

**Title:** Cerebral oxidative stress and microvasculature defects in TNF- $\alpha$  expressing transgenic and *Porphyromonas gingivalis*-infected ApoE<sup>-/-</sup> mice

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## Abstract

The polymicrobial dysbiotic subgingival biofilm microbes associated with periodontal disease appear to contribute to developing pathologies in distal body sites, including the brain. This study examined oxidative stress, in the form of increased protein carbonylation and oxidative protein damage, in the tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) transgenic mouse that models inflammatory TNF- $\alpha$  excess during bacterial infection; and in the apolipoprotein knockout (ApoE<sup>-/-</sup>) mouse brains, following *Porphyromonas gingivalis* gingival monoinfection. Following 2,4-dinitrophenylhydrazine derivatization, carbonyl groups were detected in frontal lobe brain tissue lysates by immunoblotting and immunohistochemical analysis of fixed tissue sections from the frontotemporal lobe and the hippocampus. Immunoblot analysis confirmed the presence of variable carbonyl content and oxidative protein damage in all lysates, with TNF- $\alpha$  transgenic blots exhibiting increased protein carbonyl content, with consistently prominent bands at 25 kDa ( $p = 0.0001$ ), 43 kDa and 68 kDa, over wild-type mice. Compared to sham-infected ApoE<sup>-/-</sup> mouse blots, *P. gingivalis*-infected brain tissue blots demonstrated the greatest detectable protein carbonyl content overall, with numerous prominent bands at 25 kDa ( $p = 0.001$ ) and 43 kDa ( $p = 0.0001$ ) and an exclusive band to this group between 30-43 kDa\* ( $p = 0.0001$ ). In addition, marked immunostaining was detected exclusively in the microvasculature in *P. gingivalis*-infected hippocampal tissue sections, compared to sham-infected, wild-type and TNF- $\alpha$  transgenic mice. This study revealed that the hippocampal microvascular structure of *P. gingivalis*-infected ApoE<sup>-/-</sup> mice possesses elevated oxidative stress levels, resulting in the associated tight junction proteins being susceptible to increased oxidative/proteolytic degradation, leading to a loss of functional integrity.

**Keywords:** Hippocampus, infection, microvasculature, oxidative stress/damage, *Porphyromonas gingivalis*, tight junction proteins.

## Introduction

Oxidative stress is implicated in the onset, progression and pathogenesis of numerous diseases and conditions, including periodontal disease, neurodegeneration and ageing [1-8]. Prolonged episodes of oxidative stress produces an excessive accumulation of reactive oxygen species (ROS), capable of inducing deleterious cellular and biomolecular damage to the host [1-5]. Neurons are particularly sensitive to ROS-mediated, oxidative damage, as they contain higher numbers of mitochondria along their cell bodies and axonal/dendritic tree lengths, compared to other cell types. The ROS-mediated oxidative damage within the brain can potentially lead to neuronal dysfunction, with a likely outcome of Alzheimer's disease (AD) pathology [3, 6, 8, 9]. In the ageing brain and during AD, the APOE4 genetic susceptibility further capable of increasing the possibility of recurrent infections, leakage of plasma proteins, ROS and metallic ion production (iron from erythrocyte death), adding to a growing pool of oxidative stress mediators within the brain. Furthermore, oral and extra-oral bacteria are documented to be present within AD brains [10], where these are likely to promote additional ROS generation and host tissue damage. During chronic periodontal infection, oral pathobionts, such as *Porphyromonas gingivalis* (*P. gingivalis*), drive the host's immunoinflammatory responses towards elevated levels of pro-inflammatory cytokines, such as tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL) IL-1 $\beta$  and IL-6; leading to elevated oxidative stress [11]. This is particularly achieved by the interplay between the phagocytic oxidative burst of the host's neutrophils and macrophages, coupled with the oxidative stress response initiated by bacteria [12]. Furthermore, live *P. gingivalis* infection is likely to exert its effect on the host through a diverse range of virulence factors. Apart from its lipopolysaccharide (LPS), which can cause the host to respond through oxidative burst and other mechanisms, proteolytic activity mediated by *P. gingivalis* gingipains are capable of targetting cell-cell adhesion molecules [13, 14, 15]. It is, therefore, likely that gingipains also contribute to the degradation of endothelial cell tight junction proteins and the subsequent loss of blood-brain barrier (BBB) functional integrity. Oxidative stress from host response also appears to affect a multitude of other activities relevant to AD, including activation of positive feedback between the  $\gamma$ - and  $\beta$ -secretase cleavage of the amyloid- $\beta$  protein precursor (A $\beta$ PP) metabolic product, amyloid- $\beta$  (A $\beta$ ) [16]; and the formation of the inflammasome

assembly which has subsequent effects on the liberation of mature forms of IL-1 $\beta$ /IL-18 family members [17, 18]. These cytokines modulate innate immune responses against microbes by generating a subsequent inflammatory response serving important biological and physiological functions. Inappropriate secretion of cytokines result in pro-oxidant mechanisms especially in neuroinflammatory pathologies. For example, TNF- $\alpha$  has been shown to permeabilise brain microvascular endothelial cells and degrade cell-cell adhesion proteins, such as occludin [19]; and it can induce ROS production in cultured cells [20].

Since neurodegenerative diseases are of complex etiologies, often exhibiting mixed pathologies, the contribution of specific genes is best investigated using animal models. The genetically modified apolipoprotein E knockout (ApoE<sup>-/-</sup>) mouse is a good example, in order to determine the effects of peripheral infection on a host undergoing high intrinsic stress, due to the constitutive expression of the pro-inflammatory cytokine, TNF- $\alpha$  [21, 22], lipid peroxidation [23], impaired immuno-modulatory function in macrophages and innate/adaptive immune responses [24-26]; and impaired cerebellar microvessels [27-30]. The TNF- $\alpha$  transgenic mouse represents an additional model in which to explore the direct roles of the proinflammatory cytokine, TNF- $\alpha$ , in the process of local oxidative stress within the brain. In response to chronic bacterial infection, TNF- $\alpha$  release represents a proinflammatory cytokine at the apex of the inflammatory cascade that mediates local inflammatory damage including the production of localized oxidative stress and has been shown to be upregulated in response to *P. gingivalis* LPS in vivo [13-15]. In the TNF- $\alpha$  transgenic mouse, TNF- $\alpha$  mRNA is stabilized and its expression appears to be increased within all tissues, including the brain. This makes the TNF- $\alpha$  transgenic mouse an excellent control model to explore mechanistic insights into how bacterial models of oxidative stress may be mediating their actions [31].

Whilst the cerebral microvessels in TNF- $\alpha$  transgenic and ApoE<sup>-/-</sup> mice appear to be resilient to tissue damage, the cerebellar BBB in both these mouse models was leaky when tested for mouse IgG at the beginning of the investigation. In addition, the cerebral BBB in ApoE<sup>-/-</sup> mice have been shown to become leaky either due to experimentally induced physical injury [28], or as recently reported, inflammation-mediated physical injury following infection with *P. gingivalis* [32]. Indeed, this study, demonstrated that both TNF- $\alpha$  expressing transgenic mice and ApoE<sup>-/-</sup> mice share such leaky cerebellar BBB characteristics,

thereby providing a unique opportunity to draw systematic comparisons between common biological and physiological factors involved in stress initiation.

We have previously reported associations between *P. gingivalis* LPS and AD brains [33] and subsequently established that *P. gingivalis* is capable of accessing ApoE<sup>-/-</sup> mouse brains from its primary gingival location [32, 34]. Once in the brain, *P. gingivalis* modulates innate immune responses via activation of the complement cascade, resulting in bystander injury of functional pyramidal neurons in the hippocampus, following 24 week of chronic infection [34]. A recent investigation using the same ApoE<sup>-/-</sup> *P. gingivalis*-infected mice brains (24 weeks) provided evidence of BBB defects, with numerous age-related granules and cerebral tissue damage mediated via gingipains [32]. This led us to the rationale that acute phase inflammation, in the form of oxidative stress, may precede loss of the BBB integrity in these mice at an earlier timepoint of infection which was absent in ApoE<sup>-/-</sup> *P. gingivalis*-infected mice brains (24 weeks). Therefore, this study sought to critically examine how *P. gingivalis* infection (at 12 weeks) alters acute innate immune responses by increasing oxidative exposure on cerebral parenchyma; and whether this alteration can potentially lead to tissue injury and subsequent hippocampal BBB permeability which was clearly evident at 24 week infection [32].

## **Materials and Methods**

### ***Negative and positive control mice***

C57BL/6 Tg197 (TNF- $\alpha$ ) transgenic mice were obtained from Dr. George Kollias (BSRC Fleming, Athens, Greece) [31]. At 6-8 weeks, heterozygous TNF- $\alpha$  transgenic mice develop clinical signs of systemic chronic inflammation, mediated due to overexpression of TNF- $\alpha$ . Mice were housed under controlled environmental conditions ( $20.2 \pm 2^\circ\text{C}$ , 14:10 h light:dark cycle). Experiments were performed following strict guidelines governed by the UK Animal (Scientific Procedures) Act 1986 and approved (under the Home Office Licence Number granted to the study, 70/8582) by Birmingham Ethical Review Sub Committee. At ten weeks of age, wild type and TNF- $\alpha$  transgenic ( $N = 7$  per group) animals were euthanized by the humane killing of mice under Schedule 1 Code of Practice under UK Home Office Licence conditions; and the brain

tissues were removed. One hemisphere of the brain was frozen and the other half embedded in paraffin wax blocks. All specimens (frozen and paraffin wax blocks) were sent to the University of Central Lancashire, UK, for laboratory analysis following approval (RE/15/04/SS) by their Ethical Review Committee.

### ***P. gingivalis* infection mouse model**

ApoE<sup>-/-</sup> male mice (strain B6.129P2-Apoe<sup>tm1Unc/J</sup>,  $N = 12$ ) were sham-infected or monoinfected for 12 weeks via the oral route, with the *P. gingivalis* FDC 381 strain ( $5 \times 10^9$  bacteria / mL), as described previously [35]. All experimental procedures were conducted in accordance with the guidelines of the University of Florida Institutional Animal Care and Use Committee (IACUC, Protocol #201304539). All ApoE<sup>-/-</sup> mice brain specimens were collected, stored and shipped to the University of Central Lancashire, UK, for laboratory analysis, following original approval (RE/12/04) and renewed approval (RE/15/04/SS) by their Ethical Review Committee.

## **Biochemistry**

### ***Brain tissue lysates***

Unfixed brain tissue (approximately 30 mg/mice) from a section of the frontal lobe of each mouse strain (wild-type and TNF- $\alpha$  transgenic mouse groups,  $N = 7$ ; sham- and *P. gingivalis*-infected (12 week group,  $N = 9$ ), was taken and minced in lysis buffer (50 mM Tris buffer, pH 8.0, 1 % NP40, 150 mM sodium chloride and 5 mM EDTA), containing protease inhibitors (cOmplete™ ULTRA Tablets, Roche, USA) as per manufacturer's instructions. Following incubation on ice for 20 min and vortex mixing at regular intervals, the tissue homogenates were centrifuged and supernatants collected [33]. Coomassie Blue Protein Assay (Sigma-Aldrich, UK) determined total protein concentration of each lysate; and samples stored at -20°C, until required.

### ***Western blot analysis of brain tissue lysate protein carbonyl content***

Fresh frontal brain tissue lysates were subjected to derivatization with 2,4-dinitrophenylhydrazine (2,4-DNPH) and subsequent Western blot analysis, using an Oxyblot Protein Oxidation Detection Kit (Millipore UK Ltd, UK), according to manufacturer's instructions. Derivatized samples (5 µg protein/well) were separated by SDS-PAGE on 12% linear gels and electroblotted onto polyvinylidene difluoride (PVDF) membranes, as previously described [36]; and protein carbonyl-containing proteins were detected, as described in the Oxyblot Detection Kit. Chemiluminescent substrate (SuperSignal<sup>®</sup> West Pico, ThermoFisher Scientific, UK) was prepared and applied to the membranes according to manufacturer's instructions. Specific protein signal from the membranes was visualized using a ChemicDoc<sup>®</sup> (Bio-Rad, UK) and images captured with Image Lab<sup>®</sup> Software Version 3.0.1. The amount of protein loaded/lane was determined by staining the membranes with India ink. Density of the bands of interest was quantified by first importing the blot images into ImageJ and then selecting each band for its total density value using the densitometric program in ImageJ Software (<https://imagej.nih.gov/ij/download.html>, version 64-bit Java 1.8.0\_112). **Comparisons were made between raw values of intensities/lane normalized to loading control bands.**

### ***Tissue sectioning***

Formalin fixed, paraffin wax-embedded, tissue blocks from the cerebellum and the frontotemporal lobe, inclusive of the hippocampus, were sectioned (5 µm thickness) using the Leica RM2235 Microtome. Sections were collected on adhesive coated glass microscope slides.

### **Immunohistochemistry**

#### ***Analysis of brain tissue section protein carbonyl content***

Brain tissue sections from wild-type and TNF-α transgenic mice ( $N = 7$ ); and the sham-infected and *P. gingivalis*-infected mice ( $N = 12$ ), were analysed. Sections were deparaffinized, rehydrated and derivitized with 2,4-DNPH, as previously described [37]. All sections were blocked for non-specific binding with 3%

normal horse serum and immunostaining performed by overnight incubation with anti-dinitrophenyl (anti-DNP) antibody (rabbit whole antisera, Sigma-Aldrich; diluted 1/1200). The study employed three different types of negative controls, whereby (1) 2,4-DNPH derivatization was omitted, (2) free carbonyl groups were reduced with 25 mM sodium borohydride in 80% methanol, at room temperature for 30 min, before 2,4-DNPH derivatization and (3) where the primary antibody was omitted. Immunoreactivity was visualized using the Vectastain Universal Elite ABC Kit and the DAB Peroxidase Kit (both from Vector Laboratories, UK), as per manufacturer's instructions. Following a light haematoxylin based nuclear counterstain, all sections were examined using the Nikon Eclipse E200 Microscope. Images of representative tissue sites were recorded using Nikon DS-L2 v.441 Software.

### **Statistical analysis**

Data is presented as mean  $\pm$  standard deviation (SD) ( $N \geq 3$  replicates per treatment). The data is presented as non-parametric, Mann-Whitney U test for two independent samples using the SPSS version 23 software package to compare wild-type with TNF- $\alpha$  transgenic; and sham-infected with *P. gingivalis*-infected mice. Differences were considered significant at  $p \leq 0.05$ .

## **Results**

### ***Western blot analysis***

Western blot analysis from tissue lysates from a section of the frontal lobe of all the mice tested, confirmed the presence of carbonyl content and oxidative protein damage, at variable molecular weights in the range of 25-70 kDa. Wild-type mouse brain tissue blots (Fig. 1a) consistently exhibited a prominent band around 43 kDa (Fig. 1a, broken arrow), with additional lesser bands of variable intensities at 25-40 kDa and 45-70 kDa positions. The TNF- $\alpha$  transgenic mouse brain blots (Fig. 1b), exhibited prominent bands at around 25 kDa (Fig. 1b, short arrow), 43 kDa (Fig 1b, broken arrow) and 68 kDa (Fig. 1b, arrow head); and lesser intensity bands at 50-65 kDa. Sham-infected mouse brain blots (Fig. 1c), demonstrated a prominent band at around 25 kDa (Fig. 1c, short arrow), with additional bands of highly variable degrees of intensities between 29-40



kDa and 45-70 kDa. *P. gingivalis*-infected mouse brain blots (Fig. 1d), exhibited numerous prominent bands at around 25 kDa (Fig 1d, short arrow), and 43 kDa (Fig. 1d, broken arrow); and one exclusive to this group between 30-43 kDa\* (Fig. 1d, astrix), with lesser bands at 45-70 kDa. These observations were further confirmed by the analysis of intensities for the prominent bands for each experimental group, using ImageJ Software (Fig 1e). Data is from means of triplicates  $\pm$ SD using Mann-Whitney U test for two independent samples of each 25 kDa, 43 kDa, and 30-43 kDa\* and 68 kDa bands. Significant differences between the wild-type and TNF- $\alpha$  transgenic mouse groups; and between the sham-infected and *P. gingivalis*-infected groups were identified, respectively (Fig. 1e).

### ***Immunohistochemical analysis***

The three different types of negative controls employed remained negative for protein carbonyl immunostain detection. These were (1) where 2,4-DNPH derivatization was omitted (Fig. 2a), (2) where free carbonyl groups were reduced with 25 mM sodium borohydride in 80% methanol, at room temperature for 30 min, before 2,4-DNPH derivatization (Fig. 2b); and (3) where the primary antibody was excluded (Fig. 2c). Immunostaining of protein carbonyl content within the hippocampus and microvessels of wild-type and TNF- $\alpha$  transgenic mouse brains was similar to that seen in negative controls (Fig. 2d-e), as was the case with the sham-infected mice (Fig. 2f). However, in *P. gingivalis*-infected mouse brains, protein carbonyl immunostaining was distinctly observed in the vasculature, especially localized within the hippocampal capillaries (Fig. 2g). At higher magnification, the protein carbonyl immunostaining was more distinctly observed in the microvasculature (boxed areas in Fig. 2g are shown at higher magnification in Fig. 2h-i). The microvessels adjacent to the pyramidal neurons of the CA sectors in the hippocampus (Fig. 2j, box), also showed immunostaining localised to the capillaries and endothelial cells (Fig. 2k-l, endothelial cell staining is indicated with arrows).

The frontal brains of wild-type mice demonstrated protein carbonyl immunostaining, but was much weaker at the optimal antibody dilution used (Fig. 3a-b), whereas the TNF- $\alpha$  transgenic mouse tissues exhibited weaker staining of the myelin bundles (Fig. 3c) and occasional cellular staining (Fig. 3c-d). The

sham-infected mouse tissue sections demonstrated marked staining of the myelin sheath regions (Fig. 3e) and neuronal staining (Fig. 3f). The *P. gingivalis*-infected mouse tissue sections also demonstrated staining of the myelin sheath (Fig. 3g) and neurons (Fig. 3h), whilst certain pyramidal neurons appeared to be completely damaged (Fig. 3h, insert).

## Discussion

As atherosclerosis, strokes and AD share a common APOE4 genetic susceptibility, many investigators have used the ApoE<sup>-/-</sup> mice as an animal model to assess the influences of co-morbidities of these diseases, including *P. gingivalis*-mediated infection [32, 34, 35, 38, 39]. In addition, proinflammatory cytokines, such as TNF- $\alpha$ , IL-1 $\beta$  and IL-6, are relevant to early stages of AD onset [40]. From animal models, proinflammatory cytokines, such as TNF- $\alpha$ , are known to disturb inter-endothelial cell-cell connections and thereby cause BBB permeability [19]. This makes TNF- $\alpha$  transgenic mice [31] and ApoE<sup>-/-</sup> mice brains [27, 29, 30], ideal test models to investigate if they share common oxidative stress from an intrinsic biological (proinflammatory cytokine) stressor. We first established that the leaky BBB was restricted to the cerebellum and not the cerebral microvessels in both TNF- $\alpha$  transgenic and ApoE<sup>-/-</sup> mice brains. We have previously demonstrated *P. gingivalis* hematogenous incursion from gingival epithelium to aortic vascular tissues in ApoE<sup>-/-</sup> mice [35], and into the brain tissues [32, 34]. In addition, innate immune response stimulation was demonstrated by glia via the local synthesis and release of complement cascade proteins following *P. gingivalis* infection of the brain [34]. The activated complement cascade for non-specific lysing of microbes instead, led to an inappropriate attack of pyramidal neurons [34]; and also resulted in accelerated occurrence of age-related granules [32]. Upon entry into the brain following 24 weeks of chronic infection, *P. gingivalis* infection appeared to be contributing towards hippocampal cerebral tissue injury in which impairment of the BBB was evident. This suggested a preceding negative event was taking place and was contributing to the BBB defect in the infected group. To delineate overlapping cytokine related biological events during infection, inclusion of TNF- $\alpha$  transgenic mice was essential. The rationale for the initiation of the present study was, therefore, to determine if vascular exposure to *P. gingivalis* and/or the

host's acute phase responses in the form of oxidative stress and corresponding oxidative protein damage, were responsible for the development of a leaky BBB in ApoE<sup>-/-</sup> mice at an earlier timepoint of infection (12 weeks), which was not detected at the 24 week infection timepoint. Numerous AD studies have identified the accumulation of carbonylated proteins as a consequence of elevated oxidative stress [41-44]. There are no studies at present that have attempted to address whether the resulting oxidative stress exposure from the invasion of microbes can lead to the development of leaky BBB. Therefore, to the best of our knowledge, this is the first study that has evaluated the putative role of *P. gingivalis* infection in mediating BBB deterioration and its contribution to oxidative protein damage in the ApoE<sup>-/-</sup> mouse brain model, following bacterial infection.

It is interesting to note that the extent of oxidative protein damage was evident in the wild-type mouse brain tissue lysates, as a prominent band of 43 kDa. This data suggests that even wild-type mice experience intrinsic exposure to basal levels of ROS, due to by-products of normal physiological and metabolic processes. This led to the notion that the gradual accumulation of oxidative stress-related proteins was possible in these mice, as antioxidative and repair mechanisms are not being completely (100%) efficient [45-49]. Although the extent of oxidative protein damage from the frontal lobe brain region in wild-type mice was detectable by Western blotting, this was largely restricted to a single band at 43 kDa. The Western blot profiles for the TNF- $\alpha$  transgenic mice differed to those of wild-type mice, particularly by demonstrating enhanced carbonylated proteins at around 25 kDa, 43 kDa and 68 kDa. This difference may be attributable to the endogenous expression of proinflammatory cytokine and an impaired cerebellar BBB as a consequence of enhanced oxidative stress. Therefore, the endogenous antioxidant and repair systems within the brains of TNF- $\alpha$  transgenic mice were incapable of overcoming the corresponding increases in oxidative stress caused by the enhanced proinflammatory cytokine burden, resulting in increased protein carbonylation and host tissue damage. Differences in Western blot profiles were further evident between the sham-infected mice and those infected with *P. gingivalis* at similar age. *P. gingivalis*-infected mice demonstrated the highest overall protein carbonyl content among all of the experimental groups analysed, with numerous prominent bands identified at 25 kDa and 30-43 kDa\* (exclusive to the infected group) and

at 43 kDa, with lesser intensity bands at 45-70 kDa. In contrast, sham-infected mice only demonstrated a prominent band at around 25 kDa, with additional bands of highly variable degrees of intensities between 29-40 kDa and 45-70 kDa. In addition, these observations are consistent with previous reports that have shown *P. gingivalis* can not only survive host's oxidative burst, but also has the ability to subvert its own oxidative stress via rubredoxin gene activation [12]. ApoE<sup>-/-</sup> mice are also predisposed to intrinsic stress due to constitutive TNF- $\alpha$  expression [21, 22], oxidative stress-induction, lipid peroxidation [23], impaired immuno-modulatory function in macrophages and innate/adaptive immune responses [24-26]; and an impaired cerebellar BBB [27]. Thus, the prominent band at around 25 kDa, common to TNF- $\alpha$  transgenic mice, sham- and *P. gingivalis*-infection blots from ApoE<sup>-/-</sup> mice, reflects the shared oxidative stress as a consequence of the constitutive expression of TNF- $\alpha$  and cerebellar BBB defects. However, the increased acute phase inflammatory response, as shown by detectable oxidative protein content in *P. gingivalis* mouse brains, particularly at 30-43 kDa\*, are more a consequence of infection. Accordingly, this study supports the host's phagocytic cell oxidative burst initiated by *P. gingivalis* infection/pathogenicity to initiate and perpetuate BBB damage [12].

In line with Western blot data, immunohistochemical detection of protein carbonyl content in the tissue sections taken from the frontotemporal lobe inclusive of the hippocampus, only low to negligible levels were consistently seen among the wild-type and TNF- $\alpha$  transgenic mice brains. This suggested that these mice were exposed to oxidative stress, but that was below the threshold of detection by immunohistochemistry at the optimal dilution of the primary antibody tested. TNF- $\alpha$  transgenic mouse brain tissue sections showed infrequent immunostaining on myelinated nerve bundles and occasional cellular sites, particularly in non-hippocampal areas of the brain. These findings suggest certain strain and age similarities, but equally imply the existence of differences in the levels and responses to oxidative stress within these anatomical regions of the brain in the TNF- $\alpha$  transgenic mice. As these mice are associated with a persistent low-grade exposure to TNF- $\alpha$ , this alters antioxidant defences and perpetuates oxidative stress and tissue damage within these mice [50, 51]. Therefore, the detection of increased protein carbonyl levels in these mice is expected, as these mice appear to be less efficient in counteracting the elevated levels of

oxidative stress induced by TNF- $\alpha$  and the cerebellar BBB defect. In addition, live *P. gingivalis* infection is likely to exert its effect on the host through other virulence factors, such as its proteolytic activity mediated by gingipains [13-15]. Concerning the microvascular structure of the brain, it is plausible to extrapolate relevant information from cell-cell junctional protein (catenins, occludin, E-cadherin and  $\beta_1$ -integrin) disruption from in vitro infection data already documented [13-15]. Therefore, endothelial tight junction protein disruption by this periodontal pathogen to facilitate self-accessibility across the BBB is highly plausible and confirms IgG leakage into the microvasculature at later timepoints of infection [32].

It is clear that both sham- and *P. gingivalis*-infection of ApoE<sup>-/-</sup> mice at 12 weeks exhibited significant detectable protein carbonyl content by Western blotting, but immunohistochemistry showed the major difference in its staining pattern, which was the microvasculature in the hippocampus region of the brain. This observation confirms that *P. gingivalis* infection plays a significant role in increasing the hosts' oxidative stress, which subsequently has a significant pathological impact on the cerebral microvasculature. It is reported that the cerebral BBB is breached among the elderly humans and AD subjects [52], but the reasons behind it are poorly understood. One plausible reason is that the episodic nature of periodontal disease, in which patients experience recurrent periods of quiescence and active disease. The biofilm bacteria of the active (recurring) phase of periodontitis are likely to be those that have been newly 'radicalised' as suggested by Harding et al., [53] to possess greater inflammophilic virulent properties. It is highly possible that these cyclic exposures of *P. gingivalis* infections together with its 'radicalised' companion species occur during recurrent bacteremias [54], which could contribute to eventual loss of cerebral BBB integrity and function.

These findings imply that *P. gingivalis* infection significantly increases the overall oxidative stress within the brains of ApoE<sup>-/-</sup> mice, resulting in enhanced cellular and molecular damage, culminating in an accumulation of protein carbonyl content. *P. gingivalis* is a well-established mediator of infection and host-derived, tissue damage during periodontitis, where similar to AD, oxidative stress has been implicated in the pathogenesis of disease [1-7]. Indeed, *P. gingivalis* levels have been shown to correlate with oxidative stress biomarker levels during periodontal disease and other inflammatory diseases such as atherosclerosis, where

this bacterium has been implicated [55, 56]. Therefore, the present findings suggest that *P. gingivalis* infection is capable of inducing significant immuno-inflammatory responses and associated oxidative stress in the hippocampi of ApoE<sup>-/-</sup> mice, which is characteristically prominent in the microvascular regions.

In conclusion, this study has revealed a major difference in the hippocampi of *P. gingivalis*-infected and sham-infected ApoE<sup>-/-</sup> mice, in terms of increased protein carbonyl/oxidized protein content in the hippocampal micro-vasculature. We, therefore, suggest that hippocampal microvascular structures and the homeostasis of the brain are at risk from elevated oxidative stress and oxidative protein damage, following *P. gingivalis* infection. This observation has significant implications as it suggests that following recurrent episodes of active periodontal disease there exists a possibility for the development of a defective BBB, post neuroinflammation-mediated cerebral parenchymal tissue injury. It is, therefore, highly plausible that the rising levels of intrinsic and extrinsic sources of cytokines, oxidative stress and developing BBB defects are crucial early modifiers of neurodegeneration and disease severity leading to deteriorating memory. Thus, infection with *P. gingivalis* should be interpreted as one of the plausible mechanisms by which a susceptible host can develop dementia.

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# Figures and Legends

Figure 1

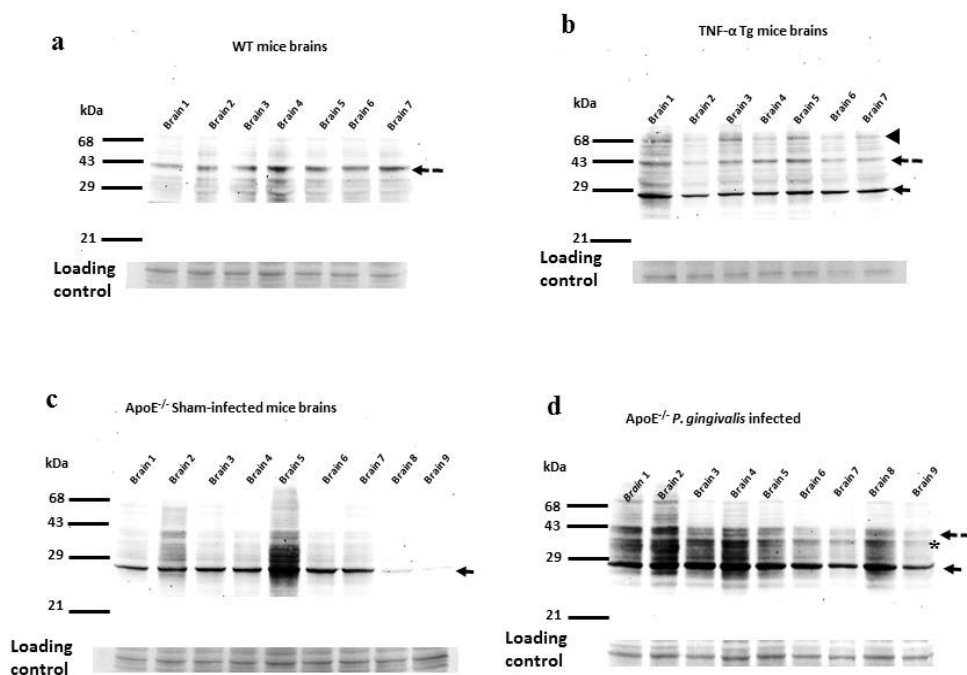
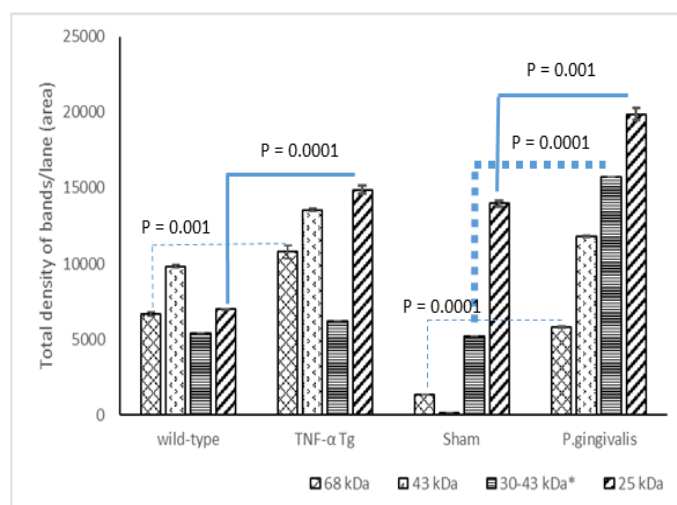


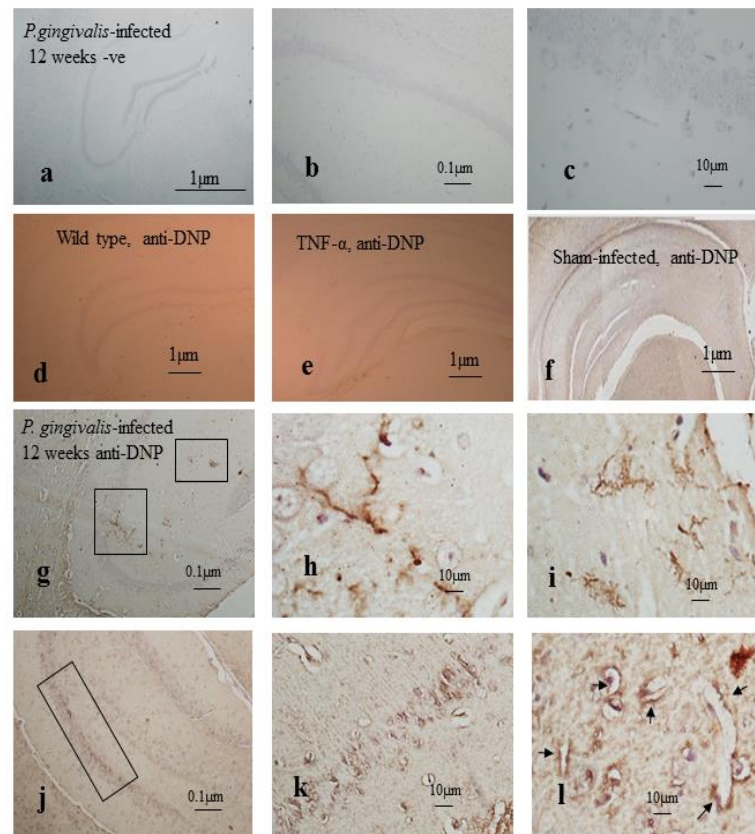
Figure 1e



## Figure 1

Representative OxyBlot profiles demonstrating protein carbonyl band intensities in wild-type (a), TNF- $\alpha$  transgenic (b), sham-infected (c), and *P. gingivalis*-infected (d) mouse brain lysates. All samples demonstrated protein carbonyl content and the presence of multiple oxidised proteins. Differences in the mean band intensities of the prominent bands at around 25 kDa 43 kDa, 30-43 kDa\* and 68 kDa between each group using Image J Software (3 replicate blots analysed). Non-parametric Mann-Whitney U (e) Shows normalised data from mean of means of triplicates  $\pm$ SD. Significance indicated (see p values in Fig. 1e) in protein carbonyl and oxidative stress levels between wild-type and TNF- $\alpha$  transgenic mice and the sham-infected and *P. gingivalis*-infected mice.

**Figure 2**

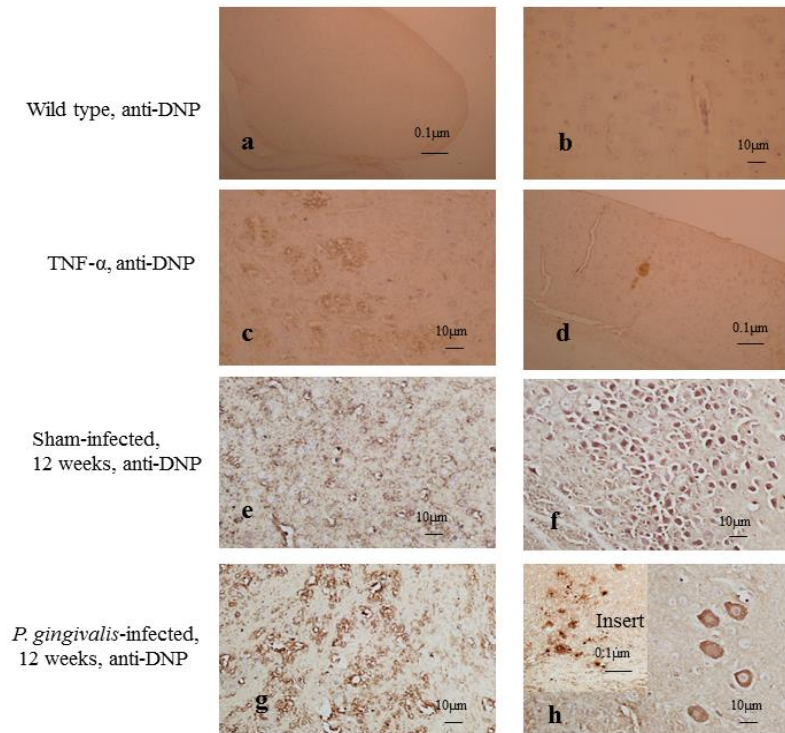


**Figure 2**

Compared with the controls (2a-c), immunohistochemical detection of protein carbonyl groups within wild-type brains and TNF- $\alpha$  transgenic mouse hippocampi generally demonstrated lower protein carbonyl immunostaining overall or within the microvessels (2d-e). This was also the case in sham-infected mouse hippocampi, although staining was observed within the pyramidal neurons (2f). *P. gingivalis*-infected mouse brains exhibited protein carbonyl immunostaining in the vasculature, especially localized within the hippocampal capillaries (2g). The boxed areas in Fig. 2g are shown at higher magnification (2h, i), to further demonstrate the microvasculature. The capillaries adjacent to the CA neurons of the hippocampus (2j, box) also showed immunostaining localised to the capillaries and endothelial cells (2k-l, arrows denote endothelial cell staining).



**Figure 3**



**Figure 3**

Immunohistochemical detection of protein carbonyl groups within the thalamus (3a) and frontal lobe (3b) of wild-type mice generally showed little immuno-staining, whereas TNF- $\alpha$  transgenic mice frontal brains demonstrated weak staining on myelin bundles (3c) and occasional cellular staining (3d). Sham-infected mice tissue sections demonstrated marked staining of the myelin sheath regions (3e) and neuronal staining (3f). *P. gingivalis*-infected mouse tissue sections also demonstrated staining of the myelin sheath (3g) and neurons (3h), whilst certain neurons appeared to be completely damaged (3h, insert).