzheimer's disease. Sci Transl Med. 2016;8(340):340ra72.
 doi:10.1126/scitranslmed.aaf1059
- 999 11. Hajishengallis G, Darveau RP, Curtis MA. The keystone-pathogen hypothesis. Nat Rev1000 Microbiol. 2012;10(10):717–725.
- 1001 12. Hajishengallis G, Lamont RJ. Beyond the red complex and into more complexity: the
 1002 polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology. Mol Oral Microbiol.
 1003 2012;27(6):409–419.
- 13. Olsen, I., Kell, D. B., & Pretorius, E. (2020). Is Porphyromonas gingivalis involved in
 Parkinson's disease?. European journal of clinical microbiology & infectious diseases : official
 publication of the European Society of Clinical Microbiology, 39(11), 2013–2018.
 https://doi.org/10.1007/s10096-020-03944-2
- 1008 14. Holmstrup P, Damgaard C, Olsen I, Klinge B, Flyvbjerg A, Nielsen CH, Hansen PR. Comorbidity
 1009 of periodontal disease: two sides of the same coin? An introduction for the clinician. J Oral
 1010 Microbiol. 2017;9(1):1332710.
- 1011 15. Kozak, M., Dabrowska-Zamojcin, E., Mazurek-Mochol, M., & Pawlik, A. (2020). Cytokines and
 1012 Their Genetic Polymorphisms Related to Periodontal Disease. Journal of clinical medicine, 9(12),
 1013 4045. https://doi.org/10.3390/jcm9124045
- 1014 16. Forner L, Larsen T, Kilian M, Holmstrup P. Incidence of bacteremia after chewing, tooth
 1015 brushing and scaling in individuals with periodontal inflammation. J Clin Periodontol. 2006
 1016 Jun;33(6):401-7. doi: 10.1111/j.1600-051X.2006.00924.x. PMID: 16677328.
- 1017 17. Dominy SS, Lynch C, Ermini F, Benedyk M, Marczyk A, Konradi A, Nguyen M, Haditsch U,
 1018 Raha D, Griffin C, Holsinger LJ, Arastu-Kapur S, Kaba S, Lee A, Ryder MI, Potempa B, Mydel P,
 1019 Hellvard A, Adamowicz K, Hasturk H, Walker GD, Reynolds EC, Faull RLM, Curtis MA, Dragunow M,
 1020 Potempa J. Porphyromonas gingivalis in Alzheimer's disease brains: Evidence for disease causation
 1021 and treatment with small-molecule inhibitors. Sci Adv. 2019 Jan 23;5(1):eaau3333. doi:
 1022 10.1126/sciadv.aau3333. PMID: 30746447; PMCID: PMC6357742.

- 1023 18. Poole S, Singhrao SK, Kesavalu L, Curtis MA, Crean S (2013) Determining the presence of
 1024 periodontopathic virulence factors in short-term postmortem Alzheimer's disease brain tissue. J
 1025 Alzheimers Dis 36, 665–677.
- 1026 19. Poole S, Singhrao SK, Chukkapalli S, Rivera M, Velsko I, Kesavalu L, Crean SJ. Active invasion
 1027 of Porphyromonas gingivalis and infection-induced complement activation in ApoE-/- mice brains. J
 1028 Alzheimers Dis. 2015;43(1):67–80.
- 1029 20. Ilievski V, Zuchowska PK, Green SJ, Toth PT, Ragozzino ME, Le K, Aljewari HW, O'Brien1030 Simpson NM, Reynolds EC, Watanabe K (2018) Chronic oral application of a periodontal pathogen
 1031 results in brain inflammation, neurodegeneration and amyloid beta production in wild type mice.
 1032 PLoS One 13, e0204941.
- 103321Dorn BR, Burks JN, Seifert KN, Progulske-Fox A. Invasion of endothelial and epithelial cells by1034strains of Porphyromonas gingivalis. FEMS Microbiol Lett. 2000;187:139–44.
- Yoshino T, Laine ML, Van Winkelhoff AJ, Dahlén G, Genotype variation and capsular
 serotypes of Porphyromonas gingivalis from chronic periodontitis and periodontal abscesses, FEMS
 Microbiology Letters, Volume 270, Issue 1, May 2007, Pages 75–81, https://doi.org/10.1111/j.15746968.2007.00651.x
- Ha JY, Choi SY, Lee JH, Hong SH, Lee HJ. Delivery of Periodontopathogenic Extracellular
 Vesicles to Brain Monocytes and Microglial IL-6 Promotion by RNA Cargo. Front Mol Biosci. 2020 Nov
 24;7:596366. doi: 10.3389/fmolb.2020.596366. PMID: 33330627; PMCID: PMC7732644.
- Veith PD, Chen YY, Gorasia DG, Chen D, Glew MD, O'Brien-Simpson NM, et al.
 Porphyromonas gingivalis outer membrane vesicles exclusively contain outer membrane and
 periplasmic proteins and carry a cargo enriched with virulence factors. J Proteome Res.
 2014;13:2420–32. doi: 10.1021/pr401227e.
- Abe N, Kadowaki T, Okamoto K, Nakayama K, Ohishi M, Yamamoto K. Biochemical and
 functional properties of lysine-specific cysteine proteinase (Lys-gingipain) as a virulence factor of
 Porphyromonas gingivalis in periodontal disease. J Biochem. 1998 Feb;123(2):305-12. doi:
 10.1093/oxfordjournals.jbchem.a021937. PMID: 9538207.
- 105026Olsen, I., & Amano, A. (2015). Outer membrane vesicles offensive weapons or good1051Samaritans?. Journal of oral microbiology, 7, 27468. https://doi.org/10.3402/jom.v7.27468
- Furuta N, Takeuchi H, Amano A. Entry of Porphyromonas gingivalis outer membrane vesicles
 into epithelial cells causes cellular functional impairment. Infect Immun. 2009;77:4761–70. doi:
 10.1128/IAI.00841-09.
- 105528Hajishengallis G. (2011). Immune evasion strategies of Porphyromonas gingivalis. Journal of1056oral biosciences, 53(3), 233–240. https://doi.org/10.2330/joralbiosci.53.233
- Meghil, M. M., Tawfik, O. K., Elashiry, M., Rajendran, M., Arce, R. M., Fulton, D. J.,
 Schoenlein, P. V., & Cutler, C. W. (2019). Disruption of Immune Homeostasis in Human Dendritic
 Cells via Regulation of Autophagy and Apoptosis by Porphyromonas gingivalis. Frontiers in
 immunology, 10, 2286. https://doi.org/10.3389/fimmu.2019.02286
- 1061 30 Deatherage BL, Cookson BT. Membrane vesicle release in bacteria, eukaryotes, and archaea:
 1062 a conserved yet underappreciated aspect of microbial life. Infect Immun. 2012;80:1948–57.

1063 31 Nativel, B., Couret, D., Giraud, P. et al. Porphyromonas gingivalis lipopolysaccharides act
1064 exclusively through TLR4 with a resilience between mouse and human. Sci Rep 7, 15789 (2017).
1065 https://doi.org/10.1038/s41598-017-16190-y

106632Muoio V, Persson PB, Sendeski MM. The neurovascular unit - concept review. Acta Physiol1067(Oxf). 2014 Apr;210(4):790-8. doi: 10.1111/apha.12250. PMID: 24629161.

1068 33 Cipolla MJ. The Cerebral Circulation. San Rafael (CA): Morgan & Claypool Life Sciences; 2009.
1069 Chapter 2, Anatomy and Ultrastructure. Available from:

1070 https://www.ncbi.nlm.nih.gov/books/NBK53086.

1071 34 Sweeney MD, Sagare AP, Zlokovic BV. Blood-brain barrier breakdown in Alzheimer disease
1072 and other neurodegenerative disorders. Nat Rev Neurol. 2018 Mar;14(3):133-150. doi:
10.1038/nrneurol.2017.188. Epub 2018 Jan 29. PMID: 29377008; PMCID: PMC5829048.

- 107435Anette Wolff, Maria Antfolk, Birger Brodin, Maria Tenje, In Vitro Blood–Brain Barrier1075Models—An Overview of Established Models and New Microfluidic Approaches, Journal of1076Pharmaceutical Sciences, Volume 104, Issue 9,2015, Pages 2727-2746, ISSN 0022-3549,
- 1077 https://doi.org/10.1002/jps.24329.
- 107836Verheggen, I.C.M., de Jong, J.J.A., van Boxtel, M.P.J. et al. Increase in blood-brain barrier1079leakage in healthy, older adults. GeroScience 42, 1183–1193 (2020).
- 1080 https://doi.org/10.1007/s11357-020-00211-2
- 1081 37 Lochhead Jeffrey J., Yang Junzhi, Ronaldson Patrick T., Davis Thomas P.,
- Structure, Function, and Regulation of the Blood-Brain Barrier Tight Junction in Central Nervous
 System Disorders, Frontiers in Physiology, 11, 2020,914,
- 1084 URL=https://www.frontiersin.org/article/10.3389/fphys.2020.00914 DOI=10.3389/fphys.2020.00914
- 1085
- 1086
 38
 Brown GC. The endotoxin hypothesis of neurodegeneration. J Neuroinflammation. 2019 Sep

 1087
 13;16(1):180. doi: 10.1186/s12974-019-1564-7. PMID: 31519175; PMCID: PMC6744684.
- 1088

Methia N, André P, Hafezi-Moghadam A, Economopoulos M, Thomas KL, Wagner DD. ApoE
deficiency compromises the blood brain barrier especially after injury. Mol Med. 2001
Dec;7(12):810-5. PMID: 11844869; PMCID: PMC1950012.

- 40 Montagne, A., Nation, D.A., Sagare, A.P. et al. APOE4 leads to blood-brain barrier
 dysfunction predicting cognitive decline. Nature 581, 71–76 (2020). https://doi.org/10.1038/s41586020-2247-3
- 1095 41 Dando SJ, Mackay-Sim A, Norton R, et al. Pathogens penetrating the central nervous system:
 1096 infection pathways and the cellular and molecular mechanisms of invasion. Clin Microbiol Rev.
 1097 2014;27(4):691–726. doi:10.1128/CMR.00118-13
- Coureuil M, Lécuyer H, Bourdoulous S, Nassif X. A journey into the brain: insight into how
 bacterial pathogens cross blood-brain barriers. Nat Rev Microbiol. 2017 Mar;15(3):149-159. doi:
 10.1038/nrmicro.2016.178. Epub 2017 Jan 16. PMID: 28090076.

Takeuchi, H., Sasaki, N., Yamaga, S., Kuboniwa, M., Matsusaki, M., & Amano, A. (2019).
Porphyromonas gingivalis induces penetration of lipopolysaccharide and peptidoglycan through the
gingival epithelium via degradation of junctional adhesion molecule 1. PLoS pathogens, 15(11),
e1008124. https://doi.org/10.1371/journal.ppat.1008124

Sheets, S. M., Potempa, J., Travis, J., Casiano, C. A., & Fletcher, H. M. (2005). Gingipains from
Porphyromonas gingivalis W83 induce cell adhesion molecule cleavage and apoptosis in endothelial
cells. Infection and immunity, 73(3), 1543–1552. https://doi.org/10.1128/IAI.73.3.1543-1552.2005

Yuhan He, Noriko Shiotsu, Yoko Uchida-Fukuhara, Jiajie Guo, Yao Weng, Mika Ikegame, Ziyi
Wang, Kisho Ono, Hiroshi Kamioka, Yasuhiro Torii, Akira Sasaki, Kaya Yoshida, Hirohiko Okamura,
Outer membrane vesicles derived from Porphyromonas gingivalis induced cell death with disruption
of tight junctions in human lung epithelial cells, Archives of Oral Biology, Volume 118, 2020, 104841,
ISSN 0003-9969, https://doi.org/10.1016/j.archoralbio.2020.104841.

1113 46 Solleiro-Villavicencio H, Rivas-Arancibia S, Effect of Chronic Oxidative Stress on

- 1114 Neuroinflammatory Response Mediated by CD4+T Cells in Neurodegenerative Diseases, Frontiers in
- 1115 Cellular Neuroscience, 12, 2018,114
- 1116 URL=https://www.frontiersin.org/article/10.3389/fncel.2018.00114
- 1117 DOI=10.3389/fncel.2018.00114

47 Banks, W.A., Gray, A.M., Erickson, M.A. et al. Lipopolysaccharide-induced blood-brain barrier
disruption: roles of cyclooxygenase, oxidative stress, neuroinflammation, and elements of the
neurovascular unit. J Neuroinflammation 12, 223 (2015). https://doi.org/10.1186/s12974-015-04341

48 Wang, X., Xue, G.-X., Liu, W.-C., Shu, H., Wang, M., Sun, Y., Liu, X., Sun, Y.E., Liu, C.-F., Liu, J.,
Liu, W. and Jin, X. (2017), Melatonin alleviates lipopolysaccharide-compromised integrity of blood–
brain barrier through activating AMP-activated protein kinase in old mice. Aging Cell, 16: 414-421.
https://doi.org/10.1111/acel.12572

Pflanzner T, Kuhlmann CR, Pietrzik CU. Blood-brain-barrier models for the investigation of
transporter- and receptor-mediated amyloid-β clearance in Alzheimer's disease. Curr Alzheimer Res.
2010 Nov;7(7):578-90. doi: 10.2174/156720510793499066. PMID: 20704558.

1129

1130 50 Kumar, S., Shaw, L., Lawrence, C., Lea, R. and Alder, J. (2014) 'P50 * Developing a

1131 Physiologically Relevant Blood Brain Barrier Model for the Study of Drug Disposition

in Glioma', Neuro-Oncology, 16(suppl 6), p. vi8-vi8. doi: 10.1093/neuonc/nou249.38

1133

1134 51 Hughes, P., Marshall, D., Reid, Y., Parkes, H. & Gelber, C. (2007). The costs of using

unauthenticated, over-passaged cell lines: how much more data do we need? Biotechniques, 43,575, 577-8, 581-2

Srinivasan, B., Kolli, A. R., Esch, M. B., Abaci, H. E., Shuler, M. L., & Hickman, J. J. (2015). TEER
measurement techniques for in vitro barrier model systems. Journal of laboratory automation, 20(2),
107–126. https://doi.org/10.1177/2211068214561025

Twain, Tamara Akeel abdulmunim, Alder, Jane Elizabeth , Sabagh, Bassem, Shaw, Andrew,
Burrow, Andrea Julie and Singh, Kamalinder (2021) Tailoring functional nanostructured lipid carriers
for glioblastoma treatment with enhanced permeability through in-vitro 3D BBB/BBTB models.
Materials Science and Engineering: C, 121 (111774). ISSN 0928-4931

Seyama M, Yoshida K, Yoshida K, Fujiwara N, Ono K, Eguchi T, Kawai H, Guo J, Weng Y, Haoze
Y, Uchibe K, Ikegame M, Sasaki A, Nagatsuka H, Okamoto K, Okamura H, Ozaki K. Outer membrane
vesicles of Porphyromonas gingivalis attenuate insulin sensitivity by delivering gingipains to the liver.
Biochim Biophys Acta Mol Basis Dis. 2020 Jun 1;1866(6):165731. doi: 10.1016/j.bbadis.2020.165731.
Epub 2020 Feb 20. PMID: 32088316.

Danaei M, Dehghankhold M, Ataei S, Hasanzadeh Davarani F, Javanmard R, Dokhani A,
Khorasani S, Mozafari MR. Impact of Particle Size and Polydispersity Index on the Clinical
Applications of Lipidic Nanocarrier Systems. Pharmaceutics. 2018 May 18;10(2):57. doi:
10.3390/pharmaceutics10020057. PMID: 29783687; PMCID: PMC6027495.

Helms HC, Abbott NJ, Burek M, Cecchelli R, Couraud PO, Deli MA, Förster C, Galla HJ,
Romero IA, Shusta EV, Stebbins MJ, Vandenhaute E, Weksler B, Brodin B. In vitro models of the
blood-brain barrier: An overview of commonly used brain endothelial cell culture models and
guidelines for their use. J Cereb Blood Flow Metab. 2016 May;36(5):862-90. doi:

1157 10.1177/0271678X16630991. Epub 2016 Feb 11. PMID: 26868179; PMCID: PMC4853841.

1158 57 <u>l</u>arosh OA. Matematicheskiĭ analiz pronitsaemosti gematoéntsefalicheskogo ber'era pri
1159 bakterial'nom meningoéntsefalite [Mathematical analysis of permeability of the blood-brain barrier
1160 in bacterial meningoencephalitis]. Zh Nevropatol Psikhiatr Im S S Korsakova. 1992;92(2):33-6.
1161 Russian. PMID: 1326169.

1162 58. Kanoh Y, Ohara T, Akahoshi T. Acute inflammatory biomarkers in cerebrospinal fluid as
1163 indicators of blood cerebrospinal fluid barrier damage in Japanese subjects with infectious
1164 meningitis. Clin Lab. 2011;57(1-2):37-46. PMID: 21391463.

Blufstein, A, Behm, C, Nguyen, PQ, Rausch-Fan, X, Andrukhov, O. Human periodontal
ligament cells exhibit no endotoxin tolerance upon stimulation with Porphyromonas gingivalis
lipopolysaccharide. J Periodont Res. 2018; 53: 589– 597. https://doi.org/10.1111/jre.12549

1168

Hirasawa, M., & Kurita-Ochiai, T. (2018). Porphyromonas gingivalis Induces Apoptosis and
Autophagy via ER Stress in Human Umbilical Vein Endothelial Cells. Mediators of inflammation, 2018,
1967506. https://doi.org/10.1155/2018/1967506

117261Wilhelm I, Fazakas C and Krizbai I A, In vitro models of the blood-brain barrier, Acta1173Neurobiol Exp 2011, 71: 113–128

Hoffmann, A., Bredno, J., Wendland, M., Derugin, N., Ohara, P., & Wintermark, M. (2011).
High and Low Molecular Weight Fluorescein Isothiocyanate (FITC)-Dextrans to Assess Blood-Brain
Barrier Disruption: Technical Considerations. Translational stroke research, 2(1), 106–111.
https://doi.org/10.1007/s12975-010-0049-x

Fabry Z, Fitzsimmons KM, Herlein JA, Moninger TO, Dobbs MB, Hart MN. Production of the
cytokines interleukin 1 and 6 by murine brain microvessel endothelium and smooth muscle
pericytes. J Neuroimmunol. 1993 Aug;47(1):23-34. doi: 10.1016/0165-5728(93)90281-3. PMID:
8376546.

- 1182 64 Behm C, Blufstein A, Abhari SY, Koch C, Gahn J, Schäffer C, Moritz A, Rausch-Fan X,
- Andrukhov O. Response of Human Mesenchymal Stromal Cells from Periodontal Tissue to LPS
 Depends on the Purity but Not on the LPS Source. Mediators Inflamm. 2020 Jul 2;2020:8704896. doi:
 10.1155/2020/8704896. PMID: 32714091; PMCID: PMC7352132.
- 1186 65 Olsen, I., & Progulske-Fox, A. (2015). Invasion of Porphyromonas gingivalis strains into 1187 vascular cells and tissue. Journal of oral microbiology, 7, 28788.
- 1188 https://doi.org/10.3402/jom.v7.28788
- 1189 66 Coats, S. R., Kantrong, N., To, T. T., Jain, S., Genco, C. A., McLean, J. S., & Darveau, R. P.
 (2019). The Distinct Immune-Stimulatory Capacities of Porphyromonas gingivalis Strains 381 and
 1191 ATCC 33277 Are Determined by the fimB Allele and Gingipain Activity. Infection and immunity,
 1192 87(12), e00319-19. https://doi.org/10.1128/IAI.00319-19
- Bergmann, S., Lawler, S. E., Qu, Y., Fadzen, C. M., Wolfe, J. M., Regan, M. S., Pentelute, B. L.,
 Agar, N., & Cho, C. F. (2018). Blood-brain-barrier organoids for investigating the permeability of CNS
 therapeutics. Nature protocols, 13(12), 2827–2843. https://doi.org/10.1038/s41596-018-0066-x
- 119668Nádházi Z, Takáts A, Offenmüller K, Bertók L. Plasma endotoxin level of healthy donors. Acta1197Microbiol Immunol Hung. 2002;49:151–157. doi: 10.1556/AMicr.49.2002.1.15.
- Kalash D, Vovk A, Huang H, Aukhil I, Wallet SM, Shaddox LM. Influence of periodontal
 therapy on systemic lipopolysaccharides in children with localized aggressive periodontitis. Pediatr
 Dent. 2015;37:35–40.
- 70 Wahaidi VY, Kowolik MJ, Eckert GJ, Galli DM. Endotoxemia and the host systemic response
 during experimental gingivitis. J Clin Periodontol. 2011;38:412–417. doi: 10.1111/j.1600051X.2011.01710.x.
- Thang R, Miller RG, Gascon R, et al. Circulating endotoxin and systemic immune activation in
 sporadic amyotrophic lateral sclerosis (sALS) J Neuroimmunol. 2009;206:121–124. doi:
 10.1016/j.jneuroim.2008.09.017.
- 1207
- 1208 72 Zhao Y, Jaber V, Lukiw WJ. Secretory products of the human GI tract microbiome and their
 1209 potential impact on Alzheimer's disease (AD): detection of lipopolysaccharide (LPS) in AD
 1210 hippocampus. Front Cell Infect Microbiol. 2017;7:318. doi: 10.3389/fcimb.2017.00318.
- 1211 73 Vargas-Caraveo, A., Sayd, A., Maus, S.R. et al. Lipopolysaccharide enters the rat brain by a
 1212 lipoprotein-mediated transport mechanism in physiological conditions. Sci Rep 7, 13113 (2017).
 1213 https://doi.org/10.1038/s41598-017-13302-6
- 1214 74 Jaeger LB, Dohgu S, Sultana R, et al. Lipopolysaccharide alters the blood–brain barrier
 1215 transport of amyloid β protein: a mechanism for inflammation in the progression of Alzheimer's
 1216 disease. Brain Behav Immun. 2009;23:507–517. doi: 10.1016/j.bbi.2009.01.017.
- 121775Bryant CE, Spring DR, Gangloff M, et al. The molecular basis of the host response to1218lipopolysaccharide. Nat Rev Microbiol. 2010;8:8–14. doi: 10.1038/nrmicro2266.
- 121976Morris MC, Gilliam EA, Li L. Innate immune programing by endotoxin and its pathological1220consequences. Front Immunol. 2015;5:680. doi: 10.3389/fimmu.2014.00680.

- 1221 77 Wendeln AC, Degenhardt K, Kaurani L, Gertig M, Ulas T, Jain G, Wagner J, Häsler LM, Wild K,
 1222 Skodras A, Blank T, Staszewski O, Datta M, Centeno TP, Capece V, Islam MR, Kerimoglu C, Staufenbiel
 1223 M, Schultze JL, Beyer M, Prinz M, Jucker M, Fischer A, Neher JJ. Innate immune memory in the brain
 1224 shapes neurological disease hallmarks. Nature. 2018;556:332–338. doi: 10.1038/s41586-018-0023-4.
- 1225 78 Sandiego CM, Gallezot JD, Pittman B, Nabulsi N, Lim K, Lin SF, Matuskey D, Lee JY, O'Connor
 1226 KC, Huang Y, Carson RE, Hannestad J, Cosgrove KP. Imaging robust microglial activation after
 1227 lipopolysaccharide administration in humans with PET. Proc Natl Acad Sci U S A. 2015;112:12468–
 12473. doi: 10.1073/pnas.1511003112.
- Skelly DT, Hennessy E, Dansereau MA, Cunningham C. A systematic analysis of the peripheral
 and CNS effects of systemic LPS, IL-1β, TNF-α and IL-6 challenges in C57BL/6 mice. PLoS One.
 2013;8:e69123. doi: 10.1371/journal.pone.0069123.
- 1232 80. Eigenmann DE, Xue G, Kim KS, Moses AV, Hamburger M, Oufir M. Comparative study of four
 1233 immortalized human brain capillary endothelial cell lines, hCMEC/D3, hBMEC, TY10, and BB19, and
 1234 optimization of culture conditions, for an in vitro blood-brain barrier model for drug permeability
 1235 studies. Fluids Barriers CNS. 2013 Nov 22;10(1):33. doi: 10.1186/2045-8118-10-33. PMID: 24262108;
 1236 PMCID: PMC4176484.
- Belardi B, Hamkins-Indik T, Harris AR, Kim J, Xu K, Fletcher DA. A Weak Link with Actin
 Organizes Tight Junctions to Control Epithelial Permeability. Dev Cell. 2020 Sep 28;54(6):792-804.e7.
 doi: 10.1016/j.devcel.2020.07.022. Epub 2020 Aug 24. PMID: 32841596; PMCID: PMC7530003.
- Tornavaca, O., Chia, M., Dufton, N., Almagro, L. O., Conway, D. E., Randi, A. M., Schwartz, M.
 A., Matter, K., & Balda, M. S. (2015). ZO-1 controls endothelial adherens junctions, cell-cell tension,
 angiogenesis, and barrier formation. The Journal of cell biology, 208(6), 821–838.
 https://doi.org/10.1083/jcb.201404140
- Stamatovic, S. M., Johnson, A. M., Keep, R. F., & Andjelkovic, A. V. (2016). Junctional proteins
 of the blood-brain barrier: New insights into function and dysfunction. Tissue barriers, 4(1),
 a1154641. https://doi.org/10.1080/21688270.2016.1154641
- 1246 e1154641. https://doi.org/10.1080/21688370.2016.1154641
- 1247